

# Dual Isolation and Profiling of Circulating Tumor Cells and Circulating Tumor DNA from a Single Liquid Biopsy Sample Using the Genesis Cell Isolation System with Celselect Slides™ and Droplet Digital PCR Protocol

## Abstract

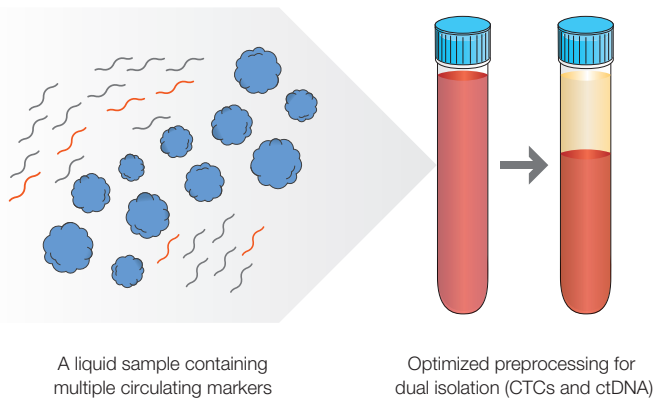
Recent advances and technology development in liquid biopsy have paved the way for noninvasive methods of cancer monitoring and diagnosis. Circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) are gaining more attention as promising biomarkers, offering molecular insights into cancer progression, overall survival, and treatment efficacy.

In this context, we introduce a protocol that describes the dual isolation of ctDNA and CTCs from the same sample. This is facilitated with the Genesis Cell Isolation System and Droplet Digital PCR (ddPCR™), along with optimized blood component separation and cell-free DNA (cfDNA) enrichment steps. After sample preparation, the Genesis System with Celselect Slides enriches CTCs based on size and deformability, and the enriched cells can be recovered for subsequent downstream analysis. Concurrently, Droplet Digital PCR can be utilized to interrogate specific loci within cfDNA to determine the presence of a range of DNA aberrations present in ctDNA, including single nucleotide variants and insertions and deletions.

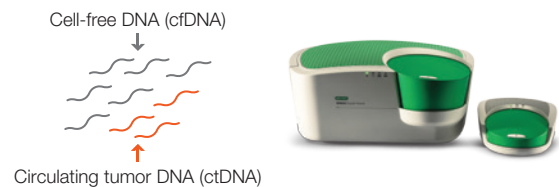
This protocol using a model CTC-ctDNA sample serves as a starting point for specific cancer-associated mutation detection. For example, *EGFR* mutations in ctDNA are a common biomarker in non-small-cell lung cancer (NSCLC) diagnostics. Their presence or absence can shape NSCLC treatment strategies and influence patient prognoses. Additionally, analyzing CTCs from the same sample can shed light on functional cancer studies and drug susceptibility assays.

A list of recommended materials, reagent preparations, and methods are provided, accompanied by example results from model CTC-ctDNA samples. Users are recommended to select and validate their assays and cell systems with this protocol prior to use. For a visual overview of the dual-isolation/profiling workflow steps, refer to Figure 1.

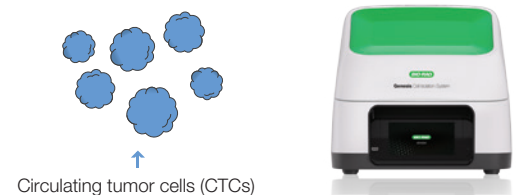
### Part 1. Model sample preparation and plasma separation



### Part 2. cfDNA extraction and ctDNA mutation analysis with Droplet Digital PCR



### Part 3. Rare cell enrichment/enumeration



**Fig. 1. Overview of dual isolation of CTCs and cfDNA from a single liquid biopsy sample.** The protocol is compatible with blood samples prepared in standard BD Vacutainer EDTA Tubes (Becton, Dickinson and Company) or Cell-Free DNA BCT Tubes (Streck).

## Instruments and Materials

### Instruments

- Genesis Cell Isolation System (Bio-Rad Laboratories, Inc., catalog #CEL20101)
- QX200™ Droplet Generator (Bio-Rad, #1864002)
- QX200 Droplet Reader (Bio-Rad, #1864003)
- QX200 AutoDG™ Droplet Digital PCR System (Bio-Rad, #1864100) (optional)
- PX1 PCR Plate Sealer (Bio-Rad, #1814000)
- C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module (Bio-Rad, #1851197)
- 2100 Bioanalyzer System (Agilent Technologies, Inc., #G2939BA)
- Sorvall Legend Micro 21R Microcentrifuge (Thermo Fisher Scientific Inc., #75002446)
- Sorvall ST 8 Small Benchtop Centrifuge (Thermo Fisher Scientific, #75007200)
- Centrifuge 5810R 15 amp version (Eppendorf SE, #022625501) with a rotor FA-45-6-30

### Materials

- Celselect Slides Enrichment Kit (Bio-Rad, #CEL80110)
- Celselect Slides Enumeration Indirect Stain Kit (Bio-Rad, #CEL80111)
- ddPCR Mutation Detection Assay: *EGFR T790M* (Bio-Rad, Assay ID #dHsaMDV2010019)
- Droplet Generation Oil for Probes (Bio-Rad, #1863005)
- Automated Droplet Generation Oil for Probes (Bio-Rad, #1864110)
- ddPCR Supermix for Probes (No dUTP) (Bio-Rad, #1863023)
- DG8 Cartridges for QX200 Droplet Generator (Bio-Rad, #1864008)
- Pierceable Foil PCR Plate Heat Seal (Bio-Rad, #1814040)
- ddPCR 96-Well Plates (Bio-Rad, #12001925)
- DNA LoBind Tubes, 1.5 ml (Eppendorf, #022431021)
- Applied Biosystems MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher Scientific, #A29319) for cfDNA extraction (refer to the MagMAX Cell-Free DNA Isolation Kit for additional materials not supplied with the kit)
- QIAamp Circulating Nucleic Acid Kit (QIAGEN, #55114) for cfDNA extraction (refer to the QIAamp Circulating Nucleic Acid Handbook for additional materials not supplied with the kit)
- Multiplexed 23 ctDNA 3% AF (Anchor Molecular, #60100101) (optional)
- Cytiva Ficoll-Paque PLUS Media (Thermo Fisher Scientific, #17144002)

**Note:** Additional materials and equipment are needed to carry out the plasma separation and cfDNA extractions using the kits mentioned here.

## Sample Preparation of Model Blood Sample\* and Plasma Separation for Dual Isolation

In order to mimic blood samples from patients with lung cancer, a model blood sample from a patient with lung cancer was prepared by spiking two model circulating markers, cancer cells (model of CTCs) and fragmented genomic DNA containing *EGFR* mutations (model of ctDNA), into an untreated healthy donor's whole blood before any plasma separation or preprocessing steps.

- CTC model: A549 is a human non-small-cell lung cancer cell line with the wild-type *EGFR*
- ctDNA model: Anchor Molecular multiplexed 23 ctDNA Standard enzymatically fragmented (~145 bp band >95%)

### Sample Preparation of Model Blood Sample from Patient with Lung Cancer (optional)\*

1. Prepare a cell stock suspension at a concentration of ~10–20 cells/μl.
2. Prepare a ctDNA standard solution at a concentration of 30 ng/μl.
3. Spike 10 μl of cell stock solution into 4 ml of whole blood.
4. Spike 2 μl of ctDNA stock solution into 4 ml of whole blood (this can lead to ~30 ng of ctDNA per 1 ml of plasma).
5. Mix well with model CTC and ctDNA samples. Store this model blood sample from a patient with lung cancer on a gentle blood rocker/shaker at room temperature until use. If it is longer than 1 hr, store at 4°C.

\* Required only for initial model sample testing. Alternatively, clinical samples with known molecular pathology can be used.

### Plasma Separation Using Two-Step Centrifugations (option A)

1. Centrifuge either 4 ml of the model sample (see Sample Preparation of Model Blood Sample from Patient with Lung Cancer instructions) or 4 ml of actual clinical blood samples at 2,000 rcf for 15 min.
2. Upon first centrifugation, transfer the upper transparent yellow plasma layer (~2 ml) to a sterile centrifuge tube.
3. Shake the plasma sample mildly by hand for 15–20 seconds and centrifuge the plasma sample at 16,000 rcf for 10 min.
4. Upon second centrifugation, transfer the clear plasma layer (~2 ml) to a new sterile centrifuge tube. Store the clear plasma layer in 4°C or in an ice bucket with ice until use.
5. The leftover layer of blood cells (~2 ml) from step 2 will be further diluted with 1x phosphate buffered saline (PBS) (for enrichment) or diluted fixing reagent (for enumeration). See the Rare Cell Enrichment/Enumeration Workflow Using the Genesis System with Celselect Slides section for details. Store the leftover layer of blood cells on a gentle blood rocker/shaker at room temperature until use. If it is longer than 1 hr, store at 4°C.

### Plasma Separation Using a Ficoll-Paque PLUS Media Solution (option B)

- To a 15 ml conical sterile centrifuge tube, add either 4 ml of the model sample (see Sample Preparation of Model Blood Sample from Patient with Lung Cancer instructions) or 4 ml of clinical sample blood. Then add 4 ml of 1x PBS to the same tube.
  - Mix the blood and PBS by inverting the tube several times.
  - To a new 15 ml conical sterile centrifuge tube, add 4 ml of Ficoll-Paque PLUS Media solution.
  - Carefully layer the diluted blood (from step 2) onto the Ficoll-Paque PLUS Media solution. It is important, when layering the sample, to not mix the Ficoll-Paque PLUS Media solution and the diluted blood sample.
  - Centrifuge at 400 g for 30 min at 18°C while the brake is set to 0.
  - Upon centrifugation, transfer the upper layer (~6 ml) containing diluted plasma and platelets to a new conical tube using a sterile pipet, leaving the buffy coat layer containing mononuclear cells undisturbed at the interface.
  - Carefully transfer the buffy coat layer containing mononucleated cells (~2 ml) to a sterile centrifuge tube using a sterile pipet.
  - Centrifuge the diluted plasma and platelets (~6 ml) at 16,000 rcf for 10 min and transfer the clear plasma layer (~6 ml) to a new sterile centrifuge tube. Store the clear plasma layer at 4°C or in an ice bucket with ice until use.
  - The layer of buffy coat (from step 7) will be further diluted with 1x PBS (for enrichment) or diluted fixing reagent (for enumeration). See the Rare Cell Enrichment/Enumeration Workflow Using the Genesis System with Celselect Slides section for details. Store the leftover layer of blood cells on a gentle blood rocker/shaker at room temperature until use. If it is longer than 1 hr, store at 4°C.
- In Preparing the Blood Sample (see user manual), use either the blood sample from step 5 in **option A** or step 9 in **option B**.
    - Label 15 ml conical tubes for each sample.
    - Pipet ~2 ml of processed, plasma-separated blood sample into its respective 15 ml conical tube.
    - Add 6 ml of 1x PBS (blood sample from **option A**) or 4 ml of 1x PBS (blood sample from **option B**) and shake the processed blood–PBS mixture vigorously by hand 15–20 seconds.
  - In Running the Genesis System (see user manual), add 6–8 ml of sample to Inlet Funnel of each Celselect Slides Station.
  - Follow the remaining steps in Running the Genesis System.

### Celselect Slides Enumeration (direct/indirect)

- Refer to the Celselect Slides CTC Enumeration Direct Staining User Manual ([bulletin 3339](#)) or Celselect Slides CTC Enumeration Indirect Staining User Manual ([bulletin 3338](#)), depending on the type of staining.
  - Performing the Genesis System Self Test
  - Celselect Slides and Slide Station Set-Up
  - Priming the Celselect Slides
  - Preparing the Reagents and Loading Reagents Cartridge
- In Preparing the Blood Sample (see relevant user manual, depending on the type of staining), use either the blood sample from step 5 in **option A** or step 9 in **option B**.
  - Label 15 ml conical tubes for each sample.
  - For each sample, add 300 µl of fixing reagent to 2.7 ml of 1x PBS. Mix well.
  - Pipet ~2 ml of processed, plasma-separated blood sample into its respective 15 ml conical tube.
  - Add 3 ml of diluted fixing reagent from step b to the ~2 ml of preprocessed blood for each sample (final volume is ~5 ml).
  - Incubate samples for 10 min on a rocker at room temperature.
  - Remove samples from the rocker and add 3 ml of 1x PBS (for diluted fixing reagent + blood sample from **option A**) (final volume is ~8 ml) or 5 ml of 1x PBS (for diluted fixing reagent + blood sample from **option B**) (final volume is ~10 ml).
  - Place blood sample on the test tube rocker until use.
- In Running the Genesis System (see relevant user manual, depending on the type of staining), add 8–10 ml of sample to Inlet Funnel of each Celselect Slides Station.
- Follow the remaining steps in Running the Genesis System.

### Rare Cell Enrichment/Enumeration Workflow Using the Genesis System with Celselect Slides

Either the sample from step 5 in **option A** or step 9 in **option B** can be used for rare cell enrichment or enumeration.

#### Celselect Slides Enrichment

- Refer to the relevant sections of the Celselect Slides Enrichment User Manual ([bulletin 3337](#)) to perform the following steps of Celselect Slides enrichment.
  - Performing the Genesis System Self Test
  - Celselect Slides and Slide Station Set-Up
  - Priming the Celselect Slides
  - Preparing the Reagents Cartridge

## Cell-Free DNA Extraction and EGFR Mutation Detection Workflow for Droplet Digital PCR

For cfDNA extraction, users can choose any circulating nucleic acid kit or cfDNA extraction kit that is available on the market. Here, two cfDNA extraction kits, MagMAX Cell-Free DNA Isolation Kit and QIAamp Circulating Nucleic Acid Kit, were each tested by following its standard protocol. Refer to each kit's standard manual for additional instruments or materials that are not supplied with the kit.

- Using the clear plasma sample from step 4 in **option A** or step 8 in **option B**, follow the instructions for manual cfDNA isolation given in the MagMAX Cell-Free DNA Isolation Kit User Guide. Either of the options for sample lysis (with or without PK) may be used. The "Prepare cell-free plasma samples" step may be skipped, as the samples have already undergone centrifugation at 16,000 rcf
- QIAamp Circulating Nucleic Acid Kit: Refer to the QIAamp Circulating Nucleic Acid Handbook for extraction protocols. Specifically, follow the Protocol: Purification of Circulating Nucleic Acids from 1 ml, 2 ml, or 3 ml Serum or Plasma using the clear plasma sample from step 4, **option A**. For the diluted plasma sample from step 8, **option B**, follow the Protocol: Purification of Circulating Nucleic Acids from 4 ml or 5 ml Serum or Plasma. If needed, the diluted plasma sample can be aliquoted into more than 2 tubes to follow the general protocols
- Elution volume from each kit is flexible and can be adapted depending on the requirements of downstream applications. Elute and make the final cfDNA extraction volume less than 50 µl

## ctDNA EGFR Mutation Detection Workflow for Droplet Digital PCR

ddPCR Mutation Detection Assays are expertly designed specifically for Droplet Digital PCR. 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.

### Reaction Setup

1. Thaw all components to room temperature. Mix thoroughly by vortexing the 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.

**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
<b>Total volume</b>	<b>20*</b>	<b>—</b>

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

3. Mix thoroughly by vortexing each tube. Centrifuge briefly to ensure that all components are at the bottom of the reaction tubes. Allow reaction tubes to equilibrate at room temperature for about 3 minutes.
4. Once the reaction mixtures are ready, load 20 µl of each reaction mix into a sample well of a DG8 Cartridge followed by 70 µl of Droplet Generation Oil for Probes into the oil wells, according to the QX200 Droplet Generator Instruction Manual (D094813). For the Automated Droplet Generator, follow instructions in the Automated Droplet Generator Instruction Manual (D112916).

### 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 protocol in 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, °C	Time	Ramp Rate	Number of Cycles
Enzyme activation	95	10 min	2°C/sec	1
Denaturation	94	30 sec	2°C/sec	40
Annealing/extension	55	1 min	2°C/sec	40
Enzyme deactivation	98	10 min	2°C/sec	1
Hold (optional)	4	Infinite	1°C/sec	1

### Data Acquisition and Analysis

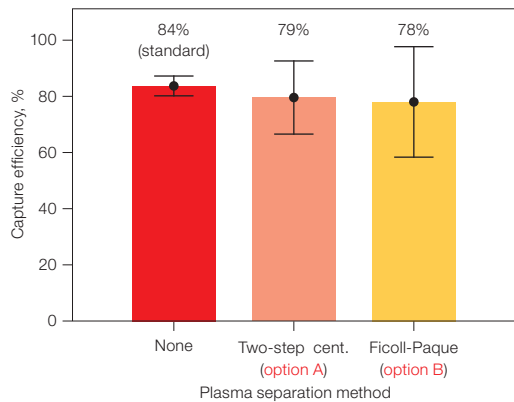
1. After thermal cycling, place the sealed 96-well plate in the QX200 Droplet Reader.
2. Open QX Manager Software Standard Edition to set up a new plate layout according to the experimental design. Refer to the QX200 Droplet Reader and QX Manager Software Standard Edition User Guide (10000107223).
3. To add a plate in 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.

- The Configure Plate window opens the window features. Fill in the plate and well information, and select the wells. Designate the sample name, experiment type, supermix type (enter ddPCR Supermix for Probes [No dUTP]), target name(s), and target (types). Select Reference Signal Ch1 for FAM and Signal Ch2 for HEX.
- Click **Apply** to load the wells and, when finished, click **OK**.
- Once the plate layout is complete, click **Run** to begin the droplet reading process. Select the appropriate dye set and run options when prompted.
- After data acquisition, select samples in the well selector under Analyze. Set the appropriate threshold for the ddPCR Mutation Detection Assay. For more detailed information about setting thresholds, refer to the Rare Mutation Detection Best Practices Guidelines (bulletin 6628).
- The concentration reported is copies/ $\mu$ l of the final 1x ddPCR reaction.

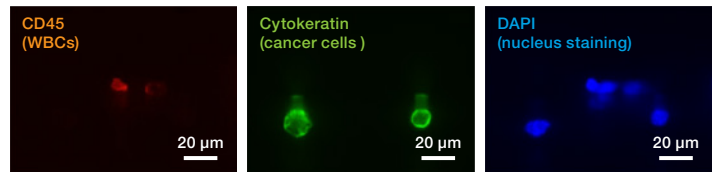
## Results

### Celselect Slides Enrichment/Enumeration (indirect)

Capture efficiency (Figure 2) and a representative image of A549 cancer cells captured in Celselect Slides and stained by CD45, cytokeratin, and 4',6-diamidino-2-phenylindole (DAPI) (Figure 3) are described here. In spite of additional plasma separation steps and liquid handlings, the Genesis System with Celselect Slides isolates cancer cells spiked in whole blood with minimal cell loss (<6%).



**Fig. 2. Capture efficiency of A549 lung cancer cells spiked in whole blood after plasma separation using the Genesis System with Celselect Slides.** The A549 cells were spiked in whole blood for a model CTC sample, which underwent either two-step centrifugations or Ficoll-Paque PLUS Media solution-based plasma separation. After plasma separation, the remaining portion of blood or buffy coat layer containing mononuclear cells was run through the Genesis System with Celselect Slides. The sample labeled "None" is a capture efficiency from standard whole blood samples without any plasma separation. Cent., centrifugation.

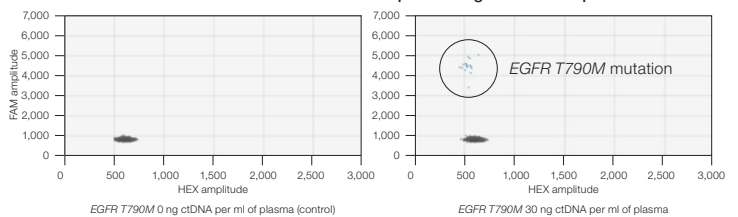


**Fig. 3. A549 cancer cells and leukocytes captured in Celselect Slides after following the indirect enumeration protocol using the Genesis System.** DAPI, 4',6-diamidino-2-phenylindole; WBCs, white blood cells.

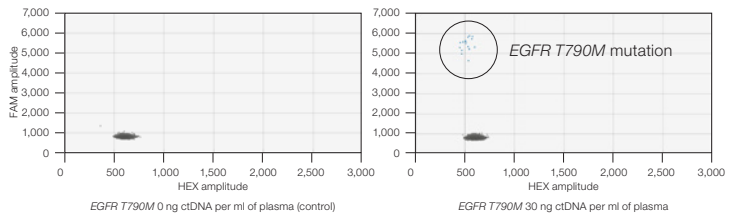
### ctDNA *EGFR T790M* Mutation Detection Using Droplet Digital PCR

2-D plots of ctDNA recovered from cell-free DNA extraction are shown in Figure 4. Bio-Rad ddPCR *EGFR T790M* Mutation Detection Assay was used. Two different ctDNA extraction methods were compatible in this study and only the blood sample with model ctDNA spiked shows positive droplets for *EGFR T790M*.

#### MAGMAX Cell-Free DNA Isolation Kit with Two-Step Centrifugation Technique



#### QIAamp Circulating Nucleic Acid Kit Isolation with Two-Step Centrifugation Technique



**Fig. 4. *EGFR T790M* DNA mutation in ctDNA enriched from whole blood.** 2-D amplitude plot of ctDNA spiked in blood in two different concentrations followed by a two-step centrifugation technique using MAGMAX Cell-Free DNA Isolation Kit and QIAamp Circulating Nucleic Acid Kit. ctDNA (30 ng) was spiked into 4 ml of plasma sample (right). High sensitivity of ddPCR Mutation Detection Assays provides precise quantification of *EGFR T790M* mutation in the cell-derived ctDNA fragments obtained from vendor. ctDNA, circulating tumor DNA.



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