PRODUCT INFORMATION SHEET

ALDEFLUOR™

Aldehyde Dehydrogenase - Based Cell Detection Kit

STEMCELL TECHNOLOGIES

VERSION 1.1.0

CATALOG #01700 1 kit

PRODUCT DESCRIPTION

ALDEFLUOR™ is a reagent kit that is used to identify human cells that express high levels of the enzyme aldehyde dehydrogenase (ALDH). The activated ALDEFLUOR™ Reagent, BODIPY™-aminoacetaldehyde (BAAA) is a fluorescent non-toxic substrate for ALDH, which freely diffuses into intact and viable cells. In the presence of ALDH BAAA is converted into BODIPY™-aminoacate (BAA), which is retained inside the cells. The amount of fluorescent reaction product is proportional to the ALDH activity in the cells and is measured using a flow cytometer. Viable ALDH-bright ($ALDH^{br}$) cells can, in principle, be isolated using a cell sorter. Active efflux of the reaction product is inhibited by an efflux inhibitor in the ALDEFLUOR™ Assay Buffer. A specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), is used to control for background fluorescence.

ALDEFLUOR™ is optimized for the detection of $ALDH^{br}$ hematopoietic cells in human blood and bone marrow, but it can also be used with non-hematopoietic cells. For a full list of ALDEFLUOR™ products, please visit www.stemcell.com.

COMPONENTS

01703	Dry ALDEFLUOR™ Reagent	50 µg
01705	ALDEFLUOR™ Diethylaminobenzaldehyde (DEAB) Reagent,1.5 mM in 95% ethanol	1 mL
01704	Hydrochloric Acid (HCl), 2N	1.5 mL
01706	Dimethylsulphoxide (DMSO)	1.5 mL
01701	ALDEFLUOR™ Assay Buffer	4 bottles x 25mL

STABILITY AND STORAGE

Product stable at 2 to 8°C until expiry date as indicated on label. Do not freeze.

Reconstituted and activated ALDEFLUOR™ Reagent should be stored at -20°C.

Activated ALDEFLUOR™ Reagent is stable at -20°C for 1 year. Repeated freezing and thawing is not recommended.

PRECAUTIONS

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

See the Material Safety Data Sheet for more information.

RELATED PRODUCTS

PRODUCT	CATALOG #
ALDEFLUOR™ Assay Buffer (55 mL)	01702
ALDECOUNT™ IVD Assay Kit with Lysis Buffer	01720
Ficoll-Paque™ PLUS (500 mL)	07957
Ammonium Chloride Solution (500 mL)	07850
RosetteSep™ Human Cord Blood Progenitor Cell Enrichment Cocktail RosetteSep™ Human Cord Blood Debulking Cocktail	15026 15166
EasySep™ Human CD34 Positive Selection Kit	18056
MammoCult™ Human Medium Kit	05620
StemSpan™ SFEM	09600
MethoCult™ H4434 Classic (24 x 3 mL)	04444
NeuroCult™ NS-A Proliferation Kit (Human)	05751

DIRECTIONS FOR USE

ALDEFLUOR™ ACTIVATION

The dry ALDEFLUOR™ Reagent is provided in a stable, inactive form (BODIPY™-aminoacetaldehyde-diethyl acetate, BAAA-DA). For use, the dry ALDEFLUOR™ Reagent is dissolved in DMSO, converted to the fluorescent activated ALDEFLUOR™ Reagent (BAAA) by treatment with 2N HCl and diluted with ALDEFLUOR™ Assay Buffer:

1. Assemble all necessary supplies and allow kit reagents to come to room temperature (RT), 15 to 25°C before use.
2. Add 25 µL of DMSO to the vial of dry ALDEFLUOR™ Reagent, mix well and let it stand for 1 minute at RT.

   Note: The dry ALDEFLUOR™ Reagent is an orange-red powder that changes to a bright yellow-green color upon addition of DMSO.
3. Add 25 µL of 2N HCl and mix well. Incubate this mixture for 15 minutes at RT.

   IMPORTANT NOTE: Adding 2N HCl <u>before</u> DMSO will render the product inactive.
4. Add 360 µL of ALDEFLUOR™ Assay Buffer to the vial and mix.

   Note: Upon addition of the ALDEFLUOR™ Assay Buffer, the solution may appear slightly cloudy. This does not affect the assay performance.
5. Keep the activated ALDEFLUOR™ Reagent at 2 to 8°C during use.
6. Aliquot the remaining activated ALDEFLUOR™ Reagent and store at -20°C (see Stability and Storage, opposite side of page).

   Note: The concentration of the activated ALDEFLUOR™ Reagent is 300 µM.

CELL SAMPLE PREPARATION

1. Prepare fresh or previously frozen cell samples according to standard procedures for the cell type.
2. If using blood samples where the red blood cell (RBC) to leukocyte ratio (RBC:WBC) of the specimen is > 2:1, lyse the

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erythrocytes with an ammonium chloride-based buffered solution that does not contain detergents or fixatives (e.g. Catalog #07800/07850) (more details available at www.stemcell.com).

3. After RBC lysis, centrifuge the sample for 5 minutes at 250 x *g*. Remove the supernatant and suspend cells in 1 mL of ALDEFLUOR™ Assay Buffer.
4. Perform a cell count.
5. If using hematopoietic cells (e.g. peripheral blood, apheresis product, bone marrow or cord blood) adjust the sample to a concentration of 1 x 10^6 cells/mL with the ALDEFLUOR™ Assay Buffer.

   Note: For other cell types, different cell concentrations may be more appropriate. See the ALDEFLUOR™ Assay Optimization section on Page 4 for suggestions for optimization of ALDEFLUOR™ staining conditions for non-hematopoietic cells.

ALDEFLUOR™ ASSAY (refer to FIGURE 1)

1. Label one "test" and one "control" tube for each sample to be tested. Place 1.0 mL of the adjusted cell suspension into each "test" sample tube.
2. Add 5 µL of ALDEFLUOR™ DEAB Reagent to the "control" tube. Recap control tube and DEAB vial immediately.

   Note: ALDEFLUOR™ DEAB is provided in 95% ethanol. Recap immediately to prevent evaporation.
3. Add 5 µL of the activated ALDEFLUOR™ Reagent per milliliter
   of sample to the first sample "test" tube. 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.
4. Add control and substrate solutions as described in Steps 2 and 3 above for each sample to be tested.
5. Incubate "test" and "control" samples for 30 to 60 minutes at 37°C (do not exceed 60 minutes).

   Note: Optimal incubation times may vary between different cell types. See the ALDEFLUOR™ Assay Optimization section on Page 4 for suggestions on optimization of ALDEFLUOR™ staining conditions for different cell types.
6. Following incubation, centrifuge all tubes for 5 minutes at 250 x *g* and remove supernatant. Resuspend cell pellets in 0.5 mL of ALDEFLUOR™ Assay Buffer and store the cells on ice or at 4°C.

   Note: If immunophenotyping is to be performed, add and incubate the antibodies after Step 6. To prevent efflux of the ALDEFLUOR™ product it is important that the antibody incubation is performed in ALDEFLUOR™ Assay Buffer. Whenever possible keep the cells chilled (4°C or on ice) as that will slow down the product efflux.
7. Optional: Perform a viability cell count. If your sample contains fewer than 90% viable cells, it is recommended to stain your cells with a DNA dye, such as propidium iodide or 7-actinoaminomycin-D, to stain dead and late apoptotic cells.

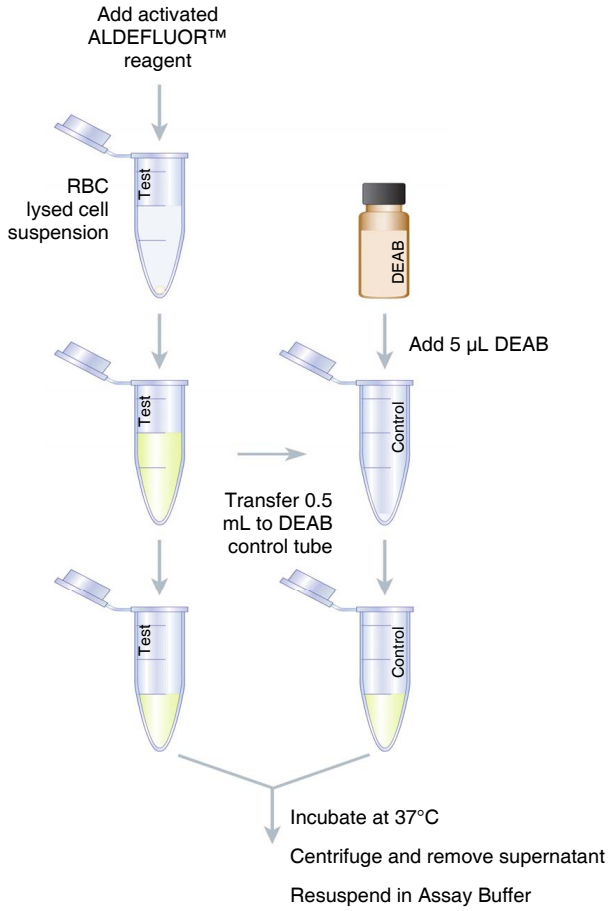

FIGURE 1. ALDEFLUOR™ ASSAY

FLOW CYTOMETER SET UP AND DATA ACQUISITION

1. Create a Forward Scatter (FSC) vs. Side Scatter (SSC) dot plot.
2. In set-up mode, place the 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. Create a region R1 that will encompass the nucleated cells based on scatter (refer to Figure 2 for sample plot).

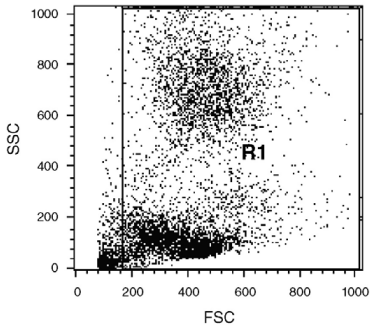

FIGURE 2. FSC VS. SSC DOT PLOT

FSC vs. SSC dot plot is created, with region R1 drawn to encompass all nucleated cells and gate out RBCs and debris. Representative data for human bone marrow mononuclear cells.

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3. Create a Fluorescence Channel 1 (FL1) vs. SSC dot plot, gated on R1.
4. On the FL1 vs. SSC plot, adjust the FL1 photo-multiplier tube voltage so that the right edge of the stained population in the DEAB control sample is placed at the second log decade on the dot plot (see Figure 3A for sample plot). Remove the tube.
5. Place the corresponding ALDH test sample on the cytometer. Create a region R2 to encompass the cell population that is ALDH bright ($ALDH^{br}$) (see Figure 3B for sample plot). Remove the tube.

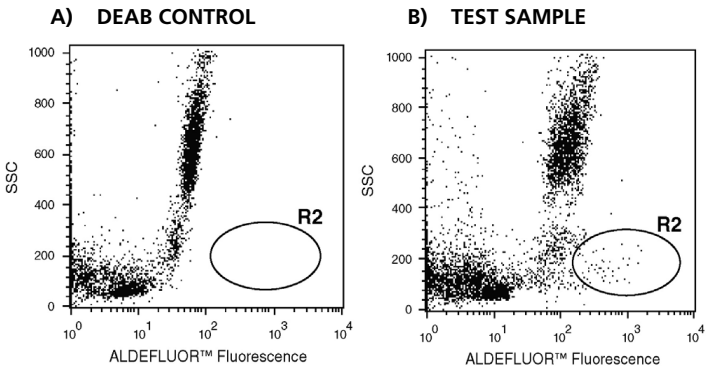

FIGURE 3. ALDEFLUOR™ FLUORESCENCE VS. SSC DOT PLOT OF HUMAN BONE MARROW MONONUCLEAR CELLS STAINED WITH ALDEFLUOR™.

ALDEFLUOR™ Fluorescence vs. SSC dot plot gated on R1 is created with control (A) and test (B) sample data. Region R2 is drawn to include all $ALDH^{br}$ cells. Representative data for human bone marrow mononuclear cells.

6. For data acquisition: remove the analyzer from set-up mode and collect 100,000 events in R1 for each test and control sample using the same instrument settings.
7. The $ALDH^{br}$ population within human blood and bone marrow typically demonstrates low side scatter as shown in Figure 3B. $ALDH^{br}$ cells in non-hematopoietic cells, such as breast cancer cell lines or primary mammary cells have different SCC properties and the gate will have to be set accordingly (see Figures 4 and 5 for sample plots).

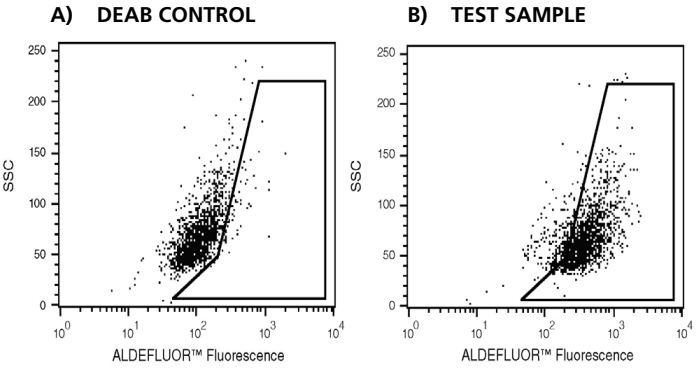

FIGURE 4. ALDEFLUOR™ FLUORSCENCE VS. SSC DOT PLOT OF SKBR3 CELL LINE STAINED WITH ALDEFLUOR™.

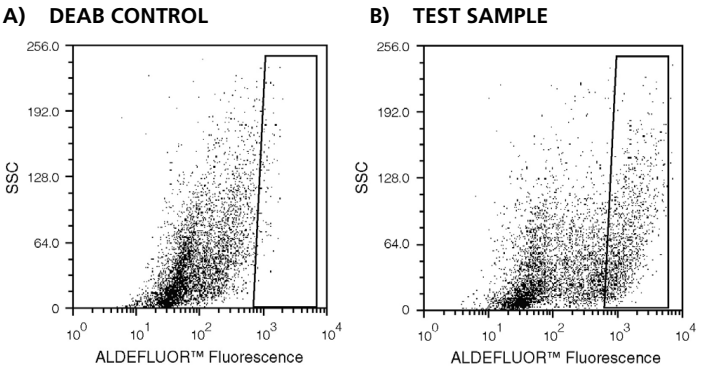

FIGURE 5. ALDEFLUOR™ FLUORESCENCE VS. SSC DOT PLOT OF PRIMARY HUMAN MAMMARY CELLS STAINED WITH ALDEFLUOR™.

The frequency of $ALDH^{br}$ cells as well as the ALDEFLUOR™ and background fluorescence intensities are dependent on the sample type. Use an independent DEAB control for every distinct sample to ensure the accuracy of your analysis.

IMPORTANT NOTE: All viable and intact cells are fluorescent after exposure to the activated ALDEFLUOR™ Reagent. A cell sample exposed to ALDEFLUOR™ in the presence of the ALDH inhibitor, DEAB, is the only appropriate negative control for this assay. Cells with high ALDH activity can only be identified in comparison with the background fluorescence levels of the DEAB control sample.

PROCEDURAL NOTES AND TIPS

- Fresh or previously frozen samples can be analyzed for $ALDH^{br}$ cells. However, the ALDEFLUOR™ kit will only detect ALDH activity in cells that are viable and have intact cell membranes.
- Removal of erythrocytes from the sample is required. Erythrocytes may be removed by lysis using reagents that do not contain detergents or fixatives (e.g. Ficoll-Paque™ PLUS Catalog #07800/0785). They may also be removed by density centrigation (for more information see: www.stemcell.com).
- 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 cell lines A549 (lung carcinoma), SKBR3 (breast cancer) and K562 (CML) express ALDH activity and can be used as positive controls for the ALDEFLUOR™ assay. In addition, commercially-available bone marrow mononuclear cells (Catalog #ABM007F, ABM010F) can also be used as positive controls.
- Identification of rare $ALDH^{br}$ cells in heterogeneous cell samples can be improved by removing mature hematopoietic cells on the basis of lineage antigen expression and enriching for $ALDH^{br}$ cells by using the RosetteSep™ Human Progenitor Cell Enrichment Kit or EasySep™ Human CD34 Positive Selection Kit prior to performing the ALDEFLUOR™ Assay. See the Related Products section on Page 1 for more information.

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ALDEFLUOR ASSAY OPTIMIZATION

The ALDEFLUOR™ assay was originally developed and optimized for detection of hematopoietic stem and progenitor cells in human cord blood, bone marrow and mobilized peripheral blood. Different assay conditions and flow cytometer setup may be required to achieve optimal results for the detection of ALDH activity in non-hematopoietic cells, cultured cells and cell lines.

OPTIMIZING CELL SAMPLE CONCENTRATION

Using ALDEFLUOR™ Assay Buffer, prepare several aliquots of the cell sample of interest containing a range of cell concentrations and perform an ALDEFLUOR™ assay. It is important that a DEAB control sample is included at each cell concentration as the background fluorescence signal of ALDEFLUOR™ stained cells may be different at different cell concentrations.

Suggested concentrations of cells per mL of ALDEFLUOR™ sample are:

1×10^5 2×10^5 5×10^5 1×10^6 2×10^6

Use the cell concentration that gives the strongest fluorescence intensity of $ALDH^{br}$ cells and the highest signal to background ratio and, for heterogeneous cell samples, the best distinction between ALDH-bright and ALDH-low cells.

For example, in the ALDH-expressing SKBR3 breast cancer cell line the optimal cell concentration was determined to be approximately 2×10^5 cells/mL(refer to Figure 6).

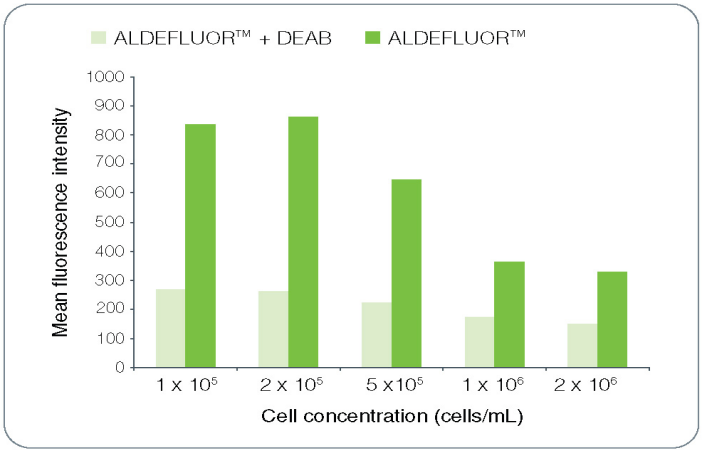

FIGURE 6: EFFECT OF CELL CONCENTRATION ON THE DETECTION OF ALDEFLUOR™ ACTIVITY IN SKBR3 CELL LINE.

OPTIMIZING INCUBATION TIME

Using an optimal sample concentration, test different ALDEFLUOR™ incubation times at 37°C.

Suggested incubation times are:

15 min 30 min 45 min 60 min

For example, in the SKBR3 cell line the optimal incubation time was determined to be 45 minutes (refer to Figure 7).

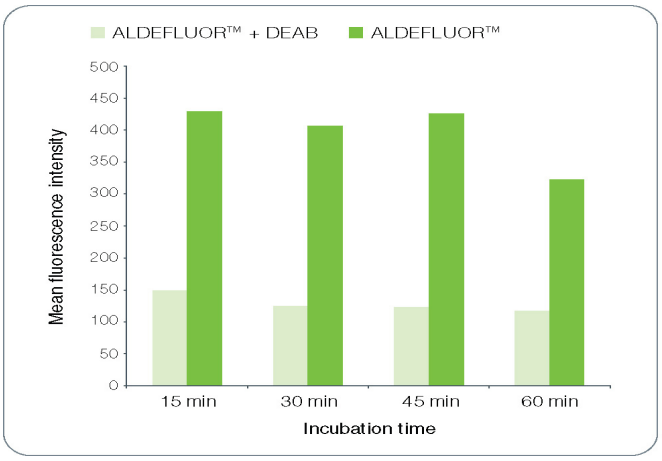

FIGURE 7: EFFECT OF INCUBATION TIME ON THE DETECTION OF ALDEFLUOR™ ACTIVITY IN SKBR3 CELL LINE.

OPTIMIZING THE USE OF EFFLUX INHIBITORS

To prevent efflux of the activated ALDEFLUOR™ Reagent (BAAA) and the reaction product (BAA), the ALDEFLUOR™ Assay Buffer contains efflux inhibitors that inhibit the activity of ABC transporters expressed in most cells. The ALDEFLUOR™ Assay Buffer formulation has been optimized to prevent efflux from hematopoietic stem and progenitor cells and also works well for many non-hematopoietic cells. However, to optimize the detection of ALDH activity in nonhematopoietic cells it may be useful to test the addition of various inhibitors at different concentrations. Recommended inhibitors and suggested concentrations include: Verapamil, 50 to 100 µM, Probenecid, 2.5 mM and 2-deoxy-D-glucose, 100 mM.

The efflux of the activated ALDEFLUOR™ Reagent and product is also inhibited at low temperature. To prevent loss of fluorescence intensity it is recommended to keep the cells on ice or at 2 to 8°C after completion of the ALDEFLUOR™ reaction. Keeping the cells chilled during cell sorting is also recommended.

OPTIMIZING THE USE OF NEGATIVE CONTROL

Increasing the concentration of the ALDH inhibitor DEAB may be useful for detecting background fluorescence in cells with very high ALDH activity. To find the optimal DEAB concentration, try doubling the amount of the inhibitor per reaction. It may be necessary to further increase the volume significantly (up to 10 fold). This, however, may have an adverse effect on the viability of the cell sample.

The effectiveness of the DEAB inhibitor can also be increased by adding the inhibitor to the cells before the addition of the activated ALDEFLUOR™ Reagent. To do this, prepare 2 tubes (1 control and 1 test) each containing an equal volume of the cell suspension at the recommended concentration. Add 10 µL of DEAB to the control tube per mL of cell sample and mix. Then add 5 µL of the activated ALDEFLUOR™ Reagent per mL of cell sample to each of the tubes and mix immediately.

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