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- Expert Report -

Validation of a Negative Selection Cell Enrichment Assay

1. Introduction and purpose

Subject of this validation audit was the performance evaluation of the automated cell enrichment platform called Walderbach manufactured by SanoLibio GmbH (Germany). The automated process is purposed to enrich rare cells in whole blood. The enrichment product comprises all benign as well as malignant, common and uncommon rare nucleated cell events and has relevancy to biomedical research and routine. The assay is based on magnetic cell separation technology employing the negative selection principle thereby depleting white blood cells (WBC) by associating magnetic beads specifically reacting to the common leukocyte antigen CD45, which then become separable in the presence of a magnetic field. In contrast, the desired cells do not express the common leukocyte

antigen CD45 and may then remain in solution. This report is purposed to verify to whom it may concern the assay performance. The enrichment quality mainly comprises two parameters; recovery of desired cells being circulating rare cells, (e.g. circulating tumor cells) and the depletion of undesired cells being WBCs. The experimental set-up, denoted as MCF7-50 was designed to simulate a cancer patient sample and likewise accommodate enrichment validation using the state of the art analysis methodology.

Within the scope of this validation, the enrichment assay was investigated in view of the following questions;

(i) A main reason of lowered sensitivity in diagnostic assays employing magnetic cell separation enrichment based on the negative selection principle is cell loss. The herein validated enrichment assay shall sufficiently answer to the question of how much desired cells are lost by the Walderbach process, when spiked into a cell suspension of pre-enriched WBC. If the assay at current state performs as expected, the so called recovery rate shall be accepted in range not lower than 80%.

(ii) A main reason of impractical analysis and inaccurate diagnosis is the commonly found high WBC carry-over. The herein validated enrichment assay shall answer to the question of how much WBCs remain after the enrichment. The so called depletion efficiency is measured in log scales and shall result in greater 3.5 log depletion as such resulting in a WBC carry-over not exceeding 5000 cells.

(iii) As basis for internal referencing of progress and comparison with state of the art technology, this validation shall answer to the question of how efficient is the enrichment. As such recovery of desired cells and likewise the depletion of WBCs must be considered in the same experiment setting. It is envisioned to achieve greater 3.5log enrichment.

(iv) Last but not least, the question shall be answered, if the assay is able to achieve purities after enrichment greater 1% that is currently considered the critical limit for molecular analysis for the given spiking amount of approximately 50 cells per mL.

MCF7 -50 Validation Experiment

The MCF7-50 Experiment was developed as a routine quality control experiment by Premise Biosystems simulating diagnostic assay performance. The breast cancer cell line MCF7 was used as model tumor cells and retrieved from ECDD Mahidol University service laboratory. The experiment has been conducted by the R&D team at the laboratory site of the Premise Biosystems in presence of the reviewer from spiking tumor cells into purified WBC suspension till analysis. The experiment consists essentially of a pre-enrichment step as to remove red blood cells, an enrichment step, as to remove WBCs and a staining step as to identify the desired or spiked cells. This report is result of one audited experiment using common fluorescence microscopy for cell counting during experimentation and fast scanning automated microscopy for comprehensive analysis.

1. Materials

Walderbach fluid handling platform (SanoLibio GmbH) and
Software Version: CD45neg Assay 1.2.13.4 (SanoLibio GmbH)
Pipettes and tips (nonsterile)
Syringe 1mL equipped with needle
Plastic containers 1.5mL
Operetta high content imaging system (PerkinElmer)
Olympus BX51 inverted fluorescence microscope equipped with filter sets for FITC, PE and Hoechst fluorescence dyes

2. Reagents

Whole blood healthy donor, 5 mL stored in Green-Top Vacutainer Tube (Sodium Heparin) stored at room temperature in the dark
Cell line MCF7 stored in 1mL DMEM complete solution at 4 degree in the dark
Walderbach buffers for enrichment procedure
Cell friendly buffer solution
a-CD45 magnetic particles (SanoLibio GmbH)
Staining reagents; a-CD45PE and a-EpCamFITC conjugated Mabs (eBioscience)

3. Sample preparation protocol

(i) Pre-Enrichment: Whole blood and model tumor cells were stored 48 hours at 4 degree before use in the spiking experiment. For WBC preparation, 4.7mL of whole blood underwent red blood cell lysis by ammonium chloride based chemical lysis procedure. The resulting white blood cell (WBC) stock was kept in 600µL cell friendly buffer and appeared in whitish color. Then, purified WBC as well as model tumor cells underwent a 30 min resting period in parallel after the RBC lysis procedure by storing the cells in a cell friendly solution at room temperature in the dark. For that purpose, 8µL of the tumor cell stock was transferred into 1mL cell friendly solution. During resting, cell counts of WBCs and tumor cells were assessed in the presence of the reviewer. Whole blood nucleated cell counts were performed by diluting whole blood 1:10 with PBS pH 7.4 and staining the suspension with Hoechst DNA staining. Cells were counted in a counting chamber slide under respective fluorescence light ultra-violet excitation. WBC stock counts were performed similarly employing a dilution factor 1:100. WBC stock counting was repeated and confirmed under 5% deviation during automated enrichment. The tumor cell count per spiking volume was assessed by dropping 10µL from the tumor cell stock onto a cover slide and counting under brightfield using an inverted microscope. The entire procedure started 8.15AM and lasted until 9.17AM.

WBC stock concentration: 4.67×10^4 per µL
Total amount of WBC in stock (600µL): 2.8×10^7 cells (5.92×10^6 cells/mL whole blood).
Whole blood WBC count: 6.50×10^6
calculated WBC loss due to RBC lysis procedure: 8.4%

Corresponding volume for $2.5x10^7$ WBCs (corresponds to 3-6 mL whole blood) : 535µL
Tumorcell stock drop count (10µL): 60cells

(ii)Spiking sample preparation. Prior to enrichment the samples have been prepared as follows. In brief, the spiking volume of MCF7 cells was adjusted to 9µL and corresponds to a theoretical count of 54 cells (see drop count 10µL=60 cells). Also, the WBC cell suspension of $2.5x10^7$ was re-adjusted to 600µL using cell friendly buffer solution. At first, tumor cells were added at equal volumes into three empty 1.5mL plastic containers (corresponding to three samples). All spike aliquots were done for each sample in parallel and in sequence following equal mixing and pipetting procedure. Then, cell suspensions or cell friendly buffer solution was added to the enrichment sample and controls, respectively.

1. 2x Positive controls: 20µL of cell friendly buffer solution was added to 9µL tumor cells. Then, the samples were stored at room temperature in the dark until staining procedure.

2. Before enrichment: 600µL WBC suspension was added to the 9µL tumor cell suspension.

3. After enrichment: Sample to measure the purity and concentration of model tumor cells after enrichment referenced against WBCs.

(iii) Walderbach Enrichment: After spiking preparation, the Walderbach platform was started by launching the Walderbach Software and placing required buffers (incubation buffer and washing buffer) and empty containers into the machine as well as mounting a syringe with needle for pipetting. During initialization, the total number of cells ($2.5x10^7$ cells) was keyed into the program as required to calculate the magnetic bead amount. During processing the removal of WBCs was obvious with observing ever smaller pellets after centrifugation of each depletion cycle. The final enriched sample was retrieved after 3 depletion cycles, measured a volume of 30µL and appeared clear and transparent. After the enrichment process, cells were immediately subjected to staining. The Walderbach enrichment process required 63 min.

(iv) Staining: As to discern tumor cells in the enriched sample from WBCs by fluorescence microscopy, a simple two color system was used, PE (yellow) for WBCs and FITC (green) for tumor cells. The exact same procedure was conducted on the two control samples. In brief, 0.6µL of a-CD45PE and a-EpcamFITC conjugated antibodies (ebioscience) were added to the samples each containing roughly 30µL. The cell suspensions were incubated for 30min in the dark at room temperature and subsequently washed by adding 1mL cell friendly buffer solution. Cells were pelleted in two steps at 500 rpm and 700 rpm, rejoined and adjusted to 100µL in cell friendly buffer. The entire process from RBC lysis till staining required 3hrs (finalization 11.15AM).

(v) Analysis: The Operetta high content imaging system (PerkinElmer) was used for fluorescence microscope analysis as to support objectivity in validation by visualization of positive events. The three samples (sample after enrichment, positive control #1 and #2) each containing 100µL were loaded into one well each of a CellCarrier-384 Ultra Microplate (PerkinElmer). The cells were spun down a last time as to be able to record all cells at the bottom layer of the well in a fine dispersed monolayer. Then, the well-plate was mounted into the Operetta automated microscope and images were recorded in bright field, PE and FITC channels at 20x magnification recording the entire area of the well requiring approximately 2 minutes per well. A so called three color stack image comprised an area of 675x508 µm or $3.43x10^5$ μm^2 (camera field, see figure 1, yellow frame). The total well bottom area measured $1.063x10^7$ μm^2 (square length 3.26 mm, see

https://www.perkinelmer.com/lab-solutions/resources/docs/TDS_CellCarrier-384_Ultra_17141.pdf) and was covered by 35 pictures. For objectivity, cell counting of desired cells was conducted at the time of recording and later on the images in presence of the reviewer.

4. Calculations

(i) Determination of Recovery

The recovery shall be understood as the percent ratio between the number of spiked cells before enrichment and the number of recovered cells after enrichment. Therefore, the recovery may never exceed 100%. In detail, the recovery is calculated as the ratio between positive events in the sample after enrichment (sample) and the average of the counts in the positive controls (control #1 and #2). Positive events are characterized as the tumor cell population by green fluorescent large near round and/or clustered events.

(ii) Determination of Depletion Efficiency

The depletion efficiency provides information of how many WBC have been removed. The assessment of the depletion efficiency requires the WBC count in the sample after enrichment (spiked sample) and is expressed in log-units to the base of 10 and calculates as the ratio between the total amount of remaining WBC and the initial amount of nucleated cells at enrichment start here, counting $2.5x10^7$ nucleated events. Thereby, the remaining undesired cells are characterized as the CD45 positive fraction and rated positive by the yellow fluorescence signal in range from near background till highest signal peaks. To do so, yellow positive events were counted manually on the pictures at 20x magnification, analysing 5 camera fields at various locations of the well area (summing up to 13% of the entire area), resulting in an average WBC count per field (after enrichment) and assuming and confirming near equal cell distribution. The WBC count per field was then extrapolated to the total well bottom area.

(iii) Determination of Enrichment Efficiency

The enrichment efficiency is the ratio between the purity of model tumor cells before and after enrichment as measured against the WBC population per given total event count and is expressed in log-units. The purity is given in % and calculates in this case as the ratio between tumor cells and WBC. Therefore, the determination of the enrichment efficiency requires knowledge of the tumor cell as well as WBC counts before and after enrichment, respectively.

5. Results

(i) The recovery

The controls #1 and #2 measured 40 and 60 tumor cell events, respectively. The enriched sample counted 40 events. An illustration of the identification of tumor cells via image counting is given in figure 2.

(ii) The depletion efficiency

The amount of WBCs before enrichment was manually assessed by counting chamber method adjusting the cell suspension to $2.5x10^7$ cells. The average WBC count per field (5 counts) after enrichment counted 97±12 cells. When extrapolated to the total area (31x a camera field), the remaining WBCs counted 3007 cells. Then, the depletion efficiency measured 3.92 log.

(iii) The enrichment efficiency

The enrichment efficiency of the Walderbach process (alone) requires the comparison with the positive controls as prepared in the experiment hereby using the spiking amount of 40 model tumor cells (control #1). Consequently, the theoretical purity of the desired cells in the sample "before enrichment" measured 0.00016% assuming exactly $2.5x10^7$ nucleated cells at the initial situation. The purity of the sample "after enrichment" measured 1.33% (40 tumor cells out of 3007 WBCs). Then, the enrichment efficiency measures 3.92log. Beyond validation purpose is the assessment of the enrichment efficiency of the entire assay comprising enrichment, staining and microscopy analysis. Hereby, the initial spiking count of 54 cells served as positive control, then calculating an enrichment efficiency of 3.79log.

The essence of the validation experiment shall be listed in the table below.

Quality Parameter	**Value**
Recovery against control 1	100%
Depletion Efficiency	3.92log
Enrichment Efficiency (Walderbach Process)	3.92log (control 2: 3.74log)
Enrichment Efficiency (Walderbach, Staining, Analysis)	3.79log
Purity (measured against WBCs)	1.33%

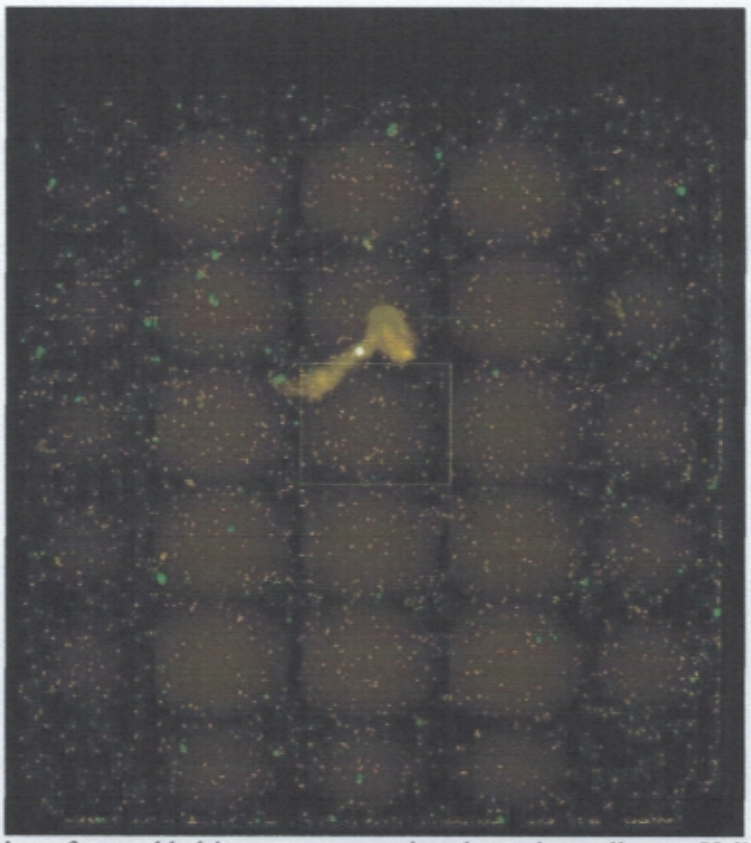

Figure 1: Illustration of assembled images representing the entire well area. Yellow dots represent CD45PE stained WBCs. The yellow squared frame represents a camera field of 675x508 µm or $3.43x10^5$ $µm^2$. The entire capture area comprises 35 camera fields.

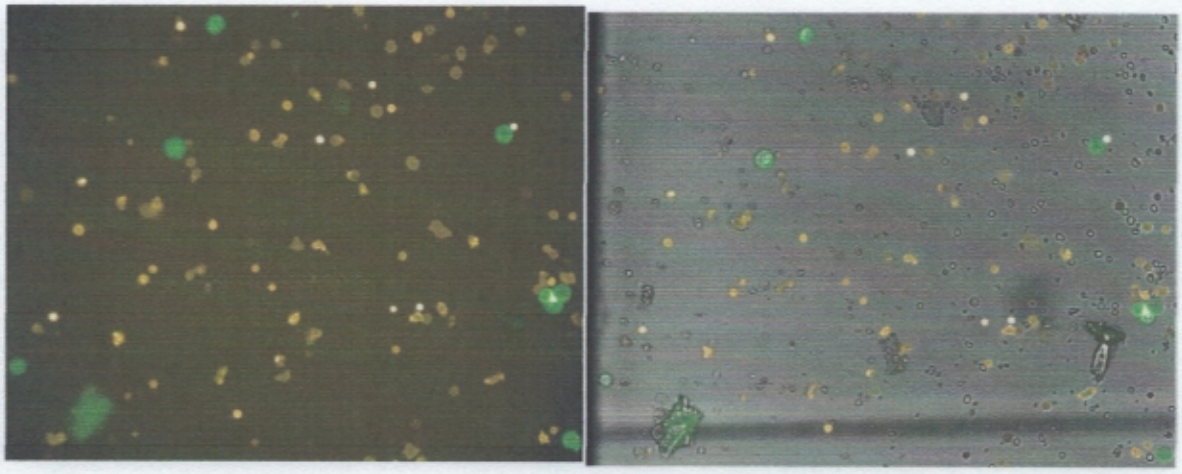

Figure 2. Detailed illustration of image record at 20x magnification in yellow color and green color channel (right) and all channel merge (left). The picture contains 6 tumor cells.

The reviewer acknowledges that the design of experimentation is scientific sound was appropriately documented and that the experiment has been carried out according to the described methodology by personal of Premise Biosystems, Co., Ltd. The subsequent sample analysis using the Operetta high content imaging system was then conducted by a specialist of the ECDD at Mahidol University.

In case of questions I may be contacted at ____pboonsakan@gmail.com_____

Reviewer
(Dr. Med. Paisarn Boonsakan)
Date: 27 / June / 2019

Managing Director, Premise Biosystems Co., Ltd.
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บริษัท เพรมมิส ไบโอซิสเต็มส์ จำกัด

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