3. The sample to be amplified and SYBR™ Green I.

With each cycle of amplification, product denaturation is ensured by monitoring sample 1 as the temperature is increased. A melting curve is determined in real-time and when the sample has denatured, the transition to the annealing step is begun. Primer annealing is monitored indirectly through the hybridization of two complementary primers in sample 2. One of the primers is 3' labeled with fluorescein and the other is 5' labeled with Cy5 or similar dye. The temperature is decreased until sample 2 shows primer hybridization as indicated by an increase in the ratio of fluorescence at 670 nm / 540 nm. This ratio increases due to resonance energy transfer between the fluorophores when they are approximated by hybridization. Product extension is followed by monitoring the fluorescence of one or more of the actual samples as demonstrated in Example 25.

Summary. From the foregoing discussion, it will be appreciated that continuous fluorescence monitoring during DNA amplification to monitor hybridization is an extraordinarily powerful analytical technique. Using the methods described herein and depending on the number of initial template copies present, product identification and quantification can be achieved in five to twenty minutes after temperature cycling has begun. The present invention achieves several advantages not heretofore available in the art. For example, the present invention provides single-color fluorescence methods to monitor product purity, relative quantitation by multiplex PCR or competitive PCR, absolute product quantification by reannealing kinetics, and an improved method for initial template quantification by fluorescence vs cycle number plots. The present invention also provides dual-color, sequence-specific methods for sequence variation detection, relative quantitation by multiplex PCR or competitive PCR, product quantification by probe annealing kinetics, and initial template quantification by fluorescence vs cycle number plots.

The following table compares double-strand-specific DNA dyes, hydrolysis probes, and hybridization probes useful in continuous monitoring of PCR. The fluorescence of double-strand-specific DNA dyes depends on the strand status of the DNA. The dual-labeled hydrolysis probes are quenched while intact and donor fluorescence increases when the probe is hydrolyzed. Hybridization probes depend on increased resonance energy transfer when hybridization brings 2 fluorophores closer together.

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Summary of Fluorescent Probes for Continuous Monitoring of PCR

		Fluorescent Probe	
	dsDNA dye	Hydrolysis	
Hybridization			
Mechanism	Strand status	Quenching	Transfer
Probe Synthesis	Unnecessary	Difficult	Simple
Specificity	Product Tm	Sequence	Sequence
Melting Analysis	Yes	No	Yes
Multicolor Analysis	No	Yes	Yes

In accordance with the present invention, time, temperature and fluorescence are acquired 1-10 times every sec and fine details of product and/or probe hybridization are observed during temperature cycling. With double-strand-specific DNA dyes, the hybridization of product with respect to temperature is used to identify products by melting curves. In addition, relative product quantification is achieved by multiplex amplification of two or more different products that differ in Tm. Further, competitive PCR is performed by altering the sequence internal to the common primers so that two or more products have different Tm's. Absolute product quantification is obtained by rapidly cooling the denatured product and observing reannealing kinetics. The sensitivity of initial template quantification with fluorescence vs cycle number plots is increased by analysis of product melting curves to control for nonspecific amplification and curve fitting algorithms. Finally, immediate fluorescence feedback for control of denaturation conditions, elongation times and product yield are obtained by monitoring product strand status with double-strand-specific DNA dyes.

The ability to monitor probe hybridization with fluorescence during temperature cycling is a powerful tool. The present invention provides dual-color fluorescence methods that depend on probe hybridization (not hydrolysis) for sequence-specific detection and quantification during PCR. The annealing kinetics and melting of hybridization probes provides information not available with probes that rely on exonuclease hydrolysis between fluorophores. Continuous monitoring of sequence-specific probe hybridization can be followed

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over temperature changes by resonance energy transfer. Probe melting occurs at a characteristic temperature determined by its sequence and complementarity to the product. Two schemes have been detailed by the present invention, (1) two adjacent hybridization probes, and (2) one labeled probe that hybridizes to a single stranded PCR product that incorporates a labeled primer. The melting temperature of sequence-specific probes identifies and discriminates products during PCR. DNA polymorphisms or mutations, including single base mutations, are detected by probe Tm shifts. In addition, relative product quantification is achieved by multiplex amplification of at least two different products with one or more probes that melt from their respective products at different temperatures. Further, competitive PCR is performed by altering the sequence internal to the primers so that one or more probes hybridize to the competitor and the natural template at different Tm's. Alternately, relative or competitive PCR are performed by multicolor analysis with probes labeled with different fluorophores, such as Cy5 and Cy5.5. Absolute product concentration is determined by analysis of probe annealing kinetics. Initial template copy number is determined from fluorescence vs cycle number plots by curve fitting algorithms.

When multiplex analysis in one PCR reaction is desired, it is common practice to use different fluorescent labels with distinguishable emission spectra to identify the multiple products. The analysis is complicated by the limited number of fluorophores available and the overlapping emission spectra of those fluorophores that are available (see HM Shapiro, supra). Analysis of product or probe hybridization with melting curves is another method to distinguish multiple PCR products. By following hybridization during temperature cycling, the number of probes and/or spectral colors needed to distinguish multiple products can be minimized..

Thepresent invention may be embodied in other specific forms without departing from its spirit or essential characteristics. The described embodiments are to be considered in all respects only as illustrative and not restrictive. The scope of the invention is, therefore, indicated by the appended claims rather than by the foregoing description. All changes which come within the meaning and range of equivalency of the claims are to be embraced within their scope.

Programming code for carrying out melting curve and other analyses is found on the following pages.

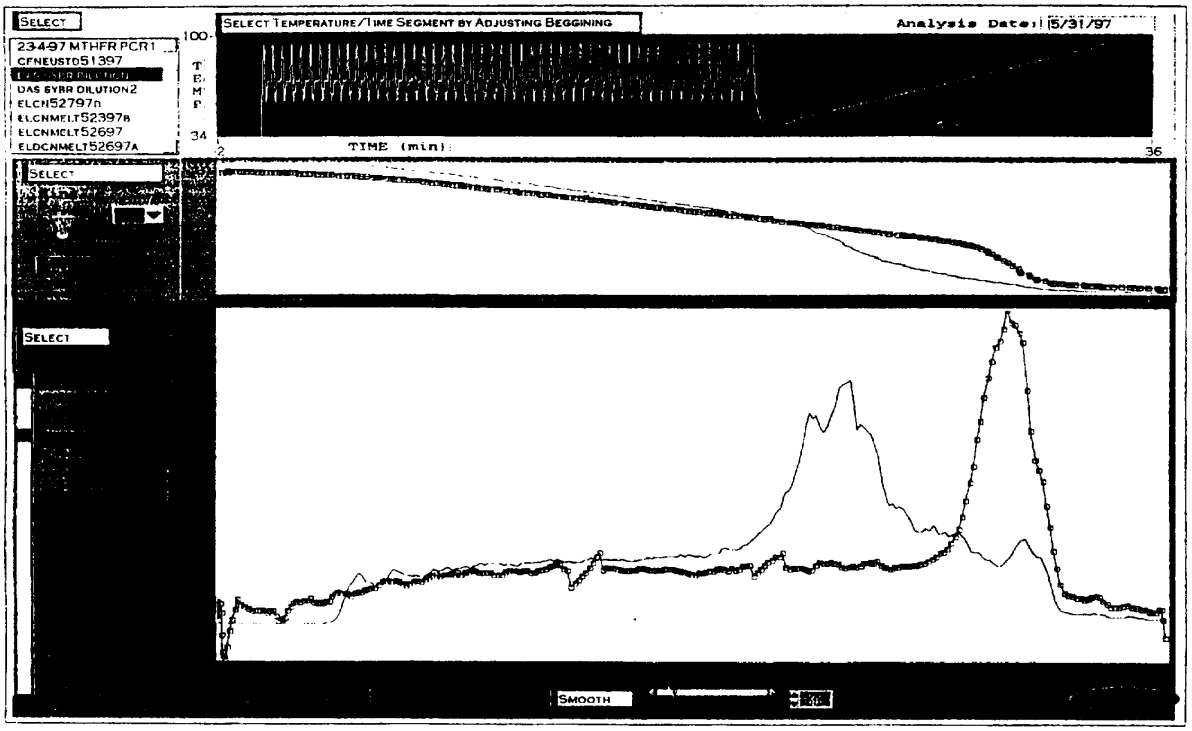
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CONNECTOR PANE

CTWMELT1 1.VI

FRONT PANEL



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- TF STOP
- [U32] COLOR PREFERENCES

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U32

U8

TF F

U8

I32 SLOPE METHOD

TF SAMPLES
CYCLES

U8 SAMPLE #

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VS T

F VS T

U32 COLOR DISPLAY

U32

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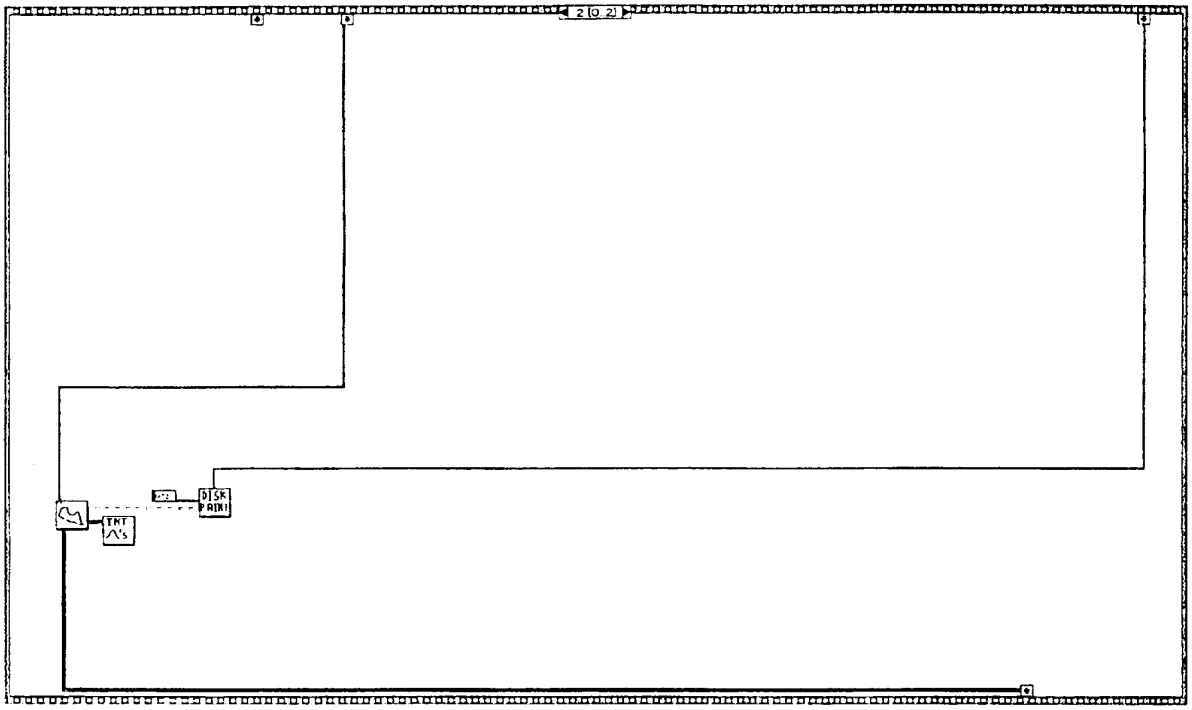
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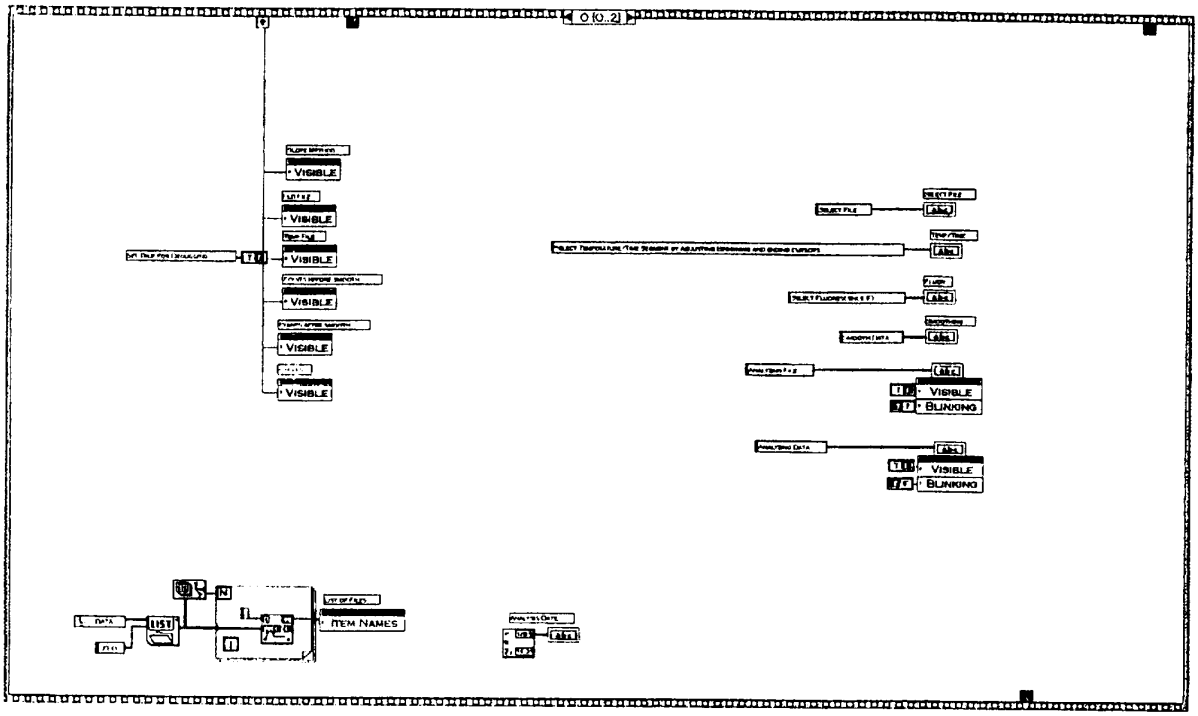
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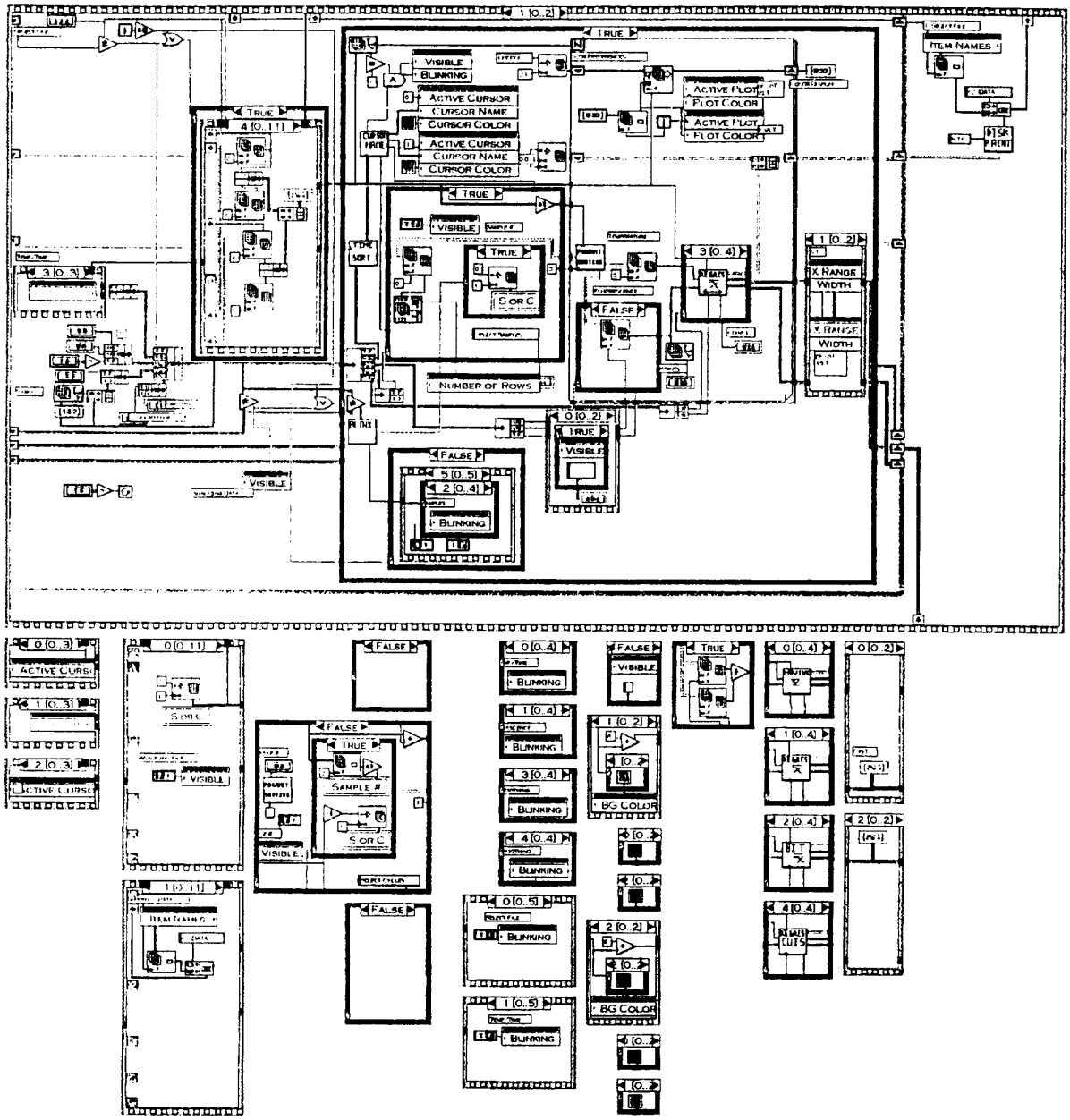


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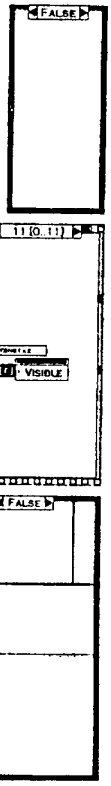


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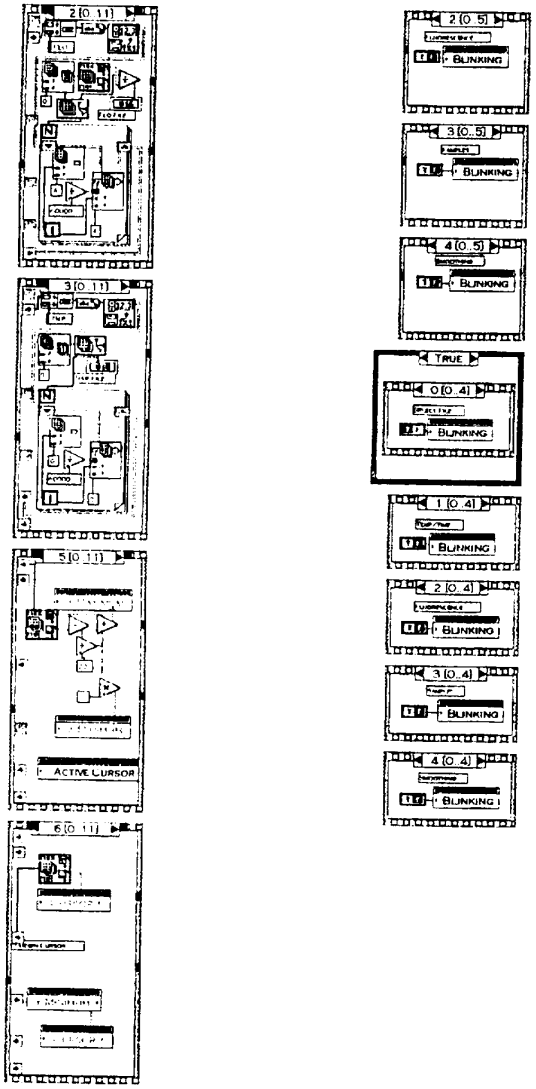


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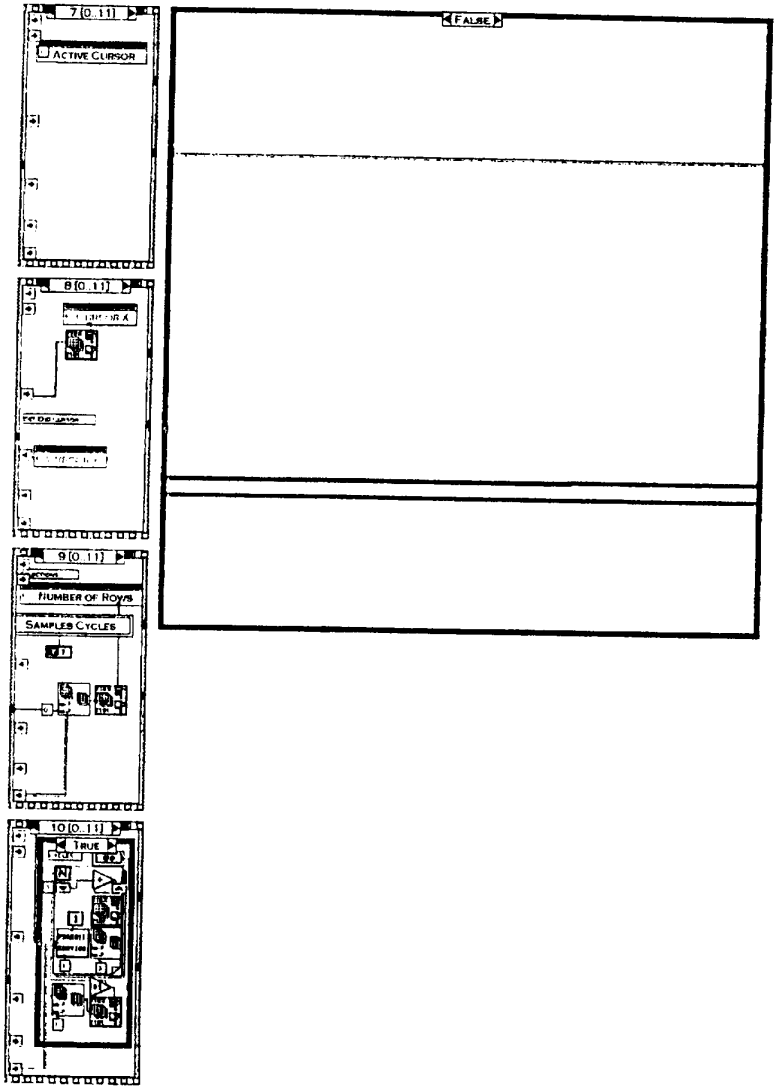


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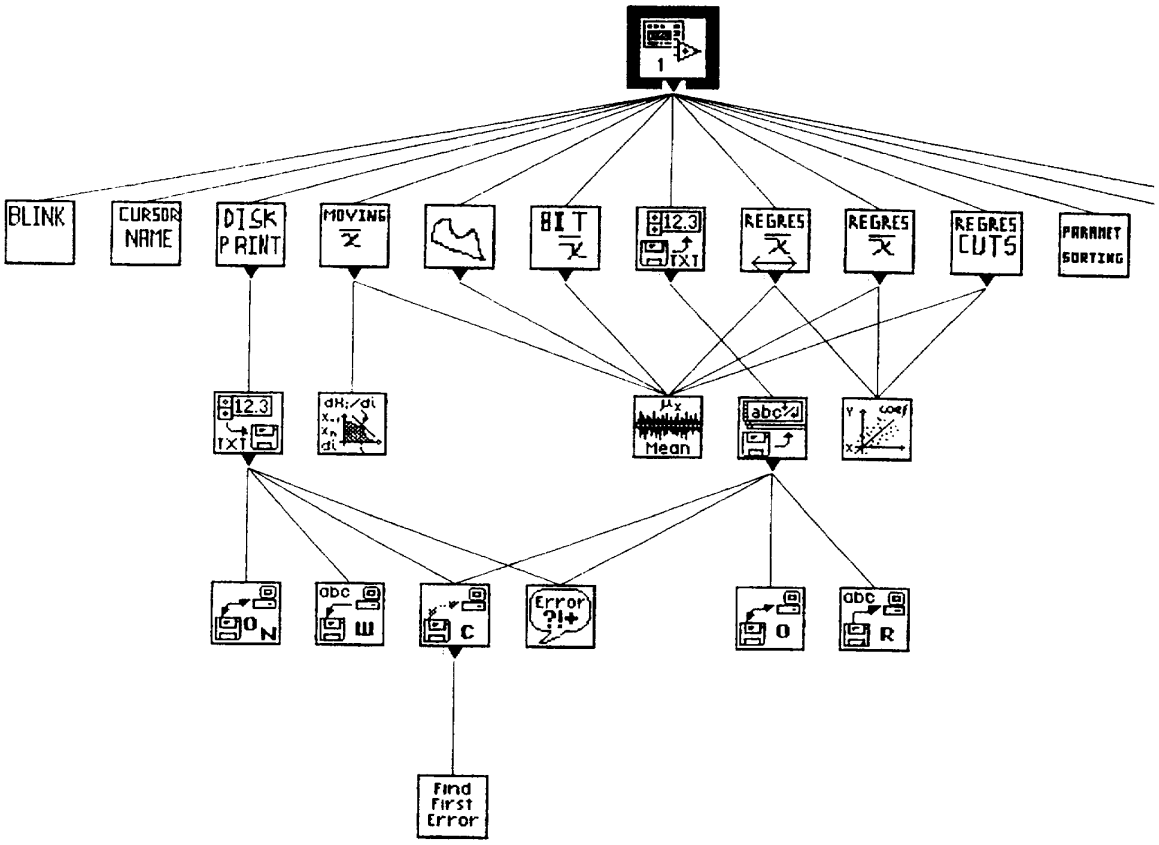


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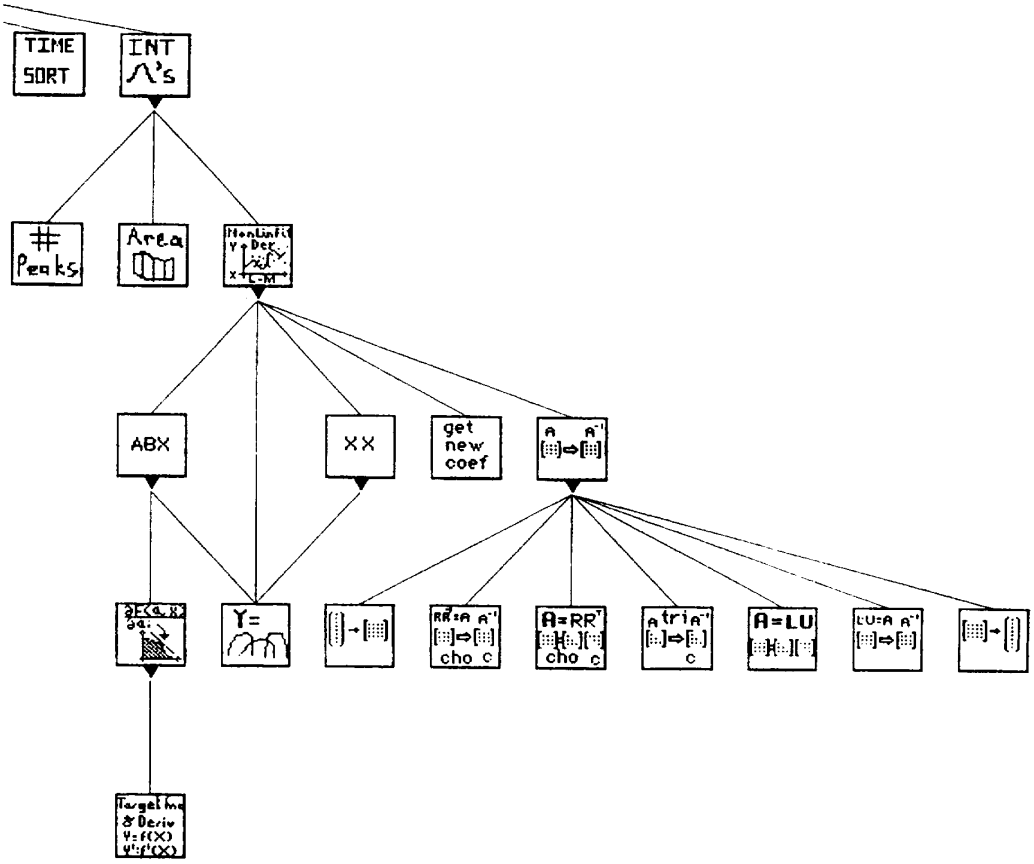


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CURSOR NAME.VI
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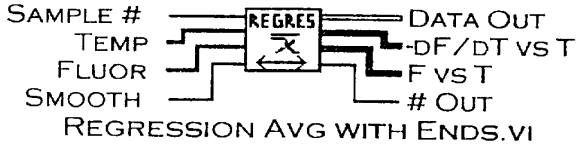
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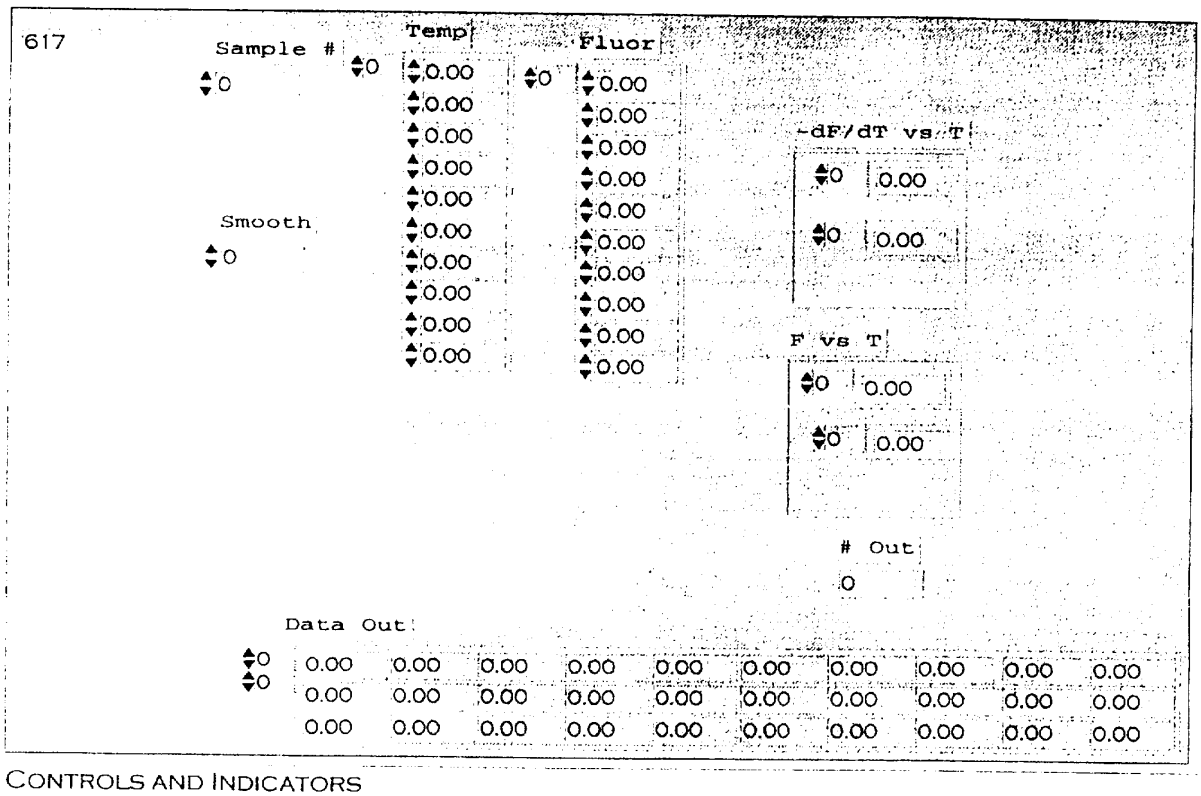
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- TEMP
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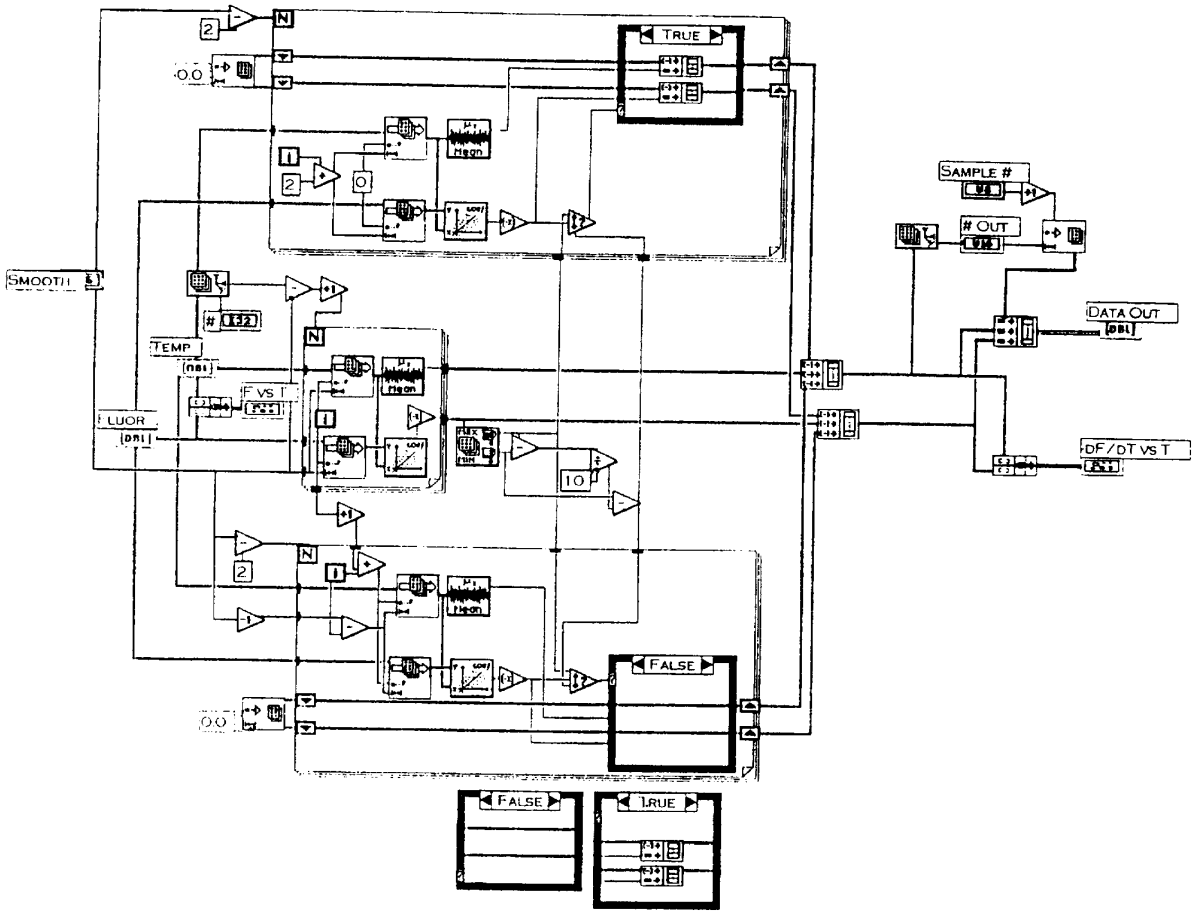
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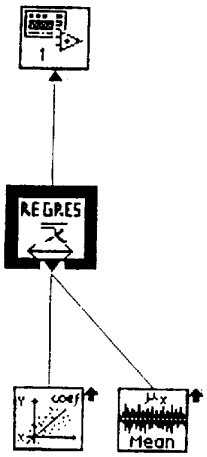
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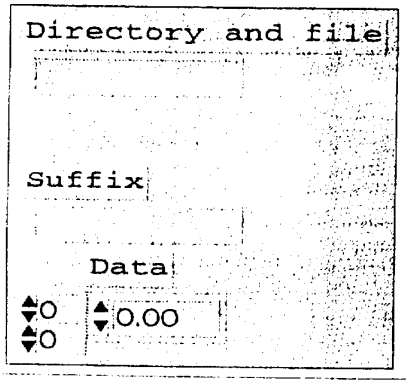
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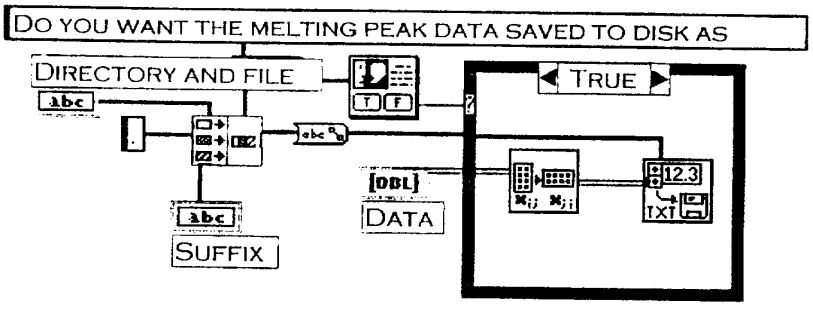
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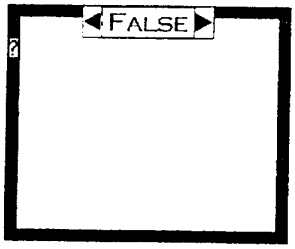
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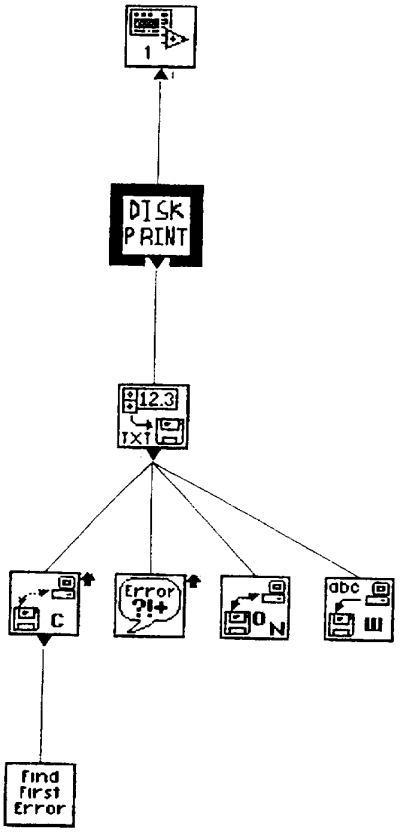
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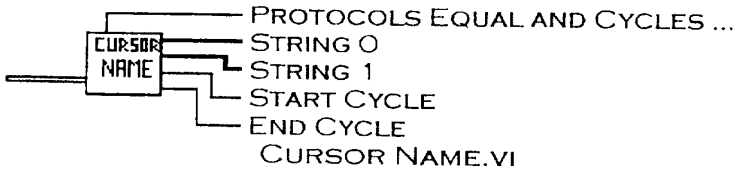
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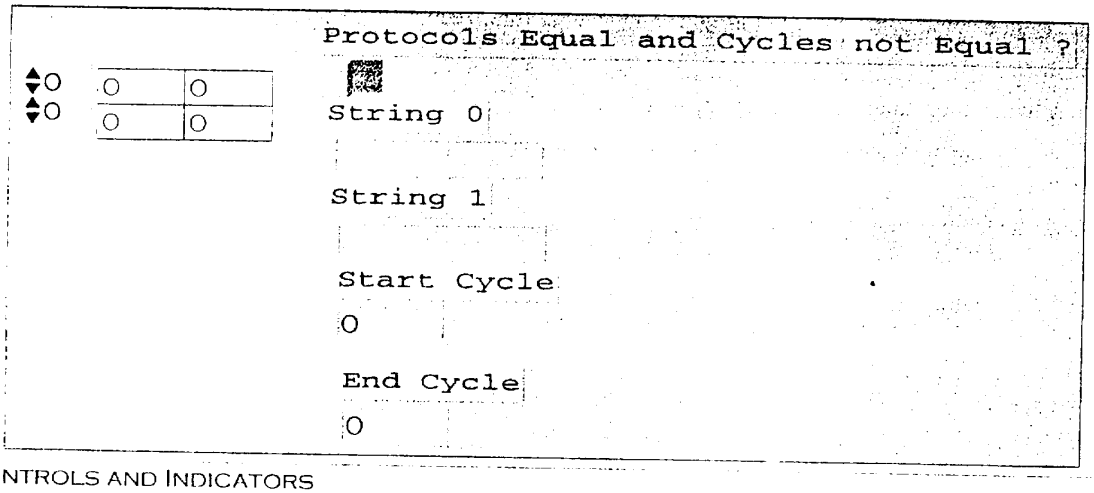
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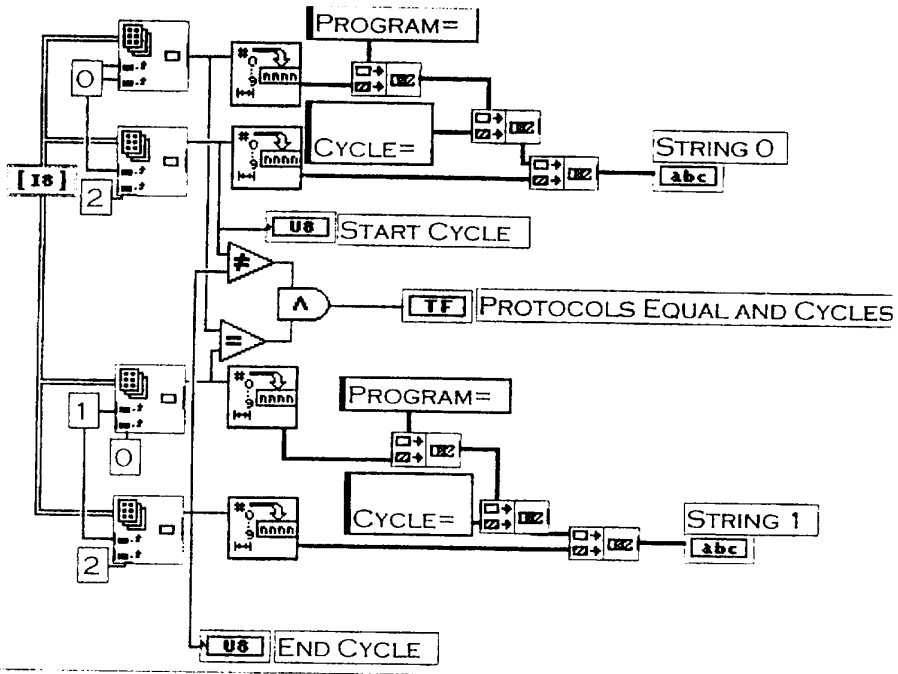
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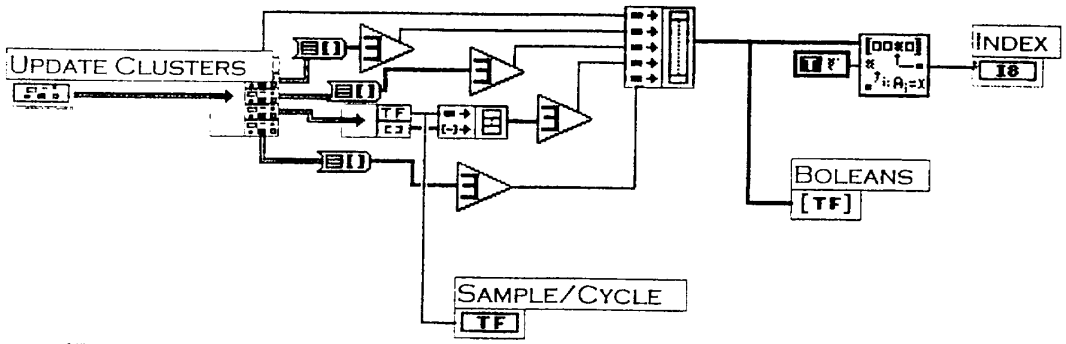
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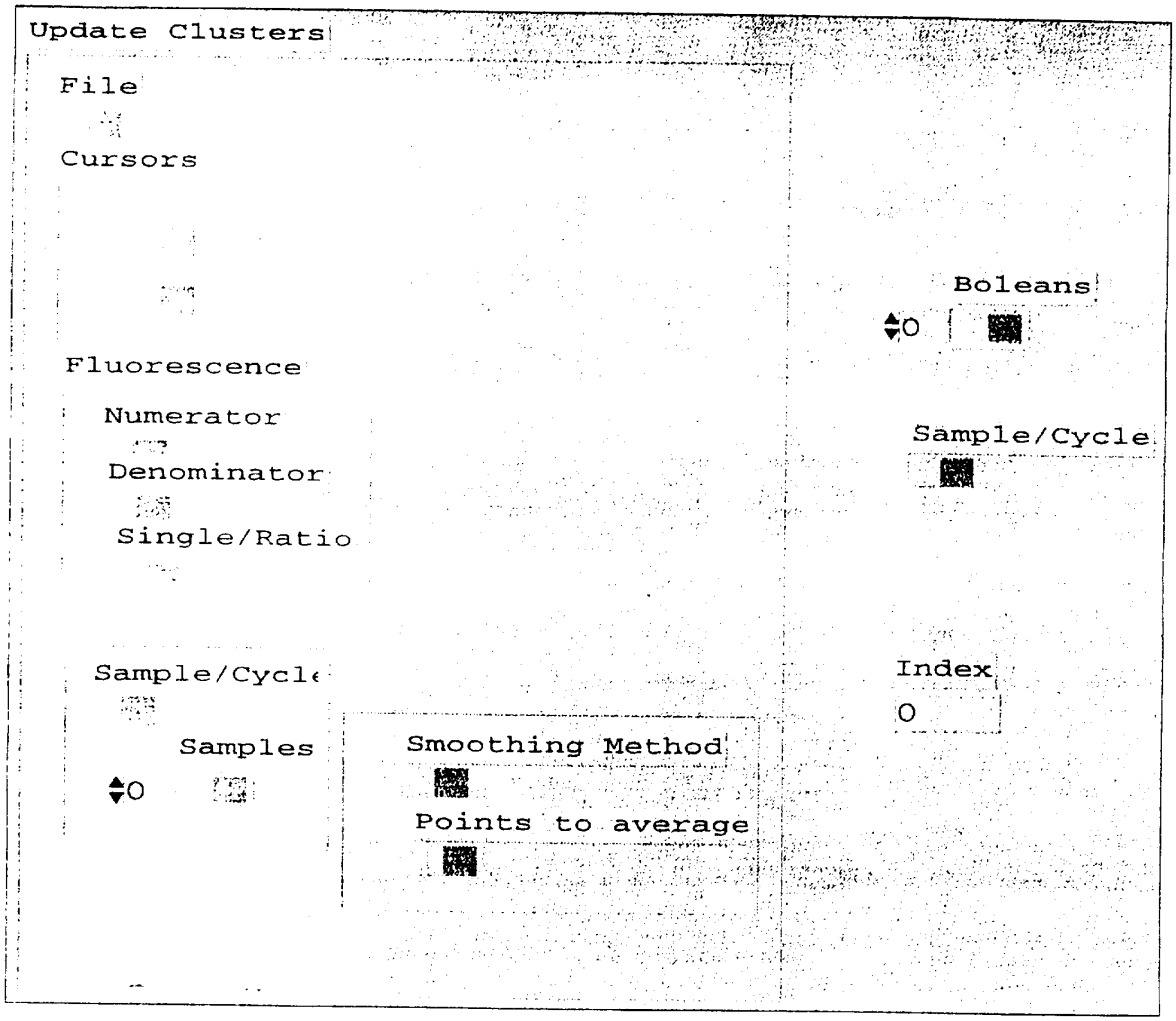
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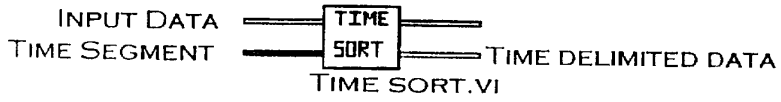
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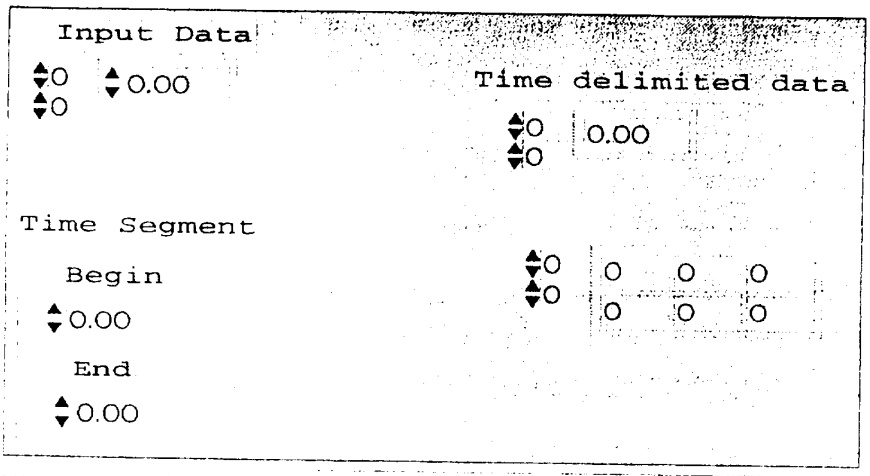
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    - SAMPLE/CYCLE
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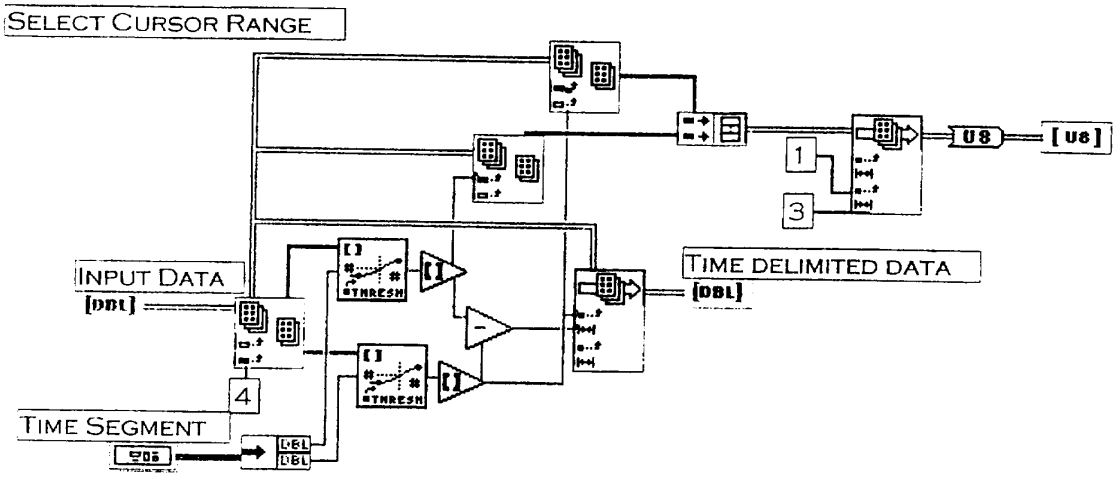
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PAGE 2 TIME SORT

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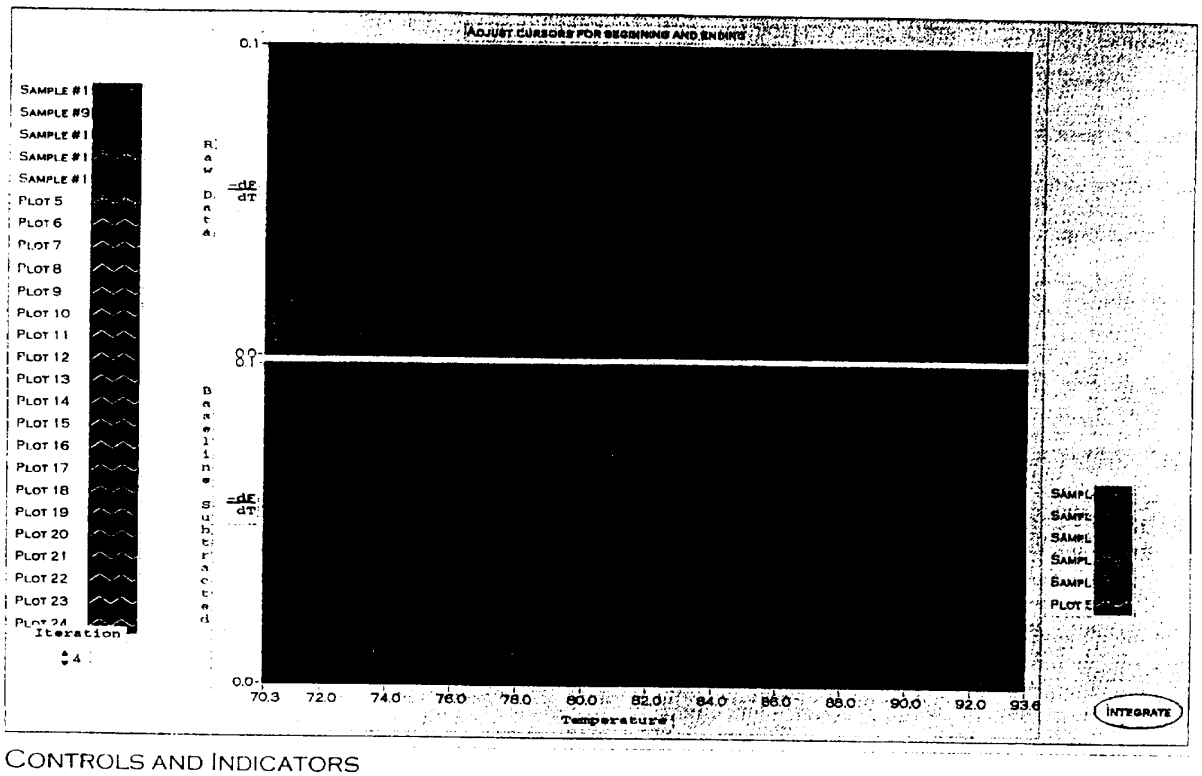
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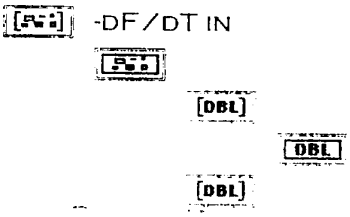
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CONTROLS AND INDICATORS





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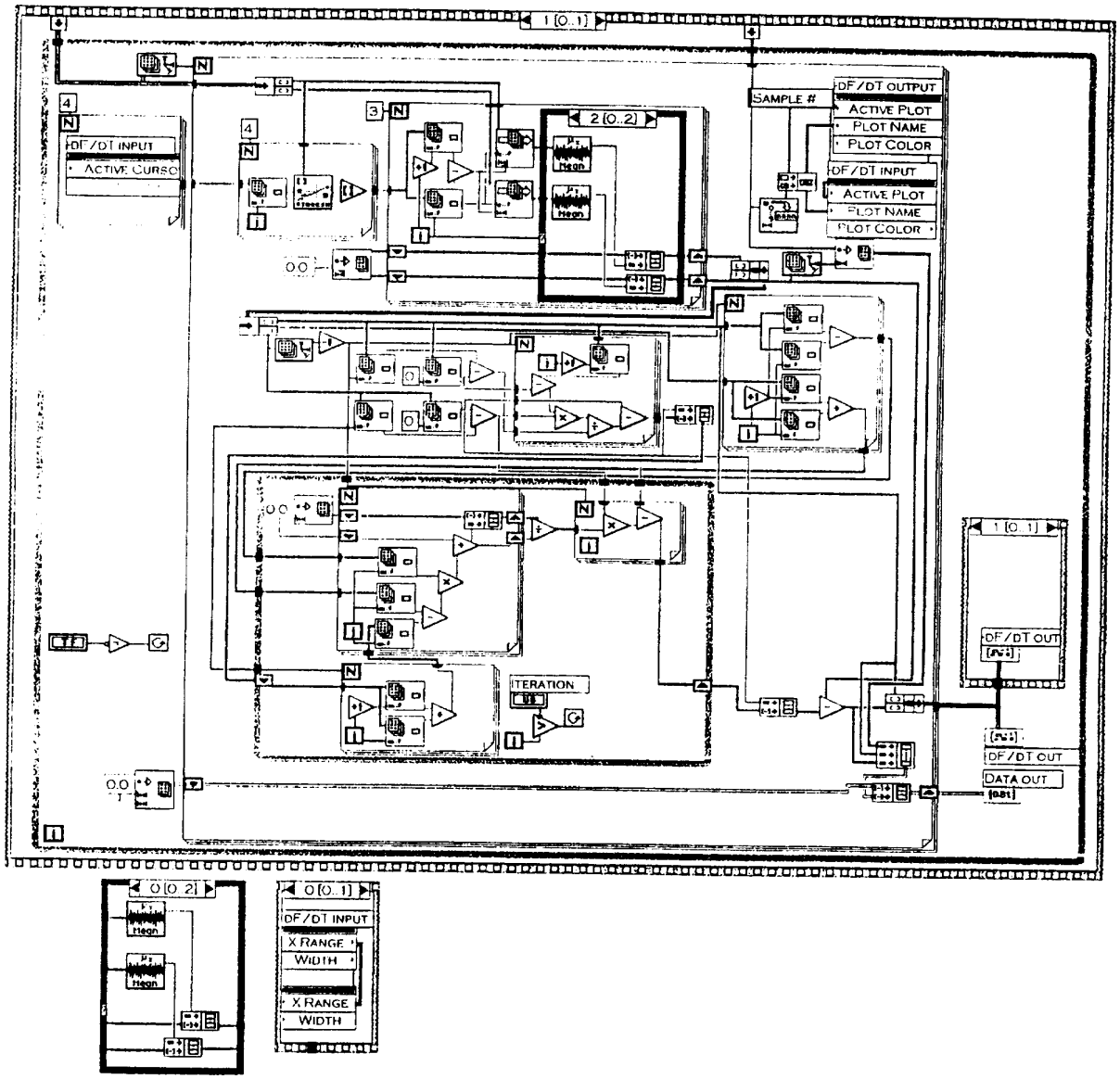
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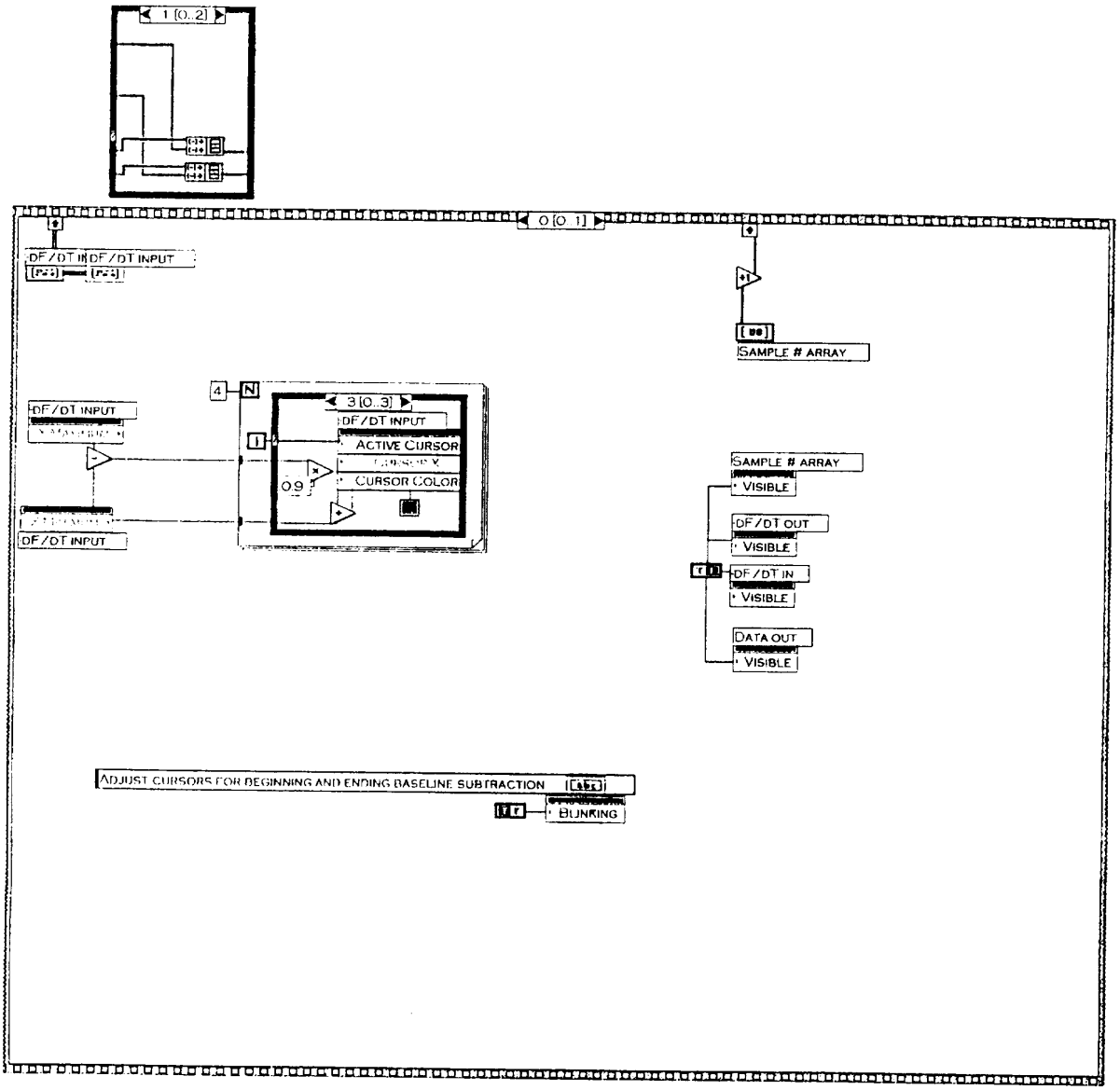
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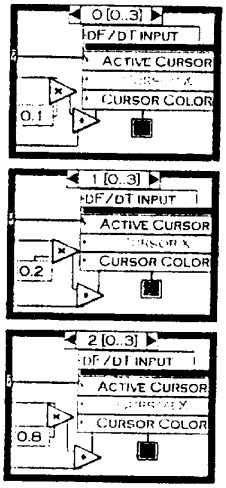
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POSITION IN HIERARCHY



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HISTORY



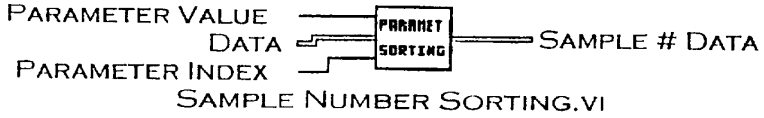
BACKGROUND.VI
LAST MODIFIED ON 6/3/97 AT 11:07 AM
PRINTED ON 6/3/97 AT 11:07 AM

"BACKGROUND.VI HISTORY"
CURRENT REVISION: 55

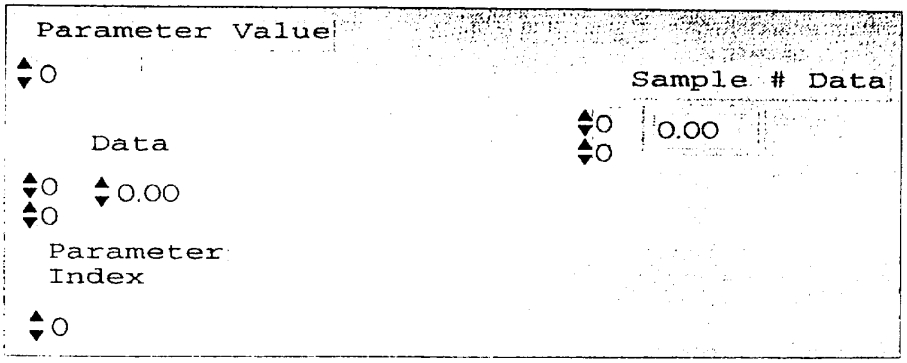
PAGE 1

SAMPLE NUMBER SORTING.VI
LAST MODIFIED ON 3/23/97 AT 10:23 PM
PRINTED ON 6/3/97 AT 11:07 AM

CONNECTOR PANE



FRONT PANEL

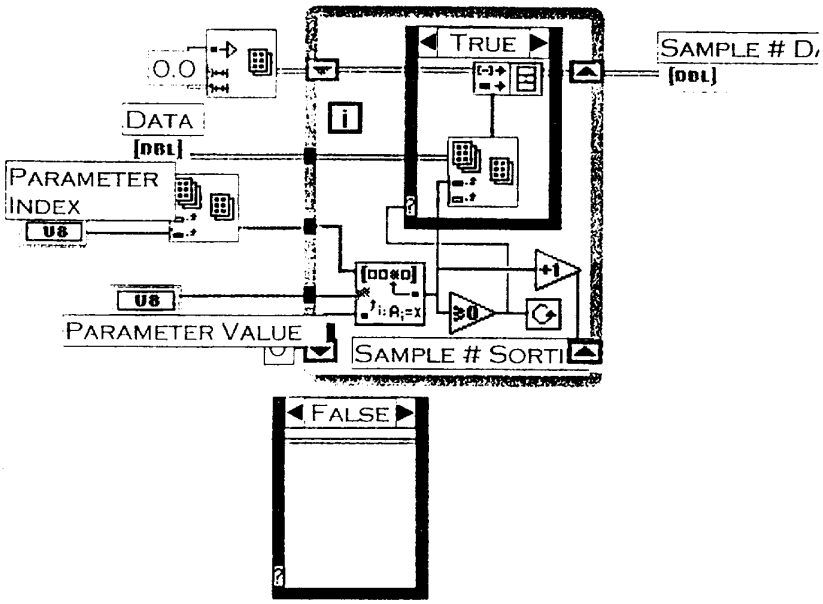


CONTROLS AND INDICATORS

[DBL] DATA
DBL
U8 PARAMETER VALUE
U8 PARAMETER INDEX
[DBL] SAMPLE # DATA
DBL

SAMPLE NUMBER SORTING.VI
LAST MODIFIED ON 3/23/97 AT 10:23 PM
PRINTED ON 6/3/97 AT 11:07 AM

BLOCK DIAGRAM



POSITION IN HIERARCHY



LIST OF SUBVIS

HISTORY

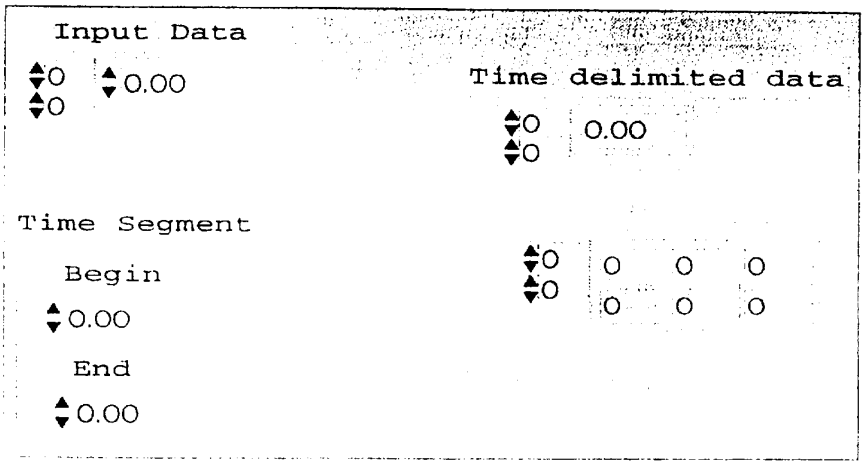
"SAMPLE NUMBER SORTING.VI HISTORY"
CURRENT REVISION: 9

TIME SORT.VI
LAST MODIFIED ON 3/23/97 AT 10:23 PM
PRINTED ON 6/3/97 AT 11:08 AM

CONNECTOR PANE



FRONT PANEL

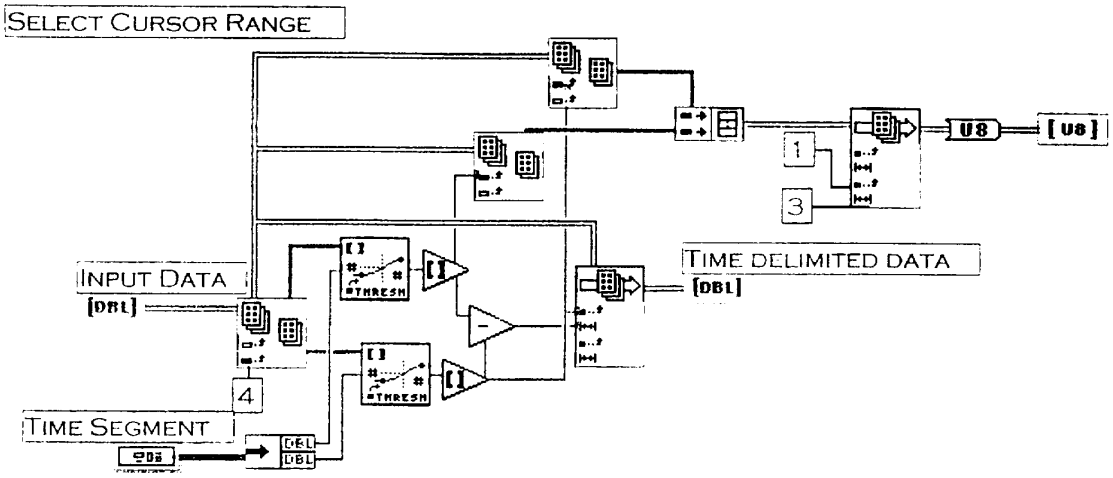


CONTROLS AND INDICATORS

- TIME SEGMENT
  - BEGIN
  - END
- INPUT DATA
  - INPUT DATA
- TIME DELIMITED DATA
- U8
- U8

TIME SORT.VI
LAST MODIFIED ON 3/23/97 AT 10:23 PM
PRINTED ON 6/3/97 AT 11:08 AM

BLOCK DIAGRAM



POSITION IN HIERARCHY



LIST OF SUBVIS

HISTORY

"TIME SORT.VI HISTORY"
CURRENT REVISION: 9

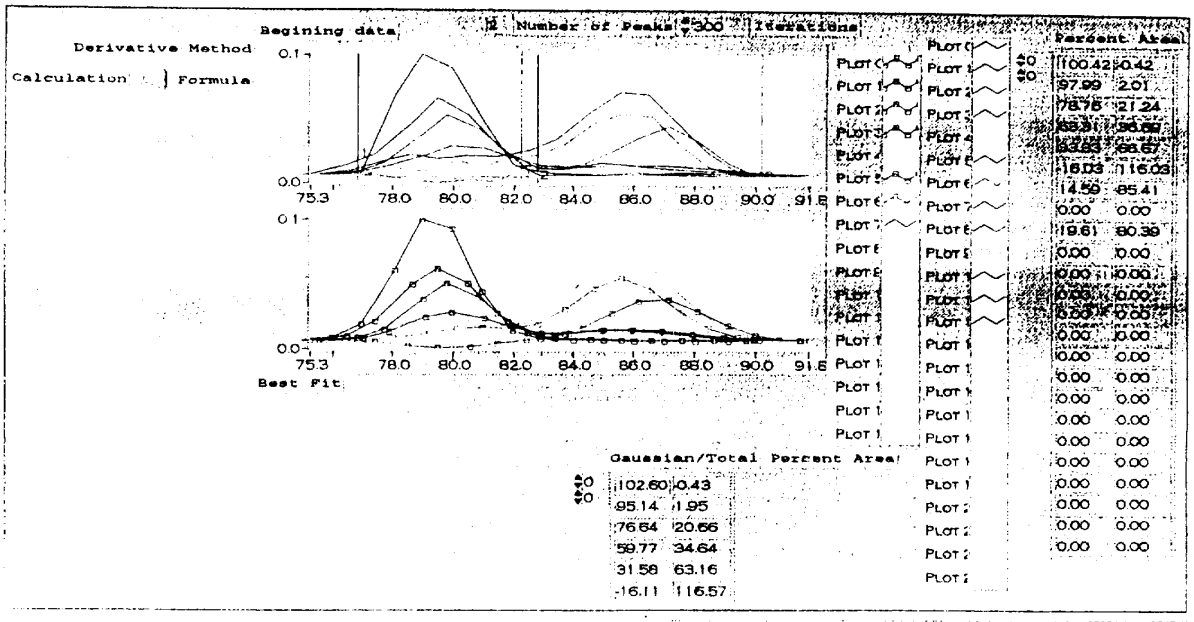
SUM OF GAUSSIANS.VI
LAST MODIFIED ON 5/19/97 AT 1:14 PM
PRINTED ON 6/3/97 AT 11:08 AM

CONNECTOR PANE

-DF/DT IN

SUM OF GAUSSIANS.VI

FRONT PANEL



CONTROLS AND INDICATORS

-DF/DT IN

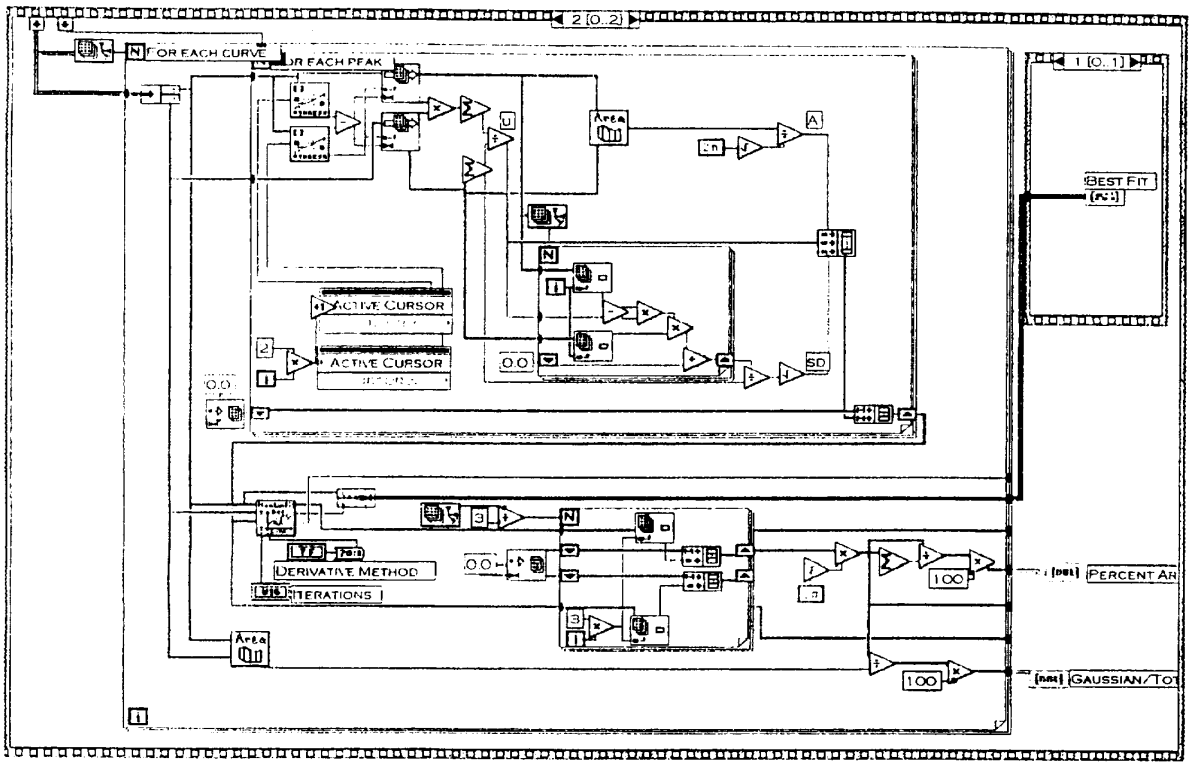
ITERATIONS

DERIVATIVE METHOD

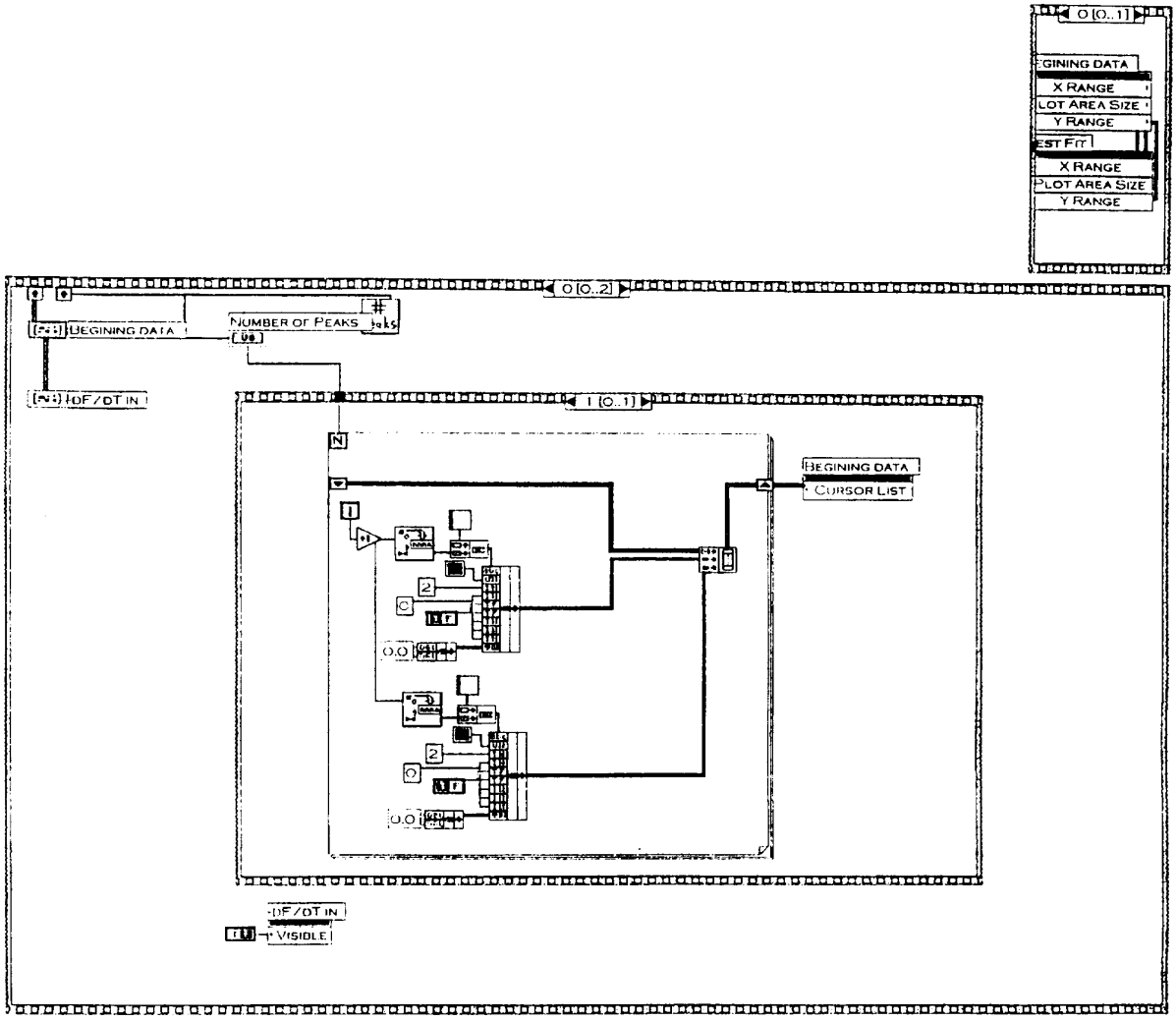
SUM OF GAUSSIANS.VI
LAST MODIFIED ON 5/19/97 AT 1:14 PM
PRINTED ON 6/3/97 AT 11:08 AM

- [SGL] BEGINING DATA
- [SGL] BEST FIT
- [U8] NUMBER OF PEAKS
- [DBL] PERCENT AREA
  - [DBL]
- [DBL] GAUSSIAN/TOTAL PERCENT AREA
  - [DBL]

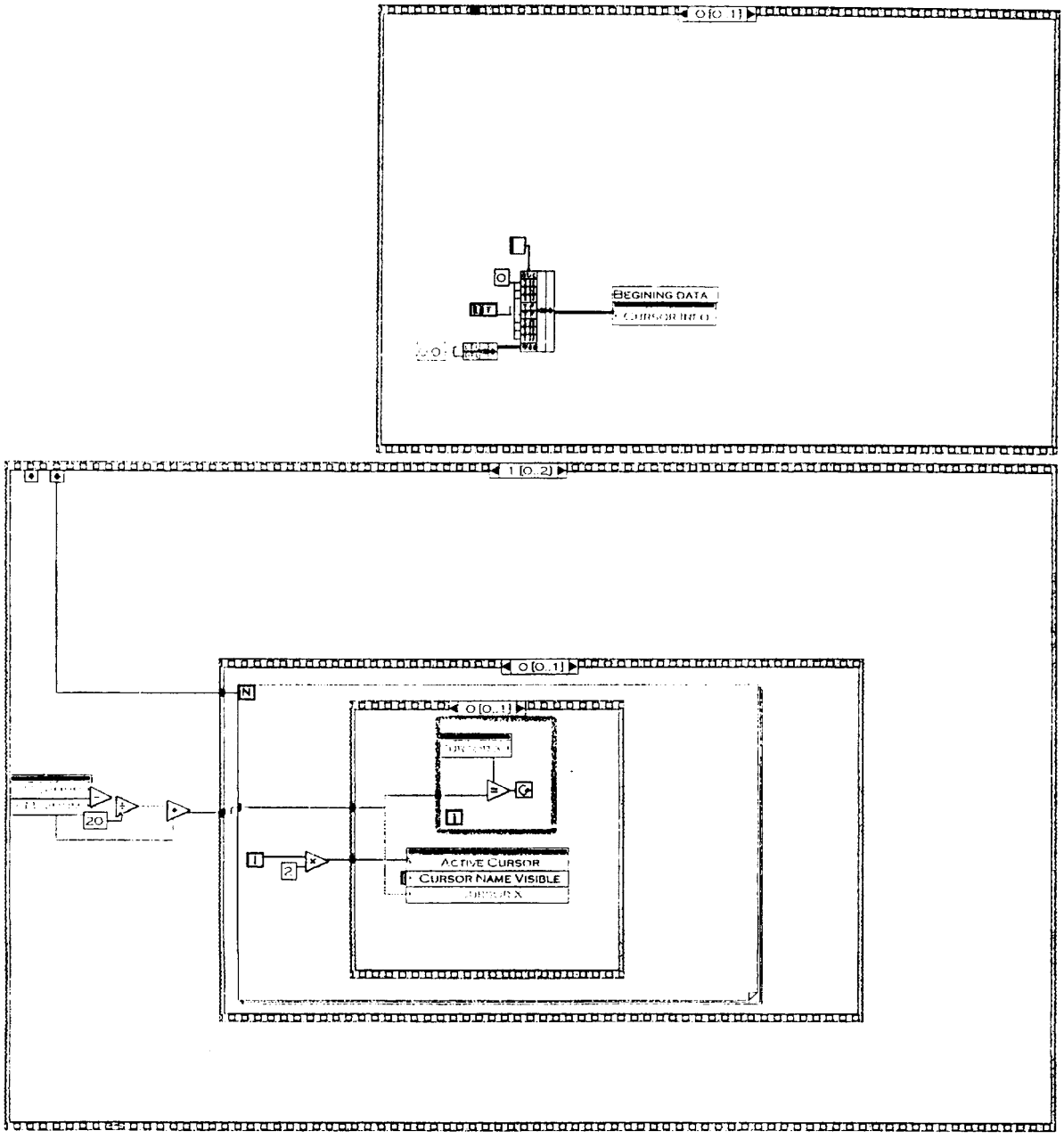
BLOCK DIAGRAM



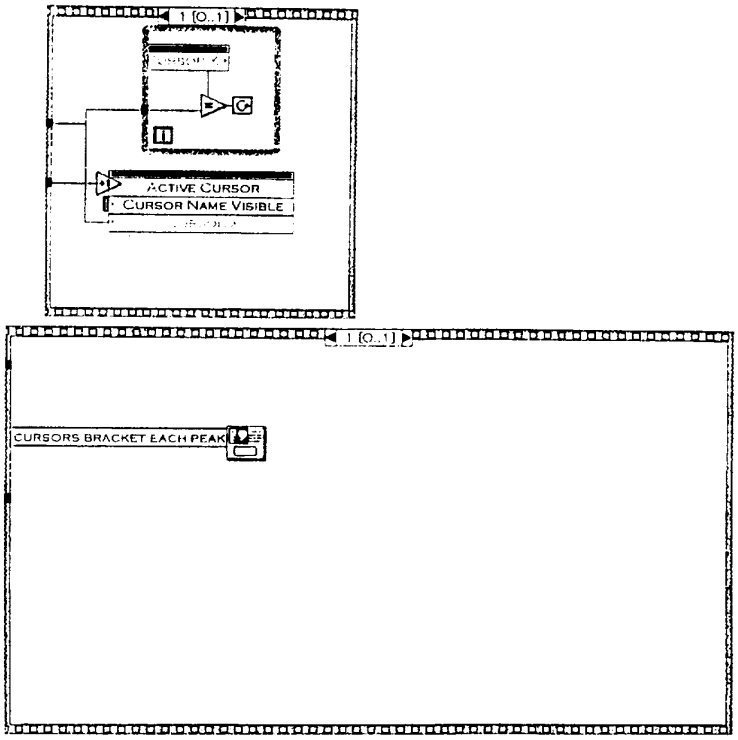
SUM OF GAUSSIANS.VI
LAST MODIFIED ON 5/19/97 AT 1:14 PM
PRINTED ON 6/3/97 AT 11:08 AM



SUM OF GAUSSIANS.VI
LAST MODIFIED ON 5/19/97 AT 1:14 PM
PRINTED ON 6/3/97 AT 11:08 AM

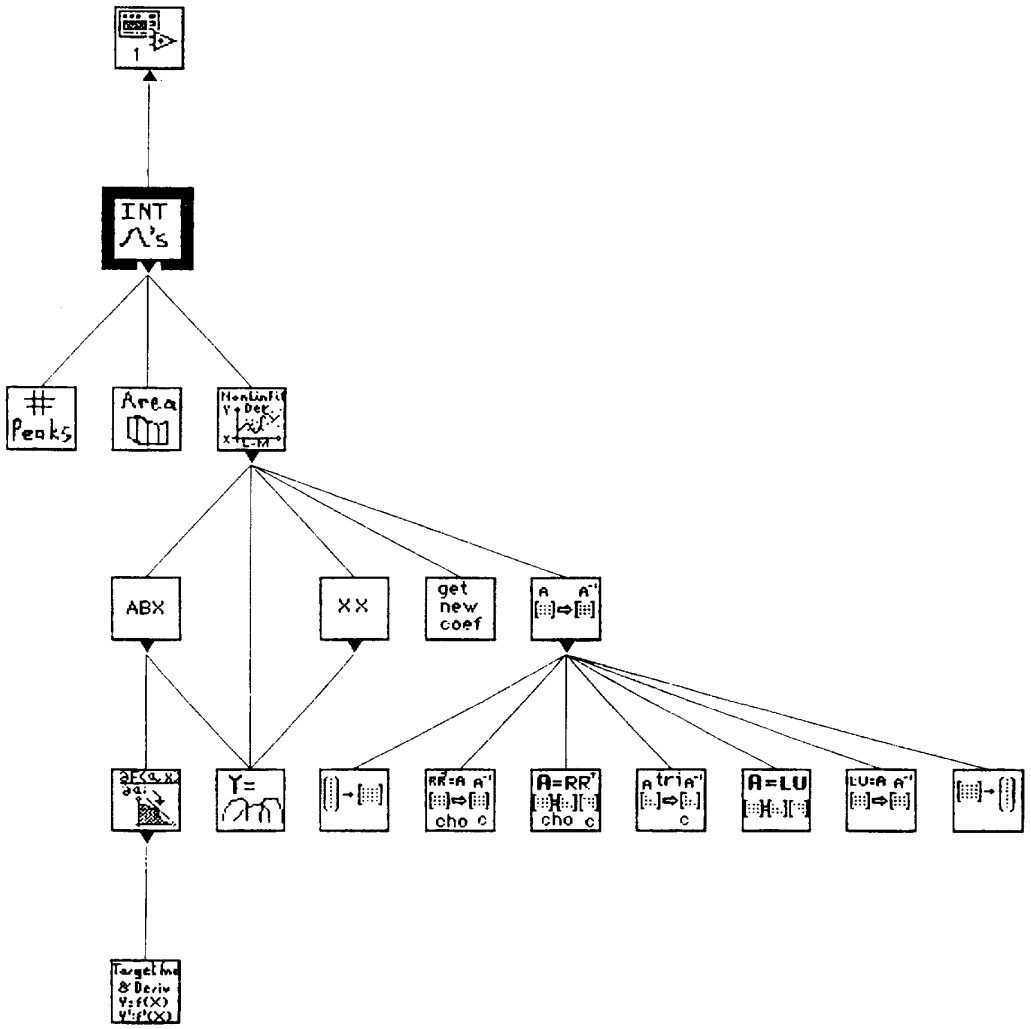


SUM OF GAUSSIANS.VI
LAST MODIFIED ON 5/19/97 AT 1:14 PM
PRINTED ON 6/3/97 AT 11:08 AM



POSITION IN HIERARCHY

SUM OF GAUSSIANS.VI
LAST MODIFIED ON 5/19/97 AT 1:14 PM
PRINTED ON 6/3/97 AT 11:08 AM



LIST OF SUBVIS

FIT SUM OF GAUSSIANS.VI
C:\DATA\FIT SUM OF GAUSSIANS.VI

PEAK#.VI
C:\DATA\PEAK#.VI

SUM OF GAUSSIANS.VI
LAST MODIFIED ON 5/19/97 AT 1:14 PM
PRINTED ON 6/3/97 AT 11:09 AM

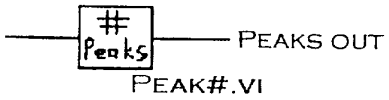
TRAPEZOID AREA.VI
C:\DATA\TRAPEZOID AREA.VI

HISTORY

"SUM OF GAUSSIANS.VI HISTORY"
CURRENT REVISION: 28

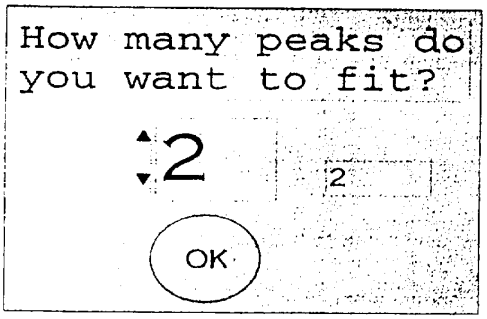
PEAK#.VI
LAST MODIFIED ON 4/16/97 AT 7:31 PM
PRINTED ON 6/3/97 AT 11:09 AM

CONNECTOR PANE



PEAK#.VI

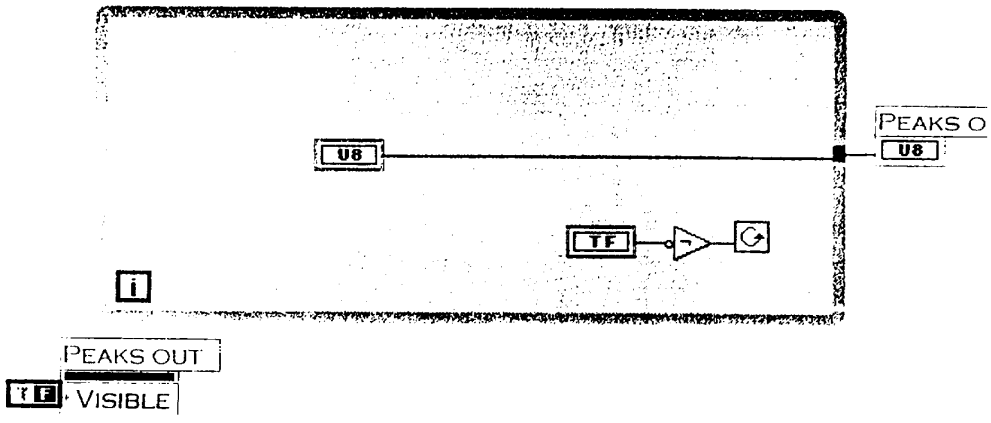
FRONT PANEL



CONTROLS AND INDICATORS

U8
TF STOP
U8 PEAKS OUT

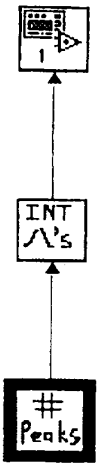
BLOCK DIAGRAM



PEAK#.VI
LAST MODIFIED ON 4/16/97 AT 7:31 PM
PRINTED ON 6/3/97 AT 11:09 AM



POSITION IN HIERARCHY



LIST OF SUBVIS

HISTORY

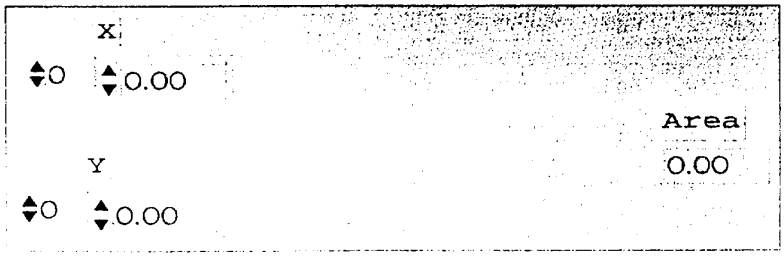
"PEAK#.VI HISTORY"
CURRENT REVISION: 6

TRAPEZOID AREA.VI
LAST MODIFIED ON 4/24/97 AT 9:26 PM
PRINTED ON 6/3/97 AT 11:09 AM

CONNECTOR PANE



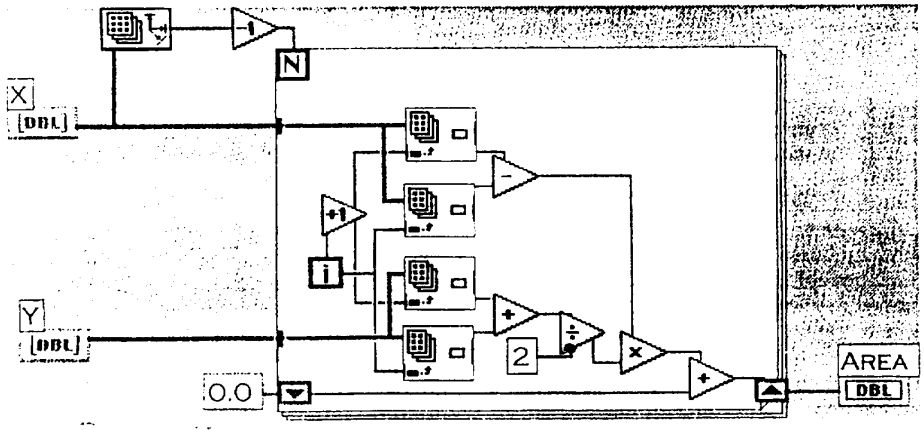
FRONT PANEL



CONTROLS AND INDICATORS

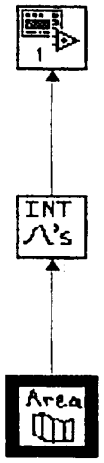
[DBL] Y
DBL
[DBL] X
DBL
DBL AREA

BLOCK DIAGRAM



TRAPEZOID AREA.VI
LAST MODIFIED ON 4/24/97 AT 9:26 PM
PRINTED ON 6/3/97 AT 11:09 AM

POSITION IN HIERARCHY



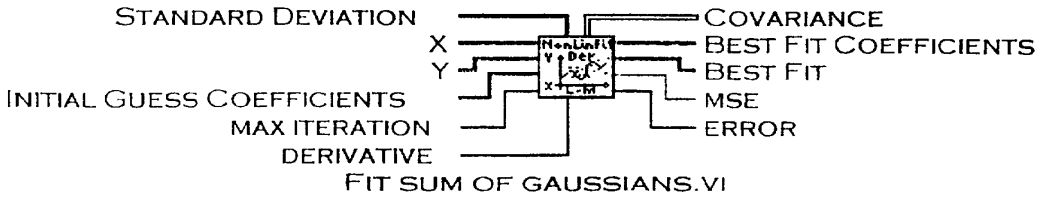
LIST OF SUBVIS

HISTORY

"TRAPEZOID AREA.VI HISTORY"
CURRENT REVISION: 1

FIT SUM OF GAUSSIANS.VI
LAST MODIFIED ON 5/16/97 AT 12:24 AM
PRINTED ON 6/3/97 AT 11:11 AM

CONNECTOR PANE





FIT SUM OF GAUSSIANS.VI
LAST MODIFIED ON 5/16/97 AT 12:24 AM
PRINTED ON 6/3/97 AT 11:11 AM

NONLINEAR LEV-MAR FIT:
==========

THIS VI USES THE LEVENBERG-MARQUARDT ALGORITHM TO DETERMINE THE LEAST SQUARES SET OF COEFFICIENTS THAT BEST FIT THE SET OF INPUT DATA POINTS(X,Y) AS EXPRESSED BY A NONLINEAR FUNCTION Y=F(X,A) WHERE A IS THE SET OF COEFFICIENTS. THIS VI ALSO GIVES THE BEST FIT CURVE Y=F(X,A).

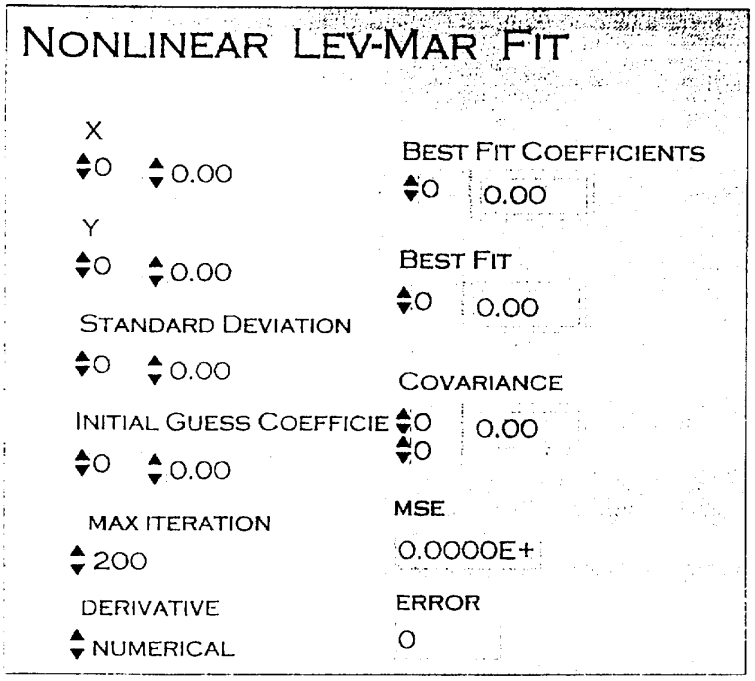
THE USER NEEDS TO OPEN A SUB-VI "TARGET FNC & DERIV NONLIN" AND INPUTS HIS/HER NONLINEAR FUNCTION F(X,A) IN THE FORMULA NODE. IF THE USER CHOOSE FORMULA CACULATION FOR DERIVATIVE METHOD, HE/SHE NEEDS TO INPUT THE PARTIAL DERIATIVE OF F(X,A) WITH RESPECT TO A AS WELL.

INPUTS:
X: EXPERIMENT DATA POINTS.
Y: EXPERIMENT DATA POINTS.
STANDARD DEVIATION: STANDARD DEVIATION OF EACH EXPERIMENT DATA.IF YOU DON'T HAVE KNOWLEDGE ABOUT THIS PARAMETER, JUST LEAVE IT EMPTY.
INITIAL GUESS COEFFICIENTS: YOUR GUESSED COEFFICIENTS. IF THIS IS TOO FAR FROM THE APPROPRIATE COEFFICIENTS, THE VI MAYBE WILL FAIL TO RETURN THE APPROPRIATE COEFICIENTS AND BEST FIT CURVE.
MAX ITERATION: MAX ITERATION THE ALGROTHIM WILL TRY. IF IT CAN NOT CONVERGE WHEN IT REACHES THE MAX ITERATION. THE VI WILL RETURN AN ERROR.
DERIVATIVE: THERE ARE TWO WAYS TO CACULATE THE PARTIAL DERIVATIVE OF F(X,A) WITH RESPECT TO COEFFICIENTS A. IF YOU CHOOSE NEMERICAL CACULATION, THE VI WILL USE NUMERICAL METHOD TO CACULATE THEM INTERNALLY. IF YOU CHOOSE FORMULA CACULATION, YOU NEED TO INPUT THE DERIVATIVE FORMULA IN THE SUBVI TARGET FNC & DERIV NONLIN. FORMULA CACULATION IS MORE PRECISE THAN NUMERICAL CACULATION.

OUTPUTS:
BEST GUESS COEFFICIENTS: THE LEAST SQUARES COEFFICIENTS.
BEST FIT: THE BEST FIT CURVE F(X,A).
COVARIANCE: COVARIANCE MATRIX OF BEST GUESS COEFFICIENTS.
MSE: MSE=1/N*SUM[(Y(I)-F(X(I),A))/STD(I)]^2)
(STD: STANDARD DEVIATION)

FIT SUM OF GAUSSIANS.VI
LAST MODIFIED ON 5/16/97 AT 12:24 AM
PRINTED ON 6/3/97 AT 11:11 AM

FRONT PANEL



CONTROLS AND INDICATORS

[DBL] X
X IS THE ARRAY OF DATA POINTS REPRESENTING THE INDEPENDENT VARIABLE X.

[DBL] X IS THE INPUT ARRAY OF DATA POINTS REPRESENTING THE DEPENDENT VARIABLE X.

[DBL] Y
Y IS THE ARRAY OF DATA POINTS REPRESENTING THE OBSERVED VALUES OF THE DEPENDENT VARIABLE Y.

[DBL]

[DBL] INITIAL GUESS COEFFICIENTS
INITIAL GUESS COEFFICIENTS IS YOUR INITIAL GUESS OF THE SOLUTION COEFFICIENTS.

[DBL]

FIT SUM OF GAUSSIANS.VI
LAST MODIFIED ON 5/16/97 AT 12:24 AM
PRINTED ON 6/3/97 AT 11:11 AM

I32 MAX ITERATION
MAX ITERATION IS THE MAXIMUM EXECUTING ITERATIONS USED IN THE NONLINEAR FIT ALGORITHM.

IF MAX ITERATIONS IS EXCEEDED AND A SOLUTION IS NOT FOUND, THIS VI WILL RETURN AN ERROR. AT THAT POINT, YOU MUST INCREASE MAX ITERATIONS, ADJUST THE INITIAL GUESS COEFFICIENTS, OR CHANGE THE TARGET FUNCTION.

DERIVATIVE
DERIVATIVE IS USED TO SPECIFY THE METHOD USED TO CALCULATE THE JACOBIAN.

0: NUMERICAL APPROXIMATION
1: FORMULA EVALUATION - YOU MUST SPECIFY THE FORMULA FOR THE JACOBIAN IN THE TARGET FNC & DERIV NONLIN VI DIAGRAM.

[DBL] STANDARD DEVIATION
STANDARD DEVIATION IS THE ARRAY OF STANDARD DEVIATIONS, SIGMA[i], FOR DATA POINT (X[i],Y[i]).

IF THE STANDARD DEVIATIONS ARE ALL EQUAL OR IF THEY ARE UNKNOWN, LEAVE THIS CONTROL UNWIRED AND THE STANDARD DEVIATIONS WILL BE SET TO 1.0.

DBL

[DBL] BEST FIT COEFFICIENTS
BEST FIT COEFFICIENTS IS THE ARRAY OF COEFFICIENTS THAT MINIMIZE THE CHI-SQUARE ERROR BETWEEN THE SOLUTION VECTOR AND THE OBSERVED Y VALUES.

DBL

[DBL] BEST FIT
BEST FIT IS THE FITTED DATA z[i] = f(x[i],A), WHERE A IS THE SET OF BEST FIT COEFFICIENTS.

DBL

DBL MSE
MSE IS THE CHI-SQUARED ERROR BETWEEN THE BEST FIT AND THE OBSERVED Y VALUES.

I32 ERROR
YOU CAN WIRE THIS OUTPUT TO THE FIND FIRST ERROR VI TO PRODUCE AN ERROR CLUSTER. THIS CLUSTER CAN THEN BE WIRED TO THE SIMPLE ERROR HANDLER VI OR THE GENERAL ERROR HANDLER VI FOR AN IMMEDIATE REPORT ON ANY ERRORS. YOU CAN ALSO LOOK UP ERROR CODES IN THE ERROR CODES APPENDIX IN THE LABVIEW CROSS REFERENCE MANUAL.

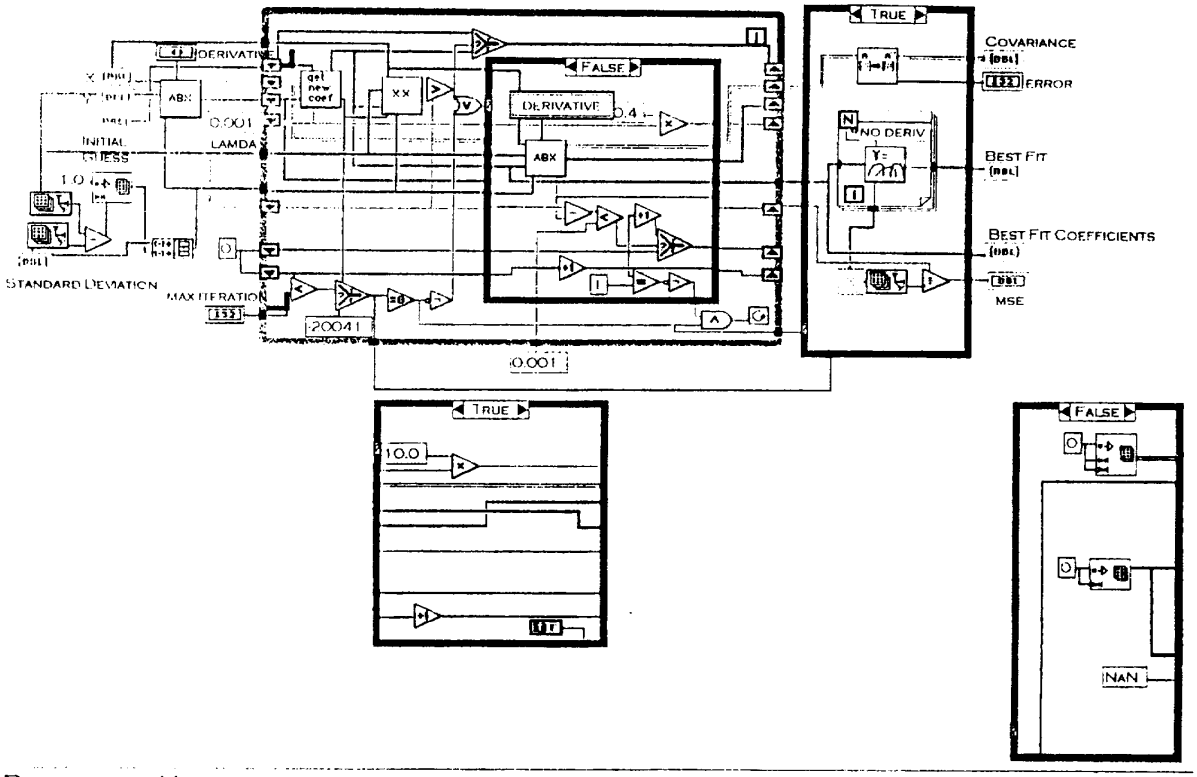
FIT SUM OF GAUSSIANS.VI
LAST MODIFIED ON 5/16/97 AT 12:24 AM
PRINTED ON 6/3/97 AT 11:11 AM

[DBL] COVARIANCE
COVARIANCE IS THE MATRIX OF COVARIANCES C.

C[J,K] IS THE COVARIANCE BETWEEN A[J] AND A[K], AND C[]J,J] IS THE VARIANCE OF A[J].

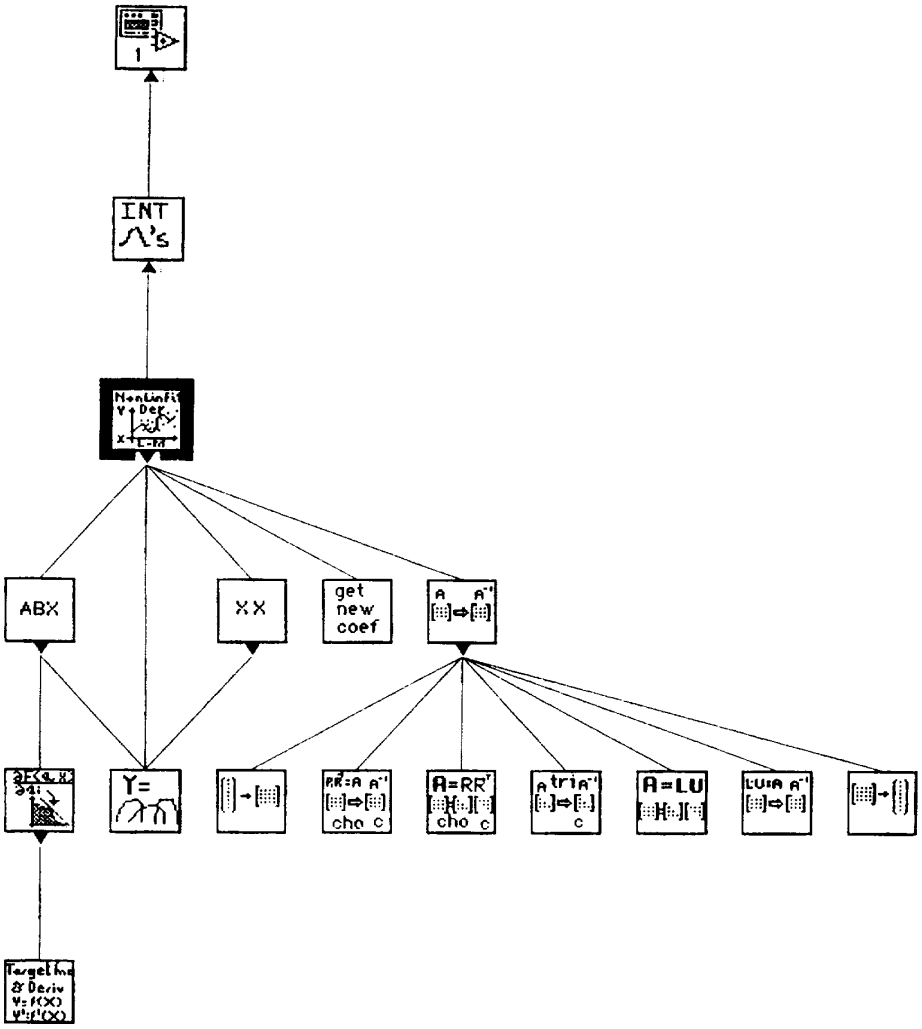
DBL

BLOCK DIAGRAM



POSITION IN HIERARCHY

FIT SUM OF GAUSSIANS.VI
LAST MODIFIED ON 5/16/97 AT 12:24 AM
PRINTED ON 6/3/97 AT 11:11 AM



LIST OF SUBVIS

INVERSE MATRIX.VI
C:\LABVIEW\VI.LIB\ANALYSIS\7LINALG.LLB\INVERSE MATRIX.VI

GET NEW COEFFICIENTS.VI
C:\LABVIEW\VI.LIB\ANALYSIS\6FITS.LLB\GET NEW COEFFICIENTS.VI

FIT SUM OF GAUSSIANS.VI
LAST MODIFIED ON 5/16/97 AT 12:24 AM
PRINTED ON 6/3/97 AT 11:11 AM



GAUSS LEV-MAR ABX.VI
C:\DATA\GAUSS LEV-MAR ABX.VI



GAUSS LEV-MAR XX.VI
C:\DATA\GAUSS LEV-MAR XX.VI



SUM OF GAUSIANS.VI
C:\DATA\SUM OF GAUSIANS.VI

HISTORY

"FIT SUM OF GAUSSIANS.VI HISTORY"
CURRENT REVISION: 24
REV. 8 SUN, SEP 25, 1994 1:48:18 PM GREGGF
VI WAS CONVERTED FROM LABVIEW VERSION 3.1B1 TO LABVIEW VERSION 3.1.

SEQUENCE LISTING

(1) **GENERAL INFORMATION:**

(i) **APPLICANT:** Wittwer, Carl T.
Ririe, Kirk M.
Rasmussen, Randy P.

(ii) **TITLE OF INVENTION:** Monitoring Hybridization During PCT

(iii) **NUMBER OF SEQUENCES:** 27

(iv) **CORRESPONDENCE ADDRESS:**
(A) **ADDRESSEE:** Thorpe, North & Western, L.L.P.
(B) **STREET:** 9035 South 700 East, Suite 200
(C) **CITY:** Sandy
(D) **STATE:** Utah
(E) **COUNTRY:** USA
(F) **ZIP:** 84070

(v) **COMPUTER READABLE FORM:**
(A) **MEDIUM TYPE:** Diskette, 3.5 inch, 1.44 Mb storage
(B) **COMPUTER:** Toshiba T2150CDS
(C) **OPERATING SYSTEM:** Windows 95
(D) **SOFTWARE:** Word Perfect 7.0

(vi) **CURRENT APPLICATION DATA:**
(A) **APPLICATION NUMBER:**
(B) **FILING DATE:**
(C) **CLASSIFICATION:**

(vii) **PRIOR APPLICATION DATA:**
(A) **APPLICATION NUMBER:** US 08/658,993
(B) **FILING DATE:** 04-JUN-96

(vii) **PRIOR APPLICATION DATA:**
(A) **APPLICATION NUMBER:** US 08/818,267
(B) **FILING DATE:** 17-MAR-97

(viii) **ATTORNEY/AGENT INFORMATION:**
(A) **NAME:** Alan J. Howarth
(B) **REGISTRATION NUMBER:** 36,553
(C) **REFERENCE/DOCKET NUMBER:** 8616.CIP7

(ix) **TELECOMMUNICATION INFORMATION:**
(A) **TELEPHONE:** (801)566-6633
(B) **TELEFAX:** (801)566-0750

(2) **INFORMATION FOR SEQ ID NO:1:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 20 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:1:

CGTGGTGGAC TTCTCTCAAT 20

(2) **INFORMATION FOR SEQ ID NO:2:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 20 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:2:

AGAAGATGAG GCATAGCAGC 20

(2) **INFORMATION FOR SEQ ID NO:3:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 35 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:3:

CAAACAGACA CCATGGTGCA CCTGACTCCT GAGGA 35

(2) **INFORMATION FOR SEQ ID NO:4:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 30 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:4:

AAGTCTGCCG TTACTGCCCT GTGGGGCAAG 30

(2) **INFORMATION FOR SEQ ID NO:5:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 27 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:5:

TCTGCCGTTA CTGCCCTGTG GGGCAAG 27

(2) **INFORMATION FOR SEQ ID NO:6:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 20 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:6:

CAACTTCATC CACGTNCACC 20

(2) **INFORMATION FOR SEQ ID NO:7:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 18 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:7:

CTGTCCGTGA CGTGGATT 18

(2) **INFORMATION FOR SEQ ID NO:8:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 18 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:8:

AAGTCCTCCG AGTATAGC 18

(2) **INFORMATION FOR SEQ ID NO:9:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 46 base pairs
(B) **TYPE:** mucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:9:

TAATCTGTAA GAGCAGATCC CTGGACAGGC GAGGAATACA GGTATT 46

(2) **INFORMATION FOR SEQ ID NO:10:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 46 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:10:

TAATCTGTAA GAGCAGATCC CTGGACAGGC AAGGAATACA GGTATT 46

(2) **INFORMATION FOR SEQ ID NO:11:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 20 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:11:

IPR2024-01451
California Institute of Technology - Ex. 2003 Page 3969

TAATCTGTAA GAGCAGATCC 20

(2) **INFORMATION FOR SEQ ID NO:12:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 20 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:12:

TGTTATCACA CTGGTGCTAA 20

(2) **INFORMATION FOR SEQ ID NO:13:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 23 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:13:

AATACCTGTA TTCCTCGCCT GTC 23

(2) **INFORMATION FOR SEQ ID NO:14:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 20 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:14:

ATGCCTGGCA CCATTAAAGA 20

(2) **INFORMATION FOR SEQ ID NO:15:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 20 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:15:

GCATGCTTTG ATGACGCTTC 20

(2) **INFORMATION FOR SEQ ID NO:16:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 20 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

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(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:16:

CGGATCTTCT GCTGCCGTCG 20

(2) **INFORMATION FOR SEQ ID NO:17:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 20 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:17:

CCTCTGACGT CCATCATCTC 20

(2) **INFORMATION FOR SEQ ID NO:18:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 31 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:18:

GAAGTCTGCC GTTACTGCCC TGTGGGGCAA G 31

(2) **INFORMATION FOR SEQ ID NO:19:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 26 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:19:

CTGCCGTACT GCCCTGTGGG GCAAGG 26

(2) **INFORMATION FOR SEQ ID NO:20:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 26 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:20:

ATGCCCTCCC CCATGCCATC CTGCGT 26

(2) **INFORMATION FOR SEQ ID NO:21:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 20 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded

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(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:21:

CAACTTCATC CACGTTCACC 20

(2) **INFORMATION FOR SEQ ID NO:22:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 27 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:22:

GTCTGCCGTT ACTGCCCTGT GGGGCAA 27

(2) **INFORMATION FOR SEQ ID NO:23:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 32 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:23:

CCTCAAACAG ACACCATGGT GCACCTGACT CC 32

(2) **INFORMATION FOR SEQ ID NO:24:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 30 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:24:

GAAGTCTGCC GTTACTGCCC TGTGGGGCAA 30

(2) **INFORMATION FOR SEQ ID NO:25:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 23 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:25:

TGAAGGAGAA GGTGTCTGCG GGA 23

(2) **INFORMATION FOR SEQ ID NO:26:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 25 base pairs
(B) **TYPE:** nucleic acid

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(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:26:

CCTCGGCTAA ATAGTAGTGC GTCGA 25

(2) **INFORMATION FOR SEQ ID NO:27:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 20 base pairs
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single-stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION:** SEQ ID NO:27:

AGGACGGTGC GGTGAGAGTG 20

CLAIMS

We claim:

1. A method for analyzing a target DNA sequence of a biological sample, said method comprising the steps of

amplifying the target sequence by polymerase chain reaction in the presence of two nucleic acid probes that hybridize to adjacent regions of the target sequence, one of said probes being labeled with an acceptor fluorophore and the other probe labeled with a donor fluorophore of a fluorescence energy transfer pair such that upon hybridization of the two probes with the target sequence, the donor and acceptor fluorophores are within 25 nucleotides of one another, said polymerase chain reaction comprising the steps of adding a thermostable polymerase and primers for the targeted nucleic acid sequence to the biological sample and thermally cycling the biological sample between at least a denaturation temperature and an elongation temperature;

exciting the biological sample with light at a wavelength absorbed by the donor fluorophore and detecting the emission from the fluorescence energy transfer pair.

2. A method for analyzing a target DNA sequence of a biological sample, said method comprising the steps of

amplifying the target sequence by polymerase chain reaction in the presence of two nucleic acid probes that hybridize to adjacent regions of the target sequence, one of said probes being labeled with an acceptor fluorophore and the other probe labeled with a donor fluorophore of a fluorescence energy transfer pair such that upon hybridization of the two probes with the target sequence, the donor and acceptor fluorophores are within 25 nucleotides of one another, said polymerase chain reaction comprising the steps of adding a thermostable polymerase and primers for the targeted nucleic acid sequence to the biological sample and thermally cycling the biological sample between at least a denaturation temperature and an elongation temperature;

exciting the sample with light at a wavelength absorbed by the donor fluorophore; and

monitoring the temperature dependent fluorescence from the fluorescence energy transfer pair.

3. The method of claims 1 or 2 wherein the donor and acceptor fluorophores are at a distance of about 0-5 nucleotides.

4. The method of claim 3 wherein the donor and acceptor fluorophores are at a distance of about 0-2 nucleotides.

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5. The method of claim 4 wherein the donor and acceptor fluorophores are at a distance of 1 nucleotide.

6. A method of real time monitoring of a polymerase chain reaction amplification of a target nucleic acid sequence in a biological sample, said method comprising the steps of

(a) adding to the biological sample an effective amount of two nucleic acid primers and a nucleic acid probe, wherein one of said primers and the probe are each labeled with one member of a fluorescence energy transfer pair comprising an acceptor fluorophore and a donor fluorophore, and wherein the labeled probe hybridizes to an amplified copy of the target nucleic acid sequence within 15 nucleotides of the labeled primer;

(b) amplifying the target nucleic acid sequence by polymerase chain reaction;

(c) illuminating the biological sample with light of a selected wavelength that is absorbed by said donor fluorophore; and

(d) detecting the fluorescence emission of the sample.

7. The method of claim 6 further comprising the step of monitoring the temperature dependent fluorescence of the sample.

8. The method of claims 6 wherein the donor and acceptor fluorophores are at a distance of about 4-6 nucleotides from one another.

9. An improved method of amplifying a target nucleic acid sequence of a biological sample, said method comprising the steps of

(a) adding to the biological sample an effective amount of a nucleic-acid-binding fluorescent entity;

(b) amplifying the target nucleic acid sequence using polymerase chain reaction, comprising thermally cycling the biological sample using initial predetermined temperature and time parameters, and then

(i) illuminating the biological sample with a selected wavelength of light that is absorbed by said fluorescent entity during the polymerase chain reaction;

(ii) monitoring fluorescence from said sample to determine the optimal temperature and time parameters for the polymerase chain reaction; and

(iii) adjusting the initial temperature and time parameters in accordance with the fluorescence.

10. The method of claim 9 wherein the fluorescent entity comprises a double strand specific nucleic acid binding dye.

11. The method of claim 9 wherein the fluorescent entity comprises a fluorescently labeled oligonucleotide probe that hybridizes to the targeted nucleic acid sequence.

12. The method of claim 9 wherein the fluorescent entity comprises a pair of oligonucleotide probes, one of said probes being labeled with an acceptor fluorophore and the other probe labeled with a donor fluorophore of a fluorescence energy transfer pair.

13. The method of claim 12 wherein the donor fluorophore emission and the acceptor fluorophore absorption spectrum overlap less than 25%, and the acceptor fluorophore has a peak extinction coefficient greater than 100,000 $M^{-1}cm^{-1}$ and upon hybridization of the two probes, the donor and acceptor fluorophores are within 15 nucleotides of one another.

14. The method of claim 13 wherein the resonance energy transfer pair comprises fluorescein as the donor and Cy5 or Cy5.5 as the acceptor.

15. A method for detecting a target nucleic acid sequence of a biological sample, said method comprising the steps of

(a) adding to the biological sample an effective amount of a pair of oligonucleotide probes that hybridize to the target nucleic acid sequence, one of said probes being labeled with an acceptor fluorophore and the other probe labeled with a donor fluorophore of a fluorescence energy transfer pair, wherein an emission spectrum of the donor fluorophore and an absorption spectrum of the acceptor fluorophore overlap less than 25%, the acceptor fluorophore has a peak extinction coefficient greater than 100,000 $M^{-1}cm^{-1}$ and upon hybridization of the two probes, the donor and acceptor fluorophores are within 25 nucleotides of one another;

(b) illuminating the biological sample with a selected wavelength of light that is absorbed by said donor fluorophore; and

(c) detecting the emission of the biological sample.

16. The method of claim 15 wherein the resonance energy transfer pair comprises fluorescein as the donor.

17. The method of claim 16 wherein the resonance energy transfer pair comprises Cy5 or Cy5.5 as the acceptor.

18. The method of claim 15 wherein the donor and acceptor fluorophores are within about 0-5 nucleotides of one another upon hybridization of the two probes with the target nucleic acid sequence.

19. The method of claim 18 wherein the donor and acceptor fluorophores are within about 0-2 nucleotides of each other.

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20. The method of claim 19 wherein the donor and acceptor fluorophores are within 1 nucleotide of each other.

21. A fluorescence energy transfer pair comprising a first probe labeled with fluorescein and a second probe labeled with Cy5 or Cy5.5.

22. A method of real time monitoring of a polymerase chain reaction amplification of a target nucleic acid sequence in a biological sample, said method comprising the steps of

amplifying the target sequence by polymerase chain reaction in the presence of two nucleic acid probes that hybridize to adjacent regions of the target sequence, one of said probes being labeled with an acceptor fluorophore and the other probe labeled with a donor fluorophore of a fluorescence energy transfer pair such that upon hybridization of the two probes with the target sequence, the donor and acceptor fluorophores are within 25 nucleotides of one another, said polymerase chain reaction comprising the steps of adding a thermostable polymerase and primers for the targeted nucleic acid sequence to the biological sample and thermally cycling the biological sample between at least a denaturation temperature and an elongation temperature;

exciting the biological sample with light at a wavelength absorbed by the donor fluorophore and detecting the emission from the biological sample; and

monitoring the temperature dependent fluorescence from the fluorescence energy transfer pair.

23. The method of claim 22 wherein the resonance energy transfer pair comprises fluorescein as the donor and Cy5 or Cy5.5 as the acceptor.

24. The method of claim 22 wherein the donor and acceptor fluorophores are within about 0-5 nucleotides of one another.

25. The method of claim 24 wherein the donor and acceptor fluorophores are within about 0-2 nucleotides of one another.

26. The method of claim 25 wherein the donor and acceptor fluorophores are within 1 nucleotide of each other.

27. The method of claim 22 wherein said monitoring step comprises determining melting profiles of the probes melting from said target sequence.

28. A method of real time monitoring of a polymerase chain reaction amplification of a target nucleic acid sequence in a biological sample, said method comprising the steps of

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amplifying the target sequence by polymerase chain reaction in the presence of SYBR™ Green I, said polymerase chain reaction comprising the steps of adding a thermostable polymerase and primers for the targeted nucleic acid sequence to the biological sample and thermally cycling the biological sample between at least a denaturation temperature and an elongation temperature;

exciting the biological sample with light at a wavelength absorbed by the SYBR™ Green I and detecting the emission from the biological sample; and

monitoring the temperature dependent fluorescence from the SYBR™ Green I.

29. The method of claim 28 wherein said monitoring step comprises determining a melting profile of the amplified target sequence.

30. A method for analyzing a target DNA sequence of a biological sample, said method comprising the steps of

(a) adding to the biological sample an effective amount of two nucleic acid primers and a nucleic acid probe, wherein one of said primers and the probe are each labeled with one member of a fluorescence energy transfer pair comprising an acceptor fluorophore and a donor fluorophore, and wherein the labeled probe hybridizes to an amplified copy of the target nucleic acid sequence within 15 nucleotides of the labeled primer;

(b) amplifying the target nucleic acid sequence by polymerase chain reaction;

(c) illuminating the biological sample with light of a selected wavelength that is absorbed by said donor fluorophore and detecting the fluorescence emission of the sample.

31. The method of claim 30 further comprising the step of monitoring the temperature dependent fluorescence of the sample.

32. The method of claim 31 wherein said monitoring step comprises determining a melting profile of the amplified target sequence.

33. The method of claim 30 wherein the resonance energy transfer pair comprises fluorescein as the donor and Cy5 or Cy5.5 as the acceptor.

34. The method of claim 30 wherein the donor and acceptor fluorophores are at a distance of about 4-6 nucleotides from one another.

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35. A method of detecting a difference at a selected locus in a first nucleic acid as compared to a second nucleic acid, comprising the steps of:

(a) providing a pair of oligonucleotide primers configured for amplifying, by polymerase chain reaction, a selected segment of the first nucleic acid and a corresponding segment of the second nucleic acid, wherein the selected segment and corresponding segment each comprises the selected locus, to result in amplified products containing a copy of the selected locus;

(b) providing a pair of oligonucleotide probes, one of said probes being labeled with an acceptor fluorophore and the other probe being labeled with a donor fluorophore of a fluorogenic resonance energy transfer pair such that upon hybridization of the two probes with the amplified products the donor and acceptor are in resonance energy transfer relationship, wherein one of the probes is configured for hybridizing to the amplified products such that said one of the probes spans the selected locus and exhibits a melting profile when the difference is present in the first nucleic acid that is distinguishable from a melting profile of the second nucleic acid;

(c) amplifying the selected segment of first nucleic acid and the corresponding segment of the second nucleic acid by polymerase chain reaction in the presence of effective amounts of probes to result in an amplified selected segment and an amplified corresponding segment, at least a portion thereof having both the probes hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship;

(d) illuminating the amplified selected segment and the amplified corresponding segment with the probes hybridized thereto with a selected wavelength of light to elicit fluorescence by the fluorogenic resonance energy transfer pair;

(e) measuring fluorescence emission as a function of temperature to determine in a first melting profile of said one of the probes melting from the amplified selected segment of first nucleic acid and a second melting profile of said one of the probes melting from the amplified corresponding segment of second nucleic acid; and

(f) comparing the first melting profile to the second melting profile, wherein a difference therein indicates the presence of the difference in the sample nucleic acid.

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36. The method of claim 35 wherein the resonance energy transfer pair comprises fluorescein as the donor and a member selected from the group consisting of Cy5 and Cy5.5 as the acceptor.

37. The method of claim 35 wherein the donor and the acceptor are coupled to the probes such that when both probes are hybridized to the amplified selected segment or the amplified corresponding segment, the donor and acceptor are separated by no more than about 25 nucleotide residues.

38. The method of claim 37 wherein the donor and the acceptor are coupled to probes such that when the probes are hybridized to the amplified selected segment or the amplified corresponding segment, the donor and acceptor are separated by about 0-5 nucleotide residues.

39. The method of claim 38 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to the probes such that when the probes are hybridized to the amplified selected segment or the amplified corresponding segment the donor and acceptor are separated by about 0-2 nucleotide residues.

40. The method of claim 39 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to the probes such that when the probes are hybridized to the amplified selected segment or the amplified corresponding segment the donor and acceptor are separated by 1 nucleotide residue.

41. A method of detecting a difference at a selected locus in a first nucleic acid as compared to a second nucleic acid, comprising the steps of:

(a) providing a pair of oligonucleotide primers configured for amplifying, by polymerase chain reaction, a selected segment of the first nucleic acid and a corresponding segment of the second nucleic acid, wherein the selected segment and corresponding segment each comprises the selected locus, to result in amplified products containing a copy of the selected locus;

(b) providing an oligonucleotide probe, wherein one of said primers and the probe are each labeled with one member of a fluorescence energy transfer pair comprising an donor fluorophore and an acceptor fluorophore, and wherein the labeled probe and labeled primer hybridize to the amplified products such that the donor and acceptor are in resonance energy transfer relationship, and wherein the probe is configured for hybridizing to the amplified products such that said probe spans the selected locus and exhibits a melting

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profile when the difference is present in the first nucleic acid that is distinguishable from a melting profile of the second nucleic acid;

(c) amplifying the selected segment of first nucleic acid and the corresponding segment of the second nucleic acid by polymerase chain reaction in the presence of effective amounts of primers and probe to result in an amplified selected segment and an amplified corresponding segment, at least a portion thereof having the labled primer and probe hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship;

(d) illuminating the amplified selected segment and the amplified corresponding segment with the labeled primer and probe hybridized thereto with a selected wavelength of light to elicit fluorescence by the fluorogenic resonance energy transfer pair;

(e) measuring fluorescence emission as a function of temperature to determine in a first melting profile of said probe melting from the amplified selected segment of first nucleic acid and a second melting profile of said probe melting from the amplified corresponding segment of second nucleic acid; and

(f) comparing the first melting profile to the second melting profile, wherein a difference therein indicates the presence of the difference in the sample nucleic acid.

42. The method of claim 41 wherein the resonance energy transfer pair comprises fluorescein as the donor and a member selected from the group consisting of Cy5 and Cy5.5 as the acceptor.

43. The method of claim 41 wherein the donor and the acceptor are coupled to the labeled primer and probe such that when both the labeled primer and probe are hybridized to the amplified selected segment or the amplified corresponding segment, the donor and acceptor are separated by no more than about 15 nucleotide residues.

44. The method of claim 43 wherein the donor and the acceptor are coupled to labeled primer and probe such that when the labeled primer and probe are hybridized to the amplified selected segment or the amplified corresponding segment, the donor and acceptor are separated by about 4-6 nucleotide residues.

45. A method of detecting heterozygosity at a selected locus in the genome of an individual, wherein the genome comprises a mutant allele and a corresponding reference allele, each comprising the selected locus, comprising the steps of:

(a) obtaining sample genomic DNA from the individual;

(b) providing a pair of oligonucleotide primers configured for amplifying, by polymerase chain reaction, a first selected

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segment of the mutant allele and a second selected segment of the corresponding reference allele wherein both the first and second selected segments comprise the selected locus;

(c) providing a pair of oligonucleotide probes, one of said probes being labeled with an acceptor fluorophore and the other probe being labeled with a donor fluorophore of a fluorogenic resonance energy transfer pair such that upon hybridization of the two probes with the amplified first and second selected segments one of the probes spans the selected locus and exhibits a first melting profile with the amplified first selected segment that is distinguishable from a second melting profile with the amplified second selected segment;

(d) amplifying the first and second selected segments of sample genomic DNA by polymerase chain reaction in the presence of effective amounts of probes to result in amplified first and second selected segments, at least a portion thereof having both the probes hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship;

(e) illuminating the amplified first and second selected segments having the probes hybridized thereto with a selected wavelength of light to elicit fluorescence by the donor and acceptor;

(f) measuring a fluorescence emission as a function of temperature to determine a first melting profile of said one of the probes melting from the amplified first selected segment and a second melting profile of said one of the probes melting from the amplified second selected segment; and

(g) comparing the first melting profile to the second melting profile, wherein distinguishable melting profiles indicate heterozygosity in the sample genomic DNA.

46. The method of claim 45 wherein the fluorogenic resonance energy transfer pair comprises fluorescein as the donor and Cy5 or Cy5.5 as the acceptor.

47. The method of claim 45 wherein the donor and the acceptor are coupled to the probes such that when the probe and reference oligonucleotide are hybridized to the amplified first selected segment or the amplified second selected segment, the donor and acceptor are separated by no more than about 25 nucleotide residues.

48. The method of claim 47 wherein the donor and the acceptor are coupled to the probes such that when the probes are hybridized to the amplified first selected segment or the amplified second

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selected segment, the donor and acceptor are separated by about 0-5 nucleotide residues.

49. The method of claim 48 wherein the donor and the acceptor are coupled to the probes such that when the probes are hybridized to the amplified first selected segment or the amplified second selected segment, the donor and acceptor are separated by about 0-2 nucleotide residues.

50. The method of claim 49 wherein the donor and the acceptor are coupled to the probes such that when the probes are hybridized to the amplified first selected segment or the amplified second selected segment, the donor and acceptor are separated by 1 nucleotide residue.

51. A method of detecting heterozygosity at a selected locus in the genome of an individual, wherein the genome comprises a mutant allele and a corresponding reference allele, each comprising the selected locus, comprising the steps of:

(a) obtaining sample genomic DNA from the individual;

(b) providing a pair of oligonucleotide primers configured for amplifying, by polymerase chain reaction, a first selected segment of the mutant allele and a second selected segment of the corresponding reference allele wherein both the first and second selected segments comprise the selected locus;

(c) providing an oligonucleotide probe, wherein one of said primers and the probe are each labeled with one member of a fluorescence energy transfer pair comprising an donor fluorophore and an acceptor fluorophore, and wherein the labeled probe and labeled primer hybridize to the amplified first and second selected segments such that one of the probes spans the selected locus and exhibits a first melting profile with the amplified first selected segment that is distinguishable from a second melting profile with the amplified second selected segment;

(d) amplifying the first and second selected segments of sample genomic DNA by polymerase chain reaction in the presence of effective amounts of primers and probe to result in amplified first and second selected segments, at least a portion thereof having both the labeled primer and probe hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship;

(e) illuminating the amplified first and second selected segments having the labeled primer and probe hybridized thereto with

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a selected wavelength of light to elicit fluorescence by the donor and acceptor;

(f) measuring a fluorescence emission as a function of temperature to determine a first melting profile of said probe melting from the amplified first selected segment and a second melting profile of said probe melting from the amplified second selected segment; and

(g) comparing the first melting profile to the second melting profile, wherein distinguishable melting profiles indicate heterozygosity in the sample genomic DNA.

52. The method of claim 51 wherein the resonance energy transfer pair comprises fluorescein as the donor and Cy5 or Cy5.5 as the acceptor.

53. The method of claim 51 wherein the donor and the acceptor are coupled to the labeled primer and probe such that when the labeled primer and probe are hybridized to the amplified first selected segment or the amplified second selected segment, the donor and acceptor are separated by no more than about 15 nucleotide residues.

54. The method of claim 53 wherein the donor and the acceptor are coupled to the labeled primer and probe such that when the labeled primer and probe are hybridized to the amplified first selected segment or the amplified second selected segment, the donor and acceptor are separated by about 4-6 nucleotide residues.

55. A method of determining completion of a polymerase chain reaction in a polymerase chain reaction mixture comprising (1) a nucleic acid wherein the nucleic acid or a polymerase-chain-reaction-amplified product thereof consists of two distinct complementary strands, (2) two oligonucleotide primers configured for amplifying by polymerase chain reaction a selected segment of the nucleic acid to result in an amplified product, and (3) a DNA polymerase for catalyzing the polymerase chain reaction, comprising the steps of:

(a) adding to the mixture (1) an effective amount of an oligonucleotide probe labeled with a resonance energy transfer donor or a resonance energy transfer acceptor of a fluorogenic resonance energy transfer pair, wherein the probe is configured for hybridizing to the amplified product under selected conditions of temperature and monovalent ionic strength, and (2) an effective amount of a reference oligonucleotide labeled with the donor or the acceptor, with the proviso that as between the probe and reference oligonucleotide one is labeled with the donor and the other is labeled with the acceptor,

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wherein the reference oligonucleotide is configured for hybridizing to the amplified product under the selected conditions of temperature and monovalent ionic strength such that the donor and the acceptor are in resonance energy transfer relationship when both the probe and the reference oligonucleotide hybridize to the amplified product;

(b) amplifying the selected segment of nucleic acid by polymerase chain reaction to result in the amplified product, at least a portion thereof having both the probe and the reference oligonucleotide hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship; and

(c) illuminating the amplified product having the probe and reference oligonucleotide hybridized thereto with a selected wavelength of light for eliciting fluorescence by the fluorogenic resonance energy pair and monitoring fluorescence emission and determining a cycle when the fluorescence emission reaches a plateau phase, indicating the completion of the reaction.

56. The method of claim 55 wherein the fluorogenic resonance energy transfer pair is fluorescein and a member selected from the group consisting of Cy5 and Cy5.5.

57. A method of determining completion of a polymerase chain reaction in a polymerase chain reaction mixture comprising (1) a nucleic acid wherein the nucleic acid or a polymerase-chain-reaction-amplified product thereof consists of two distinct complementary strands, (2) two oligonucleotide primers configured for amplifying by polymerase chain reaction a selected segment of the nucleic acid to result in an amplified product, and (3) a DNA polymerase for catalyzing the polymerase chain reaction, comprising the steps of:

(a) adding to the mixture an effective amount of a nucleic-acid-binding fluorescent dye;

(b) amplifying the selected segment of nucleic acid by polymerase chain reaction in the mixture to which the nucleic-acid-binding fluorescent dye has been added to result in the amplified product with nucleic-acid-binding fluorescent dye bound thereto; and

(c) illuminating amplified product with nucleic-acid-binding fluorescent dye bound thereto with a selected wavelength of light for eliciting fluorescence therefrom and monitoring fluorescence emission and determining a cycle when the fluorescence emission reaches a plateau phase, indicating the completion of the reaction.

58. The method of claim 57 wherein the nucleic-acid-binding fluorescent dye is a member selected from the group consisting of

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SYBR™ GREEN I, ethidium bromide, pico green, acridine orange, thiazole orange, YO-PRO-1, and chromomycin A3.

59. The method of claim 58 wherein the double-strand-specific fluorescent dye is SYBR™ GREEN I.

60. A method of controlling temperature cycling parameters of a polymerase chain reaction comprising repeated cycles of annealing, extension, and denaturation phases of a polymerase chain reaction mixture comprising a double-strand-specific fluorescent dye, wherein the parameters comprise duration of the annealing phase, duration of the denaturation phase, and number of cycles, comprising

(a) illuminating the reaction with a selected wavelength of light for eliciting fluorescence from the fluorescent dye and continuously monitoring fluorescence during the repeated annealing, extension, and denaturation phases;

(b) determining at least

(i) duration for fluorescence to stop increasing during the extension phase, or

(ii) Duration for fluorescence to decrease to a baseline level during the denaturation phase, or

(iii)number of cycles for fluorescence to reach a preselected level during the extension phase; and

(c) adjusting the length of the extension phase according to the length of time for fluorescence to stop increasing during the extension phase, the length of the denaturation phase according to the length of time for fluorescence to decrease to the baseline level during the denaturation phase, or the number of cycles according to the number of cycles for fluorescence to reach the preselected level during the extension phase.

61. The method of claim 60 wherein the double-strand specific fluorescent dye is a member selected from the group consisting of SYBR™ GREEN I, ethidium bromide, pico green, acridine orange, thiazole orange, YO-PRO-1, and chromomycin A3.

62. The method of claim 61 wherein the double-strand-specific fluorescent dye is SYBR™ GREEN I.

63. A method of determining a concentration of an amplified product in a selected polymerase chain reaction mixture comprising

(a) determining a second order rate constant for the amplified product at a selected temperature and reaction conditions by monitoring rate of hybridization of a known concentration of the amplified product;

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(b) determining rate of annealing for an unknown concentration of the amplified product; and

(c) calculating the concentration of the amplified product from the rate of annealing and the second order rate constant.

64. The method of claim 63 wherein the rate of annealing is determined after multiple cycles of amplification.

65. The method of claim 63 wherein determining the second oder rate constant comprises the steps of

raising the temperature of a first polymerase chain reaction mixture comprising a known concentration of the amplified product and an effective amount of a double-strand specific fluorescent dye, above the denaturation temperature of the amplified product to result in a denatured amplified product;

rapidly reducing the temperature of the first polymerase chain reaction mixture comprising the known amount of denatured amplified product to a selected temperature below the denaturation temperature of the amplified product while continuously monitoring the fluorescence of the first polymerase chain reaction mixture as a function of time;

plotting fluorescence as a function of time for determining maximum fluorescence, minimum fluorescence, the time at minimum fluorescence, and a second order rate constant for the known concentration of amplified product from the equation

$$F = F_{max} - \frac{F_{max} - F_{min}}{k(t-t_0)[DNA] + 1}$$

wherein F is fluorescence, F_{max} is maximum fluorescence, F_{min} is minimum fluorescence, k is the second order rate constant, t_0 is the time at F_{min}, and [DNA] is the known concentration of the amplified product.

66. The method of claim 65 wherein the double-strand specific fluorescent dye is a member selected from the group consisting of SYBR™ GREEN I, ethidium bromide, pico green, acridine orange, thiazole orange, YO-PRO-1, and chromomycin A3.

67. The method of claim 66 wherein the double-strand specific fluorescent dye is SYBR™ GREEN I.

68. A method of determining a concentration of a selected nucleic acid template by competitive quantitative polymerase chain reaction comprising the steps of:

(a) in a reaction mixture comprising:

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(i) effective amounts of each of a pair of oligonucleotide primers configured for amplifying, in a polymerase chain reaction, a selected segment of the selected template and a corresponding selected segment of a competitive template to result in amplified products thereof,

(ii) an effective amount of an oligonucleotide probe labeled with a resonance energy transfer donor or a resonance energy transfer acceptor of a fluorogenic resonance energy transfer pair, wherein the probe is configured for hybridizing to the amplified products such that the probe melts from the amplified product of the selected template at a melting temperature that is distinguishable from the melting temperature at which the probe melts from the amplified product of the competitive template,

(iii) an effective amount of a reference oligonucleotide labeled with the donor or the acceptor, with the proviso that as between the probe and transfer oligonucleotide one is labeled with the donor and the other is labeled with the acceptor, wherein the reference oligonucleotide is configured for hybridizing to the amplified products such that the donor and the acceptor are in resonance energy transfer relationship when both the probe and the reference oligonucleotide hybridize to the amplified products;

amplifying, by polymerase chain reaction, an unknown amount of the selected template and a known amount of the competitive template to result in the amplified products thereof;

(b) illuminating the reaction mixture with a selected wavelength of light for eliciting fluorescence by the fluorogenic resonance energy transfer pair and determining a fluorescence emission as a function of temperature as the temperature of the reaction mixture is changed to result in a first melting curve of the probe melting from the amplified product of the selected template and a second melting curve of the probe melting from the competitive template;

(c) converting the first and second melting curves to first and second melting melting peaks and determining relative amounts of the selected template and the competitive template from such melting peaks; and

(d) calculating the concentration of the selected template based on the known amount of the competitive template and the relative amounts of selected template and competitive template.

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69. The method of claim 68 wherein the reference oligonucleotide is one of the pair of oligonucleotide primers.

70. The method of claim 69 wherein the resonance energy transfer pair comprises fluorescein as the donor and Cy5 or Cy5.5 as the acceptor.

71. The method of claim 69 wherein the donor and the acceptor are coupled to the probe and reference oligonucleotide such that when the probe and reference oligonucleotide are hybridized to the amplified segment, the donor and acceptor are separated by no more than about 25 nucleotide residues.

72. The method of claim 71 wherein the donor and the acceptor are coupled to the probe and the reference oligonucleotide such that when the probe and reference oligonucleotide are hybridized to the amplified segment, the donor and acceptor are separated by about 0-5 nucleotide residues.

73. The method of claim 72 wherein the donor and the acceptor are coupled to the probe and reference oligonucleotide such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, the donor and acceptor are separated by about 0-2 nucleotide residues.

74. The method of claim 73 wherein the donor and the acceptor are coupled to the probe and reference oligonucleotide such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, the donor and acceptor are separated by 1 nucleotide residue.

75. The method of claim 68 wherein the reference oligonucleotide is not one of the oligonucleotide primers.

76. The method of claim 75 wherein the resonance energy transfer pair comprises fluorescein as the donor and Cy5 or Cy5.5 as the acceptor.

77. The method of claim 76 wherein the donor and the acceptor are coupled to the probe and the reference oligonucleotide such that when the probe and reference oligonucleotide are hybridized to the amplified segment, the donor and acceptor are separated by no more than about 15 nucleotide residues.

78. The method of claim 77 wherein the donor and the acceptor are coupled to the probe and the reference oligonucleotide such that when the probe and reference oligonucleotide are hybridized to the amplified segment, the donor and acceptor are separated by about 4-6 nucleotide residues.

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79. A fluorogenic resonance energy transfer pair consisting of fluorescein and Cy5.

80. A fluorogenic resonance energy transfer pair consisting of fluorescein and Cy5.5.

81. A method of determining a concentration of a selected nucleic acid template in a polymerase chain reaction comprising the steps of:

(a) in a reaction mixture comprising:

(i) effective amounts of each of a first pair of oligonucleotide primers configured for amplifying, in a polymerase chain reaction, a selected first segment of the selected template to result in an amplified first product thereof,

(ii) effective amounts of each of a second pair of oligonucleotide primers configured for amplifying, in a polymerase chain reaction, a selected second segment of a reference template to result in an amplified second product thereof,

(iii) an effective amount of a nucleic-acid-binding fluorescent dye;

amplifying, by polymerase chain reaction, an unknown amount of the selected template to result in the amplified first product and a known amount of the reference template to result in the amplified second product thereof;

(b) illuminating the reaction mixture with a selected wavelength of light for eliciting fluorescence by the nucleic-acid-binding fluorescent dye and continuously monitoring the fluorescence emitted as a function of temperature to result in a melting curve of the amplified products wherein the first product and second product melt at different temperatures;

(c) converting the melting curves to melting melting peaks and determining relative amounts of the selected template and the reference template from such melting peaks; and

(d) calculating the concentration of the selected template based on the known amount of the reference template and the relative amounts of selected template and reference template.

82. The method of claim 81 wherein the nucleic-acid-binding fluorescent dye is selected from the group consisting of of SYBR™ GREEN I, ethidium bromide, pico green, acridine orange, thiazole orange, YO-PRO-1, and chromomycin A3.

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83. The method of claim 82 wherein the double-strand specific fluorescent dye is SYBR™ GREEN I.

84. A method of monitoring amplification of a selected template in a polymerase chain reaction that also comprises a positive control template comprising the steps of:

(a) in a reaction mixture comprising:

(i) effective amounts of each of a first pair of oligonucleotide primers configured for amplifying, in a polymerase chain reaction, a selected first segment of the selected template to result in an amplified first product thereof,

(ii) effective amounts of each of a second pair of oligonucleotide primers configured for amplifying, in a polymerase chain reaction, a selected second segment of the positive control template to result in an amplified second product thereof,

(iii) an effective amount of a nucleic-acid-binding fluorescent dye;

subjecting the selected template and the positive control template to conditions for amplifying the selected template and the positive control template by polymerase chain reaction; and

(b) illuminating the reaction mixture with a selected wavelength of light for eliciting fluorescence by the nucleic-acid-binding fluorescent dye and continuously monitoring the fluorescence emitted as a function of temperature during an amplification cycle of the polymerase chain reaction to result in a first melting peak of the amplified first product, if the selected template is amplified, and a second melting peak of the amplified second product, if the positive control template is amplified;

wherein obtaining of the second melting curve indicates that the polymerase chain reaction was operative, obtaining the first melting curve indicates that the selected first segment was amplifiable, and absence of the first melting curve indicates that the selected first segment was not amplifiable.

85. The method of claim 84 wherein the nucleic-acid-binding fluorescent dye is selected from the group consisting of of SYBR™ GREEN I, ethidium bromide, pico green, acridine orange, thiazole orange, YO-PRO-1, and chromomycin A3.

86. The method of claim 85 wherein the double-strand specific fluorescent dye is SYBR™ GREEN I.

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87. A method of detecting the factor V Leiden mutation in an individual, wherein the factor V Leiden mutation consists of a single base change at the factor V Leiden mutation locus as compared to wild type, comprising the steps of:

(a) obtaining sample genomic DNA from the individual;

(b) providing wild type genomic DNA as a control;

(c) providing a pair of oligonucleotide primers configured for amplifying by polymerase chain reaction a selected segment of the sample genomic DNA and of the wild type genomic DNA wherein the selected segment comprises the factor V Leiden mutation locus to result in amplified products containing a copy of the factor V Leiden mutation locus;

(d) providing an oligonucleotide probe labeled with a resonance energy transfer donor or a resonance energy transfer acceptor of a fluorogenic resonance energy transfer pair, wherein the probe is configured for hybridizing to the amplified products such that the probe spans the mutation locus and exhibits a melting profile when the factor V Leiden mutation is present in the sample genomic DNA that is differentiable from a melting profile of the wild type genomic DNA;

(e) providing a transfer oligonucleotide labeled with the resonance energy transfer donor or the resonance energy transfer acceptor, with the proviso that as between the probe and transfer oligonucleotide one is labeled with the resonance energy transfer donor and the other is labeled with the resonance energy transfer acceptor, wherein the transfer oligonucleotide is configured for hybridizing to the amplified products such that the resonance energy transfer donor and the resonance energy transfer acceptor are in resonance energy transfer relationship when both the probe and the transfer oligonucleotide hybridize to the amplified products;

(f) amplifying the selected segment of sample genomic DNA and wild type genomic DNA by polymerase chain reaction in the presence of effective amounts of oligonucleotide probe and transfer oligonucleotide to result in amplified selected segments, at least a portion thereof having both the probe and the transfer oligonucleotide hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship;

(g) determining fluorescence as a function of temperature during an amplification cycle of the polymerase chain reaction to result in a melting profile of the probe melting from the amplified

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segment of sample genomic DNA and a melting profile of the probe melting from the amplified segment of wild type genomic DNA; and

(h) comparing the melting profile for the sample genomic DNA to the melting profile for the wild type genomic DNA, wherein a difference therein indicates the presence of the factor V Leiden mutation in the sample genomic DNA.

88. The method of claim 87 wherein the transfer oligonucleotide is one of the pair of oligonucleotide primers.

89. The method of claim 88 wherein the resonance energy transfer pair comprises fluorescein and Cy5 or Cy5.5.

90. The method of claim 89 wherein the oligonucleotide primers are SEQ ID NO:11 and SEQ ID NO:12.

91. The method of claim 90 wherein SEQ ID NO:11 is labeled with Cy5 or Cy5.5.

92. The method of claim 90 wherein the oligonucleotide probe is SEQ ID NO:13.

93. The method of claim 90 wherein SEQ ID NO:13 is labeled with fluorescein.

94. The method of claim 87 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to nucleotide residues, such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, are separated by no more than about 15 nucleotide residues.

95. The method of claim 94 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to nucleotide residues, such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, are separated by about 4-6 nucleotide residues.

96. The method of claim 86 wherein the transfer oligonucleotide is not one of the oligonucleotide primers.

97. The method of claim 96 wherein the resonance energy transfer pair comprises fluorescein and Cy5 or Cy5.5.

98. The method of claim 97 wherein the oligonucleotide primers are SEQ ID NO:11 and SEQ ID NO:12.

99. The method of claim 98 wherein the oligonucleotide probe is SEQ ID NO:13.

100. The method of claim 99 wherein SEQ ID NO:13 is labeled with fluorescein.

101. The method of claim 100 wherein the transfer oligonucleotide is labeled with Cy5 or Cy5.5.

102. The method of claim 101 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to nucleotide residues, such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, are separated by no more than about 25 nucleotide residues.

103. The method of claim 102 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to nucleotide residues, such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, are separated by no more than about 0-5 nucleotide residues.

104. The method of claim 103 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to nucleotide residues, such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, are separated by no more than about 0-2 nucleotide residues.

105. The method of claim 104 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to nucleotide residues, such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, are separated by 1 nucleotide residue.

106. The method of claim 87 wherein the difference in melting profiles represents differences in thermal melting temperature of at least about 2°C.

107. An oligonucleotide having a nucleotide sequence as in SEQ ID NO:11.

108. An oligonucleotide having a nucleotide sequence as in SEQ ID NO:12.

109. An oligonucleotide having a nucleotide sequence as in SEQ ID NO:13.

110. A method of analyzing nucleic acid hybridization comprising the steps of

(a) providing a mixture comprising a nucleic acid sample to be analyzed and a nucleic acid binding fluorescent entity; and

(b) monitoring fluorescence while changing temperature at a rate of $\geq$ 0.1°C/second.

111. The method of claim 110 wherein the nucleic acid binding fluorescent entity is SYBR™ Green I.

112. The method of claim 110 wherein the nucleic acid binding fluorescent entity comprises a pair of oligonucleotide probes wherein one of the probes is labeled with a donor and the other probes is

labeled with an acceptor of a fluorogenic resonance energy transfer pair.

113. The method of claim 112 wherein one of the probes is a primer for polymerase chain reaction amplification.

114. A method of quantitating an initial copy number of a sample containing an unknown amount of nucleic acid comprising the steps of

(a) amplifying by polymerase chain reaction at least one standard of known concentration in a mixture comprising the standard and a nucleic acid binding fluorescent entity;

(b) measuring fluorescence as a function of cycle number to result in a set of data points;

(c) fitting the data points to a given predetermined equation describing fluorescence as a function of initial nucleic acid concentration and cycle number;

(d) amplifying the sample containing the unknown amount of nucleic acid in a mixture comprising the sample and the nucleic acid binding fluorescent entity and monitoring fluorescence thereof; and

(e) determining initial nucleic acid concentration from the equation determined in step (c).

115. The method of claim 114 wherein the nucleic acid binding fluorescent entity is SYBR™ Green I.

116. The method of claim 114 wherein the nucleic acid binding fluorescent entity comprises a pair of oligonucleotide probes, one of which is labeled with a donor and the other of which is labeled with an acceptor of a fluorogenic resonance energy transfer pair.

117. The method of claim 116 wherein one of the probe is a primer for amplification.

118. A fluorescence resonance energy transfer pair comprising a donor fluorophore having an emission spectrum and an acceptor fluorophore having an absorption spectrum and an extinction coefficient greater than 100,000 $M^{-1}cm^{-1}$, wherein the donor fluorophore's emission spectrum and the acceptor fluorophore's absorption spectrum overlap less than 25%

119. The fluorescence resonance energy transfer pair wherein the donor fluorophore is fluorescein and the acceptor fluorophore is Cy5 or Cy5.5.

120. A method for analyzing a target DNA sequence of a biological sample, said method comprising the steps of

amplifying the target sequence by polymerase chain reaction in the presence of a nucleic acid binding fluorescent

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entity, said polymerase chain reaction comprising the steps of adding a thermostable polymerase and primers for the targeted nucleic acid sequence to the biological sample and thermally cycling the biological sample between at least a denaturation temperature and an elongation temperature;

exciting the sample with light at a wavelength absorbed by the nucleic acid binding fluorescent entity; and

monitoring the temperature dependent fluorescence from the nucleic acid binding fluorescent entity as temperature of the sample is changed.

121. The method of claim 120 wherein said nucleic acid binding fluorescent entity comprises a double stranded nucleic acid binding fluorescent dye.

122. The method of claim 121 wherein the double stranded nucleic acid binding fluorescent dye is SYBR™ Green I.

123. The method of claim 122 wherein the temperature dependent fluorescence is used to identify the amplified products.

124. The method of claim 123 wherein the amplified products are identified by analysis of melting curves.

125. The method of claim 124 wherein the relative amounts fo two or more amplified products are determined by analysis of melting curves.

126. The method of claim 124 wherein areas under the melting curves are found by non-linear least squares regressionof the sum of multiple gaussians.

Figure 1.

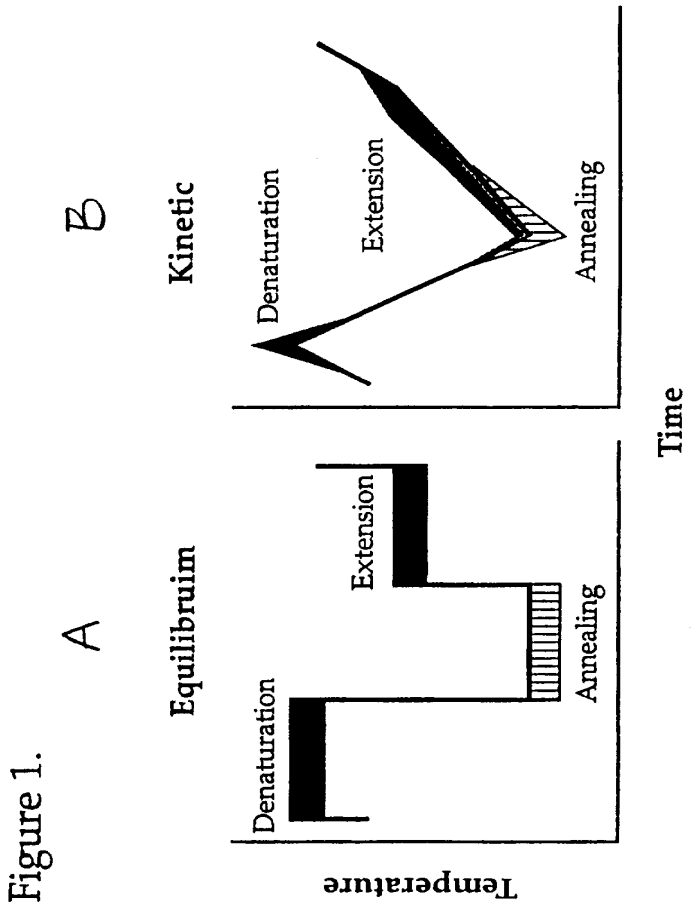
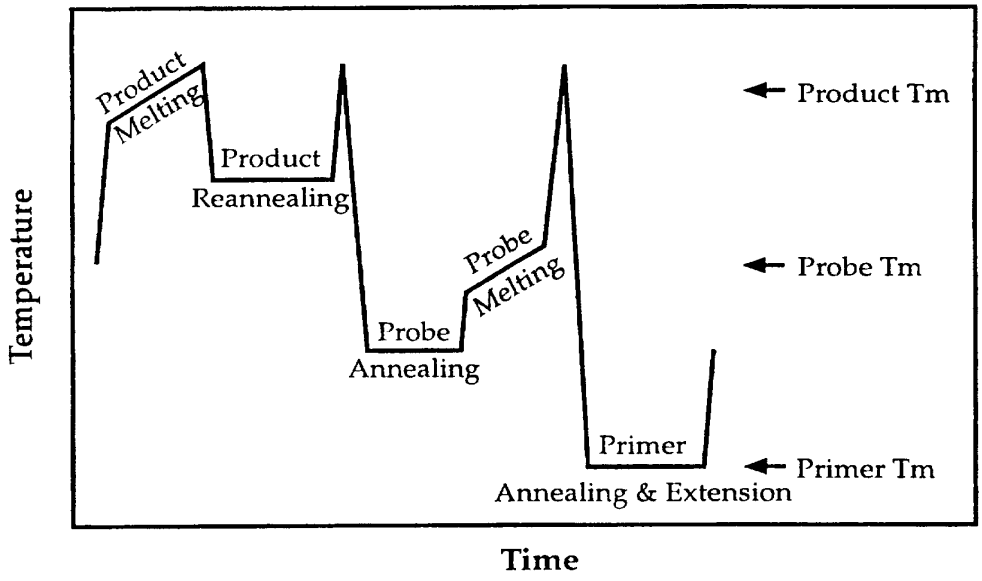

A
Equilibrium
Denaturation
Extension
Annealing
B
Kinetic
Denaturation
Extension
Annealing
Temperature
Time

Figure 2.



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FIGURE 3.

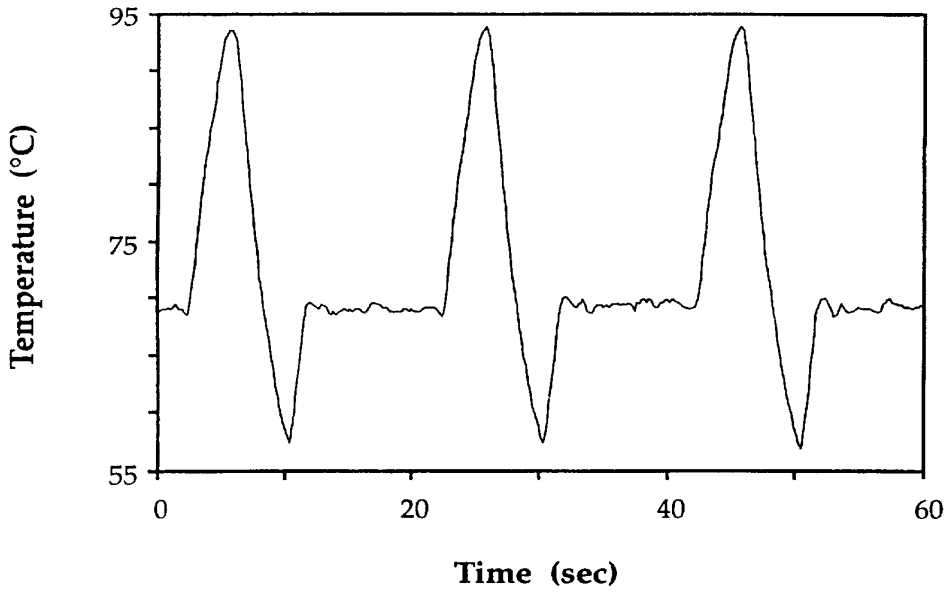


Figure 4.

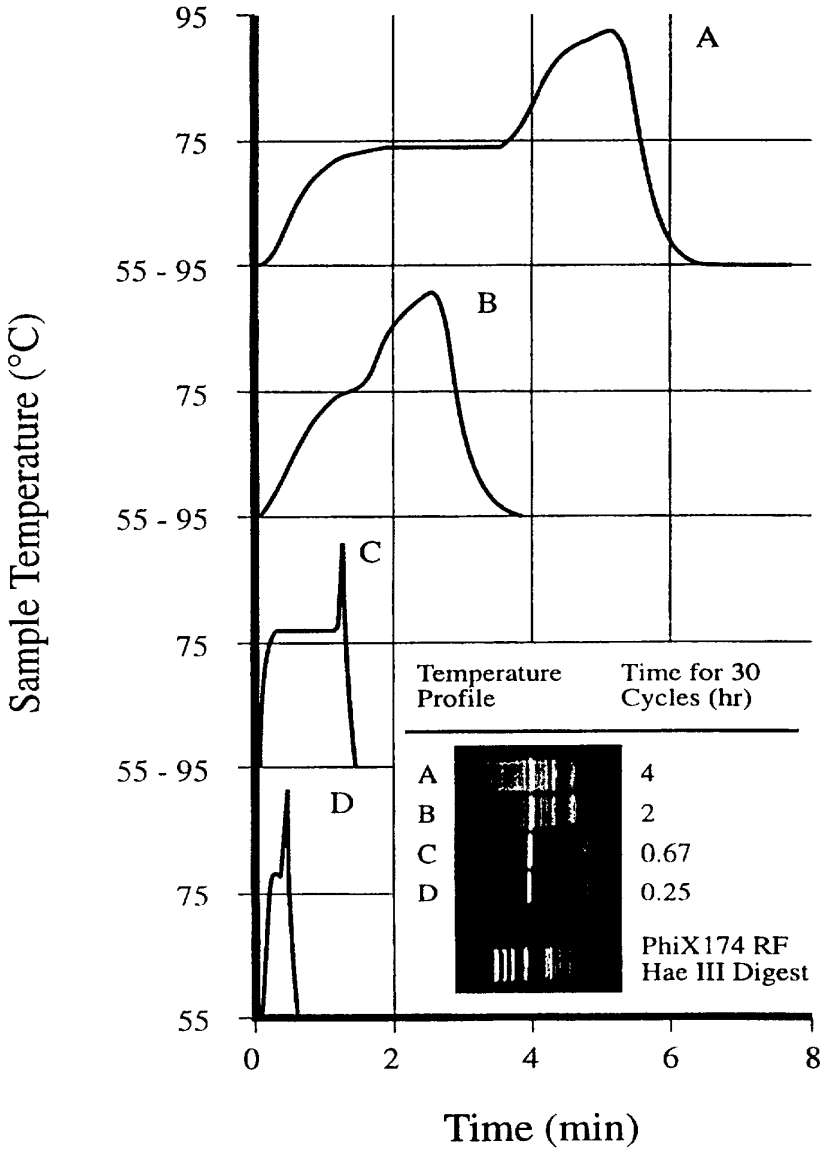
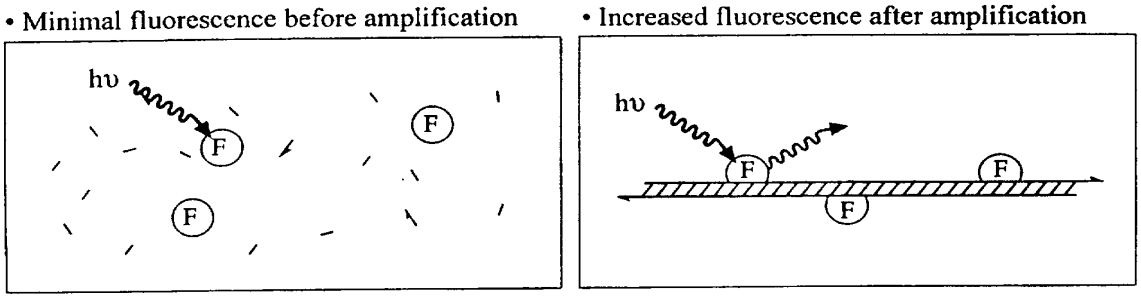
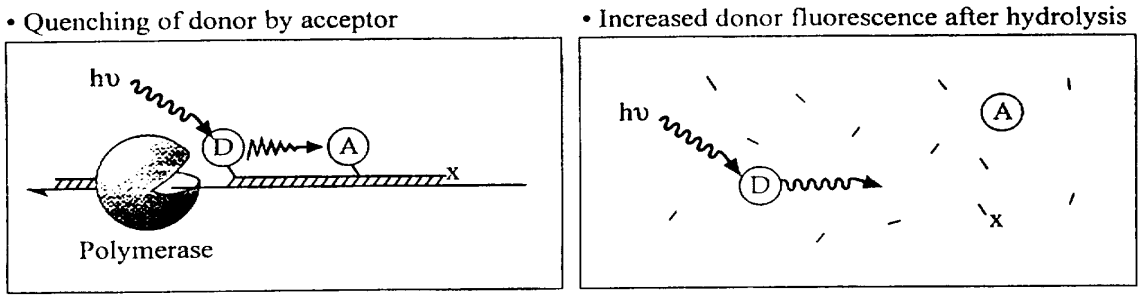

Sample Temperature (°C)
95
75
55 - 95
75
55 - 95
75
55 - 95
75
55
A
B
C
D
Temperature Profile
Time for 30 Cycles (hr)
A 4
B 2
C 0.67
D 0.25
PhiX174 RF Hae III Digest
0
2
4
6
8
Time (min)

Figure 5.

A. Increased fluorescence by binding double stranded DNA.



B. Release from quenching by hydrolysis.



C. Increased resonance energy transfer by hybridization.

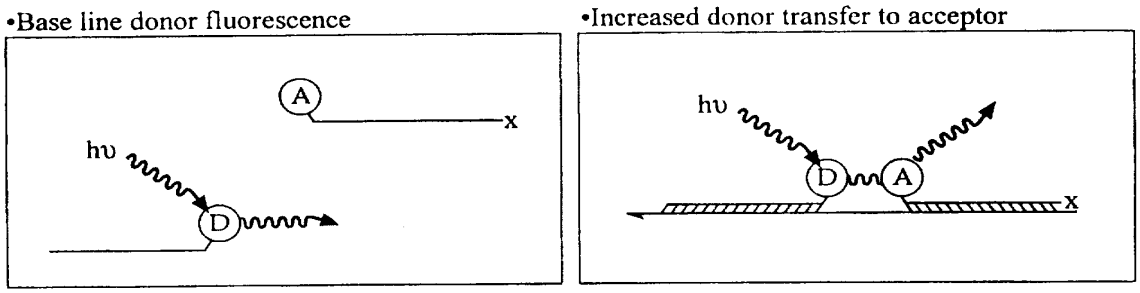


Figure 6.

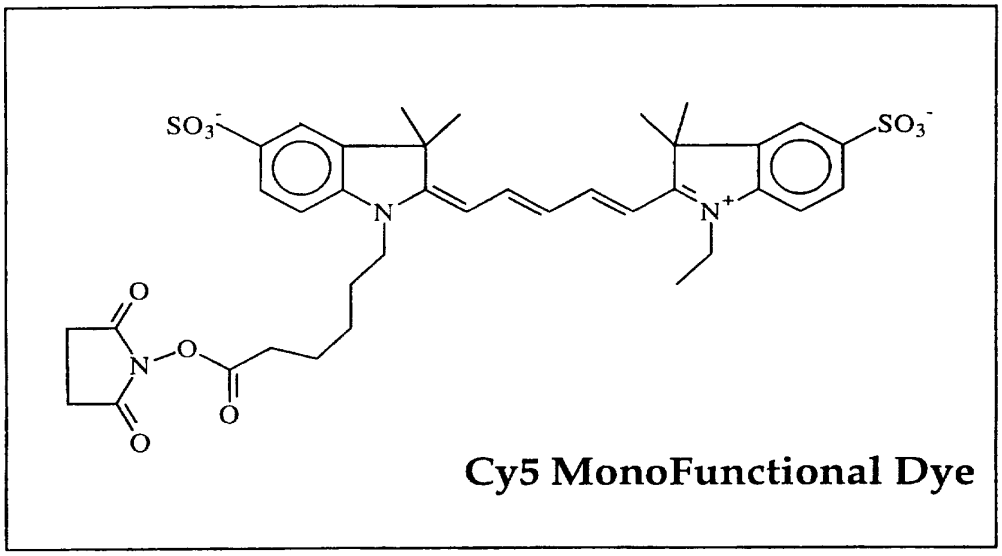


Figure 7.

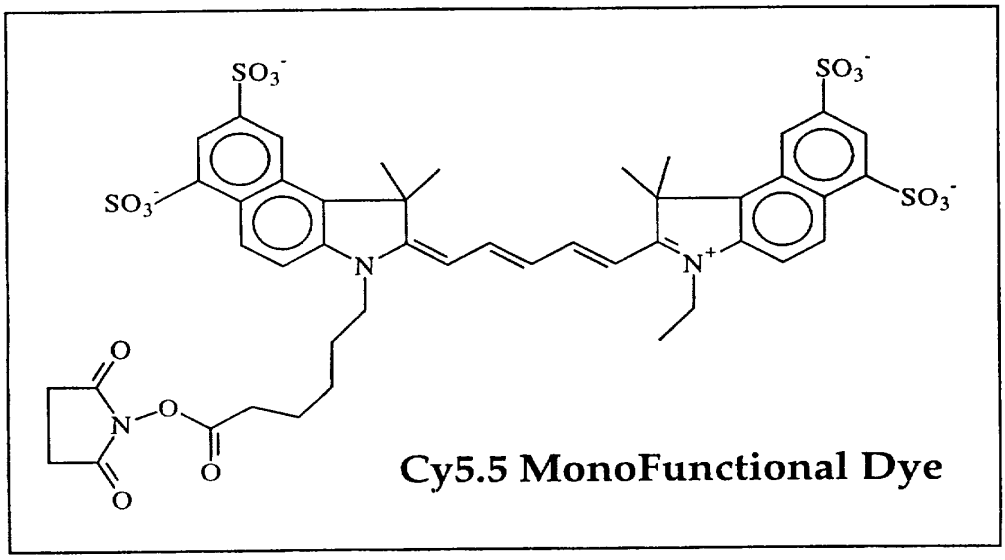
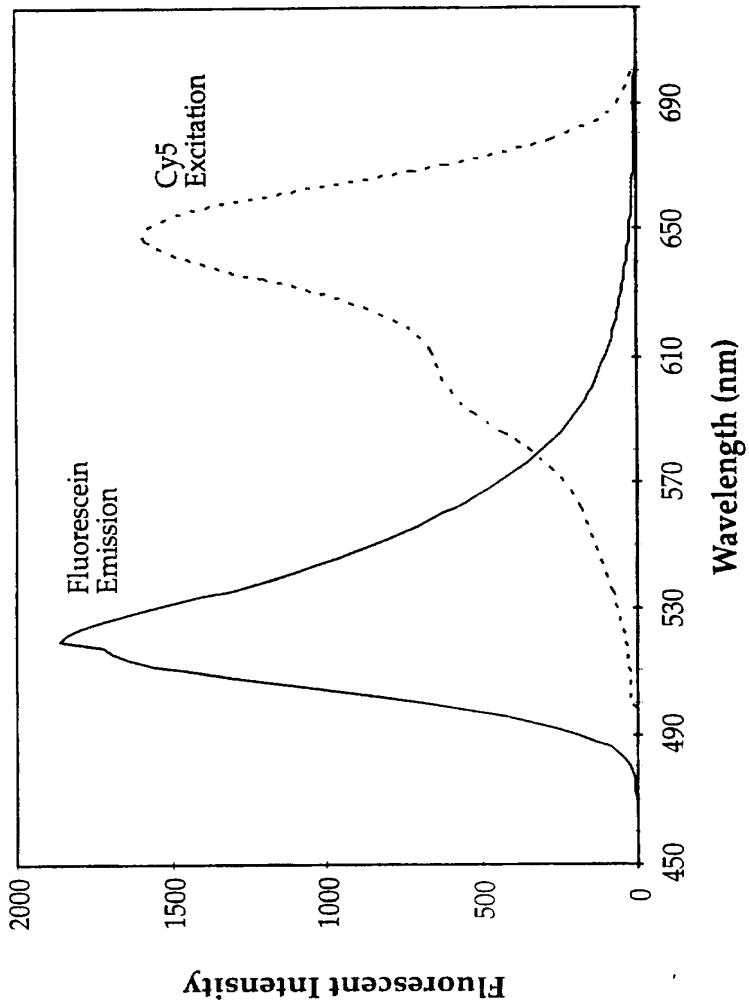


Figure 8.


Fluorescein
Emission
Cy5
Excitation
Fluorescent Intensity
2000
1500
1000
500
0
450
490
530
570
610
650
690
Wavelength (nm)

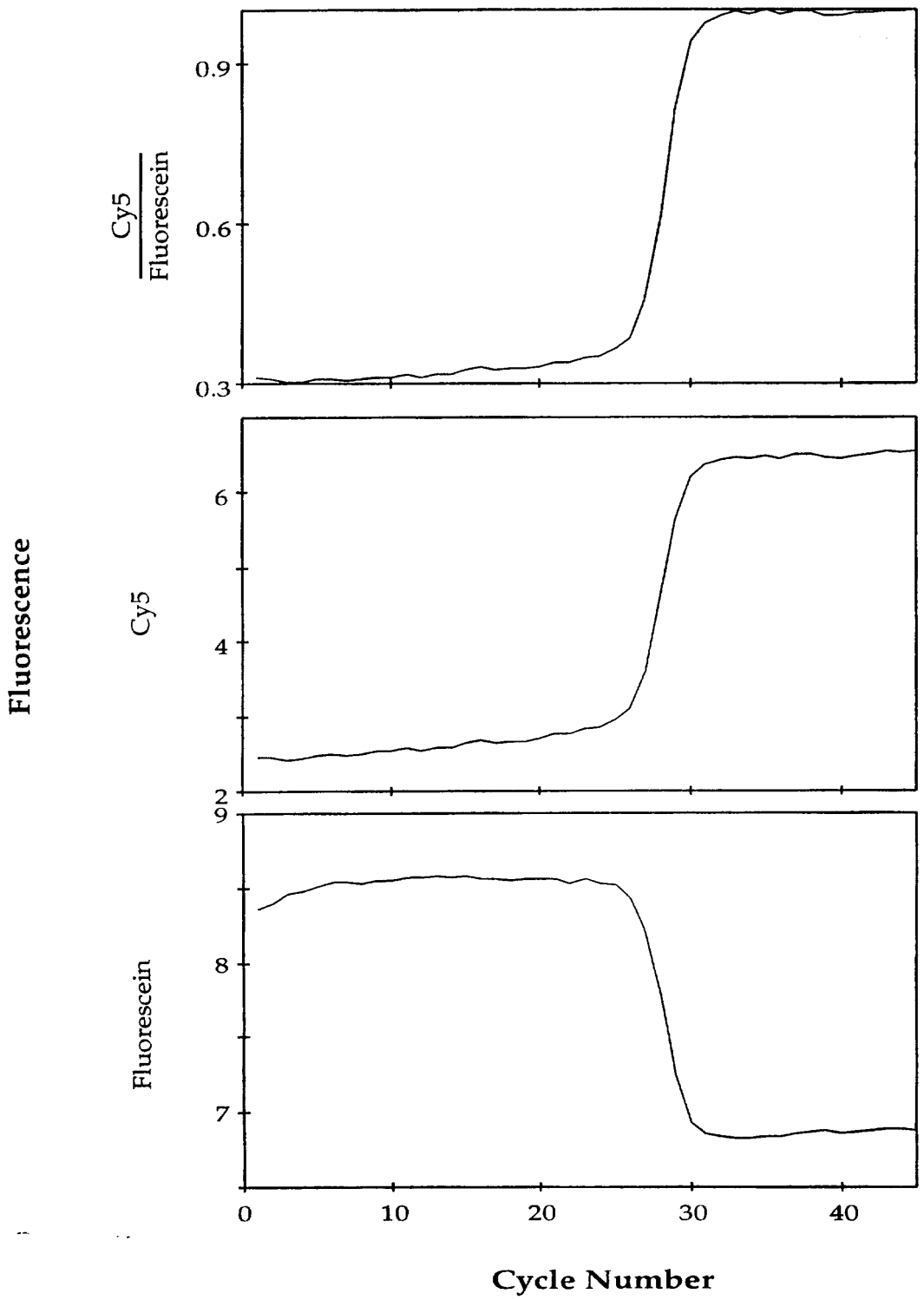

Fluorescence
Cy5 / Fluorescein
0.9
0.6
0.3
Cy5
6
4
2
Fluorescein
9
8
7
0
10
20
30
40
Cycle Number

Figure 10.

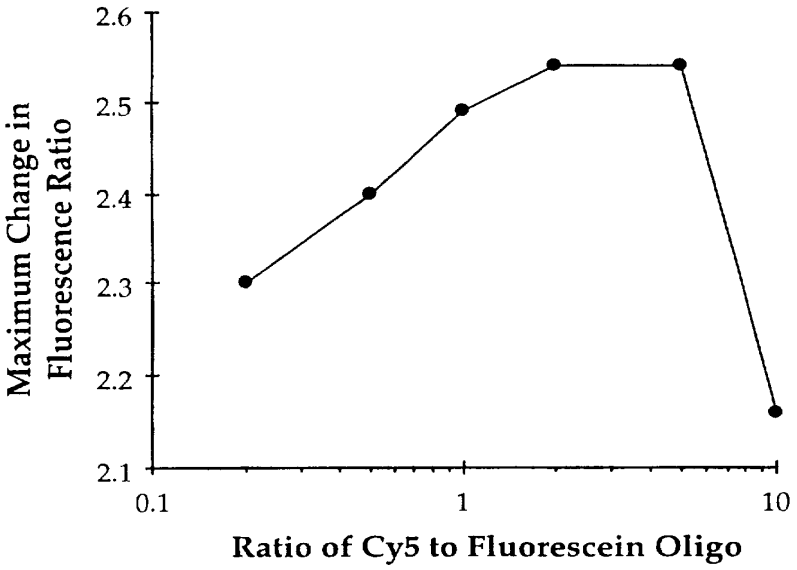

Maximum Change in Fluorescence Ratio
2.6
2.5
2.4
2.3
2.2
2.1
0.1
1
10
Ratio of Cy5 to Fluorescein Oligo

Figure 11.

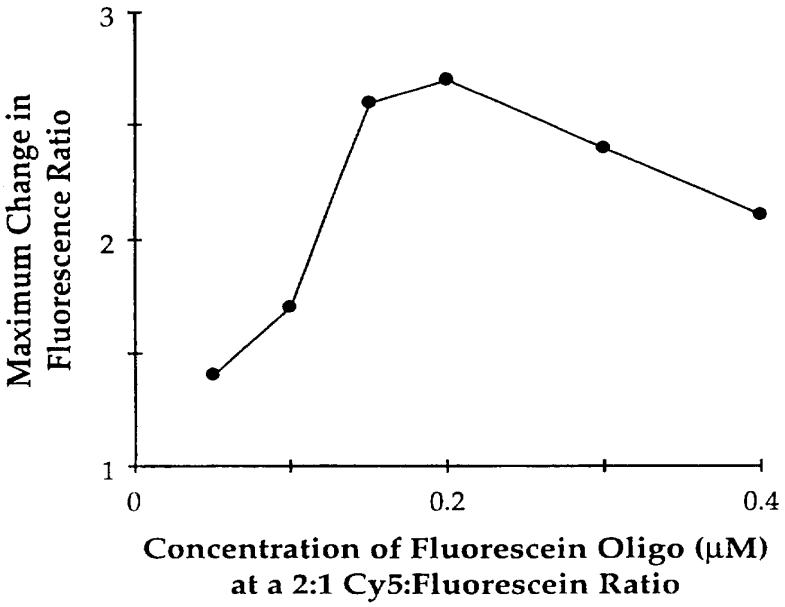
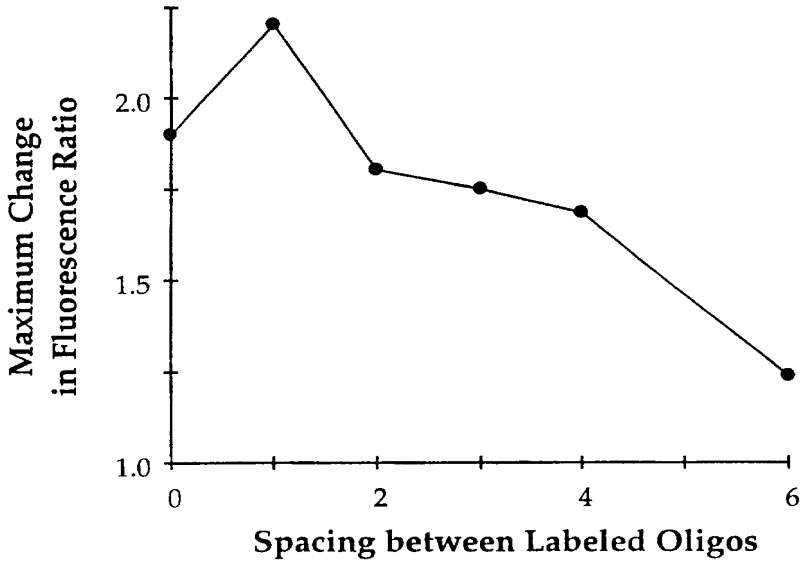

Maximum Change in Fluorescence Ratio
3
2
1
0
0.2
0.4
Concentration of Fluorescein Oligo (μM) at a 2:1 Cy5:Fluorescein Ratio

Figure 12.


Maximum Change
in Fluorescence Ratio
2.0
1.5
1.0
0
2
4
6
Spacing between Labeled Oligos

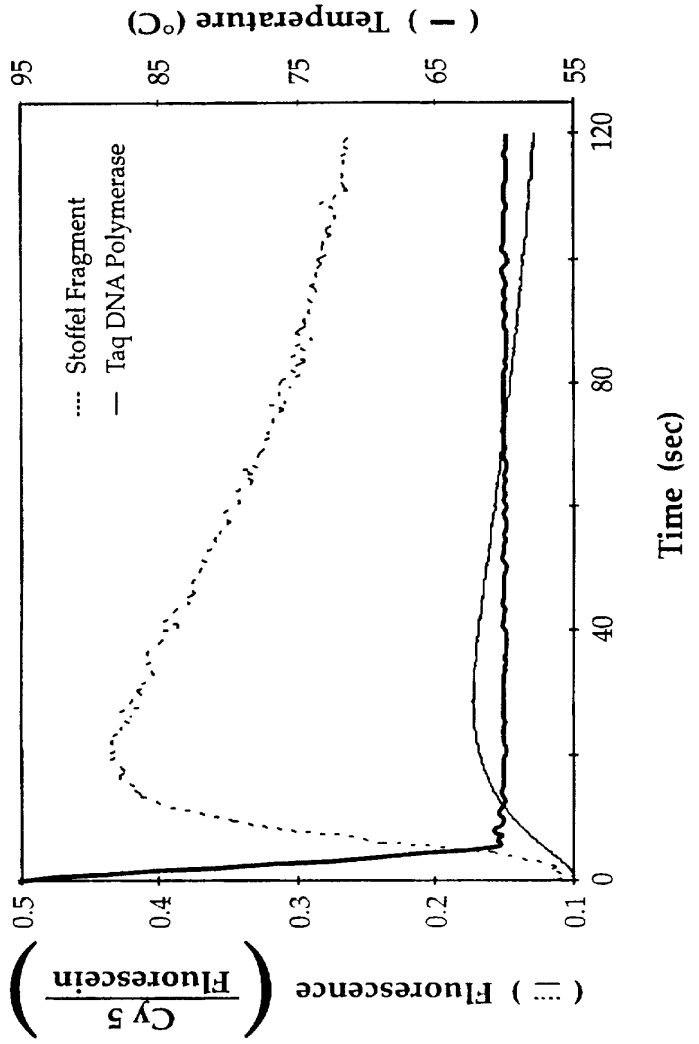
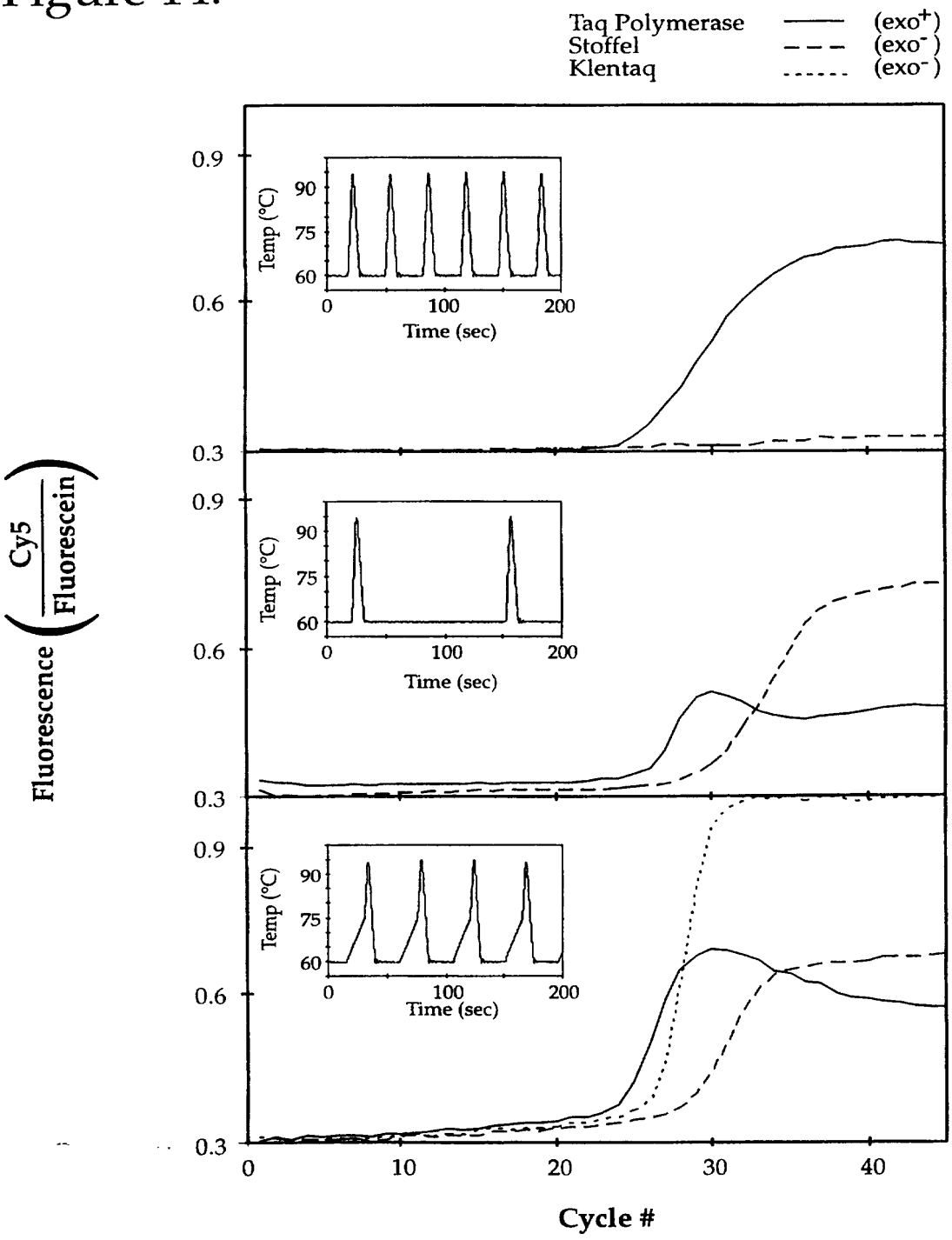

(—) Temperature (°C)
95
85
75
65
55
Stoffel Fragment
Taq DNA Polymerase
(⁻ ⁻) Fluorescence (Cy 5 / Fluorescein)
0.5
0.4
0.3
0.2
0.1
0
40
80
120
Time (sec)

Figure 13.

Figure 14.


Taq Polymerase (exo+)
Stoffel (exo-)
Klentaq (exo-)
Fluorescence (Cy5 / Fluorescein)
Temp (°C)
Time (sec)
Cycle #

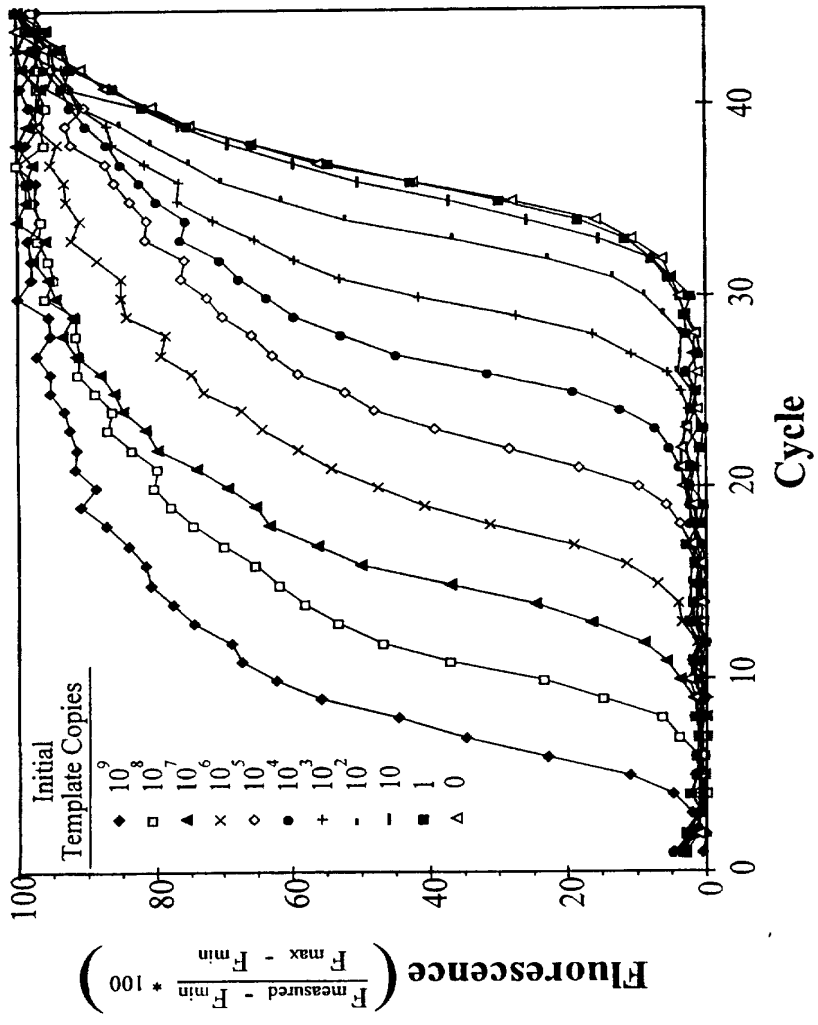

Fluorescence (F measured - F min / F max - F min * 100)
Cycle
Initial Template Copies
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
1
0
100
80
60
40
20
0
0
10
20
30
40

Figure 15.

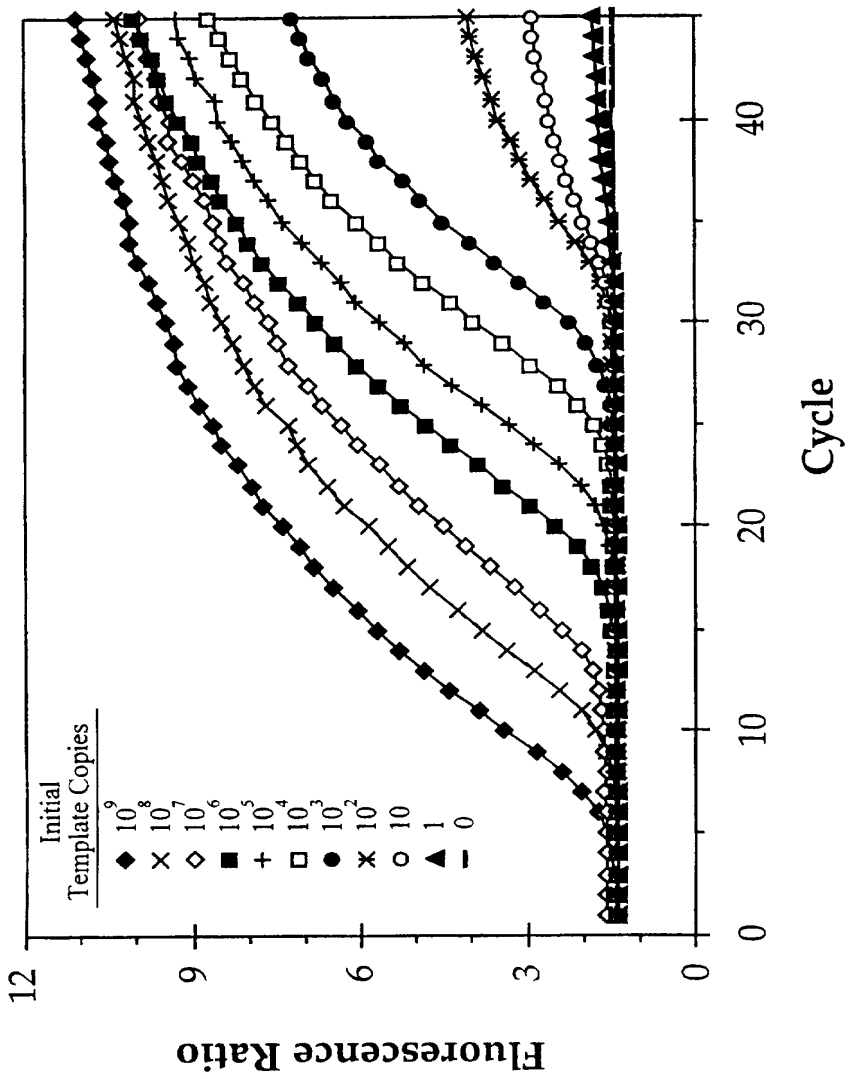

Fluorescence Ratio
12
9
6
3
0
0
10
20
30
40
Cycle
Initial Template Copies
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
1
0

Figure 16.

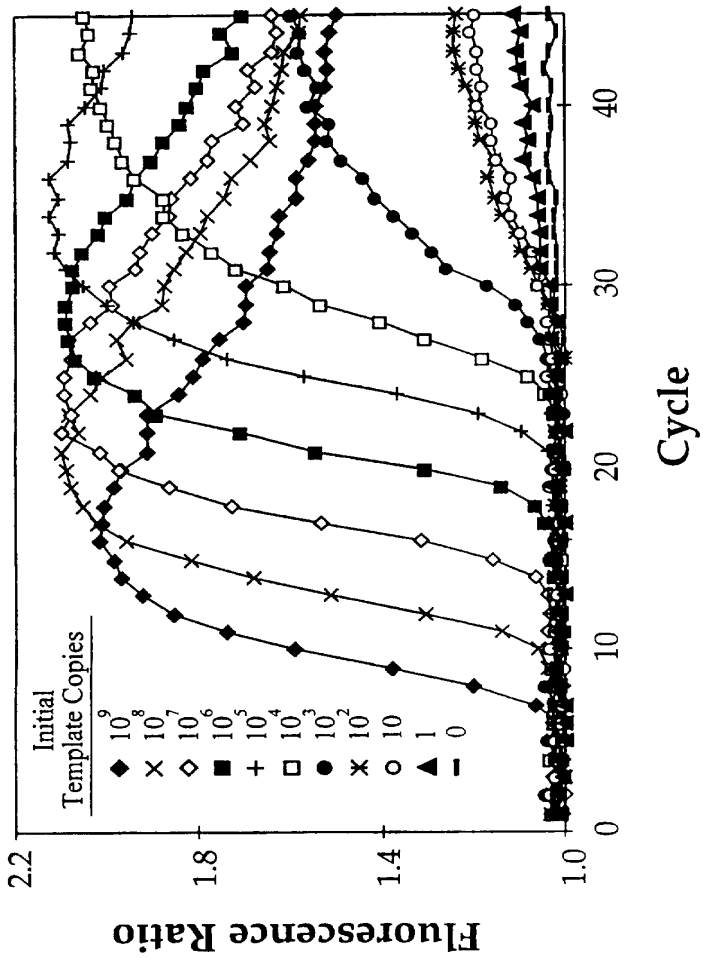

Fluorescence Ratio
2.2
1.8
1.4
1.0
0
10
20
30
40
Cycle
Initial Template Copies
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
1
0

Figure 17.

Figure 18.

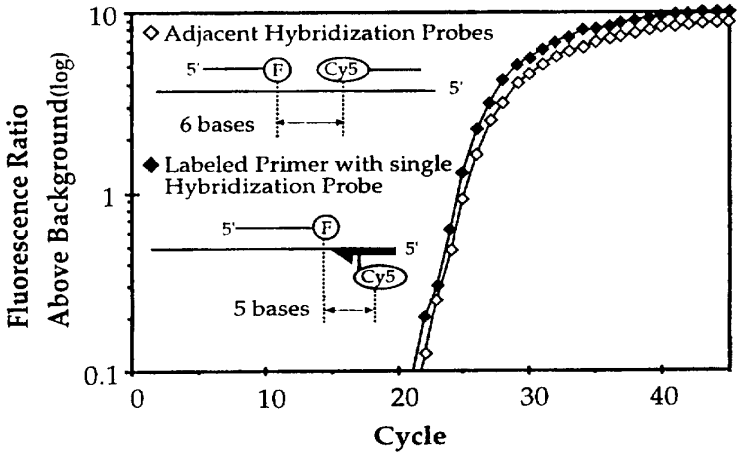
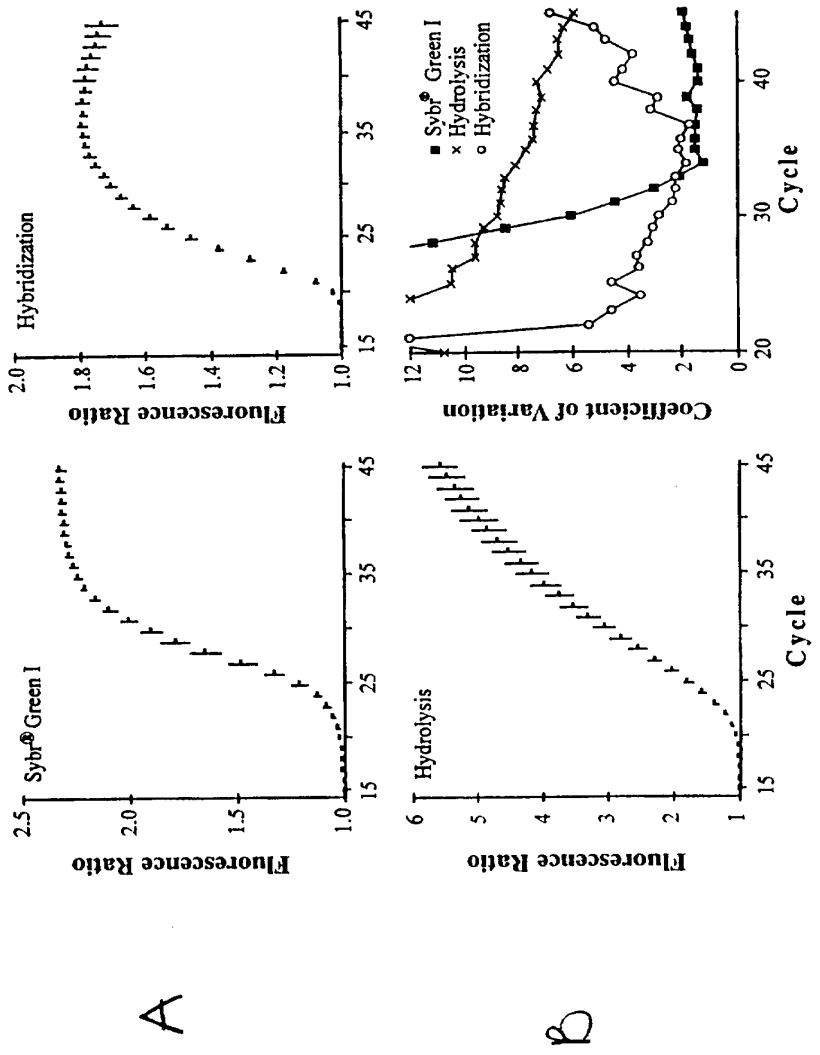

10
1
0.1
0
10
20
30
40
Cycle
Fluorescence Ratio
Above Background(log)
◇ Adjacent Hybridization Probes
5'
F
Cy5
5'
6 bases
◆ Labeled Primer with single
Hybridization Probe
5'
F
5'
Cy5
5 bases

Figure 19.


A
Sybr® Green I
Fluorescence Ratio
2.5
2.0
1.5
1.0
15
25
35
45
B
Hydrolysis
Fluorescence Ratio
6
5
4
3
2
1
15
25
35
45
Cycle
C
Hybridization
Fluorescence Ratio
2.0
1.8
1.6
1.4
1.2
1.0
15
25
35
45
D
Sybr® Green I
× Hydrolysis
o Hybridization
Coefficient of Variation
12
10
8
6
4
2
0
20
30
40
Cycle

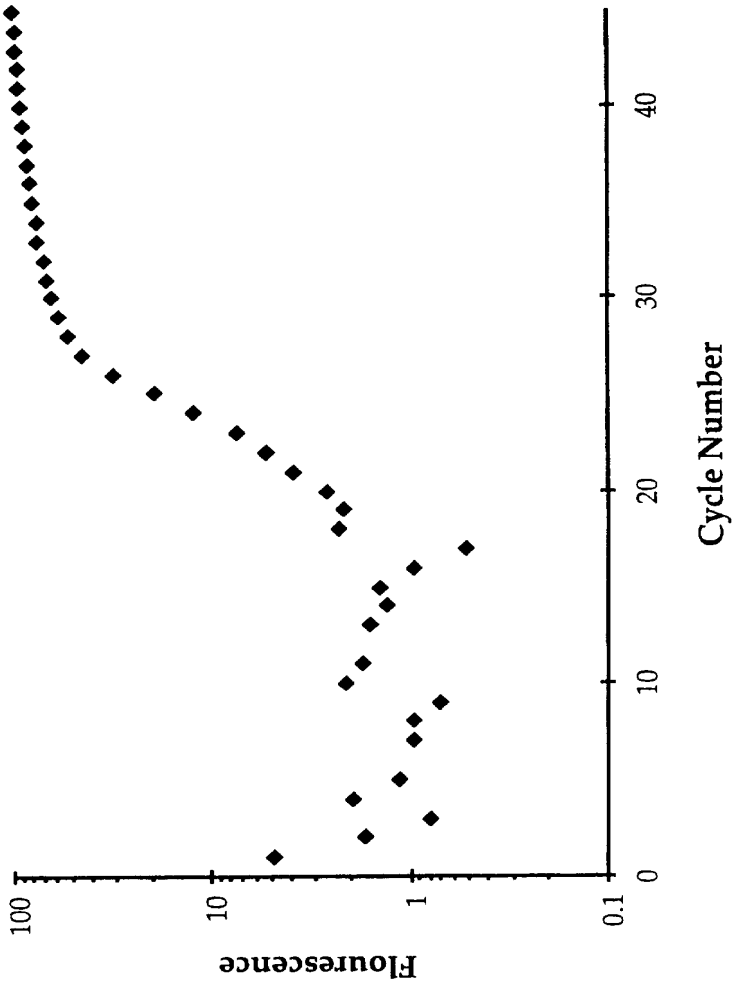


Figure 20.

Figure 21.

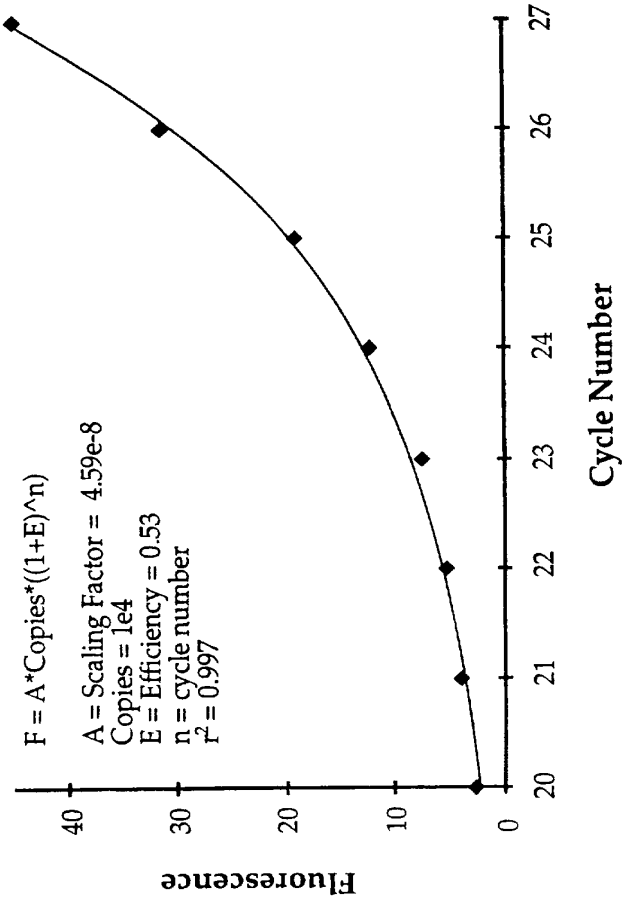

F = A*Copies*((1+E)^n)
A = Scaling Factor = 4.59e-8
Copies = 1e4
E = Efficiency = 0.53
n = cycle number
r2 = 0.997
Fluorescence
0
10
20
30
40
20
21
22
23
24
25
26
27
Cycle Number

Figure 22.

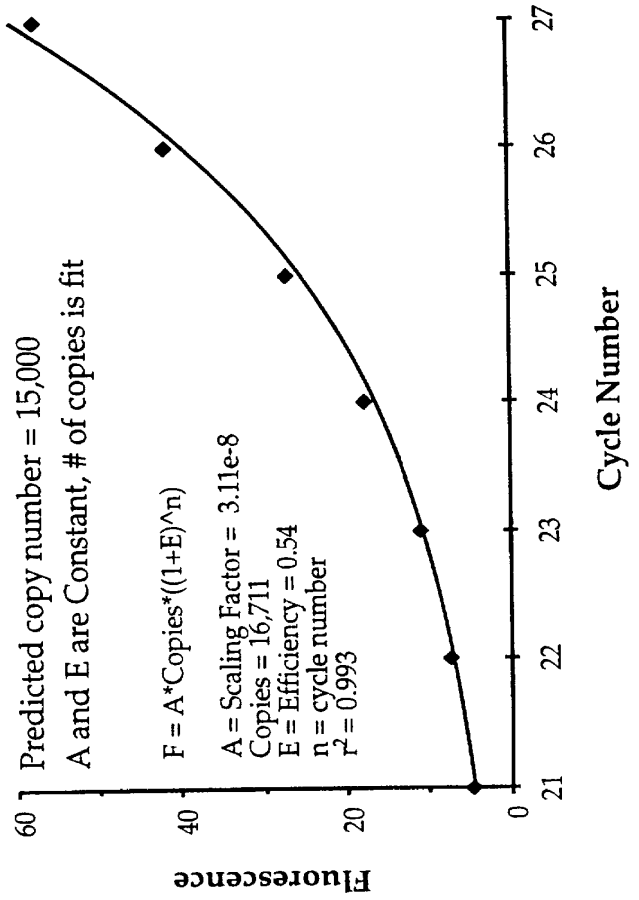

Predicted copy number = 15,000
A and E are Constant, # of copies is fit
F = A*Copies*((1+E)^n)
A = Scaling Factor = 3.11e-8
Copies = 16,711
E = Efficiency = 0.54
n = cycle number
r2 = 0.993
Fluorescence
Cycle Number
60
40
20
0
21
22
23
24
25
26
27

Figure 23.

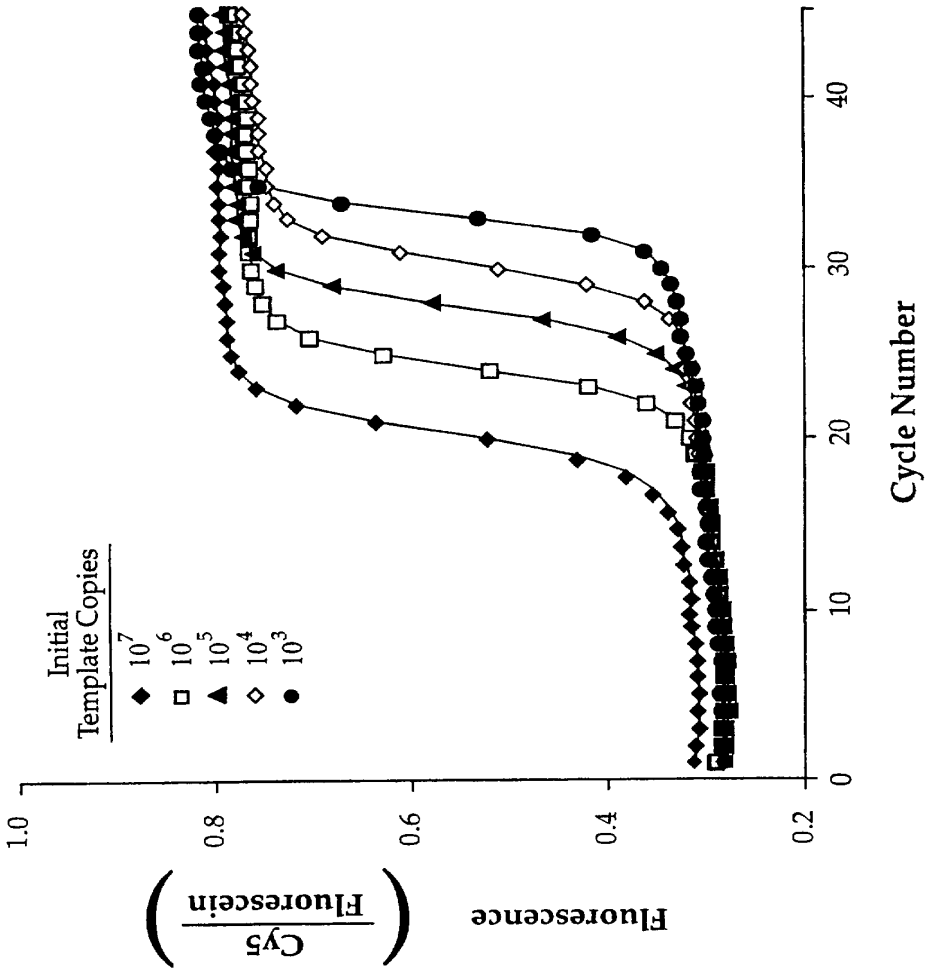
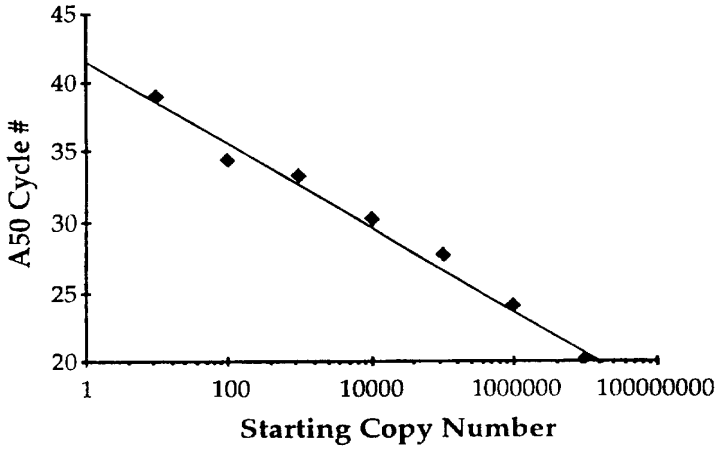

Initial
Template Copies
10^7
10^6
10^5
10^4
10^3
Fluorescence
$\left(\frac{Cy5}{Fluorescein}\right)$
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
Cycle Number

Figure 24.


45
40
35
30
25
20
A50 Cycle #
1
100
10000
1000000
100000000
Starting Copy Number

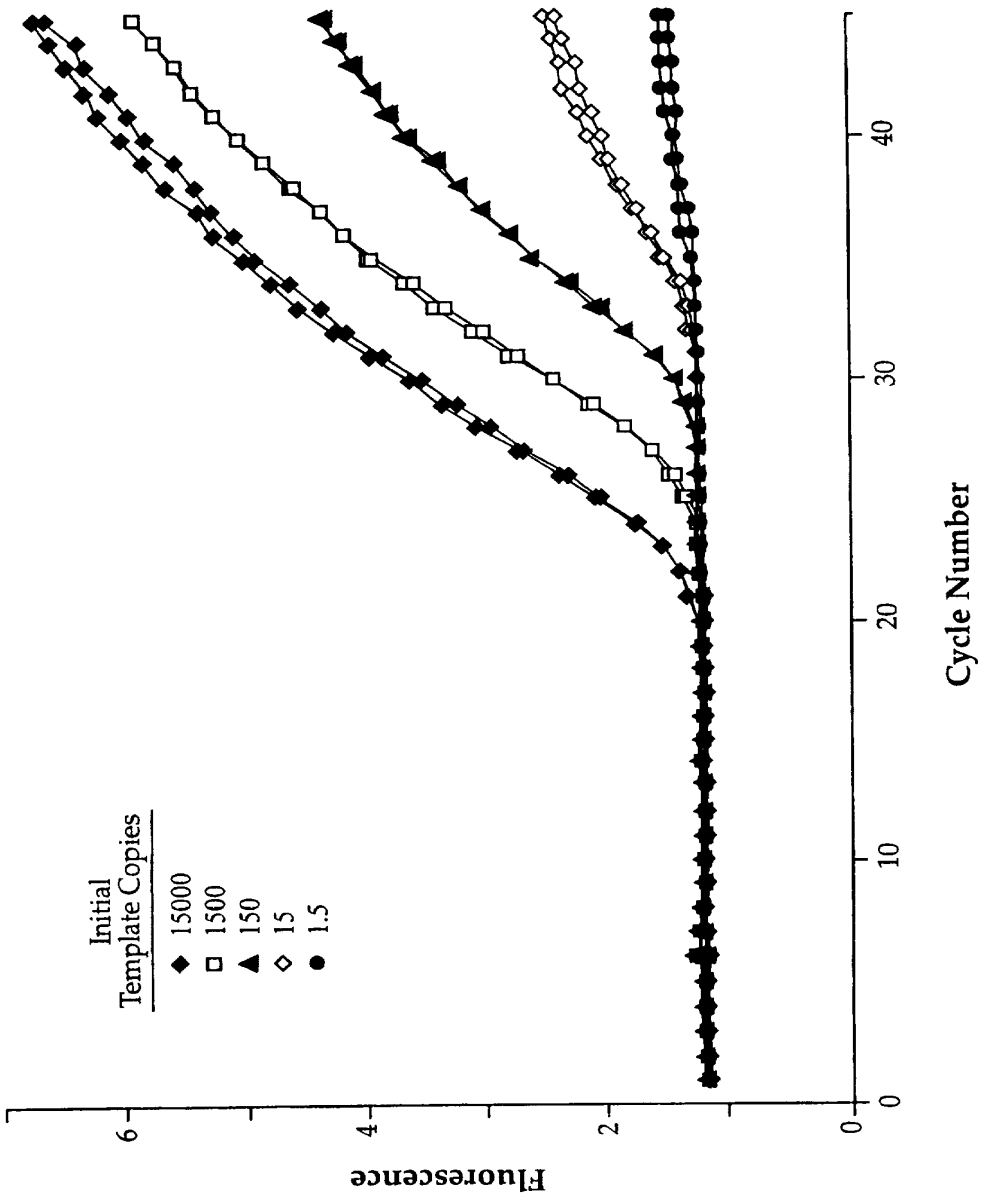

Initial
Template Copies
15000
1500
150
15
1.5
Fluorescence
0
2
4
6
0
10
20
30
40
Cycle Number

Figure 25.

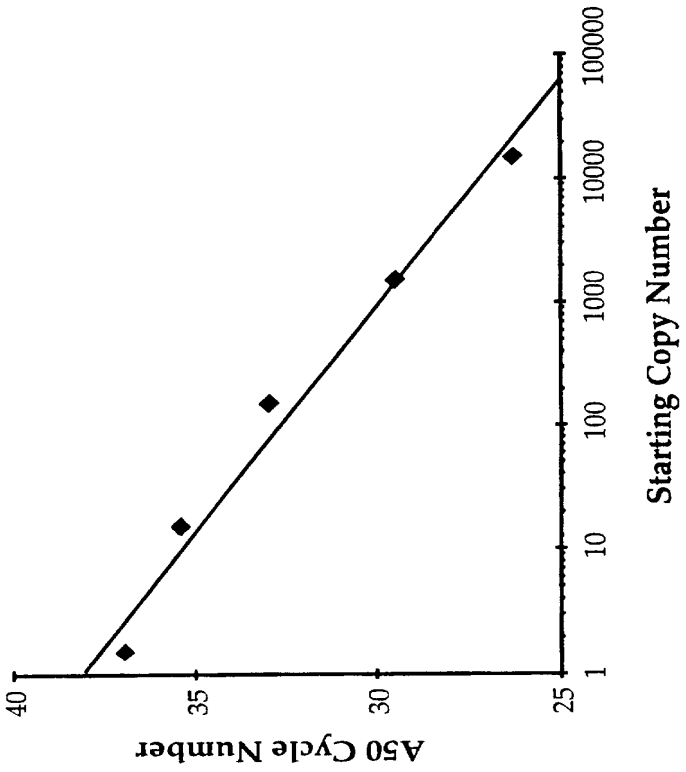

A50 Cycle Number
40
35
30
25
Starting Copy Number
1
10
100
1000
10000
100000

Figure 26.

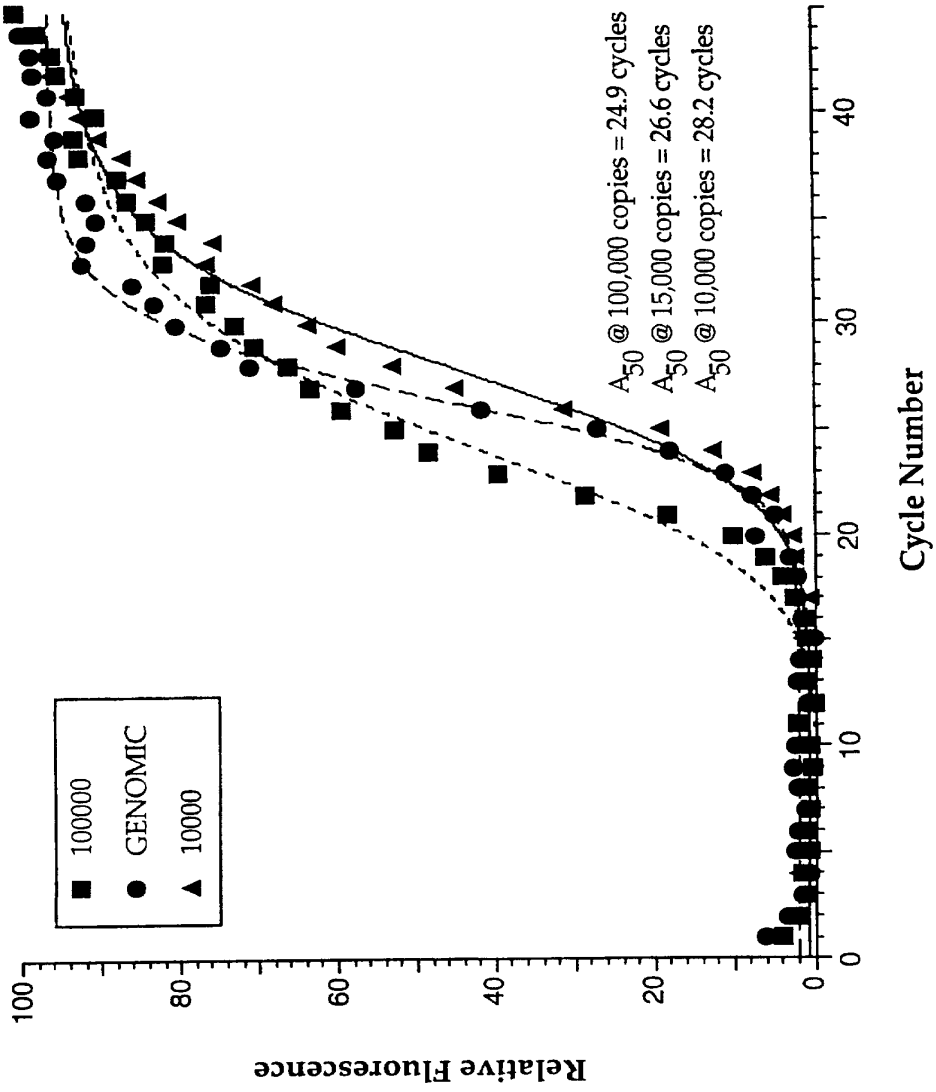

Relative Fluorescence
Cycle Number
100000
GENOMIC
10000
A_{50} @ 100,000 copies = 24.9 cycles
A_{50} @ 15,000 copies = 26.6 cycles
A_{50} @ 10,000 copies = 28.2 cycles
0
20
40
60
80
100
0
10
20
30
40

Figure 27.

Figure 28.

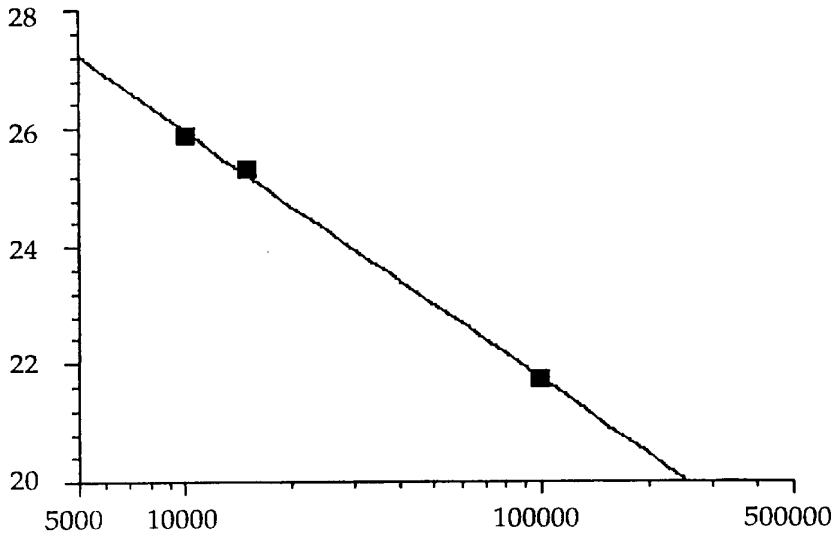


Figure 29.

A

B

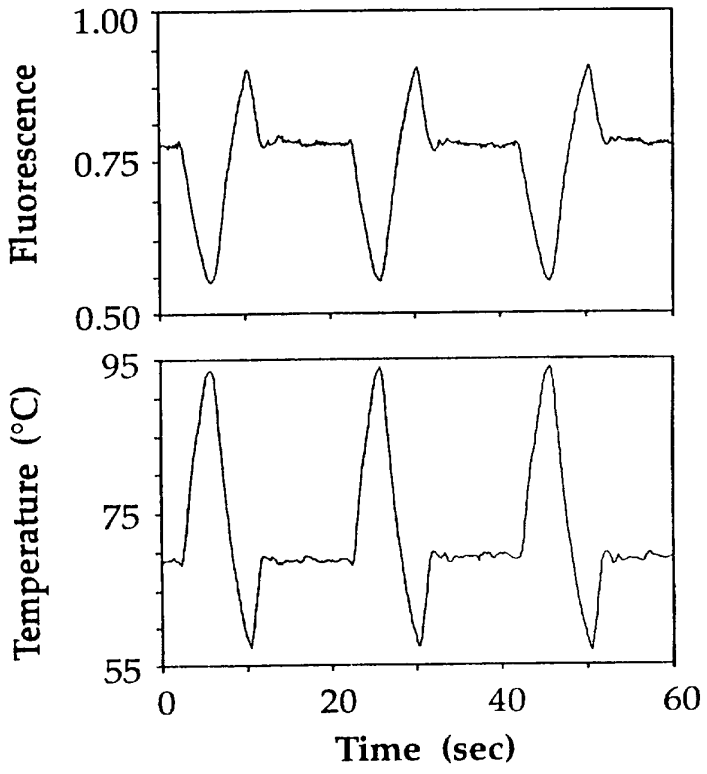
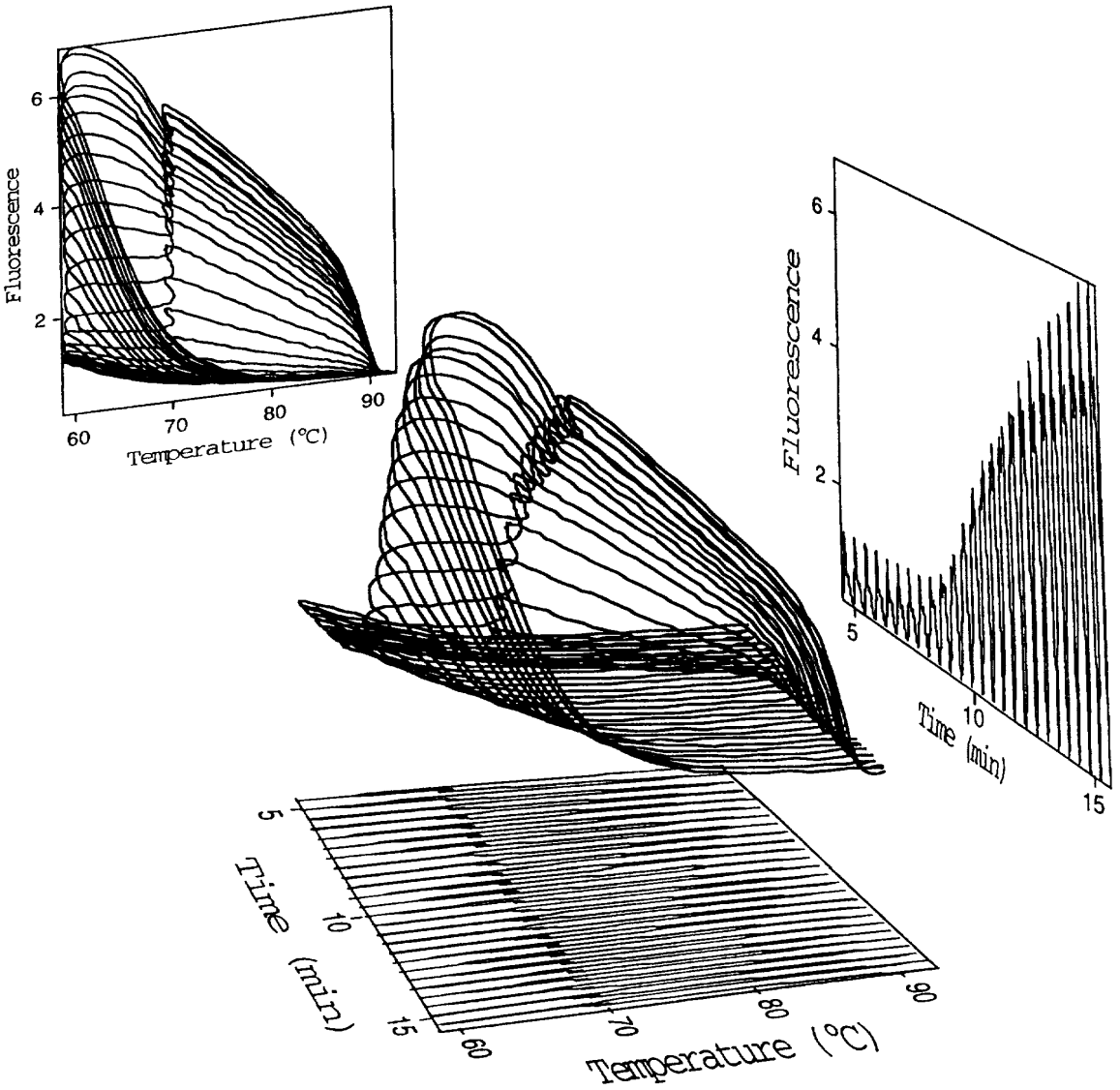

1.00
0.75
0.50
Fluorescence
95
75
55
Temperature (°C)
0
20
40
60
Time (sec)

Figure 30


Fluorescence
6
4
2
60
70
80
90
Temperature (°C)
Fluorescence
6
4
2
5
10
15
Time (min)
5
Time
10
(min)
15
60
70
80
90
Temperature (°C)

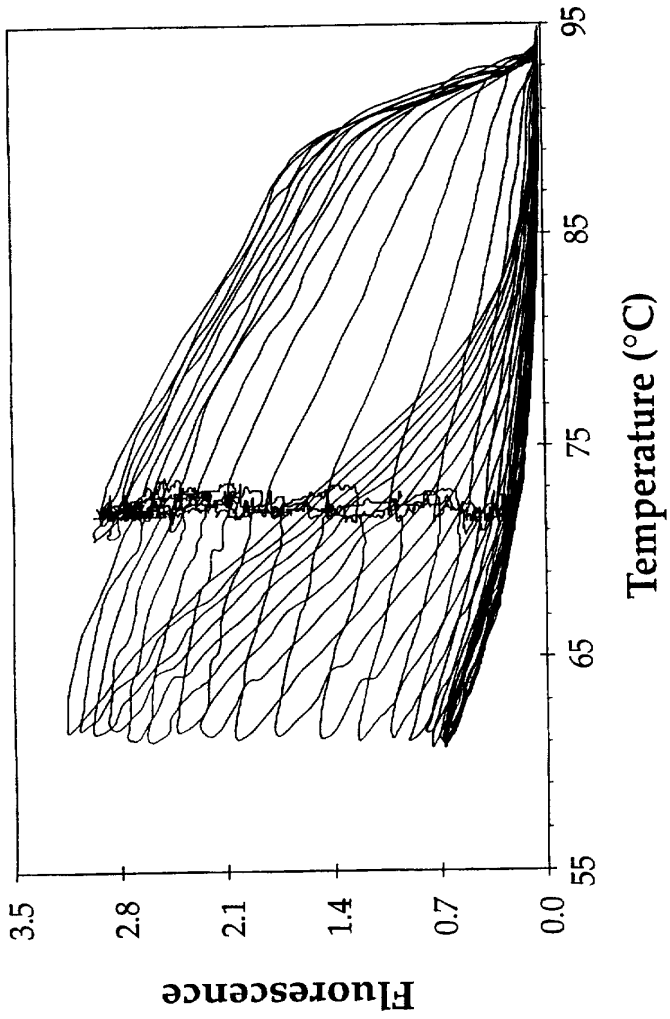
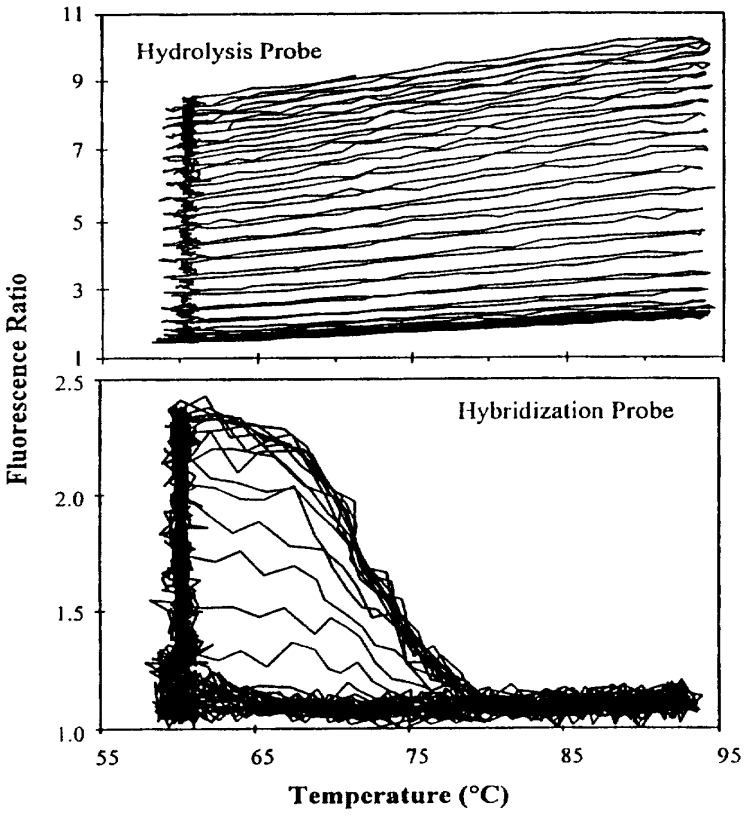


Figure 31.

Figure 32.

A

B


Hydrolysis Probe
Hybridization Probe
Fluorescence Ratio
Temperature (°C)
11
9
7
5
3
1
2.5
2.0
1.5
1.0
55
65
75
85
95

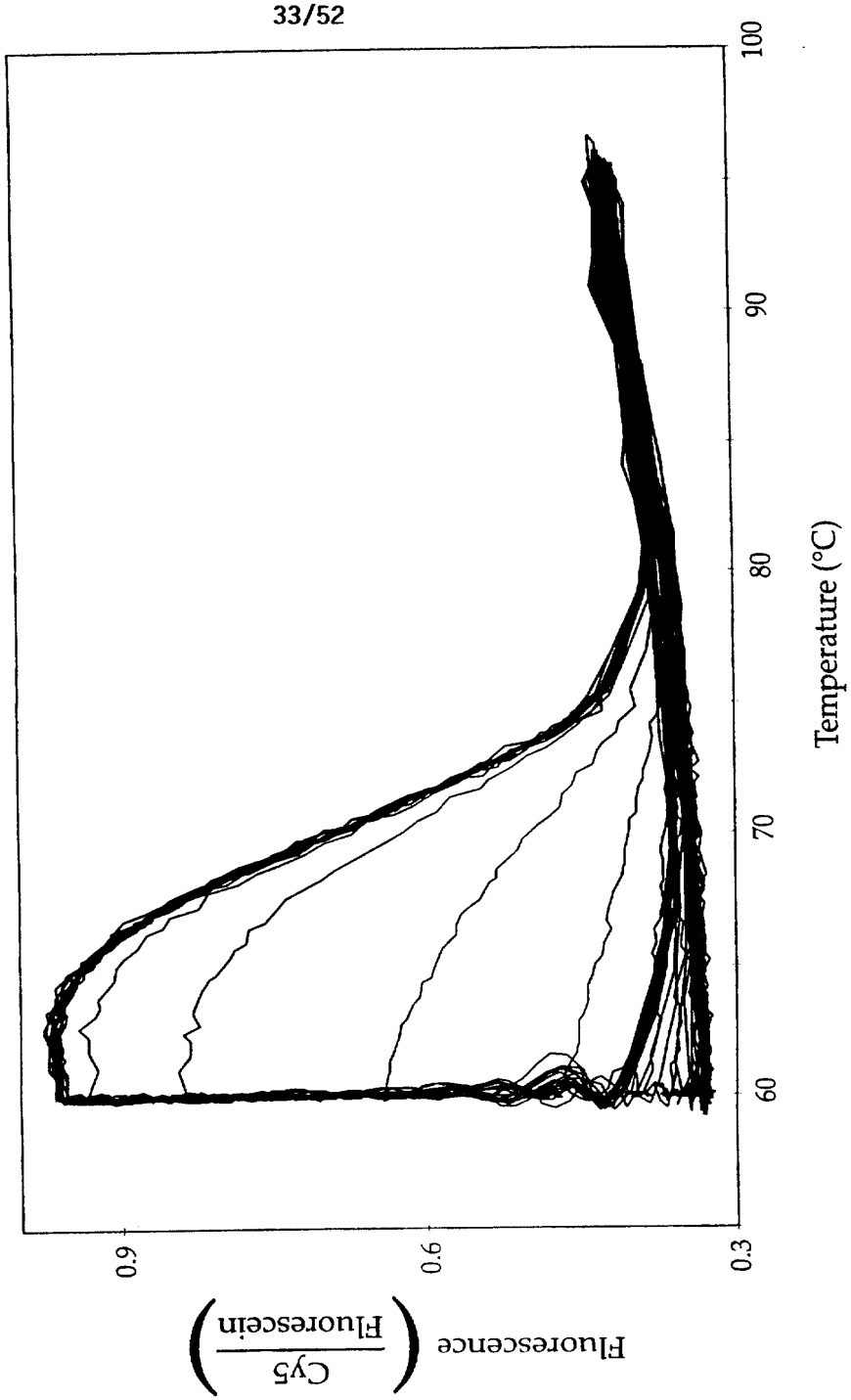

Fluorescence $\left(\frac{Cy5}{Fluorescein}\right)$
0.9
0.6
0.3
60
70
80
90
100
Temperature (°C)

Figure 33.

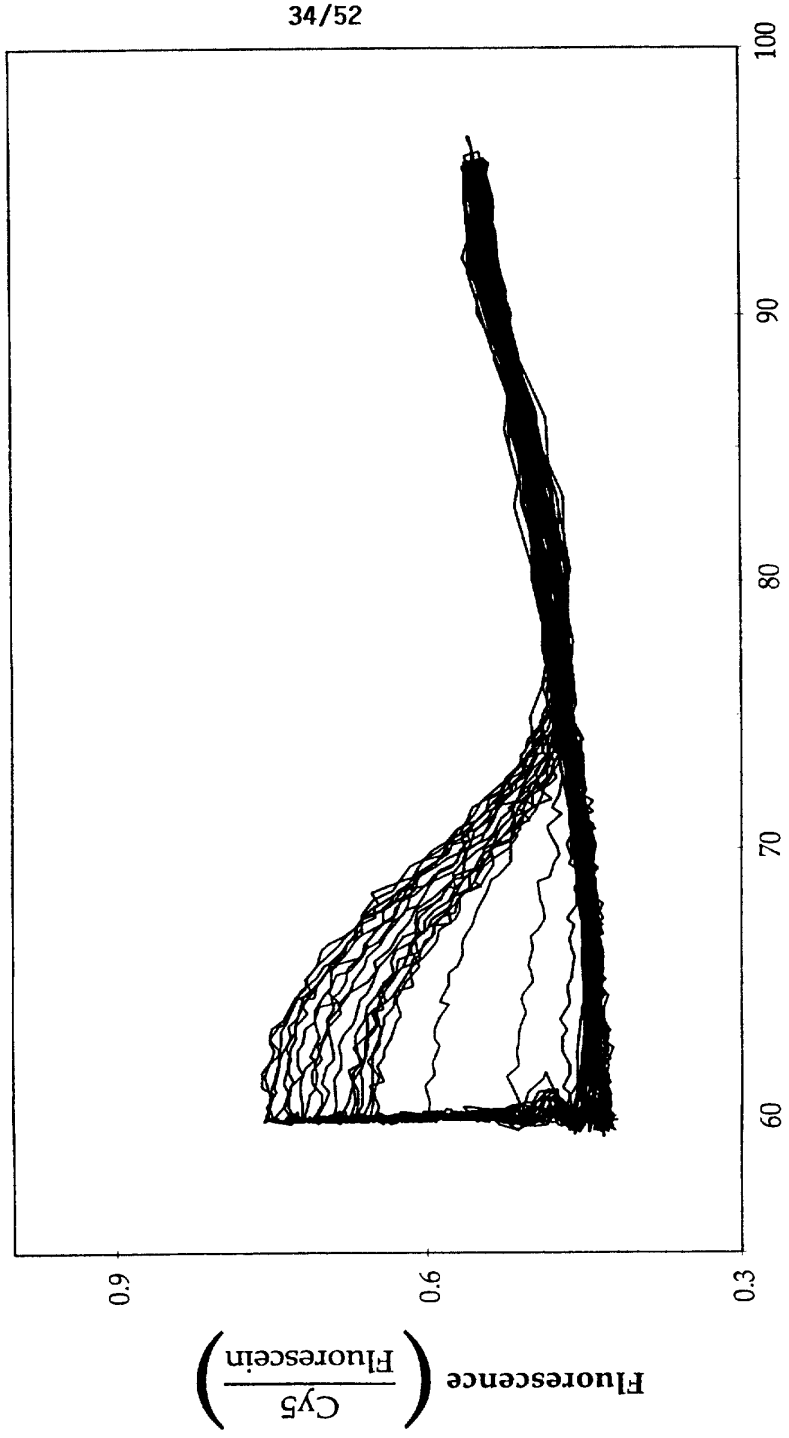

Fluorescence (Cy5/Fluorescein)
0.9
0.6
0.3
60
70
80
90
100
Temperature (°C)

Figure 34.

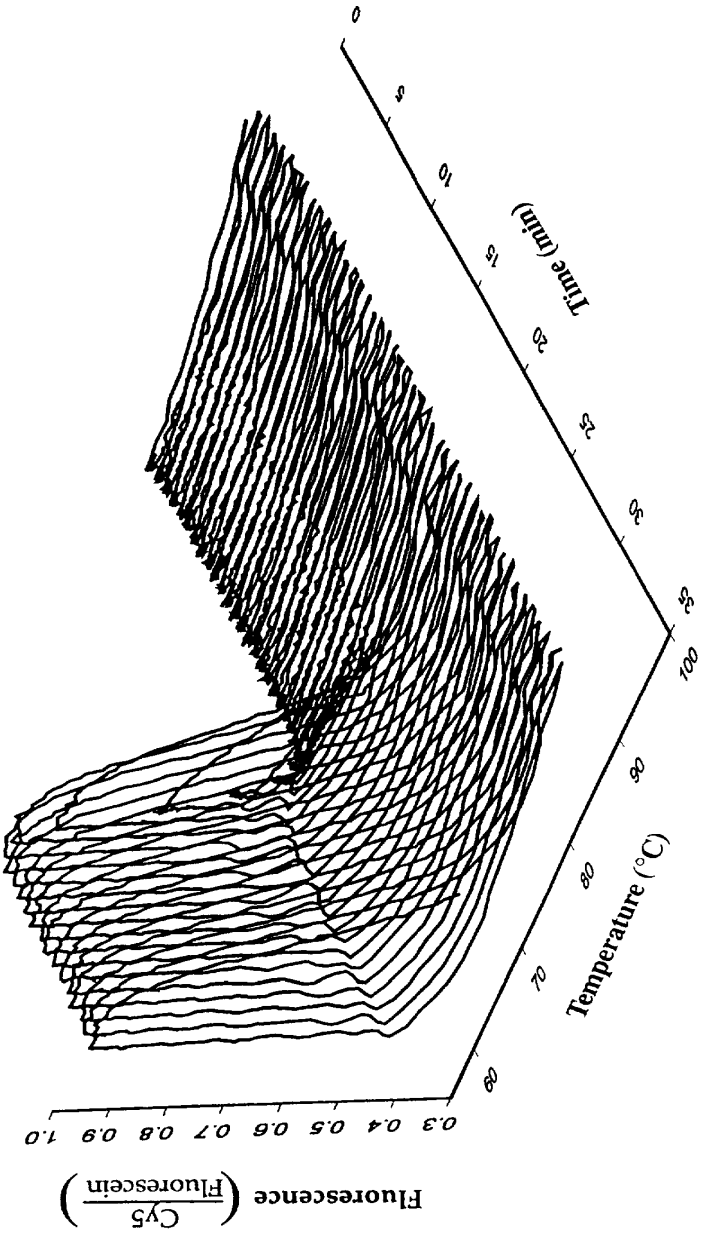

Fluorescence (Cy5/Fluorescein)
0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Temperature (°C)
60 70 80 90 100
Time (min)
0 5 10 15 20 25 30 35

Figure 35.

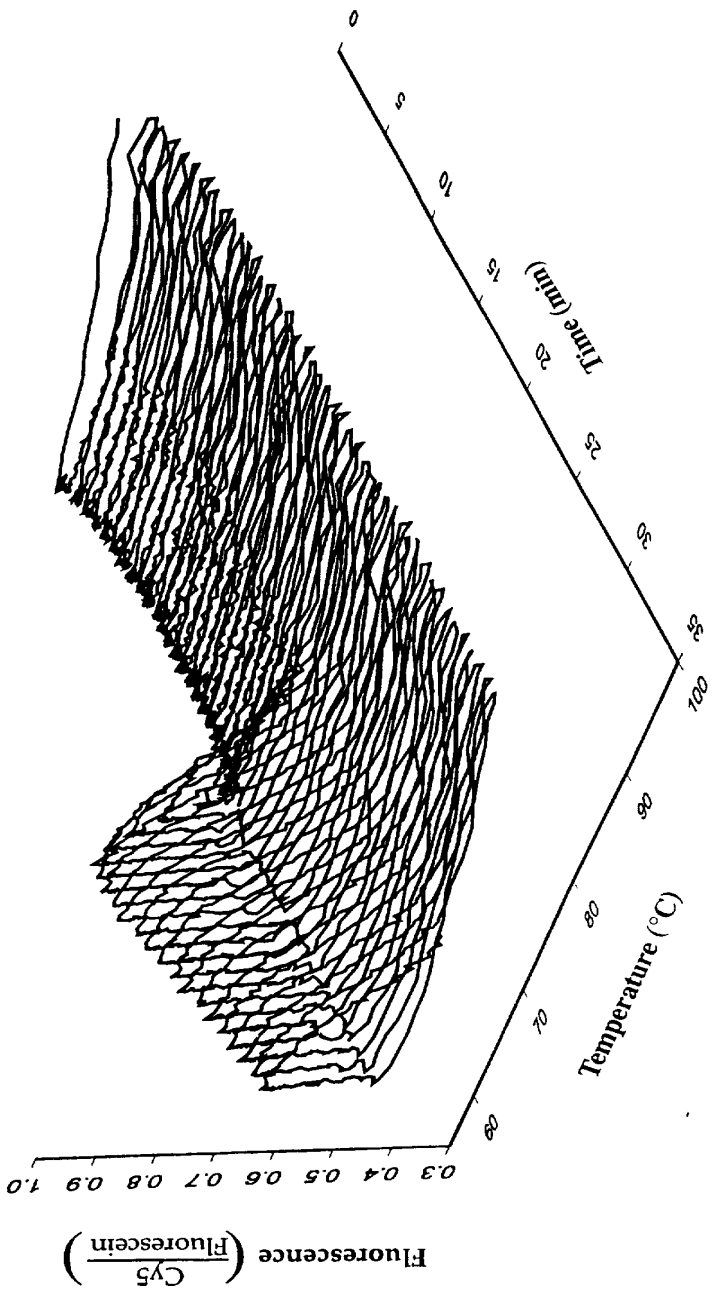

Fluorescence (Cy5/Fluorescein)
0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Temperature (°C)
60 70 80 90 100
Time (min)
0 5 10 15 20 25 30 35

Figure 36.

Figure 37

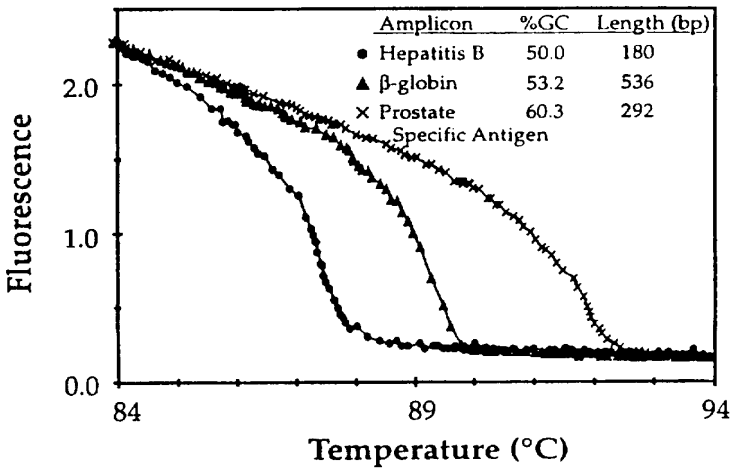

Amplicon %GC Length (bp)
Hepatitis B 50.0 180
β-globin 53.2 536
Prostate Specific Antigen 60.3 292
Fluorescence
2.0
1.0
0.0
84
89
94
Temperature (°C)

Figure 38

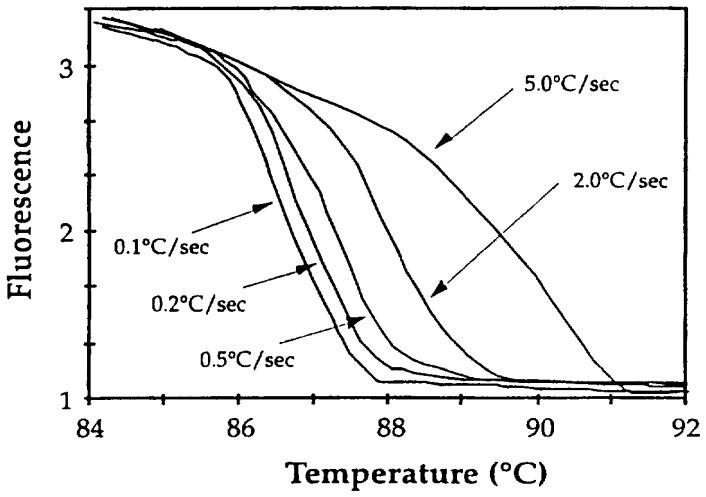

Fluorescence
3
2
1
84
86
88
90
92
Temperature (°C)
5.0°C/sec
2.0°C/sec
0.1°C/sec
0.2°C/sec
0.5°C/sec

Figure 39

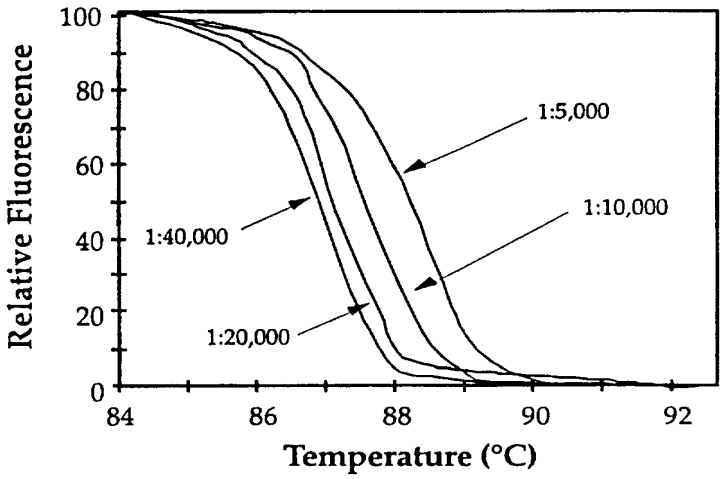
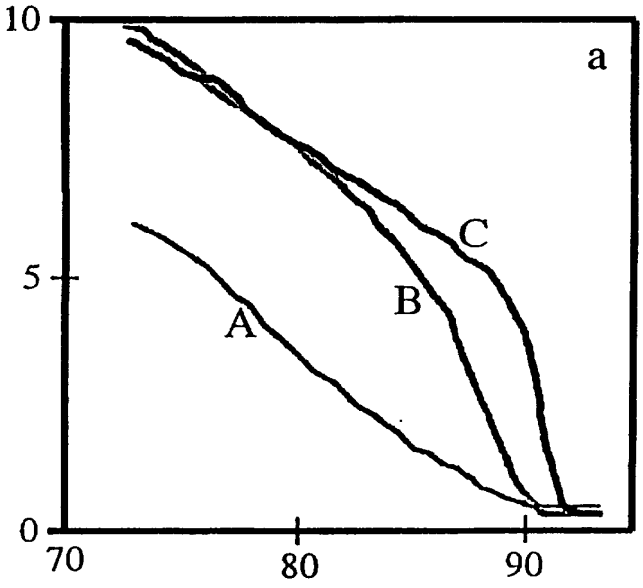

100
80
60
40
20
0
Relative Fluorescence
84
86
88
90
92
Temperature (°C)
1:5,000
1:10,000
1:40,000
1:20,000

Figure 40

A


10
5
0
70
80
90
a
A
B
C
Temperature (°C)

B

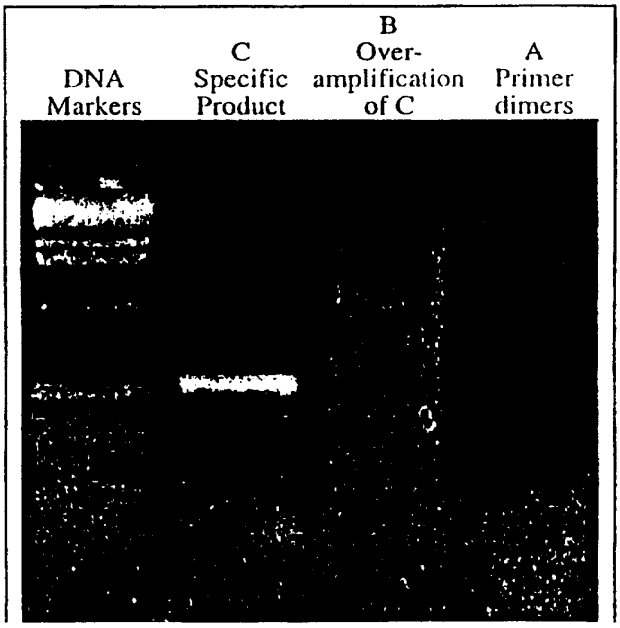
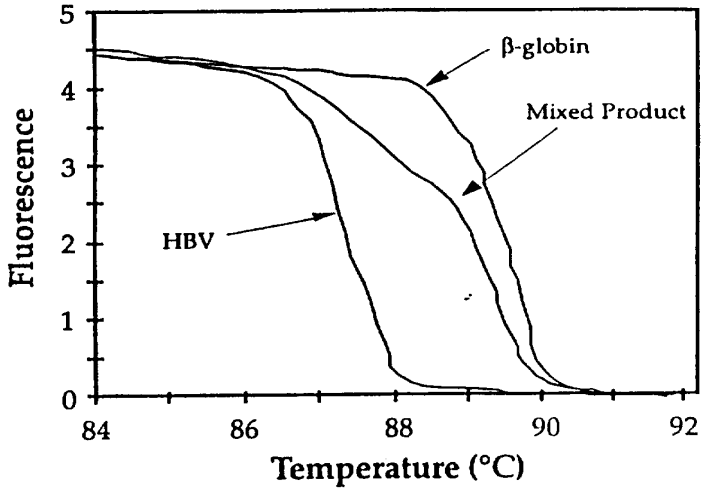

DNA Markers
C Specific Product
B Over-amplification of C
A Primer dimers

Figure 41

A


Fluorescence
Temperature (°C)
β-globin
Mixed Product
HBV
0
1
2
3
4
5
84
86
88
90
92

B

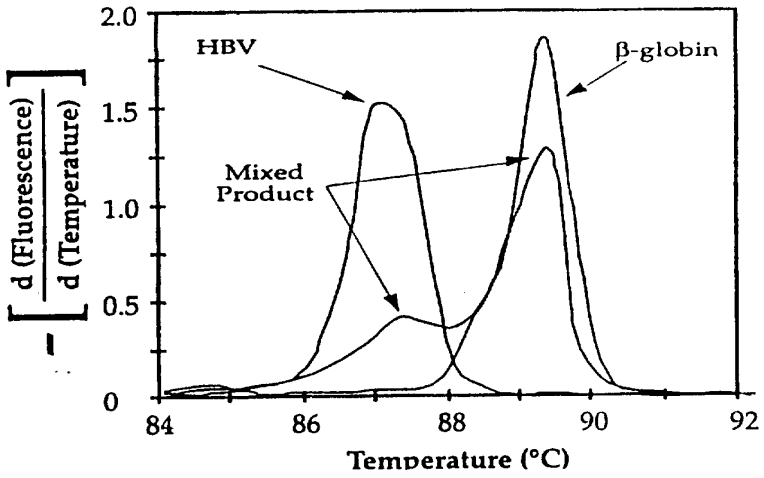

- [d (Fluorescence) / d (Temperature)]
Temperature (°C)
HBV
β-globin
Mixed Product
0
0.5
1.0
1.5
2.0
84
86
88
90
92

Figure 42

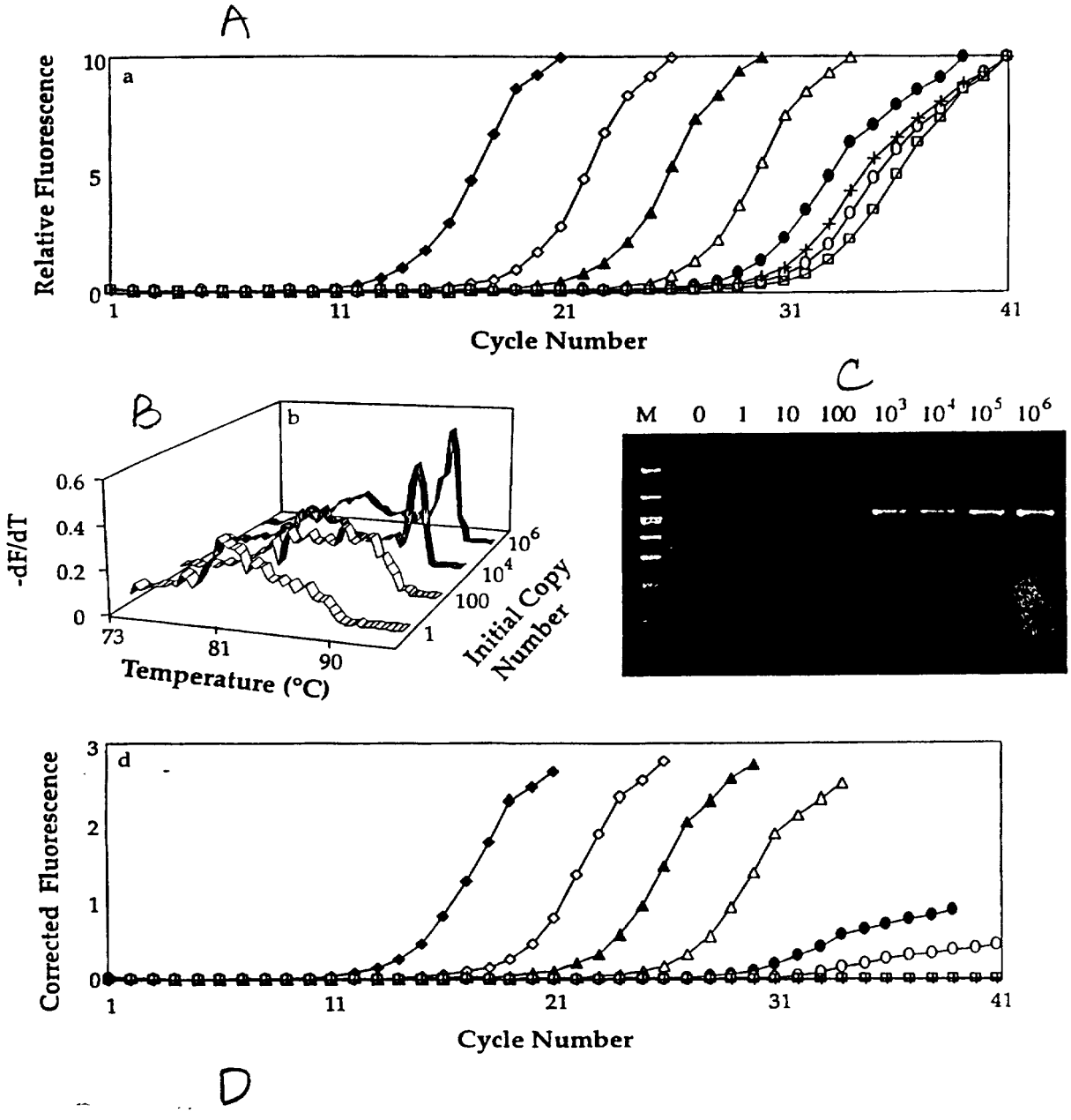

A
a
Relative Fluorescence
10
5
0
1
11
21
31
41
Cycle Number
B
b
-dF/dT
0.6
0.4
0.2
0
73
81
90
Temperature (°C)
10^6
10^4
100
1
Initial Copy Number
C
M 0 1 10 100 10^3 10^4 10^5 10^6
d
Corrected Fluorescence
3
2
1
0
1
11
21
31
41
Cycle Number
D

Figure 43

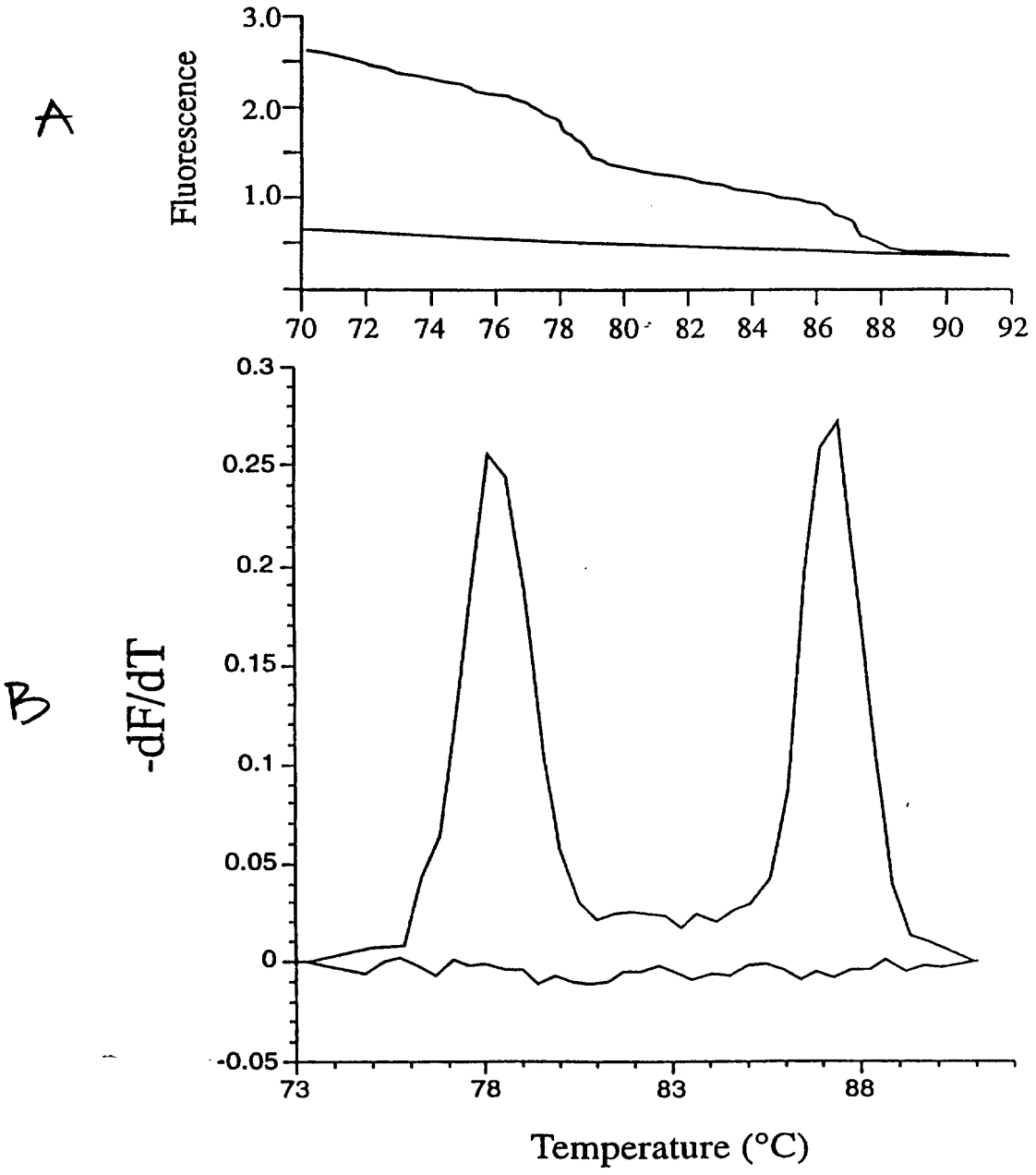

A
Fluorescence
3.0
2.0
1.0
70 72 74 76 78 80 82 84 86 88 90 92
B
-dF/dT
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
73 78 83 88
Temperature (°C)

Figure 44

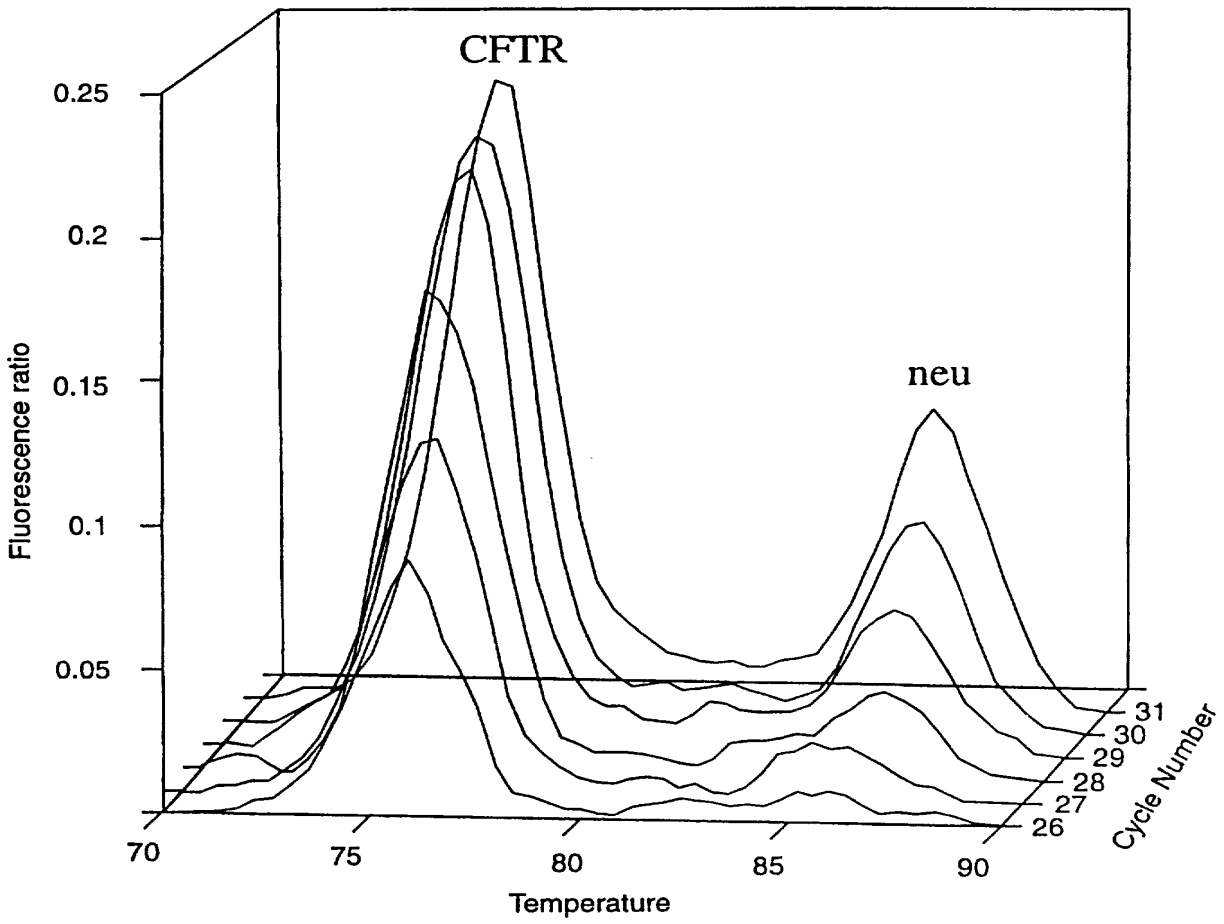

CFTR
neu
0.25
0.2
0.15
0.1
0.05
Fluorescence ratio
70
75
80
85
90
Temperature
31
30
29
28
27
26
Cycle Number

Figure 45

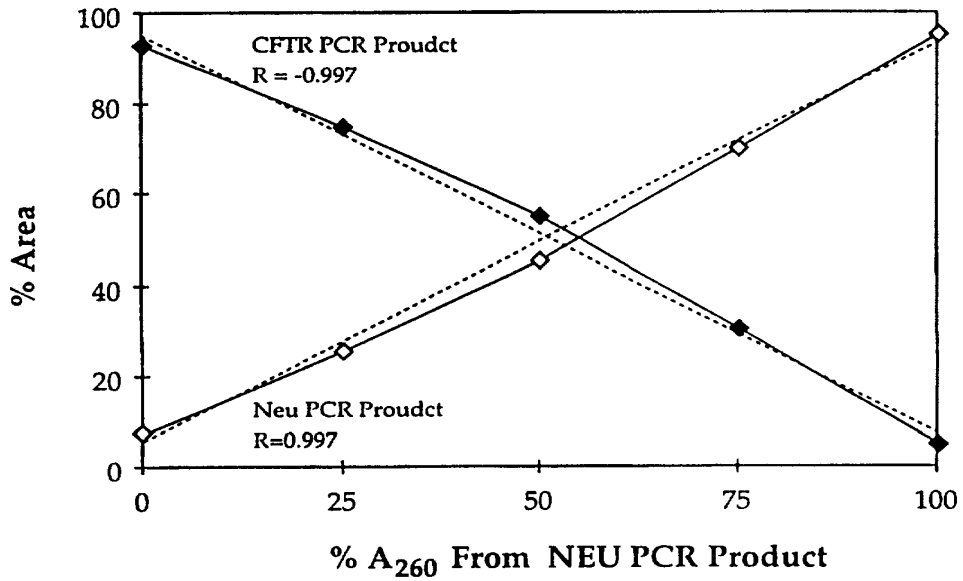

100
80
60
40
20
0
% Area
CFTR PCR Proudct
R = -0.997
Neu PCR Proudct
R=0.997
0
25
50
75
100
% A260 From NEU PCR Product

FIG. 46A

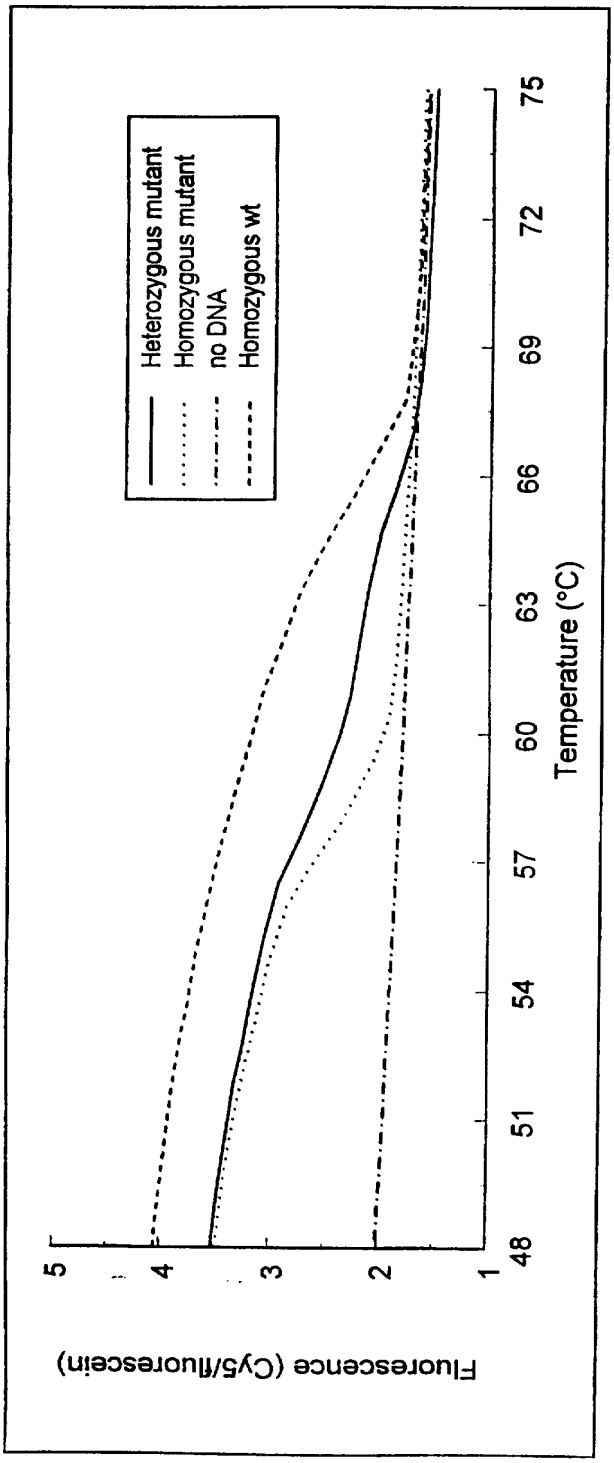
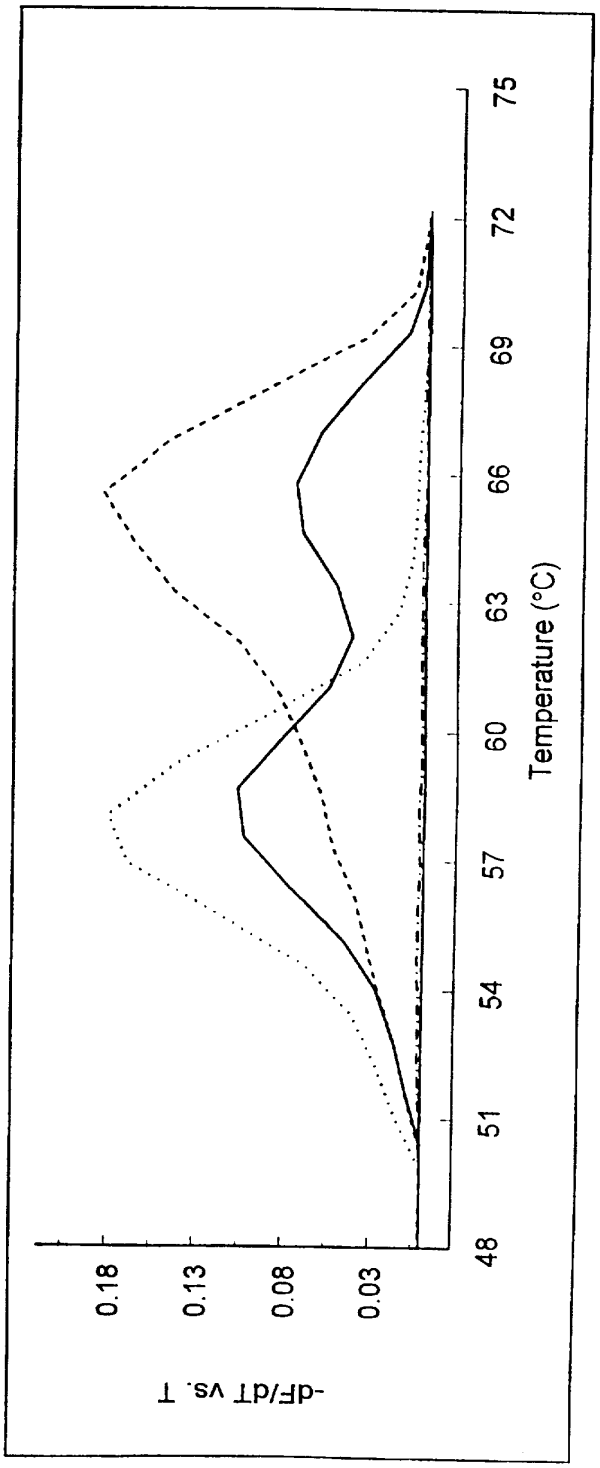


FIG. 46B



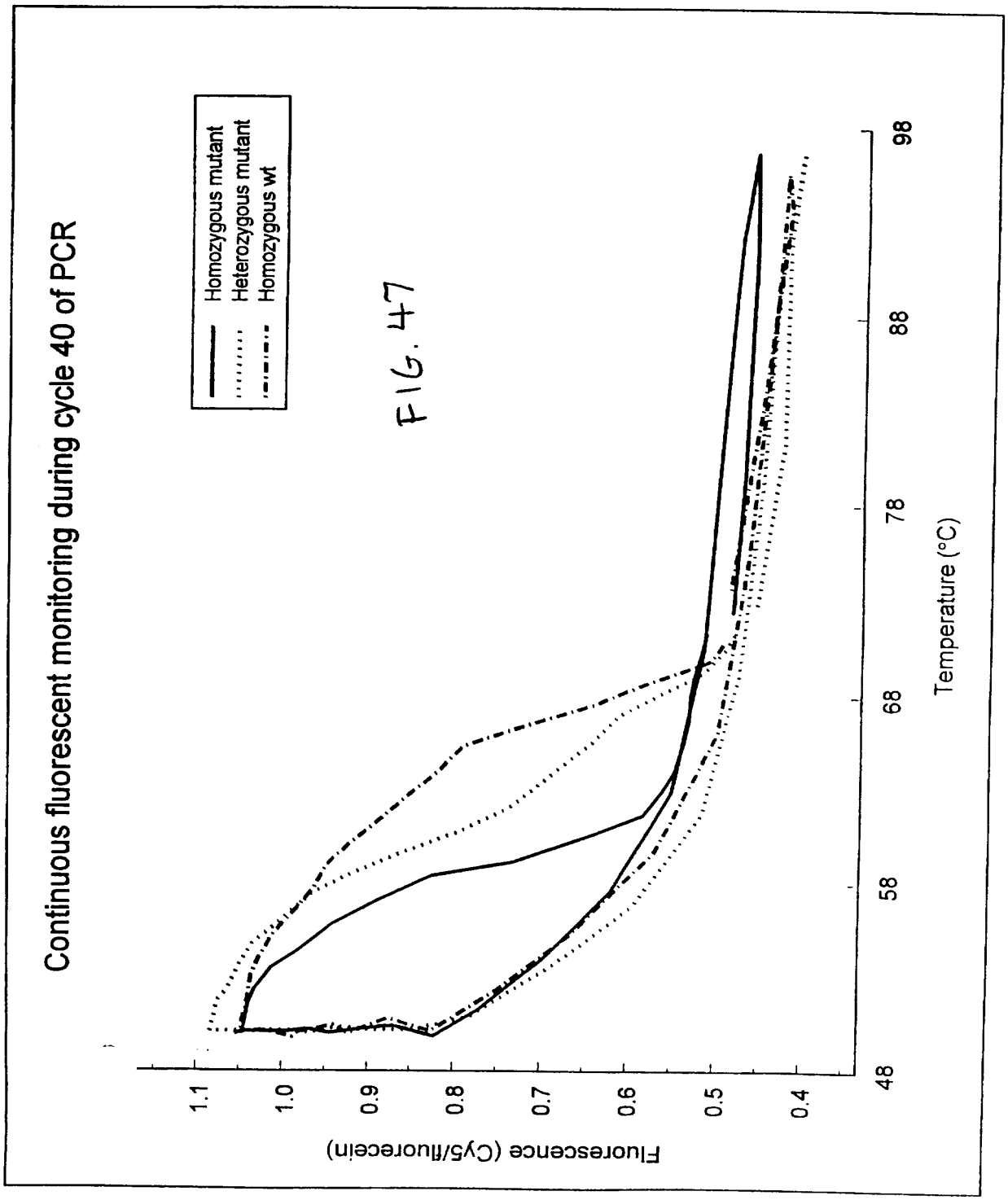

Continuous fluorescent monitoring during cycle 40 of PCR
Homozygous mutant
Heterozygous mutant
Homozygous wt
Fluorescence (Cy5/fluorecein)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
48
58
68
78
88
98
Temperature (°C)

FIG. 47

Figure 48

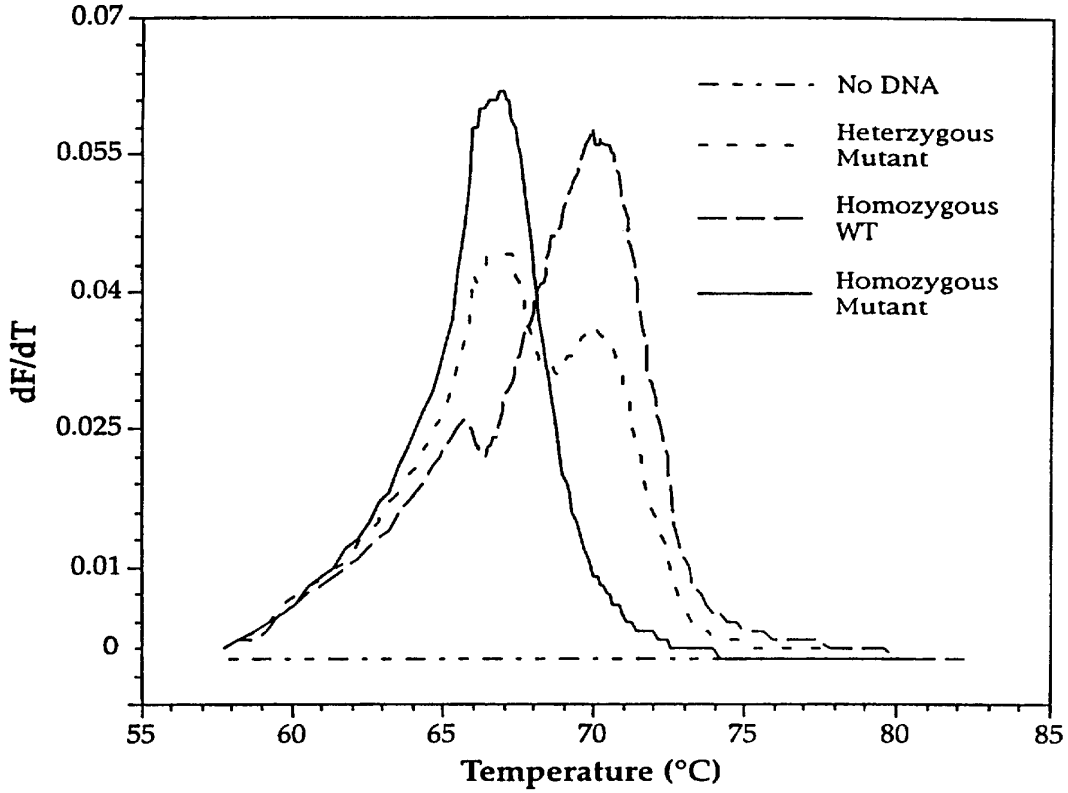

0.07
0.055
0.04
0.025
0.01
0
dF/dT
55
60
65
70
75
80
85
Temperature (°C)
No DNA
Heterzygous Mutant
Homozygous WT
Homozygous Mutant

Figure 49

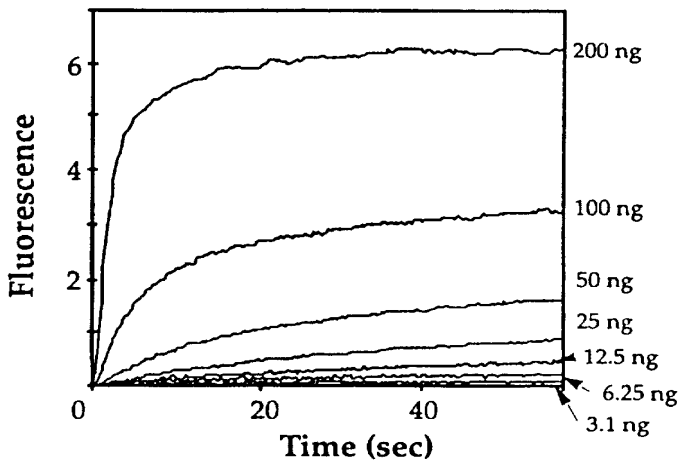
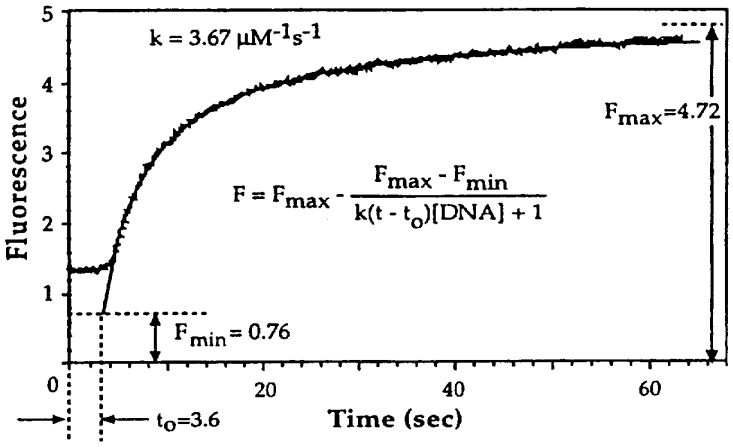

Fluorescence
6
4
2
0
20
40
Time (sec)
200 ng
100 ng
50 ng
25 ng
12.5 ng
6.25 ng
3.1 ng

FIGURE 50


k = 3.67 μM-1s-1
Fmax=4.72
F = Fmax - (Fmax - Fmin) / k(t - to)[DNA] + 1
Fmin = 0.76
Fluorescence
Time (sec)
to=3.6
0
1
2
3
4
5
20
40
60

LightCycler Functional Block Diagram

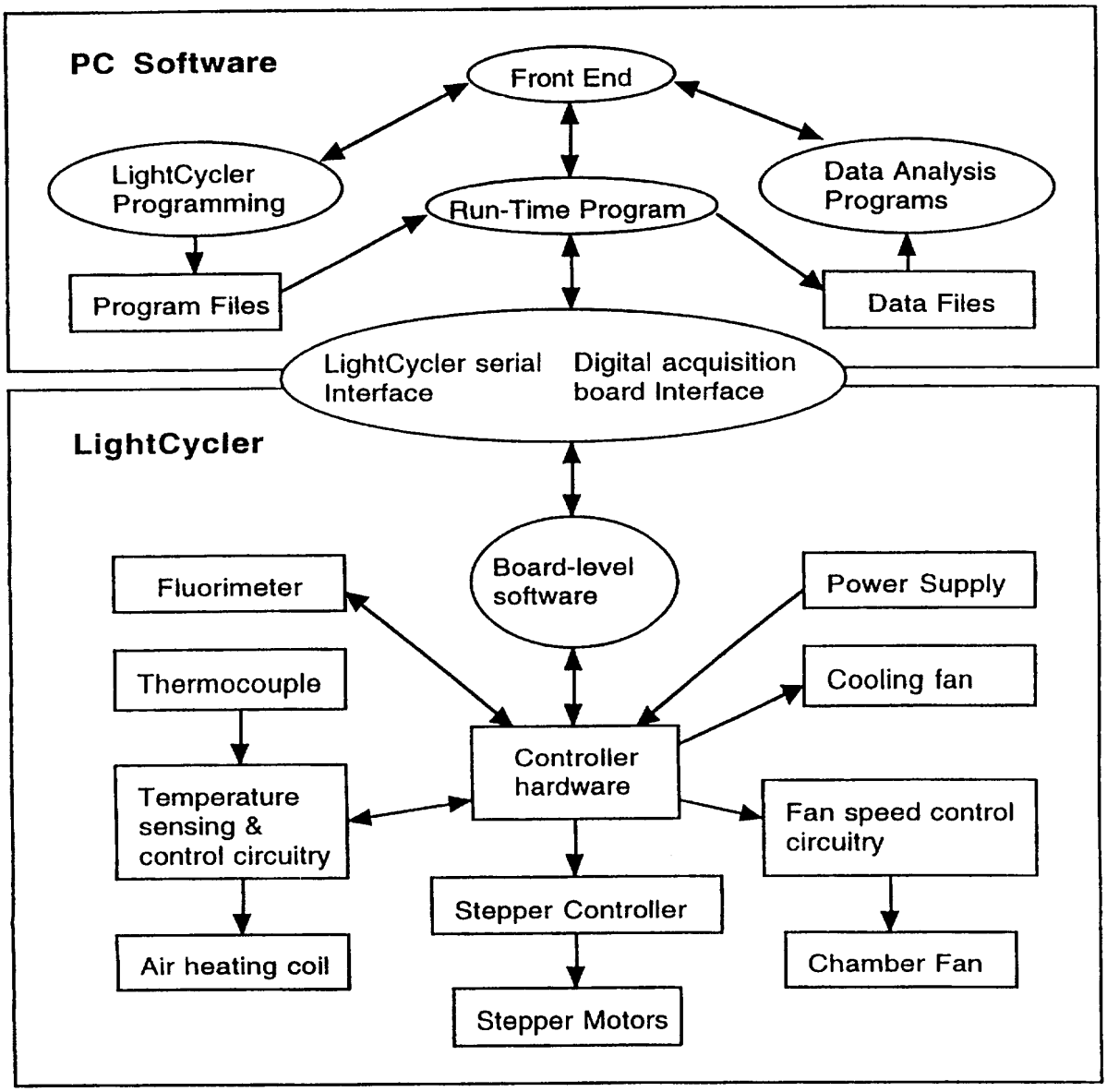
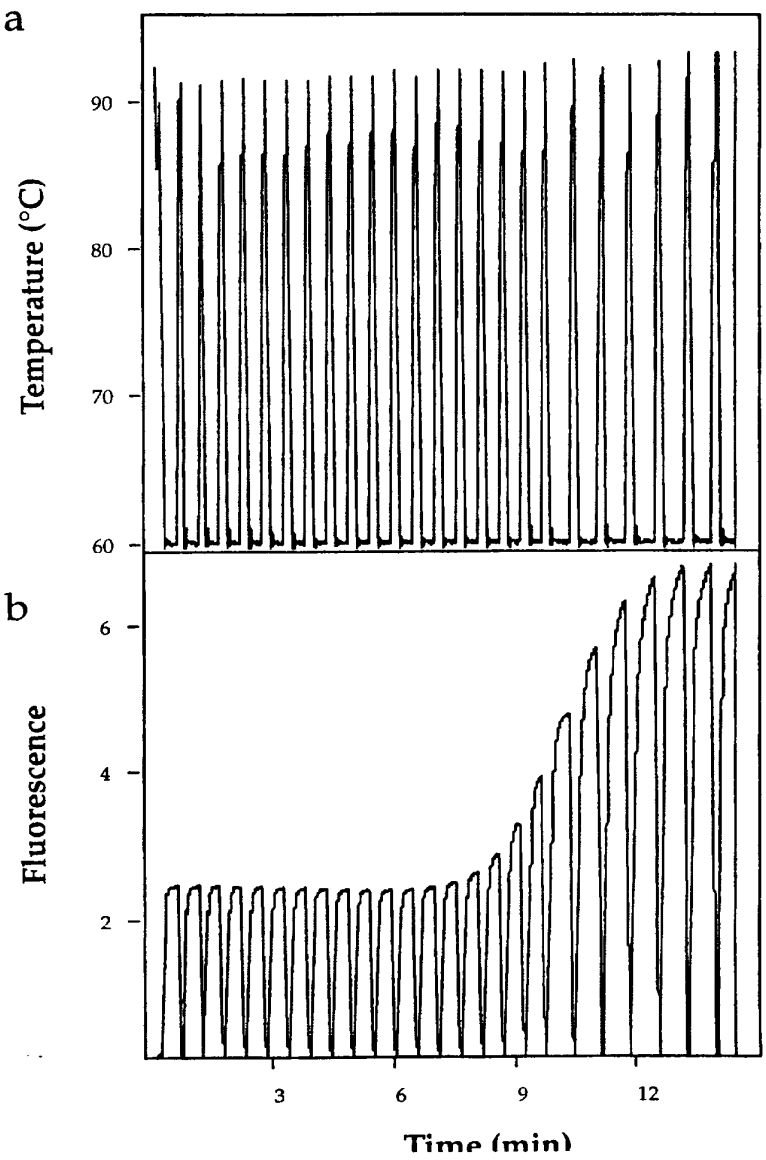


FIG. 51

Figure 52



INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/10008

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 0 566 751 A (HOFFMANN LA ROCHE) 27 October 1993 see the whole document ---	6,7,30, 34 1-5,22, 55,56
X Y	EP 0 229 943 A (MOLECULAR BIOSYSTEMS INC) 29 July 1987 see the whole document ---	15-21 1-5,22, 55,56
Y	WO 96 00901 A (UNIV CALIFORNIA) 11 January 1996 see the whole document ---	1-5,22, 55,56, 118,119
Y	WO 95 32306 A (BIOTRONICS CORP.) 30 November 1995 see the whole document --- -/--	1-5,22, 55,56

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 October 1997	18. 11. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

Form PCT/ISA/210 (second sheet) (July 1992)

IPR2024-01451
California Institute of Technology - Ex. 2003 Page 4049

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/10008

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 13399 A (PUBLIC HEALTH RESEARCH INST OF) 18 May 1995 see the whole document	1-5,22, 55,56
Y	WO 95 30139 A (PERKIN ELMER CORP) 9 November 1995 see page 9, line 20 - page 14	1-5,22, 55,56, 118,119
X	EP 0 640 828 A (HOFFMANN LA ROCHE) 1 March 1995 cited in the application	28,29, 57-60, 114,115, 120-123
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(54) Title: LIQUID LABELING WITH FLUORESCENT MICROPARTICLES

(57) Abstract

Methods of labeling liquids, solids, aerosols, and gases with fluorescent microparticles and/or with microparticles encoded with at least one other discriminator. Labeling protocols are described for such uses as adding at least one date code (for example, the expiration date of a perishable liquid), encoding routing information for one or more liquids in a pipeline having a plurality of branch points, tracing the flow of toxic or other substances into groundwater, tracing liquid flow from a point source into groundwater, tracing groundwater flow, adding a donor Social Security Number, adding a donor name, tracing flow of a liquid or a gas in a living system, adding unique identification to each of a plurality of liquid, solid or gaseous samples, encoding routing information to each of a plurality of liquid samples in a multiplexed analytical system, encoding routing information to each of a plurality of liquid reagents in a multiplexed analytical system, and combinations thereof. In one embodiment, two different fluorescent dye labels are bound to microparticles at eight independent, different concentrations each to create an 8 x 8 = 64 array of distinct microparticle sets suitable for labeling liquids, solids, and gases. When three discriminators are used at 8 levels each, an 8 x 8 x 8 = 512 array of distinct microparticle sets is created. Discriminators include such parameters as particle physical property, particle concentration, spectral property, dye type, dye concentration, label type, and combinations thereof; where particle physical property includes such attributes as particle size, particle shape, particle hydrophobicity, particle hydrophilicity, particle density, and combinations thereof; where spectral property includes such attributes as dye fluroescence absorption, dye fluorescence emission, dye absorption, dye fluorescence polarization, dye fluorescence lifetime, and combinations thereof; and where label type includes such attributes as magnetic property, reactive group, nuclear magnetic resonance, electron spin resonance, positron emission, radioactive property, fluorescence polarization, fluorescence lifetime, and combinations thereof. Kits are devised to permit users to create a large variety of sets of particles for identification purposes in liquids, solids, and gases, and microprocessor controlled, automatic pipetting of kit microparticles are also encompassed by the instant invention.

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LIQUID LABELING WITH FLUORESCENT MICROPARTICLES

1.0 Cross Reference to Related Applications

This Application claims the priority date of Provisional U.S. Application Serial No. 60/081,487, filed April 13, 1998, the disclosure of which is herein incorporated by reference in its entirety.

2.0 Field of the Invention

The present invention relates generally to a method of labeling liquids. In particular, the present invention relates to a method of labeling liquids with particles, and to flow cytometric amenable methods of encoding such labels in liquids. Such particles and methods of their use also have applications in labeling solids and gases.

3.0 Background of the Invention

Knowing the correct identity of different liquids is critical in a variety of contexts. For example, the absolute identity of different grades of aviation fuel is required for optimum performance and safety in airplanes and other aircraft. Typically, different grades of aviation fuel are identified according to color in the visual spectrum, based on dyes added exogenously.

Fluids required by automobiles, other vehicles, motorized tools and equipment in general provide additional examples of the need for correct identification of liquids. For such motorized mechanical devices, fluids therefor are colored according to the mechanical compartment for which use is intended. Such color coding clearly is designed to prevent, or at least minimize the possibility of, damage or even total destruction of the mechanical device that would result from introduction of the incorrect fluid into a given mechanical compartment. Thus, the color of the antifreeze solution is different from that of the brake fluid, etc.

In addition, as a part of a quality assurance or quality control program, there often is a need to verify the identities of various biological and other fluids that are undergoing analysis. This is particularly true in situations where integrity and identity of a sample is critical, for example, in legal situations such as court ordered testing. Examples of biological fluids that could benefit from labeling include separated or unfiltered biological fluids such as urine, cerebrospinal fluid, blood, serum, plasma, lymph fluids, tissue homogenates, interstitial fluid, cell extracts, mucus, saliva, sputum, stool, semen, vaginal secretions, lacrimal fluid, physiological secretions, or other similar

fluids. Examples can also include samples obtained from environmental sources, such as soil, water, or air; or from industrial sources, such as those taken from a waste stream, a water source, a supply line, or a production lot. Industrial sources can include such fluids as fermentation media, for example, from a biological reactor or food fermentation process, or fluids generated from foodstuffs, such as from meat, grain, produce, or dairy products.

Liquids are notoriously difficult to label and thus identify. The use of visible spectrum color indicators for fluid identification suffer from at least two inherent disadvantages: 1) a limited range of separately identified liquids due to the limited range of colors that are readily distinct to the human eye, and 2) ease of alteration and/or sabotage of the liquid for which the easy, visual based identification is sought. In addition, a significant portion of the human population suffers from color blindness, thereby increasing problems of identification related to the use of visible spectrum color indicators. This difficulty is further compounded because clinically distinct forms of color blindness have different wavelength ranges over which color perception is absent or impaired.

In addition to the perception of color, viscosity, odor, taste, and "feel" can also provide useful identification parameters. However, not only are these alternate sensory modes of identification typically impractical, or even dangerous to perform, they generally give ambiguous and inconclusive results.

A need therefore exists for a means to label liquids which is precise, tamper proof, reproducible, and easily read.

Fluorescent dyes and lasers have been used for a number of years to label and detect cells, biochemical interactions, and uniform latex particles. For such uses, laser techniques often are utilized to determine the presence or absence of a dyed constituent, and/or concentrations thereof. Fluorescent dyes have been applied to such uses as: (1) detecting the presence (and quantity) or absence of cells and/or specific types of cells in a fluid sample; (2) gauging cell vitality; (3) estimating pH and/or changes in pH in cellular compartments; (4) determining intracellular pH measurement in general; (5) identifying acidic vesicles in cells; (6) identifying DNA; (7) identifying "GC" rich or "AT" rich regions of DNA; (8) identifying cellular surface markers; (9) quantifying analytes by ELISA plate assays; (10) estimating blood flow; (11) labeling proteins for gel electrophoresis analysis; (12) labeling cellular cytoskeleton or other intracellular proteins; (13) identifying hydrophobic and hydrophilic sites on proteins and membranes; (14) measuring total amounts of protein in a sample; (15) measuring intra-molecular distances by energy transfer; (16) performing optical sectioning with confocal microscopy; (17) measuring and studying various molecular and physical chemical properties and biochemical parameters, including enzyme-substrate interactions, as well as interactions between enzymes and various modulators (activators, inhibitors,

cofactors, etc.) or other molecules, including interactions with homologous and heterologous protein subunits, and with antibodies; and (18) detecting the presence of, absence of, and/or number of, uniform dyed particles in a liquid sample.

According to the practice of earlier technologies, particles often have been labeled with a single fluorescent dye. In general, these particles are made by a copolymerization process, wherein monomers, such as unsaturated aldehyde or acrylate, are allowed to polymerize in the presence of a fluorescent dye, such as fluorescein isothiocyanate or "FITC," in the reaction mixture. For example, see U.S. Patent Nos. 4,267,234 to Rembaum, 4,267,235 to Rembaum, et al., 4,552,812 to Margel, et al. and 4,677,138 to Margel.

The use of two or more dyes of varying proportions would increase the permutation number of unique combinations of dyes in or on a single particle. Particles having varying combinations of unique characteristics, such as differences in emission wavelengths and/or fluorescence intensities, could be extremely useful for labeling liquids.

However, several problems arise when more than one dye is absorbed into or onto a particle. First, the close proximity of embedded dye molecules gives rise to significant amounts of fluorescent energy transfer (for example, Stokes shifts). This energy transfer leads to fluorescent emissions that are inconsistent with relative dye concentrations and their original emission patterns.

Second, difficulties arise when the dyes have differing solubilities in the solvent used to incorporate the dye in or on the particles. Typically, all dyes must be absorbed simultaneously; otherwise, in practice, possible dye ratios are restricted by solvent properties.

A third problem that has been encountered when multiple dyes are embedded in particles is the change in dye spectra. Specifically, it has been noted that, when the particle is composed of crosslinked polystyrene, a significant broadening of the fluorescent emission peak occurs. This can result in an overlapping of the emission spectra of neighboring dyes.

A need therefore exists for multicolored, fluorescent particles comprising a plurality of defined sets of stained multicolored microparticles, and/or a plurality of defined sets of dyed nanoparticles attached to a carrier microparticle, which can be used for labeling liquids. It would also be desirable to use such microparticles or carrier microparticles in conjunction with other labeling elements ("discriminators"), such as radioactive property, nuclear magnetic resonance, electron spin resonance, chemical reactive group, dye fluorescence absorption, dye fluorescence emission, fluorescence polarization, fluorescence lifetime, positron emission, concentration of particles, particle hydrophobicity, particle hydrophilicity, particle density, particle size, particle shape, dye concentration in or on particles, and dye type. Applications of this invention include, but are not limited to: (1) adding date codes to perishable or other liquids; (2) adding routing

information to liquids in a pipeline; (3) tracing toxic substances in groundwater; (4) adding donor's Social Security Number to blood units; (5) tracing absorption and/or secretion and excretion patterns of fluids into, out of, or within plant and animal tissues; (6) adding patient identification to clinical samples; (7) adding identifiers to clinical reagents; and (8) adding routing information to liquids in a process stream, for example, a plurality of liquid samples from one or more patients or other sources of samples, where the liquid samples are each routed to an analytical machine for a specific assay, or to an analytical machine for a panel of assays.

4.0 Summary of the Invention

It is therefore an object of the present invention to provide methods, instrumentation, and products for precisely and unambiguously labeling fluids (liquids, aerosols, and gases), and solids.

It is a further object of the present invention to provide methods, instrumentation, and products for precisely and unambiguously reading the labels within the fluids (liquids, aerosols, and gases), and solids.

The present invention uses precisely dyed microparticles and/or nanoparticles attached to carrier microparticles, and also may utilize a variety of other discriminators. In a preferred embodiment, the particles are made of polystyrene, crosslinked with 5 percent divinyl-benzene, and have a diameter of about 5.5 microns. The particles are impregnated with three different fluorescent dyes in eight different concentrations of each of the dyes, so as to create 512 subsets of labeled particles. The subset classification of each particle is identified by fluorescence measurement using appropriate instrumentation, such as a flow cytometer. Depending upon the needs of a particular application, the discriminators can be combined in a variety of ways to achieve the required number of unique labels. A first embodiment uses precisely dyed microparticles (core microparticles). In an alternative embodiment, precisely dyed nanoparticles are attached to carrier microparticles. Other embodiments incorporate such discriminators as radioactive property, nuclear magnetic resonance, electron spin resonance, chemical reactive group, dye fluorescence absorption, dye fluorescence emission, fluorescence polarization, fluorescence lifetime, positron emission, concentration of particles, particle hydrophobicity, particle hydrophilicity, particle density, particle size, particle shape, dye concentration in or on particles, and dye type. Thus, use of microparticles according to the present invention functions as an identifying means analogous to a bar code.

Multiplexed diagnostic instruments, such as those in the current art (including flow cytometers), can be programmed to recognize each specific permutation or particle set, look up the parameters in a database, and identify the sample's origin/destination and/or perform the appropriate analysis or routing function.

The present invention provides a method of labeling a gaseous, liquid, or solid material, including combinations thereof, to permit the subsequent identification of the labeled gaseous, liquid, or solid material, which comprises adding to a gaseous, liquid, or solid material, whose subsequent identification is desired, a sufficient quantity of particles belonging to one or more distinguishable sets of particles, to provide a labeled gaseous, liquid, or solid material that subsequently can be identified. In a particular embodiment of the invention, the method makes use of particles in which the particles in each set exhibit one or more discriminators that distinguish the particles of one set from those of another set. In particular, the one or more discriminators is selected from a physical, chemical, biological, magnetic, or spectral property of the particle or combinations thereof. Specific properties are described further in the detailed description of the preferred embodiments of the invention and others may be apparent to those of ordinary skill in the art.

In the present method, for example, a sufficient quantity of a combination of particles belonging to two or more distinguishable sets of particles is added to the gaseous, liquid, or solid material, whose subsequent identification is desired.

The present invention also provides a method of identifying a gaseous, liquid, or solid material, including combinations thereof, which has been labeled with a sufficient quantity of particles belonging to one or more distinguishable sets of particles. This method comprises: (a) obtaining a sample comprising a gaseous, liquid, or solid material that has been labeled with a sufficient quantity of particles belonging to one or more distinguishable sets of particles; (b) analyzing the particles found in the sample to determine which of one or more distinguishable sets of particles such particles belong to; and (c) comparing the results of the analyzing step with a key which establishes the identity of the gaseous, liquid, or solid material from which the sample was taken.

In various embodiments, the present invention makes possible the manufacture, use and sale of labeled materials which comprise (i) a gaseous, liquid, or solid material, including combinations thereof, and (ii) a sufficient quantity of particles that can be shown to belong to one or more distinguishable sets of particles. In a preferred embodiment the labeled gaseous, liquid or solid material makes use of particles in which the particles in each set exhibit one or more discriminators that distinguish the particles of one set from those of another set. Kits for labeling liquids, solids, aerosols, and gases comprise particles from a plurality of distinct particle sets, and a dispenser.

6.0 Description of the Specific Embodiments

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One method of practice of the present invention involves the use of precisely dyed core microparticles. An alternative method of practice entails the use of carrier microparticles to which nanoparticles comprising a plurality of dyes with spectrally distinct fluorescence emission spectra are attached, preferably covalently. The following summarizes exemplary but non-limiting embodiments for the production of such labeled particles suitable for use in practice of the present invention, as well as how to use such particles to label liquids, and subsequently identify such labels, each of which may be used to uniquely identify a specific liquid. As used herein: (1) the terms nanospheres and nanoparticles have equivalent meanings; (2) in the context of incorporating labels (fluorescent and otherwise) into or onto particles, the terms attach, attachment, bound, coupled, etc., are used broadly to include covalent and non-covalent incorporation of chemicals and dyes, particles, etc., as well as entrapment of such entities within the matrix of particles ("matrix entrapment"), for example, subsequent to the swelling of a particle; (3) the terms microparticles, microspheres, or microbeads bear equivalent meanings; (4) particles refers to either microparticles or nanoparticles; (5) where nanoparticles are attached to microparticles, the microparticles are referred to as carrier microparticles; and (6) where microparticles are precisely dyed, the microparticles are referred to as core microparticles.

6.1 Particles

In one embodiment, nanoparticles are coupled to carrier microparticles to form sets of distinct particles. Basically, fluorescently distinguishable particle sets are obtained through variation of the amount or type of dye in or on each nanoparticle. Then, the desired number of dye categories of nanoparticles are attached to the surface of each carrier microparticle. Ratios of differently dyed nanoparticles attached to the surfaces of the carrier microparticles are selected according to the particular need, and in accord with the encoding or identification criteria developed therefor. These ratios reflect differences in the dyes used, and in their respective quantities attached to each particle.

Similarly, and in an alternative embodiment, fluorescently distinguishable microparticle sets are obtained through variation of the amount or type of dye in or on each microparticle, and no nanoparticles are utilized. The encoding or identification criteria can be identical to those used with nanoparticles.

Nanoparticles used in this invention are commercially available in sizes ranging from about 5 to 10 nanometers (nm) to more than 1,000 nm in diameter. Optimal diameters typically will fall within the range from about 10 to about 1,000 nm, preferably from about 200 to about 500 nm. Nanoparticles as small as about 2-3 nm can also be prepared.

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Microparticles that are used as carrier particles, to which the nanoparticles are attached, typically range in diameter from about 0.1 to about 100 micrometers (μm). Even though a microparticle for practicing the present invention can be of any size, the preferred diameter usually will range from about 0.1 to about 50 μm, more preferably from about 1 to about 20 μm, and even more preferably from about 2 to about 12 μm. The particles preferably will exhibit a high degree of uniformity with respect to size, or to a set of discretely different size classes such that 1) the differences can be determined by size-dependent properties, such as light scattering, and 2) the high degree of uniformity within each class size is sufficient to permit the use of particle size as a discriminator.

Microparticles used in this invention as core particles have a diameter of less than one millimeter, and preferably in the range of about 0.1 to 1,000 micrometers (μm). Even though the microparticle can be of any size, the preferred size is 1-100 μm, more preferably 2-50 μm, more preferably 3-25 μm, and even more preferably about 5-12 μm.

Particles are made of any approximately regularly shaped material. Often, the preferred shape will be approximately spherical; however, particles of any other shape can be employed since, for most uses, this parameter will be immaterial to practice of the instant invention. However, the shape of a class of particles also can serve as an additional discriminator, which can be detected by a flow cytometric method, for example, by a high-resolution slit-scanning method that measures forward and side light scattering parameters.

Usually the nanoparticles and the microparticles are made of the same material, such as polystyrene or latex. However, other polymeric materials are acceptable, including polymers selected from chemical groups such as carbohydrate-based polymers, polyaliphatic alcohols, poly(vinyl) polymers, polyacrylic acids, polyorganic acids, polyamino acids, co-polymers, block co-polymers, tert-polymers, polyethers, naturally occurring polymers, polyimids, surfactants, polyesters, branched polymers, cyclo-polymers, polyaldehydes, and mixtures thereof. More specifically, brominated polystyrene, polyacrylic acid, polyacrylonitrile, polyamide, polyacrylamide, polyacrolein, polybutadiene, polycaprolactone, polyester, polyethylene, polyethylene terephthalate, polydimethylsiloxane, polyisoprene, polyurethane, polyvinylacetate, polyvinylchloride, polyvinylpyridine, polyvinylbenzylchloride, polyvinyltoluene, polyvinylidene chloride, polydivinylbenzene, polymethylmethacrylate, polylactide, polyglycolide, poly(lactide-co-glycolide), polyanhydride, polyorthoester, polyphosphazene, or combinations thereof, are preferable. Representative combination polymers of which the polymeric particles can be composed include, for example, poly-(styrene-co-vinylbenzyl chloride-co-acrylic acid) (in approximately an 85:10:5 molar ratio), poly(styrene-co-acrylic acid) (in approximately a 99:1 molar ratio),

poly(styrene-co-methacrylic acid) (in approximately a 90:10 molar ratio), poly(styrene-co-acrylic acid-co-m&p-divinyl-benzene) (in approximately an 89:10:1 molar ratio), poly-(styrene-co-2-carboxyethyl acrylate) (in approximately a 90:10 molar ratio), poly(methyl methacrylate-co-acrylic acid) (in approximately a 70:30 molar ratio) and poly(styrene-co-butyl acrylate-co-methacrylic acid)(in approximately a 45:45:10 weight ratio). Other exemplary polymers that can be used for the formation of microparticles include homopolymers and copolymers of lactic acid and glycolic acid (PLGA) (for example, as described in U.S. Patent Nos. 5,213,812 to Ruiz; 5,417,986 to Reid, et al.; 4,530,840, 4,897,268, 5,075,109, and 5,360,610 to Tice, et al.; 5,102,872 to Singh, et al.; 5,384,133 to Boyes, et al.; and European Patent Application Publication Number 248,531 to Southern Research Institute); block copolymers, such as tetronic 908 and poloxamer 407 (for example, as described in U.S. Patent No. 4,904,479 to Illum); and polyphosphazenes (for example, as described in U.S. Patent No. 5,149,543 to Cohen, et al.). Particles formed from synthetic polymers (such as polystyrene, polyacrylamide, polyacrylate, or latex) are commercially available from numerous sources, such as Bio-Rad Laboratories (Richmond, Calif.) and LKB Produkter (Stockholm, Sweden). Particles formed from natural macromolecules and particles, such as agarose, crosslinked agarose, globulin, deoxyribose nucleic acid, and liposomes, are commercially available from a variety of sources, such as Bio-Rad Laboratories, Pharmacia (Piscataway, N.J.), and IBF (France). Particles formed from copolymers of polyacrylamide and agarose are commercially available from various sources, including IBF and Pharmacia. Magnetic particles are commercially available from such sources as Dynal Inc. (Great Neck, N.Y.). Other materials, such as carbohydrates (e.g., carboxymethyl cellulose, and hydroxyethyl cellulose), proteinaceous polymers, polypeptides, eukaryotic and procaryotic cells, viruses, lipids, metals, resins, rubbers, silicas, and silicones (e.g., polydimethyldiphenyl siloxane, glass, ceramic, and the like), also can be utilized, depending on the specific use therefor.

Nanoparticles are preferably made of the same material as the microparticles. However, if required or advantageous, they can be made of different material.

The particles typically will also contain approximately 1% to approximately 50% by weight of one or more cross-linking agents, such as divinyl benzene, ethylene glycol dimethacrylate, trimethylol propane trimethacrylate, or N,N'methylene-bis-acrylamide, or other functionally equivalent agent(s) known in the art. Crosslinking of a carbohydrate polymer, such as hydroxypropyl cellulose, can be achieved with such agents as adipic acid, sebacic acid, succinic acid, citric acid, 1,2,3,4-butanetetracarboxylic acid, or 1,10-decanedicarboxylic acid. In a preferred embodiment, microparticles and nanoparticles are made of polystyrene, and contain about 1% to about 50% by weight of divinyl benzene.

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The particles also can have additional surface functional groups to facilitate attachment to each other or to yet another entity, examples including carboxylates, esters, alcohols, carbamides, aldehydes, amines, sulfur oxides, nitrogen oxides, and halides. For many practical applications, it is critical that the particles have surface carboxyl groups available for attachment of a reactive amine- or sulfhydryl-containing compound, for example. Such groups are preferably added to the particles by incorporating monomers containing such groups into the polymers. These monomers can include, but are not limited to, acrylic acid, methacrylic acid, itaconic acid, and the like. Alternatively, they can be added to the particles by further chemical reaction of a polymer having other precursor reactive groups, which then can be converted to carboxyl groups, for example, by hydrolysis of anhydrides, such as maleic anhydride, or by oxidation of surface methylol or aldehyde end groups. Other compounds, such as diamines, dihydrazides, mercaptoalkylamines and dimercaptans, can be attached to particles as linking moieties for later attachment of drugs, enzymes or other reactive species, including nanoparticles. Although the preferred attaching method is by covalent linkage, non-covalent binding methods, such as adsorption, can also be used.

6.2 Dyes

A dye is selected to give a detectable response that is different from that of other dyes and /or discriminators. Any fluorescence detection system (including visual inspection) can be used to detect differences in spectral properties between dyes, with differing levels of sensitivity. Such differences include, but are not limited to, differences in excitation maxima, emission maxima, fluorescence lifetimes, fluorescence emission intensity at the same excitation wavelength or at a different wavelength, absorptivity, fluorescence polarization, or combinations thereof. Thus, the detectably different dye has different spectral properties and/or different selectivity.

In one aspect of the invention, the two or more dyes forming the dye-microparticle complex have the same or overlapping excitation spectra, but possess distinctly different emission spectra, generally having emission maxima separated by greater than 10 nm, preferably greater than 20 nm, and more preferably greater than 50 nm. Simultaneous excitation of all fluorescent reagents may require excitation of the sample at a wavelength that is suboptimal for each reagent individually, but optimal for the combination of reagents. Alternatively, the additional reagent(s) can be simultaneously or sequentially excited at a wavelength that is different from that used to excite the subject dye.

A preferred category of fluorescent dyes used in this invention are of the general class known as polymethine cyanine dyes, with typical emission wavelengths falling between 550 nm and 900 nm. Spectral properties, including emission wavelengths, are strongly influenced by the number of

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methine groups. Thus, the monomethine dyes that are pyridines typically have blue to blue-green fluorescence emissions, whereas those that are quinolines typically have green to yellow-green fluorescence emissions. On the other hand, the fluorescence emissions of the trimethine dye analogs are substantially shifted toward red wavelengths, and those of pentamethine dyes are shifted even further, often exhibiting infrared fluorescence emissions (for example, see U. S. Patent No. 5,760,201). Although polymethine cyanine dyes constitute a preferred category of dyes for practice of the present invention, any dye that is soluble in an organic solvent (i.e., hydrophobic dyes) can be used when organic liquids are to be labeled.

On the other hand, when predominantly aqueous liquids are to be labeled, the above-indicated hydrophobic dyes, including the hydrophobic particles to which they are attached, may be used by subsequently or previously attaching hydrophilic groups to increase the solubility in aqueous solutions. In addition, ranges of hydrophilicity and hydrophobicity for liquids (i.e., different proportional mixtures thereof) are contemplated for mixed solvents, such as oil-in-water, water-in-oil, micelles, mixed micelles, detergent solutions, etc.

Squaric acid based fluorescent dyes can be synthesized by methods described in the literature; for example, see Sprenger, et al. *Angew. Chem.*, 79, 581 (1967); *Angew. Chem.*, 80, 541 (1968); and Maaks, et al. *Angew. Chem. Int'l. Ed.*, 5, 888 (1966). Briefly, one equivalent of squaric acid (1,2-dihydroxycyclobutenedione) is condensed with two equivalents of an active compound, such as a pyrrole, indoline, or aniline, and refluxed in a mixture of an alcohol and an aromatic solvent (such as benzene) under conditions that allow removal of water from the reaction mixture. The resulting dye can be collected and purified by any of such standard methods as recrystallization, distillation, chromatography, etc. Additionally, unsymmetrically substituted squaric acid compounds can be synthesized by methods such as those described by Law, et al., *J. Org. Chem.*, 57, 3278, (1992). Specific methods of making such dyes are well known in the art, and can be found, for example, in U.S. Patent Nos. 5,795,981; 5,656,750; 5,492,795; 4,677,045; 5,237,498; and 5,354,873. Some practical uses of such fluorescent dyes, for example, phthalocyanines, 2,3-naphthalocyanines, squaraines and croconic acid derivatives, are disclosed in U.S. Patent No. 5,525,516 to Krutak, et al.

Optionally, such dyes will contain functional groups capable of forming a stable fluorescent product with functional groups typically found in biomolecules or polymers including, for example, activated esters, isothiocyanates, amines, hydrazines, halides, acids, azides, maleimides, alcohols, acrylamides, haloacetamides, phenols, thiols, acids, aldehydes and ketones.

In addition to the specific squaric acid fluorescent dyes that are used in this preferred embodiment, related dyes can be further selected from such classes of compounds as cyclobutenedione derivatives, substituted cephalosporin compounds, fluorinated squaraine

compositions, symmetrical and unsymmetrical squaraines, alkylalkoxy squaraines, and squarylium compounds. Some of these dyes can fluoresce at near infrared as well as at infrared wavelengths -- effectively expanding the range of emission spectra up to about 1,000 nm. In addition to squaraines (i.e., derivatives of squaric acid), hydrophobic dyes, such as phthalocyanines and naphthalocyanines, also can be selected when operation at longer wavelengths is advantageous. Other classes of fluorochromes are equally suitable for use as dyes according to the present invention. Non-limiting examples of these dyes include 3-Hydroxypyrene 5,8,10-Tri Sulfonic acid, 5-Hydroxy Tryptamine (5-HT), Acid Fuchsin, Acridine Orange, Acridine Red, Acridine Yellow, Acriflavin, AFA (Acriflavin Feulgen SITSA), Alizarin Complexon, Alizarin Red, Allophycocyanin, ACMA, Aminoactinomycin D, Aminocoumarin, Anthroyl Stearate, Aryl- or Heteroaryl-substituted Polyolefin, Astrazon Brilliant Red 4G, Astrazon Orange R, Astrazon Red 6B, Astrazon Yellow 7 GLL, Atabrine, Auramine, Aurophosphine, Aurophosphine G, BAO 9 (Bisaminophenyloxadiazole), BCECF, Berberine Sulphate, Bisbenzamide, BOBO 1, Blancophor FFG Solution, Blancophor SV, Bodipy Fl, BOPRO 1, Brilliant Sulphoflavin FF, Calcien Blue, Calcium Green, Calcofluor RW Solution, Calcofluor White, Calcophor White ABT Solution, Calcophor White Standard Solution, Carbocyanine, Carbostyryl, Cascade Blue, Cascade Yellow, Catecholamine, Chinacrine, Coriphosphine O, Coumarin, Coumarin-Phalloidin, CY3.1 8, CY5.1 8, CY7, Dans (1-Dimethyl Amino Naphaline 5 Sulphonic Acid), Dansa (Diamino Naphthyl Sulphonic Acid), Dansyl NH-CH3, DAPI, Diamino Phenyl Oxydiazole (DAO), Dimethylamino-5-Sulphonic acid, Dipyrromethene boron Difluoride, Diphenyl Brilliant Flavine 7GFF, Dopamine, Eosin, Erythrosin ITC, Ethidium Bromide, Euchrysin, FIF (Formaldehyde Induced Fluorescence), Flazo Orange, Fluo 3, Fluorescamine, Fura-2, Genacryl Brilliant Red B, Genacryl Brilliant Yellow 10GF, Genacryl Pink 3G, Genacryl Yellow 5GF, Gloxalic Acid, Granular Blue, Haematoporphyrin, Hoechst 33258, Indo-1, Intrawhite Cf Liquid, Leucophor PAF, Leucophor SF, Leucophor WS, Lissamine Rhodamine B200 (RD200), Lucifer Yellow CH, Lucifer Yellow VS, Magdala Red, Marina Blue, Maxilon Brilliant Flavin 10 GFF, Maxilon Brilliant Flavin 8 GFF, MPS (Methyl Green Pyronine Stilbene), Mithramycin, NBD Amine, Nile Red, Nitrobenzoxadidole, Noradrenaline, Nuclear Fast Red, Nuclear Yellow, Nylosan Brilliant Flavin E8G, Oregon Green, Oxazine, Oxazole, Oxadiazole, Pacific Blue, Pararosaniline (Feulgen), Phorwite AR Solution, Phorwite BKL, Phorwite Rev, Phorwite RPA, Phosphine 3R, Phthalocyanine, Phycoerythrin R, Polyazaindacene Pontochrome Blue Black, Porphyrin, Primuline, Procion Yellow, Propidium Iodide, Pyronine, Pyronine B, Pyrozal Brilliant Flavin 7GF, Quinacrine Mustard, Rhodamine 123, Rhodamine 5 GLD, Rhodamine 6G, Rhodamine B, Rhodamine B 200, Rhodamine B Extra, Rhodamine BB, Rhodamine BG, Rhodamine WT, Rose Bengal, Serotonin, Sevron Brilliant Red 2B, Sevron Brilliant Red 4G, Sevron Brilliant Red B, Sevron Orange, Sevron Yellow L, SITS

(Primuline), SITS (Stilbene Isothiosulphonic acid), Stilbene, Snarf 1, sulphO Rhodamine B Can C, Sulpho Rhodamine G Extra, Tetracycline, Texas Red, Thiazine Red R, Thioflavin S, Thioflavin TCN, Thioflavin 5, Thiolyte, Thiozol Orange, Tinopol CBS, TOTO 1, TOTO 3, True Blue, Ultralite, Uranine B, Uvitex SFC, Xylene Orange, XRITC, YO PRO 1, or combinations thereof. One skilled in the art will readily know which one to select among such dyes, in part based on the emission, absorption, and hydrophobic/hydrophilic properties required for a particular application. The spectral properties of the fluorescent dyes often will be sufficiently similar in excitation wavelengths and intensity to fluorescein or rhodamine derivatives to permit the use of the same flow cytometry equipment. However, generally it is preferable that a dye have high solubility in the particular liquid that is to be "marked," and improved photostability and quantum yields also are advantageous.

More preferably, the multiple dyes used in a single application or in a related series of applications will have the same or overlapping excitation spectra, but possess distinguishable emission spectra ("spectrally distinct"). Any detection system can be used to detect differences in spectral characteristics between two or more dyes, including a solid state detector, photomultiplier tube, photographic film, or eye -- any of which can be used in conjunction with additional instrumentation or methodology, such as a spectrometer, luminometer microscope, plate reader, fluorescence scanner, flow cytometer, confocal microscope, scanning microscope, epifluoresis detector, digital (CCD) camera, video camera, photographic film, visual inspection, photodiode, quantum counter, photomultiplier tube, capillary electrophoresis detector, or any combination thereof, to complete a detection system. Preferably, dyes will have emission maxima separated by greater than 10 nm, more preferably greater than 25 nm, and even more preferably greater than 50 nm. When differentiation between two or more dyes is accomplished by visual inspection, the two or more dyes preferably have emission wavelengths of perceptibly different colors to enhance visual discrimination. When it is desirable to differentiate between two or more dyes using instrumental methods, a variety of filters and diffraction gratings are commercially available to allow the respective emission maxima to be independently detected. When two or more dyes are selected that possess relatively small differences in emission maxima, instrumental discrimination can be enhanced by ensuring that the emission spectra of the two or more dyes have similar integrated amplitudes and similar emission peak widths, and that the instrumental system's optical throughput will be equivalent across the emission peak widths of the respective two dyes. Instrumental discrimination can also be enhanced by selecting dyes with narrow emission peak widths; however, such dyes must necessarily possess high amplitude emissions, or be present in sufficient concentration that the loss of integrated signal strength is not detrimental to signal detection/discrimination, for example, as measured by the signal-to-noise ratio.

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6.3 Staining Process

The present invention describes techniques for precisely dyeing particles of varying sizes. The size of particles is immaterial to this invention since the precision of the dyeing process is not affected, except that (1) a given set of particles must be of uniform size within tight tolerances, for example, about ±10%, more preferably about ±5%, even more preferably about ±3%, and most preferably about ±2%, and (2) a particular application often will require a specific particle size, or range of sizes or size tolerance. Typically, particles are made of water-insoluble material, but may be made miscible or soluble in aqueous solvents by the addition of groups to increase hydrophilicity. The dyes employed are preferably squaric acid-based molecules that exhibit fluorescence extending into near infrared and/or infrared region, i.e., to about 1,000 nm. Use of other dyes may allow one to expand the range from the infrared to ultraviolet. The present method allows for a highly reproducible process in which two or more dyes of independent concentration are absorbed uniformly into or onto each particle, resulting in multiple spectrally distinct fluorescent signals corresponding to the number of different dyes present in or on the particle.

The following Examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These Examples are not intended in any way to limit the scope of the disclosure or the protection granted by Letters Patent hereon.

6.3.1 Example 1: General Outline of the Fluorescent Staining of Particles

A 5-10 gram (dry weight) quantity of stock particles in an aqueous medium is pipetted onto a vacuum filter membrane, and the liquid is removed and discarded. Next, a quantity of the rinse solvent (for example, 100 ml of an aliphatic alcohol, such as propanol, methanol, ethanol, etc.) is added to the particles. The particles are resuspended by placing an ultrasonic probe directly into the solution and applying power for several seconds, or as needed to effect resuspension. The suspension then is filtered, and the previous step is repeated once more. Dyeing of the particles is accomplished by adding 50 ml of a dye solution (composed of one or more dyes in an organic solvent) to the filtering cup and resuspending as before. The suspension then is allowed to sit for five minutes in the filtering cup. Next, 50 ml of rinse solvent is added to the dye suspension, sonicated and filtered. Another 100 ml of the rinse solvent is added, resuspended, and filtered. The last step is repeated once more. In order to prepare the particles for storage, 100 ml of an aqueous medium is added to the particles, the solution of which is then sonicated and filtered. Finally, 50 ml of aqueous medium is added to the particles, followed by sonication and transfer to a storage container.

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In a particular embodiment of the invention, one or more dyes are mixed in a solvent suitable for the complete dissolution of the dyes, such as chloroform. Ethanol is added to the solution to increase wetting of the particles, and to create a process-dependent, final solvent density that is less than that of the particles. The concentration of each of the one or more dyes is experimentally determined as a function of the target fluorescence intensity at each of two center wavelengths of two fluorescence channels of the measuring instrument. These concentrations maintain their relative intensity throughout this inventive process.

An important aspect of the present invention is the preparation of particles prior to the dyeing operation. Manufacturers often supply particles in an aqueous medium. It has been discovered that the surfaces of particles that have been stored in an aqueous medium must be treated to make them permeable to organic compounds. In a preferred embodiment, a quantity of a polar organic solvent, such as an alcohol, is added to the particle solution to achieve about a 50-50 mixture of the aqueous medium and the polar organic solvent. This ratio, however, can be varied and adjusted at will according to particular needs that one may have, or determined by chemical and physical properties of the medium and the solvent.

An equally efficient and precise alternate technique entails "drying" the particles through a series of alcohol rinses, e.g., with methanol, ethanol, 2-propanol, rinses. The process is begun by spinning down the aqueous suspension of particles, typically about 10% solids (v/v) in suspension. The aqueous medium is decanted, and the particles are re-suspended in methanol. The alcohol solution with about 5% solids is vortexed, sonicated, and spun down. This step is performed once or twice more. Excess alcohol is decanted from the pellet, and residual solvent is evaporated under vacuum.

Test samples consisting of 0.05 gram quantities of dried particles are used to help adjust the dye solution to its desired ratio. Each dried 0.05 gram quantity of particles is suspended in 0.5 ml of a dye mixture containing two or more dyes of interest. The suspension of particles, now at 10% solids (w/v), is vortexed, and sonicated into suspension. Once in suspension, the mixture of particles and dyes is mixed for one hour. After that hour, the particles are spun down for a period of one minute using a centrifuge. The dye solution is then decanted, and the 0.05 g quantity of particles is re-suspended in 1 ml of 90% alcohol, e.g., methanol. The rinse step uses double the volume of the dye solution, thus maintaining a 5% solid solution. The sample is vortexed, sonicated, and spun down. The methanol supernatant is decanted. The 90% methanol rinse step is repeated once more. Finally, the excess methanol is decanted from the pellet, and the particles are re-suspended in an aqueous medium. Each test sample is then tested to determine the fluorescence activity of the labeled particles.

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When the test samples show that the dye solution has the precise ratios of the desired dyes, a macro-scale batch is conducted. The principle of macro-scale work up is identical to that noted above. Briefly, (1) 25 ml of the desired dye solution is transferred to a 50 ml vial, which contains a 2.5 gram quantity of dried particles. (2) The particles, now at 10% solids (w/v), are vortexed and sonicated. (3) Once the particles are completely in suspension, the solution is mixed for an hour. After that hour, (4) the particles are separated from the dye solution by centrifugation, followed by decanting. (5) The 2.5 gram quantity of particles is then re-suspended in 50 ml of 90% methanol. The rinse step uses double the volume of the dye solution, thus maintaining a mixture of 5% solids. (6) The sample then is vortexed, sonicated, and spun down, and the methanol supernatant is decanted. (7) Step 6 is repeated once more. (8) After the final methanol rinse is decanted, the particles are put through an aqueous rinse. (9) The aqueous supernatant is decanted, and the beads are then re-suspended and stored in a fresh aqueous medium.

6.3.2 Example 2: Dyeing of 300 nm Amino Functionalized Polystyrene Particles

A 1% (w/w) organic solvent solution of particles (300 nm diameter polystyrene, amino functionalized) is stirred in a round bottom flask. To this is added a solution of the desired dye(s) in an organic solvent, such as chloroform. When the dye solution is no longer absorbed by the particles (determined empirically by prior experiments), addition of the dye is halted, and the solvent is quickly removed under reduced pressure. For some reaction conditions, the dyeing reaction can be halted within about ten minutes; however, this time depends upon a variety of reaction parameters. Alternative embodiments therefore include times shorter than and times longer than ten minutes.

6.3.3 Example 3: Use of Two (Red and Orange) Squaraine Dyes

In another embodiment, two fluorescent squaraine dyes are used, for example, a red fluorescent dye, such as 1,3-bis[(1,3-dihydro-1,3,3-trimethyl-2H-indol-2-ylidene)methyl]-2,4-dihydroxy-cyclobutenediylium, bis(inner salt), and an orange fluorescent dye, such as 2-(3,5-dimethylpyrrol-2-yl)-4-(3,5-dimethyl-2H-pyrrol-2-ylidene)-3-hydroxy-2-cyclobuten-1-one. These dyes are used to stain two separate populations of particles. Preferably, both dyes are excitable at approximately the same absorption wavelength, for example, ranging from an ultraviolet wavelength to about 800 nm, and emit fluorescent light at two distinct, essentially non-overlapping wavelengths that are separated from each other by at least 10 nm, preferably by at least 30 nm, and more preferably by at least 50 nm. For example, the emission peak of the first dye above is 585 nm, and the emission peak of the second dye is 630 nm. These dyes are chosen because they fall in the center of two of the fluorescence channels of a Becton Dickinson FACScan flow cytometer, which is

the measurement device used in a preferred embodiment of the present invention. However, the choice of fluorescence channels is relative and immaterial since different flow cytometry apparatuses can have different settings.

Once these dyes are embedded into or onto a particle, the total dye quantity will be between about 0.00001% and about 15% (w/w) of the final, total particle weight. However, this limitation is of little consequence to the present invention as long as the particles impregnated with said dyes are stable and usable for the intended purpose(s).

Optimal staining with a particular dye is dependent upon the physical and chemical parameters of the individual dye(s), the polymeric substrate(s), and the dye medium or solvent. Incubation times can vary widely depending on the desired results, the concentration of the dye(s), the chemical compositions and physical properties of the particles, and the reaction conditions. The optimal time is usually the minimum time required for the dye, in the concentration being used, to achieve the highest specific signal, while avoiding degradation of the dye and/or the particle over time, and minimizing all other undesirable fluorescent signals due to the dye(s).

In a preferred embodiment, chlorinated solvents such as chloroform are used to solubilize the dye(s). In alternative embodiments, suitable solvents are selected based on their ability to solubilize the particular class of hydrophobic dyes of interest. For example, the solvents can be acyl, aliphatic, cycloaliphatic, aromatic, or heterocyclic hydrocarbons. In further embodiments the solvents can have halogens, oxygen, sulfur, nitrogen, and/or phosphorus as either terminal groups or as internal/integral parts of a ring or chain. Specifically, solvents such as any of the alcohols, ethyl acetate, toluene, xylene, hexane, pentane, benzene, ether, acetone, various oils, carbon tetrachloride, carbon disulfide, DMSO, or methylene chloride can be used. In addition, other solvents known in the art can be used, such as the solvents listed in the *Merck Index* (Eleventh Edition; see sections MISC-63-68).

6.3.4 Example 4: General Procedure Used to Produce the X-Y Plot of Figure 1

A 5 ml quantity of undyed stock particles is placed in an aqueous medium directly on a membrane covered fritted funnel. A vacuum pump pulls air through the particles plated onto the filter paper for about one hour. Next, the dried particles are transferred to 50 ml of dye solution (having at least two dyes with spectrally distinct emission spectra), covered, and stirred at room temperature for about an hour. The particles then are separated by filtration from the dye solution, and the dyed particles are placed in a vacuum dessicator for about four hours to remove residual solvent. Next, a 200 ml quantity of Triton X-100 and water is added to the dried dyed particles in a

250 ml flask. The solution then is stirred for about three hours, followed by filtering and washing, which are repeated until no further dye is detected in the filtrate.

This procedure prepares a particular population of particles whose fluorescence emission spectra fall within a prescribed region or cluster depicted in a Fluorescence Particle Map, such as one of the ovals of Fig. 1. Typically, about 80% or greater of the individual particles within a particular set exhibit fluorescence characteristics within the desired region, preferably about 90% or greater, more preferably about 97% or greater, and most preferably about 99% or greater. When dyeing conditions are tightly controlled, a particular set of particles typically will exhibit about 1% or less of the individual particles having fluorescence characteristics that fall outside the desired region ("dispersion"), preferably about 0.5% or less, more preferably about 0.3% or less, and most preferably about 0.2% or less.

As an example, Figure 1 illustrates a 64-set particle collection, wherein each population or set differs from another by a distinct location or cluster on the X-Y plot. These locations essentially do not overlap. As opposed to the prior art methods that typically result in up to 10-20% or even higher rates of dispersion, the instant method allows production of essentially homogeneous populations or sets of particles having only about a 0.2 -0.3 % dispersion, which constitutes a large and significant improvement over the prior art.

Where nanoparticles have been dyed, the resulting dyed nanoparticles can then be linked to microparticles by any of the various well-known coupling reactions, such as carbodiimide coupling. Other methods of coupling can use such groups as carboxylates, esters, alcohols, carbamides, aldehydes, amines, sulfur oxides, nitrogen oxides, or halides.

6.4 Discriminators and Discrimination Power

Figure 1 illustrates eight levels of each of two dyes that are attached to particles according to practice of the present invention, which permits the creation of an 8 x 8 = 64 array of categories of fluorescently distinguishable particle sets. Fluorescently distinguishable discriminators thus provide the basis for creating arrays of categories of particle sets.

Additional discriminators operate similarly. For example, addition of a third dye at eight different levels requires 3-dimensional representation of an 8 x 8 x 8 = 512 array of categories of fluorescently distinguishable particle sets.

Other practical discriminators include size of particle, shape of particle, magnetic particle or particle with other magnetic property, dye type, radioactive property, chemical reactive group, nuclear magnetic resonance, dye fluorescence absorption, dye fluorescence emission , dye

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absorption, fluorescence polarization, fluorescence lifetime, electron spin resonance, positron emission, concentration of particles, particle hydrophobicity, particle hydrophilicity, and particle density. For example, if two sizes of particles and two categories of magnetism (with or without magnetism) are added to the above 3-dimensional array, the array becomes 8 x 8 x 8 x 2 x 2 = 2,048 categories of particle subsets. Sizes and shapes of particles can be determined by light scattering measurements of forward and side scatter. The size of the particle can be measured in most flow cytometry apparatuses by forward or small-angle scatter light. Particle sets can also be further distinguished by differentiating between shapes of the particles, for example, using a high-resolution slit-scanning method. Magnetic particles incorporate magnetic or magnetically responsive metal oxides (for example, superparamagnetic, paramagnetic, or ferromagnetic oxides) into the polymer or polymer matrix.

Another category of discriminators is that of reactive groups that are attached to the particles, where each reactive group is capable of reaction with a specific compound. In some cases, the use of such discriminators will require two or more additional steps (for example, addition of a compound, followed by an incubation period, and measurement of an electromagnetic parameter, such as absorption, fluorescence emission, or magnetic resonance) to determine the reactive group and, hence, the category of the particle. In some cases, chromophores can be attached to the particles so that just a single optical or other measurement is required to determine the presence or absence of the chromophore on a given particle. Such discriminators provide powerful additional discrimination power. For example, as but one conservative calculation, use of 20 such discriminators in a labeling operation increases the above discussed array to 8 x 8 x 8 x 2 x 2 x 20 = 40,960 categories of particle sets.

An additional embodiment is that of inclusion of quantum dots within, on, or attached to particles. Quantum dots are fluorescing crystals which are excitable by a large range of wavelengths of light, but which have emission wavelengths that vary according to the size of the crystal -- with short wavelengths of emission occurring with small particles, and long wavelengths occurring with large particles. Thus, a large range of sizes of the crystals is used to create a large number of discrete emission wavelengths, creating a powerful expansion of discrimination power. Many dozens of classes of particles therefore are created according to the number of size classes of the quantum dot crystals. The size classes of the crystals are created either 1) by tight control of crystal formation parameters to create each desired size class of particle, or 2) by creation of batches of crystals under loosely controlled crystal formation parameters, followed by sorting according to desired size and/or emission wavelengths. Use of quantum dots for labeling particles, in the context of the present invention, is new, but is old in the art of semiconductors. Two examples of earlier references in

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which quantum dots are embedded within intrinsic silicon epitaxial layers of semiconductor light emitting/detecting devices are United States Patent Nos. 5,293,050 and 5,354,707 to Chapple-Sokol, et al.

6.5 Methods of Labeling Liquids and Uses Therefor

The present invention basically entails the addition of particles, such as core microparticles and/or carrier microparticles having attached nanoparticles, to liquids as a labeling means.

Once a plurality of particle sets is produced, labeling a liquid simply requires adding the correct category of particle set(s) to the liquid that is to be so labeled according to that particular category of particle set(s). In a preferred embodiment, the particles are impregnated with three different fluorescent dyes in eight different concentrations or levels of each of the dyes so as to create 512 subsets of particles. Each particle is later classified into its member subset using a measurement instrument. These 512 subsets of particles comprise 512 labeling elements or discriminators. Depending on the needs of the user, these discriminators can be combined with other discriminators to increase the number of unique labels, as discussed in section 6.4. For example, the presence or absence of a labeling element yields certain permutations. Using eight levels of a discriminator at a time, the presence or absence of a discriminator gives (512!/8!(512-8)!), or approximately 10^{17} possible unique labels. In addition, varying the concentration of one or more uniquely labeled particle subsets expands the possibilities further, particularly when a uniform distribution of particles is achieved in the liquid. Also, a check digit can be added which has half or other fraction of the number of particles as the identifying particles.

In one embodiment, a signature can be created to meet the needs of a particular application by using unique particle sets. For example, a donor "Social Security Number" can be created by 1) assigning nine different order discriminators to nine different particle sets, where each particle set is respectively assigned to one of the nine ordered digit positions that make up a Social Security Number (i.e., ones, tens, hundreds, thousands, . . . to hundred millions), and 2) further categorizing each of the order class particle sets into one of ten numerical classes of digits zero through nine by assigning each of ten different levels of each of the nine order class discriminators to correspond to a selected digit that is different from the remaining nine digits. For example, order discriminators are assigned based on relative ratios of red to orange fluorescing dyes in or on particles as follows. A digit in the one's position is encoded by a first relative concentration ratio of red to orange fluorescing dyes, a digit in the ten's position is encoded by a second relative concentration ratio of red to orange fluorescing dyes, a digit in the hundred's position is encoded by a third relative concentration ratio of red to orange fluorescing dyes, etc. In a first embodiment, specific digits zero

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through nine (i.e., the ten numerical classes) are then encoded according to the absolute amounts of the two dyes ("levels of the two dyes") in or on each particle. Alternatively, in a second embodiment, ten different concentrations of the order discrimination particles (or ten different total numbers of particles/sample) may be used to represent the ten digits. In this way, a single discriminator can serve the dual role of providing discrimination with respect to two different encoding aspects. In particular, in the second embodiment, only nine sets of beads are needed to encode any Social Security Number. Of course, other discriminators can be used with equal efficiency and practicality. Obviously, variations in level of a discriminator need only be such as to permit ready discrimination by the instrumentation to be used to read the Social Security Number. Furthermore, the progression of the ten digits of the ten numerical classes need not be represented by a monotonically increasing level of a discriminator(s) for the progression; rather, a monotonically decreasing level of the discriminator(s) may be employed, or any other pattern that achieves the goal of unique classification of each of the ten numerical classes. Also, separate order and numerical discriminators can be used (a 10 x 9 = 90 array of particles); however, use of such an array to encode a Social Security Number(s) usually is not as simple and not as economical as an encoding scheme that utilizes just one category of discriminator, or a combination of discriminators, to define the nine ordered digit positions, with attendant encoding of digits zero through nine by use of levels of the selected discriminator(s). However, a second discriminator (for example, particle density) in some applications may be used advantageously to provide numerical class encoding, for example, ten different densities of particles to represent digits zero through nine within each of the nine order class particle sets; alternatively, a range of nine different particle densities can serve as the nine order discriminators. In such an application, particles with the lowest density usually will be required to have a density greater than the liquid(s) they encode.

Analogous to the coding for a patient's Social Security Number, a similar scheme can be used to encode desired alphanumeric information of a patient, such as birth date, date of sample collection, name of patient, etc. To provide the most parsimonious and efficient use of particles, order discriminators are utilized according to the number of desired alphanumeric characters for the encoding. Then, as in one encoding embodiment for encoding a Social Security Number, concentrations of particles and/or other discriminators are used. For example, if 20 character positions are required for the encoding, and only alpha characters are to be used, 26 concentrations of particles would be required; alternatively, 10 concentrations of particles x 3 levels of a second disriminator to create 30 possible alpha characters for each of the 20 character positions; or any other such combination of discriminators to create the 26 required alpha characters. Also, characters such

as "space" and apostrophe, etc., can be incorporated. In addition, if liquid labeling identifies the patient sample, virtually all laboratory errors can be detected, including inter-sample contamination.

In another embodiment, a date code is created for labeling a liquid, for example, with respect to the date that a sample is taken from the liquid, an expiration date, a birth date, an expiration date for a perishable liquid, etc. For example, one of an array of 12 particle sets is assigned to the month January, a second to February, etc.; one of an array of 31 particle sets is assigned to the date 01, a second to 02, and so on through the date 31; and one of an array of five or more particle sets is assigned to a first year of interest, for example, 1999, a second to 2000, etc., for five consecutive years. This yields over 1800 exact dates by using only distinct 48 particle sets (more if greater than 5 years are used).

An alternative embodiment for encoding dates uses the concept of a series of small arrays. For example, four different "month" particles (having $2^4 = 16$ possible combinations) can be used to encode the 12 months, leaving 4 unused combinations; five different "day" particles are then assigned to encode the 31 days ($2^5 = 32$ possible combinations, leaving one unused combination); and five different "year" particles are then used to encode 32 years. Thus, only 14 different particles need be used to encode all the days over a span of 32 years. Furthermore, use of the unused combinations would further expand this span of years. For example, simple direct use (i.e., one particle combination per year) of the five unused particle combinations could be employed to add another 5 years of encoding coverage. Alternatively, use of the 4 unused month particle combinations, together with the presence or absence of the one unused day particle, would permit encoding of an additional 8 years, or 40 years total.

The concept of use of a series of small arrays of different particles can readily be applied to other encoding situations, including for routing, alphanumeric representation, Social Security Number, etc. In addition, the concept of "present" versus "absent" for different particles, that underlies calculation of numbers of possible permutations, may be applied in the form of "high" and "low," where high and low refer, for example, to high and low concentrations of particles in a sample, to high and low absolute numbers of particles in a sample, to high and low concentrations or levels of fluorescent dyes in or on the particles, to high and low levels of any other discriminator, etc.

Another embodiment for liquid labeling of the present invention is that of labeling solids, which later are solubilized, liquified, or extracted to recover particles contained therein for purposes of encoding. As but one illustrative example, microparticles are incorporated into sodium chloride crystals for such purposes as identification of manufacturer, lot number, production date, expiration date, degree of purity, etc. Such uses also include identification and/or tracing of pirated materials and products. Applications wherein solids are benficially labeled with microparticles are essentially

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limitless, examples including glues and adhesives (for example, use on postage stamps, envelopes, memo pages that adhere to a wall, page, or other surface, etc.), ink, paint, explosives, plastics and plastic products, explosives, chemicals in general where minute quantities of an appropriate kind of microparticles do not interfere with the end use of the chemical(s), tracing dissolution attributes and flow patterns *in vivo* of pharmaceutical tablets, etc.

In yet another embodiment, microparticles are used to label gases. Typically, such a use will require microparticles of low density, unless the gas or gases are flowing sufficiently (and possibly including sufficient turbulent flow) to maintain the microparticles in suspension therein. As with applications in solids, uses of microparticles in gases have an essentially unlimited range of applications, for example, including for purposes of tracing and/or identifying pirated gases, tracing the flow of gases in an animal, and encoding of manufacturer, lot number, production date, physical properties, degree of purity, etc.

In an embodiment for encoding to route liquids through a complex of pipelines having a plurality of branch points, different particle sets are created to uniquely represent each desired destination for a plurality of liquids coursing through the complex of pipelines, and particles from a particular particle set are added in an appropriate amount (determined by experimentation) to the liquid to be encoded by that particle set. Discriminators and physical properties are selected to be consonant with the physical properties of the liquid to which the particles are to be added. For example, hydrophobic particles typically will be used in hydrophobic liquids unless the hydrophobic liquids also contain detergents or amphiphiles sufficient to evenly disperse the particles in the liquid. Similarly, the particles typically are selected to have a greater density than the liquid to promote even dispersion. Selection of the final concentration of particles in the liquid that is so encoded will depend on such factors as cost/benefit, and sensitivity of the instrumentation to perform the analyses on the particles. This embodiment functions by providing analysis at an appropriate point in the pipeline to which the particles are first added, for example, at or near and upstream from a branchpoint or decision point in a pipeline. Switches and valves are then connected to the instrumentation, and control the final routing per the desired destination, based on the one or more discriminators of the particular particle set.

Similar protocols may also be used in a clinical setting to direct one or multiple body fluid samples from one or more patients to the appropriate instrument to perform the desired clinical chemistry (multiplexed clinical assays). In such a clinical chemical setting, a plurality of body fluid samples can include the addition of a quantity of particles into each body fluid sample to correspond to the desired clinical chemical analysis and/or machine. In some cases, for example, it is desirable to route a body fluid sample to a machine that performs a panel of analyses. Typically, a flow

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cytometer will be in-line with the tubing carrying the body fluid samples to detect routing particles and to transmit data on same to the appropriate switch(es) and valve(s) to direct the body fluid samples to the desired analytical site. Flow cytometers are the preferred instrument for such use, though any instrument that is capable of detecting the particular discriminator may be used.

Historically, the use of precision dyed particles in multiplexed clinical assays is provided, for example, by WO Patent No. 97/14028 and U.S. Patent No. 5,736,330, disclosures of which are herein incorporated by reference in their respective entireties. When these earlier disclosures are combined with the current invention, it is possible to greatly improve quality assurance in the clinical laboratory. For example, by selecting a unique permutation of particle sets, and permanently assigning that permutation to a particular assay, a fail-safe assay is created. A particle set also can be coupled to an associated reagent in a GMP (General Manufacturing Practice) environment, and combined into a single reagent vial. In addition, current multiplexed diagnostic instruments can be programmed to recognize a specific permutation, look up the parameters of that assay in a database, perform that assay, and record the results, thus eliminating the possibility that incorrect reagents will be added to a patient sample.

In embodiments for tracing toxic substances in groundwater, selection of the kinds of particles and their respective concentrations will include consideration of such factors as density and hydrophobicity of the toxic liquid(s), and size of the faults and/or densities of the soil(s) connecting a point toxic liquid pollution source with one or more aquifers of interest, as well as prospective or known dilution factors from underground water sources and/or above ground water sources, for example, recent rain or generally high rain fall. In addition, the sensitivity of the instrumentation to be used to decode or read the particles taken from a site distant from the point pollution source must be considered.

In embodiments for tracing absorption patterns of fluids in plants and animals, generally smaller particles will be advantageous; however, a large variation in particle size will be required for a wide range of tissue types, body cavities, etc. Factors similar to those of the preceding examples also require consideration in plants and animals. For example, particles that would be used in studies relating to bile flow would benefit from amphiphilic particles due to the presence of amphiphilic bile salts in bile. Of course, instrumentation sensitivity and dilution during use in an experiment must also be considered.

In general, a label is constructed by selecting one or more unique particle sets according to the particular application, adding them to the liquid, and mixing. The concentration of the particle volume with respect to the liquid volume also is a variable that is application dependent, for example,

reflecting the priority of cost versus speed: as the concentration of particles is increased, so, too, is the cost increased, whereas the reading time typically will be decreased.

In general and ideally, the particles are inert in the liquid being labeled. Furthermore, criteria for matching particle types to liquids include such parameters as stability of the particle in the particular liquid, stability of the dye attachment to the particle, and non-interference of the particle(s) with the use for which the liquid is intended. Thus, hydrophobic particle types generally will be used only with hydrophobic liquids, unless the hydrophobic liquid also contains detergents, amphiphiles, and/or hydrophiles. Particles that are used in organic solvents require that the dye or other discriminator be encapsulated in, or matrix entrapped by, a solvent stable polymer, or covalently attached to the particle.

Kits can also be produced to allow a user to mix particles in predetermined proportions and thereby appropriately label a liquid according to user defined or elected parameters. For example, a kit can feature 512 distinct particle sets based on eight levels of each of three fluorescent dyes. Alternatively, or in addition, other discriminators can be featured to create the number of distinct sets of particles necessary for use in a particular liquid labeling application, which also will take into account such attributes as hydrophobicity and density of the liquid to be labeled, as well as parameters such as stability of the fluorescent dye, or stability of matrix entrapment of the fluorescent dye, in the particular liquid. Specifically, a kit features particle sets for each desired discriminator, and each particle set comprises particle subsets which represent an appropriate range of values for that particular discriminator so as to create a functional range of subsets thereof. Thus, the user simply dispenses an appropriate quantity of each of the one or more sets/subsets of particles into the liquid selected to be labeled by that particular set(s)/subset(s) of particles. Furthermore, multiple classifications can be applied concurrently, for example, including one or more sets of particles to encode the patient's name and/or Social Security Number, and a second one or more sets of particles to encode routing for test procedures, storage, etc.

Another embodiment that can be coupled to a kit entails the use of a microprocessor controlled automatic pipetting system. In this embodiment, the user types into the system, using a standard keyboard, the desired numbers or other indicators (for example, for routing, indication of test to be performed, birth date of patient, Social Security Number, etc.). The user similarly supplies the desired microparticle type that is to correspond to a selected indicator; i.e., consideration is given to the physical properties or other bases for best matching particles with the liquids at hand. A simple software program allows the user to input the desired discriminators and the alphanumeric or other symbols to which the discriminators respectively correspond. Such programs are well known in the art, and may, for example, include lookup tables for the correspondence between

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discriminators and symbols. Output signals from the microprocessor then operate to control automatic pipetting of the selected microparticles into one or more liquid or gaseous samples that are positioned in receiving modes relative to the pipetting system. Indicators indicate the purpose(s) for which the liquid or gas samples are labeled with particles.

The discriminators are used to distinguish particles based on differences in particle concentration, particle physical property, dye type, dye concentration, spectral property, label type, and combinations thereof. These broad categories of discriminators can be further categorized into other discriminators as follows. The particle physical property can include such attributes as particle size, particle shape, particle hydrophobicity, particle hydrophilicity, particle density, and combinations thereof. The spectral property can include such attributes as dye fluorescence absorption, dye fluorescence emission, dye absorption, dye fluorescence polarization, dye fluorescence lifetime, and combinations thereof. Label type refers to physicalchemical properties of chemical groups added to particles, and can include such attributes as magnetic property, (chemical) reactive group, nuclear magnetic resonance, electron spin resonance, positron emission, radioactive property, fluorescence polarization, fluorescence lifetime, and combinations thereof. Dye type refers to the chemical composition and structure of fluorescent dye molecules. Dye concentration refers to the concentration of the dye(s) on or in a particle. Particle concentration refers to the concentration of particles in the liquid being labeled.

When a liquid is ready for analysis or decoding of its label, the liquid is thoroughly mixed, and a sample is withdrawn. Next, a centrifugation, filtering, magnetic, or other separation means is used to separate the particles from the liquid thereby so encoded. The particles are then analyzed.

When encoding is achieved by the use of a series of spectrally distinct dyes, various commercially available instruments can be used to read the liquid labels. For example, a fluorescence microscope can be used on a sample of the liquid to locate and characterize the particles in the sample. In a preferred embodiment, a flow cytometer is used according to procedures known in the art, such as is disclosed in U.S. Patent No. 4,665,024 to Mansour, et al. Flow cytometry is an optical technique that analyzes particles in a fluid mixture based on the particles' optical characteristics using a flow cytometer. Background information on flow cytometry is set forth in Shapiro, Practical Flow Cytometry, Third Ed. (Alan R. Liss, Inc. 1995), incorporated herein by reference in its entirety. A flow cytometer can automatically display the clusters formed by the fluorescent emissions of the particle sets, for example, as shown herein in Fig. 1. In a preferred embodiment, the host computer that controls the flow cytometer is programmed to automatically detect the clusters and convert the particle set information into its ordinal information, such as date, "Social Security Number," patient's name, patient's birth date, or other identifying parameter.

Other means for detection and analysis can also be utilized, including, but not limited to, visual inspection, digital (CCD) cameras, video cameras, photographic film, or instrumentation such as laser scanning devices, fluorometers, photometers, luminometers, photodiodes, quantum counters, plate readers, epifluorescence microscopes, scanning microscopes, confocal microscopes, capillary electrophoresis detectors, or any other means that provides amplification of an electromagnetic signal, such as a photomultiplier tube or other light detector capable of detecting the presence, location, and intensity of excitation and emission spectra, fluorescence polarization, fluorescence lifetime, and other physical properties of a fluorescent or other electromagnetic signal.

When particles are processed by a flow cytometer, measurement of four exemplary classification parameters [forward light scatter, side light scatter, a first fluorescence emission peak (for example, red fluorescence), and a second fluorescence emission peak (for example, orange fluorescence)] can be used to identify the set, population, or cluster to which each particle belongs. Furthermore, additional chromophores, reactive groups, and other discriminators can be measured. Although methods and products of the present invention have been described in detail for purposes of general illustration, it is understood that such detail is solely for that purpose, and that variations can be made therein and thereto by those skilled in the art without departing from the scope of the invention, which is defined by the following claims. For example, the use of sets of small arrays of particles to encode respective portions of an alphanumeric string can be applied to virtually any encoding application.

WHAT IS CLAIMED IS:

1. A method of labeling a gaseous, liquid, or solid material to permit the subsequent identification of the labeled gaseous, liquid, or solid material, which comprises adding to a gaseous, liquid, or solid material, whose subsequent identification is desired, a sufficient quantity of particles belonging to one or more distinguishable sets of particles, to provide a labeled gaseous, liquid, or solid material that subsequently can be identified.

2. The method of claim 1 in which the particles in each set exhibit one or more discriminators that distinguish the particles of one set from those of another set.

3. The method of claim 2 in which a sufficient quantity of a combination of particles belonging to two or more distinguishable sets of particles is added to the gaseous, liquid, or solid material, whose subsequent identification is desired.

4. The method of claim 2 in which said one or more discriminators is selected from a physical, chemical, biological, magnetic, or spectral property of the particle or combinations thereof.

5. A method of identifying a gaseous, liquid, or solid material that has been labeled with a sufficient quantity of particles belonging to one or more distinguishable sets of particles, which comprises:

(a) obtaining a sample comprising a gaseous, liquid, or solid material that has been labeled with a sufficient quantity of particles belonging to one or more distinguishable sets of particles;

(b) analyzing the particles found in the sample to determine which of one or more distinguishable sets of particles such particles belong to; and

(c) comparing the results of said analyzing step with a key which establishes the identity of the gaseous, liquid, or solid material from which the sample was taken.

6. A labeled material which comprises (i) a gas, liquid, solid, or combinations thereof and (ii) a sufficient quantity of particles that can be shown to belong to one or more distinguishable sets of particles.

7. The labeled material of claim 6 in which the particles in each set exhibit one or more discriminators that distinguish the particles of one set from those of another set.

8. The method of claim 1 in which said material comprises a gas.

9. The method of claim 1 in which said material comprises a liquid.

10. The method of claim 1 in which said material comprises a solid.

11. The method of claim 5 in which said material comprises a gas.

12. The method of claim 5 in which said material comprises a liquid.

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13. The method of claim 5 in which said material comprises a solid.

14. The labeled material of claim 6 which comprises a gas.

15. The labeled material of claim 6 which comprises a liquid.

16. The labeled material of claim 6 which comprises a solid.

17. A kit for labeling liquids, solids, and gases comprising particles from a plurality of distinct particle sets for labeling liquids, solids, and gases, and a dispenser.

AMENDED CLAIMS

[received by the International Bureau on 18 August 1999(18.08.99);
original claim 6 amended; remaining claims unchanged (1 page)]

6. A labeled material whose subsequent identification is desired, which comprises (i) a gas, liquid, solid, or combination thereof and (ii) a sufficient quantity of particles that can be shown to belong to one or more distinguishable sets of particles.

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 264/ 5,6,7; 425/5,6; 428/402,403,407; 436/518,525,529,533

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, BIOSTIC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,723,218 A (HAUGLAND et al) 03 March 1998, see entire document.	1-17
Y	US 4,981,625 A (RHIM et al) 01 January 1991, column 3, line 63 - column 11, line 63.	1-17
A	US 5,736,330 A (FULTON) 07 April 1998, column 2 line 16 - column 7, line 12.	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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(54) Title: PRECISION FLUORESCENTLY DYED PARTICLES AND METHODS OF MAKING AND USING SAME

(57) Abstract

An improved method of making a series of bead or microsphere or particle populations characterized by subtle variation in a proportion or ratio of at least two fluorescent dyes distributed within a single bead of each population is provided. These beads, when excited by a single excitation light source are capable of giving off several fluorescent signals simultaneously. A set containing as many as 64 distinct populations of multicolored, fluorescent beads is provided and when combined with analytical reagents bound to the surface of such beads is extremely useful for multiplexed analysis of a plurality of analytes in a single sample. Thus, methods of staining polymeric particles, the particles themselves, and methods of using such particles are claimed.

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Precision Fluorescently Dyed Particles and Methods of Making and Using Same

1. Field of the Invention

The invention generally relates to multicolored, fluorescently stained small particles of generally less than 100 μm in diameter. Disclosed are methods of dyeing or staining such particles or microspheres with at least two fluorescent dyes in such a manner that intra-sample variation of dye concentrations is substantially minimized. Specifically, the invention relates to microspheres stained with at least two fluorescent dyes and methods of using said microspheres for a simultaneous analysis of a plurality of analytes.

2. Background of the Invention

Fluorescent light emitting microparticles, microspheres, microbeads, beads, or particles are now quite common and are useful for a number of practical applications especially in combination with flow cytometry based methods. As used hereinafter the terms: microparticles, microspheres, microbeads, beads, or particles are used interchangeably and bear equivalent meanings. Often, these particles are labeled with just one fluorescent dye. In general, such particles are made by copolymerization process wherein monomers, e.g., unsaturated aldehyde or acrylate, are allowed to polymerize in the presence of a fluorescent dye, e.g., fluorescein isothiocynate (FITC), in the reaction mixture (see for example U.S. Pat. Nos. 4,267,234 issued to Rembaum; 4,267,235 Rembaum et al; 4,552,812, Margel et al.; 4,677,138, Margel).

One skilled in the art would recognize that two or more dyes of varying proportions could be used to increase the permutation number of unique combinations of dyes in a single particle. These unique characteristics, i.e., emission wavelengths and fluorescence intensities could be extremely useful for multiparameter analysis of a plurality of analytes in the same sample. Three means of making multicolored, fluorescent beads have been reported, including: (a) covalent attachment of dyes onto the surface of the particle, (b) internal incorporation of dyes during particle polymerization, and (c) dyeing after the particle has been already polymerized. All three methods have been disclosed in the prior art.

The examples of the first approach are in U.S. Pat. Nos. 5,194,300 Cheung; 4,774,189 Schwartz which disclose fluorescent microspheres that are coated by covalently attaching either one or a plurality of fluorescent dyes to their surface. As such these methods are unrelated to the instant invention dealing with incorporating dyes into particles internally.

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Second approach can be found in U.S. Pat. No. 5,073,498 to Schwartz, which discloses two or more fluorescent dyes added during polymerization process and randomly dispersed within the body of the particle. However, when such particles are exposed to a single excitation wavelength only one fluorescent signal is observed at a time and thus these particles are not useful for multiparameter analysis especially in a flow cytometry apparatus with a single excitation light source. The U.S. Pat. No. 4,717,655 issued to Fulwyler discloses two dyes mixed at five different ratios and copolymerized into a particle. Although five populations of beads were claimed as being obtainable the fluorescent properties of these beads were not provided, effectively preventing one skilled in the art to make and use such beads. Thus, Fulwyler method is only a conceptual method since it was not enabled. Furthermore, any of these two methods are unrelated to the instant invention dealing with incorporating fluorescent dyes into already polymerized particles.

The principle of the third method, i.e., internally embedding or diffusing a dye after a particle has been already polymerized was originally described by L.B.Bangs (Uniform Latex Particles; Seragen Diagnostics Inc. 1984, p. 40) and relates to the instant invention as it consists of adding an oil-soluble or hydrophobic dye to stirred microparticles and after incubation washing off the dye. The microspheres used in this method are hydrophobic by nature. This allows adopting the phenomenon of swelling of such particles in a hydrophobic solvent, which may also contain hydrophobic fluorescent dyes. Once swollen, such particles will absorb dyes present in the solvent mixture in a manner reminiscent to water absorption by a sponge. The level and extent of swelling is controlled by incubation time, the quantity of cross-linking agent preventing particle from disintegration, and the nature and amount of solvent(s). By varying these parameters one may diffuse a dye throughout particle or obtain fluorescent dye-containing layers or spherical zones of desired size and shape. Removing the solvent terminates the staining process. Microparticles stained in this manner will not "bleed" the dye in aqueous solutions or in the presence of water-based solvents or surfactants such as anionic, nonionic, cationic, amphoteric, and zwitterionic surfactants.

U.S. Pat. No. 5,723,218 to Haugland et al. discloses diffusely dyeing microparticles with one or more dipyrromethene boron difluoride dyes by using a process, which is essentially similar to the Bangs method. However, when beads internally stained with two separate dipyrromethene boron dyes, were excited at 490 nm wavelength, they exhibited overlapping

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emission spectra, meaning that beads were monochromatic but not multicolored. U.S. Pat. Nos. 5,326,692 Brinkley et al; 5,716,855 Lerner et al; and 5,573,909 Singer et al. disclose fluorescent staining of microparticles with two or more fluorescent dyes. However, dyes used in their process had overlapping excitation and emission spectra allowing energy transfer from the first excited dye to the next dye and through a series of dyes resulting in emission of light from the last dye in the series. This process was intended to create an extended Stokes shift, i.e., a larger gap between excitation and emission spectra, but not the emission of fluorescence from each dye simultaneously. Thus, due to various reasons such as dye-dye interaction, overlapping spectra, non-Gaussian emission profiles and energy transfer between neighboring dyes the demand for multicolored beads simultaneously emitting fluorescence at distinct peaks was not satisfied. Zhang et al. (U.S. Pat. No. 5,786,219) devised microspheres with two-color fluorescent "rings" or microspheres containing a fluorescent spherical "disk" combined with a fluorescent ring. Nevertheless, such beads, designed for calibration purposes, cannot be used in multiparameter analysis since two dyes were mixed only at one fixed ratio. As mentioned above in regard to U.S. Pat. No. 4,717,655 issued to Fulwyler, the highest number of dyes ratios ever attempted with at least two dyes never exceeded five. Thus, until the reduction to practice of the present invention there were no reliable means of creating a series of microsphere populations or subsets in which at least two dyes were mixed at variable, precisely controlled ratios and were proven, upon exposure to a single excitation wavelength, to emit multiple fluorescent signals of variable intensity and at spaced, optically distant wavelengths.

In other words, the prior art failed to provide a reproducible method that would allow one skilled in the art to make a plurality of defined subsets of stained multicolored microparticles distinguishable by a subtle variation in fluorescence signal resulting from the combination of various dyes of distinct color and having variable intensity of color emission. As used hereinafter the term stained microspheres means that a plurality of dyes, which are used to stain a microsphere, are either uniformly diffused throughout the body of said microsphere or penetrated said microsphere in a manner that results in formation of fluorescent rings, disks, and other geometrically distinct patterns.

Clearly, it would be an important improvement to the art to have a means of precisely dyeing or staining a particle with two or more dyes premixed in a series of predetermined ratios and to have a collection of such dyed microspheres for use in multiparameter applications. This

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precision in dyeing process is commonly expressed as the coefficient of variation, which is the ratio of the standard deviation to the mean intensity of the fluorescent particle population. By minimizing this value, more subsets or populations of non-overlapping, distinctly dyed particles can be obtained. It would be a further advance in the art if the methods were repeatable or reproducible to within a minimal variation, preferably no more than about a 20% intra-sample variation, more preferably no more than about a 15% variation, and most preferably no more than about a 8% variation.

3. Summary of the Invention

An improved method is described for incorporating two or more fluorescent dyes into already polymerized microspheres. The amount of each dye absorbed by the microsphere is precisely controlled so as to give rise to two or more reproducible fluorescent signals of precise intensities and emission peaks within a given population of particles. A series of such populations or subsets of beads are dyed in batches each one of them having predetermined ratio or proportion of two or more fluorescent dyes. Due to novel and improved method of staining, the particle-to-particle variation in the same batch is greatly reduced, which allows producing an unprecedented number of distinct populations of multicolored, fluorescent microspheres residing within optically uniform, tightly defined cluster.

Accordingly, a set containing optically distinct precision stained microspheres is also claimed which would be useful for simultaneous analysis of a plurality of analytes in a same sample. In other words, said beads will provide a lot more than the use of stained beads found in the prior art since the number of analytes that can be measured simultaneously in a single tube, using a single sample aliquot is drastically increased. The fluorescent microparticle obtained by the inventive staining method is characterized by having at least two fluorescent dyes mixed within the body of the particle and each one of them capable of giving off, simultaneously, multiple fluorescent emission lights of predetermined color and intensity. The combination of notions relating to the emission peak corresponding to a given color and intensity of the fluorescent color as expressed in fluorescence channel units is generally termed as the fluorescence signal. The specific ratio or proportion of dyes at which they are mixed within a population of particles will determine the location of said populations on a fluorescence map, which allocates these populations according to fluorescent color and brightness. By using as little

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as two dyes, e.g., orange and red, as many as 64 populations of beads are made each one distinct from another by subtle variations in unique fluorescence characteristics recognized by a flow cytometry apparatus.

When each such population of beads, characterized by at least two fluorescent signals, is combined with an analytical reactant capable of binding a specific analyte in a clinical or test sample a powerful analytical tool is obtained, which can provide qualitative and quantitative assay results. The analytical method is also provided which is based on using multicolored fluorescent beads obtained by the instant invention. To achieve truly multiplexed analysis of a plurality of analytes in a sample, a third type of fluorescent signal, e.g., green fluorescent signal is provided, usually found in a label reagent, which is capable of binding the analyte of interest. Thus, methods of making multicolored beads, the beads themselves, multiple sets of such beads, and multiplexed methods of analyzing a plurality of analytes in a single sample are claimed by the instant invention.

A method of staining polymeric microspheres with two or more fluorescent dyes is disclosed, which method comprises: (a) combining at least two fluorescent dyes in a solvent mixture comprising at least one organic solvent in which the at least two fluorescent dyes are soluble and at least one alcoholic solvent in which the at least two fluorescent dyes are less soluble, to provide a solution of mixed dyes which is further characterized as having the capacity to swell at least partially but not dissolve a plurality of polymeric microspheres, which is brought into contact with the solution; (b) contacting a plurality of polymeric microspheres with the solution for a period of time sufficient to provide uniform staining of substantially all of the members of the plurality of polymeric microspheres with the at least two fluorescent dyes, the at least two fluorescent dyes being selected such that on isolation and excitation of the dyed plurality of polymeric microspheres, a distinct fluorescence signal is emitted from each dye, the intensity of which emitted signal is proportional to the amount of the dye in the dyed plurality of polymeric microspheres.

In a particular embodiment of the invention, the method further comprises dehydrating the plurality of polymeric microspheres. Such a dehydrating step is accomplished by washing the plurality of polymeric microspheres one or more times with an alcoholic solvent prior to contacting the microspheres with the solution of mixed dyes. In still a preferred method, the dehydrating step involves drying the washed microspheres or allowing the alcoholic solvent to

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evaporate from the washed microspheres prior to contacting the microspheres with the solution of mixed dyes.

Typically, the dyed plurality of polymeric microspheres is isolated by any manner well known in the art, including but not limited to filtration or centrifugation. It has been found desirable to obtain dyed plurality of polymeric microspheres in which at least one of the fluorescent dyes is diffused throughout the interior of substantially all of the members of the dyed plurality of polymeric microspheres, or in which the at least two fluorescent dyes are diffused throughout the interior of substantially all of the members of the dyed plurality of polymeric microspheres. Still other advantages can be gained by providing dyed plurality of polymeric microspheres in which at least one of the fluorescent dyes is diffused through only a portion of the interior of substantially all of the members of the dyed plurality of polymeric microspheres.

In a specific method of the invention, the staining procedure further comprises preparing a series of the solutions having differing desired ratios of the at least two fluorescent dyes and further comprises contacting separate populations of a plurality of polymeric microspheres with the series of the solutions to provide multiple distinct populations or subsets of a plurality of polymeric microspheres, each distinct population or subset having a differing desired ratio of the at least two fluorescent dyes.

It has been observed that the distinct fluorescence signals emitted from the at least two fluorescent dyes differ in their respective wavelengths by at least about 10 nm, preferably by at least about 30 nm and most preferably by at least about 50 nm.

Hence, the present invention provides a population of polymeric microspheres substantially uniformly stained with at least two fluorescent dyes, each microsphere of the population upon excitation exhibiting at least two distinct fluorescence emission signals corresponding to the at least two fluorescent dyes, the intensity of each of the at least two emitted signals (i) being proportional to the amount of its corresponding dye in the microsphere, and (ii) exhibiting a coefficient of variation among all the members of the population, which is no greater than about 20 percent. In particular, preferred populations are those in which the intensity of each of the at least two emitted signals exhibits a coefficient of variation among all the members of the population, which is no greater than about 15 percent, more preferably no greater than about 10 percent and most preferably no greater than about 8 percent. In still other embodiments, the intensity of each of the at least two emitted signals exhibits a coefficient of variation among

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all the members of the population, which is less than about 8 percent.

The present invention offers, thus, a collection of distinct populations of polymeric microspheres according to the specifications described above, each population exhibiting an emission spectrum in a Fluorescence Bead Map, which is unique to the population. In specific embodiments, the collection comprises eight or more distinct populations of polymeric microspheres, preferably sixteen or more distinct populations of polymeric microspheres, more preferably twenty-four or more distinct populations of polymeric microspheres, most preferably thirty-two or more distinct populations of polymeric microspheres and still most preferably sixty-four or more distinct populations of polymeric microspheres. Generally, the collection is further characterized in that there is substantially no overlap between any of the sixty-four or more emission spectra associated with the sixty-four or more distinct populations of polymeric microspheres.

Also contemplated by the invention, is a method of detecting simultaneously by flow cytometry a plurality of analytes in a sample, each of the analytes being recognized by a corresponding analytical reactant, comprising: (a) contacting the sample with a plurality of populations of uniformly stained microspheres, the microspheres having at least two fluorescent dyes uniformly mixed at a specific ratio within each microsphere of each the population, each population of the microspheres having a distinct analytical reactant bound to its surface, wherein, the reactant on each population of microspheres specifically interacts with one of the analytes in the sample; (b) providing a label reagent that specifically binds to the analyte and analyzing the microspheres to detect the label indicating binding of the analyte to the analytical reactant; and (c) determining the populations of microspheres having the fluorescent dyes mixed at the specific ratio within microspheres of each population to which the reactant is bound.

Other objects of the invention will become apparent from the further discussions and detailed descriptions provided herein.

Overview of the Invention and its Embodiments

Recent developments in instrumentation have necessitated the concurrent development of multiple and precisely dyed microspheres that can emit multiple fluorescent signals simultaneously. This invention describes techniques for absorbing at least two squaric acid-based fluorescent dyes into polymeric, i.e., polystyrene particles.

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The present invention describes techniques for precisely dyeing polystyrene microspheres of sizes ranging from approximately 10 nm to 100 μm in diameter. The size of particles is immaterial to this invention since the precision of the dyeing process is not affected. The only requirement is that particles are made of water-insoluble material but soluble in adequate solvents. The dyes employed are preferably squaric acid-based molecules that exhibit fluorescence extending into near infrared and/or infrared region, i.e., to ca. 1,000 nm. Use of other dyes may allow one to expand the range from the ultraviolet to infrared. This method allows for a highly reproducible process in which two or more dyes of independent concentration are absorbed uniformly into each microsphere, resulting in multiple fluorescent signals respective of the number of dyes present in the microsphere.

The technology is disclosed enabling one skilled in the art to make a series of multicolored, fluorescent particles with unique fluorescence characteristics and using such particles for multiparameter analysis of a plurality of analytes simultaneously.

4. Brief Description of Drawings

Fig. 1 showing flow chart with sequential steps of dyeing polymeric particles using the prior art technique.

Fig. 2 showing flow chart with sequential steps of dyeing polymeric particles using the instant invention.

Fig. 3 showing two-dimensional flow cytometry chart illustrating wide optical distribution of two-color-dyed microspheres using the prior art technique.

Fig. 4 showing two-dimensional flow cytometry chart illustrating tight clustering of two-color-dyed microspheres using the instant invention.

Fig. 5 showing that prior art method allows no more than 6 subsets of multicolored bead populations on a Fluorescence Bead Map.

Fig. 6 showing Fluorescence Bead Map for 64-Region Bead Set, indicating tight distribution of each bead subset fluorescence characteristics within boundaries prescribed by each region.

5. Detailed Description of Specific Embodiments

The invention provides novel polymeric beads or microspheres containing at least two fluorescent dyes. This invention further includes the improved method of making such beads by

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mixing said beads with at least two fluorescent dyes combined at predetermined ratio so that optically distinct, multiple populations of multicolored beads are formed. These bead populations are easily discriminated as essentially non-overlapping clusters by visual detection methods such as microscopy or preferably by flow cytometry. The method of simultaneous, multiparameter analysis of a plurality of analytes is also provided whereby each distinct multicolored bead population would carry an additional analytical reactant, e.g., antibody, antigen, or nucleic acid probe, which would react with a specific analyte of interest in a sample containing the plurality of analytes.

Polymeric microspheres used in this invention are commercially available from a number of vendors and range in size from 0.01 to 100 micrometers (μm) in diameter. Even though the microparticle can be of any size, the preferred size is 0.1-50 μm, more preferably 1-20 μm, and even more preferably 3-9 μm. The sizes of beads in one set can be uniform or may differ in order to distinguish and classify them into further subsets according to their size. The size of the microparticle can be measured in practically any flow cytometry apparatus by so-called forward or small-angle scatter light. These subsets can be also further distinguished by different shape of microparticles. The shape of the particle can be also discriminated by flow cytometry, e.g., by high-resolution slit-scanning method.

The preferred make of microspheres is polystyrene or latex material. However, any type of polymeric make of microspheres is acceptable including but not limited to brominated polystyrene, polyacrylic acid, polyacrylonitrile, polyacrylamide, polyacrolein, polybutadiene, polydimethylsiloxane, polyisoprene, polyurethane, polyvinylacetate, polyvinylchloride, polyvinylpyridine, polyvinylbenzylchloride, polyvinyltoluene, polyvinylidene chloride, polydivinylbenzene, polymethylmethacrylate, or combinations thereof.

The microspheres will also contain 1 to 30% of a cross-linking agent, such as divinyl benzene, ethylene glycol dimethacrylate, trimethylol propane trimethacrylate, or N,N'methylene-bis-acrylamide or other functionally equivalent agents known in the art. In preferred embodiment microspheres are made of polystyrene and contain 1 to 30% divinyl benzene.

The beads may or may not have additional surface functional groups, such as carboxylates, esters, alcohols, carbamides, aldehydes, amines, sulfur oxides, nitrogen oxides, or halides. The functionality of the microspheres' surface groups gives the microspheres their coupling capability allowing chemical binding of analytical reactants. In addition to functional

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groups on microspheres the dyes themselves can also carry chemically reactive functional groups which in addition to groups listed above can also be carboxylic acid, carboxylic acid succinimidyl ester, carboxylic acid anhydride, sulfonyl chloride, sulfonyl fluoride, hydrazine derivatives, acyl azide, isocyanate, haloacetamide, phenols, thiols, and ketones. These functional groups are useful for attachment of analytical reactants, i.e., classical, commonly used reactants such as antibody, antigen (hapten), digoxigenin, or nucleic acid probe. These may also include reactants that can form specific, high-affinity conjugates such as avidin-biotin, receptor-ligand, ligand-ligate, enzyme-substrate, lectin-carbohydrate, protein A-immunoglobulin, etc. For flow cytometry analysis the analytical reactants are commonly labeled with fluorescent tags or labels such fluorescein (FITC) or rhodamine. These light-emitting conjugates of a dye and analytical reactant are termed as label reagents.

The analytical reactants can be also selected among fluorescent reporter molecules capable to react with a variety of analytes, e.g., O_2, CO_2, pH, Ca^{++}, Na^+, K^+, or Cl^- as disclosed for example in U.S. Pat. No. 5,747,349 issued to van den Engh et al.

Suitable solvents will be selected based on their ability to solubilize the particular class of hydrophobic dyes of interest. It is preferable that their solubility characteristics are substantially similar. The solvents can be acyl, aliphatic, cycloaliphatic, aromatic or heterocyclic hydrocarbons; the solvents may or may not have halogens, oxygen, sulfur, nitrogen, and/or phosphorous as either terminal groups or as integral parts of a ring or chain. Specifically, solvents such as toluene, xylene, hexane, pentane, acetone, DMSO, or methylene chloride can be used. In a preferred embodiment, chlorinated solvents, more preferably chloroform, are used to solubilize the squaric acid class of dyes, which are preferred dyes used in this invention.

In one embodiment two fluorescent squaraine dyes are used, e.g., red dye which is 1,3-bis [(1,3-dihydro-1,3,3-trimethyl-2H-indol-2-ylidene)methyl]-2,4-dihydroxy-cyclobutenediylium, bis(inner salt) and orange dye is 2-(3,5-dimethylpyrrol-2-yl) -4-(3,5-dimethyl-2H-pyrrol-2-ylidene)-3-hydroxy-2-cyclobuten-1-one. The molar ratio between first and second dye, when present in a bead, will preferably be between about 0 and 10,000, more preferably between 0.00001 and 2,000. Both dyes would preferably be excited at the same absorption wavelength, e.g., ranging from ultraviolet to about 800 nm, and emit fluorescent light at two distinct, essentially non-overlapping wavelengths distant from each other by at least 10

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nm, preferably 30 nm, and more preferably by at least 50 nm. For example, the emission peak of the dye #1 is at 585 nm, and the peak emission of dye #2 is at 630 nm.

The squaric acid based fluorescent dyes can be synthesized by methods described in the literature. See, for example, Sprenger et al. Angew. Chem., 79, 581 (1967); Angew. Chem., 80, 541 (1968); and Maaks et al., Angew Chem. Intern. Edit., 5, 888 (1966). Briefly, one equivalent of squaric acid (1,2-dihydroxycyclobutenedione) is condensed with two equivalents of an active compound, such as a pyrrole, indoline, or aniline, and refluxed in a mixture of an alcohol and an aromatic solvent (such as benzene) under conditions that allow removal of water from the reaction mixture. The resulting dye can be collected and purified by a number of standard methods, such as recrystallization, distillation, chromatography, etc. Additionally, unsymmetrically substituted squaric acid compounds can be synthesized by methods such as those described by Law et al., J. Org. Chem. 57, 3278, (1992). Specific methods of making some of such dyes are well known in the art and can be found for example in U.S. Pat. Nos. 5,795,981; 5,656,750; 5,492,795; 4,677,045; 5,237,498; and 5,354,873. Optionally such dyes will contain functional groups capable of forming a stable fluorescent product with functional groups typically found in biomolecules or polymers including activated esters, isothiocyanates, amines, hydrazines, halides, acids, azides, maleimides, alcohols, acrylamides, haloacetamides, phenols, thiols, acids, aldehydes and ketones.

In addition to specific squaric acid dyes are used in this preferred embodiment, related dyes can be further selected from cyclobutenedione derivatives, substituted cephalosporin compounds, fluorinated squaraine compositions, symmetrical and unsymmetrical squaraines, alkylalkoxy squaraines, or squarylium compounds. Some of these dyes can fluoresce at near infrared as well as at infrared wavelengths that would effectively expand the range of emission spectra up to about 1,000 nm.

In addition to squaraines, i.e., derived from squaric acid, hydrophobic dyes such as phthalocyanines and naphthalocyanines can be also selected as operating at longer wavelengths. Other classes of fluorochromes are equally suitable for use as dyes according to the present invention. Some of these dyes are listed herein: 3-Hydroxypyrene 5,8,10-Tri Sulfonic acid, 5-Hydroxy Tryptamine, 5-Hydroxy Tryptamine (5-HT), Acid Fuhsin, Acridine Orange, Acridine Red, Acridine Yellow, Acriflavin, AFA (Acriflavin Feulgen SITSA), Alizarin Complexon, Alizarin Red, Allophycocyanin, ACMA, Aminoactinomycin D, Aminocoumarin, Anthroyl

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Stearate, Aryl- or Heteroaryl-substituted Polyolefin, Astrazon Brilliant Red 4G, Astrazon Orange R, Astrazon Red 6B, Astrazon Yellow 7 GLL, Atabrine, Auramine, Aurophosphine, Aurophosphine G, BAO 9 (Bisaminophenyloxadiazole), BCECF, Berberine Sulphate, Bisbenzamide, BOBO 1, Blancophor FFG Solution, Blancophor SV, Bodipy Fl, BOPRO 1,Brilliant Sulphoflavin FF, Calcien Blue, Calcium Green, Calcofluor RW Solution, Calcofluor White, Calcophor White ABT Solution, Calcophor White Standard Solution, Carbocyanine, Carbostyryl, Cascade Blue, Cascade Yellow, Catecholamine, Chinacrine, Coriphosphine O, Coumarin, Coumarin-Phalloidin, CY3.1 8, CY5.1 8, CY7, Dans (1-Dimethyl Amino Naphaline 5 Sulphonic Acid), Dansa (Diamino Naphtyl Sulphonic Acid), Dansyl NH-CH3, DAPI, Diamino Phenyl Oxydiazole (DAO), Dimethylamino-5-Sulphonic acid, Dipyrrometheneboron Difluoride, Diphenyl Brilliant Flavine 7GFF, Dopamine, Eosin, Erythrosin ITC, Ethidium Bromide, Euchrysin, FIF (Formaldehyde Induced Fluorescence), Flazo Orange, Fluo 3, Fluorescamine, Fura-2, Genacryl Brilliant Red B, Genacryl Brilliant Yellow 10GF, Genacryl Pink 3G, Genacryl Yellow 5GF, Gloxalic Acid, Granular Blue, Haematoporphyrin, Hoechst 33258 (bound to DNA), Indo-1, Intrawhite Cf Liquid, Leucophor PAF, Leucophor SF, Leucophor WS, Lissamine Rhodamine B200 (RD200), Lucifer Yellow CH, Lucifer Yellow VS, Magdala Red, Marina Blue, Maxilon Brilliant Flavin 10 GFF, Maxilon Brilliant Flavin 8 GFF, MPS (Methyl Green Pyronine Stilbene), Mithramycin, NBD Amine, Nile Red, Nitrobenzoxadidole, Noradrenaline, Nuclear Fast Red, Nuclear Yellow, Nylosan Brilliant Flavin E8G, Oregon Green, Oxazine, Oxazole, Oxadiazole, Pacific Blue, Pararosaniline (Feulgen), Phorwite AR Solution, Phorwite BKL, Phorwite Rev, Phorwite RPA, Phosphine 3R, Phthalocyanine, Phycoerythrin R, Polyazaindacene Pontochrome Blue Black, Porphyrin, Primuline, Procion Yellow, Propidium Iodide, Pyronine, Pyronine B, Pyrozal Brilliant Flavin 7GF, Quinacrine Mustard, Rhodamine 123, Rhodamine 5 GLD, Rhodamine 6G, Rhodamine B, Rhodamine B 200, Rhodamine B Extra, Rhodamine BB, Rhodamine BG, Rhodamine WT, Rose Bengal, Serotonin, Sevron Brilliant Red 2B, Sevron Brilliant Red 4G, Sevron Brilliant Red B, Sevron Orange, Sevron Yellow L, SITS (Primuline), SITS (Stilbene Isothiosulphonic acid), Stilbene, Snarf 1, sulphO Rhodamine B Can C, Sulpho Rhodamine G Extra, Tetracycline, Texas Red, Thiazine Red R, Thioflavin S, Thioflavin TCN, Thioflavin 5, Thiolyte, Thiozol Orange, Tinopol CBS, TOTO 1, TOTO 3, True Blue, Ultralite, Uranine B, Uvitex SFC, Xylene Orange, XRITC, YO PRO 1, or combinations thereof. One skilled in the art would certainly know which one to select among such dyes as long as desired

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emission and absorption properties as well as their hydrophobic properties are appropriate. The spectral properties of the fluorescent dyes should be sufficiently similar in excitation wavelengths and intensity to fluorescein or rhodamine derivatives as to permit the use of the same flow cytometry equipment. It is preferable that the dyes, however, have higher solubility in organic solvents and have improved photostability and quantum yields. These dyes will be combined at predetermined ratio and embedded into a microsphere vehicle and total dye quantity will be between about 0.00001% and 15% by weight to particle weight. This limitation is however of little consequence to the present invention for as long as the particle impregnated with said dyes is stable and usable for its intended purpose.

Prior art method.

The prior art method teaches staining carrier particles with a single dye only (see Fig. 1). However, for the purpose of a meaningful comparison and in order to be consistent with the thrust of the instant invention, the said method was adapted to stain with two dyes simultaneously. The prior art of dyeing large polymer particles (>5 um) as stated in "Uniform Latex Particles" by Leigh B. Bangs was performed and the general outline of the procedure is shown in Fig. 1 and obtained results are shown in Fig. 3. Briefly, the process is started by placing 5 ml of undyed stock microspheres in an aqueous medium directly on a membrane covered fritted funnel. A vacuum pump pulled air through the microspheres plated onto the filter paper for one hour. Next, the dried microspheres were transferred to 50 ml of dye solution, covered, and stirred at room temperature over night. The next day the microspheres were separated by filtration from the dye solution and the dyed particles were placed in a vacuum dessicator for about four hours to remove residual solvent. Next is added 200 ml of Triton X-100 and water solution to the dried dyed microspheres in a 250 ml flask. The solution is stirred for three hours. The solution is filtered and the washing is repeated until no further dye is detected in the filtrate. The beads stained in this manner are tested for staining uniformity by flow cytometry (Fig. 3). It can be easily seen that, based on three separate experiments (tests A, B, and C), bead-to-bead variation at FL2 and FL3 parameters (CV or coefficient of variation) is rather high and inadequate (Table 1) to satisfy the increasing demand for precisely dyed multicolored microspheres.

General outline of the instant method.

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As the demand and applications for precisely dyed multicolored microspheres increases the development of alternate processes to the aforementioned method is warranted. As a result, a modification of the prior art method has been developed which has proven to be the most efficient method for precision dyeing of the microspheres (see Fig. 2). This method takes one tenth or even less of the time of the previously mentioned method and significantly enhances its precision. As before, it is critical to remove almost all traces of water from the microspheres. To accomplish this a volume of stock microspheres in an aqueous medium is pipetted onto a vacuum filter membrane, and the liquid is removed and discarded. Next, 100 ml of the rinse solvent (an aliphatic alcohol, such as propanol, methanol, ethanol, etc.) are added to the microspheres. The microspheres are resuspended by placing an ultrasonic probe directly into the solution and applying power for several seconds or as needed to affect resuspension. The suspension is filtered and previous step is repeated once more. Dyeing of the microspheres is accomplished by adding 50 ml of a dye solution (composed of one or more dyes in an organic solvent, as described below) to the filtering cup and resuspending as before. The suspension is allowed to sit for five minutes in the filtering cup. Next, 50 ml of rinse solvent is added to the dye suspension, sonicated and filtered. Another 100 ml of the rinse solvent is added, resuspended and filtered. The last step is repeated once more. In order to prepare the microspheres for storage, 100 ml of an aqueous medium is added to the microspheres, then sonicated and filtered. Finally, 50 ml of aqueous medium is added to the microspheres, sonicated and transferred to a storage container.

In a particular embodiment of the invention, two squaric acid-based dyes are mixed in a solvent suitable for the complete dissolution of both dyes, such as chloroform. Ethanol is added to the solution to increase wetting of the microspheres, and to create a process-dependent, final solvent density that is less than that of the microspheres. The concentrations of each dye are experimentally determined as a function of the target fluorescence intensity at each of the two center wavelengths. These concentrations maintain their relative intensity throughout this inventive process.

An important aspect of the present invention is the preparation of microspheres prior to the dyeing operation. Manufacturers often supply microspheres in an aqueous medium. It has been discovered that the surface of the microsphere that had been stored in aqueous medium must be treated to make it permeable to organic compounds. In a preferred embodiment, an amount of a polar organic solvent such as an alcohol is added to the microsphere solution to achieve

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about a 50% mixture of the aqueous medium and the polar organic solvent. This ratio, however, may vary and adjusted at will according to particular needs that one may have or determined by chemical and physical properties of medium and solvent.

An equally efficient and precise technique involves "drying" the microspheres through a series of alcohol, e.g., methanol, ethanol, 2-propanol, rinses. The process begins by spinning down the aqueous suspension of microspheres, typically 10% solids in suspension. The aqueous medium is decanted, and the beads are re-suspended in methanol. The alcohol solution at ca. 5% solids is vortexed, sonicated and spun down. This step is performed once or twice more. The excess alcohol is decanted from the pellet, and residual solvent is evaporated under vacuum.

Test samples consisting of 0.05 gram of dried microspheres are used to help adjust the dye solution to its desired ratio. The dried 0.05 gram of microspheres are suspended in 0.5 ml of dye mixture containing two or more dyes of interest. The suspension of microspheres, now at 10% solids, is vortexed and sonicated into suspension. Once in suspension the mixture of microspheres and dyes is mixed for one hour. After that hour, the microspheres are spun down for a period of 1minute using a centrifuge. The dye solution is decanted back into the main flask, and the 0.05 g of microspheres are re-suspended in 1 ml of 90% of alcohol, e.g., methanol. The rinse step uses double the volume of the dye solution, thus maintaining a 5% solid solution. The sample is vortexed, sonicated and spun down. The methanol supernatant is decanted. The 90% methanol rinse step is repeated once more. Finally, the excess methanol is decanted from the pellet, and the microspheres are re-suspended in an aqueous medium. The resulting test samples are then tested to determine the fluorescence activity/intensity of the labeled beads.

When the test samples show that the dye solution, indeed, has the precise ratios of the desired dyes, a macro-scale batch is conducted. The principle of macro-scale work up is identical to that noted above. Briefly, 25 ml of the desired dye solution is transferred to a 50 ml vial, which contains 2.5 grams of dried microspheres. The microspheres, now at 10% solids, are vortexed and sonicated. Once the microspheres are completely in suspension, it is mixed for an hour. After that hour the microspheres are taken out of the dye solution by centrifugation. The dye solution is decanted back into the main flask, and the 2.5 grams of microspheres are re-suspended in 50 ml of 90% methanol. The rinse step uses double the volume of the dye solution, thus maintaining a mixture of 5% solids. The sample is vortexed, sonicated and spun down. The methanol supernatant is decanted. This step is repeated once more. After the final

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methanol rinse is decanted, the microspheres are put through an aqueous rinse. The aqueous supernatant is decanted, and the beads are then re-suspended and stored in a fresh aqueous medium.

The following Examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These Examples are not intended in any way to otherwise limit the scope of the disclosure or the protection granted by Letters Patent hereon.

Example 1.

A single solution containing two different squaric acid dyes is prepared. One dye is a red fluorescent dye 1,3-bis [(1,3-dihydro-1,3,3-trimethyl-2H-indol-2-ylidene)methyl]-2,4-dihydroxy-cyclobutenediylium, bis(inner salt) and second dye is orange fluorescent dye can be 2-(3,5-dimethylpyrrol-2-yl)
-4-(3,5-dimethyl-2H-pyrrol-2-ylidene)-3-hydroxy-2-cyclobuten-1-one. The peak emission of dye #1 is 585 nm, and the peak emission of dye #2 is 630 nm. These dyes are chosen because they fall in the center of two of the fluorescence channels of a Becton Dickinson FACScan flow cytometer, which is the measurement device used to determine the precision of prior art dyeing techniques compared with this innovative new technique. The choice of fluorescence channels is, however, relative and immaterial since another flow cytometry apparatus may have different settings.

Two samples of undyed microspheres are prepared. The first is dyed with the mixture of orange and red dyes using this innovative technique (shown Figs. 2 and 4), and the second is dyed using the prior art technique (Figs. 1 and 3). Samples are measured on the FACScan, and an X-Y plot is generated to show the relative homogeneity of each sample. X-axis represents brightness or fluorescence intensity of orange dye and Y-axis represents the same parameters of red dye. Mean intensities and coefficients of variation are also measured. It is clear that beads stained by the old method spread over much larger X-Y area, indicating that the ratio of orange and red dyes vary from particle-to-particle. In contrast, the coefficient of variation in the bead population dyed by the instant, improved method is much smaller. About 10,000 beads in each tests A, B, and C, were run in parallel with beads stained by Bangs method (Table 1).

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Example 2

To make another population of beads with different fluorescent characteristics the ratio of red/orange dyes is altered by an adequate increment in proportion of dyes so that obtained population optically does not overlap with the former population. The prior art failed to provide multiple populations of multicolored beads due to inevitable intra-sample heterogeneity resulting from inadequate staining process resulting in poor dye distribution from particle-to-particle within given staining batch. Thus, upon excitation with a light source, stained beads containing more than one dye failed to emit uniform fluorescence signals of desired intensity. The instant invention overcomes this problem and achieves construction of as many as 64 subsets of optically distinct beads by varying the ratio of just 2 dyes. This example is not in any way a limiting one since one of ordinary skill may easily generate smaller or higher number of bead subsets by using the instant teaching. One skilled in the art may appreciate that nothing even close to this achievement has ever been enabled in the actual practice. Although such an eventuality was theoretically speculated as a possible one, the prior art failed to teach one of ordinary skill how to arrive at that.

The present inventors were able, for the first time, to reduce to practice the invention and representative experimental results of obtaining 64-bead population are shown in Fig. 6 and Table 2. The results illustrated in Fig. 5 show multicolored beads by using staining procedure of the prior art method. Due to imprecision in staining technique, which results in a wide dispersion of dyes ratio from bead-to-bead, no more than 6 subsets of multicolored bead populations can be fitted on a Fluorescence Bead Map. In contrast, Fig. 6 shows Fluorescence Bead Map containing 64 populations of beads, indicating tight distribution of each bead subset fluorescence characteristics within boundaries prescribed by each region. The cross-talk between various clusters is minimal. Most of the overlap is due to the presence of bead agglomerations which emit brighter signal but they are eliminated by size discrimination based on light scatter.

In general, as can be readily glanced from Table 2, there is an unequivocal relationship between two dye concentrations in a given population of beads and location of said populations on X-Y map. Each location is assigned in terms of red (FL3) or orange (FL2) dyes intensity as expressed in linear fluorescence channels units which fall in approximately 470, 580, 690, 750, 800, 900, and 990 series. For practical reasons, i.e., space limitation in the bead cluster, the last digit "0" is omitted. The first two digits in each bead population represents fluorescence intensity

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of orange dye (FL2) and last two digits the intensity of the red dye (FL2). The fluorescent intensity increases as the numbers go higher. The beads with lowest intensity (4725) reside in lower left corner and brightest ones (9998) in upper right corner. As they move vertically up a column, both red and orange dye amounts in a bead must be increased. This is because there is a substantial amount of energy transfer from the orange dye to the red. When moving horizontally from left to right across a row, the red dye must be decreased in order to maintain a steady FL3 value. This is due to overlap of the orange dye spectrum into the red region, thus necessitating the increase in FL3 signal. In this manner multiple, non-overlapping populations of beads are constructed. Two parameters namely, a fluorescent color (red or orange) and color intensity or brightness (expressed in fluorescence channel units), are essential to classify obtained beads and are termed as a fluorescence signal.

Hence, particular populations of beads are provided whose fluorescence characteristics or signals fall within a prescribed region depicted in a Fluorescence Bead Map. Typically, about 80% or greater of the individual beads within a particular population of beads will exhibit fluorescence characteristics within the desired region, preferably about 90% or greater, more preferably about 97% or greater, most preferably about 99% or greater. For each set of beads, typically about 1% or less of the individual beads within a particular set of beads will exhibit fluorescence characteristics that fall within another, undesired region, preferably about 0.5% or less, more preferably about 0.3% or less, most preferably about 0.2% or less.

While theoretically any number of populations or subsets can be present in each Bead Map, due to the limitations in the prior art techniques it is not possible to obtain more than 6 subsets coexisting simultaneously. While theoretically it has been speculated that such subsets can be extremely valuable for multiplex analysis (see for example McHugh, "Flow Microsphere Immunoassay for the Quantitative and Simultaneous Detection of Multiple Soluble Analytes," in Methods in Cell Biology, 42, Part B, (Academic Press, 1994) so far there are no known examples in the art enabling and demonstrating the reduction to practice of tangible, multicolored beads. At best only 1 and perhaps a maximum of 5 population of beads containing various ratios of two dyes could have been possible. For example, U.S. Pat. No. 4,717,655 discloses such beads, however, the disclosure was not enabled and the method of incorporating dyes in these beads is by copolymerization process and as such it is unrelated to the instant invention. In contrast, due to a significant improvement over existing methodology it is now technically

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possible to obtain 16-subset, 32-subset, 64-subset or even higher number of bead collections using the instant methodology.

As an example 64-subset bead collection or 64 populations of beads were constructed each population differing from another by a distinct location on the X-Y plot. These locations essentially do not overlap. As opposed to the prior art methods which result in up to 10-20% or even higher rate of dispersion the instant method allows to obtain essentially homogeneous populations of beads with only 0.2 -0.3 % dispersion. As used hereinafter the term essentially non-overlaping populations means that only about 0.2-0.3 % of beads in each population may display an optical pattern or fluorescent signal which can be ascribed to the neighboring cluster of beads having the same set of fluorescent dyes but mixed at different ratio. This is a significant improvement over the prior art.

Example 3

Although multiplexed analysis capability theoretically would provide enormous benefit in the art of flow cytometry, very little progress has been previously achieved due to technical limitations in obtaining sufficient variety of multicolored, non-overlaping subsets of fluorescent beads. A review of some of these prior art techniques is provided by McHugh (see above). These methods have been unsatisfactory as applied to provide fully multiplexed assay capable of analysis of more than a few different analytes. In the prior art when beads were incorporating a combination of 2 dyes only 5 subsets of beads were allegedly obtained (U.S. Pat. No. 4,717,655 issued to Fulwyler). A set with maximum of six subsets is obtained using Bangs method (see Example 1) which is still insufficient for the purposes of truly multiplexed assay.

A series of antibodies, antigens, or nucleic acid probes, collectively named hereinafter as analytical reactants, are attached to the beads by any of a number of conventional procedures such as by chemical or physical adsorption as described by Colvin et al., "The Covalent Binding of Enzymes and Immunoglobulins to Hydrophilic Microspheres" in Microspheres: Medical and Biological Applications, 1-13, CRC, Boca Raton, FL, 1988;

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Cantarero et al., "The Adsorptive Characteristics of Proteins for Polystyrene and Their Significance in Solid-Phase Immunoassays," Anal Biochem, 105, 375-382 (1980); and Illum et al., "Attachment of Monoclonal Antibodies to Microspheres," Methods in Enzymol, 112, 67-84 (1985) 112, 67-84 (1985).

After attachment of a reactant to the beads' surface, aliquots from each subset are mixed to create a pool containing known amounts of beads within each subset. Preferably, the pooled set is prepared with equal volumes of beads from each subset, so that the set contains about the same number of beads from each subset or population. This pool is then be incubated with a fluid sample of interest, such as serum or plasma, to test for the presence of antibodies in the fluid that are reactive with antigens on the beads. Such incubation is generally performed under conditions of temperature, pH, ionic concentrations, and the like that facilitate specific reaction of antibodies in the fluid sample with antigen on the bead surface. After a sufficient period of time, the beads in the mixture are centrifuged, washed and incubated for another period of time with a "secondary" antibody such as, for example, fluorescein labeled goat anti human immunoglobulin. The secondary antibody or label reagent will bind to and fluorescently label antibodies bound to antigen on the beads. After washing (or without washing), the beads are processed by a flow cytometer and the four classification parameters forward light scatter, side light scatter, red fluorescence, and orange fluorescence are measured and used to identify the subset or population to which each bead belongs. A simultaneous measurement of green fluorescence (measurement parameter) for each bead allows one to determine whether the bead has antibody bound to it. Because the subset to which a bead belongs is correlated with the presence of a particular antigen, e.g., series of grass allergens, various substance abuse drugs, one may readily determine the specificity of the antibody bound to a bead as a function of the subset to which it belongs.

Displacement or competition assay

Assays for many substances in a clinical laboratory are based on the interference with specific ligand-ligate or antigen-antibody interactions. In these assays, one member of the ligand-ligate pair is labeled with the fluorophore or fluorochrome and one member is

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immobilized on the beads. Soluble, unlabeled analyte, which may be ligand or ligate, is added to the reaction mixture to competitively inhibit interaction of the labeled component with the immobilized component. It is usually not important which member of the pair is labeled and which is immobilized; however, in certain assays, functional advantages may dictate the orientation of the assay. In an exemplary assay of this type, each bead subset is provided with an antigen. The antigen-coated beads are then reacted with labeled antibody specific for the antigen on the bead surface. Subsequent addition of a test fluid containing soluble analyte (inhibitor) will displace the labeled antibody from the beads in direct proportion to the concentration of the soluble analyte. A standard curve of known analyte concentrations is used to provide accurate quantification of analyte in the test sample.

Nucleic acid analysis

The power and sensitivity of PCR found its application to a wide variety of analytical problems in which detection of DNA or RNA oligonucleotide sequences is required. One major difficulty with the PCR technique is the cumbersome nature of the methods of measuring end-product, i.e., amplified DNA. A flow cytometric bead-based hybridization assay permits the extremely rapid and accurate detection of genetic sequences of interest. In a preferred embodiment of this invention, a bead to which a nucleic acid segment of interest has been coupled is provided. A PCR product of interest (or any other DNA or cDNA segment) is detected by virtue of its ability to competitively inhibit hybridization between the nucleic acid segment on the bead and a complementary fluorescent DNA probe. The method is sensitive and precise and allows the detection of single point mutations in the PCR product or DNA of interest. The multiplexed DNA analysis method can be applied to detect any PCR product or other DNA of interest for specific polymorphisms or mutations and one skilled in the art will recognize that numerous applications can be imagined such as presence of histocompatibility alleles associated with susceptibility to diseases, mutations associated with genetic diseases, autoimmune diseases, or mutations of oncogenes associated with neoplasia or risk of neoplasia. In a same way nucleic acid segments from pathogenic organisms such as bacterial, viral, fungal, mycoplasmal, rickettsial, chlamydial, or protozoan pathogens can be

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detected simultaneously.

Enzyme assays

The invention is also useful for measurement of enzymes, enzyme inhibitors and other analytes. For example, bead subsets are generated with selected fluorescent substrates which are enzymatically cleaved from the bead, resulting in a loss of fluorescence. Enzymes that can be detected and measured using the invention include but are not restricted to, proteases, glycosidases, nucleotidases, and oxidoreductases. Any enzyme that results in selected bond cleavage can be measured. Alternatively, the action of the enzyme on the bead-bound substrate results in the formation or identification of a ligate for a fluorescent ligand present in the reaction mixture. The bead bearing the modified substrate then becomes fluorescent by virtue of binding of the fluorescent ligand to the newly formed ligate. Because each type of bead bearing the unique substrate can be distinguished, a mixture of bead subsets can be used to measure several enzyme activities simultaneously in the same reaction mixture.

Fluids or samples with analytes that can be analyzed using these techniques include plasma, serum, tears, mucus, saliva, urine, pleural fluid, spinal fluid and gastric fluid, sweat, semen, vaginal secretions, fluid from ulcers and other surface eruptions, blisters, and abscesses, and extracts of tissues including biopsies of normal, malignant, and suspect tissues.

The above examples can be used to perform most common immunodiagnostic and nucleic acid assays. Other applications such as high throughput screening of combinatorial chemistry libraries for discovering new drugs, environmental screening of pollutants, drug testing, food safety-related investigations, testing of multiple analytes for agricultural needs, etc, can be imagined.

It is to be understood that, while the foregoing invention has been described in detail by way of illustration and example of preferred embodiments, numerous modifications, substitutions, and alterations are possible without departing from the spirit and scope of the invention as described in the following claims.

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TABLE 1

Sample	FL2 (CV)	FL3 (CV)	Events
Old Method			
A	24.2	25.9	10000
B	27.7	25.5	10000
C	27.7	23.2	10000
Improved Method			
A	7.3	6.8	10000
B	7.3	6.7	10000
C	7.2	6.7	10000

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TABLE 2

Region 47 series	FL3 Red Dye (mM)	FL2 Orange Dye (mM)
470/250	0.0495	0.00441
470/340	0.104	0.0041
470/415	0.0685	0.004
470/470	0.388	0.00576
470/525	0.458	0.0039
470/580	0.981	0.00662
470/635	0.132	0.00557
470/690	1.831	0.00637
470/745	3.84	0.01138
470/810	11.29	0.0278
Region 58-series	FL 3 Red Dye (mM)	FL2 Orange Dye (mM)
580/250	0.0201	0.00994
580/340	0.0582	0.00979
580/415	0.0763	0.00161
580/470	1.38	0.0256
580/525	0.42	0.0114
580/580	0.613	0.0162
580/635	1.15	0.0132
580/690	2.71	0.0267
580/745	0.643	0.0248
580/810	7.5	0.0489
580/870	9.71	0.008

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TABLE 2 (Sheet 2 of 3)		
Region 69-series	FL3 Red Dye (mM)	FL3 Orange Dye (mM)
690/360	0.048	0.024
690/415	0.129	0.024
690/470	0.226	0.023
690/525	0.45	0.029
690/580	0.89	0.0459
690/635	1.11	0.0322
690/690	2.45	0.07
690/745	2.88	0.0624
690/810	5.54	0.185
690/870	5.68	0.145
750/940	10.52	1.07
Region 80-series	FL 3 Red Dye (mM)	FL2 Orange Dye (mM)
800/415	0.0465	0.15
800/470	0.123	0.131
800/525	0.226	0.061
800/580	0.504	0.0835
800/635	0.62	0.0805
800/690	1.18	0.118
800/745	1.7	0.113
800/800	3.06	0.218
800/870	5.7	0.31
800/940	7.54	0.806
950/940	7.93	0.736

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TABLE 2 (Sheet 3 of 3)		
Region 90-series	FL 3 Red Dye (mM)	FL2 Orange Dye (mM)
900/510	0.084	0.0017
900/553	0.0666	0.0146
900/596	0.138	0.0913
900/645	1.29	0.253
900/690	0.861	0.298
900/734	0.699	0.229
900/779	0.984	0.214
900/823	1.13	0.435
900/867	2.61	0.381
900/912	3.41	0.579
900/953	4.88	0.861
Region 99-series	FL 3 Red Dye (mM)	FL 2 Orange Dye (mM)
990/600	------	0.274
990/640	0.125	0.21
990/680	0.21	0.268
990/720	0.472	0.216
990/760	0.712	0.275
990/800	0.82	0.278
990/840	1.12	0.446
990/880	1.87	0.431
990/920	3.24	0.477
990/980	2.93	0.763

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What is claimed is:

1. A method of staining polymeric microspheres with two or more fluorescent dyes comprising:

(a) combining at least two fluorescent dyes in a solvent mixture comprising at least one organic solvent in which the at least two fluorescent dyes are soluble and at least one alcoholic solvent in which the at least two fluorescent dyes are less soluble, to provide a solution of mixed dyes which is further characterized as having the capacity to swell at least partially but not dissolve a plurality of polymeric microspheres, which is brought into contact with said solution;

(b) contacting a plurality of polymeric microspheres with said solution for a period of time sufficient to provide uniform staining of substantially all of the members of said plurality of polymeric microspheres with the at least two fluorescent dyes,

said at least two fluorescent dyes being selected such that on isolation and excitation of said dyed plurality of polymeric microspheres, a distinct fluorescence signal is emitted from each dye, the intensity of which emitted signal is proportional to the amount of said dye in said dyed plurality of polymeric microspheres.

2. The method of claim 1, which further comprises dehydrating said plurality of polymeric microspheres.

3. The method of claim 2 in which the dehydrating step comprises washing said plurality of polymeric microspheres one or more times with an alcoholic solvent prior to contacting same with said solution.

4. The method of claim 3, which further comprises drying said washed microspheres or allowing the alcoholic solvent to evaporate from said washed microspheres prior to contacting same with said solution.

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5. The method of claim 1, which further comprises isolating said dyed plurality of polymeric microspheres by filtration or centrifugation.

6. The method of claim 1 in which at least one of said fluorescent dyes is diffused throughout the interior of substantially all of the members of said dyed plurality of polymeric microspheres.

7. The method of claim 1 in which said at least two fluorescent dyes are diffused throughout the interior of substantially all of the members of said dyed plurality of polymeric microspheres.

8. The method of claim 1 in which at least one of said fluorescent dyes is diffused through only a portion of the interior of substantially all of the members of said dyed plurality of polymeric microspheres.

9. The method of claim 1, which further comprises preparing a series of said solutions having differing desired ratios of said at least two fluorescent dyes.

10. The method of claim 9, which further comprises contacting separate populations of a plurality of polymeric microspheres with said series of said solutions to provide multiple distinct populations or subsets of a plurality of polymeric microspheres, each distinct population or subset having a differing desired ratio of said at least two fluorescent dyes.

11. The method of claim 1 in which the distinct fluorescence signals emitted from said at least two fluorescent dyes differ in their respective wavelengths by at least about 10 nm.

12. The method of claim 1 in which the distinct fluorescence signals emitted from said at least two fluorescent dyes differ in their respective wavelengths by at least about 30 nm.

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13. The method of claim 1 in which the distinct fluorescence signals emitted from said at least two fluorescent dyes differ in their respective wavelengths by at least about 50 nm.

14. The method of claim 1 in which at least one of said emitted signals is orange and at least another emitted signal is red.

15. The method of claim 1 in which the distinct fluorescence signals emitted from said at least two fluorescent dyes exhibit wavelengths falling in a range between about 500 nm and about 1,000 nm.

16. The method of claim 1 in which said organic solvent comprises acyl group-containing, aliphatic, cycloaliphatic, aromatic, halogenated, or heterocyclic hydrocarbons.

17. The method of claim 16 in which said organic solvent is selected from the group consisting of chloroform, toluene, xylene, hexane, methylene chloride, pentane, acetone, or DMSO.

18. The method of claim 1 in which said at least two fluorescent dyes have substantially similar solubility characteristics in the organic solvent of said solvent mixture.

19. The method of claim 1 in which said alcoholic solvent is selected from the group consisting of 2-propanol, methanol, ethanol, isopropanol, butanol, or pentanol.

20. A population of polymeric microspheres substantially uniformly stained with at least two fluorescent dyes, each microsphere of said population upon excitation exhibiting at least two distinct fluorescence emission signals corresponding to the at least two fluorescent dyes, the intensity of each of said at least two emitted signals (i) being proportional to the amount of its corresponding dye in said microsphere, and (ii) exhibiting a coefficient of variation among all the members of said population, which is no greater than about 20 percent.

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21. The population of claim 20 in which the intensity of each of said at least two emitted signals exhibits a coefficient of variation among all the members of said population, which is no greater than about 15 percent.

22. The population of claim 20 in which the intensity of each of said at least two emitted signals exhibits a coefficient of variation among all the members of said population, which is no greater than about 10 percent.

23. The population of claim 20 in which the intensity of each of said at least two emitted signals exhibits a coefficient of variation among all the members of said population, which is no greater than about 8 percent.

24. The population of claim 20 in which the intensity of each of said at least two emitted signals exhibits a coefficient of variation among all the members of said population, which is less than about 8 percent.

25. The population of claim 20 in which the at least two fluorescent dyes are hydrophobic.

26. The population of claim 25 in which the at least two fluorescent dyes comprise squaric acid-based dyes.

27. The population of claim 26 in which said squaric acid-based dyes are selected from cyclobutenedione derivatives, symmetrical and unsymmetrical squaraines, substituted cephalosporin compounds, fluorinated squaraine compositions, alkylalkoxy squaraines, or squarylium compounds.

28. The population of claim 26 in which said squaric acid-based dyes are selected

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from a red fluorescent dye and an orange fluorescent dye.

29. The population of claim 28 in which the red fluorescent dye comprises 1,3-bis[(1,3-dihydro-1,3,3-trimethyl-2H-indol-2-ylidene)methyl]-2,4-dihydroxycyclobutenediyliu m, bis(inner salt) and said orange fluorescent dye comprises 2-(3,5-dimethylpyrrol-2-yl)-4-(3,5-dimethyl-2H-pyrrol-2-ylidene)-3-hydroxy-2-cyclobuten-1-one.

30. The population of claim 20 in which said microspheres comprise polystyrene, brominated polystyrene, polyacrylic acid, polyacrylonitrile, polyacrylamide, polyacrolein, polydimethylsiloxane, polybutadiene, polyisoprene, polyurethane, polyvinylacetate, polyvinylchloride, polyvinylpyridine, polyvinylbenzylchloride, polyvinyltoluene, polyvinylidene chloride, polydivinylbenzene, polyglycidylmethacrylate, polymethylmethacrylate, or copolymers, blends, composites, or combination thereof.

31. The population of claim 20 in which said microspheres further comprise at least one analytical reactant bound covalently to functional groups present on the surface of said microspheres or passively adsorbed to the surface of said microspheres.

32. The population of claim 20 in which said microspheres have a diameter between about 10 nm and 100 μm.

33. A collection of distinct populations of polymeric microspheres according to claim 20, each population exhibiting an emission spectrum in a Fluorescence Bead Map, which is unique to said population.

34. The collection of claim 33 which comprises eight or more distinct populations of polymeric microspheres.

35. The collection of claim 33 which comprises sixteen or more distinct populations

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of polymeric microspheres.

36. The collection of claim 33 which comprises twenty-four or more distinct populations of polymeric microspheres.

37. The collection of claim 33 which comprises thirty-two or more distinct populations of polymeric microspheres.

38. The collection of claim 33 which comprises sixty-four or more distinct populations of polymeric microspheres.

39. The collection of claim 38 in which there is substantially no overlap between any of the sixty-four or more emission spectra associated with said sixty-four or more distinct populations of polymeric microspheres.

40. A method of detecting a plurality of analytes in a sample, each of said analytes being recognized by a corresponding analytical reactant, comprising:

(a) contacting said sample with a plurality of populations of uniformly stained microspheres, said microspheres having at least two fluorescent dyes uniformly mixed at a specific ratio within each microsphere of each said population, each population of said microspheres having a distinct analytical reactant bound to its surface, wherein, said reactant on each population of microspheres specifically interacts with one of said analytes in said sample;

(b) providing a label reagent that specifically binds to the analyte and analyzing the microspheres to detect said label indicating binding of the analyte to the analytical reactant; and

(c) determining the populations of microspheres having said fluorescent dyes mixed at the specific ratio within microspheres of each population to which the reactant is bound.

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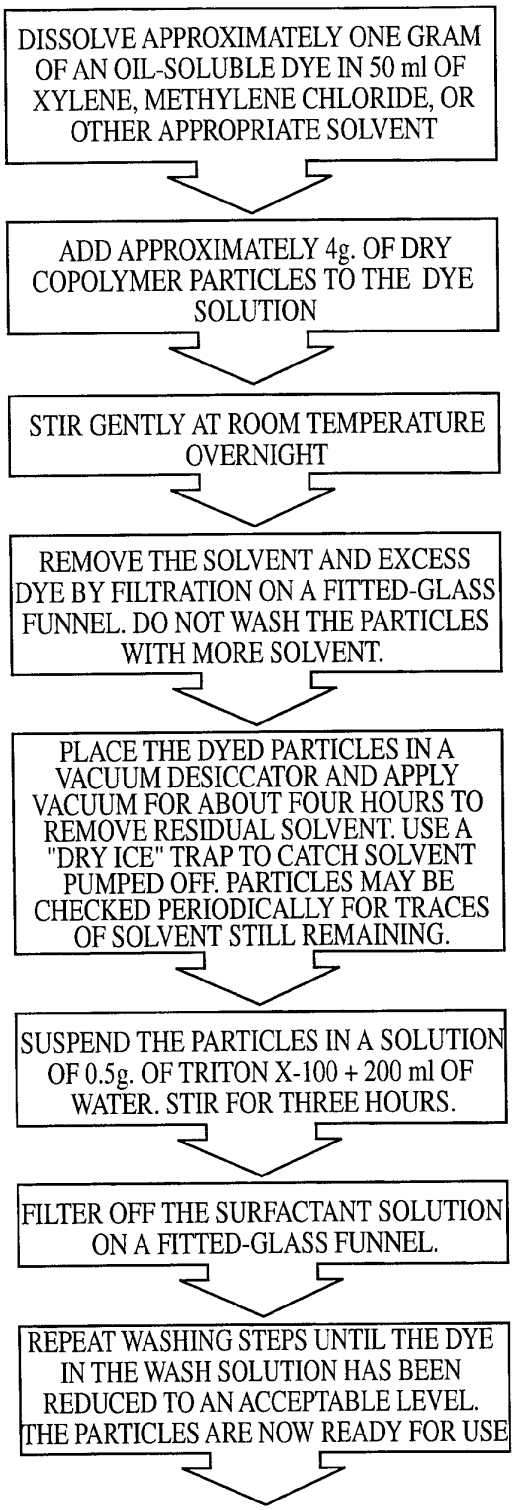


FIG. 1

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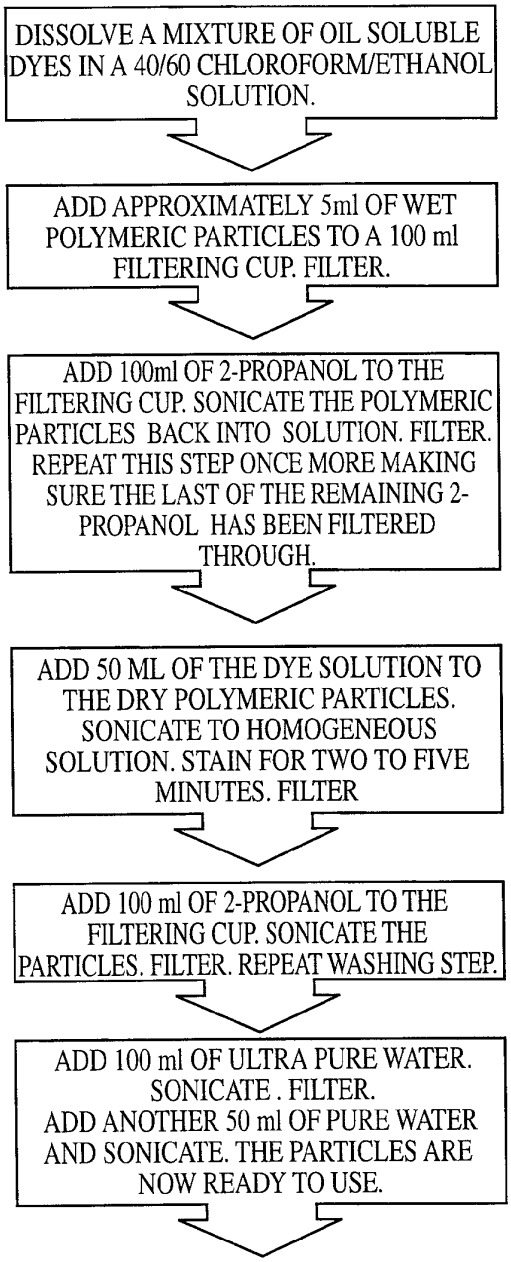


FIG. 2

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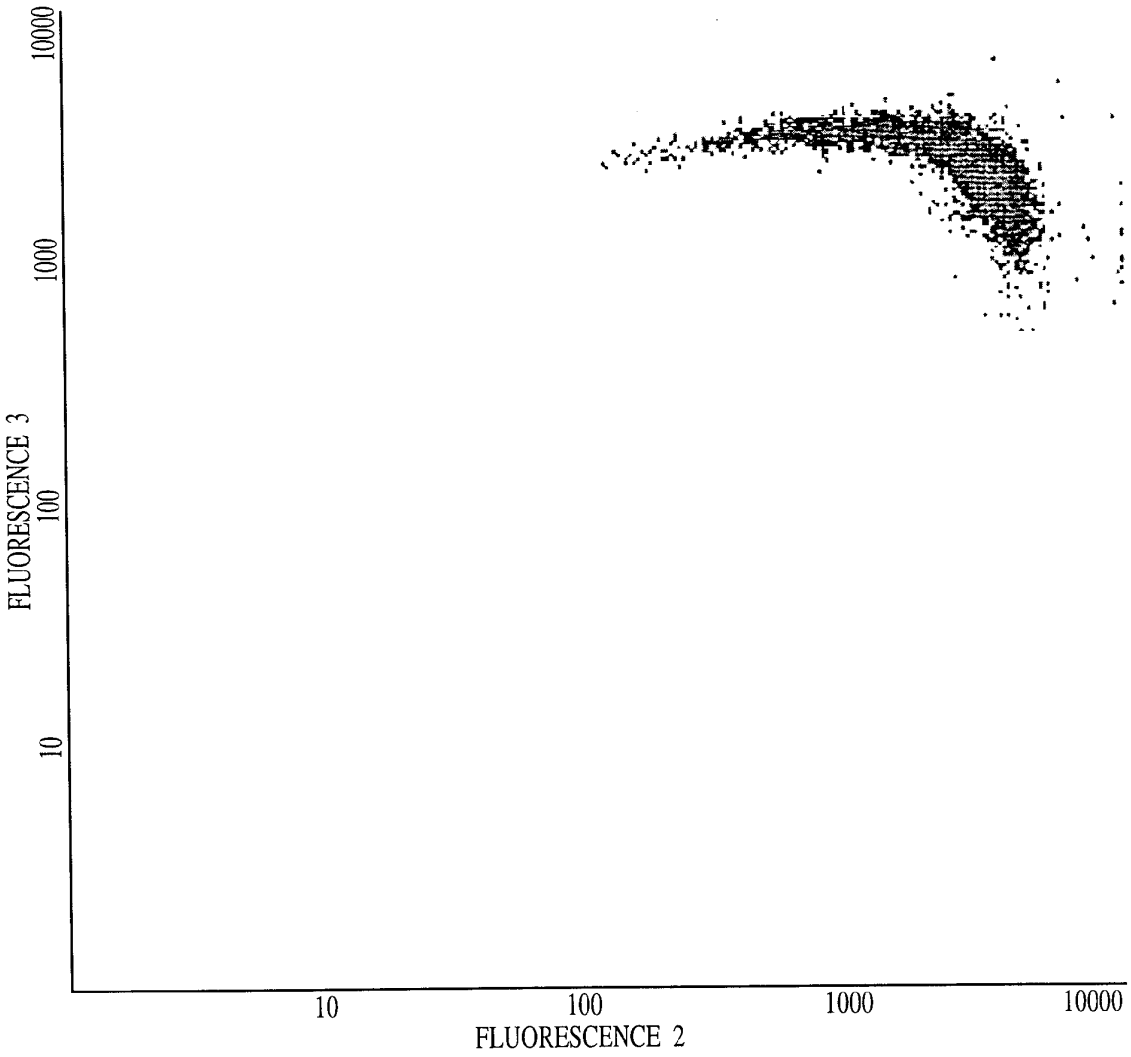


FIG. 3

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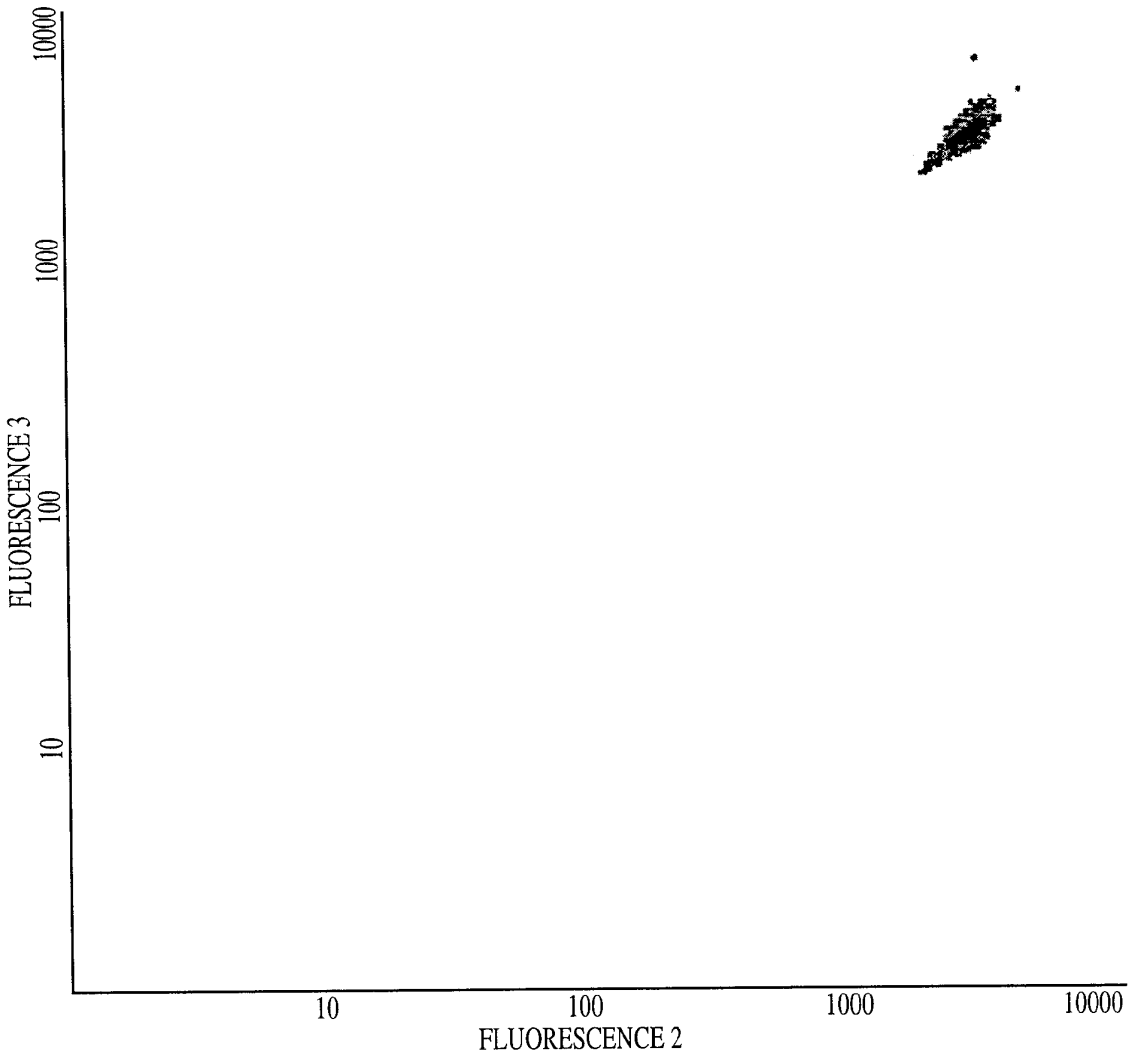


FIG. 4

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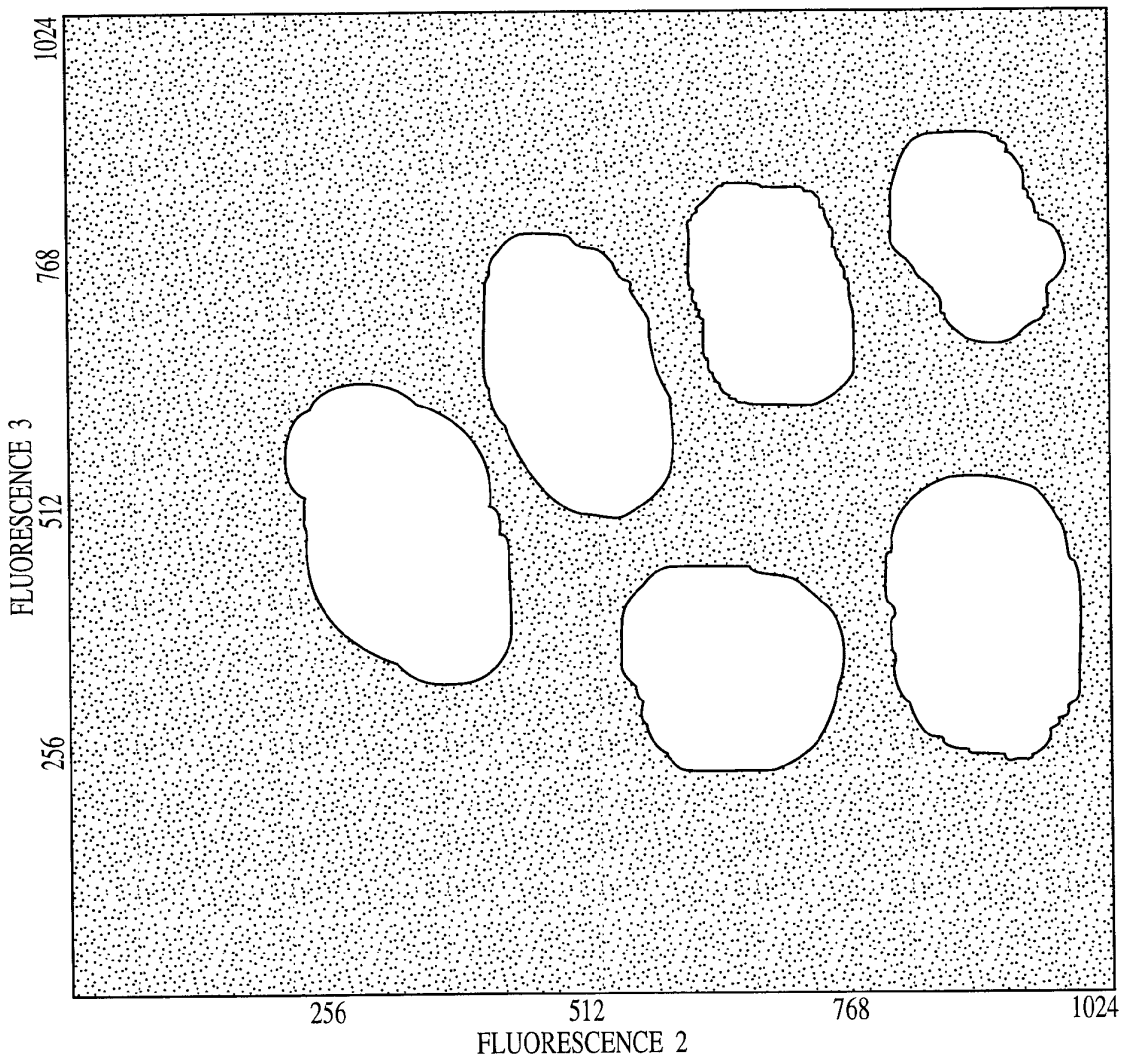


FIG. 5

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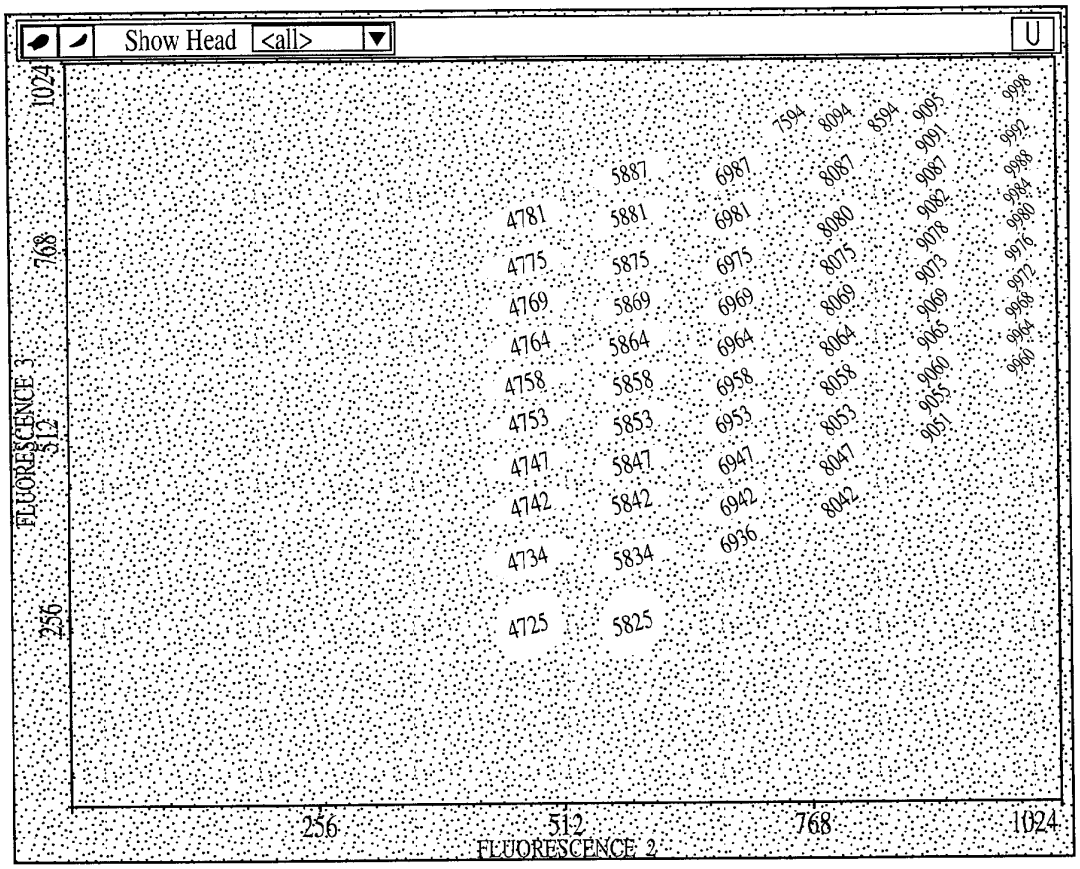


FIG. 6

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/21562

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12Q 1/68; D06P 1/90; B32B
US CL :435/6; 428/407; 8/506, 607, 611, 614, 616, 617, 638, 648
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 428/407; 8/506, 607, 611, 614, 616, 617, 638, 648

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS REG: SQUARIC ACID; SQUARAINE
APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,573,909 A (SINGER et al.) 12 November 1996 (12-11-96).	20-40
X	US 4,717,655 A (FULWYLER) 05 January 1988 (05-01-88), see examples and claims.	20-25, 30-40
A	US 3,790,492 A (FULWYLER) 05 February 1974) (05-02-74).	20-25, 30-40
P,X	US 5,723,218 A (HAUGLAND ET AL.) 03 March 1998 (03-03-98), see col 10, lines 37-62.	20-25
X	US 5,073,498 A (SCHWARTZ et al.) 17 December 1991 (17-12-91), col 4, lines 54-66 and examples.	20-25, 30-33

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 14 DECEMBER 1998	Date of mailing of the international search report 20 JAN 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARGARET EINSMANN Telephone No. (703) 308-0661

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/21562

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,326,692 A (BRINKLEY et al.) 05 July 1994	all
$\overline{X}$	Example 6 in col 21 and 22	1-8,11-13 16-25 30-32

Form PCT/ISA/210 (continuation of second sheet)(July 1992)★

Electronic Acknowledgement Receipt

EFS ID:	42287280
Application Number:	16864744
International Application Number:	
Confirmation Number:	9929
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	Nicholas Shaofeng Tiee/Christina Campbell
Filer Authorized By:	Nicholas Shaofeng Tiee
Attorney Docket Number:	44158-727.304
Receipt Date:	25-MAR-2021
Filing Date:	01-MAY-2020
Time Stamp:	20:01:30
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	ChromaCode_44158-727_304_IDS.pdf	868730 e2612249df35ce2dda1c403884dee30debdf736a	no	31

Warnings:

Information:					
This is not an USPTO supplied IDS fillable form					
2	Foreign Reference	CN101570782A-W-ABSTRACT.PDF	954701 e69d4f7bc607214dd6fa39ddfa6f6bb9043380e2	no	23
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3	Foreign Reference	CN1936019A_EFS.PDF	1207496 f5f2f8532513f83368e08dc861596db98f5d19ca	no	22
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4	Foreign Reference	EP0519338A1_EFS.PDF	1464771 b0ebfd0fef5d010f9be7acf38a6dff971fa8fb69	no	20
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5	Foreign Reference	EP1448581B1_EFS.PDF	2143373 5db7395fd2af93f4395d25c38773400998ffffd97	no	32
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6	Foreign Reference	EP1629108B1_EFS.PDF	1452383 e8c0e12d979e2b5c5e32a4774b7ad09c7c1c51e1	no	26
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7	Foreign Reference	EP1963531B1_EFS.PDF	6616099 f58e602f86958744e8023ebcd064103254014530	no	100
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8	Foreign Reference	EP2224017A1_EFS.PDF	1358540 402ed15cb6d881051d54c41da40e8ac744bb20c3	no	21
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Information:					

9	Foreign Reference	WO0107640A2.PDF	3682069 7fcdb72ee26e4ecca8331a989f3d40275539c491	no	92
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10	Foreign Reference	WO02056014A2_EFS.PDF	1645421 a361210c1ad6cd9c97f3624cdf57ce8d719dbd46	no	40
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11	Foreign Reference	WO02056014A3_EFS.PDF	154903 b63f937aeaa5d6f9eb8e3667f26edd6dbb0e3f21	no	4
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12	Foreign Reference	WO03002979A2_EFS.PDF	4855047 0b225d08ee9e13ab5f4e27c216505d95031301d4	no	87
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13	Foreign Reference	WO03020967A1_EFS.PDF	4321589 cbb7cf8147b9268086bed8bba44dfe11abfb3c5a	no	52
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14	Foreign Reference	WO2004087950A2_EFS.PDF	2587396 228e5bc4cce5f870ccbe8d336392459ee9a90e2a	no	62
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15	Foreign Reference	WO2006079049A2.PDF	8053093 cb4fee77564e10a5e559753aac779a172f694156	no	153
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16	Foreign Reference	WO2007076128-A3.PDF	217014 c202375adf182e414c54e962f77182890274dc84	no	4
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17	Foreign Reference	WO2007076128A2_EFS.PDF	9105418 02294659cabfa905f7d5ac02b5c19411999872dc	no	157
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18	Foreign Reference	WO2007076129A2_EFS.PDF	9157652 190555a2da1ccfd76db35d6d19ef082eb27e956d	no	159
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19	Foreign Reference	WO2007076129-A3.PDF	152060 fa53eb34a1c43f4f3e93a75df0d923c83f0fc095	no	4
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20	Foreign Reference	WO2007076132A2_EFS.PDF	2595056 e8e7fae0cc37d9b092136a6e29a74cc71a9c721d	no	49
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21	Foreign Reference	WO2007076132A3_EFS.PDF	121847 78bf5c1e246cb6a28467afcd403626f3b3338455	no	2
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22	Foreign Reference	WO2007139766A2_EFS.PDF	8639590 8f2d4eca31d3d8d5abd6b7ce8d9827c03fdece18	no	146
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23	Foreign Reference	WO2007139766A3_EFS.PDF	169555 05fcb44c496652de081bc14ab0b9f7c2be3c6c48	no	4
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24	Foreign Reference	WO2008118998A2_EFS.PDF	2846540 0cda152f5ba28ef26f856502321b930e207621f4	no	60
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25	Foreign Reference	WO2008124847A2_EFS.PDF	12967152 288abd6c1e6cbaad448c4e0fde724a46c5fa61ff	no	237
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26	Foreign Reference	WO2008124847A3_EFS.PDF	180023 17752cb106f99307044e3beb4e51e7c72a87ab8f	no	4
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27	Foreign Reference	WO2009036514A2_EFS.PDF	6818423 cb0369e2f63dc285383254f615cb7af0b714e99c	no	100
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28	Foreign Reference	WO2010007355A1_EFS.PDF	1802572 5927fa00fb58df4856c158acb3c961145ad4893b	no	42
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29	Foreign Reference	WO2010013017A1_EFS.PDF	5484431 af5491a3dfe54ef9a08113c9b90062dbcf59336d	no	113
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30	Foreign Reference	WO2010017543A1_EFS.PDF	1363705 78e727b1878d6c7b8a531d1f613c8781a91bee46	no	33
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31	Foreign Reference	WO2010019826A1_EFS.PDF	5478184 7587bd083e7fbcfed73467bf168065a8e4a25316	no	92
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32	Foreign Reference	WO2010128206A1.PDF	1535764 0a9926e2725d0b1ad2976e90e8c688b4c93718ff	no	42
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33	Foreign Reference	WO2011047087-A3.PDF	167560 f098495249aa226179e33e36d161dc0e789ab602	no	5
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34	Foreign Reference	WO2011047087A2_EFS.PDF	3565092 e14d713ae8dd942f2c6ce50099ea2b73bc67eaea	no	80
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35	Foreign Reference	WO2011100541A2_EFS.PDF	3577812 5fa0154184fae80ea11eb9c95f415e7aa471bd00	no	67
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36	Foreign Reference	WO2011100541-A3.PDF	161292 9777bbbe8125f16f21e13386267 6f69882f705d6	no	3
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Information:					

37	Foreign Reference	WO2011116088A2_EFS.PDF	4257144 4797db8de95aaebae18d6e9d77228bc9c450132c	no	84
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38	Foreign Reference	WO2011116088A3_EFS.PDF	164663 2795f1b939393bf7caa70cda5d2dd2dd0794ada9	no	4
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39	Foreign Reference	WO2012058638A2_EFS.PDF	12944400 7abc037e2085bbc80f7d0fd2d25f9687163544b0	no	189
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40	Foreign Reference	WO2012058638A3_EFS.PDF	210599 8dadb19ca18a26f314627bcc8de4f58fa8ce8c89	no	6
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42	Foreign Reference	WO2012135340-A3.PDF	250101 fe498b1835a16d579d8510daddfc6538bbcba250	no	6
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43	Foreign Reference	WO2012135340A2_EFS.PDF	2560330 9c2eba74d9f86a4594a9467eb41cb859d246b6c3	no	47
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Information:					

44	Foreign Reference	WO2013096851A1_EFS.PDF	6716021 5155109a566947cdc45d31360208e930731a0f06	no	128
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45	Foreign Reference	WO2013116780A1_EFS.PDF	5144926 f6b7d5713e95f9e824a2030ca30950117f4f607e	no	84
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46	Foreign Reference	WO2014022827A1_EFS.PDF	2195774 5e1026db171e6de28c7b5e705b1ae96bd2cd5dfc	no	52
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47	Foreign Reference	WO2014116884A1_EFS.PDF	6173924 d6922d081981448ea91c13e1510c7fcad17eff89	no	114
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48	Foreign Reference	WO2015147370A1_EFS.PDF	6046282 fa894fb7a4445f401fe9dfa50721b2ac81be54fc	no	136
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49	Foreign Reference	WO2016154600A1_EFS.PDF	5094322 332dae612c935f21e1f6ce070f72612230c956f6	no	86
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50	Foreign Reference	WO2017139354A1_EFS.PDF	4494139 25dc751fd2be833c858b28ad372b1d40d39e32c9	no	82
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Information:					

51	Foreign Reference	WO2017173035A1.PDF	2525583 5d8df6d2adf9a5ea0672cf50f162771e50c622d9	no	46
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Information:					
52	Foreign Reference	WO2017218777A1.PDF	4736844 1ccfea4063edf028c786314c1951a11f93bf38e3	no	88
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54	Foreign Reference	WO2019006023A1_EFS.PDF	1832060 7a77e60c38305e10c5cf89bdf83e971314edcc0b	no	32
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56	Foreign Reference	WO2019169043A1_EFS.PDF	3099873 914132ac301dd0db4fa86d74c88e0b28856549c8	no	53
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57	Foreign Reference	WO9746714A1_EFS.PDF	7002526 6ae4974d9165e2df00f37804ce062c7cd1111232	no	217
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58	Foreign Reference	WO9952708A1_EFS.PDF	1886237 54abe42c3109a6b89e4b15f30a2eaca353c8f8af	no	32
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Information:					
59	Foreign Reference	WO9919515A1_EFS.PDF	2057277 be04210e0e05b5e7760f2c2aa8096a49e2be77ed	no	42
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Information:					
60	Non Patent Literature	ARYA-BASIC-2005.PDF	1281400 8e7641c313506c6d575b3a38c2e9f3d9ee18a242	no	12
Warnings:					
Information:					
		Total Files Size (in bytes):	209531262		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

ChromaCode / 44158-706.601
VN/ KSS/ LO6/ CGD/ LKIM/ ASE/ ANN

JUN 26 2017

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: Lee Alan Johnson
Wilson Sonsini Goodrich & Rosati
650 Page Mill Road
Palo Alto, California 94304
United States of America

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

Date of mailing (day/month/year) 22 JUN 2017	
Applicant's or agent's file reference 44158-706601	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No PCT/US17/24933	International filing date (day/month/year) 30 March 2017 (30.03.2017)
Applicant CHROMACODE INC.	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46).

When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

How? Directly to the International Bureau of WIPO preferably through ePCT or on paper to: 34 chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 338 82 70

For more detailed instructions, see *PCT Applicant's Guide*, International Phase, paragraphs 9.004 – 9.011.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ **With regard to any protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that
   - ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices
   - ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made

4. **Reminders**

The applicant may **submit comments on an informal basis on the written opinion of the International Searching Authority** to the International Bureau. These comments will be made available to the public after international publication. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established.

Shortly after the expiration of **18 months from the priority date, the international application will be published** by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90*bis*.1 and 90*bis*.3).

Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for **entry into the national phase** before those designated Offices. In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months. For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

Within **19 months from the priority date, the applicant may request that a supplementary international search be carried out** by a different International Searching Authority that offers this service (Rule 45*bis*.1). The procedure for requesting supplementary international search is described in the *PCT Applicant's Guide*, International Phase, paragraphs 8.006-8.032.

Name and mailing address of the ISA/US Mail Stop PCT, Attn. ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/220 (July 2014)

YOUR PATENT DOCKET
S: FOREIGN:
DOCKETED: 6/26/17 BY:
ACTION: Search report 8/22/17
DUE DATES: written opinion 9/11/18
No related US case

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 44158-706601	FOR FURTHER ACTION	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US17/24933	International filing date *(day/month/year)* 30 March 2017 (30.03.2017)	(Earliest) Priority Date *(day/month/year)* 01 April 2016 (01.04.2016)
Applicant CHROMACODE INC		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into ..... which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).

c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☒ **Certain claims were found unsearchable** (see Box No. II)

3. ☐ **Unity of invention is lacking** (see Box No. III)

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5 With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6 With regard to the **drawings**,

a. the figure of the **drawings** to be published with the abstract is Figure No. 1

☒ as suggested by the applicant.

☐ as selected by this Authority, because the applicant failed to suggest a figure.

☐ as selected by this Authority, because this figure better characterizes the invention.

b. ☐ none of the figures is to be published with the abstract.

IPR2024-01451
California Institute of Technology - Ex. 2003 Page 4138

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/24833

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-42 and 49-82
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

IPR2024-01451
California Institute of Technology - Ex. 2003 Page 4139

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/24933

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C07H 21/00, 21/04; C12Q 1/68; G01N 21/64, 33/533, 33/542 (2017.01)

CPC - C07H 21/00, 21/04; C12Q 1/68, 1/686, 1/6806, 1/6851, 2561/101, 2561/113; G01N 21/64, 33/533, 33/542

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2006/079049 A2 (THIRD WAVE TECHNOLOGIES, INC.) 27 July 2006; page 2, lines 10-17 and 23-31; page 3, lines 13-20; page 5, lines 5-8 and 29-30; page 6, lines 18-30; page 7, lines 14-19; page 15, lines 24-31; page 16, lines 1-3 and 18-28	1-4, 5/1-3, 43-47, 48/45-47, 83-87, 88/83-87, 89/83-87, 90/83-87, 91-97, 98/91-97, 99
A	US 2009/0042735 A1 (BLAIR SM, et al.) 12 February 2009; paragraphs [0018], [0035], [0041]-[0043], [0072], [0074]	1-4, 5/1-3, 43-47, 48/45-47, 83-87, 88/83-87, 89/83-87, 90/83-87, 91-97, 98/91-97, 99
A	US 2014/0213471 A1 (CALIFORNIA INSTITUTE OF TECHNOLOGY) 31 July 2014; paragraphs [0003], [0010]-[0011], [0045], [0092]	1-4, 5/1-3, 43-47, 48/45-47, 83-87, 88/83-87, 89/83-87, 90/83-87, 91-97, 98/91-97, 99
A	US 2012/0040349 A1 (LODE PV, et al.) 16 February 2012; paragraphs [0030]-[0034], [0064]	1-4, 5/1-3, 43-47, 48/45-47, 83-87, 88/83-87, 89/83-87, 90/83-87, 91-97, 98/91-97, 99

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 June 2017 (03.06.2017)	22 JUN 2017
Name and mailing address of the ISA/	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: Lee Alan Johnson
Wilson Sonsini Goodrich & Rosati
650 Page Mill Road
Palo Alto, California 94304
United States of America

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing (*day/month/year*) 22 JUN 2017

Applicant's or agent's file reference	FOR FURTHER ACTION
44158-706601	See paragraph 2 below

International application No.	International filing date (*day/month/year*)	Priority date (*day/month/year*)
PCT/US17/24933	30 March 2017 (30.03.2017)	01 April 2016 (01.04.2016)

International Patent Classification (IPC) or both national classification and IPC
IPC - C07H 21/00, 21/04; C12Q 1/68; G01N 21/64, 33/533, 33/542 (2017.01)
CPC - C07H 21/00, 21/04; C12Q 1/68, 1/686, 1/6806, 1/6851, 2561/101, 2561/113; G01N 21/64, 33/533, 33/542

Applicant CHROMACODE INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	03 June 2017 (03.06.2017)	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (January 2015)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US17/24933

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a)).

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

a. ☐ forming part of the international application as filed:

☐ in the form of an Annex C/ST.25 text file

☐ on paper or in the form of an image file

b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

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WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US17/24933

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be not obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 6-42 and 49-82

because:

☐ the said international application, or the said claims Nos. ..... relate to the following subject matter which does not require an international search *(specify)*:

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 6-42 and 49-82 are so unclear that no meaningful opinion could be formed *(specify)*:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

☐ the claims, or said claims Nos. ..... are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. 6-42 and 49-82

☐ a meaningful opinion could not be formed without the sequence listing: the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.

☐ furnish a sequence listing on paper or in the form of an image file complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.

☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13*ter*.1(a) or (b).

☐ See Supplemental Box for further details.

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WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
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Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	***see box below***	YES
	Claims	NONE	NO
Inventive step (IS)	Claims	***see box below***	YES
	Claims	NONE	NO
Industrial applicability (IA)	Claims	***see box below***	YES
	Claims	NONE	NO

2. Citations and explanations:

Novelty (N): YES; 1-4, 5/3-4, 43-47, 48/45-47, 83-87, 88/83-87, 89/83-87, 90/83-87, 91-97, 98/91-97, 99

Inventive step (IS): YES; 1-4, 5/3-4, 43-47, 48/45-47, 83-87, 88/83-87, 89/83-87, 90/83-87, 91-97, 98/91-97, 99

Industrial applicability (IA): 1-4, 5/3-4, 43-47, 48/45-47, 83-87, 88/83-87, 89/83-87, 90/83-87, 91-97, 98/91-97, 99
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Claims 1-4, 5/3-4, 43-47, 48/45-47, 83-87, 88/83-87, 89/83 87, 90/83-87, 90/83-87, 91-97, 98/91-97, and 99 meet the criteria set out in PCT Article 33(2)-(3)

Claim 1 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest method of identifying a first target nucleic acid comprising, providing a sample comprising the first target nucleic acid, providing a first set of paired oligonucleotides with complementarity to the first target nucleic acid, said first set of paired oligonucleotides comprising a first ratio of (a) first competitive oligonucleotides to (b) first signal oligonucleotides comprising a first signal tag, wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid, amplifying the first target nucleic acid with the polymerase chain reaction, thereby degrading the first signal oligonucleotide and permitting generation of a first signal, generating the first signal, measuring intensity of the first signal, and correlating the intensity of the first signal to the first ratio, thereby identifying the first target nucleic acid.

WO 2006/079049 A2 to Third Wave Technologies, Inc. (hereinafter 'Wave') discloses method of identifying a first target nucleic acid (method of detection of a target nucleic acids (first); page 2, lines 10-14); comprising, providing a sample comprising the first target nucleic acid (providing a sample with target nucleic acids (first); page 2, lines 10-14), providing a first set of paired oligonucleotides with complementarity to the first target nucleic acid (first and second probe oligonucleotides that occupy probe hybridization site on the target nucleic acid (first); page 2, lines 23-31), said first set of paired oligonucleotides comprising a first ratio (ratio of paired oligonucleotides, first probe oligonucleotides to second probe oligonucleotides, page 3, lines 13-20; page 6, lines 28-30) of (a) first oligonucleotides (first probe oligonucleotides; page 2, lines 23-31) to (b) first signal oligonucleotides comprising a first signal tag (first oligonucleotides have a first label; page 5, lines 5-8), wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid (first probe oligonucleotides and second probe oligonucleotides are configured to bind the target nucleic acid with same affinity and identical sequences (competitive); page 15, lines 24-31; page 16, lines 6-8), amplifying the first target nucleic acid with the polymerase chain reaction (amplifying the first target nucleic acid with TAQMAN assay (PCR); page 6, lines 18-28; page 7, lines 14-19), thereby degrading the first signal oligonucleotide and permitting generation of a first signal (cleavage (degrading) of first probe oligonucleotide containing a label and allowing generation of a signal (first); page 7, lines 14-25) , generating the first signal (generating a first signal; page 18, lines 14-16), measuring intensity of the first signal (measuring the strength of the signal; page 18, lines 22-28), and thereby identifying the first target nucleic acid (identifying the target nucleic acid; page 7, lines 14-19), but Wave does not disclose (a) first competitive oligonucleotides, and correlating the intensity of the first signal to the first ratio.

US 2009/0042736 A1 to Blair, et al. (hereinafter 'Blair') discloses method of identifying a first target nucleic acid (method of identifying a target nucleic acid; paragraph [0018]) comprising, providing a sample comprising the first target nucleic acid (one target nucleic acid is present in a given sample; paragraphs [0035], [0041]), providing a first set of paired oligonucleotides with complementarity to the first target nucleic acid (providing a set (first) of paired nucleic acid probes that can hybridize (complementarity) to target (first) nucleic acids; paragraphs [0041]-[0042]), said first set of paired oligonucleotides comprising a first ratio (set of paired probe and competitive labeled probe nucleic acids (first set), would inherently have a ratio; paragraphs [0041]-[0042]) of (a) first competitive oligonucleotides to (competitive probe nucleic acid; paragraphs [0041]-[0042]) (b) first signal oligonucleotides comprising a first signal tag (labeled competitor nucleic acids (first signal); paragraphs [0041]-[0042]), wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid (probe nucleic acids (first) competes with labeled competitor nucleic acids; paragraphs [0041]-[0042]), amplifying the first target nucleic acid with the polymerase chain reaction (amplifying the first target nucleic acid with a polymerase chain reaction; paragraph [0072]), but Blair does not disclose thereby degrading the first signal oligonucleotide and permitting generation of a first signal, generating the first signal, measuring intensity of the first signal, and correlating the intensity of the first signal to the first ratio, thereby identifying the first target nucleic acid.

-***-Continued Within the Next Supplemental Box-***-

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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of

-***-Continued from: Box V: Citations and Explanations-***-

US 2014/0213471 A1 to California Institute of Technology (hereinafter 'CalTech') discloses method of identifying a first target nucleic acid (method of detecting at least one polynucleotide analyte (target); paragraph [0003]) comprising, providing a sample comprising the first target nucleic acid (providing a sample comprising a first polynucleotide analyte (target); paragraph [0003]), providing a first set of paired oligonucleotides with complementarity to the first target nucleic acid (providing a set of first primer and a first oligonucleotide specific (complementarity) for binding a first polynucleotide analyte (target; paragraph [0003]), said first set of paired oligonucleotides comprising a first ratio (set of paired probe labeled nucleic acids (first set), would inherently have a ratio; paragraph [0003]) of (b) first signal oligonucleotides comprising a first signal tag (first primer oligonucleotide comprises a first signal tag; paragraph [0011]), amplifying the first target nucleic acid with the polymerase chain reaction (amplifying the first polynucleotide analyte using a polymerase chain reaction; paragraph [0010]), generating the first signal (generating a first signal; paragraph [0011]), measuring intensity of the first signal (measuring a first signal; paragraph [0011]), thereby identifying the first target nucleic acid (identifying the first polynucleotide analyte (target); paragraph [0011]), but Caltech does not disclose (a) first competitive oligonucleotides to, wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid, thereby degrading the first signal oligonucleotide and permitting generation of a first signal, and correlating the intensity of the first signal to the first ratio.

US 2012/0040349 A1 to Lode, et al. (hereinafter 'Lode') discloses method of identifying a first target nucleic acid (method of detecting target nucleic acids; paragraphs [0031]) comprising, providing a sample comprising the first target nucleic acid (providing a sample with target nucleic acids; paragraph [0032]), providing a first set of paired oligonucleotides with complementarity to the first target nucleic acid (providing a set (first) of paired oligonucleotides that are complementary to the target nucleic acids (first); paragraph [0032]), said first set of paired oligonucleotides comprising: a first ratio of said first set of paired oligonucleotides comprising a first ratio (set (first) of paired oligonucleotides would inherently have a ratio; paragraph [0032]) of (a) first oligonucleotides (quencher probe oligonucleotide; paragraph [0032]-[0033]) to (b) first signal oligonucleotides comprising a first signal tag (label probe oligonucleotide for target nucleic acids (first); paragraphs [0032]-[0033]), amplifying the first target nucleic acid with the polymerase chain reaction (amplifying the target nucleic acids (first) by polymerase chain reaction; paragraph [0034]), but Lode does not disclose (a) first competitive oligonucleotides, wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid, thereby degrading the first signal oligonucleotide and permitting generation of a first signal, generating the first signal, measuring intensity of the first signal, and correlating the intensity of the first signal to the first ratio, thereby identifying the first target nucleic acid.

Wave, Blair, Caltech, and Lode, fail to disclose either solely or in combination the method of correlating the intensity of the first signal to the first ratio. Wave discloses ratios of a first probe oligonucleotide with a label and a second probe oligonucleotide with a label, pair of oligonucleotides, that are complementary to a target nucleic acids, amplifying the target nucleic acids, detecting the cleavage (degradation) of first and second probes (page 5, lines 23-30; page 7, lines 14-19; page 15, lines 25-31, page 16, lines 1-3), but Wave does not disclose correlating the intensity of the first signal to the first ratio. Caltech discloses method of detecting a plurality of polynucleotide analytes using oligonucleotides with attached chromophore in polymerization techniques and measuring signals to detect presence or absence of analytes in samples (paragraph [0011], [0045], [0062]), but Caltech does not disclose correlating the intensity of the first signal to the first ratio. Further there is no motivation to combine the references of record without the advantage of permissible hindsight.

Claim 2-4 and 5/3-4 meet the criteria set out in PCT Article 33(2)-(3), based on their dependency on positive claim 1.

Claim 43 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a composition comprising: a first set of paired oligonucleotides with complementarity to a first target nucleic acid, said first set of paired oligonucleotides comprising a first ratio of (a) competitive oligonucleotides to (b) signal oligonucleotides comprising a signal tag, wherein the competitive oligonucleotides are configured to compete with the signal oligonucleotides for binding to the first target nucleic acid during amplification of the first target nucleic acid with the polymerase chain reaction, wherein the signal oligonucleotides are adapted to generate a first signal having a first intensity when degraded by a nucleic acid polymerase during the polymerase chain reaction and excited by an energy source, and wherein the first intensity correlates with the first ratio.

Wave discloses a composition (composition; page 2, lines 15-17) comprising: a first set of paired oligonucleotides with complementarity to a first target nucleic acid (first and second probe oligonucleotides that occupy probe hybridization site on the target nucleic acid (first); page 2, lines 23-31), said first set of paired oligonucleotides comprising a first ratio (ratio of paired oligonucleotides, first probe oligonucleotides to second probe oligonucleotides; page 3, lines 13-20; page 6, lines 28-30) of (a) oligonucleotides (first probe oligonucleotides; page 2, lines 23-31) to (b) signal oligonucleotides comprising a signal tag (first oligonucleotides have a first label; page 5, lines 5-8), wherein the signal oligonucleotides are adapted to generate a first signal having a first intensity when degraded by a nucleic acid polymerase during the polymerase chain reaction (cleavage (degrading) of first probe oligonucleotide containing a label by a polymerase chain reaction and allowing generation of a signal (first); page 7, lines 14-25) and excited by an energy source (generating a first detectable signal in FRET (exited by an energy source); page 18, lines 14-16), but Wave does not disclose (a) competitive oligonucleotides, wherein the competitive oligonucleotides are configured to compete with the signal oligonucleotides for binding to the first target nucleic acid during amplification of the first target nucleic acid with the polymerase chain reaction, and wherein the first intensity correlates with the first ratio.

Blair discloses a composition (composition; paragraph [0016]) comprising: a first set of paired oligonucleotides with complementarity to a first target nucleic acid (providing a set of paired probe nucleic acids and labeled probe nucleic acids that competitive to target nucleic acids, paragraph [0041]-[0042]), said first set of paired oligonucleotides comprising a first ratio (set of paired probe and competitive labeled probe nucleic acids, would inherently be in a ratio; paragraphs [0042]-[0043]) of (a) competitive oligonucleotides to (competitive probe nucleic acid, paragraphs [0041]-[0042]) (b) signal oligonucleotides comprising a signal tag (labeled competitor nucleic acids (first signal); paragraphs [0041]-[0042]), wherein the competitive oligonucleotides are configured to compete with the signal oligonucleotides for binding to the first target nucleic acid (probe nucleic acids (first) competes with labeled competitor nucleic acids (first); paragraphs [0041]-[0042], [0072]), but Blair does not disclose during amplification of the first target nucleic acid with the polymerase chain reaction wherein the signal oligonucleotides are adapted to generate a first signal having a first intensity when degraded by a nucleic acid polymerase during the polymerase chain reaction and excited by an energy source, and wherein the first intensity correlates with the first ratio.

-***-Continued Within the Next Supplemental Box-***-

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INTERNATIONAL SEARCHING AUTHORITY

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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:

-***-Continued from Previous Supplemental Box-***-
Caltech discloses a composition (compositions; paragraph [0003]) comprising: a first set of paired oligonucleotides with complementarity to a first target nucleic acid (providing a set of first primer and a first oligonucleotide specific (complementarity) for binding a first polynucleotide analyte (target; paragraph [0003]), said first set of paired oligonucleotides comprising a first ratio (set of paired probe labeled nucleic acids (first set) would inherently have a ratio; paragraph [0003]) of (b) signal oligonucleotides comprising a signal tag (first primer oligonucleotide comprises a first signal tag; paragraph [0011]), but Caltech does not disclose (a) competitive oligonucleotides to, wherein the competitive oligonucleotides are configured to compete with the signal oligonucleotides for binding to the first target nucleic acid during amplification of the first target nucleic acid with the polymerase chain reaction, wherein the signal oligonucleotides are adapted to generate a first signal having a first intensity when degraded by a nucleic acid polymerase during the polymerase chain reaction and excited by an energy source, and wherein the first intensity correlates with the first ratio.
Lode discloses a composition (composition; paragraph [0054]) comprising: a first set of paired oligonucleotides with complementarity to a first target nucleic acid (providing a set (first) of paired oligonucleotides that are complementary to the target nucleic acids (first); paragraph [0032]), said first set of paired oligonucleotides comprising a first ratio (set (first) of paired oligonucleotides would inherently have a ratio; paragraph [0032]) of (a) oligonucleotides (quencher probe oligonucleotide, paragraph [0032]-[0033]) to (b) signal oligonucleotides comprising a signal tag (label probe oligonucleotide for target nucleic acids (first); paragraphs [0032]-[0033]), but Lode does not disclose (a) competitive oligonucleotides, wherein the competitive oligonucleotides are configured to compete with the signal oligonucleotides for binding to the first target nucleic acid during amplification of the first target nucleic acid with the polymerase chain reaction, wherein the signal oligonucleotides are adapted to generate a first signal having a first intensity when degraded by a nucleic acid polymerase during the polymerase chain reaction and excited by an energy source, and wherein the first intensity correlates with the first ratio.
Wave, Blair, Caltech, and Lode, fail to disclose either solely or in combination a composition during amplification of the first target nucleic acid with the polymerase chain reaction wherein the signal oligonucleotides are adapted to generate a first signal having a first intensity when degraded by a nucleic acid polymerase during the polymerase chain reaction and excited by an energy source, and wherein the first intensity correlates with the first ratio. Wave discloses ratios of a first probe oligonucleotide with a label and a second probe oligonucleotide with a label, pair of oligonucleotides, that are complimentary to a target nucleic acids, amplifying the target nucleic acids, detecting the cleavage (degradation) of first and second probes (page 5, lines 23-30; page 7, lines 14-19, page 15, lines 25-31; page 16, lines 1-3), but Wave does not disclose amplification of the first target nucleic acid with the polymerase chain reaction wherein the signal oligonucleotides are adapted to generate a first signal having a first intensity when degraded by a nucleic acid polymerase during the polymerase chain reaction and correlating the intensity of the first signal to the first ratio. Caltech discloses method of detecting a plurality of polynucleotide analytes using oligonucleotides with attached chromophore in polymerization techniques and measuring signals to detect presence or absence of analytes in samples (paragraph [0011], [0045], [0082]), but Caltech does not disclose amplification of the first target nucleic acid with the polymerase chain reaction wherein the signal oligonucleotides are adapted to generate a first signal having a first intensity when degraded by a nucleic acid polymerase during the polymerase chain reaction and correlating the intensity of the first signal to the first ratio. Further there is no motivation to combine the references of record without the advantage of permissible hindsight.

Claim 44 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest method of identifying a first target molecule comprising, providing a sample comprising the first target molecule, providing a first set of paired capture molecules that bind to the first target molecule, said first set of paired capture molecules comprising a first ratio of (a) first competitive capture molecules to (b) first signal capture molecules comprising a signal tag, wherein the competitive capture molecules compete with the signal capture molecules for binding to the first target molecule, generating the first signal, measuring intensity of the first signal, and correlating the intensity of the first signal to the first ratio, thereby identifying the first target molecule.
Wave discloses method of identifying a first target molecule (method of detection of a target nucleic acids (first target molecule); page 2, lines 10-14);comprising, providing a sample comprising the first target molecule (providing a sample with target nucleic acids (first target molecule); page 2, lines 10-14), providing a first set of paired capture molecules that bind to the first target molecule (first and second probe oligonucleotides (capture molecules) that occupy probe hybridization site on the target nucleic acid (first target molecule); page 2, lines 23-31), said first set of paired capture molecules comprising a first ratio (ratio of paired oligonucleotides (capture molecules), first probe oligonucleotides to second probe oligonucleotides; page 3, lines 13-20, page 6, lines 28-30) of (a) first capture molecules (first probe oligonucleotides; page 2, lines 23-31) to (b) first signal capture molecules comprising a signal tag (first probe oligonucleotides (capture molecules) have a first label; page 6, lines 5-8), generating the first signal (generating a first signal, page 16, lines 14-16), measuring intensity of the first signal (measuring the strength of the signal; page 18, lines 22-26), and thereby identifying the first target molecule (identifying the target nucleic acid; page 7, lines 14-19), but Wave does not disclose (a) first competitive capture molecules, wherein the competitive capture molecules compete with the signal capture molecules for binding to the first target molecule, and correlating the intensity of the first signal to the first ratio.
Blair discloses method of identifying a first target molecule (method of identifying a target nucleic acid (first target molecule; paragraph [0018]) comprising, providing a sample comprising the first target molecule (one target nucleic acid (first target molecule) is present in a given sample; paragraphs [0035], [0041]), providing a first set of paired capture molecules that bind to the first target molecule (providing a set (first) of paired nucleic acid probes (capture molecules) that can hybridize (complementarity) to target (first) nucleic acids; paragraphs [0041]-[0042]), said first set of paired capture molecules comprising a first ratio (set of paired probe and competitive labeled probe nucleic acids (first set paired capture molecules); would inherently have a ratio; paragraphs [0041]-[0042]) of (a) first competitive capture molecules (competitive probe nucleic acid; paragraphs [0041]-[0042]) to (b) first signal capture molecules comprising a signal tag (labeled competitor nucleic acids (first signal); paragraphs [0041] [0042]), wherein the competitive capture molecules compete with the signal capture molecules for binding to the first target molecule (probe nucleic acids (capture molecule) competes with labeled competitor nucleic acids (labeled capture molecule); paragraphs [0041] [0042]), but Blair does not disclose generating the first signal, measuring intensity of the first signal, and correlating the intensity of the first signal to the first ratio, thereby identifying the first target molecule.

-***-Continued Within the Next Supplemental Box-***-

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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:

-***-Continued from Previous Supplemental Box-***-
Caltech discloses method of identifying a first target molecule (method of detecting at least one polynucleotide analyte (first target molecule); paragraph [0003]) comprising, providing a sample comprising the first target molecule (providing a sample comprising a first polynucleotide analyte (first target molecule); paragraph [0003]), providing a first set of paired capture molecules that bind to the first target molecule (providing a set of first primer and a first oligonucleotide specific (complementarity) for binding a first polynucleotide analyte (first target molecule); paragraph [0003]), said first set of paired capture molecules comprising a first ratio of (set of paired probe labeled nucleic acids (first set), would inherently have a ratio; paragraph [0003]), to (b) first signal capture molecules comprising a signal tag (first primer oligonucleotide (first capture molecules) comprises a signal tag; paragraph [0011]), generating the first signal (generating a first signal; paragraph [0011]), measuring intensity of the first signal (measuring a first signal; paragraph [0011]), and thereby identifying the first target molecule (identifying the first polynucleotide analyte (target); paragraph [0011]), but Caltech does not disclose (a) first competitive capture molecules, wherein the competitive capture molecules compete with the signal capture molecules for binding to the first target molecule, and correlating the intensity of the first signal to the first ratio.
Lode discloses method of identifying a first target molecule (method of detecting target nucleic acids (first target molecule); paragraphs [0031]) comprising, providing a sample comprising the first target molecule (providing a sample with target nucleic acids (first target molecule); paragraph [0032]), providing a first set of paired capture molecules that bind to the first target molecule providing a set (first) of paired oligonucleotides (capture molecule) that are complementary to the target nucleic acids (first target molecule); paragraph [0032]), said first set of paired capture molecules comprising a first ratio (set (first) of paired oligonucleotides (capture molecules) would inherently have a ratio; paragraph [0032]) of (a) first capture molecules (quencher probe oligonucleotide; paragraph [0032]-[0033]) to (b) first signal capture molecules comprising a signal tag (label probe oligonucleotide for target nucleic acids (first); paragraphs [0032]-[0033]), but Lode does not disclose (a) first competitive capture molecules, wherein the competitive capture molecules compete with the signal capture molecules for binding to the first target molecule, generating the first signal, measuring intensity of the first signal, and correlating the intensity of the first signal to the first ratio, thereby identifying the first target molecule.
Wave, Blair, Caltech, and Lode, fail to disclose either solely or in combination the method of correlating the intensity of the first signal to the first ratio. Wave discloses ratios of a first probe oligonucleotide (capture molecule) with a label and a second probe oligonucleotide (capture molecule) with a label, pair of oligonucleotides, that are complimentary to a target nucleic acids (target molecule), amplifying the target nucleic acids, detecting the cleavage (degradation) of first and second probes (page 5, lines 23-30; page 7, lines 14-19; page 15, lines 25-31; page 16, lines 1-3), but Wave does not disclose wherein the competitive capture molecules compete with the signal capture molecules for binding to the first target molecule and correlating the intensity of the first signal to the first ratio. Caltech discloses method of detecting a plurality of polynucleotide analytes using oligonucleotides with attached chromophore in polymerization techniques and measuring signals to detect presence or absence of analytes in samples (paragraph [0011], [0045], [0082]), but Caltech does not disclose wherein the competitive capture molecules compete with the signal capture molecules for binding to the first target molecule and correlating the intensity of the first signal to the first ratio. Further there is no motivation to combine the references of record without the advantage of permissible hindsight

Claim 45-47 and 48/45-47 meet the criteria set out in PCT Article 33(2)-(3), based on their dependency on positive claim 44.

Claim 83 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a composition comprising: a first set of paired capture molecules with complementarity to a first target molecule, said first set of paired capture molecules comprising a first ratio of(a) competitive capture molecules to (b) signal capture molecules comprising a signal tag, wherein the competitive capture molecules are configured to compete with the signal capture molecules for binding to the target molecule, wherein the signal capture molecules are adapted to generate a first signal having a first intensity when excited by an energy source, and wherein the first intensity correlates with the first ratio.
Wave discloses a composition (composition; page 2, lines 15-17) comprising: a first set of paired capture molecules with complementarity to a first target molecule (first and second probe oligonucleotides (capture molecules) that occupy probe hybridization site (complimentary) on the target nucleic acid (first target molecule); page 2, lines 23-31), said first set of paired capture molecules comprising a first ratio (ratio of paired oligonucleotides (capture molecules), first probe oligonucleotides to second probe oligonucleotides; page 3, lines 13-20; page 6, lines 28-30) of (a) capture molecules (first probe oligonucleotides (capture molecules); page 2, lines 23-31) to (b) signal capture molecules comprising a signal tag (first oligonucleotides (capture molecules) have a first label; page 5, lines 5-8), but Wave does not disclose (a) competitive capture molecules, wherein the competitive capture molecules are configured to compete with the signal capture molecules for binding to the target molecule, wherein the signal capture molecules are adapted to generate a first signal having a first intensity when excited by an energy source, and wherein the first intensity correlates with the first ratio.
Blair discloses a composition (composition; paragraph [0016]) comprising: a first set of paired capture molecules with complementarity to a first target molecule (providing a set of paired probe nucleic acids and labeled probe nucleic acids that competitive to target nucleic acids (target molecule); paragraph [0041]-[0042]), said first set of paired capture molecules comprising a first ratio (set of paired probe and competitive labeled probe nucleic acids (capture molecules), would inherently be in a ratio; paragraphs [0042]-[0043]) of (a) competitive capture molecules (competitive probe nucleic acid (capture molecules); paragraphs [0041]-[0042]) to (b) signal capture molecules comprising a signal tag (labeled competitor nucleic acids (signal capture molecules); paragraphs [0041]-[0042]), wherein the competitive capture molecules are configured to compete with the signal capture molecules for binding to the target molecule (probe nucleic acids (capture molecules) competes with labeled competitor nucleic acids (signal capture molecules) for binding to target nucleic acids (target molecule); paragraphs [0041]-[0042], [0072]), but Blair does not disclose wherein the signal capture molecules are adapted to generate a first signal having a first intensity when excited by an energy source, and wherein the first intensity correlates with the first ratio.

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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
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Caltech discloses a composition (compositions; paragraph [0003]) comprising: a first set of paired capture molecules with complementarity to a first target molecule (providing a set of first primer and a first oligonucleotide (capture molecules) specific (complementarity) for binding a first polynucleotide analyte (first target molecule); paragraph [0003]), said first set of paired capture molecules comprising a first ratio (set of paired probe labeled nucleic acids (first set of paired capture molecules) would inherently have a ratio; paragraph [0003]) of (b) signal capture molecules comprising a signal tag (first primer oligonucleotide comprises a first signal tag (signal capture molecules; paragraph [0011]), but Caltech does not disclose (a) competitive capture molecule, wherein the competitive capture molecules are configured to compete with the signal capture molecules for binding to the target molecule, wherein the signal capture molecules are adapted to generate a first signal having a first intensity when excited by an energy source, and wherein the first intensity correlates with the first ratio.
Lode discloses a composition (composition; paragraph [0054]) comprising: a first set of paired capture molecules with complementarity to a first target molecule (providing a set (first) of paired oligonucleotides (capture molecules) that are complementary to the target nucleic acids (first target molecule); paragraph [0032]), said first set of paired capture molecules comprising a first ratio (set (first) of paired oligonucleotides (capture molecules) would inherently have a ratio; paragraph [0032]) of (a) competitive capture molecules (quencher probe oligonucleotide; paragraph [0032]-[0033]) to (b) signal capture molecules comprising a signal tag (label probe oligonucleotide (signal capture molecule); paragraphs [0032]-[0033]), but Lode does not disclose (a) competitive capture molecules, wherein the competitive capture molecules are configured to compete with the signal capture molecules for binding to the target molecule, wherein the signal capture molecules are adapted to generate a first signal having a first intensity when excited by an energy source, and wherein the first intensity correlates with the first ratio.
Wave, Blair, Caltech, and Lode, fail to disclose either solely or in combination wherein the signal capture molecules are adapted to generate a first signal having a first intensity when excited by an energy source, and wherein the first intensity correlates with the first ratio. Wave discloses ratios of a first probe oligonucleotide (capture molecules) with a label and a second probe oligonucleotide with a label, pair of oligonucleotides, that are complimentary to a target nucleic acids (target molecules), amplifying the target nucleic acids, detecting the cleavage (degradation) of first and second probes (page 5, lines 23-30, page 7, lines 14-19; page 15, lines 25-31; page 16, lines 1-3), but Wave does not disclose wherein the competitive capture molecules are configured to compete with the signal capture molecules for binding to the target molecule, wherein the signal capture molecules are adapted to generate a first signal having a first intensity when excited by an energy source, and wherein the first intensity correlates with the first ratio. Caltech discloses method of detecting a plurality of polynucleotide analytes using oligonucleotides with attached chromophore in polymerization techniques and measuring signals to detect presence or absence of analytes in samples (paragraph [0011], [0045], [0062]), but Caltech does not disclose wherein the competitive capture molecules are configured to compete with the signal capture molecules for binding to the target molecule, wherein the signal capture molecules are adapted to generate a first signal having a first intensity when excited by an energy source, and wherein the first intensity correlates with the first ratio. Further there is no motivation to combine the references of record without the advantage of permissible hindsight.

Claim 84-87, 88/83-87, 89/83-87, and 90/83-87 meet the criteria set out in PCT Article 33(2)-(3), based on their dependency on positive claim 83.

Claim 91 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly method of altering amplification level of a target nucleic acid comprising, providing a sample comprising the target nucleic acid, providing a set of paired oligonucleotides with complementarity to the target nucleic acid, said set of paired oligonucleotides comprising a ratio of (a) competitive oligonucleotides to (b) functional oligonucleotides, wherein the competitive oligonucleotides compete with the functional oligonucleotides for binding to the target nucleic acid and are not extendible by a nucleic acid polymerase, wherein the functional oligonucleotides are extendible by a nucleic acid polymerase as primers for amplifying the target nucleic acid by the polymerase chain reaction (PCR), and amplifying the target nucleic acid with PCR
Wave discloses method of altering amplification level of a target nucleic acid (method of altering amplification level of target nucleic acid; page 6, lines 24-30) comprising, providing a sample comprising the target nucleic acid (providing a sample with target nucleic acids (first); page 2, lines 10-14),, providing a set of paired oligonucleotides with complementarity to the target nucleic acid (first and second probe oligonucleotides that occupy probe hybridization site on the target nucleic acid, page 2, lines 23-31), said set of paired oligonucleotides comprising a ratio (ratio of paired oligonucleotides, first probe oligonucleotides to second probe oligonucleotides; page 3, lines 13-20; page 6, lines 28-30) of (a) competitive oligonucleotides to (b) functional oligonucleotides (oligonucleotides having a label (functional); page 5, lines 5-8), but Wave does not disclose wherein the competitive oligonucleotides compete with the functional oligonucleotides for binding to the target nucleic acid and are not extendible by a nucleic acid polymerase, wherein the functional oligonucleotides are extendible by a nucleic acid polymerase as primers for amplifying the target nucleic acid by the polymerase chain reaction (PCR), and amplifying the target nucleic acid with PCR
Blair discloses method of altering amplification level of a target nucleic acid (method of altering amplification of target nucleic acids paragraphs [0072], [0074]) comprising, providing a sample comprising the target nucleic acid (one target nucleic acid is present in a given sample; paragraphs [0035], [0041]), providing a set of paired oligonucleotides with complementarity to the target nucleic acid (providing a set (first) of paired nucleic acid probes that can hybridize (complementarity) to target (first) nucleic acids; paragraphs [0041]-[0042]), said set of paired oligonucleotides comprising a ratio (set of paired probe and competitive labeled probe nucleic acids (first set) would inherently have a ratio; paragraphs [0041]-[0042]) of (a) competitive oligonucleotides (competitive probe nucleic acid, paragraphs [0041]-[0042]) to (b) functional oligonucleotides (labeled competitor nucleic acids (functional); paragraphs [0041]-[0042]), but Blair does not disclose wherein the competitive oligonucleotides compete with the functional oligonucleotides for binding to the target nucleic acid and are not extendible by a nucleic acid polymerase, wherein the functional oligonucleotides are extendible by a nucleic acid polymerase as primers for amplifying the target nucleic acid by the polymerase chain reaction (PCR), and amplifying the target nucleic acid with PCR.

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WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US17/24933

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
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Caltech discloses method of altering amplification level of a target nucleic acid (method of amplification of polynucleotide analyte (target nucleic acid); paragraph [0003]) comprising, providing a sample comprising the target nucleic acid (providing a sample comprising a first polynucleotide analyte (first target molecule); paragraph [0003]), providing a set of paired oligonucleotides with complementarity to the target nucleic acid (providing a set of first primer and a first oligonucleotide specific (complementarity) for binding a first polynucleotide analyte (first target molecule); paragraph [0003]), said set of paired oligonucleotides comprising a ratio (set of paired probe labeled nucleic acids (first set), would inherently have a ratio; paragraph [0003]) of (a) competitive oligonucleotides to (b) functional oligonucleotides (nucleotide with signal (functional oligonucleotide; paragraph [0011]), but Caltech does not disclose (a) competitive oligonucleotides, wherein the competitive oligonucleotides compete with the functional oligonucleotides for binding to the target nucleic acid and are not extendible by a nucleic acid polymerase, wherein the functional oligonucleotides are extendible by a nucleic acid polymerase as primers for amplifying the target nucleic acid by the polymerase chain reaction (PCR), and amplifying the target nucleic acid with PCR.
Lode discloses method of altering amplification level of a target nucleic acid (method for amplification of detected nucleic acids, paragraph [0030]) comprising, providing a sample comprising the target nucleic acid (providing a sample with target nucleic acids; paragraph [0032]), providing a set of paired oligonucleotides with complementarity to the target nucleic acid (providing a set (first) of paired oligonucleotides that are complementary to the target nucleic acids (first); paragraph [0032]), said set of paired oligonucleotides comprising a ratio (set (first) of paired oligonucleotides would inherently have a ratio: paragraph [0032]) of (a) competitive oligonucleotides (quencher probe oligonucleotide; paragraph [0032]-[0033]) to (b) functional oligonucleotides (label probe oligonucleotide (functional); paragraphs [0032]-[0033]), but Lode does not discloses wherein the competitive oligonucleotides compete with the functional oligonucleotides for binding to the target nucleic acid and are not extendible by a nucleic acid polymerase, wherein the functional oligonucleotides are extendible by a nucleic acid polymerase as primers for amplifying the target nucleic acid by the polymerase chain reaction (PCR), and amplifying the target nucleic acid with PCR.
Wave, Blair, Caltech, and Lode, fail to disclose either solely or in combination the method wherein the competitive oligonucleotides compete with the functional oligonucleotides for binding to the target nucleic acid and are not extendible by a nucleic acid polymerase, wherein the functional oligonucleotides are extendible by a nucleic acid polymerase as primers for amplifying the target nucleic acid by the polymerase chain reaction (PCR), and amplifying the target nucleic acid with PCR. Wave discloses ratios of a first probe oligonucleotide with a label and a second probe oligonucleotide with a label (functional), pair of oligonucleotides, that are complimentary to a target nucleic acids, amplifying the target nucleic acids, detecting the cleavage (degradation) of first and second probes (page 5, lines 23-30; page 7, lines 14-19; page 15, lines 25-31; page 16, lines 1-3), but Wave does not disclose wherein the competitive oligonucleotides compete with the functional oligonucleotides for binding to the target nucleic acid and are not extendible by a nucleic acid polymerase, wherein the functional oligonucleotides are extendible by a nucleic acid polymerase as primers for amplifying the target nucleic acid by the polymerase chain reaction (PCR), and amplifying the target nucleic acid with PCR. Caltech discloses method of detecting a plurality of polynucleotide analytes using oligonucleotides with attached chromophore in polymerization techniques and measuring signals to detect presence or absence of analytes in samples (paragraph [0011], [0045], [0052]), but Caltech does not disclose wherein the competitive oligonucleotides compete with the functional oligonucleotides for binding to the target nucleic acid and are not extendible by a nucleic acid polymerase, wherein the functional oligonucleotides are extendible by a nucleic acid polymerase as primers for amplifying the target nucleic acid by the polymerase chain reaction (PCR), and amplifying the target nucleic acid with PCR. Further there is no motivation to combine the references of record without the advantage of permissible hindsight.

Claim 92-97 and 98/91-97 meet the criteria set out in PCT Article 33(2)-(3), based on their dependency on positive claim 91.

Claim 98 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly a composition comprising: a set of paired oligonucleotides with complementarity to a target nucleic acid, said set of paired oligonucleotides comprising a ratio of (a) competitive oligonucleotides to (b) functional oligonucleotides, wherein the competitive oligonucleotides are configured to compete with the functional oligonucleotides for binding to the nucleic acid target during amplification of the first target nucleic acid with the polymerase chain reaction and are not extendible by a nucleic acid polymerase.
Wave discloses a composition (composition: page 2, lines 15-17) comprising: a set of paired oligonucleotides with complementarity to a target nucleic acid (first and second probe oligonucleotides that occupy probe hybridization site on the target nucleic acid (first); page 2, lines 23-31), said set of paired oligonucleotides comprising a ratio (ratio of paired oligonucleotides, first probe oligonucleotides to second probe oligonucleotides; page 3, lines 13-20; page 6, lines 28-30) of (b) functional oligonucleotides (first oligonucleotides have a first label; page 5, lines 5-8), but Wave does not disclose (a) competitive oligonucleotides, wherein the competitive oligonucleotides are configured to compete with the functional oligonucleotides for binding to the nucleic acid target during amplification of the first target nucleic acid with the polymerase chain reaction and are not extendible by a nucleic acid polymerase.
Blair discloses a composition (composition; paragraph [0016]) comprising: a set of paired oligonucleotides with complementarity to a target nucleic acid molecule (providing a set of paired probe nucleic acids and labeled probe nucleic acids that are competitive to target nucleic acids (target molecule); paragraph [0041]-[0042]), said set of paired oligonucleotides comprising a ratio of (set of paired probe and competitive labeled probe nucleic acids (capture molecules); would inherently be in a ratio; paragraphs [0042]-[0043]) (a) competitive oligonucleotides (competitive probe nucleic acid (capture molecules); paragraphs [0041]-[0042]) to (b) functional oligonucleotides (labeled competitor nucleic acids (functional oligonucleotides); paragraphs [0041]-[0042]), wherein the competitive oligonucleotides are configured to compete with the functional oligonucleotides (probe nucleic acids (capture molecules) competes with labeled competitor nucleic acids (signal capture molecules) for binding to target nucleic acids (target molecule); paragraphs [0041]-[0042], [0072]), but Blair does not disclose for binding to the nucleic acid target during amplification of the first target nucleic acid with the polymerase chain reaction and are not extendible by a nucleic acid polymerase.

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WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US17/24933

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
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Caltech discloses a composition (compositions; paragraph [0003]) comprising: a set of paired oligonucleotides with complementarity to a target nucleic acid (providing a set of first primer and a first oligonucleotide (capture molecules) specific (complementarity) for binding a first polynucleotide analyte (first target molecule); paragraph [0003]), said set of paired oligonucleotides comprising a ratio (set of paired probe labeled nucleic acids (first set of paired capture molecules) would inherently have a ratio; paragraph [0003]) of (b) functional oligonucleotides (primer oligonucleotide (functional; paragraph [0011]), but Caltech does not disclose (a) competitive olignucleotide, wherein the competitive oligonucleotides are configured to compete with the functional oligonucleotides for binding to the nucleic acid target during amplification of the first target nucleic acid with the polymerase chain reaction and are not extendible by a nucleic acid polymerase.
Lode discloses a composition (composition; paragraph [0004]) comprising: a set of paired oligonucleotides with complementarity to a target nucleic acid (providing a set of paired oligonucleotides that are complementary to the target nucleic acids); paragraph [0032]), said set of paired oligonucleotides comprising a ratio (set (first) of paired oligonucleotides (capture molecules) would inherently have a ratio; paragraph [0032]) of (a) competitive oligonucleotides (quencher probe oligonucleotide; paragraph [0032]-[0033]) to (b) functional oligonucleotides (label probe oligonucleotide (functional oligonucleotides); paragraphs [0032]-[0033]), but Lode does not disclose wherein the competitive oligonucleotides are configured to compete with the functional oligonucleotides for binding to the nucleic acid target during amplification of the first target nucleic acid with the polymerase chain reaction and are not extendible by a nucleic acid polymerase.
Wave, Blair, Caltech, and Lode, fail to disclose either solely or in combination the composition wherein the competitive oligonucleotides are configured to compete with the functional oligonucleotides for binding to the nucleic acid target during amplification of the first target nucleic acid with the polymerase chain reaction and are not extendible by a nucleic acid polymerase. Wave discloses ratios of a first probe oligonucleotide with a label and a second probe oligonucleotide with a label (functional), pair of oligonucleotides, that are complimentary to a target nucleic acids, amplifying the target nucleic acids, detecting the cleavage (degradation) of first and second probes (page 5, lines 23-30; page 7, lines 14-19; page 15, lines 25-31; page 16, lines 1-3), but Wave does not disclose wherein the competitive oligonucleotides are configured to compete with the functional oligonucleotides for binding to the nucleic acid target during amplification of the first target nucleic acid with the polymerase chain reaction and are not extendible by a nucleic acid polymerase.
Caltech discloses method of detecting a plurality of polynucleotide analytes using oligonucleotides with attached chromophore in polymerization techniques and measuring signals to detect presence or absence of analytes in samples (paragraph [0011], [0045], [0082]), but Caltech does not disclose wherein the competitive oligonucleotides are configured to compete with the functional oligonucleotides for binding to the nucleic acid target during amplification of the first target nucleic acid with the polymerase chain reaction and are not extendible by a nucleic acid polymerase.

Claims 1-4, 43-47, 46/45-47, 83-87, 88/83-87, 89/83-87, 90/83-87, 91-97, 98/91-97, and 99 have industrial applicability as defined by PCT Article 33(4) because the subject matter can be made or used in industry.

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PCT Recordation of Search History

Case/PCT Application Number: PCT/US17/24933

CLIN Number/Technical Field of PCT Application: 008 Chemistry

Dates During Which the Search was Conducted: 25 May – 03 June 2017

Date of Completion of Recordation of Search History Form: 03 June 2017

Research Analyst Initials: TFW

Search Approval Official (SAO) Initials: DGF

Field of Search/Classification Information:

IPC(8) Classifications: A61K 31/7088, 31/711, 31/713; C07H 21/00, 21/04; C12Q 1/68; G01N 21/64, 33/532, 33/533, 33/542 (2017.01)

CPC Classifications: A61K 31/7088, 31/711, 31/713; C07H 21/00, 21/04; C12Q 1/68, 1/686, 1/6806, 1/6851, 2561/101, 2561/113; G01N 21/64, 33/532, 33/533, 33/542

Databases Searched (Patent and Non-Patent Literature (NPL), Including Sub-Databases and Files Searched) and Search Terms Used:

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, Other Countries (INPADOC), RU, AT, CH, TH, BR, PH), EBSCO, Google Scholar, PubMed;

target nucleic acid, PCR, qPCR, competitive oligonucleotides, competitive probes, dimming probes, non-fluorescing probes, target probes, signal oligonucleotides, fluorescent probe, fluorophore, functional oligonucleotides,

Database Search String Recordation, Including Dates of Searches:

Patent Database Search Strategy/Results:

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, Other Countries (INPADOC), RU, AT, CH, TH, BR, PH):

ID	Search String	Records	Date
L36	TACD:((DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs") AND ((ampli*) W10 (DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs")) AND (competit*_probe* OR competit*_oligonucleotid* OR competit*_captur*_molecul*) AND ((function*_oligonucleotid* OR signal*_oligonucleotid* OR fluoresc*_probe* OR fluoresc*_oligonucleotid*) w10 (extend*)))	97	3-Jun-17
L35	TACD:((DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs") AND ((ampli*) W10 (DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs")) AND (competit*_probe* OR competit*_oligonucleotid* OR competit*_captur*_molecul*) AND (function*_oligonucleotid* OR signal*_oligonucleotid* OR fluoresc*_probe* OR fluoresc*_oligonucleotid*))	824	3-Jun-17
L34	TAC:((DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs") AND ((ampli*) W10 (DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs")) AND (competit*_probe* OR competit*_oligonucleotid* OR competit*_captur*_molecul*) AND (function*_oligonucleotid* OR signal*_oligonucleotid* OR fluoresc*_probe* OR fluoresc*_oligonucleotid*))	2	3-Jun-17
L33	TA:((DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs") AND ((ampli*) W10 (DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs")) AND (competit*_probe* OR competit*_oligonucleotid* OR competit*_captur*_molecul*) AND (function*_oligonucleotid* OR signal*_oligonucleotid* OR fluoresc*_probe* OR fluoresc*_oligonucleotid*))	0	3-Jun-17
L32	TACD: ((amplific*) w10 ("nucleic acid" or oligonucleotides)) and ((paired or set) w5 (oligonucleotides or "nucleic acids" or probes)) and (competitive w5 (oligonucleotides or "nucleic acids" or probes)) and (functional w5 (oligonucleotides or "nucleic acids" or probes)) and ("polymerase chain reaction" or PCR or qPCR) and (extend* w10 polymerase)	197	1-Jun-17
L31	TACD: ((amplific*) w10 ("nucleic acid" or oligonucleotides)) and ((paired or set) w5 (oligonucleotides or "nucleic acids" or probes)) and (competitive w5 (oligonucleotides or "nucleic acids" or probes)) and (functional w5 (oligonucleotides or "nucleic acids" or probes)) and ("polymerase chain reaction" or PCR or qPCR)	352	1-Jun-17
L30	TACD: ((amplific*) w10 ("nucleic acid" or oligonucleotides)) and ((paired or set) w5 (oligonucleotides or "nucleic acids" or probes)) and (competitive w5 (oligonucleotides or "nucleic acids" or probes)) and (functional w5 (oligonucleotides or "nucleic acids" or probes))	356	1-Jun-17

L29	TACD: ((amplific*_level) w10 ("nucleic acid" or oligonucleotides)) and ((paired or set) w5 (oligonucleotides or "nucleic acids" or probes)) and (competitive w5 (oligonucleotides or "nucleic acids" or probes)) and (functional w5 (oligonucleotides or "nucleic acids" or probes))	0	1-Jun-17
L28	TAC: ((amplific*_level) w10 ("nucleic acid" or oligonucleotides)) and ((paired or set) w5 (oligonucleotides or "nucleic acids" or probes)) and (competitive w5 (oligonucleotides or "nucleic acids" or probes)) and (functional w5 (oligonucleotides or "nucleic acids" or probes))	0	1-Jun-17
L27	TA: ((amplific*_level) w10 ("nucleic acid" or oligonucleotides)) and ((paired or set) w5 (oligonucleotides or "nucleic acids" or probes)) and (competitive w5 (oligonucleotides or "nucleic acids" or probes)) and (functional w5 (oligonucleotides or "nucleic acids" or probes))	0	1-Jun-17
L26	TA:(target*_nucleic*_acid* or target_molecule or target_DNA or target_gene or target_polynucleotide) AND TACD: ((paired w3 (primers or oligonucleotides or "capture molecules")) and ((competit*_oligonucleotid* or competit*_polynucleotide OR competit*_probe* or Competit*_primer or capture_molecul*) AND (signal*_oligonucleotid* OR fluoresc*_oligonucleotid* OR fluoresc*_probe* or functional_oligonucleotid* or functional_primer or signal_primer or fluresc*_primer or (signal w5 capture_molecule)) and (((signal or first) w5 intensity) or amplif*)))	113	30-May-17
L25	TAC:(target*_nucleic*_acid* or target_molecule or target_DNA or target_gene) AND TAC: ((competit*_oligonucleotid* OR competit*_probe* or Competit*_primer or capture_molecul*) AND (signal*_oligonucleotid* OR fluoresc*_oligonucleotid* OR fluoresc*_probe* or functional_oligonucleotid* or functional_primer or signal_primer or fluresc*_primer or (signal w5 capture_molecule)) and (((signal or first) w5 intensity) or amplif*))	26	30-May-17
L24	TA:(target w5 (oligonucleotide or "Nucleic acid" or DNA)) AND TACD: ((competitive w5 (oligonucleotide or probe)) and (signal w5 (oligonucleotide or probe)) and (signal w5 intensity))	195	27-May-17
L23	TAC:(oligonucleotide or "Nucleic acid" or DNA) AND TACD: ((competitive w5 (oligonucleotide or probe)) and (signal w5 (oligonucleotide or probe)) and (signal w5 intensity))	917	27-May-17
L22	TAC:(oligonucleotide or "Nucleic acid" or DNA) AND TACD: (competitive w5 (oligonucleotide or probe))	4336	27-May-17
L21	TAC:(oligonucleotide or "Nucleic acid" or DNA) AND TACD: ("multi_analyte" w5 coding)	0	27-May-17
L20	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND TACD: ("multi_analyte" w5 coding)	0	27-May-17
L19	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND TACD:((competitive w5 (oligonucleotide or probes)) and ((signal or fluorscen*) w5 (probe or oligonucleotide))) AND CPC:("C12Q2561/101" or "C12Q2561/113")	76	27-May-17
L18	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND CPC:("C12Q2561/101" or "C12Q2561/113")	1925	27-May-17
L17	CPC:("C12Q2561/101" or "C12Q2561/113")	3090	27-May-17
L16	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND TACD:(("real_time" or "multi_plex" or multiplex) w5 (pcr or qpcr or "polymerase chain reaction") and (competitive w5 (oligonucleotide or probes)) and ((signal or fluorscen*) w5 (probe or oligonucleotide))) AND CPC:(("A61K31/7088" or "A61K31/711" or "A61K31/713" or "C07H21/00" or "C07H21/04") and ("C12Q1/68" or "C12Q1/686" or "C12Q1/6806" or "C12Q1/6851" or "G01N21/64" or "G01N33/532" or "G01N33/533" or "G01N33/542"))	10	27-May-17
L15	CPC:(("A61K31/7088" or "A61K31/711" or "A61K31/713" or "C07H21/00" or "C07H21/04") and ("C12Q1/68" or "C12Q1/686" or "C12Q1/6806" or	4579	27-May-17

	"C12Q1/6851" or "G01N21/64" or "G01N33/532" or "G01N33/533" or "G01N33/542"))		
L14	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND TACD:(("real_time" or "multi_plex" or multiplex) w5 (pcr or qpcr or "polymerase chain reaction") and (competitive w5 (oligonucleotide or probes)) and ((signal or fluorscen*) w5 (probe or oligonucleotide))) AND IC:(("A61K31/7088" or "A61K31/711" or "A61K31/713" or "C07H21/00" or "C07H21/04") and ("C12Q1/68" or "G01N21/64" or "G01N33/532" or "G01N33/533" or "G01N33/542"))	109	27-May-17
L13	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND TACD:(("real_time" or "multi_plex" or multiplex) w5 (pcr or qpcr or "polymerase chain reaction") and ((competitive or signal) w5 (oligonucleotide or probes))) AND IC:(("A61K31/7088" or "A61K31/711" or "A61K31/713" or "C07H21/00" or "C07H21/04") and ("C12Q1/68" or "G01N21/64" or "G01N33/532" or "G01N33/533" or "G01N33/542"))	2071	27-May-17
L12	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND TACD:(("real_time" or "multi_plex" or multiplex) w5 (pcr or qpcr or "polymerase chain reaction")) AND IC:(("A61K31/7088" or "A61K31/711" or "A61K31/713" or "C07H21/00" or "C07H21/04") and ("C12Q1/68" or "G01N21/64" or "G01N33/532" or "G01N33/533" or "G01N33/542"))	4904	27-May-17
L11	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND IC:(("A61K31/7088" or "A61K31/711" or "A61K31/713" or "C07H21/00" or "C07H21/04") and ("C12Q1/68" or "G01N21/64" or "G01N33/532" or "G01N33/533" or "G01N33/542"))	27321	27-May-17
L10	IC:(("A61K31/7088" or "A61K31/711" or "A61K31/713" or "C07H21/00" or "C07H21/04") and ("C12Q1/68" or "G01N21/64" or "G01N33/532" or "G01N33/533" or "G01N33/542"))	104578	27-May-17
L9	TA: (target w5 ("nucleic acids" or DNA)) and TACD: ((competitive w5 (probe or oligonucleotide or hybridization)) and ((detectable or signal or reporter) w5 (oligonucleotide or probe)) and (PCR or qPCR or "polymerase chain reaction") and ((signal or flourescence) w5 intensity))	74	27-May-17
L8	TA: (target w5 ("nucleic acids" or DNA)) and TAC: ((competitive w5 (probe or oligonucleotide or hybridization)) and ((detectable or signal or reporter) w5 (oligonucleotide or probe)) and (PCR or qPCR or "polymerase chain reaction") and ((signal or flourescence) w5 intensity))	0	27-May-17
L7	TACD:((target*_nucleic*_acid* or target_molecule) AND ((competit*_oligonucleotid* OR competit*_probe*) AND (signal*_oligonucleotid* OR fluoresc*_oligonucleotid* OR fluoresc*_probe*)) AND (pcr OR qpcr OR polymerase_chain_reaction) and (signal w5 intensi*))	369	27-May-17
L6	TACD:((target*_nucleic*_acid* or target_molecule) AND ((competit*_oligonucleotid* OR competit*_probe*) AND (signal*_oligonucleotid* OR fluoresc*_oligonucleotid* OR fluoresc*_probe*)) AND (pcr OR qpcr OR polymerase_chain_reaction))	762	27-May-17
L5	TAC:((target*_nucleic*_acid* or target_molecule) AND ((competit*_oligonucleotid* OR competit*_probe*) AND (signal*_oligonucleotid* OR fluoresc*_oligonucleotid* OR fluoresc*_probe*)) AND (pcr OR qpcr OR polymerase_chain_reaction))	2	27-May-17
L4	TA:((target*_nucleic*_acid* or target_molecule) AND ((competit*_oligonucleotid* OR competit*_probe*) AND (signal*_oligonucleotid* OR fluoresc*_oligonucleotid* OR fluoresc*_probe*)) AND (pcr OR qpcr OR polymerase_chain_reaction))	0	27-May-17
L3	ASN:(chromacode)	0	27-May-17
L2	TACD:("multi-analyte" or ((competitive or signal) w5 (probes or oligonucleotides))) AND INV:((Jacky w3 Lucien) or (Rajagopal w3 Aditya) or (Menge w3 Karen) or (Gosch w3 Gregory))	29	27-May-17

L1	INV:((Jacky w3 Lucien) or (Rajagopal w3 Aditya) or (Menge w3 Karen) or (Gosch w3 Gregory))	152	27-May-17

Non-Patent Literature (NPL) Search Strategy/Results:

Google Scholar; 25 May 2017 (25.05.2017)

Search Terms	Results
"target nucleic acids") AND (("competitive oligonucleotides" OR "competitive probes") AND ("signal oligonucleotides" OR "fluorescent oligonucleotides" OR "fluorescent probes")) AND (PCR OR qPCR OR "polymerase chain reaction")	3
Identification target nucleic acids competitive oligonucleotides signal oligonucleotides PCR	48,900
Identification multiple target nucleic acids "fluorescence intensity" oligonucleotides PCR	27,700
multi-plex "real-time PCR" "competitive hybridization" probes ratio-metric coding of signal intensity	2,560
qPCR multi-analyte coding techniques	827
qPCR multi-analyte coding techniques ratiometric "signal intensity"	4

PubMed; 25 May 2017 (25.05.2017)

Search Terms	Results
((((PCR) AND real-time) AND multiplex)) AND signal oligonucleotides	15
((PCR) AND Real-time) AND competitive hybridzation	57
(identification target nucleic acids) AND competitive oligonucleotides	21
(((Jacky[Author]) OR Rajagopal A[Author]) OR Menge K[Author]) OR Gosch G[Author]	47

EBSCO; 01 June 2017 (01.06.2017)

Search ID#	Search Terms	Results
S8	AB (("nucleic acids" or oligonucleotide) N5 (detection or identification or target)) AND ((paired or set) N5 (oligonucleotides or "nucleic acids" or "capture molecules)) AND signal n5 intensity	119
S7	AB (("nucleic acids" or oligonucleotide) N5 (detection or identification or target)) AND ((paired or set) N5 (oligonucleotides or "nucleic acids" or "capture molecules))	779
S6	(("nucleic acids" or oligonucleotide) N5 (detection or identification or target)) AND ((paired or set) N5 (oligonucleotides or "nucleic acids" or "capture molecules))	117,761
S5	amplification N10 (target nucleic acid) AND (paired N5 (oligonucleotides	568

	or "nucleic acids")) AND (functional n5 (oligonucleotides or "nucleic acids"))	
S4	(Target N5 ("nucleic acids" or DNA or molecules)) AND (competitive N5 (oligonucleotides or "capture molecules" or antibodies)) AND polymerase chain reaction AND (ratio N10 (label or oligonucleotides)) AND signal N5 intensity	413
S3	(Target N5 ("nucleic acids" or DNA or molecules)) AND (competitive N5 (oligonucleotides or "capture molecules" or antibodies)) AND polymerase chain reaction AND (ratio N10 (label or oligonucleotides))	2,214
S2	(Target N5 ("nucleic acids" or DNA or molecules)) AND (competitive N5 (oligonucleotides or "capture molecules" or antibodies)) AND polymerase chain reaction	6,356
S1	(Target N5 ("nucleic acids" or DNA or molecules)) AND (competitive N5 (oligonucleotides or "capture molecules" or antibodies))	20,756

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: LEE JOHNSON
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

	Date of mailing *(day/month/year)* 16 NOV 2017
Applicant's or agent's file reference 38075-729601	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US 17/37682	International filing date *(day/month/year)* 15 June 2017 (15.06.2017)
Applicant CALIFORNIA INSTITUTE OF TECHNOLOGY	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   How? Directly to the International Bureau of WIPO preferably through ePCT or on paper to, 34 chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 338 82 70

   For more detailed instructions, see *PCT Applicant's Guide*, International Phase, paragraphs 9.004 – 9.011.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ **With regard to any protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices.
   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   The applicant may **submit comments on an informal basis on the written opinion of the International Searching Authority** to the International Bureau. These comments will be made available to the public after international publication. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established.

   Shortly after the expiration of **18 months from the priority date, the international application will be published** by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90*bis*.1 and 90*bis*.3).

   Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for **entry into the national phase** before those designated Offices. In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months. For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

   Within **22 months from the priority date, the applicant may request that a supplementary international search be carried out** by a different International Searching Authority that offers this service (Rule 45*bis*.1). The procedure for requesting supplementary international search is described in the *PCT Applicant's Guide*, International Phase, paragraphs 8.006-8.032.

Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee W. Young PCT Helpdesk: 571-272-4300 Telephone No. PCT OSP: 571-272-7774

Form PCT/ISA/220 (July 2014)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
38075-729601		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 17/37682	15 June 2017 (15.06.2017)	17 June 2016 (17.06.2016)
Applicant CALIFORNIA INSTITUTE OF TECHNOLOGY		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).

c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (see Box No. II).

3. ☒ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,

a. the figure of the **drawings** to be published with the abstract is Figure No. 6

☒ as suggested by the applicant.

☐ as selected by this Authority, because the applicant failed to suggest a figure.

☐ as selected by this Authority, because this figure better characterizes the invention.

b. ☐ none of the figures is to be published with the abstract.

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California Institute of Technology - Ex. 2003 Page 4158

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/37682

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

----------Please see continuation in first extra sheet----------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

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California Institute of Technology - Ex. 2003 Page 4159

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/37682

A CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68, C12P 19/34 (2017.01)
CPC - C12Q 1/6844, C12N 15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2007/0072211 A1 (NEWTON et al.,) 29 March 2007 (29.03.2007) Claim 1; Claim 3; para [0007]	1-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 August 2017	16 NOV 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/37682

Continuation of Box No III Observations where unity of invention is lacking

Group I: Claims 1-6, drawn to a method of detecting an analyte comprising generating a kinetic signature from a signal generated during an amplification reaction .

Group II: Claims 7-17, 27-31, drawn to methods of detecting target analytes comprising performing an amplification reaction by using a primer set that comprises at least one limiting primer.

Group III: Claims 18-26, drawn to a method of detecting an analyte comprising performing a amplification on a sample by using a primer set that comprises primer pairs with different annealing temperatures.

Group IV: Claims 32-33, drawn to a method of determining an amount of nucleic acid analyte, comprising generating transformed signal by transforming a signal generated during an amplification reaction into a frequency domain.

The inventions listed as Groups I, II, III, and IV do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I requires generating a kinetic signature from a signal generated during an amplification reaction, not required by Groups II, III, and IV.

Group II requires the detection of target analytes by using a set of primers comprising at least one limiting primers, not required by Groups I, III, and IV.

Group III requires the detection of target analytes by using a set of primers by using a primer set that comprises primer pairs with different annealing temperatures, not required by Groups I, II, and IV.

Group IV requires generating transformed signal by transforming a signal generated during an amplification reaction into a frequency domain, not required by Groups I, II, and III.

Common Technical Features

The feature shared by Groups I, II, III, and IV is a method of detecting a nucleic acid analyte in a sample, the method comprising:
performing an amplification reaction on the sample;
measuring a signal generated during the amplification reaction; and
detecting the presence or absence of the target nucleic acid analyte in the sample.

Another feature shared by Groups I and IV is the method step of: comparing a test value to a reference value and determining whether the test value corresponds with the reference value.

However, these shared technical features do not represent a contribution over prior art, because the shared technical features are taught by US 2007/0072211 A1 to Newton et al., (hereinafter 'Newton').

Newton teaches a method of detecting the presence or absence of a target nucleic acid analyte in a sample (para [0011] "the invention comprises methods for identifying one or more nucleic acid targets in a sample"), the method comprising:
performing an amplification reaction on the sample (para [0011] "Such methods comprise: performing asymmetric kinetic PCR in a reaction mixture containing one or more labeled 5'-nuclease probes and one or more labeled hybridization probes");
measuring a signal generated during the amplification reaction (para [0011] "monitoring one or more growth curves from the kinetic PCR"); and
detecting the presence or absence of the target nucleic acid analyte in the sample (para [0011] "thus, identifying the nucleic acid targets in the sample").

Newton further teaches the method step of: comparing a test value to a reference value and determining whether the test value corresponds with the reference value (para [0011] "correlating the melting curve or annealing curve, thus produced, to standard melting or annealing curves of completely or partially complementary probes of known nucleic acid targets or the same probe tested against a known sample, thus, identifying the nucleic acid targets in the sample").

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

-----Please see continuation in next extra sheet----------

IPR2024-01451
California Institute of Technology - Ex. 2003 Page 4161

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/37682

Continuation of Box No III Observations where unity of invention is lacking

Another feature shared by Group II and III is the detection of target analytes by using a set of primers.

However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is taught by US 2010/0233686 A1 to Higuchi et al., (hereinafter 'Higuchi').

Higuchi teaches detection of target analytes by using a set of primers (para [0012] "The present invention is a method for amplification, detection and quantification of one or more target nucleic acids in a single sample container comprising the steps of contacting a sample, suspected of containing one or more target nucleic acids, with at least one set of oligonucleotides, each oligonucleotide within the set labeled with the same one or more reporter moieties"; para [0042] "amplification of several target nucleic acid sequences in the same reaction vessel. Each tube contains one or more sets of probes, each set labeled with the same fluorescent reporter. However, within the set, each probe is characterized by a unique melting temperature with its target nucleic acid. For the maximum multiplex ability, several sets of probes are present in the same reaction vessel"; para [0057] "In some embodiments, the present invention involves asymmetric PCR. In an asymmetric PCR mixture, one of the amplification primers is present in greater amount than the other primer. The primers are referred to as "excess primer" and "limiting primer" respectively. The nucleic acid strands resulting from the extension of these primers are referred to as "excess strand" and "limiting strand" respectively").

As the technical feature was known in the art at the time of the invention, it cannot be considered a special technical feature that would otherwise unify the groups.

Groups I, II, III, and IV therefore lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

IPR2024-01451
California Institute of Technology - Ex. 2003 Page 4162

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: LEE JOHNSON
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)* 16 NOV 2017

Applicant's or agent's file reference
38075-729601

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US 17/37682	15 June 2017 (15.06.2017)	17 June 2016 (17.06.2016)

International Patent Classification (IPC) or both national classification and IPC
IPC(8) - C12Q 1/68, C12P 19/34 (2017.01)
CPC - C12Q 1/6844, C12N 15/10

Applicant CALIFORNIA INSTITUTE OF TECHNOLOGY

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☒ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	22 August 2017	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (January 2015)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 17/37682

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a)).

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

IPR2024-01451
California Institute of Technology - Ex. 2003 Page 4164

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 17/37682

Box No. IV Lack of unity of invention

1. ☒ In response to the invitation (Form PCT/ISA/206) to pay additional fees the applicant has, within the applicable time limit:

☐ paid additional fees.

☐ paid additional fees under protest and, where applicable, the protest fee.

☐ paid additional fees under protest but the applicable protest fee was not paid.

☒ not paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose not to invite the applicant to pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rule 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-6, drawn to a method of detecting an analyte comprising generating a kinetic signature from a signal generated during an amplification reaction .

Group II: Claims 7-17, 27-31, drawn to methods of detecting target analytes comprising performing an amplification reaction by using a primer set that comprises at least one limiting primer.

Group III: Claims 18-26, drawn to a method of detecting an analyte comprising performing a amplification on a sample by using a primer set that comprises primer pairs with different annealing temperatures.

Group IV: Claims 32-33, drawn to a method of determining an amount of nucleic acid analyte, comprising generating transformed signal by transforming a signal generated during an amplification reaction into a frequency domain.

The inventions listed as Groups I, II, III, and IV do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I requires generating a kinetic signature from a signal generated during an amplification reaction, not required by Groups II, III, and IV.

Group II requires the detection of target analytes by using a set of primers comprising at least one limiting primers, not required by Groups I, III, and IV.

Group III requires the detection of target analytes by using a set of primers by using a primer set that comprises primer pairs with different annealing temperatures, not required by Groups I, II, and IV.

Group IV requires generating transformed signal by transforming a signal generated during an amplification reaction into a frequency domain, not required by Groups I, II, and III.

----------Please see continuation in first supplemental box----------

4. Consequently, this opinion has been established in respect of the following parts of the international application:

☐ all parts.

☒ the parts relating to claims Nos. 1-6

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WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 17/37682

Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	1-6	YES
	Claims	None	NO
Inventive step (IS)	Claims	None	YES
	Claims	1-6	NO
Industrial applicability (IA)	Claims	1-6	YES
	Claims	None	NO

2. Citations and explanations:

Claims 1-6 lack an inventive step under PCT Article 33(3) as being obvious over US 2007/0072211 A1 to Newton et al., (hereinafter 'Newton').

Regarding Claim 1, Newton teaches a method of detecting the presence or absence of a target nucleic acid analyte in a sample (Claim 1 - 'A method for identifying one or more nucleic acid targets in a sample,'), the method comprising:
a) performing an amplification reaction on the sample (Claim 1 - 'the method comprising: a) performing an asymmetric kinetic PCR in a reaction mixture');
b) measuring a signal generated during the amplification reaction and generating a kinetic signature from the measured signal (Claim 1 - 'b) monitoring one or more growth curves from the kinetic PCR'); and
c) comparing the generated kinetic signature to a reference signature and determining whether the generated kinetic signature corresponds with the reference signature, thereby detecting the presence or absence of the target nucleic acid analyte in the sample (Claim 1 - 'e) correlating the melting curve or annealing curve of step d) to a melting or annealing curve of a completely complementary probe of one or more known nucleic acid targets, thus, identifying the one or more nucleic acid targets in the sample.'). Newton does not state expressly that the growth curve from kinetic PCR is a kinetic signature. However, since Newton teaches the kinetic PCR is aimed at differentiating between closely related nucleic acid molecules (para [0007] - 'For example, many diseases and infections are caused by a number of, often related, pathogens. While the disease symptoms may present as similar, it can be of utmost importance to determine the actual causative pathogen in order to present an effective treatment. Not only is this true in terms of differentiation between different infectious species, but is also true, and can be even more difficult to resolve, when trying to discriminate between closely related agents, e.g., different strains of a pathogen such as the subtypes of hepatitis C virus (HCV).'), it would have been obvious to one of ordinary skill in the art that the growth curve from the kinetic PCR of Newton represents a kinetic signature enabling specific identification of a nucleic acid target.

Regarding Claim 2, Newton teaches the method of claim 1, wherein the kinetic signature comprises a signal selected from an electromagnetic signal, a wavelength, and a fluorescence emission signal (Claim 1 - 'c) monitoring one or more fluorescent signals generated from the one or more labeled hybridization probes thereby producing a melting curve or annealing curve').

Regarding Claim 3, Newton teaches the method of claim 1, wherein the kinetic signature is plotted as a curve (Claim 1 - 'd) monitoring one or more fluorescent signals generated from the one or more labeled hybridization probes thereby producing a melting curve or annealing curve').

Regarding Claim 4, Newton teaches the method of claim 1 wherein the reference signature corresponds with a kinetic signature generated by amplification of the target nucleic acid analyte under one or more members of a set of constrained amplification parameters, the set of constrained amplification parameters comprising any combination of: (i) a range of forward and/or reverse primer concentrations; (ii) a range of polymerase concentrations; (iii) polymerase type; (iv) a range of nucleotide concentrations; (v) chromophore concentration; and (vi) chromophore type; (vii) a number of thermocycles; (viii) rate of thermocycling; (ix) one or more thermocycle stage temperatures; and (x) one or more thermocycle stage time lengths; (xi) a range of target-specific molecular probes; (xii) primers associated with one or more targetspecific probes; and (xiii) a concentration of one or more enzyme cofactors (Claim 1 - 'e) correlating the melting curve or annealing curve of step d) to a melting or annealing curve of a completely complementary probe of one or more known nucleic acid targets'; Claim 3 - 'The method of claim 1, wherein the asymmetric kinetic PCR comprises a first primer and at least a second primer, wherein the amount of the first primer is greater than the amount of the second primer and wherein the hybridization probe is present in an equal or greater amount than the second primer.').

---------Please see continuation in second supplemental box----------

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WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 17/37682

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:
Box No. IV.3. Lack of unity of invention

Common Technical Features

The feature shared by Groups I, II, III, and IV is a method of detecting a nucleic acid analyte in a sample, the method comprising:
performing an amplification reaction on the sample;
measuring a signal generated during the amplification reaction; and
detecting the presence or absence of the target nucleic acid analyte in the sample.

Another feature shared by Groups I and IV is the method step of: comparing a test value to a reference value and determining whether the test value corresponds with the reference value.

However, these shared technical features do not represent a contribution over prior art, because the shared technical features are taught by US 2007/0072211 A1 to Newton et al., (hereinafter 'Newton').

Newton teaches a method of detecting the presence or absence of a target nucleic acid analyte in a sample (para [0011] "the invention comprises methods for identifying one or more nucleic acid targets in a sample"), the method comprising:
performing an amplification reaction on the sample (para [0011] "Such methods comprise: performing asymmetric kinetic PCR in a reaction mixture containing one or more labeled 5'-nuclease probes and one or more labeled hybridization probes");
measuring a signal generated during the amplification reaction (para [0011] "monitoring one or more growth curves from the kinetic PCR"); and
detecting the presence or absence of the target nucleic acid analyte in the sample (para [0011] "thus, identifying the nucleic acid targets in the sample").

Newton further teaches the method step of: comparing a test value to a reference value and determining whether the test value corresponds with the reference value (para [0011] "correlating the melting curve or annealing curve, thus produced, to standard melting or annealing curves of completely or partially complementary probes of known nucleic acid targets or the same probe tested against a known sample, thus, identifying the nucleic acid targets in the sample").

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Another feature shared by Group II and III is the detection of target analytes by using a set of primers.

However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is taught by US 2010/0233686 A1 to Higuchi et al., (hereinafter 'Higuchi').

Higuchi teaches detection of target analytes by using a set of primers (para [0012] "The present invention is a method for amplification, detection and quantification of one or more target nucleic acids in a single sample container comprising the steps of contacting a sample, suspected of containing one or more target nucleic acids, with at least one set of oligonucleotides, each oligonucleotide within the set labeled with the same one or more reporter moieties"; para [0042] "amplification of several target nucleic acid sequences in the same reaction vessel. Each tube contains one or more sets of probes, each set labeled with the same fluorescent reporter. However, within the set, each probe is characterized by a unique melting temperature with its target nucleic acid. For the maximum multiplex ability, several sets of probes are present in the same reaction vessel"; para [0057] "In some embodiments, the present invention involves asymmetric PCR. In an asymmetric PCR mixture, one of the amplification primers is present in greater amount than the other primer. The primers are referred to as "excess primer" and "limiting primer" respectively. The nucleic acid strands resulting from the extension of these primers are referred to as "excess strand" and "limiting strand" respectively").

As the technical feature was known in the art at the time of the invention, it cannot be considered a special technical feature that would otherwise unify the groups.

Groups I, II, III, and IV therefore lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:
Box No V2 Citations and Explanations

Regarding Claim 5, Newton teaches the method of claim 4, wherein the amplification reaction is carried out within one or more of the constrained amplification parameters (Claim 3 - 'The method of claim 1, wherein the asymmetric kinetic PCR comprises a first primer and at least a second primer, wherein the amount of the first primer is greater than the amount of the second primer and wherein the hybridization probe is present in an equal or greater amount than the second primer.').

Regarding Claim 6, Newton teaches the method of claim 3, wherein the at least one of an exponential region, a linear region, and a plateau region of the curve occurs over at least 4 thermocycles or over at least 8 thermocycles (para [0068] - 'FIG. 3 provides growth curves generated during an asymmetric kPCR'; and data shown in Figure 3). Newton does not state expressly at least 4 thermocycles or over at least 8 thermocycles. However, data shown in Figure 3 illustrates at least 4 thermocycles or over at least 8 thermocycles.

Claims 1-6 have industrial applicability as defined by PCT Article 33(4) because the subject matter can be made or used in industry.

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SEARCH HISTORY

Application Number	PCT/US 17/37682
Search Conducted By	AEM
Search Approved By	PP

CPC/IPC Classifications Searched	IPC(8) - C12 Q1/68, C12P 19/34 (2017.01) CPC - C12Q 1/6844, C12N 15/10
Date Conducted	22 August 2017 (22.08.2017)

Documentation Searched	Keyword limited; search terms below
Search Terms Used	Amplification, kinetic PCR or polymerase chain reaction, plot or curve, primer, electromagnetic or wavelength or fluorescence, reference or control or standard, primer set, 2 forward primers, 2 reverse primers, nested primer, limiting primer, annealing temperature, thermocycling or temperature cycle
Date Conducted	22 August 2017 (22.08.2017)

Electronic Database Searched	Patbase
Files Searched	Full-text: AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO Bibliographic: (Europe) AT BA BE BG CH CS CY CZ DD DK EE ES FI GE GR HR HU IE IS IT LT LU LV MC MD MT NL NO PL PT RO RS SE SI SK SM TR UA YU (Asia) EA GC HK ID IL IN KZ MN MY PH RU SG SU TH TJ TW UZ VN (North America) CA CR CU DO GT HN MX NI PA SV TT (South America) AR BR CL CO EC PE UY (Australasia) AU NZ (Africa) AP DZ EG KE MA MW OA ZA ZM ZW
Date Conducted	22 August 2017 (22.08.2017)
Search Logic: Classification based search	

Search 1: IC=(C12Q1/68 OR C12P19/34) (Results 128654)
Search 2: CPC=(C12Q1/6844 OR C12N15/10 OR C12Q1/68 OR C12Q1/686 OR C12Q1/6853 OR G01N21/6486 OR G01N2021/6439) (Results 14465)
Search 3: 1 OR 2 (Results 131488)
Search 4: 3 AND FT=(KINETIC W1 PCR) (Results 509)
Search 5: 4 AND FT=(LIMIT* W1 PRIMER%) (Results 9)
Search 6: 4 AND FT=(CONSTRAINED NEAR (PCR OR AMPLIFICATION OR PARAMETER%)) (Results 0)
Search 7: 3 AND FT=(CONSTRAINED NEAR (PCR OR AMPLIFICATION OR PARAMETER%)) (Results 79)
Search 8: 7 AND FT=(KINETIC NEAR PCR) (Results 0)
Search 9: 7 AND FT=(ASYMMETRIC NEAR (PCR OR AMPLIFICATION)) (Results 6)
Search 10: 4 AND FT=(NESTED NEAR PCR) (Results 159)
Search 11: 10 AND FT=(TWO NEAR (REVERSE OR FORWARD)) (Results 6)
Search 12: 11 NOT (5 OR 9) (Results 6)
Search 13: 4 AND FT=(PCR NEAR SIGNATURE) (Results 3)

Keyword only search

Search 14: FT=(KINETIC W1 PCR) (Results 639)
Search 15: 14 AND FT=(LIMIT* W1 PRIMER%) (Results 9)
Search 16: 15 NOT (5 OR 9 OR 13) (Results 0)
Search 17: 14 AND FT=(CONSTRAINED NEAR (PCR OR AMPLIFICATION OR PARAMETER%)) (Results 0)
Search 18: FT=(CONSTRAINED NEAR (PCR OR AMPLIFICATION OR PARAMETER%)) (Results 2434)
Search 19: 18 AND FT=(KINETIC NEAR PCR) (Results 0)
Search 20: 18 AND FT=(ASYMMETRIC NEAR (PCR OR AMPLIFICATION)) (Results 6)
Search 21: 20 NOT (5 OR 9 OR 13) (Results 0)
Search 22: FT=(NESTED NEAR PCR) (Results 9272)
Search 23: 22 AND FT=(TWO NEAR (REVERSE OR FORWARD)) (Results 579)
Search 24: 23 AND FT=(KINETIC W1 PCR) (Results 7)
Search 25: FT=(PCR NEAR SIGNATURE) (Results 604)
Search 26: 25 AND FT=(ASYMMETRIC NEAR (PCR OR AMPLIFICATION)) (Results 10)
Search 27: 26 NOT (5 OR 9 OR 13) (Results 9)

Forward and backward search

Search 28: PN=(US2007072211 OR US2010233686 OR US8367325) (Results 3)
Search 29: CTF 28 OR CTB 28 (Results 194)
Search 30: 29 AND FT=(KINETIC W1 PCR) (Results 6)
Search 31: 30 NOT (5 OR 9 OR 13) (Results 2)

Electronic Database Searched	Google
Files Searched	Patents
Date Conducted	22 August 2017 (22.08.2017)

Search Logic:

kinetic PCR

About 67,500 results (0.34 seconds)

kinetic PCR signature limiting primer

About 12,700 results (0.34 seconds)

constrained PCR amplification parameters

About 36,900 results (0.38 seconds)

constrained PCR amplification parameters kinetic PCR

About 16,100 results (0.61 seconds)

constrained PCR amplification kinetic PCR limiting primer

About 16,400 results (0.52 seconds)

constrained nested PCR amplification kinetic PCR limiting primer

About 2,650 results (0.52 seconds)

constrained nested PCR amplification kinetic PCR signature limiting primer

About 554 results (0.47 seconds)

kinetic PCR with 2 reverse primers

About 65,000 results (0.93 seconds)

kinetic nested PCR with 2 reverse primers

About 9,020 results (0.48 seconds)

asymmetric kinetic nested PCR with 2 reverse primers

About 1,820 results (0.52 seconds)

asymmetric kinetic nested PCR with 2 forward primers

About 1,290 results (0.58 seconds)

Electronic Database Searched	Google
Files Searched	Scholar
Date Conducted	22 August 2017 (22.08.2017)

Search Logic:

kinetic PCR
About 466,000 results (0.11 sec)

kinetic PCR signature limiting primer
About 21,700 results (0.07 sec)

constrained PCR amplification parameters
About 45,900 results (0.09 sec)

constrained PCR amplification parameters kinetic PCR
About 21,400 results (0.09 sec)

constrained PCR amplification kinetic PCR limiting primer
About 18,800 results (0.11 sec)

constrained nested PCR amplification kinetic PCR limiting primer
About 4,580 results (0.15 sec)

constrained nested PCR amplification kinetic PCR signature limiting primer
About 1,450 results (0.07 sec)

kinetic pcr with 2 reverse primers
About 235,000 results (0.09 sec)

kinetic nested pcr with 2 reverse primers
About 21,800 results (0.15 sec)

asymmetric kinetic nested pcr with 2 reverse primers
About 3,340 results (0.06 sec)

asymmetric kinetic nested pcr with 2 forward primers
About 2,050 results (0.08 sec)

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Supplemental Search

Electronic Database Searched	PatBase
Files Searched	Full-text: AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO Bibliographic: (Europe) AT BA BE BG CH CS CY CZ DD DK EE ES FI GE GR HR HU IE IS IT LT LU LV MC MD MT NL NO PL PT RO RS SE SI SK SM TR UA YU (Asia) EA GC HK ID IL IN KZ MN MY PH RU SG SU TH TJ TW UZ VN (North America) CA CR CU DO GT HN MX NI PA SV TT (South America) AR BR CL CO EC PE UY (Australasia) AU NZ (Africa) AP DZ EG KE MA MW OA ZA ZM ZW
Date Conducted	30 October 2017
Search Logic:	
1) IC=(C12Q1/68 or C12P19/34) (130470) 2) CPC=(C12Q1/6844 or C12N15/10 or C12Q1/68 or C12Q1/686 or C12Q1/6853 or G01N21/6486 or G01N2021/6439) (15934) 3) 1 or 2 (133809) 4) (PCR or polymerase chain reaction) and amplif* (168197) 5) 4 and kinetic (16544) 6) 5 and (plot or curve) (9932) 7) TAC=(detect* w5 (nucleic acid or DNA or RNA or nucleotide or oligonucleotide or polynucleotide)) (31528) 8) 7 and 6 (1344) 9) 3 and 8 (1120) 10) kinetic signature (29) 11) 10 and 8 (6) 12) 3 and 11 (6)	

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference 44158-709601	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/US2018/039846	International filing date *(day/month/year)* 27 June 2018 (27.06.2018)	Priority date *(day/month/year)* 28 June 2017 (28.06.2017)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant CHROMACODE, INC.		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☐	Box No. II	Priority
☒	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☐	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 31 December 2019 (31.12.2019)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Agnès Wittmann-Regis e-mail: pct.team6@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: KYLE BRUNER
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	14 SEP 2018
Applicant's or agent's file reference 44158-709601	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US 18/39846	27 June 2018 (27.06.2018)	28 June 2017 (28.06.2017)

International Patent Classification (IPC) or both national classification and IPC
IPC(8) - C12Q 1/70, C12Q 1/70 (2018.01)
CPC - C12Q 1/701, C12Q 1/6818, C12Q 1/68, C12Q 1/6851, C12Q 1/686, C12Q 1/703, Y10T 436/143333, C12Q 2563/103, C12Q 2563/159

Applicant CHROMACODE, INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	21 August 2018	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (January 2015)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 18/39846

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a)).

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

a. ☐ forming part of the international application as filed:

☐ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 18/39846

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 4-13, 18-29, 33-50, 54-69

because:

☐ the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international search *(specify)*:

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 4-13, 18-29, 33-50, 54-69 are so unclear that no meaningful opinion could be formed *(specify)*:

Claims 4-13, 18-29, 33-50, 54-69 are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. 4-13, 18-29, 33-50, 54-69

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

- ☐ furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
- ☐ furnish a sequence listing on paper or in the form of an image file complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
- ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13*ter*.1(a) or (b).

☐ See Supplemental Box for further details.

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 18/39846

Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	1-3, 14-17, 30-32, 51-53	YES
	Claims	None	NO
Inventive step (IS)	Claims	None	YES
	Claims	1-3, 14-17, 30-32, 51-53	NO
Industrial applicability (IA)	Claims	1-3, 14-17, 30-32, 51-53	YES
	Claims	None	NO

2. Citations and explanations:

Claims 1-3, 30-32 and 51-53 lack an inventive step under PCT Article 33(3) as being obvious over US 2014/0213471 A1 to California Institute of Technology (hereinafter 'Caltech').

Regarding claim 1, Caltech teaches a method for performing a digital assay, comprising:
(a) (i) three or more nucleic acid targets and (ii) a set of nucleic acid probes comprising a first nucleic acid probe, a second nucleic acid probe, and a third nucleic acid probe (para [0154] - 'In some cases, compositions comprise a first and a second primer or probe for the detection of at least one analyte wherein the primers are attached to either a fluorophore or a quencher at the 5' end . . . . In some cases the compositions comprise multiple pairs of first and second primers, wherein each pair of first and second primers comprise either a fluorophore or a quencher at the 5' end. In some cases each pair of first and second primers comprise a different fluorophore and quencher from the remaining set of primers. In some cases the compositions comprise at least 1, 2, 3, 4 . . . or 10000 pairs of first and second primers.'; para [0048] - 'Various combinations of oligonucleotides (e.g. primers and/or probes) can be used to detect an analyte. In some cases, a primer is used with a probe or a plurality of probes. In some cases, a plurality of primers is used with a plurality of probes. . . . In some cases, a primer and a probe is used to detect multiple analytes . . . a combination of primers and probes is used to detect multiple analytes. In some cases, a probe is used to detect an analyte. In some cases, a probe is used to detect multiple analytes. In some cases, multiple probes are used to detect an analyte.');
(b) amplifying the three or more nucleic acid targets (para [0003] 'using oligonucleotides (e.g., primers, probes) attached to chromophores (e.g., fluorophores, quenchers, etc.) in amplification or polymerization reactions in order to detect a polynucleotide analyte'; [0035] - 'the polymerase chain reaction process');
(c) generating N signals from the set of nucleic acid probes, each of which N signals corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets (para [0105] - 'The incorporation of the quencher and the fluorophore in the newly generated amplicon can lead to signal generation (e.g., quenching of fluorescence intensity from the fluorophore). With each iterative amplification or polymerization reaction, the fluorescence intensity is reduced by a factor of about 2. The amount of fluorescence detected can be used to directly determine the amount of analyte present'; para [0003] - '(b) measuring a first signal generated by the first and second chromophores; (c) performing at least one polymerization reaction with the first primer using the first polynucleotide analyte as a template; and (d) measuring a second signal generated by the first and second chromophores . . . '; para [0007] - 'further comprises combining the sample with a third primer . . . '; para [0010]-[0011] - 'the PCR process is an end-point PCR process, a digital PCR process, a real-time PCR process, a droplet digital PCR process, or a quantitative PCR process . . . methods of detecting a plurality of analytes, . . . b) measuring a first set of signals generated by the chromophores; c) performing at least one polymerization reaction with the plurality of primer pairs using the plurality of analytes as templates; and d) measuring a second set of signals generated by the chromophores; wherein the first and second set of signals are used to detect each analyte of the plurality of analytes . . . generating a set of signature profiles; wherein the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profiles'); and
(d) detecting the N signals in a single optical channel (Claim 3 - 'method of detecting multiple nucleic acid targets of differing lengths . . . .b) conducting a polymerase chain reaction (PCR); and c) measuring an endpoint fluorescence of each target, wherein the endpoint fluorescence for each target is different.'; para [0010] - 'In some cases, the PCR process is an end-point PCR process . . . or a quantitative PCR process. In some cases, the polymerization reaction is a quantitative PCR process . . . the second method is a digital PCR process'; para [0049] - 'In some cases, a single chromophore pair is used to detect an analyte'; para [0176] - 'Analytes of differing lengths (e.g., 40 bp, 60 bp, and 80 bp) are detected using a single fluorophore/quencher combination . . . By measuring fluorescence as a function of increasing temperature, multiple analytes in a single color channel are quantified.');
wherein in (a), the concentration of the second nucleic acid probe is at least about 2X, and the concentration of the third nucleic acid probe is at least about 4X (para [0030] - 'FIG. 9 exemplifies the binary coding of analytes. Analytes in a 3-plex CY3 assay were coded using binary spaced primer concentrations.'; [0173] 'Analytes in a 3-plex CY3 assay were coded using binary spaced primer concentrations'; see Figure 9).
Caltech does not expressly teach the method comprising (a) partitioning into a plurality of partitions the three or more nucleic acid targets, wherein the amplifying and generating signals steps are performed in the partitions. However, since Caltech teaches droplet digital PCR (para [0010]), it would have been obvious to one of ordinary skill in the art that Caltech's assay using a droplet digital PCR process involves (a) partitioning the nucleic acid targets into a plurality of partitions since this is a required step for carrying out droplet digital PCR, and it further would have been obvious that amplifying and signal generation would be in a partition of the plurality of partitions, in order to correctly perform the taught method using a droplet digital PCR process.

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
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Box No. V2. Citations and Explanations

Regarding claim 2, Caltech makes obvious the method of claim 1, and further teaches wherein N is 3 or more (para [0072] "In some cases, multiple color codes or chromophore combinations are assigned to one or more analytes. In some cases, 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes). In some cases, at most 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)").

Regarding claim 3, Caltech makes obvious the method of claims 1-2, and further teaches wherein N is 4 or more (para [0072] "In some cases, multiple color codes or chromophore combinations are assigned to one or more analytes. In some cases, 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes). In some cases, at most 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)").

Regarding claim 30, Caltech teaches a method for performing a digital assay, comprising:
(a) three or more nucleic acid targets (para [0154] - 'In some cases, compositions comprise a first and a second primer or probe for the detection of at least one analyte wherein the primers are attached to either a fluorophore or a quencher at the 5' end . . . . In some cases the compositions comprise multiple pairs of first and second primers, wherein each pair of first and second primers comprise either a fluorophore or a quencher at the 5' end. In some cases each pair of first and second primers comprise a different fluorophore and quencher from the remaining set of primers. In some cases the compositions comprise at least 1, 2, 3, 4, 5, . . . or 10000 pairs of first and second primers.'; para [0048] - 'Various combinations of oligonucleotides (e.g. primers and/or probes) can be used to detect an analyte. In some cases, a primer is used with a probe or a plurality of probes. In some cases, a plurality of primers is used with a plurality of probes. . . . In some cases, a primer and a probe is used to detect multiple analytes. In some cases, a combination of primers and probes is used to detect an analyte. In some cases, a combination of primers and probes is used to detect multiple analytes. In some cases, a probe is used to detect an analyte. In some cases, a probe is used to detect multiple analytes. In some cases, multiple probes are used to detect an analyte.');
(b) amplifying the three or more nucleic acid targets (para [0005] - 'In some cases, the polymerase chain reaction process is an end-point polymerase chain reaction process, a real-time polymerase chain reaction process, a digital polymerase chain reaction process, a droplet digital polymerase chain reaction process');
(c) generating N signals each of which corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets (para [0003] - '(b) measuring a first signal generated by the first and second chromophores . . . wherein the first and second signals are used to detect the first polynucleotide analyte.'; para [0007] - 'In some cases, step a) of the method described herein further comprises combining the sample with a third primer and a second oligonucleotide . . . step b) further comprises measuring a fifth signal generated by the third and fourth chromophores; step d) further comprises measuring a sixth signal generated by the third and fourth chromophores'; para [0011] - 'methods of detecting a plurality of analytes, . . . b) measuring a first set of signals generated by the chromophores . . . generating a set of signature profiles; wherein the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profiles'); and
(d) detecting the signals in a single optical channel (Claim 3 - 'method of detecting multiple nucleic acid targets of differing lengths . . . .b) conducting a polymerase chain reaction (PCR); and c) measuring an endpoint fluorescence of each target, wherein the endpoint fluorescence for each target is different.'; para [0010] - 'In some cases, the PCR process is an end-point PCR process, a digital PCR process, a real-time PCR process, a droplet digital PCR process, or a quantitative PCR process. In some cases, the polymerization reaction is a quantitative PCR process. In some cases, the detecting comprises a quantitative PCR method. In some cases, the detecting comprises a quantitative PCR method and a second method. In some cases, the second method is a digital PCR process'; para [0049] - 'In some cases, a single chromophore pair is used to detect an analyte. In some cases, a single chromophore pair is used to detect multiple analytes.'; para [0072] 'at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)'; para [0176] - 'Analytes of differing lengths (e.g., 40 bp, 60 bp, and 80 bp) are detected using a single fluorophore/quencher combination . . . .By measuring fluorescence as a function of increasing temperature, multiple analytes in a single color channel are quantified.').
Caltech does not expressly teach (a) partitioning into a plurality of partitions and amplifying in the partitions wherein N signals are generated in a partition of the plurality of partitions. However, since Caltech teaches droplet digital PCR (para [0010]), it would have been obvious to one of ordinary skill in the art that Caltech's assay using a droplet digital PCR process involves (a) partitioning the nucleic acid targets into a plurality of partitions since this is a required step for carrying out droplet digital PCR, and it further would have been obvious that amplifying and signal generation would be in a partition of the plurality of partitions, in order to correctly perform the taught method using a droplet digital PCR process.

Regarding claim 31, Caltech makes obvious the method of claim 30, and further teaches wherein N is 3 or more (para [0072] "In some cases, multiple color codes or chromophore combinations are assigned to one or more analytes. In some cases, 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes). In some cases, at most 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)").

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 18/39846

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of:
Box No. V2. Citations and Explanations

Regarding claim 32, Caltech makes obvious the method of claims 30-31, and further teaches wherein N is 4 or more (para [0072] "In some cases, multiple color codes or chromophore combinations are assigned to one or more analytes. In some cases, 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes). In some cases, at most 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)").

Regarding claim 51, Caltech teaches a method of performing a digital assay, comprising:
(a) amplifying at least three nucleic acid targets from a sample comprising a set of nucleic acid probes comprising three or more nucleic acid probes labeled with fluorophores capable of being detected in a single optical channel, wherein each nucleic acid probe is complementary to a target of the at least three nucleic acid targets (para [0154] - 'compositions comprise a first and a second primer or probe for the detection of at least one analyte wherein the primers are attached to either a fluorophore or a quencher at the 5' end . . . In some cases each pair of first and second primers comprise a different fluorophore and quencher from the remaining set of primers. In some cases the compositions comprise at least 1, 2, 3, 4, 5, 6 . . . or 10000 pairs of first and second primers.'; para [0048] - 'Various combinations of oligonucleotides (e.g. primers and/or probes) can be used to detect an analyte. In some cases, a primer is used with a probe or a plurality of probes. In some cases, a plurality of primers is used with a plurality of probes. . . . In some cases, a primer and a probe is used to detect multiple analytes. In some cases, a combination of primers and probes is used to detect an analyte. In some cases, a combination of primers and probes is used to detect multiple analytes. In some cases, a probe is used to detect an analyte. In some cases, a probe is used to detect multiple analytes. In some cases, multiple probes are used to detect an analyte.'; para [0005] - 'the polymerase chain reaction process is . . . a droplet digital polymerase chain reaction process');
(b) detecting N signals if one or more of the at least three nucleic acid targets is present, wherein each of the N signals corresponds to a unique combination of one or more of the at least three nucleic acid targets (para [0003] - '(b) measuring a first signal generated by the first and second chromophores; (c) performing at least one polymerization reaction with the first primer using the first polynucleotide analyte as a template; and (d) measuring a second signal generated by the first and second chromophores; wherein the first and second signals are used to detect the first polynucleotide analyte.'; para [0007] - 'further comprises measuring a fifth signal generated by the third and fourth chromophores; step d) further comprises measuring a sixth signal generated by the third and fourth chromophores'; para [0011] - 'Also disclosed herein are methods of detecting a plurality of analytes, . . . b) measuring a first set of signals generated by the chromophores; c) performing at least one polymerization reaction with the plurality of primer pairs using the plurality of analytes as templates . . . generating a set of signature profiles; wherein the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profiles'; Claim 3 - 'method of detecting multiple nucleic acid targets of differing lengths . . . b) conducting a polymerase chain reaction (PCR); and c) measuring an endpoint fluorescence of each target, wherein the endpoint fluorescence for each target is different.'; para [0010] - 'In some cases, the PCR process is an end-point PCR process, a digital PCR process, a real-time PCR process, a droplet digital PCR process'; para [0049] - 'In some cases, a single chromophore pair is used to detect an analyte. In some cases, a single chromophore pair is used to detect multiple analytes.'; para [0072] 'at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)'; [0176] - 'Analytes of differing lengths (e.g., 40 bp, 60 bp, and 80 bp) are detected using a single fluorophore/quencher combination . . . By measuring fluorescence as a function of increasing temperature, multiple analytes in a single color channel are quantified.'); and
(c) determining, from the N signals, the presence or absence of each of the at least three nucleic acid targets in the sample (para [0045] - 'Described herein are methods for detecting the presence or absence of at least one polynucleotide analyte in a sample . . . .In some cases, the first and second signals are used to detect the presence or absence of the amplified product (e.g. the polynucleotide analyte)').
Caltech does not expressly teach (a) partitioning into a plurality of droplets and amplifying in the droplets wherein N signals are generated in a droplet of the plurality of droplets. However, since Caltech teaches droplet digital PCR (para [0010]), it would have been obvious to one of ordinary skill in the art that Caltech's assay using a droplet digital PCR process involves (a) partitioning the nucleic acid targets into a plurality of droplet partitions since this is a required step for carrying out droplet digital PCR, and it further would have been obvious that amplifying and signal generation would be in a partition of the plurality of droplet partitions, in order to correctly perform the taught method using a droplet digital PCR process.

Regarding claim 52, Caltech makes obvious the method of claim 51, and further teaches wherein N is 3 or more (para [0072] "In some cases, multiple color codes or chromophore combinations are assigned to one or more analytes. In some cases, 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes). In some cases, at most 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)").

Regarding claim 53, Caltech makes obvious the method of claims 51-52, and further teaches wherein N is 4 or more (para [0072] "In some cases, multiple color codes or chromophore combinations are assigned to one or more analytes. In some cases, 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes). In some cases, at most 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)").

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Supplemental Box

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Box No. V2. Citations and Explanations

Claims 14-17 lack an inventive step under PCT Article 33(3) as being obvious over Caltech in view of US 2016/0108464 A1 to Bio-Rad Laboratories, Inc. (hereinafter 'Bio-rad').

Regarding claim 14, Caltech teaches a method for performing a digital assay, comprising:
(a) (i) three or more nucleic acid targets and (ii) a set of nucleic acid probes comprising a first nucleic acid probe, a second nucleic acid probe, and a third nucleic acid probe (para [0154] - 'compositions comprise a first and a second primer or probe for the detection of at least one analyte wherein the primers are attached to either a fluorophore or a quencher at the 5' end . . . In some cases the compositions comprise multiple pairs of first and second primers, wherein each pair of first and second primers comprise either a fluorophore or a quencher at the 5' end. In some cases each pair of first and second primers comprise a different fluorophore and quencher from the remaining set of primers. In some cases the compositions comprise at least 1, 2, 3, 4, 5 . . . or 10000 pairs of first and second primers.'; para [0048] - 'Various combinations of oligonucleotides (e.g. primers and/or probes) can be used to detect an analyte. In some cases, a primer is used with a probe or a plurality of probes. In some cases, a plurality of primers is used with a plurality of probes. . . . In some cases, a primer and a probe is used to detect multiple analytes. In some cases, a combination of primers and probes is used to detect an analyte. In some cases, a combination of primers and probes is used to detect multiple analytes. In some cases, a probe is used to detect an analyte. In some cases, a probe is used to detect multiple analytes. In some cases, multiple probes are used to detect an analyte.');
(b) amplifying the three or more nucleic acid targets (para [0005] - 'In some cases, the polymerase chain reaction process is an end-point polymerase chain reaction process, a real-time polymerase chain reaction process, a digital polymerase chain reaction process, a droplet digital polymerase chain reaction process');
(c) generating N signals each of which corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets (para [0003] - '(b) measuring a first signal generated by the first and second chromophores; (c) performing at least one polymerization reaction with the first primer using the first polynucleotide analyte as a template; and (d) measuring a second signal generated by the first and second chromophores; wherein the first and second signals are used to detect the first polynucleotide analyte.'; para [0007] - 'In some cases, step a) of the method described herein further comprises combining the sample with a third primer and a second oligonucleotide, wherein a third chromophore is attached to the third primer and a fourth chromophore is attached to the second oligonucleotide, . . . step b) further comprises measuring a fifth signal generated by the third and fourth chromophores; step d) further comprises measuring a sixth signal generated by the third and fourth chromophores'; para [0011] - 'Also disclosed herein are methods of detecting a plurality of analytes, . . . b) measuring a first set of signals generated by the chromophores; c) performing at least one polymerization reaction with the plurality of primer pairs using the plurality of analytes as templates; and d) measuring a second set of signals generated by the chromophores; wherein the first and second set of signals are used to detect each analyte of the plurality of analytes. In some cases, the method described herein further comprises: e) repeating step c and d at least once; and f) generating a set of signature profiles; wherein the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profiles'); and
(d) detecting the signals in a single optical channel (Claim 3 - 'method of detecting multiple nucleic acid targets of differing lengths . . . b) conducting a polymerase chain reaction (PCR); and c) measuring an endpoint fluorescence of each target, wherein the endpoint fluorescence for each target is different.'; para [0010] - 'In some cases, the PCR process is an end-point PCR process, a digital PCR process, a real-time PCR process, a droplet digital PCR process, or a quantitative PCR process. In some cases, the polymerization reaction is a quantitative PCR process. In some cases, the detecting comprises a quantitative PCR method. In some cases, the detecting comprises a quantitative PCR method and a second method. In some cases, the second method is a digital PCR process'; para [0049] - 'In some cases, a single chromophore pair is used to detect an analyte. In some cases, a single chromophore pair is used to detect multiple analytes.'; para [0176] - 'Analytes of differing lengths (e.g., 40 bp, 60 bp, and 80 bp) are detected using a single fluorophore/quencher combination . . . By measuring fluorescence as a function of increasing temperature, multiple analytes in a single color channel are quantified.'). Caltech does not expressly teach (a) partitioning into a plurality of partitions and amplifying in the partitions wherein N signals are generated in a partition of the plurality of partitions. However, since Caltech teaches droplet digital PCR (para [0010]), it would have been obvious to one of ordinary skill in the art that Caltech's assay using a droplet digital PCR process involves (a) partitioning the nucleic acid targets into a plurality of partitions since this is a required step for carrying out droplet digital PCR, and it further would have been obvious that amplifying and signal generation would be in a partition of the plurality of partitions, in order to correctly perform the taught method using a droplet digital PCR process.
Caltech also does not expressly teach wherein, in (a), the first nucleic acid probe has an intensity level of about Y, the second nucleic acid probe has an intensity level of at least about 2Y, and the third nucleic acid probe has an intensity level of at least about 4Y.
Bio-rad teaches combinatorial use of signals in a multiplexed digital assay using probes with different intensity levels on a per-probe basis (para [0196] - 'This example describes exemplary multi-labeled probes and methods of using the probes in a multiplexed digital amplification assay . . . the multi-labeled probes may be utilized for intensity-based multiplexing'; para [0198] - 'Attaching multiple fluorophores to one oligonucleotide probe allows creation of a wider range of probes, which can be used to enhance multiplexing capabilities. The ability to put multiple fluors on one probe allows the resulting emission fluorescence to be "tuned" to achieve more fluorescence signatures than are possible through single fluorophores. Probe spectra will generally be a composite result of the multiple fluorophores included . . . Addition of multiple molecules of the same fluor also may allow different fluorescence output on a per-probe basis . . . That way, when the quencher is cleaved off, the same signal is obtained from the probe, but each probe could have a different signature (e.g., probe 1-FAM; probe 2-FAM+Cy3; probe 3-FAM+HEX; probe 4-FAM+FAM; etc.)'; para [0201] - 'The multi-labeled probes of FIG. 17 are as follows: B) oligonucleotide with a 5' quencher and fluorophores (F1 and F2) at respective internal and 3'-end positions . . . D) oligonucleotide with a 5' quencher moiety and fluorophores (3 x F1, F2) at 3' and multiple internal positions'). It would have been obvious to one of ordinary skill in the art that by using probes with different fluorescence output on a per-probe basis according to Bio-rad, a combination of probes could be established wherein the first nucleic acid probe has an intensity level of about Y, the second nucleic acid probe has an intensity level of at least about 2Y, and the third nucleic acid probe has an intensity level of at least about 4Y, to perform the assay of Caltech, providing enhanced multiplexing capability as taught by Bio-rad.

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International application No.
PCT/US 18/39846

Supplemental Box

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Box No. V2. Citations and Explanations

Regarding claim 15, Caltech in view of Bio-rad teaches the method of claim 14, yet does not expressly teach wherein the first nucleic acid probe comprises one fluorophore with an intensity level of about Y, the second nucleic acid probe comprises at least two fluorophores each with an intensity level of about Y, and the third nucleic acid probe comprises at least four fluorophores each with an intensity level of about Y. Since Bio-rad teaches use of multiple fluorophores to a single probe molecule (para [0198] - 'Attaching multiple fluorophores to one oligonucleotide probe allows creation of a wider range of probes, which can be used to enhance multiplexing capabilities. The ability to put multiple fluors on one probe allows the resulting emission fluorescence to be "tuned" to achieve more fluorescence signatures than are possible through single fluorophores. Probe spectra will generally be a composite result of the multiple fluorophores included . . . Addition of multiple molecules of the same fluor also may allow different fluorescence output on a per-probe basis'), it would have been obvious to one of ordinary skill in the art that by using a single kind of fluorophore, the method of Bio-rad could provide a first nucleic acid probe that comprises one fluorophore with an intensity level of about Y, the second nucleic acid probe that comprises at least two fluorophores each with an intensity level of about Y, and the third nucleic acid probe that comprises at least four fluorophores each with an intensity level of about Y.

Regarding claim 16, Caltech in view of Bio-rad teaches the method of claim 14, yet does not expressly teach wherein the first nucleic acid probe comprises a fluorophore with an intensity level of about Y, the second nucleic acid probe comprises a fluorophore with an intensity level of at least about 2Y, and the third nucleic acid probe comprises a fluorophore with an intensity level of at least about 4Y. Since Bio-rad teaches probes for intensity-based multiplexing and further teaches a variety of fluorophores known in the art to generate different fluorescent signals (para [0196] - 'This example describes exemplary multi-labeled probes and methods of using the probes in a multiplexed digital amplification assay; see FIGS. 17-22. The multi-labeled probes may be utilized for the color-based multiplexed assays described in Section I and in Examples 6 and 7. Alternatively, or in addition, the multi-labeled probes may be utilized for intensity-based multiplexing'; para [0068] - 'Exemplary fluorophores that may be suitable include FAM, VIC, HEX, ROX, TAMRA, JOE, etc.'), it would have been obvious to one of ordinary skill in the art that by appropriate selection of fluorophores, intensity-based multiplexing according to Bio-rad could be accomplished by using a combination of probes wherein the first nucleic acid probe comprises a fluorophore with an intensity level of about Y, the second nucleic acid probe comprises a fluorophore with an intensity level of at least about 2Y, and the third nucleic acid probe comprises a fluorophore with an intensity level of at least about 4Y, for the method of Caltech.

Regarding claim 17, Caltech in view of Bio-rad teaches the method of claims 14-16, and Caltech further teaches wherein N is 3 or more (para [0072] "In some cases, multiple color codes or chromophore combinations are assigned to one or more analytes. In some cases, 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes). In some cases, at most 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)").

Claims 1-3, 14-17, 30-32 and 51-53 have industrial applicability as defined by PCT Article 33(4) because the subject matter can be made or used in industry.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference 44158-712601	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/US2018/055927	International filing date *(day/month/year)* 15 October 2018 (15.10.2018)	Priority date *(day/month/year)* 16 October 2017 (16.10.2017)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant CHROMACODE, INC.		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 10 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☐	Box No. II	Priority
☒	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☐	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 21 April 2020 (21.04.2020)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Agnès Wittmann-Regis e-mail: pct.team6@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: Kyle Bruner
Wilson Sonsini Goodrich & Rosati
650 Page Mill Road
Palo Alto, California 94304
United States of America

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	08 FEB 2019

Applicant's or agent's file reference	FOR FURTHER ACTION
44158-712601	See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US18/55927	15 October 2018 (15.10.2018)	16 October 2017 (16.10.2017)

International Patent Classification (IPC) or both national classification and IPC
IPC - C12Q 1/686, 1/68 ; G01N 21/64, 33/58, 33/533 (2019.01)
CPC - C12Q 1/686, 1/6851, 1/6811, 1/6816, 1/6827, 1/6888; G01N 21/64, 21/6428

Applicant CHROMACODE, INC.

1. This opinion contains indications relating to the following items:

- [x] Box No. I Basis of the opinion
- [ ] Box No. II Priority
- [x] Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- [ ] Box No. IV Lack of unity of invention
- [x] Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- [ ] Box No. VI Certain documents cited
- [ ] Box No. VII Certain defects in the international application
- [ ] Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	20 December 2018 (20.12.2018)	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (January 2015)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
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Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a)).

3. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

a. ☐ forming part of the international application as filed:

☐ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

An invitation to Furnish Nucleotide and/or Amino Acid Sequence Listing and to Pay, Where Applicable, Late Furnishing Fee ("ISA/225") was mailed on 26 October 2018 (26.10.2018). No approved electronic sequence listing was received in response to the ISA/225. Therefore, the international search was carried out only to the extent possible.

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

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Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 6-7, 11-17, 23-27, 31-36

because:

☐ the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international search *(specify)*:

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 6-7, 11-17, 23-27, 31-36 are so unclear that no meaningful opinion could be formed *(specify)*:

Claims Nos. 6-7, 11-17, 23-27, and 31-36 are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. 6-7, 11-17, 23-27, 31-36

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

- ☐ furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
- ☐ furnish a sequence listing on paper or in the form of an image file complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
- ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13*ter*.1(a) or (b).

☐ See Supplemental Box for further details.

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Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	1, 2, 3/1-2, 4/3/1-2, 5/4/3/1-2, 9, 10/9, 18, 20, 21/19-20, 22	YES
	Claims	8, 19, 28-29, 30/28-29	NO
Inventive step (IS)	Claims	21/19-20, 22	YES
	Claims	1-2, 3/1-2, 4/3/1-2, 5/4/3/1-2, 8-9, 10/9, 18-20, 28-29, 30/28-29	NO
Industrial applicability (IA)	Claims	-***-Please See Below-***-	YES
	Claims	NONE	NO

2. Citations and explanations:

-***-Continued from Box No. V: 1. Statement-***-

Industrial applicabillty (IA): YES: Claims 1-2, 3/1-2, 4/3/1-2, 5/4/3/1-2, 8-9, 10/9, 18-20, 21/19-20, 22, 28-29, 30/28-29

-***-Box No. V: 2. Citations and Explanations-***-

Claims 8, 19, 28-29, and 30/28-29 lack novelty under PCT Article 33(2) as being anticipated by WO 2017/173035 A1 to Chromacode Inc. (hereinafter "Chromacode").

As per Claim 8, Chromacode discloses a method of detecting the presence or absence of at least one member of a plurality of target nucleic acids (methods and compositions for uniquely and unambiguously detecting multiple targets (plurality of target nucleic acids) in a single color detection channel; paragraph [007]) in a sample (providing a sample comprising the first target nucleic acid; claim 1), the method comprising: a. providing a sample (providing a sample comprising the first target nucleic acid; claim 1) comprising, or potentially comprising, at least one member of the plurality of target nucleic acids; b. forming a mixture of the sample (providing a sample comprising the first target nucleic acid; claim 1) and i. a signal oligonucleotide probe (b. first signal oligonucleotides comprising a first signal tag; paragraph [008]); ii. a first forward oligonucleotide primer (a. first competitive oligonucleotides to b. first signal oligonucleotides comprising a first signal tag; paragraph [008]) comprising a region with complementarity to a first member of the plurality of target nucleic acids (wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid wherein it is well known that PCR requires a forward primer and reverse primer to bind to the target nucleic acid; paragraph [008]); iii. a first reverse oligonucleotide primer comprising a region with complementarity to the first member of the plurality of target nucleic acids (wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid wherein it is well known that PCR requires a forward primer and reverse primer to bind to the target nucleic acid; paragraph [008]); iv. a second forward oligonucleotide primer (the method further comprises identifying the second target nucleic acid by providing a second set of paired oligonucleotides; paragraph [008]) comprising a region with complementarity to a second member of the plurality of target nucleic acids (a. second competitive oligonucleotides to b. second signal oligonucleotides comprising a second signal tag acid wherein it is well known that PCR requires a forward primer and reverse primer to bind to the target nucleic acid; paragraph [008]); and v. a second reverse oligonucleotide primer comprising a region with complementarity to the second member of the plurality of target nucleic acids (a. second competitive oligonucleotides to b. second signal oligonucleotides comprising a second signal tag acid wherein it is well known that PCR requires a forward primer and reverse primer to bind to the target nucleic acid; paragraph [008]); c. thermally cycling the mixture under conditions appropriate to amplify each member of the plurality of target nucleic acids with polymerase chain reaction, PCR, and generating a signal if at least one member of the plurality of target nucleic acids is present in the mixture (amplifying the first target nucleic acid with the polymerase chain reaction, thereby degrading the first signal oligonucleotide and permitting generation of a first signal, amplifying the second target sequence with the polymerase chain reaction, thereby degrading the second signal oligonucleotide and permitting generation of a second signal; paragraph [008]); and d. detecting the presence or absence of the signal (measuring intensity of the first signal, and correlating the intensity of the first signal to the first ratio, thereby identifying the first target nucleic acid; claim 1).

-***-Continued Within the Next Supplemental Box-***-

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Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of:

-***-Continued from Box V: Citations and Explanations-***-

As per Claim 19, Chromacode discloses a method of detecting the presence or absence of a first and second member of a plurality of target nucleic acids (methods and compositions for uniquely and unambiguously detecting multiple targets (plurality of target nucleic acids) in a single color detection channel; paragraph [007]) in a sample (providing a sample comprising the first target nucleic acid; claim 1), the method comprising: a. providing a sample (providing a sample comprising the first target nucleic acid; claim 1) comprising, or potentially comprising, the first and second members of the plurality of target nucleic acids; b. forming a mixture comprising the sample (providing a sample comprising the first target nucleic acid; claim 1) and i. a template nucleic acid sample (providing a sample comprising the first target nucleic acid; claim 1); ii. a signal oligonucleotide probe with complementarity to the template nucleic acid (the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid (signal oligonucleotide probe with complementarity to the template nucleic acid); claim 1); iii a first oligonucleotide primer comprising a region with complementarity to the first member of the plurality of target nucleic acids (providing a first set of paired oligonucleotides with complementarity to the first target nucleic acid; claim 1); and iv a second oligonucleotide primer comprising a region with complementarity to the second member of the plurality of target nucleic acids (providing a second set of paired oligonucleotides with complementarity to the second target nucleic acid; claim 3); c. subjecting the mixture to conditions sufficient to anneal the first oligonucleotide primer to the first member of the plurality of target nucleic acids (the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid, amplifying the first target nucleic acid; paragraph [008]) and the second oligonucleotide primer to the second member of the plurality of target nucleic acids (the second competitive oligonucleotides compete with the second signal oligonucleotides for binding to the second target nucleic acid , amplifying the second target sequence with the polymerase chain reaction; paragraph [008]), such that the first (degrading the first signal oligonucleotide ;paragraph [008]) and second oligonucleotide primers are degraded (degrading the second signal oligonucleotide; paragraph [008]); d. thermally cycling the mixture under reaction conditions appropriate to amplify the template nucleic acid with polymerase chain reaction (PCR), such that a signal is generated if both the first and second members of the plurality of target nucleic acids are present in the mixture (amplifying the first target nucleic acid with the polymerase chain reaction, thereby degrading the first signal oligonucleotide and permitting generation of a first signal, amplifying the second target sequence with the polymerase chain reaction, thereby degrading the second signal oligonucleotide and permitting generation of a second signal; paragraph [008]); and e. detecting the presence or absence of the signal (measuring intensity of the first signal, and correlating the intensity of the first signal to the first ratio, thereby identifying the first target nucleic acid; claim 1).

As per Claim 28, Chromacode discloses a method of detecting the presence or absence of a first and second member of a plurality of target nucleic acids (methods and compositions for uniquely and unambiguously detecting multiple targets (plurality of target nucleic acids) in a single color detection channel; paragraph [007]) in a sample (providing a sample comprising the first target nucleic acid; claim 1), the method comprising: a. providing a sample (providing a sample comprising the first target nucleic acid; claim 1) comprising, or potentially comprising, the first and second members of the plurality of target nucleic acids b. forming a mixture comprising the sample (providing a sample comprising the first target nucleic acid; claim 1) and i. a template nucleic acid (providing a sample comprising the first target nucleic acid; claim 1); ii. a signal oligonucleotide probe with complementarity to the template nucleic acid (the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid (signal oligonucleotide probe with complementarity to the template nucleic acid); claim 1); iii. a first forward oligonucleotide primer comprising a region with complementarity to the first member of the plurality of target nucleic acids (providing a first set of paired oligonucleotides with complementarity to the first target nucleic acid; claim 1); iv. a first reverse oligonucleotide primer comprising a region with complementarity the first member of the plurallty of target nucleic acids (wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid wherein it is well known that PCR requires a forward primer and reverse primer to bind to the target nucleic acid; paragraph [008]); v. a second forward oligonucleotide primer comprising a first region with complementarity to the second member of the plurality of target nucleic acids (providing a second set of paired oligonucleotides with complementarity to the second target nucleic acid; claim 3); and vi. a second reverse oligonucleotide primer comprising a region with complementarity to the second member of the plurality of target nucleic acids (a. second competitive oligonucleotides to b. second signal oligonucleotides comprising a second signal tag acid wherein it is well known that PCR requires a forward primer and reverse primer to bind to the target nucleic acid; paragraph [008]); c. thermally cycling the mixture under reaction conditions appropriate to amplify the first and second members of the plurality of target nucleic acids with polymerase chain reaction, PCR (amplifying the first target nucleic acid with the polymerase chain reaction, amplifying the second target sequence with the polymerase chain reaction; paragraph [008]), such that the first forward oligonucleotide primer and the second forward oligonucleotide primer are degraded mixture (amplifying the first target nucleic acid with the polymerase chain reaction, thereby degrading the first signal oligonucleotide and permitting generation of a first signal, amplifying the second target sequence with the polymerase chain reaction, thereby degrading the second signal oligonucleotide and permitting generation of a second signal; paragraph [008]); d. thermally cycling the mixture under reaction conditions appropriate to amplify the template nucleic acid with PCR, such that a signal is generated if both the first and second members of the plurality of target nucleic acids are present In the mixture (the first target nucleic acid and the second target nucleic acid are identified from differing signal intensities generated during the same polymerase chain reaction; paragraph [008]); and e. detecting the presence or absence of the signal (measuring intensity of the first signal, and correlating the intensity of the first signal to the first ratio, thereby identifying the first target nucleic acid; claim 1).

As per Claim 29, Chromacode discloses the method of claim 28, and Chromacode further discloses comprising t. correlating the presence of the signal to the presence of both the first (correlating the intensity of the first signal to the first ratio, thereby identifying the first target nucleic acid; paragraph 007-) and second members of the plurality of target nucleic acids in the sample (correlating the intensity of the second signal to the second ratio, thereby identifying the second target nucleic acid; paragraph [007]).

As per Claims 30/28-29, Chromacode discloses the method of claim 28 and 29, and Chromacode further discloses wherein the signal oligonucleotide probe comprises a signal tag (generating the signal comprises exciting the signal tag; paragraph [008]).

-***-Continued Within the Next Supplemental Box-***-

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

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Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
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Claims 1, 3/1, 4/3/1, 5/4/3/1, and 18 lack an inventive step under PCT Article 33(3) as being obvious over WO 2017/ 173035 A1 to Chromacode Inc. (hereinafter "Chromacode") in view of the publication entitled "Comparison of DNA Quantification Methods for Next Generation Sequencing" by Robin, JD et al. (hereinafter "Robin")

As per Claim 1, Chromacode discloses a method of detecting the presence or absence of at least one member of a plurality of target nucleic acids (methods and compositions for uniquely and unambiguously detecting multiple targets (plurality of target nucleic acids) in a single color detection channel; paragraph [007]) in a sample (providing a sample comprising the first target nucleic acid; claim 1), the method comprising: a. providing a sample (providing a sample comprising the first target nucleic acid; claim 1) comprising, or potentially comprising, at least one member of the plurality of target nucleic acids (detecting multiple targets (plurality of target nucleic acids) in a single color detection channel; paragraph [007]); b. forming a mixture (this is achieved by creating a mixture of non-fluorescent oligo probes and the TaqMan reporter probes for every target interrogated in a qPCR reaction; paragraph [022]) comprising the sample (providing a sample comprising the first target nucleic acid; claim 1) and i. a signal oligonucleotide probe (b. first signal oligonucleotides comprising a first signal tag; paragraph [008]); ii. a first forward oligonucleotide primer (a. first competitive oligonucleotides to b. first signal oligonucleotides comprising a first signal tag; paragraph [008]) comprising: a first region complementary to a first region of a first member of the plurality of target nucleic acids (wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid wherein it is well known that PCR requires a forward primer and reverse primer to bind to the target nucleic acid; paragraph [008]); iii. a first reverse oligonucleotide primer comprising a region complementary to a second region of the first member of the plurality of target nucleic acids (wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid wherein it is well known that PCR requires a forward primer and reverse primer to bind to the target nucleic acid; paragraph [008]); iv. a second forward oligonucleotide primer comprising (the method further comprises identifying the second target nucleic acid by providing a second set of paired oligonucleotides; paragraph [008]): a first region complementary to a first region of a second member of the plurality of target nucleic acids (a. second competitive oligonucleotides to b. second signal oligonucleotides comprising a second signal tag acid wherein it is well known that PCR requires a forward primer and reverse primer to bind to the target nucleic acid; paragraph [008]); v. a second reverse oligonucleotide primer comprising a region complementary to a second region of the second member of the plurality of target nucleic acids (a. second competitive oligonucleotides to b. second signal oligonucleotides comprising a second signal tag acid wherein it is well known that PCR requires a forward primer and reverse primer to bind to the target nucleic acid; paragraph [007]); c. thermally cycling the mixture under conditions appropriate to amplify each member of the plurality of target nucleic acids with polymerase chain reaction, PCR, such that the signal oligonucleotide probe is degraded and a signal is generated if at least one member of the plurality of target nucleic acids is present in the mixture (amplifying the first target nucleic acid with the polymerase chain reaction, thereby degrading the first signal oligonucleotide and permitting generation of a first signal, amplifying the second target sequence with the polymerase chain reaction, thereby degrading the second signal oligonucleotide and permitting generation of a second signal; paragraph [008]); and d. detecting the presence or absence of the signal (measuring intensity of the first signal, and correlating the intensity of the first signal to the first ratio, thereby identifying the first target nucleic acid; claim 1). Chromacode does not disclose a second region complementary or homologous to the signal oligonucleotide probe. However, Robin discloses disclose a second region complementary or homologous to the signal oligonucleotide probe (at the 5′ end of the forward primer, a short sequence complementary to a UPL(R), Roche, (signal oligonucleotide probe) was added; figure 1). It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the PCR reaction as disclosed by Chromacode, to also include a region in the forward primer complementary to a signal probe, as disclosed by Robin, as the previous disclosure by Chromacode discloses the use of a PCR reaction to detect multiple targets in a sample reaction, and the Robin reference discloses the use of a primer with a region complementary to the target and a region complementary to a signal probe and this would provide the capability to use the forward primers together with a reverse primer to accurately quantify the presence or absence of a target analyte in a single reaction.

As per Claim 3/1, Chromacode and Robin, in combination, disclose the method of claim 1, and Chromacode further discloses wherein the signal oligonucleotide probe comprises a signal tag (a. first competitive oligonucleotides to b. first signal oligonucleotides comprising a first signal tag; paragraph [008]).

As per Claim 4/3/1, Chromacode and Robin, in combination, disclose the method of claim 3/1, and Chromacode further discloses wherein, in c, the signal is generated by the signal tag (generating the signal comprises exciting the signal tag; paragraph [008]).

As per Claim 5/4/3/1, Chromacode and Robin, in combination, disclose the method of claim 4/3/1, and Chromacode further discloses wherein the signal tag generates the signal upon degradation of the signal oligonucleotide probe (degrading the first signal oligonucleotide and permitting generation of a first signal; claim 1).

-***-Continued Within the Next Supplemental Box-***-

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In case **the space in any of the preceding boxes is not sufficient.**
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As per Claim 18, Chromacode discloses a system, rather than a kit (provided herein are compositions comprising a set of paired oligonucleotides with complementarity to a target nucleic acid; paragraph [013]) for detecting the presence or absence of at least one member of a plurality of target nucleic acids in a sample (providing a sample comprising the first target nucleic acid; claim 1), comprising: a. a signal oligonucleotide probe (b. first signal oligonucleotides comprising a first signal tag; paragraph [008]); b. a first forward oligonucleotide primer (a. first competitive oligonucleotides to b. first signal oligonucleotides comprising a first signal tag; paragraph [008]) comprising: a first region with complementarity to a first member of the plurality of target nucleic acids (wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid wherein it is well known that PCR requires a forward primer and reverse primer to bind to the target nucleic acid; paragraph [008]); c. a first reverse oligonucleotide primer comprising a region with complementarity to the first member of the plurality of target nucleic acids (wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid wherein it is well known that PCR requires a forward primer and reverse primer to bind to the target nucleic acid; paragraph [008]); d. a second forward oligonucleotide primer (the method further comprises identifying the second target nucleic acid by providing a second set of paired oligonucleotides; paragraph [008]) comprising: a first region with complementarity to a second member of the plurality of target nucleic acids (a. second competitive oligonucleotides to b. second signal oligonucleotides comprising a second signal tag acid wherein it is well known that PCR requires a forward primer and reverse primer to bind to the target nucleic acid; paragraph [008]); and e. a second reverse oligonucleotide primer comprising a region with complementarity to the second member of the plurality of target nucleic acids (a. second competitive oligonucleotides to b. second signal oligonucleotides comprising a second signal tag acid wherein it is well known that PCR requires a forward primer and reverse primer to bind to the target nucleic acid; paragraph [008]). Chromacode does not disclose a kit, and a second region complementary or homologous to the signal oligonucleotide probe. However, Robin discloses a kit for DNA quantification (page 3, 2nd paragraph), and a second region complementary or homologous to the signal oligonucleotide probe (at the 5′ end of the forward primer, a short sequence complementary to a UPL(R), Roche, (signal oligonucleotide probe) was added; figure 1). It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the system as disclosed by Chromacode, to also include a region in the forward primer complementary to a signal probe, as disclosed by Robin, as the previous disclosure by Chromacode discloses the use of a PCR reaction to detect multiple targets in a sample reaction, and the Robin reference discloses the use of a primer with a region complementary to the target and a region complementary to a signal probe and this would provide the capability to use the forward primers together with a reverse primer to accurately quantify the presence or absence of a target analyte in a single reaction. Further, it would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have packaged the components of the system disclosed by Chromacode into a kit, as commercial kits for quantification of nucleic acids are well known in the art, as shown by the use of such kits disclosed by Robin, and this would provide an improvement to ease of use for the user of the system.

Claims 9 and 10/9 lack an inventive step under PCT Article 33(3) as being obvious over Chromacode in view of the publication entitled "MNAzyme qPCR with Superior Multiplexing Capacity" by Mokany, E et al. (hereinafter "Mokany").

As per Claim 9, Chromacode discloses the method of claim 8, but Chromacode does not disclose wherein the mixture further comprises a nucleic acid enzyme. However, Mokany discloses wherein the mixture further comprises a nucleic acid enzyme (MNAzymes, nucleic acid enzymes formed from multiple partial enzymes, can be linked to PCR to provide a highly specific method for target detection and quantification; abstract). It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the PCR reaction as disclosed by Chromacode, to also include a region in the forward primer complementary to a signal probe, as disclosed by Mokany, as the previous disclosure by Chromacode discloses the use of a PCR reaction to detect multiple targets in a sample reaction, and the Mokany reference discloses the use of nucleic acid enzyme in PCR reactions and this would provide the capability to use the nucleic acid enzymes in a qPCR assay to enable multiple target identification.

As per Claim 10/9, Chromacode and Mokany, in combination, disclose the method of claim 9, and Chromacode further discloses wherein the first forward oligonucleotide primer further comprises an additional region with complementarity or homology to the signal oligonucleotide probe.

Claims 10/8 and 20 lack an inventive step under PCT Article 33(3) as being obvious over Chromacode and in view of the WO 2008/118998 A2 to Primera Biosystems, Inc. (hereinafter "Primera"),

As per Claim 10/8, Chromacode discloses the method of claim 8, but Chromacode does not disclose wherein the first forward oligonucleotide primer further comprises an additional region with complementarity or homology to the signal oligonucleotide probe. However, Primera discloses wherein the first oligonucleotide primer further comprises an additional region with complementarity to a second region of the template nucleic acid (a 3' end and a 5' end, wherein a segment of the 3' end is complementary to at least a first segment of a target sequence for probe annealing, and a segment of the 5' end is complementary to at least a second segment of the target sequence for probe annealing; claim 1). It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the PCR reaction as disclosed by Chromacode, to also include a region in the forward primer complementary to another region of the template nucleic acid, as disclosed by Primera, as the previous disclosure by Chromacode discloses the use of a PCR reaction to detect multiple targets in a sample reaction, and the Primera reference discloses the use of primers with two complementary regions to the template nucleic acid and this would provide the capability to use the primers for accurate quantification of the template nucleic acid in the PCR reaction.

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Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
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As per Claim 20, Chromacode discloses the method of claim 19, but Chromacode does not disclose wherein the first oligonucleotide primer further comprises an additional region with complementarity to a second region of the template nucleic acid. However, Primera discloses wherein the first oligonucleotide primer further comprises an additional region with complementarity to a second region of the template nucleic acid (a 3' end and a 5' end, wherein a segment of the 3' end is complementary to at least a first segment of a target sequence for probe annealing, and a segment of the 5' end is complementary to at least a second segment of the target sequence for probe annealing; claim 1). It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the PCR reaction as disclosed by Chromacode, to also include a region in the forward primer complementary to another region of the template nucleic acid, as disclosed by Primera, as the previous disclosure by Chromacode discloses the use of a PCR reaction to detect multiple targets in a sample reaction, and the Primera reference discloses the use of primers with two complementary regions to the template nucleic acid and this would provide the capability to use the primers for accurate quantification of the template nucleic acid in the PCR reaction.

Claims 2, 3/2, 4/3/2, and 5/4/3/2 lack an inventive step under PCT Article 33(3) as being obvious over Chromacode and Robin and in further view of Mokany.

As per Claim 2, Chromacode and Robin, in combination, discloses the method of claim 1, but Chromacode does not disclose wherein the mixture further comprises a nucleic acid enzyme. However, Mokany discloses wherein the mixture further comprises a nucleic acid enzyme (MNAzymes, nucleic acid enzymes formed from multiple partial enzymes, can be linked to PCR to provide a highly specific method for target detection and quantification; abstract). It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the PCR reaction as disclosed by Chromacode, to also include a region in the forward primer complementary to a signal probe, as disclosed by Mokany, as the previous disclosure by Chromacode discloses the use of a PCR reaction to detect multiple targets in a sample reaction, and the Mokany reference discloses the use of nucleic acid enzyme in PCR reactions and this would provide the capability to use the nucleic acid enzymes in a qPCR assay to enable multiple target identification.

As per Claim 3/2, Chromacode, Robin and Mokany, in combination, disclose the method of claim 2, and Chromacode further discloses wherein the signal oligonucleotide probe comprises a signal tag (a. first competitive oligonucleotides to b. first signal oligonucleotides comprising a first signal tag; paragraph [008]).

As per Claim 4/3/2, Chromacode, Robin and Mokany, in combination, disclose the method of claim 3/2, and Chromacode further discloses wherein, in c, the signal is generated by the signal tag (generating the signal comprises exciting the signal tag; paragraph [008]).

As per Claim 5/4/3/2, Chromacode, Robin and Mokany, in combination, disclose the method of claim 4/3/2, and Chromacode further discloses wherein the signal tag generates the signal upon degradation of the signal oligonucleotide probe (degrading the first signal oligonucleotide and permitting generation of a first signal; claim 1).

Claims 21/19-20 and 22/21/19-20 meet the criteria set out in PCT Article 33(2)-(3).

Claims 21/19-20 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest the method of claim 19 or 20, wherein the second oligonucleotide primer further comprises an additional region with complementarity to a third region of the template nucleic acid.

Primera discloses wherein the an oligonucleotide primer further comprises an additional region with complementarity to a region of the template nucleic acid (a 3' end and a 5' end, wherein a segment of the 3' end is complementary to at least a first segment of a target sequence for probe annealing, and a segment of the 5' end is complementary to at least a second segment of the target sequence for probe annealing; claim 1). Primera does not disclose wherein the second oligonucleotide primer further comprises an additional region with complementarity to a third region of the template nucleic acid.

The publication entitled "Two-tailed RT-qPCR: a novel method for highly accurate miRNA quantification" by Androvic, P et al. (hereinafter "Androvic") discloses , wherein the oligonucleotide primer further comprises an additional region with complementarity to a region of the template nucleic acid (it takes advantage of novel, target-specific primers for reverse transcription composed of two hemiprobes complementary to two different parts of the targeted miRNA, connected by a hairpin structure; abstract). Androvic does not disclose wherein the second oligonucleotide primer further comprises an additional region with complementarity to a third region of the template nucleic acid.

The publication entitled "An improved protocol with a highly degenerate primer targeting copper-containing membrane-bound monooxygenase genes for community analysis of methane- and ammonia-oxidizing bacteria" by Wang, JG (hereinafter "Wang") discloses a primer further comprises an additional region with complementarity to a region of the template nucleic acid (based on sequence alignment of pmoA and amoA genes, a new highly degenerate reverse primer, HD616 (Table 1), was designed at a highly conserved region to cover most of MOB and AOB; page 3, 1st column, 2nd paragraph). Wang does not disclose wherein the second oligonucleotide primer further comprises an additional region with complementarity to a third region of the template nucleic acid.

-***-Continued Within the Next Supplemental Box-***-

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US18/55927

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of:

-***-Continued from Previous Supplemental Box-***-

Thus, the prior art does not teach or fairly suggest the method of claim 19 or 20, wherein the second oligonucleotide primer further comprises an additional region with complementarity to a third region of the template nucleic acid, and sufficient motivation, based upon the previous disclosures of the Primera, Androvic and Wang references, does not exist to have determined and implemented the compositions as described in claim 21/19-20 of the instant PCT application.

Claims 22/21/19-20 meet the criteria set-out in PCT Article 33(2)-(3), due to their dependency upon positive Claims 21/19-20.

Claim 1-2, 3/1-2, 4/3/1-2, 5/4/3/1-2, 8-9, 10/8-9, 18-20, 21/19-20, 22/21/19-20, 28-29, 30/28-29 have industrial applicability as defined by PCT Article 33(4) because the subject matter can be made or used in industry.

ChromaCode | 44158-709.601
VKI | SVW | LJG | KBRU | LMJI | ABEI | ANNA

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: KYLE BRUNER
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

Date of mailing *(day/month/year)*	14 SEP 2018
Applicant's or agent's file reference: 44158-709601	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No.: PCT/US 18/39846	International filing date *(day/month/year)*: 27 June 2018 (27.06.2018)
Applicant: CHROMACODE, INC.	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

How? Directly to the International Bureau of WIPO preferably through ePCT or on paper to, 34 chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 338 82 70

For more detailed instructions, see *PCT Applicant's Guide*, International Phase, paragraphs 9.004 – 9.011.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ **With regard to any protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
   - ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices.
   - ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

The applicant may **submit comments on an informal basis on the written opinion of the International Searching Authority** to the International Bureau. These comments will be made available to the public after international publication. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established.

Shortly after the expiration of **18 months from the priority date, the international application will be published** by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90*bis*.1 and 90*bis*.3).

Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for **entry into the national phase** before those designated Offices. In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months. For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

Within **22 months from the priority date, the applicant may request that a supplementary international search be carried out** by a different International Searching Authority that offers this service (Rule 45*bis*.1). The procedure for requesting supplementary international search is described in the *PCT Applicant's Guide*, International Phase, paragraphs 8.006-8.032.

Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee W. Young Telephone No. PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/220 (July 2014)

WSGR PATENT DOCKET
U.S. FOREIGN: ✓
DOCKETED: 9/19/18 BY: ea
ACTION: Search report 4/14/18
DUE DATES: Written Opinion 4/28/19
1PS/2SR for 301 12/10/18
ATTY C/M #

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 44158-709601	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US 18/39846	International filing date *(day/month/year)* 27 June 2018 (27.06.2018)	(Earliest) Priority Date *(day/month/year)* 28 June 2017 (28.06.2017)
Applicant CHROMACODE, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).

c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☒ **Certain claims were found unsearchable** (see Box No. II).

3. ☐ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,

a. the figure of the **drawings** to be published with the abstract is Figure No. 1

☒ as suggested by the applicant.

☐ as selected by this Authority, because the applicant failed to suggest a figure.

☐ as selected by this Authority, because this figure better characterizes the invention.

b. ☐ none of the figures is to be published with the abstract.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/39846

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-13, 18-29, 33-50, 54-69
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/39846

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/70, C12Q 1/70 (2018.01)
CPC - C12Q 1/701, C12Q 1/6818, C12Q 1/68, C12Q 1/6851, C12Q 1/686, C12Q 1/703, Y10T 436/143333, C12Q 2563/103, C12Q 2563/159

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2014/0213471 A1 (CALIFORNIA INSTITUTE OF TECHNOLOGY) 31 July 2014 (31.07.2014) Claim 3; para [0003]; para [0007]; para [0010-0011]; para [0030]; para [0035]; para [0048-0049]; para [0105] ; para [0154]; para [0176]	1-3, 30-32, 51-53 --- 14-17
Y	US 2016/0108464 A1 (BIO-RAD LABORATORIES INC.) 21 April 2016 (21.04.2016) para [0068]; para [0196]; para [0198]; para [0201]	14-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 August 2018	14 SEP 2018
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: KYLE BRUNER
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	14 SEP 2018

Applicant's or agent's file reference	**FOR FURTHER ACTION**
44158-709601	See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US 18/39846	27 June 2018 (27.06.2018)	28 June 2017 (28.06.2017)

International Patent Classification (IPC) or both national classification and IPC
IPC(8) - C12Q 1/70, C12Q 1/70 (2018.01)
CPC - C12Q 1/701, C12Q 1/6818, C12Q 1/68, C12Q 1/6851, C12Q 1/686, C12Q 1/703, Y10T 436/143333, C12Q 2563/103, C12Q 2563/159

Applicant CHROMACODE, INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	21 August 2018	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (January 2015)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 18/39846

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a)).

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

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California Institute of Technology - Ex. 2003 Page 4198

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 18/39846

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 4-13, 18-29, 33-50, 54-69

because:

☐ the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international search *(specify)*:

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 4-13, 18-29, 33-50, 54-69 are so unclear that no meaningful opinion could be formed *(specify)*:

Claims 4-13, 18-29, 33-50, 54-69 are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. 4-13, 18-29, 33-50, 54-69

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.

☐ furnish a sequence listing on paper or in the form of an image file complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.

☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13*ter*.1(a) or (b).

☐ See Supplemental Box for further details.

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 18/39846

Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	1-3, 14-17, 30-32, 51-53	YES
	Claims	None	NO
Inventive step (IS)	Claims	None	YES
	Claims	1-3, 14-17, 30-32, 51-53	NO
Industrial applicability (IA)	Claims	1-3, 14-17, 30-32, 51-53	YES
	Claims	None	NO

2. Citations and explanations:

Claims 1-3, 30-32 and 51-53 lack an inventive step under PCT Article 33(3) as being obvious over US 2014/0213471 A1 to California Institute of Technology (hereinafter 'Caltech').

Regarding claim 1, Caltech teaches a method for performing a digital assay, comprising:
(a) (i) three or more nucleic acid targets and (ii) a set of nucleic acid probes comprising a first nucleic acid probe, a second nucleic acid probe, and a third nucleic acid probe (para [0154] - 'In some cases, compositions comprise a first and a second primer or probe for the detection of at least one analyte wherein the primers are attached to either a fluorophore or a quencher at the 5' end . . . . In some cases the compositions comprise multiple pairs of first and second primers, wherein each pair of first and second primers comprise either a fluorophore or a quencher at the 5' end. In some cases each pair of first and second primers comprise a different fluorophore and quencher from the remaining set of primers. In some cases the compositions comprise at least 1, 2, 3, 4 . . . or 10000 pairs of first and second primers.'; para [0048] - 'Various combinations of oligonucleotides (e.g. primers and/or probes) can be used to detect an analyte. In some cases, a primer is used with a probe or a plurality of probes. In some cases, a plurality of primers is used with a plurality of probes. . . . In some cases, a primer and a probe is used to detect multiple analytes . . . a combination of primers and probes is used to detect multiple analytes. In some cases, a probe is used to detect an analyte. In some cases, a probe is used to detect multiple analytes. In some cases, multiple probes are used to detect an analyte.');
(b) amplifying the three or more nucleic acid targets (para [0003] 'using oligonucleotides (e.g., primers, probes) attached to chromophores (e.g., fluorophores, quenchers, etc.) in amplification or polymerization reactions in order to detect a polynucleotide analyte'; [0035] - 'the polymerase chain reaction process');
(c) generating N signals from the set of nucleic acid probes, each of which N signals corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets (para [0105] - 'The incorporation of the quencher and the fluorophore in the newly generated amplicon can lead to signal generation (e.g., quenching of fluorescence intensity from the fluorophore). With each iterative amplification or polymerization reaction, the fluorescence intensity is reduced by a factor of about 2. The amount of fluorescence detected can be used to directly determine the amount of analyte present'; para [0003] - '(b) measuring a first signal generated by the first and second chromophores; (c) performing at least one polymerization reaction with the first primer using the first polynucleotide analyte as a template; and (d) measuring a second signal generated by the first and second chromophores . . . '; para [0007] - 'further comprises combining the sample with a third primer . . . '; para [0010]-[0011] - 'the PCR process is an end-point PCR process, a digital PCR process, a real-time PCR process, a droplet digital PCR process, or a quantitative PCR process . . . methods of detecting a plurality of analytes, . . . b) measuring a first set of signals generated by the chromophores; c) performing at least one polymerization reaction with the plurality of primer pairs using the plurality of analytes as templates; and d) measuring a second set of signals generated by the chromophores; wherein the first and second set of signals are used to detect each analyte of the plurality of analytes . . . generating a set of signature profiles; wherein the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profiles'); and
(d) detecting the N signals in a single optical channel (Claim 3 - 'method of detecting multiple nucleic acid targets of differing lengths . . . .b) conducting a polymerase chain reaction (PCR); and c) measuring an endpoint fluorescence of each target, wherein the endpoint fluorescence for each target is different.'; para [0010] - 'In some cases, the PCR process is an end-point PCR process . . . or a quantitative PCR process. In some cases, the polymerization reaction is a quantitative PCR process . . . the second method is a digital PCR process'; para [0049] - 'In some cases, a single chromophore pair is used to detect an analyte'; para [0176] - 'Analytes of differing lengths (e.g., 40 bp, 60 bp, and 80 bp) are detected using a single fluorophore/quencher combination . . . By measuring fluorescence as a function of increasing temperature, multiple analytes in a single color channel are quantified.');
wherein in (a), the concentration of the second nucleic acid probe is at least about 2X, and the concentration of the third nucleic acid probe is at least about 4X (para [0030] - 'FIG. 9 exemplifies the binary coding of analytes. Analytes in a 3-plex CY3 assay were coded using binary spaced primer concentrations.'; [0173] 'Analytes in a 3-plex CY3 assay were coded using binary spaced primer concentrations'; see Figure 9).
Caltech does not expressly teach the method comprising (a) partitioning into a plurality of partitions the three or more nucleic acid targets, wherein the amplifying and generating signals steps are performed in the partitions. However, since Caltech teaches droplet digital PCR (para [0010]), it would have been obvious to one of ordinary skill in the art that Caltech's assay using a droplet digital PCR process involves (a) partitioning the nucleic acid targets into a plurality of partitions since this is a required step for carrying out droplet digital PCR, and it further would have been obvious that amplifying and signal generation would be in a partition of the plurality of partitions, in order to correctly perform the taught method using a droplet digital PCR process.

----Please see continuation in first supplemental box----------

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California Institute of Technology - Ex. 2003 Page 4200

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 18/39846

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of
Box No. V2. Citations and Explanations

Regarding claim 2, Caltech makes obvious the method of claim 1, and further teaches wherein N is 3 or more (para [0072] "In some cases, multiple color codes or chromophore combinations are assigned to one or more analytes. In some cases, 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes). In some cases, at most 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)").

Regarding claim 3, Caltech makes obvious the method of claims 1-2, and further teaches wherein N is 4 or more (para [0072] "In some cases, multiple color codes or chromophore combinations are assigned to one or more analytes. In some cases, 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes). In some cases, at most 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)").

Regarding claim 30, Caltech teaches a method for performing a digital assay, comprising:
(a) three or more nucleic acid targets (para [0154] - 'In some cases, compositions comprise a first and a second primer or probe for the detection of at least one analyte wherein the primers are attached to either a fluorophore or a quencher at the 5' end . . . . In some cases the compositions comprise multiple pairs of first and second primers, wherein each pair of first and second primers comprise either a fluorophore or a quencher at the 5' end. In some cases each pair of first and second primers comprise a different fluorophore and quencher from the remaining set of primers. In some cases the compositions comprise at least 1, 2, 3, 4, 5, . . . or 10000 pairs of first and second primers.'; para [0048] - 'Various combinations of oligonucleotides (e.g. primers and/or probes) can be used to detect an analyte. In some cases, a primer is used with a probe or a plurality of probes. In some cases, a plurality of primers is used with a plurality of probes. . . . In some cases, a primer and a probe is used to detect multiple analytes. In some cases, a combination of primers and probes is used to detect an analyte. In some cases, a combination of primers and probes is used to detect multiple analytes. In some cases, a probe is used to detect an analyte. In some cases, a probe is used to detect multiple analytes. In some cases, multiple probes are used to detect an analyte.');
(b) amplifying the three or more nucleic acid targets (para [0005] - 'In some cases, the polymerase chain reaction process is an end-point polymerase chain reaction process, a real-time polymerase chain reaction process, a digital polymerase chain reaction process, a droplet digital polymerase chain reaction process');
(c) generating N signals each of which corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets (para [0003] - '(b) measuring a first signal generated by the first and second chromophores . . . wherein the first and second signals are used to detect the first polynucleotide analyte.'; para [0007] - 'In some cases, step a) of the method described herein further comprises combining the sample with a third primer and a second oligonucleotide . . . step b) further comprises measuring a fifth signal generated by the third and fourth chromophores; step d) further comprises measuring a sixth signal generated by the third and fourth chromophores'; para [0011] - 'methods of detecting a plurality of analytes, . . . b) measuring a first set of signals generated by the chromophores . . . generating a set of signature profiles; wherein the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profiles'); and
(d) detecting the signals in a single optical channel (Claim 3 - 'method of detecting multiple nucleic acid targets of differing lengths . . . .b) conducting a polymerase chain reaction (PCR); and c) measuring an endpoint fluorescence of each target, wherein the endpoint fluorescence for each target is different.'; para [0010] - 'In some cases, the PCR process is an end-point PCR process, a digital PCR process, a real-time PCR process, a droplet digital PCR process, or a quantitative PCR process. In some cases, the polymerization reaction is a quantitative PCR process. In some cases, the detecting comprises a quantitative PCR method. In some cases, the detecting comprises a quantitative PCR method and a second method. In some cases, the second method is a digital PCR process'; para [0049] - 'In some cases, a single chromophore pair is used to detect multiple analytes.'; para [0072] 'at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)'; para [0176] - 'Analytes of differing lengths (e.g., 40 bp, 60 bp, and 80 bp) are detected using a single fluorophore/quencher combination . . . .By measuring fluorescence as a function of increasing temperature, multiple analytes in a single color channel are quantified.').
Caltech does not expressly teach (a) partitioning into a plurality of partitions and amplifying in the partitions wherein N signals are generated in a partition of the plurality of partitions. However, since Caltech teaches droplet digital PCR (para [0010]), it would have been obvious to one of ordinary skill in the art that Caltech's assay using a droplet digital PCR process involves (a) partitioning the nucleic acid targets into a plurality of partitions since this is a required step for carrying out droplet digital PCR, and it further would have been obvious that amplifying and signal generation would be in a partition of the plurality of partitions, in order to correctly perform the taught method using a droplet digital PCR process.

Regarding claim 31, Caltech makes obvious the method of claim 30, and further teaches wherein N is 3 or more (para [0072] "In some cases, multiple color codes or chromophore combinations are assigned to one or more analytes. In some cases, 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes). In some cases, at most 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)").

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 18/39846

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:
Box No. V2. Citations and Explanations

Regarding claim 32, Caltech makes obvious the method of claims 30-31, and further teaches wherein N is 4 or more (para [0072] "In some cases, multiple color codes or chromophore combinations are assigned to one or more analytes. In some cases, 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes). In some cases, at most 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)").

Regarding claim 51, Caltech teaches a method of performing a digital assay, comprising:
(a) amplifying at least three nucleic acid targets from a sample comprising a set of nucleic acid probes comprising three or more nucleic acid probes labeled with fluorophores capable of being detected in a single optical channel, wherein each nucleic acid probe is complementary to a target of the at least three nucleic acid targets (para [0154] - 'compositions comprise a first and a second primer or probe for the detection of at least one analyte wherein the primers are attached to either a fluorophore or a quencher at the 5' end . . . In some cases each pair of first and second primers comprise a different fluorophore and quencher from the remaining set of primers. In some cases the compositions comprise at least 1, 2, 3, 4, 5, 6 . . . or 10000 pairs of first and second primers.'; para [0048] - 'Various combinations of oligonucleotides (e.g. primers and/or probes) can be used to detect an analyte. In some cases, a primer is used with a probe or a plurality of probes. In some cases, a plurality of primers is used with a plurality of probes. . . . In some cases, a primer and a probe is used to detect multiple analytes. In some cases, a combination of primers and probes is used to detect an analyte. In some cases, a combination of primers and probes is used to detect multiple analytes. In some cases, a probe is used to detect an analyte. In some cases, a probe is used to detect multiple analytes. In some cases, multiple probes are used to detect an analyte.'; para [0005] - 'the polymerase chain reaction process is . . . a droplet digital polymerase chain reaction process');
(b) detecting N signals if one or more of the at least three nucleic acid targets is present, wherein each of the N signals corresponds to a unique combination of one or more of the at least three nucleic acid targets (para [0003] - '(b) measuring a first signal generated by the first and second chromophores; (c) performing at least one polymerization reaction with the first primer using the first polynucleotide analyte as a template; and (d) measuring a second signal generated by the first and second chromophores; wherein the first and second signals are used to detect the first polynucleotide analyte.'; para [0007] - 'further comprises measuring a fifth signal generated by the third and fourth chromophores; step d) further comprises measuring a sixth signal generated by the third and fourth chromophores'; para [0011] - 'Also disclosed herein are methods of detecting a plurality of analytes, . . . b) measuring a first set of signals generated by the chromophores; c) performing at least one polymerization reaction with the plurality of primer pairs using the plurality of analytes as templates . . . generating a set of signature profiles; wherein the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profiles'; Claim 3 - 'method of detecting multiple nucleic acid targets of differing lengths . . . b) conducting a polymerase chain reaction (PCR); and c) measuring an endpoint fluorescence of each target, wherein the endpoint fluorescence for each target is different.'; para [0010] - 'In some cases, the PCR process is an end-point PCR process, a digital PCR process, a real-time PCR process, a droplet digital PCR process'; para [0049] - 'In some cases, a single chromophore pair is used to detect an analyte. In some cases, a single chromophore pair is used to detect multiple analytes.'; para [0072] 'at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)'; [0176] - 'Analytes of differing lengths (e.g., 40 bp, 60 bp, and 80 bp) are detected using a single fluorophore/quencher combination . . . By measuring fluorescence as a function of increasing temperature, multiple analytes in a single color channel are quantified.'); and
(c) determining, from the N signals, the presence or absence of each of the at least three nucleic acid targets in the sample (para [0045] - 'Described herein are methods for detecting the presence or absence of at least one polynucleotide analyte in a sample . . . .In some cases, the first and second signals are used to detect the presence or absence of the amplified product (e.g. the polynucleotide analyte)').
Caltech does not expressly teach (a) partitioning into a plurality of droplets and amplifying in the droplets wherein N signals are generated in a droplet of the plurality of droplets. However, since Caltech teaches droplet digital PCR (para [0010]), it would have been obvious to one of ordinary skill in the art that Caltech's assay using a droplet digital PCR process involves (a) partitioning the nucleic acid targets into a plurality of droplet partitions since this is a required step for carrying out droplet digital PCR, and it further would have been obvious that amplifying and signal generation would be in a partition of the plurality of droplet partitions, in order to correctly perform the taught method using a droplet digital PCR process.

Regarding claim 52, Caltech makes obvious the method of claim 51, and further teaches wherein N is 3 or more (para [0072] "In some cases, multiple color codes or chromophore combinations are assigned to one or more analytes. In some cases, 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes). In some cases, at most 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)").

Regarding claim 53, Caltech makes obvious the method of claims 51-52, and further teaches wherein N is 4 or more (para [0072] "In some cases, multiple color codes or chromophore combinations are assigned to one or more analytes. In some cases, 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes). In some cases, at most 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)").

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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:
Box No. V2. Citations and Explanations

Claims 14-17 lack an inventive step under PCT Article 33(3) as being obvious over Caltech in view of US 2016/0108464 A1 to Bio-Rad Laboratories, Inc. (hereinafter 'Bio-rad').

Regarding claim 14, Caltech teaches a method for performing a digital assay, comprising:
(a) (i) three or more nucleic acid targets and (ii) a set of nucleic acid probes comprising a first nucleic acid probe, a second nucleic acid probe, and a third nucleic acid probe (para [0154] - 'compositions comprise a first and a second primer or probe for the detection of at least one analyte wherein the primers are attached to either a fluorophore or a quencher at the 5' end . . . In some cases the compositions comprise multiple pairs of first and second primers, wherein each pair of first and second primers comprise either a fluorophore or a quencher at the 5' end. In some cases each pair of first and second primers comprise a different fluorophore and quencher from the remaining set of primers. In some cases the compositions comprise at least 1, 2, 3, 4, 5 . . . or 10000 pairs of first and second primers.'; para [0048] - 'Various combinations of oligonucleotides (e.g. primers and/or probes) can be used to detect an analyte. In some cases, a primer is used with a probe or a plurality of probes. In some cases, a plurality of primers is used with a plurality of probes. . . . In some cases, a primer and a probe is used to detect multiple analytes. In some cases, a combination of primers and probes is used to detect an analyte. In some cases, a combination of primers and probes is used to detect multiple analytes. In some cases, a probe is used to detect an analyte. In some cases, a probe is used to detect multiple analytes. In some cases, multiple probes are used to detect an analyte.');
(b) amplifying the three or more nucleic acid targets (para [0005] - 'In some cases, the polymerase chain reaction process is an end-point polymerase chain reaction process, a real-time polymerase chain reaction process. a digital polymerase chain reaction process, a droplet digital polymerase chain reaction process');
(c) generating N signals each of which corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets (para [0003] - '(b) measuring a first signal generated by the first and second chromophores; (c) performing at least one polymerization reaction with the first primer using the first polynucleotide analyte as a template; and (d) measuring a second signal generated by the first and second chromophores; wherein the first and second signals are used to detect the first polynucleotide analyte.'; para [0007] - 'In some cases, step a) of the method described herein further comprises combining the sample with a third primer and a second oligonucleotide, wherein a third chromophore is attached to the third primer and a fourth chromophore is attached to the second oligonucleotide, . . . step b) further comprises measuring a fifth signal generated by the third and fourth chromophores; step d) further comprises measuring a sixth signal generated by the third and fourth chromophores'; para [0011] - 'Also disclosed herein are methods of detecting a plurality of analytes, . . . b) measuring a first set of signals generated by the chromophores; c) performing at least one polymerization reaction with the plurality of primer pairs using the plurality of analytes as templates; and d) measuring a second set of signals generated by the chromophores; wherein the first and second set of signals are used to detect each analyte of the plurality of analytes. In some cases, the method described herein further comprises: e) repeating step c and d at least once; and f) generating a set of signature profiles; wherein the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profiles'); and
(d) detecting the signals in a single optical channel (Claim 3 - 'method of detecting multiple nucleic acid targets of differing lengths . . . b) conducting a polymerase chain reaction (PCR); and c) measuring an endpoint fluorescence of each target, wherein the endpoint fluorescence for each target is different.'; para [0010] - 'In some cases, the PCR process is an end-point PCR process, a digital PCR process, a real-time PCR process, a droplet digital PCR process, or a quantitative PCR process. In some cases, the polymerization reaction is a quantitative PCR process. In some cases, the detecting comprises a quantitative PCR method. In some cases, the detecting comprises a quantitative PCR method and a second method. In some cases, the second method is a digital PCR process'; para [0049] - 'In some cases, a single chromophore pair is used to detect an analyte. In some cases, a single chromophore pair is used to detect multiple analytes.'; para [0176] - 'Analytes of differing lengths (e.g., 40 bp, 60 bp, and 80 bp) are detected using a single fluorophore/quencher combination . . . By measuring fluorescence as a function of increasing temperature, multiple analytes in a single color channel are quantified.'). Caltech does not expressly teach (a) partitioning into a plurality of partitions and amplifying in the partitions wherein N signals are generated in a partition of the plurality of partitions. However, since Caltech teaches droplet digital PCR (para [0010]), it would have been obvious to one of ordinary skill in the art that Caltech's assay using a droplet digital PCR process involves (a) partitioning the nucleic acid targets into a plurality of partitions since this is a required step for carrying out droplet digital PCR, and it further would have been obvious that amplifying and signal generation would be in a partition of the plurality of partitions, in order to correctly perform the taught method using a droplet digital PCR process.
Caltech also does not expressly teach wherein, in (a), the first nucleic acid probe has an intensity level of about Y, the second nucleic acid probe has an intensity level of at least about 2Y, and the third nucleic acid probe has an intensity level of at least about 4Y.
Bio-rad teaches combinatorial use of signals in a multiplexed digital assay using probes with different intensity levels on a per-probe basis (para [0196] - 'This example describes exemplary multi-labeled probes and methods of using the probes in a multiplexed digital amplification assay . . . the multi-labeled probes may be utilized for intensity-based multiplexing'; para [0198] - 'Attaching multiple fluorophores to one oligonucleotide probe allows creation of a wider range of probes, which can be used to enhance multiplexing capabilities. The ability to put multiple fluors on one probe allows the resulting emission fluorescence to be "tuned" to achieve more fluorescence signatures than are possible through single fluorophores. Probe spectra will generally be a composite result of the multiple fluorophores included . . . Addition of multiple molecules of the same fluor also may allow different fluorescence output on a per-probe basis . . . That way, when the quencher is cleaved off, the same signal is obtained from the probe, but each probe could have a different signature (e.g., probe 1-FAM; probe 2-FAM+Cy3; probe 3-FAM+HEX; probe 4-FAM+FAM; etc.)'; para [0201] - 'The multi-labeled probes of FIG. 17 are as follows: B) oligonucleotide with a 5' quencher and fluorophores (F1 and F2) at respective internal and 3'-end positions . . . D) oligonucleotide with a 5' quencher moiety and fluorophores (3 x F1, F2) at 3' and multiple internal positions'). It would have been obvious to one of ordinary skill in the art that by using probes with different fluorescence output on a per-probe basis according to Bio-rad, a combination of probes could be established wherein the first nucleic acid probe has an intensity level of about Y, the second nucleic acid probe has an intensity level of at least about 2Y, and the third nucleic acid probe has an intensity level of at least about 4Y, to perform the assay of Caltech, providing enhanced multiplexing capability as taught by Bio-rad.

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INTERNATIONAL SEARCHING AUTHORITY

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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:
Box No. V2. Citations and Explanations

Regarding claim 15, Caltech in view of Bio-rad teaches the method of claim 14, yet does not expressly teach wherein the first nucleic acid probe comprises one fluorophore with an intensity level of about Y, the second nucleic acid probe comprises at least two fluorophores each with an intensity level of about Y, and the third nucleic acid probe comprises at least four fluorophores each with an intensity level of about Y. Since Bio-rad teaches use of multiple fluorophores to a single probe molecule (para [0198] - 'Attaching multiple fluorophores to one oligonucleotide probe allows creation of a wider range of probes, which can be used to enhance multiplexing capabilities. The ability to put multiple fluors on one probe allows the resulting emission fluorescence to be "tuned" to achieve more fluorescence signatures than are possible through single fluorophores. Probe spectra will generally be a composite result of the multiple fluorophores included . . . Addition of multiple molecules of the same fluor also may allow different fluorescence output on a per-probe basis'), it would have been obvious to one of ordinary skill in the art that by using a single kind of fluorophore, the method of Bio-rad could provide a first nucleic acid probe that comprises one fluorophore with an intensity level of about Y, the second nucleic acid probe that comprises at least two fluorophores each with an intensity level of about Y, and the third nucleic acid probe that comprises at least four fluorophores each with an intensity level of about Y.

Regarding claim 16, Caltech in view of Bio-rad teaches the method of claim 14, yet does not expressly teach wherein the first nucleic acid probe comprises a fluorophore with an intensity level of about Y, the second nucleic acid probe comprises a fluorophore with an intensity level of at least about 2Y, and the third nucleic acid probe comprises a fluorophore with an intensity level of at least about 4Y. Since Bio-rad teaches probes for intensity-based multiplexing and further teaches a variety of fluorophores known in the art to generate different fluorescent signals (para [0196] - 'This example describes exemplary multi-labeled probes and methods of using the probes in a multiplexed digital amplification assay; see FIGS. 17-22. The multi-labeled probes may be utilized for the color-based multiplexed assays described in Section I and in Examples 6 and 7. Alternatively, or in addition, the multi-labeled probes may be utilized for intensity-based multiplexing'; para [0068] - 'Exemplary fluorophores that may be suitable include FAM, VIC, HEX, ROX, TAMRA, JOE, etc.'), it would have been obvious to one of ordinary skill in the art that by appropriate selection of fluorophores, intensity-based multiplexing according to Bio-rad could be accomplished by using a combination of probes wherein the first nucleic acid probe comprises a fluorophore with an intensity level of about Y, the second nucleic acid probe comprises a fluorophore with an intensity level of at least about 2Y, and the third nucleic acid probe comprises a fluorophore with an intensity level of at least about 4Y, for the method of Caltech.

Regarding claim 17, Caltech in view of Bio-rad teaches the method of claims 14-16, and Caltech further teaches wherein N is 3 or more (para [0072] "In some cases, multiple color codes or chromophore combinations are assigned to one or more analytes. In some cases, 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes). In some cases, at most 1, 2, 3, 4, 5 . . . color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes)").

Claims 1-3, 14-17, 30-32 and 51-53 have industrial applicability as defined by PCT Article 33(4) because the subject matter can be made or used in industry.

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SEARCH HISTORY

Application Number	PCT/US 18/39846
Search Conducted By	AEM
Search Approved By	DEM

CPC/IPC Classifications Searched	IPC(8) - C12Q 1/70, C12Q 1/70 (2018.01) CPC - C12Q 1/701, C12Q 1/6818, C12Q 1/68, C12Q 1/6851, C12Q 1/686, C12Q 1/703, Y10T 436/143333, C12Q 2563/103, C12Q 2563/159
Date Conducted	21 August 2018 (21.08.2018)

Documentation Searched	Keyword limited; search terms below
Search Terms Used	Droplet PCR, digital assay, multiplexed, fluorometric measurement, multiple probes, multiple targets, optical channel, intensity gradation, signal gradation, chromophore
Date Conducted	21 August 2018 (21.08.2018)

Electronic Database Searched	Patbase
Files Searched	Full-text: AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO Bibliographic: (Europe) AT BA BE BG CH CS CY CZ DD DK EE ES FI GE GR HR HU IE IS IT LT LU LV MC MD MT NL NO PL PT RO RS SE SI SK SM TR UA YU (Asia) EA GC HK ID IL IN KZ MN MY PH RU SG SU TH TJ TW UZ VN (North America) CA CR CU DO GT HN MX NI PA SV TT (South America) AR BR CL CO EC PE UY (Australasia) AU NZ (Africa) AP DZ EG KE MA MW OA ZA ZM ZW
Date Conducted	21 August 2018 (21.08.2018)

Search Logic:

Classification based search

Search 1: IC=(C12Q1/70 OR C12Q1/70) (Results 14647)
Search 2: CPC=(C12Q1/701 OR C12Q1/6818 OR C12Q1/68 OR C12Q1/6851 OR

C12Q1/686 OR C12Q1/703 OR Y10T436/143333 OR C12Q2563/103 OR C12Q2563/159) (Results 17967)
Search 3: 1 OR 2 (Results 28884)
Search 4: 3 AND FT=(DROPLET W1 PCR) (Results 90)
Search 5: 4 AND FT=(MULTIPLEX* NEAR PCR) (Results 55)
Search 6: 5 AND FT=(FLUOR* NEAR SIGNAL%) (Results 42)
Search 7: 6 AND FT=(MULTIPLE NEAR PROBE%) (Results 19)
Search 8: 3 AND FT=(DIGITAL W1 ASSAY) (Results 28)
Search 9: 8 NOT 7 (Results 22)
Search 10: 3 AND FT=(PARTITION* NEAR (TEMPLATE% OR TARGET%)) (Results 249)
Search 11: 10 AND FT=(MULTIPLEX* NEAR PCR) (Results 48)
Search 12: 11 AND FT=(MULTIPLE NEAR PROBE%) (Results 13)
Search 13: 10 AND FT=(MULTIPLE NEAR (TARGET% OR PROBE%)) (Results 71)
Search 14: 13 AND FT=(MULTIPLE NEAR (FLUOROPHORE% OR CHROMOPHORE%)) (Results 8)

Keyword only search

Search 15: FT=(DROPLET W1 PCR) (Results 352)
Search 16: 15 AND FT=(MULTIPLEX* NEAR PCR) (Results 139)
Search 17: 16 AND FT=(FLUOR* NEAR SIGNAL%) (Results 77)
Search 18: 17 AND FT=(MULTIPLE NEAR PROBE%) (Results 31)
Search 19: 18 NOT (9 OR 7 OR 12 OR 14) (Results 12)
Search 20: FT=(DIGITAL W1 ASSAY) (Results 87)
Search 21: 20 AND FT=(MULTIPLEX* NEAR (PCR OR AMPLIF*)) (Results 31)
Search 22: 21 NOT (9 OR 7 OR 12 OR 14) (Results 9)
Search 23: FT=(PARTITION* NEAR (TEMPLATE% OR TARGET%)) (Results 4671)
Search 24: 23 AND FT=(MULTIPLEX* NEAR (PCR OR AMPLIF*)) (Results 137)
Search 25: 24 AND FT=(MULTIPLE NEAR (TARGET% OR PROBE%)) (Results 84)
Search 26: 25 AND FT=(MULTIPLE NEAR (FLUOROPHORE% OR CHROMOPHORE%)) (Results 9)
Search 27: 23 AND FT=(FLUOR* NEAR SIGNAL%) (Results 278)
Search 28: 27 AND FT=(PROBE NEAR CONCENTRATION) (Results 97)
Search 29: 28 AND FT=(INTENSIT* NEAR (DIFFERENT OR MULTIPLE)) (Results 10)

Forward and backward search

Search 30: PN=(US2014213471 OR US2017002400 OR US2016108464) (Results 3)
Search 31: CTF 30 OR CTB 30 (Results 1405)
Search 32: 31 AND FT=(DROPLET W1 PCR) (Results 37)
Search 33: 32 AND FT=(MULTIPLEX* NEAR PCR) (Results 21)
Search 34: 33 NOT (9 OR 7 OR 12 OR 14 OR 26 OR 29) (Results 10)

Electronic Database Searched	Google
Files Searched	Patents
Date Conducted	21 August 2018 (21.08.2018)

Search Logic:

droplet PCR with different intensity probes
About 68,900 results (0.29 seconds)

droplet pcr with different intensity probes multiple chromophores
About 11,000 results (0.30 seconds)

droplet pcr probes multiple copies of chromophores
About 12,500 results (0.32 seconds)

droplet pcr probes multiple copies of fluorophores
About 27,900 results (0.74 seconds)

droplet pcr probes signal intensity gradation fluorophores
About 11,900 results (0.34 seconds)

digital PCR multiplexed fluorometric signals
About 8,150 results (0.32 seconds)

digital PCR fluorophore intensity gradient
About 18,000 results (0.30 seconds)

digital PCR multiple probe concentrations
About 62,400 results (0.29 seconds)

digital PCR probe intensity gradient
About 19,700 results (0.27 seconds)

multiplexed digital PCR three targets
About 29,400 results (0.30 seconds)

multiplexed digital PCR three probes
About 53,900 results (0.38 seconds)

multiplexed digital PCR single optical channel
About 34,600 results (0.37 seconds)

multiplexed digital PCR target detection
About 59,300 results (0.35 seconds)

droplet PCR multiple targets in a single droplet
About 71,700 results (0.40 seconds)

droplet PCR multiple probes in a single droplet
About 41,000 results (0.35 seconds)

droplet PCR multiple targets and probes in a single droplet
About 37,900 results (0.31 seconds)

Electronic Database Searched	Google
Files Searched	Scholar
Date Conducted	21 August 2018 (21.08.2018)

Search Logic:

droplet PCR with different intensity probes
About 32,800 results (0.13 sec)

droplet pcr with different intensity probes multiple chromophores
About 10,600 results (0.10 sec)

droplet pcr probes multiple copies of chromophores
About 7,600 results (0.09 sec)

droplet pcr probes multiple copies of fluorophores
About 17,500 results (0.10 sec)

droplet pcr probes signal intensity gradation fluorophores
About 11,900 results (0.08 sec)

digital PCR multiplexed fluorometric signals
About 8,460 results (0.07 sec)

digital PCR fluorophore intensity gradient
About 20,800 results (0.10 sec)

digital PCR multiple probe concentrations
About 104,000 results (0.12 sec)

digital PCR probe intensity gradient
About 32,600 results (0.12 sec)

multiplexed digital PCR three targets
About 24,300 results (0.09 sec)

multiplexed digital PCR three probes
About 30,800 results (0.15 sec)

multiplexed digital PCR single optical channel
About 23,300 results (0.41 sec)

multiplexed digital PCR target detection
About 38,200 results (0.13 sec)

droplet PCR multiple targets in a single droplet

About 42,300 results (0.15 sec)

droplet PCR multiple probes in a single droplet
About 39,700 results (0.13 sec)

droplet PCR multiple targets and probes in a single droplet
About 30,100 results (0.15 sec)

SAO SUPPLEMENTAL SEARCH HISTORY

Electronic Database Searched	Patbase
Files Searched	Full-text: AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO Bibliographic: (Europe) AT BA BE BG CH CS CY CZ DD DK EE ES FI GE GR HR HU IE IS IT LT LU LV MC MD MT NL NO PL PT RO RS SE SI SK SM TR UA YU (Asia) EA GC HK ID IL IN KZ MN MY PH RU SG SU TH TJ TW UZ VN (North America) CA CR CU DO GT HN MX NI PA SV TT (South America) AR BR CL CO EC PE UY (Australasia) AU NZ (Africa) AP DZ EG KE MA MW OA ZA ZM ZW
Date Conducted	21 August 2018 (21.08.2018)

Search Logic:

Search 14: pn=(US20140213471 or US20160108464) (Results 2)
Search 15: TAC=((analyte* or nucleot* or nucleic) W4 (detec* or measur* or assay*)) (Results 30243)
Search 16: TAC=((partition* or droplet*) W5 (analyte* or nucleot* or nucleic)) (Results 663)
Search 17: 15 and 16 (Results 172)
Search 18: TAC=((probe* or primer* or label*) W9 intensit*) (Results 5318)
Search 19: 17 and 18 (Results 5)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference 44158-713601	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/US2019/019906	International filing date *(day/month/year)* 27 February 2019 (27.02.2019)	Priority date *(day/month/year)* 28 February 2018 (28.02.2018)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant CHROMACODE, INC.		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 14 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☐	Box No. II	Priority
☒	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☒	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 01 September 2020 (01.09.2020)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Xiaofan Tang e-mail: pct.team2@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: Kyle Bruner
Wilson Sonsini Goodrich & Rosati
650 Page Mill Road
Palo Alto, California 94304

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	06 MAY 2019

Applicant's or agent's file reference	FOR FURTHER ACTION
44158-713601	See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US19/19906	27 February 2019 (27.02.2019)	28 February 2018 (28.02.2018)

International Patent Classification (IPC) or both national classification and IPC
IPC - C12Q 1/6846, 1/6851, 1/6872, 1/6876; G01N 33/48 (2019.01)
CPC - C12Q 1/6846, 1/6851, 1/6872, 1/6876; G01N 33/48

Applicant CHROMACODE, INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☒ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	17 April 2019 (17.04.2019)	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (January 2015)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a)).

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

a. ☐ forming part of the international application as filed:

☐ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 18-27, 30-37, 45-56, 73-93, 97-108, 112-113, 116-117

because:

☐ the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international search *(specify)*:

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. -***-Please See Below-***- are so unclear that no meaningful opinion could be formed *(specify)*:

Claims 18-27, 30-37, 45-56, 73-93, 97-108, 112-113, and 116-117 are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. -***-Please See Above-***-

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

- ☐ furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
- ☐ furnish a sequence listing on paper or in the form of an image file complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
- ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13*ter*.1(a) or (b).

☐ See Supplemental Box for further details.

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	-***-Please See Below-***-	YES
	Claims	NONE	NO
Inventive step (IS)	Claims	NONE	YES
	Claims	-***-Please See Below-***-	NO
Industrial applicability (IA)	Claims	-***-Please See Below-***-	YES
	Claims	NONE	NO

2. Citations and explanations:

-Continued from Box No. V: 1. Statement:

Novelty (N): YES: Claims 1-14, 15/1-14, 16/15/1-14, 17/16/15/1-14, 28-29, 38-43, 44/39-43, 57-70, 71/67-70, 72/71/67-70, 94-95, 96/60-66, 109-111, 114-115, 118-125

Inventive step (IS): NO: Claims 1-14, 15/1-14, 16/15/1-14, 17/16/15/1-14, 28-29, 38-43, 44/39-43, 57-70, 71/67-70, 72/71/67-70, 94-95, 96/60-66, 109-111, 114-115, 118-125

Industrial applicability (IA): YES: Claims 1-14, 15/1-14, 16/15/1-14, 17/16/15/1-14, 28-29, 38-43, 44/39-43, 57-70, 71/67-70, 72/71/67-70, 94-95, 96/60-66, 109-111, 114-115, 118-125

-Continued from Box No. V. 2: Citations and Explanations:

Claims 1-14, 15/1-14, 16/15/1-14, 17/16/15/1-14, 28-29, 38-43, 44/39-43, 57-70, 71/67-70, 94-95, 96/60-66, 109-111, 114-115 and 118-125 lack an inventive step under PCT Article 33(3) as being obvious over US 2013/0288244 A1 to SEQUENOM, INC., (hereinafter 'Sequenom') in view of US 2013/0078626 A1 to Wasserstrom et al. (hereinafter 'Wasserstrom').

As per claim 1, Sequenom discloses a method for analyzing a size distribution of nucleic acids (method for analyzing a size distribution of nucleic acids based on amplification reactions; figure 148; paragraph [0099]), the method comprising: (A) providing a sample (DNA samples isolated from the blood; paragraph [0100]) comprising: (i) a first plurality of nucleic acids and a second plurality of nucleic acids (mixture of maternal and fetal nucleic acids; paragraph [0098]), wherein said first plurality of nucleic acids each comprise a first nucleic acid sequence of a given length and said second plurality of nucleic acids each comprise a second nucleic acid sequence longer than said given length (mixture of maternal and fetal nucleic acids, where the nucleic acids are of a given length and size-specific amplification reactions are used to enrich the fetal nucleic acids by digesting the maternal nucleic acids (longer than fetal nucleic acids); paragraphs [0098]-[0099], [0277], [0279] [0290]); (ii) a first set of paired amplification oligomers configured to amplify said first nucleic acid sequence (maternal nucleic acids can be amplified using a first set of primers; paragraphs [0294], [0302], [1166]); (iii) a second set of paired amplification oligomers, configured to amplify said second nucleic acid sequence (fetal nucleic acids can be amplified using a second set of primers; paragraphs [0099], [0281], [0294]); (iv) a first detection probe configured to anneal to a region of said first nucleic acid sequence (Taqman probe can be annealed to the nucleic acid sequence; paragraph [0230], [0232]); and (v) a second detection probe configured to anneal to a region of said second nucleic acid sequence (Taqman probe can be annealed to the nucleic acid sequence; paragraph [0230], [0232]); (B) performing an amplification reaction on (a) said first nucleic acid sequence to generate a first signal from said first detection probe (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0230], [0232]) and (b) said second nucleic acid sequence to generate a second signal from said second detection probe (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0230], [0232]); thereby analyzing said size distribution (analyzing the size distribution based on size specific amplification reactions; paragraph [0099]). Sequenom does not disclose (C) determining a ratio of a first value derived from said first signal to a second value derived from said second signal. Wasserstrom discloses (C) determining a ratio of a first value derived from said first signal to a second value derived from said second signal (calculating at least one signal ratio between signal intensities of amplification products and comparing the signal ratios to reference values; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a ratio of amplification signals and comparing to a reference level, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on size distributions (Sequenom; paragraph [0099]).

-***-Continued Within the Next Supplemental Box-***-

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Claim 60 objected to under PCT Rule 66.2(a)(iii) as containing the following defect(s) in the form or contents thereof: in claim 60, the word 'said' was repeated twice.

Form PCT/ISA/237 (Box No. VII) (January 2015)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claim 124 is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because claim [124] is indefinite for the following reason(s): [in claim 124, the trademarks "SYBR Green" and "EvaGreen" are used which makes the claim indefinite where trademarks characterize the commercial origin of goods, rather than the properties of the goods (which may change from time to time) relevant to the invention. For the purposes of this opinion, "SYBR Green" and "EvaGreen" are interpreted to mean an intercalating dye].

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of:

-***-Continued from Box V: Citations and Explanations-***-

As per claim 2, Sequenom and Wasserstrom, in combination, disclose the method of claim 1, and Sequenom further discloses wherein said first set of paired amplification oligomers (maternal nucleic acids can be amplified using a first set of primers; paragraphs [0294], [0302], [1166]) comprises: a first forward amplification oligomer (forward primers; paragraphs [0232], [0294], [0302], [1166]); and a first reverse amplification oligomer (reverse primers; paragraphs [0232], [0294], [0302], [1166]).

As per claim 3, Sequenom and Wasserstrom, in combination, disclose the method of claim 2, and Sequenom further discloses wherein said first set of paired amplification oligomers (maternal nucleic acids can be amplified using a first set of primers; paragraphs [0294], [0302], [1166]) comprises: a plurality of first forward amplification oligomers (forward primers; paragraphs [0232], [0294], [0302], [1166]); and a plurality of first reverse amplification oligomers (reverse primers; paragraphs [0232], [0294], [0302], [1166]).

As per claim 4, Sequenom and Wasserstrom, in combination, disclose the method of claim 3, and Sequenom further discloses wherein each of said plurality of first forward amplification oligomers has a different nucleic acid sequence (forward primers can be different for targeting methylated and unmethylated DNA in the maternal nucleic acids; paragraphs [0232], [0236], [0294], [0302], [1166]).

As per claim 5, Sequenom and Wasserstrom, in combination, disclose the method of claim 4, and Sequenom further discloses wherein a first forward amplification oligomer of said plurality of first forward amplification oligomers is configured to hybridize to a region of said first sequence (forward primers specifically hybridize the CpG island of methylated DNA in the maternal nucleic acids; paragraphs [0232], [0236], [0294], [0302], [1166]).

As per claim 6, Sequenom and Wasserstrom, in combination, disclose the method of claim 3, and Sequenom further discloses wherein each of said plurality of first reverse amplification oligomers has a different nucleic acid sequence (reverse primers can be different for targeting methylated and unmethylated DNA in the maternal nucleic acids; paragraphs [0232], [0236], [0294], [0302], [1166]).

As per claim 7, Sequenom and Wasserstrom, in combination, disclose the method of claim 6, and Sequenom further discloses wherein a first reverse amplification oligomer of said plurality of first reverse amplification oligomers is configured to hybridize to a region of said first sequence (reverse primers specifically hybridize the CpG island of methylated DNA in the maternal nucleic acids; paragraphs [0232], [0236], [0294], [0302], [1166]).

As per claim 8, Sequenom and Wasserstrom, in combination, disclose the method of claim 1, and Sequenom further discloses wherein said second set of paired amplification oligomers (fetal nucleic acids can be amplified using a second set of primers; paragraphs [0099], [0281], [0294]) comprises: a second forward amplification oligomer (forward primers; paragraphs [0099], [0232], [0281], [0294]); and a second reverse amplification oligomer (reverse primers; paragraphs [0099], [0232], [0281], [0294]).

As per claim 9, Sequenom and Wasserstrom, in combination, disclose the method of claim 8, and Sequenom further discloses wherein said second set of paired amplification oligomers (fetal nucleic acids can be amplified using a second set of primers; paragraphs [0099], [0281], [0294]) comprises: a plurality of second forward amplification oligomers (forward primers; paragraphs [0099], [0232], [0236], [0281], [0294]) and a plurality of second reverse amplification oligomers (reverse primers; paragraphs [0099], [0232], [0236], [0281], [0294]).

As per claim 10, Sequenom and Wasserstrom, in combination, disclose the method of claim 9, and Sequenom further discloses wherein each of said plurality of second forward amplification oligomers has a different nucleic acid sequence (forward primers can be different for targeting methylated and unmethylated DNA in the fetal nucleic acids; paragraphs [0099], [0232], [0236], [0281], [0294]).

As per claim 11, Sequenom and Wasserstrom, in combination, disclose the method of claim 10, and Sequenom further discloses wherein a second forward amplification oligomer of said plurality of second forward amplification oligomers is configured to hybridize to a region of said second sequence (forward primers specifically hybridize the CpG island of methylated DNA in the fetal nucleic acids; paragraphs [0099], [0232], [0236], [0281], [0294]).

As per claim 12, Sequenom and Wasserstrom, in combination, disclose the method of claim 9, and Sequenom further discloses wherein each of said plurality of second reverse amplification oligomers has a different nucleic acid sequence (reverse primers can be different for targeting methylated and unmethylated DNA in the fetal nucleic acids; paragraphs [0099], [0232], [0236], [0281], [0294]).

As per claim 13, Sequenom and Wasserstrom, in combination, disclose the method of claim 12, and Sequenom further discloses wherein a second reverse amplification oligomer of said plurality of second reverse amplification oligomers is configured to hybridize to a region of said second sequence (reverse primers specifically hybridize the CpG island of methylated DNA in the fetal nucleic acids; paragraphs [0099], [0232], [0236], [0281], [0294]).

As per claim 14, Sequenom and Wasserstrom, in combination, disclose the method of claim 1. Sequenom does not disclose wherein said first value and said second value provide a quantitative ratio measurement corresponding to an abundance of said first plurality of nucleic acids and said second plurality of nucleic acids in said sample. Wasserstrom discloses wherein said first value and said second value provide a quantitative ratio measurement corresponding to an abundance of said first plurality of nucleic acids and said second plurality of nucleic acids in said sample (calculating at least one signal ratio between signal intensities of amplification products and comparing the signal ratios to reference values; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a ratio of amplification signals and comparing to a reference level, as disclosed by Wasserstrom, for providing the advantage of testing the nucleic acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on size distributions (Sequenom; paragraph [0099]).

-***-Continued Within the Next Supplemental Box-***-

IPR2024-01451
California Institute of Technology - Ex. 2003 Page 4217

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of:

-***-Continued from Previous Supplemental Box-***-

As per claims 15/1-14, Sequenom and Wasserstrom, in combination, disclose the method of claims 1-14, and Sequenom further discloses wherein said first detection probe or said second detection probe comprises a non-target-hybridizing sequence (detection probes can be molecular beacons; paragraph [0319]).

As per claims 16/15/1-14, Sequenom and Wasserstrom, in combination, disclose the method of claims 15/1-14, and Sequenom further discloses wherein said first detection probe or said second detection probe is a hairpin detection probe (detection probes can be molecular beacons; paragraph [0319]).

As per claims 17/16/15/1-14, Sequenom and Wasserstrom, in combination, disclose the method of claims 16/15/1-14, and Sequenom further discloses wherein said hairpin detection probe is a molecular beacon (detection probes can be molecular beacons; paragraph [0319]).

As per claim 28, Sequenom and Wasserstrom, in combination, disclose the method of claim 1, and Sequenom further discloses said second nucleic acid sequence comprises at least a portion of said first nucleic acid sequence (where fetal nucleic acids are obtained from maternal nucleic acids by digestion; paragraph [0277], [0279]).

As per claim 29, Sequenom and Wasserstrom, in combination, disclose the method of claim 28, and Sequenom further discloses wherein said second nucleic acid sequence comprises said first nucleic acid sequence (where fetal nucleic acids are obtained from maternal nucleic acids by digestion; paragraph [0277], [0279]).

As per claim 38, Sequenom and Wasserstrom, in combination, disclose the method of claim 1. Sequenom does not disclose comprising comparing said ratio to a reference value. Wasserstrom discloses comprising comparing said ratio to a reference value (calculating at least one signal ratio between signal intensities of amplification products and comparing the signal ratios to reference values; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a ratio of amplification signals and comparing to a reference level, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on size distributions (Sequenom; paragraph [0099]).

As per claim 39, Sequenom and Wasserstrom, in combination, disclose the method of claim 38, and Sequenom further discloses comparing identifies the presence or absence of a genetic abnormality in said sample (identifying the presence or absence of a genetic abnormality like trisomy 21 in said sample based on comparing the size distribution; paragraph [0099]).

As per claim 40, Sequenom and Wasserstrom, in combination, disclose the method of claim 39, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence. Wasserstrom discloses wherein said reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence (values of reference ratios of a particular DNA category (can be third and fourth); paragraph [0050]; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a reference ratio.

As per claim 41, Sequenom and Wasserstrom, in combination, disclose the method of claim 40, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said third nucleic acid sequence and said fourth nucleic acid sequence each correspond to a region of nucleic acid not associated with said genetic abnormality. Wasserstrom discloses wherein said third nucleic acid sequence and said fourth nucleic acid sequence each correspond to a region of nucleic acid not associated with said genetic abnormality (values of reference ratios of a particular DNA category (can be third and fourth), wherein the DNA sample can be from control DNA; paragraph [0050]; claims 1, 31). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a reference ratio.

As per claim 42, Sequenom and Wasserstrom, in combination, disclose the method of claim 41, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said reference value is derived from a plurality of third values generated from a plurality of third nucleic acid sequences and a plurality of fourth values generated from a plurality of fourth nucleic acid sequences. Wasserstrom discloses wherein said reference value is derived from a plurality of third values generated from a plurality of third nucleic acid sequences and a plurality of fourth values generated from a plurality of fourth nucleic acid sequences (values of plurality of reference ratios of a particular DNA category (can be third and fourth); paragraph [0050]; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a reference ratio.

-***-Continued Within the Next Supplemental Box-***-

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:

-***-Continued from Previous Supplemental Box-***-

As per claim 43, Sequenom and Wasserstrom, in combination, disclose the method of claim 42, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said plurality of third nucleic acid sequences and said plurality of fourth nucleic acid sequences each correspond to a region of nucleic acid not associated with said genetic abnormality. Wasserstrom discloses wherein said plurality of third nucleic acid sequences and said plurality of fourth nucleic acid sequences each correspond to a region of nucleic acid not associated with said genetic abnormality (values of plurality of reference ratios of a particular DNA category (can be third and fourth), wherein the DNA sample can be from control DNA; paragraph [0050]; claims 1, 31). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a reference ratio.

As per claims 44/39-43, Sequenom and Wasserstrom, in combination, disclose the method of claims 39-43, and Sequenom further discloses wherein said genetic abnormality is a fetal aneuploidy (fetal sex chromosome aneuploidy; paragraph [0214]).

As per claim 57, Sequenom and Wasserstrom, in combination, disclose the method of claim 1, and Sequenom further discloses wherein said first value is a quantity of said first plurality of nucleic acids (nucleic acid quantification from a sample, where the nucleic acids can be maternal nucleic acids (first plurality of nucleic acids); paragraphs [0242], [0246]).

As per claim 58, Sequenom and Wasserstrom, in combination, disclose the method of claim 1, and Sequenom further discloses wherein said second value is a quantity of said second plurality of nucleic acids (nucleic acid quantification from a sample, where the nucleic acids can be fetal nucleic acids (second plurality of nucleic acids); paragraphs [0242], [0246]).

As per claim 59, Sequenom and Wasserstrom, in combination, disclose the method of claim 1. Sequenom does not disclose wherein said ratio is determined without quantifying said first plurality of nucleic acids and said second plurality of nucleic acids. Wasserstrom discloses wherein said ratio is determined without quantifying said first plurality of nucleic acids and said second plurality of nucleic acids (calculating at least one signal ratio between signal intensities (not quantities) of amplification products; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a signal ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a signal ratio.

As per claim 60, Sequenom discloses a method for identifying the presence or absence of a fetal aneuploidy (method for identifying the presence or absence of a fetal aneuploidy; paragraph [0248]), comprising: (A) providing a sample (DNA samples isolated from the blood; paragraph [0100]) comprising: (i) a plurality of fetal nucleic acids, each comprising a first nucleic acid sequence of a given length (plurality of fetal nucleic acids of a particular length; paragraph [0277]); (ii) a plurality of maternal nucleic acids, each comprising a second nucleic acid sequence longer than said given length (plurality of maternal nucleic acids of a particular length, where fetal nucleic acids are obtained from maternal nucleic acids (longer than fetal nucleic acids) by digestion; paragraphs [0277], [0279]); (iii) a first set of oligonucleotide primers configured to amplify said first nucleic acid sequence (fetal nucleic acids can be amplified using a first set of primers; paragraphs [0099], [0281], [0294]); (iv) a second set of oligonucleotide primers configured to amplify said second nucleic acid sequence (maternal nucleic acids can be amplified using a second set of primers; paragraphs [0294], [0302], [1166]); (v) a first oligonucleotide probe configured to hybridize to said first nucleic acid sequence (Taqman probe can be annealed to the nucleic acid sequence; paragraph [0232]); and (vi) a second oligonucleotide probe configured to hybridize to said second nucleic acid sequence (Taqman probe can be annealed to the nucleic acid sequence; paragraph [0232]); (B) amplifying (a) said first nucleic acid sequence to generate a first signal from said first oligonucleotide probe (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]) and (b) said second nucleic acid sequence to generate a second signal from said second oligonucleotide probe (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]); identifying said presence or absence of said fetal aneuploidy (method for identifying the presence or absence of a fetal aneuploidy; paragraph [0248]). Sequenom does not disclose (C) determining a ratio of a value derived from said first signal to a second value derived from said second signal; and (D) comparing said ratio to a reference value. Wasserstrom discloses (C) determining a ratio of a value derived from said first signal to a second value derived from said second signal (calculating at least one signal ratio between signal intensities of amplification products and comparing the signal ratios to reference ratios; claim 1); and (D) comparing said ratio to a reference value (calculating at least one signal ratio between signal intensities of amplification products and comparing the signal ratios to reference ratios; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a ratio of amplification signals and comparing to a reference level, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on size distributions (Sequenom; paragraph [0099]).

-***-Continued Within the Next Supplemental Box-***-

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California Institute of Technology - Ex. 2003 Page 4219

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

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Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
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As per claim 61, Sequenom and Wasserstrom, in combination, disclose the method of claim 60, and Sequenom further discloses wherein said first nucleic acid sequence corresponds to a region of nucleic acid potentially associated with said fetal aneuploidy (method for identifying the presence or absence of a fetal aneuploidy based on maternal and fetal nucleic acids; paragraph [0248], [0294]).

As per claim 62, Sequenom and Wasserstrom, in combination, disclose the method of claim 61, and Sequenom further discloses wherein said region comprises a region of chromosome 21 (paragraph [0269]), chromosome 18 (paragraph [0269]), chromosome 13 (paragraph [0269]), X chromosome (paragraph [0269]).

As per claim 63, Sequenom and Wasserstrom, in combination, disclose the method of claim 61, and Sequenom further discloses wherein said region comprises a region of chromosome 21 (paragraph [0269]).

As per claim 64, Sequenom and Wasserstrom, in combination, disclose the method of claim 62, and Sequenom further discloses wherein said region comprises a region of chromosome 18 (paragraph [0269]).

As per claim 65, Sequenom and Wasserstrom, in combination, disclose the method of claim 62, and Sequenom further discloses wherein said region comprises a region of chromosome 13 (paragraph [0269]).

As per claim 66, Sequenom and Wasserstrom, in combination, disclose the method of claim 62, and Sequenom further discloses wherein said region comprises a region of an X chromosome (paragraph [0269]).

As per claim 67, Sequenom and Wasserstrom, in combination, disclose the method of claim 60, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence. Wasserstrom discloses wherein said reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence (values of reference ratios of a particular DNA category (can be third and fourth); paragraph [0050]; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a reference ratio.

As per claim 68, Sequenom and Wasserstrom, in combination, disclose the method of claim 67, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said third nucleic acid sequence and said fourth nucleic acid sequence each correspond to a region of nucleic acid not associated with said fetal aneuploidy. Wasserstrom discloses wherein said third nucleic acid sequence and said fourth nucleic acid sequence each correspond to a region of nucleic acid not associated with said fetal aneuploidy (values of reference ratios of a particular DNA category (can be third and fourth), wherein the DNA sample can be from control DNA; paragraph [0050]; claims 1, 31). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of fetal aneuploidy based on a reference ratio.

As per claim 69, Sequenom and Wasserstrom, in combination, disclose the method of claim 60, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said reference value is derived from a plurality of third values generated from a plurality of third nucleic acid sequences and a plurality of fourth values generated from a plurality of fourth nucleic acid sequences. Wasserstrom discloses wherein said reference value is derived from a plurality of third values generated from a plurality of third nucleic acid sequences and a plurality of fourth values generated from a plurality of fourth nucleic acid sequences (values of plurality of reference ratios of a particular DNA category (can be third and fourth); paragraph [0050]; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a reference ratio.

As per claim 70, Sequenom and Wasserstrom, in combination, disclose the method of claim 69, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said plurality of third nucleic acid sequences and said plurality of fourth nucleic acid sequences each correspond to a region of nucleic acid not associated with said fetal aneuploidy. Wasserstrom discloses wherein said plurality of third nucleic acid sequences and said plurality of fourth nucleic acid sequences each correspond to a region of nucleic acid not associated with said fetal aneuploidy (values of plurality of reference ratios of a particular DNA category (can be third and fourth), wherein the DNA sample can be from control DNA; paragraph [0050]; claims 1, 31). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of fetal aneuploidy based on a reference ratio.

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
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As per claims 71/67-70, Sequenom and Wasserstrom, in combination, disclose the method of claims 67-70, and Sequenom further discloses wherein said region is a region of a housekeeping gene (targets are selected in a housekeeping gene; paragraph [1103]).

As per claim 94, Sequenom and Wasserstrom, in combination, disclose the method of claim 60, and Sequenom further discloses said second nucleic acid sequence comprises at least a portion of said first nucleic acid sequence (where fetal nucleic acids are obtained from maternal nucleic acids by digestion; paragraph [0277], [0279]).

As per claim 95, Sequenom and Wasserstrom, in combination, disclose the method of claim 94, and Sequenom further discloses wherein said second nucleic acid sequence comprises said first nucleic acid sequence (where fetal nucleic acids are obtained from maternal nucleic acids by digestion; paragraph [0277], [0279]).

As per claims 96/60-66, Sequenom and Wasserstrom, in combination, disclose the method of claims 60-66, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence. Wasserstrom discloses wherein said reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence (values of reference ratios of a particular DNA category (can be third and fourth); paragraph [0050]; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a reference ratio.

As per claim 109, Sequenom and Wasserstrom, in combination, disclose the method of claim 60, and Sequenom further discloses wherein said first value is a quantity of said first plurality of nucleic acids (nucleic acid quantification from a sample, where the nucleic acids can be maternal nucleic acids (first plurality of nucleic acids); paragraphs [0242], [0246]).

As per claim 110, Sequenom and Wasserstrom, in combination, disclose the method of claim 60, and Sequenom further discloses wherein said second value is a quantity of said second plurality of nucleic acids (nucleic acid quantification from a sample, where the nucleic acids can be fetal nucleic acids (second plurality of nucleic acids); paragraphs [0242], [0246]).

As per claim 111, Sequenom and Wasserstrom, in combination, disclose the method of claim 60. Sequenom does not disclose wherein said ratio is determined without quantifying said first plurality of nucleic acids and said second plurality of nucleic acids. Wasserstrom discloses wherein said ratio is determined without quantifying said first plurality of nucleic acids and said second plurality of nucleic acids (calculating at least one signal ratio between signal intensities (not quantities) of amplification products; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a signal ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a signal ratio.

As per claim 114, Sequenom and Wasserstrom, in combination, disclose the method of claim 59, and Sequenom further discloses wherein said plasma is subjected to conditions sufficient to enrich for fetal nucleic acids (digestion of maternal nucleic acids in the plasma to enrich the fetal nucleic acids; paragraphs [0099], [0130]).

As per claim 115, Sequenom and Wasserstrom, in combination, disclose the method of claim 59, and Sequenom further discloses wherein said plasma is not subjected to conditions sufficient to enrich for fetal nucleic acids (whole genome from the sample can be mapped without enrichment, wherein the sample can be plasma; paragraphs [0130], [0409]).

-***-Continued Within the Next Supplemental Box-***-

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
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As per claim 118, Sequenom discloses a method for analyzing a size distribution of nucleic acids (method for analyzing a size distribution of nucleic acids based on amplification reactions; figure 148; paragraph [0099]), the method comprising: (A) providing a sample (DNA samples isolated from the blood; paragraph [0100]) comprising: (i) a first plurality of nucleic acids and a second plurality of nucleic acids (mixture of maternal and fetal nucleic acids can be amplified using a first and second set of primers; paragraphs [0099], [0281], [0294], [0302], [1166]), wherein said first plurality of nucleic acids each comprise a first nucleic acid sequence of a given length and said second plurality of nucleic acids each comprise a second nucleic acid sequence longer than said given length (mixture of maternal and fetal nucleic acids, where the nucleic acids are of a given length and size-specific amplification reactions are used to enrich the fetal nucleic acids by digesting the maternal nucleic acids (longer than fetal nucleic acids); paragraphs [0098]-[0099], [0290]); (ii) a first set of paired oligonucleotide primers configured to amplify said first nucleic acid sequence (fetal nucleic acids can be amplified using a second set of primers; paragraphs [0099], [0281], [0294]); and (iii) a second set of paired oligonucleotide primers, configured to amplify said second nucleic acid sequence (maternal nucleic acids can be amplified using a first set of primers; paragraphs [0294], [0302], [1166]); (B) performing an amplification reaction on (a) said first nucleic acid sequence to generate a first signal (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]) and (b) said second nucleic acid sequence to generate a second signal (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]); thereby analyzing said size distribution (analyzing the size distribution based on size specific amplification reactions; paragraph [0099]). Sequenom does not disclose (C) determining a ratio of a first value derived from said first signal to a second value derived from said second signal. Wasserstrom discloses (C) determining a ratio of a first value derived from said first signal to a second value derived from said second signal (calculating at least one signal ratio between signal intensities of amplification products and comparing the signal ratios to reference values; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a ratio of amplification signals and comparing to a reference level, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on size distributions (Sequenom; paragraph [0099]).

As per claim 119, Sequenom and Wasserstrom, in combination, disclose the method of claim 118, and Sequenom further discloses wherein said sample further comprises (iv) a first oligonucleotide probe configured to hybridize to a region of said first nucleic acid sequence (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]) and (v) a second oligonucleotide probe configured to hybridize to a region of said second nucleic acid sequence (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]).

As per claim 120, Sequenom and Wasserstrom, in combination, disclose the method of claim 119, and Sequenom further discloses wherein said first signal is generated from said first oligonucleotide probe and said second signal is generated from said second oligonucleotide probe (amplification of each nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]).

As per claim 121, Sequenom and Wasserstrom, in combination, disclose the method of claim 118, and Sequenom further discloses, in a longer list of options, wherein said sample comprises an intercalating dye (paragraph [0295]). Sequenom does not explicitly disclose wherein said sample further comprises an intercalating dye. Wasserstrom discloses wherein said sample further comprises an intercalating dye (SYBR green staining; paragraph [0029]). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including an intercalating dye, as disclosed by Wasserstrom, for providing the advantage of detecting the PCR amplification signal based on staining with the dye.

As per claim 122, Sequenom and Wasserstrom, in combination, disclose the method of claim 121, and Sequenom further discloses wherein said first signal or said second signal is generated from fluorescent reporter molecule (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]). Sequenom does not disclose an intercalating dye. Wasserstrom discloses an intercalating dye (SYBR green staining; paragraph [0029]). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including an intercalating dye, as disclosed by Wasserstrom, for providing the advantage of detecting the PCR amplification signal based on staining with the dye.

As per claim 123, Sequenom and Wasserstrom, in combination, disclose the method of claim 121, and Sequenom further discloses wherein said first signal and said second signal is generated from fluorescent reporter molecule (amplification of multiple nucleic acid sequences using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraphs [0232], [0294]). Sequenom does not disclose an intercalating dye. Wasserstrom discloses an intercalating dye (SYBR green staining; paragraph [0029]). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including an intercalating dye, as disclosed by Wasserstrom, for providing the advantage of detecting the PCR amplification signal based on staining with the dye.

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:

-***-Continued from Previous Supplemental Box-***-

As per claim 124, Sequenom and Wasserstrom, in combination, disclose the method of claim 118, and Sequenom further discloses, in a longer list of options, wherein said sample comprises an intercalating dye (paragraph [0295]). Sequenom does not explicitly disclose wherein said intercalating dye is SYBR Green or EvaGreen. Wasserstrom discloses wherein said intercalating dye is SYBR Green (SYBR green staining; paragraph [0029]). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including an intercalating dye, as disclosed by Wasserstrom, for providing the advantage of detecting the PCR amplification signal based on staining with the dye.

As per claim 125, Sequenom and Wasserstrom, in combination, disclose the method of claim 118, and Sequenom further discloses wherein said first signal or said second signal are generated by mass spectrometry (amplification signal can be detected using mass spectrometry; paragraph [0294]).

Claims 72/71/67-70 lack an inventive step under PCT Article 33(3) as being obvious over Sequenom and Wasserstrom in view of US 2016/0273048 A1 to ONCODIAG.

As per claims 72/71/67-70, Sequenom and Wasserstrom, in combination, disclose the method of claims 71/67-70, and Sequenom further discloses wherein said region is a region of a housekeeping gene (targets are selected in a housekeeping gene; paragraph [1103]). Sequenom does not disclose wherein said housekeeping gene is beta-globin. Oncodiag discloses disclose wherein said housekeeping gene is beta-globin (paragraph [0108]). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a housekeeping gene, as disclosed by Oncodiag, for providing the advantage of verifying that the PCR conditions are optimum, and hence as amplification controls.

Claims 1-14, 15/1-14, 16/15/1-14, 17/16/15/1-14, 28-29, 38-43, 44/39-43, 57-70, 71/67-70, 72/71/67-70, 94-95, 96/60-66, 109-111, 114-115 and 118-125 have industrial applicability as defined by PCT Article 33(4) because the subject matter can be made or used in industry.

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: Kyle Bruner
Wilson Sonsini Goodrich & Rosati
650 Page Mill Road
Palo Alto, California 94304

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

Date of mailing *(day/month/year)*	06 MAY 2019
Applicant's or agent's file reference 44158-713601	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US19/19906	International filing date *(day/month/year)* 27 February 2019 (27.02.2019)
Applicant CHROMACODE, INC.	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   How? Directly to the International Bureau of WIPO preferably through ePCT or on paper to, 34 chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 338 82 70

   For more detailed instructions, see *PCT Applicant's Guide*, International Phase, paragraphs 9.004 – 9.011.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ **With regard to any protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices.
   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   The applicant may **submit comments on an informal basis on the written opinion of the International Searching Authority** to the International Bureau. These comments will be made available to the public after international publication. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established.

   Shortly after the expiration of **18 months from the priority date, the international application will be published** by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90*bis*.1 and 90*bis*.3).

   Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for **entry into the national phase** before those designated Offices. In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months. For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

   Within **22 months from the priority date, the applicant may request that a supplementary international search be carried out** by a different International Searching Authority that offers this service (Rule 45*bis*.1). The procedure for requesting supplementary international search is described in the *PCT Applicant's Guide*, International Phase, paragraphs 8.006-8.032.

Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 Telephone No. PCT OSP: 571-272-7774

Form PCT/ISA/220 (July 2017)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 44158-713601	FOR FURTHER ACTION	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US19/19906	International filing date *(day/month/year)* 27 February 2019 (27.02.2019)	(Earliest) Priority Date *(day/month/year)* 28 February 2018 (28.02.2018)
Applicant CHROMACODE, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).

c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☒ **Certain claims were found unsearchable** (see Box No. II).

3. ☐ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,

a. the figure of the **drawings** to be published with the abstract is Figure No. 1

☒ as suggested by the applicant.

☐ as selected by this Authority, because the applicant failed to suggest a figure.

☐ as selected by this Authority, because this figure better characterizes the invention.

b. ☐ none of the figures is to be published with the abstract.

Form PCT/ISA/210 (first sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/19906

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 18-27, 30-37, 45-56, 73-93, 97-108, 112-113, 116-117
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/19906

A. CLASSIFICATION OF SUBJECT MATTER
IPC - C12Q 1/6846, 1/6851, 1/6872, 1/6876; G01N 33/48 (2019.01)
CPC - C12Q 1/6846, 1/6851, 1/6872, 1/6876; G01N 33/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2013/0288244 A1 (SEQUENOM, INC.) 31 October, 2013; paragraphs [0098]-[0100], [0232], [0236], [0277], [0279], [0290], [0294], [0302], [1103], [1166]	1-14, 15/1-14, 16/15/1-14, 17/16/15/1-14, 28-29, 38-43, 44/39-43, 57-70, 71/67-70, 72/71/67-70, 94-95, 96/60-66, 109-111, 114-155, 118-125
Y	US 2013/0078626 A1 (WASSERSTROM, A et al.) 28 March, 2013; paragraph [0050]; claims 1, 31	1-14, 15/1-14, 16/15/1-14, 17/16/15/1-14, 28-29, 38-43, 44/39-43, 57-70, 71/67-70, 72/71/67-70, 94-95, 96/60-66, 109-111, 114-115, 118-125
Y	US 2016/0273048 A1 (ONCODIAG) 22 September, 2016; paragraph [0108]	72/71/67-70

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 April 2019 (17.04.2019)	06 MAY 2019
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: Kyle Bruner
Wilson Sonsini Goodrich & Rosati
650 Page Mill Road
Palo Alto, California 94304

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	06 MAY 2019
Applicant's or agent's file reference 44158-713601	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US19/19906	27 February 2019 (27.02.2019)	28 February 2018 (28.02.2018)

International Patent Classification (IPC) or both national classification and IPC
IPC - C12Q 1/6846, 1/6851, 1/6872, 1/6876; G01N 33/48 (2019.01)
CPC - C12Q 1/6846, 1/6851, 1/6872, 1/6876; G01N 33/48

Applicant CHROMACODE, INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☒ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	17 April 2019 (17.04.2019)	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (January 2015)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a)).

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 18-27, 30-37, 45-56, 73-93, 97-108, 112-113, 116-117

because:

☐ the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international search *(specify)*:

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. -***-Please See Below-***- are so unclear that no meaningful opinion could be formed *(specify)*:

Claims 18-27, 30-37, 45-56, 73-93, 97-108, 112-113, and 116-117 are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. -***-Please See Above-***-

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

- ☐ furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
- ☐ furnish a sequence listing on paper or in the form of an image file complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
- ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13*ter*.1(a) or (b).

☐ See Supplemental Box for further details.

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	-***-Please See Below-***-	YES
	Claims	NONE	NO
Inventive step (IS)	Claims	NONE	YES
	Claims	-***-Please See Below-***-	NO
Industrial applicability (IA)	Claims	-***-Please See Below-***-	YES
	Claims	NONE	NO

2. Citations and explanations:

-Continued from Box No. V: 1. Statement:

Novelty (N): YES: Claims 1-14, 15/1-14, 16/15/1-14, 17/16/15/1-14, 28-29, 38-43, 44/39-43, 57-70, 71/67-70, 72/71/67-70, 94-95, 96/60-66, 109-111, 114-115, 118-125

Inventive step (IS): NO: Claims 1-14, 15/1-14, 16/15/1-14, 17/16/15/1-14, 28-29, 38-43, 44/39-43, 57-70, 71/67-70, 72/71/67-70, 94-95, 96/60-66, 109-111, 114-115, 118-125

Industrial applicability (IA): YES: Claims 1-14, 15/1-14, 16/15/1-14, 17/16/15/1-14, 28-29, 38-43, 44/39-43, 57-70, 71/67-70, 72/71/67-70, 94-95, 96/60-66, 109-111, 114-115, 118-125

-Continued from Box No. V. 2: Citations and Explanations:

Claims 1-14, 15/1-14, 16/15/1-14, 17/16/15/1-14, 28-29, 38-43, 44/39-43, 57-70, 71/67-70, 94-95, 96/60-66, 109-111, 114-115 and 118-125 lack an inventive step under PCT Article 33(3) as being obvious over US 2013/0288244 A1 to SEQUENOM, INC., (hereinafter 'Sequenom') in view of US 2013/0078626 A1 to Wasserstrom et al. (hereinafter 'Wasserstrom').

As per claim 1, Sequenom discloses a method for analyzing a size distribution of nucleic acids (method for analyzing a size distribution of nucleic acids based on amplification reactions; figure 148; paragraph [0099]), the method comprising: (A) providing a sample (DNA samples isolated from the blood; paragraph [0100]) comprising: (i) a first plurality of nucleic acids and a second plurality of nucleic acids (mixture of maternal and fetal nucleic acids; paragraph [0098]), wherein said first plurality of nucleic acids each comprise a first nucleic acid sequence of a given length and said second plurality of nucleic acids each comprise a second nucleic acid sequence longer than said given length (mixture of maternal and fetal nucleic acids, where the nucleic acids are of a given length and size-specific amplification reactions are used to enrich the fetal nucleic acids by digesting the maternal nucleic acids (longer than fetal nucleic acids); paragraphs [0098]-[0099], [0277], [0279] [0290]); (ii) a first set of paired amplification oligomers configured to amplify said first nucleic acid sequence (maternal nucleic acids can be amplified using a first set of primers; paragraphs [0294], [0302], [1166]); (iii) a second set of paired amplification oligomers, configured to amplify said second nucleic acid sequence (fetal nucleic acids can be amplified using a second set of primers; paragraphs [0099], [0281], [0294]); (iv) a first detection probe configured to anneal to a region of said first nucleic acid sequence (Taqman probe can be annealed to the nucleic acid sequence; paragraph [0230], [0232]); and (v) a second detection probe configured to anneal to a region of said second nucleic acid sequence (Taqman probe can be annealed to the nucleic acid sequence; paragraph [0230], [0232]); (B) performing an amplification reaction on (a) said first nucleic acid sequence to generate a first signal from said first detection probe (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0230], [0232]) and (b) said second nucleic acid sequence to generate a second signal from said second detection probe (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0230], [0232]); thereby analyzing said size distribution (analyzing the size distribution based on size specific amplification reactions; paragraph [0099]). Sequenom does not disclose (C) determining a ratio of a first value derived from said first signal to a second value derived from said second signal. Wasserstrom discloses (C) determining a ratio of a first value derived from said first signal to a second value derived from said second signal (calculating at least one signal ratio between signal intensities of amplification products and comparing the signal ratios to reference values; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a ratio of amplification signals and comparing to a reference level, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on size distributions (Sequenom; paragraph [0099]).

-***-Continued Within the Next Supplemental Box-***-

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
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Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Claim 60 objected to under PCT Rule 66.2(a)(iii) as containing the following defect(s) in the form or contents thereof: in claim 60, the word 'said' was repeated twice.

Form PCT/ISA/237 (Box No. VII) (January 2015)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claim 124 is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because claim [124] is indefinite for the following reason(s): [in claim 124, the trademarks "SYBR Green" and "EvaGreen" are used which makes the claim indefinite where trademarks characterize the commercial origin of goods, rather than the properties of the goods (which may change from time to time) relevant to the invention. For the purposes of this opinion, "SYBR Green" and "EvaGreen" are interpreted to mean an intercalating dye].

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of:

-***-Continued from Box V: Citations and Explanations-***-

As per claim 2, Sequenom and Wasserstrom, in combination, disclose the method of claim 1, and Sequenom further discloses wherein said first set of paired amplification oligomers (maternal nucleic acids can be amplified using a first set of primers; paragraphs [0294], [0302], [1166]) comprises: a first forward amplification oligomer (forward primers; paragraphs [0232], [0294], [0302], [1166]); and a first reverse amplification oligomer (reverse primers; paragraphs [0232], [0294], [0302], [1166]).

As per claim 3, Sequenom and Wasserstrom, in combination, disclose the method of claim 2, and Sequenom further discloses wherein said first set of paired amplification oligomers (maternal nucleic acids can be amplified using a first set of primers; paragraphs [0294], [0302], [1166]) comprises: a plurality of first forward amplification oligomers (forward primers; paragraphs [0232], [0294], [0302], [1166]); and a plurality of first reverse amplification oligomers (reverse primers; paragraphs [0232], [0294], [0302], [1166]).

As per claim 4, Sequenom and Wasserstrom, in combination, disclose the method of claim 3, and Sequenom further discloses wherein each of said plurality of first forward amplification oligomers has a different nucleic acid sequence (forward primers can be different for targeting methylated and unmethylated DNA in the maternal nucleic acids; paragraphs [0232], [0236], [0294], [0302], [1166]).

As per claim 5, Sequenom and Wasserstrom, in combination, disclose the method of claim 4, and Sequenom further discloses wherein a first forward amplification oligomer of said plurality of first forward amplification oligomers is configured to hybridize to a region of said first sequence (forward primers specifically hybridize the CpG island of methylated DNA in the maternal nucleic acids; paragraphs [0232], [0236], [0294], [0302], [1166]).

As per claim 6, Sequenom and Wasserstrom, in combination, disclose the method of claim 3, and Sequenom further discloses wherein each of said plurality of first reverse amplification oligomers has a different nucleic acid sequence (reverse primers can be different for targeting methylated and unmethylated DNA in the maternal nucleic acids; paragraphs [0232], [0236], [0294], [0302], [1166]).

As per claim 7, Sequenom and Wasserstrom, in combination, disclose the method of claim 6, and Sequenom further discloses wherein a first reverse amplification oligomer of said plurality of first reverse amplification oligomers is configured to hybridize to a region of said first sequence (reverse primers specifically hybridize the CpG island of methylated DNA in the maternal nucleic acids; paragraphs [0232], [0236], [0294], [0302], [1166]).

As per claim 8, Sequenom and Wasserstrom, in combination, disclose the method of claim 1, and Sequenom further discloses wherein said second set of paired amplification oligomers (fetal nucleic acids can be amplified using a second set of primers; paragraphs [0099], [0281], [0294]) comprises: a second forward amplification oligomer (forward primers; paragraphs [0099], [0232], [0281], [0294]); and a second reverse amplification oligomer (reverse primers; paragraphs [0099], [0232], [0281], [0294]).

As per claim 9, Sequenom and Wasserstrom, in combination, disclose the method of claim 8, and Sequenom further discloses wherein said second set of paired amplification oligomers (fetal nucleic acids can be amplified using a second set of primers; paragraphs [0099], [0281], [0294]) comprises: a plurality of second forward amplification oligomers (forward primers; paragraphs [0099], [0232], [0236], [0281], [0294]) and a plurality of second reverse amplification oligomers (reverse primers; paragraphs [0099], [0232], [0236], [0281], [0294]).

As per claim 10, Sequenom and Wasserstrom, in combination, disclose the method of claim 9, and Sequenom further discloses wherein each of said plurality of second forward amplification oligomers has a different nucleic acid sequence (forward primers can be different for targeting methylated and unmethylated DNA in the fetal nucleic acids; paragraphs [0099], [0232], [0236], [0281], [0294]).

As per claim 11, Sequenom and Wasserstrom, in combination, disclose the method of claim 10, and Sequenom further discloses wherein a second forward amplification oligomer of said plurality of second forward amplification oligomers is configured to hybridize to a region of said second sequence (forward primers specifically hybridize the CpG island of methylated DNA in the fetal nucleic acids; paragraphs [0099], [0232], [0236], [0281], [0294]).

As per claim 12, Sequenom and Wasserstrom, in combination, disclose the method of claim 9, and Sequenom further discloses wherein each of said plurality of second reverse amplification oligomers has a different nucleic acid sequence (reverse primers can be different for targeting methylated and unmethylated DNA in the fetal nucleic acids; paragraphs [0099], [0232], [0236], [0281], [0294]).

As per claim 13, Sequenom and Wasserstrom, in combination, disclose the method of claim 12, and Sequenom further discloses wherein a second reverse amplification oligomer of said plurality of second reverse amplification oligomers is configured to hybridize to a region of said second sequence (reverse primers specifically hybridize the CpG island of methylated DNA in the fetal nucleic acids; paragraphs [0099], [0232], [0236], [0281], [0294]).

As per claim 14, Sequenom and Wasserstrom, in combination, disclose the method of claim 1. Sequenom does not disclose wherein said first value and said second value provide a quantitative ratio measurement corresponding to an abundance of said first plurality of nucleic acids and said second plurality of nucleic acids in said sample. Wasserstrom discloses wherein said first value and said second value provide a quantitative ratio measurement corresponding to an abundance of said first plurality of nucleic acids and said second plurality of nucleic acids in said sample (calculating at least one signal ratio between signal intensities of amplification products and comparing the signal ratios to reference values; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a ratio of amplification signals and comparing to a reference level, as disclosed by Wasserstrom, for providing the advantage of testing the nucleic acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on size distributions (Sequenom; paragraph [0099]).

-***-Continued Within the Next Supplemental Box-***-

IPR2024-01451
California Institute of Technology - Ex. 2003 Page 4234

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:

-***-Continued from Previous Supplemental Box-***-

As per claims 15/1-14, Sequenom and Wasserstrom, in combination, disclose the method of claims 1-14, and Sequenom further discloses wherein said first detection probe or said second detection probe comprises a non-target-hybridizing sequence (detection probes can be molecular beacons; paragraph [0319]).

As per claims 16/15/1-14, Sequenom and Wasserstrom, in combination, disclose the method of claims 15/1-14, and Sequenom further discloses wherein said first detection probe or said second detection probe is a hairpin detection probe (detection probes can be molecular beacons; paragraph [0319]).

As per claims 17/16/15/1-14, Sequenom and Wasserstrom, in combination, disclose the method of claims 16/15/1-14, and Sequenom further discloses wherein said hairpin detection probe is a molecular beacon (detection probes can be molecular beacons; paragraph [0319]).

As per claim 28, Sequenom and Wasserstrom, in combination, disclose the method of claim 1, and Sequenom further discloses said second nucleic acid sequence comprises at least a portion of said first nucleic acid sequence (where fetal nucleic acids are obtained from maternal nucleic acids by digestion; paragraph [0277], [0279]).

As per claim 29, Sequenom and Wasserstrom, in combination, disclose the method of claim 28, and Sequenom further discloses wherein said second nucleic acid sequence comprises said first nucleic acid sequence (where fetal nucleic acids are obtained from maternal nucleic acids by digestion; paragraph [0277], [0279]).

As per claim 38, Sequenom and Wasserstrom, in combination, disclose the method of claim 1. Sequenom does not disclose comprising comparing said ratio to a reference value. Wasserstrom discloses comprising comparing said ratio to a reference value (calculating at least one signal ratio between signal intensities of amplification products and comparing the signal ratios to reference values; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a ratio of amplification signals and comparing to a reference level, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on size distributions (Sequenom; paragraph [0099]).

As per claim 39, Sequenom and Wasserstrom, in combination, disclose the method of claim 38, and Sequenom further discloses comparing identifies the presence or absence of a genetic abnormality in said sample (identifying the presence or absence of a genetic abnormality like trisomy 21 in said sample based on comparing the size distribution; paragraph [0099]).

As per claim 40, Sequenom and Wasserstrom, in combination, disclose the method of claim 39, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence. Wasserstrom discloses wherein said reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence (values of reference ratios of a particular DNA category (can be third and fourth); paragraph [0050]; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a reference ratio.

As per claim 41, Sequenom and Wasserstrom, in combination, disclose the method of claim 40, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said third nucleic acid sequence and said fourth nucleic acid sequence each correspond to a region of nucleic acid not associated with said genetic abnormality. Wasserstrom discloses wherein said third nucleic acid sequence and said fourth nucleic acid sequence each correspond to a region of nucleic acid not associated with said genetic abnormality (values of reference ratios of a particular DNA category (can be third and fourth), wherein the DNA sample can be from control DNA; paragraph [0050]; claims 1, 31). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a reference ratio.

As per claim 42, Sequenom and Wasserstrom, in combination, disclose the method of claim 41, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said reference value is derived from a plurality of third values generated from a plurality of third nucleic acid sequences and a plurality of fourth values generated from a plurality of fourth nucleic acid sequences. Wasserstrom discloses wherein said reference value is derived from a plurality of third values generated from a plurality of third nucleic acid sequences and a plurality of fourth values generated from a plurality of fourth nucleic acid sequences (values of plurality of reference ratios of a particular DNA category (can be third and fourth); paragraph [0050]; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a reference ratio.

-***-Continued Within the Next Supplemental Box-***-

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WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US19/19906

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:

-***-Continued from Previous Supplemental Box-***-

As per claim 43, Sequenom and Wasserstrom, in combination, disclose the method of claim 42, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said plurality of third nucleic acid sequences and said plurality of fourth nucleic acid sequences each correspond to a region of nucleic acid not associated with said genetic abnormality. Wasserstrom discloses wherein said plurality of third nucleic acid sequences and said plurality of fourth nucleic acid sequences each correspond to a region of nucleic acid not associated with said genetic abnormality (values of plurality of reference ratios of a particular DNA category (can be third and fourth), wherein the DNA sample can be from control DNA; paragraph [0050]; claims 1, 31). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a reference ratio.

As per claims 44/39-43, Sequenom and Wasserstrom, in combination, disclose the method of claims 39-43, and Sequenom further discloses wherein said genetic abnormality is a fetal aneuploidy (fetal sex chromosome aneuploidy; paragraph [0214]).

As per claim 57, Sequenom and Wasserstrom, in combination, disclose the method of claim 1, and Sequenom further discloses wherein said first value is a quantity of said first plurality of nucleic acids (nucleic acid quantification from a sample, where the nucleic acids can be maternal nucleic acids (first plurality of nucleic acids); paragraphs [0242], [0246]).

As per claim 58, Sequenom and Wasserstrom, in combination, disclose the method of claim 1, and Sequenom further discloses wherein said second value is a quantity of said second plurality of nucleic acids (nucleic acid quantification from a sample, where the nucleic acids can be fetal nucleic acids (second plurality of nucleic acids); paragraphs [0242], [0246]).

As per claim 59, Sequenom and Wasserstrom, in combination, disclose the method of claim 1. Sequenom does not disclose wherein said ratio is determined without quantifying said first plurality of nucleic acids and said second plurality of nucleic acids. Wasserstrom discloses wherein said ratio is determined without quantifying said first plurality of nucleic acids and said second plurality of nucleic acids (calculating at least one signal ratio between signal intensities (not quantities) of amplification products; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a signal ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a signal ratio.

As per claim 60, Sequenom discloses a method for identifying the presence or absence of a fetal aneuploidy (method for identifying the presence or absence of a fetal aneuploidy; paragraph [0248]), comprising: (A) providing a sample (DNA samples isolated from the blood; paragraph [0100]) comprising: (i) a plurality of fetal nucleic acids, each comprising a first nucleic acid sequence of a given length (plurality of fetal nucleic acids of a particular length; paragraph [0277]); (ii) a plurality of maternal nucleic acids, each comprising a second nucleic acid sequence longer than said given length (plurality of maternal nucleic acids of a particular length, where fetal nucleic acids are obtained from maternal nucleic acids (longer than fetal nucleic acids) by digestion; paragraphs [0277], [0279]); (iii) a first set of oligonucleotide primers configured to amplify said first nucleic acid sequence (fetal nucleic acids can be amplified using a first set of primers; paragraphs [0099], [0281], [0294]); (iv) a second set of oligonucleotide primers configured to amplify said second nucleic acid sequence (maternal nucleic acids can be amplified using a second set of primers; paragraphs [0294], [0302], [1166]); (v) a first oligonucleotide probe configured to hybridize to said first nucleic acid sequence (Taqman probe can be annealed to the nucleic acid sequence; paragraph [0232]); and (vi) a second oligonucleotide probe configured to hybridize to said second nucleic acid sequence (Taqman probe can be annealed to the nucleic acid sequence; paragraph [0232]); (B) amplifying (a) said first nucleic acid sequence to generate a first signal from said first oligonucleotide probe (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]) and (b) said second nucleic acid sequence to generate a second signal from said second oligonucleotide probe (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]); identifying said presence or absence of said fetal aneuploidy (method for identifying the presence or absence of a fetal aneuploidy; paragraph [0248]). Sequenom does not disclose (C) determining a ratio of a value derived from said first signal to a second value derived from said second signal; and (D) comparing said ratio to a reference value. Wasserstrom discloses (C) determining a ratio of a value derived from said first signal to a second value derived from said second signal (calculating at least one signal ratio between signal intensities of amplification products and comparing the signal ratios to reference ratios; claim 1); and (D) comparing said ratio to a reference value (calculating at least one signal ratio between signal intensities of amplification products and comparing the signal ratios to reference ratios; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a ratio of amplification signals and comparing to a reference level, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on size distributions (Sequenom; paragraph [0099]).

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

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Supplemental Box

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As per claim 61, Sequenom and Wasserstrom, in combination, disclose the method of claim 60, and Sequenom further discloses wherein said first nucleic acid sequence corresponds to a region of nucleic acid potentially associated with said fetal aneuploidy (method for identifying the presence or absence of a fetal aneuploidy based on maternal and fetal nucleic acids; paragraph [0248], [0294]).

As per claim 62, Sequenom and Wasserstrom, in combination, disclose the method of claim 61, and Sequenom further discloses wherein said region comprises a region of chromosome 21 (paragraph [0269]), chromosome 18 (paragraph [0269]), chromosome 13 (paragraph [0269]), X chromosome (paragraph [0269]).

As per claim 63, Sequenom and Wasserstrom, in combination, disclose the method of claim 61, and Sequenom further discloses wherein said region comprises a region of chromosome 21 (paragraph [0269]).

As per claim 64, Sequenom and Wasserstrom, in combination, disclose the method of claim 62, and Sequenom further discloses wherein said region comprises a region of chromosome 18 (paragraph [0269]).

As per claim 65, Sequenom and Wasserstrom, in combination, disclose the method of claim 62, and Sequenom further discloses wherein said region comprises a region of chromosome 13 (paragraph [0269]).

As per claim 66, Sequenom and Wasserstrom, in combination, disclose the method of claim 62, and Sequenom further discloses wherein said region comprises a region of an X chromosome (paragraph [0269]).

As per claim 67, Sequenom and Wasserstrom, in combination, disclose the method of claim 60, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence. Wasserstrom discloses wherein said reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence (values of reference ratios of a particular DNA category (can be third and fourth); paragraph [0050]; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a reference ratio.

As per claim 68, Sequenom and Wasserstrom, in combination, disclose the method of claim 67, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said third nucleic acid sequence and said fourth nucleic acid sequence each correspond to a region of nucleic acid not associated with said fetal aneuploidy. Wasserstrom discloses wherein said third nucleic acid sequence and said fourth nucleic acid sequence each correspond to a region of nucleic acid not associated with said fetal aneuploidy (values of reference ratios of a particular DNA category (can be third and fourth), wherein the DNA sample can be from control DNA; paragraph [0050]; claims 1, 31). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of fetal aneuploidy based on a reference ratio.

As per claim 69, Sequenom and Wasserstrom, in combination, disclose the method of claim 60, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said reference value is derived from a plurality of third values generated from a plurality of third nucleic acid sequences and a plurality of fourth values generated from a plurality of fourth nucleic acid sequences. Wasserstrom discloses wherein said reference value is derived from a plurality of third values generated from a plurality of third nucleic acid sequences and a plurality of fourth values generated from a plurality of fourth nucleic acid sequences (values of plurality of reference ratios of a particular DNA category (can be third and fourth); paragraph [0050]; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a reference ratio.

As per claim 70, Sequenom and Wasserstrom, in combination, disclose the method of claim 69, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said plurality of third nucleic acid sequences and said plurality of fourth nucleic acid sequences each correspond to a region of nucleic acid not associated with said fetal aneuploidy. Wasserstrom discloses wherein said plurality of third nucleic acid sequences and said plurality of fourth nucleic acid sequences each correspond to a region of nucleic acid not associated with said fetal aneuploidy (values of plurality of reference ratios of a particular DNA category (can be third and fourth), wherein the DNA sample can be from control DNA; paragraph [0050]; claims 1, 31). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of fetal aneuploidy based on a reference ratio.

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As per claims 71/67-70, Sequenom and Wasserstrom, in combination, disclose the method of claims 67-70, and Sequenom further discloses wherein said region is a region of a housekeeping gene (targets are selected in a housekeeping gene; paragraph [1103]).

As per claim 94, Sequenom and Wasserstrom, in combination, disclose the method of claim 60, and Sequenom further discloses said second nucleic acid sequence comprises at least a portion of said first nucleic acid sequence (where fetal nucleic acids are obtained from maternal nucleic acids by digestion; paragraph [0277], [0279]).

As per claim 95, Sequenom and Wasserstrom, in combination, disclose the method of claim 94, and Sequenom further discloses wherein said second nucleic acid sequence comprises said first nucleic acid sequence (where fetal nucleic acids are obtained from maternal nucleic acids by digestion; paragraph [0277], [0279]).

As per claims 96/60-66, Sequenom and Wasserstrom, in combination, disclose the method of claims 60-66, and Sequenom further discloses control sequences (paragraph [0156], [0218]). Sequenom does not disclose wherein said reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence. Wasserstrom discloses wherein said reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence (values of reference ratios of a particular DNA category (can be third and fourth); paragraph [0050]; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a reference ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a reference ratio.

As per claim 109, Sequenom and Wasserstrom, in combination, disclose the method of claim 60, and Sequenom further discloses wherein said first value is a quantity of said first plurality of nucleic acids (nucleic acid quantification from a sample, where the nucleic acids can be maternal nucleic acids (first plurality of nucleic acids); paragraphs [0242], [0246]).

As per claim 110, Sequenom and Wasserstrom, in combination, disclose the method of claim 60, and Sequenom further discloses wherein said second value is a quantity of said second plurality of nucleic acids (nucleic acid quantification from a sample, where the nucleic acids can be fetal nucleic acids (second plurality of nucleic acids); paragraphs [0242], [0246]).

As per claim 111, Sequenom and Wasserstrom, in combination, disclose the method of claim 60. Sequenom does not disclose wherein said ratio is determined without quantifying said first plurality of nucleic acids and said second plurality of nucleic acids. Wasserstrom discloses wherein said ratio is determined without quantifying said first plurality of nucleic acids and said second plurality of nucleic acids (calculating at least one signal ratio between signal intensities (not quantities) of amplification products; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a signal ratio, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on a signal ratio.

As per claim 114, Sequenom and Wasserstrom, in combination, disclose the method of claim 59, and Sequenom further discloses wherein said plasma is subjected to conditions sufficient to enrich for fetal nucleic acids (digestion of maternal nucleic acids in the plasma to enrich the fetal nucleic acids; paragraphs [0099], [0130]).

As per claim 115, Sequenom and Wasserstrom, in combination, disclose the method of claim 59, and Sequenom further discloses wherein said plasma is not subjected to conditions sufficient to enrich for fetal nucleic acids (whole genome from the sample can be mapped without enrichment, wherein the sample can be plasma; paragraphs [0130], [0409]).

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Supplemental Box

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As per claim 118, Sequenom discloses a method for analyzing a size distribution of nucleic acids (method for analyzing a size distribution of nucleic acids based on amplification reactions; figure 148; paragraph [0099]), the method comprising: (A) providing a sample (DNA samples isolated from the blood; paragraph [0100]) comprising: (i) a first plurality of nucleic acids and a second plurality of nucleic acids (mixture of maternal and fetal nucleic acids can be amplified using a first and second set of primers; paragraphs [0099], [0281], [0294], [0302], [1166]), wherein said first plurality of nucleic acids each comprise a first nucleic acid sequence of a given length and said second plurality of nucleic acids each comprise a second nucleic acid sequence longer than said given length (mixture of maternal and fetal nucleic acids, where the nucleic acids are of a given length and size-specific amplification reactions are used to enrich the fetal nucleic acids by digesting the maternal nucleic acids (longer than fetal nucleic acids); paragraphs [0098]-[0099], [0290]); (ii) a first set of paired oligonucleotide primers configured to amplify said first nucleic acid sequence (fetal nucleic acids can be amplified using a second set of primers; paragraphs [0099], [0281], [0294]); and (iii) a second set of paired oligonucleotide primers, configured to amplify said second nucleic acid sequence (maternal nucleic acids can be amplified using a first set of primers; paragraphs [0294], [0302], [1166]); (B) performing an amplification reaction on (a) said first nucleic acid sequence to generate a first signal (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]) and (b) said second nucleic acid sequence to generate a second signal (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]); thereby analyzing said size distribution (analyzing the size distribution based on size specific amplification reactions; paragraph [0099]). Sequenom does not disclose (C) determining a ratio of a first value derived from said first signal to a second value derived from said second signal. Wasserstrom discloses (C) determining a ratio of a first value derived from said first signal to a second value derived from said second signal (calculating at least one signal ratio between signal intensities of amplification products and comparing the signal ratios to reference values; claim 1). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a ratio of amplification signals and comparing to a reference level, as disclosed by Wasserstrom, for providing the advantage of testing the nuclei acid sample obtained from a mother and categorizing the fetal nucleic acids for presence/absence of trisomy 21 based on size distributions (Sequenom; paragraph [0099]).

As per claim 119, Sequenom and Wasserstrom, in combination, disclose the method of claim 118, and Sequenom further discloses wherein said sample further comprises (iv) a first oligonucleotide probe configured to hybridize to a region of said first nucleic acid sequence (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]) and (v) a second oligonucleotide probe configured to hybridize to a region of said second nucleic acid sequence (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]).

As per claim 120, Sequenom and Wasserstrom, in combination, disclose the method of claim 119, and Sequenom further discloses wherein said first signal is generated from said first oligonucleotide probe and said second signal is generated from said second oligonucleotide probe (amplification of each nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]).

As per claim 121, Sequenom and Wasserstrom, in combination, disclose the method of claim 118, and Sequenom further discloses, in a longer list of options, wherein said sample comprises an intercalating dye (paragraph [0295]). Sequenom does not explicitly disclose wherein said sample further comprises an intercalating dye. Wasserstrom discloses wherein said sample further comprises an intercalating dye (SYBR green staining; paragraph [0029]). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including an intercalating dye, as disclosed by Wasserstrom, for providing the advantage of detecting the PCR amplification signal based on staining with the dye.

As per claim 122, Sequenom and Wasserstrom, in combination, disclose the method of claim 121, and Sequenom further discloses wherein said first signal or said second signal is generated from fluorescent reporter molecule (amplification of nucleic acid sequence using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraph [0232]). Sequenom does not disclose an intercalating dye. Wasserstrom discloses an intercalating dye (SYBR green staining; paragraph [0029]). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including an intercalating dye, as disclosed by Wasserstrom, for providing the advantage of detecting the PCR amplification signal based on staining with the dye.

As per claim 123, Sequenom and Wasserstrom, in combination, disclose the method of claim 121, and Sequenom further discloses wherein said first signal and said second signal is generated from fluorescent reporter molecule (amplification of multiple nucleic acid sequences using specific biased primers and the Taqman probe, during which the taq polymerase digests the probe to release a fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system; paragraphs [0232], [0294]). Sequenom does not disclose an intercalating dye. Wasserstrom discloses an intercalating dye (SYBR green staining; paragraph [0029]). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including an intercalating dye, as disclosed by Wasserstrom, for providing the advantage of detecting the PCR amplification signal based on staining with the dye.

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As per claim 124, Sequenom and Wasserstrom, in combination, disclose the method of claim 118, and Sequenom further discloses, in a longer list of options, wherein said sample comprises an intercalating dye (paragraph [0295]). Sequenom does not explicitly disclose wherein said intercalating dye is SYBR Green or EvaGreen. Wasserstrom discloses wherein said intercalating dye is SYBR Green (SYBR green staining; paragraph [0029]). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including an intercalating dye, as disclosed by Wasserstrom, for providing the advantage of detecting the PCR amplification signal based on staining with the dye.

As per claim 125, Sequenom and Wasserstrom, in combination, disclose the method of claim 118, and Sequenom further discloses wherein said first signal or said second signal are generated by mass spectrometry (amplification signal can be detected using mass spectrometry; paragraph [0294]).

Claims 72/71/67-70 lack an inventive step under PCT Article 33(3) as being obvious over Sequenom and Wasserstrom in view of US 2016/0273048 A1 to ONCODIAG.

As per claims 72/71/67-70, Sequenom and Wasserstrom, in combination, disclose the method of claims 71/67-70, and Sequenom further discloses wherein said region is a region of a housekeeping gene (targets are selected in a housekeeping gene; paragraph [1103]). Sequenom does not disclose wherein said housekeeping gene is beta-globin. Oncodiag discloses disclose wherein said housekeeping gene is beta-globin (paragraph [0108]). It would have been obvious to one of ordinary skill in the art at the time of invention to have modified the disclosure of Sequenom, by including a housekeeping gene, as disclosed by Oncodiag, for providing the advantage of verifying that the PCR conditions are optimum, and hence as amplification controls.

Claims 1-14, 15/1-14, 16/15/1-14, 17/16/15/1-14, 28-29, 38-43, 44/39-43, 57-70, 71/67-70, 72/71/67-70, 94-95, 96/60-66, 109-111, 114-115 and 118-125 have industrial applicability as defined by PCT Article 33(4) because the subject matter can be made or used in industry.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference 44158-715601	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/US2019/027751	International filing date *(day/month/year)* 16 April 2019 (16.04.2019)	Priority date *(day/month/year)* 17 April 2018 (17.04.2018)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant CHROMACODE, INC.		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 14 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☐	Box No. II	Priority
☒	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☐	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 20 October 2020 (20.10.2020)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Yukari Nakamura e-mail: pct.team7@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: KYLE BRUNER
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	26 JUN 2019
Applicant's or agent's file reference 44158-715601	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2019/027751	16 April 2019	17 April 2018

International Patent Classification (IPC) or both national classification and IPC
IPC(8) - C12Q 1/6851; C12Q 1/686; G01N 21/64; G01N 33/53 (2019.01)
CPC - C12Q 1/6851; C12Q 1/686; G01N 21/63; G01N 33/50 (2019.05)

Applicant CHROMACODE, INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	10 June 2019	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (January 2015)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2019/027751

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a)).

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2019/027751

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 21-33, 38-51, 55-64, 71-78, 82-110

because:

☐ the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international search *(specify)*:

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. see claims below are so unclear that no meaningful opinion could be formed *(specify)*:

Claims 21-33, 38-51, 55-64, 71-78, and 82-110 are multiple dependent claims not drafted in accordance with the second and third sentences of Rule 6.4(a).

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. 21-33, 38-51, 55-64, 71-78, 82-110

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

- ☐ furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
- ☐ furnish a sequence listing on paper or in the form of an image file complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
- ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13*ter*.1(a) or (b).

☐ See Supplemental Box for further details.

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2019/027751

Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	1-20, 34-37, 54, 65-70, 79-81	YES
	Claims	52, 53	NO
Inventive step (IS)	Claims	54	YES
	Claims	1-20, 34-37, 52, 53, 65-70, 79-81	NO
Industrial applicability (IA)	Claims	1-20, 34-37, 52-54, 65-70, 79-81	YES
	Claims	None	NO

2. Citations and explanations:

Claims 52 and 53 lack novelty under PCT Article 33(2) as being anticipated by University Of Washington Through Its Center For Commercialization (hereinafter Washington).

Regarding Claim 52, Washington discloses a method of quantifying target nucleic acid molecules in a sample (Paras. [0065]-[0067], methods for using digital measurements to determine a concentration of a sample…a sample can include an aqueous solution that includes a molecule of interest (e.g., a nucleic acid molecule)), the method comprising: (a) partitioning a plurality of nucleic acid molecules into a plurality of partitions (Paras. [0066]-[0067], droplets of different volumes (diameters) can be generated…a sample can include an aqueous solution that includes a molecule of interest (e.g., a nucleic acid molecule)); (b) amplifying said plurality of nucleic acid molecules in said plurality of partitions (Paras. [0066]-[0067], amplification of analyte (e.g. digital PCR) can be carried out simultaneously in all droplets of different volumes (sizes)…a sample can include an aqueous solution that includes a molecule of interest (e.g., a nucleic acid molecule)), thereby generating a plurality of signals (Paras. [0066]-[0067]); (c) detecting said plurality of signals (Para. [0066], presence of amplification product in each droplet is determined based on fluorescence and the size (volume) of each droplet is determined based on the scattering signal from the droplet); (d) based on said detecting, determining for each partition of said plurality of partitions a probability that one or more nucleic acid molecule(s) of said plurality of nucleic acid molecules is present in said partition (Para. [0066], presence of amplification product in each droplet is determined based on fluorescence and the size (volume) of each droplet is determined based on the scattering signal from the droplet; Para. [0075], sample can be distributed into droplets of variable volumes that can be analyzed. The individual volumes of the droplets (all or just some) can be determined by methods provided herein. In addition, using detection methods described herein, droplets can be analyzed for containing a detectable agent or not. For a given sample concentration, some of the variable volume droplets may contain a detectable agent and some may not. For higher sample concentrations, generally more droplets of a plurality may contain detectable agents and vice versa; for low sample concentrations, fewer droplets of a plurality may be occupied by a detectable agent. As further described herein, the probabilities of occupancy by a detectable agent in a particular volume distribution), thereby generating a plurality of probabilities (Para. [0075], probabilities of occupancy by a detectable agent in a particular volume distribution can be defined for a wide range of sample concentrations); and (e) quantifying said target nucleic acid molecules in said sample based on a function of said plurality of probabilities (Para. [0075], probabilities of occupancy by a detectable agent in a particular volume distribution can be defined for a wide range of sample concentrations, which can then be compared to real data to determine the concentration of an unknown sample; Para. [0067], a sample can include an aqueous solution that includes a molecule of interest (e.g., a nucleic acid molecule)).

Regarding Claim 53, Washington discloses the method of claim 52, wherein said method does not include a step of determining a number of nucleic acid molecules in any individual member of said plurality of partitions (Para. [0100], This example describes a non-limiting theoretical framework that can be used to describe certain aspects of the present invention described herein. It will be understood that the method described in this example is one of many ways to determine concentrations of sample using the present invention. Under this theoretical framework, it is assumed that the target molecules are present in the sample at a concentration Cs in units of molecules/volume. The sample is distributed into digital volumes of variable volumes; the distribution of target molecules into the droplets follows Poisson statistics...the amplification reaction will cause a droplet containing one or more molecules to be distinguishable from empty droplets, e.g. by its fluorescence intensity. In this method we know only whether a droplet is empty or occupied...in order to determine the concentration CS of target molecules, P(n>0, CSVi) needs to be summed over a sufficiently large number of droplets with respective volumes Vi).

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Claims 1-6, 9, 10, 13-15, 18-20, and 34-37 lack an inventive step under PCT Article 33(3) as being obvious over California Institute Of Technology (hereinafter California).

Regarding Claim 1, California discloses a method of quantifying (Para. [0036], methods described herein can provide for the direct detection of one or more analytes in a single reaction...allows for more accurate quantification of the analyte) a nucleic acid target in a sample (Para. [0083], analyte is referred to as a nucleic acid analyte), the method comprising: (a) providing a mixture (Para. [0003], combining the sample with a first primer and a first oligonucleotide) comprising: i. a plurality of nucleic acid molecules derived from, and/or corresponding with, said nucleic acid target; and ii. a plurality of oligonucleotide probes (Para. [0154], In some cases, compositions comprise a first and a second primer or probe for the detection of at least one analyte wherein the primers are attached to either a fluorophore or a quencher at the 5' end...In some cases the compositions comprise multiple pairs of first and second primers, wherein each pair of first and second primers comprise either a fluorophore or a quencher at the 5' end. In some cases each pair of first and second primers comprise a different fluorophore and quencher from the remaining set of primers. In some cases the compositions comprise at least 1, 2, 3, 4...or 10000 pairs of first and second primers; Para. [0048], Various combinations of oligonucleotides (e.g. primers and/or probes) can be used to detect an analyte. In some cases, a primer is used with a probe or a plurality of probes. In some cases, a plurality of primers is used with a plurality of probes...In some cases, a primer and a probe is used to detect multiple analytes...a combination of primers and probes is used to detect multiple analytes. In some cases, a probe is used to detect an analyte. In some cases, a probe is used to detect multiple analytes. In some cases, multiple probes are used to detect an analyte; Para. [0083], analyte is referred to as a nucleic acid target; the primers and probes are in a mixture), each of which corresponds to a different region of said nucleic acid target (Para. [0013], the first primer and the second primer are specific for the polynucleotide analyte; Para. [0045], primers to bind to specific regions of the individual template); (c) generating a plurality of signals (Para. [0011], each primer of the primer pair is attached to at least one chromophore; b) measuring a first set of signals generated by the chromophores), wherein said plurality of signals are detectable in one color channel (Para. [0049], In some cases, a single chromophore pair is used to detect an analyte; Para. [0176], Analytes of differing lengths (e.g., 40 bp, 60 bp, and 80 bp) are detected using a single fluorophore/quencher combination...By measuring fluorescence as a function of increasing temperature, multiple analytes in a single color channel are quantified), and wherein at least one signal of said plurality of signals corresponds with a unique combination of two or more of said plurality of nucleic acid molecules (Para. [0011], methods of detecting a plurality of analytes, comprising: a) combining the plurality of analytes with a plurality of primer pairs, wherein each primer pair is specific to a single analyte and each primer of the primer pair is attached to at least one chromophore; b) measuring a first set of signals generated by the chromophores...d) measuring a second set of signals generated by the chromophores; wherein the first and second set of signals are used to detect each analyte of the plurality of analytes...the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profiles); (d) detecting from said plurality of signals in said color channel (Para. [0049], In some cases, a single chromophore pair is used to detect an analyte; Para. [0176], Analytes of differing lengths (e.g., 40 bp, 60 bp, and 80 bp) are detected using a single fluorophore/quencher combination...By measuring fluorescence as a function of increasing temperature, multiple analytes in a single color channel are quantified); and (e) based on said detecting, quantifying said nucleic acid target in said sample (Para. [0011], measuring a second set of signals generated by the chromophores; wherein the first and second set of signals are used to detect each analyte of the plurality of analytes...the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profile; Para. [0083], analyte is referred to as a nucleic acid analyte; Para. [0036]), but fails to explicitly disclose (b) partitioning said mixture into a plurality of partitions; (c) generating signals in said plurality of partitions, and signals in a partition of said plurality of partitions; (d) detecting from multiple partitions of said plurality of partitions in the same embodiment. However, since in an alternate embodiment California teaches droplet digital PCR (Para. [0010]), it would have been obvious to one of ordinary skill in the art to modify the method of the primary embodiment of California with the teaching of an alternate embodiment of California so that California's assay using a droplet digital PCR process involves (b) partitioning said mixture into a plurality of partitions; (c) generating signals in said plurality of partitions, and signals in a partition of said plurality of partitions; (d) detecting from multiple partitions of said plurality of partitions, in order to correctly perform the taught method using a droplet digital PCR process.

Regarding Claim 2, modified California discloses the method of claim 1. California further discloses at least a portion of said plurality of signals is generated by said plurality of oligonucleotide probes (Para. [0003], (a) combining the sample with a first primer and a first oligonucleotide, wherein a first chromophore is attached to the first primer, a second chromophore is attached to the first oligonucleotide, the first primer and the first oligonucleotide are specific for a first polynucleotide analyte and the first chromophore is different from the second chromophore; (b) measuring a first signal generated by the first and second chromophores).

Regarding Claim 3, modified California discloses the method of claim 1. California further discloses said sample further comprises (iii) an additional plurality of nucleic acid molecules derived from, and/or corresponding with, an additional nucleic acid target and (iv) an additional plurality of oligonucleotide probes, each of which corresponds to a different region of said additional nucleic acid target (Para. [0007], In some cases, step a) of the method described herein further comprises combining the sample with a third primer and a second oligonucleotide, wherein a third chromophore is attached to the third primer and a fourth chromophore is attached to the second oligonucleotide, and the third primer and the second oligonucleotide are specific for a second polynucleotide analyte; step b) further comprises measuring a fifth signal generated by the third and fourth chromophores; step d) further comprises measuring a sixth signal generated by the third and fourth chromophores; wherein the fifth and sixth signals are used to detect the second polynucleotide analyte. In some cases, the methods disclosed herein further comprising comparing the fifth and sixth signals, wherein a change in the fifth and sixth signals indicates the presence of the second polynucleotide analyte; Paras. [0044]-[0045], terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides...primers to bind to specific regions of the individual template).

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Regarding Claim 4, modified California discloses the method of claim 3. California further discloses at least a portion of said plurality of signals is generated by said plurality of oligonucleotide probes and said additional plurality of oligonucleotide probes (Para. [0007], In some cases, step a) of the method described herein further comprises combining the sample with a third primer and a second oligonucleotide, wherein a third chromophore is attached to the third primer and a fourth chromophore is attached to the second oligonucleotide, and the third primer and the second oligonucleotide are specific for a second polynucleotide analyte; step b) further comprises measuring a fifth signal generated by the third and fourth chromophores; step d) further comprises measuring a sixth signal generated by the third and fourth chromophores; wherein the fifth and sixth signals are used to detect the second polynucleotide analyte. In some cases, the methods disclosed herein further comprising comparing the fifth and sixth signals, wherein a change in the fifth and sixth signals indicates the presence of the second polynucleotide analyte; Para. [0011], methods of detecting a plurality of analytes, comprising: a) combining the plurality of analytes with a plurality of primer pairs, wherein each primer pair is specific to a single analyte and each primer of the primer pair is attached to at least one chromophore; b) measuring a first set of signals generated by the chromophores; Para. [0044], terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides).

Regarding Claim 5, modified California discloses the method of claim 1. California further discloses said sample is derived from a biological sample (Para. [0152], sample can be a blood sample).

Regarding Claim 6, modified California discloses the method of claim 5. California further discloses said biological sample is blood (Para. [0152], sample can be a blood sample) or plasma (optionally not included).

Regarding Claim 9, modified California discloses the method of claim 1. California further discloses (c) comprises amplifying said plurality of nucleic acid molecules, thereby generating said plurality of signals (Para. [0036], methods described herein can provide for the direct detection of one or more analytes in a single reaction (such as an amplification reaction...)...once the analyte has been amplified to incorporate two different chromophores, upon excitation of one chromophore, an electron may travel through the pi-bond network of a DNA backbone to interact with a second chromophore, thereby generating a detectable signal; Para. [0003], methods generally involve using oligonucleotides (e.g., primers, probes) attached to chromophores (e.g., fluorophores, quenchers, etc.) in amplification or polymerization reactions in order to detect a polynucleotide analyte. In some embodiments, provided herein are methods of detecting at least one polynucleotide analyte in a sample, comprising: (a) combining the sample with a first primer and a first oligonucleotide, wherein a first chromophore is attached to the first primer...measuring a first signal generated by the first and second chromophores...first and second signals are used to detect the first polynucleotide analyte).

Regarding Claim 10, modified California discloses the method of claim 9. California further discloses said amplifying comprises polymerase chain reaction (Para. [0036], amplification reaction (such as a PCR reaction or PCR reaction process)).

Regarding Claim 13, modified California discloses the method of claim 1. California further discloses said plurality of signals is a plurality of fluorescent signals (Para. [0005], change in the first and second signals is a decrease in fluorescent intensity) or a plurality of chemiluminescent signals (optionally not included).

Regarding Claim 14, California discloses the method of claim 1. California further discloses said plurality of oligonucleotide probes each bind to a different region of said nucleic acid target (Para. [0013], the first primer and the second primer are specific for the polynucleotide analyte; Paras. [0044]-[0045], terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides...primers to bind to specific regions of the individual template).

Regarding Claim 15, modified California discloses the method of claim 1. California further discloses said at least one of said plurality of signals uniquely corresponds with a presence of exactly two of said plurality of nucleic acid molecules (Para. [0011], c) performing at least one polymerization reaction with the plurality of primer pairs using the plurality of analytes as templates; and d) measuring a second set of signals generated by the chromophores; wherein the first and second set of signals are used to detect each analyte of the plurality of analytes. In some cases, the method described herein further comprises: e) repeating step c and d at least once; and f) generating a set of signature profiles; Para. [0080], A signature profile is unique for each nucleotide mismatch, UU), but fails to explicitly disclose in said partition in the same embodiment. However, since in an alternate embodiment California teaches droplet digital PCR (Para. [0010]), it would have been obvious to one of ordinary skill in the art to modify the method of the primary embodiment of California with the teaching of an alternate embodiment of California so that California's assay using a droplet digital PCR process involves in said partition, in order to correctly perform the taught method using a droplet digital PCR process.

Regarding Claim 18, modified California discloses the method of claim 3. California further discloses at least a portion of said plurality of signals is generated by said plurality of oligonucleotide probes (Para. [0003], (a) combining the sample with a first primer and a first oligonucleotide, wherein a first chromophore is attached to the first primer, a second chromophore is attached to the first oligonucleotide, the first primer and the first oligonucleotide are specific for a first polynucleotide analyte and the first chromophore is different from the second chromophore; (b) measuring a first signal generated by the first and second chromophores).

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Regarding Claim 19, modified California discloses the method of claim 18. California further discloses said sample further comprises (iii) an additional plurality of nucleic acid molecules derived from, and/or corresponding with, an additional nucleic acid target and (iv) an additional plurality of oligonucleotide probes, each of which corresponds to a different region of said additional nucleic acid target (Para. [0007], In some cases, step a) of the method described herein further comprises combining the sample with a third primer and a second oligonucleotide, wherein a third chromophore is attached to the third primer and a fourth chromophore is attached to the second oligonucleotide, and the third primer and the second oligonucleotide are specific for a second polynucleotide analyte; step b) further comprises measuring a fifth signal generated by the third and fourth chromophores; step d) further comprises measuring a sixth signal generated by the third and fourth chromophores; wherein the fifth and sixth signals are used to detect the second polynucleotide analyte. In some cases, the methods disclosed herein further comprising comparing the fifth and sixth signals, wherein a change in the fifth and sixth signals indicates the presence of the second polynucleotide analyte; Paras. [0044]-[0045], terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides...primers to bind to specific regions of the individual template).

Regarding Claim 20, modified California discloses the method of claim 19. California further discloses at least a portion of said plurality of signals is generated by said plurality of oligonucleotide probes and said additional plurality of oligonucleotide probes (Para. [0007], In some cases, step a) of the method described herein further comprises combining the sample with a third primer and a second oligonucleotide, wherein a third chromophore is attached to the third primer and a fourth chromophore is attached to the second oligonucleotide, and the third primer and the second oligonucleotide are specific for a second polynucleotide analyte; step b) further comprises measuring a fifth signal generated by the third and fourth chromophores; step d) further comprises measuring a sixth signal generated by the third and fourth chromophores; wherein the fifth and sixth signals are used to detect the second polynucleotide analyte. In some cases, the methods disclosed herein further comprising comparing the fifth and sixth signals, wherein a change in the fifth and sixth signals indicates the presence of the second polynucleotide analyte; Para. [0011], methods of detecting a plurality of analytes, comprising: a) combining the plurality of analytes with a plurality of primer pairs, wherein each primer pair is specific to a single analyte and each primer of the primer pair is attached to at least one chromophore; b) measuring a first set of signals generated by the chromophores; Para. [0044], terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides).

Regarding Claim 34, California discloses a method of quantifying (Para. [0036], methods described herein can provide for the direct detection of one or more analytes in a single reaction...allows for more accurate quantification of the analyte) a first nucleic acid target and a second nucleic acid target (Para. [0011], wherein the first and second set of signals are used to detect each analyte of the plurality of analytes; Para. [0044], terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides) in a sample (Para. [0003], the sample), the method comprising: (a) providing a mixture comprising: i. a first plurality of nucleic acid molecules derived from, and/or corresponding with, said first nucleic acid target; ii. a second plurality of nucleic acid molecules derived from, and/or corresponding with, said second nucleic acid target (Para. [0011], a) combining the plurality of analytes with a plurality of primer pairs; Para. [0044], terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides); iii. a first plurality of oligonucleotide probes, each of which corresponds to a different region of said first nucleic acid target (Para. [0011], the presence of each analyte of the plurality of analyte; Para. [0019], first primer or probe is a primer...In some cases, the second primer or probe comprises an oligonucleotide sequence complementary to the sequence of a target analyte); and iv. a second plurality of oligonucleotide probes, each of which corresponds to a different region of said second nucleic acid target (Para. [0011], the presence of each analyte of the plurality of analyte; Para. [0019], the second primer or probe is a primer...In some cases, the second primer or probe comprises an oligonucleotide sequence complementary to the sequence of a target analyte); (c) generating a plurality of signals (Para. [0011], each primer of the primer pair is attached to at least one chromophore; b) measuring a first set of signals generated by the chromophores), wherein said plurality of signals are detectable in one color channel (Para. [0049], In some cases, a single chromophore pair is used to detect an analyte; Para. [0176], Analytes of differing lengths (e.g., 40 bp, 60 bp, and 80 bp) are detected using a single fluorophore/quencher combination...By measuring fluorescence as a function of increasing temperature, multiple analytes in a single color channel are quantified); (d) detecting said plurality of signals in said color channel (Para. [0049]; Para. [0176]); and (e) based on said detecting, quantifying said first nucleic acid target and said second nucleic acid target in said sample (Para. [0011], measuring a second set of signals generated by the chromophores; wherein the first and second set of signals are used to detect each analyte of the plurality of analytes...the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profile; Para. [0044], terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides; Para. [0036]), but fails to explicitly disclose (b) partitioning said mixture into a plurality of partitions; (c) signals in said plurality of partitions; (d) detecting from multiple partitions of said plurality of partitions in the same embodiment. However, since in an alternate embodiment California teaches droplet digital PCR (Para. [0010]), it would have been obvious to one of ordinary skill in the art to modify the method of the primary embodiment of California with the teaching of an alternate embodiment of California so that California's assay using a droplet digital PCR process involves (b) partitioning said mixture into a plurality of partitions; (c) signals in said plurality of partitions; (d) detecting from multiple partitions of said plurality of partitions, in order to correctly perform the taught method using a droplet digital PCR process.

Regarding Claim 35, modified California discloses the method of claim 34. California further discloses said plurality of signals is generated by said first plurality of oligonucleotide probes and said second plurality of oligonucleotide probes (Para. [0011], measuring a first set of signals generated by the chromophores...and d) measuring a second set of signals generated by the chromophores; Para. [0019], first primer or probe is a primer...In some cases, the second primer or probe comprises an oligonucleotide sequence complementary to the sequence of a target analyte).

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Regarding Claim 36, modified California discloses the method of claim 34. California further discloses at least one of said plurality of signals corresponds with a unique combination of two or more of said plurality of first nucleic acid molecules and/or said plurality of second nucleic acid molecules (Para. [0011], the first and second set of signals are used to detect each analyte of the plurality of analytes. In some cases, the method described herein further comprises: e) repeating step c and d at least once; and f) generating a set of signature profiles; wherein the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profiles; Para. [0044], terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides; Para. [0080]), but fails to explicitly disclose in a partition of said plurality of partitions in the same embodiment. However, since in an alternate embodiment California teaches droplet digital PCR (Para. [0010]), it would have been obvious to one of ordinary skill in the art to modify the method of the primary embodiment of California with the teaching of an alternate embodiment of California so that California's assay using a droplet digital PCR process involves in a partition of said plurality of partitions, in order to correctly perform the taught method using a droplet digital PCR process.

Regarding Claim 37, modified California discloses the method of claim 36. California further discloses said at least one of said plurality of signals uniquely corresponds with a presence of exactly two of said first plurality of nucleic acid molecules or said second plurality of nucleic acid molecules (Para. [0044], terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides; Para. [0011], c) performing at least one polymerization reaction with the plurality of primer pairs using the plurality of analytes as templates; and d) measuring a second set of signals generated by the chromophores; wherein the first and second set of signals are used to detect each analyte of the plurality of analytes. In some cases, the method described herein further comprises: e) repeating step c and d at least once; and f) generating a set of signature profiles; Para. [0080]. A signature profile is unique for each nucleotide mismatch, UU), but fails to explicitly disclose molecules in said partition in the same embodiment. However, since in an alternate embodiment California teaches droplet digital PCR (Para. [0010]), it would have been obvious to one of ordinary skill in the art to modify the method of the primary embodiment of California with the teaching of an alternate embodiment of California so that California's assay using a droplet digital PCR process involves molecules in said partition, in order to correctly perform the taught method using a droplet digital PCR process.

Claims 7 and 8 lack an inventive step under PCT Article 33(3) as being obvious over California Institute Of Technology (hereinafter California) in view of Lifecodexx AG (hereinafter Lifecodexx).

Regarding Claim 7, modified California discloses the method of claim 1. California fails to explicitly disclose said nucleic acid target is a chromosome. Lifecodexx is in the field of DNA detection (Lifecodexx Abstract) including with amplification (Lifecodexx Para. [0065]) and teaches said nucleic acid target is a chromosome (Lifecodexx Para. [0121], detection (or amount) of the first species of DNA (for example, from a chromosome, or part thereof, related to a chromosomal aneuploidy such as human chromosome 21)). It would have been obvious to one of ordinary skill in the art at the time of the invention to further modify California with the teaching of Lifecodexx to detail said nucleic acid target is a chromosome. The motivation for doing so would have been to identify and quantify chromosomes and thereby identify if a fetus has Downs Syndrome prior to birth (presence of an extra chromosome 21) during non-invasive pre-natal testing (Lifecodexx Para. [0081]) and thereby allow families to prepare additional resources if needed prior to birth.

Regarding Claim 8, modified California discloses the method of claim 7. California fails to explicitly disclose said chromosome is chromosome 21, chromosome 18, chromosome 15, chromosome 13, an X chromosome, or a Y chromosome. Lifecodexx teaches said chromosome is chromosome 21 (Lifecodexx Para. [0121], human chromosome 21). It would have been obvious to one of ordinary skill in the art at the time of the invention to further modify California with the teaching of Lifecodexx to detail said chromosome is chromosome 21. The motivation for doing so would have been to identify if a fetus has Downs Syndrome prior to birth (presence of an extra chromosome 21) during non-invasive pre-natal testing (Lifecodexx Para. [0081]) and thereby allow families to prepare additional resources if needed prior to birth.

Claims 11 and 12 lack an inventive step under PCT Article 33(3) as being obvious over California Institute Of Technology (hereinafter California) in view of Chromacode Inc. (hereinafter Chromacode).

Regarding Claim 11, modified California discloses the method of claim 9. California fails to explicitly disclose said amplifying degrades said plurality of oligonucleotide probes, thereby generating said plurality of signals. Chromacode is in the field of identifying nucleic acid targets with oligonucleotides by generating signals (Chromacode Abstract) and teaches said amplifying degrades said plurality of oligonucleotide probes, thereby generating said plurality of signals (Chromacode Para. [008], amplifying the up to thirty target nucleic acid sequences with the polymerase chain reaction, thereby degrading the signal oligonucleotides and permitting generation of up to thirty signals, generating up to thirty signals). It would have been obvious to one of ordinary skill in the art at the time of the invention to further modify California with the teaching of Chromacode to detail said amplifying degrades said plurality of oligonucleotide probes, thereby generating said plurality of signals. The motivation for doing so would have been to amplify the nucleic acid sequences (Chromacode Para. [008]) and thereby provide measurable signal intensities for the nucleic acid target identification (Chromacode Abstract).

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Regarding Claim 12, modified California discloses the method of claim 1. California fails to explicitly disclose said plurality of oligonucleotide probes are degraded by a nucleic acid enzyme, thereby releasing a signal tag from each of said plurality of oligonucleotide probes and generating said plurality of signals. Chromacode is in the field of identifying nucleic acid targets with oligonucleotides by generating signals (Chromacode Abstract) and teaches said plurality of oligonucleotide probes are degraded by a nucleic acid enzyme, thereby releasing a signal tag from each of said plurality of oligonucleotide probes and generating said plurality of signals (Chromacode Claims 8-10, signal oligonucleotides comprise a signal tag and a signal quenching tag...signal quenching tag is separated from the signal tag by oligonucleotide degradation during the polymerase chain reaction, thereby permitting the signal to be generated; Para. [008], degrading the signal oligonucleotides and permitting generation of up to thirty signals, generating up to thirty signals; polymerase is an enzyme that degrades nucleic acids). It would have been obvious to one of ordinary skill in the art at the time of the invention to further modify California with the teaching of Chromacode to detail said plurality of oligonucleotide probes are degraded by a nucleic acid enzyme, thereby releasing a signal tag from each of said plurality of oligonucleotide probes and generating said plurality of signals. The motivation for doing so would have been to define a pathway for the signal generation (Chromacode Para. [008]) and thereby provide measurable signal intensities for the nucleic acid target identification (Chromacode Abstract).

Claims 16 and 17 lack an inventive step under PCT Article 33(3) as being obvious over California Institute Of Technology (hereinafter California) in view of Macevicz.

Regarding Claim 16, modified California discloses the method of claim 1. California further discloses at least one signal of said plurality of signals corresponds with combinations of said plurality of nucleic acid molecules (Para. [0011], c) performing at least one polymerization reaction with the plurality of primer pairs using the plurality of analytes as templates; and d) measuring a second set of signals generated by the chromophores; wherein the first and second set of signals are used to detect each analyte of the plurality of analytes. In some cases, the method described herein further comprises: e) repeating step c and d at least once; and f) generating a set of signature profiles), but fails to explicitly disclose at least one signal corresponds with two or more unique combinations. Macevicz is in the field of signature sequences of polynucleotides determined by signals (Macevicz Abstract) and teaches at least one signal corresponds with two or more unique combinations (Macevicz Para. [0047], An important aspect of the present invention is based on the observation that when oligonucleotide tags representing different events, e.g. different nucleotides at the same locus of a template, have distinguishable labels, then the occurrence of so-called "doubles" (i.e., two different polynucleotides having the same oligonucleotide tag) can be detected by the presence of two distinct labels at the same hybridization site. Thus, the sample size may be much larger than that taught in the above references because "doubles" can simply be discarded or ignored during a detection step). It would have been obvious to one of ordinary skill in the art to further modify California with the teaching of Macevicz to detail at least one signal corresponds with two or more unique combinations. The motivation for doing so would have been to increase the sample size and thereby increase the sequencing efficiency (Macevicz Para. [0047]).

Regarding Claim 17, modified California discloses the method of claim 16. California further discloses said at least one signal of said plurality of signals (A) corresponds with a presence of one of said plurality of nucleic acid molecules (Para. [0011], methods of detecting a plurality of analytes, comprising: a) combining the plurality of analytes with a plurality of primer pairs, wherein each primer pair is specific to a single analyte and each primer of the primer pair is attached to at least one chromophore; b) measuring a first set of signals generated by the chromophores...d) measuring a second set of signals generated by the chromophores; wherein the first and second set of signals are used to detect each analyte of the plurality of analytes) and (B) corresponds with a presence of two of said plurality of nucleic acid molecules (Para. [0011], c) performing at least one polymerization reaction with the plurality of primer pairs using the plurality of analytes as templates; and d) measuring a second set of signals generated by the chromophores; wherein the first and second set of signals are used to detect each analyte of the plurality of analytes. In some cases, the method described herein further comprises: e) repeating step c and d at least once; and f) generating a set of signature profiles; Para. [0080], A signature profile is unique for each nucleotide mismatch, UU).

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Claims 65, 66, and 70 lack an inventive step under PCT Article 33(3) as being obvious over Ching et al. (hereinafter Ching) in view of University Of Washington Through Its Center For Commercialization (hereinafter Washington).

Regarding Claim 65, Ching discloses a method of quantifying target nucleic acid molecules in a sample (Para. [0012], a method for determining relative amounts of one or more target polynucleotides in a sample), the method comprising: (b) amplifying said plurality of nucleic acid molecules (Para. [0012], amplifying in the sample the one or more target polynucleotides), thereby generating a plurality of signals (Para. [0016], reading values of an optical signal from a first-stage amplification reaction, the optical signal being monotonically related to a concentration of an amplicon in the first-stage amplification reaction); (c) detecting said plurality of signals; (Para. [0016], reading values of an optical signal from a first-stage amplification reaction) (d) comparing members of said plurality of signals to one another (Para. [0016], computing a predetermined level from the values of the optical signal; (d) comparing the predetermined value with the most recent value of the optical signal; Para. [0012], (iii) comparing second amplicons of the second amplification reaction to amplicons of the at least one reference sequence in the first amplification reaction to determine relative amounts of the one or more target polynucleotides in the sample); and (e) quantifying said target nucleic acid molecules in said sample based on said comparing (Para. [0012], (iii) comparing second amplicons of the second amplification reaction to amplicons of the at least one reference sequence in the first amplification reaction to determine relative amounts of the one or more target polynucleotides in the sample), but fails to explicitly disclose (a) partitioning a plurality of nucleic acid molecules into a plurality of partitions; (b) amplifying in said plurality of partitions, said method not including a step of quantifying said plurality of nucleic acid molecules in any individual member of said plurality of partitions. Washington is in the field of digital measurements of nucleic acids (Washington Para. [0013]) and teaches (a) partitioning a plurality of nucleic acid molecules into a plurality of partitions (Washington Paras. [0066]-[0067], droplets of different volumes (diameters) can be generated…a sample can include an aqueous solution that includes a molecule of interest (e.g., a nucleic acid molecule)); (b) amplifying in said plurality of partitions (Washington Paras. [0066]-[0067], amplification of analyte (e.g. digital PCR) can be carried out simultaneously in all droplets of different volumes (sizes)…a sample can include an aqueous solution that includes a molecule of interest (e.g., a nucleic acid molecule)), and a method not including a step of quantifying a plurality of nucleic acid molecules in any individual member of said plurality of partitions (Washington Para. [0100], This example describes a non-limiting theoretical framework that can be used to describe certain aspects of the present invention described herein. It will be understood that the method described in this example is one of many ways to determine concentrations of sample using the present invention. Under this theoretical framework, it is assumed that the target molecules are present in the sample at a concentration Cs in units of molecules/volume. The sample is distributed into digital volumes of variable volumes; the distribution of target molecules into the droplets follows Poisson statistics...the amplification reaction will cause a droplet containing one or more molecules to be distinguishable from empty droplets, e.g. by its fluorescence intensity. In this method we know only whether a droplet is empty or occupied...in order to determine the concentration CS of target molecules, P(n>0, CSVi) needs to be summed over a sufficiently large number of droplets with respective volumes Vi; Para. [0067], a sample can include an aqueous solution that includes a molecule of interest (e.g., a nucleic acid molecule)). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Ching with the teaching of Washington to detail (a) partitioning a plurality of nucleic acid molecules into a plurality of partitions; (b) amplifying in said plurality of partitions, said method not including a step of quantifying said plurality of nucleic acid molecules in any individual member of said plurality of partitions. The motivation for doing so would have been to divide the sample up and allow for repetitive tests for comparison and thereby ensure testing accuracy.

Regarding Claim 66, modified Ching discloses the method of claim 65. Ching further discloses said comparing comprises generating one or more signal distribution curves from said plurality of signals (Para. [0056], FIGS. 1A and 1B, curves (1000) and (1002) represent accumulated amplicon of for example, a reference sequence and target polynucleotide, respectively, as determined by two different fluorescent signals generated by amplicon-specific probes) and analyzing said one or more signal distribution curves (Para. [0056] FIGS. 1A and 1B, a second-stage reaction is initiated when curve (1002) of the target amplicon reaches or exceeds a predetermined level (1006)).

Regarding Claim 70, modified Ching discloses the method of claim 65. Ching further discloses said plurality of nucleic acid molecules are derived from, correspond with, or are members of, said target nucleic acid molecules (Para. [0012], a method for determining relative amounts of one or more target polynucleotides in a sample).

Claims 67-69 lack an inventive step under PCT Article 33(3) as being obvious over Ching et al. (hereinafter Ching) in view of University Of Washington Through Its Center For Commercialization (hereinafter Washington) and Morgan et al. (hereinafter Morgan).

Regarding Claim 67, modified Ching discloses the method of claim 66. Ching further discloses said analyzing comprises for each of said one or more signal distribution curves (Para. [0056] FIGS. 1A and 1B, a second-stage reaction is initiated when curve (1002) of the target amplicon reaches or exceeds a predetermined level (1006)), but fails to explicitly disclose measuring an area under the curve (AUC). Morgan is in the field of molecular detection (Morgan Abstract) and teaches measuring an area under the curve (AUC) (Morgan Pg. 3 Col. 2 Para. [1] Lns. 4-5, area under the curve (AUC) represents an overall summary measure of the curve). It would have been obvious to one of ordinary skill in the art at the time of the invention to further modify Ching with the teaching of Morgan to detail measuring an area under the curve (AUC). The motivation for doing so would have been to detail the diagnostic performance of the test (Morgan Pg. 3 Col. 2 Para. [1] Lns. 1-5) and thereby best identify a target nucleic acid for treatment and/or diagnosis of a subject (Morgan Abstract).

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Regarding Claim 68, modified Ching discloses the method of claim 67. Ching further discloses (d) comprises comparing to a reference value (Para. [0059] FIG. 1B, closed-loop control, microprocessor (1080) may calculate values of characteristics of curves, such as (1013) or (1015) of FIG. 1B, at predetermined intervals so that they may be compared to a predetermined level), but fails to explicitly disclose comparing said AUC. Morgan teaches comparing said AUC (Morgan Pg. 3 Col. 2 Para. [1] Lns. 4-13, The area under the curve (AUC) represents an overall summary measure of the curve and the test's overall ability to accurately distinguish cases from non-cases. The Q* index, the highest point on the SROC curve that intersects the anti-diagonal, represents a summarization of test performance where sensitivity and specificity are equal (so the probability of an incorrect test result is the same for cases and non-cases). An AUC of one represents perfect discriminatory ability, while a Q* index of one represents perfect accuracy). It would have been obvious to one of ordinary skill in the art at the time of the invention to further modify Ching with the teaching of Morgan to detail comparing said AUC. The motivation for doing so would have been to detail the diagnostic performance of the test (Morgan Pg. 3 Col. 2 Para. [1] Lns. 1-5) and thereby best identify a target nucleic acid for treatment and/or diagnosis of a subject (Morgan Abstract).

Regarding Claim 69, modified Ching discloses the method of claim 67. Ching further discloses said one or more signal distribution curves comprises a plurality of signal distribution curves (Para. [0057] FIGS. 1A and 1B, "amplicon growth curve' means a curve, such as curves (1000), (1002), (1013), or (1015)), wherein (d) comprises comparing for each of said one or more signal distribution curves to one another (Para. [0057] FIGS. 1A and 1B, "amplicon growth curve' means a curve, such as curves (1000), (1002), (1013), or (1015); Para. [0075], it is desirable to compare readouts from different assays), but fails to explicitly disclose comparing an AUC to one another. Morgan teaches comparing an AUC to one another (Morgan Pg. 3 Col. 2 Para. [1] Lns. 4-13, The area under the curve (AUC) represents an overall summary measure of the curve and the test's overall ability to accurately distinguish cases from non-cases. The Q* index, the highest point on the SROC curve that intersects the anti-diagonal, represents a summarization of test performance where sensitivity and specificity are equal (so the probability of an incorrect test result is the same for cases and non-cases). An AUC of one represents perfect discriminatory ability, while a Q* index of one represents perfect accuracy). It would have been obvious to one of ordinary skill in the art at the time of the invention to further modify Ching with the teaching of Morgan to detail comparing an AUC to one another. The motivation for doing so would have been to detail the diagnostic performance of the test (Morgan Pg. 3 Col. 2 Para. [1] Lns. 1-5) and thereby best identify a target nucleic acid for treatment and/or diagnosis of a subject (Morgan Abstract).

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Claims 79-81 lack an inventive step under PCT Article 33(3) as being obvious over Lifecodexx AG (hereinafter Lifecodexx) in view of University Of Washington Through Its Center For Commercialization (hereinafter Washington).

Regarding Claim 79, Lifecodexx discloses a method of determining a quantity of a first target nucleic acid relative to a quantity of a second target nucleic acid in a sample (Paras. [0124]-[0125], detect a plurality (such as two or more) species of DNA in the sample, such methods can further comprise the steps of: determining the relative amount, preferably a ratio, of: (x) said first species of DNA detected with the first DMR (or first set of two or more DMR); and (y) said second species of DNA detected with the second DMR (or first set of two or more DMRs)), the method comprising: (a) providing a mixture comprising: i. a first plurality of nucleic acid molecules derived from, and/or corresponding with, said first target nucleic acid; and ii. a second plurality of nucleic acid molecules derived from, and/or corresponding with, said second target nucleic acid (Paras. [0016]-[0017], a first aspect, and as may be further described, defined, claimed or otherwise disclosed herein, the invention relates to a method for detecting in a sample from an individual an amount of a species of DNA originating from cells of a given type, which sample comprises said species of DNA in admixture with differently methylated DNA not originating from cells of said type; said method comprising the steps: (a) treating the DNA present in said sample with a reagent that differentially modifies methylated and nonmethylated DNA); (c) amplifying said first plurality of nucleic acid molecules and said second plurality of nucleic acid molecules (Para. [0065], In certain embodiments, prior to or as part of the detection that occurs as part of a step (b) and/or a step (c) of any method of present invention, each DNA region comprising said DMR(s) and/or said other region(s), respectively, is(are) amplified), thereby generating a plurality of signals; (d) detecting said plurality of signals (Para. [0123], said detected amount of each of said species of DNA is expressed as a relative concentration of said species of DNA to the respective amount of total DNA detected in said sample from the respective other region. In further of such embodiments, the methods of the present invention can further comprise the steps of detecting an amount of total DNA in a standard sample of DNA of known amount using the same other regions as used in step (c); and comparing the signal detected from said standard sample of DNA to the respective signal detected in step (c) for each of the other regions); and (e) based on said detecting, determining a ratio representative of a quantity of said first target nucleic acid relative to a quantity of said second target nucleic acid in said sample (Paras. [0124]-[0125], In those embodiments of the present invention that quantitatively detect a plurality (such as two or more) species of DNA in the sample, such methods can further comprise the steps of: determining the relative amount, preferably a ratio, of: (x) said first species of DNA detected with the first DMR (or first set of two or more DMR); and (y) said second species of DNA detected with the second DMR (or first set of two or more DMRs)), but fails to explicitly disclose (b) partitioning said mixture into a plurality of partitions; (c) amplifying in said plurality of partitions, or said method not including a step of quantifying, in any individual member of said plurality of partitions, said first plurality of nucleic acid molecules or said second plurality of nucleic acid molecules. Washington is in the field of digital measurements of nucleic acids (Washington Para. [0013]) and teaches (b) partitioning said mixture into a plurality of partitions (Washington Paras. [0066]-[0067], droplets of different volumes (diameters) can be generated...a sample can include an aqueous solution that includes a molecule of interest (e.g., a nucleic acid molecule)); (c) amplifying in said plurality of partitions (Washington Paras. [0066]-[0067], amplification of analyte (e.g. digital PCR) can be carried out simultaneously in all droplets of different volumes (sizes)...a sample can include an aqueous solution that includes a molecule of interest (e.g., a nucleic acid molecule)), and a method not including a step of quantifying, in any individual member of said plurality of partitions, a first plurality of nucleic acid molecules or a second plurality of nucleic acid molecules (Washington Para. [0100], This example describes a non-limiting theoretical framework that can be used to describe certain aspects of the present invention described herein. It will be understood that the method described in this example is one of many ways to determine concentrations of sample using the present invention. Under this theoretical framework, it is assumed that the target molecules are present in the sample at a concentration Cs in units of molecules/volume. The sample is distributed into digital volumes of variable volumes; the distribution of target molecules into the droplets follows Poisson statistics...the amplification reaction will cause a droplet containing one or more molecules to be distinguishable from empty droplets, e.g. by its fluorescence intensity. In this method we know only whether a droplet is empty or occupied...in order to determine the concentration CS of target molecules, P(n>0, CSVi) needs to be summed over a sufficiently large number of droplets with respective volumes Vi; Para. [0067], a sample can include an aqueous solution that includes a molecule of interest (e.g., a nucleic acid molecule)). It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify Lifecodexx with the teaching of Washington to detail (b) partitioning said mixture into a plurality of partitions; (c) amplifying in said plurality of partitions, and said method not including a step of quantifying, in any individual member of said plurality of partitions, said first plurality of nucleic acid molecules or said second plurality of nucleic acid molecules. The motivation for doing so would have been to divide the sample up and allow for repetitive tests for comparison and thereby ensure testing accuracy.

Regarding Claim 80, modified Lifecodexx discloses the method of claim 79. Lifecodexx further discloses further comprising, based on said ratio, quantifying said first target nucleic acid and said second target nucleic acid in said sample (Paras. [0123]-[0125], In alterative certain embodiments, one or more (preferably each) of said detection steps comprises quantitative detection and said detected amount of said species of DNA is expressed as an absolute amount of each or said species of DNA in said sample. In those embodiments of the present invention that quantitatively detect a plurality (such as two or more) species of DNA in the sample, such methods can further comprise the steps of: determining the relative amount, preferably a ratio, of: (x) said first species of DNA detected with the first DMR (or first set of two or more DMR); and (y) said second species of DNA detected with the second DMR (or first set of two or more DMRs); Para. [0198], a relative and/or absolute amount of foetal cfDNA and total cfDNA present in the plasma is determined).

Regarding Claim 81, modified Lifecodexx discloses the method of claim 79. Lifecodexx further discloses said ratio is representative of a fetal fraction (Para. [0106], cfDNA fraction is...of total cfDNA present in maternal plasma; Para. [0198]).

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Claim 54 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest:

Regarding claim 54, the prior art of record, individually or in combination, does not teach or fairly suggest the method of claim 52, wherein said method does not include a step of determining a number of partitions comprising a nucleic acid sequence corresponding to said target nucleic acid molecules.

The following prior art is made of record to support and further define the reasons for meeting the criteria set out in PCT Article 33(2)-(3) for claim 54:

(i) Washington discloses determining a number of partitions comprising a nucleic acid sequence corresponding to said target nucleic acid molecules (Paras. [0103]-[0108], The total number of occupied droplets in a simulation, NS, is counted...Results for a typical set of simulations are shown in FIG. 4. Simulations were performed with 10,000 droplets and six different concentrations...The droplet sizes were drawn from a uniform distribution of diameters between 4.0 and 190 microns. For each simulation, the droplets were sorted by volume, the droplet with the smallest volume was number 1 and the droplet with the largest volume was number 10,000. FIG. 4 plots the cumulative number of occupied droplets. The cumulative number occupied for the ith droplet is the sum of the occupied droplets for all droplet; Para. [0067], a sample can include an aqueous solution that includes a molecule of interest (e.g., a nucleic acid molecule)). Washington fails to fairly teach or suggest, either alone or in combination with the art, a method does not include a step of determining a number of partitions comprising a nucleic acid sequence corresponding to said target nucleic acid molecules.

Claims 1-20, 34-37, 52-54, 65-70, and 79-81 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.