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TITLE:

A Droplet PCR-Based Next Generation Sequencing Assay to Track Plasma DNA Mutation Dynamics in Estrogen Receptor Positive Metastatic Breast Cancer

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SUMMARY:

Here we describe a protocol that uses droplet polymerase chain reaction for target enrichment followed by next generation sequencing of blood plasma circulating tumor DNA. This technique was used to characterize mutations in the genes *ESR1* (all coding regions), *TP53* (all coding regions), *PIK3CA* (hotspots), *PIK3R1* (hotspots) and *POLE* (exonuclease domain).

ABSTRACT:

The estrogen receptor gene (*ESR1*) is expressed in approximately two-thirds of breast cancers (BCs) and predicts sensitivity to endocrine therapy. Mutations in *ESR1* have recently been associated with endocrine therapy resistance in patients with estrogen receptor positive metastatic breast cancer (ER+ MBC). Thus, monitoring the status of *ESR1* mutations may facilitate personalized therapy decisions for ER+ MBC patients. Additionally, mutations in *PIK3CA* and *TP53* are also prevalent in ER+ MBC and may influence therapeutic responses. Recent studies demonstrate mutational heterogeneity in metastatic breast cancer (MBC), highlighting a need to monitor for the emergence of new mutations over time. The analysis of blood plasma circulating tumor DNA (ctDNA) by next generation sequencing (NGS) has emerged as an attractive approach to address the mutation heterogeneity and evolution of MBC over time. However, the high costs and intensive bioinformatics required for plasma ctDNA NGS analysis limit its utility in clinical studies that require longitudinal monitoring. We have recently developed and validated an assay for plasma ctDNA mutation profiling that utilizes droplet PCR-based multiplexed target enrichment followed by NGS, which we have termed dPCR-Seq. Here we describe the protocol

for dPCR-Seq, illustrating its relative simplicity in library preparation and bioinformatics analysis to detect *ESR1* (all coding regions), *TP53* (all coding regions), *PIK3CA* (hotspots), *PIK3R1* (hotspots), and *POLE* (exonuclease domain) mutations in breast cancer patients. We have validated a subset of the *ESR1* mutations identified by dPCR-Seq using allele-specific digital PCR (dPCR) assays, demonstrating exceptional concordance in the measurement of mutant allele frequency (MAF) in clinical plasma ctDNA specimens. We anticipate that dPCR-Seq may have practical utility in future studies that investigate longitudinal monitoring of plasma ctDNA mutations as potential biomarkers of therapeutic response in ER+ MBC patients.

INTRODUCTION:

Breast cancer is the most common cancer diagnosed in women worldwide[1]. Endocrine therapy, which inhibits estrogen receptor signaling with tamoxifen, fulvestrant, or aromatase inhibitors (AIs), is a mainstay of treatment for ER+ MBC[2-5]. However, most metastatic breast cancer patients will develop resistance to endocrine therapy. Ligand-binding domain mutations in *ESR1* have been identified as a key mechanism for acquired endocrine therapy resistance[6,7]. Mutations in four other genes (i.e., *PIK3CA*[6,8,9], *PIK3R1*[10-12], *POLE*[13] and *TP53*[14-20]) are also prevalent in breast cancer patients. The ability to noninvasively profile and monitor tumor-specific mutations in plasma circulating tumor DNA (ctDNA) has immense potential to guide systemic therapy decisions that are personalized and adaptive to an evolving landscape of gene mutations in metastatic cancer patients[21,22]. Indeed, recent studies suggest that monitoring ctDNA mutation dynamics in ER+ MBC patients may be predictive of therapeutic sensitivity[23-28]. However, the optimal strategy for analyzing plasma ctDNA mutations over time in clinical plasma samples has not been established.

A cost-effective multiplexed droplet digital polymerase chain reaction (dPCR) assay based on hydrolysis probes was found to be a quick and highly sensitive method to detect *ESR1* hotspot mutations in ctDNA[29-31]. Although dPCR-based detection has excellent sensitivity and specificity, the assay is limited to detecting only the specific variant(s) for which the probes are designed. In contrast, plasma ctDNA next generation sequencing (NGS)-based assays have the potential to identify a broader genomic target region. However, concerns have arisen regarding their accuracy when measuring mutant allele fractions (MAF) of target alleles in plasma ctDNA[32]. Consequently, unique molecular identifiers were added to improve NGS accuracy when determining the absolute number of particular molecular species and their relative abundance during sequencing[33].

The dPCR technology[34,35] provides a platform for unified dPCR and NGS-based analyses of plasma ctDNA. We recently reported amplification of the complete coding sequences of *ESR1* and *TP53* and hotspot regions in *PIK3CA*, *PIK3R1*, and *POLE* from highly fragmented plasma ctDNA using a customized dPCR-based target enrichment assay. This was followed by NGS and bioinformatic analysis to accurately identify plasma ctDNA mutations with high sensitivity (79%) and specificity (100%) in a cohort of 58 breast cancer patients[36]. We estimate that the lower limit of detection for dPCR-Seq is 1.6% MAF. Significantly, we observed excellent concordance between MAF measured by dPCR-Seq versus allele-specific dPCR assays ($R^{2+} = 0.96$). This indicates that dPCR-Seq accurately maintains MAF during library preparation and subsequent NGS analyses. Our

analyses indicate that dPCR-Seq is more cost-effective, simpler, and bioinformatic analyses are easier relative to alternative plasma ctDNA NGS assays, while also maintaining a favorable analytical performance profile. Furthermore, dPCR-Seq should be adaptable to alternative platforms for dPCR analyses that may comparably provide accurate multiplexed target amplification for NGS applications. Thus, we anticipate that dPCR-Seq will be useful in future studies of plasma ctDNA biomarkers of therapeutic sensitivity. Below, the dPCR-Seq protocol is described in detail.

PROTOCOL:

The collection and analysis of blood samples follows the ethics guidelines of the University of North Carolina at Chapel Hill. Informed written consent was obtained from each patient, and our study was approved by the Institutional Review Board of University of North Carolina at Chapel Hill.

1. Blood collection from breast cancer patients

1.1. Collect 10 mL of blood from breast cancer patients in a glass blood collection tube. Please refer to Kumar et al. for patient characteristics[36].

1.2. After the blood collection, mix 8–10x by gentle inversion. Do not freeze specimens in the collection tube.

NOTE: Process the collected blood ideally on the same day within 1–4 h of collection. However, if immediate availability of the sample is not possible, the cell-free DNA (cfDNA) is stable for up to 14 days if stored at 6 °C–37 °C.

2. Plasma extraction from peripheral blood

2.1. Centrifuge the blood samples 2x, first at 1,800 x *g* followed by 2,000 x *g*, for 10 min each time and collect the plasma. Store the plasma at -80 ˚C until further use.

NOTE: Care should be taken to avoid collecting the middle whitish layer below the plasma. Discard the blood by treating it with 10% bleach for 30 min or autoclave. Alternatively, follow any other institution or company-approved protocol to process medical waste.

3. Cell-free DNA extraction from peripheral blood

3.1. Thaw the stored plasma samples in a 37 ˚C water bath for about 5 min.

3.2. Use a commercial kit (see **Table of Materials**) to extract the cfDNA from the plasma using the manufacturer's protocol with one modification at the elution step: Wait for about 15–60 min at room temperature (RT) after adding the elution buffer and then elute the sample.

Process the sample using a vacuum manifold and elute the sample in a microfuge at 14,000 x *g* for 1 min.

NOTE: A standard laboratory vacuum can be used for this step. If more concentrated cfDNA is required, then elute the sample in a small volume or concentrate it using a speed vacuum.

3.3. Use 1–2 µL of cfDNA to quantify using a fluorimeter and store the eluted cfDNA at -20 ˚C until further use.

4. Designing the NGS cancer panel

4.1. Design 272 primers to get 136 amplicons of 96 bp regions for targeted enrichment of *ESR1* (all coding regions), *TP53* (all coding regions), *PIK3CA* (hotspots), *PIK3R1* (hotspots).

NOTE: A complete list of the primers used in this assay is given in **Appendix 1**. Use any appropriate program to design the primers[37,38,39].

4.2. Synthesize each of the 272 individual primers as a 25 nM DNA oligonucleotide with standard desalted formulation. Mix sense and antisense oligonucleotides for each amplicon together to a final concentration of 100 µM in 60 µL of 10 mM Tris pH 8.0. Do this in a 96 well plate.

4.3. Divide the oligonucleotides into two sets for targeted amplification of the 68 genomic regions in each set. So, 68 wells in both 96 well plates will have 60 µL of sense and antisense oligos at a concentration of 100 µM.

NOTE: Both 96 well plates at this step will have 68 forward and 68 reverse primers.

4.4. Treat both plates separately to make two sets of oligonucleotide mixtures. Take out 1.6 µL from each well on the plate and mix the oligonucleotides to prepare a mixture consisting of 0.16 nM of each primer (0.8 µM) in 200 µL of 10 mM Tris pH 8.0.

NOTE: This step will lead to two tubes of oligonucleotide mixtures. Each tube will have 68 forward and 68 reverse primers. The primer sets for adjacent amplicons need to be separated into two distinct reactions to avoid template competition during dPCR.

5. Droplet generation and target enrichment by first round of PCR

5.1. Thaw all reagents: genotyping master mix (2x), set 1 and set 2 primers, and ctDNA samples. Vortex all reagents for 10–15 s and quickly spin to collect the contents. Keep the reagents on ice.

5.2. Prepare the master mix in a microcentrifuge tube using the following volumes per sample: 20 µL of 2x genotyping master mix, 1.6 µL of 25x droplet stabilizer, 2 µL of set 1 or set 2 primers.

NOTE: Make 10% more volume of the amount of master mix required to avoid shortage due to

potential pipetting errors.

5.3. Add 5.0 ng of ctDNA and adjust the volume to 40 µL with water.

NOTE: Because the amount of cfDNA is very limited, the protocol was standardized to use the minimum amount of cfDNA.

5.4. Load the 40 µL reaction mixture on each well of the chip (e.g., RainDrop Source chip) for droplet generation.

NOTE: Eight samples can be processed at one time using one chip. If more samples are required, then set up the reaction multiple times in a set of eight samples each time.

5.5. Transfer the droplet emulsions from the chip to PCR tubes using a multichannel pipette.

5.6. Set up a PCR reaction for the droplet emulsions with the set 1 and set 2 primers for each sample with the following conditions: 94 °C for 2 min; 55 cycles of 94 °C for 30 s, 54 °C for 30 s, 68 °C for 1 min; and finally one incubation at 68 °C for 10 min. Set a 1 °C/s temperature ramp speed between each step.

6. Recovery of DNA from the droplets after PCR amplification

6.1. To break the emulsion, add 50 µL of droplet destabilizer to the post-amplification droplets and vortex them for 30 s. Centrifuge the preparation at 2,000 x *g* for 2 min to separate the aqueous and oil phase.

6.2. Remove the oil phase from the bottom of the PCR tubes. To do so, insert the pipette tips carefully through the upper aqueous phase and carefully remove the bottom oil.

NOTE: Once the oil is removed, the samples can be stored at 20 °C for up to 7 days. A total of 40 µL of sample yields approximately 33 µL of aqueous volume.

6.3. Resuspend solid phase reversible immobilization (SPRI) magnetic beads by inversion. Add 39.6 µL of SPRI magnetic beads into each of the aqueous volumes to maintain 1.2x bead to reaction volume ratio. Mix the beads plus the aqueous sample by pipetting up and down about 10x so that the slurry appears uniform in density.

6.4. Leave the PCR tubes at RT for 5 min. Firmly position the PCR strips with samples onto a 96 well magnetic plate. Leave it for 2–3 min or until pellets are formed and the supernatant is clear.

6.5. Remove the supernatant using a multichannel pipette.

NOTE: Avoid disturbing the pellet at this step. If a bead is also aspirated into the pipet tip, then redeposit it into the well and wait for about 30 s or until the bead pellet is re-formed and the

supernatant is clear. Repipette the supernatant.

6.6. Leave the PCR tubes on the magnet and add 180 µL of freshly made 85% ethanol to each tube. Mix by pipetting 5–6x. Leave for 1 min or until the supernatant is clear. Remove all the ethanol carefully.

NOTE: The ethanol should be completely removed at this step. If any ethanol is visible, place the tubes on the magnet once again, wait for 15 s, and remove the remaining ethanol.

6.7. Remove the PCR tubes from the magnet and leave at RT for 5 min maximum to dry the bead pellets. Add 20 µL of 10 mM Tris-HCl pH 8.0 per well with a multichannel pipette. Vigorously pipette 10x to resuspend the pellets.

6.8. Place the tubes at RT for 2 min and then place on the magnet for 2 min to separate the beads. Use a multichannel pipette to collect 17 µL of eluent from each tube and deposit in a PCR tube.

7. Addition of adaptor and index sequences at the second round of PCR

7.1. Thaw all reagents: 10x buffer, 50 mM $MgSO_4$, dNTP (10 mM each), 4 M betaine, 5 µM universal forward primer, 5 µM index reverse primer and the first PCR template DNA from steps 5 and 6. Vortex all reagents for 10–15 s and quick spin to collect contents. Keep the reagents on ice.

7.2. Prepare the master mix in a microcentrifuge tube using the following volume per sample: 3.25 µL of 10x buffer, 0.875 µL of 50 mM $MgSO_4$, 1.124 µL of dNTP (10 mM each), 2.5 µL of 4 M betaine, 1.25 µL of DMSO, 1.25 µL of 5 µM universal forward primer, and 0.5 µL of high fidelity Taq polymerase.

NOTE: Make 10% more volume of the amount of master mix required to avoid shortage due to potential pipetting errors.

7.3. Add 1.25 µL of 5 µM index reverse primer and 13 µL of the first PCR template DNA to make 25 µL of secondary PCR reactions.

7.4. Set up a PCR reaction with the set 1 and set 2 primers for each sample with the following conditions: 94 °C for 2 min; 10 cycles of 94 °C for 30 s, 56 °C for 30 s, 68 °C for 1 min; and finally one incubation at 68 °C for 10 min. Set a 1 °C/s temperature ramp speed between each step.

8. Recovery of DNA after the second round of PCR

8.1. Resuspend the SPRI magnetic beads by inversion. Add 22.5 µL of SPRI magnetic beads into each of the aqueous volumes to maintain a 0.9x bead : reaction volume ratio for the second PCR.

8.2. Mix the beads plus the aqueous sample by pipetting up and down about 10x so that the

slurry appears uniform in density.

8.3. Leave the PCR tubes at RT for 5 min.

8.4. Firmly position the PCR strips with samples onto a 96 well magnetic plate. Leave it for 2–3 min or until pellets are formed and the supernatant is clear.

8.5. Remove supernatant using a multichannel pipette.

NOTE: Avoid disturbing the pellets at this step. If a bead is also aspirated into the pipet tip, then redeposit it into the well and wait for about 30 s or until the bead pellet is re-formed and the supernatant is clear. Repipette the supernatant.

8.6. Leave the PCR tubes on the magnet and add 180 µL of freshly made 85% ethanol to each tube. Mix by pipetting 5–6x.

8.7. Leave for 1 min or until the supernatant is clear.

8.8. Remove all the ethanol carefully.

NOTE: The ethanol should be completely removed at this step. If any ethanol is visible, place the tubes on the magnet once again, wait for 15 s, and remove the remaining ethanol.

8.9. Remove the PCR tubes from the magnet and leave at RT for 5 min maximum to dry the bead pellets.

8.10. Add 20 µL of 10 mM Tris-HCl pH 8.0 per well with a multichannel pipette. Vigorously pipette 10x to resuspend the pellets.

8.11. Place the tubes at RT for 2 min and then place on the magnet for 2 min to separate the beads.

8.12. Use a multichannel pipette to collect 17 µL of eluent from each tube and deposit in fresh PCR tubes.

9. DNA quantification on the bioanalyzer and pooling the libraries

9.1. Check the quantity and quality of the libraries using automated electrophoresis in accordance with the manufacturer's instructions.

9.2. Run 1.0 µL of each library on the automated electrophoresis instrument.

9.3. Add up and record all product yields with expected target amplicons between 280–320 bp along with other off target non-specific amplicons (e.g., <250 bp and >300 bp) as a function

of molarity (nM/L) for each sample.

NOTE: **Figure 1** provides more details about selecting appropriate regions for DNA quantification.

9.4. Dilute each library to 2 nM/L (2 nM) using nuclease-free water as a diluent. Mix an equal volume of each library in a tube to make an aggregate pooled sample concentration of 2 nM/L.

NOTE: The pooled library can be stored at 4 °C for up to 3 days. Pool the DNA libraries prepared from the eight samples for each MiSeq run.

9.5. Quantify the pooled library on a fluorometer and automated electrophoresis instrument with targeted sequencing.

10. Targeted sequencing protocol

10.1. Sequence each pooled library using custom sequencing primers (**Appendix 1**) and the sequencing reagent kit (see **Table of Materials**) on a Next Generation Sequencer following the manufacturer's instructions for 125 cycle paired-end sequencing.

10.2. Trim the reads in the FASTQ files to remove the adaptors and any low-quality bases at the ends using the ea-utils module fastq-mcf. Use the default parameters except for $k = 2$.

10.3. Align the sequence against the human reference genome [hg38] using Bowtie2 (bowtie2–2.2.4). Use default parameters except --local -N 1 -p 5).

10.4. Select the two groups of aligned reads.

NOTE: Filter the reads to be ≥60 nucleotides in length and select two groups of aligned reads. One group has both reads mapped to the same amplicon on the corresponding strands allowing a 1-nucleotide mismatch in the PCR primer region. The other group has both reads mapped to different amplicons within 1 kb of each other with primer sequences matching the amplicons to which they mapped allowing a 1-nucleotide mismatch.

10.5. Using Samtools, create BAM files containing each of the two categories of reads in which the 5'- and 3'-primer sequences were soft-clipped, and the alignment positions were adjusted. Merge, sort, and convert the BAM files to mpileup files (samtools-1.19; mpileup with parameters -A -B -d 1000000 -Q 30 -q 20).

10.6. Call variants using VarScan2 (VarScan.v2.3.5.jar mpileup2snp and mpileup2indel, --min-coverage 100 --min-reads2 1 --min-avg-qual 30 --min-var-freq 0 --strand-filter 1 --p-value 0.01 --output-vcf 1). Annotate the variants by snpEff (default parameters). Annotated variants unique to the patient samples are scored as true mutations.

REPRESENTATIVE RESULTS:

Detection of mutations *ESR1*, *PIK3CA*, *TP53*, *PIK3RA*, and *POLE* mutations

The dPCR-SEQ assay was used to detect mutations in 31 metastatic breast cancer patients (single time point samples from 24 patients, two time point samples from six patients and three time point samples from one patient). Mutations found in *ESR*1, *PIK3CA*, *TP53*, *POLE*, and *PIK3RA* genes by dPCR-Seq is shown in **Figure 2**. The median depth of coverage was 3,500. The clinical significance of monitoring cumulative mutations in *ESR*1, *PIK3CA*, and *TP53* calculated using this protocol is mentioned elsewhere[36].

FIGURE AND TABLE LEGENDS:

Figure 1: Calculation of the DNA yield from the chromatogram. Expected fragment sizes were between 280–320 bp. However, all individual products on chromatogram were added to calculate the total yields for each sample. The total yields were considered to make further dilutions at the time of library pooling.

Figure 2: Mutation heterogeneity in MBC patients. Lollipop plots showing the number (vertical axis) and position (horizontal axis) of mutations across the protein sequence of breast cancer relevant genes (*ESR1*, *PIK3CA*, *TP53*, *POLE*, and *PIK3R1*). The plot was originally generated using the cBioPortal. The green circle indicates missense mutations. The most prevalent missense mutations are also mentioned.

DISCUSSION:

Digital PCR has become an important tool in translational research that is used widely to track hotspot mutations in cancer patients. Genome-wide and targeted NGS of liquid and solid biopsy samples has also successfully been used to identify mutations in breast cancer patients[36]. We have developed a five gene panel to track mutations in *ESR1*, *PIK3CA*, *PIK3R1*, *POLE*, and *TP53* mutations in plasma ctDNA isolated from metastatic breast cancer patients. Alternative gene panels may be designed for different clinical scenarios. It is also possible to design patient-specific assays to monitor tumor-specific mutations that have been identified from tumor-normal genomic analyses. A critical step in the protocol is the optimal design of primers for target enrichment. These primers should be evaluated using a program such as MPD[38] or Oli2go[39] that detects primer-dimer interactions. Optimization after dPCR-Seq may be necessary to replace or adjust concentrations of primers that do not effectively amplify the genomic region of interest.

The dPCR-Seq protocol has several advantages relative to alternative NGS assays, including cost-effectiveness (processing each sample costs ~$300), rapid turnaround time (within 3 days), and simplicity of bioinformatic analyses[36]. It provides uniform amplicon coverage and high accuracy, while requiring low amounts of starting cell-free DNA material. One limitation of dPCR-Seq is the sensitivity to detect ctDNA mutations. Our analyses indicate that dPCR-Seq can reliably identify mutations that are present at greater than 1.6% mutant allele frequency, in settings of de novo discovery. It is plausible that if multiple tumor-specific mutant alleles are being monitored, this level of sensitivity may be substantially increased. Of note, a similar strategy has been used in recent studies for posttreatment surveillance and early detection of cancer recurrence[40,41]. Thus, dPCR-Seq may facilitate future studies in this clinically relevant setting.

Based on concordance analysis with allele-specific dPCR, we have found that one of the major advantages of dPCR-Seq is that it accurately measures the MAF of plasma ctDNA mutations in clinical samples from MBC patients. Of note, another recent method for multiplexed target enrichment of plasma ctDNA followed by NGS has been described[42]. In this study, microfluidics-based compartmentalization was used for multiplexed PCR to achieve target amplicon enrichment. Interestingly, although the correlation coefficient observed between dPCR and the NGS assay was favorable (R^2 = 0.64), it was not as high as what we observed with dPCR-Seq (R^2 = 0.96). Thus, we suggest that dPCR-Seq may be particularly useful in future studies investigating ctDNA mutation dynamics as a predictive biomarker of therapeutic response in ER+ MBC patients.

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DISCLOSURES:

The authors declare no conflict of interest.

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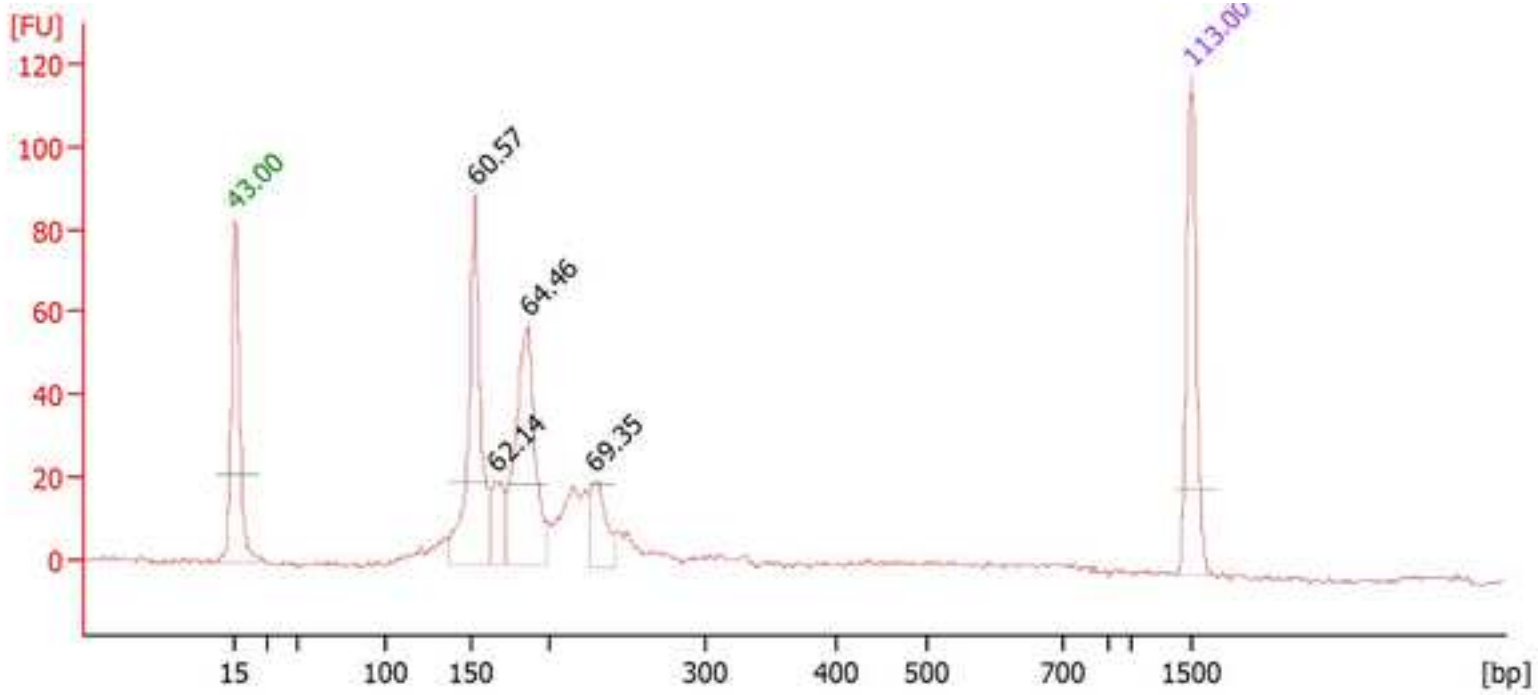
Figure 1



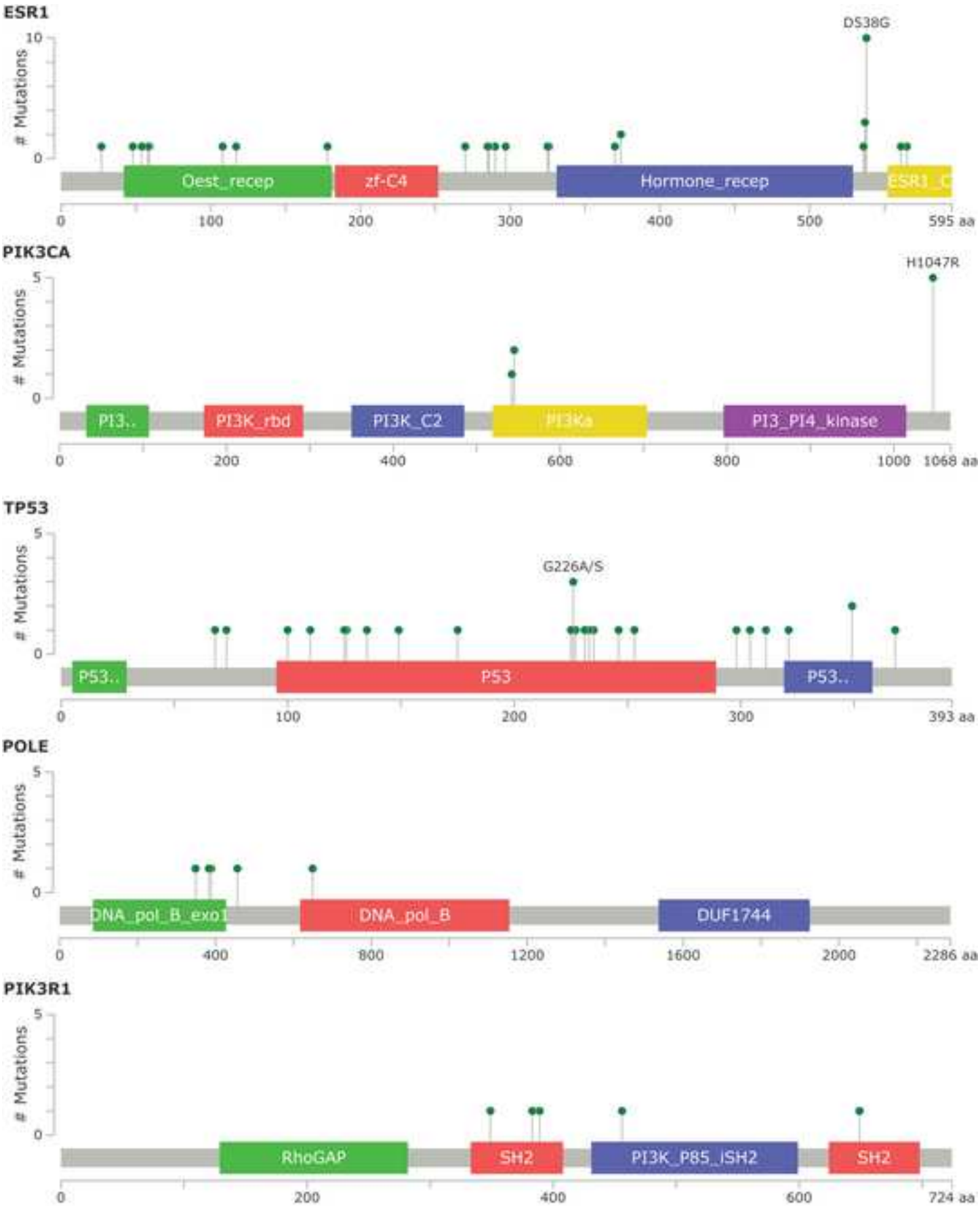
Peak		Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations
1	◀	15	4.20	424.2	Lower Marker
2		152	4.17	41.4	
3		166	0.89	8.1	
4		187	4.03	32.7	
5		230	1.02	6.7	
6	▶	1,500	2.10	2.1	Upper Marker

Yield = 41.4 + 8.1 + 32.7 + 6.7 = 88.9 nmol/l

Figure 1

Figure 2

Click here to access/download;Figure;Figure 2.jpg



Name of Material/ Equipment

10 µL Aerosol Barrier tips
10 mL Serological Pipettes
10-100 µL 8-channel pipette
1250 µL Aerosol Barrier tips
2 µL Aerosol Barrier tips
20 µL Aerosol Barrier tips
200 µL Aerosol Barrier tips
20-200 ul 8-channel pipette
2100 Bioanalyzer
5 mL Serological Pipettes
8-strip 0.2 mL PCR tubes & caps
Absolute Ethanol
Agilent DNA 1000 Kit
Betaine Anhydrous
Bio-Rad's C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module
Cell-Free DNA BCT tubes, RUO
Centrifuge 5810R
DMSO
dNTP Solution Mix
DynaMag-96 magnetic plate
Falcon 15ml Conical Centrifuge Tubes
Falcon 50mL Conical Centrifuge Tubes
Heating block (for 1.7 ml microcentrifuge tubes)
Microcentrifuge
MiSeq Reagent Kit v3
MiSeq Sequencer
Nuclease Free Water
P10 pipettes
P1000 pipettes
P2 pipettes
P20 pipettes
P200 pipettes
Platinum Taq DNA Polymerase High Fidelity
Portable Pipet-Aid XP Pipette Controller
QIAamp Circulating Nucleic Acid Kit
Qubit fluorimeter
Qubit Assay Tubes
Qubit dsDNA HS Assay Kit
RainDrop System Source Instrument
SPRIselect Reagent Kit
TaqMan Genotyping Master Mix

ThunderBolts Cancer Panel Consumables Pack
Tris Base
Vortex mixer

Company	Catalog Number
VWR	10017-062
VWR	13-675-20
Eppendorf	3125000036
VWR	10017-092
VWR	10010-364
VWR	10017-064
VWR	10017-068
Denville Scientific	463230067
Agilent	G2939BA
VWR	13-675-22
Axygen	PCR-0208-CP-C
Sigma	E7023-500ML
Agilent	5067-1504
Sigma	B2629-100G
Bio-Rad	1851197
Streck	218962
Eppendorf	22625101
Sigma	D8418-100ML
New England Biolabs	N0447L
Life Technologies	12331D
Corning	352096
Corning	352098
Denville Scientific	I0540
Eppendorf	5424
Illumina	MS-102-3003
Illumina	SY-410-1003
Integrated DNA Technologies	11-05-01-04
Denville Scientific	355022105
Denville Scientific	455060205
Denville Scientific	455010336
Denville Scientific	355032002
Denville Scientific	45505009
Life Technologies	11304-029
Drummond Scientific	4-000-101
Qiagen	55114
Thermo Fisher Scientific	Q33226
Thermo Fisher Scientific	Q32856
Thermo Fisher Scientific	Q32851
Raindance	20-04401
Beckman Coulter	B23318
Life Technologies	4371355

Raindance	20-07205
Thermo Fisher Scientific	BP152-500
Denville Scientific	Vortexer 59A

Comments/Description

Can be replaced with other equivalent product
Can be replaced with other equivalent product
Can be replaced with other equivalent product
Can be replaced with other equivalent product
Can be replaced with other equivalent product
Can be replaced with other equivalent product
Can be replaced with other equivalent product
Can be replaced with other equivalent product

Can be replaced with other equivalent product

Suitable for 15 ml conical tubes; Can be replaced with other equivalent instrument

Can be replaced with other equivalent product
Can be replaced with other equivalent product

Suitable for 1.5-2.0 ml tubes; Can be replaced with other equivalent instrument

Can be replaced with other equivalent product
Can be replaced with other equivalent product
Can be replaced with other equivalent product
Can be replaced with other equivalent product
Can be replaced with other equivalent product
Can be replaced with other equivalent product

Can be replaced with other equivalent product

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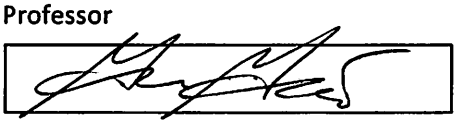
Signature:  Date: 2/21/2019

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Editorial comments:

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1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Response: We have made the appropriate changes.

2. Authors and affiliations: Please provide an email address for each author.
Response: Email address of each author is now included on title page.

3. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u and abbreviate liters to L (L, mL, µL) to avoid confusion.
Response: We have made the appropriate changes.

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Response: All the trademark symbols are now removed from the manuscript.

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
Response: We are presenting our data in 8 sections/headings. We do not have the subsections/subheading. All the steps within each section is numbered following the guidelines. We have now removed some indentations in the updated version.

6. In the JoVE Protocol format, "NOTE" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.
Response: We have made some changes in the protocol to follow this guideline. A track change version is also uploaded.

7. Line 97: What volume of blood is collected?
Response: We have made the following changes to reflect the volume of collected blood.

“Collect 10 ml of the blood from breast cancer patient in Cell-Free DNA BCT tubes, RUO”.

8. Line 276: Please describe how this is actually done. Alternatively, include a relevant reference.
Response: We have modified the text to indicate that this process is done in accordance with the manufacturer’s instructions.

9. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
Response: We have made some changes in the protocol to follow this guideline. A track change version is also uploaded.

10. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.
Response: The manuscript now follows this instruction.

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Response:

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Response:

13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
Response:

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Response:

15. References: Please do not abbreviate journal titles.
Response:

16. Table of Materials: Please remove trademark (™) and registered (®) symbols. Please sort the items in alphabetical order according to the name of material/equipment.
Response:

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors have devised a method called dPCR-seq as a new ctDNA detection method, and have described the details of the specific experimental procedure. This paper seems to provide sufficient information necessary to reproduce the experiment.

Major Concerns:

None

Minor Concerns:

None

Response: Thank you for your careful review.

Reviewer #2:

Manuscript Summary:

The authors of the manuscript "A droplet PCR based Next Generation Sequencing assay to track DNA mutation dynamics in estrogen receptor positive metastatic breast cancer" describe a method that combines digital PCR with NGS. This method is said to combine the advantages of both techniques, and uses droplet PCR for mutliplexed target enrichment which is subsequently sequenced and showed to have a lower limit of detection of 1.6% MAF. The authors described the different protocols of their dPCR-seq method well and in detail, however, only a few results were shown. It is of interest that they only need 5ng of cfDNA for their method, since often cfDNA yields are (very) low in patients.

Major Concerns:

a) The authors describe the dPCR-Seq well, however, details about the NGS is partially missing. For example, median on target reads depth coverage is an important factor in NGS (and determines costs, limit of detection, etc) but was not described or was this indicated by 125 cycles (point 10) MiSeq protocol line 301 page 6)?

Response: We have edited the manuscript to include information about median of the depth of coverage in result section. The NGS results including cost was previously discussed in separate article (NPJ Breast Cancer. 2018; 4: 39. PMID: 30534596, doi: 10.1038/s41523-018-0093-3). We also have added this information in the Discussion section.

b) Moreover, they state high sensitivity (79%) and specificity (100%) (line 80, page 1) , but what is their "gold" standard? Actually, does this mean that in 20% of cases the mutation is missed by their method? Or did the patients not have mutations in their plasma for the genes analysed?

Response: Thank you for raising this point. Sensitivity and specificity indicate specific mutations found in patients. All of the patients that were not detected by our method had mutation levels that were below the detection limit (MAF 1.6%) of dPCR-Seq. Detailed analyses of the data is now referenced to our npj Breast Cancer article (NPJ Breast Cancer. 2018; 4: 39. PMID: 30534596, doi: 10.1038/s41523-018-0093-3). The relevant reference is now included as mentioned below.

"This can be followed by NGS and bioinformatic analysis to accurately identify plasma ctDNA mutations with high sensitivity (79%) and specificity (100%) in a cohort of 58 breast cancer patients (ref 36)."

c) How is the limit of detection of the dPCR-Seq compared to allele-specific dPCR assays? Results shown do not illustrate well the performance of their method. Due to the lack of results, it is also difficult to compare their findings with those obtained with alternative (standard) methods.

Response: This manuscript mainly highlights the experimental method. However, the results from this experiment are discussed in detail at different place as below. Following reference discusses the comparison of dPCR-Seq and selected allele-specific digital PCR assay .
(NPJ Breast Cancer. 2018; 4: 39. PMID: 30534596, doi: 10.1038/s41523-018-0093-3)

Reviewer #3:

Manuscript Summary:

The research for NGS based plasma DNA mutation monitoring in blood is of vital importance for cancer prognostics and diagnostics. This paper is well written, and the experiment is demonstrated in a well-organized way. Thus, it is recommended for acceptance after minor revision.

Minor Concerns:

As readers come mostly from scientific world, rather than from commercial application field, it might be better to tell them the exact content of chemicals for droplet generation and provide wider choices of equipment and reagents for conducting experiment. For droplet generation, the oil phase, the surfactant stabilizer and the destabilizer, their exact chemical names should be provided, and their ratio and combination are the key to the success and repeatability of the experiment. Thus, it is preferable to provide these details in the video. Also, detail the structure of the chip and the peripheral equipment is important for the readers to understand the real procedures.

Response: Thank you for your careful review. There are three approach to digital PCR – (a) droplet based, (b) microfluidic based and (c) magnetic beads based. Following are the different instruments available to perform digital PCR.

1. QX200™ Droplet Digital™ PCR System by Bio-Rad (Make 20,000 nanoliter-sized droplets)
2. RainDrop Digital PCR System by RainDance Technologies (Make 10 million pico-liter sized droplets)
3. Clarity™ Digital PCR System by JN Medsys (Make 10,000 nanolitre-sized partitions)
4. Crystal Digital PCR with The Naica System by Stilla Technologies (Make 30,000 nanolitre-sized (0.59 nL) droplets) partitioned their samples into approximately 30,000 droplets; 3 fluorescent channels
5. Biomark™ HD by Fluidigm digital PCR (microfluidic-based digital PCR where sample divided into number of chambers on a chip)
6. BEAMING Digital PCR by Sysmex Inostics (Bead-based digital PCR where magnetic beads capture the target sequences)

We have used digital PCR system by RainDance technologies which is droplet based in our protocol. Although technology is now well advanced but the composition of reaction components are proprietary information which is not revealed by these companies. However, the general procedures described should be adaptable to other dPCR platforms, and we make a note of this in the revised text. Thus, we anticipate that the procedures described will be applicable to other commercial dPCR platforms, with relatively minor modifications that can be optimized by end-users.

List of SET-1 primers

Customer_Target_ID	Sense_Primer_Name
PIK3CA_01	UNC_rdt_68_L
PIK3CA_02	UNC_rdt_69_L
PIK3CA_04	UNC_rdt_70_L
PIK3CA_06	UNC_rdt_71_L
POLE_CDS_13	UNC_rdt_72_L
POLE_CDS_13	UNC_rdt_73_L
POLE_CDS_12	UNC_rdt_74_L
POLE_CDS_12	UNC_rdt_75_L
POLE_CDS_11	UNC_rdt_76_L
POLE_CDS_10	UNC_rdt_77_L
POLE_CDS_10	UNC_rdt_78_L
POLE_CDS_09	UNC_rdt_79_L
POLE_CDS_08	UNC_rdt_80_L
POLE_CDS_08	UNC_rdt_81_L
POLE_CDS_07	UNC_rdt_82_L
POLE_CDS_07	UNC_rdt_83_L
POLE_CDS_06	UNC_rdt_84_L
POLE_CDS_06	UNC_rdt_85_L
POLE_CDS_05	UNC_rdt_86_L
POLE_CDS_04	UNC_rdt_87_L
POLE_CDS_03	UNC_rdt_88_L
POLE_CDS_03	UNC_rdt_89_L
POLE_CDS_02	UNC_rdt_90_L
POLE_CDS_02	UNC_rdt_91_L
POLE_CDS_01	UNC_rdt_92_L
ESR1_CDS_01	UNC_rdt_93_L
ESR1_CDS_01	UNC_rdt_94_L
ESR1_CDS_01	UNC_rdt_95_L
ESR1_CDS_01	UNC_rdt_96_L
ESR1_CDS_01	UNC_rdt_97_L
ESR1_CDS_02	UNC_rdt_98_L
ESR1_CDS_02	UNC_rdt_99_L
ESR1_CDS_03	UNC_rdt_32_L
ESR1_CDS_03	UNC_rdt_33_L
ESR1_CDS_03	UNC_rdt_34_L
ESR1_CDS_03	UNC_rdt_35_L
ESR1_CDS_03	UNC_rdt_36_L
ESR1_CDS_04	UNC_rdt_37_L
ESR1_CDS_04	UNC_rdt_38_L

ESR1_CDS_05	UNC_rdt_39_L
ESR1_CDS_05	UNC_rdt_40_L
ESR1_CDS_06	UNC_rdt_41_L
ESR1_CDS_06	UNC_rdt_42_L
ESR1_CDS_06	UNC_rdt_43_L
ESR1_CDS_07	UNC_rdt_44_L
ESR1_CDS_07	UNC_rdt_45_L
ESR1_CDS_07	UNC_rdt_46_L
TP53_CDS_01	UNC_rdt_115_L
TP53_CDS_02	UNC_rdt_116_L
TP53_CDS_02	UNC_rdt_117_L
TP53_CDS_03	UNC_rdt_118_L
TP53_CDS_04	UNC_rdt_119_L
TP53_CDS_04	UNC_rdt_120_L
TP53_CDS_05	UNC_rdt_121_L
TP53_CDS_05	UNC_rdt_122_L
TP53_CDS_06	UNC_rdt_123_L
TP53_CDS_07	UNC_rdt_124_L
TP53_CDS_07	UNC_rdt_125_L
TP53_CDS_07	UNC_rdt_126_L
TP53_CDS_08	UNC_rdt_127_L
TP53_CDS_08	UNC_rdt_128_L
TP53_CDS_08	UNC_rdt_129_L
TP53_CDS_09	UNC_rdt_130_L
TP53_CDS_10	UNC_rdt_131_L
PIK3R1_01	UNC_rdt_64_L
PIK3R1_02	UNC_rdt_65_L
PIK3R1_03	UNC_rdt_66_L
PIK3R1_04	UNC_rdt_67_L

List of SET-2 primers

Customer_Target_ID	Sense_Primer_Name
PIK3CA_01	UNC_rdt_0_L
PIK3CA_03	UNC_rdt_1_L
PIK3CA_05	UNC_rdt_2_L
PIK3CA_06	UNC_rdt_3_L
POLE_CDS_13	UNC_rdt_4_L
POLE_CDS_13	UNC_rdt_5_L
POLE_CDS_12	UNC_rdt_6_L
POLE_CDS_11	UNC_rdt_7_L
POLE_CDS_11	UNC_rdt_8_L

POLE_CDS_10	UNC_rdt_9_L
POLE_CDS_09	UNC_rdt_10_L
POLE_CDS_09	UNC_rdt_11_L
POLE_CDS_08	UNC_rdt_12_L
POLE_CDS_07	UNC_rdt_13_L
POLE_CDS_07	UNC_rdt_14_L
POLE_CDS_06	UNC_rdt_15_L
POLE_CDS_06	UNC_rdt_16_L
POLE_CDS_05	UNC_rdt_17_L
POLE_CDS_05	UNC_rdt_18_L
POLE_CDS_04	UNC_rdt_19_L
POLE_CDS_03	UNC_rdt_20_L
POLE_CDS_02	UNC_rdt_21_L
POLE_CDS_02	UNC_rdt_22_L
POLE_CDS_01	UNC_rdt_23_L
POLE_CDS_01	UNC_rdt_24_L
ESR1_CDS_01	UNC_rdt_25_L
ESR1_CDS_01	UNC_rdt_26_L
ESR1_CDS_01	UNC_rdt_27_L
ESR1_CDS_01	UNC_rdt_28_L
ESR1_CDS_01	UNC_rdt_29_L
ESR1_CDS_02	UNC_rdt_30_L
ESR1_CDS_02	UNC_rdt_31_L
ESR1_CDS_02	UNC_rdt_100_L
ESR1_CDS_03	UNC_rdt_101_L
ESR1_CDS_03	UNC_rdt_102_L
ESR1_CDS_03	UNC_rdt_103_L
ESR1_CDS_03	UNC_rdt_104_L
ESR1_CDS_04	UNC_rdt_105_L
ESR1_CDS_04	UNC_rdt_106_L
ESR1_CDS_04	UNC_rdt_107_L
ESR1_CDS_05	UNC_rdt_108_L
ESR1_CDS_05	UNC_rdt_109_L
ESR1_CDS_06	UNC_rdt_110_L
ESR1_CDS_06	UNC_rdt_111_L
ESR1_CDS_07	UNC_rdt_112_L
ESR1_CDS_07	UNC_rdt_113_L
ESR1_CDS_07	UNC_rdt_114_L
TP53_CDS_01	UNC_rdt_47_L
TP53_CDS_02	UNC_rdt_48_L
TP53_CDS_03	UNC_rdt_49_L

TP53_CDS_03	UNC_rdt_50_L
TP53_CDS_04	UNC_rdt_51_L
TP53_CDS_04	UNC_rdt_52_L
TP53_CDS_05	UNC_rdt_53_L
TP53_CDS_06	UNC_rdt_54_L
TP53_CDS_06	UNC_rdt_55_L
TP53_CDS_07	UNC_rdt_56_L
TP53_CDS_07	UNC_rdt_57_L
TP53_CDS_08	UNC_rdt_58_L
TP53_CDS_08	UNC_rdt_59_L
TP53_CDS_08	UNC_rdt_60_L
TP53_CDS_08	UNC_rdt_61_L
TP53_CDS_09	UNC_rdt_62_L
TP53_CDS_10	UNC_rdt_63_L
PIK3R1_02	UNC_rdt_132_L
PIK3R1_03	UNC_rdt_133_L
PIK3R1_04	UNC_rdt_134_L
PIK3R1_05	UNC_rdt_135_L

Secondary PCR primers

Primer Name	Primer Sequence
Illumina_2nd_INDEX_U	AATGATACGGCGACCAC
Illumina_2nd_INDEX_0	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_0	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_0	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_0	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_0	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_0	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_0	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_0	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_0	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_1	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_1	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_1	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_1	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_1	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_1	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_1	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_1	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_1	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_1	CAAGCAGAAGACGGCAT

Illumina_2nd_INDEX_2	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_2	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_2	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_2	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_2	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_2	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_2	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_2	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_2	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_2	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_3	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_3	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_3	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_3	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_3	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_3	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_3	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_3	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_3	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_3	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_4	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_4	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_4	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_4	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_4	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_4	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_4	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_4	CAAGCAGAAGACGGCAT
Illumina_2nd_INDEX_4	CAAGCAGAAGACGGCAT

Cusom seqnecing primers

Sequencing Primer 1	ACACTCTTTCCCTACACGA
Sequencing Primer 2	GTGACTGGAGTTCAGAC

Sense_Sequence_with_Illumina_Tail	Antisense_Primer_Name
CGCTCTTCCGATCTCTGACCCCCTCCATCAACTTCTT	UNC_rdt_68_R
CGCTCTTCCGATCTCTGTTTTCAACCCTTTTTAAAAGTAATTG	UNC_rdt_69_R
CGCTCTTCCGATCTCTGAGGTGGAATGAATGGCTGAA	UNC_rdt_70_R
CGCTCTTCCGATCTCTGTCTTTTGATGACATTGCATACA	UNC_rdt_71_R
CGCTCTTCCGATCTCTGGCACACGGCAGCAGG	UNC_rdt_72_R
CGCTCTTCCGATCTCTGTCCACGGGATCATAGCCTAG	UNC_rdt_73_R
CGCTCTTCCGATCTCTGACGGACAGCAGTGAGGAG	UNC_rdt_74_R
CGCTCTTCCGATCTCTGTCCTGCTGCATGCTCAGA	UNC_rdt_75_R
CGCTCTTCCGATCTCTGAGACACAGACTCACCAGT	UNC_rdt_76_R
CGCTCTTCCGATCTCTGTCCCACAATACCGGGTAGTT	UNC_rdt_77_R
CGCTCTTCCGATCTCTGACTCAAAATCTTCAATATCTTCTGA	UNC_rdt_78_R
CGCTCTTCCGATCTCTGTCGATCATGTAGGAAATCATCA	UNC_rdt_79_R
CGCTCTTCCGATCTCTGACTGGAAATTTTAGGATGAAGGT	UNC_rdt_80_R
CGCTCTTCCGATCTCTGCGGGTGATTTCTACCGGAAA	UNC_rdt_81_R
CGCTCTTCCGATCTCTGCTCACCACGTGGATCTTCAG	UNC_rdt_82_R
CGCTCTTCCGATCTCTGGAGGTTTCCTCTTCATCAGT	UNC_rdt_83_R
CGCTCTTCCGATCTCTGCTGTGTACGCGTCGCTG	UNC_rdt_84_R
CGCTCTTCCGATCTCTGTGGAAGGACAGCCTGATGTA	UNC_rdt_85_R
CGCTCTTCCGATCTCTGTCACCAAGTCCAGATCCTCTT	UNC_rdt_86_R
CGCTCTTCCGATCTCTGATGACACACAGGTCGTCTGA	UNC_rdt_87_R
CGCTCTTCCGATCTCTGAAGTGGGTTTTAGCTTGTCG	UNC_rdt_88_R
CGCTCTTCCGATCTCTGCTGCACTGCCTAAGCGC	UNC_rdt_89_R
CGCTCTTCCGATCTCTGGCATGTTAATGAGCCAGCCT	UNC_rdt_90_R
CGCTCTTCCGATCTCTGGTTCCAGGCGCTTGAGTG	UNC_rdt_91_R
CGCTCTTCCGATCTCTGCTCCAATTGGCGCCGG	UNC_rdt_92_R
CGCTCTTCCGATCTCTGCCCTCCACACCAAAGCATC	UNC_rdt_93_R
CGCTCTTCCGATCTCTGCCTGGGCGAGGTGTACC	UNC_rdt_94_R
CGCTCTTCCGATCTCTGGCAGGTCTACGGTCAGAC	UNC_rdt_95_R
CGCTCTTCCGATCTCTGCTCCGAGCCCGCTGAT	UNC_rdt_96_R
CGCTCTTCCGATCTCTGCCAGCGGCTACACGGT	UNC_rdt_97_R
CGCTCTTCCGATCTCTGCCAAATTCAGATAATCGACGCC	UNC_rdt_98_R
CGCTCTTCCGATCTCTGGACTCGCTACTGTGCAGTG	UNC_rdt_99_R
CGCTCTTCCGATCTCTGTTTTTGTATAAAAGTTTACACGGG	UNC_rdt_32_R
CGCTCTTCCGATCTCTGGACCGAAGAGGAGGGAGAAT	UNC_rdt_33_R
CGCTCTTCCGATCTCTGTGAGAGCTGCCAACCTTTG	UNC_rdt_34_R
CGCTCTTCCGATCTCTGCTTGTTGGATGCTGAGCCC	UNC_rdt_35_R
CGCTCTTCCGATCTCTGCTGGCAGACAGGGAGCT	UNC_rdt_36_R
CGCTCTTCCGATCTCTGTGAGTCAGCAGGGTTTTTCT	UNC_rdt_37_R
CGCTCTTCCGATCTCTGCCTGGCTAGAGATCCTGATGAT	UNC_rdt_38_R

CGCTCTTCCGATCTCTGTGTCTTGTGGAAGattttctgt	UNC_rdt_39_R
CGCTCTTCCGATCTCTGCGACATGCTGCTGGCTAC	UNC_rdt_40_R
CGCTCTTCCGATCTCTGTGAGCTtctctctctcactct	UNC_rdt_41_R
CGCTCTTCCGATCTCTGATATCCACCGAGTCCTGGAC	UNC_rdt_42_R
CGCTCTTCCGATCTCTGCAGCGGCTGGCCCAG	UNC_rdt_43_R
CGCTCTTCCGATCTCTGCCTACAGTAACAAAGGCATGG	UNC_rdt_44_R
CGCTCTTCCGATCTCTGCTACATGCGCCCACTAGC	UNC_rdt_45_R
CGCTCTTCCGATCTCTGTGCAAAAGTATTACATCACGGG	UNC_rdt_46_R
CGCTCTTCCGATCTCTGTGTCAGTGGGGAACAAGA	UNC_rdt_115_R
CGCTCTTCCGATCTCTGGAGTAGGGCCAGGAAGGG	UNC_rdt_116_R
CGCTCTTCCGATCTCTGCCAAGGCCTCATTCAGCTC	UNC_rdt_117_R
CGCTCTTCCGATCTCTGCCTGAAGGGTGAAATATTCTCCA	UNC_rdt_118_R
CGCTCTTCCGATCTCTGCATAACTGCACCCTTGGTCT	UNC_rdt_119_R
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CGCTCTTCCGATCTCTGCACAGCAGGCCAGTGTG	UNC_rdt_121_R
CGCTCTTCCGATCTCTGCATGCCGCCCATGCAG	UNC_rdt_122_R
CGCTCTTCCGATCTCTGAGGCGGCTCATAGGGC	UNC_rdt_123_R
CGCTCTTCCGATCTCTGGGGACCCTGGGCAAC	UNC_rdt_124_R
CGCTCTTCCGATCTCTGCTCACAACCTCCGTCATGTG	UNC_rdt_125_R
CGCTCTTCCGATCTCTGCAGGTCTTGGCCAGTTGG	UNC_rdt_126_R
CGCTCTTCCGATCTCTGGACTTGGCTGTCCCAGAATG	UNC_rdt_127_R
CGCTCTTCCGATCTCTGCGCCGGTGTAGGAGCT	UNC_rdt_128_R
CGCTCTTCCGATCTCTGACCATTGTTCAATATCGTCCG	UNC_rdt_129_R
CGCTCTTCCGATCTCTGCAGGTCCTcagccccc	UNC_rdt_130_R
CGCTCTTCCGATCTCTGggggtggtgggCCTG	UNC_rdt_131_R
CGCTCTTCCGATCTCTGTGTTTTGTGTTTTCATTTCAGGGA	UNC_rdt_64_R
CGCTCTTCCGATCTCTGTTTACAGGAAAGGGGGAAAT	UNC_rdt_65_R
CGCTCTTCCGATCTCTGAAAATTACATGAATATAACACTCAGTT	UNC_rdt_66_R
CGCTCTTCCGATCTCTGTGACAAACGTATGAACAGCA	UNC_rdt_67_R

Sense_Sequence_Illumina_Tail	**Antisense_Primer_Name**
CGCTCTTCCGATCTCTGTCGTAAGTGTTACTCAAGAAGCA	UNC_rdt_0_R
CGCTCTTCCGATCTCTGGTTATAAATAGTGCACTCAGAATAAAA	UNC_rdt_1_R
CGCTCTTCCGATCTCTGGCTCAAAGCAATTTCTACACGA	UNC_rdt_2_R
CGCTCTTCCGATCTCTGAGATAAAACTGAGCAAGAGGC	UNC_rdt_3_R
CGCTCTTCCGATCTCTGCTGCTCCGTGGCCATC	UNC_rdt_4_R
CGCTCTTCCGATCTCTGGCCGCCTTGAGATTATGACT	UNC_rdt_5_R
CGCTCTTCCGATCTCTGCATGTGGATGCACTGGGG	UNC_rdt_6_R
CGCTCTTCCGATCTCTGAAATGCTGCCCAGTTACTCA	UNC_rdt_7_R
CGCTCTTCCGATCTCTGGTTTGGTCTCCTGGACGTG	UNC_rdt_8_R

CGCTCTTCCGATCTCTGTGAAGACACAAAAGGGGCC	UNC_rdt_9_R
CGCTCTTCCGATCTCTGATCCCAGGAGCTTACTTCCC	UNC_rdt_10_R
CGCTCTTCCGATCTCTGGGGGCAGTTTGGTCGTC	UNC_rdt_11_R
CGCTCTTCCGATCTCTGGCAAAACTTACAGGTCGTTCA	UNC_rdt_12_R
CGCTCTTCCGATCTCTGGGAAATTCATGTGAGCAGCG	UNC_rdt_13_R
CGCTCTTCCGATCTCTGGAACATCGTACTCGCGCAT	UNC_rdt_14_R
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CGCTCTTCCGATCTCTGGTTCTTCTTCACGGCAGGG	UNC_rdt_16_R
CGCTCTTCCGATCTCTGGCCTCATTTACCCCTGGAAA	UNC_rdt_17_R
CGCTCTTCCGATCTCTGCCCTGAAACTTCTTGGAGAGA	UNC_rdt_18_R
CGCTCTTCCGATCTCTGCCTTTCTGGTCGCAATGT	UNC_rdt_19_R
CGCTCTTCCGATCTCTGGGGGCTTACCTTAAATCTGCT	UNC_rdt_20_R
CGCTCTTCCGATCTCTGGCCATATTCCTGGGTGGG	UNC_rdt_21_R
CGCTCTTCCGATCTCTGTCAAAACCAAACCGCAAATCC	UNC_rdt_22_R
CGCTCTTCCGATCTCTGGTCCATCAAGAGGCGCG	UNC_rdt_23_R
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CGCTCTTCCGATCTCTGGGGGACACGGTCTGCA	UNC_rdt_25_R
CGCTCTTCCGATCTCTGTGGAGCCCCTGAACCG	UNC_rdt_26_R
CGCTCTTCCGATCTCTGTGTACAACTACCCCGAGG	UNC_rdt_27_R
CGCTCTTCCGATCTCTGGTTCGGCTCCAACGGC	UNC_rdt_28_R
CGCTCTTCCGATCTCTGTTTCCTGCAGCCCCACG	UNC_rdt_29_R
CGCTCTTCCGATCTCTGGTTTTGCTTTTCTAATGTTAATGGA	UNC_rdt_30_R
CGCTCTTCCGATCTCTGCCAGTACCAATGACAAGGGA	UNC_rdt_31_R
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CGCTCTTCCGATCTCTGTTTGACCCTCCATGATCAGGT	UNC_rdt_106_R
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CGCTCTTCCGATCTCTGACACTTTGATCCACCTGATG	UNC_rdt_111_R
CGCTCTTCCGATCTCTGCGGGTTGGCTCTAAAGTAGT	UNC_rdt_112_R
CGCTCTTCCGATCTCTGCTCTATGACCTGCTGCTGGA	UNC_rdt_113_R
CGCTCTTCCGATCTCTGCTTGGCCACTGCGGG	UNC_rdt_114_R
CGCTCTTCCGATCTCTGTCTGTCTTGAACATGAGTTTTT	UNC_rdt_47_R
CGCTCTTCCGATCTCTGCCTGGCTCCTTCCCAGC	UNC_rdt_48_R
CGCTCTTCCGATCTCTGACTTTCCACTTGATAAGAGGT	UNC_rdt_49_R

CGCTCTTCCGATCTCTGGGGGAGAGGAGCTGGTG	UNC_rdt_50_R
CGCTCTTCCGATCTCTGCTTACCTCGCTTAGTGCTCC	UNC_rdt_51_R
CGCTCTTCCGATCTCTGGGCACAAACACGCACCT	UNC_rdt_52_R
CGCTCTTCCGATCTCTGCCAGTGTGATGATGGTGAGG	UNC_rdt_53_R
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CGCTCTTCCGATCTCTGGCTCACCATCGCTATCTGAG	UNC_rdt_56_R
CGCTCTTCCGATCTCTGGGGCGGGGGTGTGGA	UNC_rdt_57_R
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CGCTCTTCCGATCTCTGGGTTTTCTGGGAAGGGACAG	UNC_rdt_59_R
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CGCTCTTCCGATCTCTGCCTTATTCCAAAATGTTAATACCTTT	UNC_rdt_132_R
CGCTCTTCCGATCTCTGAATATTGAAGCTGTAGGGAAAAA	UNC_rdt_133_R
CGCTCTTCCGATCTCTGAGATTGGAAGAAGACTTGAAGA	UNC_rdt_134_R
CGCTCTTCCGATCTCTGGCAACCGAAACAAAGCTGAA	UNC_rdt_135_R

Sequence Read	Sequence Insert
CGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG	
ATCACG	CGTGAT
CGATGT	ACATCG
TTAGGC	GCCTAA
TGACCA	TGGTCA
ACAGTG	CACTGT
GCCAAT	ATTGGC
CAGATC	GATCTG
ACTTGA	TCAAGT
GATCAG	CTGATC
TAGCTT	AAGCTA
GGCTAC	GTAGCC
CTTGTA	TACAAG
AGTCAA	TTGACT
AGTTCC	GGAACT
ATGTCA	TGACAT
CCGTCC	GGACGG
GTAGAG	CTCTAC
GTCCGC	GCGGAC
GTGAAA	TTTCAC

GTGGCC	GGCCAC
GTTTCG	CGAAAC
CGTACG	CGTACG
GAGTGG	CCACTC
GGTAGC	GCTACC
ACTGAT	ATCAGT
ATGAGC	GCTCAT
ATTCCT	AGGAAT
CAAAAG	CTTTTG
CAACTA	TAGTTG
CACCGG	CCGGTG
CACGAT	ATCGTG
CACTCA	TGAGTG
CAGGCG	CGCCTG
CATGGC	GCCATG
CATTTT	AAAATG
CCAACA	TGTTGG
CGGAAT	ATTCCG
CTAGCT	AGCTAG
CTATAC	GTATAG
CTCAGA	TCTGAG
GACGAC	GTCGTC
TAATCG	CGATTA
TACAGC	GCTGTA
TATAAT	ATTATA
TCATTC	GAATGA
TCCCGA	TCGGGA
TCGAAG	CTTCGA
TCGGCA	TGCCGA

ACGCTCTTCCGATCTCTG
GTGTGCTCTTCCGATCTGAC

Antisense_Sequence_with_Illumina_Tail	Chromosome	Sense_Start
TGCTCTTCCGATCTGACAGTCGTCTTGTTTCATCAAAAA	chr3	179,198,992
TGCTCTTCCGATCTGACGCATTTTAGAATAGGATATTGTATCAT	chr3	179,199,107
TGCTCTTCCGATCTGACACAGAGCAAATGGAAAGGCA	chr3	179,209,593
TGCTCTTCCGATCTGACTGTGCATCATTCATTTGTTTCA	chr3	179,234,200
TGCTCTTCCGATCTGACATGATCCCGTGGAGCTAGAC	chr12	132,673,535
TGCTCTTCCGATCTGACTGGGTGAAGAGGGACAGTTA	chr12	132,673,621
TGCTCTTCCGATCTGACAGAAGGACAGCCAGGGG	chr12	132,675,357
TGCTCTTCCGATCTGACATTAGAGCCTGACCTGCCC	chr12	132,675,467
TGCTCTTCCGATCTGACGCTCATCTGATCCAAAGGTG	chr12	132,675,721
TGCTCTTCCGATCTGACCACCCCCAAGCCAGAATATG	chr12	132,676,053
TGCTCTTCCGATCTGACCTTTGCAGCCTCTGACTTGT	chr12	132,676,150
TGCTCTTCCGATCTGACCCCTGTGGTTTTGGCATTTG	chr12	132,676,553
TGCTCTTCCGATCTGACTTTCCGGTAGAAATCACCCG	chr12	132,677,322
TGCTCTTCCGATCTGACTCCTGGATGTAGAACTCTAGT	chr12	132,677,388
TGCTCTTCCGATCTGACAGAAGATAGCTGACCAGTTG	chr12	132,677,573
TGCTCTTCCGATCTGACCCCTGGCAGCTGAGTTAGT	chr12	132,677,675
TGCTCTTCCGATCTGACCACTGTGGAGGATCTTGTCAA	chr12	132,679,510
TGCTCTTCCGATCTGACCCTATTTCTCTTGAACCAATGAGC	chr12	132,679,602
TGCTCTTCCGATCTGACGTTGTGAGCGAGAAGTTTCAT	chr12	132,679,950
TGCTCTTCCGATCTGACCTCTTAACAGGTGGCTTTGC	chr12	132,680,134
TGCTCTTCCGATCTGACCTTAGGCAGTGCAGTGGATT	chr12	132,680,572
TGCTCTTCCGATCTGACTCTCTAACTGTGTAGAGGATGGT	chr12	132,680,648
TGCTCTTCCGATCTGACGCCTGGAACGGAGTCAGT	chr12	132,681,143
TGCTCTTCCGATCTGACCAGGTGGCATTACAAATTAAGCA	chr12	132,681,233
TGCTCTTCCGATCTGACcaaccccacggtgcg	chr12	132,687,443
TGCTCTTCCGATCTGACTCCAGGGGGATCTTGAGC	chr6	151,807,923
TGCTCTTCCGATCTGACGGCCGCGGCGTTGAA	chr6	151,808,026
TGCTCTTCCGATCTGACCACGCTGTTGAGTGGGG	chr6	151,808,122
TGCTCTTCCGATCTGACTTCTCCAGGTAGTAGGGCAC	chr6	151,808,223
TGCTCTTCCGATCTGACctccctccTGCCGGG	chr6	151,808,319
TGCTCTTCCGATCTGACTAGCGAGTCTCCTTGGCAG	chr6	151,842,598
TGCTCTTCCGATCTGACACTTCTCTTGAAGAAGGCCT	chr6	151,842,687
TGCTCTTCCGATCTGACTCTCCCTCCTCTTCGGTCTTT	chr6	151,944,109
TGCTCTTCCGATCTGACAGCTCTCATGTCTCCAGCA	chr6	151,944,184
TGCTCTTCCGATCTGACAAGGCACTGACCATCTGG	chr6	151,944,269
TGCTCTTCCGATCTGACCTGCCAGGTTGGTCAGTAAG	chr6	151,944,366
TGCTCTTCCGATCTGACGTTCTTGAAAAGCTATTGACTCTT	chr6	151,944,457
TGCTCTTCCGATCTGACCTAGCCAGGCACATTCTAGA	chr6	152,011,623
TGCTCTTCCGATCTGACCTTACCTGTCCAAGAGCAAGT	chr6	152,011,704

TGCTCTTCCGATCTGACCACCATGCCCTCTACACATT	chr6	152,060,922
TGCTCTTCCGATCTGACCTCACCAGAATTAAGCAAAATAATAGA	chr6	152,061,030
TGCTCTTCCGATCTGACATGGTCCTTCTCTTCCAGAG	chr6	152,094,348
TGCTCTTCCGATCTGACCTGGTGCTGCTGCTGC	chr6	152,094,436
TGCTCTTCCGATCTGACGGGCATGTTTTCTTTATGTCTCT	chr6	152,094,519
TGCTCTTCCGATCTGACAGGCGGTGGGCGTCC	chr6	152,098,725
TGCTCTTCCGATCTGACGCAAGGAATGCGATGAAG	chr6	152,098,823
TGCTCTTCCGATCTGACTGCAGCAGGGATTATCTGAA	chr6	152,098,914
TGCTCTTCCGATCTGACCCAAAAAGGGTCAGTCTACC	chr17	7,669,580
TGCTCTTCCGATCTGACCTTGGAACTCAAGGATGCCC	chr17	7,670,576
TGCTCTTCCGATCTGACaggtactgtGTATATACTTACTTCTCC	chr17	7,670,664
TGCTCTTCCGATCTGACTCTTTCCTAGCACTGCCCAA	chr17	7,673,534
TGCTCTTCCGATCTGACCAAGAAAGGGGAGCCTCAC	chr17	7,673,651
TGCTCTTCCGATCTGACTGAGTAGTGGTAATCTACTGGGA	chr17	7,673,746
TGCTCTTCCGATCTGACCATGGGCGGCATGAACC	chr17	7,674,141
TGCTCTTCCGATCTGACGGCCTCATCTTGGGCCT	chr17	7,674,225
TGCTCTTCCGATCTGACCCTCAGCATCTTATCCGAGTG	chr17	7,674,862
TGCTCTTCCGATCTGACGGAGGTTGTGAGGCGCT	chr17	7,675,004
TGCTCTTCCGATCTGACGCCCTGTGCAGCTGTG	chr17	7,675,091
TGCTCTTCCGATCTGACTGTGCCCTGACTTTCAACTC	chr17	7,675,190
TGCTCTTCCGATCTGACCCCCTCCTGGCCCCT	chr17	7,676,007
TGCTCTTCCGATCTGACGTTCACTGAAGACCCAGGTC	chr17	7,676,120
TGCTCTTCCGATCTGACCTGACTGCTCTTTTCACCCA	chr17	7,676,209
TGCTCTTCCGATCTGACACTTTCTGCTCTTGTCTTTCAGA	chr17	7,676,305
TGCTCTTCCGATCTGACGCAGTCAGATCCTAGCGTC	chr17	7,676,484
TGCTCTTCCGATCTGACCGCATCTCGTACCAAAAAGG	chr5	68,293,080
TGCTCTTCCGATCTGACAATTCAACCACAGAACTGAAG	chr5	68,293,296
TGCTCTTCCGATCTGACCCTGGGATGTGCGGGT	chr5	68,293,750
TGCTCTTCCGATCTGACAGGATTCCATTTCAAATACTTACATCA	chr5	68,295,256

Antisense_Sequence_Illumina_Tail	**Chromosome**	**Sense_Start**
TGCTCTTCCGATCTGACAAGGGTTGAAAAAGCCGAAG	chr3	179,199,034
TGCTCTTCCGATCTGACAGCATTTGACTTTACCTTATCAA	chr3	179,203,715
TGCTCTTCCGATCTGACAGCACTTACCTGTGACTCCA	chr3	179,218,260
TGCTCTTCCGATCTGACTCCAATCCATTTTTGTTGTCCAG	chr3	179,234,241
TGCTCTTCCGATCTGACAGTCATAATCTCAAGGCGGC	chr12	132,673,581
TGCTCTTCCGATCTGACGCATGTTAGAATCATCCTGGC	chr12	132,673,654
TGCTCTTCCGATCTGACCCGGGCAGCAGTCCA	chr12	132,675,409
TGCTCTTCCGATCTGACCACGTCCAGGAGACCAAAC	chr12	132,675,695
TGCTCTTCCGATCTGACAGCTTGAAGAAGAGAAAGAGCA	chr12	132,675,775

TGCTCTTCCGATCTGACGCTACCTCATCACCAACAGG	chr12	132,676,108
TGCTCTTCCGATCTGACTCCTGATGCTGAGACAGACC	chr12	132,676,509
TGCTCTTCCGATCTGACGGAGGCCTAATGGGGAGTTT	chr12	132,676,608
TGCTCTTCCGATCTGACTTTGCAGGCTCATTGGTACA	chr12	132,677,352
TGCTCTTCCGATCTGACGCGAGTACGATGTTCCCTAC	chr12	132,677,537
TGCTCTTCCGATCTGACTTCTGCAGAGGGGCGG	chr12	132,677,619
TGCTCTTCCGATCTGACAGAAGAACAGGGAGCAGGAT	chr12	132,679,456
TGCTCTTCCGATCTGACCTTGGTGGGTTTGAAGCGA	chr12	132,679,544
TGCTCTTCCGATCTGACTCCAAGAAGTTTCAGGGCAA	chr12	132,679,917
TGCTCTTCCGATCTGACGGAAGGAAATGTATTTTACCAATGGA	chr12	132,680,000
TGCTCTTCCGATCTGACTCTTCTTTCTCATCACCCCT	chr12	132,680,177
TGCTCTTCCGATCTGACTCTCTTGTAGACCGAGATTTT	chr12	132,680,598
TGCTCTTCCGATCTGACTTTGGTTTTGAGCGGCT	chr12	132,681,101
TGCTCTTCCGATCTGACTTTCACTCAGGGATGATGGC	chr12	132,681,190
TGCTCTTCCGATCTGACGTCCCTGCCGGCCAC	chr12	132,687,398
TGCTCTTCCGATCTGACACATTTccggccccacc	chr12	132,687,475
TGCTCTTCCGATCTGACCTTGGATCTGATGCAGTAGGG	chr6	151,807,876
TGCTCTTCCGATCTGACTCGGGGTAGTTGTACACGG	chr6	151,807,980
TGCTCTTCCGATCTGACGACCCGGGGCCGTAG	chr6	151,808,064
TGCTCTTCCGATCTGACCAGGAAAGGCGACAGCTG	chr6	151,808,176
TGCTCTTCCGATCTGACGTACCTGTAGAATGCCGGC	chr6	151,808,269
TGCTCTTCCGATCTGACTGGTACTGGCCAATCTTTCTC	chr6	151,842,550
TGCTCTTCCGATCTGACACTCCATAATGGTAGCCTGAAG	chr6	151,842,641
TGCTCTTCCGATCTGACAGGATCTGCTCATAGGATCA	chr6	151,842,742
TGCTCTTCCGATCTGACCCTCCCCATCATCTCTCTGG	chr6	151,944,153
TGCTCTTCCGATCTGACTCTTAGAGCGTTTGATCATGAG	chr6	151,944,220
TGCTCTTCCGATCTGACTCTGGTAGGATCATACTCGGA	chr6	151,944,318
TGCTCTTCCGATCTGACCCTCTTCGCCCAGTTGATC	chr6	151,944,409
TGCTCTTCCGATCTGACCCTGATCATGGAGGGTCAA	chr6	152,011,589
TGCTCTTCCGATCTGACCTTCCCTGGGTGCTCCAT	chr6	152,011,666
TGCTCTTCCGATCTGACTGATCGTAAAGAACATGCTACT	chr6	152,011,748
TGCTCTTCCGATCTGACTGCAGATTCATCATGCGGAA	chr6	152,060,980
TGCTCTTCCGATCTGACAATAAACATTTCATCCAGCATTG	chr6	152,061,072
TGCTCTTCCGATCTGACTCAGGTGGATCAAAGTGTCTG	chr6	152,094,387
TGCTCTTCCGATCTGACATGTGCCTGATGTGGGAGA	chr6	152,094,466
TGCTCTTCCGATCTGACGCACCACGTTCTTGCACT	chr6	152,098,687
TGCTCTTCCGATCTGACGTGGCTTTGGTCCGTCTC	chr6	152,098,784
TGCTCTTCCGATCTGACGCCAGGGAGCTCTCAGAC	chr6	152,098,879
TGCTCTTCCGATCTGACTGATGTCATCTCTCCTCCCT	chr17	7,669,628
TGCTCTTCCGATCTGACCTCTGTTGCTGCAGATCCG	chr17	7,670,630
TGCTCTTCCGATCTGACCCAGCTCCTCTCCCCAG	chr17	7,673,499

TGCTCTTCCGATCTGACAGACCAAGGGTGCAGTTATG	chr17	7,673,579
TGCTCTTCCGATCTGACCCTGTCCTGGGAGAGACC	chr17	7,673,696
TGCTCTTCCGATCTGACGGTAGGACCTGATTTCCTTAC	chr17	7,673,792
TGCTCTTCCGATCTGACTGACTGTACCACCATCCACT	chr17	7,674,191
TGCTCTTCCGATCTGACTGCGTGTGGAGTATTTGGAT	chr17	7,674,830
TGCTCTTCCGATCTGACGGTCCCCAGGCCTCTG	chr17	7,674,912
TGCTCTTCCGATCTGACGTCCGCGCCATGGCC	chr17	7,675,047
TGCTCTTCCGATCTGACTTCCTACAGTACTCCCCTGC	chr17	7,675,153
TGCTCTTCCGATCTGACAACCTACCAGGGCAGCTAC	chr17	7,675,967
TGCTCTTCCGATCTGACGTGGCCCCTGCACCA	chr17	7,676,063
TGCTCTTCCGATCTGACGCAATGGATGATTTGATGCTGT	chr17	7,676,167
TGCTCTTCCGATCTGACggggg**c**tgAGGACCTG	chr17	7,676,240
TGCTCTTCCGATCTGACACCTGTGGGAAGCGAAAATT	chr17	7,676,373
TGCTCTTCCGATCTGACTTTCCTCTTGCAGCAGCC	chr17	7,676,536
TGCTCTTCCGATCTGACCATATTTCCCATCTCGATGAAA	chr5	68,293,256
TGCTCTTCCGATCTGACTGTGCGGGTATATTCTTCAT	chr5	68,293,730
TGCTCTTCCGATCTGACTCGTCTTTCTCAGCTGGATAAG	chr5	68,295,209
TGCTCTTCCGATCTGACGCATAGCAGCCCTGTTTACT	chr5	68,296,242

Antisense_Start	Amplicon_Size
179,199,091	100
179,199,206	100
179,209,675	83
179,234,297	98
132,673,633	99
132,673,706	86
132,675,455	99
132,675,566	100
132,675,820	100
132,676,148	96
132,676,249	100
132,676,651	99
132,677,407	86
132,677,486	99
132,677,672	100
132,677,759	85
132,679,601	92
132,679,695	94
132,680,045	96
132,680,232	99
132,680,661	90
132,680,741	94
132,681,241	99
132,681,325	93
132,687,533	91
151,808,019	97
151,808,110	85
151,808,221	100
151,808,313	91
151,808,418	100
151,842,695	98
151,842,780	94
151,944,201	93
151,944,276	93
151,944,368	100
151,944,463	98
151,944,556	100
152,011,712	90
152,011,799	96

152,061,021	100
152,061,129	100
152,094,437	90
152,094,521	86
152,094,618	100
152,098,824	100
152,098,916	94
152,099,008	95
7,669,679	100
7,670,668	93
7,670,761	98
7,673,618	85
7,673,750	100
7,673,844	99
7,674,237	97
7,674,321	97
7,674,960	99
7,675,102	99
7,675,190	100
7,675,278	89
7,676,105	99
7,676,210	91
7,676,299	91
7,676,425	121
7,676,583	100
68,293,161	82
68,293,395	100
68,293,835	86
68,295,347	92

Antisense_Start	Amplicon_Size
179,199,118	85
179,203,804	90
179,218,343	84
179,234,329	89
132,673,673	93
132,673,751	98
132,675,502	94
132,675,793	99
132,675,871	97

132,676,203	96
132,676,600	92
132,676,707	100
132,677,450	99
132,677,633	97
132,677,717	99
132,679,551	96
132,679,643	100
132,680,016	100
132,680,099	100
132,680,266	90
132,680,697	100
132,681,200	100
132,681,289	100
132,687,488	91
132,687,574	100
151,807,970	95
151,808,079	100
151,808,163	100
151,808,275	100
151,808,368	100
151,842,649	100
151,842,740	100
151,842,838	97
151,944,238	86
151,944,319	100
151,944,417	100
151,944,501	93
152,011,685	97
152,011,762	97
152,011,841	94
152,061,077	98
152,061,171	100
152,094,483	97
152,094,565	100
152,098,781	95
152,098,879	96
152,098,978	100
7,669,727	100
7,670,729	100
7,673,593	95

7,673,670	92
7,673,793	98
7,673,891	100
7,674,282	92
7,674,929	100
7,675,009	98
7,675,143	97
7,675,245	93
7,676,066	100
7,676,152	90
7,676,254	88
7,676,320	81
7,676,456	84
7,676,635	100
68,293,355	100
68,293,828	99
68,295,308	100
68,296,329	88