

REPRESENTATIVE DATA PLOTS FOR HUMAN BLOOD AND APHERESIS PRODUCTS

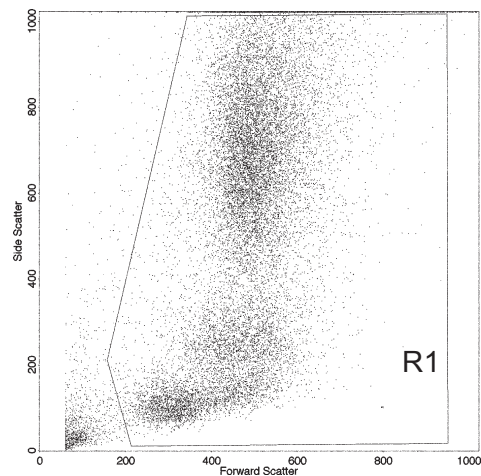


Figure 1a. FSC vs. SSC. Region R1 is drawn to encompass all nucleated cells.

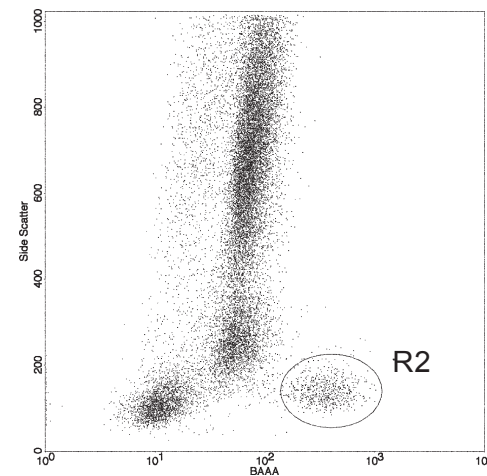


Figure 1b. Test Sample. Dot plot gated on R1. R2 should be drawn to include all ALDH^{br}SSC^{lo} cells.

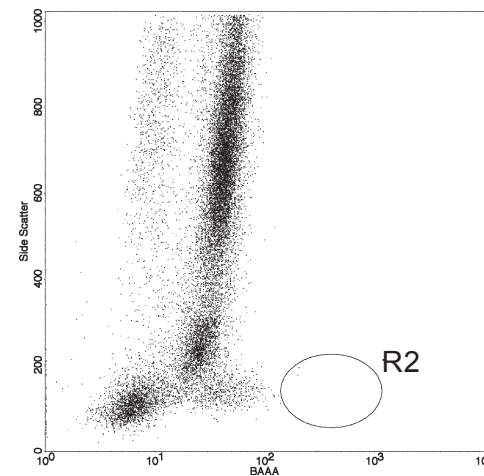


Figure 1c. DEAB Control. Dot plot is gated on R1. R2 should be adjusted so that few or no events appear in this region.

REPRESENTATIVE DATA PLOTS FOR UMBILICAL CORD BLOOD

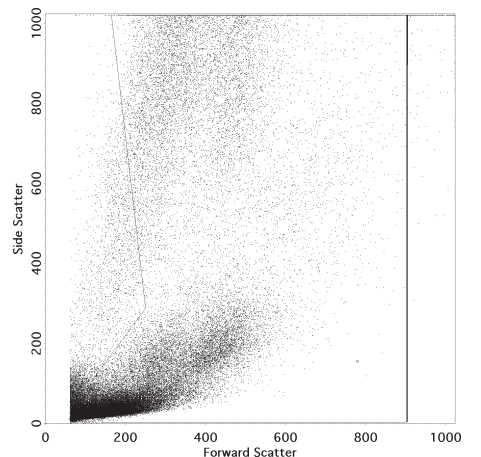


Figure 2a

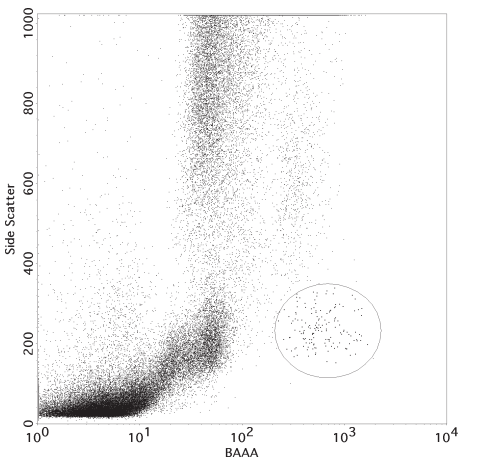


Figure 2b

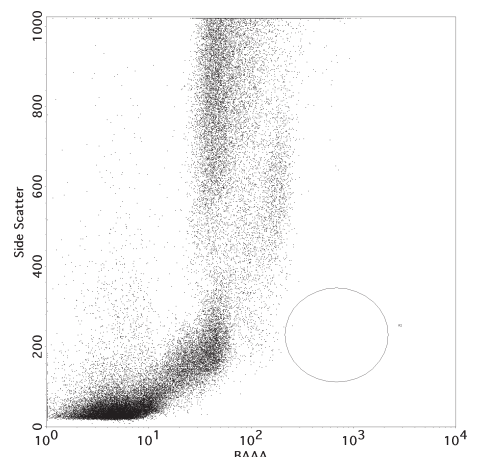


Figure 2c

REPRESENTATIVE DATA PLOTS FOR HUMAN BONE MARROW

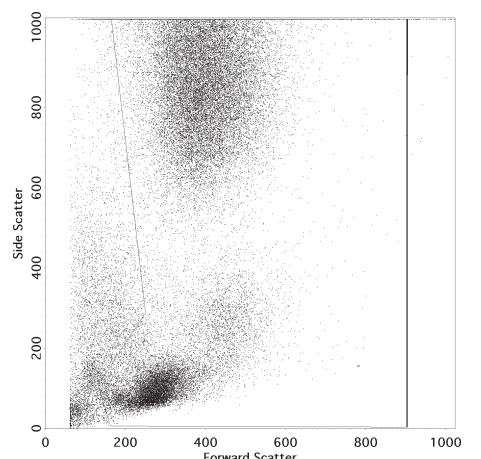


Figure 3a

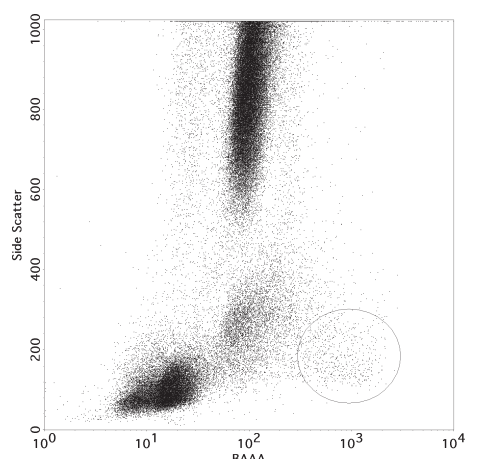


Figure 3b

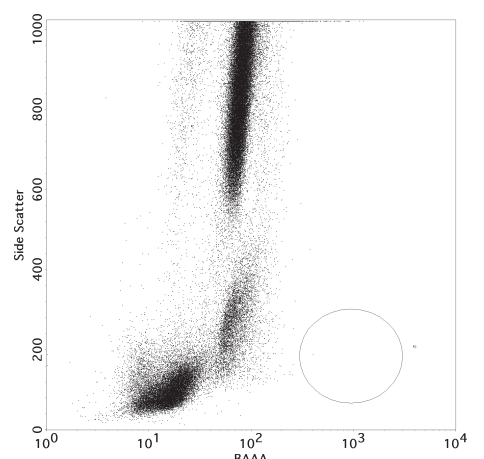


Figure 3c

ALDAGEN

ALDECOUNT

20 Tests

ALDECOUNT®

Boron, [N-(2,2-diethoxyethyl)-5-[(3,5-dimethyl-2H-pyrrol-2-ylidene-KN)methyl]-1H-pyrrole-2propanamido-KN1]difluoro-, (T-4)- (9CI)

INTENDED USE

ALDECOUNT is used to identify and enumerate by flow cytometry low side scatter cells that express high levels of aldehyde dehydrogenase (ALDH^{br}SSC^{lo}) in human peripheral blood, leukapheresis, bone marrow and umbilical cord blood samples. The test is for in vitro diagnostic use and is suited only for professional use in specialized clinical laboratories.

INDICATIONS FOR USE

This device is to be used for general diagnostic purposes to enumerate ALDH^{br}SSC^{lo} cells in fresh or frozen clinical samples (see Limitations below). These samples can be from donors mobilized with granulocyte colony stimulating factor (G-CSF) or some other mobilizing agent, or from patients that have been mobilized or treated with chemotherapeutic agents. This device is to be used by highly trained laboratory personnel skilled in cell staining and flow cytometric analysis.

SUMMARY AND PRINCIPLE

ALDH^{br}SSC^{lo} cells have been shown to have properties of stem and progenitor cells⁽¹⁻⁷⁾. Human cells with high ALDH activity become intensely fluorescent, ALDH^{br}, when exposed to ALDECOUNT reagent, a substrate for ALDH. The dry ALDECOUNT reagent is provided in a stable, inactive form. The activated form of ALDECOUNT freely diffuses into cells and is a non-toxic substrate for ALDH. The amount of fluorescent ALDH reaction product that accumulates in viable cells directly correlates to the ALDH activity in these cells⁽⁶⁻⁷⁾.

The fluorescent substrate is converted by intracellular ALDH into a charged product that, under the conditions used for the reaction, is retained only in intact cells that express high levels of ALDH. Cells that have active intracellular ALDH but are apoptotic or nonviable are not capable of retaining the charged product⁽⁶⁾.

The product is intended for use with a flow cytometer equipped with a 488 nm argon ion laser for excitation and a 525 nm band pass filter. ALDH^{br} cells are identified by flow cytometry as cells with low side scatter and high green fluorescence (expression of ALDH). Such cells are recognized by comparing the fluorescence in a test sample to that in a control containing diethylaminobenzaldehyde (DEAB), a specific inhibitor of ALDH.

The ALDECOUNT Reagent is converted to the active form by treatment with an activator. Activated substrate is diluted to a working concentration with ALDECOUNT Neutralization Buffer. To perform the assay, cells are suspended in ALDECOUNT Assay Buffer and added to the activated substrate contained in the ALDECOUNT Reagent tube. An aliquot of this cell mixture is immediately transferred to a control tube. Both tubes are incubated to allow the reaction to proceed. Throughout the assay, the cells are maintained in ALDECOUNT Assay Buffer because it contains an ATP binding cassette-mediated transport inhibitor and sodium azide, compounds that prevents active efflux of the fluorescent product from viable cells. Following incubation, cells are maintained on ice to further inhibit efflux of reaction product until analysis is complete. The amount of intracellular reaction product is measured by flow cytometry.

US Patent No. 5,876,956; 6,627,759; 6,537,807; 6,991,897. Australian Patent No. 774566; 753975. Singapore Patent No. P-81176.

MATERIALS PROVIDED

20 ALDECOUNT Reagent tubes
20 Control tubes (empty)
1 vial Activator
1 vial of DEAB Reagent in ethanol
1 bottle of Neutralization Buffer
1 bottle of ALDECOUNT Assay Buffer

MATERIALS AND EQUIPMENT NOT PROVIDED

Gloves, lab coat, biohazard bags, bench cover, 10% hypochlorite solution
Pipettes to deliver 5 to 50, 500, 1500 microliters, pipette tips
ALDECOUNT Lysis Buffer (packaged separately)
Low speed centrifuge (capable of 250 x g)
37°C water bath or heat block (incubator not recommended)
Flow cytometer equipped with a 488 nm argon ion laser for excitation and a 525 nm band-pass filter for detection in the green fluorescence (FL1) channel
Refrigerator (2 to 8°C) or ice

PROCEDURE

Read all directions before beginning the test.

Specimen Preparation:

- Effective lysis of erythrocytes in blood products is necessary for the ALDECOUNT assay to work properly. ALDECOUNT Lysis Buffer must be at room temperature before addition to peripheral blood, bone marrow and fresh cord blood specimens.
 - Peripheral blood or apheresis specimens:
 - Add 10 parts ALDECOUNT Lysis Buffer to one part sample.
 - Incubate for 15 minutes at room temperature (RT; 18 to 22°C). Do not exceed 20 minutes.
 - Bone marrow specimens:
 - Add 40 parts ALDECOUNT Lysis Buffer to one part sample.
 - Incubate for 30 minutes at RT. Do not exceed 40 minutes.
 - Fresh cord blood specimens:
 - Dilute samples with an equal volume of PBS.
 - Add 1/5th volume of hetastarch to each sample. Mix tube contents by gentle inversion.
 - Incubate for 1 hour at RT to allow erythrocyte agglutination and precipitation.
 - Draw off supernatant containing the cells of interest into a new tube.
 - Wash the cells by dilution with an equal volume of PBS and centrifuge at 250 x g for 5 minutes.
 - Suspend the cell pellets in ALDECOUNT Lysis Buffer (approximately 10 parts buffer to one part cell pellet volume).
 - Incubate for 15 minutes at 37°C. Do not exceed 20 minutes.
 - Cryopreserved cord blood specimens:
 - Partially thaw cord blood units at 37°C. Mix with an equal volume of dextran containing 5% human serum albumin (HSA).
 - Centrifuge suspension at 850 x g for 30 minutes at 4°C.
 - Suspend the cell pellet in the original volume with cold PBS, 1% HSA, 100 U DNase I.
 - Perform a cell count on an aliquot of sample. If the erythrocyte to leukocyte ratio is >10, pellet the cells by centrifugation at 250 x g for 5 minutes at 4°C.
 - Remove supernatant fluid, suspend pellet in 40 ml of cold ALDECOUNT Lysis Buffer and incubate for 30 minutes at 4°C.
- Centrifuge the cells at 250 x g for 5 minutes. Remove supernatant.
- Suspend each cell pellet with a minimum of 1 ml ALDECOUNT Assay Buffer. Obtain a cell count. Adjust cell concentration to 4 x 10⁶ cells per ml. Each test requires 0.5 ml of cell suspension.

Tube Preparation:

- Assemble all kit components and supplies; allow all reagents to come to RT before use.
- For each sample, label and prepare one ALDECOUNT reagent tube as follows:
 - Add 50 µl of Activator to each ALDECOUNT Reagent tube. Incubate the tubes at room temperature for 20 minutes. Do not exceed 30 minutes.
 - Add 1.5 ml of Neutralization Buffer to each ALDECOUNT Reagent tube and mix. Activated ALDECOUNT Reagent tubes are stable for 24 hours when stored at 2 to 8°C.
- Immediately before performing the assay, add 5 µl of DEAB solution from the kit to each negative Control tube.
NOTE: DEAB is dissolved in ethanol; recap tube immediately after use to prevent evaporation.

ALDECOUNT Assay:

Note: For each sample, prepare one test and one control tube. Process multiple samples sequentially.

- To one activated ALDECOUNT Reagent tube, add 0.5 ml of prepared cell suspension. Mix, and immediately transfer 0.5 ml of the mixture to the corresponding DEAB Control tube.
NOTE: The ALDH reaction begins immediately upon addition of the sample. It is critical that each aliquot of reaction mixture be added to the corresponding DEAB Control tube without delay.

- Repeat the above step, using a new, freshly prepared tube of ALDECOUNT Reagent and DEAB Control for each sample.
- Incubate all DEAB Control and ALDH test mixtures for 30 minutes in 37°C water bath. Do not exceed 60 minutes.
NOTE: If immunophenotyping is performed in addition to the ALDH assay, add the antibodies after step 3. See Immunophenotyping Protocol for instructions.
- Following incubation, centrifuge all test and control tubes at 250 x g for 5 minutes.
- Remove supernatant from each tube.
- Suspend each cell pellet in 0.5 ml of ALDECOUNT Assay Buffer. Place cells on ice or in the refrigerator.
NOTE: Stained samples may be capped, protected from light, and held on ice or at refrigerated temperatures for up to 24 hours before acquisition of data.
- Set up the flow cytometer per manufacturer's instructions. Perform data acquisition of each control and test sample. Acquire at least 100,000 events per sample.

Flow Cytometer Set Up and Acquisition

Prepare an acquisition template:

- Create a Forward Scatter (FSC) vs. Side Scatter (SSC) dot plot. Create a region R1 that will encompass the nucleated cells based on scatter (see Figure 1a).
 - Create a Fluorescence Channel 1 (FL1) vs. SSC dot plot, gated on R1 (see Figure 1b).
- Cytometer set up and sample acquisition:
- In set-up mode, place a DEAB control sample on the cytometer; on the FSC vs. SSC plot, adjust FSC and SSC voltages and/or gains to center nucleated cell population within the plot. Adjust the R1 region to encompass the nucleated cell population based on scatter (see Figure 1a).
 - On the FL1 vs. SSC plot, adjust the FL1 photo-multiplier tube (PMT) voltage so that the right edge of the stained population is placed at the 2nd log decade on the dot plot (see Figure 1c). Remove the tube. Note that all cells are fluorescent due to the intracellular substrate.
 - Place the corresponding ALDH test sample on the cytometer. Create a region R2 to encompass the cell population that is ALDH^{br} and SSC^{lo}. Remove the tube (see Figure 1b).
 - For data acquisition of test samples: Remove the analyzer from set-up mode and collect 100,000 events in R1 on each ALDH and DEAB sample using the same instrument settings. ALDH^{br}SSC^{lo} stem cells will appear in the R2 region.

Data Analysis

- Create an FSC vs. SSC dot plot and draw a region R1 that will encompass the nucleated cells based on scatter.
- Create two FL1 vs. SSC dot plots gated on R1. Draw a region R2 in both plots that encompasses the cell population that is ALDH^{br} and SSC^{lo}.
- Open an ALDH positive sample data file. Adjust the R1 region in the FSC vs. SSC dot plot to encompass the nucleated cell population.
- On the FL1 vs. SSC dot plot, adjust the R2 region to encompass the ALDH^{br}SSC^{lo} cells. The placement of R2 will vary among sample types (see Figures 1a-3b on back of insert) and may vary from sample to sample.
- Using the corresponding DEAB control tube, verify placement of the R2 region on the ALDH sample by making sure that there are few or no events in the R2 area of the negative control.
- Add region statistics to the plots.
- The percent gated of Region 2 represents the percentage of nucleated events (R1) that are ALDH^{br}SSC^{lo}.

IMMUNOPHENOTYPING

The emission spectrum of the ALDECOUNT Reagent overlaps that of fluorescein, so ALDECOUNT cannot be used with FITC-labeled antibodies. However, ALDECOUNT is compatible with antibodies conjugated to other fluorochromes. Appropriate compensation controls must be included as a part of the testing. ALDECOUNT-reacted cells should be included in each compensation control tube to establish the background fluorescence.

The addition of antibodies or viability markers does not affect the ALDECOUNT assay. Samples could be adequately compensated for multiparameter flow cytometry to allow analysis of ALDH^{br}SSC^{lo} cells in samples stained with ALDECOUNT alone or with ALDECOUNT plus anti-CD34-APC, anti-CD45-PE, and 7-aminoactinomycin D (7-AAD) in the same sample. Correlation of the results from the ALDH assay with and without phenotyping on 1161 paired samples of all types produced a linear regression coefficient of determination (R²) of 0.99, with a slope of 0.98.

Immunophenotyping Protocol

- Add antibodies to 12 x 75 mm tube(s) and set aside.

- Follow the assay protocol through step 3.
Note: Additional ALDECOUNT Reagent tubes may need to be set up to provide sufficient cells for immunophenotyping.
- After step 3 of the ALDECOUNT Assay, mix cells and transfer 0.5 ml to each tube of antibodies.
- Incubate samples for 15-30 minutes at 2 to 8°C.
- Centrifuge all test and control tubes at 250 x g for 5 minutes.
- Remove supernatant from each tube.
- Suspend each cell pellet in 0.5 ml of ALDECOUNT Assay Buffer. Place cells on ice or in the refrigerator.

PRECAUTIONS

ALDECOUNT Reagent is not cytotoxic. The combination of ALDECOUNT Reagent and Activator showed no cytotoxic or phototoxic effects when directly injected into animal models at concentrations 100-fold above those used in this assay. DEAB is an irritant to skin and eyes.

ALDECOUNT buffers contain 1mg/ml sodium azide. Under acidic conditions, sodium azide forms a highly toxic compound. When exposed to lead or copper, sodium azide can form explosives. The buffers containing sodium azide may be discarded into the sewage drain system but should be followed by copious amounts of water.

There are many potentially infectious etiologic agents that may be present in blood and body fluids. Because the sample may contain an active agent of infection with resulting morbidity or mortality, all specimens should be handled as though they are potentially infectious. "Standard precautions" should be observed.

LIMITATIONS

Fresh and previously frozen apheresis and cord blood samples can be analyzed for ALDH^{br}SSC^{lo} populations. Studies to determine performance characteristics of the assay with bone marrow were done only with fresh samples.

This product has not been tested with pediatric apheresis or marrow samples.

Lysis of erythrocytes in the sample is required. Lysis reagents that contain detergent or fixative will affect cell integrity and as a result, the ALDECOUNT assay will not work. Use only ALDECOUNT Lysis Buffer.

ALDECOUNT is compatible with peripheral blood or leukapheresis samples anticoagulated with acid citrate dextrose (ACD), ethylenediaminetetraacetic acid (EDTA), or heparin. Studies to validate the use of ALDECOUNT with bone marrow were done only with samples anticoagulated with heparin. Cord blood units were collected into citrose phosphate dextrose anticoagulant. Blood collection devices with improper sample fill volumes should not be used.

Upon receipt, samples should be processed and tested immediately with ALDECOUNT. If this is not possible, samples should be placed immediately on ice or in the refrigerator. Samples should not be stored for more than 48 hours before testing.

The cell concentration for ALDH test samples should not exceed 5 x 10⁶ cells per milliliter.

The proprietary ALDECOUNT Assay Buffer has been designed to optimize detection of ALDH^{br}SSC^{lo} cells in human samples. Failure to use the ALDECOUNT Assay Buffer may result in reduced signal intensity and reduced discrimination of the ALDH^{br}SSC^{lo} population.

STORAGE AND STABILITY

ALDECOUNT test kits should be refrigerated upon receipt. The ALDECOUNT kit reagents are stable for two years when stored at refrigerated temperatures (2 to 8°C).

PRECISION STUDIES

Precision studies were done with cell suspension standards engineered to contain the indicated percentages of CD34^{pos} cells in a suspension of 10⁶ purified leukocytes per milliliter. Viability of the cells in these frozen standards was 95% after thawing.

Dynamic Range

Variation in results produced by the ALDH assay over the expected range was determined by three operators who performed 30 assays with a single lot of CD34^{pos} cell standards and six lots of ALDECOUNT Reagent over a three month period. Linear regression analysis of ALDH^{br}SSC^{lo} cells to the theoretical values for CD34^{pos} cells in these standards produced an R² of 0.99. This set of experiments produced a 'limit of quantitation' for the ALDECOUNT assay and a

correlation of the ALDH^{br} and CD34^{pos} cell percentages under ideal conditions. Variation with the 0.1% standard is high (CV range 8 to 20% in experiments with at least 40 replicates), limiting predictive value below this level. The assay is capable of detecting <10 ALDH^{br} cells per 100,000 nucleated leukocytes and is linear from 0.1% to at least 18% ALDH^{br} cells.

Summary of dynamic range precision using CD34 standards

Nominal CD34 ^{pos} Cells	0.1%	0.5%	1.0%	3.0%	6.0%
Mean ALDH ^{br} SSC ^{lo} Cells	0.1%	0.5%	0.9%	2.6%	5.1%
Coefficient of Variation (CV)	28.5%	8.5%	9.2%	7.9%	4.8%

Total Imprecision

One operator tested six replicates each of the 0.1% and 6% cell standards twice a day for 20 days (n=240 for each standard). There were no appreciable differences in the mean percentages of ALDH^{br}SSC^{lo} cells produced each day; means (0.08% and 4.66% for 0.1% and 6% standards, respectively) and CV (20.4% and 5.6%) were within the expected ranges and were consistent throughout the study.

Comparison to 7-AAD Staining

Samples were split into two aliquots; one was stained with ALDECOUNT alone and the other with ALDECOUNT in combination with anti-CD34-APC, anti-CD45-PE and 7-AAD. Results from a combined 1161 apheresis, bone marrow and umbilical cord blood samples from normal and G-CSF 'mobilized' donors and patients showed that 99.8% of ALDH^{br}SSC^{lo} cells were viable (7-AAD^{neg}). In contrast, greater than 50% of the CD34^{pos} cells in some of the thawed cord blood units were nonviable (7-AAD^{pos}; Figure 4).

Instrument Comparison

Cell samples were assayed in parallel on a FACSCalibur™ (BD Biosciences) and an Epics XL-MCL™ (Beckman Coulter) flow cytometer to determine variation in values and precision produced by instrumentation from different manufacturers. Forty replicate analyses were performed by one operator on one day. Mean and CV of the percentage of ALDH^{br}SSC^{lo} cells in each sample determined with each instrument were similar.

Summary of Instrument Comparison

	Normal Donor		Cancer Patient	
INSTRUMENT	Mean (%)	CV(%)	Mean (%)	CV(%)
FACSCalibur	1	5.6	0.2	7.8
EPICS XL-MCL	1.1	5.4	0.2	8.6

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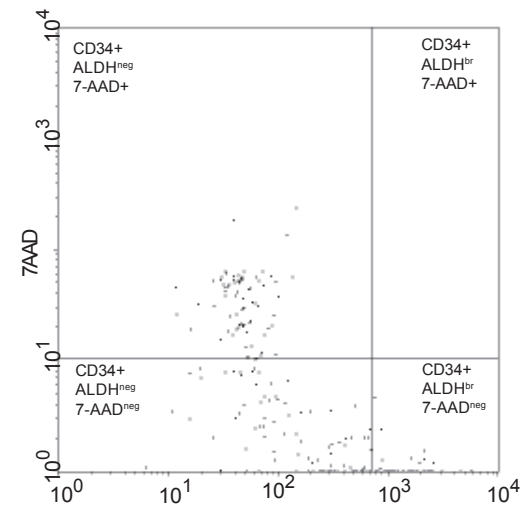


Figure 4. Representative Cytochrome of Thawed Umbilical Cord Blood With a High Proportion of CD34⁺7-AAD⁺ Cells.

Sample is gated on nucleated CD34^{pos} cells. Of total CD34^{pos} cells, 37% are positive for 7-AAD staining. In contrast, all of the ALDH^{br} cells are viable (7-AAD^{neg}).

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