

RAW SEQUENCE LISTING

The Biotechnology Systems Branch of the Scientific and Technical Information Center (STIC) no errors detected.

Application Serial Number: 11483,825A
Source: IFWO
Date Processed by STIC: 9/29/06

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IFWO

RAW SEQUENCE LISTING

DATE: 09/29/2006

PATENT APPLICATION: US/11/483,825A

TIME: 09:38:32

Input Set : A:\1306858_1.TXT

Output Set: N:\CRF4\09292006\K483825A.raw

3 <110> APPLICANT: Ericsson, Olof
 5 <120> TITLE OF INVENTION: Regulation Analysis by Cis Reactivity, RACR
 7 <130> FILE REFERENCE: 22639-3
 9 <140> CURRENT APPLICATION NUMBER: US 11/483,825A
 10 <141> CURRENT FILING DATE: 2006-07-10
 12 <150> PRIOR APPLICATION NUMBER: US 60/697,415
 13 <151> PRIOR FILING DATE: 2005-07-08
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Use of "Artificial" only as "<213> Organism" response is incomplete,
per 1.823(b) of New Sequence Rules. Valid response is Artificial Sequence.

Seq#:1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27
Seq#:28,29,30

VERIFICATION SUMMARY

DATE: 09/29/2006

PATENT APPLICATION: US/11/483,825A

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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(c) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
11/483,825	07/10/2006	Olof Ericsson	22639-3

CONFIRMATION NO. 8659

24256
DINSMORE & SHOHL, LLP
1900 CHEMED CENTER
255 EAST FIFTH STREET
CINCINNATI, OH45202

Title: Regulation analysis by cis reactivity, RACR

Publication No. US-2007-0020669-A1

Publication Date: 01/25/2007

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publicly available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently <http://www.uspto.gov/patft/>.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Office of Public Records. The Office of Public Records can be reached by telephone at (703) 308-9726 or (800) 972-6382, by facsimile at (703) 305-8759, by mail addressed to the United States Patent and Trademark Office, Office of Public Records, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently <http://pair.uspto.gov/>. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 703-305-3028.

Pre-Grant Publication Division, 703-605-4283



DTF

Docket No: 22639-3

PATENT

CERTIFICATE OF MAILING

I hereby certify that this paper is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop Amendment; Commissioner for Patents, P.O. Box 1450; Alexandria, VA 22313-1450 on February 22, 2007.

Marlene Troxle
Marlene Troxle

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

Applicant: Olof Ericsson

Serial No.: 11/483,825

Group Art Unit: TBD

Filed: July 10, 2006

Examiner: TBD

For: Regulation Analysis by Cis Reactivity, RACR

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

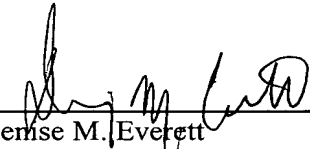
Dear Sir:

In accordance with 37 C.F.R. §§ 1.97 and 1.98 Applicant herewith submits certain additional patent references which the Patent & Trademark Office may wish to consider in examining the above-identified application. The references are listed on the attached Form PTO-1449.

Copies of the listed references are also provided. No representation is made or intended that a prior art search has been made or that no better art than that listed is available.

As this Statement is being submitted prior to issuance of the first office action on the merits, it is believed that no fee is required. Nonetheless, in the event this is mistaken or an office action has been mailed in crossing, please charge any fees required, or credit any overpayment, to Deposit Account No. 04-1133.

Respectfully submitted,

By:  _____

Denise M. Everett
Registration No. 47,552
Attorney for Applicant(s)
DINSMORE & SHOHL LLP
1900 Chemed Center
255 East Fifth Street
Cincinnati, Ohio 45202
(513) 977-8787



UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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11/483,825

07/10/2006

Olof Ericsson

22639-3

8659

24256 7590 05/22/2009
DINSMORE & SHOHL, LLP
1900 CHEMED CENTER
255 EAST FIFTH STREET
CINCINNATI, OH 45202

EXAMINER

WOOLWINE, SAMUEL C

ART UNIT

PAPER NUMBER

1637

MAIL DATE

DELIVERY MODE

05/22/2009

PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No. 11/483,825	Applicant(s) ERICSSON, OLOF	
	Examiner SAMUEL WOOLWINE	Art Unit 1637	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 1 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☐ Responsive to communication(s) filed on ____.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 1-43 is/are pending in the application.
- 4a) Of the above claim(s) ____ is/are withdrawn from consideration.
- 5) ☐ Claim(s) ____ is/are allowed.
- 6) ☐ Claim(s) ____ is/are rejected.
- 7) ☐ Claim(s) ____ is/are objected to.
- 8) ☒ Claim(s) 1-43 are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on ____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. ____.
 3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- | | |
|--|---|
| 1) ☐ Notice of References Cited (PTO-892) | 4) ☐ Interview Summary (PTO-413) |
| 2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948) | Paper No(s)/Mail Date. ____. |
| 3) ☐ Information Disclosure Statement(s) (PTO/SB/08) | 5) ☐ Notice of Informal Patent Application |
| Paper No(s)/Mail Date ____. | 6) ☐ Other: ____. |

DETAILED ACTION

Election/Restrictions

Restriction to one of the following inventions is required under 35 U.S.C. 121:

- I. Claims 1-37, drawn to methods of identifying interactions between molecules of interest, classified in class 435, subclass 6.
- II. Claims 38-43, drawn to compositions comprising "molecules of interest" coupled with nucleic acids, classified in class 536, subclass 23.1.

The inventions are distinct, each from the other because of the following reasons:

Inventions II and I are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product. See MPEP § 806.05(h). In the instant case the compositions of Group II could be used in a materially different process than is recited in the methods of Group I. Since "molecules" of interest can be proteins, such as antibodies, a composition comprising an antibody coupled to a nucleic acid could be used to detect an antigen in a sandwich immuno-PCR assay. A primary antibody is immobilized in the well of a microtitre plate. A sample is added to the well, and antigen present in the sample is captured by the primary antibody. After washing the well, the composition comprising the antibody-nucleic acid conjugate is added to the well, where it binds to the captured antigen. After washing the well, primers complementary to the nucleic acid are added, along with polymerase and other PCR reagents, including fluorescent

intercalator dye. A PCR is conducted, whereby the presence of the antibody-nucleic acid conjugate, and hence the antigen, is detected by increase in fluorescence as the intercalator binds the double-stranded PCR product.

Restriction for examination purposes as indicated is proper because all these inventions listed in this action are independent or distinct for the reasons given above and there would be a serious search and examination burden if restriction were not required because one or more of the following reasons apply:

- (a) the inventions have acquired a separate status in the art in view of their different classification;
- (b) the inventions have acquired a separate status in the art due to their recognized divergent subject matter;
- (c) the inventions require a different field of search (for example, searching different classes/subclasses or electronic resources, or employing different search queries);
- (d) the prior art applicable to one invention would not likely be applicable to another invention;
- (e) the inventions are likely to raise different non-prior art issues under 35 U.S.C. 101 and/or 35 U.S.C. 112, first paragraph.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a invention to be examined even though the requirement may be traversed (37 CFR 1.143) and (ii) identification of the claims encompassing the elected invention.

The election of an invention may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected invention.

If claims are added after the election, applicant must indicate which of these claims are readable upon the elected invention.

Should applicant traverse on the ground that the inventions are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

This application contains claims directed to the following patentably distinct species:

A1-association via ligation (claims 7, 35)

A2-association via polymerization (claims 8, 36)

The species are independent or distinct because claims to the different species recite the mutually exclusive characteristics of such species. In addition, these species are not obvious variants of each other based on the current record.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claims 1-6, 9-34 and 37 are generic.

In addition, a provisional election of species is required for one of the "molecules of interest" recited in claims 9 and 26. Note that election of any option other than "protein" may result in the withdrawal of claims reciting protein as one of the "molecules of interest" (claims 10-13, 16, 27-29).

There is an examination and search burden for these patentably distinct species due to their mutually exclusive characteristics. The species require a different field of search (e.g., searching different classes/subclasses or electronic resources, or employing different search queries); and/or the prior art applicable to one species would not likely be applicable to another species; and/or the species are likely to raise different non-prior art issues under 35 U.S.C. 101 and/or 35 U.S.C. 112, first paragraph.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species to be examined even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected species**, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

The election of the species may be made with or without traverse. To preserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the election of species requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected species.

Should applicant traverse on the ground that the species are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the species unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other species.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which depend from or otherwise require all the limitations of an allowable generic claim as provided by 37 CFR 1.141.

The examiner has required restriction between product and process claims. Where applicant elects claims directed to the product, and the product claims are subsequently found allowable, withdrawn process claims that depend from or otherwise require all the limitations of the allowable product claim will be considered for rejoinder.

Art Unit: 1637

All claims directed to a nonelected process invention must require all the limitations of an allowable product claim for that process invention to be rejoined.

In the event of rejoinder, the requirement for restriction between the product claims and the rejoined process claims will be withdrawn, and the rejoined process claims will be fully examined for patentability in accordance with 37 CFR 1.104. Thus, to be allowable, the rejoined claims must meet all criteria for patentability including the requirements of 35 U.S.C. 101, 102, 103 and 112. Until all claims to the elected product are found allowable, an otherwise proper restriction requirement between product claims and process claims may be maintained. Withdrawn process claims that are not commensurate in scope with an allowable product claim will not be rejoined. See MPEP § 821.04(b). Additionally, in order to retain the right to rejoinder in accordance with the above policy, applicant is advised that the process claims should be amended during prosecution to require the limitations of the product claims. **Failure to do so may result in a loss of the right to rejoinder.** Further, note that the prohibition against double patenting rejections of 35 U.S.C. 121 does not apply where the restriction requirement is withdrawn by the examiner before the patent issues. See MPEP § 804.01.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to SAMUEL WOOLWINE whose telephone number is (571)272-1144. The examiner can normally be reached on Mon-Fri 9:00am-5:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on (571) 272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Samuel Woolwine/
Examiner, Art Unit 1637

Docket No. 3995937-179061

PATENT

CERTIFICATE OF EFS ELECTRONIC TRANSMISSION

I hereby certify that this paper is being transmitted to the United States Patent and Trademark Office by the USPTO EFS web electronic filing system on July 20, 2009.

/Holly D. Kozlowski/

Holly D. Kozlowski

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

Applicant: Olof Ericsson : Confirmation No.: 8659
Serial No.: 11/483,825 : Group Art Unit: 1637
Filing Date: July 10, 2006 : Examiner: Samuel C. Woolwine
For: **Regulation Analysis by Cis Reactivity, RACR**

AMENDMENT AND RESPONSE TO RESTRICTION REQUIREMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Official Action dated May 22, 2009, please amend the present application as follows:

Amendments to the Claims are set forth in the Listing of Claims which begins on Page 2 of this paper.

Remarks begin on Page 15 of this paper.

Amendments to the Claims:

The following Listing of Claims replaces all prior versions and listings of the claims in this application.

Listing of the Claims:

1. (Currently Amended) A method of detecting affinity interactions between at least two molecules of interest, the method comprising:
 - a. forming a plurality of interactors by coupling each molecule of interest with at least one nucleic acid moiety comprising an identification sequence element and ~~at~~ an association element;
 - b. promoting an association between at least two nucleic acid moieties from different interactors to form a plurality of unique associated oligonucleotides, wherein each nucleic acid moiety may form more than one unique associated oligonucleotide, and wherein each unique associated oligonucleotide comprises at least two identification sequence elements derived from the at least two nucleic acid moieties;
 - c. selecting the plurality of unique associated oligonucleotides; and
 - d. subjecting the selected associated oligonucleotides to an analysis that permits detection of the at least two identification sequence elements.
2. (Original) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 1, wherein the plurality of interactors comprises more than two interactors.

3. (Original) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 1, wherein selecting the plurality of associated oligonucleotides is accomplished via enhancement.
4. (Currently Amended) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 3 2, wherein enhancement is by PCR amplification.
5. (Original) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 1, wherein coupling is covalent or noncovalent.
6. (Original) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 5, wherein coupling is covalent.
7. (Original) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 1, wherein association is via ligation.
8. (Original) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 1, wherein association is via polymerization.
9. (Original) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 1, wherein at least one molecule of interest comprises a protein, a protein domain, a peptide, a peptoid, a functional enzyme, a small molecule, a polymer, a non-proteinaceous cellular product, a virus particle, a cell metabolite, a lipid, a carbohydrate, a

nucleic acid, an inorganic compound, a reactive compound, or any combination thereof,
however, wherein all molecules of interest are not nucleic acids.

10. (Original) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 9, wherein at least one molecule of interest comprises at least one protein.

11. (Currently Amended) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 10 9, wherein the protein is an expression product of a nucleic acid molecule, and further wherein at least some of the plurality of interactors are ~~is~~ formed by coupling the protein with a nucleic acid moiety comprising an identification sequence element derived from the nucleic acid molecule.

12. (Original) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 11, wherein the nucleic acid molecule comprises ribonucleic acid or deoxyribonucleic acid.

13. (Original) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 9, wherein the at least two molecules of interest comprise at least one protein and at least one nucleic acid.

14. (Original) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 1, wherein detecting further comprises quantifying and/or determining strength of the affinity interactions.

15. (Original) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 1, wherein the analysis comprises high throughput screening.

16. (Currently Amended) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 15 ~~43~~, wherein the high throughput screening comprises a micro array analysis.

17. (Original) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 1, wherein the analysis comprises nucleic acid sequencing.

18. (Original) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 17, wherein the analysis comprises sequencing by synthesis.

19. (Original) The method of detecting affinity interactions between at least two molecules of interest as recited in claim 1, wherein promoting an association between at least two nucleic acid moieties to form a plurality of associated oligonucleotides comprises manipulating conditions to maximize cis associations and to minimize trans associations between the at least two nucleic acid moieties.

20. (Currently Amended) A method of detecting functional interactions between at least two molecules of interest, the method comprising:

- a. forming a plurality of interactors by coupling each molecule of interest with at least one nucleic acid moiety, the nucleic acid moiety comprising an identification sequence element and an association element;
- b. forming a plurality of cis-reactive cells wherein a cis-reactive cell comprises at least two interactors bound in proximity to one another by an associated oligonucleotide formed from the association between at least two nucleic acid moieties, wherein the associated oligonucleotide comprises at least two identification elements derived from the at least two nucleic acid moieties;
- c. subjecting the plurality of cis-reactive cells to conditions which stimulate a desired functional interaction having a detectable trace;
- d. selecting all cis-reactive cells exhibiting the detectable trace; and
- e. subjecting the associated oligonucleotides from the cis-reactive cells selected in step (d) to an analysis that permits detection of the at least two identification sequence elements.

21. (Original) The method of detecting functional interactions between at least two molecules of interest as recited in claim 20, wherein selecting all cis-reactive cells exhibiting the detectable trace is accomplished via enhancement.

22. (Original) The method of detecting functional interactions between at least two molecules of interest as recited in claim 21, wherein selecting all cis-reactive cells exhibiting the detectable trace is accomplished affinity purification.

23. (Original) A method of detecting functional interactions between at least two molecules of interest as recited in claim 20, further comprising enhancing the selected associated oligonucleotides by PCR amplification.

24. (Original) A method of detecting functional interactions between at least two molecules of interest as recited in claim 20, wherein coupling is covalent or noncovalent.

25. (Original) A method of detecting functional interactions between at least two molecules of interest as recited in claim 24, wherein coupling is covalent.

26. (Original) A method of detecting functional interactions between at least two molecules of interest, as recited in claim 20, wherein at least one molecule of interest comprises a protein, a protein domain, a peptide, a peptoid, a functional enzyme, a small molecule, a polymer, a non-proteinaceous cellular product, a virus particle, a cell metabolite, a lipid, a carbohydrate, a nucleic acid, an inorganic compound, a reactive compound, or any combination thereof, however, wherein not all molecules of interest are nucleic acids.

27. (Currently Amended) A method of detecting functional interactions between at least two molecules of interest, as recited in claim ~~26~~ 25, wherein at least one molecule of interest comprises at least one protein.

28. (Currently Amended) The method of detecting functional interactions between at least two molecules of interest as recited in claim 27, wherein the protein is an expression product of a

nucleic acid molecule, and further wherein at least some of the plurality of interactors are ~~is~~ formed by coupling the protein with a nucleic acid moiety comprising an identification sequence element derived from the nucleic acid molecule.

29. (Original) A method of detecting functional interactions between at least two molecules of interest, as recited in claim 26, wherein the at least two molecules of interest comprises at least one protein and at least one nucleic acid.

30. (Original) A method of detecting functional interactions between at least two molecules of interest, as recited in claim 20, wherein detecting further comprises quantifying the functional interactions.

31. (Original) A method of detecting functional interactions between at least two molecules of interest, as recited in claim 20, wherein the analysis comprises high throughput screening.

32. (Original) The method of detecting functional interactions between at least two molecules of interest as recited in claim 31, wherein the high throughput screening comprises a micro array analysis.

33. (Original) The method of detecting functional interactions between at least two molecules of interest as recited in claim 20, wherein the analysis comprises nucleic acid sequencing.

34. (Original) The method of detecting functional interactions between at least two molecules of interest as recited in claim 33, wherein the analysis comprises sequencing by synthesis.

35. (Original) The method of detecting functional interactions between at least two molecules of interest as recited in claim 20, wherein association is via ligation.

36. (Original) The method of detecting functional interactions between at least two molecules of interest as recited in claim 20, wherein association is via polymerization.

37. (Original) The method of detecting functional interactions between the at least two molecules of interest as recited in claim 20, wherein the conditions for stimulating the functional interaction between the at least two molecules of interest comprises manipulating conditions to maximize cis reactivity and to minimize trans reactivity between the at least two molecules of interest.

38. (Original) A library of interactors wherein each interactor comprises at least one molecule of interest coupled with at least one nucleic acid moiety comprising: a unique identification sequence element; and an association element, wherein each association element may associate with association elements from other interactors in the library to form a plurality of unique associated oligonucleotides, each oligonucleotide comprising at least two unique identification sequence elements derived from at least two different interactors, however wherein all molecules of interest are not nucleic acid.

39. (Original) The library of interactors according to claim 38 wherein the at least one molecule of interest comprises protein and the at least one nucleic acid moiety comprises DNA, wherein the DNA may be double or single stranded.

40. (Original) The library of interactors according to claim 38 wherein the at least one molecule of interest comprises a protein that is an expression product of a nucleic acid molecule, and further wherein the identification sequence element derives from the nucleic acid molecule.

41. (Original) A kit for assaying interactions between at least two molecules of interest, the kit comprising: a. a library of interactors as recited in claim 38; b. a plurality of oligonucleotides comprising sequence elements which enable specific detection of the unique associated oligonucleotides.

42. (Original) The kit according to claim 41, wherein at least some of the plurality of oligonucleotides is immobilized on a solid phase.

43. (Original) The kit according to claim 42, wherein the solid phase comprises a microarray.

REMARKS

The present Amendment and Response to Restriction Requirement is submitted in response to the Official Action dated May 22, 2009.

By the present amendment, claims 1, 11, 20 and 28 are amended for several matters of form and claims 4, 11, 16 and 27 are amended to change their dependency. It is believed that these changes do not involve any introduction of new matter, whereby entry is in order and is respectfully requested.

In the Official Action, the Examiner required restriction under 35 U.S.C. §121 between Group I, claims 1-37, drawn to methods of identifying interactions between molecules of interest, and Group II, claims 38-43, drawn to compositions comprising molecules of interest coupled with nucleic acids. The Examiner asserted that because the compositions of claims 38-43 could be used in a materially different process than those of claims 1-37, restriction is appropriate.

Applicant hereby elects Group I, claims 1-37. However, this restriction requirement is traversed on the basis that claims 38-43, reciting a library of interactors or a kit comprising a library of interactors, are essentially directed to the product of step (a) of claims 1 and 20, and the methods of claims 1 and 20 are directed to investigation of reactions within “libraries” of such interactors, as described, for example, at page 9, second full paragraph, of the specification. In view of this commonality, it would not be unduly burdensome for the Examiner to examine claims 38-43 simultaneously with claims 1-37. Accordingly, reconsideration of the restriction requirement and examination of claims 1-43 are respectfully requested. In the event that the restriction requirement is maintained, Applicant expressly reserves the right to file a divisional

application directed to the non-elected subject matter. It is believed that this represents a complete response to the restriction requirement.

The Examiner also asserted that the claims are directed to patentably distinct species, namely wherein the association is via ligation as in claims 7 and 35 and wherein the association is via polymerization as in claims 8 and 36, and required election under 35 U.S.C. §121 of a single species for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Applicant hereby elects the species wherein association is via ligation. Claims 1-7, 9-35, and 37-43 are believed to read on the elected species.

Finally, the Examiner indicated that a provisional election of species is required for the molecules of interest recited in claims 9 and 26. Applicant provisionally elects the species wherein at least one molecule of interest is a protein. All of claims 1-43 are believed to read on the elected species.

It is believed that the above represents a complete response to the Official Action. Examination on the merits is respectfully requested. Please charge any fees required in connection with the present communication, or credit any overpayment, to Deposit Account No. 503915.

Respectfully submitted,

/Holly D. Kozlowski/

Holly D. Kozlowski, Reg. No. 30,468
Porter, Wright, Morris & Arthur LLP
250 East Fifth Street, Suite 2200
Cincinnati, Ohio 45202
(513) 369-4224

Docket No. 3995937-179061

PATENT

CERTIFICATE OF EFS ELECTRONIC TRANSMISSION

I hereby certify that this paper is being transmitted to the United States Patent and Trademark Office by the USPTO EFS web electronic filing system on July 20, 2009.

/Holly D. Kozlowski/
Holly D. Kozlowski

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

Applicant: Olof Ericsson : Confirmation No.: 8659
Serial No.: 11/483,825 : Group Art Unit: 1637
Filing Date: July 10, 2006 : Examiner: Samuel C. Woolwine
For: **Regulation Analysis by Cis Reactivity, RACR**

REQUEST FOR EXTENSION OF TIME

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Applicant requests a one month extension of time for responding to the Official Action dated May 22, 2009, to thereby extend the deadline for response to the Official Action for one month, from June 22, 2009 to July 22, 2009. Please charge the extension fee of \$130.00 to Deposit Account No. 503915.

Respectfully submitted,

/Holly D. Kozlowski/

Holly D. Kozlowski
Registration No. 30,468
Porter Wright Morris & Arthur LLP
250 East Fifth Street, Suite 2200
Cincinnati, Ohio 45202
(513) 369-4224

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

POWER OF ATTORNEY TO PROSECUTE APPLICATIONS BEFORE THE USPTO

I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(b).

I hereby appoint:



Practitioners associated with the Customer Number:

23570

OR



Practitioner(s) names below (if more than ten patent practitioners are to be named, then a customer number must be used):

Name	Registration Number	Name	Registration Number

as attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignment documents attached to this form in accordance with 37 CFR 3.73(b)

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(b) to:



The address associated with Customer Number:

23570

OR



Firm or Individual Name

Address

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Assignee Name and Address:

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Dag Hammarskjölds väg 54A

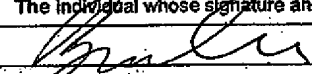
SE-751 83 Uppsala

Sweden

A copy of this form, together with a statement under 37 CFR 3.73(b) (Form PTO/SB/96 or equivalent) is required to be filed in each application in which this form is used. The statement under 37 CFR 3.73(b) may be completed by one of the practitioners appointed in this form if the appointed practitioner is authorized to act on behalf of the assignee, and must identify the application in which this Power of Attorney is to be filed.

SIGNATURE of Assignee of Record

The individual whose signature and title is supplied below is authorized to act on behalf of the assignee

Signature		Date	6 Oct 2008
Name	Björn Ekström	Telephone	+46-18-444 3971
Title	President and CEO		

This collection of information is required by 37 CFR 1.31, 1.32 and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS: SEND TO: Commissioner of Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and selection option 2.

CBCINNA/150393 v.01

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STATEMENT UNDER 37 CFR 3.73(b)Applicant/Patent Owner: Olof EricssonApplicant No./Patent No.: 11/483,825US Filing Date: July 10, 2006Entitled: Nucleic Acid EnrichmentOlink AB, a Corporation

(Name of Assignee)

(Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that it is:

1. ☒ the assignee of the entire right, title, and interest; or
 2. ☐ an assignee of less than the entire right, title and interest
 (The extent (by percentage) of its ownership interest is _____%)

in the patent application/patent identified above by virtue of either:

- A. ☒ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 018056, Frame 0075, or for which a copy thereof is attached.

OR

- B. ☐ A chain of title from the inventor(s), of the patent applicant/patent identified above, to the current assignee as follows:

1. From: _____ To: _____
 A copy of the document is attached.

2. From: _____ To: _____
 A copy of the document is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet.

☐ As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

 Signature

 Date

 Printed or Typed Name

 Telephone Number

 Title

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

CINCINNATI/157509 v.01

Electronic Patent Application Fee Transmittal

Application Number:	11483825			
Filing Date:	10-Jul-2006			
Title of Invention:	Regulation analysis by cis reactivity, RACR			
First Named Inventor/Applicant Name:	Olof Ericsson			
Filer:	Holly D. Kozlowski			
Attorney Docket Number:	22639-3			
Filed as Large Entity				
Utility under 35 USC 111(a) Filing Fees				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 1 month with \$0 paid	298 1251	1	130	130

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
Total in USD (\$)				130

Electronic Acknowledgement Receipt

EFS ID:	5729605
Application Number:	11483825
International Application Number:	
Confirmation Number:	8659
Title of Invention:	Regulation analysis by cis reactivity, RACR
First Named Inventor/Applicant Name:	Olof Ericsson
Customer Number:	24256
Filer:	Holly D. Kozlowski
Filer Authorized By:	
Attorney Docket Number:	22639-3
Receipt Date:	20-JUL-2009
Filing Date:	10-JUL-2006
Time Stamp:	11:33:54
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	\$ 130
RAM confirmation Number	7074
Deposit Account	503915
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.301 (Patent application and reexamination processing fees)

300

Alamar Exhibit 1002

Alamar v. Olink

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Response to Election / Restriction Filed	AmendmentandResponsetoRestrictionRequirement.pdf	50535	no	12
			1e4d9e41d1685d29f7ba0b6c2b3fb7ae43ddd91		
Warnings:					
Information:					
2	Extension of Time	ExtensionofTime.pdf	17119	no	1
			59c3cd177e51b13be7d37fb4937d4a4452ac06e6		
Warnings:					
Information:					
3	Power of Attorney	OlinkPowerofAttorney.pdf	55833	no	1
			5bcbf50ae8be1a29bb91e4117d7c06c8b0c377e8		
Warnings:					
Information:					
4	Assignee showing of ownership per 37 CFR 3.73(b).	373Statement.pdf	25383	no	1
			e9c90e2bd1b2510a5fbbd9518ef71c95922ebcfa		
Warnings:					
Information:					
5	Fee Worksheet (PTO-875)	fee-info.pdf	30183	no	2
			a3c3b3b3bec924b0d66fd7edd807d133b55438b2		
Warnings:					
Information:					
Total Files Size (in bytes):			179053		
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> 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. <u>New International Application Filed with the USPTO as a Receiving Office</u> 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.					

301

Alamar Exhibit 1002

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875					Application or Docket Number 11/483,825		Filing Date 07/10/2006		☐ To be Mailed	
APPLICATION AS FILED – PART I										
(Column 1)			(Column 2)		SMALL ENTITY ☐		OR		OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE (\$)	FEE (\$)	RATE (\$)	FEE (\$)				
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A		N/A					
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A		N/A					
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A		N/A					
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X \$	=	OR	X \$	=			
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X \$	=		X \$	=			
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$250 (\$125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).									
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))										
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL				
APPLICATION AS AMENDED – PART II										
(Column 1)			(Column 2)		(Column 3)		SMALL ENTITY		OR OTHER THAN SMALL ENTITY	
AMENDMENT	07/20/2009	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)	ADDITIONAL FEE (\$)	RATE (\$)	ADDITIONAL FEE (\$)		
	Total (37 CFR 1.16(i))	* 33	Minus	** 43	= 0	X \$ =	OR	X \$52=	0	
	Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0	X \$ =	OR	X \$220=	0	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))									
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0
AMENDMENT	Total (37 CFR 1.16(i))	*	Minus	**	=	X \$ =	OR	X \$ =		
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X \$ =	OR	X \$ =		
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))									
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	
* If the entry in column 1 is less than the entry in column 2, write "0" in column 3. ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.										

Legal Instrument Examiner:
/Mamye Wagstaff/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.



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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
11/483,825	07/10/2006	Olof Ericsson	22639-3

CONFIRMATION NO. 8659

POA ACCEPTANCE LETTER

23570
PORTER WRIGHT MORRIS & ARTHUR, LLP
INTELLECTUAL PROPERTY GROUP
41 SOUTH HIGH STREET
28TH FLOOR
COLUMBUS, OH 43215



Date Mailed: 09/14/2009

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 07/20/2009.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

/miwagstaff/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101



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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
11/483,825	07/10/2006	Olof Ericsson	22639-3

24256
DINSMORE & SHOHL LLP
1900 CHEMED CENTER
255 EAST FIFTH STREET
CINCINNATI, OH 45202

CONFIRMATION NO. 8659
POWER OF ATTORNEY NOTICE



Date Mailed: 09/14/2009

NOTICE REGARDING CHANGE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 07/20/2009.

- The Power of Attorney to you in this application has been revoked by the assignee who has intervened as provided by 37 CFR 3.71. Future correspondence will be mailed to the new address of record(37 CFR 1.33).

/miwagstaff/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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11/483,825

07/10/2006

Olof Ericsson

22639-3

8659

23570

7590

12/30/2009

PORTER WRIGHT MORRIS & ARTHUR, LLP
INTELLECTUAL PROPERTY GROUP

41 SOUTH HIGH STREET

28TH FLOOR

COLUMBUS, OH 43215

EXAMINER

WOOLWINE, SAMUEL C

ART UNIT

PAPER NUMBER

1637

MAIL DATE

DELIVERY MODE

12/30/2009

PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No. 11/483,825	Applicant(s) ERICSSON, OLOF	
	Examiner SAMUEL C. WOOLWINE	Art Unit 1637	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 20 July 2009.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 1-43 is/are pending in the application.
- 4a) Of the above claim(s) 38-43 is/are withdrawn from consideration.
- 5) ☒ Claim(s) 20-22 and 24-37 is/are allowed.
- 6) ☒ Claim(s) 1-19 and 23 is/are rejected.
- 7) ☐ Claim(s) _____ is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. _____.
 3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- | | |
|--|---|
| 1) ☒ Notice of References Cited (PTO-892) | 4) ☐ Interview Summary (PTO-413) |
| 2) ☐ Notice of Draftperson's Patent Drawing Review (PTO-948) | Paper No(s)/Mail Date. _____ |
| 3) ☒ Information Disclosure Statement(s) (PTO/SB/08) | 5) ☐ Notice of Informal Patent Application |
| Paper No(s)/Mail Date <u>09/25/2006;02/26/2007</u> . | 6) ☐ Other: _____ |

DETAILED ACTION

Election/Restrictions

Applicant's election with traverse of Group I, claims 1-37 in the reply filed on 07/20/2009 is acknowledged. The traversal is on the ground(s) that since products of Group II are directed to the products of step (a) of the methods of Group I, there would not be an undue burden of search. This is not found persuasive because the search for each invention would be different. A search for Group I would require searching for methods aimed at detecting affinity and functional interactions between two molecules of interest, which would not be required for Group II. A search for Group II would require a search for the basic structures involved (nucleic acids joined to "molecules of interest"), and finding such in the prior art would not indicate patentability of the methods of Group I.

The requirement is still deemed proper and is therefore made FINAL.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 3 and 21 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

It is not clear what is encompassed by the term "enhancement". Claims 4 indicates that PCR amplification is an "enhancement", and claim 22 indicates that affinity purification is an "enhancement", but the scope of claims 3 and 21 is vague and

indefinite since it cannot be determined what would constitute "enhancement" (other than PCR or affinity purification) and what would not. There is no clear definition of this use of the term "enhancement" in the specification.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.

(e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

Claims 1-12, 15-17 and 19 are rejected under 35 U.S.C. 102(a), or alternatively under 35 U.S.C. 102(e) as being anticipated by Thisted et al (WO 2005/026387 A1).

With regard to claim 1, Thisted taught *a method of detecting affinity interactions between at least two molecules of interest...*

See abstract: "In another aspect, the invention provides a method for selecting compounds which binds to a target."

See page 59, line 5: "The second aspect of the present invention relates to a method for identifying from a library a display molecule having affinity towards one or more molecular targets."

the method comprising: a. forming a plurality of interactors by coupling each molecule of interest with at least one nucleic acid moiety comprising an identification sequence element and an association element...

See abstract: "...the invention relates to a method in which a target associated with an oligonucleotide initially is mixed with a library of complexes, each complex comprising a display molecule and an oligonucleotide identifying said display module."

See page 62, line 4: "...mixing one or more molecular target(s) associated with target oligonucleotide(s) and a library of bifunctional complexes, each bifunctional complex of the library comprising a display molecule attached to an identifier oligonucleotide, which codes for said display molecule..."

In Thisted, the "interactors" are the targets and the display molecules, each having an associated nucleic acid moiety. See also figure 1, where the "association elements" of the nucleic acid moieties correspond to those portions of the target oligonucleotide and identifier oligonucleotides which anneal to the connector, while the "identification sequence elements" correspond to the remaining portions of the target oligonucleotide and identifier oligonucleotides. See also example 1, page 119, which indicates the "association elements" in bold (the "identification sequence elements" would be the remainder of the sequence in each oligonucleotide). In figure 2C, the "association elements" correspond to the complementary portions (i.e. the overlap) between the two oligonucleotides.

b. promoting an association between at least two nucleic acid moieties from different interactors to form a plurality of unique associated oligonucleotides, wherein

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each nucleic acid moiety may form more than one unique associated oligonucleotide, and wherein each unique associated oligonucleotide comprises at least two identification sequence elements derived from the at least two nucleic acid moieties...

See abstract: "...due to an increased proximity, the target oligonucleotide is coupled to the identifier oligonucleotide of complexes having a display molecule with affinity towards the target."

See page 62, line 8: "...coupling to the target oligonucleotide(s) the identifier oligonucleotide of complexes comprising display molecules binding to the target..."

See figures 1, 2 and 4 and related descriptions on pages 113-115.

c. selecting the plurality of unique associated oligonucleotides...

See discussion of claims 3 and 4 below.

d. subjecting the selected associated oligonucleotides to an analysis that permits detection of the at least two identification sequence elements...

See abstract: "...the coupled nucleotides are analysed to deduce at least the identity of the display molecule."

See page 62, line 10: "...deducing the identity of the binding display molecule(s) and/or the molecular target(s) from the coupled product between the identifier oligonucleotide(s) and the target oligonucleotide(s)."

The principle behind Thisted's method is described on page 62, lines 14-31:

The second aspect of the present invention is based on the realization that a close proximity of the identifier oligonucleotides relative to the target oligonucleotide is obtained when a display molecule has binding affinity towards a target and therefore, will be more prone to be coupled to the target oligonucleotide compared to identifier oligonucleotides of complexes not comprising a display molecule having affinity toward the target. The tendency to be coupled together depends on various factors such as (1) the affinity of the displayed molecule towards the target, (2) the length between the displayed molecule and the coupling point on the identifier oligonucleotide sequence as well as the length between the target molecule and the coupling point on the target sequence, and (3) possible steric effects resulting from the nature of the target or the oligonucleotides. The proximity effect, and thus the power of selectivity, will increase with higher local concentration of ends of nucleotides to be coupled together. Thus, various embodiments of the present invention may be envisaged to fine-tune the local concentration of the ends of the oligonucleotides, including appropriately selected lengths of target and identifier oligonucleotides, site of attachment of the target oligonucleotide to the target relative to the binding site of the display molecule, and size of target.

With regard to claim 2, Thisted clearly contemplated more than two interactors. See cited passages above and also page 64, lines 22-25. See also figure 4 and related description on page 115.

With regard to claims 3 and 4, Thisted taught "enhancement" in the form of PCR (see figures 1 and 4 and related descriptions on pages 113 and 115, for example).

With regard to claims 5 and 6, Thisted taught that the target (molecule of interest) could be associated with the target oligonucleotide (nucleic acid moiety) by either a covalent or non-covalent linkage (page 65, lines 13-15).

With regard to claims 7 and 8, Thisted taught that the "association between at least two nucleic acid moieties from different interactors" could be either via ligation or via polymerization (see page 71, line 28 through page 72, line 15, and see figures 1 and 2 and related descriptions on pages 113 and 114, for example).

With regard to claims 9 and 10, Thisted taught the target could be a protein, an enzyme, a metabolite, a lipid, a polysaccharide (which is a carbohydrate), and nucleic acid (page 63, lines 15-28). Note that proteins, polysaccharides and nucleic acid are also polymers (of amino acids, monosaccharides and nucleotides, respectively). Also, nucleic acids and lipids, for example, are "non-proteinaceous cellular product[s]". Proteins, polysaccharides, lipids and nucleic acids are also "cell metabolite[s]". Thisted also mentions peptides (page 65, line 6). Thisted also taught a heme group (page 63, line 16), which fits Applicant's definition of "small molecule" ("a non-peptidic, non-oligomeric organic compound either synthesized in the laboratory or found in nature"). Thisted also mentioned virus (paragraph spanning pages 96-97).

With regard to claims 11 and 12, Thisted taught that when the target was a protein, it could be associated with the mRNA or the cDNA (i.e. the nucleic acid) encoding it (page 66, lines 6-10).

With regard to claims 15 and 16, Thisted taught that "[t]he nucleotide sequence of the identifier sequence present in the coupled product is determined to identify the identity of the binding display molecule(s) and7or [sic and/or] optionally the molecular target(s)" (page 109, lines 14-16). Thisted taught that "[w]hen the amount is low, it is preferred to increase the amount of the coupled oligonucleotide sequence by

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polymerase chain reaction (PCR)" (page 110, lines 9-10). Thisted taught "the PCR amplified identifier oligonucleotide sequence can be analysed in a microarray" (page 110, lines 24-25).

With regard to claim 17, Thisted taught sequencing (see figure 4 and related description on page 115, especially at line 22): "This ligation will connect the target oligonucleotides with oligonucleotides that encodes for specific displayed molecules. The ligated oligonucleotides can be amplified and determined by sequencing procedures well known in the art."

With regard to claim 19, the examiner consulted the specification in order to construe the claim. The most relevant passage the examiner found was at paragraph [0058] of the published application:

The foundation of the interaction analysis is the increased probability of a reaction between molecules if they are brought in close proximity by molecular co-localization. The co-localized molecules will be prone to interact in cis rather than interact with separate molecules in trans with the presumption that the relative concentration of the co-localized molecules is higher than the concentration of other molecules in the solution. In the case of detection of affinity interactions the co-localization is introduced by the affinity interaction between the MOIs which then enhances the probability of the respective co-localized nucleic acids to associate.

From this passage then, it seems that "manipulating conditions to maximize cis associations" is achieved simply by virtue of the fact that one mixes together MOIs (molecules of interest), at least some of which have an affinity toward another MOI in the mixture, such that binding between the involved MOIs occurs. This binding brings

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the corresponding nucleic acid moieties coupled to the involved MOIs into proximity, maximizing their interaction with one another (cis) while minimizing their interactions with other nucleic acids (trans) associated with non-involved MOIs. It is asserted that this is the very same principle upon which Thisted's method is based (see passage from page 62, lines 14-31).

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 13 and 14 are rejected under 35 U.S.C. 103(a) as being unpatentable over Thisted et al (WO 2005/026387 A1) in view of Guille et al (Molecular Biotechnology 8(1):35-52, August 1997).

The teachings of Thisted have been discussed. In particular, it is noted that Thisted taught targets including nucleic acids and proteins (page 63, lines 15-28). Thisted did not teach in particular to apply the method to the detection of affinity between a protein and a nucleic acid. Thisted also did not teach quantifying and/or determining strength of the affinity interaction between the molecules of interest identified by his method.

Guille taught (first paragraph of Introduction, page 35): "Increased understanding of DNA-protein interactions has become a major goal for biologists because it is clear that these interactions play a central role in a wide range of cellular processes."

Guille also taught (page 46, first paragraph under "Quantitative Analysis of Binding"): "When working with purified and well-characterized DNA-binding proteins, one would usually like to know the equilibrium binding constant that characterizes the interaction of the protein with one or more DNA sequences."

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to apply the technique disclosed by Thisted to the identification and study of DNA-protein affinity interactions, since study of such interactions were a major goal of biologists. It would also have been obvious to quantify the strength of the binding interaction so identified, since Guille stated that this is something that the ordinary skilled practitioner would "usually like to know".

Claim 18 is rejected under 35 U.S.C. 103(a) as being unpatentable over Thisted et al (WO 2005/026387 A1) in view of Kurn (US 6,692,918).

The teachings of Thisted have been discussed. Thisted taught sequencing (see figure 4 and related description on page 115, especially at line 22): "This ligation will connect the target oligonucleotides with oligonucleotides that encodes for specific displayed molecules. The ligated oligonucleotides can be amplified and determined by sequencing procedures well known in the art." Thisted did not specifically mention "sequencing by synthesis".

Kurn taught (column 44, line 19): "Suitable sequencing methods also include sequencing by synthesis, which is a method known in the art, wherein nucleotide sequence is determined based on whether there is extension (polymerization) of a

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primer hybridized to an amplified product by a polymerase when a known dNTP type is provided in the synthesis (polymerization) reaction, wherein polymerization is indicated by the formation of pyrophosphate."

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to use a "sequencing by synthesis" technique to perform the sequencing shown in the method of Thisted, since this was a sequencing technique that was known in the art.

Allowable Subject Matter

Claims 20-22 and 24-37 are allowed. The closest prior art would be US 2009/0011957 or US 2006/0246450. These references teach the aspects of steps a-c of claim 20 except for the limitation that the desired interaction produces a detectable trace (see for example figures 1 and 2 of US 2009/0011957). While these references teach that the "identifier polynucleotide" (corresponding to the claimed "associated oligonucleotide") may comprise flanking regions that encompass a signal group, such as a fluorophore (e.g. paragraph [0272] of US 2009/0011957), the references do not teach that such a signal is the result of the functional interaction. As taught in these disclosures, this "signal" would be present in the identifier polynucleotide whether any functional interaction had occurred or not. These disclosures also fail to teach or suggest step d of claim 20.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to SAMUEL C. WOOLWINE whose telephone number is (571)272-1144. The examiner can normally be reached on Mon-Fri 9:00am-5:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on (571) 272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Samuel Woolwine/
Examiner, Art Unit 1637

Notice of References Cited	Application/Control No. 11/483,825		Applicant(s)/Patent Under Reexamination ERICSSON, OLOF	
	Examiner SAMUEL C. WOOLWINE		Art Unit 1637	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-6,692,918	02-2004	Kurn, Nurith	435/6
*	B	US-2009/0011957	01-2009	Gouliaev et al.	506/26
*	C	US-2006/0246450	11-2006	Franch et al.	435/006
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N	WO 2005026387 A1	03-2005	World Intellect	THISTED et al.	
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Guille et al. Methods for the analysis of DNA-protein interactions. Molecular Biotechnology 8(1):35-52, August 1997.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes 	Application/Control No. 11483825	Applicant(s)/Patent Under Reexamination ERICSSON, OLOF
	Examiner SAMUEL C WOOLWINE	Art Unit 1637

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Inventor name and keyword search in EAST, STN, Google (see printouts)	12/20/2009	SCW

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

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(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
24 March 2005 (24.03.2005)

PCT

(10) International Publication Number
WO 2005/026387 A1

(51) International Patent Classification⁷: **C12Q 1/68**,
C12N 15/10, C12P 1/00, C07B 61/00

TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(21) International Application Number:
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(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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60/504,748 22 September 2003 (22.09.2003) US
PA 2003 01485 8 October 2003 (08.10.2003) DK
60/509,268 8 October 2003 (08.10.2003) US

(71) Applicant (for all designated States except US): **NUEVO-LUTION A/S** [DK/DK]; Rønnegade 8, 5., DK-2100 Copenhagen (DK).

(72) Inventors; and

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(74) Agent: **HØIBERG A/S**; St. Kongensgade 59A, DK 1264 Copenhagen K (DK).

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Declarations under Rule 4.17:

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(54) Title: A METHOD FOR OBTAINING STRUCTURAL INFORMATION CONCERNING AN ENCODED MOLECULE AND METHOD FOR SELECTING COMPOUNDS

(57) Abstract: In one aspect, the present invention relates to a method for obtaining structural information about an encoded molecule. The encoded molecule may be produced by a reaction of a plurality of chemical entities and may be capable of being connected to an identifier oligonucleotide containing codons informative of the identity of the chemical entities which have participated in the formation of the encoded molecule. In a certain embodiment, primers are designed complementary to the codons appearing on the identifier oligonucleotide, and the presence, absence or relative abundance of a codon is evaluated by mixing a primer with the identifier oligonucleotide in the presence of a polymerase and substrate (deoxy)ribonucleotide triphosphates measuring the extension reaction. In another aspect, the invention provides a method for selecting compounds which binds to a target. More specifically, the invention relates to a method in which a target associated with an oligonucleotide initially is mixed with a library of complexes, each complex comprising a display molecule and an oligonucleotide identifying said display molecule. Next, due an increased proximity, the target oligonucleotide is coupled to the identifier oligonucleotide of complexes having a display molecule with affinity towards the target. In a final stage the coupled nucleotides are analysed to deduce at least the identity of the display molecule.



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A method for obtaining structural information concerning an encoded molecule and method for selecting compounds

Technical Field of the Invention

5 In one aspect, the present invention relates to a method for obtaining structural information about an encoded molecule. The encoded molecule may be produced by a reaction of a plurality of chemical entities and may be capable of being connected to an identifier oligonucleotide containing codons informative of the identity of the chemical entities which have participated in the formation of the encoded molecule. In a certain embodiment, primers are designed complementary to the codons appearing on the identifier oligonucleotide, and the presence, absence or relative abundance of a codon is evaluated by mixing a primer with the identifier oligonucleotide in the presence of a polymerase and substrate (deoxy)ribonucleotide triphosphates and measuring the extension reaction. In another aspect, the invention provides a method for selecting compounds which binds to a target. More specifically, the invention relates to a method in which a target associated with an oligonucleotide initially is mixed with a library of complexes, each complex comprising a display molecule and an oligonucleotide identifying said display molecule. Next, due an increased proximity, the target oligonucleotide is coupled to the identifier oligonucleotide of complexes having a display molecule with affinity towards the target. In a final stage the coupled nucleotides are analysed to deduce at least the identity of the display molecule.

25 A method for obtaining structural information concerning an encoded molecule

The below paragraphs up to the section entitled "Method for identifying a display molecule" relate to the first aspect of the invention.

30 The first aspect of the present invention relates to a method for obtaining structural information about an encoded molecule. The encoded molecule is usually produced by a process that comprises the reaction of a plurality of

chemical entities. The synthesis of the encoded molecule is recorded or programmed in an identifier oligonucleotide which is attached to the encoded molecule. The structural information obtained by the present method may be used to obtain the entire structure of the encoded molecule or a part thereof.

5

Background of the invention

The generation of molecules carrying new properties remains a challenging task. Recently, a number of procedures have been suggested that should allow a more efficient generation and screening of a huge number of molecules. The approach taken may involve the encoding and/or templating of molecules other than natural biopolymers and a coupling of the molecules to respective templates or identifier parts containing information about the reactants that have participated in the formation of the molecule. These approaches allow the researcher to generate and screen a huge number of molecules at the same time.

In US 5,723,598 it is suggested to prepare libraries of bifunctional molecules, in which one part of the bifunctional molecule comprises an encoded part and the other part of the molecule contains an identifying part. The identifying part is segregated into codons, i.e. stretches of nucleotides, which codes for reactants that have been involved in the synthesis of the encoded molecule. The libraries of bifunctional molecules are generally prepared by a split-and-mix method, which involves the initial reaction between a nascent bifunctional molecule and a range of different reactants in separate compartments at one end of the nascent bifunctional molecule and a corresponding range of identifier unit oligonucleotides (codons) and the other end. Subsequently, the contents of the compartments are mixed and the mixture is disposed in separate compartments and reacted again with another range of reactants and corresponding codons. Following the generation of a library of the bifunctional molecules, a partitioning with respect to affinity towards a target is conducted and the identifier part of the bifunctional molecule is decoded to establish the chemical structure of the compounds in the library that is likely to be a ligand

to the target. The decoding step implies that the identifier oligonucleotides initially are amplified by PCR. The PCR product is subsequently incorporated in to a suitable vector which is transformed to a host organism, usually E. coli. Following the incubation of the E. coli, colonies are picked and sequenced.

Halpin and Harbury have in WO 00/23458 suggested an improvement to the approach stipulated above. The approach is based on the same split-and-mix strategy for synthesis of combinatorial libraries comprising two or more synthetic steps. A plurality nucleic acid templates are used, each having at one end a chemical reactive site and dispersed throughout the stand a plurality of codon regions, each of said codon regions in turn specifying different codons. Separately, each of the strands, identified by a first codon region, is reacted at the chemical reaction sites with specific selected reagents. Subsequently, all the strands are pooled and subjected to a second partitioning based on a second codon region. The split-and-combine method is conducted an appropriate number of times to produce a library of typically between 10^3 and 10^6 different compounds. The decoding is performed utilizing the process depicted above.

Recently, a new method for encoding molecules has been suggested, which can be performed in a single "pot". WO 02/00419 and WO 02/103008 disclose methods for preparing virtually any molecule connected to a template coding for chemical entities which have reacted to form the molecule. In short, a template segregated into a plurality of codons and a plurality of building blocks comprising a transferable chemical entity and an anticodon are initially provided. Under hybridisation conditions, the template and building blocks are annealed together and the chemical entities are subsequently reacted to form the molecule. However, after a sufficient number of rounds of selections have been performed, the template must be decoded to establish the identity of the encoded molecule. The decoding step implies that the template oligonucleotides initially are amplified by PCR. The PCR product is

subsequently incorporated in to a suitable vector which is transformed to a host organism, usually *E. coli*. Following the incubation of the *E. coli*, colonies are picked and sequenced.

- 5 In an aspect of the invention, it is the object to facilitate the decoding of the coding oligonucleotide in order to obtain at least partial structural information of the encoded molecule being a ligand towards a target. In another aspect of the invention, it is desired to obtain information about which chemical entities that result in encoded molecules successful in a selection process. Such
10 chemical entities may be used in the formation of a second generation library.

Summary of the Invention

- The first aspect of the present invention concerns a method for obtaining
15 structural information about an encoded molecule produced by a process comprising reaction of a plurality of chemical entities, said encoded molecule being capable of forming part of a complex also comprising an identifier oligonucleotide containing codons informative of the identity of chemical entities which have participated in the formation of the encoded molecule, the
20 method comprises mixing a primer oligonucleotide with the identifier oligonucleotide, subjecting the mixture to a condition allowing for an extension reaction to occur when the primer is sufficient complementary to a part of the identifier oligonucleotide, and evaluating, based on measurement of the extension reaction, the presence, absence, or relative abundance of one or
25 more codons.

- The method according to the invention may be performed on a single identifier oligonucleotide or a composition of identifier oligonucleotides to obtain structural information about the encoded molecule or a composition of encoded molecules, respectively, that have been attached to the identifier oligonucleotide(s).
30

A single identifier may be analysed using the above method to verify the incorporation into the encoded molecule of one or more chemical entities or to deconvolute the identity of the entire encoded molecule. A composition of two or more identifier oligonucleotides generally results from a selection process, i.e. a process involving subjecting a library of different complexes to a condition partitioning the composition from the remainder of the library. Usually the partitioning condition includes an affinity assay in which the library of complexes is contacted with a target and the identifier oligonucleotides of the binding complexes are harvested.

The conditions allowing for an extension reaction to occur may be selected from a enzymatic or chemical means. Suitably, the condition involves one or more enzymes. In a certain embodiment of the invention, the condition which allows for an extension reaction to occur includes a polymerase or a ligase as well as suitable substrates for the enzyme used. Preferred is a polymerase together with a blend of (deoxy)ribonucleotide triphosphates. Suitably, the blend include one or more of dATP, dGTP, dCTP, and dTTP.

A library of complexes can have any appropriate size. Typically, the size is above 10^3 , typically above 10^6 different complexes. An effective, extensive, and rapid decoding is therefore desirable. The method of the present invention may be used at various stages of the process of finding a ligand to a certain target. As examples, the method of the invention may be used for controlling the quality of a starting library. The information acquired may be used to verify which codons being present, absent, and, in some embodiments, also the relative abundance. Thus, the method of the invention delivers a reliable picture of the process which has produced the library. If, for some reason, a chemical entity has not been incorporated into the encoded molecules, the absence of a codon for this chemical entity will in certain embodiments of the invention indicate this fact.

Another example of the use of the present method is following the selection. After the selection has been performed the codon profile is indicative of the chemical entities that have been used in the synthesis of encoded molecules having an affinity towards the target. In the event the selection has been sufficiently effective it may be possible directly to deduce a part or the entire structure of binding encoded molecules. Alternatively, it may be possible to deduce a structural unit appearing more frequently among the encoded molecules after the selection, which gives important information to the structure-activity-relationship (SAR). If the selection process has not narrowed the size of the library to a manageable number, the formation of a second generation library may be contemplated. In the formation of the second generation library chemical entities which have not been involved in the synthesis of encoded molecules that have been successful in the selection may be omitted, thus limiting the size of the new library and at the same time increasing the concentration of binding complexes. The second generation library may then be subjected to more stringent selection conditions to allow only the encoded molecules with a higher affinity to bind to the target. The second generation library may also be spiked with certain chemical entities suspected of increasing the performance of the final encoded molecule. The indication of certain successful chemical entities may be obtained from the SAR. The use in a second generation library of chemical entities, which have proved to be interesting for further investigation in a preceding library, may thus entail a shuffling with new chemical entities that may focus the second generation library in a certain desired direction.

The relative abundance of codons may make it possible to decode a plurality of identifiers simultaneously, even in the case when two or more identifiers contain the same codon. Thus, following the formation and selection of a first, second or further generation library, the identity of binding encoded molecules may be partly or entirely deconvoluted by the present method.

In a practical approach the library comprises complexes with identifier oligonucleotides having n codon positions each. In a certain aspect of the invention n is an integer independently selected from of 2 to 8. It may be preferred that n is constant among all the complexes in the library to facilitate the decoding process. Each of the codons in a certain position is in an aspect of the invention selected from a set of m different codons. m may vary for each codon position or may be constant among the various codon positions. It may be preferred in some embodiments to have all the codons in each position selected from the same set of m codons. However, in other embodiments, especially such involving hybridisation in the recognition between the codon and the anticodon, it may be preferred that all the codons are different.

Preferably, any member of the codon set differs from any other codons in the set with the identity of at least one nucleotide, i.e. at least one nucleotide position occurs. In some aspects of the invention it is preferred that any member of the codon set differs with at least two nucleotides nucleotide positions from any other member of the set to increase the fidelity of the method. In general, it is desired to maximize the differences between individual codons of the set. In some embodiments of the invention, a set of primers comprising a sequence of complementing the set of codons are prepared.

In a preferred aspect of the method a framing sequence is related to each of the n codon positions in a particular complex, said framing sequence positions the reaction of a chemical entity in the synthesis history of the encoded molecule. Typically, the framing sequence is identical among the complexes for each of the reaction rounds and is selected from a group of n different nucleotide sequences. In a certain aspect of the invention $n \times m$ different primers fully or in part complementing any combination of the set of m different codons and the set of n different framing sequences is prepared. The $n \times m$ primers may be used in separate compartments to reveal the identity of a chemical entity as well as the point in time of the synthesis of the encoded molecule is has reacted.

- In a particular aspect, the invention concerns a method for identifying the chemical entities utilized in the formation of an encoded molecule or a composition of encoded molecules, wherein in separate compartments, $n \times m$
- 5 primers individually are mixed with an aliquot of a composition obtained by subjecting a library of different complexes to a condition partitioning said composition from the remainder of the library, subjected to a mixture of polymerase and substrate (deoxy)ribonucleotide triphosphates under conditions allowing for an extension reaction to occur when a primer is sufficient complementary to a part of one or more identifier oligonucleotides present in the
- 10 aliquot, and evaluation, based on measurement of the extension reaction, the presence, absence, or relative abundance of one or more codons in each compartment.
- 15 The invention also concerns a set comprising a collection of oligonucleotide primers, a polymerase, a composition of (deoxy)ribonucleotide triphosphates (dNTPs), and a library of complexes composed of a display molecule part and an identifier oligonucleotide, said oligonucleotide comprising codons informative of the identity of the chemical entities which has participated in the
- 20 formation of the display molecule, wherein the oligonucleotide primers are sufficiently complementary to codons appearing on the identifier oligonucleotides in the library to allow for an extension to occur.

Detailed Description of the Invention

25 Complex

- The complex comprises an encoded molecule and an identifier oligonucleotide. The identifier comprises codons that identify the encoded molecule. Preferably, the identifier oligonucleotide identifies the encoded molecule uniquely, i.e. in a library of complexes a particular identifier is capable of distinguishing the molecule it is attached to from the rest of the molecules.
- 30

The encoded molecule and the identifier may be attached directly to each other or through a bridging moiety. In one aspect of the invention, the bridging moiety is a selectively cleavable linkage.

- 5 The identifier oligonucleotide may comprise two or more codons. In a preferred aspect the identifier oligonucleotide comprises three or more codons. The sequence of each codon can be decoded utilizing the present method to identify reactants used in the formation of the encoded molecule. When the identifier comprises more than one codon, each member of a pool of chemical entities can be identified and the order of codons is informative of the synthesis step each member has been incorporated in.
- 10

In a certain embodiment, the same codon is used to code for several different chemical entities. In a subsequent identification step, the structure of the encoded molecule can be deduced taking advantage of the knowledge of different attachment chemistries, steric hindrance, deprotection of orthogonal protection groups, etc. In another embodiment, the same codon is used for a group of chemical entities having a common property, such as a lipophilic nature, a certain attachment chemistry etc. In a preferred embodiment, however, the codon is unique i.e. a similar combination of nucleotides does not appear on the identifier oligonucleotide coding for another chemical entity. In a practical approach, for a specific chemical entity, only a single combination of nucleotides is used. In some aspects of the invention, it may be advantageous to use several codons for the same chemical entity, much in the same way as Nature uses up to six different codons for a single amino acid. The two or more codons identifying the same chemical entity may carry further information related to different reaction conditions.

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The sequence of the nucleotides in each codon may have any suitable length. The codon may be a single nucleotide or a plurality of nucleotides. In some aspects of the invention, it is preferred that each codon independently

30

comprises four or more nucleotides, more preferred 4 to 30 nucleotides. In some aspects of the invention the lengths of the codons vary.

5 A certain codon may be distinguished from any other codon in the library by only a single nucleotide. However, to facilitate a subsequent decoding process and to increase the ability of the primer to discriminate between codons it is in general desired to have two or more mismatches between a particular codon and any other codon appearing on identifier oligonucleotide. As an example, if a codon length of 5 nucleotides is selected, more than 100 nu-
10 cleotide combinations exist in which two or more mismatches appear. For a certain number of nucleotides in the codon, it is generally desired to optimize the number of mismatches between a particular codon relative to any other codon appearing in the library.

15 The identifier oligonucleotide will in general have at least two codons arranged in sequence, i.e. next to each other. Two neighbouring codons may be separated by a framing sequence. Depending on the encoded molecule formed, the identifier may comprise further codons, such as 3, 4, 5, or more codons. Each of the further codons may be separated by a suitable framing
20 sequence. Preferably, all or at least a majority of the codons of the identifier are separated from a neighbouring codon by a framing sequence. The framing sequence may have any suitable number of nucleotides, e.g. 1 to 20. Alternatively, codons on the identifier may be designed with overlapping se-
quences.

25 The framing sequence, if present, may serve various purposes. In one setup of the invention, the framing sequence identifies the position of the codon. Usually, the framing sequence either upstream or downstream of a codon comprises information which positions the chemical entity and the reaction
30 conditions in the synthesis history of the encoded molecule. The framing sequence may also or in addition provide for a region of high affinity. The high affinity region may ensure that a hybridisation event with an anti-codon will

occur in frame. Moreover, the framing sequence may adjust the annealing temperature to a desired level.

5 A framing sequence with high affinity can be provided by incorporation of one or more nucleobases forming three hydrogen bonds to a cognate nucleobase. Examples of nucleobases having this property are guanine and cytosine. Alternatively, or in addition, the framing sequence may be subjected to backbone modification. Several backbone modifications provides for higher affinity, such as 2'-O-methyl substitution of the ribose moiety, peptide nucleic acids (PNA), and 2'-4' O-methylene cyclisation of the ribose moiety, also referred to as LNA (Locked Nucleic Acid).

15 The sequence comprising a codon and an adjacent framing sequence has in a certain aspect of the invention a total length of 11 nucleotides or more, preferably 15 nucleotides or more. A primer may be designed to complementary to the codon sequence as well as the framing sequence. The presence of an extension reaction under conditions allowing for such reaction to occur is indicative of the presence of the chemical entity encoded in the codon as well as the position said chemical entity has in the entire synthesis history of the encoded molecule.

25 The identifier may comprise flanking regions around the coding section. The flanking regions can also serve as priming sites for amplification reactions, such as PCR or as binding region for oligonucleotide probe. The identifier may in certain embodiments comprise an affinity region having the property of being able to hybridise to a building block.

30 It is to be understood that when the term identifier oligonucleotide is used in the present description and claims, the identifier oligonucleotide may be in the sense or the anti-sense format, i.e. the identifier can be a sequence of codons which actually codes for the encoded molecule or can be a sequence

complementary thereto. Moreover, the identifier may be single-stranded or double-stranded, as appropriate.

5 The encoded molecule part of the complex is generally of a structure expected of having an effect on a target. When the target is of pharmaceutical importance, the encoded molecule is generally a possible drug candidate. The complex may be formed by tagging a library of different possible drug candidates with a tag, e.g. a nucleic acid tag identifying each possible drug candidate. In another embodiment of the invention, the molecule formed by a
10 variety of reactants which have reacted with each other and/or a scaffold molecule. Optionally, this reaction product may be post-modified to obtain the final molecule displayed on the complex. The post-modification may involve the cleavage of one or more chemical bonds attaching the encoded molecule to the identifier in order more efficiently to display the encoded molecule.

15 The formation of an encoded molecule generally starts by a scaffold, i.e. a chemical unit having one or more reactive groups capable of forming a connection to another reactive group positioned on a chemical entity, thereby generating an addition to the original scaffold. A second chemical entity may
20 react with a reactive group also appearing on the original scaffold or a reactive group incorporated by the first chemical entity. Further chemical entities may be involved in the formation of the final reaction product. The formation of a connection between the chemical entity and the nascent encoded molecule may be mediated by a bridging molecule. As an example,
25 if the nascent encoded molecule and the chemical entity both comprise an amine group a connection between these can be mediated by a dicarboxylic acid. A synthetic molecule is in general produced in vitro and may be a naturally occurring or an artificial substance. Usually, a synthetic molecule is not produced using the naturally translation system in an in vitro process.

30 The chemical entities that are precursors for structural additions or eliminations of the encoded molecule may be attached to a building block

prior to the participation in the formation of the reaction product leading the final encoded molecule. Besides the chemical entity, the building block generally comprises an anti-codon. In some embodiments the building blocks also comprise an affinity region providing for affinity towards the nascent
5 complex.

Thus, the chemical entities are suitably mediated to the nascent encoded molecule by a building block, which further comprises an anticodon. The anti-codon serves the function of transferring the genetic information of the
10 building block in conjunction with the transfer of a chemical entity. The transfer of genetic information and chemical entity may occur in any order. The chemical entities are preferably reacted without enzymatic interaction in some aspects of the invention. Notably, the reaction of the chemical entities is preferably not mediated by ribosomes or enzymes having similar activity.
15 In other aspects of the invention, enzymes are used to mediate the reaction between a chemical entity and a nascent encoded molecule.

According to certain aspects of the invention the genetic information of the anti-codon is transferred by specific hybridisation to a codon on a nucleic
20 acid template. Another method for transferring the genetic information of the anti-codon to the nascent complex is to anneal an oligonucleotide complementary to the anti-codon and attach this oligonucleotide to the complex, e.g. by ligation. A still further method involves transferring the genetic information of the anti-codon to the nascent complex by an extension
25 reaction using a polymerase and a mixture of dNTPs.

The chemical entity of the building block may in most cases be regarded as a precursor for the structural entity eventually incorporated into the encoded molecule. In other cases the chemical entity provides for the eliminations of
30 chemical units of the nascent encoded molecule. Therefore, when it in the present application with claims is stated that a chemical entity is transferred to a nascent encoded molecule it is to be understood that not necessarily all

the atoms of the original chemical entity is to be found in the eventually formed encoded molecule. Also, as a consequence of the reactions involved in the connection, the structure of the chemical entity can be changed when it appears on the nascent encoded molecule. Especially, the cleavage resulting in the release of the entity may generate a reactive group which in a subsequent step can participate in the formation of a connection between a nascent complex and a chemical entity.

The chemical entity of the building block comprises at least one reactive group capable of participating in a reaction which results in a connection between the chemical entity of the building block and another chemical entity or a scaffold associated with the nascent complex. The number of reactive groups which appear on the chemical entity is suitably one to ten. A building block featuring only one reactive group is used i.a. in the end positions of polymers or scaffolds, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffolds capable of being reacted further. One, two or more reactive groups intended for the formation of connections, are typically present on scaffolds. Non-limiting examples of scaffolds are opiates, steroids, benzodiazepines, hydantoines, and peptidylphosphonates.

The reactive group of the chemical entity may be capable of forming a direct connection to a reactive group of the nascent complex or the reactive group of the building block may be capable of forming a connection to a reactive group of the nascent complex through a bridging fill-in group. It is to be understood that not all the atoms of a reactive group are necessarily maintained in the connection formed. Rather, the reactive groups are to be regarded as precursors for the structure of the connection.

The subsequent cleavage step to release the chemical entity from the building block can be performed in any appropriate way. In an aspect of the invention the cleavage involves usage of a chemical reagent or an enzyme. The

cleavage results in a transfer of the chemical entity to the nascent encoded molecule or in a transfer of the nascent encoded molecule to the chemical entity of the building block. In some cases it may be advantageous to introduce new chemical groups as a consequence of linker cleavage. The new
5 chemical groups may be used for further reaction in a subsequent cycle, either directly or after having been activated. In other cases it is desirable that no trace of the linker remains after the cleavage.

In another aspect, the connection and the cleavage is conducted as a simultaneous reaction, i.e. either the chemical entity of the building block or the
10 nascent encoded molecule is a leaving group of the reaction. In some aspects of the invention, it is appropriate to design the system such that the connection and the cleavage occur simultaneously because this will reduce the number of steps and the complexity. The simultaneous connection and
15 cleavage can also be designed such that either no trace of the linker remains or such that a new chemical group for further reaction is introduced, as described above.

The attachment of the chemical entity to the building block, optionally via a
20 suitable spacer can be at any entity available for attachment, e.g. the chemical entity can be attached to a nucleobase or the backbone. In general, it is preferred to attach the chemical entity at the phosphor of the internucleoside linkage or at the nucleobase. When the nucleobase is used for attachment of the chemical entity, the attachment point is usually at the 7 position of the
25 purines or 7-deaza-purins or at the 5 position of pyrimidines. The nucleotide may be distanced from the reactive group of the chemical entity by a spacer moiety. The spacer may be designed such that the conformational spaced sampled by the reactive group is optimized for a reaction with the reactive
group of the nascent encoded molecule.

30 The encoded molecules may have any chemical structure. In a preferred aspect, the encoded molecule can be any compound that may be

synthesized in a component-by-component fashion. In some aspects the synthetic molecule is a linear or branched polymer. In another aspect the synthetic molecule is a scaffolded molecule. The term "encoded molecule" also comprises naturally occurring molecules like α -polypeptides etc,
5 however produced *in vitro* usually in the absence of enzymes, like ribosomes. In certain aspects, the synthetic molecule of the library is a non- α -polypeptide.

The encoded molecule may have any molecular weight. However, in order to
10 be orally available, it is in this case preferred that the synthetic molecule has a molecular weight less than 2000 Daltons, preferably less than 1000 Dalton, and more preferred less than 500 Daltons.

The size of the library may vary considerably pending on the expected result
15 of the inventive method. In some aspects, it may be sufficient that the library comprises two, three, or four different complexes. However, in most events, more than two different complexes are desired to obtain a higher diversity. In some aspects, the library comprises 1,000 or more different complexes, more preferred 1,000,000 or more different complexes. The upper limit for the size
20 of the library is only restricted by the size of the vessel in which the library is comprised. It may be calculated that a vial may comprise up to 10^{14} different complexes.

Extension reaction

25 The extension reaction requires a primer, a polymerase as well as a collection of deoxyribonucleotide triphosphates (abbreviated dNTP's herein) to proceed. An extension product may be obtained in the event the primer is sufficient complementary to an identifier oligonucleotide for a polymerase to recognise the double helix as a substrate. After binding of the polymerase to
30 the double helix, the deoxyribonucleotide triphosphates (blend of dATP, dCTP, dGTP, and dTTP) are incorporated into the extension product using the identifier oligonucleotide as template. The conditions allowing for the exten-

sion reaction to occur usually includes a suitable buffer. The buffer may be any aqueous or organic solvent or mixture of solvents in which the polymerase has a sufficient activity. To facilitate the extension process the polymerase and the mixture of dNTP's are generally included in a buffer which is added to the identifier oligonucleotide and primer mixture. An exemplary kit comprising the polymerase and the nNTP's for performing the extension process comprises the following: 50 mM KCl; 10 mM Tris-HCl at pH 8.3; 1.5 mM MgCl₂ ; 0.001% (wt/vol) gelatin, 200 μM dATP; 200 μM dTTP; 200 μM dCTP; 200 μM dGTP; and 2.5 units *Thermus aquaticus* (Taq) DNA polymerase I (U.S. Pat. No. 4,889,818) per 100 microliters (μl) of buffer.

The primer may be selected to be complementary to one or more codons or parts of such codons. The length of the primers may be determined by the length of the codons, however, the primers usually are at least about 11 nucleotides in length, more preferred at least 15 nucleotides in length to allow for an efficient extension by the polymerase. The presence or absence of one or more codons is indicated by the presence of or absence of an extension product. The extension product may be measured by any suitable method, such as size fractioning on an agarose gel and staining with ethidium bromide.

In a preferred embodiment the admixture of identifier oligonucleotide and primer is thermocycled to obtain a sufficient number of copies of the extension product. The thermocycling is typically carried out by repeatedly increasing and decreasing the temperature of the mixture within a temperature range whose lower limit is about 30 degrees Celsius (30°C) to about 55°C and whose upper limit is about 90°C to about 100° C. The increasing and decreasing can be continuous, but is preferably phasic with time periods of relative temperature stability at each of temperatures favouring polynucleotide synthesis, denaturation and hybridization.

When a single complex is analysed in accordance with the present method, the result may be used to verify the presence or absence of a specific chemical entity during the formation of the display molecule. The formation of an extension product is indicative of the presence of an oligonucleotide part
5 complementary to the primer in the identifier oligonucleotide. Conversely, the absence of an extension product is indicative of the absence of an oligonucleotide part complementary to the primer in the identifier oligonucleotide. Selecting the sequence of the primer such that it is complementary to one or more codons will therefore provide information of the structure of the en-
10 coded molecule coded for by this codon(s).

In a preferred aspect of the invention, in the mixture of the identifier oligonucleotide and the primer oligonucleotide, a second primer complementary to a sequence of the extension product is included. The second primer is also
15 termed reverse primer and ensures an exponential increase of the number of produced extension products. The method using a forward and reverse primer is well known to skilled person in the art and is generally referred to as polymerase chain reaction (abbreviated PCR) in the present application with claims. In one embodiment of the invention the reverse primer is annealed to
20 a part of the extension product downstream, i.e. near the 3' end of the extension product, or a part complementing the coding part of the identifier oligonucleotide. In another embodiment, the first primer (forward primer) anneals to an upstream position of the identifier oligonucleotide, preferably before the coding part, and the reverse primer anneals to a sequence of the
25 extension product complementing one or more codons or parts thereof.

The amplicons resulting from the PCR process may be stained during or following the reaction to ease the detection. A staining after the PCR process may be prepared with e.g. ethidium bromide or a similar staining agent. As
30 an example, amplicons from the PCR process is run on an agarose gel and subsequently stained with ethidium bromide. Under UV illumination bands of amplicons becomes visible. It is possible to incorporate the staining agent in

the agarose gel or to allow a solution of the staining agent to migrate through the gel. The amplicons may also be stained during the PCR process by an intercalating agent, like CYBR. In presence of the intercalating agent while the amplification proceeds it will incorporate in the double helix. The intercalation agent may then be made visible by irradiation by a suitable source.

The intensity of the staining is informative of the relative abundance of a specific amplicon. Thus, it is possible to quantify the occurrence of a codon in an identifier oligonucleotide. When a library of bifunctional complexes has been subjected to a selection the codons in the pool of identifier oligonucleotides which has been selected can be quantified using this method. As an example a sample of the selected identifier oligonucleotides is subjected to various PCR amplifications with different primers in separate compartments and the PCR product of each compartment is analysed by electrophoresis in the presence of ethidium bromide. The bands that appear can be quantified by a densitometric analysis after irradiation by ultraviolet light and the relative abundance of the codons can be measured.

Alternatively, the primers may be labelled with a suitable small molecule, like biotin or digoxigenin. A PCR-ELISA analysis may subsequently be performed based on the amplicons comprising the small molecule. A preferred method includes the application of a solid support covered with streptavidin or avidin when biotin is used as label and anti-digoxigenin when digoxigenin is used as the label. Once captured, the amplicons can be detected using an enzyme-labelled avidin or anti-digoxigenin reporter molecule similar to a standard ELISA format.

To avoid laborious post-PCR handling steps required to evaluate the amplicons, it is in a certain embodiment preferred to measure the extension process "real time". Several real time PCR processes has been developed and all the suitable real time PCR process available to the skilled person in the art can be used in the evaluating step of the present invention and are include in

the present scope of protection. The PCR reactions discussed below are of particular interest.

5 The monitoring of accumulating amplicons in real time has been made possible by labelling of primers, probes, or amplicons with fluorogenic molecules. The real time PCR amplification is usually performed with a speed faster than the conventional PCR, mainly due to reduced cycles time and the use of sensitive methods for detection of emissions from the fluorogenic labels. The most commonly used fluorogenic oligoprobes rely upon fluorescent resonance energy transfer (FRET) between fluorogenic labels or between one
10 flourophor and a dark or "black-hole" non-fluorescent quencher (NFQ), which disperse energy as heat rather than fluorescence. FRET is a spectroscopic process by which energy is passed between molecules separated by 10-100 Å that have overlapping emission and absorption spectra. An advantage of many real time PCR methods is that they can be carried out in a closed system, i.e. a system which does not need to be opened to examine the result of the PCR. A closed system implies a reduced result turnaround, minimisation of the potential for carry-over contamination and the ability to closely scrutinise the essay's performance.

20

The present real time PCR methods currently available to the skilled person can be classified into either amplicon sequence specific or non-specific methods. The basis for the non-specific detection methods is a DNA-binding fluorogenic molecule. Included in this class are the earliest and simplest approaches to real time PCR. Ethidium bromide, YO-PRO-1, and SYBR® green
25 1 all fluorescence when associated with double stranded DNA which is exposed to a suitable wavelength of light. This approach requires the fluorescent agent to be present during the PCR process and provides for a real time detection of the fluorescent agent as it is incorporated into the double
30 stranded helix.

The amplicons sequence specific methods includes, but are not limited to, the TaqMan[®], hairpin, LightCycler[®], Sunrise[®], and Scorpion[®] methods. The LightCycler[®] method also designated "HybProbes" make use of a pair of adjacent, fluorogenic hybridisation oligonucleotide probes. A first, usually the upstream oligoprobe is labelled with a 3' donor fluorophore and the second, usually the downstream probe is commonly labelled with either a Light cycler Red 640 or Red 705 acceptor fluorophore at the 5' terminus so that when both oligoprobes are hybridised the two fluorophores are located in close proximity, such as within 10 nm, of each other. The close proximity provides for the emission of a fluorescence when irradiated with a suitable light source, such a blue diode in case of the LightCycler[®]. The region for annealing of the probes may be any suitable position that does not interfere with the primer annealing. In a suitable setup, the site for binding the probes are positioned downstream of the codon region on the identifier oligonucleotide. Alternatively, when a reverse primer is used, the region for annealing the probes may be at the 3' end of the strand complementing the identifier oligonucleotide. Another embodiment of the LightCycler method includes that the pair of oligonucleotide probes are annealed to one or more codons and primer sites exterior to the coding part of the identifier oligonucleotide are used for PCR amplification.

The TaqMan[®] method, also referred to as the 5' nuclease or hydrolysis method, requires an oligoprobe, which is attached to a reporter fluorophore, such as 6-carboxy-fluorescein, and a quencher fluorophore, such as 6-carboxy-tetramethyl-rhodamine, at each end. When in close proximity, i.e. annealed to an identifier oligonucleotide, or a sequence complementing the identifier oligonucleotide, the quencher will "hijack" the emissions that have resulted from the excitation of the reporter. As the polymerase progresses along the relevant strand, it displaces and the hydrolyses the oligoprobe via its 5'→3' endonuclease activity. Once the reporter is removed from the extinguishing influence of the quencher, it is able to release excitation energy at a wavelength that can be monitored by a suitable instrument, such as ABI

Prism® 7700. The fractional cycle number at which the real-time fluorescence signal mirrors progression of the reaction above the background noise is normally used as an indicator of successful identifier oligonucleotide amplification. This threshold cycle (C_T) is defined as the PCR cycle in which the gain in fluorescence generated by the accumulating amplicons exceeds 10 standard deviations of the mean base line fluorescence. The C_T is proportional to the number of identifier oligonucleotide copies present in the sample. The TaqMan probe is usually designed to hybridise at a position downstream of a primer binding site, be it a forward or a reverse primer. When the primer is designed to anneal to one or more codons of the identifier oligonucleotide, the presence of these one or more codons is indicated by the emittance of light. Furthermore, the quantity of the identifier oligonucleotides comprising the one or more codons may be measured by the C_T value.

The Hairpin method involves an oligoprobe, in which a fluorophore and a quencher are positioned at the termini. The labels are held in close proximity by distal stem regions of homologous base pairing deliberately designed to create a hairpin structure which result in quenching either by FRET or a direct energy transfer by a collisional mechanism due to the intimate proximity of the labels. When direct energy transfer by a collision mechanism is used the quencher is usually different from the FRET mechanism, and is suitably 4-(4'-dimethylamino-phenylazo)-benzene (DABCYL). In the presence of a complementary sequence, usually downstream of a primer, or within the bounds of the primer binding sites in case of more than one a single primer, the oligoprobe will hybridise, shifting into an open configuration. The fluorophore is now spatially removed from the quencher's influence and fluorescence emissions are monitored during each cycle. In a certain aspect, the hairpin probe may be designed to anneal to a codon in order to detect this codon if present on the identifier oligonucleotide. This embodiment may be suitable if codons only differs from each other with a single or a few nucleotides, because it is well-known that the occurrence of a mismatch between a hairpin oligoprobe and its target sequence has a greater destabilising effect

on the duplex than the introduction of an equivalent mismatch between the target oligonucleotide and a linear oligoprobe. This is probably because the hairpin structure provides a highly stable alternate conformation.

- 5 The Sunrise and Scorpion methods are similar in concept to the hairpin oligoprobe, except that the label becomes irreversible incorporated in to the PCR product. The Sunrise method involves a primer (commercially available as AmplifluorTM hairpin primers) comprising a 5' fluorophore and a quencher, e.g. DABCYL. The labels are separated by complementary stretches of sequence that create a stem when the sunrise primer is closed. At the 3' terminus is a target specific primer sequence. In a preferred embodiment the target sequence is a codon, optionally more codons. The sunrise primer's sequence is intended to be duplicated by the nascent complementary strand and, in this way, the stem is destabilised, the two fluorophores are held apart, usually between 15 and 25 nucleotides, and the fluorophore is free to emit its excitation energy for monitoring. The Scorpion primer resembles the sunrise primer, but derivate in having a moiety that blocks duplication on the signalling portion of the scorpion primer. The blocking moiety is typically hexethylene glycol. In addition to the difference in structure, the function of the scorpion primers differs slightly in that the 5' region of the oligonucleotide is designed to hybridise to a complementary region within the amplicons. In a certain embodiment the complementary region is a codon on the identifier oligonucleotide. The hybridisation forces the labels apart disrupting the hairpin and permitting emission in the same way as the hairpin probes.

25

Methods for forming a library of complexes

- The complexes comprising an identifier part having two or more codons that code for reactants that have reacted in the formation of the encoded molecule part of the complex may be formed by a variety of processes. Generally, the preferred methods can be used for the formation of virtually any kind of encode molecule. Suitable examples of processes include prior art methods disclosed in WO 93/20242, WO 93/06121, WO 00/23458, WO 02/074929,

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and WO 02/103008, the content of which being incorporated herein by reference as well as methods of the present applicant not yet public available, including the methods disclosed in DK PA 2002 01955 filed 19 December 2002, and DK PA 2003 00430 filed 20 March 2003. Any of these methods
5 may be used, and the entire content of the patent applications are included herein by reference.

Below four preferred embodiments are described. A first embodiment disclosed in more detail in WO 02/103008 is based on the use of a
10 polymerase to incorporate unnatural nucleotides as building blocks. Initially, a plurality of template oligonucleotides is provided. Subsequently primers are annealed to each of the templates and a polymerase is extending the primer using nucleotide derivatives which have appended chemical entities. Subsequent to or simultaneously with the incorporation of the nucleotide
15 derivatives, the chemical entities are reacted to form a reaction product. The encoded molecule may be post-modified by cleaving some of the linking moieties to better present the encoded molecule.

Several possible reaction approaches for the chemical entities are apparent.
20 First, the nucleotide derivatives can be incorporated and the chemical entities subsequently polymerised. In the event the chemical entities each carry two reactive groups, the chemical entities can be attached to adjacent chemical entities by a reaction of these reactive groups. Exemplary of the reactive groups are amine and carboxylic acid, which upon reaction form an amide
25 bond. Adjacent chemical entities can also be linked together using a linking or bridging moiety. Exemplary of this approach is the linking of two chemical entities each bearing an amine group by a bi-carboxylic acid. Yet another approach is the use of a reactive group between a chemical entity and the nucleotide building block, such as an ester or a thioester group. An adjacent
30 building block having a reactive group such as an amine may cleave the interspaced reactive group to obtain a linkage to the chemical entity, e.g. by an amide linking group.

A second embodiment for obtainment of complexes pertains to the use of hybridisation of building blocks to an identifier oligonucleotide and reaction of chemical entities attached to the building blocks in order to obtain a reaction product. This approach comprises that templates are contacted with a plurality of building blocks, wherein each building block comprises an anti-codon and a chemical entity. The anti-codons are designed such that they recognise a sequence, i.e. a codon, on the template. Subsequent to the annealing of the anti-codon and the codon to each other a reaction of the chemical entity is effected.

The template may be associated with a scaffold. Building blocks bringing chemical entities in may be added sequentially or simultaneously and a reaction of the reactive group of the chemical entity may be effected at any time after the annealing of the building blocks to the template.

A third embodiment for the generation of a complex includes chemical or enzymatical ligation of building blocks when these are lined up on a template. Initially, templates are provided, each having one or more codons. The templates are contacted with building blocks comprising anti-codons linked to chemical entities. The two or more anti-codons annealed on a template are subsequently ligated to each other and a reaction of the chemical entities is effected to obtain a reaction product. The method is disclosed in more detail in DK PA 2003 00430 filed 20 March 2003.

A fourth embodiment makes use of the extension by a polymerase of an affinity sequence of the nascent complex to transfer the anti-codon of a building block to the nascent complex. The method implies that a nascent complex comprising a scaffold and an affinity region is annealed to a building block comprising a region complementary to the affinity section. Subsequently the anti-codon region of the building block is transferred to the nascent complex by a polymerase. The transfer of the chemical entity may

be transferred prior to, simultaneously with or subsequent to the transfer of the anti-codon. This method is disclosed in detail in DK PA 2002 01955 filed 19 December 2002.

- 5 After or simultaneously with the formation of the reaction product some of the linkers to the template may be cleaved, however at least one linker must be maintained to provide for the complex.

Nucleotides

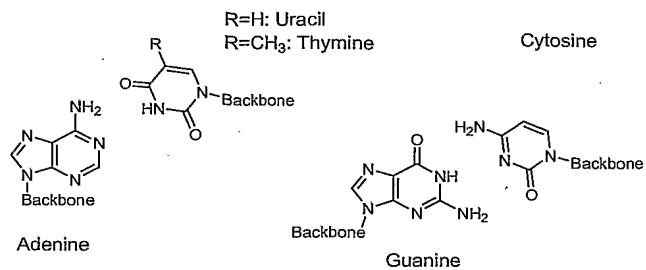
- 10 The nucleotides used in the present invention may be linked together in a sequence of nucleotides, i.e. an oligonucleotide. Each nucleotide monomer is normally composed of two parts, namely a nucleobase moiety, and a backbone. The back bone may in some cases be subdivided into a sugar moiety and an internucleoside linker.

15

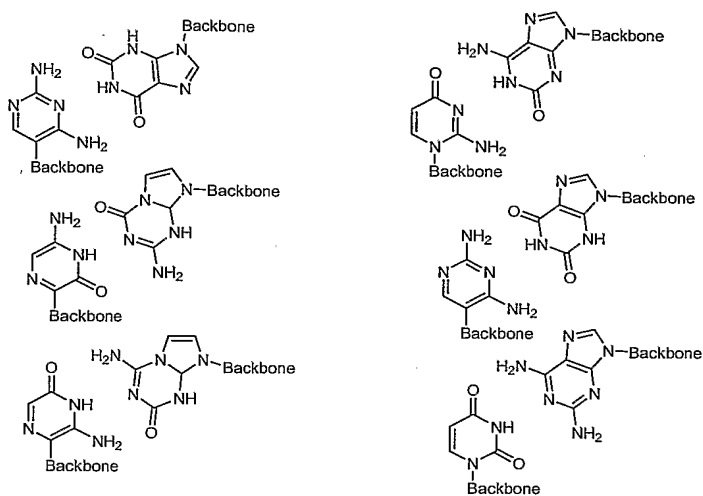
- The nucleobase moiety may be selected among naturally occurring nucleobases as well as non-naturally occurring nucleobases. Thus, "nucleobase" includes not only the known purine and pyrimidine hetero-cycles, but also heterocyclic analogues and tautomers thereof. Illustrative examples of nucleobases are adenine, guanine, thymine, cytosine, uracil, purine, xanthine, diaminopurine, 8-oxo-N⁶-methyladenine, 7-deazaxanthine, 7-deazaguanine, N⁴,N⁴-ethanocytosin, N⁶,N⁶-ethano-2,6-diamino-purine, 5-methylcytosine, 5-(C³-C⁶)-alkynylcytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridine, isocytosine, isoguanine, inosine and the
- 20 "non-naturally occurring" nucleobases described in Benner et al., U.S. Pat No. 5,432,272. The term "nucleobase" is intended to cover these examples as well as analogues and tautomers thereof. Especially interesting nucleobases are adenine, guanine, thymine, cytosine, 5-methylcytosine, and uracil, which are considered as the naturally occurring nucleobases in relation to
- 25 therapeutic and diagnostic application in humans.
- 30

Examples of suitable specific pairs of nucleobases are shown below:

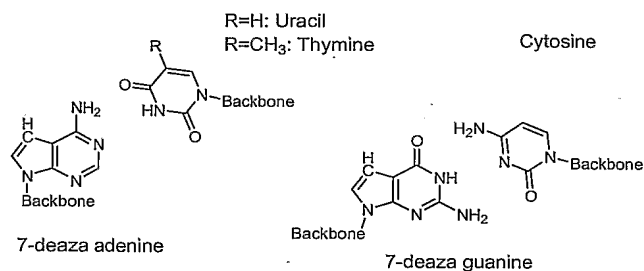
Natural Base Pairs



Synthetic Base Pairs

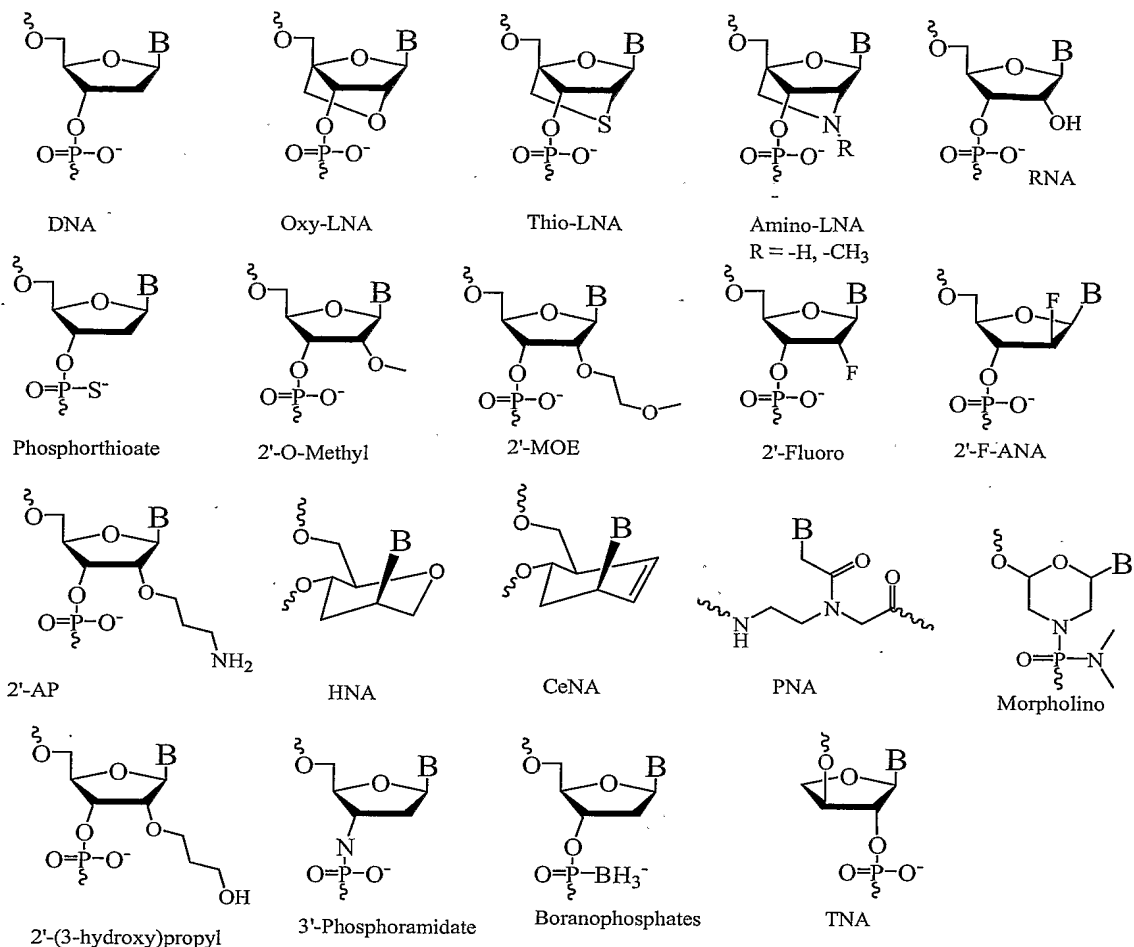


Synthetic purine bases pairing with natural pyrimidines



5 Suitable examples of backbone units are shown below (B denotes a nucleobase):

28

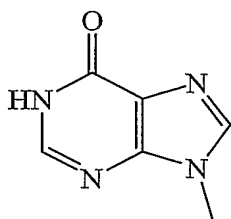
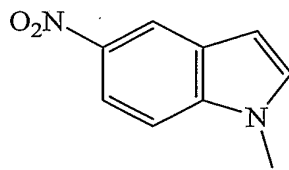
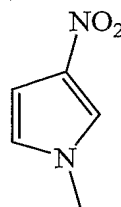
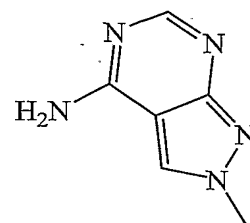
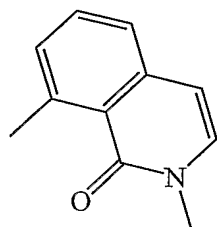
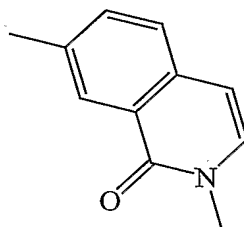
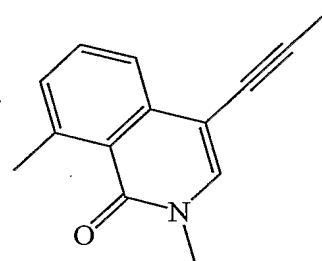
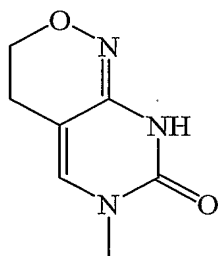
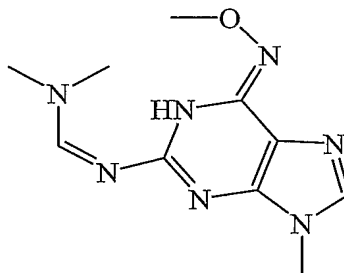
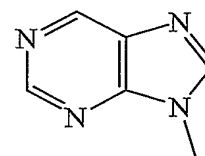


The sugar moiety of the backbone is suitably a pentose but may be the appropriate part of an PNA or a six-member ring. Suitable examples of possible pentoses include ribose, 2'-deoxyribose, 2'-O-methyl-ribose, 2'-fluor-ribose, and 2'-4'-O-methylene-ribose (LNA). Suitably the nucleobase is attached to the 1' position of the pentose entity.

An internucleoside linker connects the 3' end of preceding monomer to a 5' end of a succeeding monomer when the sugar moiety of the backbone is a pentose, like ribose or 2-deoxyribose. The internucleoside linkage may be the natural occurring phosphodiester linkage or a derivative thereof. Examples of such derivatives include phosphorothioate, methylphosphonate, phosphoramidate, phosphotriester, and phosphodithioate. Furthermore, the

internucleoside linker can be any of a number of non-phosphorous-containing linkers known in the art.

- Preferred nucleic acid monomers include naturally occurring nucleosides forming part of the DNA as well as the RNA family connected through phosphodiester linkages. The members of the DNA family include deoxyadenosine, deoxyguanosine, deoxythymidine, and deoxycytidine. The members of the RNA family include adenosine, guanosine, uridine, cytidine, and inosine. Inosine is a non-specific pairing nucleoside and may be used as a universal base because inosine can pair nearly isoenergetically with A, T, and C. Other compounds having the same ability of non-specifically base-pairing with natural nucleobases have been formed. Suitable compounds which may be utilized in the present invention includes among others the compounds depicted below

Examples of Universal Bases:**Inosine****5-Nitroindole****3-Nitropyrrole****N⁸-8aza-7deazaadenine****MICS****5MICS****PIM****dP****dK****Nebularine****Building block**

The chemical entities that are precursors for structural additions or eliminations of the encoded molecule may be attached to a building block prior to

5

the participation in the formation of the reaction product leading the final encoded molecule. Besides the chemical entity, the building block generally comprises an anti-codon.

- 5 The chemical entity of the building block comprises at least one reactive group capable of participating in a reaction which results in a connection between the chemical entity of the building block and another chemical entity or a scaffold associated with the nascent complex. The connection is facilitated by one or more reactive groups of the chemical entity. The number of reactive groups which appear on the chemical entity is suitably one to ten. A building block featuring only one reactive group is used i.a. in the end positions of polymers or scaffolds, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffolds capable of being reacted further. One, two or more reactive groups intended for the formation of connections, are typically present on scaffolds.

The reactive group of the building block may be capable of forming a direct connection to a reactive group of the nascent complex or the reactive group of the building block may be capable of forming a connection to a reactive group of the nascent complex through a bridging fill-in group. It is to be understood that not all the atoms of a reactive group are necessarily maintained in the connection formed. Rather, the reactive groups are to be regarded as precursors for the structure of the connection.

- 25 The subsequent cleavage step to release the chemical entity from the building block can be performed in any appropriate way. In an aspect of the invention the cleavage involves usage of a reagent or an enzyme. The cleavage results in a transfer of the chemical entity to the nascent encoded molecule or in a transfer of the nascent encoded molecule to the chemical entity of the building block. In some cases it may be advantageous to introduce new chemical groups as a consequence of linker cleavage. The new chemical groups may be used for further reaction in a subsequent cycle, either directly

or after having been activated. In other cases it is desirable that no trace of the linker remains after the cleavage.

5 In another aspect, the connection and the cleavage is conducted as a simultaneous reaction, i.e. either the chemical entity of the building block or the nascent encoded molecule is a leaving group of the reaction. In general, it is preferred to design the system such that the connection and the cleavage occur simultaneously because this will reduce the number of steps and the complexity. The simultaneous connection and cleavage can also be designed
10 such that either no trace of the linker remains or such that a new chemical group for further reaction is introduced, as described above.

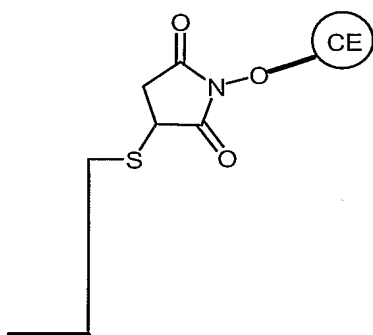
The attachment of the chemical entity to the building block, optionally via a suitable spacer can be at any entity available for attachment, e.g. the chemical entity can be attached to a nucleobase or the backbone. In general, it is
15 preferred to attach the chemical entity at the phosphor of the internucleoside linkage or at the nucleobase. When the nucleobase is used for attachment of the chemical entity, the attachment point is usually at the 7 position of the purines or 7-deaza-purins or at the 5 position of pyrimidines. The nucleotide
20 may be distanced from the reactive group of the chemical entity by a spacer moiety. The spacer may be designed such that the conformational space sampled by the reactive group is optimized for a reaction with the reactive group of the nascent encoded molecule or reactive site.

25 The anticodon complements the codon of the identifier sequence and generally comprises the same number of nucleotides as the codon. The anticodon may be adjoined with a fixed sequence, such as a sequence complementing a framing sequence.

30 Various specific building blocks are envisaged. Building blocks of particular interest are shown below.

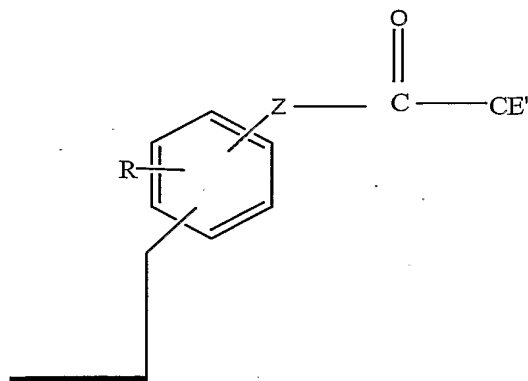
Specific Building blocks*Building blocks transferring a chemical entity to a recipient nucleophilic group*

The building block indicated below is capable of transferring a chemical entity (CE) to a recipient nucleophilic group, typically an amine group. The bold lower horizontal line illustrates the building block and the vertical line illustrates a spacer. The 5-membered substituted N-hydroxysuccinimid (NHS) ring serves as an activator, i.e. a labile bond is formed between the oxygen atom connected to the NHS ring and the chemical entity. The labile bond may be cleaved by a nucleophilic group, e.g. positioned on a scaffold



The 5-membered substituted N-hydroxysuccinimid (NHS) ring serves as an activator, i.e. a labile bond is formed between the oxygen atom connected to the NHS ring and the chemical entity. The labile bond may be cleaved by a nucleophilic group, e.g. positioned on a scaffold, to transfer the chemical entity to the scaffold, thus converting the remainder of the fragment into a leaving group of the reaction. When the chemical entity is connected to the activator through an carbonyl group and the recipient group is an amine, the bond formed on the scaffold will an amide bond. The above building block is the subject of the Danish patent application No. PA 2002 01946 and the US provisional patent application No. 60/434,439, the content of which are incorporated herein in their entirety by reference.

Another building block which may form an amide bond is

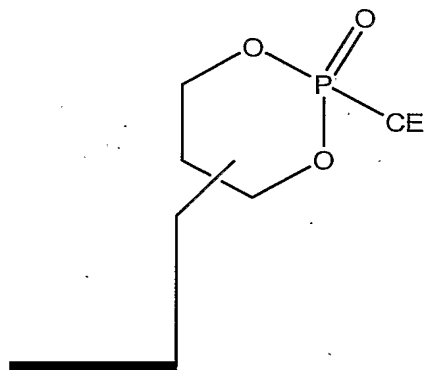


R may be absent or NO₂, CF₃, halogen, preferably Cl, Br, or I, and Z may be S or O. This type of building block is disclosed in Danish patent application No. PA 2002 0951 and US provisional patent application filed 20 December 2002 with the title "A building block capable of transferring a functional entity to a recipient reactive group". The content of both patent application are incorporated herein in their entirety by reference.

A nucleophilic group can cleave the linkage between Z and the carbonyl group thereby transferring the chemical entity $-(C=O)-CE'$ to said nucleophilic group.

Building blocks transferring a chemical entity to a recipient reactive group forming a C=C bond

A building block as shown below are able to transfer the chemical entity to a recipient aldehyde group thereby forming a double bond between the carbon of the aldehyde and the chemical entity

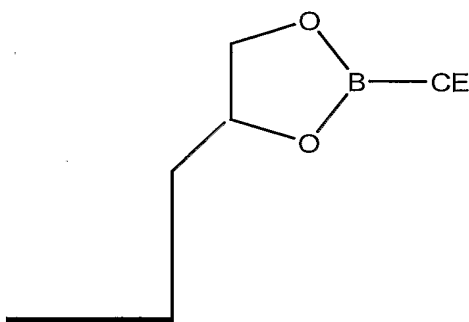


The above building block is comprised by the Danish patent application No. DK PA 2002 01952 and the US provisional patent application filed 20 December 2002 with the title "A building block capable of transferring a functional entity to a recipient reactive group forming a C=C double bond". The content of both patent applications are incorporated herein in their entirety by reference.

10 *Building blocks transferring a chemical entity to a recipient reactive group forming a C-C bond*

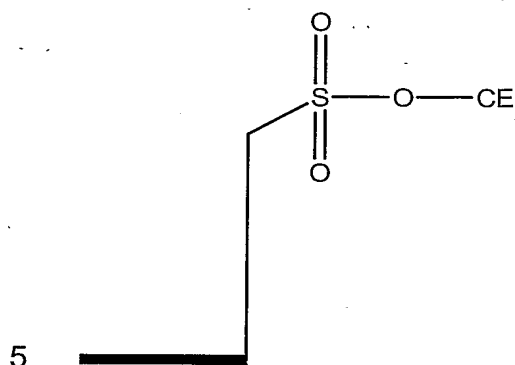
The below building block is able to transfer the chemical entity to a recipient group thereby forming a single bond between the receiving moiety, e.g. a scaffold, and the chemical entity.

15



The above building block is comprised by the Danish patent application No. DK PA 2002 01947 and the US provisional patent application No 60/434,428. The content of both patent applications are incorporated herein in their entirety by reference.

Another building block capable of transferring a chemical entity to a receiving reactive group forming a single bond is



The receiving group may be a nucleophile, such as a group comprising a hetero atom, thereby forming a single bond between the chemical entity and the hetero atom, or the receiving group may be an electronegative carbon atom, thereby forming a C-C bond between the chemical entity and the scaffold.

The chemical entity attached to any of the above building blocks may be a selected from a large arsenal of chemical structures. Examples of chemical entities are

H or entities selected among the group consisting of a C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₄-C₈ alkadienyl, C₃-C₇ cycloalkyl, C₃-C₇ cycloheteroalkyl, aryl, and heteroaryl, said group being substituted with 0-3 R⁴, 0-3 R⁵ and 0-3 R⁹ or C₁-C₃ alkylene-NR⁴₂, C₁-C₃ alkylene-NR⁴C(O)R⁸, C₁-C₃ alkylene-NR⁴C(O)OR⁸, C₁-C₂ alkylene-O-NR⁴₂, C₁-C₂ alkylene-O-NR⁴C(O)R⁸, C₁-C₂ alkylene-O-NR⁴C(O)OR⁸ substituted with 0-3 R⁹.

where R⁴ is H or selected independently among the group consisting of C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₃-C₇ cycloalkyl, C₃-C₇ cycloheteroalkyl, aryl, heteroaryl, said group being substituted with 0-3 R⁹ and

R⁵ is selected independently from -N₃, -CNO, -C(NOH)NH₂, -NHOH, -NHNHR⁶, -C(O)R⁶, -SnR⁶₃, -B(OR⁶)₂, -P(O)(OR⁶)₂ or the group consisting of

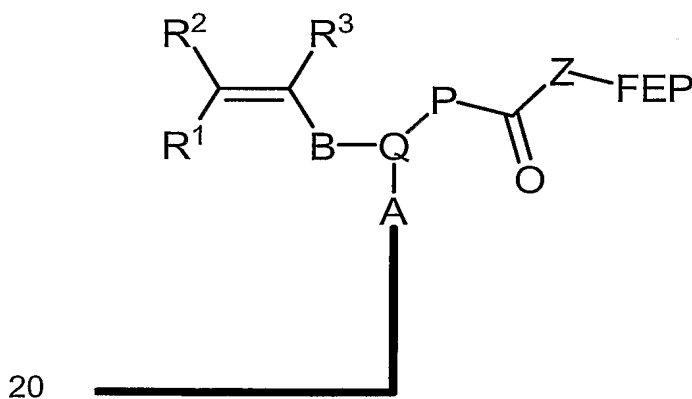
C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₄-C₈ alkadienyl said group being substituted with 0-2 R⁷,

- where R⁶ is selected independently from H, C₁-C₆ alkyl, C₃-C₇ cycloalkyl, aryl or C₁-C₆ alkylene-aryl substituted with 0-5 halogen atoms selected from -F, -Cl, -Br, and -I; and R⁷ is independently selected from -NO₂, -COOR⁶, -COR⁶, -CN, -OSiR⁶₃, -OR⁶ and -NR⁶₂.

- R⁸ is H, C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₃-C₇ cycloalkyl, aryl or C₁-C₆ alkylene-aryl substituted with 0-3 substituents independently selected from -F, -Cl, -NO₂, -R³, -OR³, -SiR³₃
- 10 R⁹ is =O, -F, -Cl, -Br, -I, -CN, -NO₂, -OR⁶, -NR⁶₂, -NR⁶-C(O)R⁸, -NR⁶-C(O)OR⁸, -SR⁶, -S(O)R⁶, -S(O)₂R⁶, -COOR⁶, -C(O)NR⁶₂ and -S(O)₂NR⁶₂.

Cross-link cleavage building blocks

- 15 It may be advantageous to split the transfer of a chemical entity to a recipient reactive group into two separate steps, namely a cross-linking step and a cleavage step because each step can be optimized. A suitable building block for this two step process is illustrated below:



- Initially, a reactive group appearing on the functional entity precursor (abbreviated FEP) reacts with a recipient reactive group, e.g. a reactive group appearing on a scaffold, thereby forming a cross-link. Subsequently, a cleavage
- 25 is performed, usually by adding an aqueous oxidising agent such as I₂, Br₂,

Cl₂, H⁺, or a Lewis acid. The cleavage results in a transfer of the group HZ-FEP- to the recipient moiety, such as a scaffold.

In the above formula

5 Z is O, S, NR⁴

 Q is N, CR¹

 P is a valence bond, O, S, NR⁴, or a group C₅₋₇arylene, C₁₋₆alkylene, C₁₋₆O-alkylene, C₁₋₆S-alkylene, NR¹-alkylene, C₁₋₆alkylene-O, C₁₋₆alkylene-S option said group being substituted with 0-3 R⁴, 0-3 R⁵ and 0-3 R⁹ or C₁₋₃ alkylene-NR⁴₂, C₁₋₃ alkylene-NR⁴C(O)R⁸, C₁₋₃ alkylene-NR⁴C(O)OR⁸,
10 C₁₋₂ alkylene-O-NR⁴₂, C₁₋₂ alkylene-O-NR⁴C(O)R⁸, C₁₋₂ alkylene-O-NR⁴C(O)OR⁸ substituted with 0-3 R⁹,

 B is a group comprising D-E-F, in which

 D is a valence bond or a group C₁₋₆alkylene, C₁₋₆alkenylene, C₁₋₆alkynylene, C₅₋₇arylene, or C₅₋₇heteroarylene, said group optionally being
15 substituted with 1 to 4 group R¹¹,

 E is, when present, a valence bond, O, S, NR⁴, or a group C₁₋₆alkylene, C₁₋₆alkenylene, C₁₋₆alkynylene, C₅₋₇arylene, or C₅₋₇heteroarylene, said group optionally being substituted with 1 to 4 group R¹¹,

20 F is, when present, a valence bond, O, S, or NR⁴,

 A is a spacing group distancing the chemical structure from the complementing element, which may be a nucleic acid,

 R¹, R², and R³ are independent of each other selected among the group consisting of H, C₁₋₆ alkyl, C₂₋₆ alkenyl, C₂₋₆ alkynyl, C₄₋₈ alkadienyl, C₃₋₇ cycloalkyl, C₃₋₇ cycloheteroalkyl, aryl, and heteroaryl, said
25 group being substituted with 0-3 R⁴, 0-3 R⁵ and 0-3 R⁹ or C₁₋₃ alkylene-NR⁴₂, C₁₋₃ alkylene-NR⁴C(O)R⁸, C₁₋₃ alkylene-NR⁴C(O)OR⁸, C₁₋₂ alkylene-O-NR⁴₂, C₁₋₂ alkylene-O-NR⁴C(O)R⁸, C₁₋₂ alkylene-O-NR⁴C(O)OR⁸ substituted with 0-3 R⁹,

30 FEP is a group selected among the group consisting of H, C₁₋₆ alkyl, C₂₋₆ alkenyl, C₂₋₆ alkynyl, C₄₋₈ alkadienyl, C₃₋₇ cycloalkyl, C₃₋₇ cycloheteroalkyl, aryl, and heteroaryl, said group being substituted with 0-3 R⁴, 0-3

R^5 and 0-3 R^9 or C_1 - C_3 alkylene- NR^4_2 , C_1 - C_3 alkylene- $NR^4C(O)R^8$, C_1 - C_3 alkylene- $NR^4C(O)OR^8$, C_1 - C_2 alkylene- $O-NR^4_2$, C_1 - C_2 alkylene- $O-NR^4C(O)R^8$, C_1 - C_2 alkylene- $O-NR^4C(O)OR^8$ substituted with 0-3 R^9 ,

where R^4 is H or selected independently among the group consisting of C_1 - C_6 alkyl, C_2 - C_6 alkenyl, C_2 - C_6 alkynyl, C_3 - C_7 cycloalkyl, C_3 - C_7 cycloheteroalkyl, aryl, heteroaryl, said group being substituted with 0-3 R^9 and

R^5 is selected independently from $-N_3$, $-CNO$, $-C(NO)NH_2$, $-NHOH$, $-NHNHR^6$, $-C(O)R^6$, $-SnR^6_3$, $-B(OR^6)_2$, $-P(O)(OR^6)_2$ or the group consisting of C_2 - C_6 alkenyl, C_2 - C_6 alkynyl, C_4 - C_8 alkadienyl said group being substituted with 0-2 R^7 ,

where R^6 is selected independently from H, C_1 - C_6 alkyl, C_3 - C_7 cycloalkyl, aryl or C_1 - C_6 alkylene-aryl substituted with 0-5 halogen atoms selected from $-F$, $-Cl$, $-Br$, and $-I$; and R^7 is independently selected from $-NO_2$, $-COOR^6$, $-COR^6$, $-CN$, $-OSiR^6_3$, $-OR^6$ and $-NR^6_2$.

R^8 is H, C_1 - C_6 alkyl, C_2 - C_6 alkenyl, C_2 - C_6 alkynyl, C_3 - C_7 cycloalkyl, aryl or C_1 - C_6 alkylene-aryl substituted with 0-3 substituents independently selected from $-F$, $-Cl$, $-NO_2$, $-R^3$, $-OR^3$, $-SiR^3_3$

R^9 is $=O$, $-F$, $-Cl$, $-Br$, $-I$, $-CN$, $-NO_2$, $-OR^6$, $-NR^6_2$, $-NR^6-C(O)R^8$, $-NR^6-C(O)OR^8$, $-SR^6$, $-S(O)R^6$, $-S(O)_2R^6$, $-COOR^6$, $-C(O)NR^6_2$ and $-S(O)_2NR^6_2$.

In a preferred embodiment Z is O or S, P is a valence bond, Q is CH, B is CH_2 , and R^1 , R^2 , and R^3 is H. The bond between the carbonyl group and Z is cleavable with aqueous I_2 .

Partitioning

The partitioning step, by which the library of bifunctional molecules is subjected to a condition partitioning one or more complexes having a certain property from the remainder of the library, may be referred to as the enrichment step or the selection step, as appropriate, and includes the screening of the library for encoded molecules having predetermined desirable characteristics. Predetermined desirable characteristics can include binding to a target,

catalytically changing the target, chemically reacting with a target in a manner which alters/modifies the target or the functional activity of the target, and covalently attaching to the target as in a suicide inhibitor.

- 5 In theory, molecules of interest can be selected based on their properties using either physical or physiological procedures. The method preferred according to the present invention is to enrich molecules with respect to binding affinity towards a target of interest. In a certain embodiment, the basic steps involve mixing the library of complexes with the immobilized target of interest.
- 10 The target can be attached to a column matrix or microtitre wells with direct immobilization or by means of antibody binding or other high-affinity interactions. In another embodiment, the target and displayed molecules interact without immobilisation of the target. Displayed molecules that bind to the target will be retained by a filter, size-exclusion chromatography *etc*, while non-
- 15 binding displayed molecules will be removed during a single or a series of wash steps. The identifiers of complexes bound to the target can then be separated by cleaving a physical connection to the encoded molecule or the entire complex may be eluted. It may be considered advantageously to perform a chromatography step after or instead of the washing step. After the
- 20 cleavage of the physical link between the synthetic molecule and the identifier, the identifier may be recovered from the media and optionally amplified before the decoding step.

- A significant reduction in background binders may be obtained with increased
- 25 washing volumes, repeating washing steps, higher detergent concentrations and prolonged incubation during washing. Thus, the more volume and number of steps used in the washing procedure together with more stringent conditions will more efficiently remove non-binders and background binders. The right stringency in the washing step can also be used to remove low-affinity
- 30 specific binders. However, the washing step will also remove wanted binders if too harsh conditions are used.

A blocking step, such as incubation of solid phase with skimmed milk proteins or other inert proteins and/or mild detergent such as Tween-20 and Triton X-100, may also be used to reduce the background. The washing conditions should be as stringent as possible to remove background binding but to
5 retain specific binders that interact with the immobilized target. Generally, washing conditions are adjusted to maintain the desired affinity binders, e.g. binders in the micromolar, nanomolar, or picomolar range.

The target can be any compound of interest. E.g. the target can be a protein,
10 peptide, carbohydrate, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, substrate, metabolite, transition state analogue, cofactor, inhibitor, drug, dye, nutrient, growth factor, cell, tissue, etc. without limitation. Suitable targets include, but are not limited to, angiotensin converting enzyme, renin, cyclooxygenase, 5-lipoxygenase, IL-10 converting enzyme,
15 cytokine receptors, PDGF receptor, type II inosine monophosphate dehydrogenase, β -lactamases, integrin, proteases like factor VIIa, kinases like Bcr-Abl/Her, phosphatases like PTP-1B, and fungal cytochrome P-450. Targets can include, but are not limited to, bradykinin, neutrophil elastase, the HIV proteins, including *tat*, *rev*, *gag*, *int*, RT, nucleocapsid etc., VEGF, bFGF,
20 TGF β , KGF, PDGF, GPCR, thrombin, substance P, IgE, sPLA2, red blood cells, glioblastomas, fibrin clots, PBMCs, hCG, lectins, selectins, cytokines, ICP4, complement proteins, etc.

A target can also be a surface of a non-biological origin, such as a polymer
25 surface or a metal surface. The method of the invention may then be used to identify suitable coatings for such surfaces.

In a preferred embodiment, the desirable synthetic molecule acts on the target without any interaction between the nucleic acid attached to the desirable
30 encoded molecule and the target. In one embodiment, the bound complex-target aggregate can be partitioned from unbound complexes by a number of methods. The methods include nitrocellulose filter binding, column chroma-

tography, filtration, affinity chromatography, centrifugation, and other well known methods. A preferred method is size-exclusion chromatography.

Briefly, the library of complexes is subjected to the target, which may include
5 contact between the library and a column onto which the target is immobilised. Identifiers associated with undesirable encoded molecules, i.e. synthetic molecules not bound to the target under the stringency conditions used, will pass through the column. Additional undesirable encoded molecules (e.g. encoded molecules which cross-react with other targets) may be
10 removed by counter-selection methods. Desirable complexes are bound to the column. The target may be immobilized in a number of ways. In one embodiment, the target is immobilized through a cleavable physical link, such as one more chemical bonds. The aggregate of the target and the complex may then be subjected to a size exclusion chromatography to separate the aggregate from the rest of the compounds in the media. The complex may then be
15 eluted from the target by changing the conditions (e.g., salt, pH, surfactant, temperature etc.). Alternatively, the complex may be provided with a cleavable linker, preferable orthogonal to a cleavable linker that attaches the target to the solid support, at a position between the synthetic molecule and the
20 identifier. Subsequent to the size exclusion chromatography this cleavable linker is cleaved to separate the identifiers of complexes having affinity towards the targets. Just to mention a single type of orthogonal cleavable linkages, one could attach the target to the solid support through a linkage that can be cleaved by a chemical agent, and the linker separating the synthetic
25 molecule and the identifier may be selected as a photocleavable linkage. More specifically, the former linkage may be a disulphide bond that can be cleaved by a suitable reducing agent like DTT (dithiothreitol) and the latter linkage may be a o-nitrophenyl group.

30 There are other partitioning and screening processes which are compatible with this invention that are known to one of ordinary skill in the art. In one embodiment, the products can be fractionated by a number of common

methods and then each fraction is assayed for activity. The fractionization methods can include size, pH, hydrophobicity, etc.

Inherent in the present method is the selection of encoded molecules on the basis of a desired function; this can be extended to the selection of molecules with a desired function and specificity. Specificity can be required during the selection process by first extracting complexes which are capable of interacting with a non-desired "target" (negative selection, or counter-selection), followed by positive selection with the desired target. As an example, inhibitors of fungal cytochrome P-450 (fungicides) are known to cross-react to some extent with mammalian cytochrome P-450 (resulting in serious side effects). Highly specific inhibitors of the fungal cytochrome could be selected from a library by first removing those complexes capable of interacting with the mammalian cytochrome, followed by retention of the remaining products which are capable of interacting with the fungal cytochrome.

Brief Description of the Figures

Fig. 11 discloses two embodiments of using a Taqman probe (5' nuclease probe) in the measurement of the presence or absence of a certain codon.

Fig. 12 discloses a standard curve used in example 3.

Fig. 13 discloses the result of two experiments reported in example 3.

Example 3 – illustrating the first aspect of the present invention

A preferred embodiment of the invention utilizing a universal Taqman probe is shown in Fig.11. Four codons are shown (P1 through P4; bold pattern) along with flanking regions (light pattern). A universal Taqman probe anneals to a region adjacent to the codon region, but within the amplicon defined by the universal PCR primers Pr.1 and Pr. 2. These primers could be the same as used for amplification of the identifier oligonucleotides encoding binders after an enrichment process on a specific target. However, if minimal length templates are preferred during the encoding process, the region involved in Taqman probe annealing could be appended to the library identifier oligonu-

cleotides by e.g. overlap PCR, ligation, or by employing a long downstream PCR primer containing the necessary sequences. The added length corresponding to the region necessary for annealing of the Taqman probe would be from 20 to 40 nts depending on the type of TaqMan probe and T_A of the PCR primers. The Q-PCR reactions are preferably performed in a 96- or 384-well format on a real-time PCR thermocycling machine.

Fig. 11A shows the detection of abundance of a specific codon sequence in position one. Similar primers are prepared for all codon sequences. For each codon sequence utilized to encode a specific BB in the library a Q-PCR reaction is performed with a primer oligonucleotide complementary to the codon sequence in question. A downstream universal reverse primer Pr. 2 is provided after the Taqman probe to provide for an exponential amplification of the PCR amplicon. The setup is most suited for cases where the codon constitutes a length corresponding to a length suitable for a PCR primer.

Fig. 11B shows the detection of abundance of a specific codon sequence in a specific codon position using a primer which is complementing a codon and a framing sequence. Similar primers are used for all the codons and framing sequences. For each codon sequence utilized to encode a specific BB at a specific codon position in the library a Q-PCR reaction is performed with an oligo complementary to the codon sequence in question as well as a short region up- or downstream of the codon region which ensures extension of the primer in a PCR reaction only when annealed to the codon sequence in that specific codon position. The number of specific primers and Q-PCR reactions needed to cover all codon sequences in all possible codon positions equals the number of codon sequences times the number of codon positions. Thus, monitoring the abundance of 96 different codon sequences in 4 different positions can be performed in a single run on four 96 wells micro titre plates (as shown in Fig. 11B) or a single 384 well plate on a suitable instrument. This architecture allows for the decoding of a $8,5 \times 10^7$ library of different encoded molecules.

Quantification is performed relative to the amount of full-length PCR product obtained in a parallel control reaction on the same input material performed with the two external PCR primers Pr.1 + Pr. 2. Theoretically, a similar rate of accumulation of this control amplicon compared to the accumulation of a product utilizing a single codon + sequence specific primer would indicate a 100% dominance of this particular sequence in the position in question.

Although the setups shown in Fig. 11A and 11B employ a Taqman probe strategy, other detection systems (SYBR green, Molecular Beacons etc.) could be utilized. In theory, multiplex reactions employing up to 4 different fluorophores in the same reaction could increase throughput correspondingly.

An example of how a deconvolution process of a library of encoded molecules occurs is described in the following. Imagine that at the end of a selection scheme a pool of 3 ligand families (and the corresponding coding templates) are dominating the population and present at approx. the same concentration. Three different chemical entities are present in the first position of the encoded compounds, and each of these chemical entities are present in combination with one unique chemical entity out of 3 different chemical entities in position P2. Only one chemical entity in position 3 gives rise to active binders, whereas any of a 20% subset of chemical entities (e.g. determined by charge, size or other characteristics) are present in position 4. The outcome of the initial codon profile analysis would be: 3 codon sequences are equally dominating in position P1, 3 other codon sequences in position P2, 1 unique codon sequence is dominant in P3 whereas somewhat similarly increased levels of 20% of the codon sequences (background levels of the remaining 80% sequences) are seen in P4. In such cases it could be relevant to use an iterative Q-PCR ("IQ-PCR") strategy to perform a further deconvolution of a library after selection. Again with reference to the example above, by taking the PCR products from the 3 individual wells that contained primers giving the high yields in position P1, diluting the product appropriately and

performing a second round of Q-PCR on each of these identifier oligonucleotides separately, it would be possible to deduce which codon sequence(s) is preferred in P2 when a given codon sequence is present in P1.

Experimental example: The 10 templates used for Q-PCR quantification

Taqman MGB probe binding region: *=AATTCCAGCTTCTAGGAAGAC

P1

P2

P3

P4

5' - CAGCTTGGACACCACGTCATACTAGCTGCTAGAGATGTGGTGATATTAGTGTGTGACGATGGTACGCACAAGTACGAACGTGCATCAGAGAGGACGAGCAGGACCTGGAACCTGGTGC*TTCCTCCACCACGTCCTCTGAC-3'

GGAAGAAGACAGAAGACCTG
TCAGGAGTCGAGAACTGAAG
TGTGTACGTCAACACGTCAG
TGTGGAACCTACCATCCAAGG
CCATCCAACATCGTTGGAAG
AACCTGTCTGTGAGATCTG
TCACGAAGCTGGATGATGAG
TAGCATCGATCGAACGTAGG
TCGAAGCTACTGTCGAGATG

CTCGACCACTGCAGGTGGAGCTCC
CGTGCTTCCTCTGCTGCACCACCG
CCTGGTGTGAGGTGAGCAGCAGC
CTCGACGAGGTCCATCCTGGTCGC
CGTGAGGAGCAGGTCTCTGTTCG
CCTGACACTGGTCGTGGTCGAGGC
CCATCTCGACGACCTGCTCCTGGG
CCACGAGGTCTCCACTGGTCCAGG
CCACTGAGCTGCTCCTCCAGGTGG

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Oligos for template synthesis:

FPv2: CAGCTTGGACACCACGTCATAC
RPv2: GTCAGAGACGTGGTGGAGGAA

Temp1-1: CAGCTTGGACACCACGTCATACCTAGAGATGTGGTGATATTAGTGTGTGACGAT
Temp1-2: CAGCTTGGACACCACGTCATACGGAGAAGACAGAAGACCTGATATTAGTGTGTGACGAT
Temp1-3: CAGCTTGGACACCACGTCATACCTCAGGAGTCGAGAAGCTGAAGATATTAGTGTGTGACGAT
Temp1-4: CAGCTTGGACACCACGTCATACCTGTGTAAGTCAACACGTCAGATATTAGTGTGTGACGAT
Temp1-5: CAGCTTGGACACCACGTCATACCTGTGGAAGTACCATCCAAGGATATTAGTGTGTGACGAT
Temp1-6: CAGCTTGGACACCACGTCATACCTCATCCAAACATCGTTGGAAGATATTAGTGTGTGACGAT
Temp1-7: CAGCTTGGACACCACGTCATACCACTGTCTGTGAGATCTGATATTAGTGTGTGACGAT
Temp1-8: CAGCTTGGACACCACGTCATACCTCAGGAAGCTGGATGATGAGATATTAGTGTGTGACGAT
Temp1-9: CAGCTTGGACACCACGTCATACCTAGCATCGATCGAAGCTAGGATATTAGTGTGTGACGAT
Temp1-10: CAGCTTGGACACCACGTCATACCTCGAAGCTACTGTGCGAGATGATATTAGTGTGTGACGAT

Temp2: GTCCTCTCTGATGCACGTTTCGTACTTGTGCGTACCATCGTCACACACTAATATC

Temp3-1: GAACGTGCATCAGAGAGGACGAGCAGGACCTGGAACCTGGTGCAATTCCAGCTTCTAGGAAGACT
Temp3-2: GAACGTGCATCAGAGAGGACTCGACCACTGCAGGTGGAGCTCCAATTCCAGCTTCTAGGAAGACT
Temp3-3: GAACGTGCATCAGAGAGGACGTGCTTCTCTGCTGCACCACCGAATTCCAGCTTCTAGGAAGACT
Temp3-4: GAACGTGCATCAGAGAGGACCTGGTGTGAGGTGAGCAGCAGCAATTCCAGCTTCTAGGAAGACT
Temp3-5: GAACGTGCATCAGAGAGGACTCGACGAGGTCCATCCTGGTCGCAATTCCAGCTTCTAGGAAGACT
Temp3-6: GAACGTGCATCAGAGAGGACGTGAGGAGCAGGTCTCTGTGCGAATTCCAGCTTCTAGGAAGACT
Temp3-7: GAACGTGCATCAGAGAGGACCTGACACTGGTGGTGGTCGAGGCAATTCCAGCTTCTAGGAAGACT
Temp3-8: GAACGTGCATCAGAGAGGACCATCTCGACGACCTGCTCCTGGGAATTCCAGCTTCTAGGAAGACT
Temp3-9: GAACGTGCATCAGAGAGGACACGAGGTCTCCACTGGTCCAGGAATTCCAGCTTCTAGGAAGACT
Temp3-10: GAACGTGCATCAGAGAGGACCACTGAGCTGCTCCTCCAGGTGGAATTCCAGCTTCTAGGAAGACT

Temp4: GTCAGAGACGTGGTGGAGGAAGTCTTCCTAGAAAGCTGGAATT

Synthesis of identifier oligonucleotides:

The 10 identifier oligonucleotides were assembled in 10 separate 50 µl PCR reactions each containing 0.05 pmol of the oligos Q-Temp1-X, Q-Temp2, Q-Temp3-X and Q-Temp4 (x=1 through 10) and 25 pmol of the external primers FPv2 and RPv2 with TA= 53°C. The 160 bp products were gel-purified using QIAquick Gel Extraction Kit from QIAGEN (Cat. No. 28706) and quantified on spectrophotometer. As a control, 20 ng of each of the templates (as estimated from these measurements) were loaded on an agarose gel.

10 Preparation of samples for Q-PCR:

Sample A: Generated by mixing 20 ng from each identifier oligonucleotide prep. Volume was adjusted to 50 µl. Concentration: 4 ng/µl = 38.46 fmol/µl (160bp x 650 Da/bp = 1.04x10⁵ g/mol. 1 ng= 9.615 fmol). Diluted to 10⁷ copies/5µl (0.00332 fmol/µl).

15

Sample B: 20 ng/20µl stocks of each template were prepared. The sample was mixed as follows:

5µl undil. Template #10

5µl 2x dil. Template #9

20 5µl 4x dil. Template #8

5µl 8x dil. Template #7

5µl 16x dil. Template #6

5µl 32x dil. Template #5

5µl 64x dil. Template #4

25 5µl 128x dil. Template #3

5µl 256x dil. Template #2

5µl 512x dil. Template #1

Concentration: 10ng/50µl= 0.20 ng/µl = 1.923 fmol/µl. Diluted 579.2-fold to 10⁷ copies/5µl (0.00332 fmol/µl).

30

Standard curve: The samples for the standard curve was prepared by diluting Sample A 116.55-fold to 10⁹ copies/5 µl (0.33 fmol/µl) and subsequently per-

forming a 10-fold serial dilution of this sample. 5 µl was used for each PCR reaction. The standard curve is shown in Fig. 12.

Q-PCR reactions

- 5 For 5 ml premix (for one 96-well plate):
 2.5 ml Taqman Universal PCR Master Mix (Applied Biosystems; includes Taq polymerase, dNTPs and optimized Taq pol. buffer)
 450 µl Rpv2 (10 pmol/ul)
 25 µl Taqman probe (6-FAM-TCCAGCTTCTAGGAAGAC-MGBNFQ; 50 µM;
 10 Applied Biosystems)
 1075 µl H₂O

- 40.5 µl premix was aliquoted into each well and 4.5 µl of relevant upstream PCR primer (FPv2 (for standard curve) or one of the codon specific primers
 15 listed below; 10 pmol/µl) and 5 µl sample (H₂O in wells for negative controls) was added. The codon-specific PCR primers were: (T_m calculations shown are from Vector NTI; matched to T_m for Rpv2 (67.7°C))

- | | | | |
|----|--------|--------------------------------|--------|
| | P1-1: | GTCATACTAGCTGCTAGAGATGTGGTGATA | 66.8°C |
| 20 | P1-2: | CATACGGAAGAAGACAGAAGACCTGATA | 67.8°C |
| | P1-3: | TCATACTCAGGAGTCGAGAACTGAAGATA | 67.6°C |
| | P1-4: | CATACTGTGTACGTCAACACGTCAGATA | 67.4°C |
| | P1-5: | CATACTGTGGAACCTACCATCCAAGGATA | 68.0°C |
| | P1-6: | CCATCCAACATCGTTGGAAGAT | 67.8°C |
| 25 | P1-7: | CATACAACCTGTCCTGTGAGATCTGATA | 67.7°C |
| | P1-8: | ATACTCACGAAGCTGGATGATGAGATA | 67.3°C |
| | P1-9: | CATACTAGCATCGATCGAACGTAGGATA | 68.1°C |
| | P1-10: | TCATACTCGAAGCTACTGTCGAGATGATA | 68.2°C |
| | P2-1: | ATATTAGTGTGTGACGATGGTACGCA | 67.8°C |
| 30 | P3-1: | ACAAGTACGAACGTGCATCAGAGA | 67.7°C |
| | P4-1: | CGAGCAGGACCTGGAACCT | 67.7°C |
| | P4-2: | TCGACCACTGCAGGTGGA | 68.3°C |

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	P4-3: GCTTCCTCTGCTGCACCA	66.7°C
	P4-4: GGTGTCGAGGTGAGCAGCA	69.1°C
	P4-5: CGACGAGGTCCATCCTGGT	68.6°C
	P4-6: GTGAGGAGCAGGTCCTCCTGT	68.0°C
5	P4-7: CTGACACTGGTCGTGGTCGA	68.8°C
	P4-8: CATCTCGACGACCTGCTCCT	67.9°C
	P4-9: ACGAGGTCTCCACTGGTCCA	68.3°C
	P4-10: ACTGAGCTGCTCCTCCAGGT	66.5°C

10 Thermocycling/measurement of fluorescence was performed on an Applied Biosystems ABI Prism 7900HT real-time instrument utilizing the standard cycling parameters:

95°C 10 min;

40 cycles of

15 95°C 15 sec;

60°C 1 min

All samples were run in duplicate.

20 Results

Fig. 12 shows the standard curve calculated by the 7900HT system software. The log of the starting copy number was plotted against the measured C_T value. The relationship between C_T and starting copy number was linear in the range from 10 to 10^9 template copies.

25

This standard curve was utilized by the system software to calculate the quantity in the "unknown" samples as shown below.

Table I: Sample A (Shown graphically in Fig. 13A)

Sample			
A: Equi-			
molar			
ratios	Observed A	Observed B	Expected
FPv2	12539947,00	11977503,00	10000000
P1-1	445841,90	480382,03	1000000
P1-2	884840,70	847478,56	1000000
P1-3	1013073,56	948770,00	1000000
P1-4	764187,94	741304,40	1000000
P1-5	1352874,60	1275155,50	1000000
P1-6	1284075,60	1337928,50	1000000
P1-7	658161,80	747371,56	1000000
P1-8	742187,20	653874,00	1000000
P1-9	824587,75	705785,75	1000000
P1-10	813550,75	836037,90	1000000
P2-1	13145159,00	14482606,00	10000000
P3-1	13263911,00	12773780,00	10000000
P4-1	1430704,80	1472576,80	1000000
P4-2	2681652,00	2481824,80	1000000
P4-3	1933106,80	2085476,40	1000000
P4-4	1359684,40	1364621,40	1000000
P4-5	2206709,80	2065813,60	1000000
P4-6	1652718,10	1873777,20	1000000
P4-7	1468208,10	1416153,00	1000000
P4-8	1664467,50	1581067,00	1000000
P4-9	1462520,60	1594593,80	1000000
P4-10	2020088,20	1912277,40	1000000

Table II: Sample B (Shown graphically in Fig. 13B)

Sample		Observed	
B: 2-fold	Observed A	B	Expected

dil.			
FPv2	4,97E+06	5,05E+06	10000000
P1-1	9955,07	10899,97	9765,625
P1-2	12732,32	13469,12	19531,25
P1-3	25542,8	25419,85	39062,5
P1-4	34748,89	44070,81	78125
P1-5	110881,41	123734,13	156250
P1-6	163687,44	166220,5	312500
P1-7	156993,81	172005,64	625000
P1-8	343176,78	374809,13	1250000
P1-9	646619,44	576151	2500000
P1-10	1,49E+06	1,72E+06	5000000
P2-1	5,19E+06	5,37E+06	10000000
P3-1	5,29E+06	5,09E+06	10000000
P4-1	(no signal)	70223,8	9765,625
P4-2	42103,32	22733,17	19531,25
P4-3	54480,62	39663,62	39062,5
P4-4	51293,07	43950,9	78125
P4-5	137946,95	115027,34	156250
P4-6	174134,64	156442,55	312500
P4-7	316505,78	283856,84	625000
P4-8	737661,44	691296,75	1250000
P4-9	1,42E+06	1,45E+06	2500000
P4-10	3,72E+06	3,52E+06	5000000

The results of the experiments show the possibility of accurately quantification of identifier oligonucleotides down to or even below 10 copies with a 9 fold dynamic range, and reliable relative quantification of the tested codons in various positions in the identifier oligonucleotide.

While the invention has been described with references to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention. All patent and literature references cited herein are hereby incorporated by reference in
5 their entirety.

The following items describe embodiments of the first aspect of the present invention:

10 Item 1: A method for obtaining structural information about an encoded molecule produced by a process comprising reaction of a plurality of chemical entities, said encoded molecule being capable of forming part of a complex also comprising an identifier oligonucleotide containing codons informative of the identity of chemical entities which have participated in the formation of the
15 encoded molecule, the method comprises

- a) mixing a primer oligonucleotide with the identifier oligonucleotide,
- b) subjecting the mixture to a condition allowing for an extension reaction to occur when the primer is sufficient complementary to a part of the identifier oligonucleotide, and
20 c) evaluating, based on measurement of the extension reaction, the presence, absence, or relative abundance of one or more codons.

25 Item 2: The method according to Item 1, wherein a composition of one, two, or more identifier oligonucleotides are processed simultaneously.

Item 3: The method according to Item 2, the composition is a result of subjecting a library of different complexes to a condition partitioning one or more
30 complexes having a certain property from the remainder of the library.

Item 4: The method according to Item 1, wherein the condition which allows for an extension reaction to occur includes a polymerase or a ligase as well as suitable substrates.

- 5 Item 5: The method according to Item 4, wherein the condition includes a polymerase and a substrate comprising a blend of (deoxy)ribonucleotide triphosphates.

- 10 Item 6: The method according to any of the preceding Items, wherein the chemical entities are precursors for structural units appearing in the encoded molecule.

- 15 Item 7: The method according to any of the Items 1 to 6, wherein the process of producing the one or more encoded molecules comprises transferring one or more chemical entities to a nascent encoded molecule by a building block which further comprises an anti-codon.

- 20 Item 8: The method of Item 7, wherein the information of the anti-codon is transferred in conjunction with the chemical entity to the nascent encoded molecule.

Item 9: The method according to any of the preceding Items 1 to 8, wherein the identifier comprises two or more codons.

- 25 Item 10: The method according to any of the preceding Items 1 to 8, wherein the identifier comprises three or more codons.

- 30 Item 11: The method according to any of the preceding Items, wherein neighbouring codons of the identifier are spaced by a framing sequence.

Item 12: The method according to Item 11, wherein the framing sequence positions the reaction of a chemical entity in the synthesis history of the encoded molecule.

5 Item 13: The method according to any of the Items 1 to 12, wherein at least a part of the primer is complementary to a codon.

Item 14: The method according to any of Items 1 to 13, wherein at least a part of the primer is complementary to a codon and an adjacent framing sequence.
10

Item 15: The method according to any of the Items 1 to 13, wherein the codons have a length of four or more nucleotides.

15 Item 16: The method according to any of the Items 1 to 15, wherein the sequence comprising the codon and an adjacent framing sequence has a total length of 11 nucleotides or more.

Item 17: The method according to any of the Items 1 to 16, wherein the extension reaction is measured using the polymerase chain reaction (PCR),
20 wherein the primer of Item 1 is involved in said PCR.

Item 18: The method according to any of the Items 1 to 17, wherein a primer is labelled.
25

Item 19: The method according to Item 18, wherein the primer is labelled with a small molecule, a radio active component, or a fluorogenic molecule.

Item 20: The method according to Item 19, wherein the small molecule label
30 is selected from biotin, dinitrophenol, and digoxigenin, and the PCR amplicons are detected using an enzyme labelled streptavidin, anti-dinitrophenol, or anti-digoxigenin, respectively, reporter molecule.

Item 21: The method according to any of the Items 1 to 19, wherein extension reaction is measured by real-time PCR.

5 Item 22: The method according to Item 21, wherein the real-time PCR involves the use of an oligonucleotide probe responsible for the generation of a detectable signal during the propagation of the PCR reaction.

10 Item 23: The method according to any of the Items 1 to 21, wherein the probe is designed to hybridise at a position downstream of a primer binding site.

Item 24: The method according to Item 22 or 23, wherein the probe is a 5' nuclease oligoprobe or a hairpin oligoprobe.

15 Item 25: The method according to Items 2 or 3, wherein the library comprises complexes with identifier oligonucleotides having n codon positions and the codons in said codon positions being selected from a set of m different codons.

20 Item 26: The method according to Item 25, wherein a framing sequence is related to each of the n codon positions in a particular complex, said framing sequence positions the reaction of a chemical entity in the synthesis history of the encoded molecule.

25 Item 27: The method according to Item 25, wherein each codon in the set of m different codons differs from any other codons in the set in at least two nucleotide positions.

30 Item 28: The method according to Item 26, wherein each framing sequence in a set of n different framing sequences differs from any other framing sequences in the set in at least two nucleotide positions.

Item 29: A method for identifying the chemical entities utilized in the formation of an encoded molecule or a composition of encoded molecules, wherein in separate compartments, $n \times m$ primers individually are mixed with an aliquot of a composition obtained by subjecting a library of different complexes to a condition partitioning said composition from the remainder of the library, subjected to a mixture of polymerase and substrate (deoxy)ribonucleotide triphosphates under conditions allowing for an extension reaction to occur when a primer is sufficient complementary to a part of one or more identifier oligonucleotides present in the aliquot, and evaluation, based on measurement of the extension reaction, the presence, absence, or relative abundance of one or more codons in each compartment.

Item 30: A set comprising a collection of oligonucleotide primers, a polymerase, a composition of (deoxy)ribonucleotide triphosphates (dNTPs), and a library of complexes composed of a display molecule part and an identifier oligonucleotide, said oligonucleotide comprising codons informative of the identity of the chemical entities which has participated in the formation of the display molecule, wherein the oligonucleotide primers are sufficient complementary to codons appearing on the identifier oligo nucleotides in the library to allow for an extension reaction to occur.

Item 31: An encoded molecule identified by a method according to Items 1 to 28.

Method for identifying a display molecule

The following pages will describe the second aspect of the invention: A METHOD FOR IDENTIFYING A DISPLAY MOLECULE, in which various patent and non-patent references cited in the present application are hereby incorporated by reference in their entirety. It is envisaged that any of the embodiments of the first aspect of the present invention may be used in combination with any of the embodiments of the second aspect of the present invention; indeed any of the features described in relation to the first aspect of

the present invention may be used in combination with any of the embodiments or features of the second aspect of the present invention, and vice versa.

- 5 The second aspect of the present invention relates to a method for identifying from a library a display molecule having affinity towards one or more molecular targets. The display molecule is a part of a complex also comprising an identifier oligonucleotide that codes for said display molecule.
- 10 Traditional drug discovery begins with a pathological phenomenon in an organism and the development of a therapeutic theory to combat this. A chemical concept follows to produce compounds for screening. Most of the processes for curing the pathological phenomenon originate with the understanding of some biological pathways and screening for an effect in tissues or
- 15 cells. This may or may not eventually reveal a "target". The target can be identified by various conventional methods, including protein expressing, protein chemistry, structure-functional studies, knowledge of biochemical pathways, and genetic studies.
- 20 In recent years, genetic information has increasingly guided the identification of molecular targets. These are derived from the knowledge of the genes of specific cell phenotypes that encode proteins that may be involved in the pathogenesis of a particular disease state.
- 25 A lead is a compound, usually a small organic molecule that demonstrates a desired biological activity on a target. Usually, a collection of compounds, referred to as a "library", is screened before a useful lead is identified. Today, many libraries are commercially available or open to public. Most pharmaceutical companies house their own compilation of compounds that have
- 30 been synthesised over several years and screened against a variety of targets.

Each compound in a library must be screened by an appropriate assay against the target. Techniques for handling the screening of several thousands compounds simultaneously have been developed and are generally referred to as high-throughput screening techniques. To push the limit of compounds possible to screen simultaneously, different manufactures have been developing instrumentation capable of handling multiple micro titer plate formats on the same platform using 384 and 1536-well plates. Advances in small volume liquid dispensing and pipetting, reliable handling of standardized plates and simplified assay formats all have made an impact on the reliability of the high-throughput screening process.

However, high-throughput screening has the disadvantage that each of the compounds has to be positioned in spatially discrete regions, usually in wells of a micro titer plate in order to observe an interaction with a target. If more than a single compound is present, it is not feasible to discern which compound displaying the appropriate biological activity. Thus, the full power of combinatorial chemistry cannot be applied because a collection of compounds usually is produced in a single container.

To be able to select a possible lead compound in a collection of compounds placed in the same container, libraries of bifunctional complexes have been evolved. Each bifunctional complex in the library comprises a potential lead compound coupled to an identifier oligonucleotide sequence. The identifier oligonucleotide sequence is suitably a nucleic acid which identifies the potential lead compound. When a library of bifunctional complexes is screened against a target, one or more of the potential leads may bind to the target. After removal of the remainder of the library, the binding bifunctional complexes can be eluated and the lead compound identified by sequencing the identifier oligonucleotide.

Various techniques for producing bifunctional complexes are known from the prior art. Some attempts to form the complex comprising a molecule as well

as the identifier oligonucleotide that codes therefore, are based on the split-and-mix principle known from combinatorial chemistry, see e.g. WO 93/06121 A1, EP 643 778 B1, and WO 00/23458.

- 5 Other attempts have focussed on the formation of encoded proteins using the natural machinery of a cell and connecting the formed protein with the template nucleic acid that has coded for the amino acid components of the protein. Examples of suitable systems are phage display, *E. coli* display, ribosome display (WO 93/03172), and protein-mRNA-fusions (WO 98/31700).
- 10 The genetic information of the nucleic acid, usually mRNA or DNA, may not necessarily be decoded between each round of selection to establish the identity of the chemical entities that has formed the protein because the nucleic acid can be amplified by known means, such as PCR, and processed for the formation of a new library enriched in respect of suitable binding proteins.
- 15

Recently, a method for encoding molecules has been suggested, which can be performed in several selection rounds without intermediate decoding, wherein the encoded molecule is not restricted to peptides and proteins. WO

20 02/00419 and WO 02/103008 disclose methods for preparing virtually any molecule connected to an identifier oligonucleotide coding for chemical entities which have reacted to form the display molecule. In short, a template segregated into a plurality of codons and a plurality of building blocks comprising a transferable chemical entity and an anticodon are initially provided.

25 Under hybridisation conditions, the template and building blocks are annealed together and the chemical entities are subsequently reacted to form the molecule.

The present invention aims at providing an efficient method for identifying

30 display molecules having affinity towards a target using a library of bifunctional complexes.

The second aspect of the present invention concerns a method for identifying display molecule(s) having affinity towards molecular target(s), comprising the steps of

- 5 mixing one or more molecular target(s) associated with target oligonucleotide(s) and a library of bifunctional complexes, each bifunctional complex of the library comprising a display molecule attached to an identifier oligonucleotide, which codes for said display molecule,
- coupling to the target oligonucleotide(s) the identifier oligonucleotide of complexes comprising display molecules binding to the target, and
- 10 deducing the identity of the binding display molecule(s) and/or the molecular target(s) from the coupled product between the identifier oligonucleotide(s) and the target oligonucleotide(s).

The second aspect of the present invention is based on the realization that a

15 close proximity of the identifier oligonucleotides relative to the target oligonucleotide is obtained when a display molecule has binding affinity towards a target and therefore, will be more prone to be coupled to the target oligonucleotide compared to identifier oligonucleotides of complexes not comprising a display molecule having affinity toward the target. The tendency to be coupled

20 together depends on various factors such as (1) the affinity of the displayed molecule towards the target, (2) the length between the displayed molecule and the coupling point on the identifier oligonucleotide sequence as well as the length between the target molecule and the coupling point on the target sequence, and (3) possible steric effects resulting from the nature of

25 the target or the oligonucleotides. The proximity effect, and thus the power of selectivity, will increase with higher local concentration of ends of nucleotides to be coupled together. Thus, various embodiments of the present invention may be envisaged to fine-tune the local concentration of the ends of the oligonucleotides, including appropriately selected lengths of target and identifier

30 oligonucleotides, site of attachment of the target oligonucleotide to the target relative to the binding site of the display molecule, and size of target.

- The molecular target may be of a biological origin or may be a synthetic molecular target. Typically, the molecular target stems from an organism selected from human and animals, especially vertebras. However, in other embodiments the target may originate from a plant. In the quest for a compound with therapeutical effect on the human or animal body, the target is usually expected to have an importance in a therapeutically theory that combats a certain disease. In the quest for discovering compounds with plant protective effect, the target is usually expected to originate from an organism that harms the crop or a competing undesired plant. The organism may be a fungus when a compound with fungicide effect is searched for or an insect when a compound having insecticide effect is desired. Optionally, a protein target stemming from a biological origin may be derivatised by altering, adding, or deleting one or more amino acids.
- The molecular target may be a protein, a small molecular hormone, a lipid, a polysaccharide, a whole cell, a nucleic acid, a metabolite, a heme group, etc. In a preferred aspect the target is a protein. The protein may serve the function in the organism of being an enzyme, a hormone, a structural element, a regulatory protein, a membrane channel or pump, a part of a signal transducing cascade, an antibody, etc. Suitable target enzymes include kinases, phosphatases, and proteases. The protein may occur as an independent entity or may be dimers, trimers, tetramers, or polymers and the protein may comprise a prosthetic group. Also, the target may be a fusion protein having two or more functionalities. Furthermore, the molecular target may be a soluble or insoluble agglomerate of one or more proteins and one or more substituents occurring in the body or artificial components. In another preferred embodiment, the molecular target is a nucleic acid, such as DNA or RNA aptamer or ribozyme.
- The target may be immobilized to a support or be present as a solution or a emulsion, as appropriate. The target optionally immobilized on the support, may also form a stable or quasi-stable dispersion in the media. In a certain

embodiment, the target is in solution and all the reactions occur in the solution too. The absence of an immobilization step reduces the background noise because there is no background surface to associate to. Thus the result of the assay may be more sensitive. In solution, the only background noise imaginable is when the oligonucleotides or display molecules of the library of complexes binds unspecific to the target molecules or the target oligonucleotides. The absence of an immobilization step generally necessitates a subsequent recovery step, eg. by chromatography.

10 In certain aspects of the invention, it is preferred to immobilize the molecular target on a solid support. The solid support may be beads of a column or the surface of a container. The immobilisation of the molecular target may ease the removal of the non-binding complexes by washing or similar means. In a certain embodiment, a cleavable linkage between the molecular target and the solid support is present. The cleavable linker is preferably selectively cleavable, that is, the linkage can be cleaved without cleaving other linkages in the target or the complexes. The cleavage of the linkage between the molecular target and the solid support may reduce the contribution from the background, such as complexes associated with the surface of the solid support and not binding to the molecular target.

A single or a multitude of different molecular targets may be mixed with the library of complexes. If two or more different targets are mixed with the library of complexes it is appropriate to provide the molecular targets with a genetic sequence coding for the identity of the target in question. Proving the targets with identifying oligonucleotides allows for a simultaneous decoding of the binding partners, i.e. the molecular target and the display molecule. The simultaneous decoding is not only suitable for finding binding partners. It is also valuable for finding a possible cross-binding interaction or to find other display molecules competing for the same target. Furthermore, appropriate selection of display molecules and/or molecular targets, can generate useful

information for preparing a structure-activity relationship (usually abbreviated SAR).

5 The molecular target may be obtained in any suitable way. A variety of targets are commercially available, either as purified protein or as the corresponding cDNA. Other protein or peptide targets may be isolated from tissues or mRNA (or the corresponding cDNA) may be extracted from a tissue. Smaller peptides may be synthesised chemically using the standard solid-phase Fmoc peptide synthesis. When nucleic acids are used or included in
10 the molecular target, it may be synthesised using the standard amedite synthesis method or by using the natural machinery.

The target can be associated with the target oligonucleotide using any suitable means and the association may involve a covalent or non-covalent linkage.
15 In an aspect of the invention the oligonucleotide is associated with the target utilizing a chemical synthesis. A protein usually comprises several groups that may be functionalized and used as attaching point. As examples the side chain of lysine contains an amino group, the side chain of serine contains a hydroxyl group and the side chain of cystein contains a thiol
20 group, all of which may serve as anchoring point for a target oligonucleotide comprising e.g. a carboxylic acid group. In another aspect of the invention, the protein target is fused to a tag, such as a His-tag, Flag-tag, antibody, or streptavidin. The tag can be selectively recognized by an anti-body or small molecule such as biotin or dinitrophenol. The anti-body or the small molecule
25 is attached to the target oligonucleotide, thereby ensuring an efficient coupling of the molecular target to the target oligonucleotide.

The target oligonucleotide can be associated to the molecular target through a cleavable linker. The cleavable linkage can be used to separate the target
30 oligonucleotide from the molecular target or the coupled product from the target at a point in time following the contacting between the target and library of complexes. The target oligonucleotide can be distanced from the

molecular target by a suitable linker, such as a polyethylene linker or a non-coding nucleotide sequence. A linker may remedy any interaction that possible can be between the molecular target and the target oligonucleotide and at the same time provide suffice space for an enzyme to perform its action.

5

A variety of methods for association of an oligonucleotide to a target is available for the skilled person in the art. An option involves the association of a target protein with the mRNA responsible for the formation thereof. This method is generally referred to as mRNA display. Optionally, the mRNA may be substituted with the corresponding cDNA. A method for generation such a single or a library of fusions between a protein and the mRNA responsible for the formation thereof is disclosed in WO 98/31700. The corresponding DNA strand may be attached to the protein using the method disclosed in WO 00/32823. The contents of both patent applications being incorporated in their entirety by reference herein. The method of WO 98/31700 includes providing a RNA stand comprising a translation initiation sequence, a start codon operable linked to a protein encoding sequence, and a peptide acceptor at the 3' end and translating the protein encoding sequence to produce a RNA-protein fusion. According to WO 00/32823 a DNA primer is covalently connected to the 3' end of the mRNA strand and extended by reverse transcriptase a to prepare the complementing DNA strand. The original RNA strand may be digested by RNase H. Another suitable method for generating a target library is disclosed in WO 01/90414, the content of which is incorporated herein by reference.

20
25

In accordance with another option, the target oligonucleotide is associated with a target using a method generally referred to as ribosome display. Ribosome display is disclosed in WO 93/03172, the content of which is included herein by reference. A still further option for association is a variation of the phage display, in which a target is displayed on the capsule of the phage and a target oligonucleotide is connected to the same capsule. Suitably, the target oligonucleotide is connected to the capsule via reactive groups positioned

30

on proteins expressed on the capsule. A suitable reactive group is -SH emanating from cysteine.

5 A further option for associating the target with an oligonucleotide includes the method disclosed in M. Yonezawa *et al*, Nucleic acid research, 2003, vol. 31, No. 19 e118 (included by reference). The method includes the initial provision of a target oligonucleotide connected to biotin and compartmentalization thereof together with a transcription and translation system. The target oligonucleotide comprises a fusion gene coding for streptavidin and a target.
10 After the formation of the fusion protein in each compartment, the streptavidin part of the fusion protein binds to the biotin moiety of the target oligonucleotide, thereby associating the target with the target oligonucleotide.

15 In case the target is a nucleic acid, it may be of the aptamer type, i.e. an aptamer or a library of aptamers comprising constant nucleic acid regions flanking a random oligonucleotide part. The random oligonucleotide part serves the function of a molecular target in the present invention and one or both the constant region serves as target oligonucleotides. Alternatively, an additional oligonucleotide sequence can be adhered to one or both the constant regions
20 to serve as the target oligonucleotide. The present invention also allows for the selection of pairs of aptamers that either binds to each other or binds to the same target but to different epitopes. The latter embodiment is of particular relevance when evolving detection assays, where aptamers that bind to different epitopes on the same target may be desired.

25 In certain embodiments, a bifunctional complex having a display molecule binding to the molecular target constitutes the target oligonucleotide associated with the molecular target. The bifunctional complexes binding to the target and serving to associate an oligonucleotide to the molecular target may
30 be provided prior to or during with the mixing step. In case the target oligonucleotide is associated with the molecular target during the mixing step the bifunctional complex may be a member of the library or may be a complex

added to the mixture. In some aspects of the invention, the display molecule is a compound known to bind to the target. The display molecule may be known to bind to the target from the prior art or from preceding screening procedures. To find a second or further binding compound, the target is generally saturated with the known display molecule prior to the mixing step. The known binding molecule may or may not be attached to a nucleic acid sequence.

In case the target oligonucleotide emanates from an identifier oligonucleotide in the library of complexes, two bifunctional complexes of a library of bifunctional complexes are associated with a common molecular target and can be discovered simultaneously. The bifunctional complexes may bind to the same binding site of the molecular target or the bifunctional complexes may bind to different binding sites. In case the bifunctional complexes bind to the same binding sites, the display molecules may be connected to each other through a covalent or non-covalent chemical bond, e.g. by a technique known as click chemistry. By way of example only, the two display molecules can be connected through a disulphide bridge. In aspects of the invention in which the display molecules binds to discrete binding domains on the same target molecule they are usually adhered together after the identification process of the present invention by a suitable linker in order to form the effective compound.

In a certain aspects of the invention an iterative method for finding the desired compound is applied. An initial display molecule or a pool of display molecules with affinity towards a target is found using the present method or another method. In the event the affinity is in the lower range of what is desired, the initial display molecule is amended by reaction with one or more chemical entities to form a second generation library, said second generation library being used in the method of the present invention.

In Nature, biochemical components interact. In a certain aspect of the present invention, two or more targets interacting in a biological context are separately subjected to the method of the invention, whereupon the identified display molecules binding to the two or more targets are linked via a suitable linker. By way of example only, blood factors, such as factor VIIa and Xa may be prevented or promoted in their interaction with each other using a compound having a part binding to a first blood factor and a part binding to a second blood factor. In other aspects of the invention, targets are linked by a suitable linker, as disclosed in Fig. 10.

A library of molecular targets may be generated by starting out from a library of DNA molecules, usually cDNA molecules, and preparing the corresponding RNA strands by a suitable RNA polymerase. In accordance with a certain aspect of the invention, the mixture step therefore includes that a molecular target library comprising different peptides each attached to the nucleic responsible for the formation thereof is mixed with a library of complexes.

The coupling of the target oligonucleotide and the identifier oligonucleotide is promoted due to the relative high local concentration of the ends of oligonucleotides. The complexes in solution, i.e. complexes not bound to the target, are relatively remote from the target oligonucleotide and the tendency of the target oligonucleotides to be coupled to such unspecific complexes is reduced. Among other things, the unspecific coupling depends on the concentration of the complexes in solution.

In an aspect of the invention the non-binding library members are either diluted or at least partly removed prior to the coupling step. Dilution may be performed by adding a suitable buffer and the removal may be performed by washing one or more times with a suitable liquid, such as a buffer. In the event the target molecule is immobilized on a bead, the beads may be retained on a filter while non-binding complexes are transported through the filter. In case the target is in solution or made in solution by cleavage of a bond

immobilising the target, optional following one or more washing step, the removal can be performed by chromatography, such as size-exclusion chromatography.

- 5 Thus, in a certain aspect of the invention, the mixing step includes the removal of non-binding library members prior to the coupling of the target oligonucleotide and the identifier nucleotide together.

10 It may be an advantage to have all or at least a part of the nucleotides on a double stranded form during the contacting with the molecular target, as certain nucleic acids may perform a binding interaction or a catalytical action on the components present during the mixing step. Thus, in one embodiment of the invention, the target oligonucleotide and/or the identifier oligonucleotide partly or fully is hybridised to a complementing oligonucleotide.

15 The coupling may be performed using any suitable means that ensures a physical connection. Suitably, the coupling is performed using means selected from the group consisting of chemical means, enzymatic means, and design means.

20 The chemical means for coupling the ends together can be selected from a large plethora. Suitable examples include that

- a) a first oligonucleotide end comprises a 3'-OH group and the second oligonucleotide end comprises a 5'-phosphor-2-imidazole group. When reacted
25 a phosphodiester internucleoside linkage is formed,
b) a first oligonucleotide end comprising a phosphoimidazolid group and the 3'-end and a phosphoimidazolid group at the 5'-end. When reacted together a phosphodiester internucleoside linkage is formed,
c) a first oligonucleotide end comprising a 3'-phosphorothioate group and a
30 second oligonucleotide comprising a 5'-iodine. When the two groups are reacted a 3'-O-P(=O)(OH)-S-5' internucleoside linkage is formed, and

d) a first oligonucleotide end comprising a 3'-phosphorothioate group and a second oligonucleotide comprising a 5'-tosylate. When reacted a 3'-O-P(=O)(OH)-S-5' internucleoside linkage is formed.

5 Suitably, the target oligonucleotide or a complementing target oligonucleotide and the identifier oligonucleotide or a complementing identifier oligonucleotide operatively are joined together, so that as to allow a nucleic acid active enzyme to recognize the coupling area a substrate. Notably, in a preferred embodiment, the coupling is performed so as to allow a polymerase to recognize the coupled strand as a template. Thus, in a preferred aspect, a
10 chemical reaction strategy for the coupling step generally includes the formation of a phosphodiester internucleoside linkage. In accordance with this aspect, method a) and b) above is preferred.

15 The enzymatic means is in some instances preferred because the coupling reaction is specific, i.e. the risk of side reactions is virtually not present. The enzymatic means is in general selected from the enzymes of the polymerase type, ligase type, and restriction enzymes, as well as any combination thereof.

20 Ligases are useful means for the coupling step. Suitable examples include Taq DNA ligase, T4 DNA ligase, T7 DNA ligase, and *E. coli* DNA ligase. The choice of the ligase depends to a certain degree on the design of the ends to be joined together. Thus, if the ends are blunt, T4 DNA ligase may be preferred, while a Taq DNA ligase may be preferred for a sticky end ligation, i.e.
25 a ligation in which an overhang on each end is a complement to each other.

In a certain aspect of the invention, a connector oligonucleotide is used. The connector oligonucleotide has a region complementing a distal part of the
30 target oligonucleotide and a region complementing a distal part of the identifier oligonucleotide. If the ends of the target and identifier oligonucleotides abut each other a ligase can ligate the ends together. If a gap exists between

the ends, a polymerase may be used to fill the gap and a ligase may subsequently perform a ligation. The regions of the connector oligonucleotide complementing the identifier oligonucleotide and target oligonucleotide, respectively, may independently be chose, e.g. in the range of 6 to 16 nucleotides, preferably in the range of 8 to 12 nucleotides. In a particular aspect of the invention the connector oligonucleotide is added in excess relative to the total target and identifier oligonucleotides to saturate the ends of complexes not bound to a target.

10 In another aspect of the invention the coupling is performed by design means. As an example, the regions at the distal ends of the target and identifier oligonucleotides are designed to be complementary to each other. Under hybridisation conditions polymerase is then allowed to extend the target oligonucleotide as well as the identifier oligonucleotide to obtain a double
15 stranded coupled product.

Still another coupling means include the design of the ends of one or more oligonucleotides with sticky ends. In a certain aspect, the target oligonucleotide and/or the identifier oligonucleotide is provided with a sticky end to allow
20 a ligase or a polymerase or a mixture thereof to adjoin the oligonucleotides. Suitably, the sticky ends can be formed by a restriction nuclease. In a practical approach, the target and the identifier oligonucleotides are initially double stranded and provide at the ends with a restriction site. Following the initial contact, the mixture is treated with a restriction nuclease to form the sticky
25 ends. After subsequent removal of the restriction enzyme from the mixture, a ligase is allowed to perform its enzymatic action to form a ligation product.

The coupled product of the identifier and target oligonucleotides comprises the information necessary for decoding the identity of the display molecule
30 and optionally also of the molecular target. The coupled product may be analysed directly in some instances to reveal the identity of the display molecules that have performed an interaction with the molecular target. As an example,

the coupled product may be detached from the target-display molecule interaction using a cut by a restriction nuclease at positions of the coupled product flanking the informative part. The informative part can be decoded in a standard sequencing machine. In general however, it is preferred to include
5 the informative part of the coupled product in to a suitable vector and transfer the vector to a host organism. The host organisms may then be cultivated on a suitable substrate and allowed to form colonies. Samples from the colonies may be used for sequencing in a sequencing machine.

10 In another approach, the target and/or the identifier oligonucleotides or sequences complementary thereto at the proximal end is provided with a priming site. The priming site may be used for annealing a primer to allow a polymerase to extend the primer using the coupled product as template. Appropriately, the extended strand is designed with another priming site which may
15 allow a second (reverse) primer to anneal thereto and subsequently a polymerase to perform an extension of the primer to produce a sequence identical to the coupled product. The extension product or the amplification product may be analysed directly or be incorporated into a vector which subsequently is transformed into a host organism as explained above. In a certain aspect
20 of the invention, the coupled oligonucleotide is amplified by PCR using priming sites positioned proximal to the display molecule and the molecular target, respectively, and flanking the informative part of the coupled product.

Following the coupling step, the target-display molecule conjugate may be
25 recovered. Any method that result in a recovery may in principle be used, including filtering, washing, elution, chromatography, etc. In a preferred aspect the target-display molecule conjugate is recovered by chromatography following the coupling of the target and the identifier oligonucleotides. Optionally, the various recovering methods may be combined. As an example, a
30 washing or elution step may precede the chromatography step. A presently preferred chromatography method is size-exclusion chromatography. The chromatography is usually performed on a sample in which the target-display

molecule conjugate is in solution. In one aspect of the invention, the target has been cleaved from a solid support prior to the chromatography step.

5 In an aspect of the invention, selective cleavable chemical moieties at each end of the coupled oligonucleotides are cleaved to liberate the coupled oligonucleotides prior to amplification. Usually, the cleavage is preceded of a step removing the non-binding complexes. The liberated product may be recovered as described above and subjected to amplification.

10 The invention also pertains to a conjugate comprising a molecular target associated with an oligonucleotide and a bifunctional complex comprising a display molecule attached to an identifier oligonucleotide, which codes for said molecule. Usually, the display molecule part of the complex is bound to the target. The target oligonucleotide and/or the identifier oligonucleotide of
15 the conjugate are in a certain embodiment joined to the molecular target and/or the display molecule, respectively, through a selectively cleavable linkage. In an embodiment of the invention, the target oligonucleotide and the identifier oligonucleotide are coupled together, whereby a product is obtained wherein a display molecule is bound to the molecular target and the identifier
20 and target oligonucleotides are coupled together. In a certain aspect, the coupled oligonucleotide is amplifiable. The amplifiability is usually obtained through a coupling that involves a phosphodiester internucleoside linkage.

25 The present invention also extends to a display molecule identified by any of the methods disclosed herein.

Detailed Description

Complex

30 The complex used in the present invention comprises a display molecule and an identifier oligonucleotide. The identifier oligonucleotide comprises identifying moieties that identifies the display molecule. Preferably, the identifier oligonucleotide identifies the molecule uniquely, i.e. in a library of complexes a

particular identifier oligonucleotide is capable of distinguishing the molecule it is attached to from the rest of the display molecules.

5 The display molecule and the identifier oligonucleotide may be attached directly to each other or through a bridging moiety. In one aspect of the invention, the bridging moiety is a selectively cleavable linkage.

10 The method may in certain embodiments be performed without amplification after the coupling step. However, when larger libraries are used and the amount of separated coupled product oligonucleotide is relatively low, it is in general preferred to use an identifier oligonucleotide and a coupled product which is amplifiable. Identifier oligonucleotides comprising a sequence of nucleotides may be amplified using standard techniques, like PCR.

15 The identifier oligonucleotide may comprise two or more codons. The sequence of codons can be decoded to identify reactants used in the formation of the molecule. When the identifier oligonucleotide comprises more than one codon, each member of a pool of chemical entities can be identified and the order of codons is informative of the synthesis step each member has been
20 incorporated in.

The sequence of the nucleotides in each codon may have any suitable length. The codon may be a single nucleotide or a plurality of nucleotides. In some aspects of the invention, it is preferred that each codon independently
25 comprises four or more nucleotides, more preferred 4 to 30 nucleotides.

The identifier oligonucleotide will in general have at least two codons arranged in sequence, i.e. next to each other. Two neighbouring codons may be separated by a framing sequence. Depending on the encoded molecule
30 formed, the identifier oligonucleotide may comprise further codons, such as 3, 4, 5, or more codons. Each of the further codons may be separated by a suitable framing sequence. Preferably, all or at least a majority of the codons

of the identifier oligonucleotide are separated from a neighbouring codon by a framing sequence. The framing sequence may have any suitable number of nucleotides, e.g. 1 to 20. Alternatively, codons on the identifier oligonucleotide may be designed with overlapping sequences.

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The framing sequence, if present, may serve various purposes. In one setup of the invention, the framing sequence identifies the position of the codon. Usually, the framing sequence either upstream or downstream of a codon comprises information which allows determination of the position of the codons. In another setup, the frames have alternating sequences, allowing for addition of building blocks from two pools in the formation of the library. The framing sequence may also or in addition provide for a region of high affinity. The high affinity region may ensure that the hybridisation of the template with an anti-codon will occur in frame. Moreover, the framing sequence may adjust the annealing temperature to a desired level.

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A framing sequence with high affinity can be provided by incorporation of one or more nucleobases forming three hydrogen bonds to a cognate nucleobase. Examples of nucleobases having this property are guanine and cytosine. Alternatively, or in addition, the framing sequence may be subjected to backbone modification. Several backbone modifications provides for higher affinity, such as 2'-O-methyl substitution of the ribose moiety, peptide nucleic acids (PNA), and 2'-4' O-methylene cyclisation of the ribose moiety, also referred to as LNA (Locked Nucleic Acid).

The identifier oligonucleotide may comprise one or two flanking regions. The flanking region can encompass a signal group, such as a fluorophore or a radio active group to allow for detection of the presence or absence of a complex or the flanking region may comprise a label that may be detected, such as biotin. When the identifier oligonucleotide comprises a biotin moiety, the identifier oligonucleotide may easily be recovered.

The flanking region(s) can also serve as priming sites for amplification reactions, such as PCR. The identifier oligonucleotide may in certain embodiments comprise an affinity region having the property of being able to hybridise to a building block.

5

It is to be understood that when the term identifier oligonucleotide is used in the present description and claims, the identifier oligonucleotide may be in the sense or the anti-sense format, i.e. the identifier oligonucleotide can be a sequence of codons which actually codes for the molecule or can be a sequence complementary thereto. Moreover, the identifier oligonucleotide may be single-stranded or double-stranded, as appropriate.

10

The display molecule part of the complex is generally of a structure expected of having an effect on the target. When the target is of pharmaceutical importance, the molecule is generally a possible drug candidate. The complex may be formed by tagging a library of different possible drug candidates with a tag, e.g. a nucleic acid tag identifying each possible drug candidate. In another embodiment of the invention, the molecule is encoded, i.e. formed by a variety of reactants which have reacted with each other and/or a scaffold molecule. Optionally, this reaction product may be post-modified to obtain the final molecule displayed on the complex. The post-modification may involve the cleavage of one or more chemical bonds attaching the encoded molecule to the identifier in order more efficiently to display the encoded molecule. In still another embodiment the display molecule is a polypeptide formed using the natural machinery, such as the methods disclosed in WO 92/02536, WO 25 91/05058, and US 6,194,550.

20

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The formation of a synthetic encoded molecule generally starts by a scaffold, i.e. a chemical unit having one or more reactive groups capable of forming a connection to another reactive group positioned on a chemical entity, thereby generating an addition to the original scaffold. A second chemical entity may react with a reactive group also appearing on the original scaffold or a

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reactive group incorporated by the first chemical entity. Further chemical entities may be involved in the formation of the final reaction product. The formation of a connection between the chemical entity and the nascent encoded molecule may be mediated by a bridging molecule. As an example, if the nascent encoded molecule and the chemical entity both comprise an amine group a connection between these can be mediated by a dicarboxylic acid. A display molecule is in general produced in vitro and may be a naturally occurring or an artificial substance. In an aspect of the invention, a display molecule is not produced using the naturally translation system in an in vitro process. In other aspects of the invention, the display molecule is a polypeptide produced using the natural translation machinery.

The chemical entities that are precursors for structural additions or eliminations of the encoded molecule may be attached to a building block prior to the participation in the formation of the reaction product leading to the final display molecule. Besides the chemical entity, the building block generally comprises an anti-codon. In some embodiments the building blocks also comprise an affinity region providing for affinity towards the nascent complex.

Thus, the chemical entities are suitably mediated to the nascent encoded molecule by a building block, which further comprises an anticodon. The anticodon serves the function of transferring the genetic information of the building block in conjunction with the transfer of a chemical entity. The transfer of genetic information and chemical entity may occur in any order, however, it is important that a correspondence is maintained in the complex. The chemical entities are preferably reacted without enzymatic interaction in some aspects of the invention. Notably, the reaction of the chemical entities is preferably not mediated by ribosomes or enzymes having similar activity. In another aspect of the invention a ribosome is used to translate an mRNA into a protein using a tRNA loaded with a natural or unnatural amino acid. In still another aspect of the invention, enzymes having catalytic activities

different from that of ribosomes are used in the formation of the display molecule.

5 According to certain aspects of the invention the genetic information of the anti-codon is transferred by specific hybridisation to a codon on a nucleic acid template. Another method for transferring the genetic information of the anti-codon to the nascent complex is to anneal an oligonucleotide complementary to the anti-codon and attach this oligonucleotide to the complex, e.g. by ligation. A still further method involves transferring the
10 genetic information of the anti-codon to the nascent complex by an extension reaction using a polymerase and a mixture of dNTPs.

The chemical entity of the building block may in certain cases be regarded as a precursor for the structural entity eventually incorporated into the encoded
15 molecule. In other cases the chemical entity provides for the eliminations of chemical units of the nascent encoded molecule. Therefore, when it in the present application with claims is stated that a chemical entity is reacted with a nascent encoded molecule it is to be understood that not necessarily all the atoms of the original chemical entity is to be found in the eventually formed
20 encoded molecule. Also, as a consequence of the reactions involved in the connection, the structure of the chemical entity can be changed when it appears on the nascent encoded molecule. Especially, the cleavage resulting in the release of the entity may generate a reactive group which in a subsequent step can participate in the formation of a connection between a nascent
25 complex and a chemical entity.

The chemical entity of the building block comprises at least one reactive group capable of participating in a reaction which results in a connection between the chemical entity of the building block and another chemical entity or
30 a scaffold associated with the nascent complex. The number of reactive groups which appears on the chemical entity is suitably one to ten. A building block featuring only one reactive group is used *i.a.* in the end positions of

polymers or scaffolds, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffolds capable of being reacted further. One, two or more reactive groups intended for the formation of connections, are typically present on scaffolds. Non-limiting
5 examples of scaffolds are opiates, steroids, benzodiazepines, hydantoines, and peptidylphosphonates.

The reactive group of the chemical entity may be capable of forming a direct connection to a reactive group of the nascent complex or the reactive group
10 of the building block may be capable of forming a connection to a reactive group of the nascent complex through a bridging fill-in group. It is to be understood that not all the atoms of a reactive group are necessarily maintained in the connection formed. Rather, the reactive groups are to be regarded as precursors for the structure of the connection.

15 The subsequent cleavage step to release the chemical entity from the building block can be performed in any appropriate way. In an aspect of the invention the cleavage involves usage of a chemical reagent or an enzyme. The cleavage results in a transfer of the chemical entity to the nascent encoded molecule or in a transfer of the nascent encoded molecule to the chemical
20 entity of the building block. In some cases it may be advantageous to introduce new chemical groups as a consequence of linker cleavage. The new chemical groups may be used for further reaction in a subsequent cycle, either directly or after having been activated. In other cases it is desirable that
25 no trace of the linker remains after the cleavage.

In another aspect, the connection and the cleavage is conducted as a simultaneous reaction, i.e. either the chemical entity of the building block or the nascent encoded molecule is a leaving group of the reaction. In some aspects
30 of the invention, it is appropriate to design the system such that the connection and the cleavage occur simultaneously because this will reduce the number of steps and the complexity. The simultaneous connection and

cleavage can also be designed such that either no trace of the linker remains or such that a new chemical group for further reaction is introduced, as described above.

5 The attachment of the chemical entity to the building block, optionally via a suitable spacer can be at any entity available for attachment, e.g. the chemical entity can be attached to a nucleobase or the backbone. In general, it is preferred to attach the chemical entity at the phosphor of the internucleoside linkage or at the nucleobase. When the nucleobase is used for attachment of
10 the chemical entity, the attachment point is usually at the 7 position of the purines or 7-deaza-purins or at the 5 position of pyrimidines. The nucleotide may be distanced from the reactive group of the chemical entity by a spacer moiety. The spacer may be designed such that the conformational space sampled by the reactive group is optimized for a reaction with the reactive
15 group of the nascent encoded molecule.

The display molecules of the invention may have any chemical structure. In a preferred aspect, the display molecule can be any compound that may be synthesized in a component-by-component fashion. In some aspects the
20 display molecule is a linear or branched polymer. In another aspect the display molecule is a scaffolded molecule. The term "display molecule" also comprises naturally occurring molecules like α -polypeptides etc, however produced *in vitro* usually in the absence of enzymes, like ribosomes. In certain aspects, the display molecule of the library is a non- α -polypeptide.

25 The display molecule may have any molecular weight. However, in order to be orally available, it is in this case preferred that the display molecule has a molecular weight less than 2000 Daltons, preferably less than 1000 Dalton, and more preferred less than 500 Daltons.

30 The size of the library may vary considerably pending on the expected result of the inventive method. In some aspects, it may be sufficient that the library

comprises two, three, or four different complexes. However, in most events, more than two different complexes are desired to obtain a higher diversity. In some aspects, the library comprises 1,000 or more different complexes, more preferred 1,000,000 or more different complexes. The upper limit for the size of the library is only restricted by the size of the vessel in which the library is comprised. It may be calculated that a vial may comprise up to 10^{14} different complexes.

Methods for forming libraries of complexes

The complexes comprising an identifier oligonucleotide having two or more codons that codes for reactants that have reacted in the formation of the molecule part of the complex may be formed by a variety of processes. Generally, the preferred methods can be used for the formation of virtually any kind of encode molecule. Suitable examples of processes include prior art methods disclosed in WO 93/20242, WO 93/06121, WO 00/23458, WO 02/074929, and WO 02/103008, the content of which being incorporated herein by reference as well as methods of the present applicant not yet public available, including the methods disclosed in DK PA 2002 01955 filed 19 December 2002, and DK PA 2003 00430 filed 20 March 2003. Any of these methods may be used, and the entire content of the patent applications are included herein by reference.

Below four preferred embodiments are described. A first embodiment disclosed in more detail in WO 02/103008 is based on the use of a polymerase to incorporate unnatural nucleotides as building blocks. Initially, a plurality of template oligonucleotides is provided. Subsequently primers are annealed to each of the templates and a polymerase is extending the primer using nucleotide derivatives which have appended chemical entities. Subsequent to or simultaneously with the incorporation of the nucleotide derivatives, the chemical entities are reacted to form a reaction product. The encoded molecule may be post-modified by cleaving some of the linking moieties to better present the encoded molecule.

Several possible reaction approaches for the chemical entities are apparent. First, the nucleotide derivatives can be incorporated and the chemical entities subsequently polymerised. In the event the chemical entities each carry two reactive groups, the chemical entities can be attached to adjacent chemical entities by a reaction of these reactive groups. Exemplary of the reactive groups are amine and carboxylic acid, which upon reaction form an amide bond. Adjacent chemical entities can also be linked together using a linking or bridging moiety. Exemplary of this approach is the linking of two chemical entities each bearing an amine group by a bi-carboxylic acid. Yet another approach is the use of a reactive group between a chemical entity and the nucleotide building block, such as an ester or a thioester group. An adjacent building block having a reactive group such as an amine may cleave the interspaced reactive group to obtain a linkage to the chemical entity, e.g. by an amide linking group.

A second embodiment for obtainment of complexes pertains to the use of hybridisation of building blocks to a template and reaction of chemical entities attached to the building blocks in order to obtain a reaction product. This approach comprises that templates are contacted with a plurality of building blocks, wherein each building block comprises an anti-codon and a chemical entity. The anti-codons are designed such that they recognise a sequence, i.e. a codon, on the template. Subsequent to the annealing of the anti-codon and the codon to each other a reaction of the chemical entity is effected.

The template may be associated with a scaffold. Building blocks bringing chemical entities in may be added sequentially or simultaneously and a reaction of the reactive group of the chemical entity may be effected at any time after the annealing of the building blocks to the template.

A third embodiment for the generation of a complex includes chemical or enzymatical ligation of building blocks when these are lined up on a template.

Initially, templates are provided, each having one or more codons. The templates are contacted with building blocks comprising anti-codons linked to chemical entities. The two or more anti-codons annealed on a template are subsequently ligated to each other and a reaction of the chemical entities is effected to obtain a reaction product. The method is disclosed in more detail in DK PA 2003 00430 filed 20 March 2003.

A fourth embodiment makes use of the extension by a polymerase of an affinity sequence of the nascent complex to transfer the anti-codon of a building block to the nascent complex. The method implies that a nascent complex comprising a scaffold and an affinity region is annealed to a building block comprising a region complementary to the affinity section. Subsequently the anti-codon region of the building block is transferred to the nascent complex by a polymerase. The transfer of the chemical entity may be transferred prior to, simultaneously with or subsequent to the transfer of the anti-codon. This method is disclosed in detail in DK PA 2002 01955 filed 19 December 2002 and DK PA 2003 01064, filed 11 July 2003.

Thus, the codons are either pre-made into one or more templates before the encoded molecules are generated or the codons are transferred simultaneously with the formation of the encoded molecules.

After or simultaneously with the formation of the reaction product some of the linkers to the template may be cleaved, however at least one linker must be maintained to provide for the complex.

Nucleotides

The nucleotides used in the present invention may be linked together in a sequence of nucleotides, i.e. an oligonucleotide. Each nucleotide monomer is normally composed of two parts, namely a nucleobase moiety, and a backbone. The back bone may in some cases be subdivided into a sugar moiety and an internucleoside linker.

The nucleobase moiety may be selected among naturally occurring nucleobases as well as non-naturally occurring nucleobases. Thus, "nucleobase" includes not only the known purine and pyrimidine hetero-cycles, but also
5 heterocyclic analogues and tautomers thereof. Illustrative examples of nucleobases are adenine, guanine, thymine, cytosine, uracil, purine, xanthine, diaminopurine, 8-oxo-N⁶-methyladenine, 7-deazaxanthine, 7-deazaguanine, N⁴,N⁴-ethanocytosin, N⁶,N⁶-ethano-2,6-diamino-purine, 5-methylcytosine, 5-(C³-C⁶)-alkynylcytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-
10 hydroxy-5-methyl-4-triazolopyridine, isocytosine, isoguanine, inosine and the "non-naturally occurring" nucleobases described in Benner et al., U.S. Pat No. 5,432,272. The term "nucleobase" is intended to cover these examples as well as analogues and tautomers thereof. Especially interesting nucleobases are adenine, guanine, thymine, cytosine, 5-methylcytosine, and uracil,
15 which are considered as the naturally occurring nucleobases in relation to therapeutic and diagnostic application in humans.

Examples of suitable specific pairs of nucleobases are shown on p27 of this application, entitled "Natural base pairs", "synthetic base pairs", "synthetic
20 purine bases pairing with natural pyrimidines".

Suitable examples of backbone units are shown diagrammatically on p28 of this application (B denotes a nucleobase). The sugar moiety of the backbone is suitably a pentose but may be the appropriate part of an PNA or a six-
25 member ring. Suitable examples of possible pentoses include ribose, 2'-deoxyribose, 2'-O-methyl-ribose, 2'-fluor-ribose, and 2'-4'-O-methylene-ribose (LNA). Suitably the nucleobase is attached to the 1' position of the pentose entity.

30 An internucleoside linker connects the 3' end of preceding monomer to a 5' end of a succeeding monomer when the sugar moiety of the backbone is a pentose, like ribose or 2-deoxyribose. The internucleoside linkage may be

the natural occurring phosphodiester linkage or a derivative thereof. Examples of such derivatives include phosphorothioate, methylphosphonate, phosphoramidate, phosphotriester, and phosphodithioate. Furthermore, the internucleoside linker can be any of a number of non-phosphorous-containing
5 linkers known in the art.

Preferred nucleic acid monomers include naturally occurring nucleosides forming part of the DNA as well as the RNA family connected through phosphodiester linkages. The members of the DNA family include deoxyadenosine, deoxyguanosine, deoxythymidine, and deoxycytidine. The
10 members of the RNA family include adenosine, guanosine, uridine, cytidine, and inosine. Inosine is a non-specific pairing nucleoside and may be used as universal base because inosine can pair nearly isoenergetically with A, T, and C. Other compounds having the same ability of non-specifically base-
15 pairing with natural nucleobases have been formed. Suitable compounds which may be utilized in the present invention includes among others the compounds depicted on p30 of this application, entitled "Examples of Universal Bases".

20 Building block

The chemical entities that are precursors for structural additions or eliminations of the encoded molecule may be attached to a building block prior to the participation in the formation of the reaction product leading the final encoded molecule. Besides the chemical entity, the building block generally
25 comprises an anti-codon.

The chemical entity of the building block comprises at least one reactive group capable of participating in a reaction which results in a connection between the chemical entity of the building block and another chemical entity or
30 a scaffold associated with the nascent complex. The connection is facilitated by one or more reactive groups of the chemical entity. The number of reactive groups which appear on the chemical entity is suitably one to ten. A

building block featuring only one reactive group is used i.a. in the end positions of polymers or scaffolds, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffolds capable of being reacted further. One, two or more reactive groups intended for the formation of connections, are typically present on scaffolds.

The reactive group of the building block may be capable of forming a direct connection to a reactive group of the nascent complex or the reactive group of the building block may be capable of forming a connection to a reactive group of the nascent complex through a bridging fill-in group. It is to be understood that not all the atoms of a reactive group are necessarily maintained in the connection formed. Rather, the reactive groups are to be regarded as precursors for the structure of the connection.

The subsequent cleavage step to release the chemical entity from the building block can be performed in any appropriate way. In an aspect of the invention the cleavage involves usage of a reagent or an enzyme. The cleavage results in a transfer of the chemical entity to the nascent encoded molecule or in a transfer of the nascent encoded molecule to the chemical entity of the building block. In some cases it may be advantageous to introduce new chemical groups as a consequence of linker cleavage. The new chemical groups may be used for further reaction in a subsequent cycle, either directly or after having been activated. In other cases it is desirable that no trace of the linker remains after the cleavage.

In another aspect, the connection and the cleavage is conducted as a simultaneous reaction, i.e. either the chemical entity of the building block or the nascent encoded molecule is a leaving group of the reaction. In general, it is preferred to design the system such that the connection and the cleavage occur simultaneously because this will reduce the number of steps and the complexity. The simultaneous connection and cleavage can also be designed

such that either no trace of the linker remains or such that a new chemical group for further reaction is introduced, as described above.

5 The attachment of the chemical entity to the building block, optionally via a suitable spacer can be at any entity available for attachment, e.g. the chemical entity can be attached to a nucleobase or the backbone. In general, it is preferred to attach the chemical entity at the phosphor of the internucleoside linkage or at the nucleobase. When the nucleobase is used for attachment of the chemical entity, the attachment point is usually at the 7 position of the purines or 7-deaza-purines or at the 5 position of pyrimidines. The nucleotide may be distanced from the reactive group of the chemical entity by a spacer moiety. The spacer may be designed such that the conformational space sampled by the reactive group is optimized for a reaction with the reactive group of the nascent encoded molecule or reactive site.

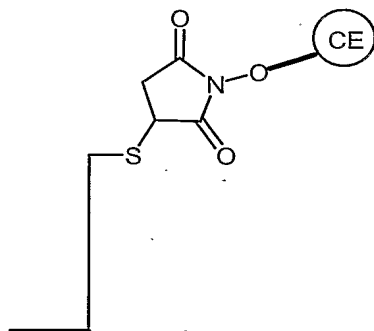
15 The anticodon complements the codon of the identifier oligonucleotide sequence and generally comprises the same number of nucleotides as the codon. The anticodon may be adjoined with a fixed sequence, such as a sequence complementing a framing sequence.

20 Various specific building blocks are envisaged. Building blocks of particular interest are shown below.

Building blocks transferring a chemical entity to a recipient nucleophilic group

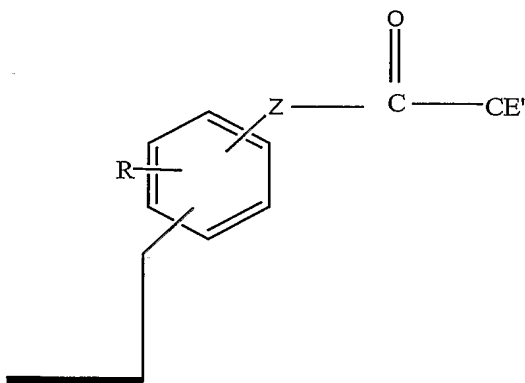
25 The building block indicated below is capable of transferring a chemical entity (CE) to a recipient nucleophilic group, typically an amine group. The bold lower horizontal line illustrates the building block and the vertical line illustrates a spacer. The 5-membered substituted N-hydroxysuccinimide (NHS) ring serves as an activator, i.e. a labile bond is formed between the oxygen atom connected to the NHS ring and the chemical entity. The labile bond may be cleaved by a nucleophilic group, e.g. positioned on a scaffold

30



The 5-membered substituted N-hydroxysuccinimid (NHS) ring serves as an activator, i.e. a labile bond is formed between the oxygen atom connected to the NHS ring and the chemical entity. The labile bond may be cleaved by a nucleophilic group, e.g. positioned on a scaffold, to transfer the chemical entity to the scaffold, thus converting the remainder of the fragment into a leaving group of the reaction. When the chemical entity is connected to the activator through an carbonyl group and the recipient group is an amine, the bond formed on the scaffold will an amide bond. The above building block is the subject of WO03078627A2, the content of which is incorporated herein in their entirety by reference.

Another building block which may form an amide bond is



R may be absent or NO₂, CF₃, halogen, preferably Cl, Br, or I, and Z may be S or O. This type of building block is disclosed in WO03078626A2. The content of this patent application is incorporated herein in the entirety by reference.

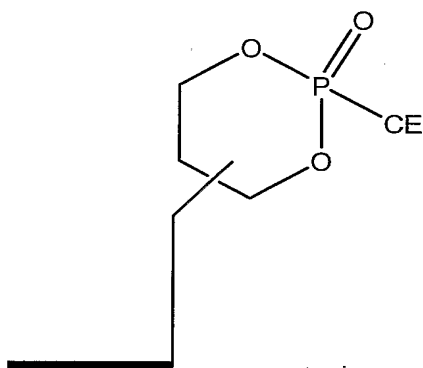
A nucleophilic group can cleave the linkage between Z and the carbonyl group thereby transferring the chemical entity $-(C=O)-CE'$ to said nucleophilic group.

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Building blocks transferring a chemical entity to a recipient reactive group forming a C=C bond

A building block as shown below are able to transfer the chemical entity to a recipient aldehyde group thereby forming a double bond between the carbon of the aldehyde and the chemical entity

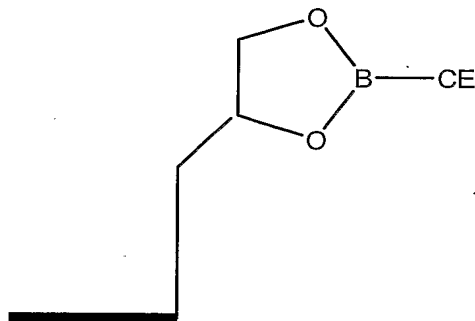
10



15 The above building block is disclosed in WO03078445A2, the content of which being incorporated herein in the entirety by reference.

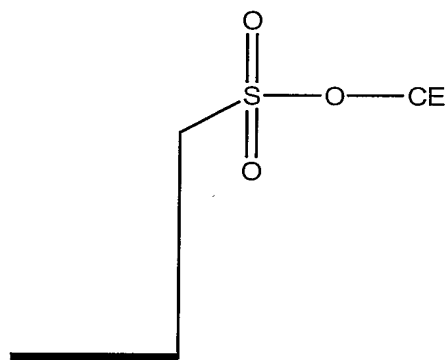
Building blocks transferring a chemical entity to a recipient reactive group forming a C-C bond

20 The below building block is able to transfer the chemical entity to a recipient group thereby forming a single bond between the receiving moiety, e.g. a scaffold, and the chemical entity.



The above building block is disclosed in WO03078445A2, the content of which being incorporated herein in the entirety by reference.

- 5 Another building block capable of transferring a chemical entity to a receiving reactive group forming a single bond is



- 10 The receiving group may be a nucleophile, such as a group comprising a hetero atom, thereby forming a single bond between the chemical entity and the hetero atom, or the receiving group may be an electronegative carbon atom, thereby forming a C-C bond between the chemical entity and the scaffold. The above building block is disclosed in WO03078446A2, the content of
 15 which is incorporated herein by reference.

The chemical entity attached to any of the above building blocks may be a selected from a large arsenal of chemical structures. Examples of chemical entities are

- 20 H or entities selected among the group consisting of a C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₄-C₈ alkadienyl, C₃-C₇ cycloalkyl, C₃-C₇ cycloheteroal-

kyl, aryl, and heteroaryl, said group being substituted with 0-3 R^4 , 0-3 R^5 and 0-3 R^9 or C_1 - C_3 alkylene- NR^4_2 , C_1 - C_3 alkylene- $NR^4C(O)R^8$, C_1 - C_3 alkylene- $NR^4C(O)OR^8$, C_1 - C_2 alkylene- $O-NR^4_2$, C_1 - C_2 alkylene- $O-NR^4C(O)R^8$, C_1 - C_2 alkylene- $O-NR^4C(O)OR^8$ substituted with 0-3 R^9 .

5 where R^4 is H or selected independently among the group consisting of C_1 - C_6 alkyl, C_2 - C_6 alkenyl, C_2 - C_6 alkynyl, C_3 - C_7 cycloalkyl, C_3 - C_7 cycloheteroalkyl, aryl, heteroaryl, said group being substituted with 0-3 R^9 and

R^5 is selected independently from $-N_3$, $-CNO$, $-C(NO)NH_2$, $-NHOH$, $-NHNHR^6$, $-C(O)R^6$, $-SnR^6_3$, $-B(OR^6)_2$, $-P(O)(OR^6)_2$ or the group consisting of
10 C_2 - C_6 alkenyl, C_2 - C_6 alkynyl, C_4 - C_8 alkadienyl said group being substituted with 0-2 R^7 ,

where R^6 is selected independently from H, C_1 - C_6 alkyl, C_3 - C_7 cycloalkyl, aryl or C_1 - C_6 alkylene-aryl substituted with 0-5 halogen atoms selected from $-F$, $-Cl$, $-Br$, and $-I$; and

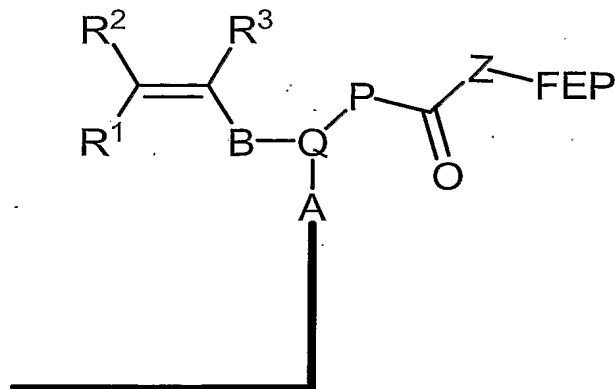
15 R^7 is independently selected from $-NO_2$, $-COOR^6$, $-COR^6$, $-CN$, $-OSiR^6_3$, $-OR^6$ and $-NR^6_2$.

R^8 is H, C_1 - C_6 alkyl, C_2 - C_6 alkenyl, C_2 - C_6 alkynyl, C_3 - C_7 cycloalkyl, aryl or C_1 - C_6 alkylene-aryl substituted with 0-3 substituents independently selected from $-F$, $-Cl$, $-NO_2$, $-R^3$, $-OR^3$, $-SiR^3_3$

20 R^9 is $=O$, $-F$, $-Cl$, $-Br$, $-I$, $-CN$, $-NO_2$, $-OR^6$, $-NR^6_2$, $-NR^6-C(O)R^8$, $-NR^6-C(O)OR^8$, $-SR^6$, $-S(O)R^6$, $-S(O)_2R^6$, $-COOR^6$, $-C(O)NR^6_2$ and $-S(O)_2NR^6_2$.

Cross-link cleavage building blocks

25 It may be advantageous to split the transfer of a chemical entity to a recipient reactive group into two separate steps, namely a cross-linking step and a cleavage step because each step can be optimized. A suitable building block for this two step process is illustrated below:



Initially, a reactive group appearing on the functional entity precursor (abbreviated FEP) reacts with a recipient reactive group, e.g. a reactive group appearing on a scaffold, thereby forming a cross-link. Subsequently, a cleavage is performed, usually by adding an aqueous oxidising agent such as I₂, Br₂, Cl₂, H⁺, or a Lewis acid. The cleavage results in a transfer of the group HZ-FEP- to the recipient moiety, such as a scaffold.

In the above formula

Z is O, S, NR⁴

Q is N, CR¹

P is a valence bond, O, S, NR⁴, or a group C₅₋₇arylene, C₁₋₆alkylene, C₁₋₆O-alkylene, C₁₋₆S-alkylene, NR¹-alkylene, C₁₋₆alkylene-O, C₁₋₆alkylene-S option said group being substituted with 0-3 R⁴, 0-3 R⁵ and 0-3 R⁹ or C₁₋₃alkylene-NR⁴, C₁₋₃alkylene-NR⁴C(O)R⁸, C₁₋₃alkylene-NR⁴C(O)OR⁸, C₁₋₂alkylene-O-NR⁴, C₁₋₂alkylene-O-NR⁴C(O)R⁸, C₁₋₂alkylene-O-NR⁴C(O)OR⁸ substituted with 0-3 R⁹,

B is a group comprising D-E-F, in which

D is a valence bond or a group C₁₋₆alkylene, C₁₋₆alkenylene, C₁₋₆alkynylene, C₅₋₇arylene, or C₅₋₇heteroarylene, said group optionally being substituted with 1 to 4 group R¹¹,

E is, when present, a valence bond, O, S, NR⁴, or a group C₁₋₆alkylene, C₁₋₆alkenylene, C₁₋₆alkynylene, C₅₋₇arylene, or C₅₋₇heteroarylene, said group optionally being substituted with 1 to 4 group R¹¹,

F is, when present, a valence bond, O, S, or NR⁴,

A is a spacing group distancing the chemical structure from the complementing element, which may be a nucleic acid,

R^1 , R^2 , and R^3 are independent of each other selected among the group consisting of H, C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₄-C₈ alkadienyl, C₃-C₇ cycloalkyl, C₃-C₇ cycloheteroalkyl, aryl, and heteroaryl, said group being substituted with 0-3 R^4 , 0-3 R^5 and 0-3 R^9 or C₁-C₃ alkylene-NR⁴₂, C₁-C₃ alkylene-NR⁴C(O)R⁸, C₁-C₃ alkylene-NR⁴C(O)OR⁸, C₁-C₂ alkylene-O-NR⁴₂, C₁-C₂ alkylene-O-NR⁴C(O)R⁸, C₁-C₂ alkylene-O-NR⁴C(O)OR⁸ substituted with 0-3 R^9 ,

FEP is a group selected among the group consisting of H, C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₄-C₈ alkadienyl, C₃-C₇ cycloalkyl, C₃-C₇ cycloheteroalkyl, aryl, and heteroaryl, said group being substituted with 0-3 R^4 , 0-3 R^5 and 0-3 R^9 or C₁-C₃ alkylene-NR⁴₂, C₁-C₃ alkylene-NR⁴C(O)R⁸, C₁-C₃ alkylene-NR⁴C(O)OR⁸, C₁-C₂ alkylene-O-NR⁴₂, C₁-C₂ alkylene-O-NR⁴C(O)R⁸, C₁-C₂ alkylene-O-NR⁴C(O)OR⁸ substituted with 0-3 R^9 ,

where R^4 is H or selected independently among the group consisting of C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₃-C₇ cycloalkyl, C₃-C₇ cycloheteroalkyl, aryl, heteroaryl, said group being substituted with 0-3 R^9 and

R^5 is selected independently from -N₃, -CNO, -C(NOH)NH₂, -NHOH, -NHNHR⁶, -C(O)R⁶, -SnR⁶₃, -B(OR⁶)₂, -P(O)(OR⁶)₂ or the group consisting of C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₄-C₈ alkadienyl said group being substituted with 0-2 R^7 ,

where R^6 is selected independently from H, C₁-C₆ alkyl, C₃-C₇ cycloalkyl, aryl or C₁-C₆ alkylene-aryl substituted with 0-5 halogen atoms selected from -F, -Cl, -Br, and -I; and R^7 is independently selected from -NO₂, -COOR⁶, -COR⁶, -CN, -OSiR⁶₃, -OR⁶ and -NR⁶₂.

R^8 is H, C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₃-C₇ cycloalkyl, aryl or C₁-C₆ alkylene-aryl substituted with 0-3 substituents independently selected from -F, -Cl, -NO₂, -R³, -OR³, -SiR³₃

R^9 is =O, -F, -Cl, -Br, -I, -CN, -NO₂, -OR⁶, -NR⁶₂, -NR⁶-C(O)R⁸, -NR⁶-C(O)OR⁸, -SR⁶, -S(O)R⁶, -S(O)₂R⁶, -COOR⁶, -C(O)NR⁶₂ and -S(O)₂NR⁶₂.

In a preferred embodiment Z is O or S, P is a valence bond, Q is CH, B is CH₂, and R¹, R², and R³ is H. The bond between the carbonyl group and Z is cleavable with aqueous I₂.

5

Contacting between target and library

The contacting step, by which the library of bifunctional molecules is subjected under binding conditions to a target associated with a target oligonucleotide, may be referred to as the enrichment step or the selection step, as appropriate, and includes the screening of the library for display molecules having predetermined desirable characteristics. Predetermined desirable characteristics can include binding to a target, catalytically changing the target, chemically reacting with a target in a manner which alters/modifies the target or the functional activity of the target, and covalently attaching to the target as in a suicide inhibitor.

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In theory, display molecules of interest can be selected based on their properties using either physical or physiological procedures. The method preferred according to the present invention is to enrich molecules with respect to binding affinity towards a target of interest. In a certain embodiment, the basic steps involve mixing the library of complexes with the immobilized target of interest. The target can be attached to a column matrix or microtitre wells with direct immobilization or by means of antibody binding or other high-affinity interactions. In another embodiment, the target and displayed molecules interact without immobilisation of the target. Displayed molecules that bind to the target will be retained on this surface, while nonbinding displayed molecules in a certain aspect of the invention will be removed during a single or a series of wash steps. The identifier oligonucleotides of complexes bound to the target can then be coupled to the target oligonucleotide. It may be considered advantageously to perform a chromatography step after or instead of the washing step, notably in cases where the target is not immobilized. After the coupling between the identifier oligonucleotide and the

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target oligonucleotide, the coupled oligonucleotide may be recovered and optionally amplified before the decoding step.

5 A significant reduction in background binders may be obtained with increased washing volumes, repeating washing steps, higher detergent concentrations and prolonged incubation during washing. Thus, the more volume and number of steps used in the washing procedure together with more stringent conditions the more efficiently the non-binders and background binders will be removed. The right stringency in the washing step can also be used to re-
10 move low-affinity specific binders. However, the washing step will also remove wanted binders if too harsh conditions are used.

A blocking step, such as incubation of solid phase with skimmed milk proteins or other inert proteins and/or mild detergent such as Tween-20 and Triton X-100, may also be used to reduce the background. The washing conditions should be as stringent as possible to remove background binding but to retain specific binders that interact with the immobilized target. Generally, washing conditions are adjusted to maintain the desired affinity binders, e.g. binders in the micromolar, nanomolar, or pocomolar range.

20 In traditional elution protocols, false positives due to suboptimal binding and washing conditions are difficult to circumvent and may require elaborate adjustments of experimental conditions. However, an enrichment of more than 100 to 1000 is rarely obtained. The present invention alleviates the problem with false positive being obtained because the non-specific binding complexes to a large extent remain in solution or attached to the reaction chamber such that the identifier oligonucleotide of non-binding complexes will be in a low concentration compared to the identifier oligonucleotides of binding
25 complexes relative to the concentration of the target oligonucleotide.

30 The target can be any compound of interest. E.g. the target can be a protein, peptide, carbohydrate, polysaccharide, glycoprotein, hormone, receptor, an-

tigen, antibody, virus, substrate, metabolite, transition state analogue, cofactor, inhibitor, drug, dye, nutrient, growth factor, cell, tissue, etc. without limitation. Suitable targets include, but are not limited to, angiotensin converting enzyme, renin, cyclooxygenase, 5-lipoxygenase, IL-10 converting enzyme, cytokine receptors, PDGF receptor, type II inosine monophosphate dehydrogenase, β -lactamases, integrin, proteases like factor VIIa, kinases like Bcr-Abl/Her, phosphatases like PTP-1B, and fungal cytochrome P-450. Targets can include, but are not limited to, bradykinin, neutrophil elastase, the HIV proteins, including *tat*, *rev*, *gag*, *int*, RT, nucleocapsid etc., VEGF, bFGF, TGF β , KGF, PDGF, GPCR, thrombin, substance P, IgE, sPLA2, red blood cells, glioblastomas, fibrin clots, PBMCs, hCG, lectins, selectins, cytokines, ICP4, complement proteins, etc.

Certain targets comprise one or more discrete binding domains. Proteins that contain these domains are involved in a variety of processes, such as cellular transporters, cholesterol movement, signal transduction and signaling functions which are involved in development and neurotransmission. See Herz, *Lipoprotein receptors: beacons to neurons?*, (2001) Trends in Neurosciences 24(4):193-195; Goldstein and Brown, *The Cholesterol Quartet*, (2001) Science 292:1310-1312. The function of a discrete binding domain is often specific but it also contributes to the overall activity of the protein or polypeptide. For example, the LDL-receptor class A domain (also referred to as a class A module, a complement type repeat or an A-domain) is involved in ligand binding while the gamma-carboxyglutamic acid (Gla) domain which is found in the vitamin-K-dependent blood coagulation proteins is involved in high-affinity binding to phospholipid membranes. Other discrete binding domains include, e.g., the epidermal growth factor (EGF)-like domain in tissue-type plasminogen activator which mediates binding to liver cells and thereby regulates the clearance of this fibrinolytic enzyme from the circulation and the cytoplasmic tail of the LDL-receptor which is involved in receptor-mediated endocytosis.

Individual target proteins can possess one or more discrete monomer domains, as discussed above. These proteins are often called mosaic proteins. For example, members of the LDL-receptor family contain four major structural domains: the cysteine rich A-domain repeats, epidermal growth factor precursor-like repeats, a transmembrane domain and a cytoplasmic domain.

5 The LDL-receptor family includes members that: 1) are cell-surface receptors; 2) recognize extracellular ligands; and 3) internalize them for degradation by lysosomes. See Hussain et al., *The Mammalian Low-Density Lipoprotein Receptor Family*, (1999) Annu. Rev. Nutr. 19:141-72. For example, some

10 members include very-low-density lipoprotein receptors (VLDL-R), apolipoprotein E receptor 2, LDLR-related protein (LRP) and megalin. Family members have the following characteristics: 1) cell-surface expression; 2) extracellular ligand binding consisting of A-domain repeats; 3) requirement of calcium for ligand binding; 4) recognition of receptor-associated protein and

15 apolipoprotein (apo) E; 5), epidermal growth factor (EGF) precursor homology domain containing YWTD repeats; 6) single membrane-spanning region; and 7) receptor-mediated endocytosis of various ligands. See Hussain, *supra*. Yet, the members bind several structurally dissimilar ligands. The present invention offers the possibility of identifying two or more ligands against

20 the same target as discussed elsewhere herein.

In some aspects of the invention, a dimer compound binding with two targets normally interacting in a biological context is identified using the methods of the present invention. Examples of such targets are factor Xa and factor VIIa.

25 The method includes separately screening a library of bifunctional complexes and identifying suitable display molecules that binds to both targets. The two display molecules pools are then linked together. In the event m display molecules are identified having an affinity above a certain threshold (i.e. a low Kd) to a first target and n display molecules having an affinity above another or the same threshold, a dimer compound array of n times m molecules

30 are formed. The dimer compound are subsequently screened for the ability to bind to the first and the second target molecule, thereby identifying a dimer or

a range of dimers that specifically bind to the first and the second target molecule with a certain affinity.

5 A target can also be a surface of a non-biological origin, such as a polymer surface or a metal surface. The method of the invention may then be used to identify suitable coatings for such surfaces.

10 In a preferred embodiment, the desirable display molecule acts on the target without any interaction between the nucleic acid attached to the desirable encoded molecule and the target. In one embodiment, the bound complex-target aggregate can be partitioned from unbound complexes prior to or subsequent to the coupling step by a number of methods. The methods include nitrocellulose filter binding, column chromatography, filtration, affinity chromatography, centrifugation, and other well known methods. A preferred method
15 is size-exclusion chromatography.

Briefly, the library of complexes is subjected to the target, which may include contact between the library and a column onto which the target is immobilised. Identifier oligonucleotides associated with undesirable display molecules, i.e. display molecules not bound to the target under the stringency
20 conditions used, will pass through the column. Additional undesirable display molecules (e.g. display molecules which cross-react with other targets) may be removed by counter-selection methods. Desirable complexes are bound to the column. The target may be immobilized in a number of ways. In one
25 embodiment, the target is immobilized through a cleavable physical link, such as one more chemical bonds. Following the interaction of the display molecule and the target, the respective oligonucleotides are coupled. The aggregate of the target and the complex may then be subjected to a size exclusion chromatography to separate the aggregate from the rest of the compounds in
30 the media.

The complex may be provided with a cleavable linker at a position between the display molecule and the identifier oligonucleotide. When the target is immobilized the cleavable linker of the complex is preferable orthogonal to the cleavable linker that attached the target to the solid support. Subsequent to the optional size exclusion chromatography, this cleavable linker is cleaved to separate the identifier oligonucleotides of complexes having affinity towards the targets. Just to mention a single type of orthogonal cleavable linkages, one could attached to target to the solid support through a linkage that can be cleaved by a chemical agent, and the linker separating the display molecule and the identifier oligonucleotide may be selected as a photo-cleavable linkage. More specifically, the former linkage may be a disulphide bond that can be cleaved by a suitable reducing agent like DTT (dithiothreitol) and the latter linkage may be an o-nitrophenyl group.

There are other partitioning and screening processes which are compatible with this invention that are known to one of ordinary skill in the art. Such known process may be used in combination with the present inventive method. In one embodiment, the coupling products can be fractionated by a number of common methods and then each fraction is assayed for activity. The fractionization methods can include size, pH, hydrophobicity, etc.

Inherent in the present method is the selection of encoded molecules on the basis of a desired function; this can be extended to the selection of molecules with a desired function and specificity. Specificity can be required during the selection process by first extracting complexes which are capable of interacting with a non-desired "target" (negative selection, or counter-selection), followed by positive selection with the desired target. As an example, inhibitors of fungal cytochrome P-450 are known to cross-react to some extent with mammalian cytochrome P-450 (resulting in serious side effects). Highly specific inhibitors of the fungal cytochrome could be selected from a library by first removing those complexes capable of interacting with the

mammalian cytochrome, followed by retention of the remaining products which are capable of interacting with the fungal cytochrome.

5 The present invention can be used to identify compounds that bind to different molecular targets. A small library of preferred compounds can be directly linked to an oligonucleotide that identifies the structure of the said compound. This can be done manually or using a more automatic system such as robotic equipments. These tagged compounds can then be mixed with one or more target molecules to select for compound and target pairs that bind to each other. For example, a library of compounds designed to bind preferably to protein kinases could be mixed with a library of various protein kinases to identify compounds that specifically interact with a certain protein kinase. The use of the present proximity selection procedure will in this instance generate an extensive structure activity relationship (SAR) where different binding compound are match simultaneously against different related target molecules. The above method can also be used for other target classes such as proteases, phosphatases, GPCRs, nuclear receptors and corresponding compound libraries. The information for these selections can be used to study the selectivity and specificity and to design sub-libraries with potential binding compounds.

20 In a prior art selection where the target is immobilized to a surface, for example in a well or a bead, the effective concentration of the target will be high locally on the surface but infinitively low in the solution. This restriction will result in low recovery of binding molecules because most of the binding molecules are free in the solution and removed in future washing steps. The present invention allows selection in solution at true equilibrium conditions between the molecular target and the binding molecules. The invention is not dependent on washing- or separation-step as is most prior art selection protocols. The amount of captured binding molecules can be varied using a suitable concentration of the target. Most of the binding molecules with a certain binding constant can be captured if the target concentration is higher than the

binding constant of the binding molecules. Also the concentration of the target can be adjusted to capture binders with a certain binding constant. A high target concentration will also increase the likelihood of selecting specific binders that are present in low copy number. By using high concentration of target, the solution selection can also be used to identify binders with low binding affinity. This is especially important with screening for small fragments which normally possess low affinity but still holds important structural information that can be used in second generation library design.

In a certain embodiment, a binding platform may be constructed that can be used for almost any target. The binding platform should preferably be small enough to only allow association of a few or a single target molecule. This to ensure a solution based selection procedure with adjustable target concentration. The binding platform is primarily composed of two components; a small surface allowing association of the target molecule, and an association area/site for the target oligonucleotide. This binding platform may be designed to mediate the association of the target and target oligonucleotide to allow proximity selection in solution.

Cleavable linkers

A cleavable linker may be positioned between the target and a solid support, between the potential drug candidate and the identifier oligonucleotide, between the molecular target and the target oligonucleotide or any other position that can provide for a separation of the identifier oligonucleotide from successful complexes from non-specific binding complexes. The cleavable linker may be selectively cleavable, i.e. conditions may be selected that only cleave that particular linker.

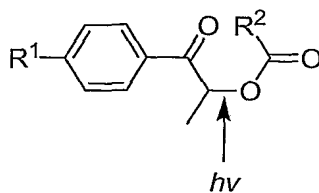
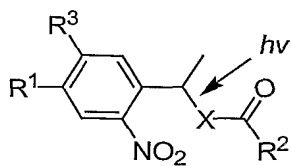
The cleavable linkers may be selected from a large plethora of chemical structures. Examples of linkers includes, but are not limited to, linkers having an enzymatic cleavage site, linkers comprising a chemical degradable component, and linkers cleavable by electromagnetic radiation, such as light.

Examples of linkers cleavable by electromagnetic radiation (light)

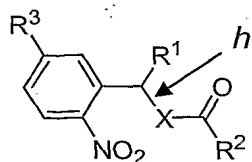
o-nitrobenzyl

p-alkoxy

5

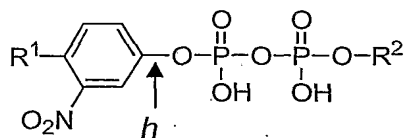


o-nitrobenzyl in exo position



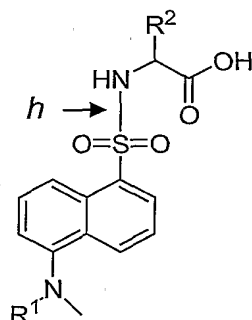
For more details see Holmes CP. J. Org. Chem. **1997**, 62, 2370-2380

5 3-nitrophenyloxy



For more details see Rajasekharan Pillai, V. N. Synthesis. **1980**, 1-26

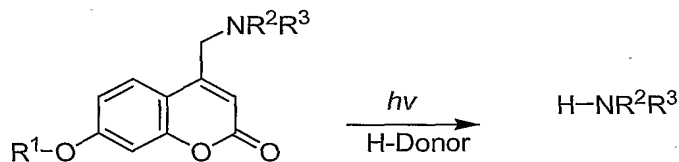
Dansyl derivatives:



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For more details see Rajasekharan Pillai, V. N. Synthesis. **1980**, 1-26

Coumarin derivatives



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For more details see R. O. Schoenleber, B. Giese. Synlett **2003**, 501-504

R^1 and R^2 can be either of the potential drug candidate and the identifier oligonucleotide, respectively. Alternatively, R^1 and R^2 can be either of the target or a solid support, respectively.

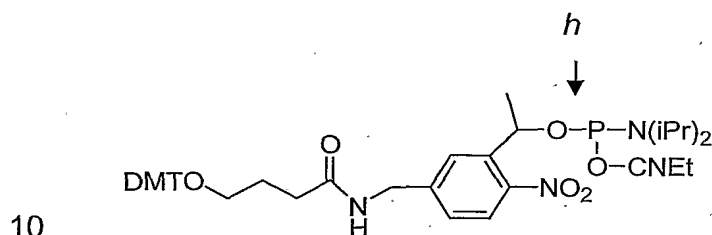
20

$R^3 = \text{H or OCH}_3$

If X is O then the product will be a carboxylic acid

If X is NH the product will be a carboxamide

One specific example is the PC Spacer Phosphoramidite (Glen research catalog # 10-4913-90) which can be introduced in an oligonucleotide during synthesis and cleaved by subjecting the sample in water to UV light (~ 300-350 nm) for 30 seconds to 1 minute.



DMT = 4,4'-Dimethoxytrityl

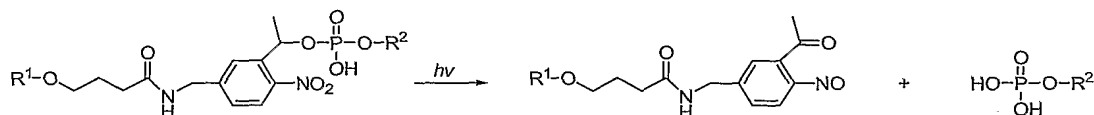
iPr = Isopropyl

CNEt = Cyanoethyl

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The above PC spacer phosphoramidite is suitable incorporated in a library of complexes at a position between the identifier and the potential drug candidate. The spacer may be cleaved according to the following reaction.

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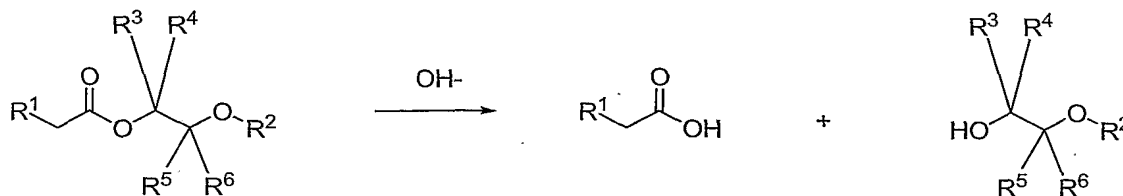


25

R^1 and R^2 can be either of the encoded molecule and the identifying molecule, respectively. In a preferred aspect R^2 is an oligonucleotide identifier and the R^1 is the potential drug candidate. When the linker is cleaved a phosphate group is generated allowing for further biological reactions. As an example, the phosphate group may be positioned in the 5' end of an oligonucleotide allowing for an enzymatic ligation process to take place.

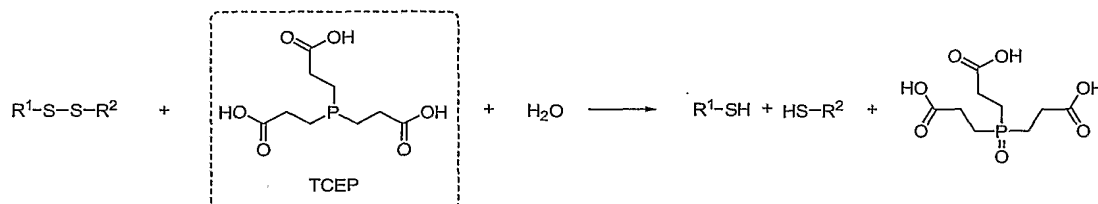
Examples of linkers cleavable by chemical agents:

Ester linkers can be cleaved by nucleophilic attack using e.g. hydroxide ions. In practice this can be accomplished by subjecting the target-ligand complex to a base for a short period.



- 10 R^1 and R^2 can be either of be the potential drug candidate or the identifier oligonucleotide, respectively. R^{4-6} can be any of the following: H, CN, F, NO_2 , SO_2NR_2 .

- 15 Disulfide linkers can efficiently be cleaved / reduced by Tris (2-carboxyethyl) phosphine (TCEP). TCEP selectively and completely reduces even the most stable water-soluble alkyl disulfides over a wide pH range. These reductions frequently required less than 5 minutes at room temperature. TCEP is a non-volatile and odorless reductant and unlike most other reducing agents, it is resistant to air oxidation. Trialkylphosphines such as TCEP are stable in
- 20 aqueous solution, selectively reduce disulfide bonds, and are essentially un-reactive toward other functional groups commonly found in proteins.



- 25 More details on the reduction of disulfide bonds can be found in Kirley, T.L.(1989), Reduction and fluorescent labeling of cyst(e)ine-containing pro-

teins for subsequent structural analysis, *Anal. Biochem.* **180**, 231 and Levinson, M.E., *et al.* (1969), Reduction of biological substances by water-soluble phosphines: Gamma-globulin. *Experientia* **25**, 126-127.

5 *Linkers cleavable by enzymes*

The linker connecting the potential drug candidate with the identifier oligonucleotide or the solid support and the target can include a peptide region that allows a specific cleavage using a protease. This is a well-known strategy in molecular biology. Site-specific proteases and their cognate target amino
10 acid sequences are often used to remove the fusion protein tags that facilitate enhanced expression, solubility, secretion or purification of the fusion protein.

Various proteases can be used to accomplish a specific cleavage. The specificity is especially important when the cleavage site is presented together
15 with other sequences such as for example the fusion proteins. Various conditions have been optimized in order to enhance the cleavage efficiency and control the specificity. These conditions are available and known in the art.

Enterokinase is one example of an enzyme (serine protease) that cuts a specific amino acid sequence. Enterokinase recognition site is Asp-Asp-Asp-Asp-Lys (DDDDK), and it cleaves C-terminally of Lys. Purified recombinant Enterokinase is commercially available and is highly active over wide ranges
20 in pH (pH 4.5-9.5) and temperature (4-45°C).

The nuclear inclusion protease from tobacco etch virus (TEV) is another commercially available and well-characterized protease that can be used to cut at a specific amino acid sequence. TEV protease cleaves the sequence Glu-Asn-Leu-Tyr-Phe-Gln-Gly/Ser (ENLYFQG/S) between Gln-Gly or Gln-Ser with high specificity.
25

Another well-known protease is thrombin that specifically cleaves the sequence Leu-Val-Pro-Arg-Gly-Ser (LVPGS) between Arg-Gly. Thrombin has
30

also been used for cleavage of recombinant fusion proteins. Other sequences can also be used for thrombin cleavage; these sequences are more or less specific and more or less efficiently cleaved by thrombin. Thrombin is a highly active protease and various reaction conditions are known to the public.

Activated coagulation factor FX (FXa) is also known to be a specific and useful protease. This enzyme cleaves C-terminal of Arg at the sequence Ile-Glu-Gly-Arg (IEGR). FXa is frequently used to cut between fusion proteins when producing proteins with recombinant technology. Other recognition sequences can also be used for FXa.

Other types of proteolytic enzymes can also be used that recognize specific amino acid sequences. In addition, proteolytic enzymes that cleave amino acid sequences in an un-specific manner can also be used if only the linker contains an amino acid sequence in the complex molecule.

Other type of molecules such as ribozymes, catalytically active antibodies, or lipases can also be used. The only prerequisite is that the catalytically active molecule can cleave the specific structure used as the linker, or as a part of the linker, that connects the encoding region and the displayed molecule or, in the alternative the solid support and the target.

A variety of endonucleases are available that recognize and cleave a double stranded nucleic acid having a specific sequence of nucleotides. The endonuclease Eco RI is an example of a nuclease that efficiently cuts a nucleotide sequence linker comprising the sequence GAATTC also when this sequence is close to the nucleotide sequence length. Purified recombinant Eco RI is commercially available and is highly active in a range of buffer conditions. As an example the Eco RI is working in in various protocols as indicted below (NEBuffer is available from New England Biolabs):

NEBuffer 1 : [10 mM Bis Tris Propane-HCl, 10 mM MgCl₂, 1 mM dithiothreitol (pH 7.0 at 25°C)],

NEBuffer 2 : [50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol (pH 7.9 at 25°C)],

5 NEBuffer 3 : [100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol (pH 7.9 at 25°C)],

NEBuffer 4 : [50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (pH 7.9 at 25°C)].

10 Extension buffer : mM KCl, 20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM (NH₄)₂SO₄, 2 mM MgSO₄ and 0.1% Triton X-100, and 200 µM dNTPs.

Determining the identifier oligonucleotide sequence

15 The nucleotide sequence of the identifier sequence present in the coupled product is determined to identify the identity of the binding display molecule(s) and/or optionally the molecular target(s). In a certain embodiment of the invention, chemical entities that participated in the formation of the display molecules that binds to the target are identified. The synthesis method of the display molecule may be established if information on the chemical

20 entities as well as the point in time they have been incorporated in the display molecule can be deduced from the identifier oligonucleotide. It may be sufficient to obtain information on the chemical structure of the various chemical entities that have participated in the display molecule to deduce the full molecule due to structural constraints during the formation. As an example, the

25 use of different kinds of attachment chemistries may ensure that a chemical entity on a building block can only be transferred to a single position on a scaffold. Another kind of chemical constraints may be present due to steric hindrance on the scaffold molecule or the chemical entity to be transferred. In general however, it is preferred that information can be inferred from the

30 identifier oligonucleotide sequence that enable the identification of each of the chemical entities that have participated in the formation of the encoded

molecule along with the point in time in the synthesis history the chemical entities have been incorporated in the (nascent) display molecule.

5 Although conventional DNA sequencing methods are readily available and useful for this determination, the amount and quality of isolated bifunctional molecule may require additional manipulations prior to a sequencing reaction.

10 Where the amount is low, it is preferred to increase the amount of the coupled oligonucleotide sequence by polymerase chain reaction (PCR) using PCR primers directed to primer binding sites present in the identifier oligonucleotide sequence.

15 In one embodiment, the different coupled oligonucleotide sequences are cloned into separate sequencing vectors prior to determining their sequence by DNA sequencing methods. This is typically accomplished by amplifying the different coupled oligonucleotide sequences by PCR and then using a unique restriction endonuclease sites on the amplified product to directionally clone the amplified fragments into sequencing vectors. The cloning and sequencing of the amplified fragments then is a routine procedure that can be
20 carried out by any of a number of molecular biological methods known in the art.

25 Alternatively, the bifunctional complex or the PCR amplified identifier oligonucleotide sequence can be analysed in a microarray. The array may be designed to analyse the presence of a single codon or multiple codons in a identifier oligonucleotide sequence.

30 In still another approach, the coupled oligonucleotide product is analysed by QPCR. Preferably, the QPCR affords information as to the chemical moieties that has participated in the formation of the display molecules and optionally the identity of the target. The QPCR approach also allows a direct investiga-

tion of the enrichment factor if two samples are analysed in parallel, one with target and the other with the target plus library. The difference in signal from these to samples will illustrate how much coupling that is due to the target mediated coupling compared to the background coupling. Various conditions
5 can be investigated to obtain the most optimal selection procedure before the sequences are analysed to identify the precise structures of the binding molecules.

Synthesis of nucleic acids

10 Oligonucleotides can be synthesized by a variety of chemistries as is well known in the art. For synthesis of an oligonucleotide on a substrate in the direction of 3' to 5', a free hydroxy terminus is required that can be conveniently blocked and deblocked as needed. A preferred hydroxy terminus blocking group is a dimethoxytrityl ether (DMT). DMT blocked termini are first de-
15 blocked, such as by treatment with 3% dichloroacetic acid in dichloromethane (DCM) as is well known for oligonucleotide synthesis, to form a free hydroxy terminus.

Nucleotides in precursor form for addition to a free hydroxy terminus in the
20 direction of 3' to 5' require a phosphoramidate moiety having an aminodiisopropyl side chain at the 3' terminus of a nucleotide. In addition, the free hydroxy of the phosphoramidate is blocked with a cyanoethyl ester (OCNET), and the 5' terminus is blocked with a DMT ether. The addition of a 5' DMT-, 3' OCNET-blocked phosphoramidate nucleotide to a free hydroxyl requires
25 tetrazole in acetonitrile followed by iodine oxidation and capping of unreacted hydroxyls with acetic anhydride, as is well known for oligonucleotide synthesis. The resulting product contains an added nucleotide residue with a DMT blocked 5' terminus, ready for deblocking and addition of a subsequent blocked nucleotide as before.

30 For synthesis of an oligonucleotide in the direction of 5' to 3', a free hydroxy terminus on the linker is required as before. However, the blocked nucleotide

to be added has the blocking chemistries reversed on its 5' and 3' termini to facilitate addition in the opposite orientation. A nucleotide with a free 3' hydroxyl and 5' DMT ether is first blocked at the 3' hydroxy terminus by reaction with TBS-Cl in imidazole to form a TBS ester at the 3' terminus. Then the DMT-blocked 5' terminus is deblocked with DCA in DCM as before to form a free 5' hydroxy terminus. The reagent (N,N-diisopropylamino)(cyanoethyl) phosphonamidic chloride having an aminodiisopropyl group and an OCNET ester is reacted in tetrahydrofuran (THF) with the 5' deblocked nucleotide to form the aminodiisopropyl-, OCNET-blocked phosphonamidate group on the 5' terminus. Thereafter the 3' TBS ester is removed with tetrabutylammonium fluoride (TBAF) in DCM to form a nucleotide with the phosphonamidate-blocked 5' terminus and a free 3' hydroxy terminus. Reaction in base with DMT-Cl adds a DMT ether blocking group to the 3' hydroxy terminus.

The addition of the 3' DMT-, 5' OCNET-blocked phosphonamidated nucleotide to a linker substrate having a free hydroxy terminus then proceeds using the previous tetrazole reaction, as is well known for oligonucleotide polymerization. The resulting product contains an added nucleotide residue with a DMT-blocked 3' terminus, ready for deblocking with DCA in DCM and the addition of a subsequent blocked nucleotide as before.

Brief Description of the Figures

Fig. 1 discloses an embodiment for proximity-dependent selection,
Fig. 2 discloses different approaches for accomplishing coupling,
Fig. 3 discloses four different approaches for producing a coupling
Fig. 4 discloses a library versus library screening,
Fig. 5 discloses an embodiment in which a target oligonucleotide association is performed in solution.
Fig. 6 discloses a target oligonucleotide association on cell surface.
Fig. 7 discloses a target with multiple binding sites which may associate with members of the bifunctional molecule library.

Fig. 8 discloses a target with one binding site for association with a pair of displayed molecules.

Fig. 9 discloses a 2nd generation-library driven proximity selection.

5 Fig. 10 discloses multiple targets for simultaneously subjected to a library of complexes.

Detailed Disclosure of the Figures

Fig. 1 outlines an embodiment for a proximity-dependent selection (PDS).

10 The molecular target is linked to a target oligonucleotide, which in some embodiment may be unique for the target molecule. This target sequence comes in close proximity with a specific identifier oligonucleotide when the displayed molecule of a bifunctional complex binds to the target molecule. This proximity will promote the coupling between the bifunctional complex molecules that bind to the target compare to bifunctional complex in solution.

15 Thus, there will be a selection for coupling products that contain display molecules that possess affinity for the target molecule. The final ligation product is amplified using two primers that only amplify ligated products.

20 In a first step, the target associate with a target oligonucleotide is mixed with a library of complexes, in which each complex comprises a display molecule attached to an identifier oligonucleotide. The display molecules are then incubated with the target. The display molecules which have an affinity towards the molecular target will bind, while the complexes not having affinity will remain in solution. Subsequent to the incubation, a connector oligonucleotide is

25 added. The connector oligo nucleotide comprises parts that hybridise to sequences near the ends of the target and the identifier oligonucleotides, respectively. Subsequent to the addition of the connector oligonucleotide, a ligation is effected by chemical or enzymatic means. Preferably a ligase is used to ligate the target and the identifier oligonucleotides together. The connector oligonucleotide is generally added in excess to saturate the complexes in solution to avoid unspecific ligation.

30

After the ligation, the ligation product is amplified by PCR. Thus, a forward primer is annealed to the ligation product at the 3' end thereof and extended using a polymerase. The transcribed product comprises a site to which a second (or reverse) primer can anneal so as to provide for an extension of the second primer using the transcribed product as template. Using forward and reverse primers as indicated above together with a polymerase and suitable substrates produces amplicons, which comprises information about the display molecule as well as the molecular target. The ligated product can be introduced into a host organism using a suitable vector. The host vector may be allowed to form colonies and the colonies can be sequenced to establish the identity of the display molecule.

Fig. 2 shows various options to perform coupling between the target oligonucleotide and the identifier oligonucleotide. **A.** The ligation is promoted using a connector oligonucleotide that anneals both to the target oligonucleotide and the identifier oligonucleotide. The connector oligonucleotide is designed such that the ends of the identifier oligonucleotide and the target oligonucleotides are abutted. A ligase is subsequently allowed to ligate the ends together. **B.** A connector oligonucleotide is used to promote fill in of a gap using a polymerase and finally ligation using a ligase. **C.** The distal end of the target oligonucleotide overlaps the distal end of the identifier oligonucleotide, which allows a polymerase to extend the target oligonucleotide as well as the identifier oligonucleotide thereby forming a double stranded product. **D.** Blunt-ended ligation of single-stranded or double stranded DNA using a suitable enzyme like T4 DNA ligase.

Fig. 3 shows various methods for preparing a coupling area on an existing bifunctional complex. Conjugates between molecular targets associated with an oligonucleotide and complexes comprising a display molecule and an identifier oligonucleotide can be modified to allow a ligase to couple the oligonucleotides together. **A.** The identifier oligonucleotide is extended with a primer with an overhang that creates the coupling area. The extension is

suitably conducted before the selection process to obtain the benefit of a double stranded nucleotide sequence. A target oligonucleotide can be ligated to the blunt end of the extended primer or a connector oligonucleotide can be used to connect the target oligonucleotide and the extended primer prior to ligation with a suitable ligase. **B.** The identifier oligonucleotide is annealed to a primer that binds internally. The primer is subsequently extended, suitably before the selection process. The extension forms a coupling area directly on the identifier oligonucleotide, which allows a target oligonucleotide to be annealed and ligated. **C.** The first step is identical to the procedure as describe in B but the target sequence has a free 5'-end that allow ligation to the 3'-end of the identifier oligonucleotide. A blunt ended single stranded ligation can be performed. Alternatively, this variation can be performed using a connector oligonucleotide and subsequent ligation. **D.** A primer is annealed to a identifier oligonucleotide and extended to produce a double-stranded DNA which is subsequently cut with an enzyme (e.g. restriction enzyme) to produce a single-stranded DNA portion that can be used as handle in the coupling process.

Fig. 4 shows a library versus library selection method. Different targets specifically encoded by the attached target oligonucleotides are mixed with a library of bifunctional complexes. The displayed molecules will bind to specific targets and promote the ligation through the proximity effect. This ligation will connect the target oligonucleotides with oligonucleotides that encodes for specific displayed molecules. The ligated oligonucleotides can be amplified and determined by sequencing procedures well known in the art. The ligated sequences will reveal which display molecules that bind to which target.

Fig. 5 discloses *inter alia* the association of the target oligonucleotide to the target. One way of associating the target oligonucleotide with the target molecule is to link the oligonucleotide through a tag introduced on the target molecule. The tag can be attached before the target is produced (e.g. a short

amino acid sequence such as HIS-tag or FLAG-tag) or be modified after the target is produced. The target sequence can then be associated through the tag using a tag-binding molecule such as an antibody or other type of molecules that binds to the tag.

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Fig. 6 discloses target oligonucleotide association on a cell surface. Specific receptors can be engineered to express a specific tag on the cell surface.

Different tags can be used such as HIS- or FLAG-tags or other types of tags that become bound with the receptor. The tag will only be displayed on the cell surface together with the specific receptor. The target oligonucleotide is then associated with the receptor target using a mediator molecule that carries the target oligonucleotide and binds to the tag. A mediator molecule could be an antibody that binds to the tag (e.g. anti-HIS or anti-FLAG antibodies) that is associated with the target oligonucleotide. This procedure will specifically associate the target oligonucleotide with a receptor target on the cell surface which will promote a ligation between oligonucleotides of the binding displayed molecules and the target oligonucleotide.

Fig. 7 shows a target molecule with several sites for binding of ligands. The target is subjected to a library of complexes of bifunctional molecules. Display molecules of the complexes binds to the discrete sites of the molecular target thus promoting a high local concentration of the ends of the oligonucleotides which have bound to the target. Subsequently a connector oligonucleotide is added to adjoin the distal ends of the oligonucleotides together. Usually, the connector oligonucleotide is added in excess to saturate the ends of the identifier oligonucleotides free in the solution. The ends of the oligonucleotides kept together by the connector oligonucleotide are ligated together forming a coupled product. The coupled product is amplified by PCR using primers annealing to each end of the coupled product. The amplified coupled product is decoded to identify the display molecules which have bound to the in the target. In a step not shown on the figure, the two binding display molecules are coupled together via a suitable linker to form a ligand

which binds to two sites of the target. Suitably, the dimer comprising the two revealed display molecules and the linker is synthesised by organic synthesis.

5 Libraries of bifunctional complexes can also be screened against each other using the present invention. Such an embodiment allows the detecting of pairs of displayed molecules that bind to the same target at different or the same binding site or pair of displayed molecules that bind to different targets. The power of the screening libraries in the above fashion is indicated by the
10 fact that a library of e.g. 10^4 different displayed molecules generates a total combination of display molecules of 10^8 when pair of binders are searched for.

Fig. 8 discloses a library of bifunctional complexes which is presented to a
15 target having a site possible to be occupied by two display molecules. Initially, the target is mixed with the library of bifunctional complexes under conditions which promote a binding interaction to take place. A first bifunctional complex associates with the target to form the target associated with the target oligonucleotide. Subsequently to or simultaneously with the binding of the
20 first display molecule, a second complex binds to the same site of the target. The display molecules may or may not be reacted with each other to form a covalent linkage between the display molecules. In another embodiment, the two display molecules are connected *via* a suitable linker or reacted with an external reactant so as to form a single molecule. After the binding interaction
25 of the library of complexes with the target, the ends of the complexes which comprises display molecules that binds to the target are joint together. In an aspect of the invention, the ends are joined together using a connector polynucleotide. The connector polynucleotide is preferably added in excess to saturate ends of identifier oligonucleotides which are not part of a binding
30 complex. After the hybridisation event between the ends of the identifier oligonucleotides and the connector oligonucleotide a ligation is conducted. Suitably the ligation is performed by a ligase to form a coupled product,

which can be used as a template by a polymerase. After the ligation, the coupled product is amplified by PCR to form PCR amplicons comprising information of the display molecules which have participated in the binding interaction.

5

Fig. 9 discloses a two (or more) step identification method. In a first step the method as disclosed in figure 1 is conducted and in the second step a new library prepared upon the knowledge harvested in the first library is used to generate the second generation library. Initially, a library of complexes are presented to a target having a binding site. In the library, display molecules having a binding affinity above a certain threshold is not present, illustrated on the drawing with a display molecule only having a partial fit in the binding site of the target. In the synthesis of the second generation library components used in the synthesis of the low binding display molecule are shuffled with further components and/or the low binding display molecule is added or subtracted a structural unit. As an example, a further round of addition of chemical entities can be conducted. For systems for complex generation relying on the natural translation system a deletion, alteration or addition of nucleic acid can be performed. The second generation library is presented to the a target again. In the event the alteration of the initial low binding molecule has been successful display molecules are generated which binds with a higher affinity towards the target.

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Fig. 10 discloses two targets which are attached to each other prior to the mixing with the library of complexes. The attachment can be natural, i.e. the association between target 1 and target 2 occur in a biological context or the attachment can be artificial, i.e. the association between target 1 and target 2 is obtained by a chemical synthesis. In the latter instance, the association between the targets may be obtained by any chemical or enzymatic means which ensure a linkage. The association of the targets may also be obtained by expressing target 1 and target 2 as a fusion protein, i.e. a single protein having two distinct targets or monomer domains. In an embodiment, one of

the targets in the fusion protein is a capturing protein, like streptavidin. In the event the library is spiked with complexes having a ligand against the capturing protein, like biotin, it is feasible to form a connection between the fusion protein and a member of the library. The further functionality, i.e. target 2, of the fusion protein may be then be subjected to a screening process to find binder from the library.

During the mixing step, the two attached targets are contacted with the library of complexes under binding conditions. The library may be spiked with a complex comprising a compounds known to bind to the one of the targets in order to find suitable binders against another target. If the library comprises suitable binding display molecules, two ends of the binding complexes is positioned in close proximity. The addition of a connector oligonucleotide ensures that the ends are kept close together when a ligase is allowed to perform the action of ligating the ends together. The resulting PCR product comprises genetic information which encodes both the display molecules that have participated in the binding interaction with target 1 and target 2.

20

EXAMPLES illustrating the second aspect of the present invention

Example 1:

Oligonucleotide sequences

25

Target Sequence (ES-1)

5'-X-TAGTC GATGT AGCTA GCTAG TGCGC CAATG **CCTTA TCAGC**

Identifier Sequence (IS-1) (extension part)

5'-**GATCG ATGAC** TGACG CCGGT AAATCTACCGTCTAAGCTG-Y-3'

30

Underlined sequence is reverse primer binding site

Control Identifier Sequence (CIS-1) (extension part)

120

5'-**GATCG ATGAC** TGACG CCGGT *gacgt cgtag atata gatgc*
AAATCTACCGTCTAAGCTG-Z-3'

Underlined sequence is reverse primer

5

Connector Sequence (CS-1)

5'-AAAAGGA**ATAGTCG-CTAGCTACTG**TTTT

Primers (Forward PR-1 and reverse PR-2)

10

PR-1: 5'-TAGTC GATGT AGCTA GCTAG

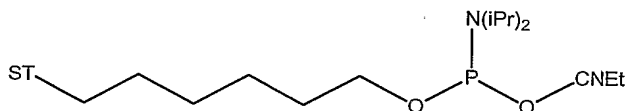
PR-2: 5'-CAGCT TAGAC GGTAG ATTT

Target labelling with oligonucleotide sequence.

15

The target molecule (streptavidin) is modified with an oligonucleotide sequence using a terminus modifier that allow direct coupling to the target(s) molecule. The oligonucleotide sequence ES-1 is synthesised with the 5'-Thiol-Modifier (Glen Research, #10-1926-90) to produce a oligonucleotide that can be coupled to the target(s) through the 5'-end (designated X in the ES-1).

20



5'-Thiol-Modifier C6

25

The covalent attachment of the oligonucleotide at the target is carried out with the aid of the heterobispecific crosslinker Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sSMMCC). The ε-amino groups of lysine side chains of the target(s) are first derivatized with sSMMCC cross linker to provide a maleimide functionality, which subsequently is reacted with the thiolated oligonucleotide.

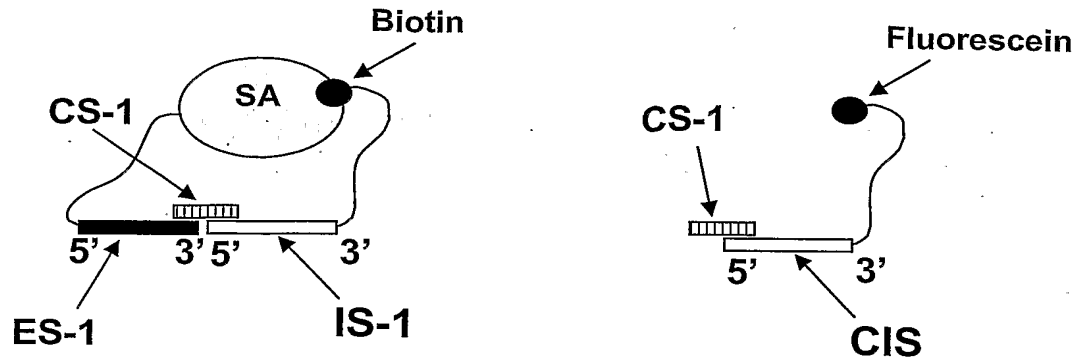
30

Protocol: Dissolve approx. 2 mg of sSMCC in 60 μ l of DMF. Add the sSMCC solution to 200 μ l of a 100 μ M solution of streptavidin in PBS buffer pH 7.3 and incubate in the dark at room temperature for about 1 hour. The excess sSMCC is removed using NAP5 or NAP10 (Pharmacia) using a PBSE buffer. The thiolated oligo (ES-1) is activated in TE buffer, pH 7.4 using 1 mM DDT and excess removed using spin column (BioRad). The activated streptavidin and oligonucleotide are preferably used directly in the cross linking reaction by mixing and incubation for about 1 hour in the dark at room temperature. The modified streptavidin is preferably purified on a size-exclusion column or a anion-exchange column (MonoQ HR5/5, Pharmacia).

Bifunctional complex molecules.

Bifunctional complexes are preferably molecules that are composed of a nucleotide sequence that encodes for the displayed molecule. These complexes can be generated using various procedures, as disclosed elsewhere herein. The bifunctional complexes preferably contain an oligonucleotide that can be ligated or otherwise connected to the oligonucleotide sequence on the target mediated by the binding of the display molecule to the target.

This example describes the bifunctional complex as the IS-1 sequence which is labelled with a biotin in the 3'end (designated Y in the sequence). This oligonucleotide is synthesized using Biotin-dT (Glen Research, # 10-1038-95), which will function as the display molecule and the oligonucleotide sequence encoding the biotin moiety. The displayed biotin molecule has high affinity for streptavidin which will bring the coding oligonucleotide (CS-1) in close proximity of the identifier sequence (IS-1). This will promote the ligation between the ES-1 and the IS-1 oligonucleotides as shown below. The ligation is mediated by the connector sequence (CS-1).



A different bifunctional complex with a Fluorescein as display molecule is also present. The fluorescein will not bind to the target molecule (streptavidin) resulting in no proximity ligation between the ES-1 and the IS-1 oligonucleotides.

Selection through proximity ligation

20 pM bifunctional complex (IS-1) is mixed with 100 pM conjugated target molecule to allow binding of the biotin molecule to streptavidin. Another bifunctional complex (CIS-1) was used as a control (100 pM) with a different display molecule (Fluorescein) included in the synthesis of the oligonucleotide as a Fluorescein-dT (Glen Research, # 10-1056-95). This displayed molecule is encoded by another unique sequence in the CIS-1 oligonucleotide. However, the ligation region is identical to the IS-1 oligonucleotide allowing ligation if proximity is achieved. The CIS-1 oligonucleotide is 10 nucleotides longer permitting the distinction from the IS-1 oligonucleotide by running an agarose gel and determining the length of the oligonucleotides. The mixtures were incubated in 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 0.15 mM ATP, pH 7.4 for 1 hour to allow association of the Biotin (or Fluorescein) to streptavidin.

The connector sequence (CS-1) is then added at 400 nM concentration to promote the connection between the ES-1 and IS-1 oligonucleotides together with 2 U T4 DNA ligase to start the ligation. The reaction was kept at 30°C

for 5 min and then 80°C for 20 min. The relatively high concentration of the connector oligonucleotide will saturate all IS-1 that have not been brought into proximity of an encoding sequence.

5 Amplification of the ligated products was performed with the polymerase chain reaction (PCR) using primers corresponding to the 5'-end of the encoding sequence (PR-1: 5'-TAGTC GATGT AGCTA GCTAG) and the 3'-end of the identifier oligonucleotide sequence (PR-2: 5'-CAGCT TAGAC GGTAG ATTT). The primers are design to only amplify the ligated product. PCR was
10 performed using Ready-To-Go (RTG) PCR beads (Amersham Biosciences) and 10 pmol of each primer in a reaction volume of 25 µl. The PCR reaction consisted of an initial denaturation step of 94°C for 2 minutes followed by 20-45 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 1 minute and extension at 72°C for 1 minute. A final extension step of 2 min-
15 utes at 72°C was included. The PCR products were resolved by agarose gel electrophoresis and the band corresponding to the expected size was cut from the gel and purified using QIAquick Gel Extraction Kit (QIAGEN).

The different length of the Biotin and Fluorescein identifier oligonucleotide
20 sequences (IS-1 and CIS-1) is used to verify that the bifunctional complex with Biotin have been enriched through the binding to streptavidin. This example describes a selection using two different bifunctional complexes. The same approach can be used for larger libraries, at least up to 10^{14} different molecules. The same approach is used when screening library
25 versus library. In this case the targets are encoded by different sequences but with identical coupling area.

Cloning/sequencing

To sequence individual PCR fragments the purified PCR products were
30 cloned into the pCR4-TOPO vector (Invitrogen) according to the manufacturer's instructions. The resulting mixture was used for transformation of TOP10 *E. coli* cells (Invitrogen) using standard procedures. The cells were

plated on growth medium containing 100 µg/ml ampicillin and left at 37°C for 12-16 hours. Individual *E.coli* clones were picked and transferred to PCR wells containing 50 µl water. These wells were then boiled for 5 minutes and 20 µl mixture from each well was used in a PCR reaction using RTG PCR beads and 5 pmol each of M13 forward and reverse primers according to the manufacturer's instructions. A sample of each PCR product was then treated with Exonuclease I (USB) and Shrimp Alkaline Phosphatase (USB) to remove degraded single stranded DNA and dNTPs and sequenced using the DYEnamic ET cycle sequencing kit (Amersham Biosciences) according to the manufacturer's instructions and the reactions were analyzed on a MegaBace 4000 capillary sequencer (Amersham Biosciences). Sequence outputs were analyzed with ContigExpress software (Informax Inc.).

Example 2: Library versus library

A library of bifunctional complexes is screened against another library of encoded peptides or proteins. Examples of other encoded peptides are ribosome displayed peptides or mRNA displayed peptides. This example describes the use of bifunctional complexes together with mRNA displayed targets to perform library versus library screening. Any other library, where the genotype is associated with the phenotype, can be used together with bifunctional complexes as described in this invention.

A freshly transcribed mRNA (0.5 – 2.5 nmol) is prepared from an appropriate library. The transcribed mRNA library is hybridized to biotinylated puromycin-linker (about 0.5 nmol) in 300 µl binding buffer (30 mM Tris, pH 7.0, 250 mM NaCl) by heating to 85° C for 30 sec followed by cooling to 4° C in 5 min. 100 µl pre-washed Neutravidin beads (Pierce) is then added to the hybridization mixture and incubated for 30 min at 4 °C under rocking. Subsequently, the beads are washed in 3x 100 µl binding buffer followed by centrifugation to remove the liquid phase. The moist beads were then irradiated for 15 min at room temperature with a 25W UV-lamp (Pyrex-filter, $\lambda > 300\text{nm}$). Subsequently, the beads are washed with 100 µl plain water to yield the photo-

crosslinked mRNA-puromycin template which is directly used for mRNA-peptide fusion formation in rabbit reticulocyte lysate to produce the mRNA-protein fusions library according to the literature (Kurz et al., Nucleic Acids Res. 2000, 28:83). The puromycin-linker is also prepared according to Kurz et al. and biotinylated by carbamate bond formation between the puromycin amino group (50 μ M) and the photo-cleavable biotin-reagent (NHS-PC-Biotin, 5 mM, EZ-LinkTM-Biotin, Pierce Chemicals) in 25% DMSO/water for 2 h at room temperature followed by NaCl/EtOH precipitation.

10 The mRNA-peptide fusion library is then converted by a suitable primer to contain a coupling area. The coupling area is formed by a primer partly complementary to the distal region of the mRNA and which is able to promote an extension on the mRNA strand with the coupling area. This coupling area is then used in the selection procedure to couple the target oligonucleotide with
15 the identifier oligonucleotide mediated by the binding of displayed molecules to the mRNA-peptide fusions.

1-100 pmol bifunctional complex library molecules are mixed with 1-100 pmol mRNA-peptide fusion library in a binding buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 0.15 mM ATP, pH 7.4) for 1 hour to allow binding of the displayed molecules to the mRNA-peptide fusion molecules. The connector sequence (an oligonucleotide which is complementary to the coupling area is then added at about 400 nM concentration to promote the connection between the target and identifier oligonucleotides. Subsequently, 2 U T4 DNA
25 ligase is added to start the ligation. The reaction is kept at 30°C for 5 min and then 80°C for 20 min. Amplification of the ligated products is performed using a polymerase chain reaction (PCR) using primers corresponding to the 5'-end of the target oligonucleotide and the 3'-end of the identifier oligonucleotide. The primers are design to only amplify the ligated product. PCR is performed using Ready-To-Go (RTG) PCR beads (Amersham Biosciences) and
30 10 pmol each primer in a reaction volume of 25 μ l. The PCR reaction consisted of an initial denaturation step of 94°C for 2 minutes followed by 20-45

cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 1 minute and extension at 72°C for 1 minute. A final extension step of 2 minutes at 72°C was included. The PCR products are resolved by agarose gel electrophoresis and the band corresponding to the expected size is cut from the gel and purified using QIAquick Gel Extraction Kit (QIAGEN).

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

Claims

1. A method for identifying display molecule(s) having affinity towards molecular target(s), comprising the steps of
mixing one or more molecular target(s) associated with target oligonucleotide(s) and a library of bifunctional complexes, each bifunctional complex of the library comprising a display molecule attached to an identifier oligonucleotide, which codes for said display molecule,
coupling to the target oligonucleotide(s) the identifier oligonucleotide of complexes comprising display molecules binding to the target, and
deducing the identity of the binding display molecule(s) and/or the molecular target(s) from the coupled product between the identifier oligonucleotide(s) and the target oligonucleotide(s).
2. The method of claim 1, wherein the display molecule is a reaction product of two or more chemical entities and the identifier oligonucleotide comprises codons identifying the chemical entities.
3. The method of claim 1, wherein one or more members of the library are potentially binding compounds tagged with identifier oligonucleotides.
4. The method according to claim 1, 2 or, 3, wherein the chemical entities are precursors for a structural unit appearing in the display molecule.
5. The method according to any of the claims 1 to 4, wherein some or all of the chemical entities are not naturally occurring α -amino acids or precursors thereof.
6. The method according to claim 1 or 2, wherein each codon comprises 4 or more nucleotides.
7. The method according to claim 1 or 2, wherein the display molecules of the library complexes are non- α -polypeptides.
8. The method according to claim 1 to 4, wherein the display molecules of the library complexes are non-nucleic acids.
9. The method according to any of the preceding claims, wherein the display molecule has a molecular weight less than 2000 Dalton, preferably less than 1000 Dalton, and more preferred less than 500 Dalton.

10. The method according to any of the preceding claims, wherein the identifier oligonucleotide uniquely identifies the display molecule.

11. The method according to any of the claims 1 to 10, wherein one or more chemical entities are transferred to the nascent display molecule by a chemical building block further comprising an anti-codon.

12. The method according to claim 11, wherein the information of the anti-codon is transferred in conjunction with the chemical entity to the nascent complex.

13. The method according to any of the preceding claims, wherein the chemical entities are reacted without enzymatic interaction.

14. The method according to any of the claims 1 to 13, wherein the codons are separated by a framing sequence.

15. The method according to any of the claims 1 to 14, wherein the display molecule and the identifier oligonucleotide are joined by a selectively cleavable linker.

16. The method according to claim 15, wherein the linker is cleaved by irradiation.

17. The method according to any of the claims, wherein the library comprises one, two or more different complexes.

18. The method according to any of the claims 1 to 16, wherein the library comprises 1,000 or more different complexes.

19. The method according to claim 1, wherein the molecular target is of a biological origin.

20. The method according to any of the claims 1 to 19, wherein the molecular target is immobilized on a solid support.

21. The method according to claim 20, wherein the target immobilized on the support forms a stable or quasi-stable dispersion.

22. The method according to claim 21, wherein a cleavable linker is present between the solid support and the molecular target.

23. The method according to any of the claims 1 to 22, wherein the molecular target is a protein.

24. The method according to claim 23, wherein the protein is selected from the group consisting of kinases, proteases, phosphatases, and antibodies.

5 25. The method according to any of the claims 1 to 24, wherein the molecular target is a nucleic acid.

26. The method according to claim 24, wherein the nucleic acid is an DNA or RNA aptamers.

10 27. The method according to any of the claims 23 to 26, wherein the target protein is attached to the nucleic acid responsible for the formation thereof.

28. The method according to any of the claims 1 to 27, wherein the mixture step includes that a molecular target library comprising different peptides each attached to the nucleic acid responsible for the formation thereof is mixed with a library of complexes.

15 29. The method according to claim 28, wherein the library of complexes comprises a single bifunctional complex.

30. The method according to any of the claims 1 to 29, wherein the target oligonucleotide is associated by a chemical synthesis to the molecular target.

20 31. The method according to claim 30, wherein the molecular target is associated with the target oligonucleotide through one or more covalent or non-covalent bonds.

25 32. The method according to any of the claims 1 to 31, wherein a bifunctional complex having a display molecule binding to the molecular target constitutes the target oligonucleotide associated with the molecular target.

33. The method according to claim 32, wherein the display molecule is a compound known to bind to the target.

34. The method according to claim 33, wherein a target is saturated with a known ligand prior to the mixing step.

30 35. The method according to claim 34, wherein the target oligonucleotide is associated with the molecular target during the mixing step.

36. The method according to claim 35, wherein two bifunctional complexes of a library of bifunctional complexes are associated with a common molecular target.

5 37. The method of claim 36, wherein the bifunctional complexes bind to the same binding site of the molecular target.

38. The method of any of the claims 31 to 36, wherein the bifunctional complexes bind to discrete binding sites.

10 39. The method according to any of the preceding claims, wherein an initial ligand or a pool of ligands with potential affinity towards a target is amended by reaction with one or more chemical entities to form a second generation library, said second generation library being used in the method according to any of the claims 1 to 39.

15 40. The method according to any of the preceding claims, wherein two or more targets interacting in a biological context separately are subjected to the method of claim 1, whereupon the identified display molecules binding to the two or more targets are linked via a suitable linker. ,

20 41. The method according to any of the claims 1 to 40, wherein two or more molecular targets or type of molecular targets are involved in the mixing step and the target oligonucleotide identifies the molecular targets or the type of molecular targets.

42. The method according to any of the claims 1 to 41, wherein the mixing step includes the removal of non-binding library members prior to the coupling of the target oligonucleotide and the identifier oligonucleotide together.

25 43. The method according to any of the previous claims, wherein the target oligonucleotide and/or the identifier oligonucleotide partly or fully is hybridised to a complementing oligonucleotide.

30 44. The method according to any of the claims 1 to 43, wherein the coupling is performed using means selected from the group consisting of chemical means, enzymatic means, and design means.

45. The method according to any of the claims 1 to 44, wherein the target oligonucleotide or a complementing target oligonucleotide and the identi-

fier oligonucleotide or a complementing identifier oligonucleotide operatively are joined together so as to allow for a polymerase to recognise the coupled strand as a template.

5 46. The method according to any of the preceding claims, wherein the enzymatic means are selected from enzymes of the type polymerase, ligase and restriction enzyme, and any combination thereof.

47. The method according to claim 46, wherein a ligase is used to join the target oligonucleotide and the identifier oligonucleotide together.

10 48. The method of claim 47, wherein a connector oligonucleotide having a region complementing a distal part of the target oligonucleotide and a region complementing a distal part of the identifier oligonucleotide is used during the coupling step so as to allow a ligase or a combination of a ligase and a polymerase to join the identifier and target oligonucleotides together.

15 49. The method according to claim 48, wherein the ends of the oligonucleotides abut each other.

50. The method according to claim 49, wherein the region of the connector oligonucleotide complementing a distal part of the identifier and/or target oligonucleotide is 6 to 16 nucleotides.

20 51. The method according to claims 49, wherein the region is 8 to 12 nucleotides.

52. The method according to claim 48, wherein the connector oligonucleotide is added in excess.

25 53. The method according to any of the claims 1 to 46, wherein a region at the distal ends of the target and identifier oligonucleotides are complementary to each other and a polymerase is allowed to extend the target and/or the identifier oligonucleotide.

30 54. The method according to any of the claims 1 to 47, wherein the target oligonucleotide and/or the identifier oligonucleotide is provided with a sticky end to allow a ligase or a polymerase or a mixture thereof to adjoin the oligonucleotides.

55. The method according to claim 54, wherein the sticky end is formed by a restriction nuclease.

56. The method according to any of the claims 1 to 55, wherein the target and the identifier oligonucleotide or sequences complementary thereto at the proximal end is provided with a priming site.

5 57. The method according to any of the claims 1 to 56, wherein the target-display conjugate is recovered by chromatography following the coupling of the target and the identifier oligonucleotides.

58. The method according to claim 57, wherein the chromatography is size-exclusion chromatography.

10 59. The method according to claim 1, wherein the coupled identifier and target oligonucleotide is amplified prior to decoding the identity of the display molecule.

60. The method according to any of the claim 1, wherein the coupled oligonucleotide is amplified by PCR using priming sites positioned proximal to the display molecule and the molecular target, respectively.

15 61. The method according to claim 59, wherein selective cleavable chemical moieties in each end of the coupled oligonucleotides are cleaved to liberate the coupled oligonucleotide prior to amplification.

62. The method according to any of the claims 1 to 61, wherein the coupled oligonucleotide is recovered and subjected to amplification.

20 63. A conjugate comprising a molecular target associated with an oligonucleotide and a bifunctional complex comprising a display molecule attached to an identifier oligonucleotide, which codes for said display molecule.

64. The conjugate of claim 63, wherein the display molecule is bound to the target.

25 65. The conjugate according to claims 63 or 64, wherein the target oligonucleotide and/or the identifier oligonucleotide are joined to the molecular target and/or the display molecule, respectively, through a selectively cleavable link.

30 66. The conjugate according to any of the claims 63 to 65, wherein the target oligonucleotide is coupled to the identifier oligonucleotide.

67. The conjugate according to claims 66, wherein the coupled oligonucleotide is amplifiable.

68. A display molecule identified by the method according to any of the claims 1 to 62.

Fig. 1

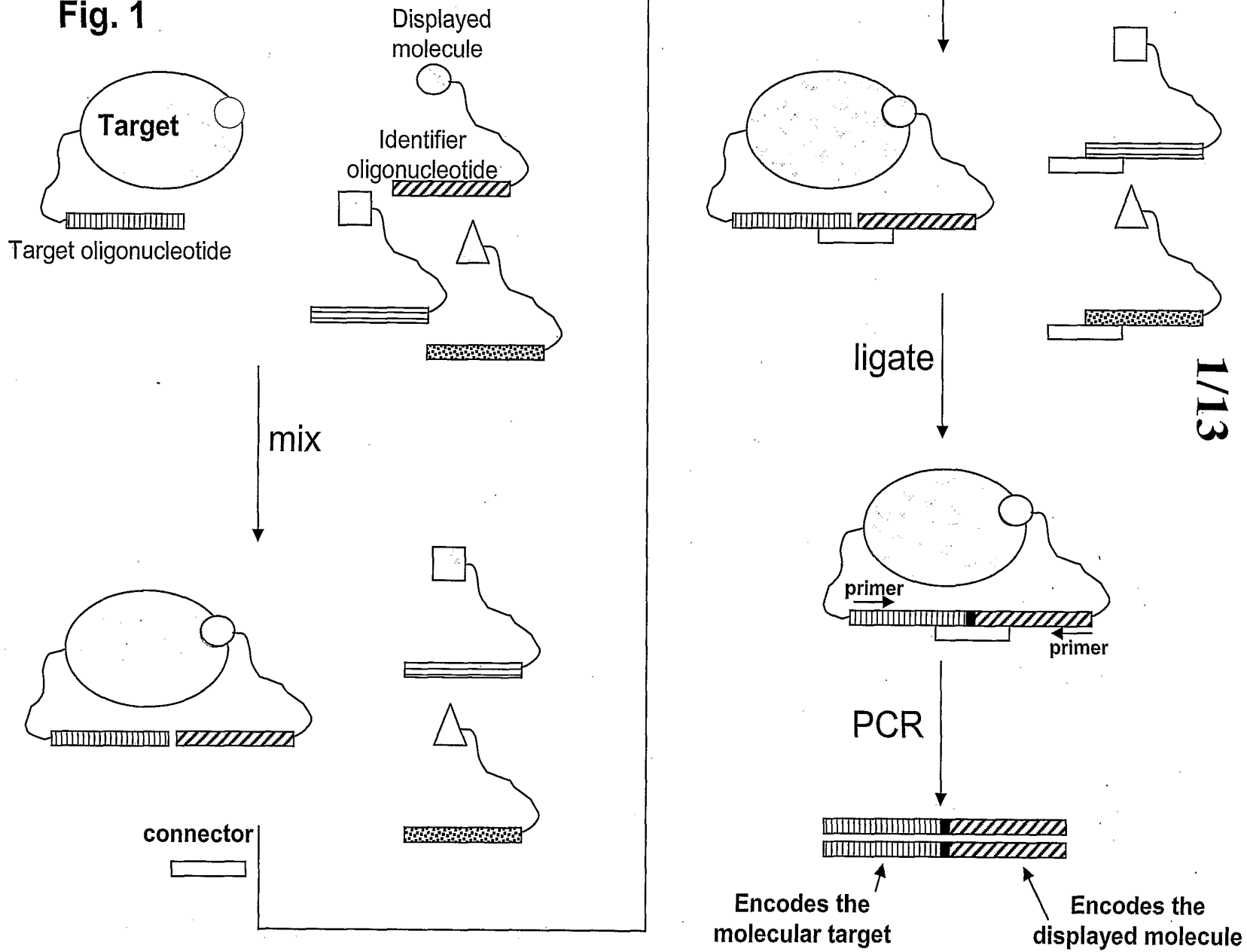
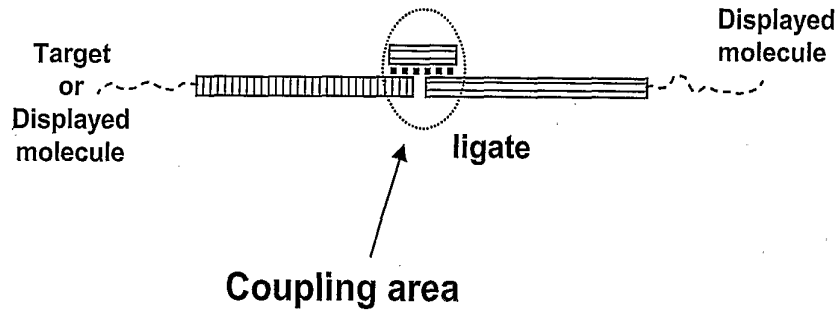
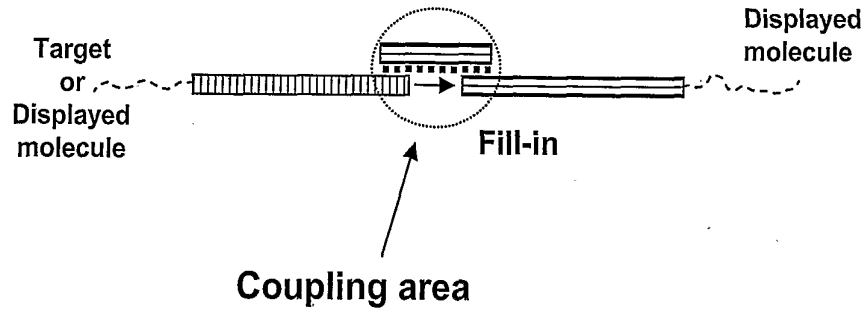


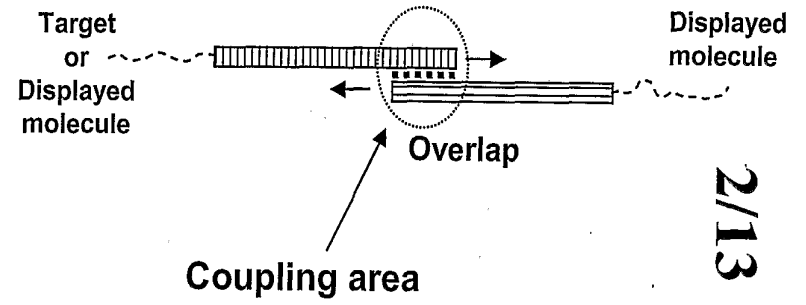
Fig. 2
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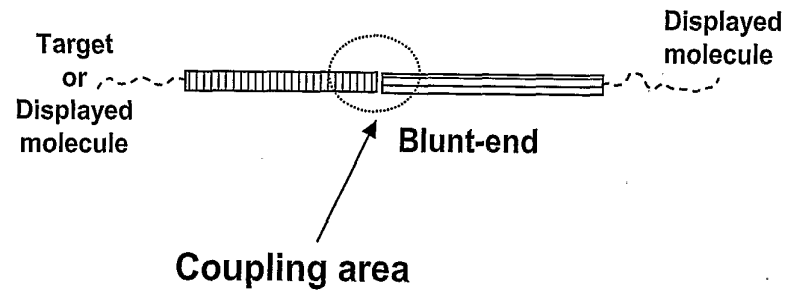
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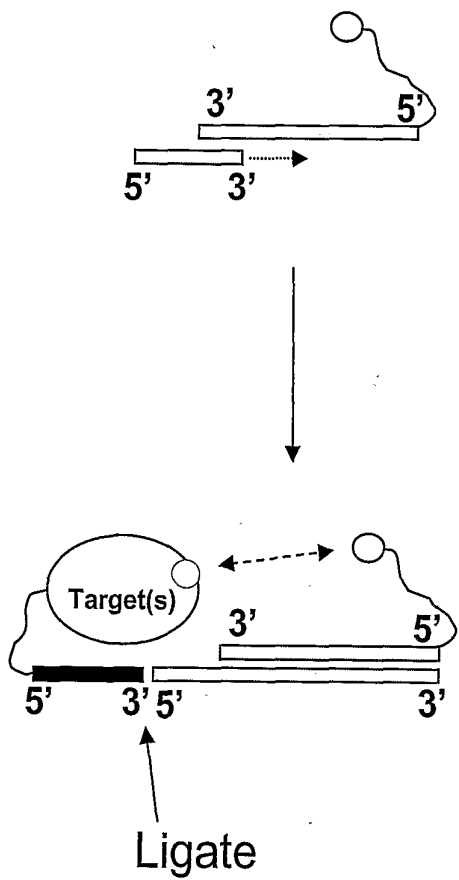
C



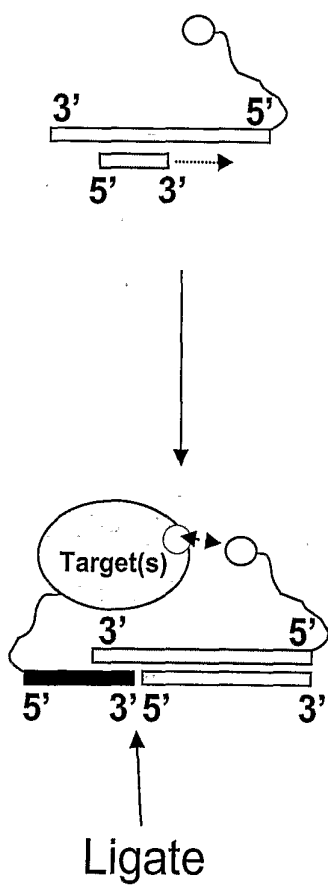
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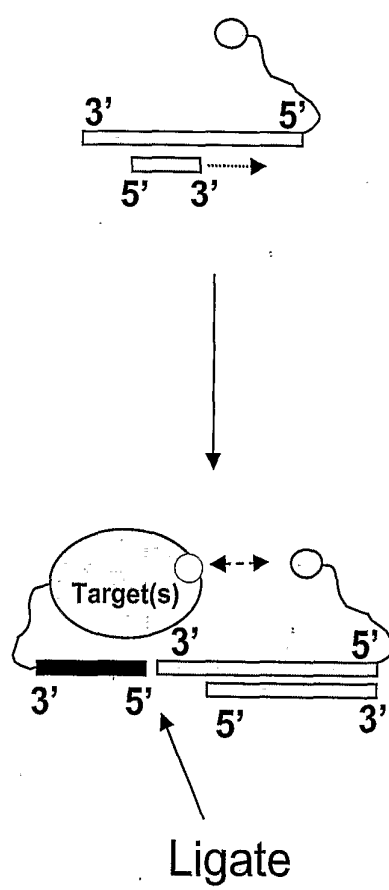
A Fig. 3



B



C



D

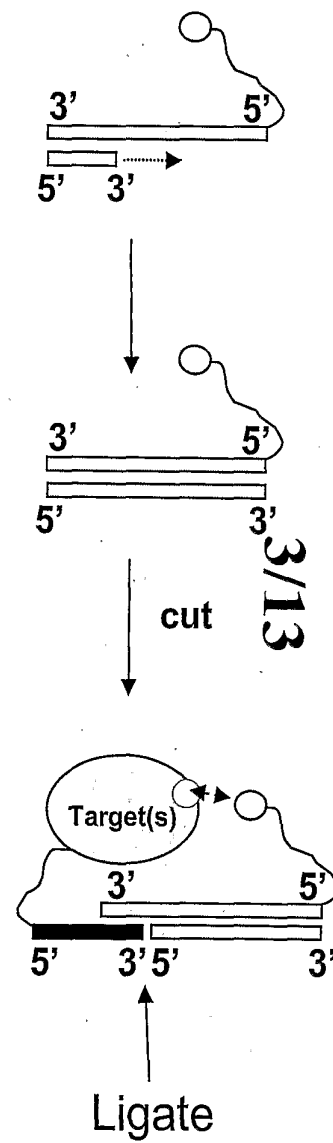
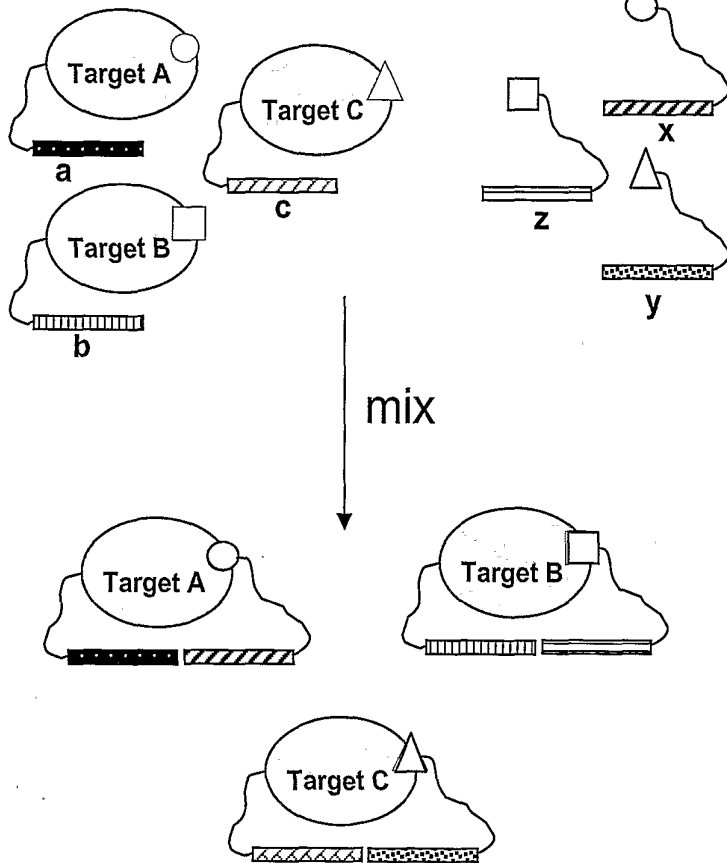


Fig. 4
Library of molecular
Target molecules



Add
connector

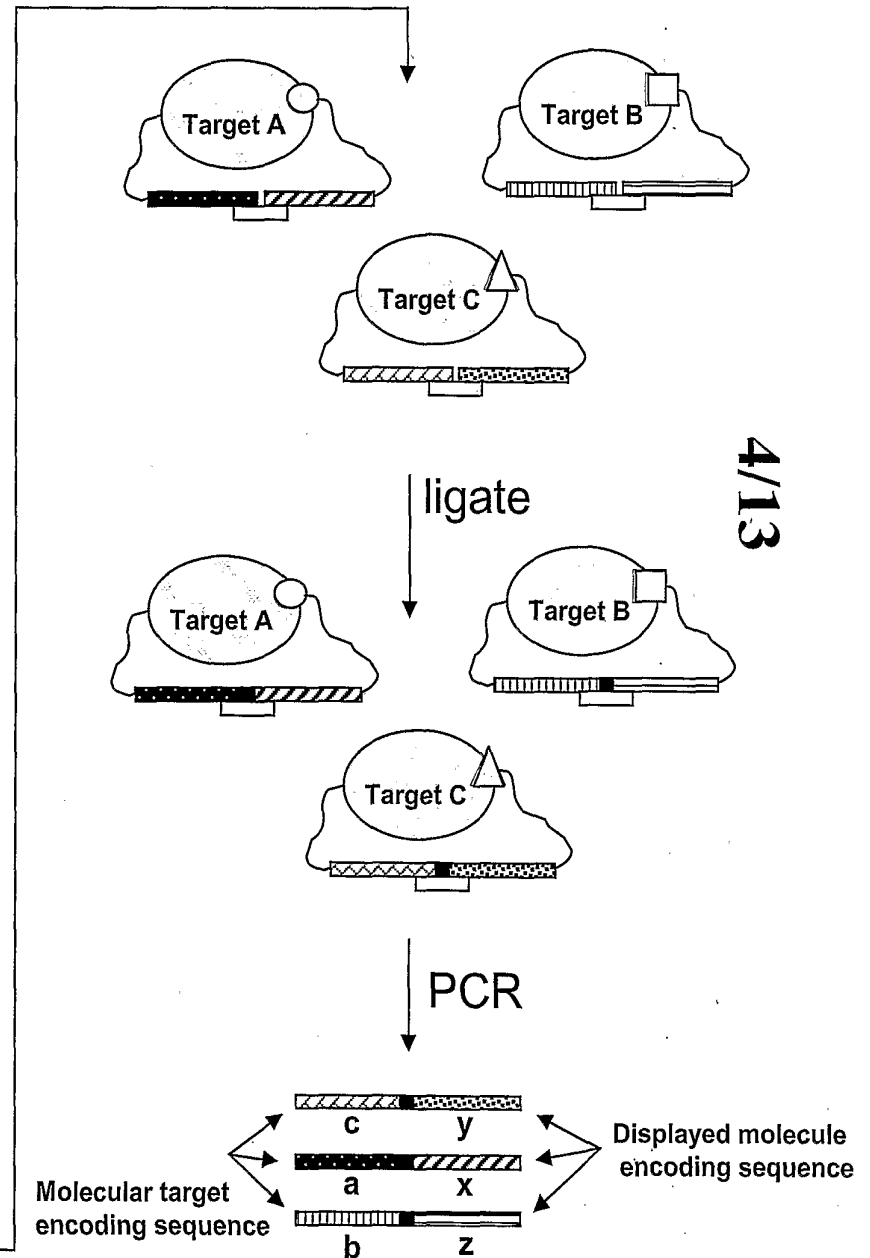
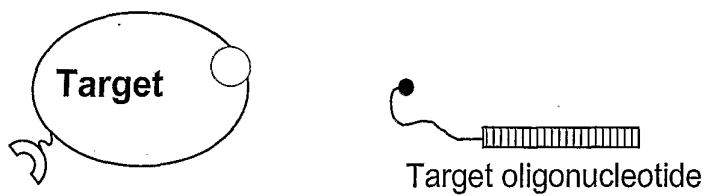
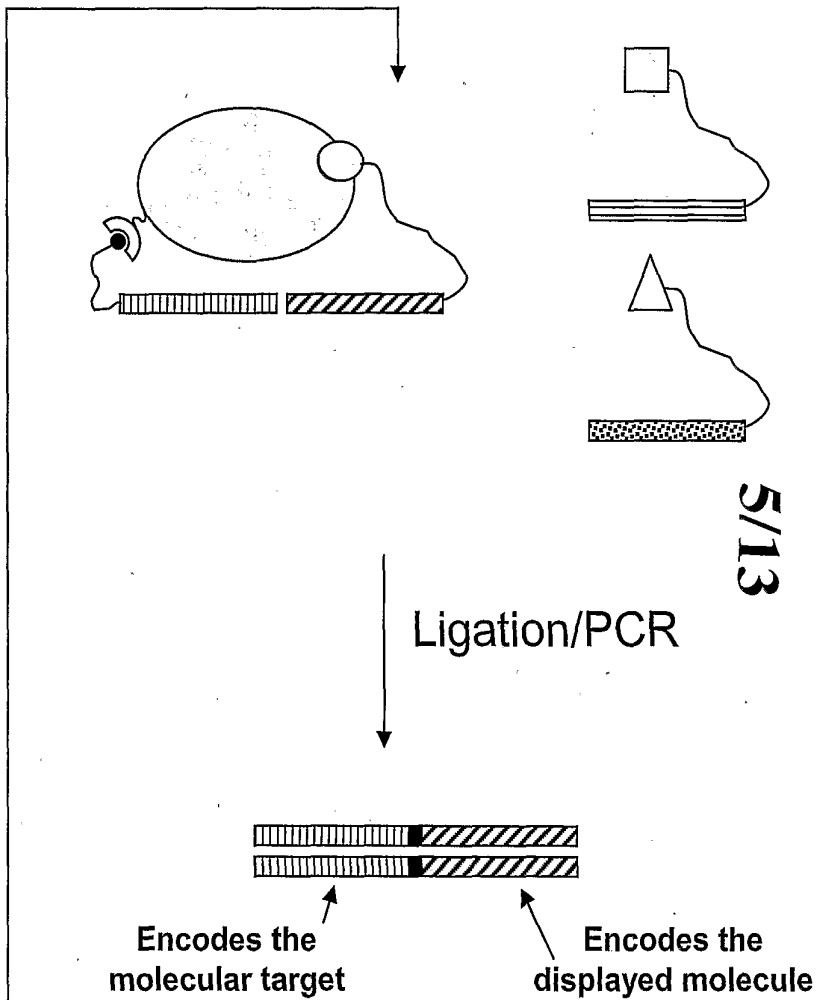
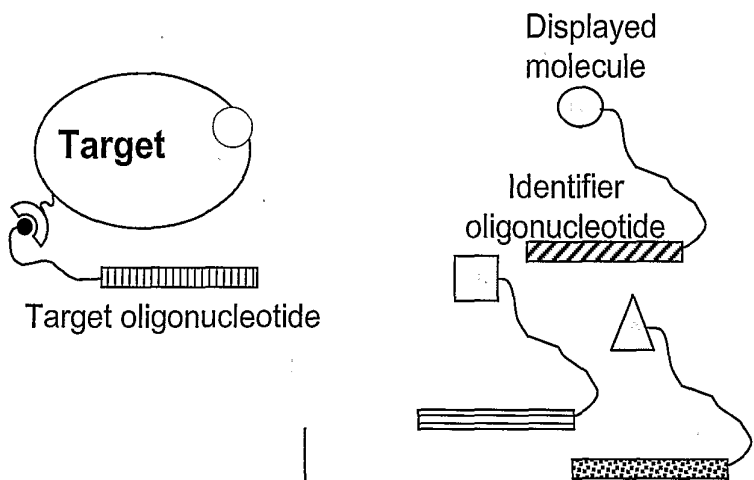


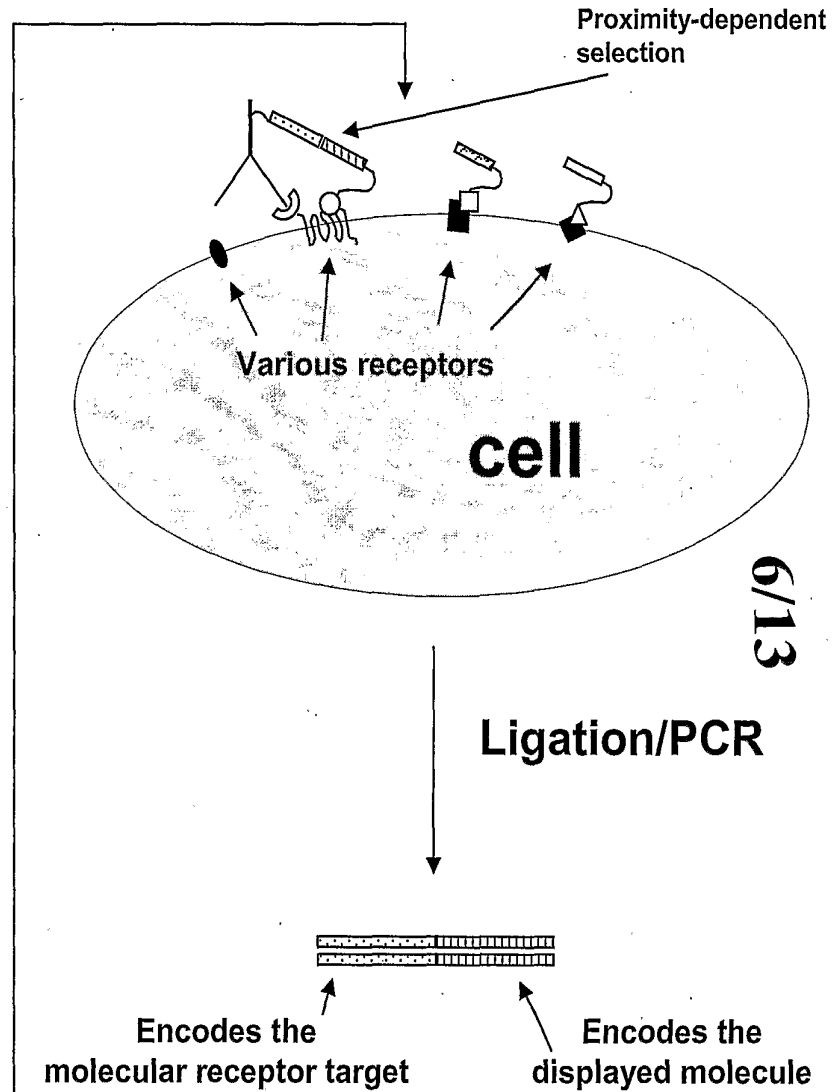
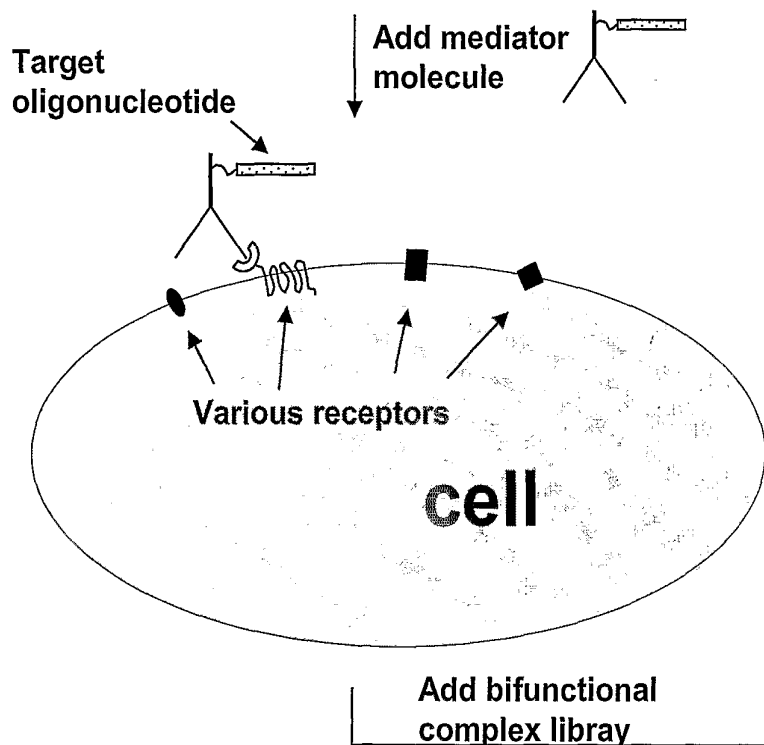
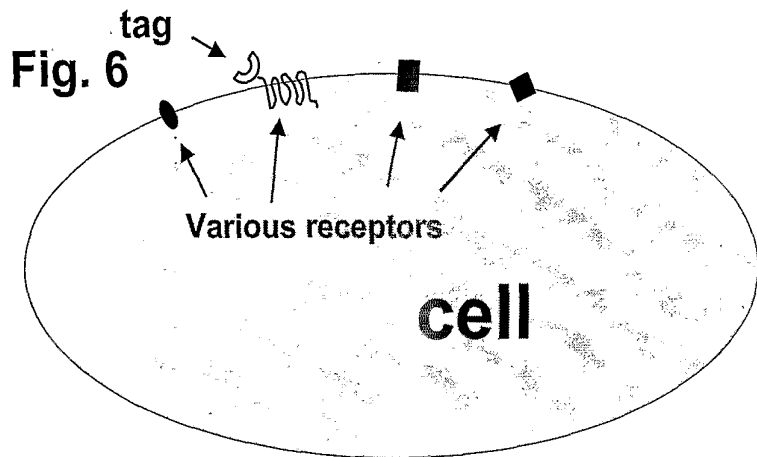
Fig. 5



mix



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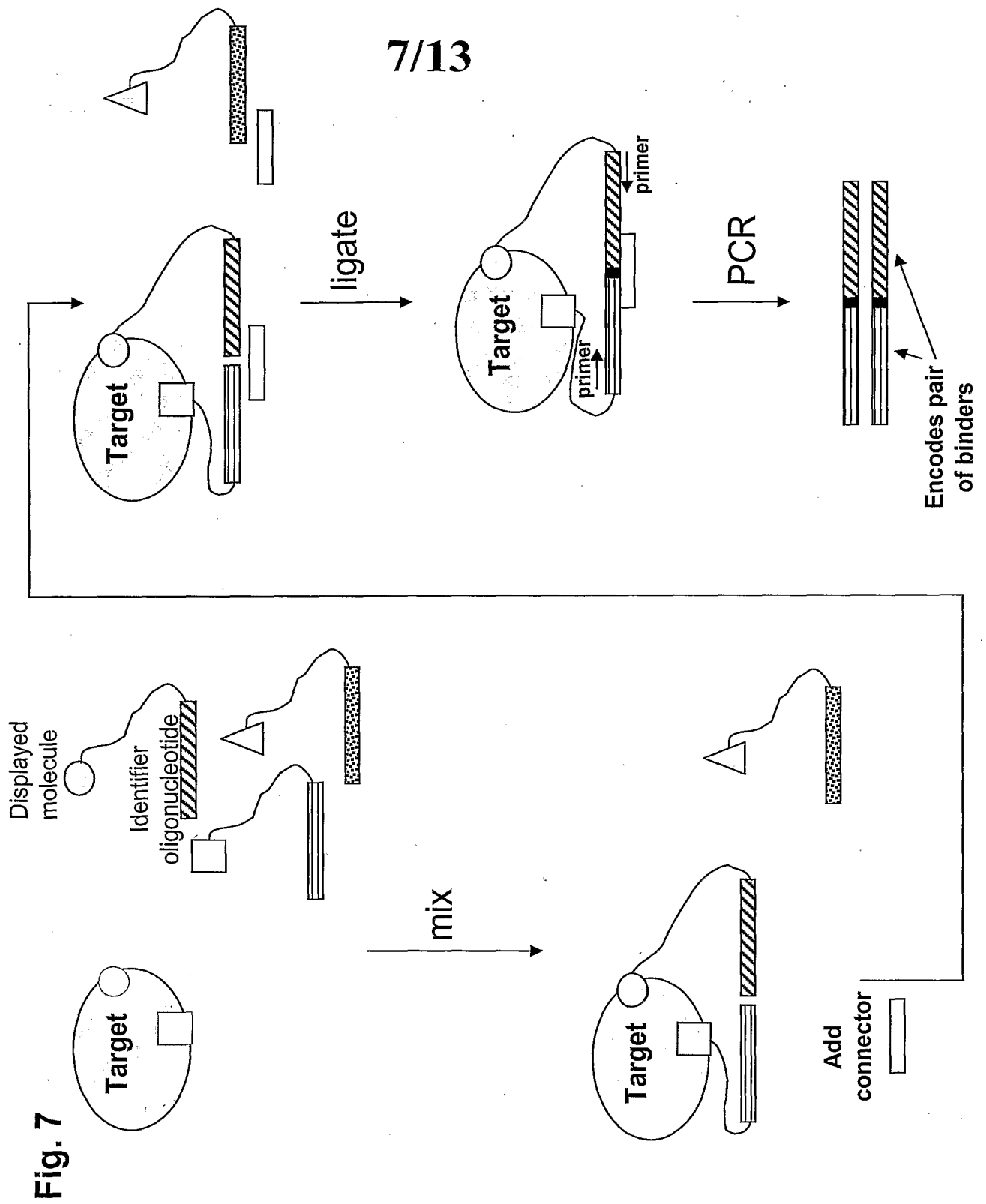


Fig. 7

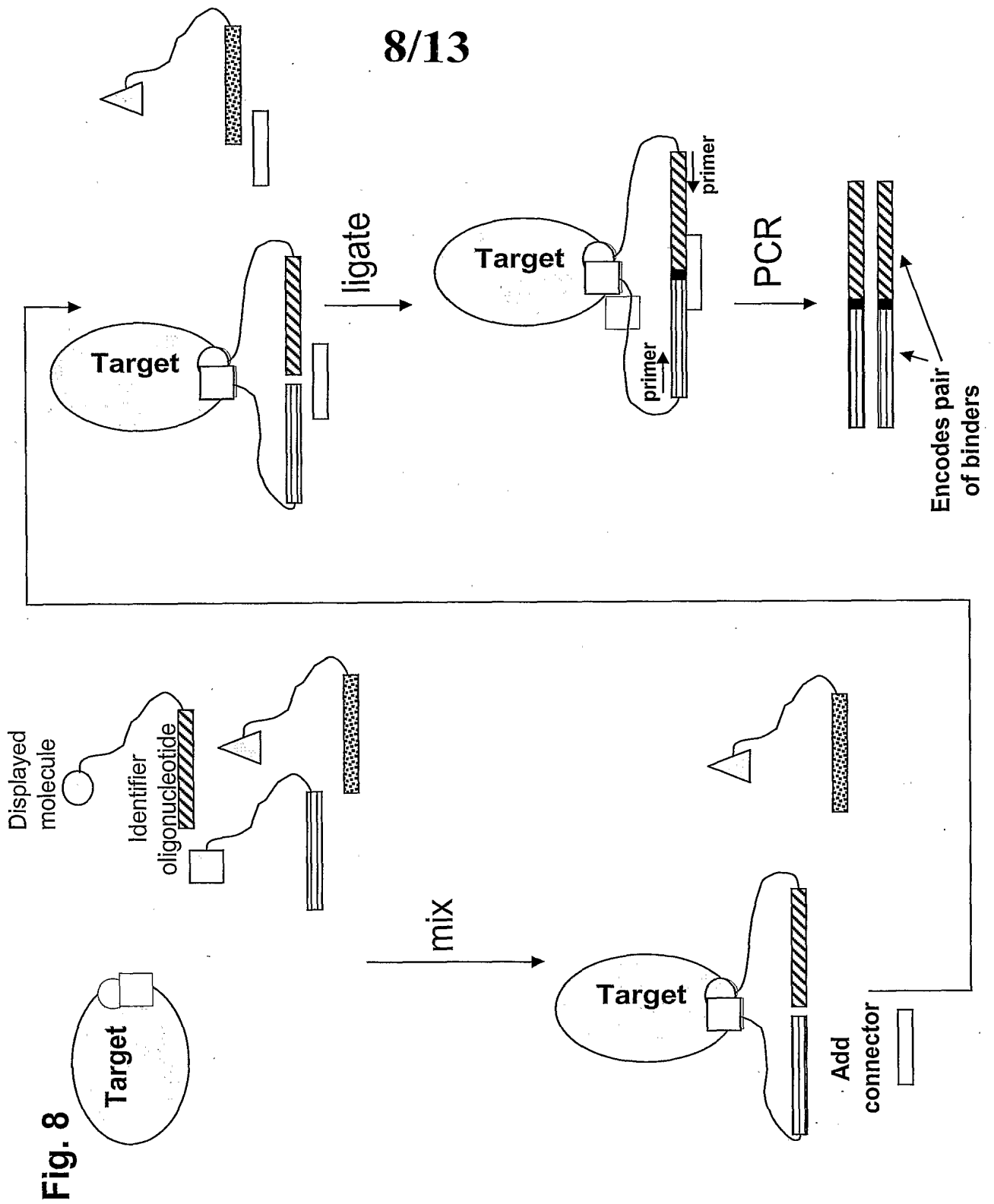
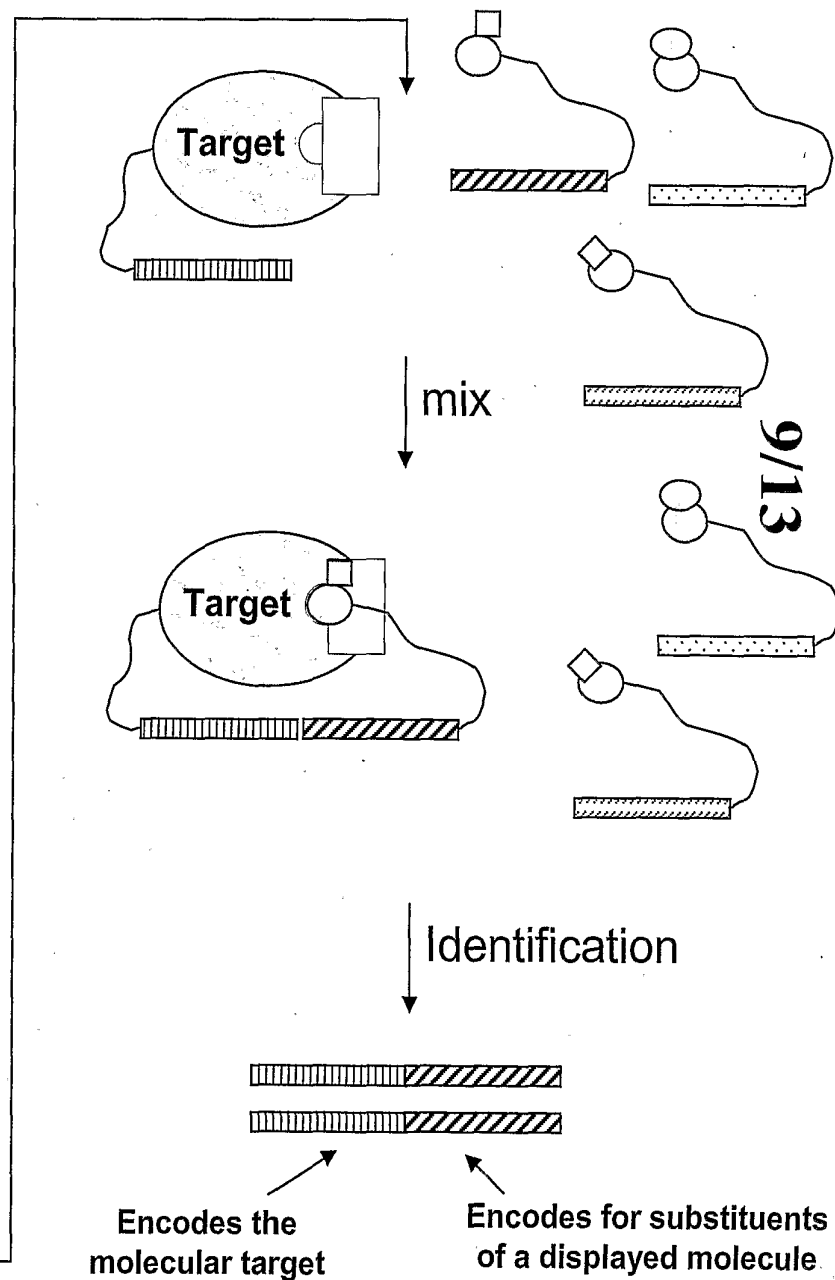
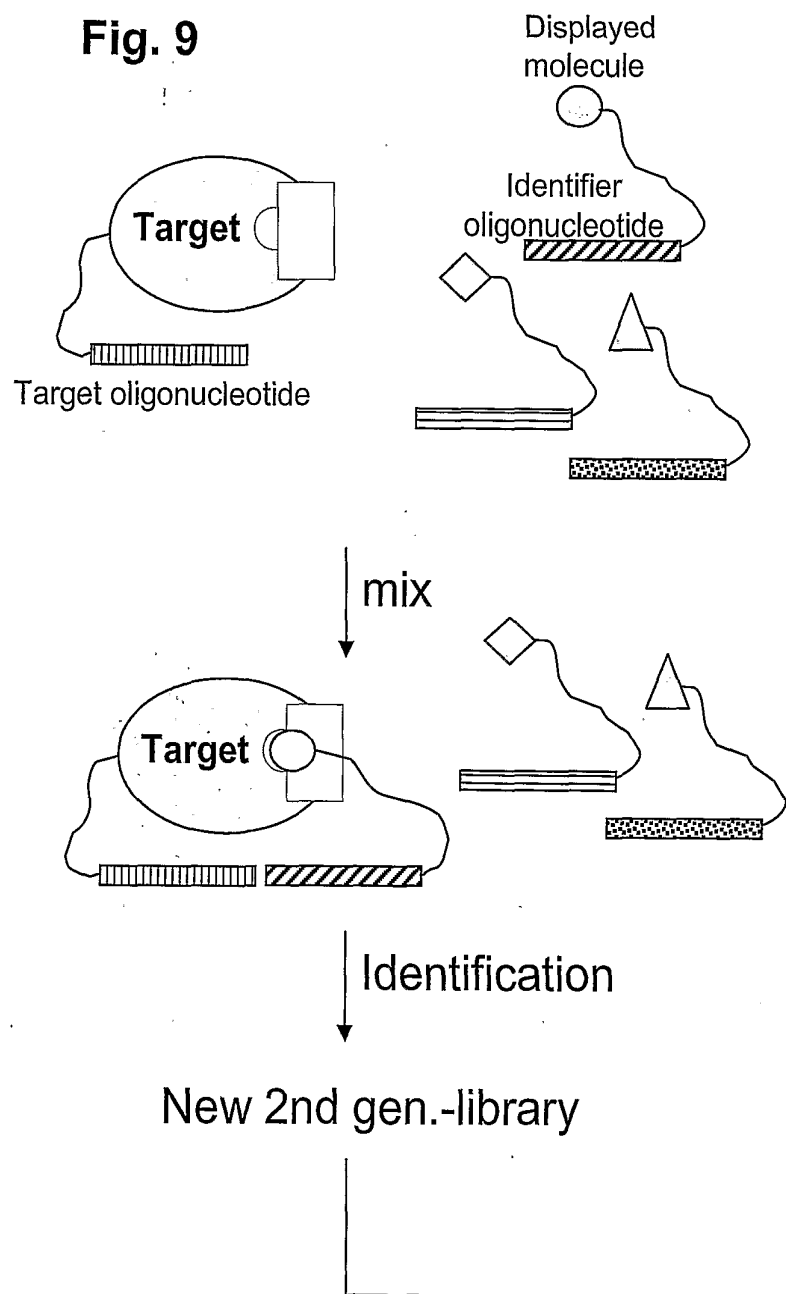
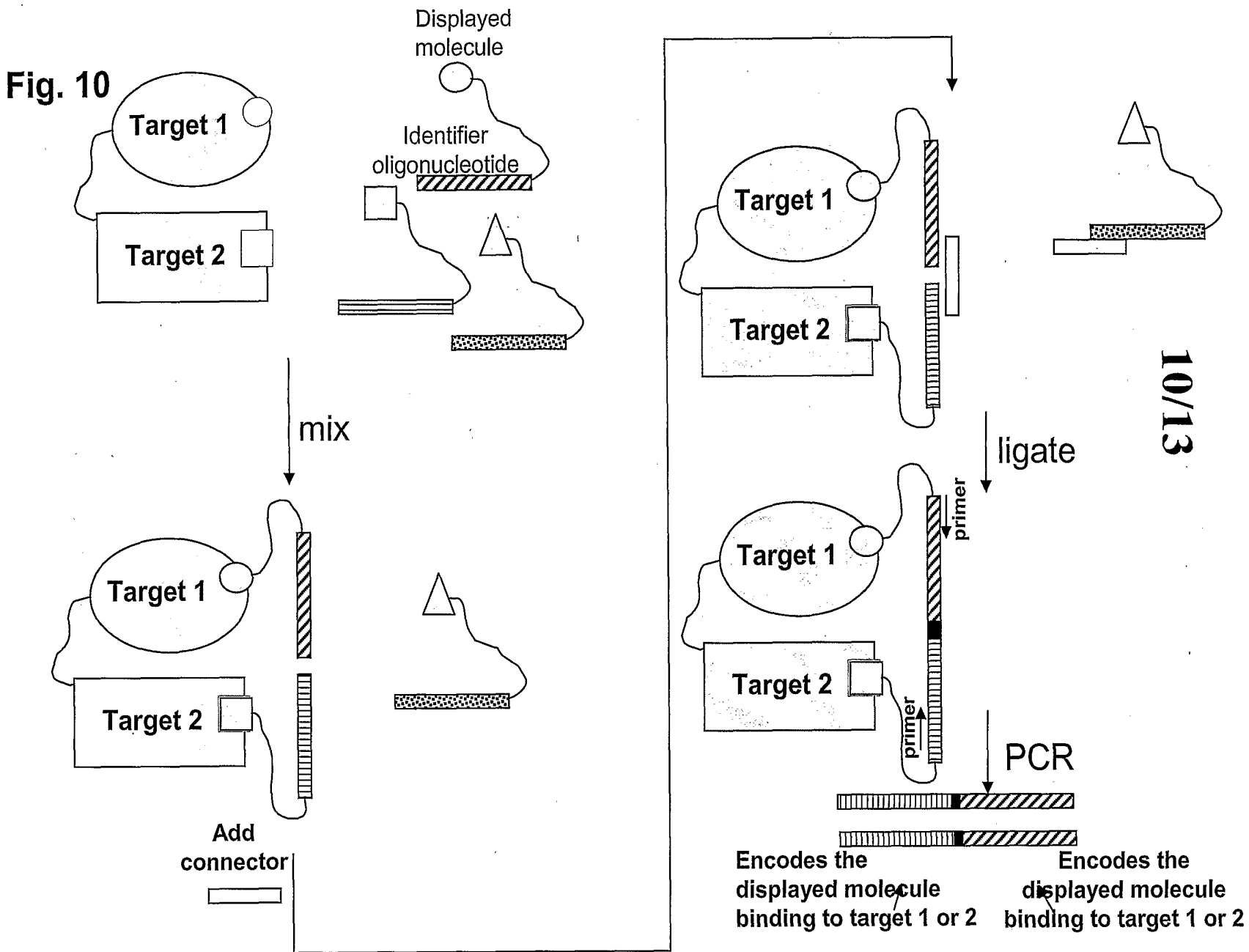


Fig. 9



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Fig. 11A

5

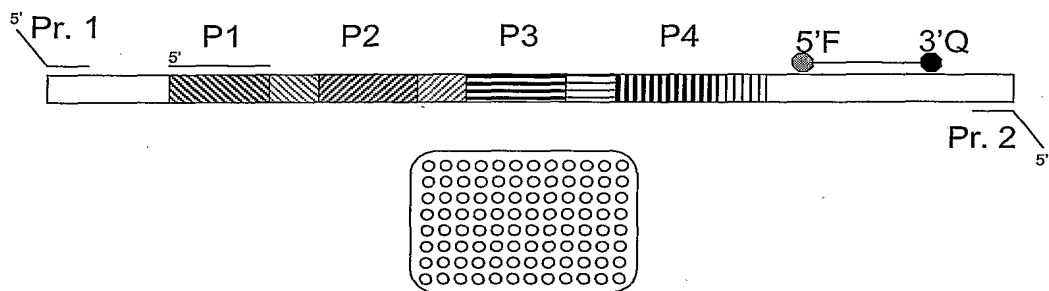
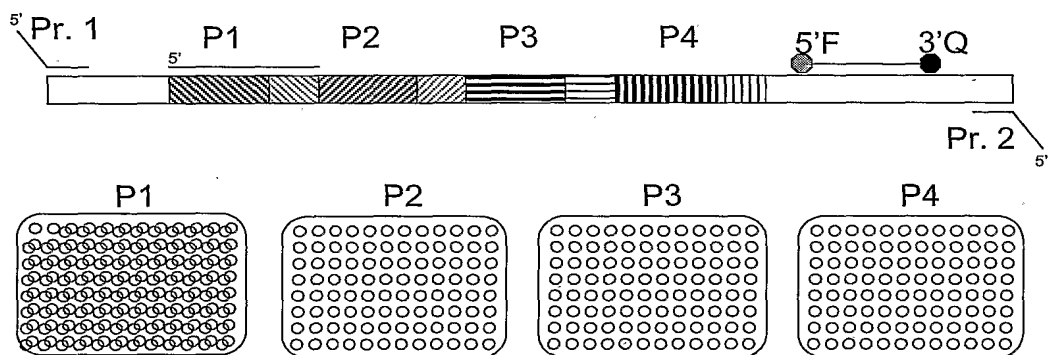
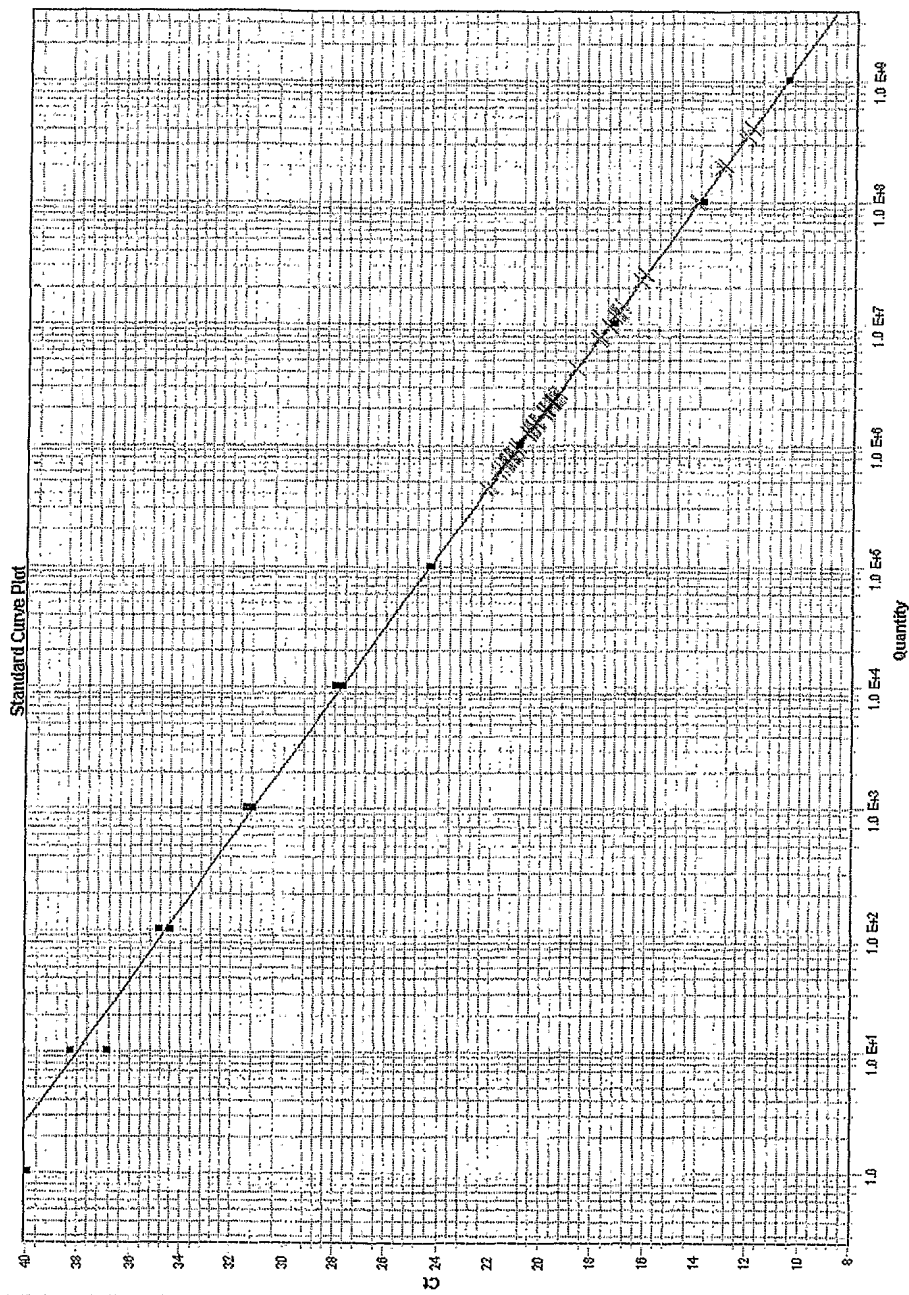


Fig. 11B



12/13

Fig. 12



13/13

Fig. 13A

5

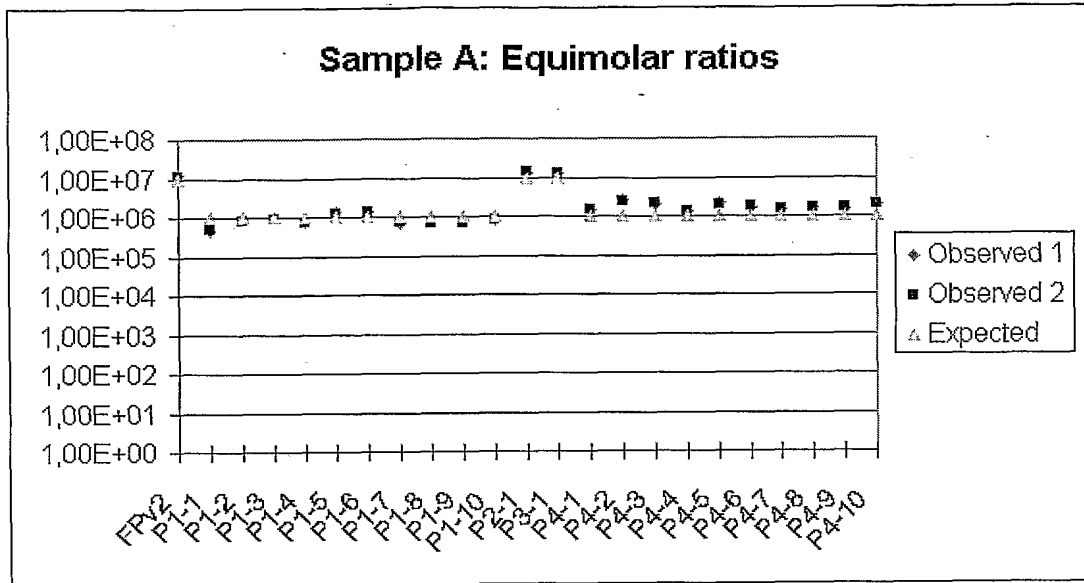
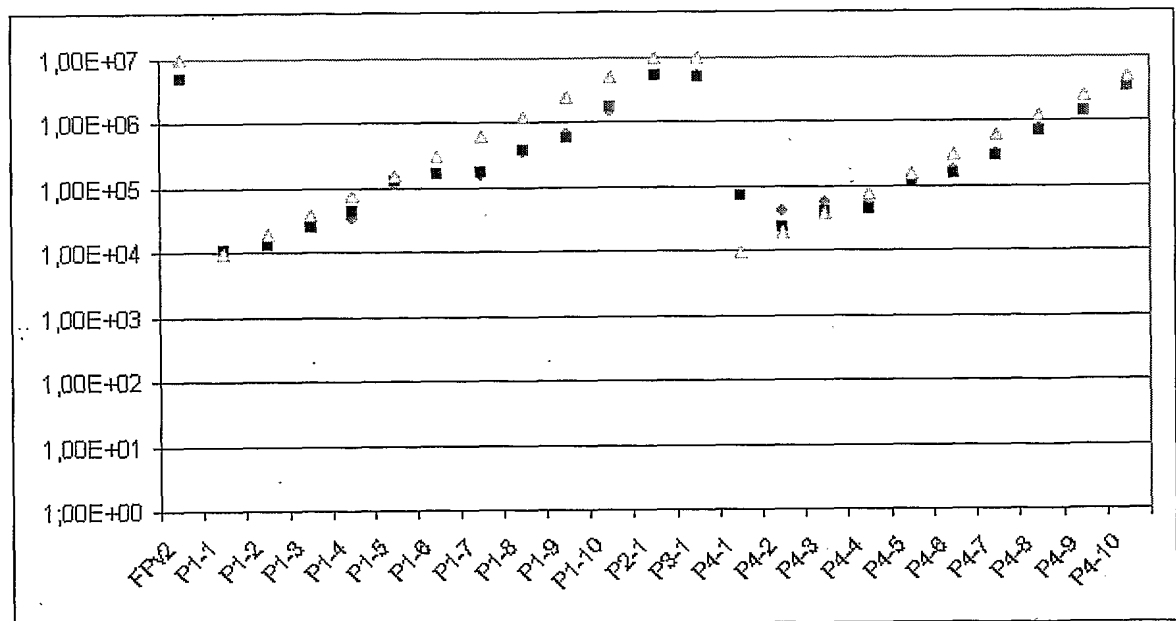


Fig. 13B Sample B



INTERNATIONAL SEARCH REPORT

International Application No.
PCT/DK2004/000630

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68 C12N15/10 C12P1/00 C07B61/00

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 7 C12Q C12N C12P C07B

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)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/025567 A (BERNARD, ANDRE; DUEBEL, STEFAN) 27 March 2003 (2003-03-27)	63-68
A	figures 1,2	1-62
X	WO 02/103008 A (NUEVOLUTION A/S; PEDERSEN, HENRIK; GOUILAEV, ALEX, HAAHR; SAMS, KLARNE) 27 December 2002 (2002-12-27)	63-68
A	figures 3-6	1-62
X	WO 00/32823 A (PHYLOS, INC) 8 June 2000 (2000-06-08)	63-68
A	page 1 - page 21; figures 1-20	1-62
X	WO 02/083951 A (NORTHEASTERN UNIVERSITY; GIESE, ROGER, W) 24 October 2002 (2002-10-24)	63-68
A	figures 2,3	1-62
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4 February 2005

Date of mailing of the international search report

14/02/2005

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Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 00/23458 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 27 April 2000 (2000-04-27) the whole document -----	1-68
A	WO 01/53539 A (PHYLOS, INC) 26 July 2001 (2001-07-26) the whole document -----	1-68

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/DK2004/000630

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20 DEC 2009

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L2 0 SEA ABB=ON PLU=ON (OLOF AND ERICSSON)
L3 0 SEA ABB=ON PLU=ON (OLAF AND ERICSSON)
L4 199 SEA ABB=ON PLU=ON ERICSSON O/AU

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 D 151-205 TI
 D 19,26,33,37,56,91,115,132,138,148,149,165,171,179,197,203 IBI



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CONFIRMATION NO. 8659

SERIAL NUMBER 11/483,825	FILING or 371(c) DATE 07/10/2006 RULE	CLASS 435	GROUP ART UNIT 1637	ATTORNEY DOCKET NO. 22639-3	
APPLICANTS Olof Ericsson, Uppsala, SWEDEN; ** CONTINUING DATA ***** This appln claims benefit of 60/697,415 07/08/2005 ** FOREIGN APPLICATIONS ***** ** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ** 07/28/2006					
Foreign Priority claimed ☐ Yes ☒ No 35 USC 119(a-d) conditions met ☐ Yes ☐ No Verified and /SAMUEL C WOOLWINE/ Acknowledged Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY SWEDEN	SHEETS DRAWINGS 9	TOTAL CLAIMS 43	INDEPENDENT CLAIMS 3
ADDRESS PORTER WRIGHT MORRIS & ARTHUR, LLP INTELLECTUAL PROPERTY GROUP 41 SOUTH HIGH STREET 28TH FLOOR COLUMBUS, OH 43215					
TITLE Regulation analysis by cis reactivity, RACR					
FILING FEE RECEIVED 2150	FEES: Authority has been given in Paper No. _____ to charge/credit DEPOSIT ACCOUNT No. _____ for following:		☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit		

EAST Search History**EAST Search History (Prior Art)**

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
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L2	2	("011438" "785657").ap. and landegren.in.	US-PGPUB	OR	OFF	2009/12/20 15:08
L3	1	"496385".ap. and fredriksson.in.	US-PGPUB	OR	OFF	2009/12/20 15:13
L4	1	"6878515".pn.	USPAT	OR	OFF	2009/12/20 15:14
L7	2	"20010041333"	US-PGPUB	OR	OFF	2009/12/20 15:18
L8	1	"20020004205"	US-PGPUB	OR	OFF	2009/12/20 15:19
L13	1	"004275".ap. and bamdad.in.	US-PGPUB	OR	OFF	2009/12/20 16:10
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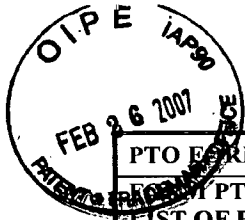
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FORM PTO - 1449

LIST OF PATENTS AND PUBLICATIONS
FOR APPLICANT'S INFORMATION
DISCLOSURE STATEMENT

ATTY DOCKET: 22639-3

APPLICANT: Olof Ericsson

FILING DATE: July 10, 2006

FOR: Regulation Analysis by
Cis Reactivity, RACR

SERIAL NO.:

11/483,825

GROUP: TBD

UNITED STATES PUBLISHED PATENT DOCUMENTS

Exr. Init.	APPL. DOCUMENT NUMBER							PUBL. DATE	NAME	CLASS	SUB CLASS
/S.W./	11	0	1	1	4	3	8	October 20, 2005	Landegren		
	09	7	8	5	6	5	7	May 30, 2002	Landegren et al.		
	10	4	9	6	3	8	5	January 6, 2005	Fredriksson		

UNITED STATES ISSUED PATENT DOCUMENTS

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	6	8	7	8	5	1	5	April 12, 2005	Landegren		

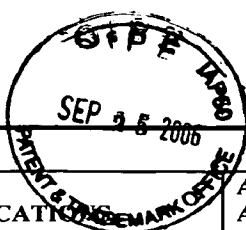
OTHER ART (INCLUDING AUTHOR, TITLE DATE, PERTINENT PAGES, ETC.)

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12/12/2009



PTO FORM 4/92										Page 1 of 1			
FORM PTO - 1449 LIST OF PATENTS AND PUBLICATIONS FOR APPLICANT'S INFORMATION DISCLOSURE STATEMENT										ATTY DOCKET: 22639-3 APPLICANT: Olof Ericsson FILING DATE: July 10, 2006 FOR:		SERIAL NO.: 11/483,825 GROUP: TBD	
UNITED STATES PUBLISHED PATENT DOCUMENTS													
Exr. Init.	APPL. DOCUMENT NUMBER							PUBL. DATE	NAME	CLASS	SUB CLASS		
/S.W./	02	0	5	1	9	8	6	May 2, 2002	Baez et al.				
/S.W./	03	0	8	2	8	3	0	May 1, 2003	Schrieber et al.				
FOREIGN PATENT DOCUMENTS													
	DOCUMENT NUMBER							DATE	COUNTRY	CLASS	SUB CLASS		
OTHER ART (INCLUDING AUTHOR, TITLE DATE, PERTINENT PAGES, ETC.)													
/S.W./	Burbulis, I, et al. "Using protein-DNA Chimeras to detect and count small numbers of molecules. Nat Methods. 2005 Jan; 2(1):31-37 e-pub: 2004 Dec. 21												
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12/12/2009

Methods for the Analysis of DNA-Protein Interactions

*Matthew J. Guille and G. Geoff Kneale**

Abstract

The interaction of proteins with DNA is a central theme of molecular biology. In this article, we review some of the principal techniques currently used for the identification and characterization of DNA binding proteins, and for investigation of the molecular interactions that are responsible for the recognition of specific DNA sequences.

Index Entries: DNA-binding proteins; transcription factors; DNA modification; DNA footprinting.

1. Introduction

Increased understanding of DNA-protein interactions has become a major goal for biologists because it is clear that these interactions play a central role in a wide range of cellular processes. Examples include transcription regulation, DNA replication, genetic recombination, DNA repair, and the restriction and modification of DNA. The types of investigation carried out on DNA-protein interactions fall into two main categories. In the first type, a DNA sequence has been shown to have function, for example, by deletion of a promoter region of a gene, and information is needed about the protein and its interaction with this sequence in order to understand its role in transcription control. In the second type, the protein is already well-characterized and the investigation concentrates on the precise molecular nature of the contacts between the protein and DNA, and the possible conformational changes in either component that might result, often as a prelude to fuller structural studies of the complex.

In this article, we review both of these types of investigation, which have substantial areas of overlap. The techniques described permit the identification of DNA-binding proteins in cell extracts, and give information on the size of the protein(s) involved, the location of the DNA-binding site, the strength and specificity of binding, the identities of

individual groups or specific bases involved in binding, and the effects of protein binding on gross conformation or local structure of DNA.

The recognition of DNA sequences by proteins is a complex phenomenon, involving specific hydrogen bonding contacts to the DNA bases and/or interactions with the sugar-phosphate backbone. The latter interactions can also be highly specific because of the sequence-dependent structural polymorphism of DNA. In addition, intercalation of planar aromatic amino acid side chains between the DNA bases can occur, most notably with single-stranded DNA binding proteins. Furthermore, when bound, many DNA-binding proteins induce drastic structural changes in the DNA that are essential to their function. The advantages of using complementary methods to investigate the complexities of protein-DNA interactions will be clear, as each has advantages and disadvantages for specific tasks. For this reason, it is unwise to rely on a single technique when interpreting the results of such investigations.

2. Identification and Preliminary Characterization of DNA-Binding Proteins

2.1. The Electrophoretic Mobility Shift Assay (EMSA) in Crude Protein Extracts

If initial studies (perhaps from functional or genetic analysis) have suggested that a particular

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DNA sequence is recognized by a protein within the cell, EMSA is often undertaken using this DNA sequence as a probe in crude cell extract. There are a number of reasons for this:

1. To confirm that one or more proteins interact with this region of DNA specifically;
2. To test the distribution of this complex between different cell-types or growth states; and
3. To identify which subcellular compartment the protein is in.

The EMSA (also known as a gel retardation assay, or "gel-shift" assay) is relatively straightforward (1,2). A radioactively labeled DNA duplex, either a short restriction fragment (25–75 bp) or a synthetic oligonucleotide, is incubated with whole-cell, cytoplasmic or nuclear extract in the presence of competitors to prevent nonspecific binding. (Poly[dI.dC], tRNA, and salmon sperm DNA may all be used.) DNA–protein complexes are then allowed to form and the free DNA and complexes are resolved on a low ionic strength, low percentage polyacrylamide gel. The complexes and free DNA are then visualized by autoradiography (Fig. 1). In these assays of crude extracts, a number of complexes are often formed with only a subset specific for the DNA sequence under test. Specificity of binding is assessed by inclusion of an excess of unlabeled competitor DNA in the binding reaction. An excess of unlabeled probe competes for specific complexes, but point-mutant versions of the probe or unrelated DNA sequences do not (Fig. 1).

The simplicity of this assay, its high sensitivity, and the fact that many protein samples may be assayed simultaneously make it the usual method of choice for assaying DNA–protein complexes from a number of cell-types; for example, when testing the tissue distribution of a transcription factor or when a time course of binding activity is needed (3). The small amount of protein needed for EMSA has also made this the method of choice for following the subcellular distribution of DNA-binding proteins (3–5). This is particularly important when analyzing transcription factors, many of which are regulated by cytoplasm to nucleus translocation (reviewed in ref. 6).

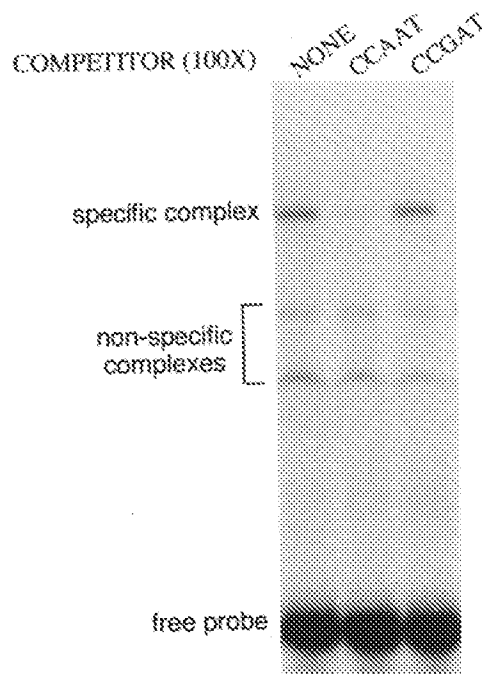


Fig. 1. Electrophoretic mobility shift assay of *Xenopus* CBTF. Extract from 0.5 stage 15 *Xenopus* embryos were incubated at 4°C with an end-labeled DNA probe corresponding to nucleotides –66 to –31 of the *Xenopus* GATA-2 promoter (3). Where added, the competitor was at 100-fold molar excess over the probe and both were added simultaneously. After 15 min the reactions were loaded onto a 4%, 0.25X TBE polyacrylamide gel. The free DNA and DNA–protein complexes were separated by electrophoresis at 200V for 2 h, the gel was dried, and the complexes visualized by autoradiography.

Although EMSA using crude cell extracts as a protein source provides very limited information about the nature of the interaction between the DNA and protein, it does provide a very useful assay for the amount of binding activity in any given sample. It is not possible, however, to be absolutely quantitative using this assay, when nonspecific competitors are included; these will have an affinity for the DNA-binding protein being assayed and necessarily cause errors in the estimation of activity present. Nevertheless, EMSA is an important semi-quantitative assay and is usually chosen to follow DNA-binding protein activity during purification (7–9).

Before embarking on the purification or detailed biochemical characterization of an unknown DNA-binding protein, it is prudent to undertake a preliminary analysis. In such a study, we attempt to discover how many subunits are in the DNA-binding complex and their size. Two basic strategies are used to identify the composition of such complexes from crude cell extracts, both relying on the bound DNA sequence to detect the proteins. A variety of techniques can be used to crosslink proteins covalently to the DNA probe. Alternatively, a blot of protein from a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel may be incubated with a radiolabeled double-stranded oligonucleotide-binding site under conditions where the interaction is specific, and the resulting complex visualized by autoradiography.

2.2. DNA-Protein Photocrosslinking

Covalent crosslinks between protein and DNA have been produced by a variety of methods (10). Although a number of the techniques have relied on photocrosslinking, for example, ultraviolet (UV) laser irradiation of unmodified DNA and UV-irradiation of azido-modified DNA, the technique most commonly applied to analysis of a complex in a crude extract relies on substitution of 5-bromo-2'-deoxyuridine (BrdU) for thymidine in the oligonucleotide binding site and covalent crosslinking by UV irradiation. In our experience, when working with crude or partially purified preparations of protein, it is necessary to use preparative EMSA to obtain sufficiently pure DNA-protein complex for clear results. The BrdU-substituted probe may be produced in two ways. If the exact nucleotides involved in contact with the protein component are not known, then a mixture of substituted probes are produced by "filling in" a partial oligonucleotide duplex with Klenow fragment and a nucleotide triphosphates (NTP) mixture containing dTTP and BrdUTP (typically at a 3:1 molar ratio) and one or more radiolabeled NTPs. If, however, a T in the binding site is known to contact the protein, then an oligonucleotide may be synthesized with the BrdU substitution at this position. The resulting duplex

is then radiolabeled as usual. Either type of probe will work, but the "mixed probe" approach is more likely to reveal proteins in a complex not directly involved in contact with the DNA. However, this type of probe may contain more than one substitution and hence crosslink to two proteins, or more than once to a single polypeptide, causing anomalous migration in subsequent SDS-PAGE analysis.

The DNA-protein complexes are then formed on the substituted probe as usual and separated from free probe and nonspecific complexes by EMSA. The resulting gel is exposed to UV on a transilluminator or crosslinker to link the DNA and protein covalently. The gel is subsequently exposed wet for autoradiography, and the specific complex cut out of the gel and eluted. This DNA-protein complex is then precipitated prior to running on SDS-PAGE. After fixing, the gel is dried and the complex visualized by autoradiography.

The attachment of DNA to a protein may cause it to run anomalously on an SDS gel, although often it has little effect (*see Fig. 2*). With internally labeled probes, it is possible to remove much of the DNA by gentle treatment of the complex with DNase I prior to SDS-PAGE, but this often results in a much reduced signal. Although crosslinking can be criticized for sometimes giving an inaccurate estimate of the molecular weight of the protein(s) in a complex with DNA, it does have the advantage of identifying components of a complex that are not in immediate contact with DNA. This type of information is vital when designing purification strategies for DNA-protein complexes. A similar crosslinking approach has been used to determine which nucleotides in a binding site contact which protein in a multi-subunit transcription factor. In this case, as well as BrdU substitution, BrdC and BrdG were used to replace bases at known DNA-protein contact points (revealed by interference techniques). After crosslinking and SDS-PAGE of each singly substituted oligonucleotide, it was possible to identify which base substitutions resulted in crosslinking to which molecular weight species of the complex (11).

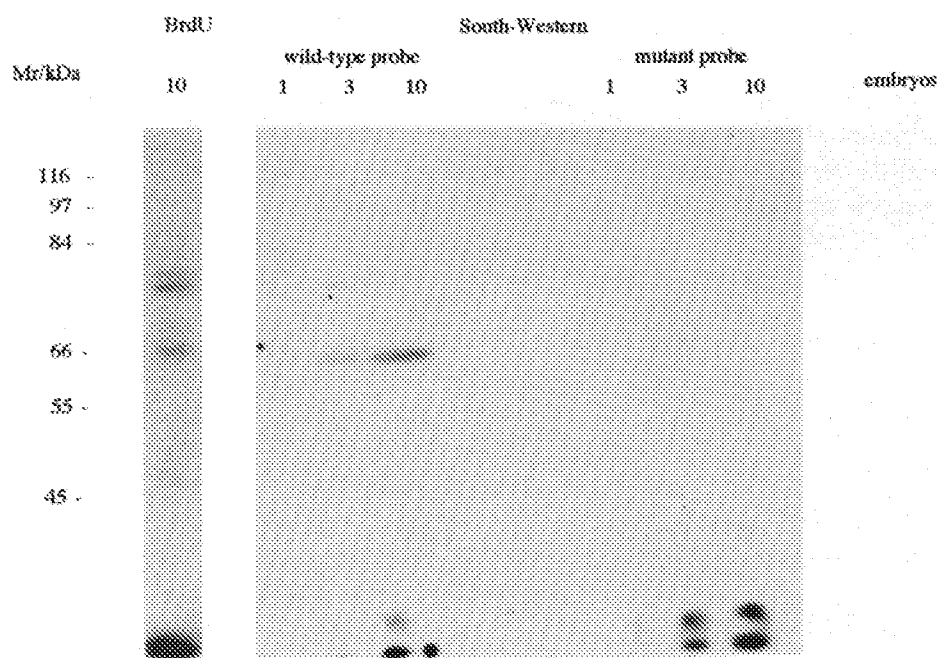


Fig. 2. **(Left)** Bromodeoxyuridine crosslinking of *Xenopus* CBTF. Extract from 10 oocytes was mixed with an internally labeled, BrdU-substituted DNA probe corresponding to –66 to –31 of the *Xenopus* GATA-2 promoter (3). Specific DNA–protein complexes were separated on a 4%, 0.25X TBE gel and irradiated with UV at 256 nm for 20 min. The complexes were visualized by autoradiography, cut from the gel, eluted into SDS-PAGE loading buffer and run on a 10% Laemmli gel. The gel was dried and the protein–DNA conjugate visualized after exposure on a storage phosphor screen. **(Right)** South-Western analysis of *Xenopus* CBTF. Extract from 1, 3, or 10 oocytes was run in duplicate on the same 10% Laemmli gel as the BrdU crosslinked products and transferred to nitrocellulose overnight in 10 mM CAPS, pH 9.8, 10% methanol. The duplicate filters so prepared were immediately blocked and probed. The probe was end-labeled DNA corresponding to nucleotides –66 to –31 of the GATA-2 promoter for the “wild-type” half of the filter and the same sequence but with the core altered from CCAAT to CCGAT for the “mutant” half. This point mutation inactivates the GATA-2 promoter in vivo and abolishes binding of CBTF in EMSA. Washing was for four by 15 min, this extended wash removed all nonspecific binding. The DNA–protein complexes were then visualized by autoradiography.

2.3. South-Western Blotting

A complementary method that gives accurate information about the molecular weight of the DNA-binding protein of a DNA–protein complex (but none on proteins not in direct contact with the DNA) is South-Western blotting (12). To obtain reliable results from the technique, it is necessary to have available as control a point-mutated version of the oligonucleotide probe that does not form the specific complex of interest. This is usually tested by EMSA prior to embarking on a South-Western investigation.

Crude cell protein is first separated by SDS-PAGE and then transferred electrophoretically to a

nitrocellulose membrane. During the transfer process, a fraction of the protein may re-fold sufficiently to bind its cognate DNA. If this does not occur, correct re-folding may be enhanced by treating the filter with Guanidinium-HCl to denature the protein and then re-folding in decreasing concentrations of this agent (13). Duplicate filters are prepared and nonspecific DNA-binding sites blocked by incubation with salmon-sperm DNA, Denhardt’s reagent, and poly(dI.dC). The filters are then incubated with radioactively labeled oligonucleotide probes, either the bona fide binding site or the mutant. Filters are washed to remove unbound or weakly bound probe and exposed for autoradiography. Specific com-

plexes will show as differences between the bona fide and mutant binding sites.

South-Western blotting will fail if a heteromeric protein complex is necessary for DNA binding. Also, if multiple, specific bands are observed, then this technique shows that a family of proteins can recognize a particular DNA sequence. In the example shown, the complex is seen to include at least two proteins by BrdU crosslinking and the smaller of these is demonstrated by a South-Western blot to be the DNA-binding subunit of this transcription factor (**Fig. 2**).

2.4. Functional Analysis of Transcription Factors

Relating a DNA-protein complex visualized by EMSA or footprinting to a particular biological function is a difficult but very necessary undertaking when DNA-protein interactions in less well-characterized systems are being considered. If transcription factors are under study, it may be possible to make use of interference footprinting data to test whether a complex has function. By making point mutations in the DNA-binding site of a complex based on such data, it is possible to generate and test by EMSA a series of binding sites with various abilities of complex formation. The functional analysis of this series of binding sites is then carried out by transfection.

The mutations are either introduced into the promoter/enhancer under test or placed upstream of an heterologous promoter. In either case, these control a reporter gene. The constructs are transfected into the cell type under study and the effect of the mutations of the binding site on transcription assessed. If the ability of a DNA sequence to form complex and its transcription-regulation activity correlate, this is strong evidence that the observed complex is responsible for the activity seen. Such analysis showed that the ISGF3 complex was the most important of three formed on the interferon stimable response element (ISRE) during stimulation of transcription of the 6–16 gene by type I interferons (**14**). Although this is strong evidence for a functional role of a particular protein, it is sometimes possible to obtain more direct evidence.

If purified transcription factor is available in sufficient quantity then supporting evidence to that of the aforementioned type can be gained from adding the factor to an in vitro transcription system and thus assaying its activity directly. A nuclear extract is prepared from cells either lacking the factor under test or from which the factor has been removed by DNA-affinity chromatography (**15,16**). A variety of methods for making transcriptionally active extracts may need to be tried for a particular DNA-protein complex. Into this extract, a DNA template containing the binding site of interest within an appropriate promoter and reporter context is added, together with NTPs. Transcription is then allowed to occur in the presence and absence of the putative transcription factor. Transcripts may be assayed in a number of ways. If a supercoiled template is used, then primer extension (**17**) or RNase mapping (**18**) of the resulting transcript may be used to test both the amount of transcript produced and that the in vivo start of transcription is used. Transcription initiation at cryptic start sites present in plasmids is prevalent in eukaryotic extracts.

Alternatively the “run-off” transcription assay may be employed. Here, the template is cut with a restriction enzyme at a point downstream (usually 100–250 bp) of the transcription start site. One or more of the nucleotide triphosphates added is radioactively labeled; the radioactive transcript synthesized is then of a discrete length as the polymerase “runs off” the end of the template. The amount of transcript and start of transcription may then be assayed directly by running the products on a sequencing-type gel. In practice, the tendency for transcription to start at cryptic initiation sites within plasmids means that both of these types of analysis give a high background of nonspecific signal. This may be overcome by placing a G-free cassette downstream of the promoter sequences under test. This is a stretch of DNA that contains no guanosine residues. The run-off transcription reaction is then carried out in the absence of guanosine triphosphate and, as all other transcripts will rapidly abort when transcribing a G, only those initiating at the correct site will be seen. This method of transcription factor assay is now widely used.

A final method for assaying the activity of a transcription factor relies on isolating the cDNA and the gene encoding it, and then inactivating the gene by insertional mutagenesis (19,20) either in cultured somatic cells, or embryonic stem cells from which mutant embryos lacking a functional transcription-factor gene may later be generated. Subsequent comparison of transcription levels of target genes of the transcription factor in normal and mutant cells will give information on the functional significance of that factor, but any effects observed may be indirect. If animals containing the inactivated gene are made, then phenotypic effects may be noted. Such a strategy, although long-term and involved, is often the only way of analyzing the function of, for example, a developmentally important transcription factor, (21).

In practice, a number of DNA-protein complexes may interact at one binding site (22), possibly controlling gene transcription in response to more than one intracellular signaling pathway. This complexity makes it extremely difficult to be sure, in some cases, of the exact contribution of any single DNA-protein complex to the final transcription rate of a gene and all three of the types of analysis previously outlined may be needed before any firm conclusions may be drawn.

3. Locating the DNA-Binding Site: DNA Footprinting

In order to confirm that a protein is indeed binding to a particular region of DNA and to discover the precise sequence to which it binds, footprinting techniques are most frequently used. The DNA region under investigation is uniquely end-labeled and allowed to interact with a source of protein, which may range from crude nuclear extract to a pure preparation. The DNA-protein complex is then treated with an enzymatic or chemical reagent that partially cleaves the DNA. The bound protein protects the region of the DNA with which it interacts from attack by the reagent. Denaturing electrophoresis and autoradiography of the degraded DNA (in parallel with a DNA sequencing ladder) identifies the protected region as a gap in the continuous bands of the digestion products (Fig. 3). If one is to gain maximum

advantage from the technique, then wherever possible the bands on the sequencing gel should be analyzed quantitatively (e.g., on a phosphor-imager) because densitometry of autoradiographs can give misleading results owing to nonlinearity of X-ray film.

3.1. Enzymatic Footprinting Methods

3.1.1. DNase I Footprinting

DNase I was the first reagent to be used in a DNA footprinting experiment (23). Because DNase I is large when compared to most other footprinting reagents, its attack on the DNA is more readily prevented by steric hindrance. Moreover, the reaction is carried out under mild conditions that are unlikely to perturb the complex. Thus, this reagent is the most likely of all those used for footprinting to detect a specific protein-DNA interaction. For example, the interaction of *Xenopus* upstream binding factor with the ribosomal DNA enhancer can only be detected at present using DNase I as a footprinting reagent (24). Similarly, DNase I footprinting can be used to detect a protein-DNA complex which is undetectable by EMSA and has been used as an alternative to that assay for analysis of proteins in crude cellular extracts.

DNase I does, however, have some disadvantages as a footprinting reagent. It has a diameter of roughly 40Å; its size, therefore, prevents it from cutting immediately adjacent to a bound protein. This means, of course, that the footprint observed will extend beyond the genuine contacts between the DNA and the protein, although it is possible to allow for this effect (25). In addition, DNase I footprinting generally requires an excess of DNA-binding protein over the DNA fragment, to ensure the DNA-binding site is saturated; this may limit the application of this technique where only small amounts of protein are available—for example, in developmental studies when only small numbers of embryos can be obtained. Finally, cleavage of DNA by this enzyme is variable; some sequences are very rapidly digested, while others (principally A or T tracts) are untouched even with prolonged incubation. Thus, an uneven ladder of digestion products is seen after electrophoresis, sometimes limiting the interpretation of results, because protection within a poorly cleaved region may not be readily apparent.

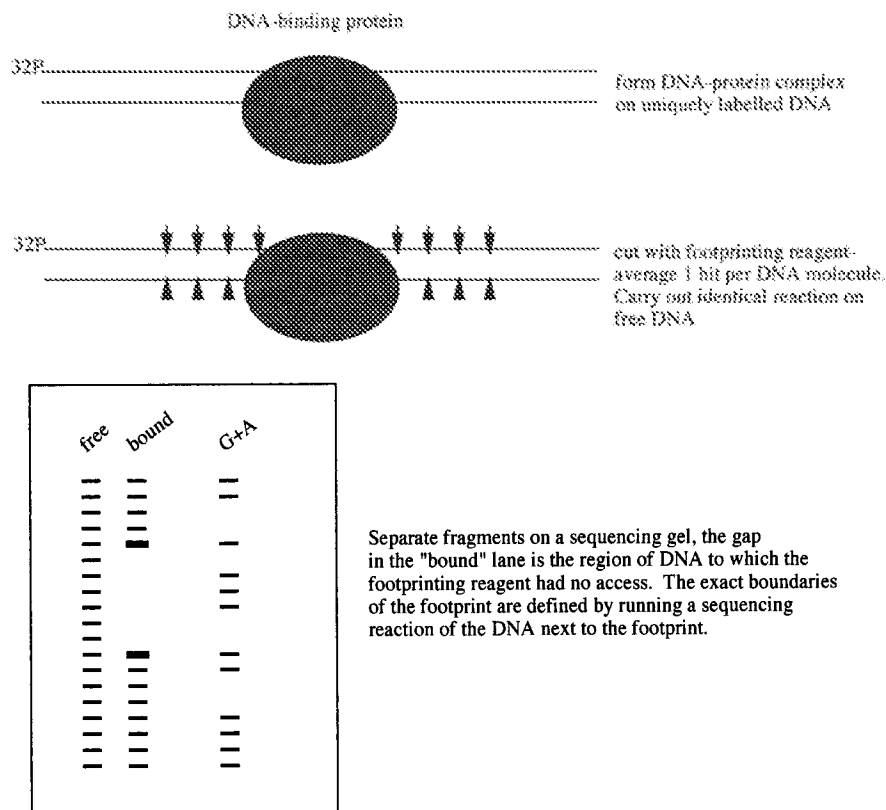


Fig. 3. Schematic diagram of a footprinting reaction.

3.1.2. Exonuclease III (*ExoIII*)

A second enzyme commonly used for footprinting is *ExoIII* which has 3'-5' exonuclease activity (26). After a protein-DNA complex has been formed, this enzyme is used to digest both strands processively. The protein blocks the action of *ExoIII* and a double-stranded DNA region protected by the protein remains. The labeled DNA is then run on a sequencing gel and the size of the remaining fragments corresponds to the ends of the protected region. Because all the free DNA is digested, there is no background ladder of digestion products observed, providing a distinct advantage over other footprinting techniques. Assigning the observed bands to the + or - DNA strands can be achieved either by labeling a unique end of the DNA fragment, or cleavage of the label from one end if a convenient restriction enzyme site is present. The processive mode of action of *ExoIII* allows

it to cut into a region of DNA with low affinity for its cognate-binding protein. This potential disadvantage can be overcome by carrying out a time-course of *ExoIII* digestion whenever a new interaction is being studied. Indeed, the ability of *ExoIII* footprinting to reveal (and distinguish) both strong and weak interactions can be an advantage. As with DNase I, *ExoIII* can be used to detect protein-DNA complexes that are not revealed by EMSA (27). Nevertheless, only interactions with a half-life longer than the application time of the *ExoIII* can be detected by this method (26,28). One further limitation of *ExoIII* footprinting is that it is only able to reveal the protected region at the 3' end of each strand (which need not be equivalent to the 5' boundary on the opposite strand, although it is often assumed to be so). However, for preliminary (low-resolution) studies, this is often sufficient.

3.2. Chemical Footprinting Methods

Chemical footprinting methods all have one clear advantage over enzymatic ones: The small size of the reagents used means that they can cut the DNA very closely indeed to the bound protein, resulting in higher resolution than can be achieved with DNase I or *ExoIII*. The possibility should be borne in mind, however, that chemical probes, unlike enzyme probes, may chemically modify the protein component as well as the DNA component of a complex. The hydroxyl radical is one chemical reagent commonly used, and is particularly useful for high resolution studies because it is comparable in size to a water molecule. The exact mechanism whereby these radicals cleave DNA is unclear, but is thought to involve the abstraction of a hydrogen atom from a sugar in the DNA backbone and the breakage of the strands by secondary reactions of the sugar radical (29). The principle involved is exactly the same as DNase I footprinting discussed earlier. However, unlike DNase I, hydroxyl radicals have almost no sequence preferences for cutting and will work in a wide variety of conditions. One disadvantage of using hydroxyl radicals as a footprinting reagent is when purified protein samples have been stored in glycerol; this is a radical scavenger and inhibits cutting when present at concentrations higher than 0.5%. Other chemical-footprinting reagents include dimethyl sulfate (DMS), a reagent that is fairly specific for purine bases, modifying the N7 of guanine and the N3 of adenine (30).

One problem that is often encountered when attempting to footprint a DNA binding protein is that of low occupancy, i.e., if not all the DNA molecules in the sample have a protein bound, then there will be a large background owing to free DNA, which will reduce the clarity of the footprint. This will often be the case when using crude extracts, but problems can also occur with pure proteins, especially if the complexes are not very stable. One possible solution is to ensure that there is a large excess of the protein compared to the DNA, but this can itself give rise to problems of multiple binding, where the protein binds to additional sites on the DNA in a relatively non-

specific manner. (This becomes more likely as the size of the DNA fragment is increased.) Another approach is to purify the free- and bound-DNA fractions following an EMSA on a nondenaturing gel, before carrying out the footprinting reaction; this does at least ensure that the protein and DNA are present in stoichiometric amounts, and may be particularly useful if working with crude extracts of protein, or if the concentrations are unknown. However, if the complexes are weak or unstable, there is no guarantee that the protein will remain fully bound to the DNA following extraction from the gel.

A novel variation on this theme is to perform the footprinting reaction *in situ* in the nondenaturing gel. This approach has been used very successfully with the footprinting reagent copper phenanthroline (31) and has found application in studying a wide variety of DNA-protein interactions (*see, e.g., ref. 32*). A major advantage of this variation on the standard footprinting procedure is that kinetically weak complexes should be maintained in the gel. Additionally, if multiple complexes are formed, DNA from each can be separately extracted from the gel after footprinting and the protected sites in each complex can be investigated.

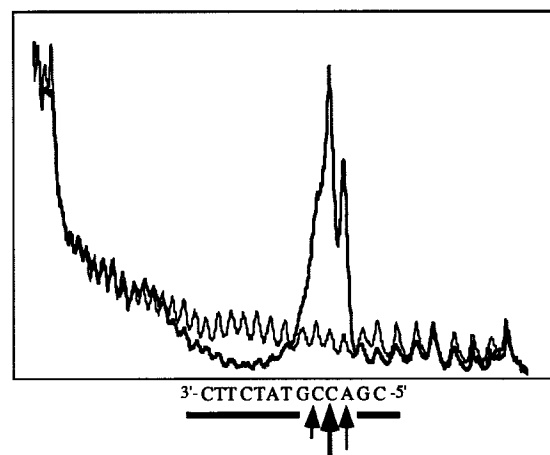
3.3. DNA "Footprinting" as a Probe of Structure

Traditionally, DNA footprinting has been considered to be a relatively straightforward procedure for mapping the protected sites on the DNA, as judged by weak or absent bands in a DNA sequencing ladder where the protein is bound. However, proteins can also distort the structure of DNA when they bind, sometimes quite severely, and in such cases, what might be seen is an increase in the intensity of certain bands, rather than a decrease.

A very clear example of this phenomenon is provided by the hydroxyl radical footprinting of the type I DNA methyltransferase *M.EcoR124I* (33). In this case, binding of the protein to a DNA fragment containing its recognition sequence yields a clear and extensive footprint, amounting to over two turns of the double helix on both strands. Within this footprint, however, is found a region of 2–3 nt that is very highly modified,

Markedly increased intensity of bands in the DNA ladder are often seen with DNase I footprinting, particularly at the 3' boundary of the footprint. Even though the bands are much more intense than in the free DNA track, this is unlikely to be owing to a conformational change in the DNA because the effect is very common, and typically occurs at the edge of the footprint, i.e., outside of the binding site rather than within it. It most likely represents a favorable interaction between the DNase I and the bound protein, which would enhance the cutting frequency at that site. Nevertheless, such an effect is quite helpful in visualizing the precise boundaries of the footprint.

Although the footprinting techniques previously discussed can identify precisely the binding site for protein on a DNA duplex, they all suffer from the drawback that the complex is formed *in vitro*. As a result, cofactors may be missing or the precise ionic conditions found within the cell may not be duplicated and thus the interaction is in some way artifactual. This criticism can be overcome by carrying out DMS modification of DNA *in vivo*, because DMS can penetrate plasma and nuclear membranes (36).



ing-type gel and transferred electrophoretically to a nylon filter. The particular sequences of interest are then visualized by hybridization of the filter to a

radioactively labeled single-stranded DNA probe and subsequent autoradiography. Using this method, it has been possible to identify footprinted regions of promoters that are not seen *in vitro* (36), and this method has proven particularly useful in studying allele-specific transcriptional activity (39).

4. Identification of Specific Bases Involved in Protein Binding

The DNA footprinting procedures previously referred to give information on the region of the DNA that is protected when the protein binds. An alternative (and complementary) approach is to modify the bases of the DNA and assess which base modifications prevent (or reduce) binding. Such methods give a direct measure of the contribution of individual nucleotides to the binding affinity, and can thus identify specific molecular interactions in the complex. Moreover, because often it is only specific groups of the nucleotide that are modified in the DNA (for example, the phosphate group, the sugar, or a specific group on the base), then information is provided on the precise nature of the intermolecular contacts.

4.1. Interference Footprinting

The most common experiments of this kind are represented by the "interference" footprinting techniques, in which the DNA sequence is chemically modified at a low level (typically yielding approximately a single modified nucleotide per DNA fragment). The protein is then bound to the modified DNA, and the mixture subjected to nondenaturing gel electrophoresis to separate free and bound DNA, both of which are then subjected to a strand-cleavage reaction (in which the DNA is cleaved specifically at the sites of modification) and subsequently applied to a sequencing gel. By determining the sites of modification in the free and bound DNA, the contribution of each nucleotide to the binding affinity for the protein will be established. Those nucleotides modified at crucial sites will be under-represented (or absent) in the DNA sequences from the bound fraction, whereas those nucleotides whose modification does not affect protein-DNA complex formation will be well represented in the bound DNA sequences.

The "interference" method may be thought of as the reverse of "protection" footprinting, the

crucial difference being that in the latter case, protein binding is carried out before chemical modification of the DNA, whereas in the former, the DNA is modified first and then "challenged" for protein binding. As in the "protection" footprinting experiments described earlier, the DNA strands must be separately end-labeled with ^{32}P ; chemical modification, DNA binding, and subsequent DNA sequencing should be carried out in parallel reactions with each strand separately labeled, together with a Maxam and Gilbert sequencing reaction on the DNA fragment to enable identification of the sites of modification. Again, if possible, the bands on the sequencing gel should be analyzed on a phosphorimager to obtain reliable data on the relative population of modified DNA molecules in the "free" and "bound" fractions.

The principle of the interference technique is similar, regardless of the reagent used, although the experimental details differ, as does the interpretation of the results. Hydroxyl radical modification, for instance, itself leads to cleavage of the DNA, and thus no separate step is required prior to running the sequencing gel. Most other commonly used reagents (e.g., DMS or ethyl nitrosourea [ENU]), on the other hand, require an additional step to convert the nucleotide modification into a strand cleavage so that the position of the modification can be determined on a sequencing gel (30,40).

Hydroxyl radical modification leads to cleavage via the removal of the entire nucleoside, and thus eliminates all interactions of the protein with the missing base and/or sugar (41,42). Because the reagent is relatively insensitive to base sequence, it has the advantage of being able to probe all nucleotides in a DNA-binding site. However, the missing nucleoside can also change the conformation of the DNA or enhance its flexibility, which can complicate the interpretation of a hydroxyl radical interference experiment. For example, the perturbed DNA structure may actually enhance binding in some cases, as seen in the case of RNA polymerase (43).

More localized contacts of a DNA-binding protein with specific groups on the bases can be probed by interference experiments using the reagent DMS. As stated earlier, DMS methylates

the N7 position of guanine and the N3 of adenine and thus is able to probe interactions in the major and minor grooves of DNA. This does, however, mean that DMS is unable to provide any information on the interaction of a DNA-binding protein with pyrimidine bases in a DNA-binding site. Nevertheless, methylation interference has proved a very useful technique in studying numerous DNA-binding proteins; for example, in the case of the *c-fos* serum-response element binding factors, methylation interference experiments identify a subset of the bases suggested by methylation protection as being essential for binding (44).

Although not generally leading to sequence specificity, the interaction of the phosphate groups on the DNA backbone with a DNA binding protein are often critical in maintaining the high affinity of binding. ENU is widely used to probe such interactions, and can identify critical phosphate groups that are important for DNA binding. Ethylation interference experiments have been used to probe the interaction of the *Escherichia coli* MetJ repressor and the phage-434 repressor with their respective operator sequences (45,46). In both cases, they identified phosphate groups on the DNA that correlated with interactions that could be deduced from the crystal structure of the DNA-protein complexes.

4.2. Base Analogs

The interference methods previously described can be carried out using either a natural (cloned) DNA-binding site, or a synthetic-oligonucleotide duplex bearing the appropriate sequence. It is worth noting that an alternative way to probe the effect of individual base modifications on the DNA-binding affinity of a protein is to incorporate modified bases at specific sites during the synthesis of the two strands that are used to form the duplex. This, naturally, is more time-consuming (and costly!) than the chemical modification procedures previously described, because individual DNA duplexes have to be constructed for each modification to be studied. Moreover, each modified DNA duplex has to be tested individually for DNA binding, usually by a gel-retardation assay (EMSA).

Thus, this type of analysis is generally most useful when a DNA-binding protein has already been characterized in some detail, and the potential base changes to be analyzed are clearly indicated by earlier experiments. The results are generally more clear cut than those obtained by interference "footprinting," and can yield quite accurate values for the relative affinity of the protein for DNA sequences lacking specific groups on a base (or even the entire base), from which the contribution of the relevant interaction(s) to the binding energy can be deduced (47). There are now a large number of base analogs that can be used in this way in which functional groups are deleted (e.g., inosine, purine, 2-amino purine) or modified (e.g., 6-thio G, and various methylated bases such as 5-methyl C, N6-methyl G), as well as the more traditional approach of simply substituting one base by another of the standard bases A, G, C, or T (or indeed, U). If uracil has been incorporated into the sequence, then treatment of the oligonucleotide with the enzyme uracil-N-glycosylase provides a convenient way to remove the entire base from a given site.

4.3. Binding-Site Selection

At the other extreme, it is possible to synthesize an oligonucleotide that is degenerate at a number of sites, i.e., one which contains a random mixture of the four standard bases at certain positions in a given sequence. By selecting those sequences that are preferentially bound by the protein, typically using EMSA, the preferred bases at selected sites in the sequence can be established. Many variations of the basic technique can be found, known by various names such as binding site selection analysis (BSSA) or sequence selection by exponential enrichment (SELEX). Using low-degeneracy oligonucleotide mixtures, and direct chemical sequencing of bound and free DNA following EMSA, Gogos et al. (48) were able to identify preferred bases in the binding site of the CF2 zinc-finger protein from *Drosophila*. In this method, the use of solid-phase sequencing considerably simplifies the procedure (49). Other strategies can be adopted that make use of PCR or DNA cloning after the initial selection step, using a highly degenerate

oligonucleotide mixture initially; multiple rounds of selection can thus be used to amplify small sequence preferences to determine the optimum DNA-binding site (50,51).

5. Quantitative Analysis of Binding

When working with purified and well-characterized DNA-binding proteins, one would usually like to know the equilibrium binding constant that characterizes the interaction of the protein with one or more DNA sequences. Many techniques are available for such studies, the most commonly employed being gel-retardation assays (EMSA) which, as discussed in **Subheading 2.1.**, can also be used more qualitatively to characterize interactions in protein extracts or to aid in the separation of free and bound protein when using many of the techniques referred to already. Spectroscopic methods (principally fluorescence and circular dichroism), and quite unrelated techniques such as surface plasmon resonance, each have advantages for the analysis of binding.

Whichever method is used, it is vital to know the protein concentration fairly accurately. Clearly, the protein must be pure according to SDS-PAGE; it should also have a good UV spectrum with no sign of contaminating nucleic acid. It is surprising how often this is overlooked; not only would contaminating DNA affect the observed binding to the DNA-binding site, but it would also give rise to errors in determination of protein concentration. Assuming the sample is spectroscopically pure, a reasonable estimate of the protein concentration will be obtained if the molar-extinction coefficient of the protein is calculated simply by summing the product of the number and extinction coefficient of each aromatic amino acid at a given wavelength, normally 280 nm.

For many purposes, it is often necessary to know the accurate concentration of the DNA. For defined sequences, this can be done by adding the contribution from each of the bases present in the sequence. However, hyperchromicity should be taken into account and it is advisable to carry out snake-venom phosphodiesterase treatment of the DNA to allow correction for this factor (52) because the absorbance of the DNA will be lower

than the sum of its component bases, even if the DNA duplex is "melted" at high temperature (when some residual hyperchromicity will always remain owing to base stacking).

5.1. EMSA of Purified Proteins

EMSA are widely used to quantify the affinity of a protein for a given DNA sequence. Two rather different approaches can be used, both relying on the titration of a protein of known concentration into a fixed concentration of DNA. Often, the DNA concentration is very low (compared with the K_d for the interaction) and is not accurately known. In this case, relatively high concentrations of protein will be required to achieve significant binding, and the free protein concentration will be close to that of the total protein concentration (53). If so, the analysis is very similar to the Michaelis-Menton analysis for enzyme-substrate reactions (in which it is assumed that the enzyme concentration is negligible compared with the substrate concentration). Similar procedures for fitting the data can be used, and likewise an estimate of the K_d can be obtained from the concentration of protein needed to shift 50% of the DNA (cf. the relationship between K_m and V_{max}).

One potential drawback of this approach, however, is that a 1:1 stoichiometry is assumed (together with the assumption that all the protein is fully active). In order to check this, it is advisable to carry out the gel mobility assay at a higher concentration of DNA (and consequently, of protein). At sufficiently high concentrations ($[DNA] \gg K_d$), binding will be linear with protein concentration until the stoichiometric point is reached in the titration, at which point all the DNA will be bound (**Fig. 5**). If both the DNA and protein concentrations are known, the ratio of the two concentrations at this point gives the stoichiometry, n . Apparent values of $n > 1$ could indicate that more than one protein is binding to the DNA; an alternative explanation is that not all of the protein is active. (One way to attempt to distinguish these possibilities is to repeat the experiment with different preparations of the protein.) It is often apparent when more than

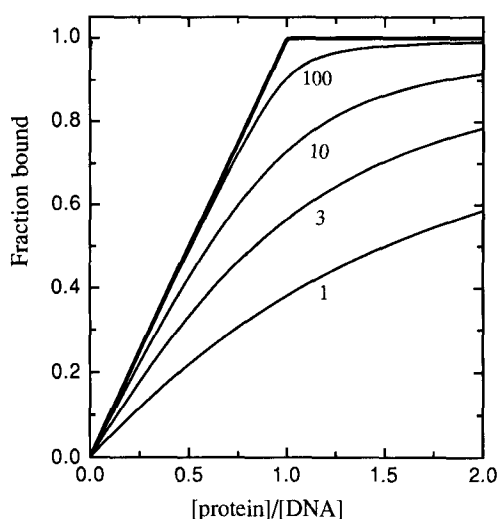


Fig. 5. Theoretical curves for protein binding to a single site on DNA for different (fixed) DNA concentrations. The binding curves for DNA concentrations equal to 1, 3, 10, and 100 times the K_d are shown. The curve is also shown for infinitely tight binding (heavy line).

one protein is binding to the DNA as multiple bands may be seen corresponding to the various complexes that can form (53). However, the lack of such bands does not necessarily indicate a 1:1 stoichiometry; for example, a single band is observed for highly cooperative interactions such as the multiple binding of gene 5 protein to oligonucleotides (54).

At intermediate DNA concentrations, only a fraction (f) of the DNA will be bound at protein concentration (P_0) corresponding to the stoichiometric point (Fig. 5). In this case, it can be shown that $K_d = P_0(1 - f)^2 / f$ and the equilibrium constant can be obtained without any approximations (55). This method does require the concentration of the DNA to be known, and for this reason, is often done with synthetic oligonucleotides, which are prepared in sufficient quantities for spectroscopic determination of concentration after labeling. For the former approach, small quantities of a cloned DNA fragment will suffice.

It is also possible to measure the kinetics of dissociation of a protein-DNA complex by EMSA. To do this, one can form a 1:1 complex, and compete off the protein from the labeled DNA by incubation with an excess of unlabeled DNA for

varying times. The amount of labeled complex should decrease exponentially with time, and a log plot will yield the dissociation rate constant, K_d (as long as the latter is significantly longer than the "dead" time of the experiment).

Competition assays can be used quantitatively to determine the relative binding affinity of a protein for two or more DNA sequences, for example, to compare binding to DNA sequences containing base analogs or point mutations. A stoichiometric (1:1) complex is formed with the canonical DNA sequence at a fixed concentration (C_1). From the concentration (C_2) of unlabeled competitor needed to dissociate 50% of the complex, the ratio of binding affinities for the two sequences can be obtained simply from the formula $K_1/K_2 = [2(C_2/C_1) - 1]$ (see ref. 52).

5.2. Surface Plasmon Resonance

Surface plasmon resonance (SPR) or related techniques such as resonant mirror devices can be employed to study the kinetics of protein-DNA interactions by attaching a DNA probe to the surface of a chip. Usually, the oligonucleotide is biotinylated so that it can be linked to streptavidin on the chip surface. The protein is allowed to flow over the surface of the chip, and the increase in mass due to protein binding measured (usually in arbitrary resonance units, or [RU]) as a function of time. Once bound, the protein can be dissociated from the DNA by flowing buffer over the chip, and the signal will decrease. Thus analysis of the time dependence of these two processes can, in principle, allow the kinetics of the interaction to be investigated, giving the rate constants for association and dissociation (k_{on} and k_{off}). Figure 6 shows one such example.

For complex binding (e.g., if a number of proteins bind cooperatively), analysis is not at all straightforward. Even for simple equilibria, however, where numbers are easy to obtain by fitting the curves to appropriate equations, one must exercise great caution in interpreting the "apparent" values obtained, which can be subject to errors of many different kinds. A number of self-consistency checks are available that should always be used before taking the derived parameters too seriously (56).

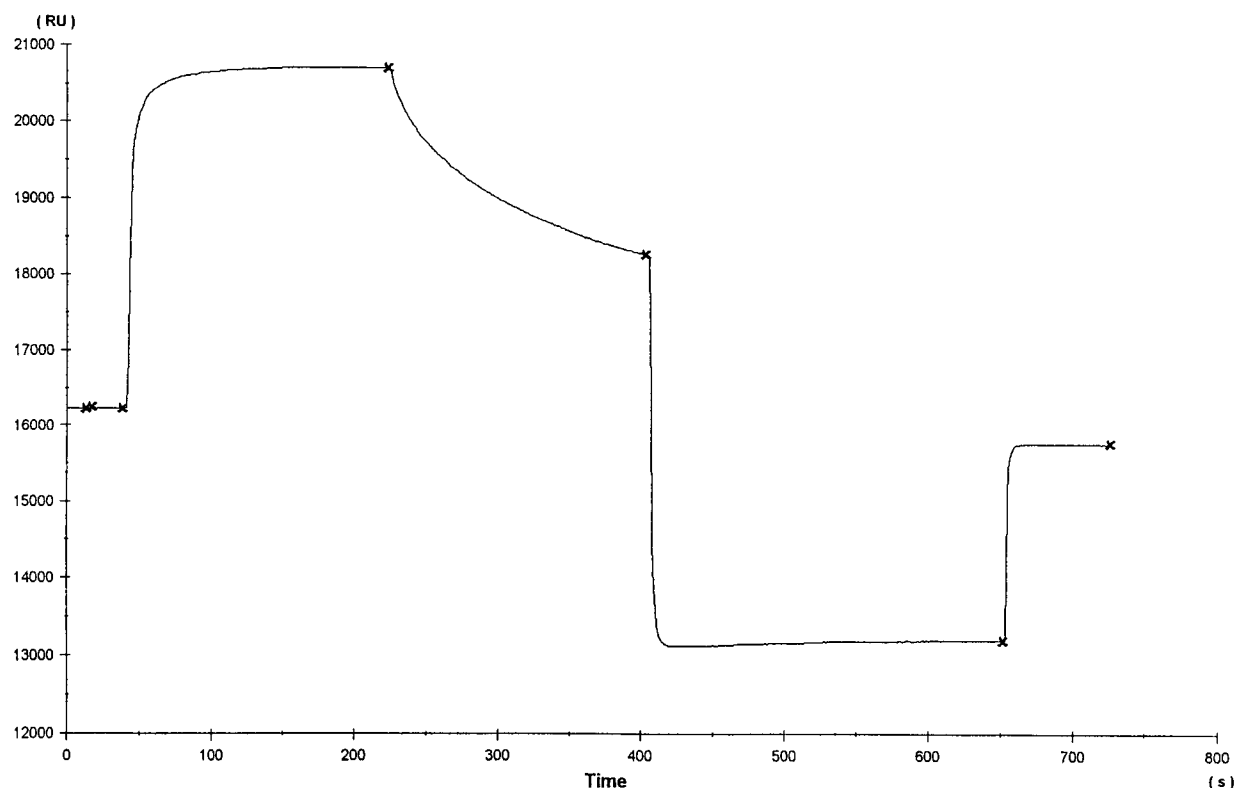


Fig. 6. Analysis of the binding kinetics of the DNA methyltransferase *M.EcoR124* by SPR. The first two phases of the sensorgram show binding of the protein to a 30-bp DNA sequence containing the recognition site, followed by dissociation of the protein from the DNA. Subsequent experiments in which the concentration of protein is varied allow the kinetic constants of the interaction to be obtained. In this example, the curves could be fitted with the values $k_{on} = 2.5 \times 10^5/s/M$ and $k_{off} = 2.6 \times 10^{-3}/s$ in good agreement with the measured equilibrium binding constant of $10^8/M$.

Nevertheless, SPR provides a very useful technique for comparative analysis of DNA binding proteins (e.g., using different DNA-binding protein mutants), and there are many ways in which the technique can be adapted. For example, the DNA-binding protein itself can be immobilized on the surface of the chip, and a variety of DNA sequences can be tested for their affinity to the protein. Although there are a number of techniques available for obtaining equilibrium binding constants, or dissociation-rate constants, SPR provides one of the few methods for the determination of association-rate constants directly.

5.3. Spectroscopic Methods

Virtually all the biophysical techniques that can be applied to proteins can also be applied very effectively to analyze protein–DNA interactions.

Among the most valuable are fluorescence and circular dichroism, because they are relatively straightforward. Nuclear magnetic resonance and X-ray crystallography, of course, are able to provide far more detail if circumstances are favorable, but are much more time consuming and dependent on very expensive instrumentation.

5.3.1. Fluorescence Spectroscopy

Fluorescence can be used in many different ways to study the interaction of a protein with DNA. Most simply, one looks for a quenching or a wavelength shift in the intrinsic protein fluorescence as DNA is titrated in (55), which might arise if a tyrosine or tryptophan were involved in the interaction. The resulting binding curve can be analyzed by the methods described in **Subheading 5.1**. Alternatively, a fluorescent probe

can be attached (for example, to a cysteine at a specific site in the protein). This has the advantages of greater sensitivity (assuming one sees any change at all), but one should use some other methods to check that the probe has not perturbed DNA binding.

An alternative is to use a fluorescence-competition assay; for example, the fluorescent probe ANS binds at the DNA-binding site of the methyltransferase *M.EcoR124I*, and competition with the specific DNA sequence produces a large decrease in signal as the probe is displaced into free solution (57). Fluorescence polarization provides another approach; the increase in molecular weight of the complex, compared to the free protein, will often lead to a decrease in the extent of depolarization of the emitted light, especially in the case of large complexes (58).

5.3.2. Circular Dichroism (CD) Spectroscopy

CD is less sensitive than fluorescence and thus is not particularly useful for deriving binding constants for DNA-protein interactions. It does, however, have a number of important uses in analyzing the structure of DNA-protein complexes (59). The far UV CD (170–250 nm) is sensitive to changes in the secondary structure of proteins and thus any structural changes due to DNA binding can be studied. In the near UV CD (250–300 nm), the signal derives principally from the DNA component, and changes here reveal conformational changes in the DNA when the protein binds. For example, the gene 5 protein produces a large decrease in the peak at 276 nm on binding to single-stranded DNA (58), suggesting a loss of base-stacking interactions owing to intercalation of aromatic side chains of the protein. In contrast, the methyltransferase *M.EcoR124I* shows an increase of over 50% in the CD signal of the DNA (60), which may be related to the large distortion in the DNA-binding site suggested by hydroxyl-radical footprinting of this complex (33).

6. Conclusion

We have aimed to provide a brief overview of a wide variety of techniques that can be usefully employed to study DNA-protein interactions,

from the identification of novel DNA binding proteins in crude cell extracts, to the detailed analysis of the purified proteins and their mode of interaction with specific DNA sequences. Of necessity, we have had to be selective in the methods chosen for inclusion. For the sake of illustration, many of the examples described are taken from studies being undertaken in our own work, but the literature in this area is vast.

Many topics have been left out owing to restrictions of space; for example, many of the traditional techniques of protein chemistry and protein structure analysis are all widely used in the study of DNA-protein interactions; e.g., chemical modification and limited proteolysis (61,62). Overexpression of DNA-binding proteins and their domains, though not covered here, are also increasingly important tools. For details of these and other relevant techniques, the reader is referred to ref. 63.

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Docket No. 3995937-179061

PATENT

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/Holly D. Kozlowski/
Holly D. Kozlowski

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

Applicant: Olof Ericsson : Confirmation No.: 8659
Serial No.: 11/483,825 : Group Art Unit: 1637
Filing Date: July 10, 2006 : Examiner: Samuel C. Woolwine
For: **Regulation Analysis by Cis Reactivity, RACR**

AMENDMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Official Action dated December 30, 2009, please amend the present application as follows:

Amendments to the Claims are set forth in the Listing of Claims which begins on page 2 of this paper.

Remarks begin on page 7 of this paper.

Amendments to the Claims:

The following Listing of Claims replaces all prior versions and listings of the claims in this application.

Listing of the Claims:

1. - 19. (Cancelled).

20. (Previously Presented) A method of detecting functional interactions between at least two molecules of interest, the method comprising:

- a. forming a plurality of interactors by coupling each molecule of interest with at least one nucleic acid moiety, the nucleic acid moiety comprising an identification sequence element and an association element;
- b. forming a plurality of cis-reactive cells wherein a cis-reactive cell comprises at least two interactors bound in proximity to one another by an associated oligonucleotide formed from the association between at least two nucleic acid moieties, wherein the associated oligonucleotide comprises at least two identification elements derived from the at least two nucleic acid moieties;
- c. subjecting the plurality of cis-reactive cells to conditions which stimulate a desired functional interaction having a detectable trace;
- d. selecting all cis-reactive cells exhibiting the detectable trace; and
- e. subjecting the associated oligonucleotides from the cis-reactive cells selected in step (d) to an analysis that permits detection of the at least two identification sequence elements.

21. (Currently Amended) The method of detecting functional interactions between at least two molecules of interest as recited in claim 20, wherein selecting all cis-reactive cells exhibiting

the detectable trace is accomplished via selective separation or selective detection of the cis-reactive cells ~~enhancement~~.

22. (Currently Amended) The method of detecting functional interactions between at least two molecules of interest as recited in claim 21, wherein selecting all cis-reactive cells exhibiting the detectable trace is accomplished by affinity purification.

23. (Original) A method of detecting functional interactions between at least two molecules of interest as recited in claim 20, further comprising enhancing the selected associated oligonucleotides by PCR amplification.

24. (Original) A method of detecting functional interactions between at least two molecules of interest as recited in claim 20, wherein coupling is covalent or noncovalent.

25. (Original) A method of detecting functional interactions between at least two molecules of interest as recited in claim 24, wherein coupling is covalent.

26. (Original) A method of detecting functional interactions between at least two molecules of interest, as recited in claim 20, wherein at least one molecule of interest comprises a protein, a protein domain, a peptide, a peptoid, a functional enzyme, a small molecule, a polymer, a non-proteinaceous cellular product, a virus particle, a cell metabolite, a lipid, a carbohydrate, a nucleic acid, an inorganic compound, a reactive compound, or any combination thereof, however, wherein not all molecules of interest are nucleic acids.

27. (Previously Presented) A method of detecting functional interactions between at least two molecules of interest, as recited in claim 26, wherein at least one molecule of interest comprises at least one protein.

28. (Previously Presented) The method of detecting functional interactions between at least two molecules of interest as recited in claim 27, wherein the protein is an expression product of a nucleic acid molecule, and further wherein at least some of the plurality of interactors are formed by coupling the protein with a nucleic acid moiety comprising an identification sequence element derived from the nucleic acid molecule.

29. (Original) A method of detecting functional interactions between at least two molecules of interest, as recited in claim 26, wherein the at least two molecules of interest comprises at least one protein and at least one nucleic acid.

30. (Original) A method of detecting functional interactions between at least two molecules of interest, as recited in claim 20, wherein detecting further comprises quantifying the functional interactions.

31. (Original) A method of detecting functional interactions between at least two molecules of interest, as recited in claim 20, wherein the analysis comprises high throughput screening.

32. (Original) The method of detecting functional interactions between at least two molecules of interest as recited in claim 31, wherein the high throughput screening comprises a micro array analysis.

33. (Original) The method of detecting functional interactions between at least two molecules of interest as recited in claim 20, wherein the analysis comprises nucleic acid sequencing.

34. (Original) The method of detecting functional interactions between at least two molecules of interest as recited in claim 33, wherein the analysis comprises sequencing by synthesis.

35. (Original) The method of detecting functional interactions between at least two molecules of interest as recited in claim 20, wherein association is via ligation.

36. (Original) The method of detecting functional interactions between at least two molecules of interest as recited in claim 20, wherein association is via polymerization.

37. (Original) The method of detecting functional interactions between the at least two molecules of interest as recited in claim 20, wherein the conditions for stimulating the functional interaction between the at least two molecules of interest comprises manipulating conditions to maximize cis reactivity and to minimize trans reactivity between the at least two molecules of interest.

38. – 43. (Cancelled).

44. (New) The method of detecting functional interactions between at least two molecules of interest as recited in claim 21, wherein the selective detection comprises labeling the cis-reactive cells with an affinity reagent.

45. (New) The method of detecting functional interactions between at least two molecules of interest as recited in claim 21, wherein the selective detection comprises introducing a modified molecule wherein the modification permits the selective detection.

REMARKS

The Official Action dated December 30, 2009 has been carefully considered. Accordingly, the present response is believed sufficient to place this application in condition for allowance. Reconsideration is respectfully requested.

By the present amendment, claims 1-19 and 38-43 are cancelled. Claim 21 is amended to define the selection step in accordance with the teachings in the specification at page 37, and claim 22 is amended for a matter of form. Claims 44 and 45 are added, support for which may be found in the specification, for example, at page 37, first full paragraph. It is believed that these changes do not involve any introduction of new matter, whereby entry is in order and is respectfully requested.

In the Official Action, in both the Summary and at page 11, the Examiner indicated that claims 20-22 and 24-37 are allowed. However, claim 21 was also rejected at page 3 of the Official Action, while no specific rejection of claim 23 was made in the Official Action. Accordingly, it appears that claims 20 and 22-37 should have been indicated as allowable in the Official Action and the Examiner's confirmation in this regard is respectfully requested.

The previous restriction requirement was maintained in the Official Action and claims 38-43 were withdrawn from consideration. The withdrawn claims 38-43 have now been cancelled.

Claims 3 and 21 were rejected under 35 U.S.C. §112, second paragraph, as being indefinite. The Examiner asserted that it is not clear what is encompassed by the term "enhancement".

This rejection is traversed with respect to claim 21, claim 3 having been cancelled, and reconsideration is respectfully requested. More particularly, claim 21 has been amended to omit

reference to enhancement and to recite that selecting all cis-reactive cells exhibiting the detectable trace is accomplished via selective separation or selective detection of the cis-reactive cells. Both selective separation and selective detection are techniques well known in the art, whereby claim 21 is definite and clear to one of ordinary skill in the art. Accordingly, the rejection under 35 U.S.C. §112, second paragraph, has been overcome and reconsideration is respectfully requested.

Claims 1-12, 15-17 and 19 were rejected under 35 U.S.C. §102(a) or 102(e) as being anticipated by the Thisted et al PCT publication WO 2005/026387 A1. Claims 13 and 14 were rejected under 35 U.S.C. §103(a) as being obvious and unpatentable over Thisted et al in view of Guille et al, *Molecular Biotechnology*, 8(1):35-52 (1997), and claim 18 was rejected under 35 U.S.C. §103(a) as being obvious and unpatentable over Thisted et al in view of the Kurn U.S. Patent No. 6,692,918. As claims 1-19 have been cancelled, this rejection is rendered moot.

It is believed that the above represents a complete response to the Official Action and places the application in condition for allowance. Reconsideration and an early allowance are requested. Please charge any fees required in connection with the present communication, or credit any overpayment, to Deposit Account No. 503915.

Respectfully submitted,

/Holly D. Kozlowski/

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/Holly D. Kozlowski/
Holly D. Kozlowski

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

Applicant: Olof Ericsson : Confirmation No.: 8659
Serial No.: 11/483,825 : Group Art Unit: 1637
Filing Date: July 10, 2006 : Examiner: Samuel C. Woolwine

For: **Regulation Analysis by Cis Reactivity, RACR**

REQUEST FOR EXTENSION OF TIME

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Applicant requests a three month extension of time for responding to the Official Action dated December 30, 2009, to thereby extend the deadline for response to the Official Action for three months, from March 30, 2010 to June 30, 2010. Additionally, Applicant claims small entity status in accord with 37 CFR 1.27. Please charge the extension fee of \$555.00 to Deposit Account No. 503915.

Respectfully submitted,

/Holly D. Kozlowski/

Holly D. Kozlowski
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250 East Fifth Street, Suite 2200
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Electronic Patent Application Fee Transmittal

Application Number:	11483825			
Filing Date:	10-Jul-2006			
Title of Invention:	Regulation analysis by cis reactivity, RACR			
First Named Inventor/Applicant Name:	Olof Ericsson			
Filer:	Holly D. Kozlowski			
Attorney Docket Number:	22639-3			
Filed as Small Entity				
Utility under 35 USC 111(a) Filing Fees				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 3 months with \$0 paid	507 2253	1	555	555

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
Total in USD (\$)				555

Electronic Acknowledgement Receipt

EFS ID:	7915766
Application Number:	11483825
International Application Number:	
Confirmation Number:	8659
Title of Invention:	Regulation analysis by cis reactivity, RACR
First Named Inventor/Applicant Name:	Olof Ericsson
Customer Number:	23570
Filer:	Holly D. Kozlowski
Filer Authorized By:	
Attorney Docket Number:	22639-3
Receipt Date:	29-JUN-2010
Filing Date:	10-JUL-2006
Time Stamp:	14:56:10
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	\$555
RAM confirmation Number	13446
Deposit Account	503915
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.09 (Patent application and reexamination processing fees)

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Amendment/Req. Reconsideration-After Non-Final Reject	June-29-2010-Amendment.pdf	51799 065d81c80b9332aa439ea8c6159f5ae5c101266a	no	8
Warnings:					
Information:					
2	Extension of Time	June-29-2010-Extension-of-Time.pdf	18235 9e42e739482a251b456992a6f99d050e6ca00650	no	1
Warnings:					
Information:					
3	Fee Worksheet (PTO-875)	fee-info.pdf	30189 dec04a29b716ec62eb9569b8d90e9731238b4ba5	no	2
Warnings:					
Information:					
Total Files Size (in bytes):			100223		
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> 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. <u>New International Application Filed with the USPTO as a Receiving Office</u> 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.					

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875					Application or Docket Number 11/483,825		Filing Date 07/10/2006		☐ To be Mailed		
APPLICATION AS FILED – PART I											
(Column 1)			(Column 2)		SMALL ENTITY ☐		OR		OTHER THAN SMALL ENTITY		
FOR	NUMBER FILED	NUMBER EXTRA	RATE (\$)	FEE (\$)	RATE (\$)	FEE (\$)					
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A		N/A						
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A		N/A						
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A		N/A						
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X \$	=	OR	X \$	=				
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X \$	=		X \$	=				
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$250 (\$125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).										
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))											
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL					
APPLICATION AS AMENDED – PART II											
(Column 1)		(Column 2)		(Column 3)		SMALL ENTITY		OR		OTHER THAN SMALL ENTITY	
AMENDMENT	06/29/2010	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)	ADDITIONAL FEE (\$)	RATE (\$)	ADDITIONAL FEE (\$)		
	Total (37 CFR 1.16(i))	* 20	Minus	** 43	= 0	X \$	=	OR	X \$52=	0	
	Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0	X \$	=	OR	X \$220=	0	
	☐ Application Size Fee (37 CFR 1.16(s))										
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR			
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0	
(Column 1)		(Column 2)		(Column 3)		SMALL ENTITY		OR		OTHER THAN SMALL ENTITY	
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)	ADDITIONAL FEE (\$)	RATE (\$)	ADDITIONAL FEE (\$)		
	Total (37 CFR 1.16(i))	*	Minus	**	=	X \$	=	OR	X \$	=	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X \$	=	OR	X \$	=	
	☐ Application Size Fee (37 CFR 1.16(s))										
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR			
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE		
* If the entry in column 1 is less than the entry in column 2, write "0" in column 3. ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.											

Legal Instrument Examiner:
/ANNETTE SMITH/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

CERTIFICATE OF EFS ELECTRONIC TRANSMISSION

I hereby certify that this paper is being transmitted to the United States Patent and Trademark Office by the USPTO EFS web electronic filing system on July 8, 2010.

/Holly D. Kozlowski/
Holly D. Kozlowski

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

Applicant: Olof Ericsson : Confirmation No.: 8659
Serial No.: 11/483,825 : Group Art Unit: 1637
Filing Date: July 10, 2006 : Examiner: Samuel C. Woolwine

For: **Regulation Analysis by Cis Reactivity, RACR**

WITHDRAWAL OF CLAIM FOR SMALL ENTITY STATUS

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Applicant withdraws its claim for small entity status under 37 C.F.R. 1.27. Additionally, as the claim of small entity status made in the Extension of Time filed June 29, 2010 was inadvertently made in error, please charge the remainder of the large entity extension fee of \$555.00 to Deposit Account No. 503915. No deceptive intent was involved in the claim of small entity status.

Respectfully submitted,

/Holly D. Kozlowski/

Holly D. Kozlowski
Registration No. 30,468
Porter Wright Morris & Arthur LLP
250 East Fifth Street, Suite 2200
Cincinnati, Ohio 45202
(513) 369-4224

Electronic Patent Application Fee Transmittal

Application Number:	11483825			
Filing Date:	10-Jul-2006			
Title of Invention:	Regulation analysis by cis reactivity, RACR			
First Named Inventor/Applicant Name:	Olof Ericsson			
Filer:	Holly D. Kozlowski			
Attorney Docket Number:	22639-3			
Filed as Large Entity				
Utility under 35 USC 111(a) Filing Fees				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 3 months with \$555 paid	513 1253	1	\$555	\$555

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
Total in USD (\$)				555

Electronic Acknowledgement Receipt

EFS ID:	7972813
Application Number:	11483825
International Application Number:	
Confirmation Number:	8659
Title of Invention:	Regulation analysis by cis reactivity, RACR
First Named Inventor/Applicant Name:	Olof Ericsson
Customer Number:	23570
Filer:	Holly D. Kozlowski
Filer Authorized By:	
Attorney Docket Number:	22639-3
Receipt Date:	08-JUL-2010
Filing Date:	10-JUL-2006
Time Stamp:	10:31:43
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	\$555
RAM confirmation Number	7046
Deposit Account	503915
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 5.15 (Patent application and reexamination processing fees)

Alamar Exhibit 1002

Alamar v. Olink

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	Withdrawal-of-Small-Entity-Status.pdf	17278 6605c054f9065598eb7db67945c57a95d2699339	no	1
Warnings:					
Information:					
2	Fee Worksheet (PTO-875)	fee-info.pdf	30121 942429043457c994f1f166fc89ebf85525d16596	no	2
Warnings:					
Information:					
Total Files Size (in bytes):			47399		
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> 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. <u>New International Application Filed with the USPTO as a Receiving Office</u> 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.					

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 07/09/2010

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NOTICE OF ALLOWANCE AND FEE(S) DUE

23570 7590 09/17/2010

PORTER WRIGHT MORRIS & ARTHUR, LLP
INTELLECTUAL PROPERTY GROUP
41 SOUTH HIGH STREET
28TH FLOOR
COLUMBUS, OH 43215

EXAMINER

WOOLWINE, SAMUEL C

ART UNIT

PAPER NUMBER

1637

DATE MAILED: 09/17/2010

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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11/483,825

07/10/2006

Olof Ericsson

3995937-179061

8659

TITLE OF INVENTION: REGULATION ANALYSIS BY CIS REACTIVITY, RACR

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	\$1510	\$300	\$0	\$1810	12/17/2010

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status above is to be removed, check box 5b on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above, or

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check box 5a on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and 1/2 the ISSUE FEE shown above.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: **Mail** Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

23570 7590 09/17/2010

PORTER WRIGHT MORRIS & ARTHUR, LLP
 INTELLECTUAL PROPERTY GROUP
 41 SOUTH HIGH STREET
 28TH FLOOR
 COLUMBUS, OH 43215

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/483,825	07/10/2006	Olof Ericsson	3995937-179061	8659

TITLE OF INVENTION: REGULATION ANALYSIS BY CIS REACTIVITY, RACR

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	\$1510	\$300	\$0	\$1810	12/17/2010

EXAMINER	ART UNIT	CLASS-SUBCLASS
WOOLWINE, SAMUEL C	1637	435-006000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

- (1) the names of up to 3 registered patent attorneys or agents OR, alternatively, 1 _____
- (2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed. 2 _____
- 3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

- ☐ Issue Fee
- ☐ Publication Fee (No small entity discount permitted)
- ☐ Advance Order - # of Copies _____

4b. Payment of Fee(s); (Please first reapply any previously paid issue fee shown above)

- ☐ A check is enclosed.
- ☐ Payment by credit card. Form PTO-2038 is attached.
- ☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. Change in Entity Status (from status indicated above)

- ☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27. ☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature _____

Date _____

Typed or printed name _____

Registration No. _____

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

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UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/483,825	07/10/2006	Olof Ericsson	3995937-179061	8659
23570	7590	09/17/2010	EXAMINER	
PORTER WRIGHT MORRIS & ARTHUR, LLP INTELLECTUAL PROPERTY GROUP 41 SOUTH HIGH STREET 28TH FLOOR COLUMBUS, OH 43215			WOOLWINE, SAMUEL C	
			ART UNIT	PAPER NUMBER
			1637	
			DATE MAILED: 09/17/2010	

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 569 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 569 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (<http://pair.uspto.gov>).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

Notice of Allowability	Application No.	Applicant(s)	
	11/483,825	ERICSSON, OLOF	
	Examiner	Art Unit	
	SAMUEL C. WOOLWINE	1637	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to amendment filed 06/29/2010.
2. ☒ The allowed claim(s) is/are 20-37,44 and 45.
3. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
 - a) ☐ All b) ☐ Some* c) ☐ None of the:
 1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. ____.
 3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

* Certified copies not received: ____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.

THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

4. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.
5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
 - (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
 - 1) ☐ hereto or 2) ☐ to Paper No./Mail Date ____.
 - (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date ____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

- | | |
|---|---|
| <ol style="list-style-type: none"> 1. ☒ Notice of References Cited (PTO-892) 2. ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948) 3. ☐ Information Disclosure Statements (PTO/SB/08),
Paper No./Mail Date ____ 4. ☐ Examiner's Comment Regarding Requirement for Deposit
of Biological Material | <ol style="list-style-type: none"> 5. ☐ Notice of Informal Patent Application 6. ☐ Interview Summary (PTO-413),
Paper No./Mail Date ____. 7. ☒ Examiner's Amendment/Comment 8. ☒ Examiner's Statement of Reasons for Allowance 9. ☐ Other ____. |
|---|---|

/Samuel Woolwine/
Primary Examiner, Art Unit 1637

ALLOWANCE

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

The application has been amended as follows: in claim 20, replace each step designation "a.", "b.", "c.", etc with "a)", "b)", "c)", etc to conform to MPEP 608.01(m): "Each claim begins with a capital letter and ends with a period. Periods may not be used elsewhere in the claims except for abbreviations. See *Fressola v. Manbeck*, 36 USPQ2d 1211 (D.D.C. 1995)."

The following is an examiner's statement of reasons for allowance: The claims are directed to methods for identifying functional interactions between molecules of interest. The methods involve the formation of "cis-reactive cells", wherein a "cis-reactive cell" comprises at least two "interactors" each comprising a molecule of interest coupled to at least one nucleic acid moiety. The "interactors" within a "cis-reactive cell" are bound in proximity by an "associated oligonucleotide" formed by association of at least two nucleic acid moieties from separate interactors. After the functional interaction between two molecules of interest within a "cis-reactive cell" to produce a detectable trace, the "associated oligonucleotide" of that "cis-reactive cell" is analyzed to identify at least two "identification sequence elements" derived from each of the at least two nucleic acid moieties comprised by the "associated oligonucleotide".

In addition to the closest prior art cited in the Office action mailed 12/30/2009, another body of close prior art is exemplified by Liu et al (US 2009/0203530, priority date June 7, 2005). Liu taught forming polymers by nucleic acid templated synthesis (e.g. figure 2). In some cases, the polymers were selected by binding to a target (the formation of a polymer having target-binding ability could serve as a "detectable trace" of the functional interaction of two molecules, i.e. monomers, that created the polymer, and the selection of polymers that bind to a target could serve as the "selecting" of instant claim 20, step (d)). The templates associated with polymers that bound the target were then subject to analysis (i.e. sequencing) to identify the polymer (figure 2, paragraphs [0029]-[0030]). The polymers were formed by hybridizing transfer units, each comprising a monomeric subunit and an oligonucleotide, on a template (e.g. figure 1, paragraph [0007]). In some cases the template comprised a reactive unit to initiate polymer growth (e.g. figure 3A, paragraph [0032]). However, Liu did not teach or suggest the formation of an "associated oligonucleotide" comprising the nucleic acid moieties attached to two interacting molecules (in Liu's context, "monomeric subunits"), or analyzing such an "associated oligonucleotide". Even if one considers the original nucleic acid template to correspond to the claimed "nucleic acid moiety" of one of the claimed "interactors", this template is not "formed from the association between at least two nucleic acid moieties [of at least two interactors]" and so cannot be considered as the claimed "associated oligonucleotide". Hence, Liu's sequencing of the template does not constitute an analysis of an "associated oligonucleotide" as set forth in the claimed invention.

In addition, to clarify the record, Applicant is correct in the statements made on page 7 of the response filed 06/29/2010: the previous Office action should have indicated claims 20 and 22-37 as allowable. In any event, the amendment of 06/29/2010 places claims 20-37 and new claims 44-45 in condition for allowance.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Any inquiry concerning this communication or earlier communications from the examiner should be directed to SAMUEL C. WOOLWINE whose telephone number is (571)272-1144. The examiner can normally be reached on Mon-Fri 9:00am-5:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on (571) 272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Art Unit: 1637

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Samuel Woolwine/
Primary Examiner
Art Unit 1637

Notice of References Cited	Application/Control No. 11/483,825	Applicant(s)/Patent Under Reexamination ERICSSON, OLOF	
	Examiner SAMUEL C. WOOLWINE	Art Unit 1637	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-2009/0203530	08-2009	Liu et al.	506/1
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

EAST Search History**EAST Search History (Prior Art)**

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	3	"483825".ap.	US-PGPUB	OR	OFF	2010/09/11 13:15
S2	14	(david near3 liu).in. and (templated adj1 synthesis)	US-PGPUB	OR	OFF	2010/09/11 14:09
S3	13	S2 and (binding with target)	US-PGPUB	OR	OFF	2010/09/11 14:10

EAST Search History (I nterference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	0	(functional and interaction and nucleic and "cis- reactive").clm.	USPAT; UPAD	OR	OFF	2010/09/11 15:49
L2	0	(nucleic and "detectable trace").clm.	USPAT; UPAD	OR	OFF	2010/09/11 15:49

9/ 11/ 2010 3:50:46 PM

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	1	"20090011957"	US-PGPUB	OR	OFF	2010/09/11 19:14
L2	1	"20060246450"	US-PGPUB	OR	OFF	2010/09/11 19:16
L7	6	(liu near2 david).in. and template and "transfer unit"	USPAT	OR	OFF	2010/09/11 20:04
L8	6	(liu near2 david).in. and template and "reactive unit"	USPAT	OR	OFF	2010/09/11 20:05
L9	5	(liu near2 david).in. and template and "reactive proximity"	USPAT	OR	OFF	2010/09/11 20:05
L10	14	(liu near2 david).in. and template and "reactive proximity"	US-PGPUB; USPAT	OR	OFF	2010/09/11 20:05
L11	125	(pedersen).in. and template same react\$3	US-PGPUB; USPAT	OR	OFF	2010/09/11 20:06
L12	15	(pedersen).in. and nuevolution.as.	US-PGPUB; USPAT	OR	OFF	2010/09/11 20:06
L13	32436	((detect\$3 identif\$7) near3 (interact\$4 bind\$3)) and proximity	US-PGPUB; USPAT	OR	OFF	2010/09/11 20:08
L14	4092	((detect\$3 identif\$7) near3 (interact\$4 bind\$3)) and (proximity same (oligos oligonucleotides "nucleic acid"))	US-PGPUB; USPAT	OR	OFF	2010/09/11 20:08
L15	2590	((detect\$3 identif\$7) near3 (interact\$4 bind\$3 react \$4)) and (proximity same (oligos oligonucleotides "nucleic acid") same (react \$4 interact\$4 bind\$3))	US-PGPUB; USPAT	OR	OFF	2010/09/11 20:09
L16	2499	l15 and ((interact\$4 bind\$3 react\$4) with (oligos oligonucleotides "nucleic acid"))	US-PGPUB; USPAT	OR	OFF	2010/09/11 20:10

L17	491	l16 and ("2 hybrid" "2-hybrid" "two hybrid" "two-hybrid")	US-PGPUB; USPAT	OR	OFF	2010/09/11 20:12
L18	396	l17 and (@ad<"20050708" @pd<"20050708")	US-PGPUB; USPAT	OR	OFF	2010/09/11 20:13

EAST Search History (I nterference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L3	0	"nucleic acid".clm. and "detectable trace".clm. and identif \$7.clm.	USPAT; UPAD	OR	OFF	2010/09/11 19:59
L4	395	"nucleic acid".clm. and "detectable".clm. and identif\$7.clm.	USPAT; UPAD	OR	OFF	2010/09/11 20:00
L5	46	"nucleic acid".clm. and "detectable signal".clm. and identif \$7.clm.	USPAT; UPAD	OR	OFF	2010/09/11 20:00
L6	21	"nucleic acid".clm. and "detectable signal".clm. and identif \$7.clm. and interact \$3.clm.	USPAT; UPAD	OR	OFF	2010/09/11 20:00

9/ 11/ 2010 9:23:02 PM

Search Notes 	Application/Control No. 11483825	Applicant(s)/Patent Under Reexamination ERICSSON, OLOF
	Examiner SAMUEL C WOOLWINE	Art Unit 1637

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Inventor name and keyword search in EAST, STN, Google (see printouts)	12/20/2009	SCW
Update search in EAST (see printouts)	09/11/2010	SCW

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner
	Text Search in EAST (see printouts)	09/11/2010	SCW

--	--

Holly D. Kozlowski
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porter wright

CINCINNATI
CLEVELAND
COLUMBUS
DAYTON
HAMPSHIRE
WASHINGTON, DC

November 23, 2010

VIA FACSIMILE (571-273-6500)

Director of U.S. Patent and
Trademark Office
Attention: Refund Branch
P.O. Box 1450
Alexandria, VA 22313-1450

Re: U.S. Patent Application Serial No. 11/483,825

To Whom It May Concern:

With regard to the above-referenced patent application, it appears a duplicate charge of \$555 was made to our Deposit Account No. 503915.

1. On June 29, a Request for Extension of Time was filed claiming small entity status. A fee of \$555 was charged to Deposit Account No. 503915, RAM Confirmation No. 13446.

2. On July 8, 2010, a Withdrawal of Claim for Small Entity Status was filed and an additional \$555 was charged to Deposit Account No. 503915, RAM Confirmation No. 7046.

3. On July 9, 2010, an additional \$555 was charged without further authorization and in error to Deposit Account No. 503915.

Copies of the receipts for 1. and 2. and the charge 3. are attached for your reference. We respectfully request a refund of \$555 to be credited to Deposit Account No. 503915.

If you have any questions regarding the above, please don't hesitate to contact us.

Sincerely yours,



Holly D. Kozlowski
Porter Wright Morris & Arthur LLP

HDK:law
Enclosures

CINCINNATI 11/24/2010 9:01

Electronic Patent Application Fee Transmittal

Application Number:

11483825

Filing Date:

10-Jul-2006

Title of Invention:

Regulation analysis by cis reactivity, RACR

Adjustment date: 12/01/2010 EEKURBY1
06/30/2010 INTEFSW 00013446 503915 11483825
01 FC:2453 555.00 CR

First Named Inventor/Applicant Name:

Olof Ericsson

Filer:

Holly D. Kozlowski

Attorney Docket Number:

22639-3

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 3 months with \$0 paid	2253	1	555	555

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
Total in USD (\$)				555

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: **Mail** Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

23570 7590 09/17/2010

PORTER WRIGHT MORRIS & ARTHUR, LLP
 INTELLECTUAL PROPERTY GROUP
 41 SOUTH HIGH STREET
 28TH FLOOR
 COLUMBUS, OH 43215

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/483,825	07/10/2006	Olof Ericsson	3995937-179061	8659

TITLE OF INVENTION: REGULATION ANALYSIS BY CIS REACTIVITY, RACR

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	\$1510	\$300	\$0	\$1810	12/17/2010

EXAMINER	ART UNIT	CLASS-SUBCLASS
WOOLWINE, SAMUEL C	1637	435-006000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. Use of a Customer Number is required.

2. For printing on the patent front page, list
 (1) the names of up to 3 registered patent attorneys or agents OR, alternatively,
 (2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

PORTER, WRIGHT, MORRIS & ARTHUR, LLP

1 _____
 2 _____
 3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

OLINK AB

Uppsala, Sweden

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

- ☒ Issue Fee
☒ Publication Fee (No small entity discount permitted)
☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): (Please first reapply any previously paid issue fee shown above)

- ☐ A check is enclosed.
☐ Payment by credit card. Form PTO-2038 is attached.
☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number 503915 (enclose an extra copy of this form).

5. Change in Entity Status (from status indicated above)

- ☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27. ☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature /HOLLY D. KOZLOWSKI/

Date 12/15/2010

Typed or printed name Holly D. Kozlowski

Registration No. 30468

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Electronic Patent Application Fee Transmittal

Application Number:	11483825			
Filing Date:	10-Jul-2006			
Title of Invention:	REGULATION ANALYSIS BY CIS REACTIVITY, RACR			
First Named Inventor/Applicant Name:	Olof Ericsson			
Filer:	Holly D. Kozlowski/Belinda Reynolds			
Attorney Docket Number:	3995937-179061			
Filed as Large Entity				
Utility under 35 USC 111(a) Filing Fees				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Utility Appl issue fee	1501	1	1510	1510
Publ. Fee- early, voluntary, or normal	1504	1	300	300

Alamar Exhibit 1002

Alamar v. Olink

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				1810

Electronic Acknowledgement Receipt

EFS ID:	9043668
Application Number:	11483825
International Application Number:	
Confirmation Number:	8659
Title of Invention:	REGULATION ANALYSIS BY CIS REACTIVITY, RACR
First Named Inventor/Applicant Name:	Olof Ericsson
Customer Number:	23570
Filer:	Holly D. Kozlowski/Belinda Reynolds
Filer Authorized By:	Holly D. Kozlowski
Attorney Docket Number:	3995937-179061
Receipt Date:	15-DEC-2010
Filing Date:	10-JUL-2006
Time Stamp:	16:28:58
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	\$ 1810
RAM confirmation Number	3549
Deposit Account	503915
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 5.38 (Patent application and reexamination processing fees)

Alamar Exhibit 1002

Alamar v. Olink

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	ptol.pdf	89530	no	1
			21eccfae6df580cdfa64d283bd0cf002b3e1339		

Warnings:

Information:

2	Fee Worksheet (PTO-875)	fee-info.pdf	32305	no	2
			c86d60e2a8c9bb1af273ecb9c7ff01e30cce3779		

Warnings:

Information:

Total Files Size (in bytes):			121835
------------------------------	--	--	--------

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.



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/483,825	02/08/2011	7883848	3995937-179061	8659

23570 7590 01/19/2011
PORTER WRIGHT MORRIS & ARTHUR, LLP
INTELLECTUAL PROPERTY GROUP
41 SOUTH HIGH STREET
28TH FLOOR
COLUMBUS, OH 43215

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (application filed on or after May 29, 2000)

The Patent Term Adjustment is 1029 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (<http://pair.uspto.gov>).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site <http://pair.uspto.gov> for additional applicants):

Olof Ericsson, Uppsala, SWEDEN;

CERTIFICATE OF EFS ELECTRONIC TRANSMISSION

I hereby certify that this paper is being transmitted to the United States Patent and Trademark Office by the USPTO EFS web electronic filing system on September 14, 2011.

/Holly D. Kozlowski/

Holly D. Kozlowski

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

Patent No.: 7,883,848 B2 : Issue Date: February 8, 2011
Applicant: Olof Ericsson : Confirmation No.: 8659
Serial No.: 11/483,825 : Group Art Unit: 1637
Filing Date: July 10, 2006 : Examiner: Samuel C. Woolwine

For: **Regulation Analysis by Cis Reactivity, RACR**

REQUEST FOR CERTIFICATE OF CORRECTION

Attention: Certificate of Correction Branch

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

A Certificate of Correction is requested for the present patent owing to an error occurring during printing of the patent. A review of the Issue Notification shows the error was made during printing. The requested correction is set forth in the attached Form PTO/SB/44. Since this correction is necessitated by a Patent Office printing error, no fee is believed to be required with this Request. However, in the event that a fee is required, please charge the fee to Deposit Account No. 503915.

Respectfully submitted,

/Holly D. Kozlowski/

Holly D. Kozlowski, Reg. No. 30,468
Porter, Wright, Morris & Arthur LLP
250 East Fifth Street, Suite 2200
Cincinnati, Ohio 45202
(513) 369-4224

UNITED STATES PATENT AND TRADEMARK OFFICE CERTIFICATE OF CORRECTION

Page 1 of 1

PATENT NO. : 7,883,848 B2

APPLICATION NO.: 11/483,825

ISSUE DATE : February 8, 2011

INVENTOR(S) : Olof Ericsson

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Claim 19, Column 44, line 13, change "12," to --2,--

MAILING ADDRESS OF SENDER (Please do not use customer number below):

Porter Wright Morris & Arthur LLP
250 East Fifth Street, Suite 2200
Cincinnati, Ohio 45202

This collection of information is required by 37 CFR 1.322, 1.323, and 1.324. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1.0 hour to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Attention Certificate of Corrections Branch, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	10948807
Application Number:	11483825
International Application Number:	
Confirmation Number:	8659
Title of Invention:	REGULATION ANALYSIS BY CIS REACTIVITY, RACR
First Named Inventor/Applicant Name:	Olof Ericsson
Customer Number:	23570
Filer:	Holly D. Kozlowski/Teresa Wilson
Filer Authorized By:	Holly D. Kozlowski
Attorney Docket Number:	3995937-179061
Receipt Date:	14-SEP-2011
Filing Date:	10-JUL-2006
Time Stamp:	16:20:08
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	Request for Certificate of Correction	Request-For-Certificate-Of-Correction.pdf	20091 f4f01822998f3ad3d6698b1296ea055f591e b104	no	1

Warnings:

Information:	544	Alamar Exhibit 1002
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Alamar v. Olink

2	Request for Certificate of Correction	Certificate-Of-Correction.pdf	164239 dbde54cd67820cc6675c8c3107c631518db83d38	no	2
Warnings:					
Information:					
Total Files Size (in bytes):				184330	
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> 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. <u>New International Application Filed with the USPTO as a Receiving Office</u> 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.					

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

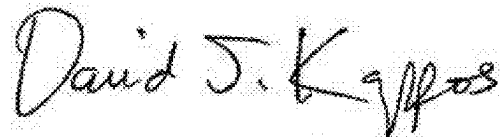
PATENT NO. : 7,883,848 B2
APPLICATION NO. : 11/483825
DATED : February 8, 2011
INVENTOR(S) : Ericsson

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 44, line 13, change "12," to --2,--

Signed and Sealed this
Twenty-second Day of November, 2011

A handwritten signature in black ink, reading "David J. Kappos". The signature is written in a cursive, flowing style with a large initial "D".

David J. Kappos
Director of the United States Patent and Trademark Office