INSTRUCTION MANUAL

nPAC™ CSC IF Kit Cat.# OL5080012 Not for diagnostic use.

MATERIALS PROVIDED

Materials provided	Quantity for 12 tests	Color Code
nPAC™ CSC Antibody Mix (contains mouse anti-Vim-550 / anti-CD45-490 NHS-Ester conjugated antibodies)	12x 50 µl	Orange
DAPI	24 µl	White
nPAC™ Blocking Buffer	12x 100 µl	Yellow
nPAC™ Washing Buffer (2X)	12x 2000 µl	Green
nPAC™ Fixative	1200 µl	Purple
nPAC™ Permeabilization Buffer	12 ml	-
Filter Tubes	1 pack	-
Low-retention Tubes	1 pack	-

STORAGE CONDITIONS

The kit is shipped at 4°C. The expiry date for unopened reagents is one year after manufacture when stored at 4°C, protected from light. Filter tubes and low-retention tubes can be stored at room temperature. nPAC™ Antibody Mix, Blocking Buffer, and Washing Buffer (2X) are designed for single-use and should not be stored after opening. Fixative should be brought to room temperature before use. Washing Buffer needs to be diluted to 1X with demineralized H_2O before use. DAPI, Fixative, and Permeabilization Buffer can be re-used and should be stored at 4°C for up to 1 year after opening. Do not use expired or precipitated reagents. The kit components are manufactured and tested as a master lot. Do not mix and match reagents from different kits.

ADDITIONAL MATERIAL REQUIRED

Required Consumables

Blood Collection Kit (OncoLab, OL6010112)
CRC IF Materials Kit (OncoLab, OL5090012)
PBS (without Ca++ or Mg++), demineralized H_2O

Required Equipment

Cell Counting Device
Tube Rotator (360°)
Vacuum generator to draw down cells on membranes
Standard laboratory equipment (swing bucket centrifuge to spin 130 mm long CPT™ Tubes [1600 RCF], benchtop centrifuge with fixed angle rotor [1600 RCF], pipettes, reaction tubes)
Fluorescence Microscope (e.g., nCyteDx®)

WARNINGS & PRECAUTIONS

Please read the full package insert before testing samples. Collect blood into a CellSave Preservation Tube only and apply to storage and transport conditions specified by the manufacturer. Refrigerating samples prior to processing could adversely affect sample integrity. All biological specimens are considered biohazardous. Handle as if capable of transmitting infection. Microbial contamination of reagents can cause erroneous results and should be avoided. Treat and dispose waste using proper precautions and in accordance with local, state, and federal regulations. MSDS for CellSave Tubes (Menarini) and CPT™ Tubes (BD) can be found on the manufacturer's homepage. Visit bd.com and cellsearchctc.com for further information. Antibody Mix / Blocking Buffer contain traces (<0.1%) of Triton X-100, H_3BO_3, and NaN_3. Permeabilization Buffer contains 0.1% Triton X-100. Fixative contains 4% PFA. Washing Buffer contains <1% H_3BO_3. DAPI contains 4,6'-diamidino-2-phenylindole. MSDS for Antibody Mix, Blocking Buffer, Fixative, and Permeabilization Buffer can be found on axondx.com. MSDS for Washing Buffer and DAPI can be found on oncolab.at. All personnel should follow universal precautions and use laboratory safety equipment (i.e., safety glasses, laboratory coat, gloves). Perform cleaning procedures after each sample batch to prevent carrying over cells from one batch to another.

INTRODUCTION

This kit is used to fluorescently label vimentin (Vim)-positive circulating stromal cells (CSC) and CD45+ white blood cells (WBC). The kit is to be used with the CellSave tubes for sample preparation and the nCyte® platform (AxonDx®) for enumeration and identification of Vim+ populations. Stromal cells from the primary tumor are shed into the bloodstream. Analysis of these cells may be useful to elucidate biological mechanisms of the tumor environment. Analysis and enumeration of CSC is performed using the nAble™ software (AxonDx®). This kit contains nPAC™ immunofluorescent reagents that were designed to standardize and optimize the sample preparation for use with CellSave and CPT™ tubes. After peripheral blood mononuclear cell (PBMC) isolation, fluorescent reagents are added for identification and enumeration of CSC. The fluorescent reagents include antibodies directed against panVim, CD45, and other propriety markers that are restricted to WBC. DAPI (4', 6-diamidino-2-phenylindole, dihydrochloride) is used to stain cell nuclei. The nAble® software automatically scans the entire surface of the Isopore™ membrane (Merck Millipore), acquires images, and displays any event to the user where Vim+ and DAPI+ are co-located. Images are presented to the user in a gallery format for final classification of the captured cell. An event is classified as a CSC when its morphological features are consistent with that of a cell and it exhibits the correct phenotypes, i.e., Vim+, DAPI+, and CD45-.

PREPROTOCOL CONSIDERATIONS

Users may also analyze specimens stained and prepared with this kit on other fluorescence microscopes besides the nCyte® platform from AxonDx®. However, this will require further optimization work. User-defined reagents must be conjugated with an NHS ester type fluorochrome and will need to be optimized for use on the nCyte® platform prior to generating test results. For better accuracy at cell counting, it is recommended to record the cell number of DAPI+ cells; however, cell number may also be determined by counting unstained cells with a light microscope. It is recommended to perform a test run by spiking whole blood with a positive control (Vim+ cell line).

ASSAY PROCEDURE

COLLECT BLOOD SPECIMENS WITH THE **ONCOLAB BLOOD COLLECTION KIT CE-IVD (OL6010112)**:

1. If this Blood Collection Kit is used to isolate rare circulating tumor-associated cells with epithelial marker expression, first draw blood into a 2 ml EDTA tube before using CellSave Preservation Tube / BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube for blood collection. This will prevent blood contamination with skin epithelial cells. Discard the 2 ml EDTA tube afterward.
   Note: If other blood collection tubes are used (i.e., for routine blood draws) before the CellSave Tube will be used, there is no need to draw blood into the EDTA tube first.
2. Collect blood aseptically by venipuncture or from a venous port. Fill the CellSave Preservation Tube until blood flow stops to ensure the correct ratio of sample to anticoagulant and preservative. Immediately mix by gently inverting the tube 8 times. Store the blood sample at room temperature for up to 72 h. Please refer to the CellSave Preservative Tube instructions for use, storage, and handling instructions. Do not refrigerate samples.
   Note: Tube inversion prevents clotting. Inadequate or delayed mixing may result in inaccurate test results. Visually inspect each sample for clotting before processing. Clotted samples should be discarded.
3. Mix the blood in the CellSave Preservation tube by manually inverting 5 times and transfer 6 ml of blood into a correspondingly labelled 8 ml BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube. Invert the CPT™ Tube 10 times. Please refer to the CPT™ Tube instructions for use, storage, and handling.
4. Centrifuge the CPT™ Tube at 1600 RCF for 20 min.
5. Carefully remove most of the plasma layer and discard (leaving a small layer of plasma above the buffy coat will minimize disruption of buffy coat layer and potential cell loss). Collect the buffy coat cell layer (containing WBC and rare circulating cells) and transfer it into 2x 1.5 ml low-retention tubes. Rinse the CPT™ plug with 500 µl PBS and combine in low-retention tubes.
6. Centrifuge in a fixed angled microfuge at 900 RCF for 1 min, rotate tube 180°, and repeat centrifugation for additional min. Repeat the 180° rotation and centrifugation step 3 more times. This will ensure all cells travel to the bottom of the tube without damaging the cells. Centrifuge at room temperature using a room temperature capable centrifuge. Following sample centrifugation, visually inspect each sample tube to ensure no cells remain on the wall of the tube. If cells remain rotate and centrifuge one more time and recheck.
7. Discard the supernatant and further process the mononuclear cell fraction.

STAIN THE SAMPLES USING THE **ONCOLAB CSC IF STAINING KIT (OL5080012)**:

8. Resuspend pellet in 100 µl nPAC™ Fixative (purple cap) and incubate for 15 min at room temperature.
9. After fixing, add 1000 µl of nPAC™ Permeabilization Buffer and incubate for 30 min at room temperature.
10. Centrifuge sample at 1,600 RCF for 1 min, rotate tube 180° and centrifuge for additional min. Repeat 180° spin and centrifuge 3 more times. This will ensure all cells travel to the bottom of the tube without damaging the cells. Centrifuge at room temperature using a room temperature capable centrifuge. Following sample centrifugation, visually inspect each sample tube to ensure no cells remain on the wall of the tube. If cells remain rotate and centrifuge one more time and recheck.
11. Remove supernatant and discard in hazardous waste container, add 1000 µl 1X diluted nPAC™ Washing Buffer (green cap) and resuspend well.

For DAPI+ cell counting at later time point, remove 10 µl of the cells and resuspend in 90 µl 1:100 DAPI dilution (in PBS). Therefore, dilute 2 µl of DAPI (white cap) with 18 µl of PBS (1:10 dilution). Take 10 µl of the 1:10 DAPI dilution and add 90 µl of PBS (1:100 dilution). Keep the rest of the 1:10 DAPI solution for later (protected from light). *Alternatively, you may also determine the number of cells on a conventional light microscope. In that case, dilute 10 µl of cells with 90 µl of PBS and count without trypan blue. If the sample is too dense to count, you may have to dilute it further. Record the cell number.*
Note: Label all reaction tubes accordingly to avoid mixing up samples!

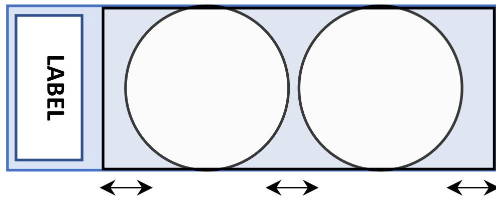
12. Centrifuge the cell / Washing Buffer solution 5 times at 1,600 RCF for 1 min, rotating tube 180° after each spin. During wash, transfer 100 µl of nPAC™ Blocking Buffer (yellow cap) into a filter tube. Use a second filter tube to combine 50 µl nPAC™ CSC Antibody Mix (orange cap) and 1.5 µl of the 1:10 DAPI dilution. Centrifuge both filter tubes at 13,000 RCF for 1 min. Remove the filters afterward. Store Antibody Mix / DAPI solution at room temperature, protected from light until use.
13. After the last centrifugation step of cell / Washing Buffer solution, discard the supernatant and resuspend the cell pellet with the total volume of the *filtered* Blocking Buffer.
14. Incubate the cell / Blocking Buffer solution on a 360° tube rotator for 15 min.

In the case of DAPI+ cell counting: Resuspend cells in 1:100 DAPI dilution and count on a device that can detect DAPI+ cells. Dilute the sample further if necessary. Record the cell number.

15. Add 50 µl *filtered* Antibody Mix + DAPI Solution to the cell / Blocking Buffer solution. Incubate for 30 min at room temperature.
16. Centrifuge 3 times at 1,600 RCF for 1 min, rotating tube 180° after each spin.
17. Remove supernatant and adjust the cell number to 1×10^6 cells/100 µl in 1X Washing Buffer (i.e., for 10×10^6 cells counted add 1000 µl 1X Washing Buffer).

MOUNT SAMPLES USING THE **ONCOLAB CRC IF MATERIALS KIT (OL5090012):**

18. Place membrane on dampened manifold support, one membrane for every 1×10^6 cells (i.e., 10×10^6 cells would need 10 membranes).
    Note: Ensure the manifold lid is securely attached to the base, for proper vacuum.
19. Add 1000 µl ml 1X Washing Buffer to the membrane.
20. Resuspend the cell suspension with a pipette several times and transfer 100 µl of cell suspension onto each membrane.
21. Apply gentle vacuum to draw down cells.
    Note: It is mandatory to keep the membranes hydrated while on manifold support. If the liquid on some membranes was already pulled through while others still have 1X Washing Buffer onside, add some 1X Washing Buffer to the blank membranes to keep them wet.
22. Add 1000 µl ml 1X Washing Buffer and draw down cells, ensuring all liquid has been pulled through before removing the manifold cover. Avoid drying out of membranes.
23. Place two membranes with cells facing upwards on a labelled slide by using forceps, add 140 µl of aqueous mounting medium per membrane, and apply clean coverslip.
    Note: Only add membranes to one slide at a time, so they do not dry out before mounting.



- Label with sample name, slide number, LOT, and date
- Place membranes with cells showing upwards on slide
- Keep at least 1 mm distance between membranes
- Centrally place cover glass above two membranes

24. Allow to sit for 30 min to set, then transfer to humidity chamber if stored overnight.
25. Analyze the samples using the nCyte® platform (**OL90100**) or a fluorescence microscope.

DATA ANALYSIS

Specimens can but do not have to be analyzed with the nCyte® platform (AxonDx®) for enumeration and identification of Vim+ populations. Results are reported as the number of CSC per ml when using 6 ml of blood.

DISPOSAL

Dispose of in accordance with local regulations. Dispose of waste in accordance with environmental legislation.

DISCLAIMER

This product is intended for research purposes only and not intended to be used for therapeutic or diagnostic purposes in humans or animals. CellSave is a product of Menarini Silicon Biosystems Inc. Trademarks: CPT® (BD), nCyte®, nAble®, and nPAC™ (AxonDx). This technology, including products and/or associated components thereof, and procedures and instrument systems described herein, are protected by United States patents and corresponding international patents and pending patent applications, including one or more of the following: US Patent Numbers 10, 147, 180 and EU patent #3083979 for cell detection capture, isolation, and apparatus. Additional patent applications have been filed for improvements to the base technology and further coverage (PCT).

NOTES