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(54) Title: METHODS OF ATTACHING ADAPTERS TO SAMPLE NUCLEIC ACIDS

(57) Abstract: Methods of preparing double-stranded nucleic acids with single-stranded overhangs for amplification and sequencing are disclosed. Contacting a blunt-ended double-stranded nucleic acid molecules with Taq results in non-templated directed addition of a single nucleotide to the 3' ends of the nucleic acid with A added most frequently followed by G followed by C and T. G tailing is sufficiently frequent that the efficiency of ligation of nucleic acid molecules to adapters can be significantly increased by including adapters tailed with T and C. The ligation efficiency can be increased even further with blunted-ended adapters to ligate to blunt-ended nucleic acid molecules that failed to undergo tailing.

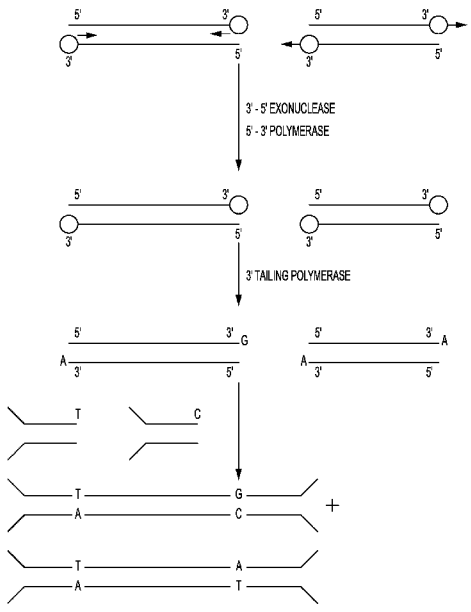

FIG. 1

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[Continued on next page]

Declarations under Rule 4.17:

— *as to the identity of the inventor (Rule 4.17(i))*
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

Published:

— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

(88) Date of publication of the international search report:
21 February 2019 (21.02.2019)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/27632

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2018.01)
CPC - C12Q 1/6806, C21Q 1/6869

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2011/0319299 A1 (OSBORNE et al.) 29 December 2011 (29.12.2011) abstract, para [0049], [0113], [0114]	1-4, 22-24, 27
Y	OHTSUBO et al., "Efficient N-tailing of blunt DNA ends by Moloney murine leukemia virus reverse transcriptase" Scientific Reports, 02 February 2017, Vol 7, page 41769 (pp 1-10). abstract, pg 2, para 9, Fig. 1, legend	1-4, 22-24, 27
Y	WO 2016/019360 A1 (DOVETAIL GENOMICS LLC) 04 February 2016 (04.02.2016) para [0008], [0012], [0014], [0015]	24, 27

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
16 October 2018	11 JAN 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/27632

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

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

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

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

3. ☒ Claims Nos.: 5-21, 25, 26, 28-39
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: claims 1-4, 22-24, 27, drawn to a method of preparing nucleic acids for analysis comprising annealing an adapter to a double stranded DNA.

Group II: claims 40-42, drawn to a kit comprising a pair of at least partially double stranded adapters.

----continued on extra sheet----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4, 22-24, 27

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/27632

Continuation of:
Box No. III. Observations where unity of invention is lacking

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I includes the special technical feature of a method comprising annealing a partially double stranded adapter to a double stranded DNA, not required by Group II.

Group II includes the special technical feature of a composition comprising a pair of adapters, not required by Group I.

Common Technical Features

The inventions of Groups I and II share the technical feature of partially double stranded adapters with T and C overhang at a 3'-end.

However, these shared technical features do not represent a contribution over prior art in view of US 2011/0319299 A1 to Osborne et al., (hereinafter 'Osborne') and the article "Efficient N-tailing of blunt DNA ends by Moloney murine leukemia virus reverse transcriptase" by Ohtsubo et al., (hereinafter 'Ohtsubo') (Scientific Reports, 02 February 2017, Vol. 7, No. 41769, pages 1-10).

Osborne teaches (instant claim 1) a method of preparing nucleic acids for analysis (abstract - "Aspects of the present invention are drawn to methods and compositions for the genetic analysis of regions of interest (ROI) from one or more starting polynucleotide sample(s). In certain aspects, adapter tagged polynucleotide fragments from a plurality of initial polynucleotide samples are pooled, circularized and amplified to produce an immortalized library"), comprising:
(a) blunt-ending double-stranded nucleic acids with single-stranded overhangs in a sample by the action of one or more enzymes providing a 5'-3' polymerase activity and 3'-5' proofreading activity, and four standard nucleotide types, wherein single-stranded overhangs with 5' ends serve as templates for extension of a complementary strand by the polymerase activity and single-stranded overhangs with 3' ends are digested by the proofreading activity producing blunt-ended nucleic acids (para [0049] - " Preparing the ends of fragments for ligation, e.g., to an adapter, plasmid, other fragments, etc., is sometimes referred to herein as "polishing"...As such, polishing refers to any step or steps used to produce ligation-compatible ends. Ligation-compatible ends may have any configuration, e.g., be blunt ends"; para [0113] - "The ends of fragments 302 are made blunt by treatment with T4 polynucleotide kinase, T4 DNA polymerase and/or Klenow enzyme...T4 DNA polymerase and Klenow provide both 3'-5' exonuclease activity and polymerase (or 5' "fill-in") activity"; FIG. 3A step 302 shows end repair using a T4 DNA polymerase and/or Klenow DNA polymerase to generate blunt-end DNA);
(b) without separating the blunt-ended nucleic acids from other components of the sample, end-tailing the blunt-ended nucleic acids by action of a polymerase without a 3'-5' proofreading function, which preforms a directed addition of a nucleotide to the 3' ends of blunt-ended nucleic acids, wherein A is added preferentially (para [0065] - "one could add a specific functional domain onto a polynucleotide of interest using a tailed primer in an extension reaction at any point in the workflow"; para [0113] - "Blunt fragments 303 are then A-tailed by treatment with dATP and Klenow fragment lacking 3'-5' exonuclease activity"; para [0068] - "In certain other embodiments, non-proofreading polymerases are employed"; FIG. 3A step 303 shows A-tailing the 3' end of the blunt DNA);
(c) annealing the nucleic acids from step (b) with at least partially double stranded adapters with a single nucleotide T or C overhang at a 3'-end; and (d) ligating the nucleic acids to the adapters (para [0098] - "combinatorial adapter can be produced by annealing two oligos, both of which have MID sequences (e.g., MID1' and MID2' as shown in the adapter of FIG. 2D)"; para [0052] - "Ligation-compatible ends (e.g., of a fragment and an adapter) can be ligated to one another under appropriate ligation conditions, for example in the presence of an enzyme having DNA ligase activity in appropriate buffering conditions and co-factors"; para [0090] - "As shown in FIG. 2, each adapter includes two nucleic acid strands - a top strand and a bottom strand - that hybridize to one another to form clamp region 201. When ligated to a compatible end of a nucleic acid fragment via ligation site 204 (which in this case includes a 3' T overhang"; para [0114] - "As shown in FIG. 3B, these A-tailed fragments 304 are then ready for ligation to adapters 305 having a compatible ligation site (e.g., having a 3' T overhang; adapter 305 is shown after attachment to fragments 304)"; FIG. 3B step 304 shows ligating adapters to the ends of the DNA).

Osborne does not specifically teach a method of preparing nucleic acids for analysis, comprising: end-tailing the blunt-ended nucleic acids by action of a polymerase without a 3'-5' proofreading function, which performs a non-template directed addition of a nucleotide to the 3' ends of blunt nucleic acids, wherein A is added preferentially to G preferentially to C or T. Ohtsubo teaches Moloney murine leukemia virus (MMLV-RT) shows strong template-independent polymerase activity using blunt DNA ends as substrate that generates 3' overhangs of A, C, G or T, wherein nucleotides were appended efficiently in the order of: A>G>T>C (abstract - "Moloney murine leukemia virus reverse transcriptase (MMLV-RT) is a widely used enzyme for cDNA synthesis. Here we show that MMLV-RT has a strong template-independent polymerase activity using blunt DNA ends as substrate that generates 3' overhangs of A, C, G, or T. Nucleotides were appended efficiently in the order A > G > T > C, and tail lengths varied from 4 to 5, 2 to 7, 2 to 4, and 2 to 3 for A, C, G, and T, respectively"). Given that Osborne teaches a method of preparing nucleic acids for analysis, comprising: blunt-ending double-stranded nucleic acids with single-stranded overhangs; end-tailing the blunt-ended nucleic acids by action of a polymerase without a 3'-5' proofreading function, which performs a directed addition of a nucleotide to the 3' ends of blunt-ended nucleic acids, wherein A is added preferentially; annealing the nucleic acids from step (b) with at least partially double-stranded adapters with a single nucleotide T overhand at a 3'-end; and ligating the nucleic acids to the adapters, and Ohtsubo teaches MMLV-RT shows strong template-independent polymerase activity, wherein nucleotides were appended efficiently in the order of: A>G>T>C, one of ordinary skill in the art would have found it obvious to combine the method taught by Osborne with the template-independent polymerase activity of MMLV-RT taught by Ohtsubo, and thus, to have an improved method of preparing nucleic acids for analysis.

As said technical features were known in the art at the time of the invention, these cannot be considered special technical features that would otherwise unify the groups.

Groups I and II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.