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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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Author Questionnaire:

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **(Y/N) N**

Can you record movies/images using your own microscope camera? **(Y/N) N/A**

If no, JoVE will need to record the microscope images using our scope kit (through a camera port or one of the oculars). Please list the make and model of your microscope.

N/A

2. Does your protocol include software usage? **(Y/N) Y**

If yes, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps.

Dennis demonstrating the bioinformatics analysis.

3. Which steps from the protocol section below are the most important for viewers to see? Please list 4-6 individual steps using the step numbers listed in this document. This information is important to prepare your Videographer for your shoot. (You do not need to include steps that will be screen captured. Please do not list entire sections.)

Step 2.2, Step 4.1-4.4, Step 5.1, Step 6.1, Step 6.3, Step 7.2,

4. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1-2 individual steps using the step numbers listed in this document. (Please do not list entire sections.)

Step 4 and Step 7.2

5. Will the filming need to take place in multiple locations? **(Y/N) N**

If yes, how far apart are the locations?

Section - Introduction

Videographer: Interviewee Headshots are required. Take a headshot for each interviewee.

Authors, these headshots will be used for the JoVE Dedicated Author Webpage. Here is one example if you wish to take a look.

1. REQUIRED Interview Statements: (Said by you on camera) - All interview statements may be edited for length and clarity.

1.1. **Gaorav P. Gupta:** Our droplet PCR based next generation sequencing protocol offers a relatively simple and cost-effective way to accurately measure mutations in plasma circulating tumor DNA **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking towards camera.

1.2. **Sunil Kumar:** Analysis of plasma DNA is challenging because the amount of tumor DNA in the starting material can be very limited. Droplet PCR based target enrichment overcomes this challenge **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking towards camera.

Ethics title card: (for human subjects or animal work, does not count toward word length total)

1.3. Procedures involving human subjects have been approved by the Institutional Review Board (IRB) at University of North Carolina at Chapel Hill **[1]**.

1.3.1. Title Card

Section - Protocol

2. Plasma Extraction and Cell-free DNA Extraction from Peripheral Blood

2.1. Collect 10 milliliters of blood from breast cancer patients in a glass blood collection tube **[1]**. After the blood collection, mix 8 to 10 times by gentle inversion. Do not freeze specimens in the collection tube **[2]**.

2.1.1. Talent retrieves the collected blood in the glass blood collection tube.

2.1.2. Talent mixes the tubes 8 to 10 times.

2.2. Centrifuge the blood samples at 1,800 x g for 10 minutes and collect the plasma **[1]**. Take care to avoid collecting the middle whitish layer below the plasma **[2]**. Then, centrifuge at 2,000 x g for another 10 minutes and collect the plasma **[3-TXT]**.

2.2.1. Talent places the blood samples into the centrifuge, shuts lid and starts run. *Videographer, the authors consider this an important step for visualization.*

2.2.2. Centrifuged sample as talent collects the plasma, taking care to avoid the layer below the plasma. *Videographer, the authors consider this an important step for visualization.*

2.2.3. Talent removes the centrifuged samples from the centrifuge and motions to collect plasma. *Videographer, the authors consider this an important step for visualization.* **TEXT: Store the plasma at -80 ˚C until further use**

2.3. Use a commercial kit to extract the cell free DNA from the plasma using the manufacturer's protocol **[1-TXT]**. Process the sample using a vacuum manifold and elute the sample in a microfuge at 14,000 x g for 1 minute **[2]**.

2.3.1. Talent pulls reagents out of commercial extraction kit. **TEXT: See Table of Materials in text**

2.3.2. Talent pulls the microfuge with the eluted sample out of the microcentrifuge.

2.4. Use 1 to 2 microliters of cell free DNA to quantify using a fluorimeter and store the eluted cell free DNA at minus 20 degrees Celsius until further use **[1]**.

2.4.1. Talent quantifies the cfDNA using a fluorimeter.

3. Designing the Next Generation Sequencing Cancer Panel

3.1. Design and synthesize 272 primers to get 136 amplicons of 96-basepair regions for targeted enrichment of all coding regions in ESR1 and TP53 *(T-P-fifty three)* and for hotspots in PIK3CA *(pick 3 C-A)* and PIK3R1 *(pick 3 R-one)* **[1-TXT]**.

3.1.1. Primer design_2.png. **TEXT: See text for complete list of primers**

3.2. Divide the oligonucleotides into two sets for targeted amplification of the 68 genomic regions in each set so that 68 wells in both 96-well plates have 60 microliters of sense and antisense oligos at a concentration of 100 microMolar **[1]**.

3.2.1. Primer design_1.png *– Video editor, if possible, transition from the previous figure into a zoomed in view of one set of primers (a set of horizontal checkmarks that are opposite each other) in this figure. Then zoom out to the full figure.*

3.3. Treat both plates separately to make two sets of oligonucleotide mixtures. Take out 1.6 microliters from each well on the plate and mix the oligonucleotides to prepare a mixture consisting of 0.16 nanoMolar of each primer in 200 microliters of 10 milliMolar Tris pH 8.0 **[1]**.

3.3.1. Talent removes 1.6 microliters from each well on the plate and mixes the oligonucleotide.

4. Droplet Generation and Target Enrichment by First Round of PCR

4.1. **Sunil Kumar:** Target enrichment by droplet PCR is the key step in this protocol. It achieves desired amplification of minor mutant alleles **[1]**.

4.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking towards camera.

4.2. Keeping the reagents on ice, prepare the master mix in a microcentrifuge tube by combining 20 microliters of 2x genotyping master mix, 1.6 microliters of 25x droplet stabilizer, and 2 microliters of either set 1 or set 2 primers. **[1]**.

4.2.1. Talent prepares the master mix in a microcentrifuge tube. Use labeled containers. *Videographer, the authors consider this an important step for visualization.*

4.3. Add 5.0 nanograms of circulating tumor DNA and adjust the volume to 40 microliters with water. Because the amount of cell free DNA is very limited, the protocol was standardized to use the minimum amount **[1]**.

4.3.1. Talent adds 5.0 ng of ctDNA and adjusts the volume to 40 microliters. Use labeled containers. *Videographer, the authors consider this an important step for visualization.*

4.4. Load the 40-microliter reaction mixture on each well of the chip for droplet generation **[1]**. Transfer the droplet emulsions from the chip to PCR tubes using a multichannel pipette **[2]**.

4.4.1. Chip as talent loads 40 microliters of the reaction mixture to each well of the chip. *Videographer, the authors consider this an important step for visualization.*

4.4.2. Talent transfers the droplet emulsions from the chip to the PCR tubes using a multichannel pipette. *Videographer, the authors consider this an important step for visualization.*

4.5. Set up a PCR reaction for the droplet emulsions with the set 1 and set 2 primers for each sample as described in the text protocol **[1]**.

4.5.1. Talent sets up the PCR reaction for the droplet emulsions with the set 1 and set 2 primers for each sample. *Videographer, the authors consider this an important step for visualization.*

5. Recovery of DNA From the Droplets After PCR Amplification

5.1. To break the emulsion, add 50 microliters of droplet destabilizer to the post-amplification droplets and vortex them for 30 seconds **[1]**. Centrifuge the preparation at 2,000 x g for 2 minutes to separate the aqueous and oil phase **[2]**.

5.1.1. Talent adds 50 microliters of droplet destabilizer to the post-amplification droplets and vortexes them. *Videographer, the authors consider this an important step for visualization.*

5.1.2. Talent places the preparation in the centrifuge and starts run. *Videographer, the authors consider this an important step for visualization.*

5.2. 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 **[1]**.

5.2.1. ECU: Pipette tip as talent inserts it through the upper aqueous phase and carefully removes the bottom oil.

5.3. Now, resuspend SPRI magnetic beads by inversion **[1-TXT]**. Add 39.6 microliters of SPRI magnetic beads into each of the aqueous volumes to maintain a 1.2x bead to reaction volume ratio **[2]**.

5.3.1. Talent resuspends the SPRI magnetic beads by inversion. **TEXT: SPRI = solid phase reversible immobilization**

5.3.2. Talent adds 39.6 microliters of SPRI magnetic beads into each of the aqueous volumes.

5.4. Mix the beads plus the aqueous sample by pipetting up and down about 10 times so that the slurry appears uniform in density **[1]**.

5.4.1. Sample as talent mixes the beads plus aqueous sample by pipetting up and down.

5.5. After leaving the PCR tubes at room temperature for 5 minutes, firmly position the PCR strips with samples onto a 96-well magnetic plate **[1]**. Leave it for 2 to 3 minutes or until pellets are formed and the supernatant is clear **[2]**.

5.5.1. 96-well plate as talent firmly positions the PCR strips with sample there.

5.5.2. Talent starts a timer to count down from 2 or 3 minutes.

5.6. Then, remove the supernatant using a multichannel pipette **[1]**.

5.6.1. Talent removes the supernatant using a multichannel pipette.

5.7. Leave the PCR tubes on the magnet and add 180 microliters of freshly made 85% ethanol to each tube **[1]**. Mix by pipetting five to six times **[2]**. After leaving for 1 minute or until the supernatant is clear, remove all the ethanol carefully **[3]**.

5.7.1. Talent places the PCR tubes on the magnet and adds 180 microliters of freshly made 85% ethanol.

5.7.2. Talent mixes the tube by pipetting.

5.7.3. Talent removes the ethanol.

5.8. Remove the PCR tubes from the magnet and leave at room temperature for 5 minutes maximum to dry the bead pellets **[1]**. Add 20 microliters of 10 milliMolar Tris-HCl pH 8.0 per well with a multichannel pipette and then place the tubes at room temperature for 2 minutes **[2]**.

5.8.1. Talent removes the PCR tubes from the magnet and leaves at room temperature.

5.8.2. Talent adds 20 microliters of 10 mM Tris-HCl with a multichannel pipette.

5.9. Now, place the tubes on the magnet for 2 minutes to separate the beads **[1]**. Use a multichannel pipette to collect 17 microliters of eluent from each tube and deposit in a PCR tube **[2]**.

5.9.1. Talent places the tubes on the magnet.

5.9.2. Tubes as talent uses a multichannel pipette to collect 17 microliters of eluent from each tube and deposit in the PCR tube.

6. Addition of Adaptor and Index Sequences at the Second Round of PCR and Subsequent Recovery of DNA

6.1. For the second round of PCR, prepare the master mix in a microcentrifuge tube as described in the text protocol **[1]**. Add 1.25 microliters of 5 microMolar index reverse primer and 13 microliters of the first PCR template DNA to make 25 microliters of secondary PCR reactions **[2]**.

6.1.1. Talent prepares the master mix. Use labeled containers. *Videographer, the authors consider this an important step for visualization.*

6.1.2. Talent adds 1.25 microliters of 5 microMolar index reverse primer and 13 microliters of the first PCR template DNA. Use labeled containers. *Videographer, the authors consider this an important step for visualization.*

6.2. Perform a PCR reaction with the set 1 and set 2 primers for each sample as detailed in the text protocol **[1]**.

6.2.1. Talent sets up the PCR reaction tube as described in the text protocol.

6.3. Following the PCR reaction, add 22.5 microliters of resuspended SPRI magnetic beads into each of the aqueous volumes to maintain a 0.9x bead to reaction volume ratio for the second PCR **[1]**.

6.3.1. Talent adds 22.5 microliters of resuspended SPRI magnetic beads into each of the aqueous volumes. *Videographer, the authors consider this an important step for visualization.*

6.4. After processing the samples as described in the text protocol, use a multichannel pipette to collect 17 microliters of eluent from each tube and deposit in fresh PCR tubes **[1]**.

6.4.1. Talent uses a multichannel pipette to collect 17 microliters of eluent from each tube and deposit in fresh PCR tubes.

7. DNA Quantification on the Bioanalyzer and Targeted Sequencing Protocol

7.1. Check the quantity and quality of the libraries using the Bioanalyzer in accordance with the manufacturer's instructions **[1]**.

7.1.1. ScreenCapture_Bioanalyzer_v2.mp4 *– Video editor, please show 0:16-0:33.*

7.2. After recording all product yields, dilute each library to 2 nanoMolar using nuclease-free water as a diluent **[1]**. Mix an equal volume of each library in a tube to make an aggregate pooled sample concentration of 2 nanoMolar **[2]**.

7.2.1. Talent dilutes the libraries using nuclease-free water. *Videographer, the authors consider this an important step for visualization.*

7.2.2. Talent mixes an equal volume of each library in a tube. *Videographer, the authors consider this an important step for visualization.*

7.3. Then, quantify the pooled library on a fluorometer and Bioanalyzer with targeted sequencing **[1]**.

7.3.1. Talent inserts the sample into the Bioanalyzer or fluorometer.

7.4. Sequence each pooled library using custom sequencing primers and the sequencing reagent kit on a Next Generation Sequencer following the manufacturer's instructions for 125 cycle paired-end sequencing **[1-TXT]**.

7.4.1. Talent prepares samples to send out to central facility. **TEXT: See text for primers and reagent kit**

7.5. Trim the reads in the FASTQ *(fast-Q)* files to remove the adaptors and any low-quality bases at the ends using the ea-utils *(E-A u-tils)* module fastq-mcf *(fast-Q-M-C-F)*. Use the default parameters except for k equals 2. Align the sequence against the human reference genome using Bowtie2. Use default parameters except local N 1 p 5 **[1]**.

7.5.1. ScreenCapture_Bioinformatic 20analysis.mp4 *– Video editor, please show 0:00-0:12.*

7.6. Using Samtools, create BAM *(bam)* files containing each of the two categories of reads in which the 5-prime- and 3-prime primer sequences were soft-clipped, and the alignment positions were adjusted. Merge, sort, and convert the BAM files to mpileup *(M-pile-up)* files **[1]**.

7.6.1. ScreenCapture_Bioinformatic 20analysis.mp4 *– Video editor, please show 0:17-0:55.*

7.7. Call variants using VarScan2. Then, annotate the variants by snpEff *(snip-eff)*. Annotated variants unique to the patient samples are scored as true mutations **[1]**.

7.7.1. ScreenCapture_Bioinformatic 20analysis.mp4 – *Video editor, please show 1:29-2:03.*

Section – Results

8. Results: Mutation Heterogeneity in Metastatic Breast Cancer Patients

8.1. The dPCR-SEQ *(D-P-C-R-seeq)* assay was used to detect mutations in 31 metastatic breast cancer patients **[1]**. Shown here are lollipop plots showing the number… **[2]** and position … **[3]** of mutations across the protein sequence of breast cancer relevant genes **[4]**

8.1.1. Figure 2

8.1.2. Figure 2 – *Video editors, please emphasize the vertical axes.*

8.1.3. Figure 2 – *Video editors, please emphasize the horizontal axes.*

8.1.4. Figure 2

8.2. Mutations were found in *ESR*1… **[1]**, *PIK3CA*… **[2]**, *TP53*… **[3]**, *POLE (pole)*… **[4]**, and *PIK3R1* **[5]** genes by dPCR-Seq . The median depth of coverage was 3,500 **[6]**.

8.2.1. Figure 2 – *Video editors, please emphasize the row labeled "ESR1".*

8.2.2. Figure 2 – *Video editors, please emphasize the row labeled "PIK3CA".*

8.2.3. Figure 2 – *Video editors, please emphasize the row labeled "TP53".*

8.2.4. Figure 2 – *Video editors, please emphasize the row labeled "POLE".*

8.2.5. Figure 2 – *Video editors, please emphasize the row labeled "PIK3R1".*

8.2.6. Figure 2

Section - Conclusion

9. Conclusion Interview Statements: (Said by you on camera) - All interview statements may be edited for length and clarity.

9.1. **Gaorav P. Gupta:** It is critical to evaluate for balanced amplification with newly designed custom primer sets by assessing read depth for the different amplicon regions, and adjusting primer concentrations or redesigning primers as needed **[1]**.

9.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

9.2. **Sunil Kumar:** The detection threshold of dPCR-Seq assay is 1.6% and the calculated mutation frequency matches very well with digital PCR assays for individual target alleles **[1]**.

9.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

9.3. **Dennis A. Simpson:** dPCR-Seq can be adapted to any other gene of interest, and allows investigators to perform NGS on plasma DNA samples in a time- and cost-effective manner **[1]**.

9.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.