ANGLE

CELLS FOR PRECISION MEDICINE.

Application Note

Testing Parsortix™ System enrichment of cancer cell lines

Introduction

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

This document describes a procedure for testing the performance of the Parsortix™ system at enriching spiked cancer cell lines from healthy volunteer blood samples, evidencing system capture and harvest efficiency, as well as harvest purity.

The document summarises how to:

1. Collect and store a healthy volunteer blood sample.
2. Label a cultured cell line with CellTracker™ Green.
3. Spike CellTracker™ Green-labelled cells into a blood sample.
4. Process the blood sample using the Parsortix™ system, and evaluate the cell capture and harvest efficiency.

Reagents and Consumables

Product	Catalogue Number	Manufacturer
10 ml K2 EDTA Vacutainer	367525	BD
CellTracker™ Green CMFDA Dye	C2925	Life Technologies
DMSO	D2650	Sigma-Aldrich
1x PBS	14190-136	Life Technologies
Fetal Bovine Serum	F2442-6x500ml	Sigma-Aldrich
Trypsin EDTA 10x	59418c-100ml	Sigma-Aldrich
15 ml Falcon Tube	352099	Corning
Cell Culture Medium	Dependent on cell line.	Dependent on cell line.
C-Chip Counting Chamber (haemocytometer)	DHC-N01	NanoEnTek
Corning CellBIND 96 well Flat Clear Bottom Microplates	3340	Corning
NucBlue Live Ready Probes Reagent	R37605	Life Technologies
Aluminium Foil	-	-
Decomatic or ProKlenz 120	-/143205WR	Decon Laboratories ltd. /Steris Life Sciences
Absolute Ethanol	E/0650DF/15	Fisher Chemical

⚠ **CAUTION: The end-user is responsible for following all appropriate safety and user guidelines given by the manufacturers.**

Equipment

Product	Catalogue Number	Supplier
Cell Culture Facilities	-	-
Fluorescence Microscope (FITC and DAPI channels)	DMI6000 or equivalent	Leica or equivalent
Parsortix™ PR1 Instrument	PR1	ANGLE Europe Ltd./ ANGLE North America Inc.
Parsortix™ Cell Separation Cassette	GEN3D6.5	ANGLE Europe Ltd./ ANGLE North America Inc.
Parsortix™ Cleaning Cassette	GEN3P10RUO	ANGLE Europe Ltd./ ANGLE North America Inc.

Methods

Collection and Storage of Healthy Volunteer Blood Sample

Blood samples should be processed either the same day as collection or within 24 h of collection.

1. Collect 10 ml of blood from a healthy volunteer into a 10 ml EDTA vacutainer. Invert the tube 8-10 times. Follow relevant safety and ethical considerations.
2. If processing the blood sample on the same day as collection, the blood sample can be stored at room temperature for up to 8 h.
3. If processing the blood sample on the day after collection, store the blood sample at 4 °C overnight. The next day, place the blood sample at room temperature for 30 min to warm. Mix the blood sample by placing on a blood roller or by inverting 8-10 times.

CellTracker™ Green Labelling of Cells

Prepare stock dye solution

1. Allow CellTracker™ Green CMFDA to warm to room temperature, and dissolve in DMSO to a final concentration of 6 mM.
2. Aliquot to 20 µl and store covered at -20 °C.
3. Working concentration: 6 µM (1:1,000)

Staining protocol – adherent cells

Cells should be sub-cultured 24 h prior to staining. The cells should be at approximately 70 % confluence on the day of spiking.

1. Prepare CellTracker™ working solution (per T75: 8 ml pre-warmed 1x PBS and 8 µl stock CellTracker™ Green CMFDA solution (giving 6 µM working concentration)).
2. Remove culture medium from the cell flask and then wash the cells with 1x PBS.
3. Gently add 8 ml of CellTracker™ working solution to the flask and incubate for 10 min at 37 °C in a cell culture CO_2 incubator.
4. Remove the medium and add 10 ml of full medium (ie. the standard cell culture medium used for the cell line. The medium must include serum to quench the reaction.)
5. Remove the medium and then wash the cells with 1x PBS.

6. Trypsinise the cells by adding 2 ml of a 1x Trypsin/EDTA solution. Incubate the cells for 5 min at 37 °C in a cell culture CO_2 incubator. Add 8 ml of cell culture medium containing serum and transfer the entire cell solution to a 15 ml Falcon tube.
7. Centrifuge the cells at 300 g for 5 min to pellet the cells. Remove the supernatant and re-suspend the cell pellet in 1 ml of 1x PBS.
8. Confirm that the CellTracker™ Green has been taken up by the cells by examining the cells using a fluorescence microscope (FITC channel). Excitation: 492 nm, emission: 517 nm.
9. Proceed immediately to spiking of the blood sample.

Spiking CellTracker™ Green-Labelled Cells into Blood Sample

Preparation of a 50 cells/µl stock suspension of cells

1. Pipette 10 µl of the cell suspension into a haemocytometer and determine the cell concentration in cells per µl (ie. count the four outer corner squares of the counting grid, and then divide this number by 4 to get the average, then multiply the number by 10 to obtain cells per µl. Use the following formula: n/4*10 = number of cells/µl)
2. Dilute an aliquot of the labelled cell suspension in 1x PBS, calculated to achieve a final concentration of 50 cells/µl. For example, if original cell suspension is 500 cells/µl, then dilute 1:10 by adding 100 µl of the cell suspension to 900 µl of 1x PBS.
3. Confirm that 50 cells/µl solution is achieved by pipetting 10 µl of the diluted cell suspension into a haemocytometer. Determine the cell concentration as described in step 1 above. If necessary adjust to the desired concentration of cells/µl and confirm the corrected concentration.

Transfer (Spiking) 200 labelled cells into blood sample and control wells

1. Prepare the control plate by adding 200 µl of 1x PBS to six wells of a 96-well plate.
2. Prepare the blood sample by inverting the sample several times.
3. Re-suspend the 50 cells/µl solution before starting the spiking, and then pipette 4 µl of the cell suspension into three of the control wells. Resuspend the cell suspension again by pipetting, and then pipette 4 µl of the cell suspension into the blood sample, and then into each of the remaining three control wells.
4. Cover the control plate with aluminium foil and incubate at room temperature for at least 60 min to allow the cells to settle.
5. Count the cells in each well using a fluorescence microscope (FITC channel).
6. Calculate the mean number of cells in the six wells. This value will act as the assumed number of cells spiked into the blood sample. Ensure that the % CV of the control wells does not exceed 12 %. If it is above 12 %, consider repeating the spiking with another blood sample.

Processing the Spiked Blood Sample using Parsortix™ and Counting

Follow the instrument set up instructions in the Parsortix™ User Manual, and then process the blood sample using the following protocols:

1. PX2_PF
2. PX2_S99F
3. PX2_CT2
4. PX2_H
5. PX2_CT

Counting the cell capture

1. After the separation is complete (PX2_S99F), remove the cassette from the instrument and examine using a fluorescence microscope (FITC channel).
2. Start visualising the cassette, using the x10 microscope objective, at one end and follow along the steps (see pattern designated by the red arrows in Figure 1).
3. Count the pre-labelled cells (capture rate). **Note:** *Most of the pre-labelled cells will be located along the steps of the cassette, but the cells can also be found in the areas directly to the left or right of the steps.*

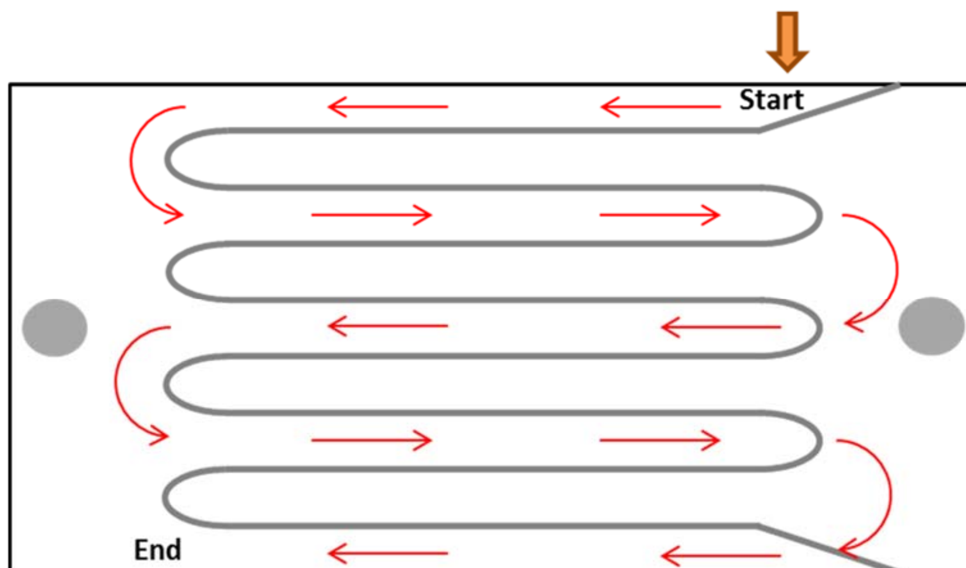
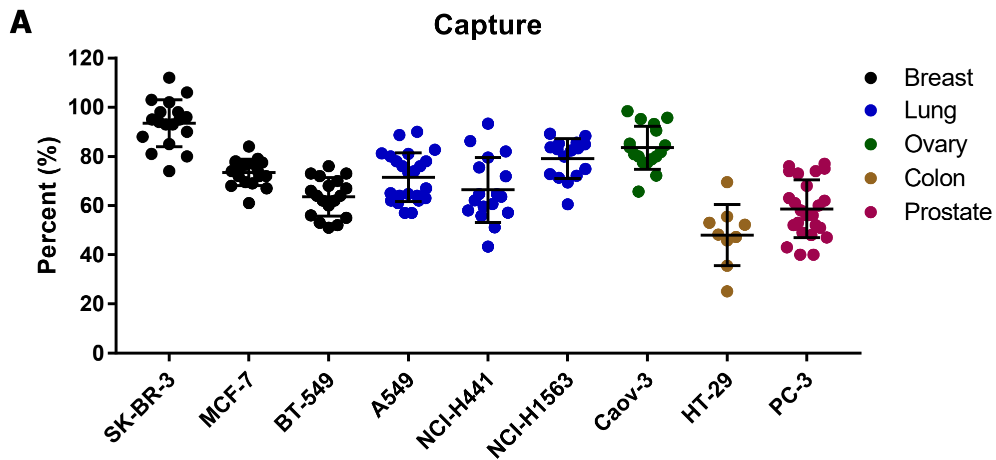


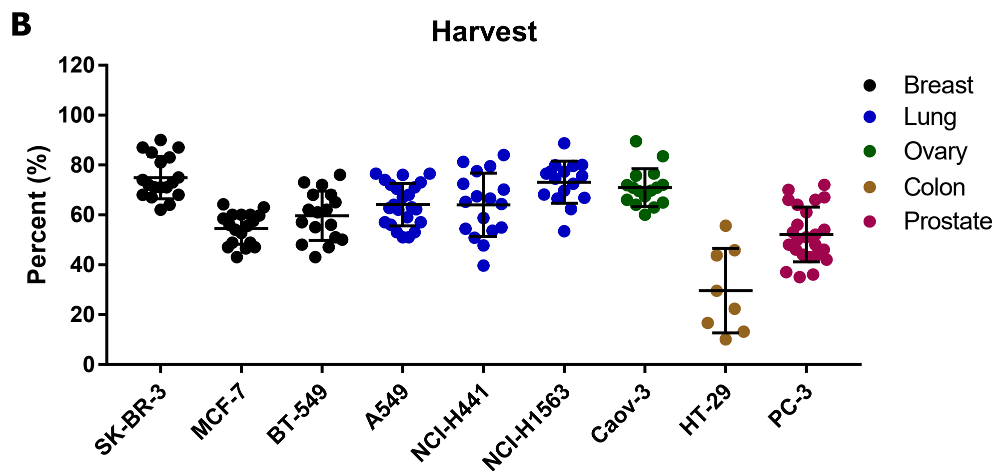
Figure 1: Cassette Diagram Indicating Procedure for Capture Enumeration.

Counting the cell harvest

1. Harvest the cells from the Parsortix™ instrument into a 96-well plate or an 8-well chamber slide (PX2_H).
2. If interested in harvest purity, add 2 drops of NucBlue reagent to the well/slide to enable for the examination of white blood cells.
3. Allow the cells to settle by leaving them protected from light, at room temperature, for at least 60 min.
4. Count the cells using a fluorescence microscope. Spiked cells (green-labelled) will be positive in both the FITC and DAPI channels. White blood cells in the harvest will be positive in the DAPI channel only.

Sample Outcomes





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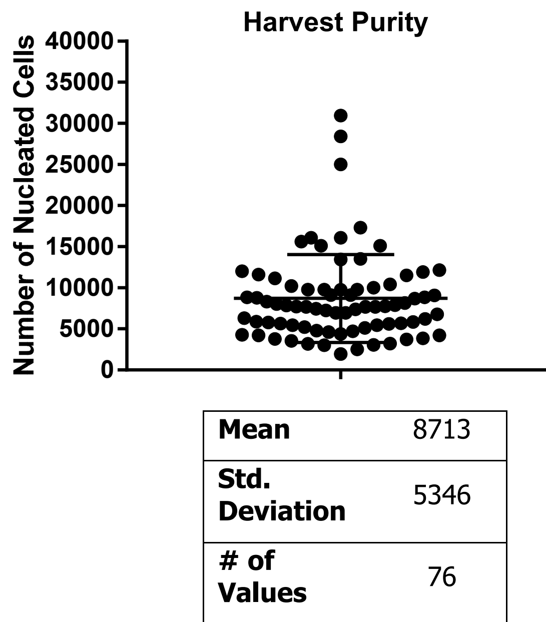
	Breast			Lung			Ovary	Colon	Prostate
Cell Line	**SK-BR-3**	**MCF-7**	**BT-549**	**A549**	**NCI-H441**	**NCI-H1563**	**Caov-3**	**HT-29**	**PC-3**
Mean % Capture	93.5	73.6	63.6	71.6	66.5	79.2	83.6	48.0	58.7
±SD	±9.5	±5.4	±7.8	±9.9	±13.2	±8.1	±8.7	±12.5	±11.8
Mean % Harvest	74.9	54.6	59.7	64.2	64.0	73.1	71.0	29.6	52.1
±SD	±8.5	±6.4	±9.9	±8.5	±12.7	±8.4	±7.6	±17.0	±11.0
# of Values	18	18	18	23	17	16	17	9	24

Figure 2: Cell Line Capture and Harvest Data. *CellTracker™ Green-labelled cells were spiked into healthy volunteer EDTA blood samples and processed using the Parsortix™ System.* ***(A)*** *Percent Capture* ***(B)*** *Percent Harvest* ***(C)*** *Table of values shown in above graphs.*



Mean	8713
Std. Deviation	5346
# of Values	76

Figure 3: Harvest Purity – EDTA 24 h Blood Samples. *Number of nucleated cells in the harvest after 10 ml healthy volunteer EDTA blood samples are processed using the Parsortix™ system.*

Troubleshooting

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.