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Procedure

IsoFlux KRAS/BRAF mutation detection using CAST-PCR

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Revision: C

Summary

This document is intended to guide the user through genomic DNA mutation (KRAS/BRAF) detection CTs isolated by the IsoFlux system by CAST-PCR.

Principle

CTCs are isolated from human peripheral blood with IsoFlux™ System. WGA is performed on IsoFlux™ enriched samples. Amplified DNA is isolated and tested for a panel of KRAS and BRAF mutations by cast-PCR™

Competitive Allele-Specific TaqMan® PCR (castPCR™) technology, developed by Life Technologies, is a highly specific and sensitive method of detecting and quantitating rare mutations in samples that contain large amounts of normal, wild type genomic DNA (DNA). CastPCR™ technology combines allele-specific TaqMan® qPCR with allele-specific MGB blockers in order to suppress non-specific amplification from wild type alleles, resulting in better specificity than traditional allele-specific PCR. CastPCR™ technology allows the detection of rare mutant targets in a background of abundant wild-type targets from small amounts of precious clinical samples like CTCs.

Materials and Reagents

1. Whole genome amplification: (Qiagen, REPLI-g Ultrafast Mini kit, Catalog No. 150033)

2. DNA extraction: (Qiagen, DNA QIAamp DNA Micro Kit, Catalog No. 56304)

3. TaqMan® Mutation detection assays(Life Technologies Catalog No. 4465804)

   Assay ID: Hs00000113_mu Assay Name: KRAS_516_mu, Amino Acid Change: p. G12C
   Assay ID: Hs00000115_mu Assay Name: KRAS_517_mu Amino Acid Change: p. G12S
   Assay ID: Hs00000117_mu Assay Name: KRAS_518_mu Amino Acid Change: p.G12R

Assay ID: Hs00000119_mu Assay Name: KRAS_520_mu Amino Acid Change: p.G12V
Assay ID: Hs00000121_mu Assay Name: KRAS_521_mu Amino Acid Change: p. G12D
Assay ID: Hs00000123_mu Assay Name: KRAS_522_mu Amino Acid Change: p.G12A
Assay ID: Hs00000131_mu, Assay Name: KRAS_532_mu Amino Acid Change: p.G13D
Assay ID: Hs00000111_mu Assay Name: BRAF_476_mu Amino Acid Change: p. V600E

4. TaqMan® Mutation Detection Reference Assays (Life Technologies, Catalog No. 4465807)

   Assay ID: Hs00000174_rf, Assay Name: KRAS_rf
   Assay D: Hs00000172_rf, Assay Name: BRAF_rf

5. TaqMan® Genotyping Master Mix, (Life Technologies, Catalog No. 4371355)

6. Real time PCR system (with FAM and ROX fluorescence channels).

7. General Supplies
    1. Hemocytometer
    2. Micropipettes
    3. Pipet tips (pipet tips with aerosol barriers for preventing cross-contamination are recommended)
    4. 1.5 or 2mL microcentrifuge tubes (low retention recommended)
    5. PCR tubes and plates
    6. Nuclease-free water
    7. Microcentrifuge
    8. Vortexer
    9. 96-100% ethanol
    10. PBS (phosphate buffered saline)

Prepare clinical cancer patient CTC samples or cancer cell line spiked samples

1. Clinical sample: isolate CTCs with IsoFlux system, recover CTC with low recovery holder and proceed to mutation detection.

2. Cancer cells spiked normal human blood sample: KRAS/BRAF mutation cell lines

should be used, e.g., MDA-MB-231 cell line (heterozygous for KRAS G13D mutation) (ATCC: ATCC® HTB-26).

3. Labeling model cell lines with CellTracker dyes
    1. Select a subconfluent T25 flask of model cells to be labeled.
    2. Remove tissue culture medium
    3. Replace with 10mL of pre-warmed serum free base medium (all additives except for FBS)
    4. Add freshly prepared cell tracker stock to a final concentration of 2μM a in the flask. [Add 20μl/10mL of medium]. Cell tracker dyes are supplied in small vials that can be prepared at a stock concentration of 10mM in DMSO - make a new vial each day.
    5. Incubate under tissue culture conditions for 30 minutes
    6. Remove base medium and replace with pre-warmed complete medium 7. Incubate for an additional 30 minutes under tissue culture conditions 8. After 30 minutes, cells can be harvested by trypsinization and prepared for counting

Counting model cells using modified droplet method

1. Prepare a standard dilution series of cells in IsoFlux binding buffer, 1mL per dilution - typically 1:10 and 1:100 is sufficient for T25 flasks. Resuspend the cells after each dilution
2. Prepare 2 double-glass Brightline® hemocytometers by wiping the surface with 70% isopropanol and tissue paper. Dry thoroughly
3. Using the 1:100 dilution, deposit a 20uL drop onto the center of each hemocytometer grid. Do not apply coverslip
4. Allow cells to settle for 10 minutes (cover hemocytometer with a box to prevent desiccation).
5. Count all cells in the entire droplet. Scan up and down the rows to keep your place. Record the cell number for each droplet/ grid. Take the average of these for the total cell number per 20μL. Use cells accordingly for spiking.
6. Spiking cells into Ficoll tubes for model experiments
7. Prepare Ficoll tube as directed in the IsoFlux protocol
8. Dispense whole blood into tube
9. Add desired number of cells from the above counted dilution
10. Proceed with density separation

Procedure for mutation detection

Whole genome amplification (WGA) REPLI-g® UltraFast Mini Kit (Qiagen)

1. *Things to do before: Reconstitute DLB buffer with 500μL nuclease free water. Prepare

Buffer D2 by mixing 5µL DTT (1M) with 55µL of reconstituted DLB. Make 0.5x PBS buffer by mixing PBS with equal volume of nuclease free water.

2. After IsoFlux isolation, transfer recovered CTCs (beads/cells) to a 0.2mL PCR tubes in 20µL PBS, remove PBS with the aid of a magnet. Process immediately or snap-freeze the beads/cells pellet on dry ice, store at -80°C. The sample may be thawed on ice just before processing.
3. Add 3.5µL 0.5x PBS to beads/cells. Mix by vortexing and centrifuge briefly 4. Add 3.5µL D2, Mix by vortexing and centrifuge briefly. Incubate on ice for 10 minutes. 5. Add 3.5µL stop solution, vortex briefly to mix and centrifuge briefly.
6. Add 19.5µL Master mix. Mix by vortexing and centrifuge briefly. (Master Mix: 15µL Reaction buffer, 1µL DNA polymerase and 3.5µL nuclease-free water)
7. Run thermocycler program:

    30°C 2-4 hour

    65°C 3 minutes

    4°C Hold

8. Amplified DNA can be stored at -20°C.

Purification of amplified DNA with QIAamp® DNA Micro Kit (Qiagen)

1. Buffer AW1 and AW2 are supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96-100%) as indicated on the bottles. Buffer AW1 and AW2 are stable for 1year when stored closed at room temperature.
2. Equilibrate REPLI-g amplified DNA to room temperature (15-25°C). 3. Add 100µL nuclease-free water. Mix by vortexing and centrifuge briefly. Transfer all sample to a microfuge tube
4. Add 130µL Buffer AL to the sample. Mix by vortexing for 15 seconds and centrifuge briefly. (In order to ensure efficient binding of the DNA to the QIAamp spin column membrane, it is essential that the REPLI-g amplified DNA and Buffer AL are mixed thoroughly to yield a homogeneous solution.
5. Add 130µL ethanol (96-100%) to the sample. Mix by vortexing for 15 seconds and centrifuge briefly.
6. Carefully apply the mixture from the last step to the QIAamp spin column (in a 2mL collection tube) without wetting the rim, close the cap, and centrifuge at 6000xg for 1 minute. Place the QIAamp spin column in a clean 2mL collection tube (provided), and discard the tube containing the filtrate. (Centrifugation is performed at 6000xg in order to reduce impurities. Centrifugation at full speed will not affect the yield or purity of the DNA. If the sample has not completely passed through the column after centrifugation, centrifuge again at higher speed until the QIAamp spin column is empty).

7. Carefully open the QIAamp spin column and add 500µL Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000xg for 1 min. Place the QIAamp spin column in a clean 2mL collection tube (provided), and discard the collection tube containing the filtrate.
8. Carefully open the QIAamp spin column and add 500µL Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000xg) for 3 minutes. 9. Place the QIAamp spin column in new microfuge collection tube (not provided with the kit) and discard the collection tube with the filtrate. Centrifuge at 20,000xg for 1 minute. 10. Place the QIAamp MinElute column in a clean 1.5mL microfuge tube (not provided with the kit) and discard the collection tube containing flow-through. Carefully open the lid of the QIAamp MinElute column and apply 50µL Buffer AE (diluted 10 times with nuclease free water) to the center of the membrane. (Important: Ensure that the elution solution is equilibrated to room temperature (15-25°C).
11. Close the lid and incubate at room temperature (15-25°C) for 5 minutes. Centrifuge at full speed (20,000xg) for min.
12. Store purified DNA at -20°c.

Running CAST-PCR

1. In a mutation detection experiment, run the test sample with both mutant allele assay and corresponding gene reference assay. Technical replicates are not required. 2. Prepare the PCR mix and the PCR plate as follows for a total 20µL reaction volume: 1. 10µL of TaqMan® Genotyping Master Mix
    2. 2µL of TaqMan® Mutation Detection Assay (mutant allele or gene reference assay)
    3. 8µL of amplified DNA (1:8 dilution with diluted AE buffer)
3. Cover the plate with an optical adhesive film.
4. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles.
5. Run mode - Standard, choose FAM fluorescent for read out.
6. Sample volume 20µL (96-we plates)
7. Thermal-cycling profile:

    Hold at 95°C for 10 minutes
    Cycles of 92°C for 15 seconds and 58° C for 1 minute
    Cycles of 92°C for 15 seconds and 60°C for 1 minutes (record fluorescent data)

8. Load the reaction plate into the real-time PCR instrument, then start the run.

Data analysis

1. Analyze the data in the real-time PCR system software, using the following analysis settings: Manual Ct (threshold cycle): 0.2, Automatic Baseline: On
2. The real-time PCR system software determines the Ct values for the mutation detection assays.

3. View the amplification plots and/or Ct values for all reaction wells as follows: the reference assay Ct should be similar between all sample if WGA were successful, typically 20 to 21. Non-spiked samples typically showed no amplification (or very high Ct) for mutant assay. Spiked samples typically have Ct values at high 30s or under.
4. Export the Results or Results Table from the real-time PCR system software as Excel file (or text file)
5. Calculate delta Ct (dCt) of each sample (the Ct difference of reference assay and mutation assay)
6. The dCt of samples spiked with cell line with mutation should be lower than non-spiked samples by at least 2 Ct.