liquidbio

Procedure

Instructions for Use: IsoFlux CTC Enumeration Kit

Applies to 910-0093

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Revision: I

INTENDED USE

The intended use for the IsoFlux™ Circulating Tumor Cell Enumeration Kit (CTC Enumeration Kit) is as a laboratory reagent for the fluorescence staining of cells to identify circulating tumor cells (CTCs) that are cytokeratin (CK) positive, CD45 negative, and nucleated. The kit is designed for use with the IsoFlux System, a benchtop instrument for semi-automated rare cell isolation. The kit contains antibodies and reagents for immunofluorescence staining. The CTC Enumeration Kit is for Research Use Only.

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. This document is intended to guide the user through a typical procedure of immunofluorescence staining, fluorescent microscope imaging, and image analysis to enumerate circulating tumor cells (CTCs) of epithelial origin isolated using the IsoFlux instrument.

PRINCIPLES OF THE PROCEDURE

Upon enrichment with the IsoFlux system, CTCs are fixed and stained with the fluorescent reagents. The fluorescent reagents include the following: anti-CK-fluorescein isothiocyanate (FITC) specific for the intracellular protein cytokeratin (characteristic of epithelial cells), anti CD45- Indocarbocyanine (Cy3) specific for leukocytes and Hoechst 33342, which stains the cell nucleus. Fluorescent images of stained samples are acquired with a microscope with an automatic stage. Images are processed by imaging software and CTCs are enumerated as morphologically intact CK+/CD45-/nucleated cells.

KIT COMPONENTS

- Fixative Solution (2 X 6mL store protect from light at 4°C)
- Normal donkey serum (NDS) (freeze-dried powder, 2mL for blocking; see preparation below)
- Rabbit anti-human CD45 primary antibody (1µg/µL, 100µL; see preparation below, store at -20°C)
- Donkey anti-rabbit IgG Cy 3-conjugated secondary antibody (freeze-dried powder, 0.5mL; see preparation below, store protect from light at -80 to -20°C)
- Triton X-100 10% solution (10mL, store at room temperature)
- Mouse anti human-pan-Cytokeratin FITC-conjugated antibody 50X concentrated (200µL, store protect from light at 4°C)
- Mounting medium (store protect from light at 4°C)
- Hoechst 33342 (see preparation below, store protect from light at 4°C) Binding Buffer (BB) (2 X 12mL)

REAGENT STORAGE AND HANDLING

- Protect reagents from exposure to light.
- When properly stored, reagents are stable until the expiration date printed on the reagent container or kit box. Do not use expired reagents.
- Do not mix and match reagents from different kits.

REAGENTS PREPARATION PRIOR TO USE

- Normal Donkey Serum (NDS): Reconstitute the freeze dried NDS in 2mL double distilled sterile water. This is considered 100% NDS. Store as aliquots at -20°C. Avoid repeated freeze-thaw.
- Rabbit anti CD45 antibody: Vortex for 10 seconds. Briefly spin down the tube. Store as small aliquots (5 to 10µL) at -20°C. Thawed antibody may be kept at 4°C and should be used within 6 weeks. Working concentration is 1-4µg/mL (~1:100 dilution v/v) from the stock antibody as supplied.
- Donkey anti-rabbit IgG Cy 3-conjugated secondary antibody: Reconstitute the freeze dried antibody with 0.5mL of double distilled sterile water (reconstitution in 20-50% glycerol may prolong the shelf life). Store as small aliquots (10 to 50µL) at -80°C. Avoid repeated freeze-thaw. After thawing, store in the dark at 4°C and use within 6 weeks. Working concentration is 1:200 dilution v/v from the reconstituted antibody.
- Hoechst 33342: Dissolve the entire content of 25mg powder in 1.25mL double distilled sterile water to make 20mg/mL stock solution (5000X final concentration). Mix well by vortexing. Briefly centrifuge the vial to bring down liquid from lid. Further dilute a small quantity in water to make 1000X concentration. Store in the dark at 4°C. To use, add the appropriate amount to the sample to 1X final concentration.

For all the reagents above and anti CK antibody, when ready to use, spin at maximum

speed (14000xg) on a bench top centrifuge. Use only the top portion of the reagents to minimize debris and staining artifacts.

MATERIALS REQUIRED, NOT PROVIDED

- IsoFlux Instrument (Liquidbio 950-0100)
- Fluorescence microscope equipped with automatic stage, excitation/emission filters for FITC (495 nm/521 nm), Cy™3 (550 nm/570nm) and Hoechst 33342 (361 nm/497 nm) Imaging analysis software (MetaMorph by Molecular Devices or similar software) Pipettes and tips
- Microfuge tubes (clear)
- Permanent magnets (accessory parts included with IsoFlux instrument, large round and small cylindrical magnet), or a magnetic rack for microfuge tubes
- Optional: 24-well Glass Bottom plates (e.g., SensoPlate™ Greiner Bio-One 662892) or glass slides
- Round glass cover slips 12mm diameter
- Optional: Isoflux Sample Mounting Kit (Liquidbio 910-0124). Please refer to Isoflux Sample Mounting Kit for Isoflux CTC Samples IFU

WARNINGS AND PRECAUTIONS

- For Research Use Only
- Please read the entire contents of the Instructions for Use before processing samples.
- **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.

Avoid pipetting less than 2µL of antibodies per each staining batch.

Enumeration Procedure

Preparation of staining solutions (suffice for 8 samples):

Prepare the following solutions before staining. Mix well by briefly vortex and centrifuge down to remove liquid from lids. Keep in the dark. Perform staining procedure at room temperature (18 to 25°C). Use of Isoflux Sample Mounting Kit (Liquidbio 910-0124) is highly recommended. Please refer to document number 630-0144 Isoflux Sample Mounting Kit for Isoflux CTC
Samples IFU.

Fix (1:1): combine 200µL of Binding Buffer (BB) + 200µL of Fixative solution (3.7% formaldehyde solution)
NDS (10%): combine 100µL of normal donkey serum + 900µL of BB
CD45 (1:100): combine 4µL of Rabbit anti human CD45 + 396µL of BB **Cy3 (1:200)**: combine 2µL of Donkey anti rabbit IgG-Cy3 + 398µL of BB **Perm buffer (0.2% TX-100)**: combine 980µL BB + 20µL 10% TX-100 solution
CK-FITC (1:50): combine 8µL of mouse anti human Cytokeratin-FITC (50X concentrated) + 40µL Perm buffer + 352µL BB
Wash+H buffer (1x Hoechst 0.02% TX-100): combine 900µL Binding Buffer + 100µL Perm buffer + 1µL of 1000X Hoechst 33342 solution

Sample staining

Note: During liquid handling steps, be mindful of retaining cells/beads pellet (i.e. be sure not to lose beads in pipet tips). Direct pipetting of cells/beads should be minimized and be very gentle. All staining procedures are to be performed at room temperature and protect from light.

1. After CTC isolation with the IsoFlux system, recover and transfer the cells/beads into a clear microfuge tube pre-coated with BB. Place the tube on the large round magnet for 30 seconds. Remove and discard the buffer. Remove the tube from the magnet. Immediately dispense 40µL of **Fix (1:1)** solution into the tube, gently dispersing the cells/beads. Do not pipette up and down. Rather, dispense the fixative to disperse the cells/beads.
2. Incubate at room temperature for 20 minutes.
3. Place the tube on the large round magnet for 15 seconds. Remove and discard the **Fix (1:1)** solution. Remove the tube from the magnet.
4. Immediately add 100µL of BB to the cells/beads. Briefly use the magnet to mix the beads.
   (***This is a potential stopping point. The sample may be stored at 4°C for up to 14 days.)***
5. Place the tube on the large round magnet for 15 seconds. Remove and discard the

buffer. Add 40µL of **NDS (10%)**. Incubate at room temperature for 5 minutes.

6. Place the tube on the large round magnet for 15 seconds. Remove and discard the **NDS** buffer. This is called the magnet pull-down method.

7. Add 40µL of **CD45 (1:100)** and incubate at room temperature for 20 minutes with occasional gentle mixing of the beads.

8. Place the tube on the large round magnet for 15 seconds. Remove and discard the buffer. Remove the tube from the magnet. Wash the cells/beads once with 100µL of BB using the magnet pull-down method.

9. Add 40µL **Cy3 (1:200)** and incubate in the dark at room temperature for 20 minutes with occasional gentle mixing of the beads.

10. Wash the cells/beads once with 100µL of BB using the magnet pull-down method.

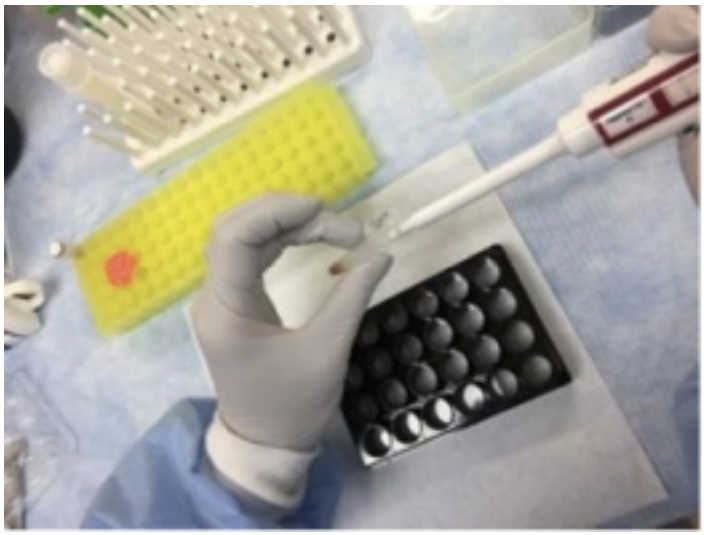
11. Permeabilize the cells with 40µL of **Perm buffer (0.2% TX-100)** for 5 minutes at room temperature.

12. Place the tube on the large round magnet for 15 seconds. Remove and discard the buffer. Add 40µL of **CK-FITC (1:50)** and incubate in the dark for 40 to maximum 60 minutes at room temperature with occasional gentle mixing of the beads.

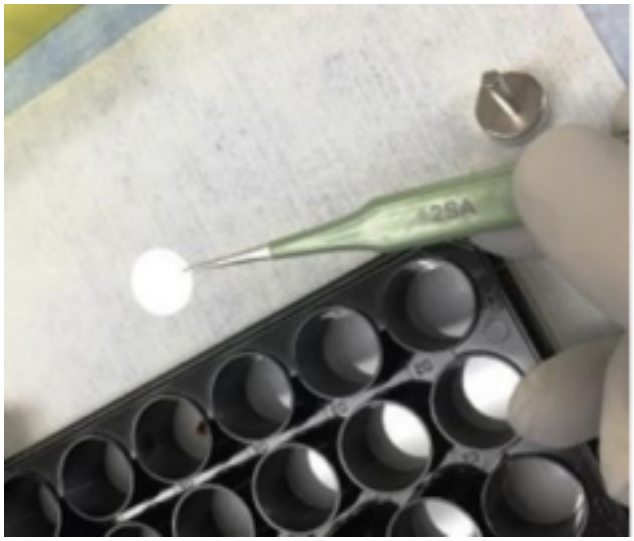
13. Place the tube on the large round magnet for 15 seconds. Remove and discard the buffer. Remove the tube from the magnet. Wash the cells/beads once with 100µL of **Wash+H buffer (1x Hoechst 0.02% TX-100)** using the magnet pull-down method.

14. Add 100µL of BB to the cells/beads.

15. Transfer the stained cells/beads into a SensoPlate™ (alternatively, glass slide may be used) well centered on a small cylindrical magnet. Remove the buffer while keeping the stained cells/beads at the center of the well over the magnet.



16. Remove the plate from the magnet. Dispense 2µL of Mounting Media over the cells/beads spot. Immediately place the cells/beads over the larger round magnet. Place the glass coverslip over the cells/beads spot. Remove the plate from the magnet.




17. Cover the SensoPlate™ with plate sealer and aluminum foil. The sample is now ready for imaging and can be stored at 4°C for up to 2 weeks if kept moist and away from light.

Image acquisition

Note: A fluorescence microscope equipped with automatic stage and excitation/emission filters FITC (495 nm/521 nm), Cy™3 (550 nm/570nm) and Hoechst 33342 (361 nm/497 nm) is needed for cell imaging and CTC enumeration.

1. Images are acquired using an automatic stage with a stage list covering the whole area of the cells/beads sample spot.
   Typically, magnifying power of 100X (e.g., 10X eyepiece and 10X objective) is needed for reliable CTC enumeration. Increased magnifying power of 400 (e.g. 10X eyepiece and 40X objective) is necessary to acquire a more resolved image of individual CTCs.
2. Acquire images (10X objective) of stained sample for all three fluorescent channels: CK-FITC, CD45-Cy3 and Nucleus-Hoechst 33342.

Image analysis

Note: Imaging analysis software (MetaMorph or similar) is required for image analysis for CTC enumeration.

Image analysis and CTC enumeration with MetaMorph software is performed as following: 1. Open images (10X objective) as a separate stack for each of the three fluorescent channels.

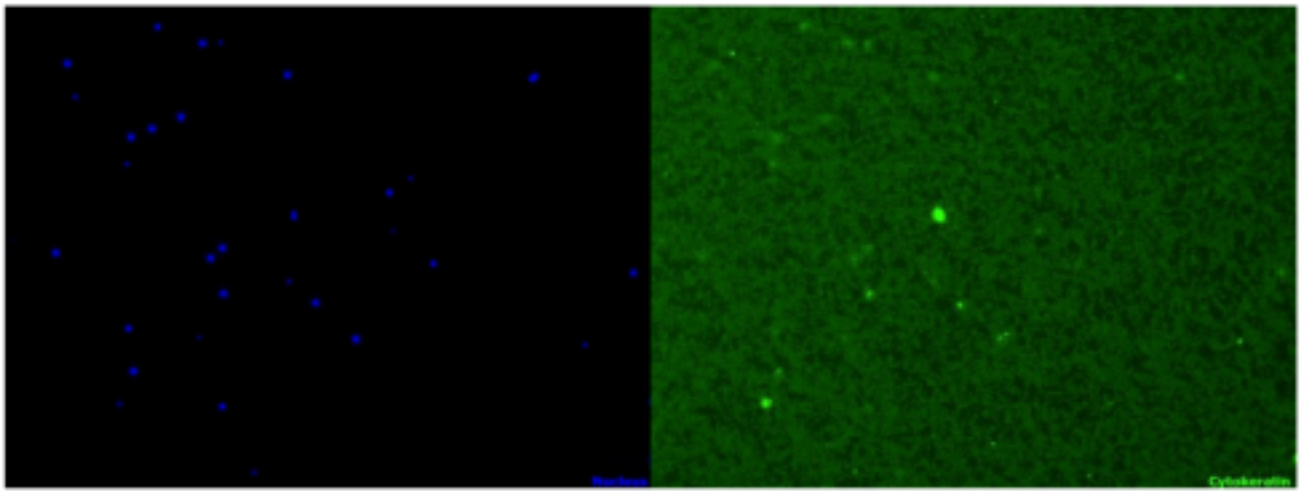
2. Adjust the fluorescent intensity and threshold appropriately for all raw images of the three fluorescent channels to remove auto-fluorescent signal from the beads (see Appendix for representative images).

3. Overlay the three stacks of images of the three fluorescent channels 3.4. Enumerate CTCs as morphologically intact CK+/CD45-/nucleated cells.
4. Total recovered cells (CTCs and background leukocytes) are enumerated as nucleated cell (Hoechst 33342+) counts using MetaMorph integrated morphology analysis. 5. Images of representative CTCs can be acquired by going back to the original stage and get an image at higher magnifying power (e.g. 40X objective).

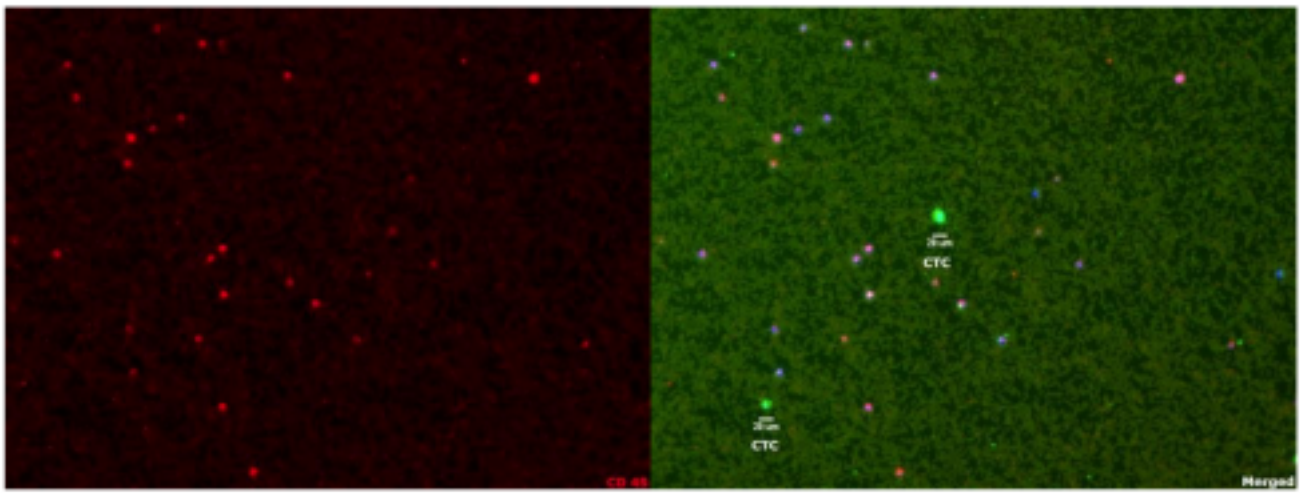
Typical CTC images (raw and adjusted)

10X Magnification

Raw Image



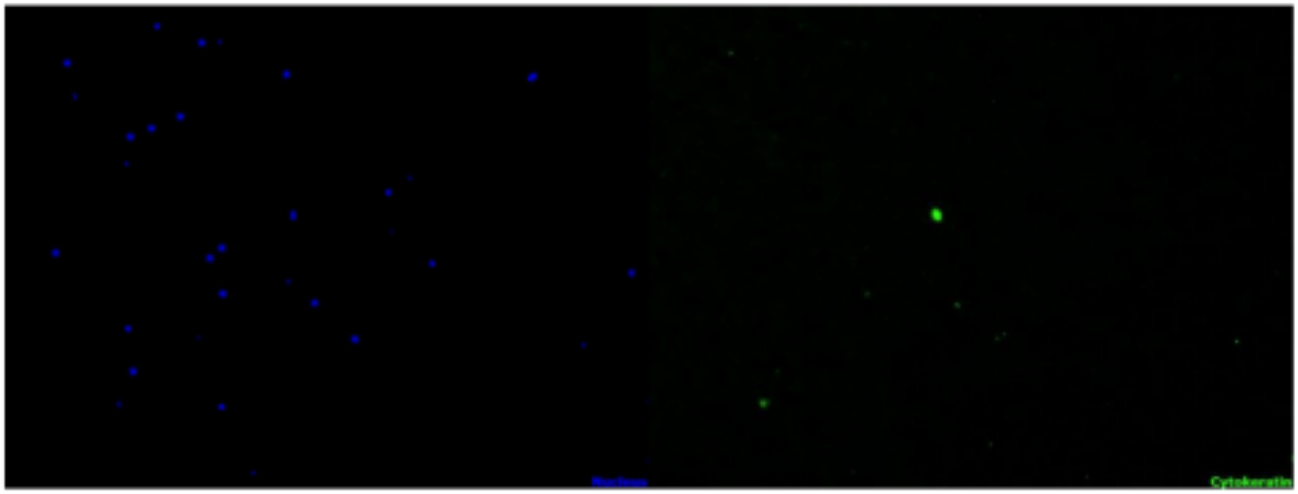
Nucleus CytoKeratin



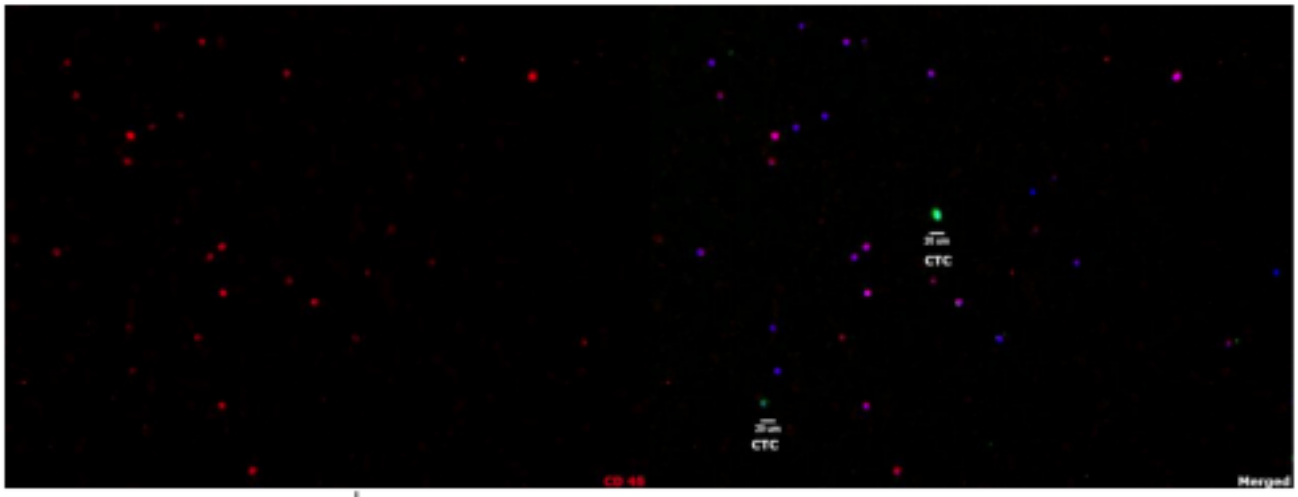
CD45 Merged

10X Magnification

Adjusted (background subtraction) images:



Nucleus CytoKeratin



CD45 | Merged

40X Magnification