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(54) Title: ENRICHMENT METHOD FOR SEQUENCING

(57) Abstract: The present invention provides advantageous enrichment methods that are suitable in the field of next generation sequencing, wherein particles comprising an amplicon to be sequenced are separated and thereby enriched from particles that do not comprise an amplicon. The method blocks the single-stranded oligonucleotides present on the particles not comprising an amplicon by addition of a hybridizing agent that forms a hybrid with the single-stranded oligonucleotide, whereby unspecific interactions resulting in unwanted carry-over of the particles not comprising an amplicon into the enriched fraction of particles comprising an amplicon are reduced.



WO 2020/141144 A1

“Enrichment method for sequencing”

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FIELD OF THE INVENTION

The present invention belongs to the field of sample purification, in particular for enriching particles comprising desired nucleic acids such as surface-bound double-stranded amplicons. The described technology is useful for a variety of applications and assays, in particular sequencing applications, e.g. for enriching particles comprising surface-bound clonally amplified template molecules.

BACKGROUND OF THE INVENTION

Small particles, also referred to as microspheres, are a commonly used tool for nucleic-acid based applications in the fields of basic biological research, biomedical research, applied testing, and molecular diagnostics. Applications include, but are not limited to, clonal amplification of specific DNA fragments on the surface of microspheres by polymerase chain reaction or other amplification methods, and specific isolation of nucleic acids/nucleic acids with oligo-conjugated microspheres by hybridization-based methods. Such particles are widely used in sequencing applications.

Next generation sequencing (NGS), or massively parallel sequencing, where millions to hundreds of millions of reads can be generated in the same sequencing run, is a technology that has found numerous applications in research and clinical areas. Different NGS technologies and methods exist such as pyrosequencing, sequencing by synthesis or sequencing by ligation. NGS technologies, NGS platforms and common applications/fields for NGS technologies are e.g. reviewed in Voelkerding et al (Clinical Chemistry 55:4 641-658, 2009), Metzker (Nature Reviews/ Genetics Volume 11, January 2010, pages 31-46), Goodwin et al., (Nature Reviews, June 2016, Vol. 17: pp. 333 – 351 “Coming of age: ten years of next-generation sequencing technologies”), Yohe et al. (Arch Pathol Lab Med, November 2017, Vol. 141: pp. 1544 – 1557 “Review of Clinical Next-Generation Sequencing”) and Masoudi-Nejad (Chapter 2 “Emergence of Next-Generation Sequencing in

“Next Generation Sequencing and Sequence Assembly” SpringerBriefs in Systems Biology, 2013). In order to sequence nucleic acids they need to be prepared in a suitable manner. Current next-generation sequencing approaches share common sample preparation steps to provide the nucleic acid template that is then sequenced and usually include steps such as
5 nucleic acid extraction, optionally target enrichment, sequencing library preparation (wherein the comprised template molecules are usually provided with adapter sequences at one or both ends) and clonal amplification.

Clonal amplification is required because common sequencing methods such as sequencing
10 by synthesis are not sensitive enough for sequencing single molecules. Thus, common next generation sequencing methods require prior clonal amplification of single nucleic acid template molecules to get sufficient copies of the template molecule for sequencing. DNA is usually clonally amplified on a solid surface. To achieve this, many NGS platforms employ particle-based clonal amplification of nucleic acids by polymerase chain reaction (PCR). In a
15 particle-based emulsion PCR (see e.g. WO 2005/073410), an emulsion is prepared with aqueous compartments in form of droplets inside oil medium. Emulsion PCR is widely used in next generation sequencing (e.g. QIAGEN GeneReader System or ThermoFisher IonTorrent PGM). The particles used in emulsion PCR comprise a single-stranded (capture) oligonucleotide that can hybridize to the template molecules, usually to the adapter sequence
20 that is incorporated into the template molecule during library preparation, and serves as a primer during amplification.

In emulsion PCR, small reaction compartments are formed in which (ideal case) one single template molecule and one single bead is present; thereby, this single molecule can be
25 bound to the particle and amplified by PCR. A single-strand of the template molecule becomes associated with the particle, followed by PCR-based amplification of the template molecule. The droplet essentially provides a closed reaction compartment for the PCR and contains after amplification one particle associated with amplified nucleic acids (amplicons) that derived from the same template molecule. Both particles and template are statistically
30 distributed in the droplet. As a consequence, situations could arise in which (a) multiple templates or (b) multiple particles are present in a droplet. This would lead to un-desired outcomes, either (a) polyclonal beads having a mixture of different templates being amplified on the particle in a droplet or (b) the same template being amplified on multiple beads. To minimize this, both particles and template concentrations are reduced by dilution. Hence,
35 when preparing the emulsion, one of such droplets statistically contains one particle and less than one library molecule per droplet (thereby trying to ensure that no droplet contains two library molecules), as well as the components which are required to perform a PCR (such as polymerase, nucleotides, primer, etc.). The high dilution of the sequencing library is important to achieve that single library molecules are amplified on single particles, as non-clonally
40 amplified libraries cannot be sequenced. As a consequence of this mixture ratio, multiple droplets are created that contain a particle but no template molecule. Therefore, after

emulsion PCR there are compartments which comprise an amplicon- containing particle (also referred to in the art as “live bead”) and compartments that comprise a particle that lacks an amplicon (also referred to in the art as “null bead”).

5 Subsequent to the emulsion PCR, the oil phase is removed or the emulsion is “broken” in order to release the particles comprising the amplified nucleic acids (see e.g. WO2014/186152 and EP 3 170 903). This breaking step releases live beads as well as null beads. To ensure a high throughput of the subsequent NGS sequencing reaction, the null beads must be depleted by enriching the live beads. Removal of the null beads is
10 challenging. One method to enrich the live beads is to selectively bind the live beads to another type of particle, also referred to as “enrichment bead”, whereby a larger particle complex is formed. Null beads do not specifically bind to the enrichment beads and are therefore depleted in a subsequent size selective purification step, which enriches the complexes comprising the enrichment beads and the live beads due to their larger size (see
15 e.g. WO2014/186152). However, despite performing such purification/enrichment step, null beads are still present at a relatively high concentration in the enriched live bead sample (e.g. up to 50%). In a downstream sequencing process the non-depleted null beads create significant problems. Each null bead without usable sequence will be deposited onto the available spaces on the flow cell (thereby taking up available spaces in the flow cell), thereby
20 competing with (decreasing the number of) live beads that will provide usable sequence. As a consequence of the inefficient depletion of null beads, sequencing efficiency can be significantly reduced. In order to improve the depletion of null beads, WO 2015/170187 teaches to add enzymes such as single-stranded nucleases, to degrade the capture oligonucleotides on the null beads. Yet, there is still a need for further methods that improve
25 the purification of the live beads.

The invention aims at avoiding drawbacks of the prior art methods. In particular, it is an object to increase the efficiency of the enrichment of amplicon-covered particles from non-amplicon covered particles for next generation sequencing (NGS) applications.

SUMMARY OF THE INVENTION

The present invention is based on the finding that enrichment of a first type of particles comprising double-stranded nucleic acid molecules (e.g. live beads) from a composition
35 additionally containing a second type of particles comprising a single-stranded oligonucleotide (e.g. null beads) can be significantly improved by adding a hybridizing agent capable of forming a hybrid with the single-stranded oligonucleotide prior to and/or during separation of the first type of particles. Addition of the hybridizing agent advantageously reduces the carry-over of the second type of particles during the enrichment process.
40 Without wishing to be bound in theory, it is believed that by forming a hybrid with the single-stranded oligonucleotide present on the second type of particles, the hybridizing agent blocks the single-stranded oligonucleotide and thereby hinders it from interacting with the first type

of particles and/or the third type of particles which may be used during the enrichment process. The hybridizing agent thereby improves the depletion of the second type of particles during the enrichment process of the first type of particles. The technology is particularly suitable for enriching live beads from null beads after clonal amplification using e.g. emulsion PCR. It provides particles of the first type with higher purity allowing for a more efficient downstream sequencing.

The technology of the invention is effective, reliable and can be easily integrated into existing enrichment and sequencing workflows. Additional time-consuming processing steps are not required because the hybridizing agent blocking the single-stranded oligonucleotide present on the second type of particles (null beads) can be easily integrated into existing workflows and may even be included in commonly used processing reagents, such as a wash reagent and/or enrichment reagent. The hybridizing agent can be easily combined with automated sequencing platforms without requiring an additional processing step and/or space in the platform. It thereby makes an important contribution to the art.

According to a first aspect, the present disclosure provides a method for enriching a first type of particles comprising double-stranded nucleic acid molecules from a composition additionally containing a second type of particles comprising a single-stranded oligonucleotide, wherein the method comprises

- adding a hybridizing agent and forming a hybrid with the single-stranded oligonucleotide; and
- separating first type of particles from second type of particles.

According to a second aspect, the present disclosure provides a method of amplifying a template and enriching the provided amplicon, wherein the method comprises:

(a) providing an emulsion comprising one or more aqueous compartments in oil, at least a portion of said compartments comprising

(i) amplification agents,

(ii) a particle comprising a single-stranded oligonucleotide, wherein said single-stranded oligonucleotide is capable of acting as a primer and wherein the particle comprises multiple copies of said single stranded oligonucleotide on the particle surface, and

(iii) a template molecule;

wherein other compartments comprise one or both of (i) and (ii) but do not comprise (iii) a template molecule;

(b) exposing said emulsion to amplification conditions, wherein after amplification at least two types of particles are provided, wherein the particles of the first type comprise a double-stranded amplicon and the particles of second type comprise the single-stranded oligonucleotide but no double-stranded amplicon;

(c) optionally recovering comprised particles from the emulsion;

(d) enriching particles of the first type using the method according to the first aspect.

According to a third aspect, the present disclosure provides a kit for enriching a first type of particles comprising double-stranded nucleic acid molecules from a composition additionally containing a second type of particles comprising a single-stranded oligonucleotide, wherein the kit comprises:

- particles of a third type;
- a porous device, capable of retaining the third type of particles;
- a hybridizing agent capable of forming a hybrid with the single-stranded oligonucleotide of the second type of particles.

According to a fourth aspect, the present disclosure is directed to the use of a hybridizing agent in a method for enriching a first type of particles comprising double-stranded nucleic acid molecules from a composition which additionally contains a second type of particles comprising a single-stranded oligonucleotide for blocking the single-stranded oligonucleotide of the second type of particles by forming a hybrid. As disclosed herein, the double-stranded nucleic acid molecules are preferably double-stranded amplicons as are provided after clonal amplification.

Other objects, features, advantages and aspects of the present application will become apparent to those skilled in the art from the following description and appended claims. It should be understood, however, that the following description, appended claims, and specific examples, while indicating preferred embodiments of the application, are given by way of illustration only.

DESCRIPTION OF THE FIGURES

Fig. 1: Illustrates amplification by particle-based emulsion PCR. Aqueous compartments inside oil are produced that serve as small PCR reaction units as is known in the art. **Fig. 1a** illustrates a single compartment (e.g. aqueous droplet in oil) which comprises a single template molecule carrying e.g. sequencing adapters at each end and a base particle which comprises a single-stranded oligonucleotide on its surface. Illustrated is a single strand of the template molecule. A double-stranded template molecule can be rendered single-stranded by denaturation before preparing the emulsion so that a single-stranded template molecule is introduced into the compartment or a double-stranded template molecule can be introduced into the compartment which is then denatured e.g. by heating within the compartment to provide two separate strands so that one strand of the template molecule can anneal via a complementary sequence to the single-stranded oligonucleotide present on the surface of the base particle. Suitable embodiments are well-known in the art and can be used in the context of the present invention. The base particle is preferably provided as a microsphere, also referred to herein as bead. The base particle comprises multiple copies of the single-stranded oligonucleotide, also referred to as single-stranded capture oligonucleotide, as it is capable of capturing the template molecule when single-stranded by hybridizing to a

complementary sequence, e.g. to an adapter sequence, of the template molecule. A preferred embodiment of the single-stranded (capture) oligonucleotide is illustrated in the enlarged box. The single-stranded (capture) oligonucleotide may comprise a spacer region adjacent to the particle surface (the spacer region may be provided by a stretch of the same nucleotide, e.g. T) and a hybridizing region that is complementary to a sequence comprised in the template molecule, e.g. an adapter sequence. The compartment furthermore comprises agents necessary to perform the PCR, such as a polymerase, dNTPs, a buffer and preferably a second primer which may comprise an affinity partner of an interaction pair, such as preferably biotin. Also a mixture of accordingly modified (e.g. biotin) and unmodified primers may be used.

As is illustrated in **Fig. 1b**, the single-stranded template molecule hybridizes via the adapter sequence to the hybridizing region of the single-stranded (capture) oligonucleotide, whereby the template is captured to the base particle. As shown in **Fig. 1c**, the 3' end of the single-stranded oligonucleotide serves as a primer and is elongated during polymerization using the sequence of the template molecule as template, thereby providing a complementary strand. As a result, a double-stranded amplicon is formed comprising the original strand of the template molecule and the complementary strand resulting from the elongation of the single-stranded (capture) oligonucleotide which served as first primer. As shown in **Fig. 1d-e**, the double-stranded amplicon is then denatured, whereby the original strand of the template molecule is released while the elongated complementary strand remains via the elongated single-stranded (capture) oligonucleotide tightly anchored to the particle surface. The original strand of the template molecule can hybridize again to a "free" single-stranded (capture) oligonucleotide, where it serves again as template for the single-stranded (capture) oligonucleotide, thereby generating again a double-stranded amplicon. A second primer in solution may hybridize to the 3' end of the elongated complementary strand that is anchored to the particle surface, and is extended towards the particle, thereby also providing a double-stranded amplicon. By performing repetitive cycles, a high density of clonally amplified target nucleic acids associated to the particle is provided within the compartment. The thereby generated particle of the first type comprises multiple copies of the double-stranded amplicon, e.g. thousands to millions copies.

As explained in the background, template nucleic acid molecules are provided in the emulsion at a concentration that is below one template molecule per compartment to avoid that multiple template molecules are present per compartment. Therefore, after performing the emulsion PCR, there are compartments comprising a particle of the first type, which comprises multiple copies of the same double-stranded amplicon ("live bead"), as well as compartments comprising a particle of the second type which comprise a single-stranded oligonucleotide but no double-stranded amplicon as there is no template for amplification ("null bead"). The particles of the second type correspond to the base particles. The different types of particles are illustrated in **Fig. 1f**. The first type of particles must be enriched from

the second type of particles, as second type of particles would take up valuable space in the subsequent sequencing process. The present invention advantageously improves the enrichment of the first type of particles comprising the double-stranded amplicon by adding a hybridizing agent that blocks the single-stranded oligonucleotide on the null beads thereby avoiding unspecific binding that can lead to unwanted carry-over of the null beads.

Fig. 2: Illustrates the workflow for enriching the first type of particles (live beads) from the second type of particles (null beads). **Fig. 2a** illustrates the emulsion provided after performing the emulsion PCR. It comprises compartments comprising a particle of a first type (live bead) and compartments comprising a particle of the second type (null beads, corresponding to the base particle). As shown in **Fig. 2a**, the particles of the first type may comprise a first affinity partner of an interaction pair, in the shown embodiment biotin. The first affinity partner, preferably biotin, can be introduced by using a correspondingly modified reverse (second) primer during amplification, which incorporates the affinity partner into the double-stranded amplicon, whereby the particle of the first type becomes covered with said affinity partner (preferably biotin). The particles of the second type do not comprise a double-stranded amplicon resulting from amplification (as there was no template) and therefore, do not comprise said affinity partner. The particles of the second type correspond to the base particles and accordingly, only comprise the single-stranded (capture) oligonucleotide. As is illustrated in **Fig. 2b**, the particles are released from the compartments, e.g. by breaking the emulsion. The released particles may be further purified, e.g. by performing one or more washing steps to remove the oil. Thereby, a composition is provided which comprises particles of the first type as well as particles of the second type. An embodiment for enriching the particles of the first type from said composition while depleting particles of the second type is illustrated in **Fig. 2c**. For enrichment, the composition comprising the particles of the first and second type is contacted with a third type of particles (also referred to herein as enrichment beads). The particles of the third type may be spherical. The third type of particles is preferably larger than the first type of particles and the second type of particles (e.g. 10-15µm versus 0.5-3µm, e.g. 15µm versus 1µm). The enrichment particles specifically bind the first type of particles, whereby an association of particles is formed, also referred to herein as complex or aggregate. Due to its larger size, this complex comprising the third type of particles with the bound first type of particles can be separated from the second type of particles e.g. using a porous device, such as a nylon mesh, as is illustrated in **Fig. 2**. For specific binding of the first type of particles, the third type of particles may comprise a second affinity partner of an interaction pair, e.g. a biotin binding polypeptide (such as streptavidin or avidin) in case the particles of the first type comprise biotin as is shown in the illustrated embodiment. As the first affinity partner (e.g. biotin) is only introduced and thus present in case of successful template amplification, the particles of the second type cannot effectively bind to the third type of particles and are thus removed in the size-selective separation step.

However, as explained in the background, a considerable amount of particles of the second type are nevertheless found in the enriched fraction of the first type of particles. Without wishing to be bound by theory, it is believed that the second type of particles non-specifically bind or interact with the first type of particles and/or the third type of particles via the single-stranded (capture) oligonucleotide that is present on the surface of the second type of particles. Due to this unspecific binding/interaction, second type particles become associated with the complexes and are carried over into the enriched fraction of the first type of particles. The inventors identified the single-stranded (capture) oligonucleotide present on the surface of the null beads as root cause for non-efficient removal of the null beads. The single-stranded oligonucleotide which is usually present in a high density on the particles may cause unspecific binding of the second type particles, so that they are carried over into the fraction of enriched first type particles. These non-specific interactions negatively affect the separation and thus enrichment efficiency. The present invention advantageously reduces the amount of carry-over of null beads by blocking the single-stranded (capture) oligonucleotide that is present in a large amount on the surface of the second type particles. This is achieved by adding a hybridizing agent that forms a hybrid with the single-stranded oligonucleotide, whereby the oligonucleotide can no longer interact with the first type of particles and/or third type of particles. The present invention thereby renders the enrichment process significantly more efficient, as the hybridizing agent diminishes the unspecific interaction of second type particles. By adding a hybridizing agent as taught herein, preferably provided by one or more oligonucleotides reverse complimentary to the single-stranded oligonucleotide present on the second type of particles, second type particles (null beads) are more efficiently depleted in the size-selective separation step of the enrichment process. The hybridizing agent may be added during one or more of the steps that are illustrated in **Fig. 2**. E.g. the hybridizing agent may be added when breaking the emulsion and/or during washing of the particles that were separated from the broken emulsion (see **Fig. 2b**). Additionally or alternatively, the hybridizing agent may be added when adding or processing the third type of particles in order to enrich the first type of particles (see **Fig. 2c**). The hybridizing agent may be simply included into one or more of the processing reagents, e.g. a washing reagent or the enrichment reagent. This is advantageous, because the technology of the present invention can be easily implemented into existing processing workflows. There is not requirement for additional, time consuming processing steps. All steps of the enrichment process can be performed at room temperature as the hybridizing agent can hybridize to the single-stranded oligonucleotide at room temperature.

Fig. 3: Shows different embodiments of the hybridizing agent and the resulting hybrid that can be formed with the single-stranded (capture) oligonucleotide of the second type of particles. The hybridizing agent may comprise and may thus be provided by one or more oligonucleotides capable of hybridizing to the single-stranded (capture) oligonucleotide. As is illustrated by **Fig. 3a**, the hybridizing agent may be provided by a single-stranded oligonucleotide that hybridizes over the entire length with the single-stranded (capture)

oligonucleotide (i.e. the hybridizing region and, if present, the spacer region – see also **Fig. 1a**, enlargement) thereby forming a double-stranded hybrid with a blunt end. As is illustrated in **Fig. 3b**, the oligonucleotide of the hybridizing agent may also hybridize to the outer end of the single-stranded (capture) oligonucleotide, thereby providing an outer blunt end wherein a portion of the single-stranded (capture) oligonucleotide is not involved in hybrid formation. E.g. the spacer region may not be involved in the formation of the hybrid or may only be partially involved in hybrid formation. In one embodiment, the oligonucleotide of the hybridizing agent may hybridize over the entire hybridizing region of the single-stranded (capture) oligonucleotide, as is illustrated in **Fig. 3b**, or at least a portion thereof as long as an outer blunt end is formed. **Fig. 3c** and **Fig. 3d** illustrate embodiments that are related to the embodiment illustrated in **Fig. 3a**, where the oligonucleotide of the hybridizing agent is either longer or shorter than the single-stranded (capture) oligonucleotide, thereby providing a hybrid with a sticky end. The oligonucleotide may hybridize over the entire length of the single-stranded (capture) oligonucleotide (as shown in **Figs. 3c** and **3d**) or may hybridize to a portion thereof whereby a portion of the single-stranded (capture) oligonucleotide is not involved in the formation of the hybrid (as illustrated in **Figs. 3e** and **3f**). Hence, in one embodiment the oligonucleotide of the hybridizing agent is longer than the single-stranded (capture) oligonucleotide. In another embodiment it is complementary to a portion of the single-stranded oligonucleotide, wherein this portion is less than the entire length of the single-stranded oligonucleotide. For instance, as depicted in **Figs. 3d** and **3f** the outer end of the single-stranded (capture) oligonucleotide may remain single-stranded after hybridization, thereby providing a short sticky end. In such a case, the hybridizing agent is complementary to the hybridizing region and optionally, at least a portion of the spacer region. A sticky end, if present in the formed hybrid, is short, preferably $\leq 5\text{nt}$, such as $\leq 4\text{nt}$, $\leq 3\text{nt}$, preferably $\leq 2\text{nt}$ or 1nt , and therefore does not contribute to unspecific binding of the second type of particles (null beads) to the first type of particles (live beads) and/or the third type of particles (enrichment beads).

Furthermore, the hybridizing agent may comprise and thus may be provided by two or more different oligonucleotides, wherein each oligonucleotide is capable of hybridizing to a different region of the single-stranded (capture) oligonucleotide (embodiments are illustrated in **Figs. 3g** to **3j**). When hybridized to the single-stranded (capture) oligonucleotide, the two or more different oligonucleotides of the hybridizing agent are located adjacent to each other in the formed double-stranded hybrid. When all oligonucleotides of the hybridizing agent hybridize to their target region of the single-stranded oligonucleotide of the second type of particles, a double-stranded hybrid is generated that may span at least a portion of or the entire hybridizing region (and optionally a portion of or the entire spacer region, if present) of the single-stranded (capture) oligonucleotide. Gaps may be present between the hybridized oligonucleotides of the hybridizing agent. If nucleotide gaps are present between the individual adjacent oligonucleotides, they are smaller than the length of the oligonucleotides. Adjacent oligonucleotides are preferably spaced apart not more than 5nt , 4nt , 3nt , 2nt or 1nt .

from each other in the formed hybrid. Preferably, all or at least two oligonucleotides of the hybridizing agent are contiguous to each other in the formed double-stranded hybrid and thus, the first oligonucleotide directly follows and is next to the terminal nucleotide of the previous oligonucleotide. When hybridized to the single-stranded (capture) oligonucleotide, no nucleotide gaps are present between the adjacent probe molecules in such a contiguous setting. Such contiguous short oligonucleotides closely resemble a longer oligonucleotide molecule, wherein however, no phosphodiester bond is present between the contiguous nucleotides of adjacent oligonucleotides, also referred to as nick. Portions of the single-stranded (capture) oligonucleotide may remain single-stranded, e.g. at the outer or inner end. E.g. in embodiments, the spacer region of the single-stranded (capture) oligonucleotide or a portion thereof (if present) may remain single-stranded. Furthermore, a sticky end may be formed at the outer end, as has been explained above. Several embodiments of a hybridizing agent comprising at least two oligonucleotides are illustrated in **Figs. 3g to 3i**, **Fig. 3i** illustrates an embodiment with two contiguous oligonucleotides spanning at least the hybridizing region of single-stranded (capture) oligonucleotide and forming an outer blunt end. **Fig. 3j** illustrates an embodiment, wherein three antisense oligonucleotides are used as hybridizing agent. Furthermore, the hybridizing agent may make use of a combination of the above described embodiments.

Fig. 4 illustrates the workflow used in the example. Compared to the prior art method, an antisense oligonucleotide hybridizing to the single-stranded capture oligonucleotide was added to the enrichment buffer to reduce unspecific binding of the null beads during the enrichment process (see large arrow). The example demonstrates that more live beads were recovered by the workflow employing the present invention, leading to an increase in output of the whole sequencing system. The present invention therefore significantly improves existing sequencing workflows.

DETAILED DESCRIPTION OF THE INVENTION

The disclosed technology is now explained in further detail.

METHOD ACCORDING TO THE FIRST ASPECT

According to a first aspect, the present disclosure provides a method for enriching a first type of particles comprising double-stranded nucleic acid molecules from a composition additionally containing a second type of particles comprising a single-stranded oligonucleotide, wherein the method comprises

- adding a hybridizing agent and forming a hybrid with the single-stranded oligonucleotide; and
- separating first type of particles from second type of particles.

The method according to the first aspect improves the enrichment of first type of particles comprising double-stranded nucleic acid molecules from a composition comprising the first type of particles and a second type of particles comprising a single-stranded oligonucleotide by improving the depletion of the second type of particles. This is achieved by the addition of the hybridizing agent which forms a hybrid with the single-stranded oligonucleotide on the second type of particles, whereby said oligonucleotide is blocked. This reduces the carry-over of particles of the second type during the enrichment process as is demonstrated in the examples.

The method is particularly suitable for enriching and purifying clonally amplified templates that are attached to a particle (live beads) by removing particles without an amplicon (null beads). Such particle compositions comprising first type and second type of particles as defined herein are e.g. formed in the preparation of samples for next generation sequencing. Such particles are e.g. generated during clonal amplification, using e.g. particle-based emulsion PCR, which is widely applied in next generation sequencing platforms. Therefore, the double-stranded nucleic acid molecule present on the surface of a particle of the first type is preferably a double-stranded amplicon. An "amplicon" is a product of an amplification reaction. An amplicon is typically double-stranded, but can be rendered single-stranded if desired. As disclosed herein, a single particle usually comprises multiple copies of the same double-stranded amplicon on its surface. As usually multiple different template molecules (e.g. library) are clonally amplified, a mixture of different particles of the first type is provided, wherein each particle of the first type comprises multiple copies of the same double-stranded amplicon but wherein different particles of the first type comprise different double-stranded amplicons, as they originate from different template molecules. The particles of the second type comprise multiple copies of the same single-stranded oligonucleotides, but no double-stranded amplicon. All embodiments disclosed herein particularly apply and refer to this preferred embodiment.

Core steps and features were explained and illustrated in conjunction with the Figs. The individual steps of the method and suitable and preferred embodiments are explained in further detail below.

The composition

The method allows and improves enriching particles of a first type comprising double-stranded nucleic acid molecules from a composition additionally comprising a second type of particles comprising a single-stranded oligonucleotide. The method preferably comprises providing a composition including particles comprising a double-stranded amplicon (first type) and particles comprising a single-stranded oligonucleotide but no double-stranded amplicon (second type).

Such composition is e.g. provided/obtained after performing a particle-based emulsion PCR. The particles of the first type comprising the double-stranded amplicon are obtained when the template is clonally amplified on the particle surface (also referred to as "live beads"), while the second type of particle comprises a single-stranded oligonucleotide but does not
5 comprise a clonally amplified template (also referred to as "null beads"). An according composition is provided by the emulsion as such or is provided after breaking the emulsion and separating the first and second type of particles from the broken emulsion. As disclosed herein, the hybridizing agent may be added to this composition, e.g. during a washing step, to form a hybrid with the single-stranded oligonucleotide that is present on the surface of the
10 second type of particles.

As described herein, the first type of particles and the second type of particles may originate from the same type of base particles which comprises the single-stranded oligonucleotide. It is preferred that the base particle comprises only one unique single-stranded oligonucleotide.
15 The surface of the base particles comprises multiple copies of the single-stranded oligonucleotide. In embodiments, the base particle comprises at least 500 or at least 1000 copies of the single-stranded oligonucleotide, e.g. at least 5000, at least 10,000, at least 15,000, at least 20,000, at least 50,000 or at least 100,000 copies. For certain applications, such as sequencing applications, a high copy number of the single-stranded oligonucleotide
20 on the base particles is preferred. The number may lie e.g. in a range of 1000 to 15,000,000 copies of the single-stranded oligonucleotide per base particle. In embodiments, the density of the surface bound single-stranded oligonucleotides is selected from 500 to 500,000 oligonucleotides/ μm^2 , 750-200,000 oligonucleotides/ μm^2 , or 1000-100,000 oligonucleotides/ μm^2 . Suitable embodiments are well-known in the art and can be used in the
25 context of the present invention. The surface of the base particles may be coated with the single-stranded oligonucleotide as is known in the art.

The first type of particles may differ from the second type of particles in that said particles comprise a double-stranded amplicon, while the particles of the second type correspond to
30 the base particles that were e.g. used for clonal amplification (see also Fig. 1). The base particle (and accordingly the second type of particles) may comprise a large number of the same single-stranded oligonucleotide that is complementary to a region of the template molecule and that can serve as a primer for amplification. Extension of the single-stranded oligonucleotide by a polymerase using the hybridized strand of the template molecule as
35 template provides a double-stranded amplicon that is attached to the particle. The single-stranded oligonucleotide that serves as a primer is in the first type of particles partially or fully incorporated into the double-stranded amplicon. In one embodiment, the presence of the double-stranded amplicon is the only difference between the first and second type of particles. As disclosed herein and known in the art, a single particle of the first type usually
40 comprises multiple copies of the same double-stranded amplicon covalently attached to the

particle surface and different particles of the first type may comprise different double-stranded amplicons (originating from different template molecules).

Core characteristics of the particles are described in the following. The description applies to the first type of particles, the second type of particles as well as to the base particles if not indicated otherwise. The particles may be of any convenient size and material. They may be made of an inorganic material, a natural polymer or synthetic polymer. Examples include but are not limited to cellulose, cellulose derivatives, acrylic resins, silica materials, glass, silica gel, polystyrene, gelatine, polyvinyl pyrrolidone, copolymers of vinyl and acrylamide, polystyrene, polystyrene cross-linked with divinylbenzene or the like, polyacrylamides, latex gels, dextran, rubber, plastics, nitrocellulose, metals, cross-linked dextrans (e.g., Sephadex) agarose gel (Sephacrose), and other solid support materials known to those of skill in the art. The particles may have an average size within a range of 0.1 μm to 50 μm . In embodiments, the average size is within the range of 0.3 μm to 20 μm , 0.5 μm to 10 μm and preferably 0.5 μm to 5 μm . A particle size in the range of 0.5 μm to 3 μm , such as 0.7 μm to 3 μm such as e.g. 1 μm is particularly suitable for sequencing applications.

The particles may be magnetic and therefore respond in a magnetic field. The use of magnetic particles simplifies the processing of the particles by using a magnet. The particles may be superparamagnetic, paramagnetic, ferromagnetic or ferrimagnetic.

First type of particles

The first type of particles comprises double-stranded nucleic acid molecules on the particle surface. Preferably, a particle of the first type comprises a double-stranded amplicon on its surface. When referring to the amplicon, this includes a reference to multiple copies of the same amplicon. A single particle of the first type of particles preferably comprises multiple copies of the same double-stranded amplicon. Usually and preferably, a particle comprises only a single type of amplicon, as is e.g. provided as result of clonal amplification in a particle-based emulsion PCR (see Fig. 1). The number of double-stranded amplicons present on the surface of a particle of a first type correlates with the number of single-stranded oligonucleotides present on the surface of a base particle. It is referred to the above disclosure which also applies to the number of amplicons on the surface of a particle of a first type. Typically, hundreds or thousands to millions of double-stranded amplicons are present on the surface of a particle of a first type after successful amplification, which is preferred for sequencing applications. As disclosed herein, the first type of particles may originate from the same type of base particles as the second type of particles. The originally single-stranded oligonucleotide of the base particle is in the first type of particles extended during the amplification process using a strand of the template molecule as template and becomes at least partially incorporated in a double-stranded hybrid. A spacer region, if present, may or may not remain single-stranded when the amplicon is generated. The first type of particles may additionally comprise a single-stranded oligonucleotide, if not all single-stranded

oligonucleotides were incorporated in a double-stranded amplicon. If present, such remaining residual single-stranded oligonucleotides are preferably present in a low amount.

As disclosed herein, the particles of the first type may be magnetically responsive.

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Second type of particles

The second type of particles may have the same characteristics as the base particles disclosed herein. A particle of the second type comprises multiple copies of the same single-stranded oligonucleotide. The particles of the second type may be coated with the same single-stranded oligonucleotide. The second type of particles (and base particles) may also comprise more than one type of single-stranded oligonucleotide on the same particle surface. E.g. the single-stranded oligonucleotides may comprise the same hybridizing region but may differ in their spacer region. Preferably, all particles of the second type comprise multiple copies of the same single-stranded oligonucleotide(s). Methods for attaching single-stranded oligonucleotides to a given particle surface are known in the art. Preferably, the single-stranded oligonucleotide is covalently attached.

A single-stranded oligonucleotide may have an overall length within the range of 10nt to 200nt. It may have a length within a range of 10nt to 150nt or 15nt to 100nt. Preferred ranges include 15nt to 75nt and 20nt to 50nt. Particularly preferred is a length within the range of 20nt to 35nt or 25nt to 30nt. These ranges are particularly suitable for use as single-stranded (capture) oligonucleotide for clonal amplification.

The single-stranded oligonucleotide is exposed on the particle surface and is accessible for hybridization. As is illustrated in Fig. 1, the single-stranded oligonucleotide may serve as a single-stranded capture oligonucleotide for capturing a single-stranded template molecule during clonal amplification in an emulsion PCR. If a template is present in the compartment, a particle of the first type is provided from the base particle comprising the single-stranded (capture) oligonucleotides. If no template is present, no amplicons are provided and the base particle corresponds to a particle of the second type. Hence, in contrast to the particles of the first type, the particles of the second type do not comprise a double-stranded amplicon. Preferably, the second type of particles only comprise the single-stranded oligonucleotide(s).

As is described herein, a single-stranded oligonucleotide provided on the surface of the second type of particles (and base particles) may comprise different functional regions, such as a spacer region and a hybridizing region. In one embodiment, the single-stranded oligonucleotide comprises a spacer region nearby the particle surface and a hybridizing region located 3' thereto (see Fig. 1a, enlargement). Being located at the proximity of the particle surface the spacer region may compensate irregularities on the particle surface and thereby ensures that the hybridizing region of the oligonucleotide is well exposed on the particle surface and is sterically accessible for hybridization to e.g. a template molecule.

However, such spacer region is not required for the purpose of the present invention. Adequate exposure of the single-stranded oligonucleotide can also be achieved by appropriate attachment strategies which ensure that the single-stranded oligonucleotide is exposed and well-accessible for hybridization.

The spacer region of the single-stranded oligonucleotide, if present, may have a length within the range of 3nt to 50nt, preferably 5nt to 20nt, more preferably 7nt to 15nt. Typically, it will have a length around 10nt. For the spacer region, any sequence may be chosen that suits the purpose of spacing the particle surface and the hybridizing region of the single-stranded oligonucleotide. In one embodiment the spacer region comprises of consists of a nucleotide stretch of the same nucleotide, e.g. A, T/U, G, or C, preferably T.

Furthermore, the single-stranded oligonucleotide may comprise a hybridizing region. The hybridizing region can be used to capture a template molecule for clonal amplification. It may be complementary to a portion of the template molecule, said portion preferably corresponding to an adapter sequence of a sequencing library provided at the end of the template molecule (see e.g. Fig. 1). The hybridizing region is designed to enable base pairing with the target molecule, preferably a comprised adapter sequence. The hybridizing region may be of any suitable length and may lie within a range of 10nt to 200nt or 10nt to 100nt. Preferably, the range is selected from 10nt to 50nt, 12nt to 30nt and 12nt to 25nt, more preferably 15nt to 25nt and 15nt to 20nt. Preferably, the hybridizing region hybridizes without mismatches to the complementary portion of the template molecule, such as e.g. an adapter sequence provided at the end of the template molecule.

As is described herein, the single-stranded oligonucleotide that is exposed on the particle surface may lead to unspecific binding during the enrichment process, resulting in a carry-over of the second type of particles into the enriched fraction comprising the first type of particles. This unspecific binding is in the present technology reduced by adding the hybridizing agent which forms a hybrid with the single-stranded oligonucleotide. Due to this hybrid formation, unspecific binding of the second type of particles is reduced. As disclosed herein, the hybridizing agent may be added at one or more steps of the enrichment method prior to and/or during separation of the first type of particles from the second type of particles (see also Fig. 2). Before discussing further details of the hybridizing agent and the formed hybrid, the separation step is described in further detail which results in an enrichment of the first type of particles.

Separation of the first type of particles

For enrichment, particles of the first type which preferably comprise a double-stranded amplicon (e.g. live beads) are separated from second type of particles (e.g. null beads). The separation provides an enriched fraction of first type of particles. Suitable embodiments of

the separation step were illustrated in Fig. 2 and are also known in the art (see e.g. WO 2014/186152).

5 In a preferred embodiment, the separation comprises binding the first type of particles to a third type of particles and separating the third type of particles with the bound first type of particles from second type of particles. Preferably, at least a portion of said third type of particles bind two or more particles of said first type of particles (see Fig. 2). The third type of particles with the bound first type of particles provides an association of particles, also referred to herein as complex of aggregate. The formed complex/association of third type of particles and bound first type of particles is larger than the second type of particles. This facilitates separation by a size-selective separation step (e.g. filtration or density gradient centrifugation).

15 The third type of particles, also referred to as enrichment particles, is preferably larger than the first type of particles and the second type of particles (which may originate from the same base particles). In embodiments, the third type of particles is at least 5 times larger. It may be at least 7 times or at least 10 times larger. E.g. the third type of particles may be between approx. 10 to 20 times larger. In embodiments, the particles of the third type may have an average size of at least 5 μ m or at least 7 μ m. Preferably, the particles of the third type have a size of at least 10 μ m or at least 12 μ m, such as about 15 μ m. In one embodiment, the first and second type of particles have a size of approximately 0.7 μ m to 3 μ m in diameter and the third type of particles have a size of at least 10 μ m (e.g. in the range of 10 μ m to 20 μ m, such as 15 μ m).

25 Suitable materials for providing particles are known in the art and were also described above. The third type of particles may be magnetic and accordingly may be magnetically-responsive. The particles may be superparamagnetic, paramagnetic, ferrimagnetic or ferromagnetic. The use of magnetic particles allows separation of the third type of particles with the bound first type of particles using a magnet.

30 Binding of the first type of particles to the third type of particles may be reversible, thereby allowing to recover the first type of particles from the third type of particles. Embodiments are known in the art and are also described herein.

35 According to one embodiment, the first type of particles specifically binds to the third type of particles. The second type of particles is not capable of specifically binding to the third type of particles. Hence, the third type of particles may selectively interact with the first type particles, thereby increasing the overall size in comparison to the first and/or second type particles. Yet, despite the specific interaction, unspecific binding of the second type of particles to the third type of particles and/or the first type of particles nevertheless occurs in prior art methods, leading to an inefficient separation and hence, inefficient enrichment of

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first type particles. This unspecific interaction is reduced according to the teachings of the invention by adding a hybridizing agent at one or more steps of the process.

According to one embodiment, the hybridizing agent is added to the composition when adding the third type of particles and/or the reagent (e.g. enrichment solution/buffer) that supports the binding of the first type particles to the third type particles. Advantageously, the hybridizing agent, which may be provided by at least one oligonucleotide reverse complementary to the single-stranded oligonucleotide of the second type of particles, may be included in the enrichment reagent that is used in order to provide suitable conditions for binding the first type of particles to the third type of particles.

According to a preferred embodiment, specific binding of the first type of particles to the third type of particles is mediated by an interaction pair. The interaction pair may be an affinity interaction pair, wherein one partner specifically binds the other partner of the interaction pair. The interaction pair may be selected from (i) biotin and a biotin binding polypeptide, (ii) antibody and antigen and (iii) ligand and receptor. Other options are readily available to the skilled person. The interaction pair is preferably provided by biotin and a biotin binding polypeptide such as streptavidin or avidin.

According to one embodiment, the first partner of the interaction pair is associated with the double-stranded nucleic acid (preferably amplicon) of the first type of particles and the second partner of the interaction pair is associated with the third type of particles. When the partners of the interaction pair interact with each other, the first type of particles is specifically bound to the third type of particles. As the particles of the second type do not comprise the first partner of the interaction pair, they cannot bind specifically to the third type of particles. As described herein, the first partner of the interaction pair (e.g. biotin) may be introduced into the double-stranded amplicon during the amplification process. E.g. amplification may involve the use of a primer in solution that comprises the first partner of the interaction pair (e.g. biotin). Thereby, one strand of the double-stranded amplicon comprises the first partner. As the primer is incorporated into the double-stranded amplicon during the amplification process, the resulting double-stranded amplicon provided on a particle of the first type comprises the first partner (e.g. biotin), usually at its outer end (see Fig. 2). Also a mixture of primers comprising the first partner and unmodified primers may be used as second primer. As a result of the amplification process, the particles of the first type which comprise a double-stranded amplicon become covered with the first partner of the interaction pair, while the second type of particles, which do not comprise a corresponding amplicon, lack the first partner and therefore, cannot specifically bind to the third type of particles during enrichment. The third type particles which comprise the second partner of the interaction pair (e.g. streptavidin) specifically bind therefore the first type particles via the first partner of the interaction pair that this comprised in the double-stranded amplicon. According to one embodiment, the first partner of the interaction pair is biotin which is associated with the

double-stranded amplicon of the first type of particles and the second partner is a biotin binding polypeptide, preferably streptavidin or avidin, associated with the third type of particles (see e.g. Fig. 2). Other interaction pairs are also described in the art (see e.g. WO2014/186152).

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Separation and thus enrichment of first type of particles from second type of particles preferably comprises size selection. Separation may comprise separating the third type of particles with the bound first type of particles based on their larger size compared to second type of particles.

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According to one embodiment, the third type of particles with the bound first type of particles are separated by passing the composition through a porous device, wherein the third type of particles with the bound first type of particles are retained by the porous device, while particles of the second type pass through. The porous device can be a filter. The filter may be a mesh, preferably a single layer mesh. It may be a nylon mesh. The suitable pore size depends on the size of the second type of particles (as they must pass through the pores) and the third type of particles, because at least the formed association of the third type of particles with bound first type of particles must be retained by the porous device (so that it does not pass through the pores). Preferably, the third type of particles cannot pass the pores. Suitable pore sizes can be chosen by the skilled person in view of the chosen size of the first, second and third type of particles. The pore size may be e.g. within a range of 1µm to 50µm, e.g. 5µm to 25µm, 7µm to 20µm or preferably 8µm to 15 µm, such as 10-11µm. The porous device may be comprised in a column, e.g. spin column. The spin column may be centrifuged so as to facilitate separation. Alternatively, vacuum could be applied to the column to suck the composition with the particles of the second type through the porous device, while the third particles with the bound first type of particles are retained.

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According to one embodiment, separation of the third type of particles with the bound first type of particles comprises density centrifugation. For example, the composition may be contacted with a relatively dense medium (e.g. glycerol) and subjected to centrifugation, whereby the third type of particles with the bound first type of particles are separated from the second type of particles. According to a further embodiment, separation of the third type of particles with the bound first type of particles is performed using a magnet. This is e.g. feasible, if the third type of particles are magnetic and the first and second type of particles are not magnetic. As the first type of particles specifically bind to the third type of particles, they are separated together with the magnetic third type of particles while the unbound second type of particles are not recovered by the magnetic separation.

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The separated third particles with the bound particles of the first type may be further processed. E.g. they may be washed after separation by performing at least one washing step. The washing step may further deplete second type of particles. The method may further

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comprise releasing the particles of the first type from the third type of particles. The release step comprises reversing the specific binding of the first type of particles from the second type of particles. Suitable methods are known to the skilled person. For example, particles of the first type labelled with biotin may be released from the third type of particles comprising a
5 biotin binding protein such as streptavidin by denaturation, e.g. via treatment with a base such as NaOH.

The particles of the third type may then be separated from the first type of particles. E.g. when using a column comprising a porous device, the first type of particles that are eluted
10 from the third type of particles may pass through the pores, while the third type of particles are retained, thereby achieving separation. Density centrifugation and/or magnetic separation may also be used.

Suitable embodiments for enrichment are also described in WO 2014/186152 to which it is
15 referred.

As disclosed herein, the third type of particles may be used for enriching the first type particles after clonal amplification, preferably after breaking the emulsion and after recovering and purifying the particles, whereby a purified composition comprising first and
20 second type particles is provided.

The hybridizing agent and the hybrid formed with the single-stranded oligonucleotide

One drawback of prior art enrichment methods is the persistent presence of large amounts of second type particles in the enriched fraction of first type of particles. Although not being
25 bound by theory, it is believed that this is the result of unspecific interactions of the second type particles, in particular of the comprised single-stranded oligonucleotide, resulting in inefficient separation and carry-over of second type of particles. A crucial step in the method according to the first aspect therefore is the addition of a hybridizing agent capable of hybridizing to the single-stranded oligonucleotide that is present on the second type of
30 particles, whereby a hybrid is formed. Blocking the single-stranded oligonucleotide by forming a hybrid advantageously reduces the carry-over of second type of particles that results from unspecific binding/interactions mediated by the single-stranded oligonucleotide thereby rendering the depletion of said particles more efficient as is demonstrated by the examples. Hybrid formation blocks and thereby hinders the single-stranded oligonucleotide
35 from non-specifically interacting with e.g. the first and/or the third type of particles. It thereby reduces the amount of second type of particles in the separated fraction of the first type of particles.

The hybridizing agent may hybridize to a portion or the full length of the single-stranded
40 oligonucleotide present on the second type particles. The hybridizing agent shall not be limited to a particular molecule structure. Any hybridizing agent capable of forming a hybrid

with the single-stranded oligonucleotide of second type particles may be applied according to this invention. Hybridization may be achieved by hydrogen bonding between nucleobases. Such base pairing may be based on Watson-Crick base pairing or other modes of pairing, such as Hoogsteen base pairing. Therefore, double helices or triple helices may be formed.

5 In a preferred embodiment, the hybridizing agent is provided by at least one single-stranded oligonucleotide. The oligonucleotide may be an antisense oligonucleotide that is reverse complementary to the single-stranded oligonucleotide or a portion thereof (see Fig. 3), resulting in formation of a hybrid structure. The at least one oligonucleotide may be selected
10 from various types, such as DNA, RNA, PNA, LNA or HNA, wherein preferably the oligonucleotide is selected from the group consisting of DNA or RNA, more preferably DNA. The hybridizing agent may comprise nucleobases selected from A, G, T/U, and C, as well as from any chemical moiety capable of hybridizing to one or more of A, G, T/U and C. Chemical moieties that are capable of hybridizing to one or more of A, G, T/U and C may be
15 selected from artificial nucleic acids, such as peptide nucleic acids, morpholino nucleic acids, locked nucleic acids, glycol nucleic acids and threose nucleic acids, or from derivatives of natural nucleic acids, such as iso-configurations, daminopyrimidine, and xanthine.

Suitable embodiments of the hybridizing agent comprising one or more single-stranded
20 oligonucleotides are illustrated in Fig. 3 and were explained above and it is referred to this disclosure. The at least one oligonucleotide is at least partially complementary to the single-stranded oligonucleotide of the second type of particles. A single-stranded oligonucleotide of the hybridizing agent may have a length within a range of 5nt to 200nt, 5nt to 150nt, 10nt to 100nt and 10nt to 50nt. Preferably, the length is within a range of 10nt to 35nt, e.g. 15nt to
25 30nt. The length depends on the length of the single-stranded oligonucleotide of the second type particles and/or whether two or more oligonucleotides provide the hybridizing agent and are involved in hybrid formation with the single-stranded oligonucleotide. If two or more oligonucleotides are used, the individual oligonucleotides may be shorter compared to using a single oligonucleotide as the combination of oligonucleotides when hybridized to the single-
30 stranded oligonucleotide provide the hybrid and block the single-stranded oligonucleotide.

In one embodiment, the hybridizing agent is provided by a single oligonucleotide capable of forming a hybrid with the single-stranded oligonucleotide of the second type particles. The length and thus the number of nucleotides (nt) of the oligonucleotide may be adjusted to the
35 number of nucleotides of the single-stranded oligonucleotide of the second type of particles. In one embodiment the single-stranded oligonucleotide of the hybridizing agent and the single-stranded oligonucleotide of the second type of particles are identical in length so that the hybrid is formed over the entire length of the single-stranded oligonucleotide of the second type of particles. In a further embodiment the oligonucleotide has a length that differs
40 from the length of the single-stranded oligonucleotide of the second type of particles wherein the difference in the number of nucleotides is $\leq 15\text{nt}$, $\leq 10\text{nt}$, $\leq 7\text{nt}$ or $\leq 5\text{nt}$. Thereby, a hybrid

is formed with a portion of the single-stranded oligonucleotide of the second type of particles. Preferably, the length of the oligonucleotide is within a range of 10nt to 35nt, e.g. 15nt to 30nt and the single-stranded oligonucleotide present on the surface of the second type of particles has a length within the same range.

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According to a further embodiment, the hybridizing agent comprises two or more different single-stranded oligonucleotides capable of hybridizing at least to a portion of the single-stranded oligonucleotide of the second type of particles. The two or more different single-stranded oligonucleotides may differ in at least one nucleotide from each other. The at least

10 two single-stranded oligonucleotides may hybridize to different regions of the single-stranded oligonucleotide of the second type of particles. Preferably, they hybridize adjacent to each other to the single stranded oligonucleotide. Suitable embodiments were also described in conjunction with Fig. 3 to which it is referred.

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According to one embodiment, the hybridizing agent forms a hybrid over at least 60%, at least 70%, preferably at least 80%, at least 90% or over the entire length of the single-stranded oligonucleotide of the second type particles. As discussed in conjunction with Fig. 3, to reduce the unspecific interaction of said single-stranded oligonucleotide it can be sufficient to block only a portion thereof by hybrid formation. Preferably, at least the outer end

20 of the single-stranded oligonucleotide may form part of the resulting hybrid and preferably provides a hybrid with an outer blunt end.

20

According to one embodiment, a double-stranded hybrid is formed over $\geq 60\%$, $\geq 70\%$, $\geq 80\%$, $\geq 90\%$ or $\geq 95\%$ of the single-stranded oligonucleotide. The hybrid may be formed over

25 the entire length (100%) of the single-stranded oligonucleotide of the second type particles.

25

According to one embodiment, the formed hybrid leaves a portion of the single-stranded oligonucleotide single-stranded. The remaining single-stranded region may be $\leq 40\%$, $\leq 30\%$, $\leq 20\%$ or $\leq 10\%$. The single-stranded portion may have a length of $\leq 10\text{nt}$, $\leq 8\text{nt}$ or $\leq 5\text{nt}$. The

30 single-stranded portion may be provided at the 5' end of the single-stranded oligonucleotide, wherein the 5' end corresponds to the inner end directed towards the particle surface. A single-stranded portion may also be located at the outer (3') end of the single-stranded oligonucleotide, wherein the outer end correspond to the end of the oligonucleotide directed away from the particle surface. Thereby, a sticky end is formed. However, as discussed in

35 conjunction with Fig. 3, the sticky end is too short to result in unspecific binding. According to one embodiment, a partially single-stranded region is flanked by double-stranded hybrid regions. Suitable embodiments are described above in conjunction with Fig. 3.

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According to one embodiment, the formed hybrid comprises a blunt end, wherein the blunt end is formed at the outer end of the single-stranded oligonucleotide, wherein the outer end corresponds to the end of the oligonucleotide that is directed away from the particle surface.

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It corresponds to the 3' end of the single-stranded oligonucleotide that can be elongated during amplification.

In one embodiment, the formed hybrid comprises at least one mismatch. In a different embodiment, the formed hybrid does not comprise a mismatch in the formed double-stranded region.

The hybridizing agent may be added e.g. prior to and/or during separation of the first type of particles. It may be added in two or more steps of the enrichment process to improve the depletion result. According to one embodiment, the hybridizing agent is contacted two or more times with the particles prior to separation and/or is used in excess during at least one step. It is thereby ensured that the single-stranded oligonucleotides present on the second type of particles are efficiently blocked by the hybridizing agent prior to separation. Suitable embodiments are described in conjunction with Fig. 2 and also elsewhere herein.

The hybridizing agent is used within the method in an amount so that at least a portion of the single-stranded oligonucleotides present on the second type of particles form a hybrid with the hybridizing agent. A hybrid is preferably formed with the majority of the single-stranded oligonucleotides present on the surface of the second type particles. Suitable concentration ranges for the hybridizing agent can be determined by the skilled person and can be adjusted by the skilled person to the assumed number of second type particles in the composition. According to one embodiment, the % of blocked single-stranded oligonucleotides present during separation lies in the range of 50-100%, 60-100%, 70-100% , 80-100% and preferably 90-100%. Typically, the hybridizing agent is added in an oversaturated regime, which means that the amount of hybridizing agent is usually higher than the amount of single-stranded oligonucleotide molecules expected to be present after amplification. Hence, the molar concentration ratio of hybridizing agent to single-stranded oligonucleotide may be greater than 1.

The hybridizing agent may also hybridize to any remaining single-stranded oligonucleotides if present on the first type particles (e.g. residual amounts present after clonal amplification).

According to one embodiment, the amount of second type particles in the separated fraction of first type particles is $\leq 40\%$, $\leq 35\%$ or $\leq 30\%$. The amount of second type particles in the separated fraction of first type particles may be $\leq 25\%$ or $\leq 20\%$.

The method according to the first aspect is preferably carried out subsequent to an emulsion amplification reaction. The principle of the particle-based emulsion PCR is illustrated in Fig. 1. The hybridizing agent preferably is provided by one or more oligonucleotides reverse complementary to the single-stranded oligonucleotide. It may be added after amplification to the composition comprising first and second type particles. In one embodiment, the

hybridizing agent is comprised in a reagent that is added for processing to the composition. Examples of reagents which may comprise the hybridizing agent include but are not limited to an emulsion breaking reagent, a wash reagent, a buffer exchange solution, a reagent comprising the third type particles and the enrichment reagent that may be added to support

5 binding of the first type of particles to the third type of particles. In one embodiment, the concentration of the hybridizing agent in a reagent is in a range selected from 1 nM and 100 mM, 10 nM and 50 mM, 100 nM and 1 mM. In one embodiment, a reagent comprises the hybridizing reagent, such as e.g. a single antisense oligonucleotide or a combination of two or more oligonucleotides, in a final concentration selected from 2 μ M to 50 μ M, 5 μ M to 35 μ M

10 and 7 μ M to 25 μ M, such as e.g. 10 μ M. The suitable concentration for the hybridizing agent may be adjusted depending on the number of applied reagents that comprise the hybridizing agent. For instance, if the hybridizing agent is applied in a single solution, the concentration of the oligonucleotide may be adjusted to a higher concentration compared to an embodiment wherein the hybridizing agent is comprised in more than one applied reagent.

15 The hybridizing agent may be added at least once, or two or more times. It may be added at different steps of the method, respectively during processing of the particles. Therefore, the hybridizing agent may be supplied multiple times by using multiple solutions comprising the hybridizing agent, which can be used in sequential or non-sequential steps. In an exemplary embodiment, the hybridizing agent is present in a wash reagent that is used for washing the

20 particles after breaking the emulsion and/or in an enrichment reagent (e.g. buffer) used before and/or during the size-selective separation of the third type of particles to which the first type of particles are bound. Conditions are used that allow hybridization of the hybridizing agent to the single-stranded oligonucleotides.

25 The hybridizing agent hybridizes to the single-stranded oligonucleotides which are usually present at a high density on the second type particles that do not comprise double-stranded amplified template nucleic acids, thereby reducing unspecific binding and unwanted carry-over of the second type particles during the enrichment step. The present method thereby overcomes prior art drawbacks. It can be easily integrated into existing sequencing

30 workflows because the hybridizing agent, which is preferably provided by at least one reverse complementary antisense oligonucleotide, can be added as additive to reagents that are already used in the workflow. It can be used at room temperature. The hybridizing agent is effective and easy to store. Therefore, the enrichment step of prior art methods (e.g. WO 2014/186152) is improved, also in comparison to alternative methods such as an enzyme-

35 based removal of the capture oligonucleotide (e.g. WO 2015/170187). The hybridizing agent is inexpensive, in particular in comparison to enzyme-based strategies. It is moreover very effective. The small oligonucleotides of the hybridizing agent can easily and quickly reach the single-stranded particles on the cell surface. Furthermore, a separate processing step is not required with the present invention as the hybridizing agent may be added to one or more

40 processing reagents that are anyhow used. This is an important advantage, in particular for automated workflows. Another important advantage is that the hybridizing agent can be

readily applied at various time points of existing processing and enrichment protocols, due to its high compatibility and robust functionality. The method of the invention thus enables an efficient enrichment of first type particles through a cost-effective, simple and highly efficient reduction of the unspecific interaction of second type particles. By adding a hybridizing agent according to the invention, the single-stranded oligonucleotide of second type particles is blocked, disabling unspecific interaction via arbitrary base pairing.

METHOD ACCORDING TO THE SECOND ASPECT

According to a second aspect, the present disclosure provides a method of amplifying a template and enriching the provided amplicon, wherein the method comprises:

(a) providing an emulsion comprising one or more aqueous compartments in oil, at least a portion of said compartments comprising

(i) amplification agents,

(ii) a particle comprising a single stranded oligonucleotide, wherein said single-stranded oligonucleotide is capable of acting as a primer and wherein the particle comprises multiple copies of said single-stranded oligonucleotide on the particle surface, and

(iii) a template molecule;

wherein other compartments comprise one or both of (i) and (ii) but do not comprise (iii) a template molecule;

(b) exposing said emulsion to amplification conditions, wherein after amplification at least two types of particles are provided, wherein the particles of the first type comprise a double-stranded amplicon and the particles of the second type comprise the single-stranded oligonucleotide but no double-stranded amplicon;

(c) optionally recovering comprised particles from the emulsion;

(d) enriching particles of the first type using the method according to the first aspect.

The method according to the first aspect is particularly suitable in order to enrich particles comprising a clonally amplified template ("live beads") from particles not comprising a clonally amplified template ("null beads"). Effective depletion of second type particles not comprising an amplified template improves the subsequent sequencing. E.g. the space in a flow cell of a NGS sequencing device is less occupied by second type particles, which are irrelevant for nucleic acid sequencing, but are more occupied by first type particles comprising the amplified target nucleic acid. Hence, more clonally amplified nucleic acid molecules can be sequenced per sequencing run, enhancing the overall throughput and cost-efficiency.

In the second aspect of the invention, particle-based emulsion amplification is performed to amplify target nucleic acids for subsequent sequencing. The steps of the present method are explained in the following:

Step (a)

In step (a) an emulsion is provided comprising one or more aqueous compartments in oil. The emulsion may be formed according to any suitable method known in the art. As these methods are well-known and systems are also commercially available, they do not need any
5 detailed description (see e.g. WO2005/073410 and WO2014/186152, herein incorporated by reference). The emulsion may be a heat stable water-in-oil emulsion. In a preferred embodiment, the emulsion is heat stable to allow thermal processing/cycling, e.g., to at least 94°C, at least 95°C, or at least 96°C. The compartments may be provided by microdroplets having a size between 1 and 100µm.

At least a portion of said compartments comprise in a single compartment (i) amplification agents, (ii) a particle comprising a single-stranded oligonucleotide, wherein said single-stranded oligonucleotide is capable of acting as a primer and wherein the particle comprises multiple copies of said single stranded oligonucleotide on the particle surface, and (iii) a
15 template molecule. These compartments provide upon amplification a particle of the first type, comprising multiple copies of the double-stranded amplicon on its surface. Other compartments comprised in the emulsion comprise (i) and (ii) but do not comprise (iii) a template molecule. As these compartments lack a template molecule, no double-stranded amplicon can be provided on the particle upon amplification.

The particles comprising the single-stranded (capture) oligonucleotide are suspended as base particles in a heat stable water-in-oil emulsion. Suitable and preferred embodiments of the base particles (see description of the second type of particles) and the single-stranded oligonucleotide were described above in conjunction with the method according to the first
25 aspect and it is referred to the above disclosure. In a preferred embodiment, the template nucleic acids and the base particles are configured to associate with each other to reversibly bind. In an advantageous embodiment, a template nucleic acid molecule binds to the base particle via hybridization. The template nucleic acids preferably comprise adapter sequences 5' and/or 3' of the target nucleic acid, i.e. at the respective ends of the template nucleic acids.
30 Moreover, preferably different template nucleic acids comprise the same adapter sequences 5' and/or 3', preferably at both ends, as are usually provided in a sequencing library. At least one of the adapter sequences of the template molecule is capable of hybridizing to the single-stranded (capture) oligonucleotide. The base particles comprise multiple copies of the same single-stranded oligonucleotide thereby ensuring that after clonal amplification, the
35 double-stranded amplicons are present on the particle surface in high density. Suitable embodiments are also described above. The single-stranded (capture) oligonucleotide may hybridize to various different template nucleic acids of a sequencing library, as the hybridization is mediated through the adapter sequence of the target nucleic acid sequence, which can be configured be the same for the provided template nucleic acids.

As disclosed herein, the surface of the base particles (and second type of particles) may be provided with two or more different single-stranded oligonucleotides. If different types of single-stranded oligonucleotides are used, they may differ e.g. in the sequence and/or length of the spacer region, if present. The hybridizing region of the different single-stranded oligonucleotides is preferably identical to allow efficient hybridization of the template molecule during amplification, e.g. to the adapter sequence of the template molecule, and to provide the generated amplicons in a high density at the particle surface.

As explained above, for clonal amplification it is required to control the concentration of the template molecule (e.g. by limiting dilution) and the particle concentration such that each compartment comprises on average less than one template molecule. The emulsion can be configured to statistically comprise one base particle per compartment and/or less than one template nucleic acid molecule per compartment. Such a dilution ratio maximizes the frequency of amplifying only one template molecule per compartment in order to generate a clonally amplified template on a single particle that is homogeneous. Thereby, a particle of a first type is generated that comprises multiple copies of the same double stranded amplicon derived from the same template molecule. The majority of the compartments comprised in the emulsion may include only one template molecule and one base particle. In addition, there are compartments that comprise a base particle but do not contain a template molecule. There may also be compartments that comprise a template but no base particle.

The compartment furthermore comprises amplification agents and thus the agents that are required for amplifying the template nucleic acid within the compartment. Amplification reactions include thermal amplification reactions, preferably polymerase chain reaction (PCR) or isothermal reactions. A preferred example would be amplification agents for performing a PCR. The agents preferably comprise a polymerase (preferably DNA polymerase), dNTPs, salts and buffers. Embodiments are known to the skilled person and do not need any detailed description. A reverse transcriptase is additionally required for a RNA template.

Advantageously, the amplification agents may comprise a primer, also referred to herein as second primer. It may function as reverse primer in solution (see also Fig. 1). This second primer is capable of hybridizing to a portion of the template nucleic acid molecule. As described herein, it is preferred that the template molecule comprises sequencing adapters at the 5' and 3' end. The single-stranded (capture) oligonucleotide of the base particle preferably hybridizes to the adapter sequence at the 3' end of the single-stranded template molecule where it functions as a first primer. Details were described above and it is referred to this disclosure. Preferably, the second primer hybridizes to the 3' end of a newly synthesized strand, wherein this newly synthesized strand comprises the single-stranded oligonucleotide bound to the particle. In case a second primer is applied that hybridizes to the 3' end of the newly synthesized strand, a polymerization reaction may be induced. The

second primer in solution may thus hybridize to the 3' end of the reverse strand of the template molecule that is obtained by elongation of the single-stranded capture oligonucleotide using the single-stranded template molecule as template (see Fig. 1). This portion may correspond to the adapter sequence in the reverse strand. Thereby, multiple copies of the double-stranded amplicon may be formed quickly.

The second primer may comprise a first partner of an interaction pair, such as e.g. biotin. It is referred to the above detailed disclosure which also applies here. In one embodiment, the second primer is biotinylated such that one strand of the amplified template comprises, preferably terminates with a biotin. Thereby, an amplicon is provided on the particle that comprises the first partner of an interaction pair. Labelling the generated amplicon with a first partner of an interaction pair may also be achieved by using e.g. labelled nucleotides for amplification. The amplicon may also be provided with the first partner of the interaction pair after amplification.

As disclosed above, providing the generated amplicon with a first partner of an interaction pair facilitates the subsequent enrichment of the successfully amplified first type of particles (live beads) which can specifically bind to third type of particles comprising the second partner of the interaction pair (e.g. a biotin binding polypeptide such as streptavidin or avidin).

Step (b)

Step (b) comprises exposing said emulsion to amplification conditions. In a preferred embodiment, nucleic acid amplification is performed by PCR. The emulsion may be exposed to any suitable thermocycling conditions and/or processes known in the art. The amplification of the template nucleic acid by PCR in a particle-based emulsion PCR may be performed as is known in the art (see also Fig. 1).

For instance, 15 to 140 cycles, such as 25 to 100 cycles or preferably 30 to 70 cycles such as e.g. 50-60 cycles of amplification may be performed. It is desirable to provide first type particles comprising a high density of double-stranded amplicons where a compartment comprises a template molecule. This is achieved by a high density of the single-stranded oligonucleotides on the surface of the base particles and a sufficiently high number of amplification cycles to achieve that the majority or all provided single-stranded oligonucleotides are after successful amplification provided with a double-stranded amplicon. Routinely, the template DNA is amplified until hundreds to million copies, i.e. double-stranded amplicons, are provided on the surface of the particle. Embodiments were disclosed above and it is referred to the respective disclosure. A portion of the single-stranded oligonucleotides bound to the particle may remain single-stranded without an associated double-stranded amplicon after amplification. However, the amount, if at all present, is usually minor considering that many copies of the template molecule are provided

during amplification which will then hybridize to the single-stranded oligonucleotides present on the particle surface. In embodiments, substantially all single-stranded oligonucleotides are after amplification provided with a double-stranded amplicon.

Accordingly, after amplification at least two types of particles are provided in the compartments, wherein the first type of particles comprises a double-stranded amplicon ("live beads") and the second type of particles comprises the single-stranded oligonucleotide but no double-stranded amplicon ("null beads"). The second type of particles essentially corresponds to the base particles that were added when forming the emulsion.

Step (c)

Optional step (c) comprises recovering the comprised particles from the emulsion prior to performing enrichment step (d). Step (c) is preferably performed and may comprise breaking the emulsion and recovering the comprised particles from the broken emulsion. After separating the particles from the broken emulsion, step (c) may comprise one or more washing steps to remove residual traces of the oil phase from the nucleic acid molecules. Hence, the recovered particles may optionally be further purified in step (c), e.g. washed, prior to performing step (d).

Thus, in one embodiment, following particle-based emulsion amplification, the emulsion is "broken". This step is well-known in the art and is also referred to as "de-emulsification". The breaking or de-emulsification leads to the complete or partial separation of the water-in-oil emulsion into oil and water layers.

Methods for breaking such emulsions are known in the prior art and therefore need no detailed description. Breaking the emulsion may involve the use of an inorganic or organic de-emulsifier, and processes that treat emulsions mechanically. Breaking the emulsion may involve the use of additional oil to cause the emulsion to separate into two phases. The oil phase is then removed, and a suitable organic solvent is added. After mixing, the oil/organic solvent phase is removed. This step may be repeated several times. Finally, the aqueous layers above the particles are removed. The particles may be washed, e.g. with an organic solvent and buffer and then washed again in buffer. Suitable organic solvents include alcohols such as methanol, ethanol, isopropanol and the like. In another embodiment, the emulsion is broken by the addition of organic phase that solubilizes both aqueous phase and the oil/detergent and the homogenous solution removed after centrifugation or magnetic separation. The workup is usually then followed by washes with aqueous buffers, such as PBS with additional detergent (e.g. Tween-20). Such method is e.g. described in WO 2014/186152. A further method for breaking an emulsion after performing a particle-based emulsion PCR is described in EP 3 170 903 and may be used. Therefore, an anionic surfactant may be added which may be selected from the group consisting of: alkyl sulfates and alkyl carboxylates. In an embodiment the anionic surfactant is provided to a water-in-oil

emulsion in a final concentration of between approx. 1 - 50 wt.-%, preferably approx. 2 - 40 wt.-%, more preferably approx. 3 - 30 wt.-%, more preferably approx. 4 - 20 wt.-%, more preferably approx. 5 - 15 wt.-%, most preferably approx. 10 wt.-%. The anionic surfactant is added to the water-in-oil emulsion in a concentration which ensures an effective breaking and removal of the oil phase. It is to be understood that the surfactant may be added to the emulsion in several steps where, possibly, different amounts or concentrations may be added.

Several means can be used to separate the particles from the broken emulsion. E.g. the particles may be recovered from the broken emulsion by one or more of sedimentation, preferably centrifugation, filtration or application of a magnetic field (if the particles are magnetic). Separation may be achieved by sedimenting the particles, preferably assisted by centrifugation, and removal of the supernatant. Hence, step (c) may encompass centrifugation to separate the comprised particles from the broken emulsion. According to one embodiment the particles comprise magnetic properties, as described above, thereby allowing magnetic processing for separation and optionally washing. The particles may be collected by a magnet and the supernatant removed. Examples for magnetic processing of the particles for recovery from the broken emulsion are described in EP 3 170 903. This measure has the advantage that a convenient and simple separation of the captured nucleic acids can be achieved by applying a magnetic field to the broken solution.

Step (c) may comprise multiple breaking and washing cycles.

According to one embodiment, the hybridizing agent is added in step (c). In embodiments, it is added during breaking the emulsion.

According to a preferred embodiment, the particles are washed one or more times in step (c) and the hybridizing agent is added during washing the particles. It may be added during one or more of the washing steps performed in step (c). Suitable washing steps and wash reagents for washing the particles are described in WO2014/186152 and EP 3 170 903. As described therein, the particles may be washed e.g. with a mixture of an organic solvent (e.g. methanol, ethanol, isopropanol) and annealing buffer (e.g. 100mM Tris-HCL, 1M NaCl, 0.2% non-ionic detergent (e.g. Tween 20), pH 8), followed by washing with annealing buffer. Also described is the washing with aqueous buffers, such as PBS with additional detergent (e.g. a non-ionic detergent such as Tween 20). Also described therein is washing with an aqueous buffer, such as TE buffer, comprising an anionic surfactant such as ammonium lauryl sulfate followed by washing with TE buffer. Also described is the washing of the particles after recovery from the emulsion with the enrichment buffer, that is subsequently used for enriching the first type of particles from the second type of particles using a third type of particles.

As disclosed above the hybridizing agent may advantageously be included into at least one wash reagent that is used for washing the particles in step c). This is convenient, because treatment of the second type particles with the hybridizing agent for blocking the single-stranded oligonucleotide by forming hybrids can be performed during washing of the mixture of the first and second type of particles so that the number of processing steps is not increased. The hybridizing agent is added to a wash reagent that establishes conditions that allow hybridization of the hybridizing agent to the single-stranded oligonucleotides of the second particles. Suitable wash reagents and conditions can also be determined by the skilled person following the disclosure of the present application. As disclosed above, the hybridizing agent may be comprised in a wash reagent in a final concentration of at least 1 μ M, at least 5 μ M or at least 10 μ M. The hybridizing agent is preferably included in a high concentration in the wash reagent to achieve good saturation of the single-stranded oligonucleotides by forming a hybrid.

Step (d)

Step (b) provides with the emulsion a composition comprising first type of particles and second type of particles. Optional but preferably performed step (c) provides a further purified composition comprising first and second type of particles. As usually multiple different template molecules (e.g. a library) are clonally amplified, a mixture of different particles of the first type is provided, wherein each particle of the first type comprises multiple copies of the same double-stranded amplicon but wherein different particles of the first type comprise different double-stranded amplicons, as they originate from different template molecules. The second type of particles essentially corresponds to the base particles.

Step (d) comprises enriching particles of the first type using the method according to the first aspect. This method, the hybridizing agent and the formed hybrid were described in detail above and it is referred to the respective disclosure which also applies here. Step (d) depletes second type particles.

As discussed above, enriching preferably comprises specifically binding the first type of particles to a third type of particles. In one embodiment, the hybridizing agent is added when adding the third type of particles and/or when separating said third type of particles with the bound first type of particles from the remaining particles. According to one embodiment, the hybridizing agent is comprised in an enrichment reagent that is added to the composition when adding the third type of particles. The enrichment reagent supports binding of the first type of particles to the third type of particles. Preferably, the enrichment reagent establishes conditions supporting the specific binding of the first type of particles to the third type of particles. As disclosed herein, binding is preferably mediated by an interaction pair such as preferably biotin and a biotin binding polypeptide such as streptavidin. Including the hybridizing agent in the enrichment reagent is advantageous, because no additional steps need to be performed and the hybridizing agent can be integrated into existing work-flows.

Suitable enrichment buffers are disclosed e.g. in WO 2014/186152 and are also commercially available. As disclosed above, the hybridizing agent may be comprised in the enrichment reagent such as e.g. an enrichment buffer in a final concentration of at least 1 μ M, at least 5 μ M or preferably at least 10 μ M.

The recovered particles of the first type comprising the double-stranded amplicons can be subsequently used for sequencing. The sequencing steps are preferably performed on each individual bead. For sequencing by synthesis applications, the complement strand of the amplicon may dissociate, thereby rendering particle-bound single-stranded DNA templates for sequencing.

KIT ACCORDING TO THE THIRD ASPECT

According to a third aspect, a kit is provided for enriching a first type of particles comprising double-stranded nucleic acid molecules from a composition additionally containing a second type of particles comprising a single-stranded oligonucleotide, wherein the kit comprises:

- particles of a third type;
- a porous device, capable of retaining the third type of particles;
- a hybridizing agent capable of forming a hybrid with the single-stranded oligonucleotide of the second type of particles.

The kit can be advantageously used in the methods according to the first and second aspect.

Details of the particles of the third type, the porous device, which may be comprised in a column, and the hybridizing agent were described above and it is referred to the respective disclosure which also applies here. Additional compounds may be one or more buffers for washing and/or a buffer suitable for allowing the interaction of the interaction pair, e.g. an enrichment buffer. As disclosed above, one or more of these buffers may comprise the hybridizing agent described above. A further compound may be a buffer suitable for allowing releasing associated particles. Moreover, the kit may comprise components to conduct the size-selective separation step, such as an enrichment column.

As described above, the third type of particles may comprise a biotin binding polypeptide, such as streptavidin or avidin. Suitable embodiments are known in the art and are also commercially available. The kit may furthermore include a reagent suitable for allowing binding between biotin and the biotin binding protein. The reagent may be an enrichment buffer as disclosed above. The reagent may comprise the hybridizing agent, or the hybridizing agent may be added thereto before the reagent is used. Embodiments and suitable concentrations were disclosed above and it is referred to the respective disclosure. The hybridizing agent may be comprised in the enrichment reagent in a final concentration of

at least 1 μ M, at least 5 μ M or preferably at least 10 μ M. Suitable concentration ranges include 2 μ M to 50 μ M, 5 μ M to 35 μ M and 7 μ M to 25 μ M, such as e.g. 10 μ M.

The kit may comprise a reagent for breaking an emulsion. Details of such reagent for breaking an emulsion obtained in a particle-based emulsion PCR were described above. The reagent may comprise the hybridizing agent, or the hybridizing agent may be added thereto before the reagent is used.

The kit may furthermore comprise at least one wash reagent. The wash reagent may comprise the hybridizing agent, or the hybridizing agent may be added thereto before the reagent is used. The hybridizing agent may be comprised in the wash reagent in a final concentration of at least 1 μ M, at least 5 μ M or at least 10 μ M. Suitable concentration ranges include 2 μ M to 50 μ M, 5 μ M to 35 μ M and 7 μ M to 25 μ M, such as e.g. 10 μ M. The wash reagent may be for washing the first type of particles and second type of particles after breaking the emulsion. Suitable embodiments were disclosed above and it is referred to the respective disclosure.

The kit may comprise a reagent suitable for releasing bound particles of the first type from the particles of the third type. Details were described above and it is referred to the respective disclosure.

The kit may furthermore comprise base particles comprising a single-stranded oligonucleotide. The hybridizing agent comprised in the kit is capable of forming a hybrid with the single-stranded oligonucleotide of the base particles. Details were described above and it is referred to the respective disclosure which also applies here. A base particle comprises multiple copies of the single-stranded oligonucleotide and preferably is coated therewith. The base particles may correspond to the second type particles described in detail above and it is referred to the respective disclosure. The base particles may be used in a particle-based emulsion PCR as described above.

The kit may furthermore comprise amplification agents, such as in particular PCR agents. Details were described above. The amplification agents may comprise a second primer, which preferably comprises a first partner of an interaction pair, such as e.g. biotin.

USE ACCORDING TO THE FOURTH ASPECT

According to a fourth aspect, the present disclosure is directed to the use of a hybridizing agent in a method for enriching a first type of particles comprising double-stranded nucleic acid molecules from a composition which additionally contains a second type of particles comprising a single-stranded oligonucleotide for blocking the single-stranded oligonucleotide of the second type of particles by forming a hybrid. As disclosed herein, the double-stranded

nucleic acid molecules are preferably double-stranded amplicons as are provided after clonal amplification. Hence, also provided is the use of a hybridizing agent in a method for enriching a first type of particles comprising a double-stranded amplicon from a composition which additionally contains a second type of particles comprising a single-stranded oligonucleotide but no double-stranded amplicon for blocking the single-stranded oligonucleotide of the second type of particles by forming a hybrid.

Details of the hybridizing agent and the associated advantages were discussed in detail above and it is referred to the above disclosure which also applies here.

FURTHER EMBODIMENTS AND CHARACTERISTICS OF THE METHODS ACCORDING TO THE PRESENT DISCLOSURE

Further general characteristics and embodiments of the invention as defined according to the first, second, third and fourth aspect are disclosed in the following. Nucleic acid templates for sequencing as well as their preparation are well known in the art and therefore, do not need to be described herein in detail. Common steps for preparing the nucleic acid template for sequencing usually include steps such as nucleic acid isolation from a biological sample and library preparation, optional target enrichment, and clonal amplification (described in detail above). The technology of the present invention may be integrated into a sequencing workflow comprising the steps of (1) nucleic acid extraction, (2) optionally but preferably target enrichment, (3) library preparation, (4) clonal amplification, (5) next generation sequencing and (6) data analysis.

The nucleic acid template and preparation of a sequencing library

Nucleic acids may first be isolated from a biological sample or an artificial nucleic acid sample e.g. generated by PCR may be provided. Biological samples from which nucleic acids can be isolated include but are not limited to cell-containing samples and cell-free biological samples. Exemplary biological samples include, but are not limited to, whole blood; blood derived samples such as plasma, serum, blood cells, buffy coat; swabs; urine; sputum; saliva; semen; lymphatic fluid; amniotic fluid; cerebrospinal fluid; peritoneal effusions; pleural effusions; biopsy samples; fluid from cysts; synovial fluid; vitreous humor; aqueous humor; bursa fluid; eye washes; eye aspirates; pulmonary lavage; lung aspirates; animal, including human or plant tissues, including but not limited to, liver, spleen, kidney, lung, intestine, brain, heart, muscle, pancreas, cell cultures, as well as lysates, extracts, or materials and fractions obtained from the samples described above or any cells and microorganisms and viruses that may be present on or in a sample and the like. Materials obtained from clinical or forensic settings that contain nucleic acids are also within the intended meaning of the term "sample". Preferably, the biological sample derived from a human, animal, plant, bacteria or fungi. The extracted nucleic acids may be DNA and/or RNA. The isolated nucleic acid may be extracellular nucleic acids, such as extracellular DNA, that can be isolated from cell-free or cell-depleted body fluid sample. RNA is usually transcribed into cDNA.

The step of nucleic acid isolation may be followed by target enrichment, as is well-known in the art. The target region corresponds to the region which is supposed to be sequenced and accordingly, which is supposed to be covered by the enriched target sequences in order to obtain the sequence information for the target region of interest. The regions of interest (e.g. of clinical relevance) may be amplified using PCR. If the sequencing library is made of genomic DNA, a target region of interest usually consists of one or more genomic regions. The present invention can be applied not only to coding exons in a genome but to any arbitrarily defined sequence portion of a genome or even metagenome. The present invention can also be applied to the transcriptome and to cDNAs derived from the transcriptome. Accordingly, the target region of interest may also correspond to one or more transcripts, miRNAs, tumor derived nucleic acids or any other nucleic acid sequences of interest that are supposed to be sequenced.

According to one embodiment, the nucleic acid template is a DNA template. The DNA template may have been prepared from RNA by reverse transcription. The DNA template may have a length in a range selected from 50 to 1000, 50 to 800 and 50 to 500 nucleotides (the length refers to bp (base pairs) in case of a double-stranded molecule). Suitable sizes can be obtained by fragmentation. Suitable techniques are well-known in the art.

The method may comprise preparing a sequencing library. Methods are well-known in the art. A sequencing library comprises a plurality of double-stranded nucleic acid molecules and is suitable for massive parallel sequencing and accordingly, is suitable for next generation sequencing. The plurality of double-stranded nucleic acid molecules present in the sequencing library may be linear. A sequencing library which is suitable for next generation sequencing can be prepared using methods known in the prior art. Usually, methods for preparing a sequencing library suitable for next generation sequencing includes fragmenting the DNA followed by DNA repair and end polishing and, finally, often NGS platform-specific adaptor ligation.

For example, the DNA, such a genomic DNA, cell-free DNA or cDNA, can be fragmented for example by shearing, such as sonification, hydro-shearing, ultrasound, nebulization or enzymatic fragmentation, in order to provide DNA fragments that are suitable for subsequent sequencing. The length of the fragments can be chosen based on the sequencing capacity of the next generation sequencing platform that is subsequently used for sequencing. Usually, the obtained fragments have a length of 1500bp or less, 1000bp or less, 750bp or less, 600bp or less and preferably 500bp or less as this corresponds to the sequencing capacity of most current next generation sequencing platforms. The obtained fragments may have a length that lies in a range of 20 to 550bp, 50 to 500bp, preferably 100 to 400bp, more preferred 150 to 350bp. Respective fragment sizes are particularly suitable for genomic DNA, also considering that the size of an exon is approx. 150bp to 200bp in length and respective short fragments can be efficiently sequenced using common next generation sequencing

platforms. However, also longer fragments can be used, e.g. if using next generation sequencing methods which allow longer sequence reads. Furthermore, also smaller fragment sizes (e.g. starting from 15bp) can be feasible depending on the starting material for preparing the sequencing library and the sequences of interest. The fragmented DNA can be repaired afterwards and end polished using methods known in the prior art, thereby providing for example blunt ends or A overhangs.

Preferably, adapters are ligated at the 5' and/or 3' ends of the DNA fragments, preferably at both ends of the obtained fragments. The specific design of the adapters depends on the next generation sequencing platform to be used and for the purposes of the present invention, basically any adapters used for preparing sequencing libraries for next generation sequencing can be used. In a preferred embodiment, at least one adapter sequence is provided which allows hybridization to at least a portion of the single-stranded oligonucleotide of the base particles. The adapter sequences provide a known sequence composition allowing e.g. subsequent library amplification and/or sequencing primer annealing. As adapters, double-stranded or partially double-stranded nucleic acids of known sequence can be used. The adapters may have blunt ends, cohesive ends with 3' or 5' overhangs, may be provided by Y shaped adapters or by stem-loop shaped adapters. The adapters have a length of at least 7, preferably at least 10, preferably at least 15 bases. The adapter length preferably lies in a range of 10 to 100 bases, preferably 15 to 75 bases, more preferred 20 to 60 bases. Either the same or different adapters can be used at the 3' and 5' end of the fragments. In a preferred embodiment of the invention, at least one of the adapters is at the sense or antisense strand complementary to the single-stranded oligonucleotide of the base particles. Optionally, the adapters may also provide an individual index thereby allowing the subsequent pooling of two or more target enriched sequencing libraries prior to sequencing.

The nucleic acid template to be amplified by emulsion PCR may thus be a population of DNA such as e.g. a DNA library. It is preferred that each member of the DNA population has a common nucleic acid sequence at the first end and a common nucleic acid sequence at the second end. This can be achieved by providing an adapter DNA sequence at each end. The nucleic acid template may be provided by a PCR product comprising adapter sequences at each end.

Sequencing

As disclosed above, the present method can be embedded within a sequencing workflow. After enrichment, an enriched fraction of first type of particles is provided, wherein the particles comprise double-stranded amplicons. The double-stranded amplicons result from clonal amplification so that the amplicons on a single particle are the same or are essentially the same (in view of potential amplification errors) as they were obtained from the same original template molecule. Thereby particles are generated, wherein each particle comprises

only one type of amplicon with a high density. Particles without any amplified target nucleic acids (second type particles, also referred to as “null beads”) are depleted in the enrichment step.

5 The methods may further comprise using the enriched fraction of first type of particles as a sample for sequencing. The method may accordingly encompass sequencing the amplicons attached to the particle. Sequencing may be performed using any known sequencing method and sequencing device. For instance, a GeneReader System (Qiagen) may be used. Importantly, this invention is not limited to a particular applied sequencing device capable of
10 sequencing amplicons attached to a particle as substrate. In one embodiment, sequencing is performed by a sequencing by synthesis method.

In one embodiment, next generation sequencing of the amplicons bound to the first type particles is performed. Hence, the amplified nucleic acids comprised in the first type nucleic
15 acids may be sequenced. Before sequencing, the enriched particles of the first type may be subjected to conditions that induce denaturation of the double-stranded amplicons to generate single-stranded template nucleic acids. Additionally, primers may be present when denaturation occurs. An exemplary sequencing process comprises the hybridization of a primer, and the preparation of a flow cell. Afterward the sample is loaded into the device and
20 sequencing is started. In one embodiment, the GeneReader sequencing method is applied, which may comprise FASTQ-file generation.

The sequencing may be followed by a data analysis step.

25 **Automation**

In one embodiment, the various methods and processes described above are automated. For example, the enriching method may be performed using an automated sample processing system. The system may have regions for particular tasks, e.g. centrifugation, to which and from which materials, e.g. tubes containing particles, are moved by a robotic arm
30 or the like. The regions may have platforms, drawers, or decks. The commercially available QIAcube from QIAGEN is equipped with an automated centrifuge and pipetting system which can be programmed to do all or a portion of the method steps with limited human intervention. Automated systems suitable for processing magnetic particles are also known in the art.

35 The methods of the present invention may also be applied for particles used for other nucleic-acid based applications in the fields of basic biological research, biomedical research, applied testing, and molecular diagnostics. Applications include, but are not limited to, clonal amplification of specific DNA fragments on the surface of microspheres by
40 polymerase chain reaction or other amplification methods, and specific isolation of nucleic acids/ nucleic acid with oligonucleotide-conjugated particles by hybridization-based methods.

The method could also be used for decreasing unspecific binding observed for other hybridization based products of nucleic acids (e.g. Oligotex mRNA kits). Further potential applications include enrichment of emulsion PCR live beads (i.e. first type particles), enrichment of particles with desired DNA/RNA sequences, and/or capture of specific DNA and RNA targets (the method is applicable to non-particle based methods for capturing nucleic acids, e.g. via the surface of a tube).

The term "particle" refers to discrete, small objects that may be in various shapes, such as a sphere, capsule, polyhedron, and the like. It preferably is a sphere. In case of a sphere, it is not contemplated to have a perfectly round sphere but rather an approximate round or ellipsoidal shape, which may have a rough and/or structured surface. Moreover, the particles may be polydisperse having different properties within one or multiple populations of applied particles. Moreover, the term "bead" may be interchangeably used with the term "particle". The term "first type particle" or "particles of a first type" may be interchangeably used with the term "first type of particles", the term "second type particle" or "particles of a second type" may be interchangeably used with the term "second type of particles", and the term "third type particle" or "particles of a third type" may be interchangeably used with the term "third type of particles".

As used herein, the term "comprising" is to be construed as encompassing both "including" and "consisting of", both meanings being specifically intended, and hence individually disclosed embodiments in accordance with the present invention.

This invention is not limited by the exemplary method disclosed herein, and any methods, uses, systems and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of this invention. Numeric ranges are inclusive of the numbers defining the range. The headings provided herein are not limitations of the various aspects or embodiments of this invention which can be read by reference to the specification as a whole.

As used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Reference to "the disclosure" and "the invention" and the like includes single or multiple aspects taught herein; and so forth. Aspects taught herein are encompassed by the term "invention".

It is preferred to select and combine preferred embodiments described herein and the specific subject-matter arising from a respective combination of preferred embodiments also belongs to the present disclosure.

EXAMPLES

1. Material and methods

5 The technology of the present invention is now illustrated by the following examples, wherein side-by-side experiments were performed in comparison with a prior art method using the QIAGEN GeneReader workflow:

10 Prior art method: Enrichment using the commercially available QIAGEN Clonal Amplification workflow (Clonal Amplification kit, Cat. No. 185001, 2016; GeneRead QIAcube, Cat. No. 9002345).

15 Method according to the invention: A hybridizing agent consisting of an antisense oligonucleotide is added to the enrichment buffer at 10 μ M final concentration. The enrichment buffer is part of Clonal Amplification kit, Cat. No. 185001.

The antisense oligonucleotide is reverse complementary to the single-stranded (capture) oligonucleotide of the base particles used in the particle based emulsion PCR. The antisense oligonucleotide of the hybridizing agent has the following sequence: 5' ACT GGC CGT CGT TTT ACA AAA AAA AAA 3'

20 All other parameters are identical to the control condition. Hence, in the illustrated embodiment, the antisense oligonucleotide (hybridizing agent) is added to the particles (composition comprising live beads and null beads) generated using the QIAGEN GeneReader workflow after breaking the emulsion and before separating the live beads from the null beads.

25 All samples were processed through the GeneReader workflow and sequenced side-by-side using the QIAGEN GeneRead Advanced Sequencing Q Kit (3), Cat. No. 185231; QIAGEN GeneReader with APF Instrument Configuration.

30 The exact configuration (kits and protocols) of the set-up of the examples is given in **Fig. 4**:

- Prior art configuration: according to the overview in the figure.
- Method according to the invention: same configuration, however with the modification that the antisense oligonucleotide was added as hybridizing agent to the enrichment buffer prior to starting the enrichment (process step is highlighted in Fig. 4 by a box and arrow).

35

2. Results

40 A total of 6 Flow Cells were generated for the prior art (control) and the method according to the invention, whereon an antisense oligonucleotide was added as hybridizing agent to the enrichment buffer. The results (summarized in Table 1) clearly indicate the benefit of the

invention. The enrichment of live beads by effectively depleting null beads was improved compared to the prior art when adding the hybridizing agent that forms a hybrid with the single-stranded oligonucleotide present on the null beads, thereby reducing unspecific binding. Both the number of output bases and the number of mapped reads was increased with the invention. The normalized output bases and mapped reads are higher with the invention than with the prior art condition for the enriched sample (35% and 36% higher, respectively). The results demonstrate the effectiveness of the present invention in depleting null beads. This increased usable output will improve any NGS application. For example, it will allow mixing more patient samples in a single sequencing run (multiplexing).

Sequencing Chemistry	Panel	Condition	Number of flow cells*	output (bases)	mapped reads	error rate
GeneRead Advanced Sequencing Q Kit	GeneRead QIAact Actionable Insights Tumor Panel	Prior art	6	100%	100%	100%
		Invention	6	135%	136%	100%

Table 1: Results of evaluation of hybridizing agent in the GeneReader workflow. All values are normalized to the control condition. *Each flow cell is corresponding to an independent enrichment experiment.

CLAIMS

1. A method for enriching a first type of particles comprising double-stranded nucleic acid molecules from a composition additionally containing a second type of particles comprising a single-stranded oligonucleotide, wherein the method comprises
- adding a hybridizing agent and forming a hybrid with the single-stranded oligonucleotide; and
 - separating first type of particles from second type of particles.
2. The method according to claim 1, wherein the separation comprises binding the first type of particles to a third type of particles and separating the third type of particles with the bound first type of particles from second type of particles, optionally wherein the hybridizing agent is comprised in a composition comprising the third type of particles and/or an enrichment reagent that is added to support binding of the first type of particles to the third type of particles.
3. The method according to claim 2, wherein binding of the first type of particles to the third type of particles is mediated by an interaction pair, optionally wherein
- (i) the first partner of the interaction pair is associated with the double-stranded nucleic acid molecules of the first type of particles and
 - (ii) the second partner of the interaction pair is associated with the third type of particles.
4. The method according to claim 3, wherein the interaction pair has one or more of the following characteristics:
- (aa) the interaction pair is an affinity interaction pair, preferably selected from (i) biotin and an biotin binding polypeptide, (ii) antibody and antigen and (iii) ligand and receptor;
 - (bb) the first partner of the interaction pair is biotin associated with the double-stranded nucleic acid molecules of the first type of particles and the second partner is a biotin binding polypeptide, preferably streptavidin or avidin, associated with the third type of particles.
5. The method according to any one of claims 2 to 4, wherein the separation step comprises one or more of the following:
- (aa) separating the third type of particles with the bound first type of particles based on their larger size compared to second type of particles;

(bb) separating the third type of particles with the bound first type of particles by passing the composition through a porous device, wherein the third type of particles with the bound first type of particles are retained, while particles of the second type pass through;

5 (cc) separation comprises density centrifugation;

(dd) separating the third type of particles with the bound first type of particles is performed using a magnet.

10 6. The method according to one or more of claims 1 to 5, wherein the particles of the first type have one or more of the following characteristics:

(aa) the double-stranded nucleic acid molecule is a double-stranded amplicon;

(bb) different particles of the first type comprise different double-stranded amplicons;

15 (cc) each particle of the first type comprises multiple copies of the same double-stranded amplicon; and/or

(dd) the particles are magnetically responsive.

20 7. The method according to one or more of claims 1 to 6, wherein the second type of particles has one or more of the following characteristics:

(aa) each particle comprises multiple copies of and is preferably coated with the single-stranded oligonucleotide;

(bb) the single-stranded oligonucleotide is exposed on the particle surface;

25 (cc) the single-stranded oligonucleotide has a length within the range of 10nt to 75nt, preferably 15nt to 50nt or 20nt to 35nt; and/or

(dd) the particles are magnetically responsive.

30 8. The method according to one or more of claims 1 to 7, wherein the first type of particles and the second type of particles are provided by the same type of base particle comprising the single-stranded oligonucleotide, wherein preferably, the surface of the base particle comprises multiple copies of the single-stranded oligonucleotide, and wherein the first type of particles differs from the second type of particles in that the particles of the first type comprise a double-stranded amplicon and wherein the second type of particles corresponds to the base particle.

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9. The method according to one or more of claims 1 to 8, wherein the hybridizing agent has one or more of the following characteristics:

(aa) it is provided by at least one single-stranded oligonucleotide,

- (bb) it is provided by two or more different single-stranded oligonucleotides capable of hybridizing at least to a portion of the single-stranded oligonucleotide of the second type of particles;
- (cc) it is provided by at least two single-stranded oligonucleotides that hybridize to different regions of the single-stranded oligonucleotide of the second type of particles;
- (dd) it hybridizes at least to the outer end of the single-stranded oligonucleotide and preferably provides a hybrid with a blunt end;
- (ee) it forms a hybrid over at least 60%, preferably at least 70%, more preferably at least 80%, at least 90% or over the entire length of the single-stranded oligonucleotide;
- (ff) it is selected from the group consisting of DNA, RNA, PNA, LNA and HNA, preferably from the group consisting of DNA and RNA, more preferably DNA;
- (gg) it comprises nucleobases, wherein the nucleobases are selected from A, G, T/U, C, and chemical derivatives thereof; and/or
- (hh) it comprises chemical moieties capable of hybridizing to one or more of A, G, T/U, or C.

10. The method according to one or more of claims 1 to 9, wherein the hybridizing agent forms a hybrid with the single-stranded oligonucleotide of the second type of particle that has one or more of the following characteristics:

- (aa) a double-stranded hybrid is formed over $\geq 60\%$, $\geq 70\%$, $\geq 80\%$, $\geq 90\%$ or $\geq 95\%$ of the single-stranded oligonucleotide or is formed over the entire length (100%) of the single-stranded oligonucleotide of the second type particles;
- (bb) the formed hybrid leaves a portion of the single-stranded oligonucleotide single-stranded, optionally wherein
- (i) the single-stranded portion may be $\leq 40\%$, $\leq 30\%$, $\leq 20\%$ or $\leq 10\%$ of the single-stranded oligonucleotide;
- (ii) the single-stranded portion may have a length of $\leq 10\text{nt}$, $\leq 8\text{nt}$ or $\leq 5\text{nt}$; and/or
- (iii) single-stranded portion may be provided at the 5' end of the single-stranded oligonucleotide, wherein the 5' end corresponds to the inner end directed towards the particle surface.
- (cc) the formed hybrid comprises a blunt end, wherein the blunt end is formed at the outer end of the single-stranded oligonucleotide, wherein the outer end corresponds to the end of the oligonucleotide that is directed away from the particle surface;
- (dd) the formed hybrid comprises or does not comprise at least one mismatch in the formed double-stranded region;

- (ee) it comprises a blunt end, wherein the blunt end is formed at the outer end of the single-stranded oligonucleotide, wherein the outer end corresponds to the end of the oligonucleotide directed away from the particle.

5 11. The method according to one or more of claims 1 to 10, wherein the hybridizing agent is contacted two or more times with the composition prior to separating the first type of particles for enrichment.

10 12. The method according to one or more of claims 1 to 11, wherein the method is carried out subsequent to a particle-based emulsion polymerase chain reaction, preferably after breaking the emulsion.

13. A method of amplifying a template and enriching the provided amplicon, wherein the method comprises:

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(a) providing an emulsion comprising one or more aqueous compartments in oil, at least a portion of said compartments comprising

(i) amplification agents,

20 (ii) a particle comprising a single-stranded oligonucleotide, wherein said single-stranded oligonucleotide is capable of acting as a primer and wherein the particle comprises multiple copies of said single-stranded oligonucleotide on the particle surface, and

(iii) a template molecule;

25 wherein other compartments comprise one or both of (i) and (ii) but do not comprise (iii) a template molecule;

(b) exposing said emulsion to amplification conditions, wherein after amplification at least two types of particles are provided, wherein the particles of the first type comprise a double-stranded amplicon and the particles of the second type comprise the single-stranded oligonucleotide but no double-stranded amplicon;

30 (c) optionally recovering comprised particles from the emulsion;

(d) enriching particles of the first type using the method according to any one of claims 1 to 11.

35 14. The method according to claim 13, wherein the amplification agents have one or more of the following characteristics:

(aa) they comprise agents selected from a polymerase, dNTPs, buffer and a second primer; and/or

40 (bb) they comprise a second primer comprising a first partner of an interaction pair, and wherein a third type of particles is used in step (d) for separation which comprises the second partner of said interaction pair, optionally wherein said interaction pair is biotin and streptavidin.

15. The method according to claim 13 or 14, wherein step c) is performed and comprises
(i) breaking the emulsion and recovering the comprised particles from the broken
emulsion, and
5 (ii) optionally further purifying, e.g. washing, the recovered particles,
thereby providing a composition comprising the first and second type of particles.

16. The method according to any one of claims 13 to 15, wherein the single-stranded
oligonucleotide has one or more of the following characteristics:

- 10 (i) it has a length within the range of 10nt to 75nt, preferably 15nt to 50nt or 20nt to 35nt;
(ii) it comprises a hybridizing region that is complementary to a portion of the template,
wherein said portion preferably corresponds to an adapter sequence of a sequencing
15 library, optionally wherein said hybridizing region has a length of 50nt or less,
preferably 30nt or less, more preferably 25nt or less; and/or
(iii) it comprises a spacer region comprising of consisting of a nucleotide stretch of the
same nucleotide, preferably T, wherein said spacer region is at the proximity of the
surface of the particle.

20 17. The method according to one or more of claims 13 to 16, wherein the hybridizing agent is
added (i) in step (c) during purification of the particles after breaking the emulsion, preferably
during washing the particles; (ii) when adding the third type of particles, wherein the
hybridizing agent is preferably comprised in an enrichment solution; and/or (iii) when
25 separating the third type of particles with the bound first type of particles from the remaining
particles.

18. The method according to one or more of claims 13 to 17, wherein the hybridizing agent is
comprised in at least one reagent that is used in step (c) and/or step (d), preferably in a
30 reagent selected from a washing solution, the composition comprising the third type of
particles, and the enrichment solution, wherein preferably the hybridizing agent is added to at
least one, at least two or more preferably all three reagents.

19. The method according to any one of claims 13 to 18, further comprising using the
35 enriched particles comprising the double-stranded amplicons for sequencing, wherein
preferably, the sequencing steps are performed on each individual bead.

20. A kit for enriching a first type of particles comprising double-stranded nucleic acid
molecules from a composition additionally containing a second type of particles comprising a
40 single-stranded oligonucleotide, wherein the kit comprises:
- particles of a third type;

- a porous device, capable of retaining the third type of particles;
- a hybridizing agent capable of forming a hybrid with the single-stranded oligonucleotide of the second type of particles.

5 21. The kit according to claim 20, having one or more of the following characteristics

- (i) the third type of particles comprises a biotin binding polypeptide;
- (ii) it comprises a reagent suitable for allowing binding between biotin and a biotin binding protein, optionally wherein the reagent comprises the hybridizing agent;
- 10 (iii) it comprises a wash reagent, optionally wherein the wash reagent comprises the hybridizing agent;
- (iv) it comprises a reagent for breaking an emulsion, optionally wherein the reagent comprises the hybridizing agent;
- (v) it comprises a reagent suitable for releasing bound particles of the first type from the particles of the third type;
- 15 (vi) it comprises base particles comprising a single-stranded oligonucleotide, wherein the hybridizing agent is capable of forming a hybrid with said single-stranded oligonucleotide; and/or
- 20 (vii) it comprises amplification agents, preferably PCR reagents.

Fig. 1

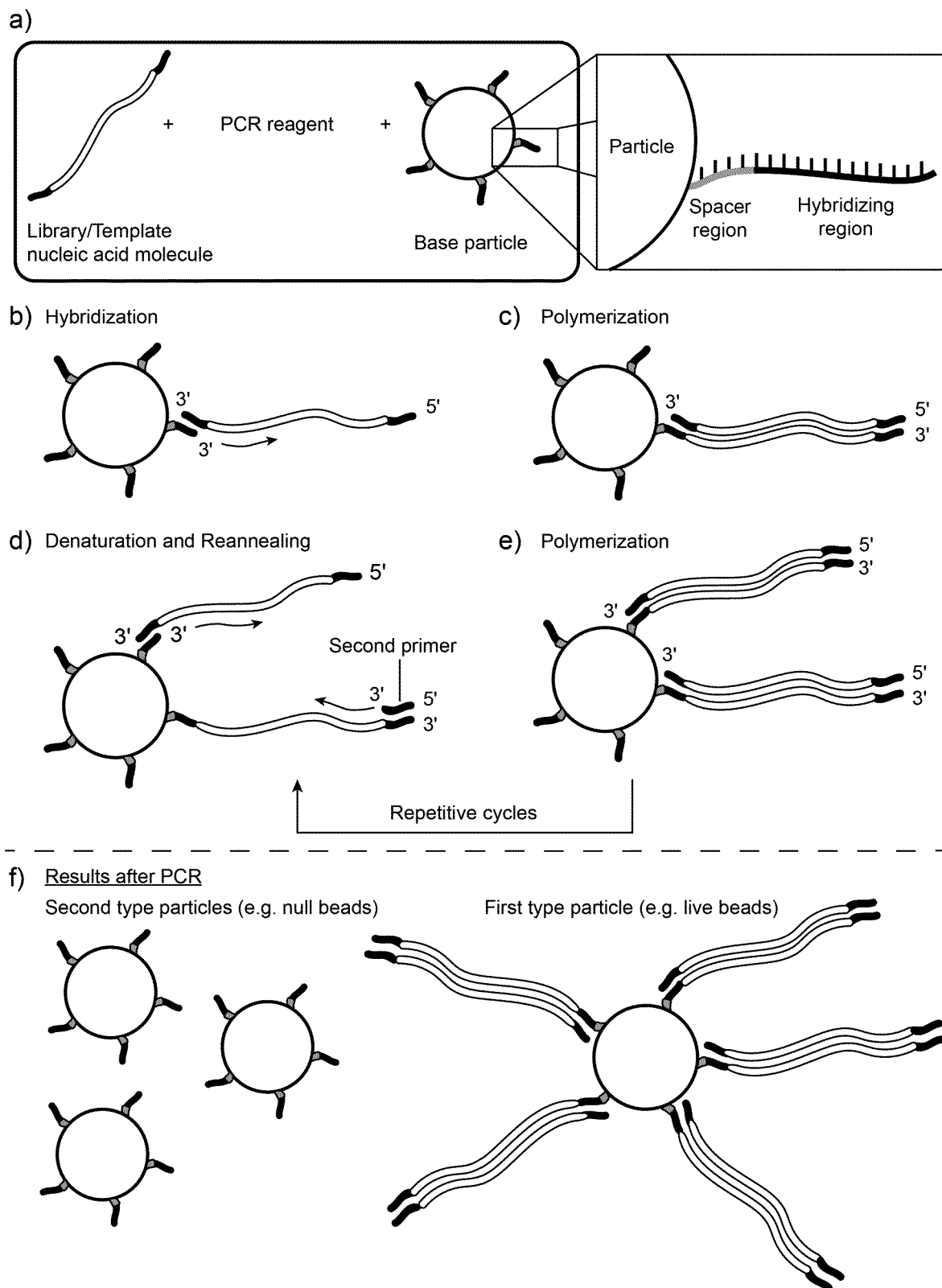


Fig. 2

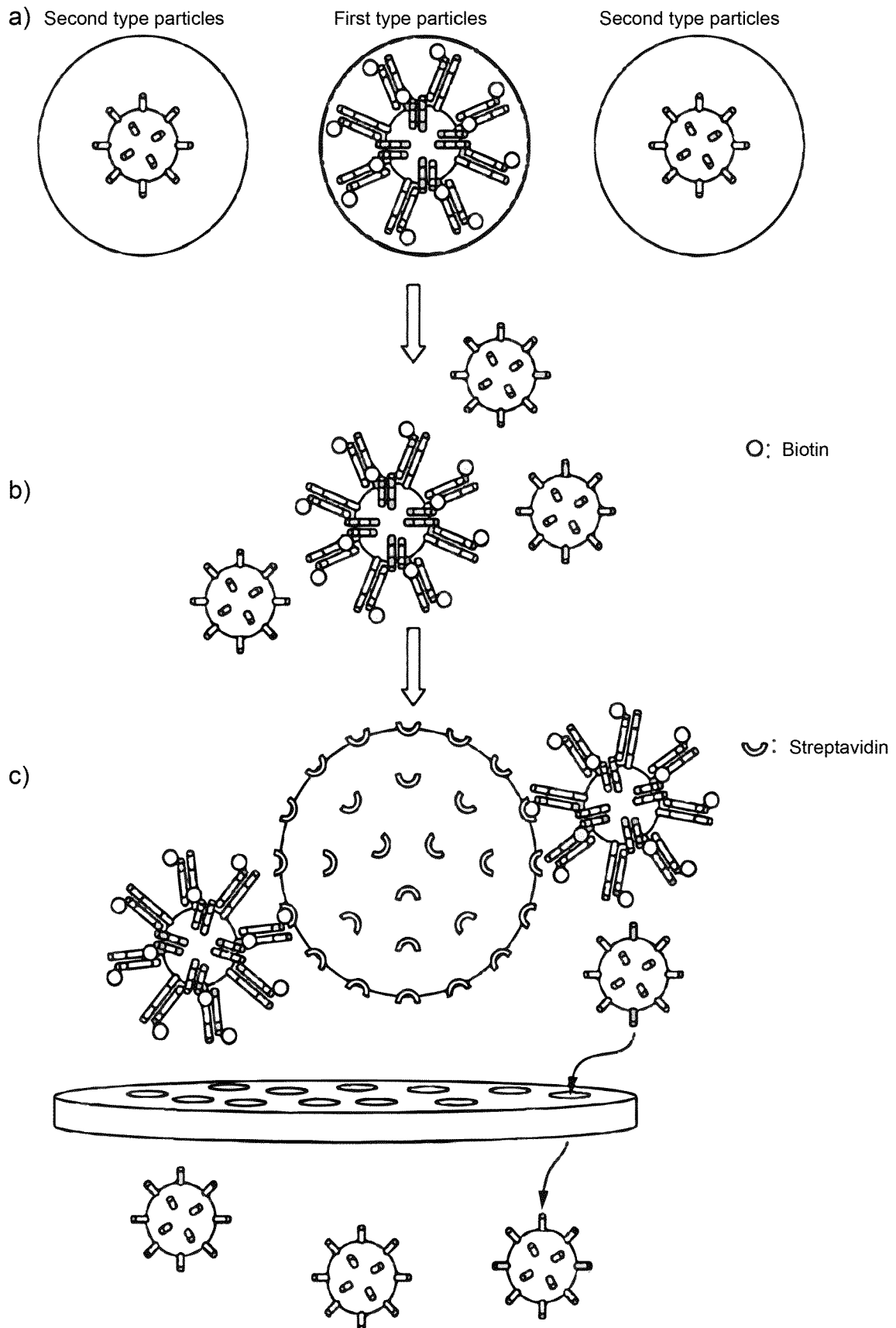


Fig. 3

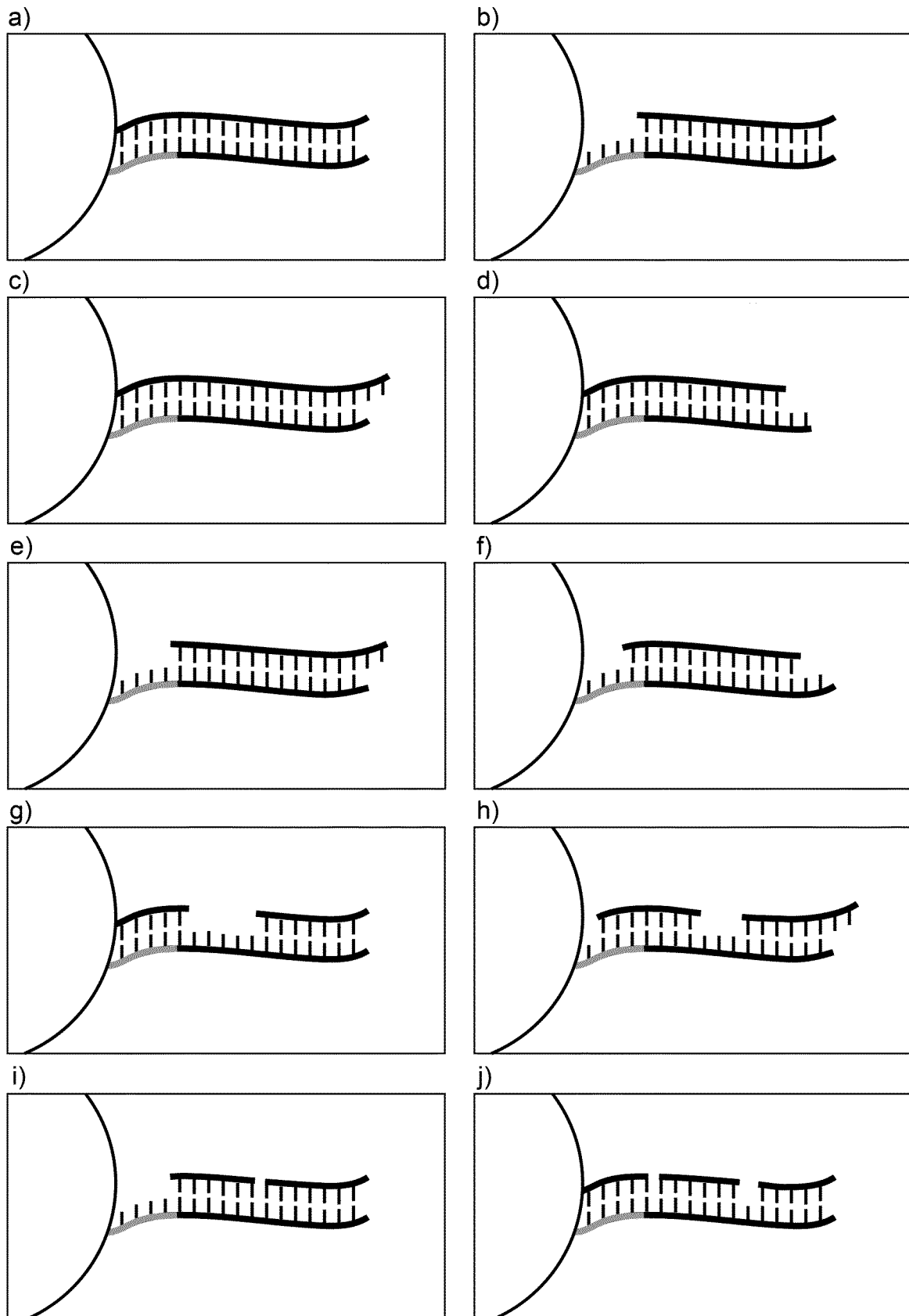


Fig. 4

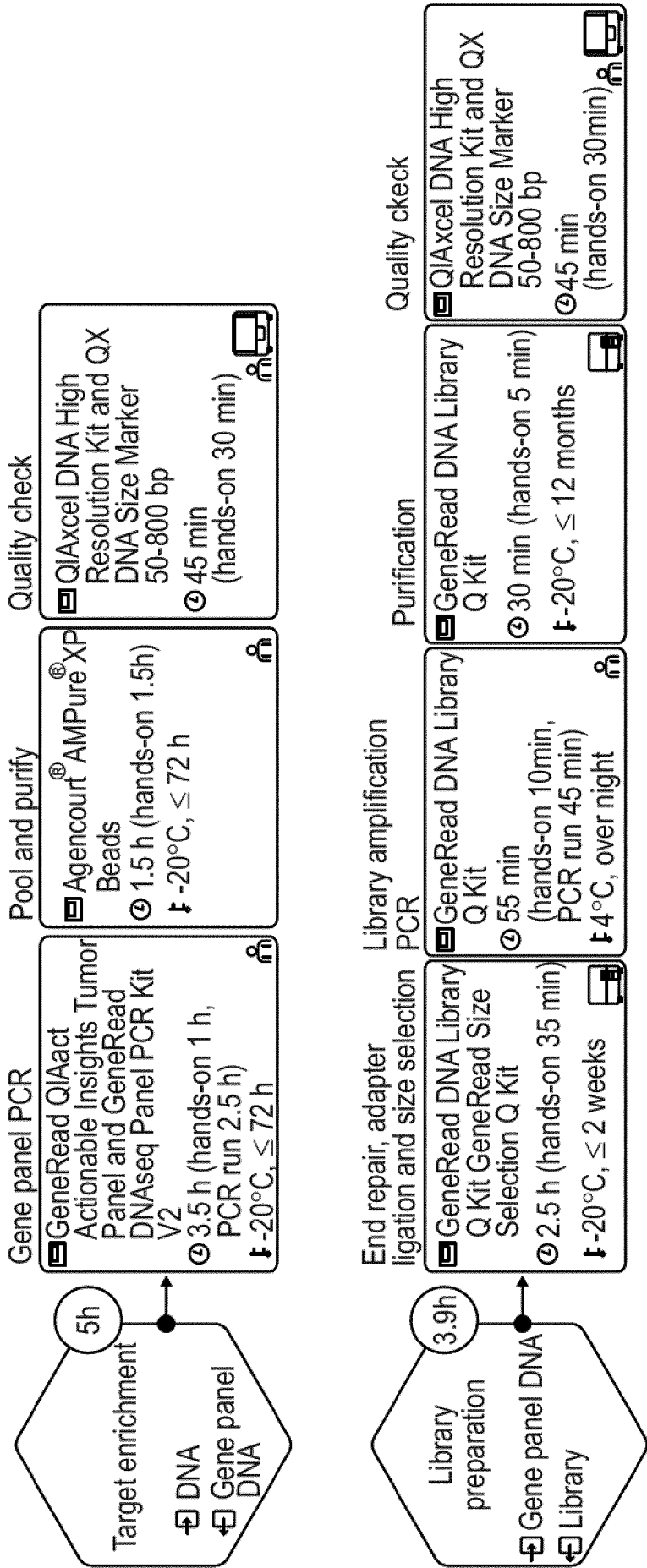
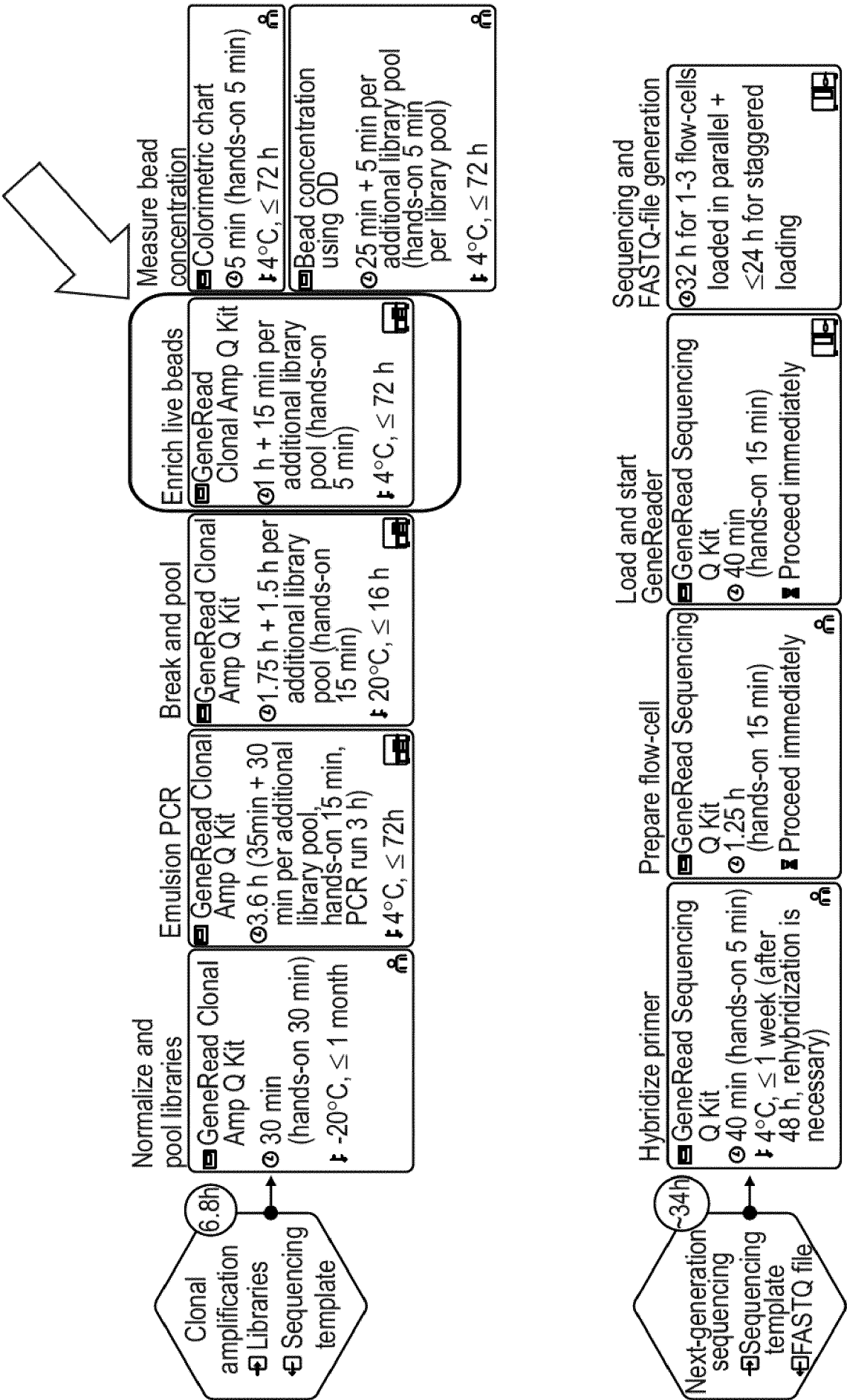


Fig. 4 - continued



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/087061

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/6806
ADD.

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)
C12Q

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

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

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y A	WO 2015/191877 A1 (LIFE TECHNOLOGIES CORP [US]) 17 December 2015 (2015-12-17) paragraph [0004]; figures 1,3,5; examples 1,2 paragraph [0004] paragraph [0014] paragraph [0032] - paragraph [0034] -----	1,6-19 2-5 20,21
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Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search

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31/03/2020

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/087061

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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