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TITLE:

Detection and Monitoring of Tumor Associated Circulating DNA in Patient Biofluids

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KEYWORDS:

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SUMMARY:

Here, we present a protocol to detect tumor somatic mutations in circulating DNA present in patient biological fluids (biofluids). Our droplet digital polymerase chain reaction (dPCR)-based method enables quantification of the tumor mutation allelic frequency (MAF), facilitating a minimally invasive complement to diagnosis and temporal monitoring of tumor response.

ABSTRACT:

Complications associated with upfront and repeat surgical tissue sampling present the need for minimally invasive platforms capable of molecular sub-classification and temporal monitoring of tumor response to therapy. Here, we describe our dPCR-based method for the detection of tumor somatic mutations in cell free DNA (cfDNA), readily available in a patient biofluids. Although limited in the number of mutations that can be tested for in each assay, this method provides a high level of sensitivity and specificity. Monitoring of mutation abundance, as calculated by MAF, allows for the evaluation of tumor response to therapy, thereby providing a much-needed supplement to radiographic imaging.

INTRODUCTION:

Due to the limitations in the availability of the tumor tissue for molecular analyses, there is a need for the development of highly sensitive methods capable of detecting tumor somatic mutations in patient biofluids, including plasma, serum and cerebrospinal fluid (CSF). For example, pediatric diffuse midline gliomas (DMGs) present a challenge due to their neuroanatomical location. Over the past decade, molecular profiling of DMG tissue specimens has uncovered driver mutations in this tumor type[1] and has revealed spatial and temporal heterogeneity of mutations, making biopsy necessary for characterizing a patient within a disease subgroup and promoting molecularly-targeted therapies[2]. As such, clinical trials advocate for integrating surgical biopsies for tumor molecular profiling at upfront diagnosis[3-6]. During the treatment, monitoring of the therapeutic response in DMGs is limited to MRI, which lacks sensitivity to detect small changes or tumor genomic evolution. MRI is also prone to detect pseudoprogression, transient inflammation at the site of the tumor that mimics true progression on imaging and may misinform interpretation of tumor response[7]. Thus, DMGs represent a tumor type with a high need for an alternative, minimally invasive means of detecting tumor mutations and monitoring clinical response. In order to address these needs, we developed and optimized a protocol for detecting, and quantifying the allelic frequency of, tumor mutations in cfDNA from plasma, serum and CSF of children with midline gliomas[8]. Given that childhood DMGs often (>80%) harbor a somatic lysine-to-methionine mutation at position 27 of histone variant H3.1 (H3.1K27M, 20% of cases) or H3.3 (H3.3K27M, 60% of cases)[1], these and other mutations characteristic of DMGs (i.e., *ACVR1, PIK3R1*) were targeted for mutant allele quantification using cfDNA[8]. This assay can be tailored to detect hotspot mutations in other tumor types using sequence-specific primers and probes. The versatility of this approach makes it applicable to a variety of cancers which would benefit from the integration of cfDNA-based diagnostic tools and therapeutic monitoring.

To sensitively detect low allelic frequency mutations in cfDNA, we employ a dPCR-based approach. In this method, the PCR reaction mixture is partitioned into a theoretical maximum of 10 million droplets using the dPCR platform (e.g., RainDance), with 7-9 million droplets per PCR well typically seen experimentally. Due to the high degree of the sample partitioning, at most only one to two DNA molecules can be present in each droplet. PCR amplification and sequence-specific fluorescent probe hybridization can occur in each droplet, such that millions of reactions take place. This maximizes the sensitivity and enables detection of rare mutant alleles, which might otherwise go undetected against a background of wildtype DNA. The utilization of locked nucleic acid (LNA) probes enhances the specificity of the assay by restricting mismatch hybridization and supporting accurate mutation detection[9-11]. Here, we describe our methods of sample processing, cfDNA extraction, preamplification of target alleles, dPCR and data analysis.

PROTOCOL:

All methods described here have been approved by the Institutional Review Board of the University of California San Francisco (San Francisco, CA; IRB #14-13895) and the Children's National Health System (Washington, D.C.; IRB #1339). Specimens were collected and studied

after obtaining informed consent from the patient or patient's guardian.

1. Consenting patients under IRB protocol for collection and storage of biological specimens

1.1. Collect patient samples after written informed consent is obtained from each patient, or the patient's guardian, for participation in a clinical trial or for biorepository as approved by the respective Institutional Review Board.

1.1.1. For this study, include patients that were diagnosed with a brain tumor radiographically. No exclusion criteria were used. Specimens were collected and studied after obtaining informed consent from the patient or patient's guardian.

2. Blood collection and separation of plasma or serum

2.1. Collect the blood sample using a 10.0 mL draw into blood collection tubes. If collecting blood for plasma, use K_2- or K_3-EDTA tubes, heparin or cfDNA blood collection tubes. If collecting blood for serum, use gel-barrier tubes containing the clot activator and gel to separate serum.

NOTE: While 10 mL is recommended to enable replicate experiments, a minimum of 3 mL will suffice.

2.2. Carefully invert the collection tube 10 times to mix the blood sample with the collection tube reagents.

2.3. Leave the blood samples from which the serum will be collected at room temperature for 30 min to allow blood to clot. If processing blood to obtain plasma, proceed immediately to step 2.4.

NOTE: Samples being processed for plasma may be kept on ice for a maximum of 2 h prior to centrifugation. However, a rapid transition from the blood draw to processing minimizes cfDNA degradation.

2.4. Centrifuge blood collection tubes at 2,000 *x g* for 15 min in a centrifuge set to 4 °C to separate plasma or serum from white and red blood cell layers.

2.5. Taking care not to disturb the white blood cell layer (which forms a thin line between the upper plasma/serum layer and lower layer of packed red blood cells), pipette the top layer of plasma/serum (which may be clear, yellow, or pink in color) equally in 1 mL aliquots into sterile polypropylene screw-cap cryovials.

2.6. Record the subject identification number, specimen type (plasma or serum), and collection date on the label of the cryovials.

2.7. Store cryovials in the -80 °C freezer until use. If an -80 °C freezer is not available, store the samples at -20 °C for up to one week.

3. CSF collection and processing

3.1. Collect CSF from a shunt, lumbar puncture, or surgery.

NOTE: Such procedures should only be conducted if deemed necessary by clinicians. Extra sample beyond that which is necessary for clinical use may be used for research purposes.

3.2. Measure the volume of CSF collected and make note of its color (bloody, yellow, clear).

3.3. Centrifuge the collection tube at 10,000 *x g* for 10 min at 4 °C to pellet the cellular debris.

3.4. Transfer the CSF supernatant equally in 500 µL aliquots into polypropylene screw cap cryovials.

3.5. Record the subject identification number, specimen type, and date of specimen collection on the label of the cryovials. Store at -80 °C until use.

4. Extraction of cfDNA from liquid specimens

4.1. Perform cfDNA extraction protocol as per the instructions in the cfDNA extraction kit handbook[12].

4.1.1. Clean the bench space and equipment with 10% bleach and 70% ethanol.

NOTE: Use of a PCR hood, regular changing of gloves and frequent decontamination of the bench space and equipment with 10% bleach and 70% ethanol during all steps of the protocol is important to avoid sample contamination.

4.1.2. Prepare aliquots of reagents, e.g., wash buffers, from the extraction kit to avoid cross-contamination.

4.2. Thaw patient samples on ice prior to beginning the extraction. For plasma/serum, thaw 1 mL aliquot of sample. For CSF, thaw 500 µL aliquot of sample.

NOTE: Lysis steps (4.5 – 4.8.2) of the extraction protocol differ between plasma/serum and CSF, but from step 4.9 on the protocol is identical for both types of biofluids.

4.3. Set a heating block to 60 °C for use during the lysis step (4.6).

4.4. Prepare the lysis buffer and carrier RNA mixture in a 15 mL tube by adding 5.6 µL of carrier RNA and 0.9 mL of lysis buffer per sample[12].

4.5. For plasma/serum, add 100 µL proteinase K, 1 mL of sample, and 0.8 mL of lysis buffer/carrier

RNA mixture prepared in step 4.4 to a labeled 2.0 mL tube. Mix by pulse vortexing for 30 s at high speed[12].

4.5.1. For CSF, add 125 µL proteinase K, 500 µL of the sample, 1 mL of lysis buffer/carrier RNA mixture, and 250 µL tissue lysis buffer to a 2.0 mL tube. Bring the full volume up to 2 mL by adding 125 µL of 1x phosphate-buffered saline (PBS). Mix by pulse vortexing for 30 s at high speed[12].

4.6. Incubate samples for 30 min at 60 °C in the heating block[12].

4.7. Remove samples from the heating block and lower the temperature to 56 °C. Return samples to the bench and transfer the lysate into new labeled 15 mL tubes.

4.8. For plasma/serum, add 1.8 mL of nucleic acid binding buffer and mix for 30 s by pulse vortexing[12].

4.8.1. For CSF, add 3.6 mL of nucleic acid binding buffer and mix for 30 s by pulse vortexing[12].

4.9. Incubate samples for 5 min on ice[12].

4.10. Insert the column into the vacuum connector. Open the column and insert a 20 mL tube extender into the column[12].

4.11. Pipette the contents of the 15 mL tube directly into the bottom of the tube extender, avoiding leaving droplets on the walls of the tube extender.

4.12. With the switch of the vacuum connector closed, switch on the vacuum pump. Once the pressure has built to 900 mbar, open the valve of the vacuum connector. The sample will now flow through the column.

4.13. Once all the lysate has been drained from the column, turn off the pump and open the trapdoor to the vacuum connector, relieving the pressure gradient. Once the pressure reaches 0 mbar, close the valve of the vacuum connector and the trapdoor.

4.14. Remove the 20 mL tube extender, being careful not to pass extenders over adjacent columns. Dispose of the extender. Remove the tube extender and discard. Do not pass tube extender of one column over a neighboring column, as this can cross-contaminate samples.

4.15. Add 600 µL of first wash buffer to the column[12].

4.16. Leave lids of tubes open and turn on the vacuum pump. Let the pressure gauge reach 900 mbar, then turn the switch on the vacuum connector to the open position to draw the wash buffer through the column.

4.17. Turn off the vacuum pump, open the trapdoor to release pressure, and relieve the pressure

to 0 mbar. Turn valve of the vacuum connector to the closed position.

4.18. Add 750 µL of second wash buffer to the column and repeat steps 4.16-4.17[12].

4.19. Add 750 µL of MB grade ethanol to the column and repeat steps 4.16-4.17[12].

4.20. Close the lid of the column and place the column inside a new 2.0 mL collection tube. Dispose of the vacuum connector[12].

4.21. Centrifuge the tube with the column at 20,000 *x g* for 3 min at room temperature[12].

4.22. Place the column into a new collection tube and open the lid of the tube. Place a laboratory tissue over the top and incubate at 56 °C for 10 min[12].

4.23. Place the column into a clean 1.5 mL PCR-clean tube and pipette 100 µL of elution buffer (or molecular biology grade H_2O (MB H_2O)) directly into the center of the column. Do not touch the column with a pipette tip. Close the lid and leave at room temperature for 3 min.

4.24. Centrifuge at 20,000 *x g* at room temperature for 1 min to elute cfDNA[12].

4.25. Pipette the eluate back into the column and incubate at room temperature for 3 min.

4.26. Repeat the centrifugation at full speed for 1 min to elute cfDNA a second time to increase yield (as per manufacturer's recommendation).

4.27. Store cfDNA in labeled DNase/RNase free PCR-clean tubes at 4 °C or proceed directly to step 5.

5. Vacuum concentration of cfDNA

5.1. Insert and balance the tubes containing eluted cfDNA into holders on the vacuum concentrator. Open the lids of the tubes. Set the temperature of vacuum concentrator to 23 °C.

5.2. Turn on the vacuum concentrator, then turn on the vapor trap. Centrifuge samples for 40 min or until a final volume of 10.5-11 µL is reached.

5.2.1. If the volume is below 10.5 µL, add DNA suspension buffer or MB H_2O to reach a final volume of 10.5 µL.

5.3. Pipette the full volume along the walls of the tube to remove residual DNA fragments.

5.4. Briefly centrifuge the samples on a tabletop centrifuge to collect droplets on the walls of the tubes.

5.5. Proceed directly to the preamplification step (step 8) or store samples at 4 °C.

5.5.1 Wrap lids of tubes in a laboratory film to avoid contamination if they are stored for later use.

6. Design and preparation of primers and probes

6.1. Design forward and reverse primers, and wildtype and mutant LNA probes, for the target(s) of interest. Commercially order lyophilized primers and probes (refer to Table of Materials).

NOTE: Sequences for primers and probes targeting *H3F3A* p.K27M and other target mutations for pediatric midline gliomas can be found in Supplementary Table 3 of Panditharatna et al., 2018[8].

6.2. Prior to opening the lyophilized primers and probes, briefly centrifuge the tubes. Add the appropriate volume of DNA suspension buffer or MB H_2O, as noted on the product specification sheet, to resuspend the lyophilized primers and probes to a concentration of 100 µM.

6.3. Gently vortex, pipette up and down several times to mix and briefly centrifuge primers and probes using a tabletop centrifuge.

6.4. Prepare 10 µM aliquots of primers and probes by adding 10 µL of 100 µM stock to 90 µL of DNA suspension buffer or MB H_2O.

6.5. Prepare 1 µM aliquots of forward and reverse primers (to be used for preamplification), by adding 10 µL of the 10 µM primer to 90 µL of DNA suspension buffer or MB H_2O.

6.6. Store primers and probes at 4 °C in air-tight dark containers. Wrap laboratory film around lids to avoid contamination, and cover probes in aluminum foil to avoid exposure to light. Store probes at -20°C for long term storage if they are not being used regularly.

7. Selection of positive controls

7.1. Select tumor tissue genomic DNA (gDNA) sample(s) with known DNA concentration, which harbors the mutation of interest at a known allelic frequency to be used as a positive control.

7.1.1. Use 0.025 ng of positive control tumor tissue DNA in the preamplification step (step 8).

NOTE: This DNA input provides a robust positive signal on dPCR (as determined by performing serial dilutions of tumor tissue gDNA harboring *H3F3A* p.K27M mutation[8]) while minimizing the amount of sample required.

8. Preamplification of target alleles

NOTE: Preamplification protocol is as per Jackson et al., 2016[13].

8.1. Clean the bench space and equipment with 10% bleach and 70% ethanol.

8.2. Obtain 1 µM forward and reverse primers, cfDNA samples, gDNA positive controls, and DNA polymerase master mix, and set on ice.

8.3. Calculate the volume of reagents needed to preamplify the desired number of cfDNA samples and positive control, for either singleplex or multiplex preamplification as follows:

8.3.1. For singleplex preamplification (to preamplify wildtype and mutant alleles for a single mutation of interest), prepare PCR reaction mixture by adding 17.5 µL of DNA polymerase master mix, and 3.5 µL of forward and reverse primers (100 nM) per sample[13].

8.3.2. For multiplex preamplification (to preamplify two sets of wildtype and mutant alleles for two mutations of interest), prepare PCR reaction mixture by adding 17.5 µL of DNA polymerase master mix and 1.75 µL of each set of forward and reverse primers (50 nM) per sample[13].

NOTE: Prepare enough PCR reaction mixture for the number of samples plus 10% extra to account for any pipetting error.

8.4. Dispense 24.5 µL of PCR reaction mixture into each well of an 8-well PCR strip tube[13].

8.5. Dispense 10.5 µL of DNA sample into the appropriate well to bring the total reaction volume to 35 µL[13]. Gently pipette the full volume up and down 10 times to mix.

8.6. Perform PCR amplification at 98 °C for 3 min; 9 cycles of 98 °C for 10 s, annealing temperature for 3 min, 72 °C for 30 s; and an extension at 72 °C for 2 min[13].

8.7. Transfer the full volume of preamplified product to labeled DNase/RNase free PCR-clean tubes and dilute in 140 µL TE DNA suspension buffer (pH 8.0)[13].

8.8. Wrap the lids of the tubes in laboratory film. Store the preamplified product at 4 °C. For long term preservation, store at -20 °C.

9. Detection and quantification of target DNA using dPCR

9.1. Clean the bench space and equipment with 10% bleach and 70% ethanol.

9.2. Set 10 µM primers and probes, genotyping master mix, and DNA samples on ice. Store the droplet stabilizing oil at room temperature.

9.3. Gently vortex and briefly centrifuge all reagents except droplet stabilizing oil.

9.4. Ensure that the compressed nitrogen gas cylinder attached to the droplet-generating instrument and the quantification instrument (**Table of Materials**) is set at 90 psi.

9.5. Turn on the drop generating instrument and the computer with the instrument software. Launch the software and click **Start Initializing**.

9.5.1. Run a high-pressure flush on the drop generating instrument if it has not been used in one week or more.

9.6. Prepare the dPCR reaction mixture for 8 wells of the PCR strip tube plus 10% extra, by adding 220 µL of genotyping master mix, 8.8 µL each of 10 µM wildtype and mutant probes, 39.6 µL each of 10 µM forward and reverse primers, and 17.6 µL of droplet stabilizing oil[14].

9.7. Gently mix the dPCR reaction mixture by pipetting the full volume up and down 10-20 times. Do not vortex or centrifuge after droplet stabilizing oil has been added to the reaction mixture.

9.8. Obtain a PCR 8-well strip tube and dispense 38 µL of dPCR reaction mixture directly into the bottom of each well[14]. Remove any bubbles with a clean pipette tip.

NOTE: PCR strip tubes that are interlocked or packed tightly together can result in static electric charges, causing coalescence and noisy signal when analyzing dPCR data. To avoid this, aliquot strip tubes into separate biohazard bags, avoid over-packing the bags, and keep the tubes from rubbing together.

9.9. Add 12 µL of preamplified DNA sample to the appropriate wells of the PCR strip tube. For the negative control, add 12 µL of MB H_2O.

NOTE: Analyze cfDNA samples in replicate wells. For CSF, analyze at least in duplicate, and for plasma/serum analyze each sample in triplicate.

9.10. Gently pipette the full volume up and down 10 times to mix the reaction mixture with the DNA sample.

9.11. Obtain a new droplet-generating instrument chip and pipette the full volume from wells 1-8 of the PCR strip tube into the corresponding channels A-H in the chip. Avoid touching the bottom of the channels with a pipette tip.

9.12. Obtain a new clean PCR strip tube and insert into the droplet-generating instrument. Scan or manually enter the droplet-generating instrument chip ID into the software on the instrument computer. Enter names for each of the 8 channels. Click **Start the run** to begin dropletizing samples.

9.13. When the dropletization has completed, remove the PCR strip tube and apply strip tube caps.

9.14. Transfer the strip tube to a thermal cycler and balance with another strip tube containing 80 µL of water per well.

9.15. Program the thermal cycling conditions[14] as: 95 °C for 10 min; 45 cycles of two temperatures: 95 °C for 30 s and annealing temperature for 2 min; 98 °C for 10 min; and hold at 10 °C.

9.15.1. Set the ramp rate to 0.5 °C/s and set the sample volume to 80 µL[14].

9.16. When thermal cycling is complete, remove the strip tube and transfer to the quantification instrument. Remove PCR strip caps and replace with high speed caps.

9.17. Turn on the quantification instrument and the connected computer with instrument software. Launch the instrument software and click **Setup a Run**.

9.18. Place the strip tube into the quantification instrument.

9.19. Obtain a new quantification instrument chip and scan or manually enter the chip ID into the instrument. Insert the chip into the machine.

9.20. Place the metal shield on top of the chip and close the lid of the instrument.

9.21. In the computer software, enter a name for the dPCR run and for each of the 8 channels (A-H). Select **Fast Mode** (which must be used with high speed caps) and click **Start** to begin the quantification.

9.22. When quantification is complete, remove the metal shield (to be saved and reused) and dispose of the PCR strip tube and chip. On the instrument computer, the Run Log will contain result files for each run. Use the .fcs files for each sample to analyze with the analyst software.

10. Data Analysis

10.1. Launch the analyst software and open the .fcs files to analyze raw spectral data. Under Analysis View, select **Intact**. Under Sample View, click the boxes next to each of the 8 samples so that there is a check mark in each box. The software will plot signals for the mutant (FAM) and wildtype (HEX) alleles along the x- and y-axes, respectively**.**

10.2. Use the calculated matrix function to apply for the spectral compensation on intact droplets, as per the manufacturer's instructions[15].

10.3. Adjust the axis settings under Axis Options. Set the x-axis to a minimum of 0 and maximum of 30,000, and the y-axis to a minimum of -5000 and maximum of 10,000. When droplet clusters have been identified, adjust axes to reduce empty space in the graph.

NOTE: The position of mutant and wildtype clusters will depend on the probes used, the fluorophore and their concentration.

10.4. Select the sample corresponding to the positive control tumor tissue gDNA to set the negative (cluster closest to the origin), mutant (cluster closest to the x-axis), and wildtype (cluster closest to the y-axis) gates. Ensure that the clusters are distinct and easily identified in a successful assay.

10.5. Apply the gate settings for the positive control to all samples by right-clicking on the positive control sample and clicking the option to **Apply all settings to selected samples**.

10.6. Under the graphical view, click **Multiple Samples** tab to view plots for all selected samples. Export the image as a .TIF file.

10.7. Under the **Workspace** tab at the top left of the screen, select **Export Analysis** (csv) and save the analyzed data file as a .csv file.

10.8. Open the .csv file in spreadsheet software to view negative, wildtype, and mutant droplet counts for each sample. Calculate the MAF by dividing the Poisson corrected mutant droplet count by the sum of the Poisson corrected mutant plus wildtype droplet counts for each sample.

NOTE: Use the Poisson corrected count because Poisson statistics account for that fact that positive droplets may contain more than a single copy of target DNA, and thus summing the positive droplets may not yield an accurate count[16].

REPRESENTATIVE RESULTS:

Figure 1 shows representative results for successful detection of *H3F3A* p.K27M mutation in preamplified plasma (top left panel) and CSF (top right panel) cfDNA from two children with DMG. cfDNA samples were analyzed in replicate but only one representative graph is shown for each sample type. The dPCR plots show successful droplet generation, with 7-9 million droplets per PCR well (most of which are negative droplets that do not contain target DNA). A minimum of 7 million droplets per PCR well indicates a successful dropletization, while fewer than 7 million indicate failure of the assay, in which case the user should not proceed with data analysis. **Figure 1** shows a clear separation between mutant and wildtype clusters along the x and y-axes, respectively. Robust wildtype clusters indicate that the cfDNA extraction was successful because template DNA is present. Mutant clusters show a MAF of 1.60% and 39.92% for the plasma and CSF samples, respectively, demonstrating positive detection of the mutation. For these patients, dPCR results are in accordance with tumor mutation status, as confirmed by genomic analysis of biopsy tumor tissue[8]. The negative control (bottom left panel) shows 0 mutant and 0 wildtype droplets, indicating that there was no contamination of the PCR reaction mixture. The positive control tumor tissue gDNA (bottom right panel) shows the mutation being detected at the expected allelic frequency for the particular tumor sample selected.

In **Figure 2**, representative results are shown for unsuccessful detection of the mutation of interest, *H3F3A* p.K27M, in plasma cfDNA from a child with DMG. Mutation status was confirmed by genomic analysis of tumor tissue[8]. Representative results from two replicate PCR wells are shown (top panels). The total number of droplets, including negative droplets, is between 7-9 million per PCR well, indicating successful dropletization. The wildtype clusters, plotted along the y-axis, show approximately 7-8,000 wildtype droplets per PCR well for the plasma cfDNA, indicating successful cfDNA extraction (as there is target wildtype DNA in the sample). However, 0 mutant droplets are detected in the plasma sample. The bottom left panel shows negative control (MB H_2O) with no wildtype or mutant droplets; and the bottom right panel shows the positive control preamplified tumor tissue gDNA (0.025 ng) with positive mutation detection. As shown in this figure, the absence of mutation detection in plasma may not necessarily mean the patient is wildtype for the mutation of interest, as false negatives do occur[8]. In some cases, when the mutation is missed in the plasma collected at upfront biopsy, it is detected in the plasma obtained from the same patient at a later time point[8].

FIGURE AND TABLE LEGENDS:

Figure 1. Successful detection of *H3F3A* p.K27M mutation in preamplified plasma and CSF cfDNA from children with midline gliomas.

Figure 2. False negative results obtained from analysis of a preamplified plasma cfDNA sample from a patient known to harbor the mutation of interest, *H3F3A* p.K27M, in the tumor.

DISCUSSION:

Here we have presented our method for detecting and quantifying the allelic frequency of tumor mutations in cfDNA from the patient liquid biopsy. We emphasize critical steps for the success of this method, including pre-analytical sample processing, cfDNA extraction, PCR assay design, and data analysis. To limit the sample volume used, cfDNA is extracted from 1 mL of plasma but only 500 µL of CSF. When extracting from CSF, the protocol for extraction from 1 mL of urine (following the cfDNA extraction kit handbook[12]) is used, as per the manufacturer's recommendation. The difference in sample volume required between plasma and CSF is due to lower levels of tumor-specific cfDNA in plasma compared to CSF of patients with brain tumors, necessitating larger sample volumes for mutation detection[8]. If the sample is available, more than 1 mL of plasma may be extracted from to produce higher DNA yield. However, in the case of pediatric patients, it is important to minimize the amount of blood used whenever possible, as even a simple procedure such as blood draw is fatiguing to pediatric patients with cancers undergoing radiation therapies. Extracting from 1 mL aliquots of plasma also enables replicate extractions, such that the assay can be repeated (e.g., in cases of failure in DNA extraction or sample contamination).

In an effort to reduce any sample use that is not strictly necessary, cfDNA is typically not quantified. However, we have found a range of cfDNA concentrations between 0.2-2 ng/µL and 0.6-13 ng/µL in plasma and CSF, respectively. Given the low amount of cfDNA, and the fact that tumor-specific mutant alleles are present at a low frequency, a pre-amplification step using the same set of primers used for dPCR is necessary to significantly increase the amount of target DNA

in the sample, aiding in mutation detection[8]. Diluting the pre-amplified product in DNA suspension buffer provides sufficient volume for technical replicates, which aids in distinguishing true positives. Because the number of mutant droplets can be low (for example, between 0-2 mutant droplets in a plasma sample), the inclusion of technical replicates is key for resolving mutation status. While one PCR well may yield 0 mutant droplets, the other two may yield 1-2 for a single plasma sample analyzed in triplicate; thus, the inclusion of replicates allows for greater accuracy when determining mutation status. The MAF is then calculated as the average of the replicate values.

Multiplexed pre-amplification (preamplifying wildtype and mutant alleles of two mutations of interest) increases the utility of a single sample, as the same starting material can be used to test for two mutations. Importantly, a multiplex preamplification product can be analyzed in singleplex during dPCR for greater simplicity, as described here. However, both preamplification PCR and dPCR may be multiplexed. When multiplexing, conditions must be optimized for both sets of primers and probes: primer annealing temperatures must be similar to run together in PCR amplification, and probes should be designed to generate distinct clusters (based on fluorescent signal and intensity). When validating a new set of primers and probes, run an annealing temperature gradient to determine optimum annealing temperature based on the range suggested by the manufacturer. Before testing probes on patient cfDNA specimens, validate them using synthetic DNA constructs and/or tumor tissue gDNA of different inputs (i.e., up to 10 ng).

For the detection of mutant alleles with greater specificity and reduced mismatches in the hybridization of probe to the target DNA, locked nucleic acid (LNA) probes are used. LNA is a nucleic acid analog with a methylene bridge connecting the 2'-oxygen and 4'-carbon of the ribose ring[9]. The methylene bridge locks the nucleic acid into a rigid bicyclic conformation that restricts flexibility, increases thermal duplex stability, and improves the specificity of probe hybridization to target DNA[10,18-19]. If a single base mismatch exists between LNA probe and template DNA, duplex formation between probe and target will destabilize. As such, LNA probes improve the specificity of probe binding and result in a higher signal-to-noise ratio[11]. Analysis of plasma and CSF from non-CNS-diseased pediatric patients has established the specificity of our assay with LNA probes targeting *H3F3A* p.K27M, to determine that an allelic frequency of equal to or less than 0.001% is considered a false positive[8]. For additional considerations for optimizing the design of probes and primers, including GC content, amplicon size, probe reporter dyes, and quenchers, refer to the dPCR manufacturer guidelines[14,17]. Although our method is optimized for use with the RainDance system, the protocol may be adapted for use with other dPCR platforms.

The method presented here draws strength from the high sensitivity and target enrichment achieved by dPCR, which remains the platform of choice for detecting rare tumor mutations in cfDNA. Although powerful, dPCR is limited in the number of mutations that can be tested for in a single assay. An alternative to dPCR is next generation sequencing (NGS), which may detect multiple mutations across many genes, increasing the utility of a single sample. NGS can detect mutations in CSF and plasma of patients with brainstem gliomas[20], however, is currently less sensitive than dPCR at detecting tumor mutations in cfDNA, with detection limits of 0.1-10% MAF

compared to 0.001% in PCR-based approaches[21]. dPCR also achieves faster turnaround time than NGS, enabling rapid detection of mutations of interest. The PCR approach is applicable across cancer types and can be expanded to detect hotspot mutations and methylated cfDNA[22].

The liquid biopsy is indeed in its infancy and will require further development for tailoring to specific diseases. Tumor monitoring in the context of tumor evolution will be the next challenge, where a platform capable of detecting emerging mutations with high sensitivity would be required. Additionally, platforms capable of detecting a variety of biomolecules (peptides, cytokines, RNA) will be highly beneficial in assessing tumor response to treatment, as well as advancing personalized clinical interventions.

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DISCLOSURES:

The method described in this manuscript is included in an International patent application "Methods for detecting cancer biomarkers" by Javad Nazarian & Eshini Panditharatna.

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Figure 1

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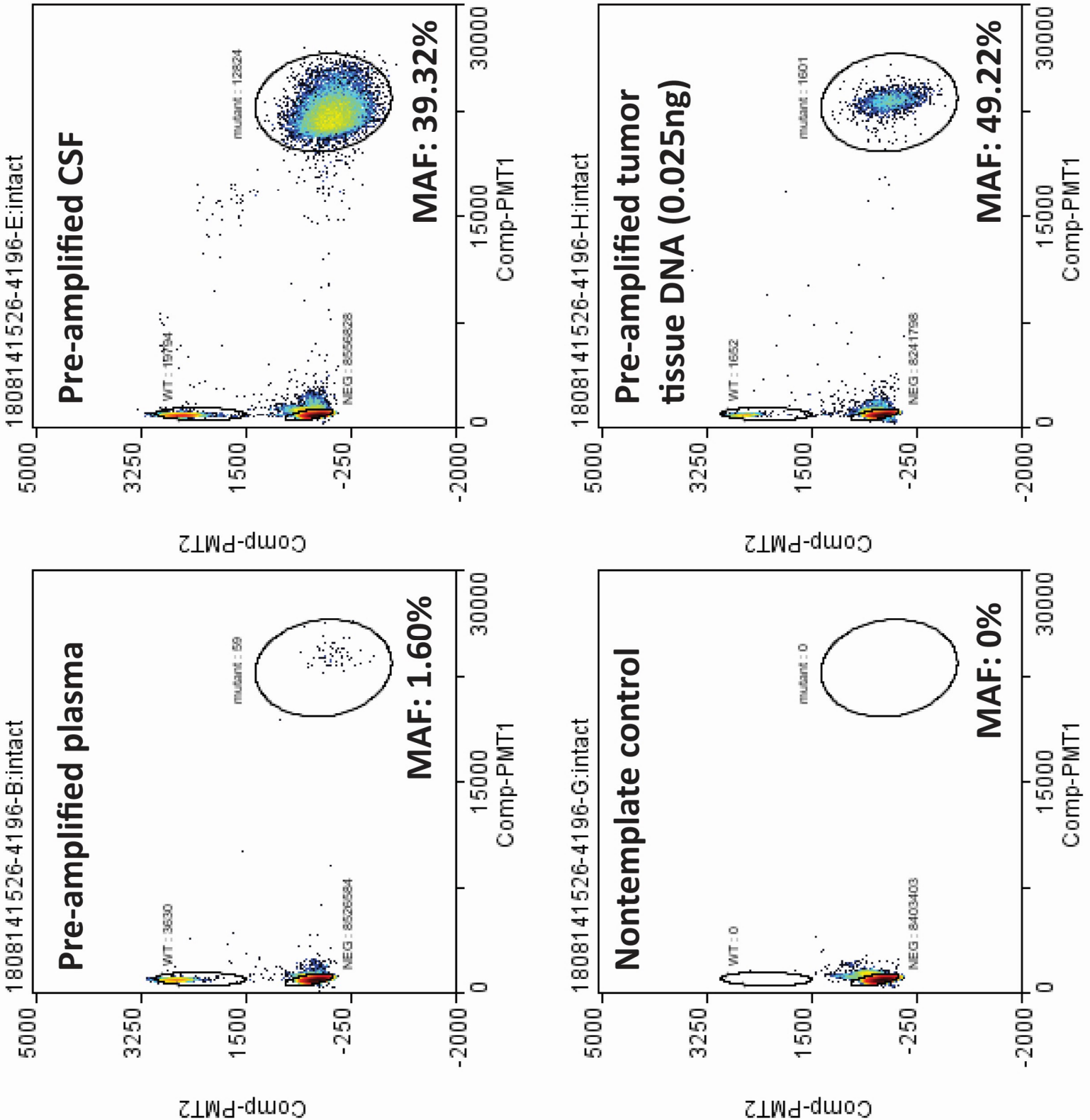
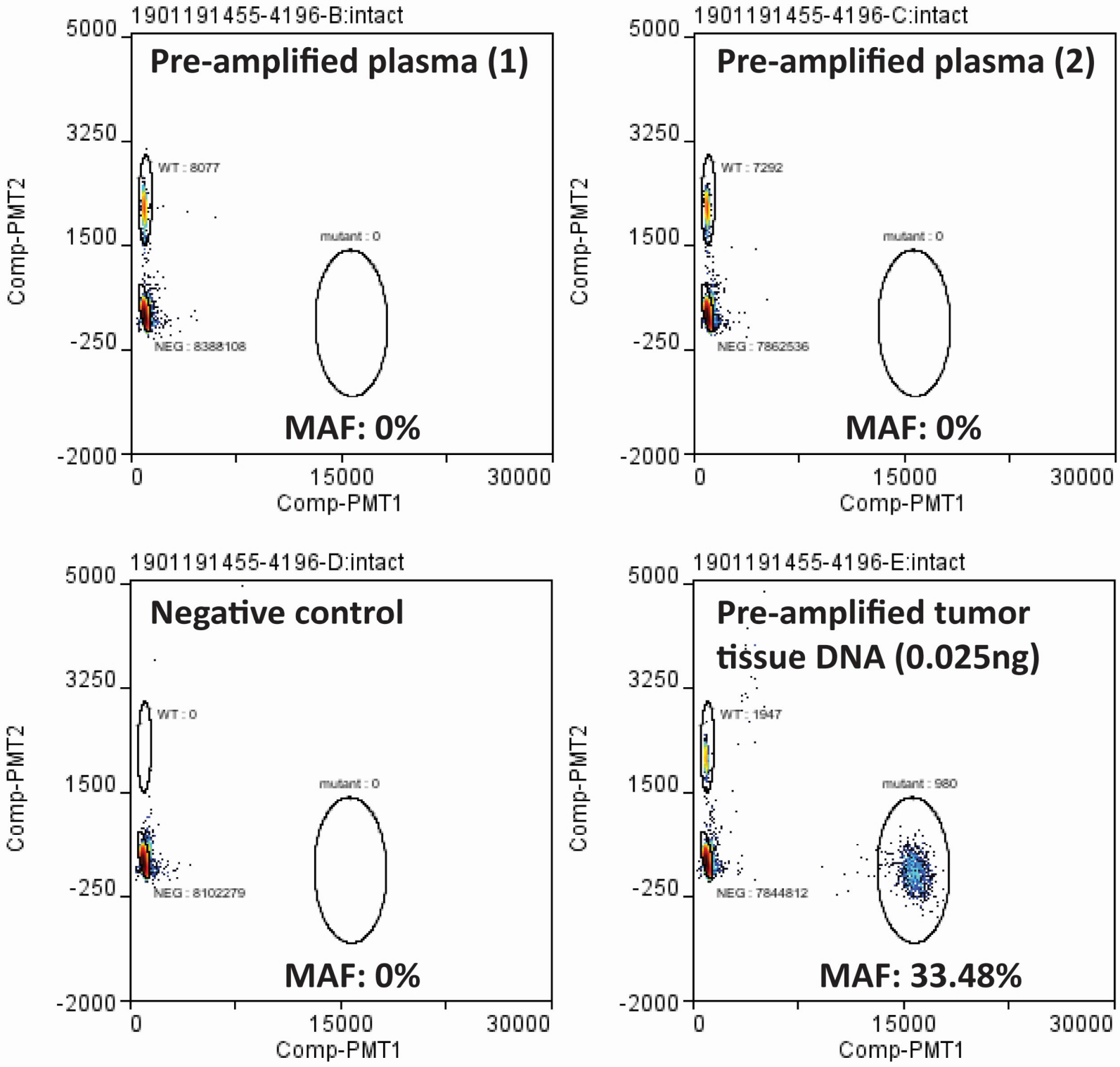


Figure 2



Name of Material/ Equipment

10mM Tris-HCl, 0.1mM EDTA DNA Suspension Buffer (pH 8.0)
BD Vacutainer K2-EDTA 7.2mg Blood Collection Tubes (10mL)
BD Vacutainer Plastic Serum tube with Red BD Hemogard Closure (10mL)
Bleach
Buffer ATL
Cell-Free DNA Blood Collection Tubes
CentriVap Concentrator
Forward Primer
MiniAmp Thermal Cycler
Molecular Biology Grade Absolute Ethanol (200 proof)
Mutant Probe
PAXgene Blood ccfDNA tubes
PCR 8 well strip tube caps
PCR 8 well strip tubes
Pipette (p20, p200, p1000)
Pipette filter tips (p20, p200, p1000)
Q5 Hot Start High-Fidelity 2X Master Mix
QIAamp Circulating Nucleic Acid HandBook
QIAamp Circulating Nucleic Acid Kit
QIAamp MinElute Virus Spin Kit
Qiavac 24 Plus
Raindance Consumable Kit
Raindance Sense Instrument
Raindance Source Instrument
RainDrop Analyst II Software
Refrigerated Vapor Trap
Reverse Primer
Smartblock 2mL
TaqMan Genotyping Master Mix
Thermomixer C
WT Probe

Company	Catalog Number
Teknova	T0227
Becton Dickinson Diagnostics	367525
Becton Dickinson Diagnostics	367895
General Lab Supplier	
Qiagen	19076
Streck	218961
Labconco	7810010
Integrated DNA Technologies, Inc.	
Thermofisher Scientific	A37834
General Lab Supplier	
Integrated DNA Technologies, Inc.	
PreAnalytiX	768115
VWR	10011-786
Axygen	PCR-0208-C
General Lab Supplier	
General Lab Supplier	
New England Biolabs Inc	M0494S
Qiagen	
Qiagen	55114
Qiagen	57704
Qiagen	19413
Bio-Rad	20-04411
Bio-Rad	
Bio-Rad	
RainDance Technologies	
Savant	RVT5105-115
Integrated DNA Technologies, Inc.	
Eppendorf	05 412 506
Thermofisher Scientific	4371353
Eppendorf	14 285 562PM
Integrated DNA Technologies, Inc.	

Comments/Description

For plasma collection

For serum collection

cfDNA blood collection tubes (optional)

Custom design

Custom design
cfDNA blood collection tubes (optional)

cfDNA extraction kit handbook (2013 edition)
cfDNA extraction kit (Protocol Step 4)
Optional kit for DNA extraction from small (< or =200µL) sample volumes
For use with QIAamp Circulating Nucleic Acids kit
Contains droplet-generating instrument chips, quantification instrument chips, PCR strip caps including
Quantification instrument (used in Protocol Step 9)
Droplet-generating instrument (used in Protocol Step 9)
Analyst software used for Data Analysis (Protocol Step 10)

Custom design

Custom design

; high-speed caps, and droplet stabilizing oil

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Vineeta Bajaj, Ph.D.
Review Editor
Journal of Visualized Experiments

March 12, 2019

Dear Dr. Bajaj,

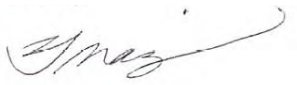
We appreciate the opportunity to revise and resubmit our manuscript "Detection and monitoring of tumor associated circulating DNA in patient biofluids."

We thank the Reviewers for their insightful comments and suggestions. We made our best effort to duly address all of the comments in the revised version of our manuscript. Whenever necessary, we included additional information to address reviewers' suggestions and have amended the text to support these suggestions. Below we address each of the reviewers' comments in italics.

We believe that our response and introduced revisions fully address raised concerns and satisfy reviewers' expectations. We would appreciate if our modified manuscript could be accepted further for publication. Once again, we would like to express our gratitude for considering our manuscript for publication in the *Journal of Visualized Experiments*.

Sincerely,

Javad Nazarian



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202-476-6022 P
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Editor's Comments

- Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

- Please provide an email address, at least 6 keywords or phrases

Response: *Thank you. We have thoroughly proofread the manuscript to correct any spelling or grammatical errors, included the email address for each author and have revised the keywords section to include the following 6 keywords or phrases: Liquid biopsy, digital droplet PCR, circulating cell free DNA, plasma, serum, cerebrospinal fluid.*

- Please rephrase the Short Abstract/Summary to clearly describe the protocol

Response: *We have rephrased the Short Abstract to clearly state the protocol and its applications in detecting tumor mutations using plasma, serum and CSF of cancer patients, within the 50-word limit.*

- Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section,

- and remove all commercial language.

Response: *We have corrected the formatting of the manuscript to fit these criteria, removed commercial language from the manuscript text and replaced with generic terms. We have referenced all commercial products in the Table of Materials and Reagents.*

- Please define all abbreviations during the first-time use

- move the ethics statement before the numbered steps of the protocol.

Response: *Thank you. We have revised the text to define all abbreviations during first-time use. We have moved the Ethics statement to before the numbered steps of the protocol.*

- Please ensure that all text in the protocol section is written in the imperative tense

Response: *We have edited the Protocol section so that all steps of the protocol are written in the imperative tense in complete sentences. In few instances where text cannot be written in the imperative tense, we have added concise Notes.*

- The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Response: *We have removed discussion about to protocol to the Discussion section, so that the Protocol contains only action items directing the reader to do something.*

- Please revise the text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

- 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 or dashes

Response: *We have removed the use of personal pronouns from the Protocol and included personal pronouns only sparingly in the Introduction and Discussion sections.*

We have also adjusted the numbering of the Protocol as per JoVE Instructions for Authors and following the provided Template.

- The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.

Response: *Thank you, we have revised and simplified the Protocol to include discrete steps, with only 2-3 actions per step and have removed large paragraphs of text between sections.*

- Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Response: *We have revised the entire protocol to include more detail on how each step was performed.*

- Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).

Response: *In the Protocol steps in which computer software is used, we have added explicit instructions such as "click", "select" and press "start" for more clarity.*

- 1.1: what kind of patients are used in the study? Inclusion and exclusion criteria?

Response: *We have included the types of patients used in the study, and inclusion and exclusion criteria, in Protocol Step 1.1.1. Please refer to Page 2.*

- 2.3: How was serum or plasma obtained from blood?

- 3.1: How was the CSF sample collected?

Response: *We have described how serum or plasma was obtained from blood in Protocol section 2, steps 2.1-2.5, on Page 2.*

We have listed sources of CSF collection (shunt, surgery, lumbar puncture) in Protocol section 3 (Page 3), and clarified that CSF collection should only be conducted if deemed necessary by clinicians, with any extra sample being collected for research purposes.

- 4: we need discrete experimental steps for the protocol section, please explain how the extraction was performed.

Response: *We have revised the Protocol to contain discrete experimental steps and a more complete explanation of how the extraction procedure is performed.*

- 5.1: How was this done?

Response: *We have edited Protocol section 5 to more clearly explain how the vacuum concentration protocol is performed to concentrate cfDNA samples (please refer to Pages 5-6).*

- 7.5: Please explain how this was done in your experiment. Please provide concentrations and volume of each reagent used here.

Response: *We have revised the Protocol on preparation of PCR master mix in steps 8.4.1 and 8.4.2. These steps list the volumes and concentrations of each reagent to use per sample when singleplexing (8.3.1) and when multiplexing (8.3.2) preamplification (Page 7).*

- 7.8: How?

Response: *We have revised this step (Step 9.11 on Page 8) to clarify that we mean to pipette (rather than transfer) the full volume from wells 1-8 of the strip tube into the corresponding channels on the dropletizer chip.*

- 8: Please provide steps of how the analysis was performed. We need button clicks, graphical user interfaces, codes etc.

Response: *We have rewritten the Protocol on analysis of ddPCR data in a new section, Protocol section 10, with more detail including button clicks and other direct useful information (Page 9-10).*

- Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: *Thank you, we have highlighted 2.75 pages of Protocol representing essential steps to be used for filming.*

- Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

Response: *We have included a paragraph to explain the Representative Results in Figures 1 and 2 as per these criteria on Pages 10-11. We have shown an example of both successful and unsuccessful results from the ddPCR assay described in our manuscript.*

- Please describe the result with respect to your experiment, you performed an experiment, how did it helped you to conclude what you wanted to and how is it in line with the title.

Response: *We have described the result and how to interpret the result with respect to our experiment on Pages*

10-11 in the Representative Results section.

- Please include all the Figure/Table Legends together at the end of the Representative Results in the manuscript text.

Response: *We have included all the Figure/Table legends in one section together at the end of the Representative Results (please refer to Page 11).*

- Please obtain explicit copyright permission to reuse any figures from a previous publication.

Response: *We have included only figures that were generated by the first author of this manuscript (ddPCR plots seen in Figures 1 and 2) for use with this manuscript and have not been published before.*

- Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response: *Thank you. We have revised the Discussion section to address each of these points throughout the 6 paragraphs (Pages 11-13).*

- Please alphabetically sort the materials table.

Response: *We have alphabetically sorted the Materials table.*

Reviewer #1:

Manuscript Summary:

This submission by Bonner et al. reports on a protocol for the detection and monitoring of tumor associated circulating DNA in patient bio-fluids. The protocol is generally well written and provides important details for an increasingly used molecular platform for the detection and quantification of cell free nucleic acid in liquid samples from cancer patients. However, there are some issues that should be addressed prior to publication.

Response: *We thank the reviewer for their comments and constructive feedback on our manuscript. We agree that the platform described in our manuscript for detection and quantification of cfDNA in liquid samples from cancer patients is an important and increasingly used approach. We have addressed the questions raised below in the comments and feel that the inclusion of these revisions has strengthened our manuscript.*

Major Concerns:

-The main issue with this paper is that the authors provide detailed protocol information for a platform (RainDance) that has been purchased by another company (Biorad). It is my understanding RainDance is no longer being produced and soon won't be serviced [please correct me if this is incorrect]. The authors need to address the relevance of this protocol for labs that have different/newer ddPCR machines, or are thinking of obtaining a new (non-RainDance) device. The authors should update the protocol (+ subsequent video) to include reference to steps with differences if work is performed on a newer platform.

Response: *Thank you for your comment. We have described our protocol for ddPCR using the RainDance system to detect and quantify allelic frequency of tumor mutations in patient biofluids. The reviewer is correct that the RainDance platform is currently no longer in commission, having been purchased by BioRad. However, as there are still groups using the RainDance platform, we feel that our protocol remains relevant. We have noted in the Discussion section (Page 12) that the protocol may be adapted for use with BioRad systems, however, have not included an explicit protocol for BioRad platform because we do not currently use this platform. The method we use and have described here is based on our lab's recently published work in Clinical Cancer Research, Panditharatna et al (2018) "Clinically relevant and minimally invasive tumor surveillance of pediatric diffuse midline gliomas using patient-derived liquid biopsy."*

- The authors switch back and forth between whether this is a protocol for any tumor type or just pediatric diffuse midline glioma. The long abstract mentions all tumor types (no reference to CNS tumors), the short abstract mentions brain tumors, and the intro provides detailed info about pediatric diffuse midline glioma. If this platform is relevant to all tumor types, then these should all be consistent and introduction should address ddPCR background data from multiple pediatric and adult settings (and potentially reduce discussion of pediatric diffuse midline glioma)

Response: *Thank you for pointing this out. We have revised the text to make our focus more broadly applicable to all tumor types and minimized our emphasis on its utility for pediatric diffuse midline gliomas (our disease of interest). We agree that the method is more powerful when generalizable to different tumor types. However, we have mentioned that pediatric midline gliomas represent a tumor type that exemplify high need for this form of minimally invasive mutation detection and tumor monitoring on Page 1, to explain our rationale for developing and optimizing this protocol.*

Minor Concerns:

- It is unclear why author's use a urine DNA isolation kit - do they recommend this for non-urine samples? Why?

Response: *The use of the urine protocol for extraction from CSF is recommended by the manufacturer (Qiagen) of the QIAamp Circulating Nucleic Acid Kit. We have clarified this in the Discussion (Page 12) and have made a note in the Protocol section (Page 3) that the CSF and plasma/serum protocols differ in only the initial lysis steps.*

- It is unclear why authors' use different sample sizes for plasma and spinal fluid. If this is related to event level, is there a rule of thumb for determining sample sizes that is generalizable?

Response: *We use different sample sizes for plasma and CSF because of the higher level of tumor cfDNA present in CSF of patients with brain tumors. In order to limit the use of sample, we have found that only 500 µL of CSF is needed to generate robust mutant signal when the mutation is present (Panditharatna et al. 2018). However, because of the lower levels of ctDNA in plasma/serum, a larger input volume is needed, and thus we extract from 1mL of plasma/serum. We thank you for this comment and have added the above information to the Discussion on*

Page 11.

- Do the authors elute twice with the sample liquid? Have they demonstrated that this increases yield? Wouldn't eluting twice with two smaller batches of elute buffer make more sense (i.e., potentially losing some tDNA in column on second pass).

Response: *We elute twice with the same sample liquid according to instructions from the manufacturer of the QIAamp Circulating Nucleic Acid Kit (Qiagen). We have clarified this in the manuscript text on Page 5.*

- Why is CSF performed in duplicate and plasma is in triplicate? Again, is there a generalizable rule that can be applied?

Response: *We analyze CSF in duplicate because of the higher tumor-specific cfDNA levels in CSF of patients with brain tumors, requiring fewer technical replicates to discern mutation status. We analyze plasma in triplicate because in many cases very few (0-2) mutant alleles are detected and the inclusion of more technical replicates allows for more accurate determination of mutation status. We have included more detail on our rationale for choosing this number of replicates in Paragraph 2 of the Discussion (Page 12).*

- Is there a minimum droplet count that is recommended for analysis?

Response: *We thank you for raising this question. In paragraph 2 of the Introduction (Page 1), we have clarified that RainDance platform creates up to 10 million droplets, but that experimentally we see 7-9 million droplets per well of the PCR strip tube. In the Representative Results section on Page 11, we have included the important point that less than 7 million droplets indicate failure of the assay and that the user should not proceed with analysis of the sample.*

Reviewer #2:

Manuscript Summary:

The manuscript by Bonner et al. details a method for monitoring ctDNA using ddPCR-based analysis. A number of points need to be addressed to strengthen the manuscript.

Response: *Thank you for your thorough review and constructive comments on our manuscript.*

Major Concerns:

- Line 45 introduction, what are the other major mutations associated with pediatric CNS cancers? Would these mutations be worth including in a ddPCR-based analysis for a greater coverage of driver mutations?

Response: *We appreciate your comment. We have revised the introduction to include examples of other major mutations in pediatric CNS cancers we have targeted using sequence-specific probes with this platform (Page 1).*

- Line 49 introduction, are the authors correct in indicating their assay is based on 10 million droplets? Typically, related ddPCR assays, such as the BioRad assay, are based on counting up to 20,000 droplets.

Response: *Thank you for the comment. Rain Dance system is set apart from other dPCR assays in its ability to generate up to 1x10^7 droplets. We have included this information in the Introduction section (Page 1).*

- Given a 10 ml blood draw, resulting in 4-5 ml plasma ,is specified in the methods why was only 1 ml of plasma used in the DNA extraction? Was this because there were actually replicate extractions? Otherwise, why not use the full amount available to maximise sensitivity in the ddPCR or at least perform a dose response?

Response: *We appreciate the response. Although using 4-5 mL of plasma will most likely increase the level of detection in ddPCR, every attempt should be made to minimize the amount of blood used because even a simple procedure such as blood draw is fatiguing to pediatric patients with CNS tumors undergoing radiation therapies. It is also important to have 1 mL aliquots available for replicate extractions; this is important for cases when the extraction needs to be repeated due to technical issues or sample contamination. We have addressed these points in Paragraph 2 of the Discussion section (Page 11-12).*

- How did the authors determine the quantity of DNA template used in the preamp reaction and why was 0.025 ng chosen?

Response: *We have included a section in the protocol explaining the selection of 0.025ng of genomic DNA as positive control, which can be found in Protocol step 7 (Page 6). 0.025ng of DNA template had been determined to be the optimal quantity that results in a distinct cluster of mutant allele population, as shown in our previously published work (Panditharatna et al. 2018). We refer to Supplementary Figure 1B of Panditharatna et al (2018) in which a serial dilution of mutant tumor tissue genomic DNA was performed to determine 0.025ng of input DNA provides robust mutant signal while limiting the amount of DNA needed for each assay, and thus was chosen as positive control.*

- Line 111 methods, why was the DNA eluted in such a large volume and then concentrated? This could impact on yield? The Qiagen protocol does allow for elution in as small a volume as 20-25 ul. Was a comparison made to eluting in smaller volumes without concentration to justify a concentration step?

Response: *The elution of DNA in a large volume, 100 µL, was a manufacturer recommendation on the part of Qiagen. We concentrate the eluted sample so that the entire eluate can be used for preamplification reaction, with an input volume of 10.5uL. We have clarified that the repeated elution step is a manufacturer recommendation on Page 5 (line 253-254).*

- Methods and Line 281 discussion, why was a preamplification step which could introduce PCR-specific errors included? No appropriated justification was provided in the form of data comparing no preamp vs preamp.

Response: *In Panditharatna et al (2018) Supplementary Figure 1B there is a comparison between non-preamplified and pre-amplified tumor tissue DNA for varying inputs. We have added a reference to this publication in the text (Page 12, lines 540-542), as it supports the inclusion of a preamplification step to significantly increase the amount of mutant and wildtype alleles being detected in this assay.*

- Line 241 methods, need to provide more information on threshold/gate settings eg was a mutation call based on at least 2 positive droplets etc

Response: *Thank you for pointing this out. A clearer description of how gates are set has been added to Protocol steps 10.3-10.5 (Page 10). Clusters defining where gates should be set all must be distinctly identifiable in the positive control for an assay to have been considered successful.*

- In the results why is the MAF significantly different (49 vs 33%) between figures 1 and 2 for the positive control preamplified tumour tissue DNA?

Response: *The MAF of positive control will depend on the tumor gDNA sample used and the allelic frequency of the mutation in that sample. Typically between 35-50% MAF is seen for our positive control for the heterozygous H3F3A p.K27M mutation.*

- Line 284, discussion, based on the statement "in that two mutations can be tested…." Can the authors clarify the differences between H3F3A and HIST1H3B? They were preamplified using gene specific primers but then it appears the K27M mutation was analysed using the one set of ddPCR primer/probes? This indicates the sequence is identical around this mutation although they are two separate genes? A diagram would help here.

Response: *Thank you for your comment.* H3F3A *and* HIST1H3B*, both genes encoding variants of histone H3, differ in nucleotide sequences and primers for each target are distinct. However, we often multiplex during preamplification to preamplify target alleles of both H3F3A and HIST1H3B, because the annealing temperatures of the primers are the same (58°C) allowing them to run together during PCR. For ddPCR, we singleplex the reaction to test for the mutation in only one variant at a time, most commonly in H3F3A because this is the more frequently mutated variant of the two. We use multiplexed preamplification product as input DNA, but test in singleplex for one mutation at a time, for greater simplicity in the ddPCR assay. We have revised the Discussion section to clarify this point (please refer to Page 12).*

- The authors should show different samples with different MAF and varying amounts of template DNA to truly ascertain the LOD for their assay. Values for sensitivity, specificity and LOD would help in comparison to other platforms such as the BioRad system and provide justification for statements such as line 325 referring to high sensitivity of their platform.

Response: *Thank you for this suggestion, we agree that including more data on sensitivity, specificity and limit of detection will justify the claim that our platform has high sensitivity for mutation detection. We have revised our Discussion section to address this, by including a reference in paragraph 4 of the Discussion (Page 12) to Panditharatna et al. (2018) Supplementary Table 1, in which specificity of the assay was determined using non-CNS-diseased patient plasma and CSF.*

- Line 336 discussion, if you consider targeted NGS panels vs ddPCR then improving NGS technologies now provides sensitivity comparable to ddPCR. It would be hard to imagining any assay having the sensitivity (LOD) of 0.001%? Realistically 0.01-0.1 % is "believable" and probably sufficient in a clinical setting.

Response: *Thank you for your comment. In our experience analyzing plasma and CSF cfDNA from patients with known mutation status, the ddPCR platform described in our manuscript is sufficient to achieve a limit of detection of 0.001% for H3F3A p.K27M (Panditharatna et al., 2018). We also reference a Review article on Page 13 which supports our claim that PCR-based approaches reach a sensitivity of 0.001% compared to 0.1-10% in NGS-based approaches.*

Minor Concerns:

- Line 49 introduction, can the authors clarify the statement "each of which may contain only a few DNA templates"?

Response: *We have clarified this statement to explain that due to the high partitioning of PCR reaction mixture, only one to two molecules of DNA can be found in each droplet (please refer to Page 1).*

- Line 164 methods, the supplier of the probes should be included.

Response: *Thank you, we have included that Integrated DNA Technologies (IDT) is the supplier of our probes and primers in step 6.1 of the Protocol section (Page 6).*

- Line 169 methods, should read "primers".

Response: *Thank you. We have corrected this spelling error so that the text now reads "primers".*

Reviewer #3:

Manuscript Summary:

The manuscript entitled "Detection and monitoring of tumor associated circulating DNA in patient biofluids" by Bonner et al. describes protocol and method for detection of somatic DNA mutations from liquid biopsies, specifically from plasma, serum and cerebrospinal fluid, using digital droplet PCR.

The topic is certainly of high interest as the ddPCR method is becoming widely used both for research and in the clinics, therefore a detailed description of the protocol is most welcomed. However, it is this reviewer's opinion that this manuscript leaves a lot to be desired for - the methods are not sufficiently detailed to enable reproducibility nor have adequate controls been shown and/or used.

Response: *Thank you for your comments and thorough review of our manuscript. With your input we have revised the text to add more detail on the methods and ensure reproducibility of the assay. We thank the reviewer for their input and feel that the inclusion of the suggested details has strengthened our manuscript.*

Major Concerns:

- It is not clear which system has been used for the ddPCR - nothing is mentioned in the abstract, the protocol describes the procedure for the Rain Drop system, but also mentions Biorad DigitalDriplet PCR system, while in Material/Equipment list it lists Sense and Source Instrument from Biorad (I assume this to be RainDrop Sence and RainDrop Source instruments) without catalog numbers.

Response: *Thank you for the comment. We have revised the manuscript to make it clear that we have used the RainDance ddPCR system and that our method is based on this platform. However, we have mentioned in the Discussion (Page 13, lines 576-578) that the protocol may be adapted for use with other ddPCR systems (BioRad).*

- For cfDNA extraction, no quality control or quantification method has been described. Authors didn't describe or use any methods to check sample quantity, quality or contamination with gDNA that might arise from cell lysis. This is of particular concern when working with serum samples as they are frequently contaminated with gDNA from peripheral blood cells. Furthermore, due to sampling error statistical probability of detection of a mutated ctDNA is directly proportional to the number of genome equivalents in the analyte. If too little cfDNA is obtained in the start, pre-amplification cannot rescue the assay and may result in false negatives. This aspect is not discussed in the discussion section lines 317-328.

Response: *In the Discussion section on Page 12 (lines 537-539) we have added a sentence stating that we typically do not quantify cfDNA in an effort to preserve all sample volume when analyzing patient samples. However, we have included a range of concentrations we have found from quantifying a small cohort of CSF and plasma cfDNA. We also determine success of cfDNA extraction from the number of wildtype droplets detected in the sample (as discussed in the Representative Results on Page 10-11). This provides a way to validate that cfDNA extraction was successful without using the already-low volume of cfDNA eluate for quantification. Because our targets are mutations that are present only in tumor cells and not in healthy cells, the contamination of genomic DNA from non-tumor cells would be expected to increase only the number of wildtype droplets detected, but not mutant droplets and therefore would not be expected to induce false positives.*

- The authors don't show the required input of cfDNA for ddPCR reaction, but instead report input volumes. Again, related to the issue raised above it is important to know the number of genome equivalents going into the reaction. This can be estimated either by qPCR/ddPCR or by Qubit.

Response: *Thank you for your suggestion. Although the cfDNA level in different samples is highly variable between patients, we have found that generally 1 mL of plasma and 500 µL of CSF provide sufficient cfDNA levels for mutation detection for our target of interest (Panditharatna et al., 2018).*

- Design of the probes and primers is only generally described. It would be preferred to show this on an example of the priers used, and to show primer sequences.

Response: *We have included a section in the Protocol with more detail on primer and probe design and preparation (Protocol step 6, Page 6) and listed considerations and resources for primer and probe design in the Discussion section on Page 12. The primer sequences are not included in this manuscript as they have been previously published in Panditharatna et al. (2018). However, we have included a reference to the supplementary table from this publication that lists the primer and probe sequences for pediatric midline glioma mutations of*

interest we have targeted using this ddPCR assay, on Page 6 of the Protoco (lines 286-288).

- Lowest MAF of 0.001% was determined using positive control sample (using 0.025ng pre-amplified positive control DNA). However, given that the sample was pre-amplified and starting concentrations are unknown, this MAF doesn't reflect true limit of detection from liquid biopsy sample, since to detect 1 mutated fragment among 100000 wild-type fragments a total of 300000 genome equivalents is required amounting to ~1ug of starting DNA. It would be useful to show assay results on a sample before and after pre-amplification.

Response: *Thank you for your comment. We clarify that the lowest MAF of 0.001% was determined using patient plasma of known mutation status (mutant versus wildtype). We also reference Panditharatna et al (2018) Supplementary Figure 1B in which a comparison of ddPCR results for non-preamplified versus preamplified DNA is shown. We have more explicitly referred to this finding in our Discussion to support the inclusion of a preamplification step (Page 12).*

- To estimate false positive droplets, authors used only no-template control, but did not use or discus using mutation-negative control to determine the Limit of Detection that can be affected by assay cross-reactivity and/or PCR errors (or particular concern if using pre-amplification)

Response: *We thank you for this comment. In Panditharatna et al (2018) the limit of detection was determined by analyzing 29 plasma and 27 CSF samples from non-CNS-diseased pediatric patients in duplicate, to determine specificity of the platform (Supplementary Table 1, Panditharatna et al., 2018; Clinical Cancer Research). When testing patient samples for mutation status we use molecular biology grade water as a negative control rather than non-CNS-diseased pediatric patient plasma or CSF due to lack of sample availability, and because we have established the specificity of the assay using the cohort described from our lab's previous publication. We have more explicitly referred to how we have determined the limit of detection in the Discussion on Page 12.*