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Sabot et al.(10) **Pub. No.: US 2009/0226975 A1**(43) **Pub. Date: Sep. 10, 2009**(54) **CONSTANT CLUSTER SEEDING****Publication Classification**(75) Inventors: **Andrea Sabot**, Essex (GB);
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(57) **ABSTRACT**

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SAN DIEGO, CA 92121-1975 (US)(73) Assignee: **Illumina, Inc.**, San Diego, CA (US)(21) Appl. No.: **12/395,299**(22) Filed: **Feb. 27, 2009****Related U.S. Application Data**(60) Provisional application No. 61/035,254, filed on Mar.
10, 2008.

The invention provides methods for controlling the density of different molecular species on the surface of a solid support. A first mixture of different molecular species is attached to a solid support under conditions to attach each species at a desired density, thereby producing a derivatized support having attached capture molecules. The derivatized support is treated with a second mixture of different molecular species, wherein different molecular species in the second mixture bind specifically to the different capture molecules attached to the solid support. One or more of the capture molecules can be reversibly modified such that the capture molecules have a different activity before and after the second mixture of molecular species are attached. In particular embodiments, the different molecular species are nucleic acids that are reversibly modified to have different activity in an amplification reaction.

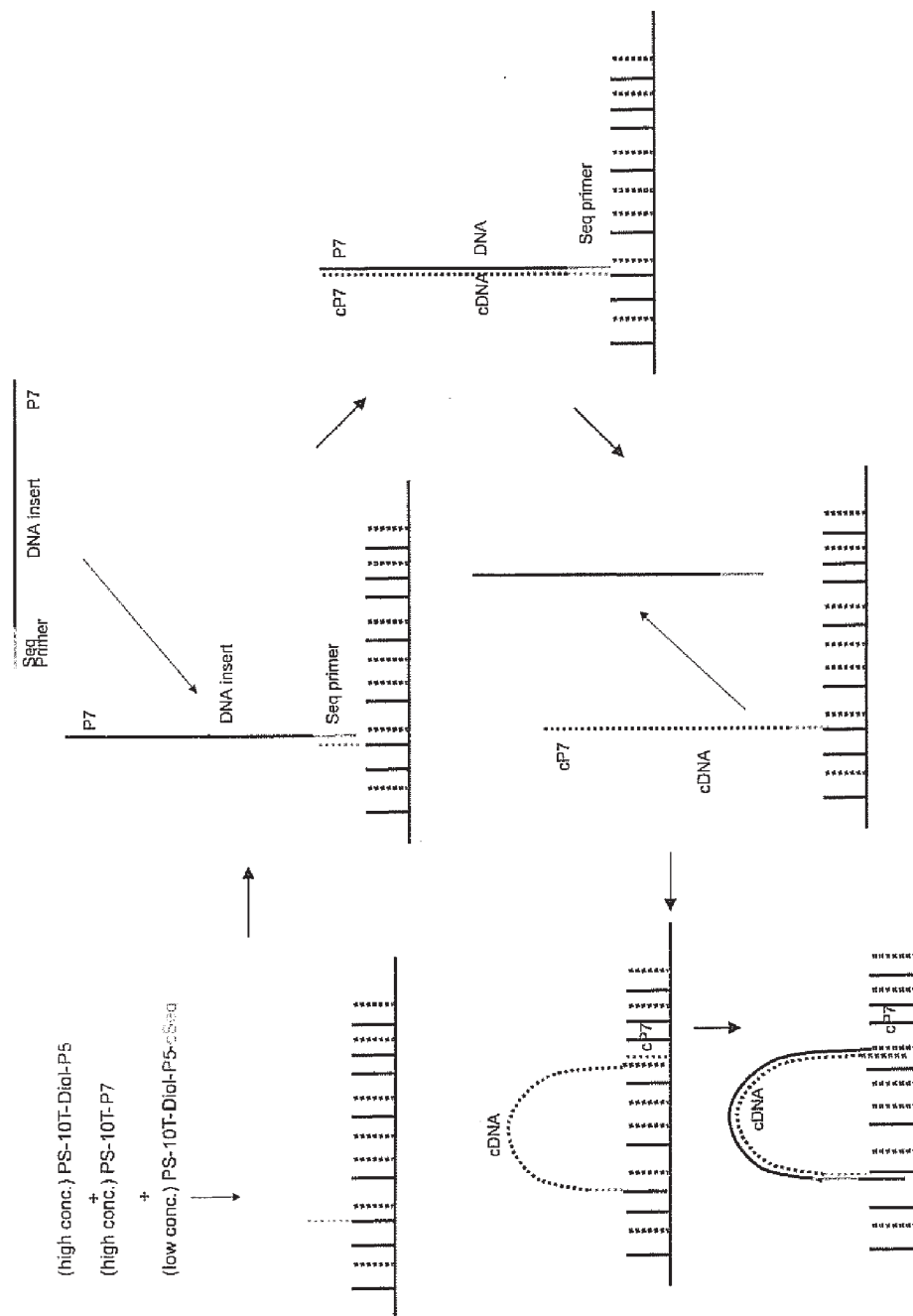


Fig 1

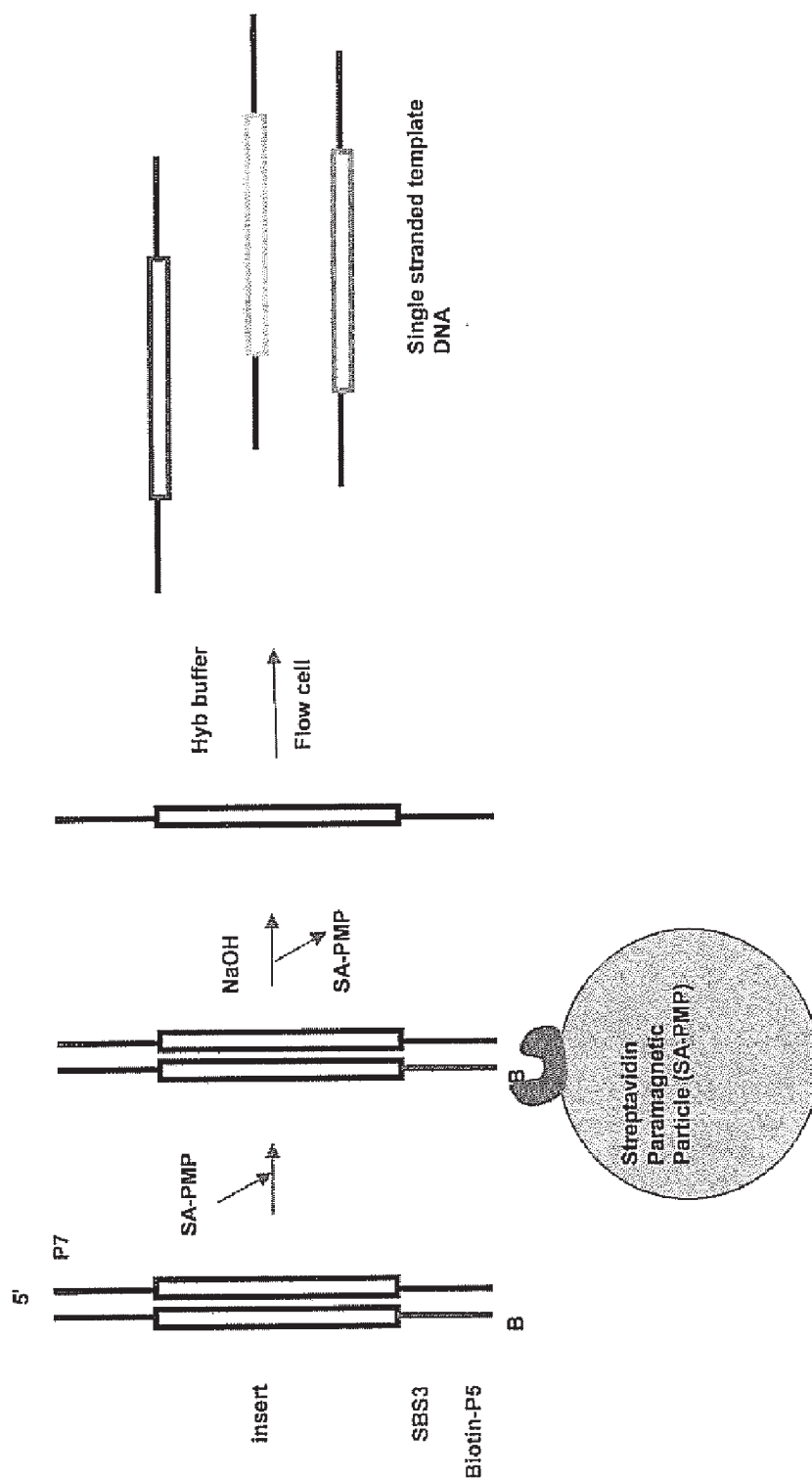


Fig 2

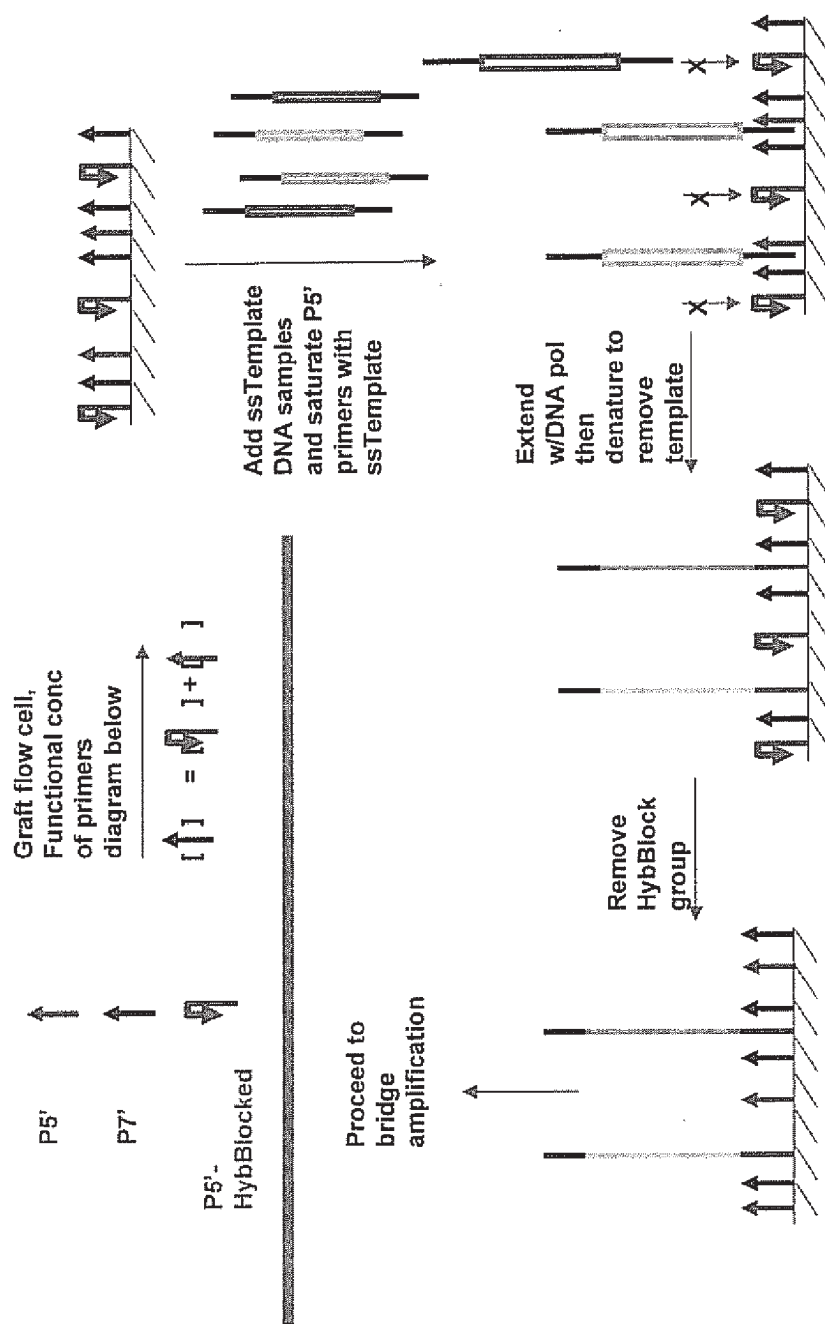


Fig 3

CONSTANT CLUSTER SEEDING

[0001] This application is based on, and claims the benefit of, U.S. Provisional Application Ser. No. 61/035,254, filed Mar. 10, 2008 and entitled "Constant Cluster Seeding," the entirety of which is hereby incorporated by reference.

FIELD OF THE INVENTION

[0002] The current invention relates to the field of nucleic acid amplification. More specifically, the present invention provides methods for optimising the density of nucleic acid clusters produced on a solid support whilst eliminating the need for multiple sample titration steps.

BACKGROUND TO THE INVENTION

[0003] Several publications and patent documents are referenced in this application in order to more fully describe the state of the art to which this invention pertains. The disclosure of each of these publications and documents is incorporated by reference herein.

[0004] A number of methods for high throughput nucleic acid sequencing rely on a universal amplification reaction, whereby a DNA sample is randomly fragmented, then treated such that the ends of the different fragments all contain the same DNA sequence. Fragments with universal ends can then be amplified in a single reaction with a single pair of amplification primers. Separation of the library of fragments to the single molecule level prior to amplification ensures that the amplified molecules form discrete populations that can then be further analysed. Such separations can be performed either in emulsions, or on a surface.

[0005] Polynucleotide arrays have been formed based on 'solid-phase' nucleic acid amplification. For example, a bridging amplification reaction can be used wherein a template immobilised on a solid support is amplified and the amplification products are formed on the solid support in order to form arrays comprised of nucleic acid clusters or 'colonies'. Each cluster or colony on such an array is formed from a plurality of identical immobilised polynucleotide strands and a plurality of identical immobilised complementary polynucleotide strands. The arrays so formed are generally referred to herein as 'clustered arrays.'

[0006] In common with several other amplification techniques, solid-phase bridging amplification uses forward and reverse amplification primers which include 'template specific' nucleotide sequences which are capable of annealing to sequences in the template to be amplified, or the complement thereof, under the conditions of the annealing steps of the amplification reaction. The sequences in the template to which the primers anneal under conditions of the amplification reaction may be referred to herein as 'primer binding' sequences.

[0007] Certain embodiments of clustering methods make use of 'universal' primers to amplify a variable template portion that is to be amplified and that is flanked 5' and 3' by common or 'universal' primer binding sequences. The 'universal' forward and reverse primers include sequences capable of annealing to the 'universal' primer binding sequences in the template construct. The variable template portion, or 'target' may itself be of known, unknown or partially known sequence. This approach has the advantage that it is not necessary to design a specific pair of primers for each

target sequence to be amplified; the same primers can be used for amplification of different templates provided that each template is modified by addition of the same universal primer-binding sequences to its 5' and 3' ends. The variable target sequence can therefore be any DNA fragment of interest. An analogous approach can be used to amplify a mixture of templates (targets with known ends), such as a plurality or library of target nucleic acid molecules (e.g. genomic DNA fragments), using a single pair of universal forward and reverse primers, provided that each template molecule in the mixture is modified by the addition of the same universal primer-binding sequences.

[0008] Such 'universal primer' approaches to PCR amplification, and in particular solid-phase bridging amplification, are advantageous since they enable multiple template molecules of the same or different, known or unknown sequence to be amplified in a single amplification reaction, which may be carried out on a solid support bearing a single pair of 'universal' primers. Simultaneous amplification of a mixture of templates of different sequences can otherwise be carried out with a plurality of primer pairs, each pair being complementary to each unique template in the mixture. The generation of a plurality of primer pairs for each individual template can be cumbersome and expensive for complex mixtures of templates.

[0009] In preparing a clustered array, typically the higher the concentration of template used, the higher the density of clusters that will be produced on a clustered array. If the density of clusters is too great, it may be difficult to individually resolve each cluster and overlapping colonies may be formed. A titration can be performed to determine the optimal template concentration to achieve an optimal cluster density on the array wherein each cluster can be separately resolved. However, such titrations can lead to a loss of valuable flow cell channels due to a cluster density that is too high or too low, a loss of template sample, an increase in the level of reagents required or increase in sample processing time.

[0010] Thus, there is a need for a method of controlling and achieving desired cluster density that is independent of the concentration of the original nucleic acid sample and avoids nucleic acid titration steps. The present invention satisfies this need and provides other advantages as well.

SUMMARY OF THE INVENTION

[0011] The invention provides a method of amplifying polynucleotides. The method can include (a) providing a plurality of oligonucleotides immobilised to a solid support, wherein the plurality of oligonucleotides includes first species having a capture sequence and second species having an amplification sequence, wherein the first species are immobilised at a lower density than the second species; (b) applying a plurality of single stranded template polynucleotides to the solid support under conditions wherein the single stranded template polynucleotide molecules hybridise to the capture sequence but do not hybridise to the amplification sequence; (c) extending the first species to generate polynucleotide complements of the single stranded polynucleotide template molecules, wherein the polynucleotide complements are immobilised on the solid support; and (d) amplifying the polynucleotide complements, wherein the amplifying includes hybridizing the amplification sequences to the complements.

[0012] In a particular aspect, the invention provides a method of controlling the density of colonies of amplified

single stranded polynucleotides formed on a solid support. The method can include the steps of (a) providing a plurality of single stranded template polynucleotides; (b) providing a plurality of at least three oligonucleotides immobilised to a solid support wherein at least one of the oligonucleotides is a capture sequence capable of hybridising to the single stranded template polynucleotides, and at least two of the oligonucleotides are amplification sequences which are incapable of hybridising to the single stranded template polynucleotides, wherein the capture sequences are immobilised at a lower density than the amplification sequences; (c) applying the single stranded template polynucleotides to the solid support under suitable conditions such that the single stranded template polynucleotide molecules hybridise to the capture sequences; (d) extending the capture sequences using a nucleic acid polymerase to generate double stranded extension products complementary to the single stranded template polynucleotides; (e) denaturing the double stranded extension products to remove the hybridised single stranded polynucleotide template molecules from the extension products to produce single stranded template molecules immobilised on the solid support; and (f) amplifying the single stranded template molecules immobilised on the solid support using the two or more amplification sequences immobilised on the solid support; wherein the density of the immobilised colonies is controlled by the density of the capture primers rather than the concentration of the single stranded template polynucleotides.

[0013] The invention further provides a flow cell uniformly grafted with a plurality of oligonucleotides, wherein the plurality includes three species of oligonucleotides having different sequences, wherein one of the three species is present at a lower density than the other two species.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] FIG. 1 shows a method of the invention wherein the capture sequence is longer than the amplification sequences, and the template selectively hybridises to the capture sequence that extends beyond the amplification sequence. The capture sequence is extended opposite the template strand, and the template strand is denatured and removed. The immobilised template copy can hybridise to one of the immobilised amplification sequences, and the amplification sequence can be extended. The capture sequence also comprises a sequence corresponding to one of the amplification sequences, and hence upon synthesising a duplex from the immobilised template copy, both ends of the immobilised duplex can comprise sequences complementary to one of the amplification sequences.

[0015] FIG. 2 shows an exemplary method of preparing a single stranded template library suitable for amplification.

[0016] FIG. 3 shows an exemplary method of the invention wherein one of the amplification primers is initially blocked from strand elongation. After extending the immobilised template strand, the block is removed and the sample can proceed through cycles of bridge amplification.

DETAILED DESCRIPTION OF THE INVENTION

[0017] The invention relates to methods for controlling the density of different molecular species derivatized on a surface. In particular embodiments, the molecular species are nucleic acids having different sequences. The invention is particularly useful for controlling the density of nucleic acid

clusters produced on a solid support. An advantage of the methods is the reduction or even elimination of the need for multiple sample titration steps for controlling density of molecules on surfaces.

[0018] In embodiments wherein surfaces are derivatized with nucleic acids for subsequent formation of amplified clusters, the density of the cluster on the support is controlled by the density of one of the immobilised primers used for capturing the template samples. The density of primers on every chip can be controlled during manufacturing, simply by the ratio of the three or more immobilised primers, and hence the density of clusters is independent of the concentration or dilution of the template sample. This concentration independence removes the need to accurately measure the initial concentration of double stranded template, and is independent of the accurate dilution of the sample. The density of clusters on multiple chips can be made substantially uniform by controlling the ratio and concentration of capture sequences to amplification sequences attached to the chip surface. Because primers can typically be synthesized and manipulated under more controlled conditions than template samples that are derived from different biological sources, the methods set forth herein provide increased reproducibility in creating cluster arrays. Further advantages are provided by creating pools of primers in a desired ratio that can be reused for creating multiple cluster arrays having reproducible density.

[0019] In accordance with the methods set forth herein a plurality of oligonucleotides can be immobilised to a solid support. The plurality can include different species of oligonucleotide molecule each having a different sequence. For example, a plurality of oligonucleotides can include at least two different species, at least three different species or more, wherein a first species has a different sequence than the other species in the plurality. It will be understood that different species of oligonucleotide can share a common sequence so long as there is a sequence difference between at least a portion of the different species. For example, as shown in FIG. 3, the two species identified as P5' and P5'HybBlocked share a common sequence but the P5'HybBlocked species has an additional hairpin forming sequence not found in the P5' species.

[0020] The term 'immobilised' as used herein is intended to encompass direct or indirect attachment to a solid support via covalent or non-covalent bond(s). In certain embodiments of the invention, covalent attachment may be used, but generally all that is required is that the molecules (for example, nucleic acids) remain immobilised or attached to a support under conditions in which it is intended to use the support, for example in applications requiring nucleic acid amplification and/or sequencing. Typically oligonucleotides are immobilized such that a 3' end is available for enzymatic extension and at least a portion of the sequence is capable of hybridizing to a complementary sequence. Immobilization can occur via hybridization to a surface attached oligonucleotide. Alternatively, immobilization can occur by means other than base-pairing hybridization, such as the covalent attachment set forth above.

[0021] The term 'solid support' as used herein refers to any insoluble substrate or matrix to which molecules can be attached, such as for example latex beads, dextran beads, polystyrene surfaces, polypropylene surfaces, polyacrylamide gel, gold surfaces, glass surfaces and silicon wafers. The solid support may be a planar glass surface. The solid support

may be mounted on the interior of a flow cell to allow the interaction with solutions of various reagents.

[0022] In certain embodiments the solid support may comprise an inert substrate or matrix which has been 'functionalised', for example by the application of a layer or coating of an intermediate material comprising reactive groups that permit covalent attachment to molecules such as polynucleotides. By way of non-limiting example such supports may include polyacrylamide hydrogel layers on an inert substrate such as glass. In such embodiments the molecules (for example, polynucleotides) may be directly covalently attached to the intermediate layer (for example, a hydrogel) but the intermediate layer may itself be non-covalently attached to other layers of the substrate or matrix (for example, a glass substrate). Covalent attachment to a solid support is to be interpreted accordingly as encompassing this type of arrangement.

[0023] 'Primer oligonucleotides' or 'amplification sequences' are polynucleotide sequences that are capable of annealing specifically to a single stranded polynucleotide sequence to be amplified under conditions encountered in a primer annealing step of an amplification reaction. Generally, the terms "nucleic acid," "polynucleotide" and "oligonucleotide" are used interchangeably herein. The different terms are not intended to denote any particular difference in size, sequence, or other property unless specifically indicated otherwise. For clarity of description the terms may be used to distinguish one species of molecule from another when describing a particular method or composition that includes several molecular species.

[0024] A polynucleotide sequence that is to be amplified is generally referred to herein as a "template." A template can include primer binding sites that flank a template sequence that is to be amplified. In particular embodiments, as set forth in further detail below, a plurality of template polynucleotides includes different species that differ in their template sequences but have primer binding sites that are the same for two or more of the different species. The two primer binding sites that flank a particular template sequence can have the same sequence, such as a palindromic sequence or homopolymeric sequence, or the two primer binding sites can have different sequences. Accordingly, a plurality of different template polynucleotides can have the same primer binding sequence or two different primer binding sequences at each end of the template sequence. Thus, species in a plurality of template polynucleotides can include regions of known sequence that flank regions of unknown sequence that are to be evaluated, for example, by sequencing.

[0025] Generally amplification reactions use at least two amplification primers, often denoted 'forward' and 'reverse' primers. Generally amplification sequences are single stranded polynucleotide structures. They may also contain a mixture of natural or non-natural bases and also natural and non-natural backbone linkages, provided, at least in some embodiments, that any non-natural modifications do not permanently or irreversibly preclude function as a primer—that being defined as the ability to anneal to a template polynucleotide strand during conditions of an extension or amplification reaction and to act as an initiation point for the synthesis of a new polynucleotide strand complementary to the annealed template strand. That being said, in certain embodiments the present invention may involve the use of a subset of primers, either forward or reverse, that have been modified to preclude hybridisation to a template polynucleotide strand,

the modification being altered or reversed at some point such that hybridisation is no longer precluded.

[0026] Primers may additionally comprise non-nucleotide chemical modifications, for example to facilitate covalent attachment of the primer to a solid support. Certain chemical modifications may themselves improve the function of the molecule as a primer or may provide some other useful functionality, such as providing a cleavage site that enables the primer (or an extended polynucleotide strand derived therefrom) to be cleaved from a solid support. Useful chemical modifications can also provide reversible modifications that prevent hybridisation or extension of the primer until the modification is removed or reversed. Similarly, other molecules attached to a surface in accordance with the invention can include cleavable linker moieties and/or reversible modifications that alter a particular chemical activity or function of the molecule.

[0027] A plurality of oligonucleotides used in the methods set forth herein can include species that function as capture oligonucleotides. The capture oligonucleotides may include a 'template specific portion', namely a sequence of nucleotides capable of annealing to a primer binding sequence in a single stranded polynucleotide molecule of interest such as one that is to be amplified. The primer binding sequences will generally be of known sequence and will therefore be complementary to a region of known sequence of the single stranded polynucleotide molecule. The capture oligonucleotides may include a capture sequence and an amplification sequence. For example, as shown in FIG. 1, a capture oligonucleotide may be of greater length than amplification primers that are attached to the same substrate, in which case the 5' end of the capture sequences may comprise a region with the same sequence as one of the amplification primers. A portion of a template, such as the 3' end of the template, may be complementary to the 3' of the capture sequences. The 5' end of the template may contain a region that comprises a sequence identical to one of the amplification primers such that upon copying the template, the copy can hybridise to the immobilised amplification primer. Thus, an oligonucleotide species that is useful in the methods set forth herein can have a capture sequence, an amplification sequence or both. Conversely, an oligonucleotide species can lack a capture sequence, an amplification sequence or both. In this way the hybridization specificity of an oligonucleotide species can be tailored for a particular application of the methods.

[0028] The length of primer binding sequences need not be the same as those of known sequences of polynucleotide template molecules and may be shorter, being particularly 16-50 nucleotides, more particularly 16-40 nucleotides and yet more particularly 20-30 nucleotides in length. The desired length of the primer oligonucleotides will depend upon a number of factors. However, the primers are typically long (complex) enough so that the likelihood of annealing to sequences other than the primer binding sequence is very low. Accordingly, known sequences that flank a template sequence can include a primer binding portion and other portions such as a capture sequence, tag sequence or combination thereof.

[0029] 'Solid phase amplification,' when used in reference to nucleic acids, refers to any nucleic acid amplification reaction carried out on or in association with a solid support. Typically, all or a portion of the amplified products are synthesised by extension of an immobilised primer. In particular the term encompasses solid phase amplification reactions

analogous to standard solution phase amplifications except that at least one of the amplification primers is immobilised on the solid support.

[0030] As will be appreciated by the skilled reader, a given nucleic acid amplification reaction can be carried out with at least one type of forward primer and at least one type of reverse primer specific for the template to be amplified. However, in certain embodiments, the forward and reverse primers may include template specific portions of identical sequence. In other words, it is possible to carry out solid phase amplification using only one type of primer and such single primer methods are encompassed within the scope of the invention. The one type of primer may include (a) subset(s) of modified primer(s) that have been modified to preclude hybridisation to a template polynucleotide strand, the modification being removed, altered or reversed at some point such that hybridisation is no longer precluded. Other embodiments may use forward and reverse primers which contain identical template specific sequences but which differ in some structural features. For example, one type of primer may contain a non-nucleotide modification which is not present in the other. In still yet another embodiment, the template specific sequences are different and only one primer is used in a method of linear amplification. In other embodiments of the invention the forward and reverse primers may contain specific portions of different sequence.

[0031] In certain embodiments of the invention, amplification primers for solid phase amplification are immobilised by covalent attachment to the solid support at or near the 5' end of the primer, such that a portion of the primer is free to anneal to its cognate template and the 3' hydroxyl group is free to function in primer extension. Again, in certain embodiments there is provided a subset of modified primers that are prevented from hybridisation and/or extension until the modification is removed, reversed or altered. In particular embodiments, the amplification primers will be incapable of hybridisation to the initial single stranded template. In such embodiments, hybridisation of the single stranded template will typically be specific for the capture sequences such that the amount of capture sequences on the surface determines the amount of template captured and thus the density of the resulting amplified clusters.

[0032] The chosen attachment chemistry will typically depend on the nature of the solid support and any functionalization or derivatization applied to it. In the case of nucleic acid embodiments, the primer itself may include a moiety which may be a non-nucleotide chemical modification to facilitate attachment. For example, the primer may include a sulphur containing nucleophile such as a phosphorothioate or thiophosphate at the 5' end. In the case of solid supported polyacrylamide hydrogels, this nucleophile may bind to a bromoacetamide group present in the hydrogel. In one embodiment, the means of attaching primers to the solid support is via St phosphorothioate attachment to a hydrogel comprised of polymerised acrylamide and N-(5-bromoacetamidylpentyl) acrylamide (BRAPA).

[0033] A uniform, homogeneously distributed 'lawn' of immobilised oligonucleotides may be formed by coupling (grafting) a solution of oligonucleotide species onto the solid support. The solution can contain a homogenous population of oligonucleotides but will typically contain a mixture of different oligonucleotide species. The mixture can include, for example, at least two, three or more different species of oligonucleotide. Each surface that is exposed to the solution

therefore reacts with the solution to create a uniform density of immobilised sequences over the whole of the exposed solid support. As such, a portion of the surface having a mixture of different immobilized sequences can be surrounded by an area of the surface having a mixture of the same immobilized sequences. A suitable density of amplification oligonucleotides is at least 1 fmol/mm² (6×10^{10} per cm²), or more optimally at least 10 fmol/mm² (6×10^{11} per cm²). The density of the capture oligonucleotides can be controlled to give an optimum cluster density of 10^6 - 10^9 clusters per cm². The ratio of capture oligonucleotide species to the amplification oligonucleotide species can be any desired value including, but not limited to at least 1:100, 1:1000 or 1:100000 depending on the desired cluster density and brightness. Similar densities or ratios of other molecular species can be used in embodiments where molecules other than nucleic acids are attached to a surface.

[0034] Previously, the density of attached single stranded polynucleotide molecules and hence the density of clusters has been controlled by altering the concentration of template polynucleotide molecules applied to a support. It has now been discovered that by utilising a modified primer or capture sequence, the density of clusters on the amplified array can be controlled without relying on careful titration of the starting concentration of template polynucleotide strand applied to the solid support. This has the significant advantage that the methods need not rely on accurate concentration measurements and dilutions of the template polynucleotide molecules, thereby leading to increased reliability, reduction in dilution errors and a reduction in time and quantity of reagents required in downstream processes. For each solid support that contains too many or too few clusters, there is a reduction in the amount of data generated for an analysis of the clusters. This can mean that to generate the required depth of coverage of the sample may require additional analytical runs that would not be required if the cluster density was optimal. Too many clusters gives optical saturation and an increase in overlap between two amplified molecules; too few clusters gives undesirably high amounts of dark space that do not generate any data, thereby wasting reagents that are more efficiently used with a densely populated surface.

[0035] In a particular embodiment, for each cluster, an immobilised complementary copy of a single stranded polynucleotide template molecule is attached to the solid support by a method of hybridisation and primer extension. Methods of hybridisation for formation of stable duplexes between complementary sequences by way of Watson-Crick base-pairing are known in the art. The immobilised capture oligonucleotides can include a region of sequence that is complementary to a region or template specific portion of the single stranded template polynucleotide molecule. An extension reaction may then be carried out wherein the capture sequence is extended by sequential addition of nucleotides to generate a complementary copy of the single stranded polynucleotide sequence attached to the solid support via the capture oligonucleotide. The single stranded polynucleotide sequence not immobilised to the support may be separated from the complementary sequence under denaturing conditions and removed, for example by washing.

[0036] The terms 'separate' and 'separating,' when used in reference to strands of a nucleic acid, refer to the physical dissociation of the DNA bases that interact within for example, a Watson-Crick DNA-duplex of the single stranded polynucleotide sequence and its complement. The terms also

refer to the physical separation of these strands. Thus, the term can refer to the process of creating a situation wherein annealing of another primer oligonucleotide or polynucleotide sequence to one of the strands of a duplex becomes possible. After the first extension reaction, the duplex is immobilised through a single 5' attachment, and hence strand separation can result in loss of one of the strands from the surface. In cases where both strands of the duplex are immobilised, separation of the strands means that the duplex is converted into two immobilised single strands.

[0037] In one aspect of the invention, one or more of the amplification primers can be modified to prevent hybridisation of a region or template specific portion of the single stranded polynucleotide molecule. Alternatively or additionally, one or more of the amplification primers may be modified to prevent extension of the primer during one or more extension reactions, thus preventing copying of the hybridised templates. These modifications can be temporary or permanent.

[0038] Generally, the capture sequences will include a region of the same sequence as the plurality of amplification oligonucleotides. Once the 3' end of the extended immobilised template copy has hybridised to one of the amplification primers and been extended, the resulting duplex will be immobilised at both ends and all of the bases in the capture oligonucleotide sequence will have been copied. Thus the capture oligonucleotide may include both the amplification primer sequence, plus a further sequence that is complementary to the end of the template. Typically the sequence complementary to the end of the template will not be present in any of the amplification primers. Alternatively, the amplification primers can contain the sequences complementary to the ends of the single stranded templates, but the amplification primers can be reversibly blocked to prevent hybridisation and/or extension during one or more extension step, such as a first extension step in a particular amplification process.

[0039] According to one aspect of the invention, one or more of the amplification primers may include a modification that acts as a reversible block to either template hybridisation or extension or both. By way of non-limiting example, such modifications can be presence of an additional sequence of nucleotides that is complementary to the amplification primer. This additional sequence can be present in a portion of the amplification primer and thus acts as an intramolecular hairpin duplex, or a 3' blocking group that prevents extension of the primer. Alternatively, the additional sequence can be found on a separate oligonucleotide that hybridizes to the amplification primer. A particular feature of such a modification is that it can be removed, altered or reversed such that the functionality of the modified primer oligonucleotide is restored and the primer is able to undergo hybridisation and extension during later steps of the methods. Among other examples, the blocking group may be a small chemical species such as a 3' phosphate moiety that can be removed enzymatically, may be an abasic nucleotide such that the 3' end of the primer is not capable of hybridisation (and thereby extension), or may be a sequence of nucleotides that can be selectively excised from the immobilised strands, for example, using restriction endonucleases that selectively cleave particular sequences or deglycosylases that selectively cleave oligonucleotides having exogenous bases such as uracil deoxyribonucleotides or 8-oxoguanine.

[0040] In one embodiment a plurality of three types of oligonucleotides (for example comprising capture sequences,

forward and reverse primers) are immobilised to a solid support. Alternatively the three oligonucleotides may be forward amplification, blocked forward amplification and reverse amplification, where the unblocked forward primer acts as the capture sequence.

[0041] The single stranded polynucleotide molecules may have originated in single-stranded form, as DNA or RNA or may have originated in double-stranded DNA (dsDNA) form (e.g. genomic DNA fragments, PCR and amplification products and the like). Thus a single stranded polynucleotide may be the sense or antisense strand of a polynucleotide duplex. Methods of preparation of single stranded polynucleotide molecules suitable for use in the method of the invention using standard techniques are well known in the art. The precise sequence of the primary polynucleotide molecules may be known or unknown during different steps of the methods set forth herein. It will be understood that a double stranded polynucleotide molecule can be hybridized to an immobilized capture oligonucleotide as exemplified herein for single stranded polynucleotide molecules, so long as a single stranded region of the double stranded polynucleotide is available and complementary to the capture oligonucleotide sequence.

[0042] An exemplary method for the isolation of one strand of a double stranded molecular construct is shown in FIG. 2. A sample of unknown sequence may be fragmented, and adapters attached to the ends of each fragment. One strand of the adapters may contain a moiety for surface immobilisation, for example a biotin that can be captured onto a streptavidin surface. The adapters may be mismatch adapters, for example as described in copending application US 2007/0128G24, the contents of which are incorporated herein by reference in their entirety. Amplification of the mismatch or forked adapters using a pair of amplification primers, one of which carries a biotin modification means that one strand of each duplex carries a biotin modification. Immobilisation of the strands onto a streptavidin surface means that the non-biotinylated strand can be eluted simply by denaturation/strand separation. The eluted constructs will be in single stranded form and upon exposure to hybridisation conditions can be used to hybridise against the immobilised capture sequences which can be extended.

[0043] In a particular embodiment, the single stranded polynucleotide molecules are DNA molecules. More particularly, the single stranded polynucleotide molecules represent genomic DNA molecules, or amplicons thereof, which include both intron and exon sequence (coding sequence), as well as non-coding regulatory sequences such as promoter and enhancer sequences. Still yet more particularly, the single stranded polynucleotide molecules are human genomic DNA molecules, or amplicons thereof.

[0044] In a particular embodiment, a single stranded target polynucleotide molecule has two regions of known sequence. Yet more particularly, the regions of known sequence will be at the 5' and 3' termini of the single stranded polynucleotide molecule such that the single stranded polynucleotide molecule will be of the structure:

5'[known sequence I]-[target polynucleotide sequence]-[known sequence II]-3'

[0045] Typically "known sequence I" and "known sequence II" will consist of more than 20, or more than 40, or more than 50, or more than 100, or more than 300 consecutive nucleotides. The precise length of the two sequences may or may not be identical. The primer binding sequences generally

will be of known sequence and will therefore particularly be complementary to a sequence within known sequence I and known sequence II of the single stranded polynucleotide molecule. The length of the primer binding sequences need not be the same as those of known sequence I or II, and may be shorter, being particularly 16-50 nucleotides, more particularly 16-40 nucleotides and yet more particularly 20-30 nucleotides in length. Known sequence I can be the same as known sequence II or the two can be different.

[0046] Methods of hybridisation for formation of stable duplexes between complementary sequences by way of Watson-Crick base pairing are known in the art. A region or part of the single stranded polynucleotide template molecules can be complementary to at least a part of the immobilised capture sequence oligonucleotides. Since the amplification oligonucleotides are either modified to prevent hybridisation and/or extension, or are non-complementary to the known ends of the template strands, only the capture sequences will be capable of hybridisation and extension. An extension reaction may then be carried out wherein the capture sequence primer is extended by sequential addition of nucleotides to generate a complementary copy of the single stranded template polynucleotide attached to the solid support via the capture sequence oligonucleotide. The single stranded template polynucleotide sequence not immobilised to the support may be separated from the complementary sequence under denaturing conditions and removed, for example by washing. The distance between the individual capture sequence oligonucleotides on the surface therefore controls the density of the single stranded template polynucleotides and hence the density of clusters formed later on the surface is also controlled.

[0047] In embodiments such as that shown in FIG. 3 wherein the modified forward primer oligonucleotides are blocked and are unable to be extended, generally all of the amplification primer oligonucleotides will hybridise to the single stranded template polynucleotides. When the extension reaction is carried out only the unmodified forward capture primer oligonucleotides are extended by sequential addition of nucleotides to generate a complementary copy of the single stranded template polynucleotide attached to the solid support via the unmodified forward primer oligonucleotide. The single stranded template polynucleotide sequences not hybridised to the support may be separated from the unextended blocked forward primer oligonucleotides under denaturing conditions and removed, for example by washing with a chemical denaturant such as formamide. The distance between the individual unmodified forward primer oligonucleotides on the surface therefore controls the density of the single stranded template polynucleotides and hence the density of clusters formed later on the surface is also controlled.

[0048] Following the attachment of the complementary single stranded template polynucleotides, the modified/blocked primers can be treated to reverse, remove or alter the modification such that they become functionally equivalent to the unmodified forward primer oligonucleotides. For example, the double stranded structure may be removed either by denaturation, for example by heating or treatment with an alkaline solution when it is formed by a separate hybridised polynucleotide. Alternatively, where the hybridised polynucleotide is covalently linked, enzymatic digestion could be used to sequence-selectively cleave the strand, followed by denaturation. Such methods for removing the double stranded structure are known in the art and would be apparent to the skilled person (Sambrook and Russell,

Molecular Cloning, A Laboratory Manual, third edition, Cold Spring Harbor Laboratory Press (2001)).

[0049] In one embodiment of the invention, the single stranded template polynucleotide molecule can be attached to the solid support by ligation to double stranded primers immobilised to the solid support using ligation methods known in the art (Sambrook and Russell, *supra*). Such methods utilise ligase enzymes such as DNA ligase to effect or catalyse the joining of the ends of the two polynucleotide strands, in this case, the single stranded template polynucleotide molecule and the primer oligonucleotide ligate such that covalent linkages are formed. In this context "joining" means covalent linkage of two polynucleotide strands that were not previously covalently linked. Thus, an aim of the invention can also be achieved by modifying the 3' end of a subset of primer oligonucleotides such that they are unable to ligate to the single stranded template polynucleotides. By way of non-limiting example, the addition of 2'3'dideoxy AMP (dideoxyAMP) by the enzyme terminal deoxynucleotidyl transferase (TdT) effectively prevents T4 DNA ligase from ligating treated molecules together.

[0050] An alternative method would be to have the capture sequences as duplex strands and the amplification sequences as single strands. Upon ligation of the single strands to the capture duplexes (which would be the only immobilised species carrying a free 5' phosphate) the 3' end of the immobilised strand can be extended as described above. Upon denaturation of the hybridised template sequence, amplification of the immobilised strand can proceed as described. Other such methods for attaching single strands will be apparent to others skilled in the art.

[0051] In a next step according to particular embodiments of the present invention, suitable conditions are applied to the immobilised single stranded polynucleotide molecule and the plurality of amplification primer oligonucleotides such that the single stranded polynucleotide molecule hybridises to an amplification primer oligonucleotide to form a complex in the form of a bridge structure. Suitable conditions such as neutralising and/or hybridising buffers are well known in the art (See Sambrook et al., *supra*; Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Md. (1998)). The neutralising and/or hybridising buffer may then be removed.

[0052] Next by applying suitable conditions for extension an extension reaction is performed. The primer oligonucleotide of the complex is extended by sequential addition of nucleotides to generate an extension product complementary to the single stranded polynucleotide molecule. The resulting duplex is immobilised at both 5' ends such that each strand is immobilised.

[0053] Suitable conditions such as extension buffers/solutions comprising an enzyme with polymerase activity are well known in the art (See Sambrook et al., *supra*; Ausubel et al. *supra*). In a particular embodiment dNTP's may be included in the extension buffer. In a further embodiment dNTP's could be added prior to the extension buffer. This bridge amplification technique can be carried out as described, for example, in U.S. Pat. No. 7,115,400 and US 2005/0100900 A1, the contents of which are incorporated herein by reference.

[0054] Examples of enzymes with polymerase activity which can be used in the present invention are DNA polymerase (Klenow fragment, T4 DNA polymerase), heat-stable DNA polymerases from a variety of thermostable bacteria

(such as Taq, VENT, Pfu, or Tfi DNA polymerases) as well as their genetically modified derivatives (TaqGold, VENTexo, or Pfu exo). A combination of RNA polymerase and reverse transcriptase can also be used to generate the extension products. Particularly the enzyme has strand displacement activity, more particularly the enzyme will be active at a pH of about 7 to about 9, particularly pH 7.9 to pH 8+, yet more particularly the enzymes are Est or Klenow.

[0055] The nucleoside triphosphate molecules used are typically deoxyribonucleotide triphosphates, for example dATP, dTTP, dCTP, dGTP, or are ribonucleoside triphosphates for example ATP, UTP, CTP, GTP. The nucleoside triphosphate molecules may be naturally or non-naturally occurring.

[0056] After the hybridisation and extension steps, the support and attached nucleic acids can be subjected to denaturation conditions. A flow cell can be used such that, the extension buffer is generally removed by the influx of the denaturing buffer. Suitable denaturing buffers are well known in the art (See Sambrook et al., supra; Ausubel et al. supra). By way of example it is known that alterations in pH and low ionic strength solutions can denature nucleic acids at substantially isothermal temperatures. Formamide and urea form new hydrogen bonds with the bases of nucleic acids disrupting hydrogen bonds that lead to Watson-Crick base pairing. In a particular embodiment the concentration of formamide is 50% or more. These result in single stranded nucleic acid molecules. If desired, the strands may be separated by treatment with a solution of very low salt (for example less than 0.01 M cationic conditions) and high pH (>12) or by using a chaotropic salt (e.g. guanidinium hydrochloride). In a particular embodiment a strong base is used. A strong base is a basic chemical compound that is able to deprotonate very weak acids in an acid base reaction. The strength of a base is indicated by its pK_b value, compounds with a pK_b value of less than about 1 are called strong bases and are well known to one skilled in the art. In a particular embodiment the strong base is Sodium Hydroxide (NaOH) solution used at a concentration of from 0.05 M to 0.25 M, particularly 0.1 M.

[0057] Following the hybridization, extension and denaturation steps exemplified above, two immobilised nucleic acids will be present, the first being the first template single stranded polynucleotide molecule (that was initially immobilised) and the second being a nucleic acid complementary thereto, extending from one of the immobilised primer oligonucleotides. Both the original immobilised single stranded polynucleotide molecule and the immobilised extended primer oligonucleotide formed are then able to initiate further rounds of amplification by subjecting the support to further cycles of hybridisation, extension and denaturation.

[0058] It may be advantageous to perform optional washing steps in between each step of the amplification method. For example an extension buffer without polymerase enzyme with or without dNTP's could be applied to the solid support before being removed and replaced with the full extension buffer.

[0059] Such further rounds of amplification can be used to produce a nucleic acid colony or "cluster" comprising multiple immobilised copies of the single stranded polynucleotide sequence and its complementary sequence.

[0060] The initial immobilisation of the single stranded polynucleotide molecule means that the single stranded polynucleotide molecule can hybridise with primer oligonucleotides located at a distance within the total length of the single

stranded polynucleotide molecule. Other surface bound primers that are out of reach will not hybridize to the polynucleotide. Thus the boundary of the nucleic acid colony or cluster formed is limited to a relatively local area surrounding the location in which the initial single stranded polynucleotide molecule was immobilised.

[0061] Once more copies of the single stranded polynucleotide molecule and its complement have been synthesised by carrying out further rounds of amplification, i.e. further rounds of hybridisation, extension and denaturation, then the boundary of the nucleic acid colony or cluster being generated will be able to be extended further, although the boundary of the colony formed is still limited to a relatively local area around the location in which the initial single stranded polynucleotide molecule was immobilised. For example the size of each amplified cluster may be 0.5-5 microns.

[0062] It can thus be seen that the method of the present invention allows the generation of a plurality of nucleic acid colonies from multiple single immobilised single stranded polynucleotide molecules and that the density of these colonies can be controlled by altering the proportions of modified capture/amplification oligonucleotides used to graft the surface of the solid support.

[0063] In one embodiment, the hybridisation, extension and denaturation steps are all carried out at the same, substantially isothermal temperature. For example the temperature is from 37° C. to about 75° C., particularly from 50° C. to 70° C., yet more particularly from 60° C. to 65° C. In a particular embodiment the substantially isothermal temperature may be the optimal temperature for the desired polymerase.

[0064] In a particular aspect, the method according to the first aspect of the invention is used to prepare clustered arrays of nucleic acid colonies, analogous to those described in U.S. Pat. No. 7,115,400, US 2005/0100900 A1, WO 00/18957 and WO 98/44151 (the contents of which are herein incorporated by reference), by solid-phase amplification.

[0065] In yet another aspect more than one capture sequences and more than two amplification sequences, for example, at least three or four or more, different amplification primer sequences may be grafted to the solid support. In this manner more than one library, with common sequences which differ between the libraries, could be utilised to prepare clusters, such as, for example libraries prepared from two different patients. Whilst the cluster may overlap in space, they would be able to be sequenced one after the other due to the differences between the ends of the templates. For example, two different samples can be captured using two different capture sequences. These can be amplified from the same two amplification primers. The samples can be differentiated due to the two different capture sequences, which can be used as the sites for hybridisation of two different sequencing primers. The use of different capture sequences thereby gives rise to a method of sample indexing using different sequencing primers.

[0066] Clustered arrays formed by the methods of the invention are suitable for use in applications usually carried out on ordered arrays such as micro-arrays. Such applications by way of non-limiting example include hybridisation analysis, gene expression analysis, protein binding analysis, sequencing, genotyping, nucleic acid methylation analysis and the like. The clustered array may be sequenced before

being used for downstream applications such as, for example, hybridisation with fluorescent RNA or binding studies using fluorescent labelled proteins.

Sequencing Methods

[0067] The invention also encompasses methods of sequencing amplified nucleic acids generated by solid-phase amplification. Thus, the invention provides a method of nucleic acid sequencing comprising amplifying a pool of nucleic acid templates using solid-phase amplification as described above and carrying out a nucleic acid sequencing reaction to determine the sequence of the whole or a part of at least one amplified nucleic acid strand produced in the solid-phase amplification reaction.

[0068] Sequencing can be carried out using any suitable sequencing technique. A particularly useful method is one wherein nucleotides are added successively to a free 3' hydroxyl group, resulting in synthesis of a polynucleotide chain in the 5' to 3' direction. The nature of the nucleotide added may be determined after each nucleotide addition or at the end of the sequencing process. Sequencing techniques using sequencing by ligation, wherein not every contiguous base is sequenced, and techniques such as massively parallel signature sequencing (MPSS) where bases are removed from, rather than added to the strands on the surface are also within the scope of the invention.

[0069] The initiation point for the sequencing reaction may be provided by annealing of a sequencing primer to a product of the solid-phase amplification reaction. In this connection, one or both of the adaptors added during formation of the template library may include a nucleotide sequence which permits annealing of a sequencing primer to amplified products derived by whole genome or solid-phase amplification of the template library.

[0070] The products of solid-phase amplification reactions wherein both forward and reverse amplification primers are covalently immobilised on the solid surface are so-called 'bridged' structures formed by annealing of pairs of immobilised polynucleotide strands and immobilised complementary strands, both strands being attached to the solid support at the 5' end. Arrays comprised of such bridged structures provide inefficient templates for typical nucleic acid sequencing techniques, since hybridisation of a conventional sequencing primer to one of the immobilised strands is not favoured compared to annealing of this strand to its immobilised complementary strand under standard conditions for hybridisation.

[0071] In order to provide more suitable templates for nucleic acid sequencing, it may be advantageous to remove or displace substantially all or at least a portion of one of the immobilised strands in the 'bridged' structure in order to generate a template which is at least partially single-stranded. The portion of the template which is single-stranded will thus be available for hybridisation to a sequencing primer. The process of removing all or a portion of one immobilised strand in a 'bridged' double-stranded nucleic acid structure may be referred to herein as 'linearization', and is described in further detail in WO07010251, the contents of which are incorporated herein by reference in their entirety.

[0072] Bridged template structures may be linearized by cleavage of one or both strands with a restriction endonuclease or by cleavage of one strand with a nicking endonuclease. Other methods of cleavage can be used as an alternative to restriction enzymes or nicking enzymes, including

inter alia chemical cleavage (e.g. cleavage of a diol linkage with periodate), cleavage of abasic sites by cleavage with endonuclease (for example 'USER', as supplied by NEB, part number M5505S), or by exposure to heat or alkali, cleavage of ribonucleotides incorporated into amplification products otherwise comprised of deoxyribonucleotides, photochemical cleavage or cleavage of a peptide linker.

[0073] Following the cleavage step, regardless of the method used for cleavage, the product of the cleavage reaction may be subjected to denaturing conditions in order to remove the portion(s) of the cleaved strand(s) that are not attached to the solid support. Suitable denaturing conditions, for example sodium hydroxide solution, formamide solution or heat, will be apparent to the skilled reader with reference to standard molecular biology protocols (Sambrook et al., supra; Ausubel et al. supra). Denaturation results in the production of a sequencing template which is partially or substantially single-stranded. A sequencing reaction may then be initiated by hybridisation of a sequencing primer to the single-stranded portion of the template.

[0074] Thus, the invention encompasses methods wherein the nucleic acid sequencing reaction comprises hybridising a sequencing primer to a single-stranded region of a linearized amplification product, sequentially incorporating one or more nucleotides into a polynucleotide strand complementary to the region of amplified template strand to be sequenced, identifying the base present in one or more of the incorporated nucleotide(s) and thereby determining the sequence of a region of the template strand.

[0075] One sequencing method which can be used in accordance with the invention relies on the use of modified nucleotides having removable 3' blocks, for example as described in WO04018497, US 2007/0166705A1 and U.S. Pat. No. 7,057,026, the contents of which are incorporated herein by reference in their entirety. Once the modified nucleotide has been incorporated into the growing polynucleotide chain complementary to the region of the template being sequenced there is no free 3'-OH group available to direct further sequence extension and therefore the polymerase can not add further nucleotides. Once the nature of the base incorporated into the growing chain has been determined, the 3' block may be removed to allow addition of the next successive nucleotide. By ordering the products derived using these modified nucleotides, it is possible to deduce the DNA sequence of the DNA template. Such reactions can be done in a single experiment if each of the modified nucleotides has a different label attached thereto, known to correspond to the particular base, to facilitate discrimination between the bases added during each incorporation step. Alternatively, a separate reaction may be carried out containing each of the modified nucleotides separately.

[0076] The modified nucleotides may carry a label to facilitate their detection. A fluorescent label, for example, may be used for detection of modified nucleotides. Each nucleotide type may thus carry a different fluorescent label, for example, as described in U.S. Provisional Application No. 60/801,270 (Novel dyes and the use of their labelled conjugates), published as WO07135368, the contents of which are incorporated herein by reference in their entirety. The detectable label need not, however, be a fluorescent label. Any label can be used which allows the detection of an incorporated nucleotide.

[0077] One method for detecting fluorescently labelled nucleotides comprises using laser light of a wavelength spe-

cific for the labelled nucleotides, or the use of other suitable sources of illumination. The fluorescence from the label on the nucleotide may be detected by a CCD camera or other suitable detection means. Suitable instrumentation for recording images of clustered arrays is described in U.S. Provisional Application No. 60/788,248 (Systems and devices for sequence by synthesis analysis), published as WO07123744, the contents of which are incorporated herein by reference in their entirety.

[0078] The invention is not intended to be limited to use of the sequencing method outlined above, as essentially any sequencing methodology which relies on successive incorporation of nucleotides into a polynucleotide chain can be used. Suitable alternative techniques include, for example, Pyrosequencing™, FISSEQ (fluorescent in situ sequencing), MPSS and sequencing by ligation-based methods, for example as described in U.S. Pat. No. 6,306,597 which is incorporated herein by reference.

[0079] The nucleic acid sample may be further analysed to obtain a second read from the opposite end of the fragment. Methodology for sequencing both ends of a cluster are described in co-pending applications WO07010252 and PCTGB2007/003798, the contents of which are incorporated by reference herein in their entirety. In one example, the series of steps may be performed as follows; generate clusters, linearize, hybridise first sequencing primer and obtain first sequencing read. The first sequencing primer can be removed, a second primer hybridised and the tag sequenced. The nucleic acid strand may then be 'inverted' on the surface by synthesising a complementary copy from the remaining immobilised primers used in cluster amplification. This process of strand resynthesis regenerates the double stranded cluster. The original template strand can be removed, to linearize the resynthesized strand that can then be annealed to a sequencing primer and sequenced in a third sequencing run.

[0080] In the cases where strand resynthesis is employed, both strands can be immobilised to the surface in a way that allows subsequent release of a portion of the immobilised strand. This can be achieved through a number of mechanisms as described in WO07010251, the contents of which are incorporated herein by reference in their entirety. For example, one primer can contain a uracil nucleotide, which means that the strand can be cleaved at the uracil base using the enzymes uracil glycosylase (UDG) which removes the nucleoside base, and endonuclease VIII that excises the abasic nucleotide. This enzyme combination is available as USER™ from New England Biolabs (NEB part number M5505). The second primer may comprise an 8-oxoguanine nucleotide, which is then cleavable by the enzyme FPG (NEB part number M0240). This design of primers gives control of which primer is cleaved at which point in the process, and also where in the cluster the cleavage occurs. The primers may also be chemically modified, for example with a disulfide or diol modification that allows chemical cleave at specific locations.

Flow Cells

[0081] The invention also relates to flow cells for the preparation of amplified arrays of nucleic acids wherein the flow cells contain a uniform coating of three or more immobilised primers. Thus a substrate described herein can occur within or as a part of a flow cell and the methods set forth herein can be carried out in a flow cell. In contrast to spotted arrays of multiple sequences, the three or more oligonucleotides can be

coated over the whole of the array surface rather than in discreet locations that comprise different sequences in each small location. The arrays may be of a size of 1 cm² or greater whereby the whole 1 cm² or greater comprises a homogeneous coating of multiple copies of the same three or more sequences. A flow cell can be distinguished from a 'spotted array' or photolithographically synthesised array due to the fact that the oligonucleotides are attached to each and every surface; top, bottom, walls and ends of the flow cell chamber, rather than being an array that is mounted in a housing. However, if desired a flow cell that is used in a method set forth herein can have surfaces with different reactivity for oligonucleotides such that the oligonucleotides are only attached to one or a subset of the aforementioned surfaces or even to only a subset of regions within these surfaces.

[0082] The flow cell may be coated with exactly three oligonucleotide species of different sequence composition, namely two amplification primers and a capture primer. The capture primer may be present at a lower concentration than the amplification primer, for example at least 100, 1000 or 100,000 fold lower relative concentration. The two amplification primers may be present at similar ratios to each other, for example varying by less than a factor of two. The capture primers may be longer than the amplification primers, and may comprise the amplification primer sequence region plus a capture sequence region, as shown for example in FIG. 1. Alternatively or additionally, the amplification primers may be blocked to prevent hybridisation and/or extension.

[0083] Although the invention has been exemplified herein for embodiments using nucleic acid species, it will be understood that the same principles can be applied to other molecular species. For example, surfaces of substrates can be derivatized with other synthetic molecules such as peptides, small molecule ligands, saccharides or the like. By controlling the amount of different species of such molecules in the derivatization step, a desired density of each species can result. Samples of molecules that bind to one or more of these solid phase molecules can be used without the need for titrating the samples because the density of molecules from the sample that bind to the surfaces will be controlled by the density of their binding partners on the surface. Accordingly, attachment of molecules from the sample can be controlled thermodynamically in a process that is allowed to proceed to equilibrium as opposed to a kinetic process that requires more precise control of reaction conditions and incubation times. Once bound to the surface the molecules from the sample can be subsequently modified or detected. In such embodiments, the surface can include reversibly modified synthetic molecules such that altering or removing the modification can allow the molecules from the sample to be modified or detected for a particular analytical assay or step.

[0084] While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques and apparatus described above may be used in various combinations. All publications, patents, patent applications, or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, or other document were individually indicated to be incorporated by reference for all purposes.

1. A method of amplifying polynucleotides, comprising:
 - (a) providing a plurality of oligonucleotides immobilised to a solid support, wherein the plurality of oligonucleotides comprises first oligonucleotide species comprising a capture sequence and second oligonucleotide species comprising an amplification sequence, wherein the first oligonucleotide species are immobilised on the solid support at a lower density than the second oligonucleotide species;
 - (b) applying a plurality of template polynucleotide molecules to the solid support under conditions wherein the template polynucleotide molecules hybridise to the capture sequence of the first oligonucleotide species but do not hybridise to the second oligonucleotide species;
 - (c) extending the first oligonucleotide species to generate polynucleotide complements of the template polynucleotide molecules, wherein the polynucleotide complements are immobilised on the solid support; and
 - (d) amplifying the polynucleotide complements under conditions wherein the polynucleotide complements hybridize to the amplification sequences of the second oligonucleotide species.
2. The method of claim 1, wherein the plurality of oligonucleotides further comprises a third oligonucleotide species.
3. The method of claim 2, wherein the third oligonucleotide species comprises a second amplification sequence.
4. The method of claim 3, wherein the amplifying occurs under conditions wherein the template polynucleotide molecules hybridize to the second amplification sequences of the third oligonucleotide species.
5. The method of claim 1, wherein the first oligonucleotide species is immobilised at a density of at least 1000 fold lower than the amplification sequences.
6. The method of claim 1, wherein the oligonucleotide species is immobilised at a density of 10^6 - 10^9 copies per cm^2 .
7. The method of claim 1, wherein the capture sequence is longer than the amplification sequences.
8. The method of claim 1, wherein the second oligonucleotide species comprises a reversible blocking group.
9. The method of claim 8, wherein the reversible blocking group comprises a chemical species attached to the 3' end of the second oligonucleotide species.
10. The method of claim 9, wherein the chemical species is a phosphate group.
11. The method of claim 9, wherein the reversible blocking group comprises a self-complementary hairpin structure.
12. The method of claim 11, wherein the second oligonucleotide species is cleaved after extension of the capture sequence, thereby removing the self-complementary hairpin structure from the amplification sequence on the solid support.
13. The method of claim 1, wherein the amplification is isothermal.
14. The method of claim 13, wherein the isothermal amplification comprises cycles of polymerase extension and chemical denaturation.
15. The method of claim 1, wherein the template polynucleotide molecules that are applied to the solid support are single stranded.

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