(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 July 2018 (19.07.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/132392 A3

(51) International Patent Classification:
C12Q 1/68 (2018.01) *G01N 33/53* (2006.01)

(21) International Application Number:
PCT/US2018/013019

(22) International Filing Date:
09 January 2018 (09.01.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

62/444,734	10 January 2017 (10.01.2017)	US
62/546,418	16 August 2017 (16.08.2017)	US
62/546,836	17 August 2017 (17.08.2017)	US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: MULTIPLEXED SIGNAL AMPLIFICATION

(57) Abstract: Provided herein, in some embodiments, are methods and compositions for highly multiplexed *in situ* signal amplification via hairpin-mediated concatemerization.

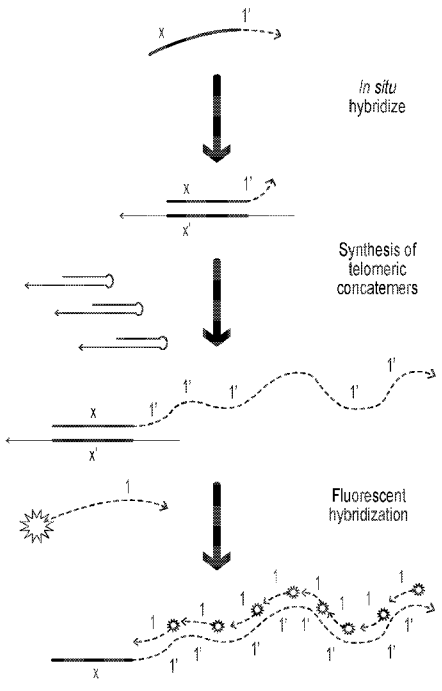

FIG. 4

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[Continued on next page]

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:
23 August 2018 (23.08.2018)

INTERNATIONAL SEARCH REPORT

International application No.
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Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

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

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

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

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

3. ☒ Claims Nos.: 4-17, 22-29
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 1-3, 30, 32, 33, drawn to a multiplexed target detection method for detecting a plurality of nucleic acid targets, and a composition comprising a nucleic acid target bound to a concatemers of tandem repeat sequence.

Group II: claims 18-21, 31, drawn to a multiplexed target detection method for detecting a plurality of protein or peptide targets, and a composition comprising a protein target indirectly linked to a concatemers of tandem repeat sequence.

- Please see extra sheet for continuation -

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

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

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

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

Remark on Protest

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

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

☒ No protest accompanied the payment of additional search fees.

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International application No.
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A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68, G01N 33/53 (2018.01)
CPC - C12Q 1/6853, C12Q 1/6806, C12Q 1/6804, G01N 33/5308, G01N 2458/10

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2003/0165917 A1 (Ullman et al.) 04 September 2003 (04.09.2003) para [0023], [0019], [0049], [0051], [0056], Figs. 1, 5	30 ----- 1-3, 18-21, 32, 33
X -- Y	US 2007/0003950 A1 (Shen et al.) 04 January 2007 (04.01.2007) para [0023], [0086], [0095], [0162]	31 ---- 18-21
Y	WO 2016/123419 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 04 August 2016 (04.08.2016) pg 9, ln 14 to pg 10, ln 7, Fig. 3A	1-3, 18-21
Y	Wu et al. "A Nonenzymatic Hairpin DNA Cascade Reaction Provides High Signal Gain of mRNA Imaging inside Live Cells" J. Am. Chem. Soc. 2015, 137, 4900-4903; pg 4900, col 2, para 3, to pg 4901, col 1, para 1, Fig. 1	32, 33

☐ 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
03 July 2018	11 JUL 2018

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

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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 for detecting nucleic acid targets, not required by Group II.

Group II includes the special technical feature of a method for detecting protein targets, not required by Group I.

Common Technical Features

The inventions of Groups I and II share the technical feature of a multiplexed target detection method and a composition comprising a target linked to a concatemers of tandem repeat sequence.

However, these shared technical features do not represent a contribution over prior art in view of US 2003/0165917 A1 to Ullman et al. (hereinafter 'Ullman') and WO 2016/123419 A1 to PRESIDENT AND FELLOWS OF HARVARD COLLEGE (hereinafter 'Harvard').

Ullman teaches (instant claim 1) a multiplexed target detection method (para [0023], The method...in detecting one or more target nucleic acids, particularly where multiplexing is desirable.), comprising:
(a) combining a sample containing a plurality of nucleic acid targets with a plurality of probe strands, each probe strand comprising (i) an unpaired 5' target domain complementary to one of the nucleic acid targets (para [0051], The first example is shown in FIG. 1. This example is best suited to detection of RNA and mixtures of RNA....The long strand of the stem/loop probe can hybridize to target nucleic acid.) and (ii) an unpaired 3' primer domain (para [0051], The short strand is initially hybridized to the long strand except for its 3' end, which comprises a 1-3 base sequence that does not hybridize to the long strand.), and producing a first reaction mixture comprising molecular targets bound to probe strands (para [0051], Upon binding to the target, the short strand is displaced and becomes available for binding to the hybridizing reagent.);
(b) combining the first reaction mixture produced in step (a) with dNTPs, strand-displacing polymerase, and a plurality of catalytic molecules (para [0019], the stem/loop probe is contacted with the sample comprising a complex mixture of nucleic acids and the hybridizing reagent, the enzyme(s) appropriate for the protocol and, when needed, the appropriate nucleotide triphosphates for nucleic acid formation.), each catalytic molecule comprising a circular DNA, and producing a second reaction mixture comprising nucleic acid concatemers bound to molecular targets (para [0056] In FIG. 5, as in the prior examples the target polynucleotide binds to a stem/loop probe with the short strand at the 3' end. Upon release of the short strand from hybridization, the short strand is available to bind to the hybridizing reagent, which in this case is circular DNA. In the presence of a DNA polymerase, the short strand is extended indefinitely with concatenated versions of the complement of the circular hybridizing reagent.);
(c) combining the second reaction mixture produced in step (b) with a plurality of signal strands, each signal strand linked to a different detectable molecule and comprising a domain complementary to the unpaired 3' primer domain of one of the probe strands, and producing concatemers labeled by a plurality of signal strands (para [0049], The amplified product may then serve to bind a labeled complementary sequence at each site, or labeled antibodies to RNA/DNA hybrids, or other conventional technique may be employed for detection.).
Ullman does not specifically teach applying a catalytic molecule comprising, 5' to 3', a first domain, a second domain, and a third domain wherein the first domain is bound to the second domain, and the third domain is an unpaired 3' toehold domain complementary to the unpaired 3' primer domain of one of the probe strands (see instant Specification, Figs. 3-4, a hairpin with 3' toehold). Harvard teaches that contacting free probe with hairpin having a complimentary region to said free probe would have produced concatemers of polynucleotide (pg 9, ln 14 to pg 10, ln 7, Fig. 3A shows a schematic overview of the autocyclic copy-and-release of an example of a hairpin-encoded DNA template domain t onto multiple copies of primer strand a, producing sequences a* - t*;....Fig. 3D shows DNA records indicative of barcodes having different lengths and different proximities to one another.....while isolated probes generate half - records.). Although Harvard does not specifically teach that concatemers of polynucleotide are generated form the autocyclic copy-and-release of hairpin-encoded DNA-template, however, concatemers of polynucleotide would have generated from the reaction (see instant Specification, Figs. 3-4). Accordingly, it would have been obvious to one of the ordinary skill in the art to have applied hairpin-encoded DNA template of Harvard for the circular DNA template of Ullman, because Harvard teaches that polynucleotides can be generated from hairpin-encoded DNA template by strand displacement.

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.