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(88) **Date of publication of the international search report**:
30 December 2009

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(54) **Title**: DETECTION OF TARGET NUCLEIC ACID SEQUENCES USING FLUORESCENCE RESONANCE ENERGY TRANSFER

(57) **Abstract**: A method for identifying a plurality of target nucleic acid molecules in a sample. The method provides a plurality of oligonucleotide probe sets. Each set comprises a first and a second probe, each having a target-specific portion and a tunable portion with an acceptor or a donor group. The first probe further comprises an endcapped hairpin. A reaction comprises a denaturation and hybridization cycle. Under the hybridization, the set of probes hybridize in a base-specific manner to their respective target nucleotide sequences, and ligate to one another to form a ligation product. Under conditions that permit hybridization of the tunable portions of the ligation product to one another, an internally hybridized ligation product formed, which allows the detection of the fluorescence resonance energy transfer (FRET). A method comprising PCR amplification is also disclosed.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/39855

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C40B 20/04; C40B 30/04 (2009.01)
USPC - 506/4; 506/9
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C40B 20/04; C40B 30/04 (2009.01)
USPC - 506/4; 506/9

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/91.2; 435/6, 435/91.21, 506/2 - see keyword below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Search terms: target, nucleic acid, first, second, oligonucleotide probe, hairpin, endcap, donor, acceptor, ligase, ligation, hybridization, denature, FRET, junction, mismatch, complementary, allele, SNP, melting temperature, differ, common target, aromatic, aliphatic

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Wabuyele et al. Approaching Real-Time Molecular Diagnostics: Single-Pair Fluorescence Resonance Energy Transfer (spFRET) Detection for the Analysis of Low Abundant Point Mutations in K-ras Oncogenes. J. AM. CHEM. SOC. 2003, 125:6937-6945; Abstract; pg 6938, col 2, last para; pg 6939, col 1 and col 2; pg 6940, col 2, middle para; pg 6941, col 1; pg 6942, col 1, col 2 para 2 and last para; pg 6943, col 1; and Fig 1, 3, 4 and 5	1-17, 52-57, and 65
Y	PINGLE et al. Synthesis of endcap dimethoxytrityl phosphoramidites for endcapped oligonucleotides. Curr Protoc Nucleic Acid Chem. 2003, Chapter 5, Unit 5.6, pg 1-15. pg 1, para 1	1-17, 52-57, and 65
Y	US 2007/0254289 A1 (LI et al.) 01 Nov 2007 (01.11.2007), para [0146], [0150], [0153], [0156], [0163], and [0197]	3-8

☐ Further documents are listed in the continuation of Box C. ☐

* 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

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Form PCT/ISA/210 (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/39855

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.:
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:

Group I: claims 1-17, 52-57, 65, drawn to a method for identifying one or more of a plurality of target nucleic acid molecules in a sample, comprising:
-- providing a sample;
-- providing a plurality of oligonucleotide probe sets, each set characterized by
(a) a first oligonucleotide probe, comprising a target-specific portion and a tunable portion with an endcapped hairpin and
(b) a second oligonucleotide probe comprising a target specific portion and a tunable portion;
-- providing a ligase;
-- blending the sample, the plurality of oligonucleotide probe sets, and the ligase to form a ligase detection reaction mixture;
-- subjecting the ligase detection reaction mixture to one or more ligase detection reaction cycles; and
-- detecting the FRET between the donor and acceptor groups of the internally hybridized ligation product.

**********Continued in 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.:

claims 1-17, 52-57, and 65

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.
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********** SUPPLEMENTAL BOX **********

Continuation of: Box No III (unity of invention is lacking)

Group II: claims 18-51, 55, 58-67, drawn to a method for identifying one or more of a plurality of target nucleic acid molecules in a sample, comprising
-- providing a sample;
-- providing a plurality of oligonucleotide probe sets;
--performing PCR;
-- providing a ligase;
-- blending the sample, the plurality of oligonucleotide probe sets, and the ligase to form a ligase detection reaction mixture;
-- subjecting the ligase detection reaction mixture to one or more ligase detection reaction cycles; and
-- detecting the fluorescence resonance energy transfer (FRET) between the donor and acceptor groups of the internally hybridized ligation product.

The inventions listed as Groups I-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:

Group I does not include the inventive concept of combining FRET analysis with PCR, as required by Group II.

Groups I and II share the technical feature of a method for identifying one or more of a plurality of target nucleic acid molecules in a sample. However, said method is obvious over the publication "Approaching Real-Time Molecular Diagnostics: Single-Pair Fluorescence Resonance Energy Transfer (spFRET) Detection for the Analysis of Low Abundant Point Mutations in K-ras Oncogenes" by Wabuyele et al. (hereinafter "Wabuyele") in view of the publication "Protein-DNA footprinting by endcapped duplex oligodeoxyribonucleotides" by Ng et al. (hereinafter "Ng") as follows:
Wabuyele teaches a method for identifying one or more of a plurality of target nucleic acid molecules in a sample, comprising:
-- providing a sample potentially containing one or more target nucleic acid molecules (abstract; "specificity and sensitivity to detect single-point mutations in as little as 600 copies of human genomic DNA directly without PCR at a level of 1 mutant per 1000 wild type sequences using 20 LDR [i.e. ligase detection reactions] thermal cycles") providing a plurality of oligonucleotide probe sets, each set characterized by
(a) a first oligonucleotide probe, comprising a target-specific portion and a tunable portion (pg 6938 fig 1; pg 6938 right col para 3- "A ligase-based point mutation detection assay that uses an allele-specific discriminating primer and a common primer, each having a 10 base pair (bp) complementary arm with fluorescent labels at their 5'- and 3'-ends") and
(b) a second oligonucleotide probe comprising a target specific portion and a tunable portion, wherein one of the first and second oligonucleotide probes has an acceptor group and the other of the first and second probes has a donor group pg 6938 fig 1; pg 6938 right col para 3- "A ligase-based point mutation detection assay that uses an allele-specific discriminating primer and a common primer, each having a 10 base pair (bp) complementary arm with fluorescent labels at their 5'- and 3'-ends");
-- providing a ligase (pg 6938 fig 1);
-- blending the sample, the plurality of oligonucleotide probe sets, and the ligase to form a ligase detection reaction mixture (pg 6938 fig 1);
-- subjecting the ligase detection reaction mixture to at least one ligase detection reaction cycles, each cycle comprising a denaturation treatment,
-- wherein any hybridized oligonucleotides are separated from the target nucleic acid sequences, and a hybridization treatment (abstract; "using 20 LDR [i.e. ligase detection reactions] thermal cycles"),
-- wherein the set of oligonucleotide probes hybridize in a base-specific manner to their respective target nucleotide sequences (pg 6938 fig 1), if present in the sample, and ligate to one another to form a ligation product containing the tunable portions, the target-specific portions, the acceptor group, and the donor group (pg 6938 fig 1);
-- subjecting the ligation products to conditions effective to permit hybridization of the tunable portions of the ligation product to one another to form an internally hybridized ligation product (pg 6938 right col para 3, "After ligation and denaturation of the duplex formed between the target DNA and ligated product, the rMB [i.e. reverse molecular beacon] is formed because the stem is designed to have a higher thermal stability compared to the target/LDR-product duplex, bringing the dye labels in close proximity. Fluorescence emission resulting"); and
-- detecting the fluorescence resonance energy transfer (FRET) between the donor and acceptor groups of the internally hybridized ligation product (pg 6938 right col para 3; " bringing the dye labels in close proximity. Fluorescence emission resulting"), thereby indicating the presence of a target nucleic acid molecule in the sample (pg 6938 fig 1).
Wabuyele does not teach including an endcapped hairpin as part of the oligonucleotide construct. Ng teaches endcapped hairpin (pg 1 left col para 1, "Placing crosslinks (or endcaps) at each end allows one to construct very short duplexes that if not endcapped would melt and exist as separate single strands in aqueous solution at room temperature"). An artisan of ordinary skill would readily appreciate the value of an endcapped oligonucleotide would enable higher melting temperatures to be employed in an assay. Consequently, it would have been obvious to one of ordinary skill in the art to combine first and second oligonucleotide probes with tunable regions that are complimentary to one another with acceptor and donor dyes at the 3' and 5' respectively enabling FRET under certain conditions, and oligonucleotide probe with an endcapped hairpin region, as taught by Ng. As said method was obvious at the time of the invention, as evidenced by the combination of Wabuyele and Ng, this cannot be considered a special technical feature 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.