Video Article

Employing Digital Droplet PCR to Detect BRAF V600E Mutations in Formalin-fixed Paraffin-embedded Reference Standard Cell Lines

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Abstract

ddPCR is a highly sensitive PCR method that utilizes a water-oil emulsion system. Using a droplet generator, an extracted nucleic acid sample is partitioned into ~20,000 nano-sized, water-in-oil droplets, and PCR amplification occurs in individual droplets. The ddPCR approach is in identifying sequence mutations, copy number alterations, and select structural rearrangements involving targeted genes. Here, we demonstrate the use of ddPCR as a powerful technique for precisely quantitating rare *BRAF* V600E mutations in FFPE reference standard cell lines, which is helpful in identifying individuals with cancer. In conclusion, ddPCR technique offers the potential to precisely profile the specific rare mutations in different genes in various types of FFPE samples.

Video Link

The video component of this article can be found at http://www.jove.com/video/53190/

Introduction

The accumulation of genetic mutations in key regulatory genes alters normal cell programing like cell proliferation, differentiation, and survival, leading to cancer[1]. The RAS-RAF-MAP kinase pathway mediates cellular responses to growth signals. Oncogenic BRAF mutations can result from driver mutations in the *BRAF* gene, which may cause the BRAF oncoprotein to become overactive[2]. Mutations in the *BRAF* gene also result in overactive downstream signaling via MEK and ERK[3], which, in turn, leads to excessive cell growth and proliferation independently of growth factor-mediated regulation[4-6].

Several tools are available for DNA mutation profiling, such as quantitative real-time *BRAF* V600E mutations in formalin-fixed, paraffin-embedded (FFPE) reference standard cell lines by ddPCR. ddPCR is an PCR-based method for absolute quantification offering higher accuracy compared to conventional quantitative real-time PCR (qPCR)[7,8]. ddPCR also provides higher resolving power and accuracy for the detection of rare mutations in DNA templates, enabling more informative cancer research and diagnosis[9]. Additional advantages of ddPCR over conventional qPCR include its enhanced sensitivity and accuracy when studying low template copy numbers[10-12]. Herein, a protocol for automatically extracting DNA from FFPE reference standard cell lines, followed by determining the presence or absence of *BRAF* V600E mutations by ddPCR is demonstrated. The usage of software for data analysis and a graphical representation of the results are also described. The entire procedure is relatively simple and totally depends on the number of samples to be profiled and the number of conventional PCR and ddPCR machines available.

The following protocol describes standard procedures for *BRAF* V600E-positive FFPE reference standard cell lines (HD598, HD593, HD617, HD273 and wildtype (WT)) is performed in a fully automated instrument using the Tissue Preparation System (TPS) protocol. Subsequently, isolated DNA samples are analyzed for the presence of *BRAF* V600E mutations using ddPCR system. Targeted mutation analysis is performed after all samples have been profiled and the data has been loaded into the data analysis software. Depending on the number of samples/groups studied, data analysis may require from one to several hours. The experimental component of the methodology requires accuracy in handling DNA and pipetting into 96 well plates, while data analysis is performed using software.

Protocol

1. DNA Extraction from FFPE Reference Standard Cell Lines

Note: For this procedure, DNA extraction was performed from FFPE reference standard cell lines (HD598, HD593, HD617, HD273 and wildtype (WT)) using the FFPE Tissue DNA isolation kit as described in the protocol below. Automated DNA extraction was achieved by following the manufacturer's instructions for total DNA isolation.

1. Using a microtome and the original FFPE block, manually prepare fresh sections prior to DNA extraction and analysis, according to established procedures. Ensure that the sample input for the tissue section(s) analyzed does not exceed a combined total thickness of 10 µm and that the surface area does not exceed 600 mm^2 for a single section.

1.2 TPS protocol

Note: The volumes shown in Table 1 correspond to the minimum required to process 48 samples, and the procedure shown is in accordance with the TPS guidelines. Before starting the experiment settle down the FFPE samples in the e-tube by centrifugation at 600 x g, to avoid loss of samples during the automated program.

1. Turn on the automated DNA isolation instrument and computer. Open the Run control software and insert an auto load tray into the TPS deck loading area.
2. Dispense reagents into their corresponding troughs as shown in **Figure 1**.
    1. Place the 4 samples (*BRAF* WT, *BRAF* V600E 50%, 5% and 1%) in the sample carrier racks.
    2. Place the tip boxes in the troughs in columns 2 and 3 and check the tips for presence of more than one filter per tip, which will abort the run.
    3. Ensure proper mixing of the lysis buffer and wash buffer by inverting them 3–5 times and load them into the respective troughs in column 4 (**Figure 1**).
    4. After inverting for few times or mild vortexing, load the elution buffer, magnetic beads, and FFPE buffer in the small troughs in column 5, leaving 1 slot empty where indicated (**Figure 1, Table 1**).
    5. Load a 2 ml deep well plate (DWP) onto the plate carrier (**Figure 1**).
3. Perform the following final steps before starting a run:
    1. Uncap all tubes and reagent troughs. Confirm that sufficient capacity is available in the liquid waste bottle. Confirm that the solid waste bottle is empty and lined with a biohazard bag. Confirm that the tip eject plate is centered in the waste assembly.
    2. Close the front cover.
4. Start the software. Open the NA_Prep_Main_MLSTARlet.med file.
5. Click “Start.” The instrument status will switch from idle to running.
6. Enter the number of samples for this run. Choose the desired method for this run (DNA = 0). Enter the position of the first high volume tip, selecting “1” if all trays are full. Enter the position of the first standard volume tip, selecting “1” if all trays are full.
   Note: The instrument will then run through automated steps without user intervention. A detailed workflow is shown in **Figure 2**. Once the experiment is finished, reagent waste and tips are injected into the waste assembly.
7. Quantify purified genomic DNA by using a fluorometric method.
   Note: DNA samples which contain a minimum concentration of 3.3 ng/µl are subjected to ddPCR analysis (section 2).

2. DNA Mutation Profiling: ddPCR Protocol

Note: The protocol for DNA mutation profiling consists of 3 major steps:1) Droplet generation, 2) Conventional PCR amplification, 3) Droplet reading and 4) DNA mutation profiling.

2.1. Droplet generation

Note: ddPCR supermix is recommended for ddPCR, as this mix contains reagents required for droplet generation.

1. To avoid contamination, follow standard precautions, such as wearing gloves, using a clean PCR hood, clean pipets, and low-protein-binding tubes.
2. Ensure that the minimum concentration of human genomic DNA sample is 3.3 ng/µl. Note: The amount of purified DNA sample concentration will vary based on the analysis of % mutation frequency detection.
3. Assemble reaction mixtures in 96-well PCR plates. Thaw and equilibrate the reaction components to RT. Prepare PCR reactions by combining 2X ddPCR supermix (10 µl) and 20 primers (forward and reverse, 900 nM) and probe (250 nM) with each purified DNA sample (66ng/2 µl) make up to 20 µl with distilled water (as per the droplet generator protocol)
4. Vortex the mix thoroughly to ensure homogeneity and briefly centrifuge to collect the contents at the bottom of the tube before dispensing. Load 20 µl onto the cartridge for droplet formation.
5. Operation of the droplet generator, per the manufacturer’s recommended protocol
    1. Insert the cartridge into the holder with the notch in the cartridge positioned on the upper left side of the holder. Add 20 µl of reaction mix containing samples into the middle, and 70 µl of generator oil into the bottom wells.
    2. Attach the gasket across the top of the cartridge. Ensure that the gasket is securely hooked on both ends of the holder; otherwise, sufficient pressure for droplet generation will not be achieved.

3. Open the droplet generator by pressing the green button on the top of the instrument and insert the cartridge. When the holder is in the correct position, both the power (left right) and holder (middle right) indicator lights are green.
4. Press the top button on the instrument again to close the door and initiate droplet generation. Note: After pressing the button, a manifold positions itself over the outlet wells, drawing oil and samples through the microfluidic channels, where droplets are created. Droplets flow into the droplet well, where they accumulate. The droplet indicator light (at right) flashes green after 10 sec to indicate that droplet generation is in progress.
5. When droplet generation is complete, all 3 indicator lights change to solid green; open the door by pressing the button, and remove the holder from the unit. Remove the disposable gasket from the holder and discard it. Note: The top wells of the cartridge contain droplets, and the middle and lower wells are nearly empty, with a small amount of residual oil.

2.2. Preparation for PCR

1. For each sample, pipet out 40 µl of the droplet contents from the top well the cartridges into a single well of a recommended 96-well PCR plate as indicated in the manufacturer's instrument protocol. Note: Using a multi-channel pipette is ideal for transferring the droplets emulsions. Slow and gentle aspiration of droplets is recommended to minimize droplet shearing during transfers.
2. Seal the PCR plate with foil immediately after transferring droplets to avoid evaporation. Use pierceable foil plate seals that are compatible with the PCR plate sealer and the needles in the droplet reader. Follow the instructions in the PCR plate sealer instruction manual.
3. Set the plate sealer temperature to 180 °C and the time to 5 sec.
4. Touch the arrow to open the tray door. Position the support block on the tray with the 96-well side facing up. Place the 96-well plate onto the support block and ensure that all plate wells are aligned with the support block.
5. Cover the 96-well plate with 1 sheet of pierceable foil seal. Once the 96-well plate is secured on the support block and covered with the pierceable foil seal, touch the seal button. The tray will close and heat sealing will initiate.
6. When heat sealing is complete, the door opens automatically; remove the plate from the heat block for thermal cycling and then remove the heat block.
7. Ensure that all the wells in the plate are sealed by checking that depressions in the foil are readily apparent over each well. Once sealed, the plate is ready for thermal cycling.
8. Once droplets are removed, press the latches on the cartridge holder to open it. Remove the empty cartridge and discard it.
9. Perform conventional PCR amplification by following the parameters detailed in **Table 2**.

2.3 Droplet reading (as per the manufacturer's recommended protocol)

Note: Following PCR amplification of the nucleic acid target in the droplets, the droplet reader instrument analyzes each droplet individually using a 2-color detection system[13]. We typically set to detect FAM and VIC reporter fluorophores.

1. Click "Flush system" to prime the droplet reader and make it ready for ddPCR analysis.
2. Load the plate into the droplet reader and click "start." The droplet reader aspirates each sample, singulates the droplets, and streams them in single file past a 2-color detector. The detector reads the droplets to enumerate positive and negative samples.
3. When droplet reading is complete, open the door and remove the plate holder from the unit. Remove the 96-well PCR plate from the holder and discard it.
4. For proper maintenance, replace the droplet reader oil and empty the waste receptacle as needed. Add 50 ml of 10% bleach to the waste bottle to prevent microbial growth.

2.4 DNA mutation profiling (as per the manufacturer's recommended protocol)

Note: PCR-positive and PCR-negative droplets are counted to provide absolute quantification of target *BRAF* V600E DNA mutations in digital form, using data analysis software.

1. Click Setup to enter information about the samples, assays, and experiments.
2. In the Setup window, load a plate (filename.qlp), then click Analyze to open and analyze the data. The data analysis interface is separated into three windows: Results Table, Well Selector and Processed Data/Graphical Display.
3. In the Processed Data window, concentration data for each target appear in the wells in the plate map and are tabulated in the Results Table. Click Concentration to visualize data in concentration plots.

Representative Results

For our ddPCR analysis, we studied the *BRAF* V600E mutation FFPE reference standard cell lines. The droplet reader connects to a laptop computer running data analysis software. Each individual droplet is defined on the basis of fluorescent amplitude as being either positive or negative. The software provided by manufacturer also allows a user-defined cutoff to be entered to define the threshold between the positive and negative droplets. The number of positive and negative droplets in a sample is used to calculate the concentration of target in terms of copies/µl.

Fluorescence was detected and processed into a two-dimensional scatter plot display, custom software was used to draw appropriate gates for each droplet endpoint cluster, and the number of droplets within each gate was counted. As shown in **Figure 3A**, droplets represented by blue dots (FAM fluorescence signal) above the cut-off line for all samples (pink line) were positive for mutated *BRAF* V600E. Droplets represented by blue dots in the *BRAF* WT (NTC; Lane 2) sample could be due to a non-specific signal (false positive). False positive signals (*BRAF* WT) were normalized with other mutation samples. As shown in **Figure 3B**, WT *BRAF* droplets are represented by green dots (VIC fluorescence signal). In both plots, the grey dots at the bottom are considered as the fluorescence background. The overall mutant allele frequencies were calculated using the data shown in **Figure 3C**, based on the relative percentages of WT *BRAF* and *BRAF* V600E templates detected. Obtained ddPCR results contain the droplet event counts and calculated wild-type and mutant DNA molecule counts for the *BRAF* V600E (50%, 10%, 5%, 1%, 0.5% , 0.1% and 0.05%) samples calculated by using the below mentioned formula .

% of Mutant frequency = (Mutant copy / (Wildtype + Mutant copy)) x 100

Accordingly, *BRAF* V600E mutations were identified and verified with reference standard (*BRAF* WT). Defined *BRAF* V600E mutation allelic frequencies of 50%, 10%, 5%, 1%, 0.5%, 0.1% and 0.05% were used to test the sensitivity and reproducibility of the ddPCR system. From our analysis with known sample concentrations, we confirmed that ddPCR is able to detect as low as 0.05% of *BRAF* V600E mutation. The detection of false positive mutant count in NTC or WT might possibly be due to non-specific probe hydrolysis as reported earlier [14]. Detection of more than two copies in a sample has been considered as positive in tumor tissue [15].

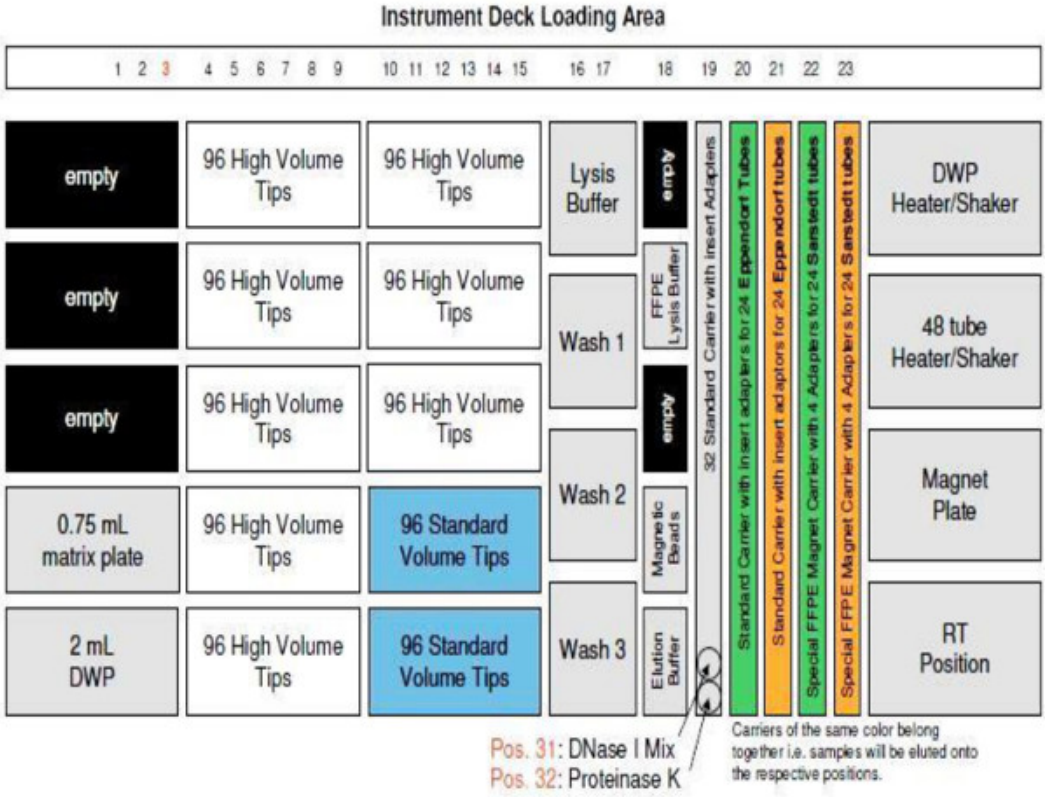

Figure 1. Schematic representation describing reagent and sample loading in preparation for automated DNA extraction instrument. Place the samples in a carrier racks and dispense the reagents into corresponding troughs as mentioned. Employing automated TPS protocol that supports multiple sample types, delivers accurate, and reliable results with maximum productivity. Re-printed with permission from Siemens Healthcare Diagnostics. (courtesy of Siemens Healthcare Diagnostics).

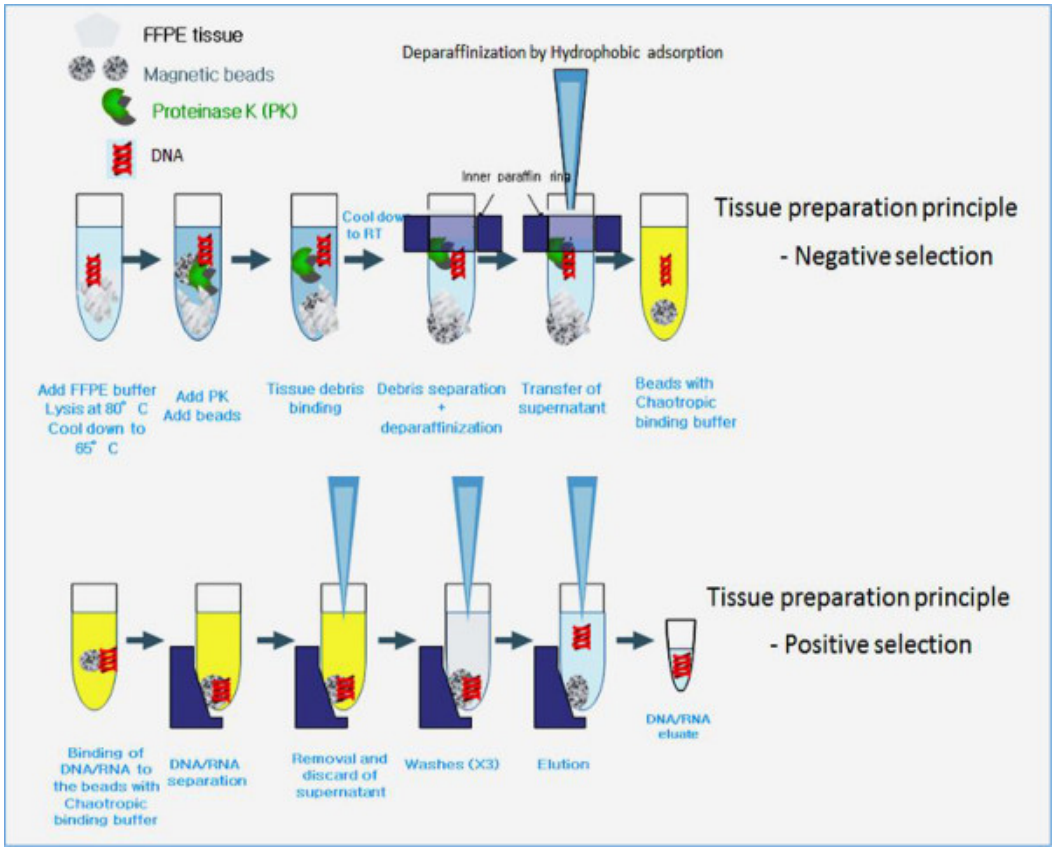

Figure 2. Schematic representation of the Tissue Preparation System Workflow for automated DNA extraction. Fully automated DNA isolation procedure for FFPE tissues sections including negative selection steps of paraffin, tissue debris removal and positive selection steps of binding and elution are shown. Re-printed with permission from Siemens Healthcare Diagnostics. (courtesy of Siemens Healthcare Diagnostics).

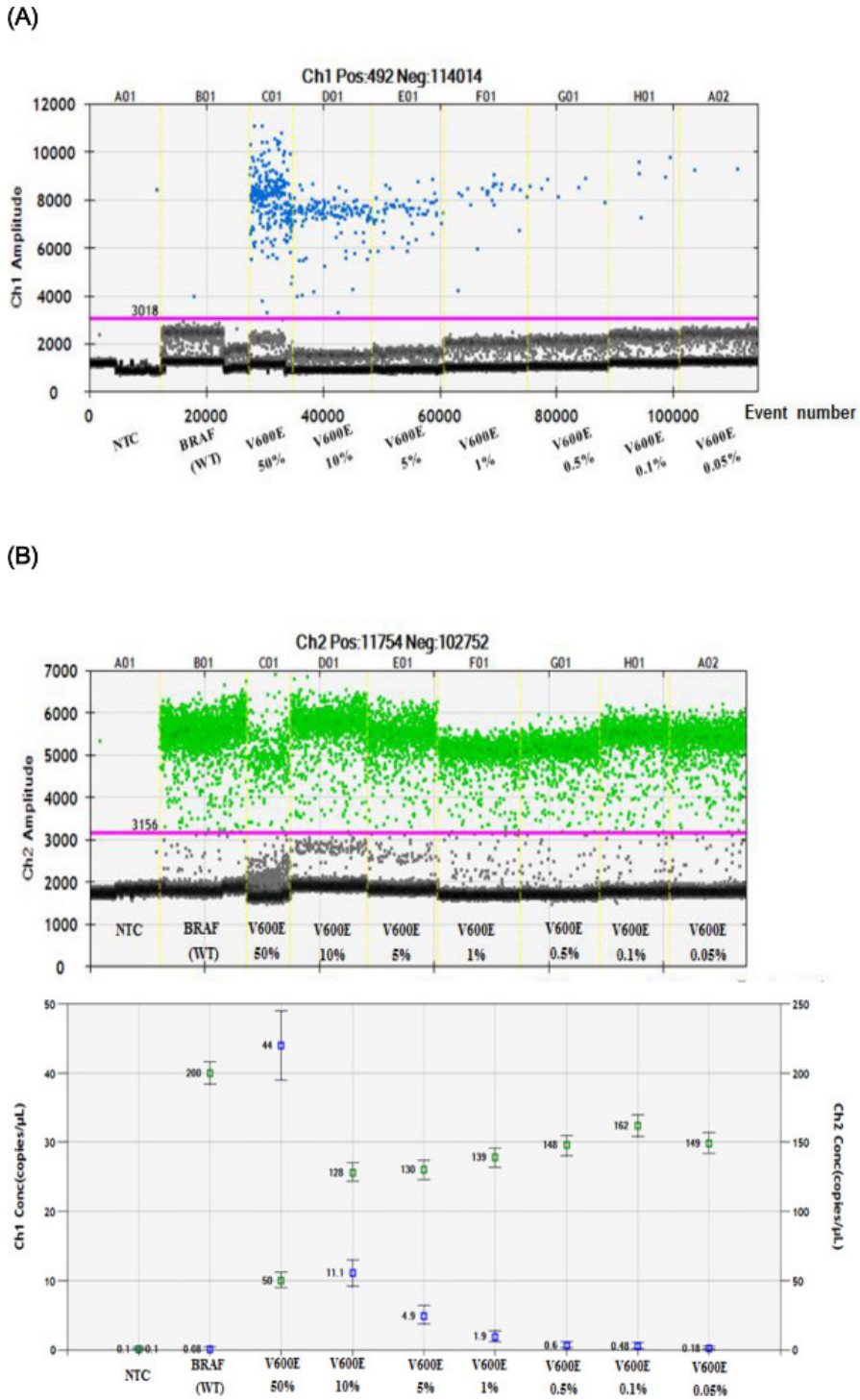

Figure 3. Use of the ddPCR system for precise quantification of the *BRAF* V600E mutation in FFPE reference standard cell line samples. **(A, B)** Visualization of positive fluorescence amplitudes in 1D plots (1dot -1droplet). Blue dots (**A**, FAM positive) represent mutant *BRAF* V600E-positive droplets, while green dots (**B**, VIC positive) represent WT *BRAF*-positive droplets. This determination enables precise mutation quantification in FFPE reference standard cell lines. The pink line is the discrimination threshold between positive and negative signals of the droplets. **(C)** The fractional abundance plot shows blue markers that indicate the concentration (copies/µl) of *BRAF* V600E mutation, and the green markers indicate the concentration (copies/µl) of *BRAF* (WT). All error bars generated by data analysis software represent the 95% confidence interval.

Reagents	Volume (ml)
Lysis Buffer	106 ml
Wash Buffer 1	101 ml

Wash Buffer 2	72 ml
Wash Buffer 3	106 ml
Elution Buffer	19 ml
Magnetic beads	8 ml
FFPE buffer	15 ml
Proteinase K	3.3 ml

Table 1. Total volume of reagents (TPS kit) required for DNA extraction with 48 samples.

Cycling Step	Temperature	Time	# Cycles
Enzyme activation	95 °C	10 min	1
Denaturation	94 °C	30 sec	40
Annealing/extension	60 °C	1 min *	
Hold	98 °C	10 min	1
Hold	4 °C	Forever	1
* Adjust ramp rate settings to 2-2.5 °C/sec. Use a heated lid set to 105 °C and set the sample volume to 40 µl			

Table 2. Conventional PCR thermocycling conditions

Discussion

Here, we highlight the applicability of ddPCR and DNA isolation from FFPE reference standard cell line samples for a specific gene mutation assessment. In this study, TPS automated DNA isolation method is used which can be readily adapted, automated, and can accommodate up to 48 different samples simultaneously, allowing for larger scale experiments and lower variability. One of the limitations of the DNA isolation in the present work is that every FFPE sample is unique, and will vary one another in surface contaminants, microbial flora, and/or human genetic backgrounds. In general, extracted DNA quality and quantity and the success of whole genomic DNA amplification are dependant upon various parameters before, during and after extraction. These include, type and amount of tissue, type of fixative used for tissue preservation, duration of fixation, age of the paraffin block and storage conditions, as well as the length of the desired DNA segment to be analysed[16]. Removal of paraffin from the tissue is the most critical step for successful extraction as undissolved paraffin leads to poor sample quality. During the droplet generation, care must be taken to prevent bubble formation – this is another critical step for successful mutation detection. Considering the sample to sample variation that might arise between sample populations and based on the motive of the experiment, certain modifications in the procedure might be required to obtain desired result.

Another advantage is that DNA isolation and ddPCR is conducted using automated systems in this protocol, and hence there is negligible error and user intervention required is very minimal. Isolating whole-genome-amplified DNA from paraffin-embedded tissue/cells was obtained by using TPS system. One of the drawbacks in using automated DNA isolation system is that, it is not cost efficient to use small number of samples. Instead of this automated step, other standard DNA isolation procedure could also be performed for limited samples

A recent study stated that using droplet digital PCR (ddPCR) is able to determine the relative copy number of specific genomic loci even in the presence of intermingled normal tissue obtained from FFPE tissues. By using a control dilution series, Nadauld, L. *et al.* determined the limits of detection (LOD) for the ddPCR assay and reported its improved sensitivity on minimal amounts of DNA compared to standard real-time PCR[17]. Here, FFPE reference standard cell lines are used to demonstrate the mutation detection capability of the ddPCR system. The ddPCR system results indicated the possibility to detect rare mutation allelic frequencies down to 0.05% mutation. Collectively, these data indicate that the ddPCR system also enables quantitative analysis of the percentages of various mutant alleles and relative differences in heterozygous clinical tumor samples. Large number of FFPE samples can be analyzed for specific gene mutations simultaneously and this is an optimal technique for population wide genetic studies.

Finally, it should be taken into account that the mutation frequencies are represented here are absolute quantification and should not be considered as relative value of mutation rate or frequency. ddPCR readout provides absolute quantification of target DNA mutation. These values can be used for validating mutation frequencies of samples prepared under the same condition, and sequenced over the same region. However, these absolute values are reproducible and can be used for quantitative comparison of mutation distribution and frequency when optimal parameters are controlled. In conclusion, ddPCR has recently emerged as a robust tool that gives absolute quantitation of nucleic acids in FFPE and biopsy samples and also can be duplexed with reference assays for determination of either normalized transcript concentrations or DNA copy number.

Disclosures

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