liquidbio

Instructions for Use

IsoFlux EMT CTC Enrichment Kit

Applies to: 910-0106

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Revision: F

INTENDED USE

The intended use for the IsoFlux™ EMT CTC Enrichment Kit is as a general-purpose laboratory reagent for preparation of blood samples to enrich circulating tumor cells (CTCs). The kit is used with the IsoFlux System, a bench-top instrument for semiautomated cell isolation. The kit contains immunomagnetic beads and reagents targeted towards cells of epithelial origin. The EMT CTC Enrichment kit is for Research Use Only, and is recommended for use as preparation for molecular analysis (NGS, ddPCR).

SUMMARY AND EXPLANATION

Circulating tumor cells (CTCs) are cancer cells that shed from a primary or metastatic tumor and enter the peripheral circulation. Carcinomas are cancers of epithelial origin and include breast, prostate, lung, and colorectal cancers. These tumors shed CTCs that are of epithelial origin. CTCs are distinct from other blood cells since cells of epithelial origin are not normally found in the circulation.

The IsoFlux EMT CTC Enrichment Kit is designed to standardize and automate the enrichment of CTCs from biological samples using the IsoFlux System. CTCs are enriched from the sample using an immunomagnetic capture reagent while the sample flows through a microfluidic cartridge designed for cellular isolation. The kit produces an enriched cell pellet that can subsequently be used for further testing.

PRINCIPLES OF THE PROCEDURE

The IsoFlux EMT CTC Kit contains immunomagnetic capture beads (CTC beads), microfluidic cartridges, and additional reagents required for performing CTC enrichments. The CTC beads consist of two types micro-scale particles with a magnetic core surrounded by a polymeric layer; they are coated with antibodies targeting the EpCAM antigen and a

second bead type with a linker chemistry that allows them to also be conjugated to mouse monoclonal EGFR and other epithelial to mesenchymal transition (EMT) antibodies. CTCs can be isolated from mononuclear cell suspensions of whole blood or other similar cell samples. CTC beads are then mixed with the cell sample to bind to the target cells during a period of incubation.

The cell sample and beads mixture is loaded onto the microfluidic cartridge and processed with the IsoFlux instrument, where the cells pass through the fluidic channel of the cartridge. Midway through the fluidic channel is a cell isolation zone that is exposed to an external magnetic field inside the instrument. The target cells having CTC beads attached are attracted towards the magnetic field. The target cells are collected on a removable disk that forms the roof of the isolation zone. After the sample is processed, the enriched cells are transferred inside the instrument to a low volume recovery holder or a microfuge tube. The enriched CTCs are ready for further analysis.

MATERIALS PROVIDED

Instructions for Use
8 sterile microfluidic cartridges (includes 8 low-volume recovery holders, 8 microfuge tubes for cell recovery)
1 tube of 500µL CTC beads* (EpCAM pre-conjugated)
2 tubes of 500µL RCE (pan mouse IgG) beads* (un-conjugated)
1 tube of 140µL EGFR antibody (EGFR Ab)
1 tube of 40µL EMT antibody 1 (EMT Ab1)
1 tube of 10µL EMT antibody 2 (EMT Ab2)
1 tube of 40µL EMT antibody 3 (EMT Ab3)
1 tube of 500µL Fc blocker reagent*
4 tubes of 12mL sterile preservative free Binding Buffer

*Contains 0.02% sodium azide as a preservative.

REAGENT STORAGE AND HANDLING

- EMT Ab1 and EMT Ab2 should be used immediately or stored at -20°C. Once thawed, the antibodies may be kept at 2° to 8°C and used within 60 days. Avoid refreezing. EGFR Ab and EMT Ab3 should be used immediately or store at 2° to 8°C. Do not freeze.
- CTC, RCE beads and Fc blocker reagents should be stored at 2° to 8°C and used within 60 days after opening. Do not freeze.
- Binding Buffer should be stored unopened at 2° to 8°C. After opening, unused buffer may be stored frozen at -20°C, thawed once, and used within 60 days. When properly stored, reagents are stable until the expiration date printed on the reagent container, kit box, or otherwise specified above. Do not use expired reagents.
- Protect reagents from heat in excess of 35°C.
- Protect reagents from exposure to light.

- Microfluidic cartridges should be stored unopened at room temperature. Do not mix and match reagents from different kits.

MATERIALS REQUIRED, NOT PROVIDED

- IsoFlux Instrument (Catalog No. 950-0100)
- Permanent magnets (accessory parts included with IsoFlux instrument: large round and small cylindrical magnets)
- Swing bucket centrifuge capable of 1500xg (with brake settings)
- Test tube racks
- Calibrated micropipettes and tips
- Serological pipettes and pipettor
- 5 or 2mL microfuge tubes (preferably low retention)
- Microfuge tube rotator
- 50mL Leucosep® tubes (with frit) (Greiner, Catalog No. 227290)
- Phosphate Buffer Saline without Ca2+ Mg2+ (PBS-CMF)
- 50mL conical tubes
- Ficoll-Paque™ Plus (GE Healthcare, Catalog No. 17-1440-02)
- Optional: CTL-Wash™ Supplement (CTL, Catalog No. CTLW-010)
- Optional: Benzonase® Nuclease (Sigma, Catalog No. E8263)
- Optional: Nylon Mesh Cell Strainer, 40 µm (BD, Catalog No. 352340)

WARNINGS AND PRECAUTIONS

- For Research Use Only
- Please read the entire contents of the Instructions for Use before processing samples. **Caution:** Care should be taken to collect and transfer blood samples before processing. Cells are fragile and can be damaged or lost if not handled properly. **Caution:** All personnel should follow universal precautions for biological sample handling and use personal protective equipment (i.e., safety glasses, laboratory coat, gloves, etc.).
- **Caution:** Microbial contamination of reagents can cause erroneous results and should be avoided.
- **Warning:** All biological specimens, cartridges and other materials coming into contact with the specimen(s) are considered bio-hazardous. Handle as if capable of transmitting infection. Treat and dispose of waste using proper precautions and in accordance with local, state, and federal regulations. Never pipette by mouth.
- **Warning:** Some of the reagents contain sodium azide as a preservative. If swallowed, seek medical advice immediately. Keep out of reach of children. Keep away from food and drink. Wear suitable protective clothing. Contact with acids liberates very toxic gas. Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in lead or copper plumbing where explosive conditions can develop. Operator training is required to perform the test procedure.

ENRICHMENT PROCEDURE

Specimen collection and preparation

1. Collect biological samples aseptically into an appropriate sample collection tube. 2. If samples are being shipped or transported, pack the samples accordingly to control exposure to excessive temperatures or agitation. Typically, samples can be shipped in an insulated Styrofoam shipping container with cold (not frozen) gel packs as a buffer to temperature fluctuations.
3. Depending on the sample type, a pre-processing step might be required such as density centrifugation or red blood cell lysis.
4. A typical pre-processing procedure for human whole blood collected in 10mL K2EDTA tube (mononuclear cell fraction preparation)is provided in Section 3. Please consult the manufacturer's instructions for pre-processing procedure for other cell sample types.

Antibody to Beads Coupling reaction: coating RCE beads with antibody cocktails and CTC beads preparation

Note: This preparation suffices for **5 samples**. Antibody-coupled beads are stable at 4°C and should be used within 4 weeks. This preparation is to be used in conjunction with the CTC (EpCAM) beads prepared below. Scale up or down as appropriate.

1. Thaw all antibody tubes on ice. Briefly centrifuge all the antibody tubes to bring the solutions to the bottom of the tube. Set aside on ice until use.
2. Briefly centrifuge the RCE beads (IgG beads) tube to bring down any liquid or beads from under the lid. Carefully open the lid and resuspend the RCE beads stock to a uniform suspension with a micropipette.
3. Dispense all 500µL of RCE beads stock into a new 1.5mL microfuge tube. Place the tube on the round magnet for 15 seconds until all the beads accumulate. With the magnet in place, remove and discard the storage buffer. Remove the tube from the magnet. Wash the bead with 1mL of Binding Buffer, using the force of dispensing the buffer to resuspend the beads. Place the tube on the magnet for 15 seconds and discard the supernatant. Remove the tube from the magnet. Resuspend the washed beads in **400µL** Binding Buffer.
4. Add to the washed beads tube
    1. 60µL of EGFR Ab
    2. 13µL of EMT Ab1
    3. 4µL of EMT Ab2
    4. 13µL of EMT Ab3
5. Close the lid. Gently invert to mix.
6. Place the round magnet on one side of the tube so that the beads accumulate. Remove the tube from the magnet and invert several times to resuspend the beads.

Repeat this process 10 times. This is referred to as ACTIVE MIXING.

7. Incubate for 1 hour at room temp (or overnight at 4°C) with gentle tilting and rotation.The antibody cocktail conjugated beads are now referred as EMT beads. *Beads are now ready to be washed and used. Use 90µL of beads suspension per sample. The preparation below suffices for 5 samples. Unused (unwashed) beads may be stored at 4°C and should be used within 4 weeks.*

Wash EMT Beads

1. Briefly centrifuge the tube for 5 second to bring down any liquid or beads that may have remained under the lid.
2. Place the tube on the round magnet for 15 seconds until all the beads accumulate.
3. With the magnet in place, remove and discard the antibody buffer.
4. Remove the tube from the magnet.
5. Wash the bead with 1mL of Binding Buffer, using the force of dispensing the buffer to resuspend the beads.
6. Place the tube on the magnet for 15 seconds and discard the supernatant.
7. Remove the tube from the magnet. Repeat the wash one more time. Place the tube on the magnet for 15 seconds remove and discard the wash.
8. Resuspend the washed beads in 500µL Binding Buffer.
9. Keep on ice until use. *Be mindful not to lose the beads. Keep the magnet in contact with the tube whenever washing is required.*

Wash CTC (EpCAM) Beads

This preparation suffices for 5 samples to be used in conjunction with the EMT beads prepared above. Use 30µL of CTC beads suspension per sample. Prepare 40µL per sample to allow room for pipetting. Scale up or down as appropriate.

1. Re-suspend the CTC (EpCAM) beads stock to a uniform suspension with a micropipette.
2. Dispense 200µL of beads stock into a new 1.5mL microfuge tube.
3. Place the tube on the round magnet for 15 seconds until all the beads accumulate.
4. With the magnet in place, remove and discard the buffer.
5. Remove the tube from the magnet. Wash the bead with 1mL of Binding Buffer, using the force of dispensing the buffer to re-suspend the beads.
6. Place the tube on the magnet for 15 seconds remove and discard the wash.
7. Remove the tube from the magnet.
8. Re-suspend the washed beads in 200µL Binding Buffer. Keep on ice until use.

Pre-processing of whole blood sample (mononuclear cell fraction preparation)

1. For each sample to be processed coat a microfuge tube with 500µL of Binding Buffer

and rotate at 4°C (or room temp) until use. *Alternatively if using Protein LoBind tubes (Eppendorf Cat. No. 022431081), coating is not required.*

2. Prepare the 50mL Leucosep® (with frit) tube by adding 15.2mL of FicollPaque™ PLUS and centrifuging the tube at 1000xg for 30 seconds with the brake setting to ON.
3. Only when ready to process the blood sample, gently add 5mL of PBS-CMF to the Leucosep® tube.
4. Immediately decant the blood from the blood collection tube into the Leucosep® tube.
5. Gently rinse down the wall of the blood collection tube with 10mL of PBS-CMF.
6. Re-cap and gently invert the blood collection tube several times to mix.
7. Add the rinse to the same Leucosep® tube. Repeat the rinse once more.
8. Immediately centrifuge the tubes at 800xg for 15 minutes with the brake setting to OFF.
9. Decant the supernatant from Leucosep® tube into a new 50mL conical tube, leaving about 5 to 10mL remaining.
10. Gently swirl the remaining supernatant to dislodge any cells that may be stuck to the wall of the Leucosep® tube and then decant it into the same conical tube. 11. Rinse the wall of the Leucosep® tube with 10mL of PBS-CMF and add that to the same 50mL conical tube.

Be careful not to suction the Ficoll-Paque™ PLUS through the frit; avoid pressing the pipette against the frit. Optional: CTL-Wash™ Supplement may be added to improve cell viability.

12. Centrifuge at 280xg for 10 minutes with the brake setting to ON.
13. Use a 25 or 50mL serological pipette to gently aspirate off the supernatant as much as possible without disturbing the pellet. Use a 5mL pipette to remove the remaining supernatant closer to the bottom of the tube. Alternatively, a vacuum system set-up may be used. Gently aspirate off the supernatant and avoid disturbing the pellet (up to ~500µL buffer may be left remaining).

This is considered **PBMC**. *We recommend that you do not decant the supernatant, because the pellets might be very loose in clinical samples.*

Optional: *Benzonase® Nuclease (up to 1000 Units per sample) may be added.Addition of nucleases is required for samples that have been stored for ≥24 hours or severely lysed to minimize cell aggregation due to lysis.*

14. Add 40µL of Fc Blocking Reagent to the PBMC sample in the 50mL conical tube above.
15. Gently tap the tube on the bench a few times to loosen pellet. Tap patiently until the pellet is completely resuspended. As necessary, add up to 300µL of Binding Buffer to the tube. All cell clumps must be dispersed as they may clog the micro-channel during isolation. Incubate for 5 minutes on ice.
16. Remove and discard the Binding Buffer from the microfuge tube prepared in Step 1.
17. Gently resuspend the cell in the 50mL conical tube with a micropipette setting to no more than 300µL.
18. Transfer the cell suspension into the microfuge tube.

19. Rinse the residual cells in the 50mL conical tube with Binding Buffer and transfer to the same microfuge tube. The final volume should be no more than 1mL.

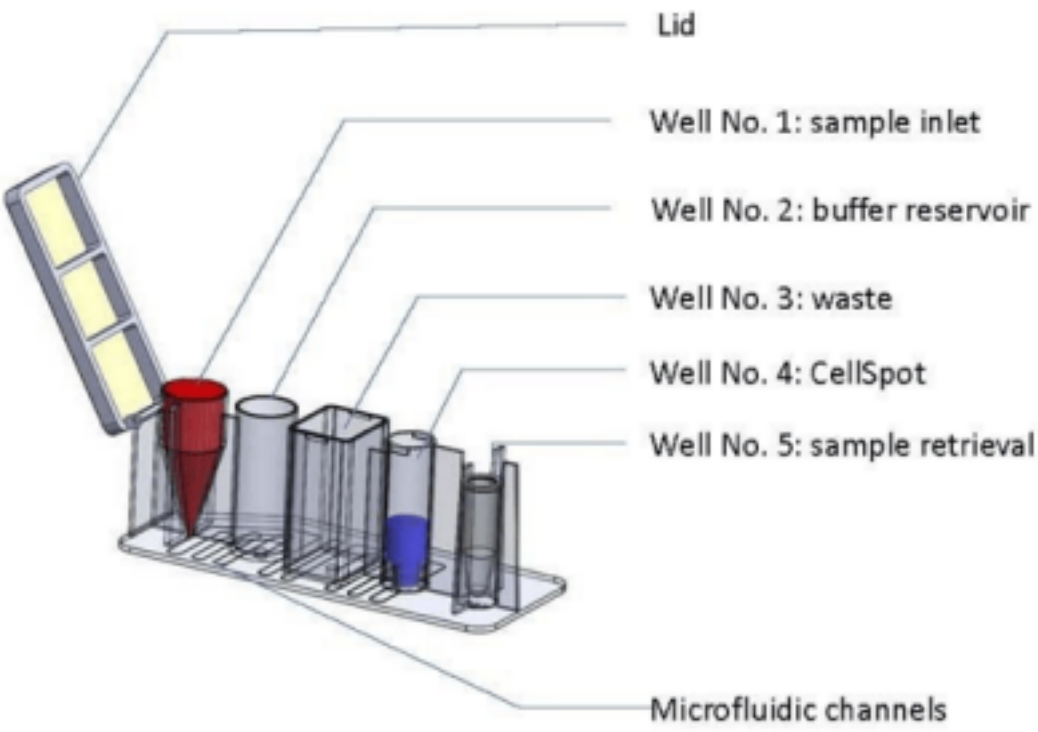
Caution: maximum sample volume is 1 mL when loading onto the cartridge. Try to prepare the sample at the above step so that the volume is about 800µL to allow for additional rinsing when loading onto the cartridge. We recommend using a 200µL micropipette with wide-bored tip when transferring to minimize shear force, and allow for estimating the sample volume.

Coupling reaction of beads and cell sample:

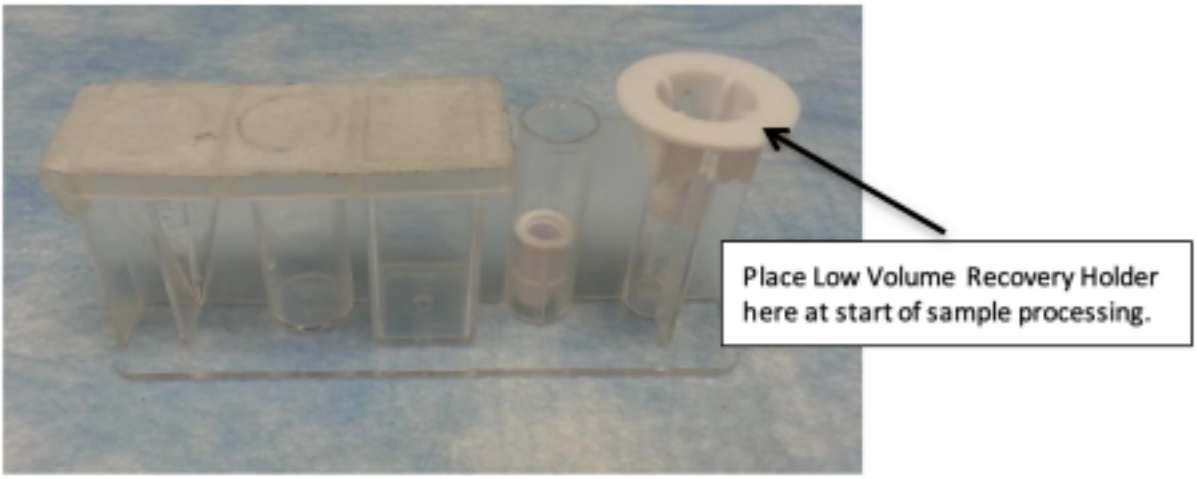
1. Resuspend the washed CTC beads with a micropipette. Add 30µL of washed CTC beads to each sample of cell suspension.
2. Resuspend the washed EMT beads with a micropipette. Add 90µL of washed EMT beads to each sample of cell suspension.
3. Invert the tube to mix. ACTIVE MIX
4. Incubate for 1.5 hours at 4°C with gentle tilting and rotation. The cell sample is now ready for CTC enrichment with the IsoFlux System. *Optimum incubation time is 2 hours. At 1.5 hours after incubation, the preparation for enrichment can begin.*

CTC enrichment with IsoFlux System

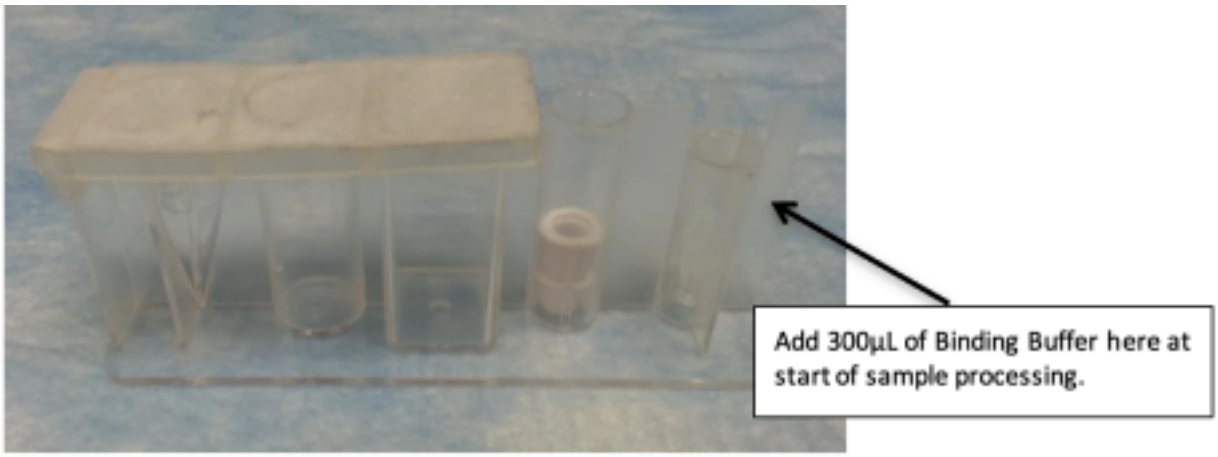
1. Refer to the IsoFlux System **Instructions for Use** and on-screen commands for full instructions to process samples for cell enrichment.
2. Power on the IsoFlux instrument. The touch screen panel will light up; the instrument will initialize and perform automatic routine system check.
3. The touch screen will display "**Run Protocol**" and "**Select Protocol**" icons when the instrument is ready for use. Choose one the options below:
    1. "**Run Protocol**" to run the most recent protocol used (shown at the bottom of the touch screen).
    2. "**Select Protocol**" to select the appropriate protocol and then press "**Run Protocol**".
4. Select the **Number of Samples to Run**. Cartridge loading carriage(s) will slide out automatically. A total 4 samples can be processed simultaneously. Positions No. 1 and 2 are on the left carriage. Positions No. 3 and 4 are on the right carriage. Samples should be loaded from left to right sequentially from Position No. 1 to 4.
5. Remove the microfluidic cartridge from the pouch and position it upright on a flat surface (see drawing below).



6. Sample retrieval Microfuge Tube is in well No. 5.
7. The Low-Volume Recovery Holder is in well No. 3.
8. Remove and keep the Low-Volume Recovery Holder to be used in the next step.
9. Decide if the enriched cells will be recovered with Low-Volume Recovery Holder or in the Microfuge Tube.
10. If the enriched cells will be recovered with Low-Volume Recovery Holder, remove the Microfuge Tube in well No. 5 and insert the Low Volume Recovery Holder as shown below:



11. If using the Microfuge tube, add 300µL of Binding Buffer to the Microfuge tube in the sample retrieval position on the cartridge (well No. 5) as shown below.



12. Carefully open the cartridge lid and add 3mL Binding Buffer to the buffer reservoir (well No. 2) of each cartridge. Carefully snap close the cartridge lid.
13. Load cartridge(s) onto the carriage(s). Press **Prime**. Cartridge loading carriage(s) will slide in automatically. Machine will prime for about 6 minutes.
14. After priming is completed, the touch screen will show **Ready to Load Sample**. Press **Ready to Load Sample**. Left carriage will slide out automatically. Carefully open cartridge lids.
15. Gently add the beads-coupled cell samples from Step 4 of the ***Coupling reaction of beads and cell sample*** section to the Sample well (well No. 1) and avoid forming bubbles. Carefully snap close the cartridge lid and load onto the carriage.
16. After all cell samples are loaded for the left carriage, press **Load**. Left carriage will slide in automatically. If running one or two samples, cell isolation will start at this point. If running more than two samples, right carriage will slide out automatically. Load the rest of the samples and press **Load**, right carriage will slide in automatically. Cell isolation will start.
17. Cell isolation typically takes about 45 minutes but it may vary for different samples.
18. After cell isolation is completed, touch screen will show **Extract Sample**. Press **Extract Sample**. Carriage(s) will slide out automatically.
19. The instrument will place the CellSpot into either the Low-Volume Recovery Holder or the Microfuge Tube.

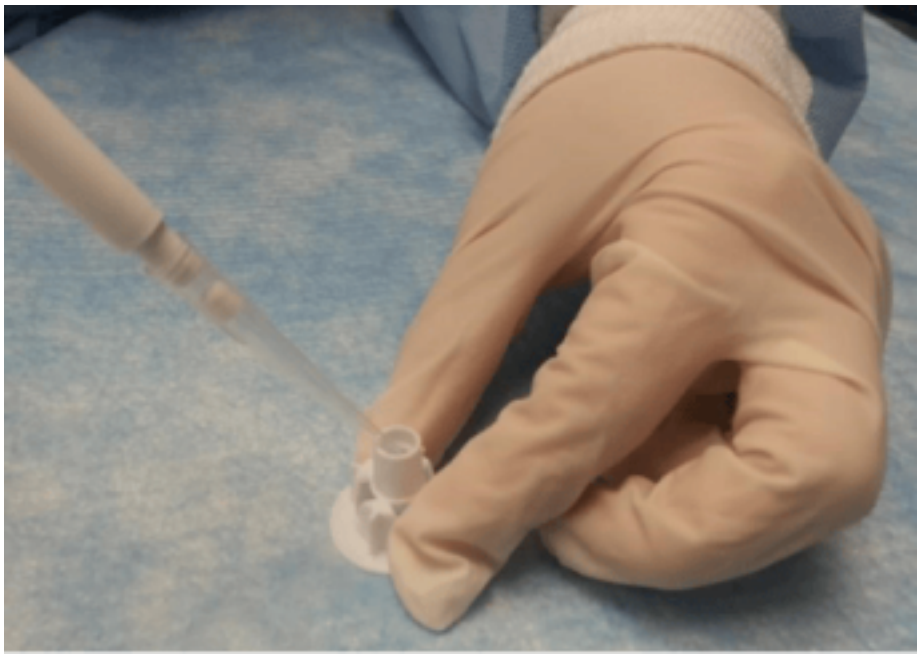
Warning: Recover the sample within 5 minutes after the isolation is finished.
DO NOT ALLOW SAMPLE TO DRY

Cell retrieval (if using Low-Volume Recovery Holder)

1. Remove and invert the holder such that the enriched cells are facing up.
2. Immediately add 20µL (a drop) of Binding Buffer to the CellSpot to prevent cells from drying.
3. If necessary, place the CellSpot over the small cylindrical magnet for 5 seconds to center the cells/beads pellet. Remove the CellSpot from the magnet.
4. Rinse the micropipette tip with Binding Buffer to minimize cells sticking to the tip.

Gently aspirate the cells/beads into the pipette tip. Dispense the collected cells/beads into a new microfuge tube (not provided). *We recommend doing the liquid-to-liquid transfer (i.e. the microfuge tube should also contain a small volume of Binding Buffer).*

5. Place the microfuge tube on the large magnet.
6. Aspirate most of the supernatant and rinse the CellSpot to collect any residual cells/beads. Repeat the previous steps 4-6 until no visible cells/beads are observed on the CellSpot.



Cell retrieval (if using Microfuge Tube)

1. Gently invert the tube 2-3 times until all the cells/beads are suspended in the Binding Buffer at the bottom of the tube.
2. Centrifuge the microfuge tubes briefly to collect all cells. Enriched CTCs are now ready for further testing.