IMMUNOPHENOTYPING

The emission spectrum of the ALDEFLUOR Reagent overlaps that of fluorescein, so ALDEFLUOR cannot be used with FITC-labeled antibodies. However, ALDEFLUOR is compatible with antibodies conjugated to other fluorochromes. When using ALDEFLUOR with cell lines, we recommend the inclusion of a viability marker to improve resolution of the ALDH^{br} population. Appropriate compensation controls must be included as a part of the testing. ALDEFLUOR-reacted cells should be included in each compensation control tube to establish the background fluorescence (for more information see ALDEFLUOR Technical Notes on Immunophenotyping and compensation at www.stemcell.com/technical/aldh/aspx).

Immunophenotyping Protocol

1. Add antibodies to 12 x 75 mm tube(s) and set aside.
2. Follow the assay protocol through step 4.
3. After step 4 of the ALDEFLUOR Assay, mix cells and transfer 0.5 ml to each tube of antibodies.
4. Incubate samples for 15 to 30 minutes at 2 to 8°C.
5. Centrifuge all test and control tubes at 250 x *g* for 5 minutes.
6. Remove supernatant from each tube.
7. Suspend each cell pellet in 0.5 ml of ALDEFLUOR Assay Buffer. Place cells on ice or in the refrigerator.

PRECAUTIONS

ALDEFLUOR reagent is not cytotoxic. The combination of ALDEFLUOR reagent, DMSO and HCl showed no cytotoxic or phototoxic effects at concentrations 100-fold above those used in this assay. DEAB is an irritant to skin and eyes.

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.

PROCEDURAL NOTES AND TIPS

Fresh or previously frozen samples can be analyzed for ALDH^{br} populations. ALDEFLUOR will detect only cells with intact membranes that express high levels of ALDH. However, the inclusion of a viability marker can be helpful, especially when testing cryopreserved cells, cell lines and cultured cells.

Lysis of erythrocytes in the sample is required. Lysis reagents that contain detergent or fixative will affect cell integrity and, as a result, the ALDEFLUOR assay will not work. Erythrocyte lysing recommendations may be found at www.stemcell.com/technical/aldh/aspx. Alternatively, erythrocytes can be removed using immunomagnetic systems, such as RosetteSep® and EasySep®.

When frozen aliquots of the activated ALDEFLUOR reagent are thawed, a small precipitate (pellet) may be observed. Before use, mix the thawed reagent to suspend the precipitate. This precipitate does not affect assay performance.

The ALDH enzymatic reaction begins immediately upon addition of the activated substrate to the cell suspension. It is imperative that an aliquot of the ALDEFLUOR-reacted cells be added to the DEAB control tube without delay.

All ALDH test samples should have a cell concentration of approximately 1 x 10^6 cells per milliliter. Laboratory studies have shown that cell concentrations of up to 5 x 10^6 human leukocytes per milliliter may be used without altering the concentration or amount of ALDEFLUOR reagent added. Recommended cell concentrations have not been established for cells from other tissue or species.

The cell lines A549 (lung carcinoma) and K562 (CML) express ALDH activity and can be used as positive controls for the ALDEFLUOR assay. In addition, commercially-available bone marrow or mononuclear cell preparations can be used as positive controls.

Identification of rare ALDH^{br} cells in heterogeneous cell samples can be improved by removing mature cells prior to testing. Removal of differentiated cells using StemSep® or EasySep can result in significant enrichment of the ALDH^{br} population.[8,11,17]

After staining, the samples should be kept on ice or refrigerated at 2 to 8°C to prevent loss of fluorescent signal from active efflux. When sorting ALDEFLUOR-reacted cells, samples should be kept cold throughout the sort process.

The proprietary ALDEFLUOR Assay Buffer has been designed to optimize signal with human blood cells and contains an ABC transport inhibitor that prevents active efflux of the ALDEFLUOR product from viable cells. Failure to use the ALDEFLUOR Assay Buffer with samples may result in reduced signal intensity and reduced discrimination of the ALDH^{br} population.

The graphs are provided to illustrate representative scatter patterns for human blood apheresis products. Different scatter patterns may be observed with other cell types.

STORAGE AND STABILITY

ALDEFLUOR kit reagents do not contain antimicrobial agents; therefore, aseptic techniques should be used. ALDEFLUOR test kits should be refrigerated upon receipt. Following activation of the ALDEFLUOR reagent, any unused portion should be divided into aliquots and stored at or below -20°C. Activated ALDEFLUOR reagent is stable for one year stored frozen at or below -20°C.

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ALDEFLUOR

For Research Use Only
40 Tests

ALDEFLUOR®

Boron, [N-(2,2-diethoxyethyl)-5-[(3,5-dimethyl-2H-pyrrol-2-ylidene-κN)methyl]-1H-pyrrole-2-propanamidato-κN1]difluoro-, (T-4)- (9CI)

For Research Use Only. Not for use in diagnostic procedures.

KIT COMPONENTS

Dry ALDEFLUOR reagent, 50 µg; activated reagent 300 µM
Diethylaminobenzaldehyde (DEAB), 1.5 mM in 95% ethanol, 1 ml
Hydrochloric Acid (HCl), 2N, 1.5 ml
Dimethylsulphoxide (DMSO), 1.5 ml
ALDEFLUOR Assay Buffer, 4 bottles of 25 ml each
ALDEFLUOR Quick Reference Guide

SUMMARY AND PRINCIPLE

ALDEFLUOR is used to identify, evaluate, and isolate stem and progenitor cells that express high levels of aldehyde dehydrogenase (ALDHbright or ALDH^{br})[1-18]. The fluorescent ALDEFLUOR Reagent freely diffuses into cells and is a non-toxic substrate for ALDH (Figure 1). The amount of fluorescent ALDH reaction product that accumulates in cells directly correlates to the ALDH activity in these cells. The negative charge of this reaction product prohibits diffusion from the cells, however it can be actively pumped (effluxed) from cells via the ATP-binding cassette (ABC) transporter system. This active efflux is inhibited by the special formulation of the ALDEFLUOR Assay Buffer. Therefore, *the ALDEFLUOR reaction product will be retained only by cells with intact membranes and fixed, permeabilized or dead cells will appear ALDH negative.*

With the ALDEFLUOR assay, viable stem and progenitor cells are identified by flow cytometry as cells with higher 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 assay reaction is then measured in the green fluorescence channel of a standard flow cytometer.

ALDEFLUOR is optimized for the detection of ALDH^{br} cells from human blood products but is not restricted to the identification of hematopoietic stem and progenitor cells[16]. ALDEFLUOR is useful for the identification of stem and progenitor cells of various lineages and from multiple species, such as primitive hematopoietic progenitor cells in mouse bone marrow[11,17,18], multipotent neural cells in embryonic rats[10], neural stem cells in murine brain and spinal cord tissue[13-14] and stem cells from the tunicate Botryllis[12]. Information and procedures provided here pertain mostly to the optimized assay with human blood products. **Additional advice concerning how to adapt ALDEFLUOR for use with other cell types can be found in the ALDEFLUOR FAQ document at www.stemcell.com/technical/faq.aspx.**

The ALDEFLUOR reagent is provided in a stable, inactive form (BODIPY®-aminoacetaldehyde-diethyl acetate, BAAA-DA). For use, dry ALDEFLUOR reagent (Figure 2) is dissolved in DMSO, converted to the active substrate (BODIPY-aminoacetaldehyde, BAAA) by treatment with 2N HCl and diluted to the working concentration with ALDEFLUOR Assay Buffer. To perform the assay, an aliquot of the activated substrate is added to the cells suspended in ALDEFLUOR Assay Buffer. An aliquot of this cell mixture is immediately transferred to a tube containing DEAB for the control. These mixtures are incubated to allow conversion of the substrate to the fluorescent product (BODIPY-aminoacetate, BAA). The amount of intracellular fluorescent product is then measured using a flow cytometer.

* 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. Other patents pending.

FIGURE 1. ALDEFLUOR Assay Schematic

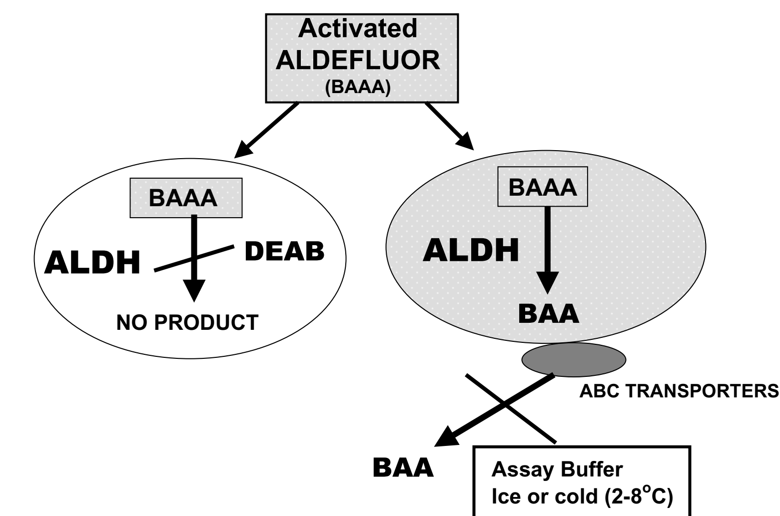
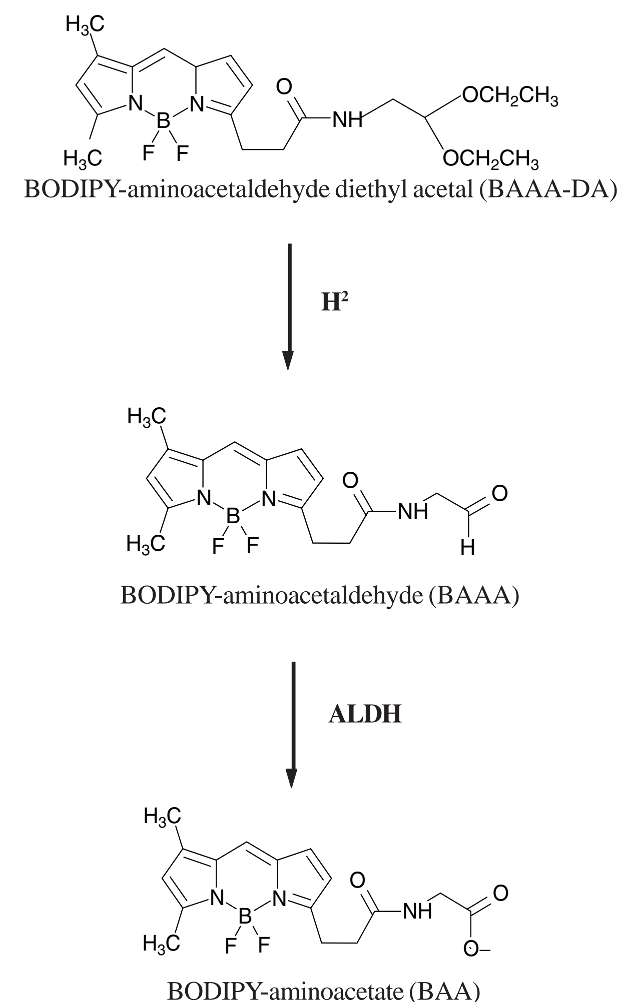


FIGURE 2. ALDEFLUOR Assay Chemistry



MATERIALS AND EQUIPMENT NOT PROVIDED

Gloves, lab coat, biohazard bags, bench covers, 10% hypochlorite solution (bleach)
Pipettes to deliver 1 - 1000 microliters, pipette tips
12 x 75 mm tubes compatible with the cytometer used
Low speed centrifuge (capable of 250 x *g*)
37°C heating device (water bath or heat block)
Flow cytometer equipped with a 488 nm blue argon ion laser for excitation and an optical filter set to detect 515-545 nm (green) fluorescence
Refrigerator (2 to 8°C) or ice
Erythrocyte lysing agent (without detergent or fixatives), e.g. Ammonium Chloride Solution (StemCell Technologies Catalog #07800).

INSTRUCTIONS FOR USE

Reagent Preparation (Figure 3)
(performed only with initial use of kit)

1. Assemble all necessary supplies and allow kit reagents to come to room temperature (RT), 18 to 22°C before use.
2. Activate the ALDEFLUOR reagent:
   a. Add 25 µl of DMSO to the vial of dry ALDEFLUOR reagent and mix well. Dry ALDEFLUOR Reagent is an orange-red powder that changes to a bright yellow-green upon addition of DMSO.
   b. Let stand for 1 min at RT.
   c. Add 25 µl of 2N HCl and mix well.
   d. Incubate this mixture for 15 min at RT. Do NOT exceed 30 minutes.
3. Add 360 µl of ALDEFLUOR Assay Buffer to the vial and mix.
   Note: Upon addition of the Assay Buffer, the solution may appear slightly cloudy. This does not affect the assay performance.
4. Keep the activated reagent at 2 to 8°C during use.
5. Store remaining activated ALDEFLUOR substrate at or below -20°C (see Storage and Stability).

Sample Preparation

1. Prepare fresh or frozen test samples according to standard procedures for the sample type.
2. If using samples containing blood and the erythrocyte to leukocyte ratio (RBC:WBC) of the specimen is > 2:1, lyse the erythrocytes with an ammonium chloride-based buffered solution that does not contain detergents or fixatives (more detail available in ALDEFLUOR Technical Note - RBC Lysis at www.stemcell.com/technical/aldh.aspx).
3. After lysis, centrifuge the sample for 5 min at 250 x *g*, remove the supernatant and suspend cells in 1 ml of ALDEFLUOR Assay Buffer.
4. Perform a cell count.
5. Adjust sample to a concentration of 1 x 10^6 cells/ml with ALDEFLUOR Assay Buffer (refer to Calculations section for more information).

ALDEFLUOR Assay (Figure 4)

1. Label one "test" and one "control" 12 x 75 mm tube for each sample to be tested. Place 1.0 ml of the adjusted cell suspension into each "test" sample tube.
2. Beginning with the first sample:
   a. Add 5 µl of DEAB solution to the "control" tube. Recap control tube and DEAB vial immediately.
   Note: DEAB is provided in 95% ethanol. Recap immediately to prevent evaporation.
   b. Add **5 µl** of activated ALDEFLUOR substrate **per milliliter of sample** to the first sample "test" tube.
   c. Mix and immediately transfer 0.5 ml of the mixture to the DEAB "control" tube.
   Note: The ALDH enzymatic reaction begins immediately upon addition of the activated substrate to the cell suspension. It is imperative that an aliquot of the ALDEFLUOR–reacted cells be added to the DEAB control tube without delay.
3. Add control and substrate solutions as described in step 2 for each sample to be tested.
4. Incubate "test" and "control" samples for 30 to 60 minutes at 37°C (do not exceed 60 minutes).
   Note: If immunophenotyping is to be performed, add and incubate the antibodies after step 4. Refer to Immunophenotyping section (next page).
5. Following incubation, centrifuge all tubes for 5 min at 250 x *g* and remove supernatant. Resuspend cell pellets in 0.5 ml of ALDEFLUOR Assay Buffer.

FIGURE 3. ALDEFLUOR Reagent Activation

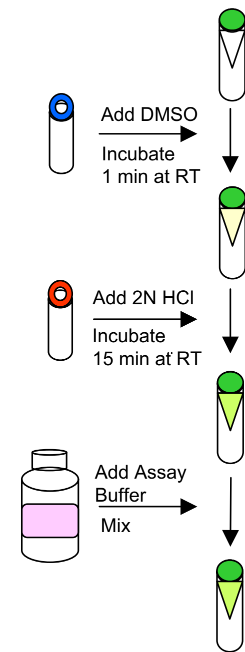
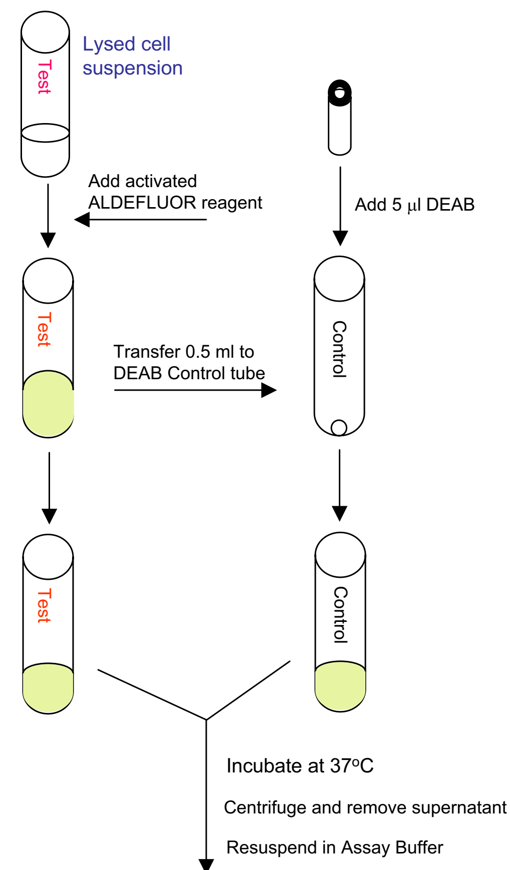


FIGURE 4. ALDEFLUOR Assay



Note: The ALDEFLUOR Assay Buffer has been specially formulated to prevent efflux. Cells can be resuspended in other media but the addition of efflux inhibitor(s) is recommended.

6. Cap samples and place on ice or in the refrigerator immediately. Samples are stable for 24 hours at 2 to 8°C.
7. Set up the selected flow cytometer instrument per manufacturer's instructions. Acquire at least 100,000 events per sample.

FLOW CYTOMETER SET UP AND DATA ACQUISITION

Prepare an acquisition template:

1. Create a Forward Scatter (FSC) vs. Side Scatter (SSC) dot plot. Create a region R1 that will encompass the nucleated cells based on scatter.
2. Create a Fluorescence Channel 1 (FL1) vs. SSC dot plot, gated on R1.
   Note: Refer to Figure 5 for examples.

To set-up analyzer and acquire data:

1. In set-up mode, place a DEAB control sample on the cytometer. Adjust FSC and SSC voltages and gains to center the nucleated cell population within the FSC vs. SSC plot. Adjust the R1 region to encompass the nucleated cell population based on scatter (see Data Plot 1).
2. On the FL1 vs. SSC plot, adjust the FL1 photo-multiplier tube voltage so that the right edge of the stained population is placed at the second log decade on the dot plot. Remove the tube. Note that all cells are fluorescent due to the intracellular pool of fluorescent substrate (see Data Plot 3).
3. Place the corresponding ALDH test sample on the cytometer. Create a region R2 to encompass the cell population that is $ALDH^{br}$. Remove the tube (see Data Plot 2).
   Note: The $ALDH^{br}$ population within blood products typically demonstrates low side scatter as shown in Figure 5. The side scatter profile for cell lines will be more heterogeneous so R2 should be drawn to include higher side scatter events.
4. For data acquisition of test samples: Remove the analyzer from set-up mode and collect 100,000 events in R1 for each ALDH and DEAB sample using the same instrument settings.

DATA HANDLING

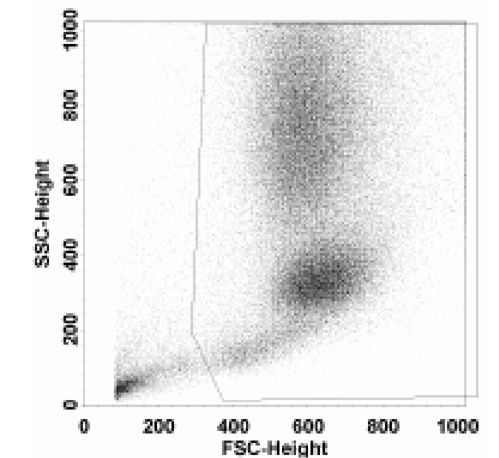
1. Create an FSC vs. SSC dot plot and create a region R1 that will encompass the nucleated cells based on scatter.
2. Create two FL1 vs. SSC dot plots gated on R1.
3. Open an ALDH test sample data file. Adjust the R1 region in the FSC vs. SSC dot plot to encompass the nucleated cell population.
4. On the first FL1 vs. SSC dot plot, create a R2 region to encompass the cell population that is $ALDH^{br}$.
   Note: $ALDH^{br}$ events may be very rare, and their frequency is sample type dependent.
5. On the second FL1 vs. SSC dot plot, use the corresponding DEAB control data file to verify placement of the R2 region on the ALDH sample. Make sure that there are few or no events in the R2 region of the control tube.
6. Add region statistics to the plots.
7. The percentage of cells gated in R2 represents the nucleated events (R1) that are $ALDH^{br}$.

CALCULATIONS

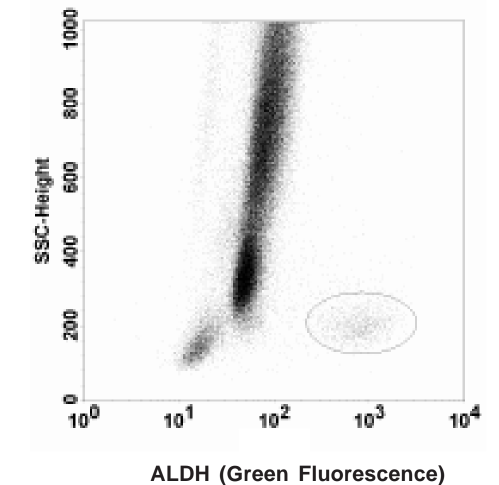
To calculate and prepare the cell dilution to achieve 1 x 10^6 cells/ml:

1. Determine WBC and RBC counts.
2. If the RBC:WBC ratio of human peripheral blood or apheresis sample is greater than 2:1 after initial lysis, repeat the lysis step and cell counts.
3. The total sample volume for this example is 1.0 ml.
4. Divide the CELL CONCENTRATION REQUIRED (1 x 10^6 cells/ml) by the CURRENT CELL CONCENTRATION and multiply by 1000 to get the number of microliters of cell suspension needed.
5. Subtract this number from 1000 microliters (final volume = 1 ml) to get the amount of Assay Buffer needed.
   Example: WBC from hematology analyzer = 17.2
   Divide: 1.0 x 10^6 cells/ml by 17.2 x 10^6 cells/ml = 0.058 x 1000 = 58 µl of cells required
   Subtract: 1000 µl – 58 µl = 942 µl Assay Buffer needed
6. To calculate the absolute concentration of $ALDH^{br}$ cells (# $ALDH^{br}$/ml) in the original sample:
   Multiply: WBC count by percentage of $ALDH^{br}$ cells in R2.

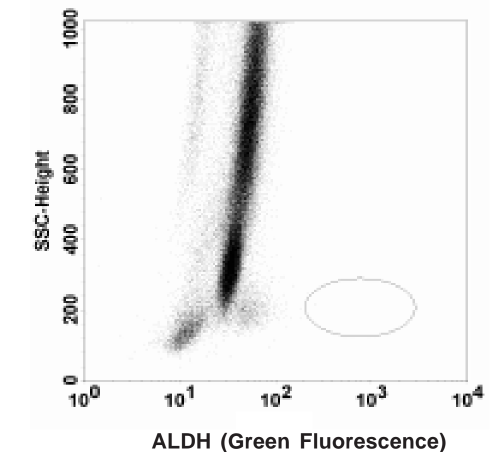
Figure 5. Representative Data Plots For Human Apheresis Products



Plot 1. FSC vs. SSC
FSC vs. SSC dot plot is created, with region R1 drawn to encompass all nucleated cells.



Plot 2. FL1 vs. SSC with test sample
FL1 vs. SSC dot plot gated on R1 is created. Wtih test sample data, region 2 is drawn to include all $ALDH^{br}$ cells.



Plot 3. FL1 vs. SSC with DEAB sample
FL1 vs. SSC dot plot gated on R1. R2 should be adjusted so that few or no events appear in the R2 region.