Technical Note

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# QuantiGene ViewRNA Circulating Tumor Cell (CTC) Assay

## About CTCs

Circulating Tumor Cells (CTCs) are derived from primary and metastatic cancers and only small number of CTCs are observed in blood making their isolation and characterization a major technological challenge. CTCs are pivotal to understanding the biology of metastasis and promise potential as a biomarker to noninvasively evaluate tumor genotypes during disease progression and response to treatment. Technologies that provide purer CTC populations amenable to better characterization, both cellular and molecular will enable clinical applications, including early disease detection and biomarker discovery to predict treatment response and disease progression (Yu M et al 2011).

### Detection and Isolation of CTCs

Isolation of an adequate number of CTCs in a reproducible manner has been limited due to their extreme rarity with approximately 1 per $10^9$ cells in peripheral blood of patients (1-100 CTCs/mL whole blood) with metastatic cancer (Krivacic RT et al 2004). Several methods have been developed to detect and isolate CTCs in the peripheral blood of patients with cancer (Ross AA et al [1993]; Hardingham JE et al [1993]; Naume B et al [1997]; Racila E et al [1998]; Zigeuner RE et al [2000]; Bilkenroth U et al [2001]; Kagan M et al [2002]; Allard WJ et al [2004]; Muller V et al [2005]; Wu CH et al [2006]; Maheswaran S et al [2008]; Fehm T et al [2009]). These methods depend on cytometric/immunological characteristics, although CTCs have been be isolated by size (Vona G et al 2000). In addition, CTCs can be captured using magnetic bead-conjugated antibodies against epithelial-cell adhesion molecule (EpCAM); however, its use in affecting treatment decisions remains a point of discussion (Braun S and Marth C [2004]; Cristofanilli M et al [2004]; Cristofanilli M et al [2005]). To date it is unknown to what extent these different technologies compare in terms of sensitivity, specificity and reproducibility.

Recently, ScreenCell introduced innovative devices that use a filter to isolate and sort tumor cells by size that is simple to use and efficient at capturing of CTCs from human whole blood (Desitter I et al 2011). The ScreenCell technology enables characterization of CTCs at the single cell level: (i) their identification and enumeration and (ii) their characterization through immunocytochemistry, fluorescence *in situ* DNA & RNA hybridization (FISH) assays.

### Analyzing CTCs

Affymetrix offers two types of filtration devices for different applications:

- *QuantiGene ViewRNA Fixed Cell Isolation Kit.* Comprised of 4 ScreenCell Cyto filtration devices, enabling capture of fixed CTCs for subsequent RNA FISH analysis.
- *QuantiGene ViewRNA Live Cell Isolation Kit*. Comprised of 3 ScreenCell CC filtration devices, enabling capture of live CTCs for cell culture and subsequent RNA FISH analysis.

Captured CTCs, using both filter types, are analyzed using QuantiGene ViewRNA multiplex *in situ* hybridization assay for visualization of 1 to 4 targets at the single-molecule/single-cell level. Using probes specific to CTC identification markers, for example, cytokeratins, in combination with other genes of interest, you can enumerate and characterize individual CTCs. Thousands of QuantiGene ViewRNA probes have been validated and custom probes to any RNA sequence are readily available upon request.

### About this Technical Note

The procedures in this Technical Note describe how to:

- Capture CTCs using ScreenCell filtration devices
- Perform multiplex RNA FISH using QuantiGene ViewRNA ISH Cell Assay on the captured CTCs
- Image and enumerate/characterize the CTCs on ScreenCell filters

## Required Materials

**Table 1** Required Materials for Running a QuantiGene ViewRNA ISH Cell Assay in a 24-Well Format:

| Item | Source | Part Number |
|---|---|---|
| QuantiGene ViewRNA CTC-CC Kit or<br>QuantiGene ViewRNA CTC-Cyto Kit | Affymetrix | QVC0050<br>QVC0051 |
| QuantiGene ViewRNA Probe Sets | Affymetrix | |
| 37% formaldehyde | Sigma | 1635 (**Do Not Substitute**) |
| 1X PBS, pH 7.0-7.2 (for using ScreenCell filters) | Life Technologies | 14190-144 or equivalent |
| Prolong Gold Antifade Reagent | Life Technologies | P36930 |
| 24-well culture plates | Nunc | 142475 or equivalent |
| Forceps | Affymetrix | QVC0501 or equivalent |
| 75 mm x 25 mm glass microscope slides | Major Laboratory Supplier (MLS) | |
| 15 mL sterile conical tubes | MLS | |
| Plate shaker | MLS | |
| Incubator validated to maintain temperatures at 40 ± 1 °C and 50 ± 1 °C | MLS | |
| Automated fluorescent microscope with the following specifications:<br>▪ Automated fluorescent microscope system with autofocus function and motorized stage<br>▪ 100 W mercury lamp<br>▪ 10X (N.A. 0.45) and 40X (N.A. 0.8) objectives, or better<br>▪ Filter sets for DAPI, ALEXA 488, ALEXA 546, ALEXA 647and ALEXA 750 (depending on the channel for detection)<br>▪ CCD-camera suited for fluorescent imaging<br>▪ Software for image acquisition with z-stacking | MLS | |

## Before You Start

If you have not performed the QuantiGene ViewRNA ISH Cell Assay previously, we strongly recommend that you perform the following before running precious CTC samples:

- Perform the QuantiGene ViewRNA ISH Cell Assay with a positive control cell line on coverslips. This cell line should express the genes of interest. Probe Sets should include those for a CTC biomarker, for example, cytokeratins 8, 18, and 19 and a housekeeping gene, for example, ACTB or GAPDH. HeLa and PC9 cells are examples of positive control cells expressing cytokeratins 8, 18, and 19, ACTB and GAPDH. Refer to the QuantiGene ViewRNA ISH Cell Assay User Manual for the assay procedure.
- Perform an experiment in which you spike whole blood with the positive control cells. Add about 1000 positive control cells into 3 mL whole blood. Process the sample with ScreenCell filtration device, and perform the QuantiGene ViewRNA ISH Cell Assay as described below.

After you obtain satisfactory results and are familiar with the assay procedures for CTC isolation and RNA FISH, you can confidently begin processing precious CTC samples.

# Capturing CTCs with ScreenCell Filtration Devices

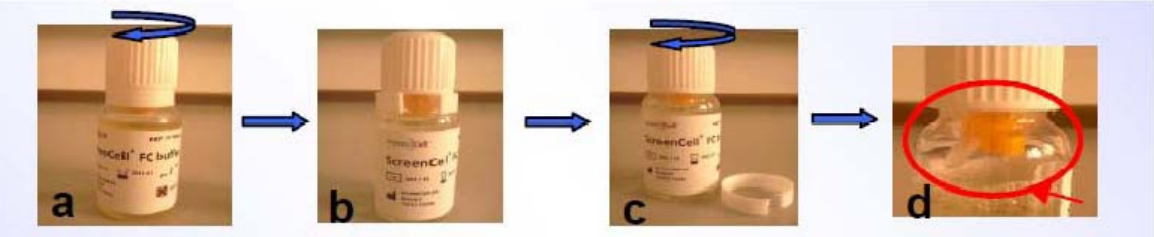
## Sample Requirements

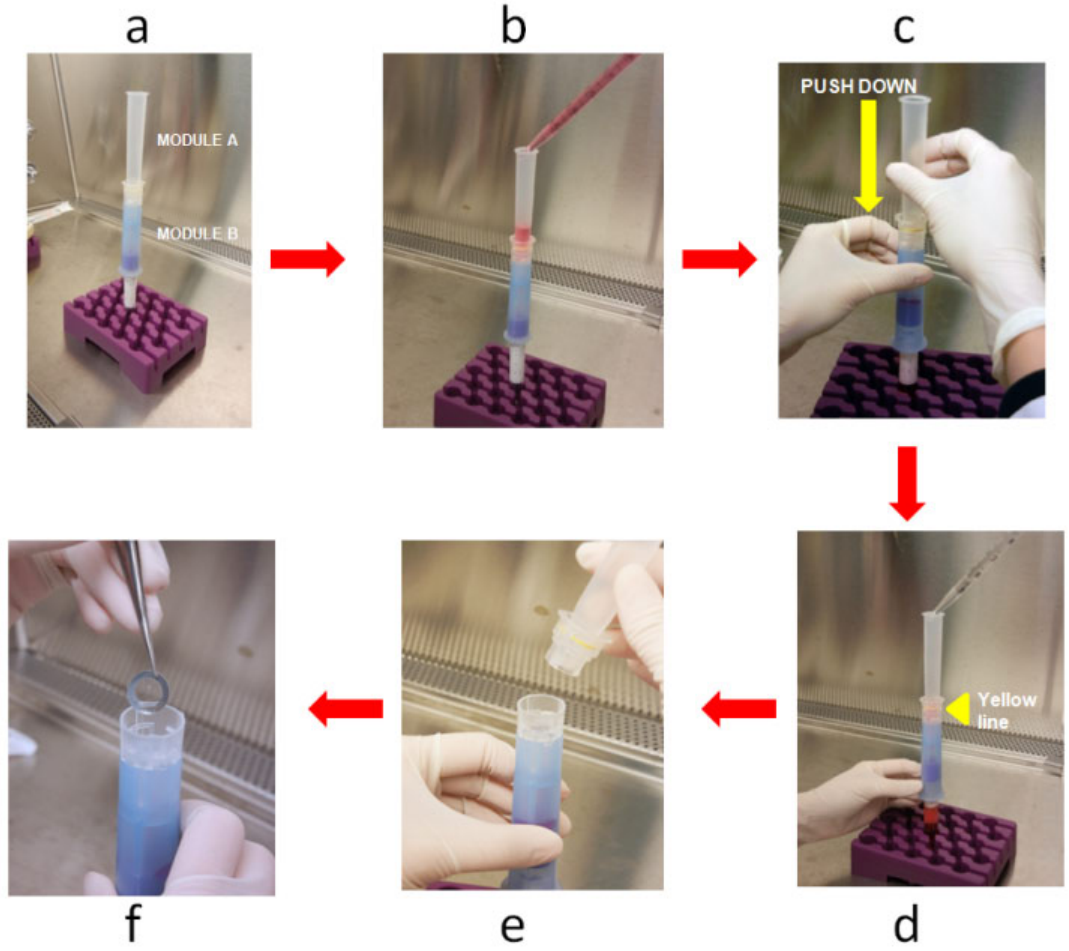
- When drawing blood, the first mL of blood collected must be eliminated because it is carrying cutaneous epithelial cells taken by the needle during the sampling. The blood should be inverted in the collection tube 5 times to ensure uniform mixing with the anti-coagulant.
- Human whole blood must be drawn in a K2-EDTA collection tube and stored at 4°C until processing with a ScreenCell filtration device. Blood samples must be processed within 4 hours of blood draw.
- Purified human PBMCs can be used instead of whole blood.

**NOTE:** **Blood samples of other species have not been validated.**

## Procedure for Fixed Cell Isolation using ScreenCell Cyto Filtration Device

We have made important changes to the ScreenCell procedure, as outlined in the ScreenCell instruction for use Package Insert. These changes were made to facilitate the QuantiGene ViewRNA ISH Cell Assay. Please read through the following procedure before starting.

| Step | Action |
|---|---|
| **Step 1. Prepare Buffer** | **A.** Reconstitute FC Buffer:<br>**1.** Screw the cap fully on the vial to break apart the molded tamper-evident ring (a, b).<br>**2.** Make sure that the cap is screwed tightly (c) and agitate the vial thoroughly.<br>**3.** Verify the clear cap of the yellow inside chamber has been opened to release the contents (d).<br>**4.** In a fume hood, add all formaldehyde (provided) from the amber tube to the bottle, and invert 10 times to mix.<br>**5.** Keep the reconstituted FC Buffer at room temperature (RT). It should be used within 24 hrs.<br>  <br>**B.** Prepare 4% formaldehyde solution (to be used in step 4 below):<br>In a fume hood, prepare 15 mL of fresh 4% formaldehyde solution by diluting 1.62 mL of a 37% stock formaldehyde with 13.38 mL of 1X PBS and vortexing briefly to mix (sufficient for 12 filters). |
| **Step 2. Blood Dilution and RBC Lysis** | **A.** Transfer up to 3 mL of whole blood into a 15 mL sterile conical tube. If using less sample, bring up the volume to 3 mL with 1X PBS containing 5 mM EDTA.<br>**B.** Add 4 mL of reconstituted FC Buffer.<br>**C.** Close the tube, and invert several times to mix. Then incubate for 8 min at RT. During incubation, assemble the filtration unit as described in Steps 3A and 3B. |

| Step | Action |
|---|---|
| **Step 3. Blood Filtration** | **A.** Place the purple-capped vacuum collection tube in a rack to keep it in an upright position.<br>**B.** Remove the yellow protective cover at the bottom of the filtration unit (Module B), and gently put the filtration unit on top of the collection tube (a). DO NOT PUSH DOWN AND PREMATURELY PUNCTURE MEMBRANE OF THE VACUUM COLLECTION TUBE.<br>**C.** Load samples to the top module of the filtration unit (Module A) (b).<br>**D.** Push down on the filtration unit until needle punctures the membrane of the vacuum collection tube to start the filtration (c).<br>**E.** When blood sample reaches the yellow level line, **immediately** add 1.6mL 1X PBS to the filter unit to rinse out the filter (d).<br>**F.** After all liquid has passed through the filter, unscrew Module A counterclockwise and pull to separate (e). Using forceps, transfer the filter (label on metal ring facing up) onto a piece of blotting paper (f). Blot quickly to remove excess solution.<br><br>**IMPORTANT: Filtration is usually completed within 3 min. If clogging occurs, the blood is coagulated and the filter should be discarded.**<br><br>  |

| Step | Action |
|---|---|
| **Step 4. Fixation** | **A.** Use forceps to transfer each filter (label on metal ring facing up) to a well of a 24-well plate containing 4% Formaldehyde (1 mL/well). The filter may float. Make sure that the filter is fully submerged in the solution by pushing it down with forceps on the metal ring, avoiding the filter membrane.<br>**B.** Cover the 24-well plate, place in a fume hood and fix cells for 60 min at RT.<br>**C.** Aspirate formaldehyde solution, and wash the filters with 2 mL/ well 1X PBS for a total of 3 times.<br>  |
| **Step 5. Baking** | **A.** Aspirate all of the last PBS wash. Using forceps, tilt the filter against the wall of the well (see figure below) to facilitate drying in the next step.<br>**B.** Place uncovered 24-well plate, with filters in tilted position, in a 50 °C incubator for 30 min or until filters are completely dry.<br>**C.** Let the filter cool to RT for 10-15 min. Lay the filter flat in the well. The filter can be used immediately in the QuantiGene ViewRNA assay. Alternatively, seal the plate containing filters with parafilm, and store at –80 °C for up to one week.<br>  |

## Procedure for Live Cell Isolation using ScreenCell CC Filtration Device

The following procedures should be performed in a sterile laminar flow cell culture hood.

Different cancer cells have specific requirements for cell growth. Hence, the ability of the captured CTCs to expand will vary from sample to sample.

| Step | Action |
|---|---|
| **Step 1. Prepare Buffer** | **A.** LC Buffer is ready to use. Store at RT and use it in a sterile environment. |
| **Step 2. Blood Dilution** | **A.** Transfer up to 6 mL whole blood into a 15 mL sterile conical tube. If using less sample volume, bring up the volume to 6 mL with 1X PBS containing 5 mM EDTA.<br>**B.** Add 1 mL of LC Buffer.<br>**C.** Close the tube, invert the tube a few times to mix, and incubate for 2 min at RT. During incubation, assemble the filtration unit as described in Steps 3A and 3B.<br>**D.** After the incubation, add 1.6 mL culture medium without serum. Invert the tube to mix. |

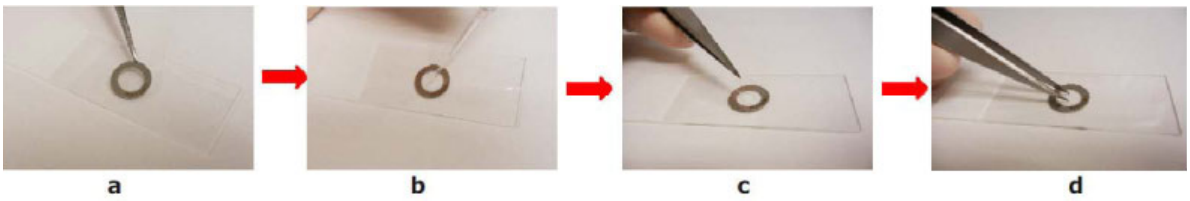
| Step | Action |
|---|---|
| **Step 3. Blood Filtration** | **A.** Place the purple-capped vacuum collection tube in a rack to keep it in an upright position.<br>**B.** Remove the yellow protective cover at the bottom of the filtration unit (Module B), and gently put the filtration unit on top of the collection tube. DO NOT PUSH DOWN AND PREMATURELY PUNCTURE MEMBRANE OF THE VACUUM COLLECTION TUBE.<br>**C.** Load the samples into the top module of the filter unit (Module A).<br>**D.** Push down on the filtration unit until needle punctures the membrane of the vacuum collection tube to start the filtration.<br>**E.** After all liquid has passed through the filter, unscrew Module A counterclockwise and pull to separate. Using sterilized forceps, retrieve the filter with the label on the metal ring facing up.<br>**IMPORTANT: Filtration is usually completed within 3 min. If clogging occurs, the blood is coagulated and the filter should be discarded.**<br>**NOTE: Refer to step 3 of the previous section, *Procedure for Fixed Cell Isolation using ScreenCell Cyto Filtration Device*, for images of corresponding steps. Note that the PBS wash is not required for ScreenCell CC filter.** |
| **Step 4. Cell Culture** | **A.** Place each filter (label on metal ring facing up) into a well of a 24-well plate containing 1 mL/well of appropriate culture medium with growth factors. Make sure the filter is fully submerged under the culture medium.<br>**B.** Culture cells over night in a humidifying $CO_2$ incubator at 37 °C.<br>**C.** After overnight culture, aspirate approximately 800 µL of the existing culture medium from the bottom corner of the well with a 1 mL pipette and then dispense the medium directly on top of the filter surface to dislodge the settled red blood cells on the filter surface.<br>**D.** Examine the filter under the phase contrast microscope after each cycle of flushing. Repeat the flushing procedure without changing the medium until the filter surface looks sufficiently clean (generally takes about 5-10 cycles).<br>**E.** Replace the sample with fresh culture medium and continue to culture the cells for as long as needed. |
| **Step 5. Fixation** | **A.** After cell culture is complete, aspirate the media and wash the filter gently with 2mL/well 1X PBS once.<br>**B.** In a fume hood, prepare 15 mL of 4% formaldehyde solution by diluting 1.62 mL of 37% formaldehyde with 13.38 mL of 1X PBS. Vortex briefly to mix (sufficient for 12 filters).<br>**C.** Remove all PBS, add freshly prepared 4% formaldehyde solution (1 mL/well) and fix cells for 60 min at RT. Make sure that the filter is fully submerged in the solution. The filter may float. Push it down with forceps on the metal ring, avoiding the filter membrane.<br>**D.** Aspirate formaldehyde solution, and wash the filters with 2 mL/ well of 1X PBS for a total of 3 times. |
| **Step 6. Baking** | **A.** Aspirate all of the last PBS wash. Using forceps, tilt the filter against the wall of the well (see figure below) to facilitate drying in the next step.<br>**B.** Place uncovered 24-well plate, with filters in tilted position, in a 50 °C incubator for 30 min or until filters are completely dry.<br>**C.** Let the filter cool to RT for 10-15 min. Lay the filter flat in the well. The filter can be used immediately in the QuantiGene ViewRNA assay. Alternatively, seal the plate containing filters with parafilm, and store at –80 °C for up to one week. |

## Performing the QuantiGene ViewRNA ISH Cell Assay

Refer to the *QuantiGene ViewRNA ISH Cell Assay User Manual* for assay principle, procedural details, and troubleshooting.

We recommend using ALEXA 546 channel for specific CTC biomarker. The ALEXA 546 channel has less interference with cellular auto-fluorescence, and is excited more efficiently by mercury light source, thus facilitating the detection of CTCs.

## Procedure

| Step | Action |
|---|---|
| **Step 1. Equilibrate Filters** | Equilibrate the filters to RT if they have been stored at –80 °C. |
| **Step 2. Rehydrate Cells** | Add 2 mL/well of 1X PBS to each well and incubate at RT for 10 min to rehydrate the cells. |
| **Step 3. Assay Procedure Modifications** | Follow procedures as described in the *QuantiGene ViewRNA ISH Cell Assay User Manual*, starting from the Permeabilize Cells with Detergent Solution step to the DAPI staining with the following modifications:<br>▪ Digest samples with Protease QS diluted at 1:4000 for 60 min at RT.<br>▪ Apply gentle shaking in all washing steps with Wash Buffer using a shaker. |
| **Step 4. Mount Filters and Image** | After the final wash, mount the filters as follows:<br>**1.** Label the frosted area of the glass slides with a pencil.<br>**2.** Place the filter on a piece of clean blotting paper for a few seconds to remove excess PBS (label on metal ring facing up).<br>**3.** Transfer the filter to a labeled microscopic glass slide (label on metal ring facing up). Make sure the filter is laying flat on the glass slide (a).<br>**4.** Place a small drop (approximately 4 µL) of Prolong Gold Antifade Reagent onto the center of the filter. Avoid air bubbles (b).<br>**5.** Gently place a 7 mm coverslip (provided) on top of the mounting medium (c). Let the coverslip slowly settle down for 30-60 seconds, then use forceps to gently press down the coverslip towards the filter. Make sure the coverslip is level with the filter (d).<br>**6.** Cure the slides overnight at RT, protected from light. Samples can then be stored at 2–8 °C protected from light. Fluorescent signals will be stable for up to one week when properly stored.<br>For best results, wait for the mounting medium to completely cure before viewing under a microscope.<br>  <br>a b c d |

# Imaging and CTC Enumeration/Characterization

**NOTE: This section is available as a PowerPoint presentation at www.panomics.com.**

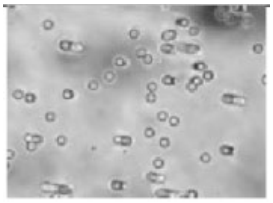
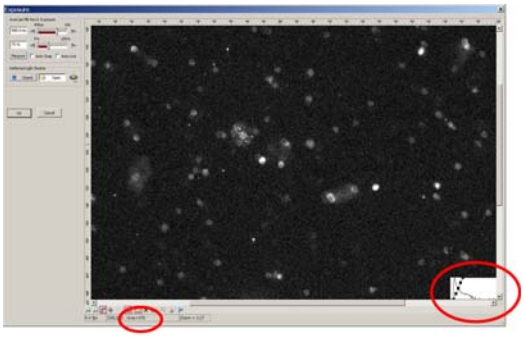
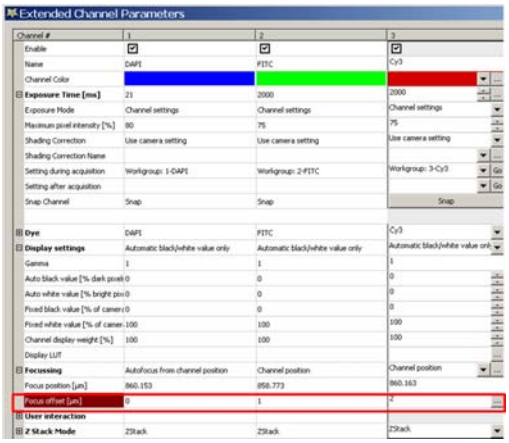
After the assay, the cells on the filter can be imaged and quantified. However, the process of CTC enumeration is greatly facilitated by using an automated fluorescent microscope system. The automated microscope system should include motorized focusing, XY-stage and filter switching, as well as a CCD-camera optimized for fluorescence imaging. In addition, software is needed to control the microscope and CCD-camera with functionality for autofocus, Z-stacking, image-stitching (also called mosaic or montage), multi-channel imaging as well as point-of-interest marking and counting.

In this section, we will outline the workflow for: filter scanning, CTC enumeration and, acquisition of high-resolution multiplex fluorescent image for identified CTCs. Consult your imaging specialist and microscope vendor for specific parts needed and further support. Vendors offering such systems include BioView, MetaSystems, and Nikon Instruments Inc. and Leica Microsystems.

The following procedure uses Zeiss AxioVision software (v. 4.8.1.0) as an example. Your software may look different, but should be able to perform similar functions. Refer to the user manual of your software for details.

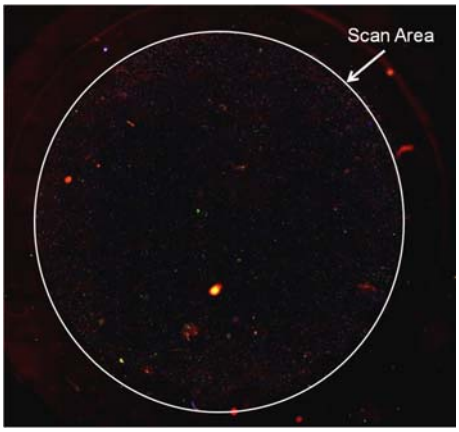
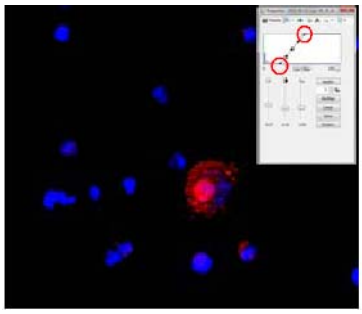
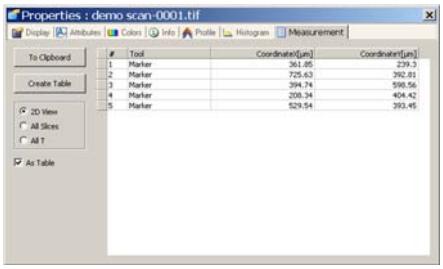
## Filter Scanning

**To scan the filter:**

| Step | Action | Image |
|---|---|---|
| **Step 1. Identify the Boundary of Filter** | **A.** Place a mounted filter on the stage.<br>**B.** Identify the filter boundary manually in brightfield, and determine the area for scanning. |  |
| **Step 2. Locate Positive Cells and Set Exposure Time** | **A.** Manually identify a positive cell expressing the CTC biomarker with the proper fluorescent filter.<br>**B.** Using a 10X objective (N.A. 0.45), determine optimal exposure time for DAPI and the channel (ALEXA 546 channel recommended) for CTC biomarker. Avoid overexposure by checking the signal intensity profile and the pixel value of the signal to keep it under the saturation point. |  |
| **Step 3. Determine Autofocus Offset** | The optimal focal planes for each fluorophore may be different. This step is to find out the optimal focusing positions for each channel.<br>**A.** Focus on the cell with DAPI filter.<br>**B.** Switch to appropriate filter for the CTC biomarker and refocus to determine the offset value in the scanning parameters.<br>**C.** Record the difference in the offset control in the scanning parameters.<br>**D.** We recommend autofocusing with DAPI at every field. |  |
| **Step 4. Scan Whole Filter** | **A.** Set up scan to capture images for DAPI and the CTC biomarker for the entire filter with predetermined coordinates from Step 1, using the 10X objective (N.A. 0.45).<br>**B.** Start the scan. |  |

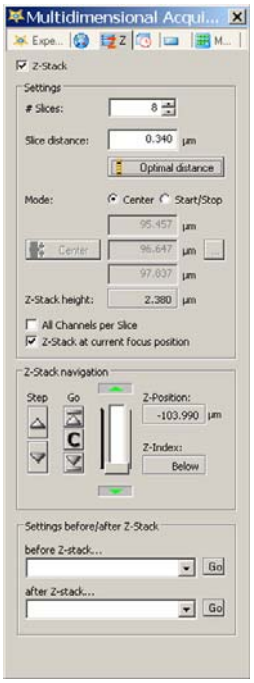
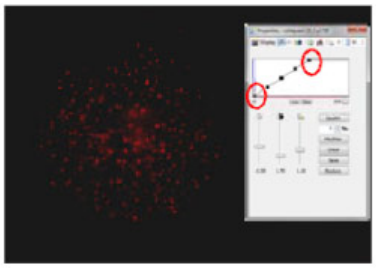
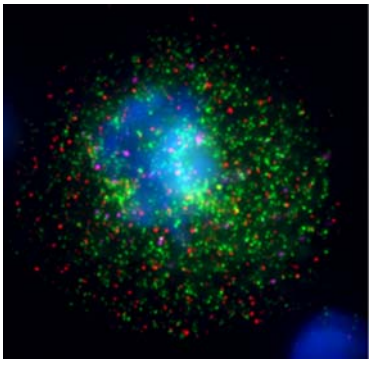
## CTC Enumeration

**To enumerate CTCs:**

| Step | Action | Image |
|---|---|---|
| **Step 1. Stitch Images** | **A.** Using the image acquisition software, convert the acquired multiple images to a single image. This function is referred to as image-stitching, mosaic or montage.<br>**B.** Display the image on a monitor for viewing. | 10X mosaic filter image<br>  |
| **Step 2. Adjust Thresholds** | Adjust the upper and lower thresholds of the stitched image for optimal viewing of the specific signal of the CTC biomarker gene. |  |
| **Step 3. Cell Count** | **A.** Count positive cells manually for CTC enumeration.<br>**B.** Alternatively, set up software to mark and count “point of interest”. Make sure to record coordinates of these points. Click on the positive cells individually. The software will record the total number of positive cells on the filter and the positions of individual cells. |  |

## Acquisition of high-resolution multiplex fluorescent image

**To acquire an image:**

| Step | Action | Image |
|---|---|---|
| **Step 1. Return Stage Position to Positive Cells** | Using the recorded coordinates, move microscope stage to the field with desired CTC to image. | |
| **Step 2. Set Exposure Time and Acquire z-stack images** | **A.** Switch to a 40X (N.A. 0.8 or higher) objective. Set the proper exposure time and offset for individual channels. Avoid overexposure by checking the pixel value of the signal and keep it under the saturation point.<br>**B.** Select *z*-stack function and adjust parameters (optical depth of each *z*-step and number of *z*-plane).<br>Optimal optical depth is typically determined by software and is related to objective NA. The number of *z*-planes to acquire depends on sample thickness and can be determined by the z-stack start/stop function. For the first time, test a few settings to achieve the best result.<br>**C.** After imaging, move to the next field. It is possible to automate this step by the software. |  |
| **Step 3. Adjust Thresholds** | **A.** Convert the images of a z-stack into a single image.<br>**B.** Adjust thresholds of individual channels.<br>**C.** Merge individual channels into the final image. | Adjust individual channel<br>  <br>Final composite image<br>  |

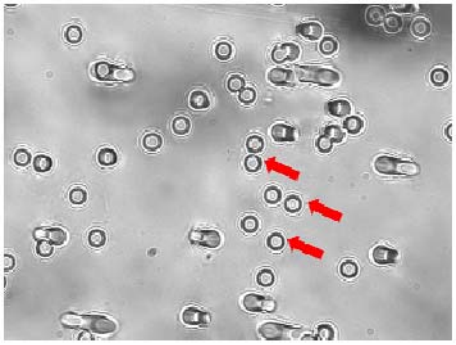
## Image Interpretation

This section illustrates how to interpret the images from your assay. In a typical assay with 3 mL of blood sample, up to a few hundred CTCs (for 1-100 CTCs per mL of blood) and a few thousand leukocytes will be present on the filter. The presence of leukocytes is the result of normal filter operation.

A positive CTC should:

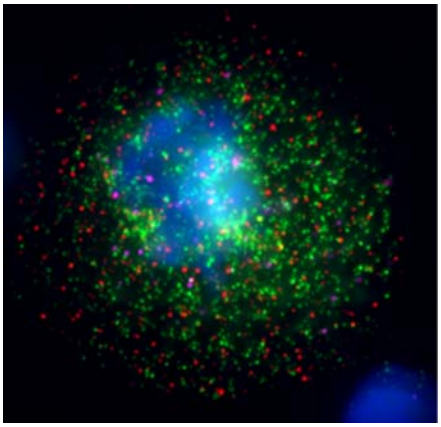
- Express the specific CTC biomarker, for example, cytokeratin for CTC of epithelial origin.
- Stain positive with DAPI indicating the presence of nucleus.
- Exhibit a cell size typically larger than the pore size of the filter (approximately 7 µm). (The pore of the filter can be observed under brightfield. The cell size can be confirmed by overlaying the brightfield and DAPI images.)

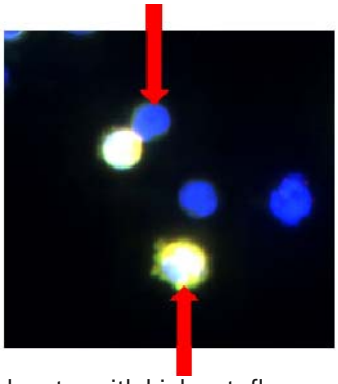
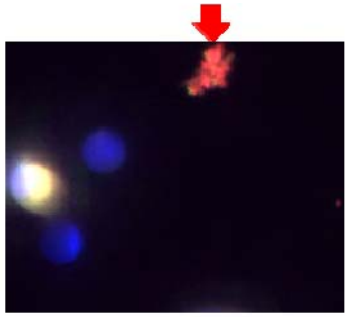
**Filter observed in brightfield showing the pores (see arrows)**



**Objects that can be observed on the filter**

Typically, you will observe a low number of CTCs, depending on the sample, and a few thousand leukocytes on the filter. This section illustrates the objects that might be present on the filter and how to interpret them correctly.

| Object | Description |
|---|---|
| CTC  | This is a CTC showing positive signals which appear as distinct dots (or puncta). Each dot is representing one RNA molecule. The identity of a CTC is confirmed by the expression of CTC biomarker, the presence of the nucleus, and bigger cell size. |

| Object | Description |
|---|---|
| Leukocytes with no background<br>  <br>Leukocytes with high autofluorescence | These are leukocytes from the blood. There are typically several thousand leukocytes captured from 3 mL of blood. The nuclei (as shown in DAPI stain) are about the same size of the filter pores. The leukocytes are normally stained negative in this assay, since they are often trapped inside the pores limiting the accessibility of assay reagents. About 10-15% of the leukocytes on the filter may show high autofluorescence. It varies with individual samples and is related to the storage time of the blood sample before capture. These autofluorescent leukocytes can be distinguished from the CTCs based on (1) the autofluorescence signal is present in all detection channels and appears as uniform staining in the whole cell, and (2) the leukocytes are smaller in size and are often present at the same location of the filter pores. |
| Cell debris<br>  | The filter may retain cell debris that interacts with assay reagents non-specifically. As a result, it is usually stained strongly in all detection channels. It can be distinguished by the irregular size and the lack of DAPI stain. |

## References


Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, Tibbe AGJ, Uhr JW and Terstappen LWMM: Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res 10: 6897-6904, 2004.

Bilkenroth U, Taubert H, Riemann D, Rebmann U, Heynemann H and Meye A: Detection and enrichment of disseminated renal carcinoma cells from peripheral blood by immunomagnetic cell separation. Int J Cancer 92: 577-582, 2001.

Braun S and Marth C: Circulating tumor cells in metastatic breast cancer – Toward individualized treatment? N Engl J Med 351: 824-826, 2004.

Kagan M, Howard D, Bendele T, Mayes J, Silvia J, Repollet M, Doyle J, Allard J, Tu N, Bui T, Russell T, Rao C, Hermann M, Rutner H and Terstappen LWMM: A sample preparation and analysis system for identification of circulating tumor cells. J Clin Ligand Assay 25: 104-110, 2002.

Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW and Hayes DF: Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 351: 781-791, 2004.

Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, Doyle GV, Matera J, Allard WJ, Miller MC, Fritsche HA, Hortobagyi GN and Terstappen LW: Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol 23: 1420-1430, 2005. [Erratum, J Clin Oncol 23:4808, 2005.]

Desitter I, Guerrouahen BS, Benali-Furet N, Wechsler J, Jänne PA, Kuang Y, Yanagita M, Wang L, Berkowitz JA, Distel RJ, Cayre YE: A new device for rapid isolation by size and characterization of rare circulating tumor cells. Anticancer Res 31(2):427-41, 2011.

Fehm T, Hoffmann O, Aktas B, Becker S, Solomayer EF, Wallwiener D, Kimmig R and Kasimir-Bauer S: Detection and characterization of circulating tumor cells in blood of primary breast cancer patients by RT-PCR and comparison to status of bone marrow disseminated cells. Breast Cancer Res 11: 109, 2009.

Hardingham JE, Kotasek D, Farmer B, Butler RN, Mi JX, Sage RE and Dobrovic A: Immunobead-PCR: a technique for the detection of circulating tumor cells using immunomagnetic beads and the polymerase chain reaction. Cancer Res 53: 3455-3458, 1993.

Krivacic RT, Ladanyi A, Curry DN, Hsieh HB, Kuhn P, Bergsrud DE, Kepros JF, Barbera T, Ho MY, Chen LB, Lerner RA and Bruce RH: A rare-cell detector for cancer. Proc Natl Acad Sci USA 101: 10501-10504, 2004.

Maheswaran S, Sequist LV, Nagrath S, Ulkus L, Brannigan B, Collura CV, Inserra E, Diederichs S, Iafrate AJ, Bell DW, Digumarthy S, Muzikansky A, Irimia D, Settleman J, Tompkins RG, Lynch TJ, Toner M and Haber DA: Detection of mutations in EGFR in circulating lung-cancer cells. N Engl J Med 359: 366-377, 2008.

Min Yu, Shannon Stott, Mehmet Toner, Shyamala Maheswaran, and Daniel A. HaberCirculating tumor cells: approaches to isolation and characterization. J Cell Biol. Vol. 192 No. 3 373–382, 2011.

Muller V, Stahmann N, Riethdorf S, Rau T, Zabel T, Goetz A, Janicke F and Pantel K: Circulating tumor cells in breast cancer: correlation to bone marrow micrometastases, heterogeneous response to systemic therapy and low proliferative activity. Clin Cancer Res 11: 3678-3685, 2005.

Naume B, Borgen E, Beiske K, Herstad TK, Ravnas G, Renolen A, Trachsel S, Thrane-Steen K, Funderud S and Kvalheim G: Immunomagnetic techniques for the enrichment and detection of isolated breast carcinoma cells in bone marrow and peripheral blood. J Hematother 6: 103-114, 1997.

Racila E, Euhus D, Weiss AJ, Rao C, McConnell J, Terstappen LW and Uhr JW: Detection and characterization of carcinoma cells in the blood. Proc Natl Acad Sci USA 95: 4589-4594, 1998.

Ross AA, Cooper BW, Lazarus HM, Mackay W, Moss T J, Ciobanu N, Tallman M S, Kennedy MJ, Davidson NE and Sweet D: Detection and viability of tumor cells in peripheral blood stem cell collections from breast cancer patients using immunohistochemical and clonogenic assay techniques. Blood 82: 2605-2610, 1993.

Vona G, Sabile A, Louha M, Sitruck V, Romana S, Schutze K, Capron, Franco D, Pazzagli M, Vekemans M, Lacour B, Brechot C and Paterlini-Brechot P: Isolation by size of epithelial tumor cells: a new method for the immunopharmological and molecular characterization of circulating tumor cells. Am J Pathol 156: 57- 63, 2000.

Wu CH, Lin SR, Yu FJ, Wu DC, Pan YS, Hsieh JS, Huang SY and Wang JY: Development of a high-throughput membrane array method for molecular diagnosis of circulating tumor cells in patients with gastric cancers. Int J Cancer 119: 373-379, 2006.

Zigeuner RE, Riesenberg R, Pohla H, Hofstetter A and Oberneder R: Immunomagnetic cell enrichment detects more disseminated cancer cells than immunocytochemistry in vitro. J Urol 164: 1834-1837, 2000.

## Technical Help

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