Isolation and Transcriptomic Analysis of Circulating Tumor Cells Using the Genesis Cell Isolation System with Celselect Slides™ and the ddSEQ™ Single-Cell 3′ RNA-Seq Kit

Protocol

Abstract

Circulating tumor cells (CTCs) show promise as a liquid biopsy biomarker, offering molecular insights into cancer progression, survival, and treatment response due to their innate expression of cancer-associated molecules. Transcriptomic analysis of CTCs can reveal genes associated with cancer progression and metastasis. However, bulk analysis of transcriptomes or quantitative PCR (qPCR) involving specific genes in CTCs can overlook critical transcripts in cancer, and these methods offer only limited insight into the relationship between gene expression and conventional CTC enumeration data using protein markers. In contrast, single-cell RNA sequencing (scRNA-Seq) enables identification of various cell subtypes and can assist in the comprehensive study of diseases at the genetic level. Here, we present an integrated workflow to combine a label-free CTC capture system with in-droplet barcoding scRNA-Seq for efficient, cost-effective analysis of heterogeneous CTC populations. By utilizing droplet technology to capture and profile individual cells with high sensitivity, the ddSEQ Single-Cell 3′ RNA-Seq Kit offers a streamlined solution for whole transcriptome analysis at the single-cell level, even for applications with rare cell types (see Figure 1). In this protocol for CTC isolation and scRNA-Seq, we use two cancer cell types — epithelial and mesenchymal — to model CTCs at different stages of the epthelial–mesenchymal transition (EMT) and reveal distinct transcriptomic profiles using Omnition Analysis Software. We recommend that users carefully review their blood sample preparation and validate their own sample handling conditions prior to using this protocol.

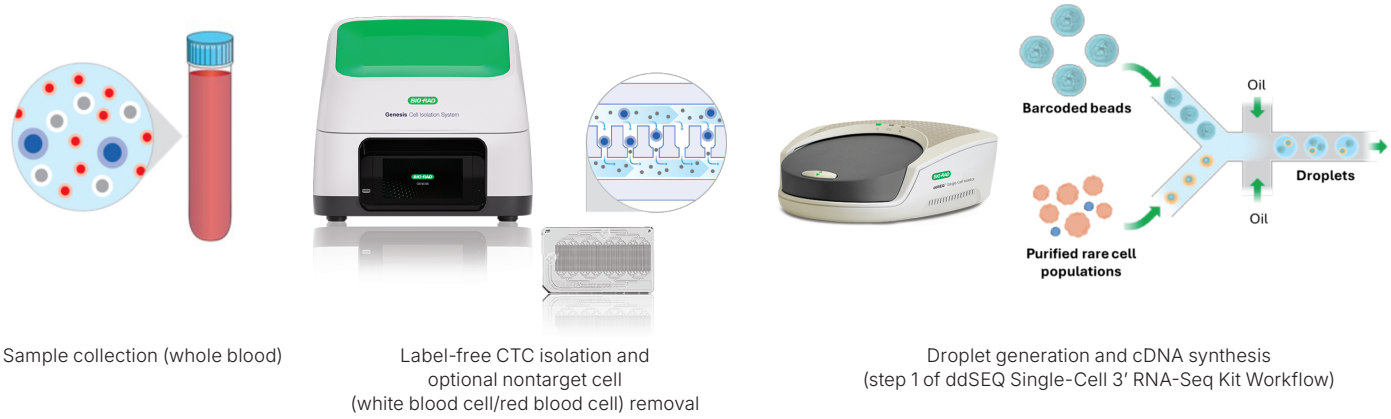

Fig. 1. Overview of single-cell CTC RNA-Seq using the Genesis Cell Isolation System with Celselect Slides and the ddSEQ Single-Cell 3′ RNA-Seq Kit.

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Instruments and Materials

Instruments and Accessories

- Genesis Cell Isolation System (Bio-Rad Laboratories, Inc., catalog #12019822)
- ddSEQ Single-Cell Isolator (Bio-Rad, #12004336)
- ddSEQ Cartridge Holder (Bio-Rad, #12004739)
- C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module (Bio-Rad, #1851197)
- TC20 Automated Cell Counter (Bio-Rad, #1450102)
- DynaMag-5 Magnet (Thermo Fisher Scientific Inc., catalog #12303D)
- ThermoMixer Temperature Control Device (Eppendorf, catalog #2231001127)
- Sorvall ST 8 Small Benchtop Centrifuge (Thermo Fisher, #75007200)

Materials

- ACK (Ammonium-Chloride-Potassium) Lysing Buffer (Thermo Fisher, #A1049201)
- Dynabeads CD45 (Thermo Fisher, #11153D)
- Bovine Serum Albumin, 10% Aqueous Solution, Nuclease-Free (Sigma-Aldrich, catalog #126615)
- DNA LoBind Tubes, 1.5 mL (Eppendorf, catalog #022431021)
- DNA LoBind Tubes, snap cap, 5 mL (Eppendorf, #0030108310)
- DNA LoBind Tubes, screw cap, conical, 15 mL (Eppendorf, #0030122208)
- Cell Counting Slides for TC10/TC20 Cell Counter, Dual-Chamber (Bio-Rad, #1450011)
- Celselect Slides Enrichment Kit (Bio-Rad, #CEL80110)
- ddSEQ Single-Cell 3′ RNA-Seq Kit (Bio-Rad, #17009671)
- ddSEQ Single-Cell 3′ RNA-Seq cDNA Module (Bio-Rad, #17010272)
- ddSEQ Single-Cell 3′ RNA-Seq Library Module (Bio-Rad, #12020413)

Note: Additional materials and equipment are needed to carry out 3′ RNA-Seq library preparation, library purification, library quality check, and sequencing. Please refer to the ddSEQ Single-Cell 3′ RNA-Seq Kit User Guide for details.

Sample Collection for Rare Single-Cell RNA Sequencing

For high-quality scRNA-Seq results, liquid samples containing rare cancer cells should be handled with caution. In order to mimic blood samples from patients with breast cancer, a blood sample was prepared by spiking breast cancer cells into an untreated, healthy donor's whole blood before any preprocessing steps.

- Rare cell input number estimation — this protocol uses 10–100 target cancer cells per 1 mL of whole blood to mimic CTCs in blood samples from patients with cancer. As this protocol does not have a step for immunostaining captured cells to detect CTCs, users should run a parallel enumeration protocol separately, if needed, to evaluate potential rare cell input to this workflow. We recommend using clinical samples with a minimum of 50 cancer cells per 1 mL of whole blood when following this protocol. Please note that scRNA-Seq results may vary based on the user's proficiency in handling rare cell samples
- Blood tube compatibility — this protocol recommends standard ethylenediaminetetraacetic acid (EDTA) tubes for blood collection. Streck tubes (Cell-Free DNA BCT RUO) have shown good stability for standard CTCs and ctDNA applications, including Celselect Slides enumeration. However, the fixative in the tube may impact the scRNA-Seq library quality and final sequencing results. If the use of Streck tubes is unavoidable, refer to notes on the Agilent Bioanalyzer result comparison and precautions for Streck tube samples in the Results section Blood Collection Tube Type Compatibility below
- Safe stops and post-enrichment sample storage — before following this protocol, make sure you are familiar with the general Single-Cell 3′ RNA-Seq Kit Workflow (page 7 in ddSEQ Single-Cell 3′ RNA-Seq Kit User Guide Version 1.0). We recommend completing step 1, Droplet Generation and cDNA Synthesis, on the same day as the rare cell isolation using the Genesis Isolation System with Celselect Slides. Post-enrichment samples should not be stored at –20°C; if needed, they can be stored at 4°C for no more than 12 hr. Do not fix post-enrichment samples for long-term storage. For optimal results, plan to perform the rare cell enrichment, droplet generation, and cDNA synthesis on the same day

Sample Preparation of Model Blood Sample from Cancer Patient (optional)*

1. Prepare a cell stock suspension at a concentration of ~10–20 cells/µL.
2. Spike 10–100 µL of the cell stock solution into 7.5 mL of whole blood collected in EDTA blood collection tubes (Streck tubes can be used with caution).
3. Mix well with model CTC samples. Keep the model blood sample on a gentle blood rocker/shaker at room temperature until use, but store at 4°C if longer than 1 hr.

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

Label-Free CTC Isolation and Nontarget Cell Removal (optional)

Although Celselect Slides effectively remove 99.9% of red blood cells (RBCs), white blood cells (WBCs), and other blood components, small amounts of residual contamination in samples with extremely rare CTCs may remain, which could interfere with droplet generation of rare cells and single-cell 3′ RNA library preparation. This optional nontarget cell removal step is designed to improve the overall quality of the library.

- RBC removal — ACK Lysing Buffer can be added to two chambers of the enrichment reagents cartridge. RBCs will be lysed during the Genesis System enrichment protocol
- WBC removal — for post-enrichment samples, a manual magnetic bead–based WBC removal step can be used. This 30 min step removes an additional 93% of WBCs after enrichment while minimizing loss of target rare cells (less than 15%)

On-System Red Blood Cell Lysis Using ACK Lysing Buffer (optional)

1. Pierce the top foil of the Reagents Cartridge with the Reagents Cartidge Venter, making sure to twist the venter to open the foil.

2. Add 2 mL of ACK Lysing Buffer to chambers 7 and 8 of each Reagents Cartridge.

Note: Protect the ACK Lysing Buffer and Reagents Cartridges from light and store at room temperature until use.

3. Follow the standard enrichment protocol with the prepared model sample or clinical sample.

White Blood Cell Removal Using Dynabeads CD45 (optional)

1. Prepare 4 mL of 1x phosphate buffered saline (PBS) + 0.1% bovine serum albumin (BSA) immediately before the Cell Recovery procedure (refer to page 6 of Celselect Slides Enrichment Kit 2.0 User Manual, bulletin 3337).
2. During the Cell Recovery step, prepare 100 µL of Dynabeads CD45 (100 µL for one sample) as follows:
   a. Dispense a 100 µL aliquot into a 1.5 mL LoBind microcentrifuge tube and place it on a magnetic rack.
   b. After pelleting for 1–2 min, discard the supernatant.
   c. Resuspend the beads in 500 µL of 1x PBS + 0.1% BSA and magnetize again.
   d. Discard the supernatant and resuspend the beads in 100 µL of 1x PBS + 0.1% BSA.
3. Upon completion of enrichment, collect the entire volume of post-enrichment sample into a 5 mL LoBind Tube.
4. Add 100 µL of washed Dynabeads CD45 from step 2 to the tube from step 3 and mix well by vortexing.
5. Incubate at room temperature or 4°C for 30 min on a rocker.
6. Place the tube on the magnet; collect and transfer the entire volume to a new 5 mL LoBind Tube.

ddSEQ Library Preparation

1. Prepare 1 mL of a 1x PBS + 0.1% BSA solution.
2. Either the post-enrichment sample (~4 mL) or the sample from step 6 of White Blood Cell Removal Using Dynabeads CD45 can be applied as the input sample for the ddSEQ library preparation.
3. Centrifuge the tube at 600 rcf for 5 min. Remove the supernatant, leaving approximately 100 µL of the supernatant and cell pellet undisturbed.
4. Add 400 µL of 1x PBS + 0.1% BSA to the tube from step 3 and resuspend well.
5. Centrifuge the tube at 600 rcf for 5 min and remove the supernatant, leaving 5.2 µL.

Note: Despite RBC removal, users may still see a red pellet at the bottom of the tube where the target cells are. Do not remove this pellet.

Care should be taken when removing the supernatant. The remaining 5.2 µL of the pellet will go directly to the input sample volume for droplet generation without cell counting to minimize cell loss. Adjust the cell suspension accordingly to ≤5.2 µL.

6. Refer to the page 7 of the ddSEQ Single-Cell 3′ RNA-Seq Kit User Guide for an overview of the workflow.
7. For library preparation, follow the instructions on page 30 of the ddSEQ Single-Cell 3′ RNA-Seq Kit User Guide.

Note: For optimal results, we recommend performing step 1, Droplet Generation and cDNA Synthesis, on the same day as the rare cell isolation using the Genesis System with Celselect Slides.

Important: For samples with low cell numbers (cell number ≤2,500), the addition of 1 µL of carrier DNA (page 61 of ddSEQ Single-Cell 3′ RNA-Seq Kit User Guide) and using 14 PCR cycles for the cDNA index (page 65 of ddSEQ Single-Cell 3′ RNA-Seq Kit User Guide) are necessary.

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Results

Blood Collection Tube Type Compatibility

In order to mimic the CTCs collected in different types of blood collection tubes, cancer cells (MCF7) were spiked into blood from a healthy donor collected in either an EDTA tube or a Streck tube and incubated for 30 min to 1 hr. All samples underwent both RBC removal and WBC removal as described in the protocol. Upon completion of the scRNA-Seq library preparation, the quality of cDNA libraries was checked and confirmed using an Agilent 2100 Bioanalyzer (Figure 2). The samples collected in EDTA tubes showed a trace between the 200–1,200 bp region with one peak. Conversely, the samples collected in Streck tubes showed a wider range of cDNA, with one or two peaks (200–5,000 bp).

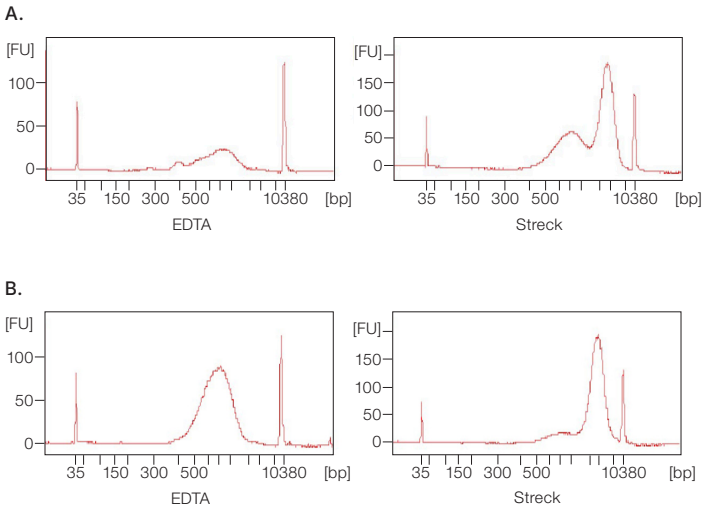

Fig. 2. Sample Agilent Bioanalyzer traces for cDNA after library preparation using the ddSEQ Single-Cell 3' RNA-Seq Kit. A total of 100 (**A**) or 500 (**B**) cancer cells (MCF7) were spiked into blood from a healthy donor collected in an EDTA tube or a Streck tube. EDTA, ethylenediaminetetraacetic acid.

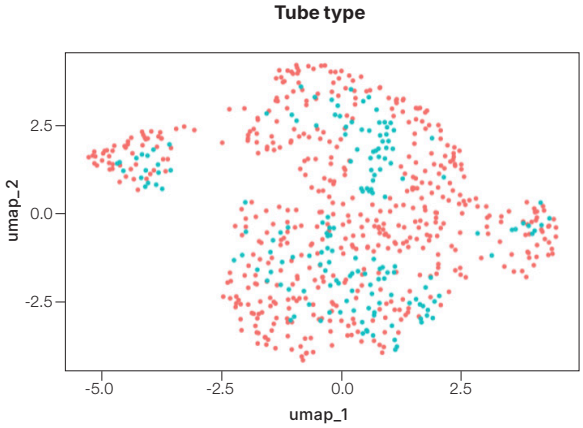

Fig. 3. Clustering analysis of model circulating tumor cells (CTCs) from blood collected in two different tubes. EDTA (●) and Streck (●).

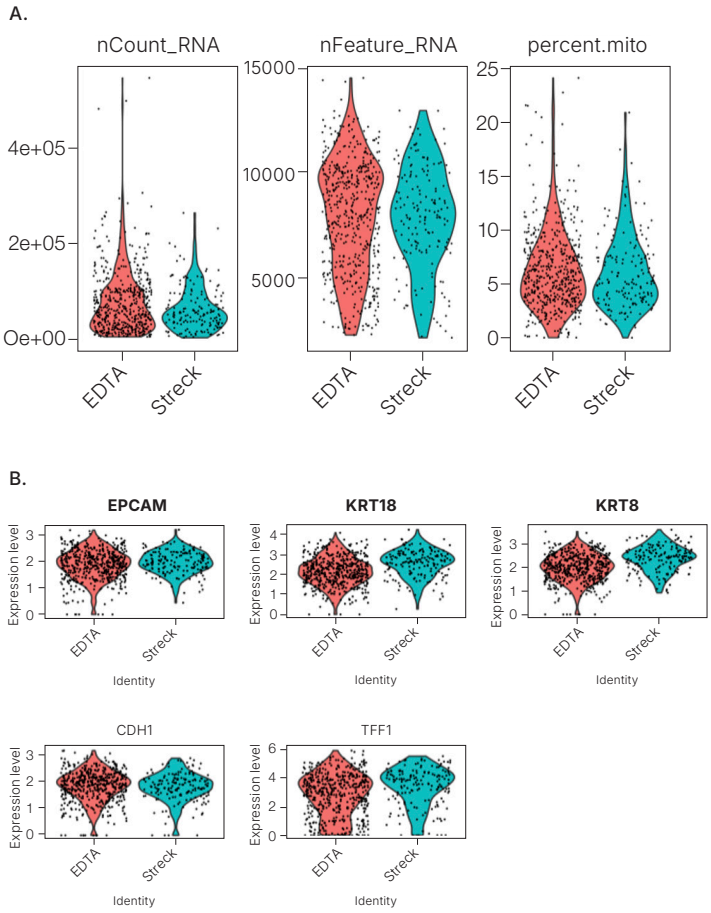

Fig. 4. Tertiary analysis of model CTCs (MCF7) spiked into blood from a healthy donor collected in EDTA or Streck tubes. A, violin plots for the number of unique UMIs and number of unique genes; **B**, violin plots of representative epithelial gene expression. CTCs, circulating tumor cells; EDTA, ethylenediaminetetraacetic acid; UMI, unique molecular identifier.

Important: To determine library size ranges and concentrations for sequencing loading:

For cDNA libraries using samples collected in Streck tubes, capture the 200–5,000 bp region. For deconvolution oligonucleotide (DO) libraries using samples collected in Streck tubes, capture the 200–500 bp region (same as the standard recommendation).

For cDNA and DO libraries using samples collected in EDTA tubes, follow the standard instructions described on page 78 of the ddSEQ Single-Cell 3' RNA-Seq Kit User Guide.

The clustering analysis (Figure 3) and tertiary analysis (Figure 4) of model CTCs demonstrated that both tubes are compatible with the protocol used to detect cancer-associated genes at a single-cell level. However, results for clinical samples collected in these tubes with longer incubation times (>1 hr) may vary.

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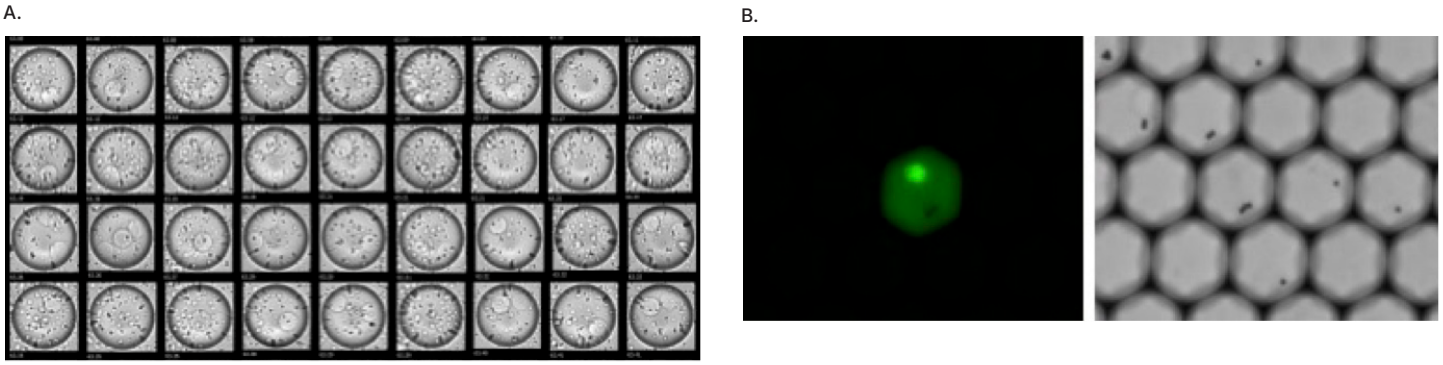

Fig. 5. ddSEQ droplet size evaluation and model CTCs in ddSEQ droplets with beads for scRNA-Seq. A, individual droplets with model CTCs and bead(s) were evaluated using a FlowCam; the median droplet size was determined as 98.3 ± 1.6 µm, which is sufficient to capture a small to large individual CTC or CTC cluster. **B**, MCF7 cells were pre-dyed using CellTracker FITC Dye (Thermo Fisher) before cell isolation using the Genesis System. Post-enrichment model CTCs were captured in individual droplets at the single-cell level together with beads for single-cell RNA sequencing. CTCs, circulating tumor cells; FITC, fluorescein isothiocyanate.

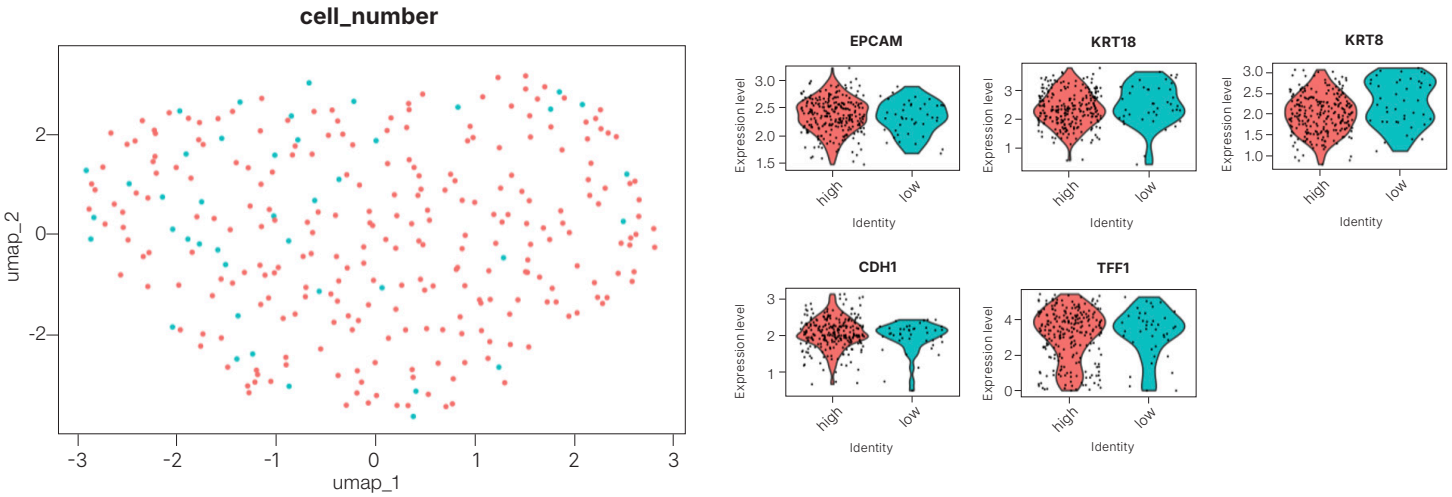

Fig. 6. Transcriptomic analysis of model circulating tumor cells (CTCs; MCF7) spiked into blood at different concentrations. After tertiary analysis, both high cell input (500–1,000 cells/7.5 mL of blood) and low cell input (100–499 cells/7.5 mL of blood) groups showed representative genes. **A**, clustering analysis of model CTCs spiked into high and low cell number groups; **B**, violin plot of representative epithelial gene expression in cells.

Transcriptomic Analysis of Model CTCs Using Cancer Cells Spiked into Blood at Various Concentrations

We performed a transcriptomic analysis of model CTCs spiked into blood at varying concentrations. As this protocol integrates both single-cell isolation and library preparation, some cell loss may occur at each step. Two groups were tested: a low-cell group (100–500 cells per 7.5 mL of blood) and a high-cell group (501–1,000 cells per 7.5 mL). Both groups underwent red and white blood cell removal post-enrichment, followed by droplet encapsulation with bead(s) (Figure 5); the prepared libraries were analyzed using Omnition Analysis Software v1.1.0 and Seurat v5.0.3. Cells with a high proportion of mitochondrial transcripts were excluded from downstream analysis. Comparisons between tube types and input cell numbers were performed without sample integration.

Tertiary analysis demonstrated the feasibility of profiling cancer-associated genes, even in samples with low cell numbers (Figure 6).

Evaluation of the EMT/MET Status of Circulating Rare Cells

Transcriptomic analysis of CTCs can identify candidate genes associated with cancer progression and metastasis. In addition, scRNA-Seq of CTCs facilitates the study of their inherent heterogeneity. In order to model heterogeneous

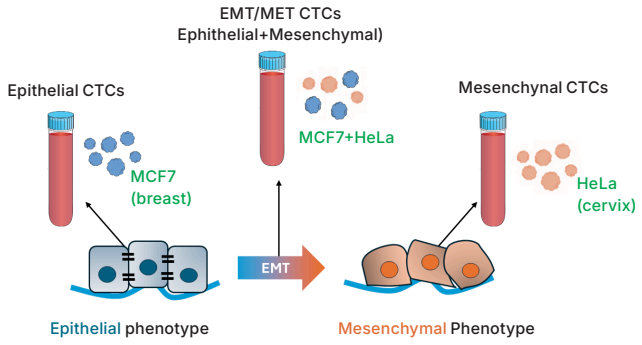

Fig. 7. Contrived sample preparation for mimicking CTCs in different EMT/MET status. CTCs, circulating tumor cells; EMT, epithelial–mesenchymal transition; MET, mesenchymal–epithelial transition.

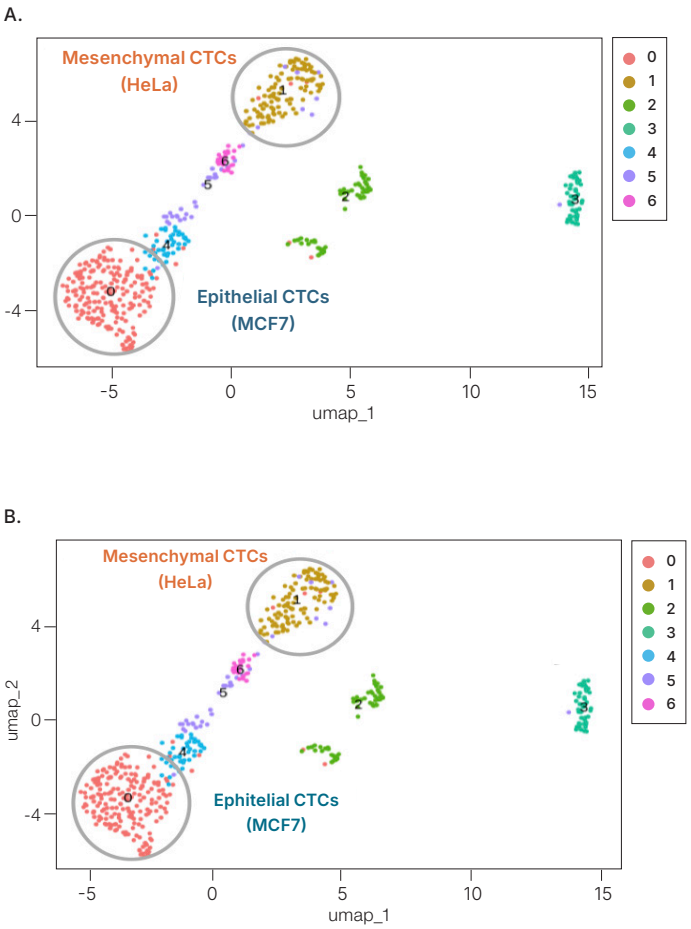

Fig. 8. Clustering analysis of model CTCs. After scRNA-Seq and Omnition Software analysis, individual cells were visualized using a UMAP plot. **A**, clustering of the heterogenous model CTC sample; **B**, UMAP without clustering labeled by sample type, one containing only MCF7 cells and the other containing MCF7 and HeLa cells. CTCs, circulating tumor cells; EMT, uniform manifold approximation and projection.

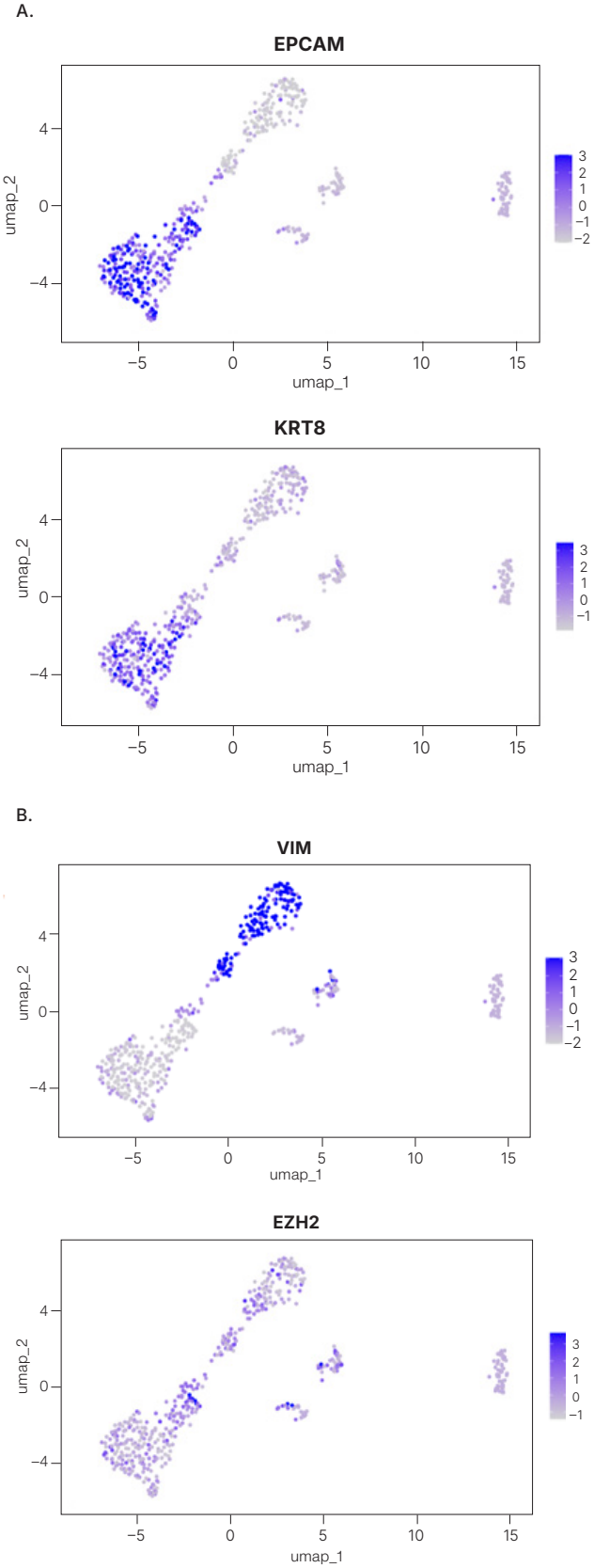

Fig. 9. Gene expression of model circulating tumor cells (CTCs) spiked into blood samples. Further clustering of each different cell population revealed expression of representative genes for each cell type: **A**, EpCAM and KRT18 for epithelial CTCs and **B**, VIM and EZH2 for mesenchymal CTCs.

CTC subpopulations at different EMT statuses, samples were prepared by spiking two types of cancer cells — MCF7 (epithelial CTCs) and HeLa (mesenchymal CTCs) cells — into whole blood from a healthy donor at a concentration of 10–100 cells/mL (Figure 7). A total of 7.5 mL of whole blood was used for model sample preparation. The model sample was subjected to the WBC removal procedure specified in White Blood Cell Removal Using Dynabeads CD45, and the library was prepared by following the current protocol.

The prepared library was sequenced using an Illumina® NextSeq® 2000 System and primarily analyzed using Omnition Analysis Software v1.1.0.

Tertiary analysis (Figures 8 and 9) was carried out using Seurat v5.0.3. Cells with a high percentage of their transcriptome originating from mitochondria were removed from downstream analysis. Analysis of MCF7 and HeLa cells spiked into blood sample was integrated using Seurat's SCT integration method. Clustering analysis (Figure 8) showed distinctive clusters between two different samples, and gene expression analysis (Figure 9) revealed representative mesenchymal and epithelial gene expression in each sample.

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