

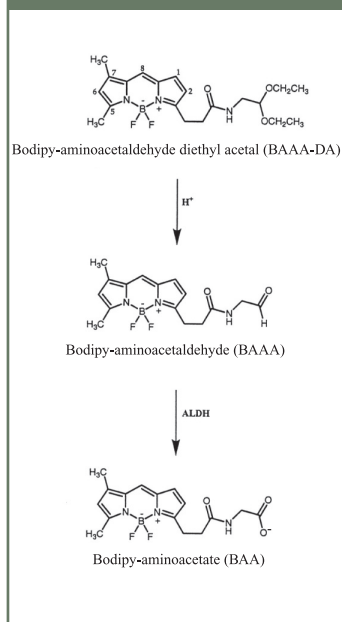
# TECHNICAL BULLETIN

## IDENTIFICATION OF VIABLE STEM AND PROGENITOR CELLS WITH ALDEFLUOR®

### Introduction

Primitive hematopoietic cells are relatively resistant to alkylating agents such as the active derivatives of cyclophosphamide (e.g. 4-hydroxyperoxycyclophosphamide (4-HC) and mafosfamide).<sup>1</sup> This resistance is due to the selective expression in primitive hematopoietic cells of the enzyme aldehyde dehydrogenase (ALDH).<sup>2,3</sup> Fluorescent ALDH-substrates can be used to identify, quantitate and isolate hematopoietic cells by flow cytometry.<sup>4,5</sup> The ALDEFLUOR reagent system offers a non-immunological way to identify human stem cells and progenitors from bone marrow (BM), mobilized peripheral blood (MPB) and umbilical cord blood (UCB) on the basis of their ALDH activity. The purpose of this technical bulletin is to provide an overview of the assay principle, applications and technical considerations of ALDEFLUOR, as well as to discuss recent publications in which ALDEFLUOR has been used to identify, isolate and characterize stem cells and progenitor cells from hematopoietic, as well as, nonhematopoietic tissues.

**Figure 1. Structure of BAAA-DA, BAAA and BAA**



Prior to use, BAAA-DA is dissolved in DMSO and converted to BAAA when exposed to acid (1 N HCl) for 15-30 minutes at room temperature. BAAA can freely diffuse into viable cells and is converted by ALDH into BAA, which is negatively charged and retained inside the cells.

(DEAB) results in a significant decrease in the fluorescence intensity of ALDH<sup>hi</sup> cells and is commonly used as a negative staining control to set up the flow cytometer and to distinguish between ALDH<sup>hi</sup> cells and more dimly stained cells that take up BAAA passively and/or have low levels of ALDH activity.<sup>5</sup> The fluorescence of BAAA-treated cells is much brighter

### Assay Principle

ALDEFLUOR is supplied in the form of Bodipy<sup>™</sup>-aminoacetaldehyde diethyl acetal (BAAA-DA) (Figure 1) which by itself is not a substrate of ALDH. BAAA-DA is dissolved in dimethylsulfoxide (DMSO) and exposed to acid to convert it into Bodipy-aminoacetaldehyde (BAAA) which is a fluorescent substrate for ALDH. BAAA is not stable for long periods at room temperature, but can be stored for several months when frozen in aliquots. Treatment of cells is usually performed at 37°C with BAAA at a concentration of 1.5 μM. BAAA is uncharged and can diffuse freely across the plasma membrane of intact viable cells. Intracellular ALDH converts BAAA into Bodipy-aminoacetate (BAA), which is retained intra-cellularly because of its net negative charge, which disallows free diffusion. The assay buffer (supplied with the ALDEFLUOR kit) contains a transport inhibitor, which prevents efflux of the BAA from the cells. As a result, cells expressing high levels of ALDH retain BAA and thus fluoresce. They can then be detected using the green fluorescence (FL1) channel of a standard flow cytometer.

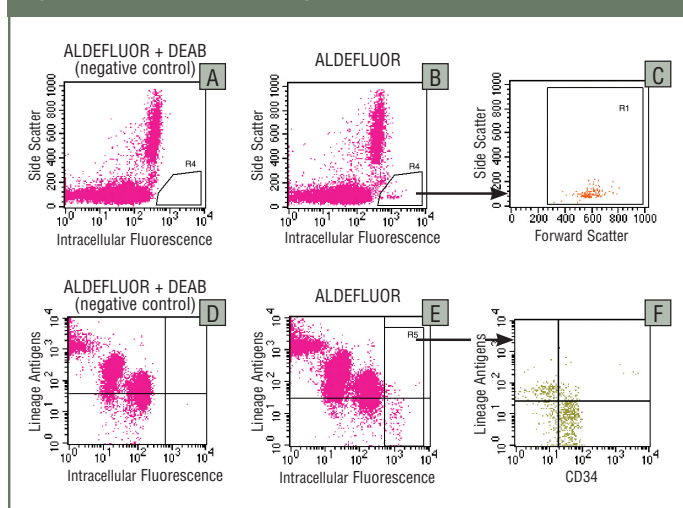
Incubation of cells with BAAA in the presence of a 10-fold molar excess of the ALDH inhibitor diethylamino-benzaldehyde

than usually obtained in standard flow cytometry staining experiments with fluorochrome-labeled antibodies. For this reason the amplification of the green fluorescence detector of the flow cytometer needs to be adjusted to much lower levels than for detection of fluorescent antibody stained cells. For a detailed description of the experimental procedure for ALDEFLUOR treatment of hematopoietic cells and for setting up the flow cytometer for optimal detection of ALDH<sup>hi</sup> cells please refer to the ALDEFLUOR package insert.

### Frequency of ALDH<sup>hi</sup> Hematopoietic Cells

The frequency of ALDH<sup>hi</sup> MPB cells was reported to be variable ( $3.1 \pm 4.8\%$ , n=14),<sup>6</sup> which probably reflects the large differences in stem cell and progenitor cell frequencies between MPB preparations obtained from different donors undergoing mobilization treatment. Figure 2 shows representative staining results of low-density UCB cells treated with ALDEFLUOR alone (Figure 2B,C,E,F) or as a negative control, with ALDEFLUOR and DEAB (Figure 2A,D). A small population of brightly fluorescent cells with low side scatter (SSC) (region R4 in Figure 2B) was clearly detected in the BAAA-stained population, but was absent in the negative control (Figure 2A). In several reported studies the frequency of ALDH<sup>hi</sup>SSC<sup>low</sup> UCB cells was found to be ~1% (Table 1).<sup>7,8</sup>

**Figure 2. Detection of ALDH expression in human cord blood cells**



Human low-density UCB cells were treated with ALDEFLUOR in the presence of the ALDH inhibitor DEAB (A,D) or with ALDEFLUOR alone (B,C,E,F). A fraction of the cells were analyzed by flow cytometry without further treatments (A-C), whereas a second fraction was stained with a panel of PE-labeled antibodies against lineage-specific antigens (CD2, CD3, CD11b, CD14, CD16, CD19, CD24, CD56 and Glycophorin-A) and Cy-5 labeled anti-CD34 (D-F). A and B show the intracellular fluorescence versus the Side Scatter of cells gated in an electronic window to exclude debris and dead, propidium iodide-stained cells. Region R4 in B shows a brightly fluorescent ALDH<sup>hi</sup> population (0.3% of cells) that is absent from the DEAB control in A. The forward versus Side Scatter profile of the ALDH<sup>hi</sup> cells gated in R4 is shown in C. D and E show the intracellular fluorescence versus lineage antigen expression for all viable cells. F shows CD34 expression versus lineage antigen expression for gated ALDH<sup>hi</sup> cells (Region R5 in E).

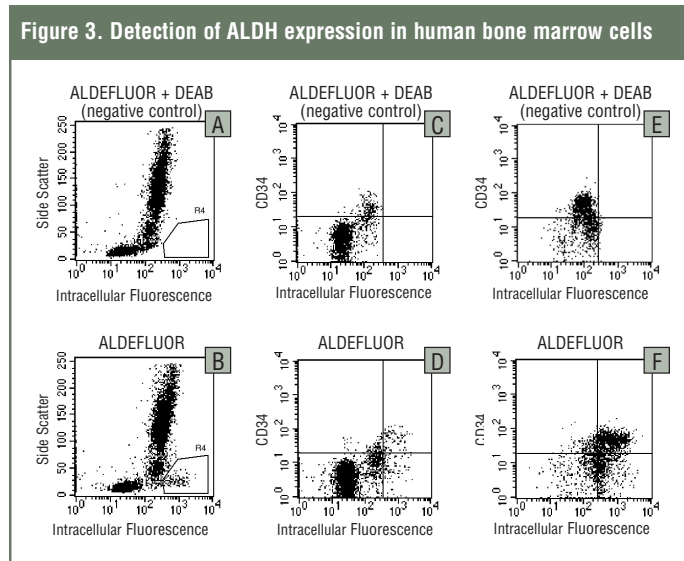
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Results of preliminary experiments on ALDH expression in human BM showed that the fluorescence profiles for ALDEFLUOR-treated BM cells (Figure 3A,B) and the frequency of ALDH<sup>hi</sup> BM cells were very similar to the profiles obtained for UCB cells (Figure 2A,B and Table 1).

### Multiparameter Analysis of ALDH<sup>hi</sup> Cells

ALDEFLUOR-treated cells can be stained with fluorescently-conjugated antibodies against cell surface markers and analyzed by multiparameter flow cytometry to correlate ALDH expression with other phenotypic properties of hematopoietic cells. In principle, all fluorescent labels that are commonly used in conjunction with Bodipy or other green fluorochromes can be used in combination with ALDEFLUOR. These include R-phycoerythrin (PE), PE-tandem conjugates, PerCP, Cyanine-5 (Cy-5) and allophycocyanin (APC), which are detected in the FL2, FL3 or FL4 detectors of most commonly used flow cytometers. However, as the green fluorescence of ALDEFLUOR-treated cells is very strong, it is even more important than in standard multicolor experiments that electronic fluorescence compensations between the



Low-density BM cells (A-D) and lineage-depleted\* BM cells (E,F) were treated with ALDEFLUOR in the presence (A,C,E) or absence (B,D,F) of the ALDH-inhibitor DEAB, and immunostained with Cy-5-conjugated anti-CD34.

green fluorescence channel used to measure ALDH and the other fluorescence channels are set accurately using appropriate single color stained control cells.

### Expression of CD34, CD133 and Other Hematopoietic Markers on ALDH<sup>hi</sup> Cells

In various studies the reported average frequencies of ALDH<sup>hi</sup>SSC<sup>low</sup> cells that express CD34 ranged between 73 and 95% with little difference between UCB and MPB (Table 1).<sup>7-9</sup> The frequency of ALDH<sup>hi</sup>SSC<sup>low</sup> human BM cells that expressed CD34 was found to be somewhat lower, 49-59% (Figure 3C-F, Table 1), but additional studies, directly comparing hematopoietic cells from different tissues, will be needed to determine whether these differences are significant.

A relatively large proportion of ALDH<sup>hi</sup>SSC<sup>low</sup> UCB and BM cells displays the CD34<sup>+</sup>CD38<sup>-</sup> phenotype, which is consistent with the selective expression of ALDH in primitive

hematopoietic cells (Table 1). The frequency of ALDH<sup>hi</sup>SSC<sup>low</sup> cells that are CD34<sup>+</sup>CD38<sup>-</sup> was reported to be only 0.75 ± 1.2% in MPB, which is more than 10-fold lower than the frequency reported for UCB and BM (Table 1). Additional studies will be needed to determine whether these differences represent lower ALDH expression in very primitive MPB cells or different CD38 distribution on hematopoietic subsets in MPB compared to UCB and BM. A large proportion of ALDH<sup>hi</sup>SSC<sup>low</sup> cells also displays other phenotypic markers of primitive hematopoietic cells, i.e., CD117 and CD133 (Table 1).<sup>7-9</sup> The ALDH<sup>hi</sup>SSC<sup>low</sup> CD133<sup>+</sup> cells include the small subset of CD133<sup>+</sup>CD34<sup>-</sup> cells which may represent very primitive multipotent stem cells.<sup>6,10</sup>

Analysis of ALDEFLUOR-treated UCB cells stained with a panel of PE-labeled antibodies against lineage-specific antigens expressed on lineage-committed progenitors and mature cells (CD2, CD3, CD11b, CD14, CD16, CD19, CD24, CD56 and Glycophorin-A) and Cy-5 labeled anti-CD34 showed that a large proportion (63% in the experiment shown in Figure 2) of ALDH<sup>hi</sup> cells expressed little or no lineage antigens (Figure 2E). Most Lin<sup>-</sup>ALDH<sup>hi</sup>SSC<sup>low</sup> cells were CD34<sup>+</sup> (Figure 2F). CD34<sup>+</sup>ALDH<sup>hi</sup>SSC<sup>low</sup> cells were heterogeneous with respect to lineage antigen expression. Most ALDH<sup>hi</sup>CD34<sup>-</sup> cells expressed lineage antigens, but at much lower levels than most ALDH<sup>dim</sup> or ALDH<sup>-</sup> cells (upper left quadrant in Figure 2F), and may represent committed precursors that are in the process of differentiating into lineage<sup>+</sup> cells. A small population of ALDH<sup>hi</sup>CD34<sup>-</sup> cells (less than 0.04% of the UCB cells) has undetectable lineage antigen expression (lower left quadrant in Figure 2F). Similar populations of ALDH<sup>hi</sup>CD34<sup>-</sup> UCB and BM cells have also been reported in other studies and were found to have low or absent expression of CD38.<sup>11,12</sup> Functional characterization of sorted ALDH<sup>hi</sup>CD34<sup>-</sup> cells in culture and transplantation assays are needed to determine if these ALDH<sup>hi</sup>CD34<sup>-</sup>Lin<sup>-</sup> and CD38<sup>-</sup> cells represent very primitive hematopoietic stem cell (HSC) or progenitor cells, similar to the CD34<sup>-</sup> HSCs reported previously.<sup>13,14</sup> If confirmed, ALDEFLUOR treatment might thus provide a relatively straightforward approach for the isolation of these rare CD34<sup>-</sup> HSCs.

### Enrichment of ALDH<sup>hi</sup> Cells by Depletion of Lineage-Antigen Expressing Cells

Removal of lineage<sup>+</sup> cells prior to ALDEFLUOR treatment increases the proportion of ALDH<sup>hi</sup> UCB cells by >10 fold to, on average, a frequency of 12-23% of the lineage<sup>-</sup> cells (Table 1).<sup>7,9</sup> Lineage depletion can be achieved by immunomagnetic separation using the EasySep® or StemSep® methods or by immunosetting using the RosetteSep® method.\*\* Representative flow cytometry diagrams are shown of CD34 vs. ALDH expression before (Figure 3C,D) and after (Figure 3E,F) immunomagnetic depletion of lineage<sup>+</sup> human BM cells using the StemSep® method. In this experiment depletion of lineage<sup>+</sup> cells increased the frequency of ALDH<sup>hi</sup>SSC<sup>low</sup> cells from 1 to 47% (Table 1C). By increasing the frequency of ALDH<sup>hi</sup> cells, lineage depletion prior to ALDEFLUOR staining will greatly reduce the time needed to further purify ALDH<sup>hi</sup> cells by FACS sorting. Lineage depletion also removes ALDH<sup>low</sup> monocytes, granulocytes and other mature cells and this will allow more selective gating of ALDH<sup>hi</sup> cells and improve the purity of ALDH<sup>hi</sup> hematopoietic cells isolated by FACS sorting.

### Functional Properties of ALDH<sup>hi</sup> Cells

Exposure of hematopoietic cells to ALDEFLUOR does not affect cell viability and proliferation. ALDEFLUOR-treated cell suspensions show normal colony forming cell (CFC) numbers and colony size distributions compared to untreated samples in standard hematopoietic colony assays in semi-solid media (MethoCult® GF H4434, Catalog #04434). Sorted ALDH<sup>hi</sup> cells show high frequencies of progenitors and HSCs identifiable in CFC assays, long-term cultures and transplantation assays in immunodeficient NOD/SCID mice, whereas sorted ALDH<sup>low</sup> cells are relatively depleted of such cells. In various studies, CFC and long-term culture-initiating cell (LTC-IC) frequencies in human UCB or mobilized blood samples were

\* Human low-density BM cells were incubated with a panel of antibodies against lineage antigens (CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b and Glycophorin-A; StemSep® progenitor enrichment cocktail, Catalog #14066) and lineage<sup>+</sup> cells were removed using the StemSep® immunomagnetic separation method.

\*\* For further information on the cell separation products offered by STEMCELL Technologies please contact a representative or consult our website ([www.stemcell.com](http://www.stemcell.com)).

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Table 1. Frequencies of ALDH-expressing subsets in human cord blood, mobilized peripheral blood and bone marrow

A. Cord Blood			
Phenotype	Frequency	n	Reference
ALDH <sup>hi</sup> SSC <sup>low</sup>	% of total MNC:	1.1 ± 0.2	4 7
	% of lin-depleted:	12 ± 3	6 7
		22.6 ± 3.0	6 - 9 9
CD34 <sup>+</sup>	% of ALDH <sup>hi</sup> :	95 ± 1	4 7
		74 ± 20	11 5
		91.0 ± 2.9	6 - 9 9
CD34 <sup>+</sup> CD38 <sup>-</sup>	% of ALDH <sup>hi</sup> :	65 ± 4	4 7
		46 ± 22	6 5
		25.5 ± 3.7	6 - 9 9
CD133 <sup>+</sup>	% of ALDH <sup>hi</sup> :	31 - 57	3 7
		72.9 ± 4.2	6 - 9 9
CD117 <sup>+</sup>	% of ALDH <sup>hi</sup> :	83.1 ± 2.7	6 - 9 9
B. Mobilized Peripheral Blood			
Phenotype	Frequency	n	Reference
ALDH <sup>hi</sup> SSC <sup>low</sup>	% of total MNC:	3.1 ± 4.8	14 6
CD34 <sup>+</sup>	% of ALDH <sup>hi</sup> SSC <sup>low</sup> :	73.4 ± 11.7	6 - 16 6
CD34 <sup>+</sup> CD38 <sup>-</sup>	% of ALDH <sup>hi</sup> SSC <sup>low</sup> :	0.75 ± 1.2	6 - 16 6
CD133 <sup>+</sup>	% of ALDH <sup>hi</sup> SSC <sup>low</sup> :	68.2 ± 15.8	6 - 16 6
C. Bone Marrow			
Phenotype	Frequency	n	Reference
ALDH <sup>hi</sup> SSC <sup>low</sup>	% of total MNC:	1.1	1 StemCell
	% of lin-depleted:	46.9	1 StemCell
CD34 <sup>+</sup>	% of ALDH <sup>hi</sup> SSC <sup>low</sup> :	49.2 - 58.8	3 StemCell
CD34 <sup>+</sup> CD38 <sup>-</sup>	% of ALDH <sup>hi</sup> SSC <sup>low</sup> :	6.5, 21.9	2 StemCell
CD34 <sup>-</sup> CD38 <sup>-</sup>	% of ALDH <sup>hi</sup> SSC <sup>low</sup> :	2.1, 4.3	2 StemCell

enriched between 50 and 220-fold in sorted ALDH<sup>hi</sup> cells relative to unfractionated cells, with CFC-frequencies up to 1 in 4.5 ALDH<sup>hi</sup>SSC<sup>low</sup> cells. ALDH<sup>low</sup> fractions showed little or no CFC and LTC-IC activity when cultured at densities of up to 2 x 10<sup>5</sup> cells.<sup>5,7,9</sup>

Human HSCs detected by their ability to give multi-lineage cell engraftment in genetically immunodeficient NOD/SCID and NOD/SCID/b2-microglobulin-knockout mice have been found in the ALDH<sup>hi</sup>SSC<sup>low</sup> and ALDH<sup>hi</sup>Lin<sup>-</sup> subsets.<sup>7,9</sup> ALDH<sup>hi</sup>SSC<sup>low</sup> cells supported human lympho-myelopoiesis for >16 weeks in transplanted mice whereas ALDH<sup>low</sup> cells did not engraft at cell doses up to 5 x 10<sup>5</sup> per mouse,<sup>9</sup> or only showed myeloid repopulation for 4-8 weeks after transplantation.<sup>7</sup> Using limiting dilution analysis, the frequency of long-term NOD/SCID repopulating cells was estimated to be >1/4000 ALDH<sup>hi</sup>SSC<sup>low</sup> cells.<sup>7</sup>

The in vitro and in vivo data demonstrate that ALDH activity identifies a heterogeneous population of cells which is enriched for committed progenitors with in vitro colony-forming ability, as well as distinct sub-populations of human hematopoietic stem cells that can generate mature blood cells with different kinetics and duration in vivo.

In one study, the number of transplanted ALDH<sup>hi</sup>SSC<sup>low</sup> cells in autologous MPB stem cell grafts correlated with the time to neutrophil and platelet reconstitution.<sup>5</sup> These results indicate that enumeration of ALDH<sup>hi</sup> cells may provide an alternative to measurements of CD34<sup>+</sup> cell content to identify and enumerate hematopoietic stem cells and progenitor cells in clinical grafts on the basis of functional characteristics rather than phenotype.

### Detection of ALDH Activity in Mouse Hematopoietic Stem Cells and Progenitor Cells

The utility of ALDEFLUOR for hematopoietic cells from mice and other species has not been determined. However, high levels of ALDH activity have been identified in mouse hematopoietic cells in experiments that used a different ALDH substrate, dansyl aminoacetaldehyde (DAAA).<sup>4</sup> Detection of the fluorescence of DAAA-treated cells requires the use of flow cytometers equipped with a UV laser, which are less commonly available than flow cytometers used to detect ALDEFLUOR treated cells. Using DAAA treatment in combination with elutriation and depletion of lineage<sup>+</sup> cells, a rare population of small ALDH<sup>hi</sup> cells was identified that was capable of long-term multi-lineage repopulation after transplantation, but lacked radioprotection and spleen colony forming ability.<sup>4</sup> Of interest, these cells expressed low to undetectable levels of Thy-1, Sca-1, c-Kit and CD34, and may represent a different class of stem cells than the cells that express the "classical" Lin<sup>-</sup> Sca1<sup>+</sup>kit<sup>+</sup>CD34<sup>-</sup> phenotype. Further analysis of ALDH activity in mouse BM subsets will be important to characterize these different stem cell populations.

### Expression of ALDH in Nonhematopoietic Stem Cells

Information on ALDH expression in nonhematopoietic cells is scarce. One group reported high ALDH expression in CD45<sup>-</sup>CD31<sup>+</sup> human UCB cells, which might represent endothelial cells,<sup>8</sup> but further characterization of these cells is needed to confirm and expand this finding. Immunofluorescence microscope analysis of fetal rat brain cells treated with ALDEFLUOR indicated that neural stem cells, identified at day 10.5 of gestation (E10.5), express ALDH.<sup>15</sup> The E10.5 neural cells displayed other characteristics of neural stem cells, i.e., they were CD24<sup>-</sup>, expressed nestin and the transcription factor Sox-1 and also expressed the ATP-binding cassette transporter, ABCG2, which has been shown to be responsible for the SP-phenotype of primitive hematopoietic cells. ALDH<sup>hi</sup> cells were rare among E14.5 cells, which mainly consist of neural progenitor cells, suggesting that ALDH activity could be used to distinguish between neural stem and progenitor cells in rodent fetal brain tissue.<sup>15</sup>

### Expression of ALDH in Tumor Cells

Human tumor cell lines that are resistant to alkylating agents express the ALDH-1 and ALDH-3 isozymes.<sup>16</sup> Both isozymes are enzymatically active on the BAAA substrate. ALDH-1A1 is the isozyme that is expressed in human hematopoietic cells.<sup>17</sup> Expression levels of ALDH-1A1 in some human tumor cell lines, as measured by real-time PCR and spectrophotometric methods, appear to correlate with their ability to oxidize BAAA.<sup>17</sup> Measurement of ALDH activity by ALDEFLUOR staining may be useful to screen tumor cell samples for resistance to alkylating agents and to identify heterogeneity within tumor cell populations. However, not all cell lines that express ALDH-1A1 are able to oxidize BAAA, and, conversely, not all cell lines that oxidize BAAA express ALDH-1A1. This suggests that other factors influence ALDH1-mediated BAAA oxidation and that other ALDH isozymes may oxidize BAAA as well.<sup>17</sup> As ALDH activity in tumor cell lines appears to be higher than in normal hematopoietic cells, cell selection on the basis of ALDH expression may be a potential purging strategy for leukemia and other cancers. The development of such therapeutic approaches, however, will require further research into ALDH expression and activity in leukemia-initiating cells and other primary tumor cell populations obtained from patients.

### Further Information

Further information on ALDEFLUOR including a detailed description of the technical procedure, can be found at [www.stemcell.com/technical/aldh.aspx](http://www.stemcell.com/technical/aldh.aspx). Please click on the ALDEFLUOR link.

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