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Canadian Intellectual Property Office

CA 2926317 C 2023/05/23

(11)(21) 2 926 317

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 2014/10/30
(87) Date publication PCT/PCT Publication Date: 2015/05/07
(45) Date de délivrance/Issue Date: 2023/05/23
(85) Entrée phase nationale/National Entry: 2016/04/04
(86) N° demande PCT/PCT Application No.: GB 2014/053238
(87) N° publication PCT/PCT Publication No.: 2015/063498
(30) Priorité/Priority: 2013/10/30 (GB1319180.4)

(51) Cl.Int./Int.Cl. *C07H 21/00* (2006.01), *C07H 21/04* (2006.01), *C12P 19/34* (2006.01)

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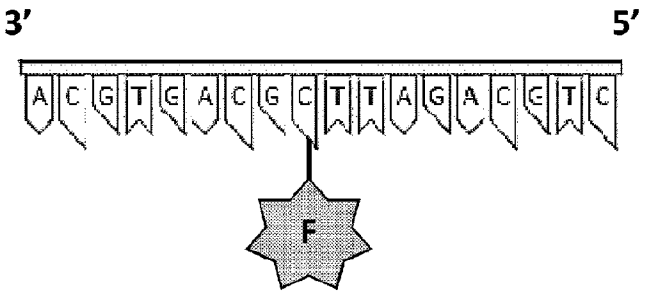
(54) Titre : SONDE A L'ACIDE NUCLEIQUE COMPRENANT UN SEUL MARQUEUR DE FLUOROPHORE LIE A UNE CYTOSINE INTERNE POUR L'UTILISATION DANS UNE AMPLIFICATION ISOTHERME MEDIEE PAR LES BOUCLES

(54) Title: NUCLEIC ACID PROBE WITH SINGLE FLUROPHORE LABEL BOUND TO INTERNAL CYTOSINE FOR USE IN LOOP MEDIATED ISOTHERMAL AMPLIFICATION

Schematic of MAST DNA probe



5' T G C A C T G C G A A T C T G C A G 3'

(57) Abrégé/Abstract:
The present invention provides novel probes for use in LAMP detection methods. The probes contain a single fluorophore label bound to an internal cytosine residue of the probe. The probes are particularly useful in the detection of chlamydia and gonorrhea infections in a patient.

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 May 2015 (07.05.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/063498 A3

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/GB2014/053238

(22) International Filing Date:
30 October 2014 (30.10.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1319180.4 30 October 2013 (30.10.2013) GB

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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, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, 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).

Published:

— *with international search report (Art. 21(3))*

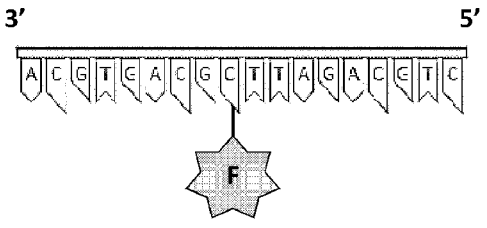
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(88) Date of publication of the international search report:
16 July 2015

(54) Title: NUCLEIC ACID PROBE WITH SINGLE FLUOROPHORE LABEL BOUND TO INTERNAL CYTOSINE FOR USE IN LOOP MEDIATED ISOTHERMAL AMPLIFICATION

FIG1

Schematic of MAST DNA probe



5' T GCA CT GCGAA TC TG CAG 3'

(57) Abstract: The present invention provides novel probes for use in LAMP detection methods. The probes contain a single fluorophore label bound to an internal cytosine residue of the probe. The probes are particularly useful in the detection of chlamydia and gonorrhea infections in a patient.

WO 2015/063498 A3

NUCLEIC ACID PROBE WITH SINGLE FLUOROPHORE LABEL BOUND TO INTERNAL CYTOSINE FOR USE IN LOOP MEDIATED ISOTHERMAL AMPLIFICATION

The present invention relates to a probe for the detection of a nucleic acid, a method using said probe and a kit of parts. Preferably the probe of the invention is useful in a method for the detection of nucleic acids derived from *Chlamydia trachomatis* and/or *Neisseria gonorrhoeae* and may be used in the diagnosis of Chlamydia and/or Gonorrhoea infections.

Nucleic acid amplification is one of the most valuable tools in the life sciences field, including application-oriented fields such as clinical medicine, in which diagnosis of infectious diseases, genetic disorders and genetic traits is particularly benefited. In addition to the widely used PCR-based detection (Saiki R.K., Scharf,S., Faloona,F., Mullis,K.B., Horn,G.T., Erlich,H.A. and Arnheim,N. (1985) Science, 230, 1350–1354), several amplification methods have been invented. Examples include nucleic acid sequence-based amplification (NASBA), self-sustained sequence replication (3SR) and loop-mediated isothermal amplification (LAMP). PCR uses heat denaturation of double-stranded DNA products to promote the next round of DNA synthesis. 3SR and NASBA eliminate heat denaturation by using a set of transcription and reverse transcription reactions to amplify the target sequence.

These methods can amplify target nucleic acids to a similar magnitude, all with a detection limit of less than 10 copies and within an hour or so. They require either a precision instrument for amplification or an elaborate method for detection of the amplified products due to poor specificity of target sequence selection. Despite the simplicity and the obtainable magnitude of amplification, the requirement for a high precision thermal cycler in PCR prevents this powerful method from being widely used, such as in private clinics as a routine diagnostic tool. In contrast, LAMP is a method that can amplify a few copies of DNA to over 100 in less than an hour under isothermal conditions and with greater specificity.

As with other molecular-probe based technologies identified above, loop-mediated isothermal amplification (LAMP) assays can be used to detect the presence of specific

Date Reçue/Date Received 2022-05-09

microorganisms in a sample. However, the detection methods are based on direct visual detection, turbidity or via a non-specific DNA intercalating dye. Direct visual measurement is end point measurement and is unable to provide real time analysis. Turbidity and non-specific intercalating dyes do provide real time analysis of amplification which occurs however this is non-specific i.e. all amplification is detected whether this is true positive amplification or false amplification due to mis-priming, cross specificity.

In accordance with a first aspect of the present invention there is provided a probe for isothermal nucleic acid amplification comprising an oligonucleotide probe sequence complementary to a region of a target nucleic acid sequence, wherein said oligonucleotide probe sequence has only one fluorophore ligand and which ligand is bound to an internal cytosine base and wherein said oligonucleotide probe sequence does not have a 3' end terminator.

In a preferred embodiment to oligonucleotide probe sequence is a DNA sequence and the target nucleic acid sequence is a DNA sequence.

Preferably, fluorescence increases to indicate the presence of the target nucleic acid in a sample.

The cytosine base is preferably substantially centrally disposed along the oligonucleotide's length. There are particular benefits associated with labeling the probe internally at a cytosine base. The specificity of the DNA product amplified in an isothermal reaction may be confirmed using a melt curve analysis. However due to a large number of product variants generated in this reaction and a low resolution of melt curve analysis, using intercalating dyes like V13, it is very difficult to distinguish between specific and unspecific DNA products generated under isothermal conditions. Commonly used probes such as TaqMan® probe are not compatible with LAMP technology due to the strand displacement activity of BST polymerase. The probe of the invention is elongated and becomes incorporated into a DNA

product during isothermal amplification, which allows for performing a melt curve analysis on the generated product. In the probe of the invention, the fluororphore is conjugated to an internal cytosine complementary to guanine in the antisense strand. Guanine affects the excitation state of many fluorophores resulting in a formation of unique melt curve signatures and allows distinguishing between specific and unspecific products generated under isothermal conditions.

The oligonucleotide does not contain a ddNTP at its 3' end which enables incorporation of the labelled oligonucleotide into the amplicon. Thus, the 3' end of the probe is not "blocked".

The fluorophore may comprise any one or more selected from the following: FAM, JOE, TET, HEX, TAMRA, ROX, ALEXA and ATTO.

The probe may comprise the following sequence:

5' Xn C* Xm 3' (SEQ ID NO. 1)

Where n is >1, m is >3, X is nucleotide base; and * is a fluorophore. Preferably, the nucleotide base is selected from A, T, C and G. Preferably, n is more than 1 to 20 or less, more preferably more than 1 to 10 or less. Preferably, m is more than 3 to 20 or less, more preferably more than 3 to 10 or less. It is contemplated that all combinations of lengths of probe covered by the possible number of nucleotides that n or m make take by the preceding ranges are disclosed.

Preferably, the probe may comprise a sequence selected from any one of the following sequences:

SEQ ID NO. 3: TAAGATAAC[C-FAM]CCGCACGTG (CT PB1-FAM internal)

SEQ ID NO. 5: GCGAACATA [C-ALEXA546] CAGCTATGATCAA (GC porA7-joe loopF) or

SEQ ID NO. 6: ATGTTCA [C-JOE] CATGGCGGAG (GC glnA7-ALEXA546 loopB).

The fluorescence is preferably increased when the oligonucleotide is incorporated into the target nucleic acid sequence which results in a change in the configuration of the amplicon-probe complex leading to an alteration of the fluorophore excitation state.

The cytosine bound to the fluorophore ligand is not disposed at or proximate to the 5' or 3' end. More preferably it is not disposed in the first 3 bases from either the 5' or 3' end. Preferably the cytosine bound to the fluorophore is disposed at the middle base of the probe.

In accordance with a further aspect of the present invention, there is provided an isothermal nucleic acid amplification probe as described hereinabove.

In accordance with a further aspect of the present invention, there is provided a loop-mediated isothermal amplification probe as described above.

Methods and compositions for determining at least one target nucleic acid in a mixture of nucleic acids generally employ a probe, a hybridizing reagent, and one or more phosphate bond-forming enzymes associated with any required nucleotide triphosphates to form a nucleic acid chain.

These methods usually involve amplification, such as including the use of a promoter in conjunction with a RNA polymerase, a restriction site where only one strand is cleaved and is then displaced by extension with a DNA polymerase, or a circular hybridizing reagent, where concatenated repeats are produced. Detection of the amplified nucleic acid may take many forms but preferably via a fluorophore.

In accordance with a further aspect of the present invention, there is provided a method of detecting a target nucleic acid in a sample comprising:

a. amplifying a target nucleic acid in the sample to provide an amplified nucleic acid;
b. probing the amplified nucleic acid with a probe as described hereinabove; and

c. detecting the presence of a single or multiple target nucleic acids.

The target nucleic acid may be that from a micro-organism, fungi, yeast, virus, human, animal, plant etc. The target nucleic acid for LAMP is known to enable LAMP primers and appropriately specific probes to be synthesised. Thus, the presence or absence of said micro-organism, fungi, yeast, virus, human, animal or plant in a sample can be determined. Preferably the target nucleic acid is from *Chlamydia trachomatis or Neisseria gonorrhoeae.*

Preferably, fluorescence increases to indicate the presence of the target nucleic acid in a sample.

The process is isothermal, and allows for amplification in a single stage or sequential stages in a single vessel, where all of the reagents are compatible.

In a further aspect, the present invention provides a method of diagnosing Chlamydia and/or Gonorrhea in a patient, comprising

providing a sample derived from the patient;

adding one or more probes of the present invention to the sample; and

detecting the presence of a nucleic acid derived from *Chlamydia trachomatis* and/or *Neisseria gonorrhoeae* wherein an increase in the fluorescence of the probe indicates the presence of a *Chlamydia trachomatis* and/or *Neisseria gonorrhoeae* infection.

The sample may be treated by routine methods to enable the probe to bind with any target nucleotide present in the sample. Such treatment may include centrifuging and lysing the sample to release any target nucleic from the infecting microorganism.

In one embodiment, a single type of probe specific for a nucleic acid from either *Chlamydia trachomatis* or *Neisseria gonorrhoeae* is used in the method such that either only *Chlamydia trachomatis* or only *Neisseria gonorrhoeae* is detected in the sample.

In a preferred embodiment, at least two different probes are added to the sample wherein a first probe is labelled with a first fluorescent label and is specific for probing *Chlamydia trachomatis* nucleic acid and a second probe is labelled with a different fluorescent label to the first probe and is specific for probing *Neisseria gonorrhoeae* nucleic acid. In this embodiment, it is possible to simultaneously detect a Chlamydia and a Gonorrhea infection in a single sample derived from a patient.

In one aspect of the method of the invention, the sample from the patient may be a blood sample, urine sample, serum sample or saliva sample.

In accordance with a further aspect of the present invention there is provided a kit comprising a probe as described hereinabove, LAMP reaction buffer containing a polymerase enzyme, dNTPS and LAMP primers for the target.

In one embodiment a positive and negative control may be included in the kit. The reagents may be presented as wet reagents or in lyophilised form.

The buffer used in the method or kit of the invention comprises dNTPs at a concentration of from 1-10mM, one or more salts at a concentration of from 2-20mM, Tris pH8.8 at a concentration of from 10- 100mM, Trehalose at a concentration of from 10-100mM, BST polymerase at an amount of from 1U-12U and 0.01%-1% 1,2 propanediol.

Abbreviations

CT – *Chlamydia trachomatis*

GC - *Neisseria gonorrhoeae*

GlnA7 - Glutamine synthetase

PorA7 – porin protein A7

LAMP –loop mediated isothermal amplification

PCR – polymerase chain reaction.

The present invention will now be described, by way of example only, with reference to the following examples and figures.

LAMP reaction

V13 based detection of the target CT and GT DNA by LAMP was performed using LAMP V6.21 reaction buffer developed by the Applicant. Probe based detection of the target DNA was performed in V6.21p (without V13). The LAMP primer concentrations were as follows: CT PB1 - 0.8µM FIP & BIP primer, 0.2µM F3 & B3 and 0.4µM Loop primers, GC porA7 and GC glnA7 – 2 µM FIP & BIP primer, 0.25µM F3 & B3 and 0.5µM Loop primers. All probes were used at a final concentration of 0.625µM. LAMP reactions were run for 60mins at a constant temperature of 63C using ABI7500 real-time PCR machine. Readouts of the fluorescent signal were obtained in SybrGreen/FAM, Joe or Cy3 channel as appropriate.

Probe sequences

SEQ ID NO. 2: GTGCACGC[C-FAM]CCAATAGAAT

SEQ ID NO. 3: TAAGATAAC[C-FAM]CCGCACGTG (CT PB1-FAM internal)

SEQ ID NO. 4: TCGAGCAA[C-FAM]CGCTGTGAC[ddC] (CT PB1-FAM terminal)

SEQ ID NO. 5: GCGAACATA [C-ALEXA546] CAGCTATGATCAA (GC porA7-joe loopF)

SEQ ID NO. 6: ATGTTCA [C-JOE] CATGGCGGAG (GC glnA7-ALEXA546 loopB) or

SEQ ID NO. 7: CCA GGG TAT CTA ATC CTG TTT G [C-FAM].

Target Sequences

The target DNA sequences used in the Examples are

SEQ ID No. 8: *Chlamydia trachomatis* G/SotonG1 plasmid pSotonG1 complete sequence (GenBank: HE603235.1)

```
   1 tttgcaactc ttggtggtag actttgcaac tcttggtggt agactttgca actcttggtg
  61 gtagacttgg tcataatgga cttttgttaa aaaatttctt aaaatcttag agctccgatt
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5521 cggggtttgt tatgcaaaat aaaagaaaag tgagggacga ttttattaaa attgttaaag
5581 atgtgaaaaa agatttcccc gaattagacc taaaaatacg agtaaacaag gaaaaagtaa
5641 ctttcttaaa ttctcccttа gaactctacc ataaaagtgt ctcactaatt ctaggactgc
5701 ttcaacaaat agaaaactct ttaggattat tcccagactc tcctgttctt gaaaaattag
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5941 acttggccca atttttaggg aaaaaagtgt tacttgctga cctagacccg caatccaatt
6001 tatcttctgg attgggggct agtgtcagaa ataaccaaaa aggcttgcac gacatagtat
6061 acaaatcaaa cgatttaaaa tcaatcattt gcgaaacaaa aaaagatagt gtggacctaa
6121 ttcctgcatc atttttatcc gaacagttta gagaattgga tattcataga ggacctagta
6181 acaacttaaa gttatttctg aatgagtact gcgctccttt ttatgacatc tgcataatag
6241 acactccacc tagcctagga gggttaacga aagaagcttt tgttgcagga gacaaattaa
6301 ttgcttgttt aactccagaa ccttttttcta ttctagggtt acaaaagata cgtgaattct
6361 taagttcggt cggaaaacct gaagaagaac acattcttgg aatagctttg tctttttggg
6421 atgatcgtaa ctcgactaac caaatgtata tagacattat cgagtctatt tacaaaaaca
6481 agctttttc aacaaaaatt cgtcgagata tttctctcag ccgttctctt cttaaagaag
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6601 atgaaatagc aaatattttg catatcgaat atgaacgaga ttactctcag aggacaacgt
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7201 aagggtgttg gatcaatttc ttccttcatc tagaaacaaa gacgttagag aaacgataga
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7441 tttatttagg gatttgattt tacgagagag a

SEQ ID No. 9: *Neisseria gonorrhoeae* partial porA gene for class 1 outer membrane protein, isolate GC3 (GenBank: HE681886.1)

```
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 121 gatccttggg acagcaacaa tgatgtggct tcgcaattgg gtattttcaa acgccacgac
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 301 tataacacta acggtactac tactactttc gttccggctg ttgtcggcaa gcccggatcg
 361 gatgtgtatt atgccggtct gaattacaaa aatggcggtt ttgccgggaa ctatgccttt
 421 aaatatgcga gacacgccaa tgtcggacgt aatgcttttg agttgttctt gctcggcagt
 481 gggagtgatg aagccaaagg taccgatccc ttgaaaaacc atcaggtaca ccgcctgacg
 541 ggcggctatg gggaaggcgg cttgaatctc gccttggcgg ctcagttgga tttgtctgaa
 601 aatgccgaca aaaccaaaaa cagtacgacc gaaattgccg ccactgcttc ctaccgcttc
 661 ggtaatacag tcccgcgcat cagctatgcc catggtttcg actttgtcga acgcagtcag
 721 aaacgcgaac ataccagcta tga
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SEQ ID No. 10: *Neisseria gonorrhoeae* glutamine synthetase (glnA) gene, glnA-14 allele, partial cds

(GenBank: AF520262.1)

```
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 121 gctcgtccat cggcggctgg aaaggcattg aggcttccga tatgcagctg cgtcccgatg
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 301 ccgaagccta tttgaaatct tccggtatcg gcgacaccgc ctatttcggc cccgaacccg
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 421 tcacgtccga aagcggcgcg tgggcaagcg gcctgcatat ggacggtcaa aacaccggcc
 481 accgccccgc cgtcaaaggc ggctacgcgc ccgtcgcgcc gattgactgc ggtcaagatt
 541 tgcgctccgc catggtgaac attttggaag gactcggcat cgaagtcgaa gtccaccaca
 601 gcgaagtcgg taccggcagc caaatggaaa tcggcacccg tttcgccact ttggtcaaac
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 721 aaaccgccac ctttatgccc aaaccgatta tgggcgacaa cggcagcggt atgcacgtcc
 781 accaatccat ttggaaagac ggtcaaaacc tgttcgcagg cgacggctat gccggtttgt
 841 ccgataccgc gctctactac atcggcggca tcatcaaaca cgccaaagcc ctgaacgcga
 901 ttaccaatcc gtccaccaac tcctacaaac gcctcgtgcc gcactttgaa gcaccgacca
 961 aattggccta ttccgccaaa aaccgttccg cttccatccg tatcccgtct gtgaacagca
1021 gcaaggcgcg ccgcatcgaa gcgcgtttcc ccgacccgac cgccaacccg tatttggcat
1081 ttgccgccct gctgatggcc ggtttggacg gcattcaaaa caaaatccat ccgggcgacc
1141 ctgccgataa aaacctgtac gacctgccgc cggaagaaga cgcgctcgtc ccgaccgtct
1201 gcgcttcttt ggaagaagca cttgccgccc tcaaggtcga ccacgaattc ctgctgcgcg
1261 gcggcgtgtt cagcaaagac tggatcgaca gctacatcgc ctttaaagag gaagatgtcc
1321 gccgcatccg tatggcgccg cacccgctgg aatttg
```

The primer sequences used in the LAMP reaction are as follows:

CT plasmid

F3	TCTACAAGAGTACATCGGTCA (SEQ ID No. 11)
B3	TGAAGCGTTGTCTTCTCG (SEQ ID No. 12)
FIP	GCAGCTTGTAGTCCTGCTTGAGTCTTCGTAACTCGCTCC (SEQ ID No. 13)
BIP	TCGAGCAACCGCTGTGACCCTTCATTATGTCGGAGTCTG (SEQ ID No. 14)
LF1	CGGGCGATTTGCCTTAAC (SEQ ID No. 15)
LB1	TACAAACGCCTAGGGTGC (SEQ ID No. 16)

GC porA7

F3	ACCAAAAACAGTACGACCGA (SEQ ID No. 17)
B3	AAGTGCGCTTGGAAAAATCG (SEQ ID No. 18)
	FIPATGGGCATAGCTGATGCGCGAATTGCCGCCACTGCTTC (SEQ ID No. 19)
BIP	TCGACTTTGTCGAACGCAGTCAAATCGACACCGGCGATGA (SEQ ID No. 20)
LoopF1	GCGAACATACCAGCTATGATCAA (SEQ ID No. 21)

GC glnA7

F3	TCATATCTTGGGTTTGGTCG (SEQ ID No. 22)
B3	CTGCATATGGACGGTCAAA (SEQ ID No. 23)

FiP	CGAAGTCCACCACAGCGAATTTGACCAAAGTGGCGAA (SEQ ID No. 24)
BiP	CTTCGATGCCGAGTCCTTCCGATTGACTGCGGTCAAGAT (SEQ ID No. 25)
LF	CAAATGGAAATCGGCACCC (SEQ ID No. 26)
LB	ATGTTCACCATGGCGGAG (SEQ ID No. 27)

Buffer

The Applicant has developed a buffer system for use with the probes of the invention and is designated V6.21 (or V6.21p without V13 dye present) in the following Examples. The concentrations of the buffer components are after buffer reconstitution:

V6.21

4-10mM dNTP's, 10mM salt, 30mM Tris pH8.8, 30mM Trehalose, 1-8U Bst polymerase, Dye and 0.05% propanediol.

V6.21p

4-10mM dNTP's, 10mM salt, 30mM Tris pH8.8, 30mM Trehalose, 1-8U Bst polymerase, and 0.05% propanediol.

PCR

CT/GC detection in clinical samples by real-time PCR was performed using APTIMA CT/GC multiplex (Gen-Probe) according to the manufacturer's instructions.

Agarose Gel Electrophoresis

DNA electrophoresis was conducted in 1% agarose gel 1xTAE buffer at 100V. LAMP DNA products were vitalized with GelRed (Invitrogen) with transilluminator.

V6.21 and V6.21p buffer were developed by the Applicant. LAMP primers were obtained from Eurofins. Fluorophore-labelled oligonucleotides were purchased from Integrated DNA technologies. Tris buffer, agarose gel and PCR grade water were purchased from Sigma. CT and GC DNA standards were obtained from ATCC.

Figures

Figure 1 is a schematic of DNA probe of the invention. The probe consists of an oligonucleotide with an internal cytosine conjugated with a defined fluorophore. The probe may be complementary to the internal region of the amplicon flanked by Fip and Bip primers or it may be a modified LoopF or LoopB primer internally labeled with a fluorophore.

Example 1

Figures 2A to 2F shows amplification plots generated with the CT PB1 (Figure 2A and Figure 2D), GC glnA7 (Figure 2B and Figure 2E) and GC porA7 (Figure 2C and Figure 2F) primers in V6.21 buffer containing V13 (Figures 2A, 2B and 2C) or V6.21p buffer without V13 dye (Figures 2D, 2E and 2F). The target sequences shown in SEQ ID NOs. 8 to 10 with CT PB1 internal probe conjugated with FAM, GC glnA7 loop probe conjugated with Joe and GC porA7 loop probe conjugated with Alexa546 respectively. All reactions were performed for 60mins at a constant temperature of 63C with ABI7500 machine.

Example 2

Figures 3A and 3B are melt curve analyses of LAMP products generated with CT PB1 primers in the presence of CT PB1 internal probe conjugated with FAM. 100pg per reaction of ATTC CT DNA standard was used as a positive control. A – normalized reporter plot, B – derivative reporter plot. Melt curve plots were generated based on the readouts in FAM channel with ABI7500 machine.

Example 3

Figures 4A and B are melt curve analyses of LAMP product generated with GC glnA7 primers in the presence of GC glnA7 loop probe conjugated with JOE. 100pg per reaction of ATTC GC DNA standard was used as a positive control. Figure 4A shows a normalized reporter plot and Figure 4B shows a derivative reporter plot. Melt curve plots were generated based on the readouts in JOE channel with ABI7500 machine.

Example 4

Figures 5A and 5B are melt curve analyses of LAMP product generated with GC porA7 primers in the presence of GC porA7 loop probe conjugated with ALEXA546. 100pg per reaction of ATTC GC DNA standard was used as a positive control. Figure 5A shows a normalized reporter plot, Figure 4B shows a derivative reporter plot. Melt curve plots were generated based on the readouts in Cy3 channel with ABI7500 machine.

Example 5

Figures 6A to 6D show the results of a test to confirm the DNA product specificity with a probe of the invention in loop mediated isothermal amplification. The late amplification time of the false positives (more than 30mins after the lowest target DNA concentration detectable in the LAMP reaction (100fg GC DNA) indicates that the unspecific amplification may be a result of primer dimer formation. The standard melt curve analysis does not allow to distinguish between the specific and unspecific product in this LAMP reaction, but the unspecific product may be recognized with the probe of the invention. GC DNA was amplified with GC porA7 primers and visualized with V13 dye or GC porA7-ALEXA546 probe as appropriate.

Example 6

Figure 7 shows the amplification plots generated with CT PB1 primers in V6.21 buffer containing V13 or V6.21p buffer without V13 dye but in the presence of CT PB1 terminal probe (complementary to loop region) with an internal C conjugated with FAM and 3' terminator (3'ddC). Despite a successful amplification of the target DNA confirmed by excitation of the V13 dye in the control reaction, CT PB1 probe with 3' terminator did not generate a positive signal.

Example 7

Figures 8A and 8B shows the amplification plots generated in V6.21p buffer containing ROX in the presence of CT PB1 primers and CT PB1 terminal probe with an internal cytosine conjugated with FAM (Figure 8A), and universal primers and 3'UP probe with 3' terminal cytosine conjugated with FAM (Figure 8B). The first line represents signals generated by ROX, and the second line corresponds to the signal generated in the FAM channel. Binding of the probe with an internally labeled C to the target DNA results in FAM excitation. Binding of the probe with a 3' end C labeled to the target does not alter the FAM excitation state.

Example 8

Figures 9A to 9C show the amplification plots generated with CT PB1 primers in V6.21p buffer without V13 in the presence of CT PB1 internal probe with an internal C conjugated with FAM and a reference dye (ROX). Figure 9A show raw data, readouts from the FAM channel in the first line and from the ROX channel in a second line. Figure 9B shows amplification plots (generated in FAM channel) normalized to ROX. Figure 9C shows derivative reporter melt curve plots.

Example 9

Figures 10A to 10C show the validation of CT PB1-FAM probe specificity. Figure 10A shows amplification plots generated with CT PB1-FAM probe in the presence of CT DNA and CT

primers. As a control, two sets of reactions were performed where unspecific genes, GC glnA7 and GC porA7 were amplified with the corresponding LAMP primers in the presence of CT PB1-FAM probe. In V6.21p buffer the amplification plots in the presence of CT PB1 probe in the FAM channel were generated only when CT DNA was present in the reaction and no signal was generated when unspecific genes (GC glnA7 and GC porA7) were amplified. No signal was also generated when an unspecific probe was used in a reaction where CT DNA was amplified with CT primers. Figure 10C shows data obtained in an analogous experiment but conducted in V6.21 buffer containing an intercalating dye V31. Figure 10C shows DNA products generated in the experiment described in Figure 10A.

Example 10

Figures 11A and 11B shows the validation of CT PB1-FAM probe against APTIMA CT assay. Fifty clinical samples confirmed to be positive (n=29) (Figure 11A) or negative (n=21) (Figure 11B) for CT were tested in V6.21p buffer with CT PB1-FAM probe. Out of 50 samples 24 tested negative (Figure 11A) and 26 tested positive (figure 11B) for CT with CT PB1-FAM probe. There was 86% agreement between the Aptima and CT PB-FAM tests.

Example 11

Figures 12A and 12B show the amplification plots generated in CT/GC multiplex with CT PB1-FAM + GC porA7-Alexa546 probes. CT and GC DNA was amplified in separate reactions or in conjugation in V6.21p buffer in the presence of CT PB1-FAM and GC porA7-Alexa546 probes. The readouts were taken in Cy3 (Figure 12A) and FAM (Figure 12B) channels. The experiment revealed that two DNA targets may be amplified and detected in a simultaneous reaction with FAM and Alexa546 labeled probes and that there was no cross reactivity between CT PB1 and GC porA7 primers and probes.

Example 12

Table1 shows a comparison between V13 LAMP for CT and GC, CT/GC Aptima and CT/GC multiplex (CT PB1-FAM + GC porA7-Alexa546). DNA extracted from 136 clinical samples was tested with CT/GC Aptima multiplex, CT PB1 and GC porA7 primers in V6.21 buffer containing V13 or in a multiplex reaction in v6.21p buffer in the presence of CT PB1 and GC porA7 primers and CT PB1-FAM and GC porA7-Alexa546 probes. In a control experiment the samples were also tested in a simplex reaction with GC glnA7-joe probe. The table shows the agreement scores between the tests.

CLAIMS

1. A method of detecting a target nucleic acid sequence in a sample comprising:

amplifying a target nucleic acid in the sample by loop-mediated isothermal amplification; probing the amplified nucleic acid with a probe comprising; an oligonucleotide probe sequence complementary to a region of the target nucleic acid sequence, wherein said oligonucleotide probe sequence has only one fluorophore label and which label is bound to an internal cytosine base and wherein said oligonucleotide probe sequence does not have a 3' end terminator, wherein the cytosine base is substantially centrally disposed along the oligonucleotide probe sequence's length except for the positions 1-3 at the 3' end and the position 1 at the 5' end; and

detecting the presence of a single or multiple target nucleic acids, wherein an increase in fluorescence of the probe indicates the presence of the target nucleic acid in the sample.

2. The method of claim 1 wherein the oligonucleotide probe sequence is a DNA sequence and the target nucleic acid sequence is a DNA sequence.

3. The method as claimed in any one of claims 1 or 2, wherein the fluorophore comprises FAM, JOE, TET, HEX, TAMRA, ROX, ALEXA or ATTO.

4. The method as claimed in claim 3, wherein the fluorophore is FAM, Joe or Alexa546.

5. The method as claimed in any one of claims 1 to 4, wherein the oligonucleotide probe sequence comprises the following sequence:

5' Xn C * Xm 3'

wherein n is > 1, m>3, X is nucleotide base; and * is fluorophore, and

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wherein the nucleotide base is selected from A, T, C and G, n is $1 < n \leq 20$, and m is $3 < m \leq 20$.

6. The method as claimed in any one claims 1 to 5, wherein the oligonucleotide probe sequence comprises:

SEQ ID NO. 3: TAAGATAAC[C-FAM]CCGCACGTG (CT PB1-FAM internal)

SEQ ID NO. 5: GCGAACATA [C-ALEXA546] CAGCTATGATCAA (GC porA7-joe LoopF)

or

SEQ ID NO. 6: ATGTTCA [C-JOE] CATGGCGGAG (GC glnA7-ALEXA546 loopB).

7. The method as claimed in any one of claims 1 to 6, wherein the target nucleic acid is from a micro-organism, fungi, yeast or virus.

8. The method of claim 1, wherein the target nucleic acid is from *Chlamydia trachomatis or Neisseria gonorrhoeae*.

9. A method of diagnosing Chlamydia and/or Gonorrhea infection in a patient, comprising

providing a sample derived from the patient;

carrying out the method of claim 1 ; and

detecting the presence of a nucleic acid derived from *Chlamydia trachomatis* and/or *Neisseria gonorrhoeae*, wherein an increase in the fluorescence of the probe indicates the presence of a *Chlamydia trachomatis* and/or *Neisseria gonorrhoeae* infection.

10. The method of claim 9, wherein a single type of probe specific for a nucleic acid from either *Chlamydia trachomatis* or *Neisseria gonorrhoeae* is added to the sample.

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11. The method of claim 9, wherein at least two different probes are added to the sample wherein a first probe is labelled with a first fluorescent label and is specific for probing *Chlamydia trachomatis* nucleic acid and a second probe is labelled with a different fluorescent label to the first probe and is specific for probing *Neisseria gonorrhoeae* nucleic acid.

12. The method of claim 1, wherein the probe is provided in a buffer system comprising dNTPs at a concentration from 1-10mM, one or more salts at a concentration of each salt from 2-20mM, Tris pH8.8 at a concentration from 10-100mM, Trehalose at a concentration from 10-100mM, BST polymerase at an amount from 1U-12U and 0.01%-1% 1,2 propanediol.

13. The method of claim 12, wherein the one or more salts are selected from the group consisting of KCl, $(NH_4)_2SO_4$ and $MgSO_4$.

14. A probe for isothermal nucleic acid amplification comprising an oligonucleotide probe sequence complementary to a region of a target nucleic acid sequence, wherein said oligonucleotide probe sequence has only one fluorophore label and which label is bound to an internal cytosine base and wherein said oligonucleotide probe sequence does not have a 3' end terminator, wherein the cytosine base is substantially centrally disposed along the oligonucleotide's length except for the positions 1-3 at the 3' end and the position 1 at the 5' end; wherein the probe sequence comprises:

SEQ ID NO. 3: TAAGATAAC[C-FAM]CCGCACGTG (CT PB1-FAM internal)

SEQ ID NO. 5: GCGAACATA [C-ALEXA546] CAGCTATGATCAA (GC porA7-joe LoopF)

or

SEQ ID NO. 6: ATGTTCA [C-JOE] CATGGCGGAG (GC glnA7-ALEXA546 loopB).

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15. A kit for detecting a target nucleic acid according to the method of any one of claims 1 to 13 comprising a probe as specified in a method of any one of claims 1 to 13 or as claimed in claim 14, loop-mediated isothermal amplification reagent buffer, BST polymerase, dNTPs and one or more loop-mediated isothermal amplification primers.

16. The kit as claimed in claim 15, further comprising a positive and negative control.

17. The kit of claim 16, wherein the reagent buffer comprises the dNTPs at a concentration from 1-10mM, one or more salts at a concentration from 2-20mM, Tris pH8.8 at a concentration from 10- 100mM, Trehalose at a concentration from 10-100mM, the BST polymerase at an amount of from 1U-12U and 0.01%-1% 1,2 propanediol.

18. The kit of claim 17, wherein the one or more salts are selected from the group consisting of KCl, $(NH_4)_2SO_4$ and $MgSO_4$.

19. The method as claimed in claim 5, wherein n is $1 < n \leq 10$.

20. The method as claimed in claim 5 or 19, wherein m is $3 < m \leq 10$.

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FIG1

Schematic of MAST DNA probe

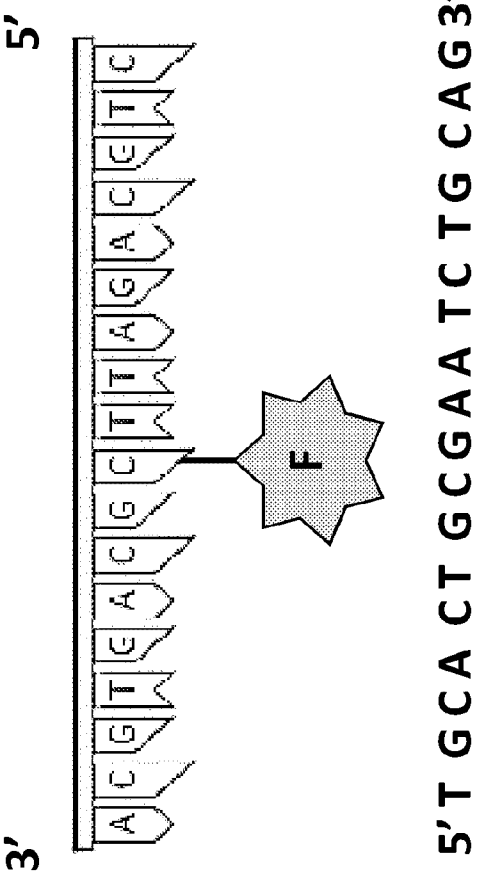
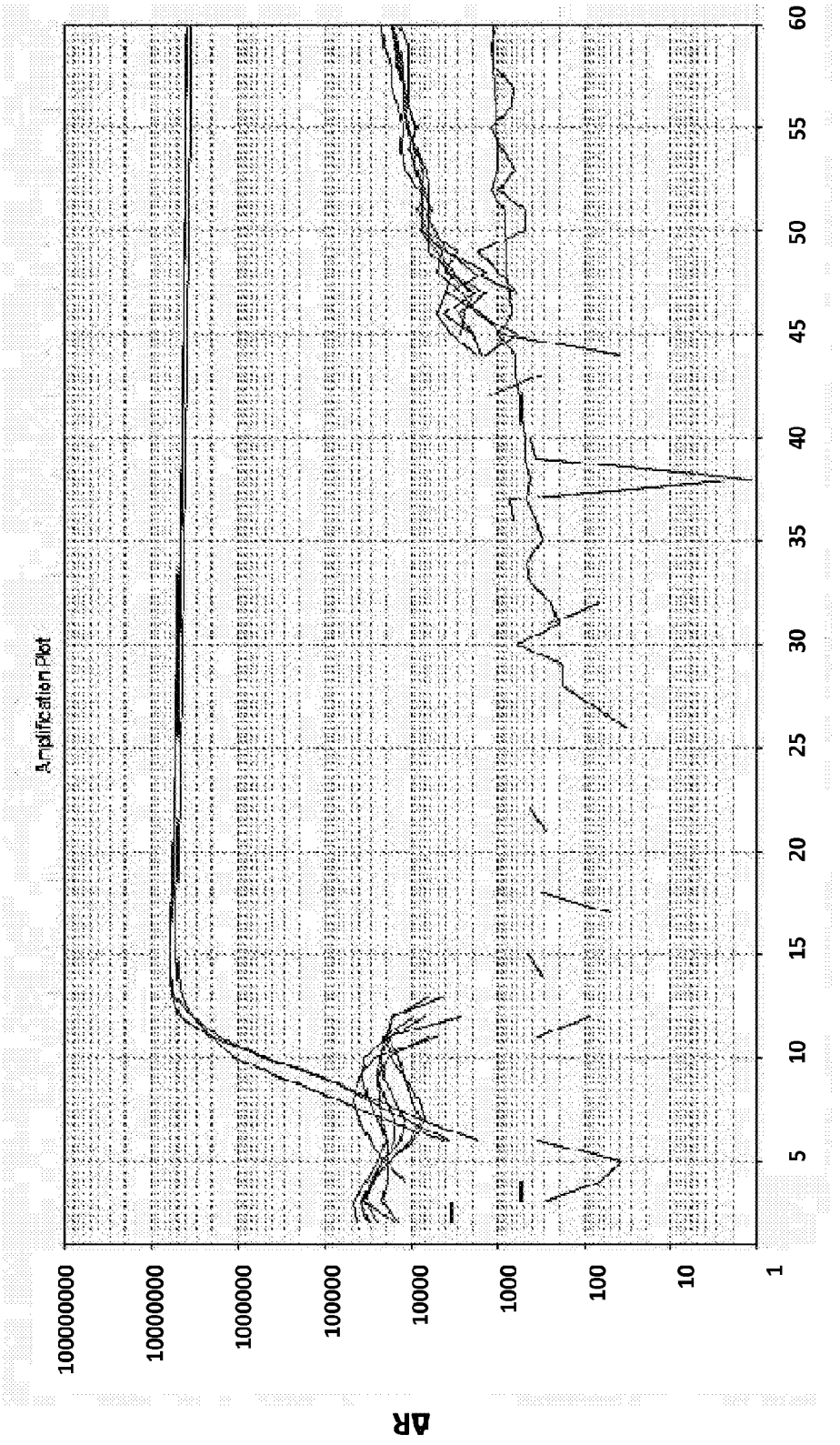


FIG 2A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1000000
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10000
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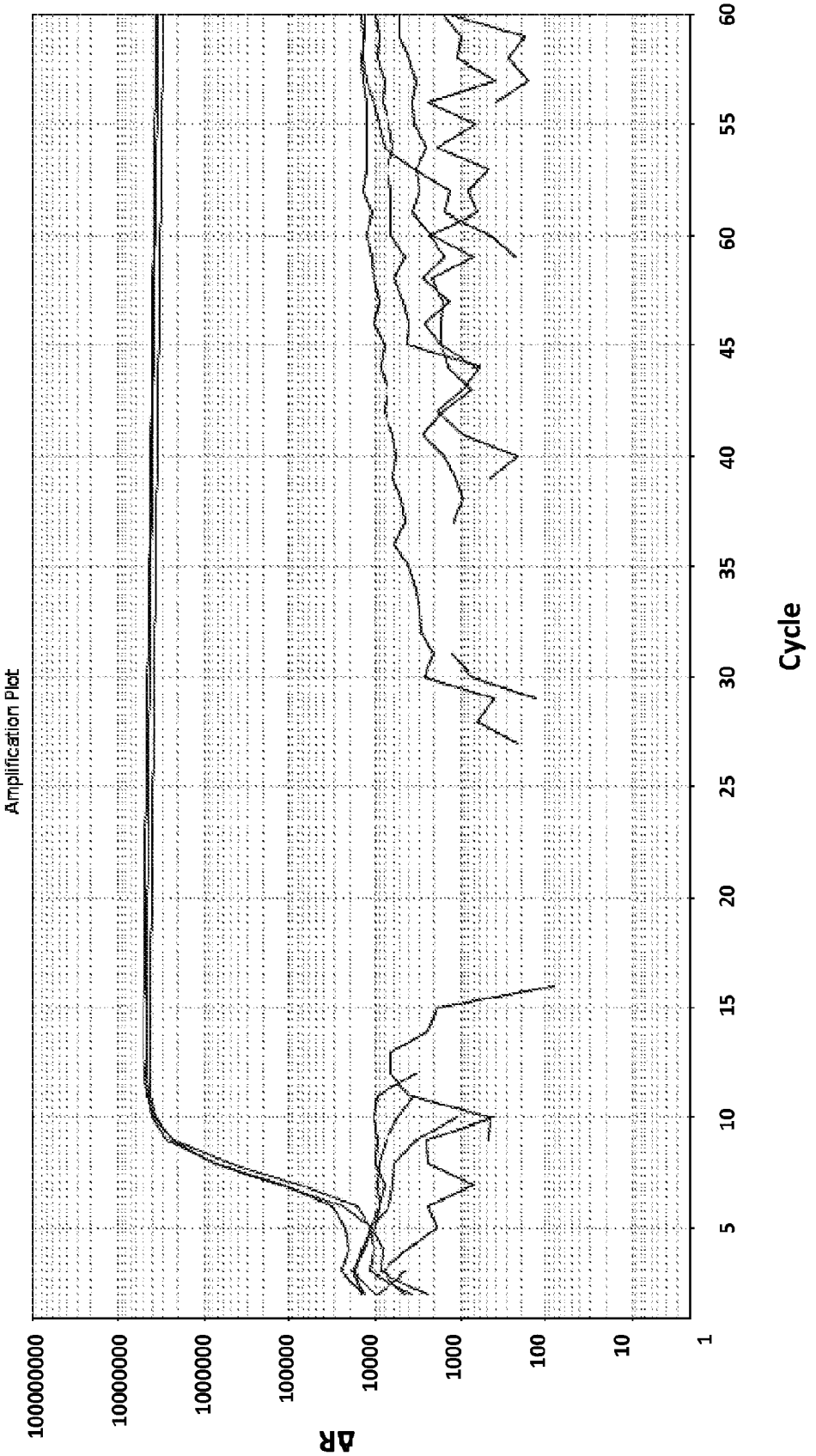
55
60

FIG 2B


Amplification Plot
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1000000
100000
10000
1000
100
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5
10
15
20
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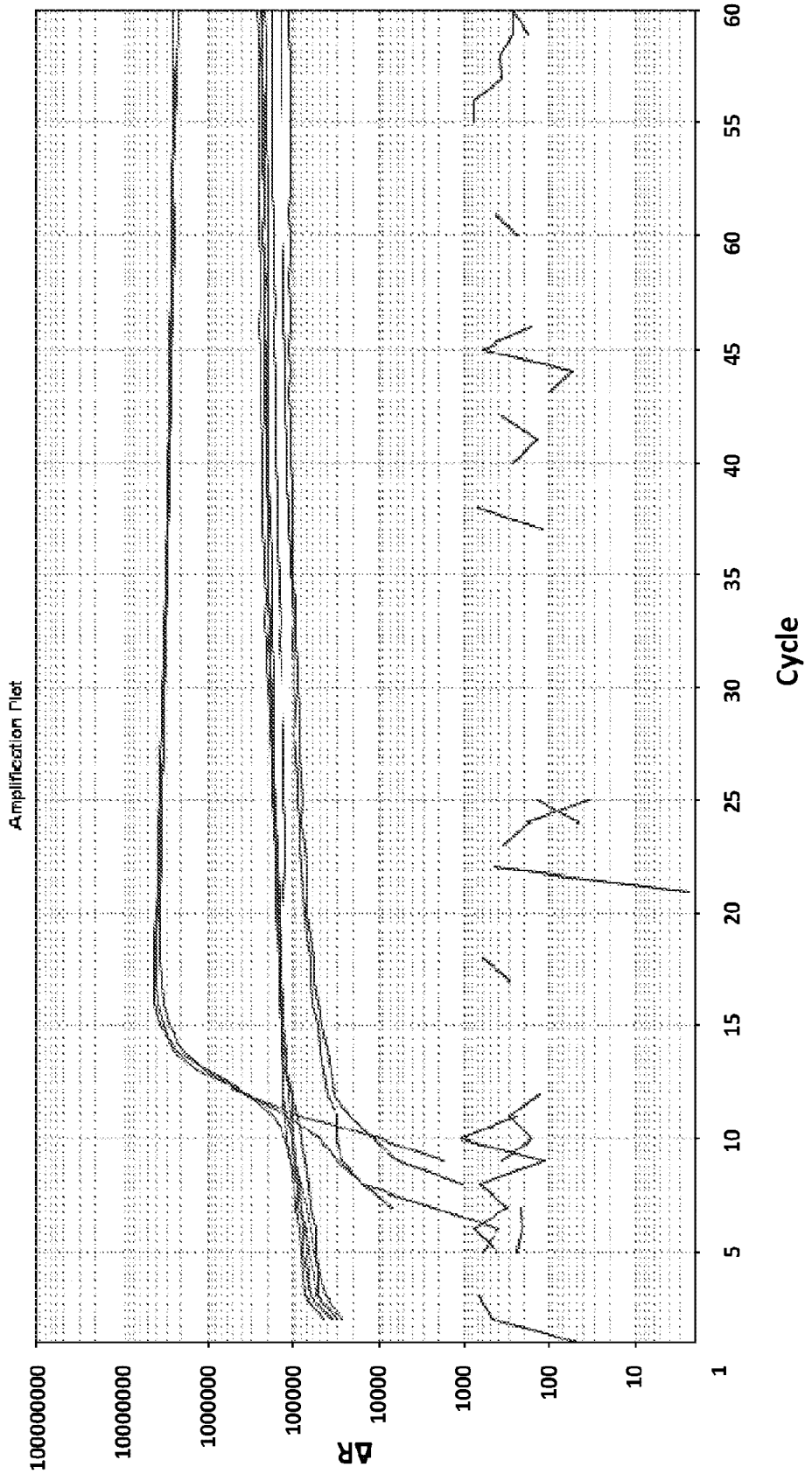
FIG 2C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60

FIG 2D

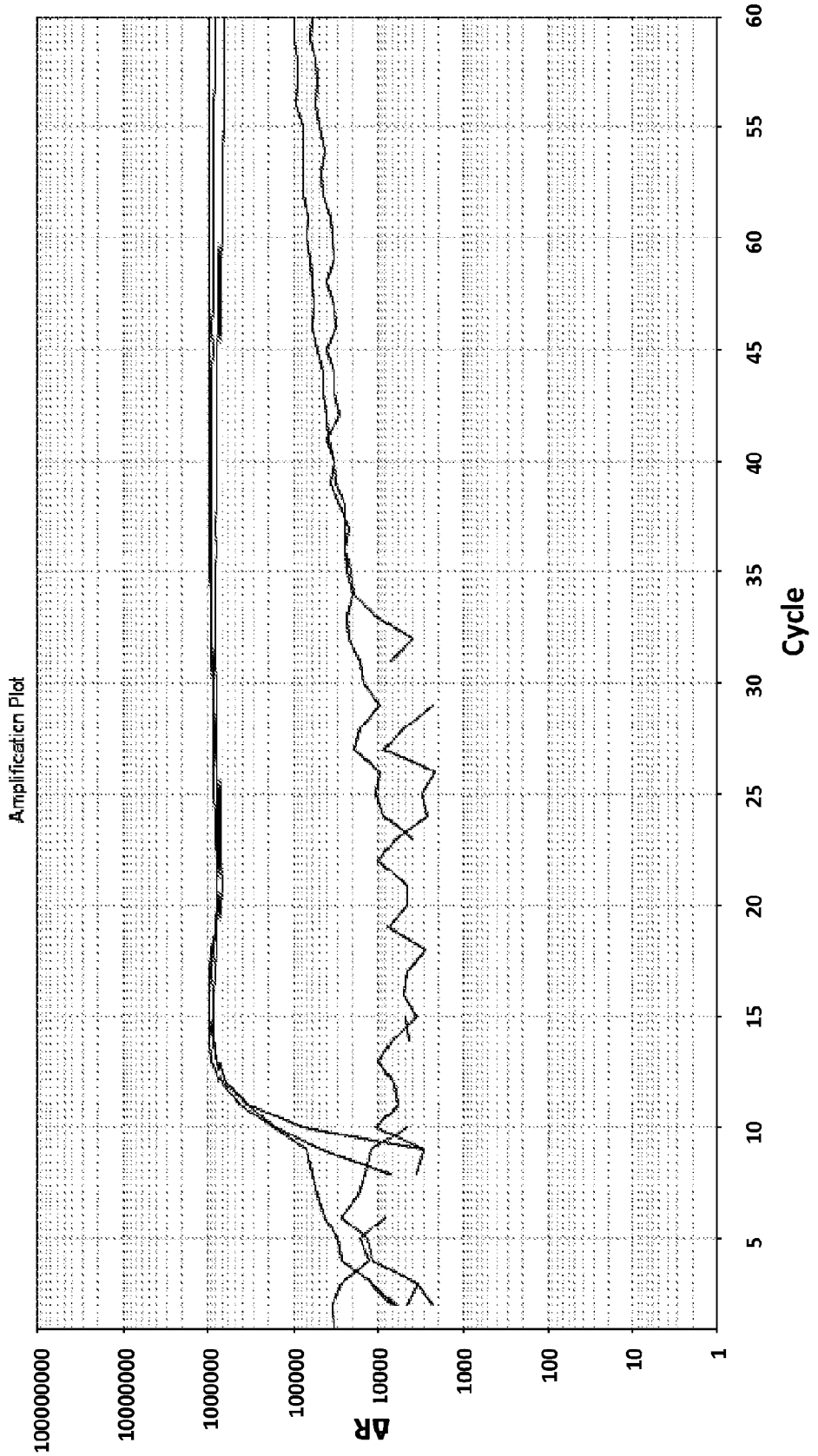
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FIG 2E

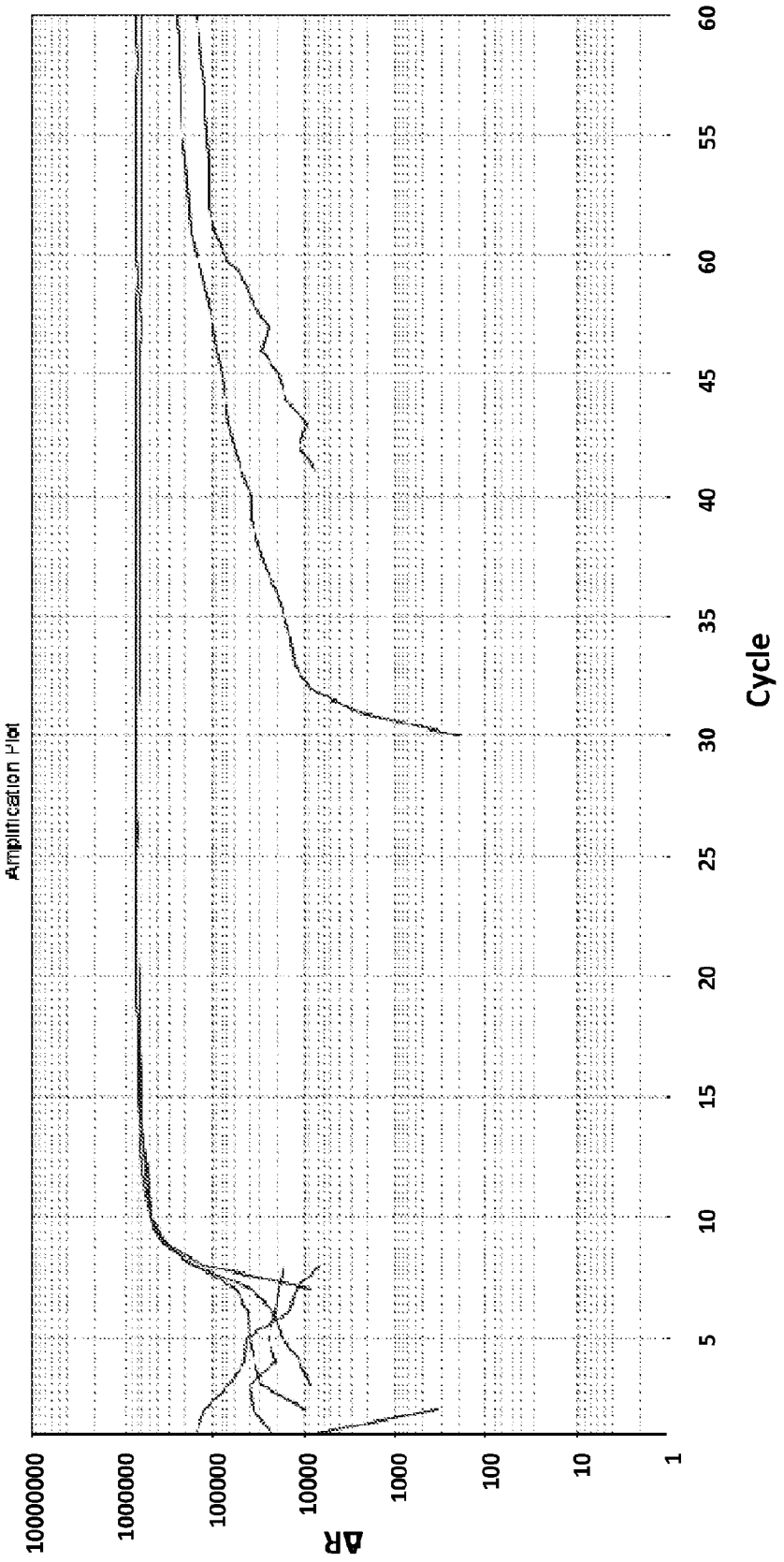
Amplification Plot
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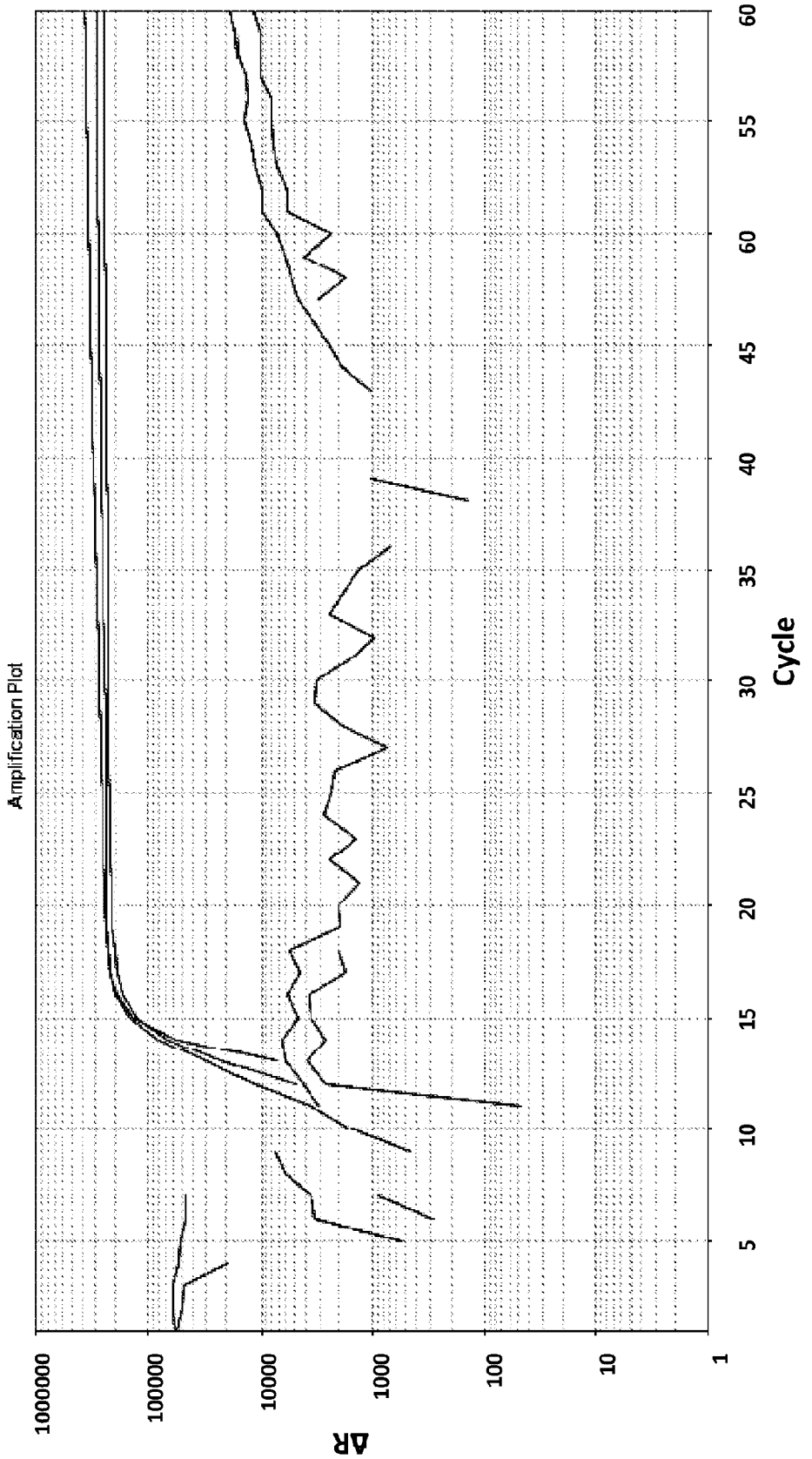
FIG 2F


Amplification Plot
ΔR
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10000
1000
100
10
1
5
10
15
20
25
30
35
40
45
50
55
60
Cycle

CT PB1– FAM melt curve

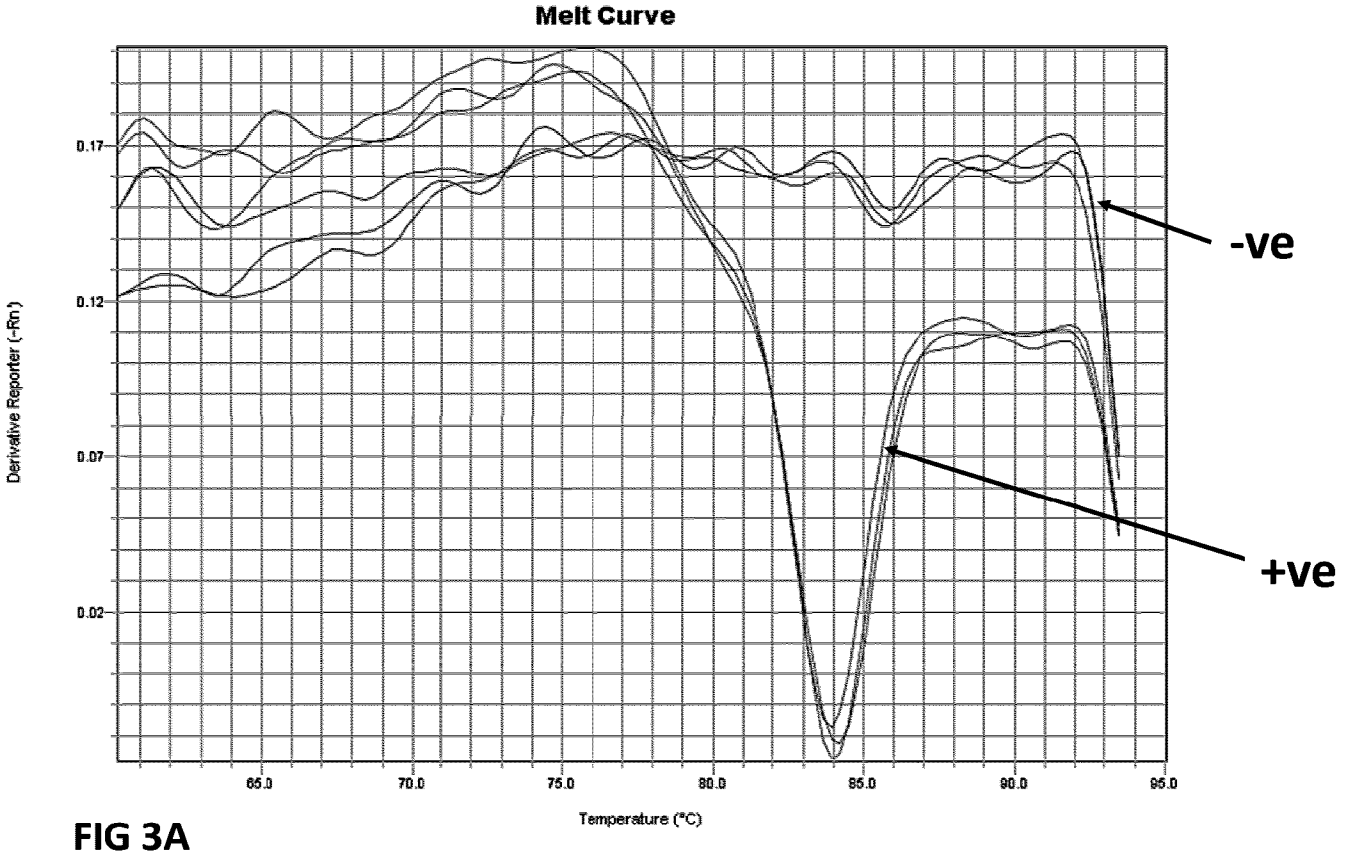


FIG 3A

Date Reçue/Date Received 2021-02-05

CT PB1– FAM melt curve

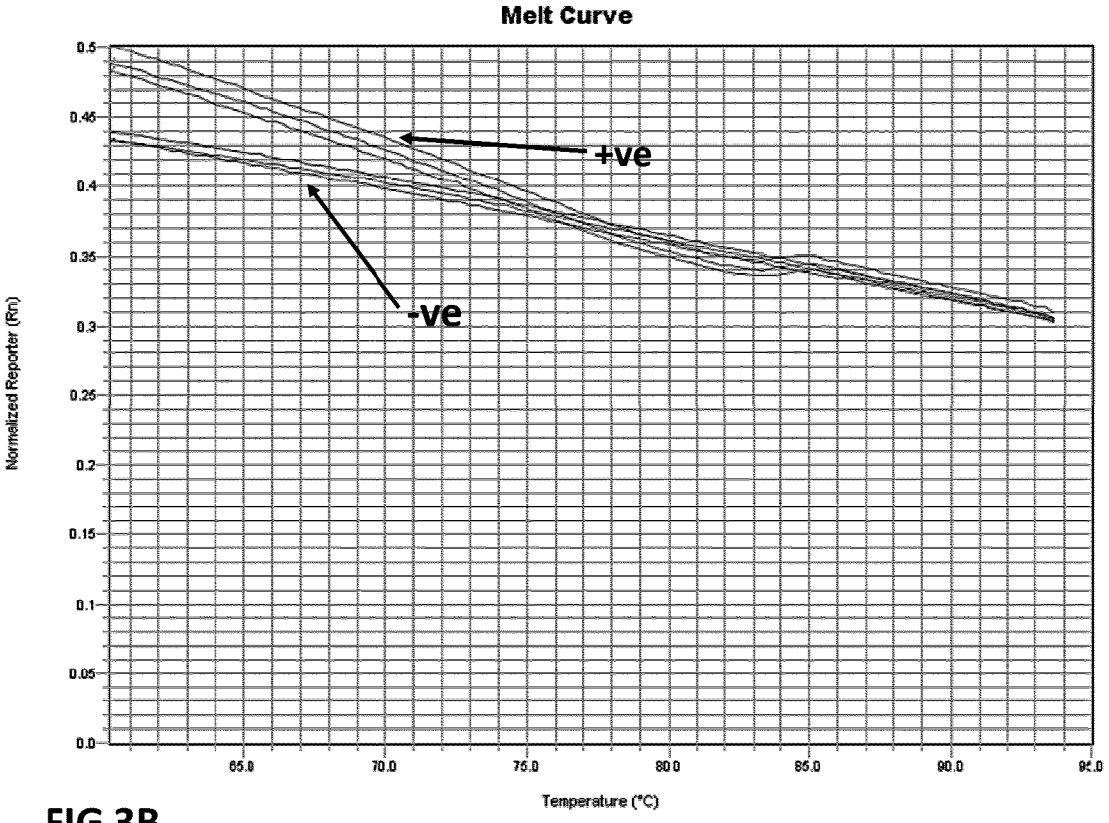


FIG 3B

Date Reçue/Date Received 2021-02-05

GC glnA7 – Joe melt curve

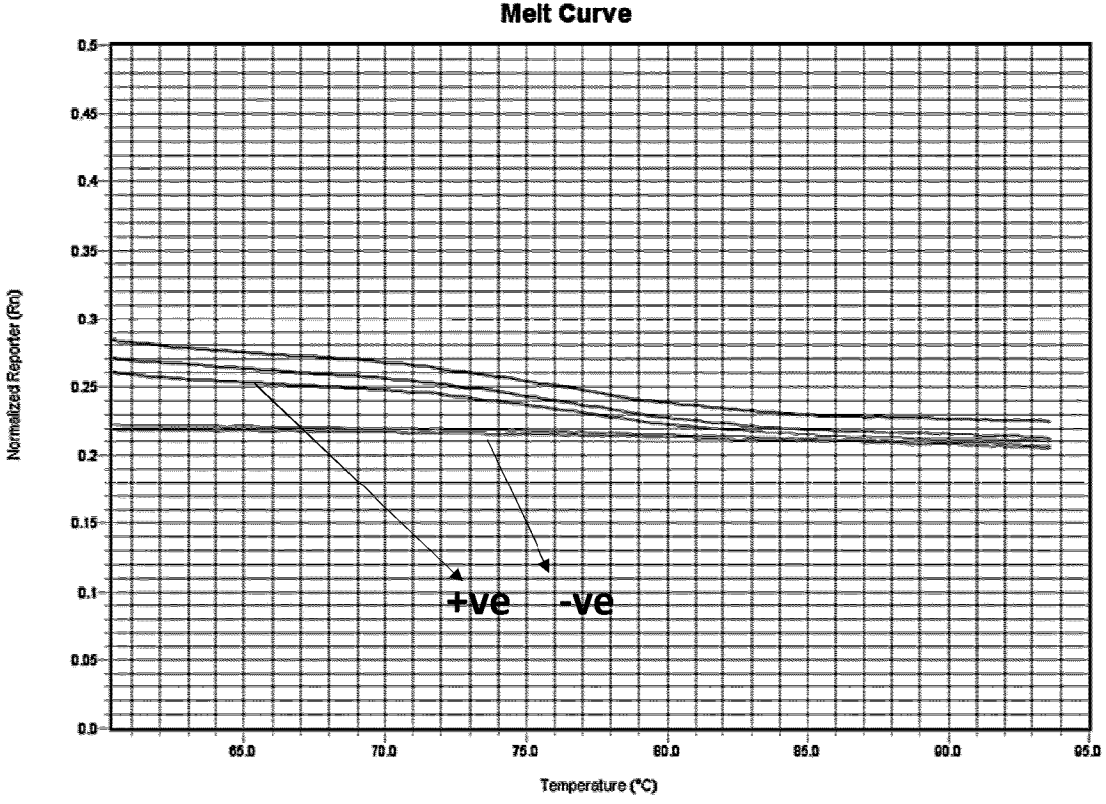


FIG 4A

Date Reçue/Date Received 2021-02-05

GC glnA7 – Joe melt curve

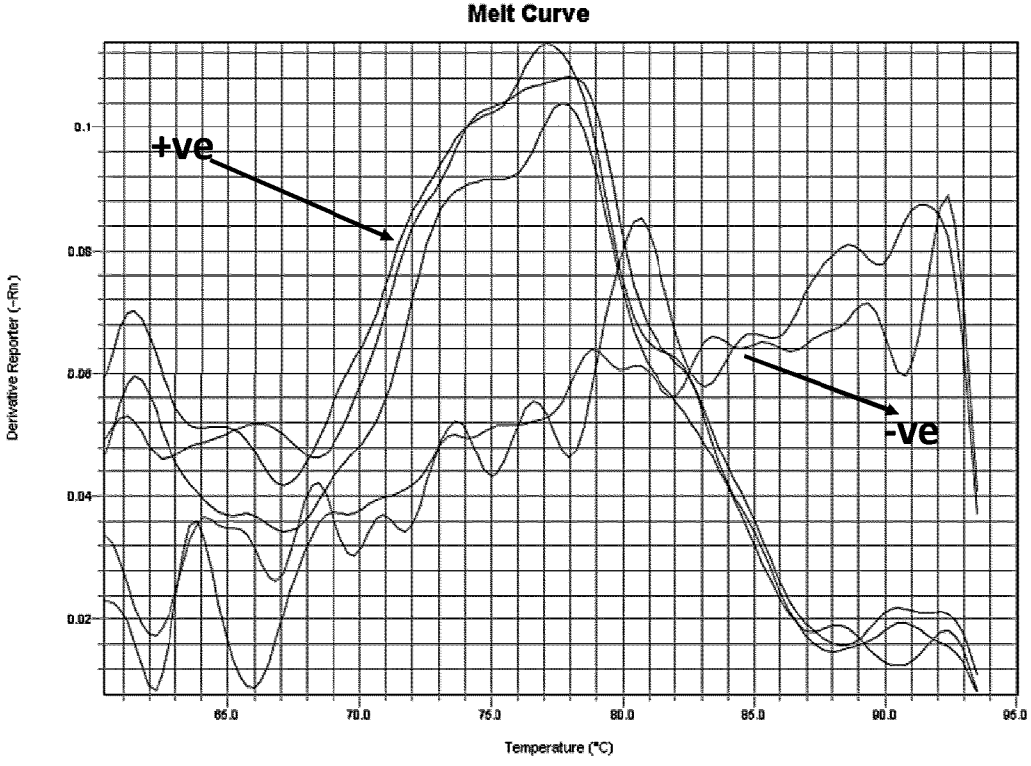


FIG 4B

Date Reçue/Date Received 2021-02-05

GC porA7 – Alexa546 melt curve

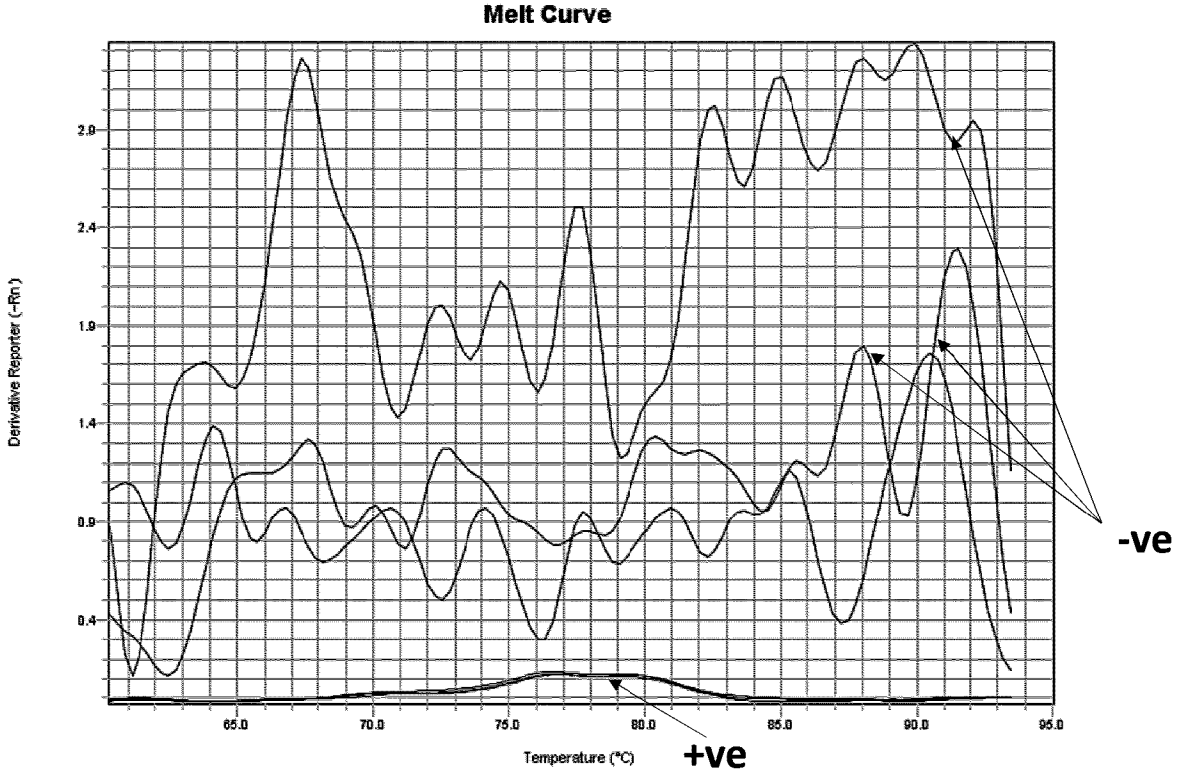


FIG 5A

Date Reçue/Date Received 2021-02-05

GC porA7 – Alexa546 melt curve

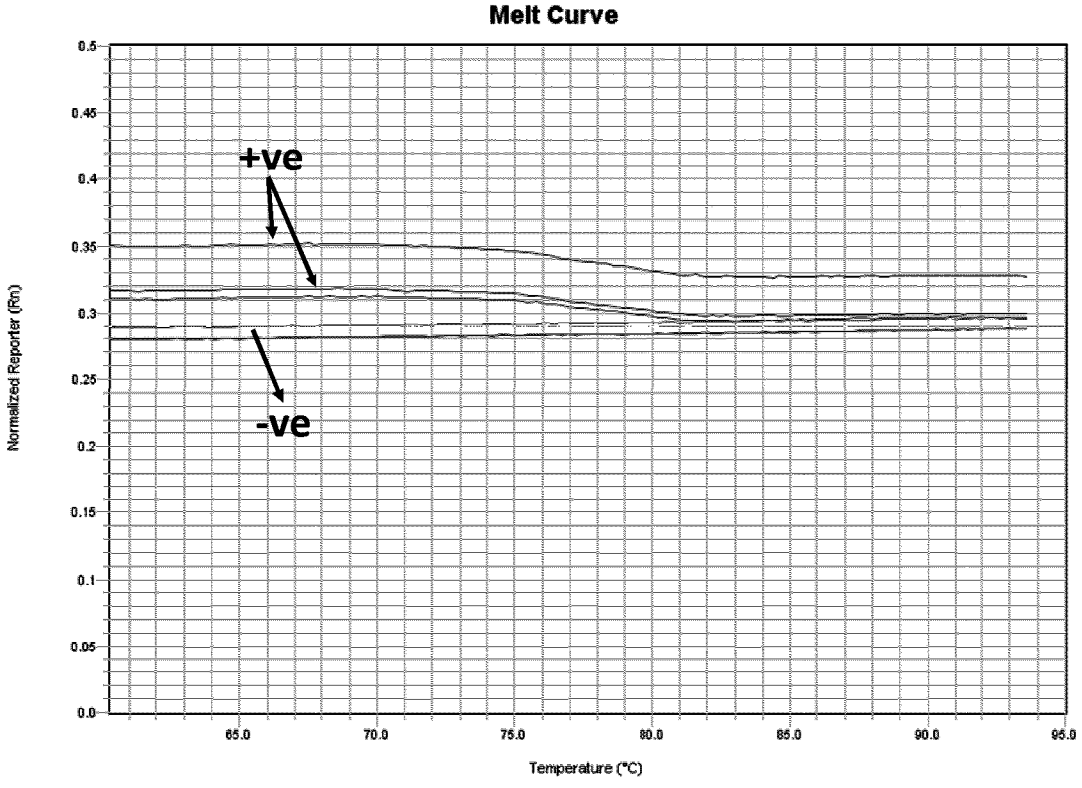


FIG 5B

Date Reçue/Date Received 2021-02-05

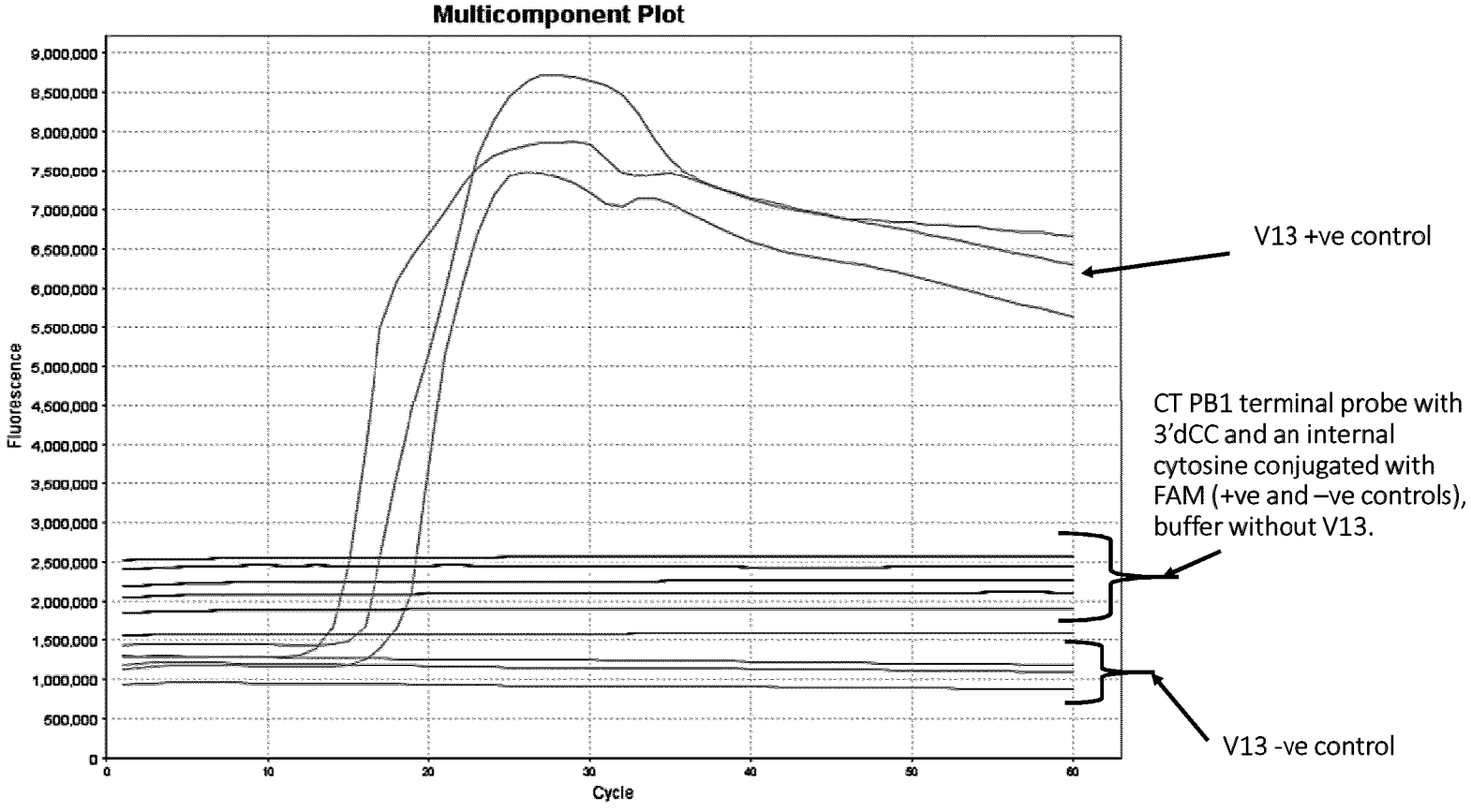


FIG 6

Date Reçue/Date Received 2021-02-05

FIG 6B

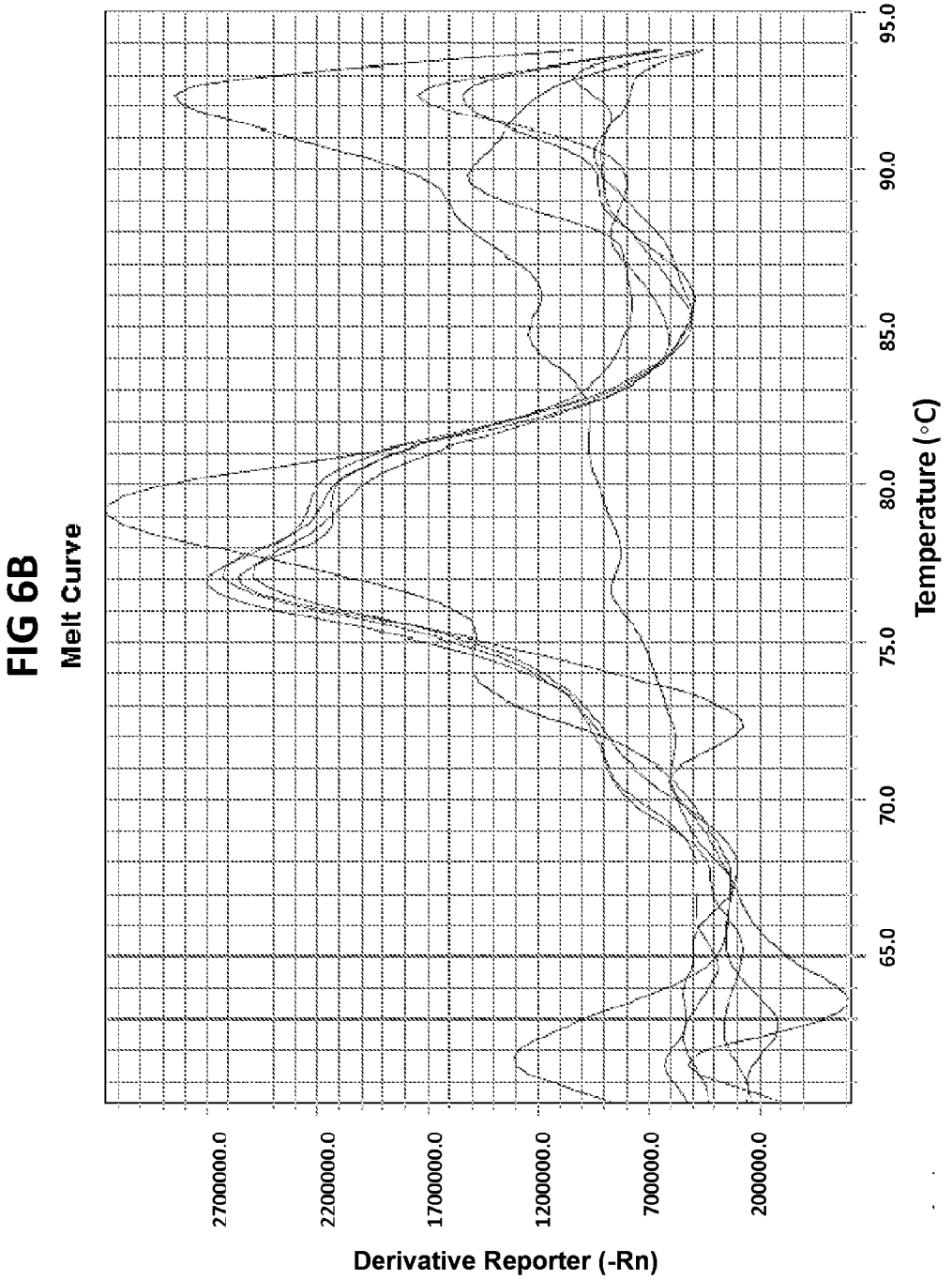

Melt Curve
Derivative Reporter (-Rn)
2700000.0
2200000.0
1700000.0
1200000.0
700000.0
200000.0
Temperature (°C)
95.0
90.0
85.0
80.0
75.0
70.0
65.0

FIG 6C

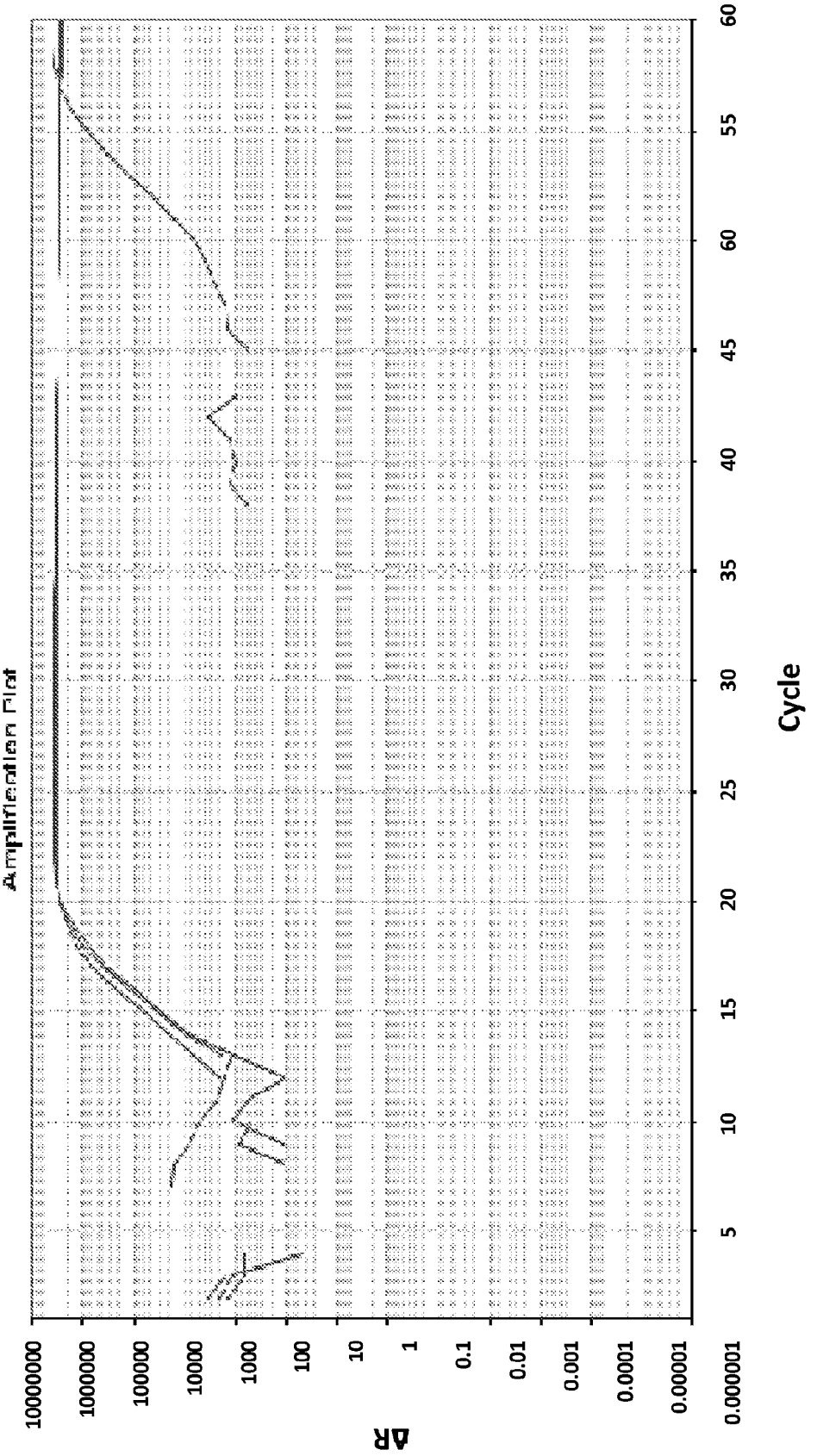

Amplification Plot
ΔR
Cycle
10000000
1000000
100000
10000
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
5
10
15
20
25
30
35
40
45
50
55
60

FIG 6D

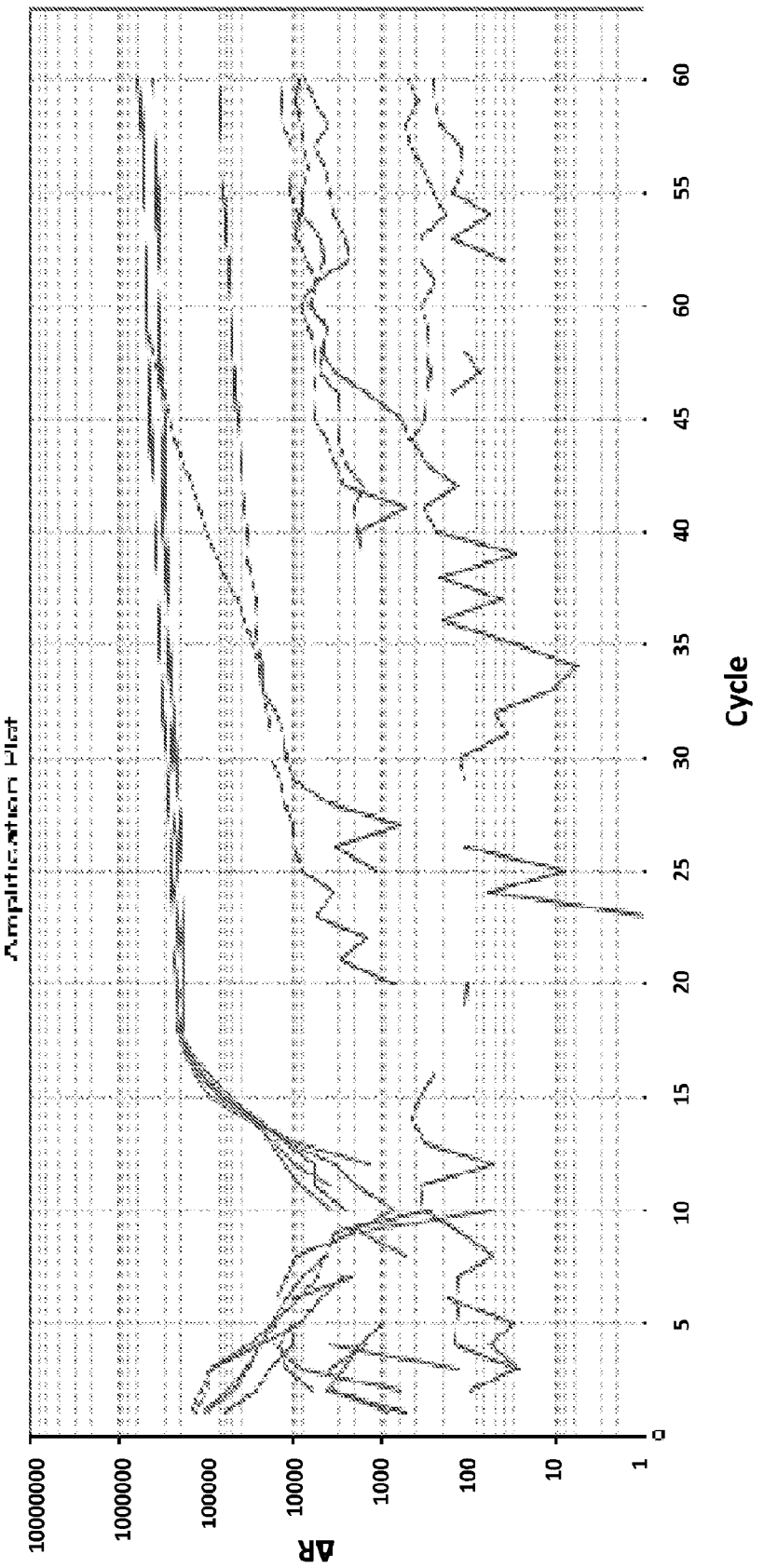
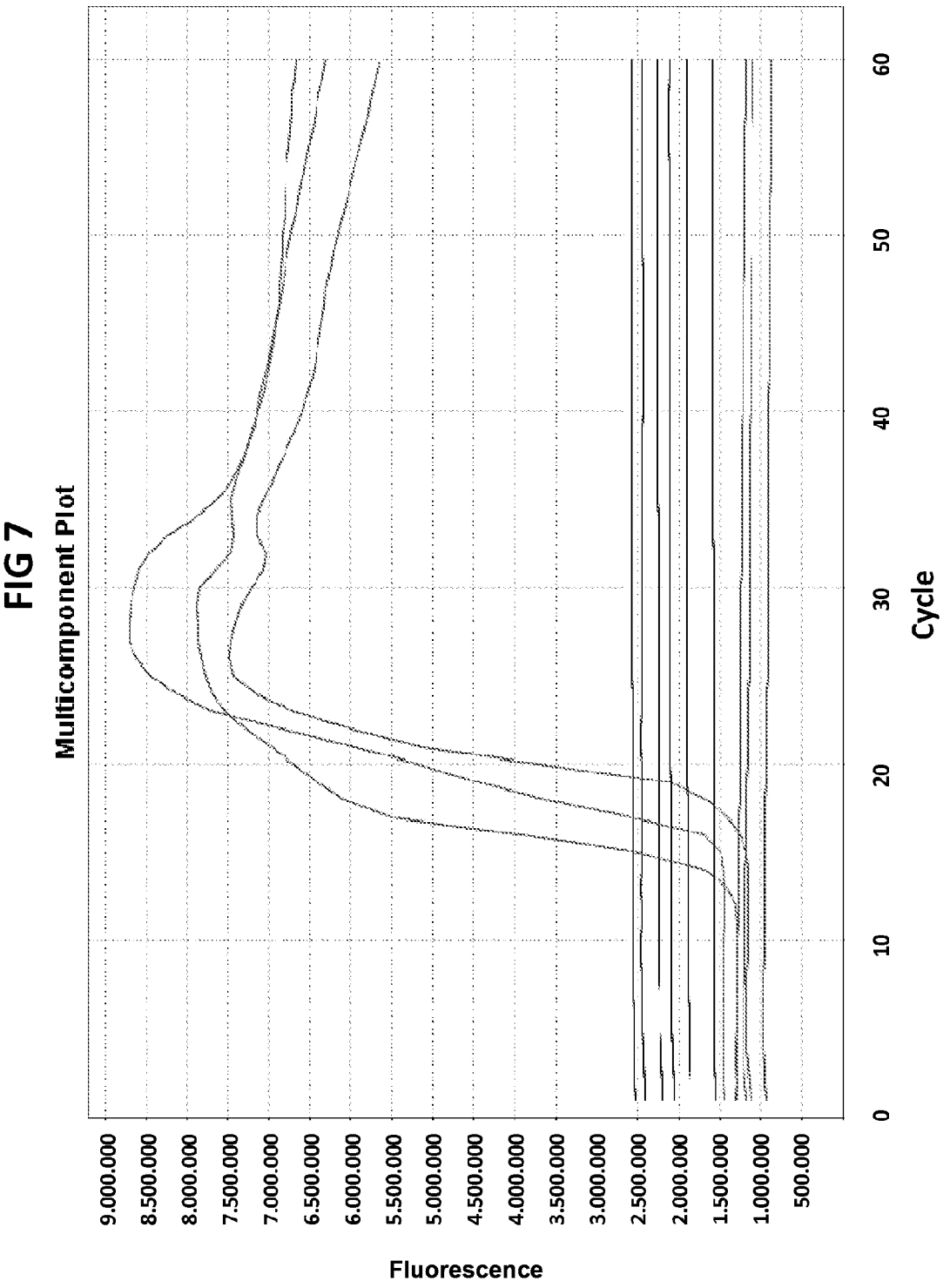

Amplification Plot
ΔR
10000000
1000000
100000
10000
1000
100
10
1
0
5
10
15
20
25
30
35
40
45
60
55
60
Cycle

FIG 7



Date Reçue/Date Received 2022-05-09

FIG 8A

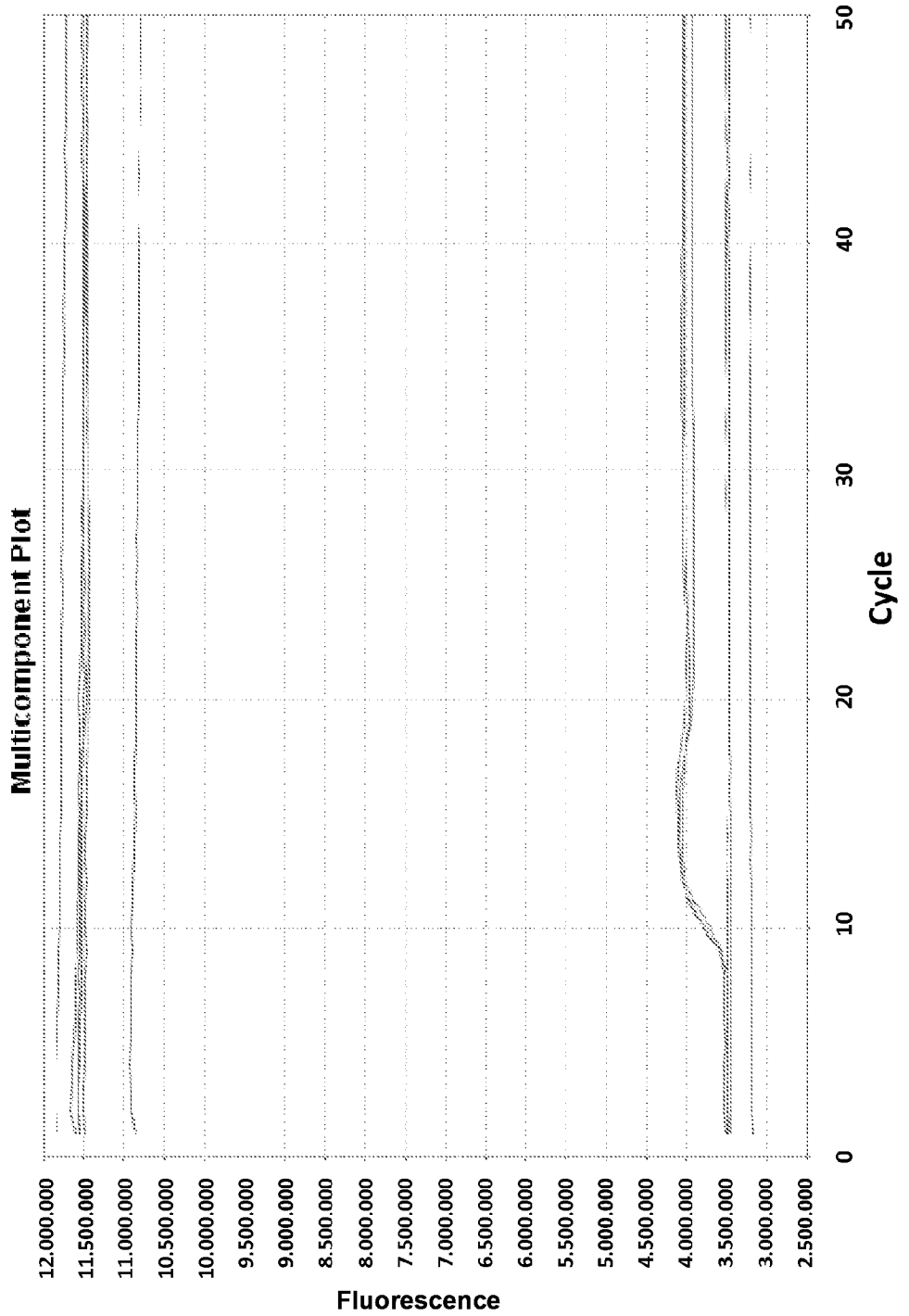

Multicomponent Plot
Fluorescence
Cycle
12.000.000
11.500.000
11.000.000
10.500.000
10.000.000
9.500.000
9.000.000
8.500.000
8.000.000
7.500.000
7.000.000
6.500.000
6.000.000
5.500.000
5.000.000
4.500.000
4.000.000
3.500.000
3.000.000
2.500.000
0
10
20
30
40
50

FIG 8B

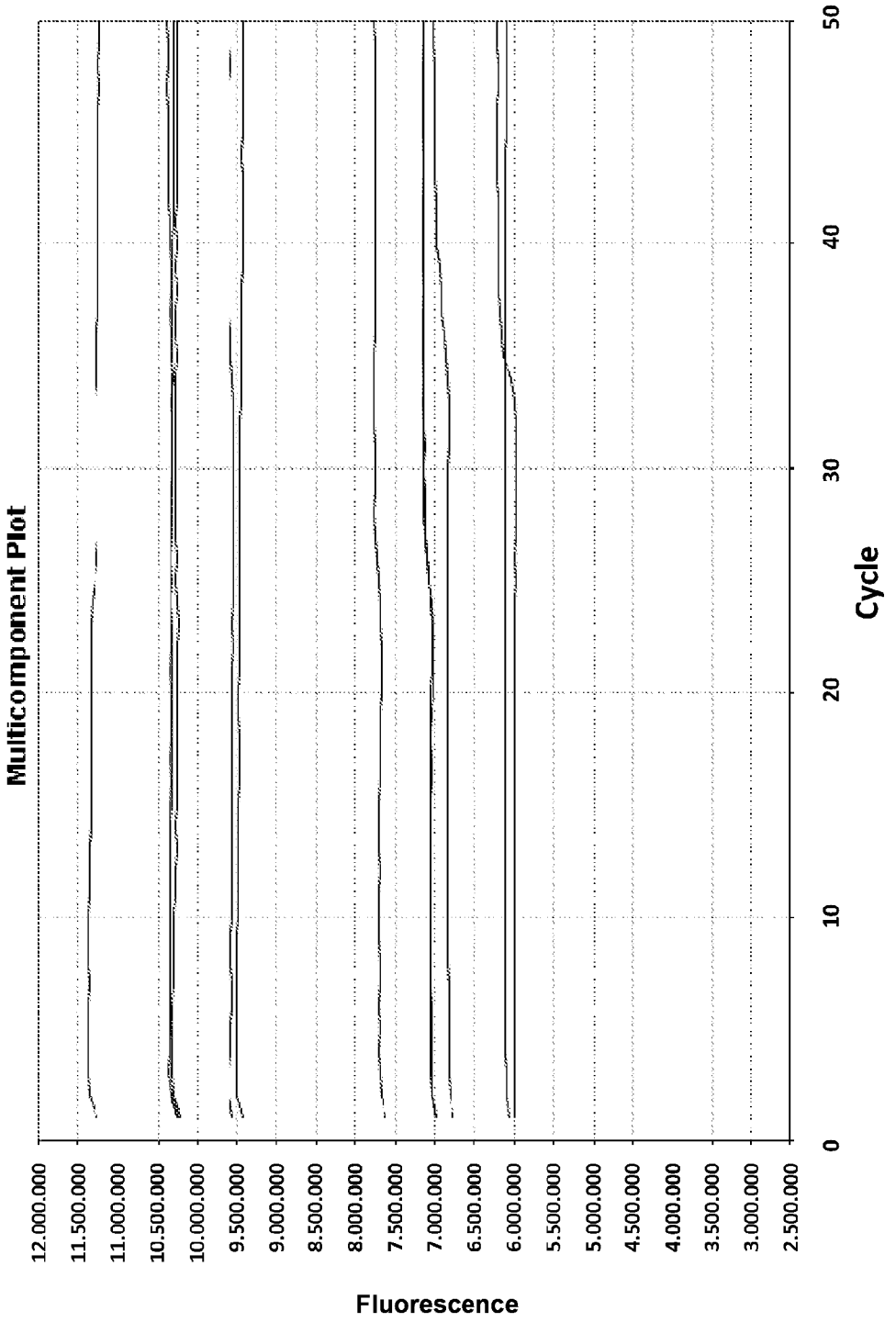
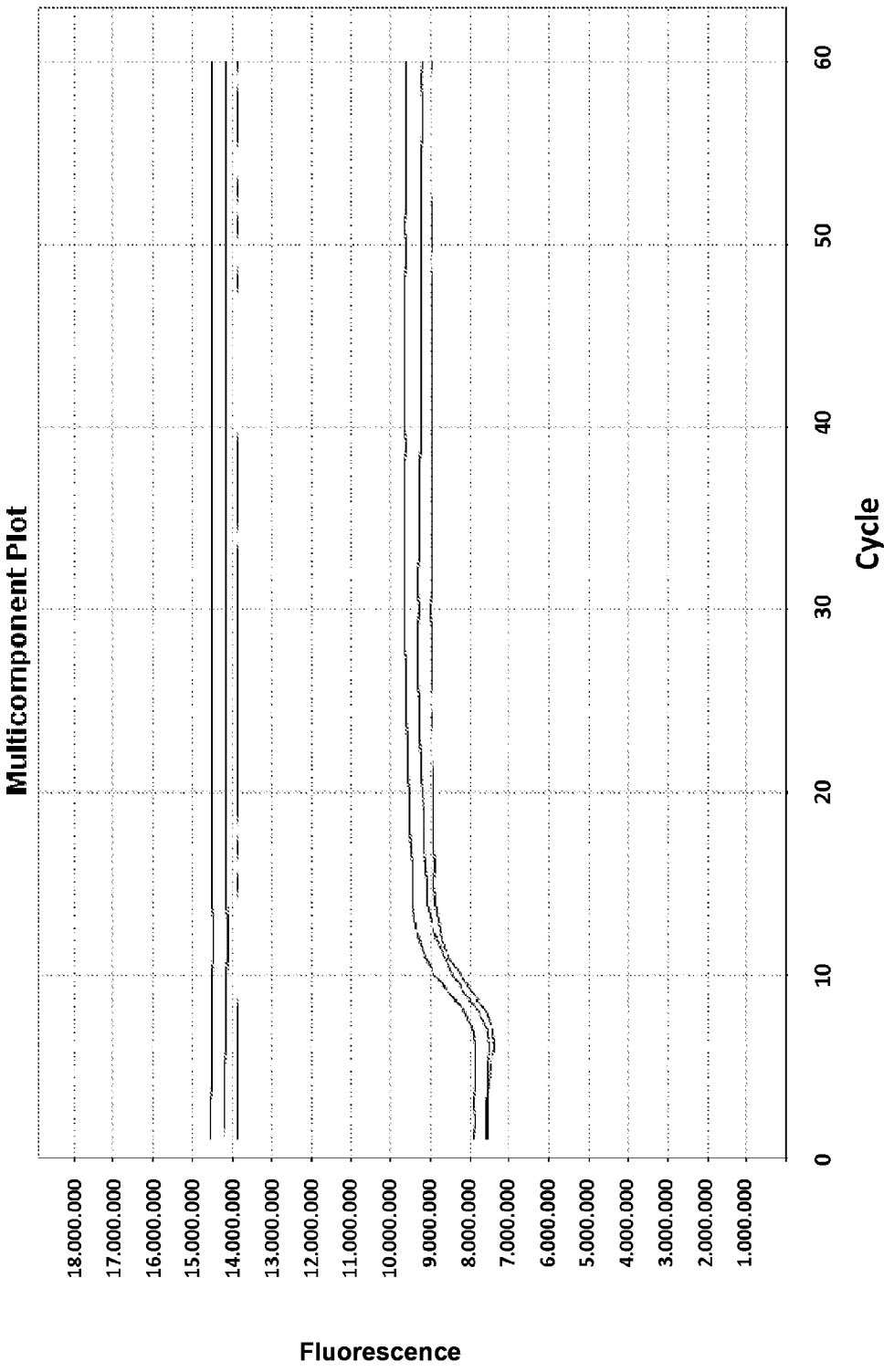

Multicomponent Plot
Fluorescence
Cycle
12.000.000
11.500.000
11.000.000
10.500.000
10.000.000
9.500.000
9.000.000
8.500.000
8.000.000
7.500.000
7.000.000
6.500.000
6.000.000
5.500.000
5.000.000
4.500.000
4.000.000
3.500.000
3.000.000
2.500.000
0
10
20
30
40
50

FIG 9A



Date Reçue/Date Received 2022-05-09

FIG 9B

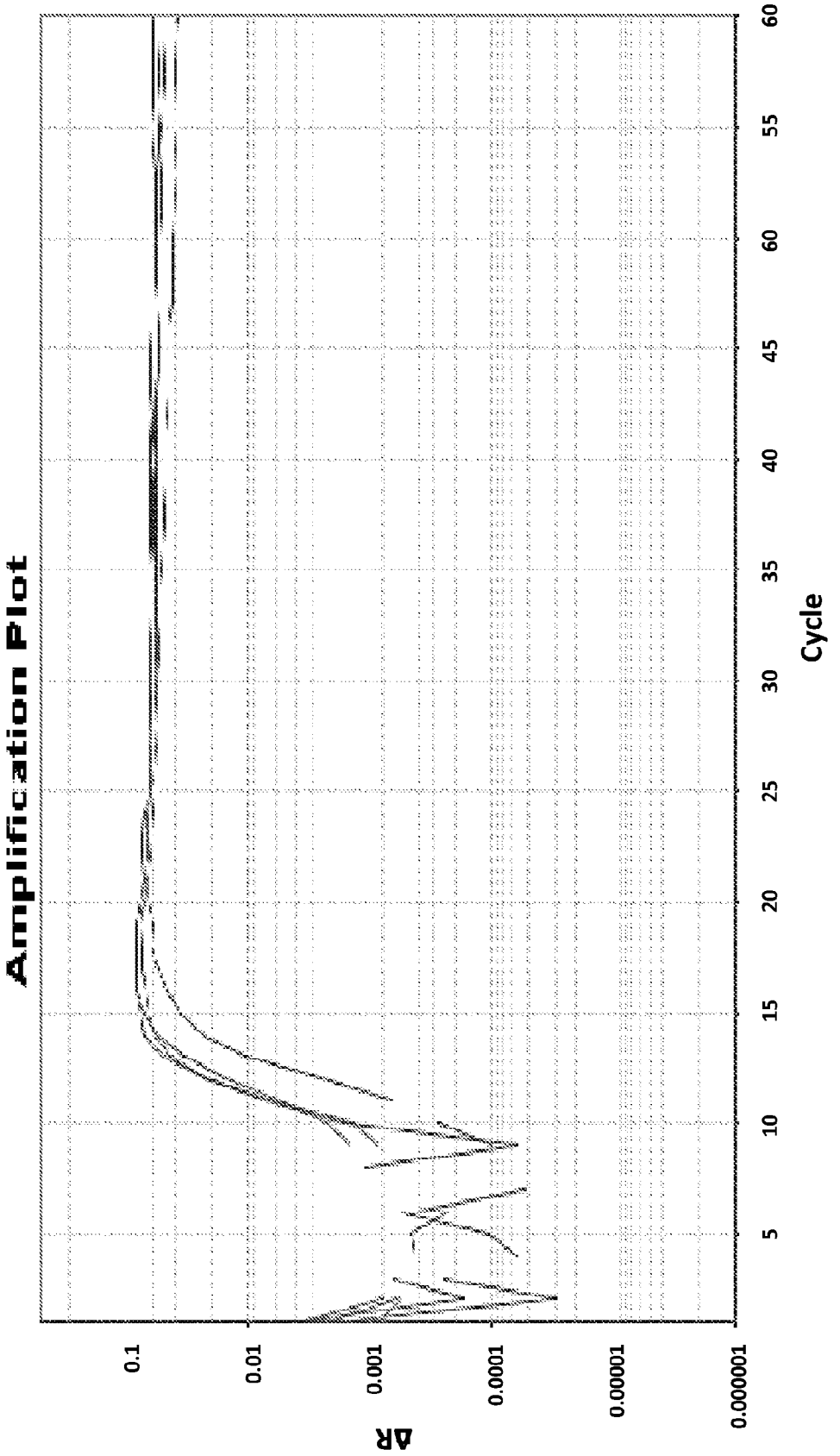
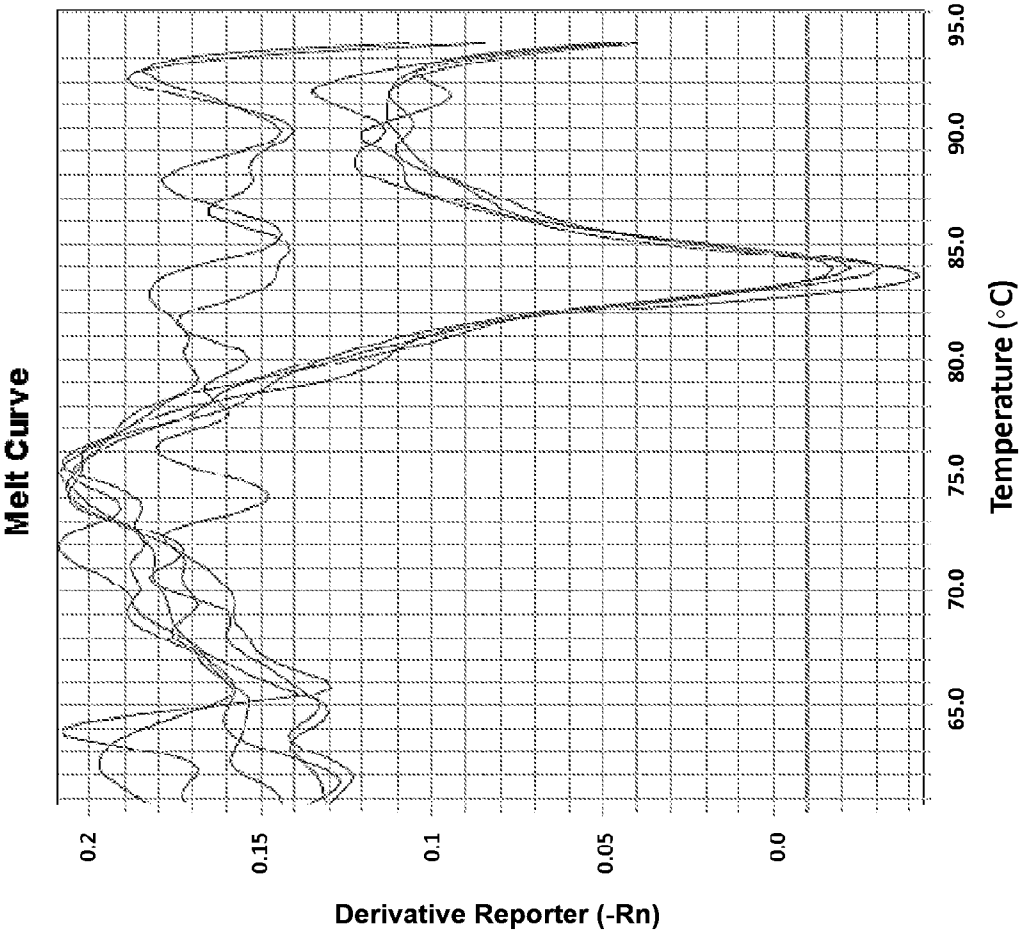

Amplification Plot
ΔR
0.1
0.01
0.001
0.0001
0.00001
0.000001
5
10
15
20
25
30
35
40
45
55
60
Cycle

FIG 9C



Date Reçue/Date Received 2022-05-09

FIG 10A

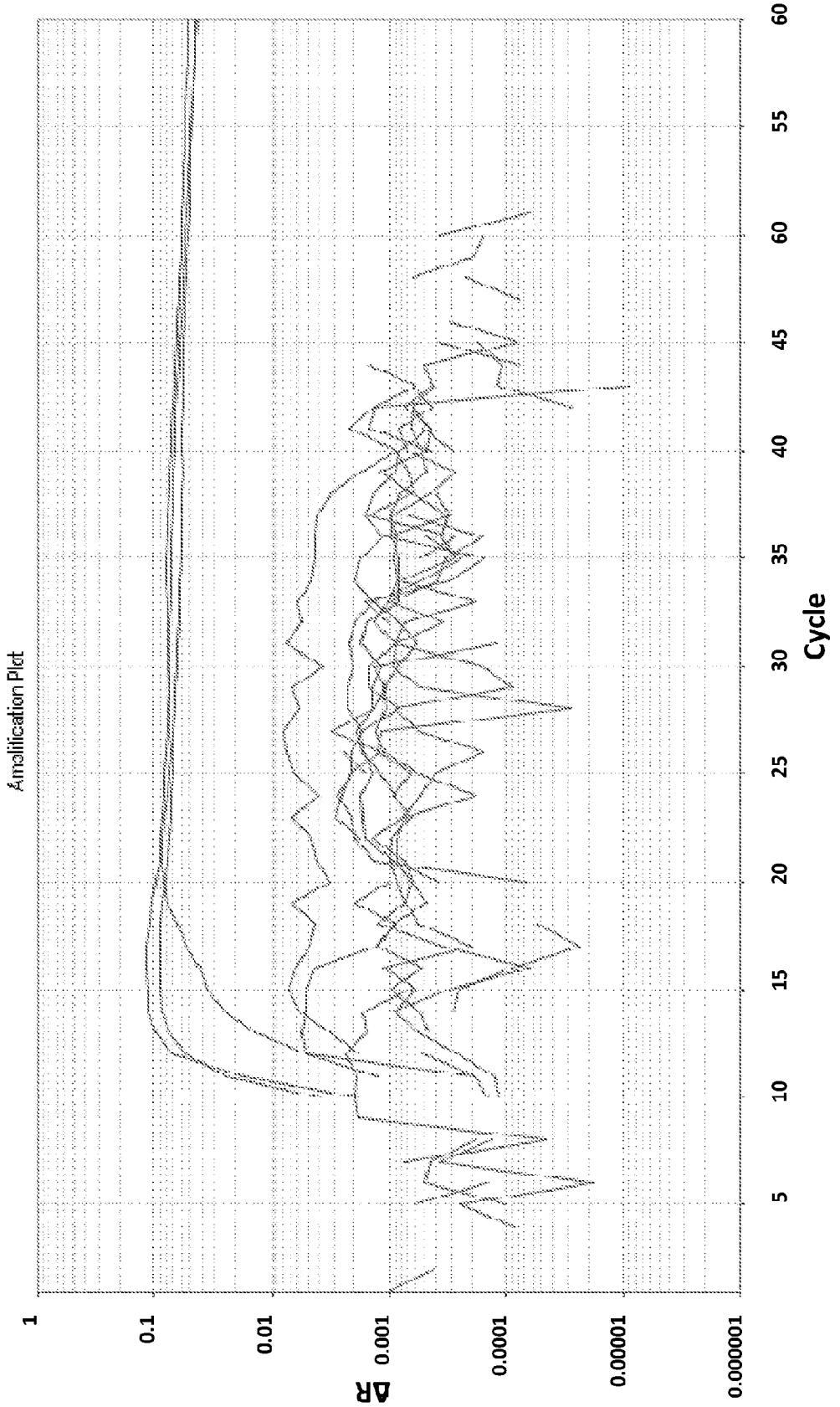

Amplification Plot
ΔR
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
5
10
15
20
25
30
35
40
45
50
55
60
Cycle

FIG 10B

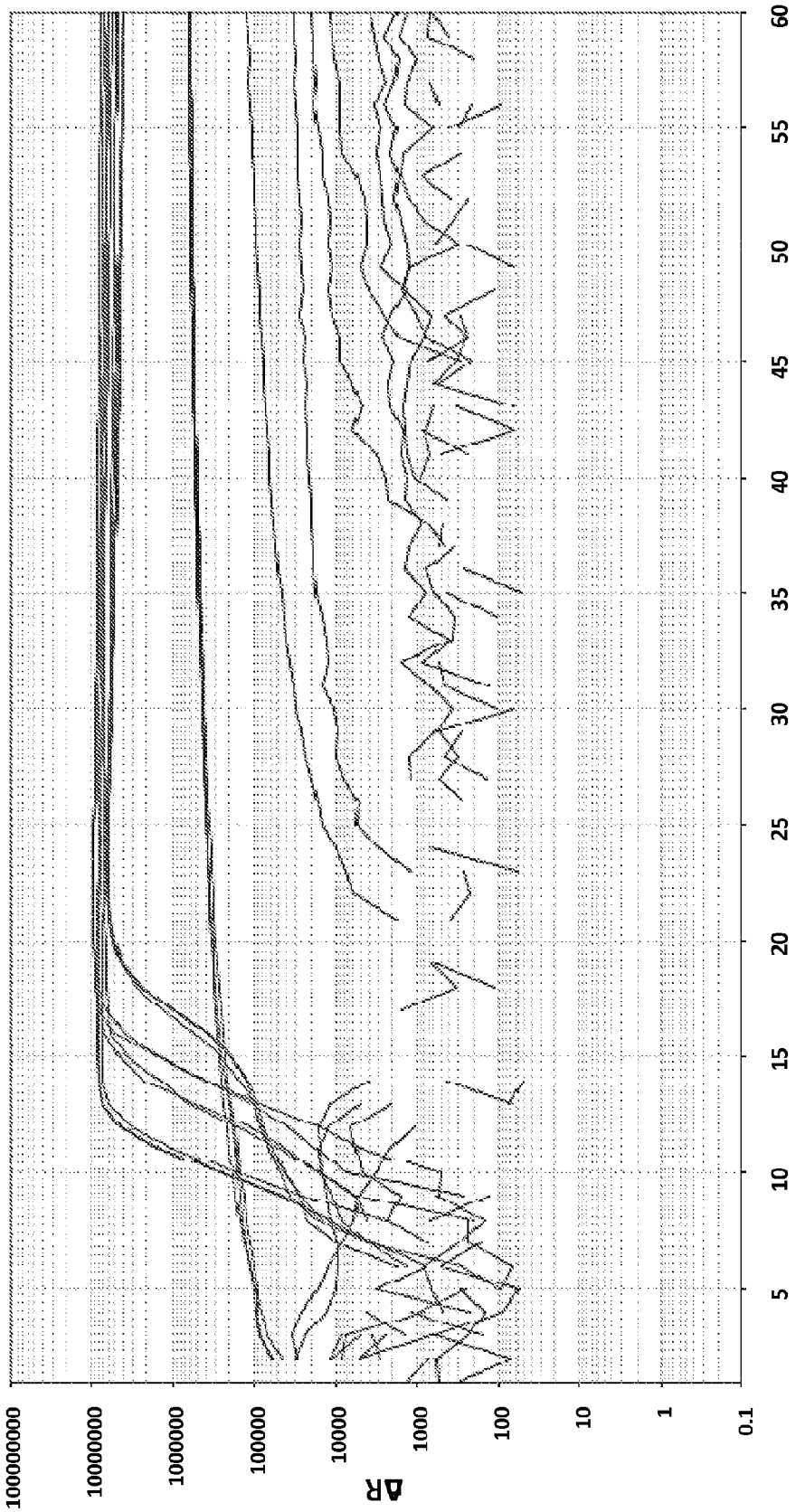

Amplification plot
ΔR
100000000
10000000
1000000
100000
10000
1000
100
10
1
0.1
5
10
15
20
25
30
35
40
45
50
55
60
Cycle

FIG 10C

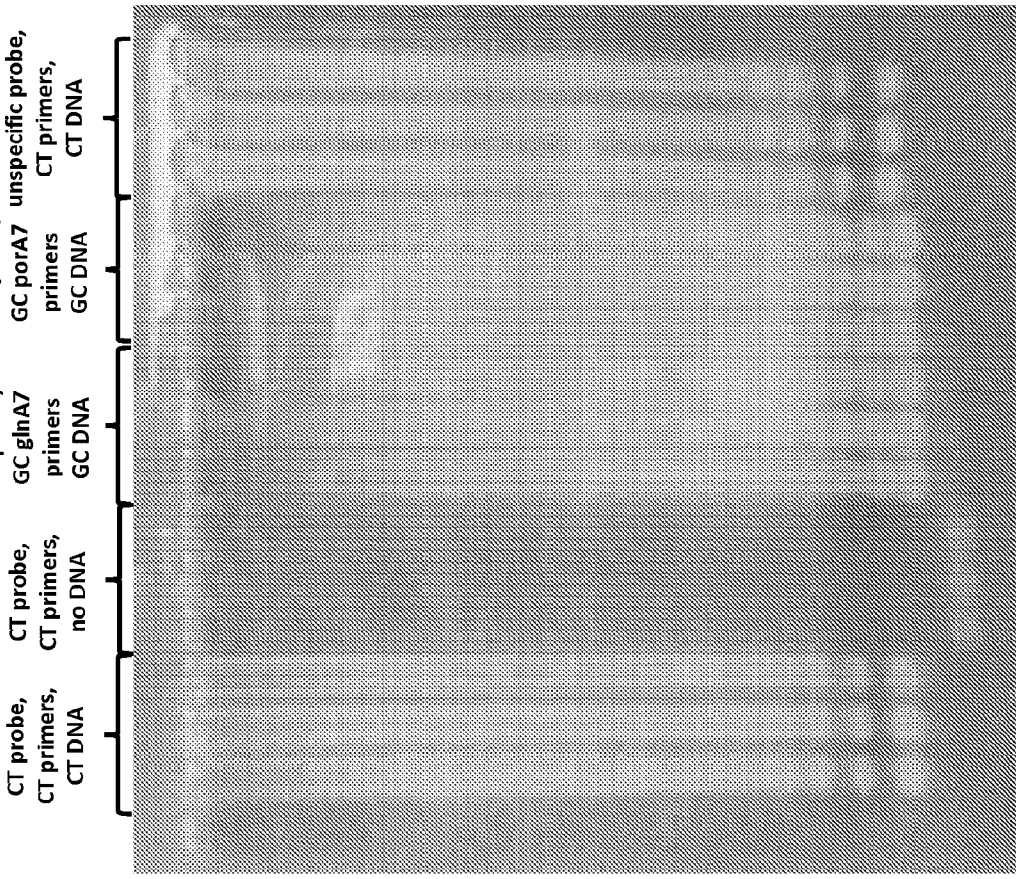

CT probe, CT primers, CT DNA
CT probe, CT primers, no DNA
CT probe, GC glnA7 primers GC DNA
CT2 probe, GC porA7 primers GC DNA
unspecific probe, CT primers, CT DNA

FIG 11A

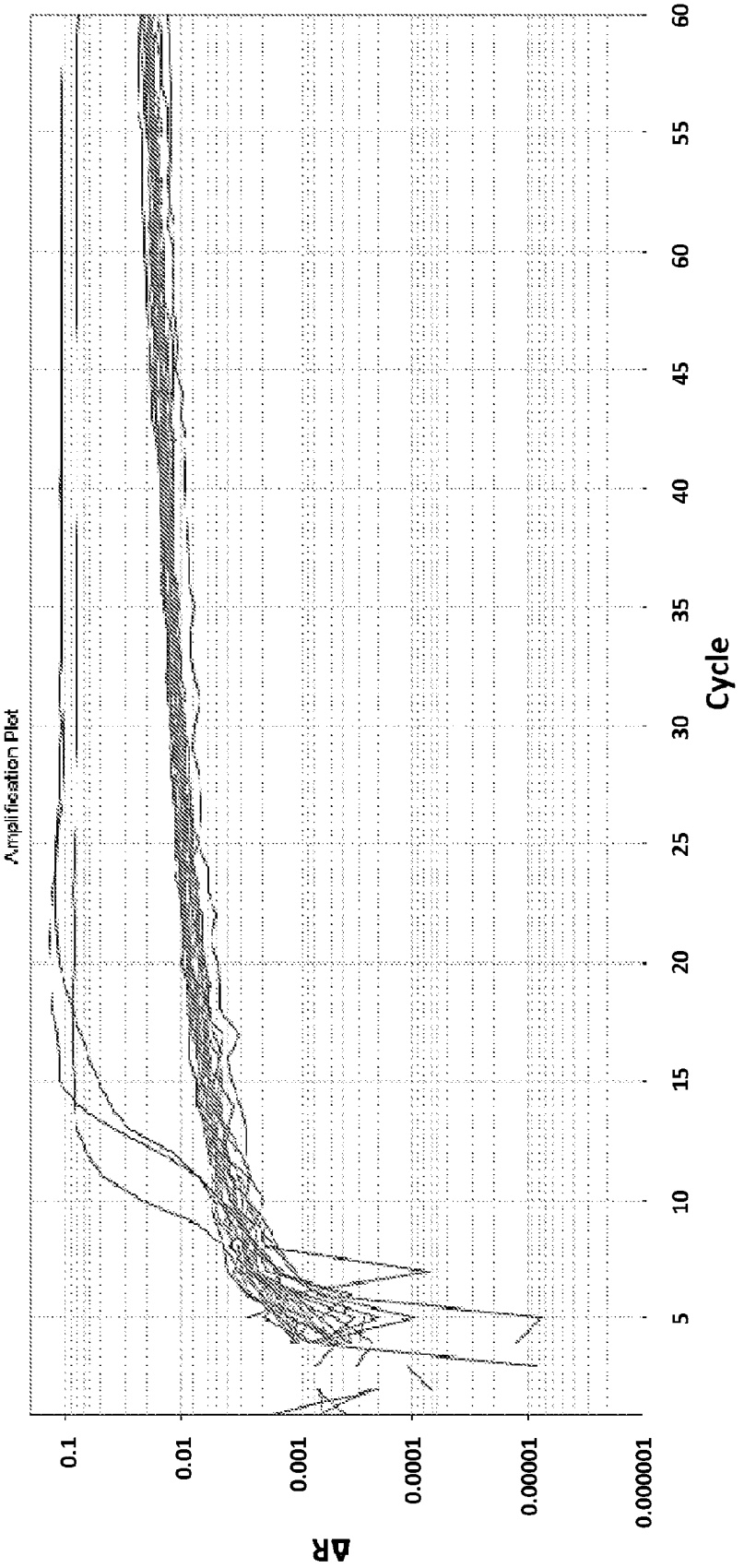

Amplification Plot
ΔR
0.1
0.01
0.001
0.0001
0.00001
0.000001
5
10
15
20
25
30
35
40
45
50
55
60
Cycle

FIG 11B

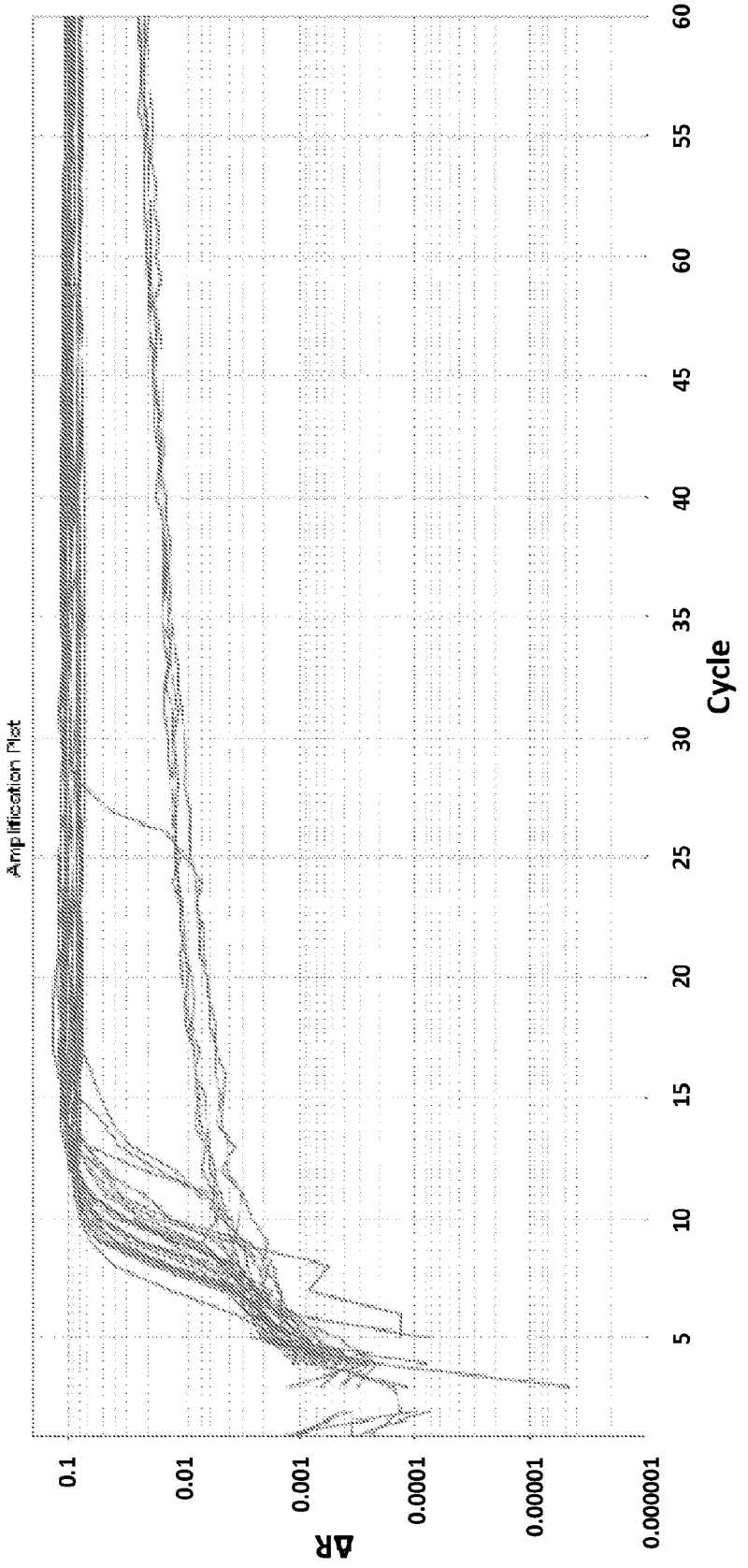
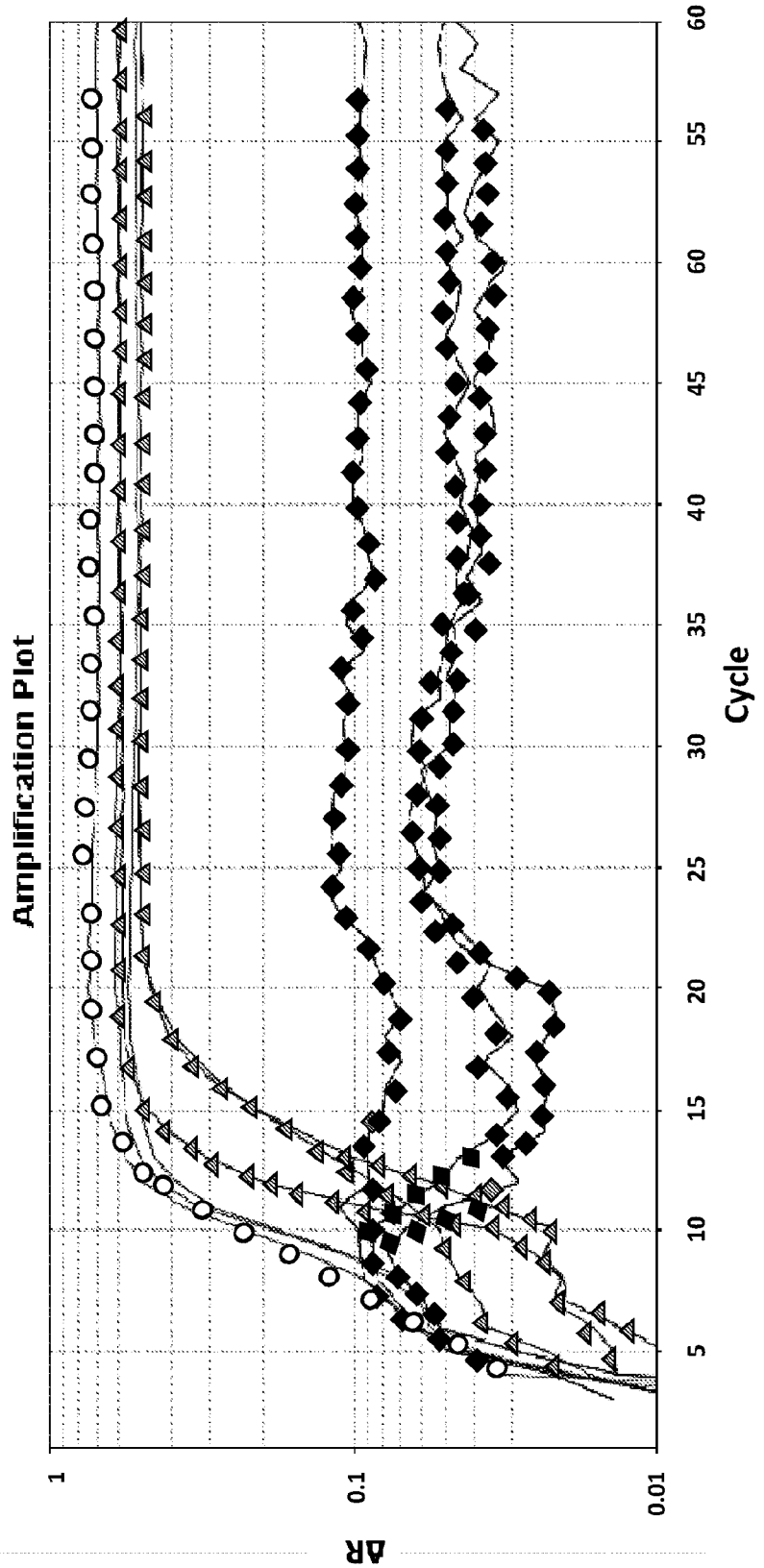

Amplification Plot
ΔR
0.1
0.01
0.001
0.0001
0.00001
0.000001
5
10
15
20
25
30
35
40
45
50
55
60
Cycle

FIG 12A


Amplification Plot
ΔR
1
0.1
0.01
5
10
15
20
25
30
35
40
45
60
55
60
Cycle

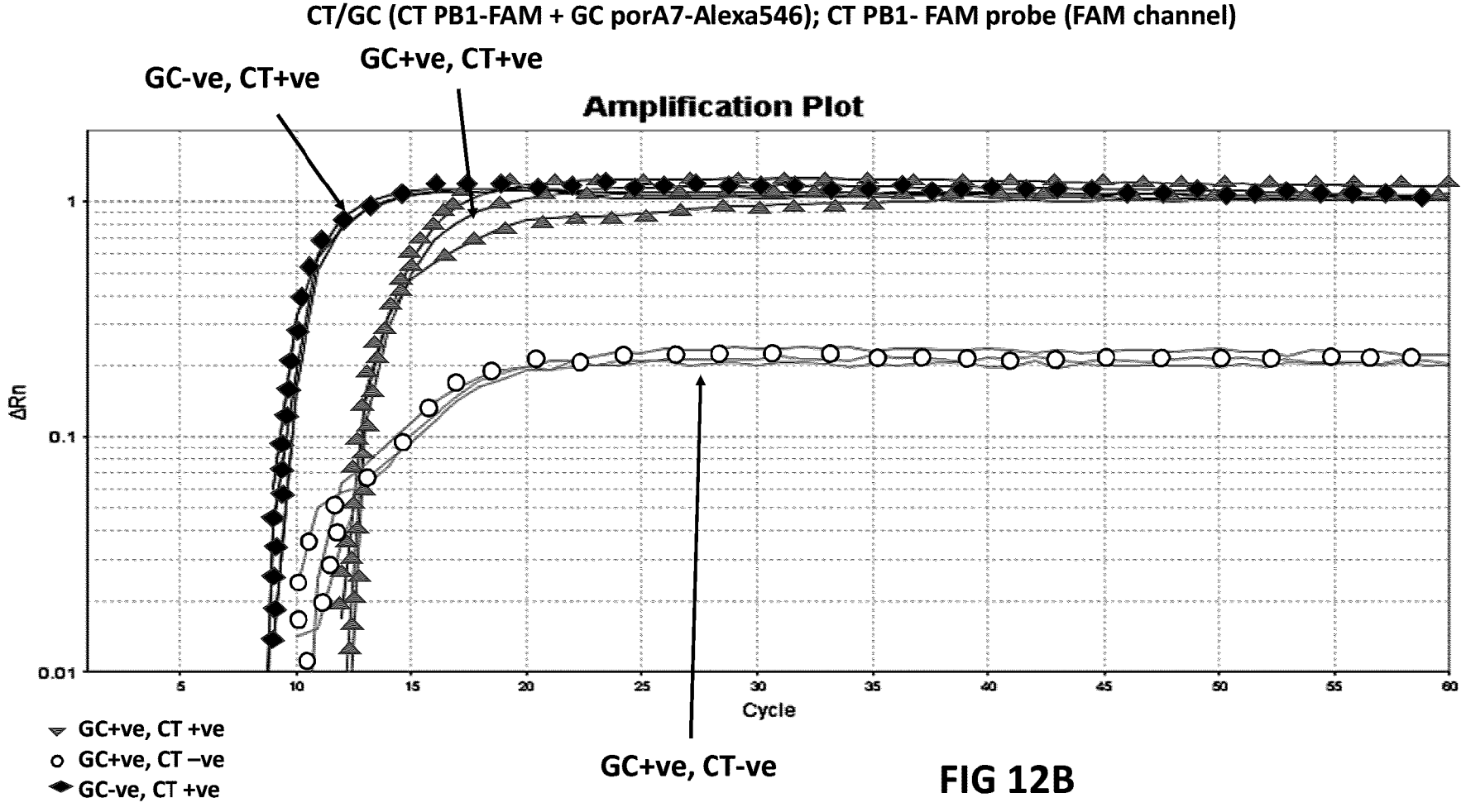


FIG 12B

Date Reçue/Date Received 2021-02-05

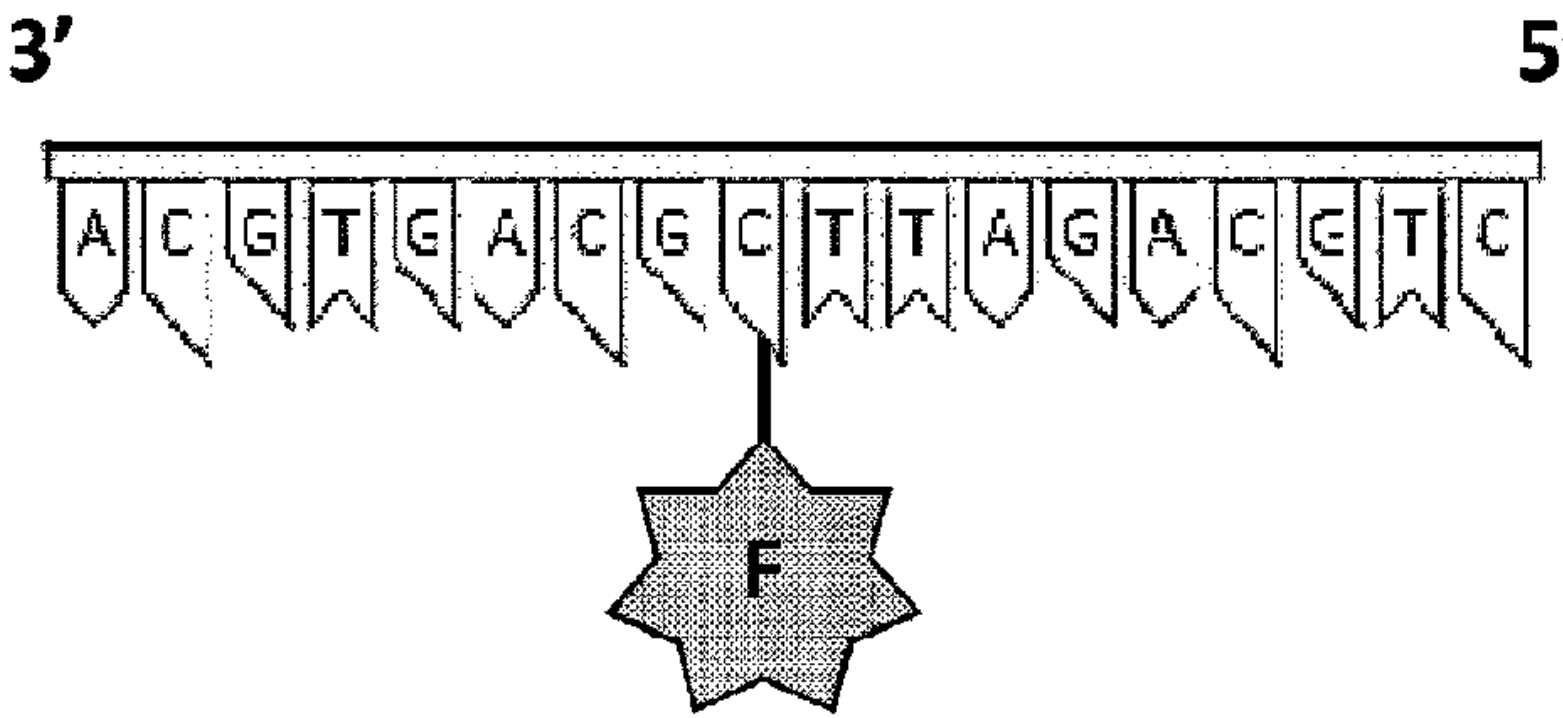
Table1

Comparison between V13-based LAMP for CT and GC, CT/GC Aptima multiplex and CT/GC MAST multiplex (CT PB1-FAM + GC porA7-Alexa546).

Test on 136 clinical samples

Tests confirmed	Agreement score
CT LAMP vs CT PB1-FAM in multiplex	92%
GC LAMP vs GC porA7-Alexa546 in multiplex	94%
CT in multiplex vs CT Aptima	83%
GC in multiplex vs GC Aptima	86%

Schematic of MAST DNA probe



5' T GCA CT GCGAA TC TG CAG 3'