

Profiling *EGFR* DNA Mutation Analysis in Circulating Rare Cells Using the Genesis Cell Isolation System with Celselect Slides™ and Droplet Digital™ PCR (ddPCR™)

Protocol

Abstract

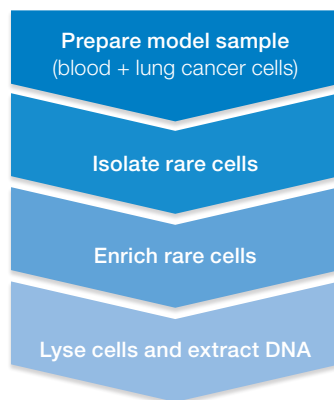
The epidermal growth factor (*EGFR*) mutation is critical for determining the most effective treatment for patients with lung cancer. Identifying the mutation in rare circulating tumor cells (CTCs) found in peripheral blood can offer crucial insights into the efficacy of certain drugs and patient prognosis, especially in non-small cell lung cancer (NSCLC). Unfortunately, the rarity of these cells presents a significant detection challenge.

The Genesis Cell Isolation System with Celselect Slides can efficiently capture CTCs based on size and deformability. These cells can then be recovered for further analysis. To detect these cells, Droplet Digital PCR can be used for direct quantification of DNA mutation.

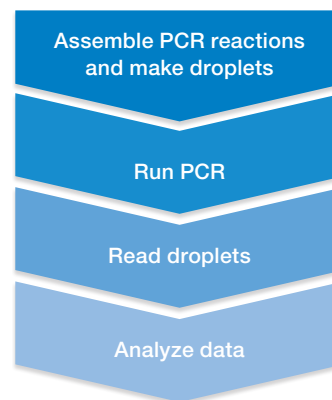
This optimized protocol using a model CTC sample is designed as a starting point for specific cancer-associated genetic mutation detection. We recommend end users choose and validate their assays with this protocol before use.

Workflow Overview

Part 1. Rare Cell Enrichment



Part 2. *EGFR* Mutation Detection



Instruments and Materials

Instruments*

- Genesis Cell Isolation System (catalog #CEL20101)
- QX200™ Droplet Generator (#1864002)
- QX200 Droplet Reader (#1864003)
- QX200 AutoDG™ Droplet Digital PCR System (#1864100) (optional)
- PX1 PCR Plate Sealer (#1814000)
- C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module (#1851197)
- Qubit 4 Fluorometer (Thermo Fisher Scientific Inc., #Q33226)

Materials*

- Celselect Slides Enrichment Kit (#CEL80110)
- DNAzol BD (Molecular Research Center, Inc., #DN129)
- ddPCR *EGFR* Exon 19 Deletions Screening Kit (#12002392)
- ddPCR Mutation Detection Assay: *EGFR* T790M (Assay ID #dHsaMDV2010019)
- ddPCR Mutation Detection Assay: *EGFR* L858R (Assay ID #dHsaMDV2010021)
- Qubit 1X dsDNA High Sensitivity Assay Kit (Invitrogen, #Q33230)
- DG8 Cartridges for QX200 Droplet Generator (#1864008)
- Droplet Generation Oil for Probes (#1863005)
- Automated Droplet Generation Oil for Probes (#1864110) (optional)
- ddPCR Supermix for Probes (No dUTP) (#1863023)
- PCR Plate Heat Seal (pierceable foil seal; #1814040)
- DNA LoBind Microcentrifuge Tube, 1.5 ml (Eppendorf, #022431021)
- ddPCR 96-Well Plates (#12001925)

* Source is Bio-Rad Laboratories, Inc. unless otherwise noted.

Rare Cell Enrichment Workflow Using the Genesis System with Celselect Slides

For *EGFR* mutation detection using the Genesis System with Celselect Slides and Droplet Digital PCR, three different model CTC samples were prepared with three different non–small cell lung cancer cell lines at different *EGFR* mutations.

- NCI-H1975 is a human non–small cell lung cancer cell line harboring the *EGFR* L858R mutation and the T790M mutation
- NCI-H1650 is a human non–small cell lung cancer cell line with a deletion mutation on exon 19 of the *EGFR* gene
- A549 is a human non–small cell lung cancer cell line with the wild-type *EGFR*

Model CTC Sample Preparation

1. Prepare a cell stock suspension at a concentration of 100, 400, and 1,000 cells/10 μ l.
2. Spike the 10 μ l of cell stock solution into 4 ml of whole blood to prepare a model CTC sample.

3. Mix well with 4 ml of 1x phosphate buffered saline (PBS). Store this PBS + blood solution at room temperature on a blood rocker/shaker that is gently agitating until use. If it is longer than 1 hr, store at 4°C.

Celselect Slides Enrichment and DNA Extraction

1. Refer to the following sections in the Celselect Slides Enrichment User Manual (bulletin 3337) to perform Celselect Slides enrichment:
 - a. Performing the Genesis System Self-Test
 - b. Celselect Slides and Slide Station Set-Up
 - c. Priming the Celselect Slides
 - d. Preparing the Reagents Cartridge
 - e. Preparing the Blood Sample
 - 1) Use the blood sample prepared according to instructions in the Model CTC Sample Preparation section
 - f. Running the Genesis System
 - 1) At step 15, the recovered cell sample can be centrifuged at 600 rcf for 5 min before cell lysis
2. Remove the supernatant and add 1 ml of DNAzol BD directly into the conical tube.
3. Transfer the entire sample to an Eppendorf DNA LoBind 1.5 ml Microcentrifuge Tube.
4. Shake vigorously by hand for 15–20 seconds and store at room temperature for 5 min.
5. Add 400 μ l of isopropanol to the DNAzol BD-lysate sample mixture.
6. Vortex or shake vigorously and store at room temperature for 4 min.
7. Sediment the precipitated DNA by centrifugation at 10,000 rcf for 6 min.
8. Upon centrifugation, remove the supernatant and gently add 0.5 ml of DNAzol BD to the DNA pellet.
9. Vortex/shake the DNA pellet until completely dispersed.
10. Centrifuge the resulting mixture at 10,000 rcf for 5 min.
11. Remove the supernatant and wash the DNA pellet by mixing with 1 ml of 75% ethanol.
12. Repeat steps 10–11 for a total of two DNA washes.
13. Remove any residual ethanol with a micropipet. Do not allow the pellet to completely dry.
14. Solubilize DNA by adding 20 μ l of DNase-/RNase-free distilled water.
15. Measure a dsDNA quantity in the sample by following the Qubit 1X dsDNA High Sensitivity Assay Kit protocol.

EGFR Mutation Detection Workflow Using Droplet Digital PCR

ddPCR Mutation Detection Assays are expertly designed specifically for Droplet Digital PCR using proprietary computational algorithms. The assays used in this protocol consist of mutant target and wild-type assays mixed and provided in a single tube, with FAM targeting the mutant allele and HEX targeting the wild-type allele. This assay is made up of a single primer set plus two competitive probes. This assay takes advantage of the high sensitivity and precision of Droplet Digital PCR.

Reaction Setup

1. Thaw all components to room temperature. Mix thoroughly by vortexing each tube to ensure homogeneity because a concentration gradient may form during -20°C storage. Centrifuge briefly to collect contents at the bottom of each tube and store protected from light.
2. Prepare samples at the desired concentration before setting up the reaction mix according to the guidelines in Table 1. If multiple samples are to be assayed using the same target and reference duplex assay, prepare a master reaction mix without sample template, dispense equal aliquots into the reaction tubes, and add the sample template to each reaction tube as the final step.
3. Prepare a negative control: at least one well containing only wild-type template at a concentration similar to the concentration of unknown samples.

Table 1. ddPCR reaction setup.

| Component | Volume per Reaction, μl | Final Concentration |
|--|------------------------------------|---------------------|
| 2x ddPCR Supermix for Probes (No dUTP) | 10 | 1x |
| 20x target (FAM) and wild-type (HEX) primers/probe | 1 | 1x |
| Restriction enzyme, diluted | 1 | 2–5 U/reaction |
| Target sample and/or DNase-free water | Variable | Variable |
| Total volume | 20* | – |

* For the Automated Droplet Generator, prepare 22 μl per reaction.

4. Mix thoroughly by vortexing the reaction tubes. Centrifuge briefly to ensure that all components are at the bottom of each reaction tube. Allow the reaction tubes to equilibrate at room temperature for about 3 min.
5. Once the reaction mixtures are ready, load 20 μl of each reaction mix into a sample well of a DG8 Cartridge (#1864008) followed by 70 μl of Droplet Generation Oil for Probes (#1863005) into the oil wells, according to the QX200 Droplet Generator Instruction Manual (10031907). For the Automated Droplet Generator, follow instructions in the Automated Droplet Generator Instruction Manual (10043138).

Thermal Cycling Conditions

1. After droplet generation with the QX200 Droplet Generator, carefully transfer droplets into a clean ddPCR 96-Well Plate or remove the plate containing ddPCR droplets from the Automated Droplet Generator. Seal the plate with the PX1 PCR Plate Sealer.

2. Proceed to thermal cycling (see Table 2) and subsequent reading of droplets in the QX200 Droplet Reader.

Table 2. Recommended thermal cycling conditions for the C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module.*

| Cycling Step | Temperature, $^{\circ}\text{C}$ | Time | Ramp Rate, $^{\circ}\text{C}/\text{sec}$ | Number of Cycles |
|---------------------|---------------------------------|----------|--|------------------|
| Enzyme activation | 95 | 10 min | 2 | 1 |
| Denaturation | 94 | 30 sec | 2 | 40 |
| Annealing/extension | 55 | 1 min | 2 | 40 |
| Enzyme deactivation | 98 | 10 min | 2 | 1 |
| Hold (optional) | 4 | Infinite | 1 | 1 |

* For the C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module, use a heated lid set to 105°C and set the sample volume to 40 μl .

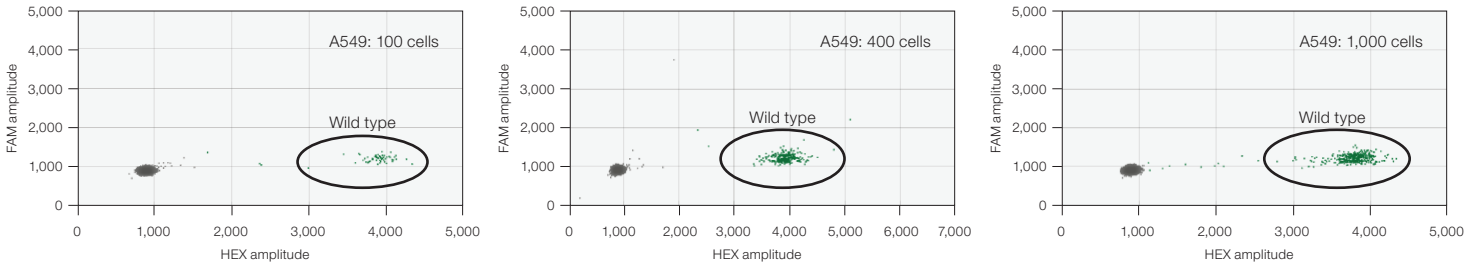
Data Acquisition and Analysis

1. After thermal cycling, place the sealed 96-well plate in the QX200 Droplet Reader.
2. Open QX Manager Software Standard Edition to set up a new plate layout according to the experimental design. Refer to the QX200 Droplet Reader and QX Manager Software Standard Edition User Guide (10000107223).
3. To add a plate in the QX Manager Software, click the **Add Plate** tab. The software should enable the Configure Plate button. Click **Configure Plate** to open the plate configuration window.
4. Plate features show in the plate configuration window. Fill in the plate and well information, select the wells, designate the sample name, experiment type, ddPCR Supermix for Probes (No dUTP) as the supermix type, target name(s), target type(s), and reference: Ch1 for FAM and Ch2 for HEX.
5. Select **Apply** to load the wells and, when finished, select **OK**.
6. Once the plate layout is complete, select **Run** to begin the droplet reading process. Select the appropriate dye set and run options when prompted.
7. After data acquisition, select samples in the well selector under Analyze. Set appropriate threshold for the ddPCR Mutation Detection Assay. For more detailed information about setting thresholds, please refer to the Rare Mutation Detection Best Practices Guide (bulletin 6628).
8. The concentration reported is copies/ μl of the final 1x ddPCR reaction.

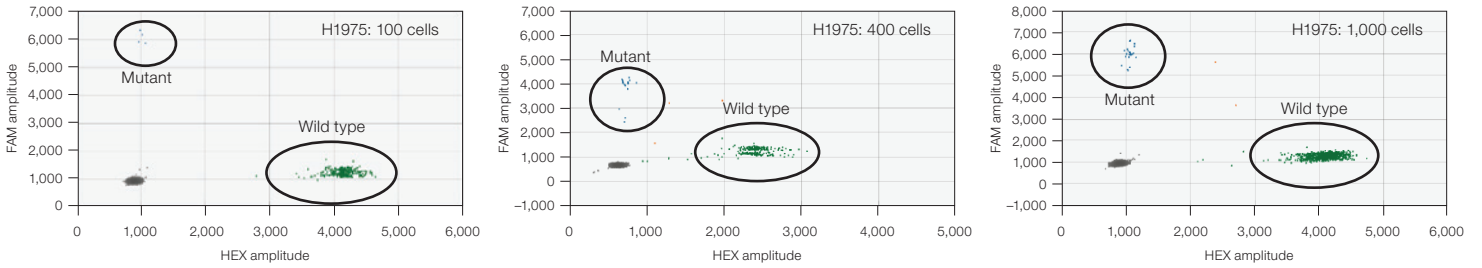
Results

ddPCR plots of cancer cell lines H1975 (L858R/T790M), H1650 (exon 19 deletion), and A549 (wild type) for the three tested *EGFR* mutations are shown in Figure 1. For the exon 19 deletion, the Bio-Rad ddPCR Mutation Detection Assay screens for 15 common deletions (nondiscriminatory detection in the FAM channel) but in this protocol, one of the exon 19 deletions is being tested in the H1650 cell line.

EGFR T790M



EGFR L858R



Exon 19 Deletion

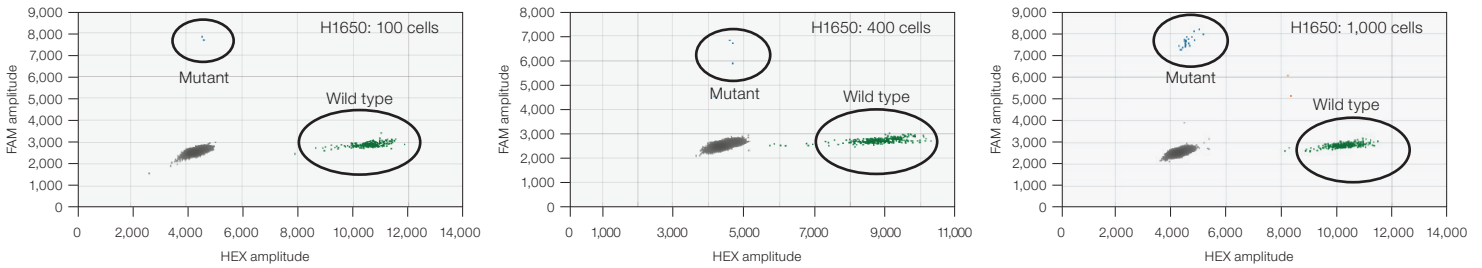


Fig. 1. EGFR DNA mutation in cancer cells enriched using the Genesis System with Celselect Slides. 2-D amplitude plot of lung cancer cells spiked in blood followed by DNA extraction shows H1975 (*EGFR* L858R/T790M), H1650 (exon 19 deletion), and A549 (wild type) with different concentrations of cells collected from the rare cell isolation steps using the Genesis System with Celselect Slides. High sensitivity of ddPCR Mutation Detection Assays provides precise quantification of T790M, L858R, and exon 19 deletion mutation in the presence of wild-type and mutant DNA. The black cluster on the plots represents the negative droplets, the green cluster represents the droplets that are positive for wild-type DNA only, and the blue cluster represents the droplets that are positive for mutant DNA only.

Visit [bio-rad.com/Celselect](https://www.bio-rad.com/Celselect) for more information.

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