
# Application Note

## Introduction

The procedures described in this document are for RESEARCH USE ONLY and not for use in clinical or diagnostic procedures.

### Immunofluorescence

Immunofluorescence Microscopy (IF) is a commonly used technique that can be performed on cells harvested using the Parsortix™ platform. It enables identification and phenotypic characterisation of circulating tumour cells (CTCs) which are captured and eluted from the Parsortix system.

Here we describe a workflow using IF to label CTCs for analysis via the accepted criteria of Cytokeratin positive ($CK^+$), CD45 negative ($CD45^-$) that have a well-defined nucleus.

## Reagents and equipment

| Product | Catalog number | Supplier |
|---|---|---|
| DPBS | 21-030-CV | Corning |
| Triton X-100 | X-100 | Sigma |
| 16% Paraformaldehyde (PFA) | 15710 | Electron Microscopy Sciences |
| Bovine Serum Albumin (BSA) | A7030 | Sigma |
| Pan-Keratin (clone C11) Mouse mAb Alexa488 | 4523S | Cell Signalling |
| PE/Dazzle anti-human CD45 (Clone HI60) | 304052 | BioLegand |
| NucBlue Live Cell Stain [Hoechst33342] | 37605 | Invitrogen |
| LabTek II $CC^2$ 2-Chamber Slides | 154852 | Fisher Scientific |
| Fluorescence cube (Green) | 39002 (or equivalent) | Chroma |
| Fluorescence cube (Red) | 39010 (or equivalent) | Chroma |
| Fluorescence cube (UV/Blue) | 39000 (or equivalent) | Chroma |
| Optional | | |
| Cell Staining Buffer | 420201 | Biolegand |

Buffers:

Staining Buffer: PBS with 0.1% Triton and 3% BSA. Store at 4°C when not in use.

8% PFA: 1 to 1 of 16% PFA in PBS; make fresh on a weekly basis

# Methods

## Parsortix Separation

1. Perform cell separation on Parsortix instrument, following the instructions in the User Manual and Quick Reference Guide
2. Harvest cells, using 210 µL (i.e. do not use second, 1 ml option in harvest protocol) into two chamber slides.

## Staining Process

1. Add 200µL of 8% PFA to harvested sample. Incubate at room temperature for 5 minutes.
2. Place chamber slide into swing bucket centrifuge with slide adaptor. Spin for 5 minutes at 400xg. Ensure centrifuge rotor is balanced.
3. Carefully remove the slide and remove supernatant from the corner of the chamber well.
4. Place 200µL of staining buffer in the well and incubate for 10 minutes at room temperature. Optionally, BioLegand Cell Staining buffer may be used for this step.
5. Add 3µL of undiluted Pan-Keratin antibody (1:50) and 2µL of CD45 antibody (1:100) to each well. Incubate at 37°C for 1 hour.

   Note that recommended antibody concentrations should only be considered a guideline. Antibody titers should be determined to ensure optimal staining for different cell types.
6. Add 1 drop of NucBlue to each well and incubate at 37°C for 10 minutes.
7. Place chamber slide into swing bucket centrifuge with slide adaptor. Spin for 5 minutes at 400xg.
8. Carefully remove the sample slide and remove supernatant from the corner of the chamber well.
9. Place 200µL of PBS into each well.
10. Visualize with fluorescence microscope. $CK^+$ cells visually appear green; with emission and excitation peaks at 499 nm and 520 nm, respectively. $CD45^+$ cells visually appear red; with emission and excitation peaks at 566 nm and 610 nm.

## Sample outcomes

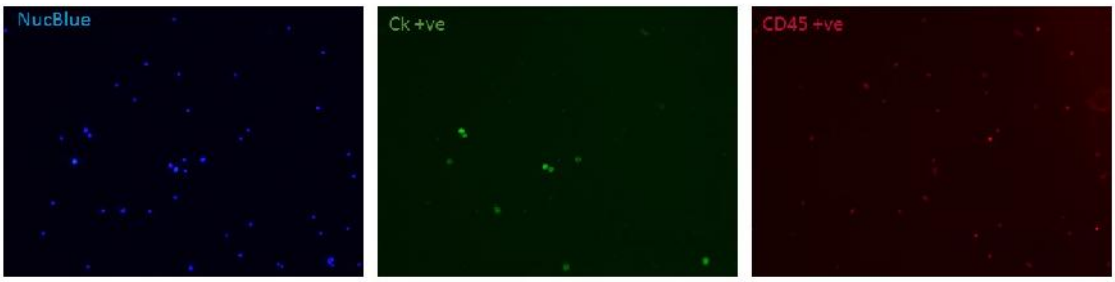


Figure 1: SKBR3 cultured cells separated, harvested, and stained for identification Representative images of results. Taken using Leica DMI5000 fluorescence microscope with 20X objective coupled.

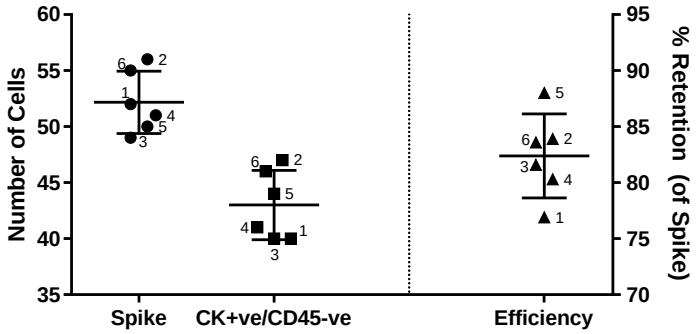


Figure 2: Efficiency of assay for staining of harvested cells from Parsortix. This data shows the number of spiked and identified Sk-Br-3 cells for each of 6 individual experiments. The right y-axis shows the calculated % retention of the 6 samples processed with this assay.

## Troubleshooting

### Assay Troubleshooting

Concern: There are no cells visible at the end of staining assay

When working with rare cells there is always a risk of cell loss. It is extremely important to be careful throughout the process to ensure best results. When a new lab or user begins work with this assay, ANGLE recommends running a control sample with 500 spiked cells alongside experimental conditions and controls to help troubleshoot the source of any potential loss. These cells can be added directly to a separate chamber slide, then fixed/stained alongside any other samples.

### Concern: There is a fluorescent blue haze when visualizing the sample

The presence of a fluorescent haze usually means the sample has not been washed sufficiently. Continuing to visualize the sample may result in miscounting the total number of cells. ANGLE's recommendation is:

1. Add 200µL of PBS to the well and then spin
2. Place chamber slide into swing bucket centrifuge with slide adaptor. Spin for 5 minutes at 400 xg.
3. Carefully remove the sample and remove supernatant from the corner of the chamber well.
4. Place 200µL of PBS into each well.

## Instrument Troubleshooting

See Parsortix User Manual for separation, harvesting or general instrument troubleshooting.

Contact ANGLE technical support at us-support@angleplc.com or eu-support@angleplc.com for additional troubleshooting.