

TRANSPLANTATION

Development and validation of a rapid, aldehyde dehydrogenase bright–based cord blood potency assay

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Key Points

- Cord blood content of ALDH^{br} cells correlates well with CFUs and may act as a surrogate potency assay for cord blood units.
- ALDH^{br} cells in segments are assayed rapidly, allowing potency results to be used for release of the unit from a public cord blood bank.

Banked, unrelated umbilical cord blood provides access to hematopoietic stem cell transplantation for patients lacking matched bone marrow donors, yet 10% to 15% of patients experience graft failure or delayed engraftment. This may be due, at least in part, to inadequate potency of the selected cord blood unit (CBU). CBU potency is typically assessed before cryopreservation, neglecting changes in potency occurring during freezing and thawing. Colony-forming units (CFUs) have been previously shown to predict CBU potency, defined as the ability to engraft in patients by day 42 posttransplant. However, the CFU assay is difficult to standardize and requires 2 weeks to perform. Consequently, we developed a rapid multiparameter flow cytometric CBU potency assay that enumerates cells expressing high levels of the enzyme aldehyde dehydrogenase (ALDH bright [ALDH^{br}]), along with viable CD45⁺ or CD34⁺ cell content. These measurements are made on a segment that was attached to a cryopreserved CBU. We validated the assay with prespecified criteria testing accuracy, specificity, repeatability, intermediate precision, and linearity. We then prospectively examined the

correlations among ALDH^{br}, CD34⁺, and CFU content of 3908 segments over a 5-year period. ALDH^{br} ($r = 0.78$; 95% confidence interval [CI], 0.76-0.79), but not CD34⁺ ($r = 0.25$; 95% CI, 0.22-0.28), was strongly correlated with CFU content as well as ALDH^{br} content of the CBU. These results suggest that the ALDH^{br} segment assay (based on unit characteristics measured before release) is a reliable assessment of potency that allows rapid selection and release of CBUs from the cord blood bank to the transplant center for transplantation. (*Blood*. 2016;127(19):2346-2354)

Introduction

Banked unrelated donor cord blood is an important source of allogeneic cells for hematopoietic stem cell transplantation (HSCT). Benefits of cord blood include rapid procurement, minimal risk to the donor, lower risk of graft-versus-host disease in the recipient, and more permissive HLA matching.¹⁻⁵ Despite these benefits, delays in engraftment or primary graft failure are observed in 10% to 15% of patients after transplant.⁶⁻⁸ Many graft failures may be due to low potency, the ability of the cord blood unit (CBU) to restore hematopoiesis after HSCT.

CBUs are selected by transplant centers (TCs) using HLA matching along with CBU characteristics of potency measured before cryopreservation, including the total nucleated cell count (TNCC), myeloerythroid and mixed hematopoietic progenitor colony-forming units (CFUs), and CD34⁺ cell content, often adjusted for cell dose. Freezing, storage, and thawing of CBUs reduce the overall potency. Measurements taken before cryopreservation would not reflect potential insults that could affect the potency of the transplanted unit.⁹⁻¹¹ Potency markers measured on a representative sample of the thawed CBU closer to the time of transplant would more accurately predict the capacity of the unit to engraft in the transplant setting. Previous work in our

laboratory showed that CFUs measured on thawed CBUs are predictive of neutrophil engraftment and survival posttransplant.¹² However, CFU assays are typically scored 2 weeks after the unit has been transplanted. Therefore, an assay is needed to assess the potency of the CBU before final selection and release to the TC.

Aldehyde dehydrogenase (ALDH) is highly expressed (ie, ALDH bright [ALDH^{br}]) in hematopoietic stem and progenitor cells.¹³⁻¹⁷ Flow-sorted ALDH^{br} CBU cells coexpress CD34 and CD133 antigens that are associated with primitive repopulating and progenitor cell functions.¹⁵ Furthermore, ALDH^{br} populations isolated from CBUs are highly enriched for CFUs, whereas ALDH^{dim} cells possess little colony-forming ability.¹⁸ Transplant of purified ALDH^{br} human cord blood progenitors into immunodeficient, interleukin-2 receptor- γ null mice results in lymphoid and myeloid reconstitution.¹⁹ Accordingly, we developed a potency assay based on ALDH^{br} expression in cord blood cells.

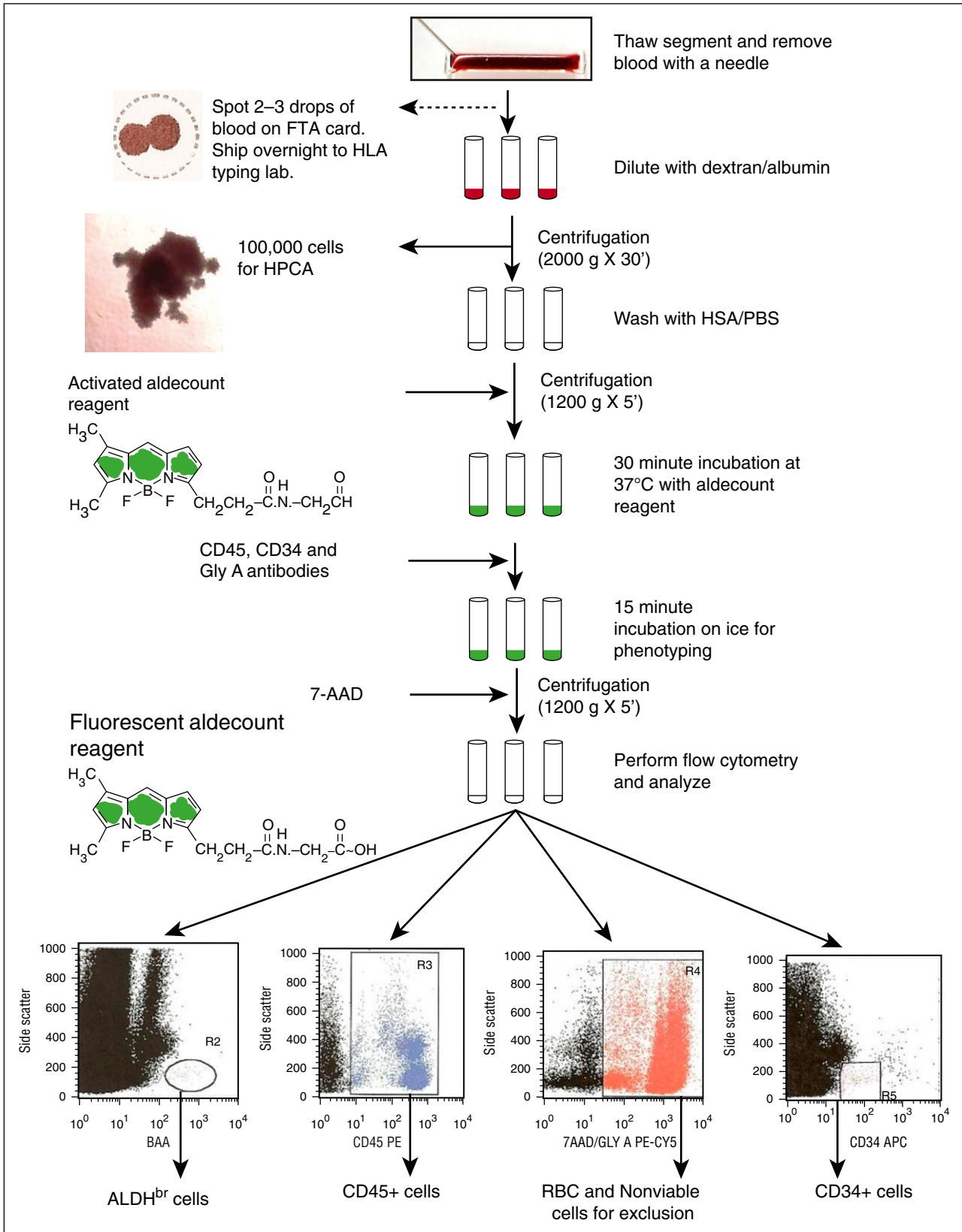
For the past 10 years, it has been common practice at cord blood banks to heat-seal the tubing through which a cryobag is filled so that a series of blood-containing segments remain attached to the unit. These segments can be removed individually without compromising the

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Figure 1. Flowchart of the ALDH potency assay performed on attached segments of CBUs requested for CT for donor selection. 7-AAD, 7-aminoactinomycin D; FTA, Fast Technology Analysis; GlyA, glycophorin A; HSA/PBS, human serum albumin/phosphate-buffered saline; HPCA, hematopoietic progenitor cell assay.

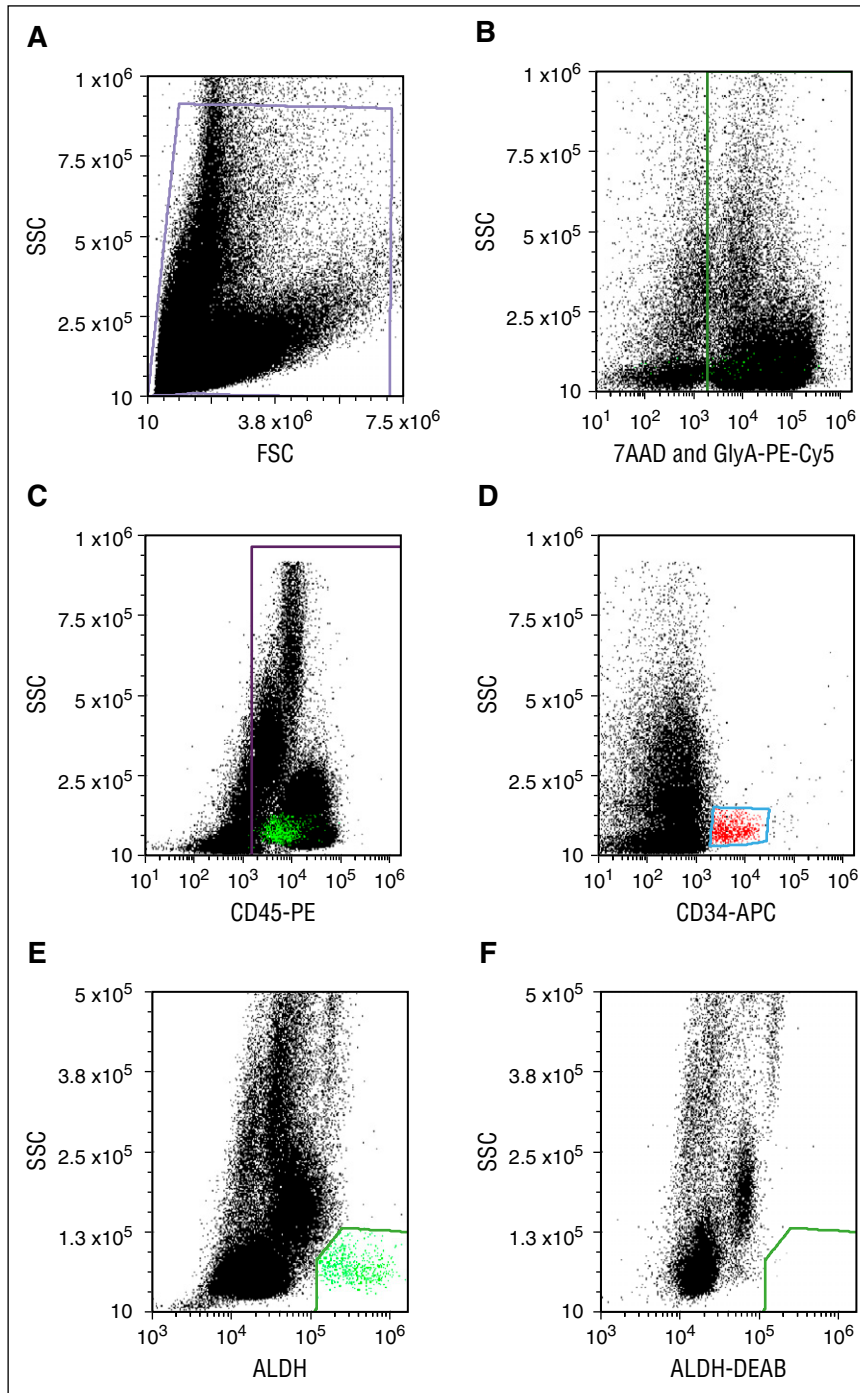


Figure 2. Potency assay gating. (A) Debris-free gate. (B) Gating of the nonviable (7-AAD⁺) and RBC (PE-Cy5-GlyA⁺) cells. (C) Gating of the PE-CD45⁺ cells. (D) Gating of APC-CD34⁺ cells. (E) Gating of ALDH^{br} cells. (F) Gating of cells stained with the Aldecount reagent in the presence of the inhibitor DEAB. BAA, BODIPY aminoacetate; FSC, forward scatter; SSC, side scatter.

integrity of the CBU and are used for confirmatory typing (CT) of the HLA match prior to final selection of a CBU for transplantation. Because HLA-typing requires only a small amount of blood, the remainder of blood in the segment is available for potency testing.

We now describe the development and validation of an in vitro flow cytometric assay measuring ALDH^{br} cell content of a CBU segment for evaluating the potency of a CBU during CT. We consider the CFU assay the best available laboratory surrogate for clinical engraftment; therefore, we prospectively compared the correlation of CFU content of 3908 segments from a public cord blood bank with several flow parameters. Our results suggest that the ALDH^{br} cell content of segments is the parameter that is most highly correlated with CFU.

Therefore, ALDH^{br} content is likely to be the best parameter measured to predict the ability of a CBU to engraft.

Materials and methods

Control segment preparation

Fresh CBUs excluded from banking in the Carolinas Cord Blood Bank (CCBB) were processed to generate segments that served as assay controls. Hetastarch (6%; Hospira, Lake Forest, IL) was sterilely added to the cord blood collection bag at a 1:5 ratio. The cord blood bag was docked to a Sepax cell separation

Table 1. Statistics for segments used for accuracy, linearity, and repeatability

Test ALDH ^{br} concentration, %	Actual ALDH ^{br} concentration, %			Mean	Standard deviation	CV	Meets specifications?
	Segment 1	Segment 2	Segment 3				
0.05	0.052	0.054	0.049	0.051	0.002	3.3	Yes
0.10	0.108	0.108	0.105	0.106	0.001	1.2	Yes
0.50	0.524	0.535	0.481	0.513	0.023	4.5	Yes
1.00	0.974	1.101	1.133	1.069	0.069	6.4	Yes
3.00	2.692	3.084	2.596	2.791	0.211	7.5	Yes

kit (CS-530.1; Biosafe Systems, Houston, TX) and processed on a Sepax-1 system using the program for cord blood volume reduction and red blood cell (RBC) depletion. Mononuclear cells were collected in a volume of 20 mL. The output bag was inserted into a Coolmix device (Biosafe), and 5 mL of dimethyl sulfoxide (DMSO; Akron Biotech, Boca Raton, FL) (55% DMSO in 5% dextran 40) was added (1 mL/min) using a Fusion One Syringe Infusion Pump (Chemyx, Stafford, TX).

Segments with ALDH^{br} cells (purified by magnetic-activated cell sorting [MACS] according to the manufacturer’s protocol) spiked into stocks of CBU cells were prepared for validation experiments. Fresh CBUs were processed using Ficoll-Paque Premium (GE Healthcare Life Sciences, Piscataway, NJ). Cells from the mononuclear layer were incubated with Fc receptor blocking reagent, CD34, and CD133 MicroBeads (100 µL each; Miltenyi Biotec, Bergisch Gladbach, Germany), and passed over a LS column (Miltenyi Biotec). MACS buffer (75 mL of MACS BSA Stock Solution added to 1450 mL of autoMACS Rinsing Solution (both Miltenyi Biotec) was used for washing and elution. The column pass-through, containing unbound cells, and the column elute, containing purified cells, were collected. The percentage of ALDH^{br} cells in each fraction was determined by flow cytometry using Aldecoum and cell counts obtained with an XE-1000i Automated Hematology System (Sysmex America, Lincolnshire, IL). The eluate was spiked into the pass-through to create segments with specific ALDH^{br} values and cryopreserved for validation studies.

Segments were prepared using ethylene vinyl acetate tubing (provided by Medsep, Pall, Port Washington, NY), the same tubing used in CBU cryopreservation bags. Four-inch lengths of tubing were heat-sealed at one end. Processed cord blood was dispensed into the tubing using a syringe and a 16-gauge blunt needle (BD Biosciences, San Jose, CA), and segments were created by heat-sealing the tubing in thirds. The segments were inserted in cryotubes, placed into a Cryo 1°C Freezing Container (Nalgene, Rochester, NY), and placed in a -80°C freezer. The resulting freezing rate of 1°C/min simulated control-rate freezers. The following day, the segments were transferred to long-term storage in the vapor phase of liquid nitrogen (-196°C).

Thawing, washing, and staining of cord blood removed from CBU segments

After removal from liquid nitrogen storage, the segments were kept on dry ice until ready to be thawed. A segment was thawed by holding it between the

fingers, and then it was wiped with alcohol and placed on a sterile gauze pad. The blood was aspirated into a syringe using a 25-gauge needle and dispensed into dextran-albumin (DA, 100 µL). DA was prepared by adding human serum albumin (25%; Octapharma, Hoboken, NJ) to dextran 40 (10% in 0.9% sodium chloride, Hospira) for a final concentration of 5% human serum albumin. All segments were processed before proceeding to the next steps. The blood-and-DA mixture was shaken to mix and then incubated at room temperature for 10 minutes, followed by step-wise DA additions, mixing, and incubations: 200 µL of DA for 3 minutes, 400 µL of DA for 3 minutes, and 200 µL of DA for 3 minutes.

An aliquot (120 µL) was removed and placed into a separate tube (CFU assay tube). The remaining sample was centrifuged at 2000g for 30 minutes at 4°C. The cell pellet was washed in 2 mL of Aldecoum Assay Buffer (STEMCELL Technologies, Vancouver, BC, Canada) and recentrifuged at 250g for 5 minutes at 4°C. The cell pellet was resuspended in 0.5 mL of Aldecoum buffer for ALDH staining (Figure 1), which was done according to the manufacturer’s instructions. The sample was added to an activated Aldecoum Reagent tube (STEMCELL Technologies), and then 0.5 mL of sample was transferred to the diethylaminobenzaldehyde (DEAB) control tube. All tubes were incubated in a water bath at 37°C for 30 minutes.

Multiparameter flow antibody staining

After the 37°C incubation, all of the tubes were placed in an ice bath. The Aldecoum Reagent tubes were stained with the phenotyping antibodies anti-CD45-phycoerythrin (PE) (mouse, clone 5B1; Miltenyi Biotec), anti-GlyA-PE-Cy5 (diluted 100-fold from antibody stock, mouse, clone GA-R2; BD Biosciences), and anti-CD34-allophycocyanin (APC) (mouse, clone 8G12, BD Biosciences). The tubes were incubated in an ice bath for 15 minutes, followed by centrifugation (250g for 5 minutes at 4°C). The cell pellet in each tube was resuspended in Aldecoum Assay buffer. Via-Probe viability solution (7-AAD, 10 µL; BD Bioscience) was added to each phenotyping tube prior to flow cytometry analysis.

Flow cytometry

Flow cytometry was performed on an Accuri C6 digital flow cytometer (BD Biosciences) equipped with 488- and 635-nm lasers with appropriate color

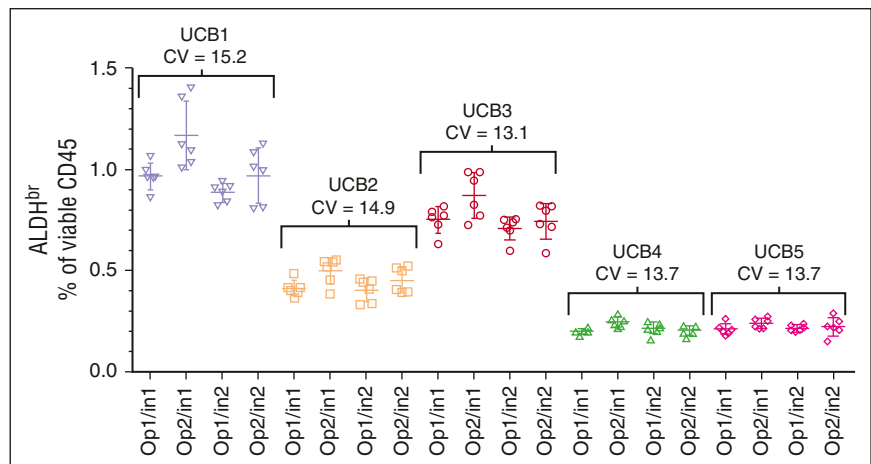


Figure 3. Intermediate precision assessment. CV of segments from 5 CBUs (UCB1-UCB5) analyzed over 6 days using 2 operators (Op1 and Op2) and 2 Instruments (In1 and In2). The target specification was a CV ≤20%.

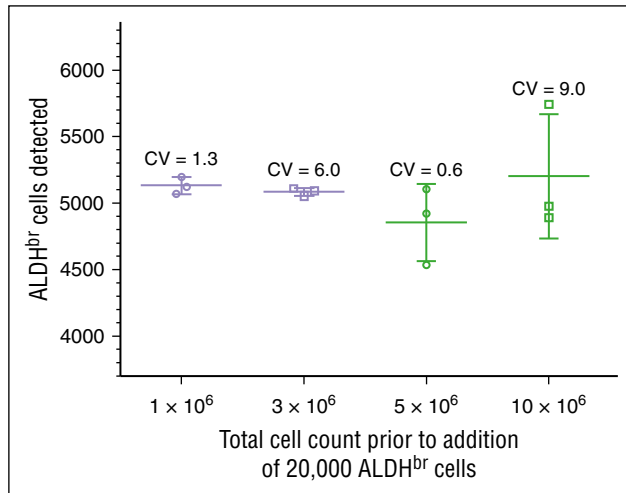


Figure 4. Validation of specificity. Purified ALDH^{br} cells (20 000) were spiked into varying amounts of CBU cells (1×10^6 , 3×10^6 , 5×10^6 , and 10×10^6). Segments were created and cryopreserved. The segments were subsequently thawed and analyzed with the potency assay, with a target of collecting 5000 ALDH^{br} cells. The target of 4000 to 6000 cells was met at all concentrations.

detectors/filters. Gates for antigen-expressing cells were set using appropriate isotype-specific antibody control tubes. For each sample, 100 000 viable CD45⁺ events were collected. Data were analyzed using FCS Express (De Novo Software, Glendale, CA). All dot plots were gated off R1 in a side-scatter vs forward-scatter light-scatter cytogram; Figure 2A). Lysis of RBCs in thawed segments caused high cell death and cell debris, which interfered with flow cytometric analysis. We circumvented the problem of RBC interference by staining RBCs with GlyA-PE-Cy5 and creating a dump gate. Detection of GlyA and the viability stain in the same channel (Figure 2B) allowed simultaneous exclusion of RBCs and nonviable cells. CD45⁺ (Figure 2C) and CD34⁺ populations (Figure 2D) were identified after excluding events in the dump gate. CD34⁺ cells were low in SSC and medium-bright for APC. CD45⁺ cells were spread across the SSC spectrum. ALDH^{br} cells were detected as a well-separated low-SSC and BODIPY aminoacetate–bright population (Figure 2E). The cells in this group were mostly CD34⁺ and CD45⁺, though characteristically dim in CD45-PE fluorescence. This cell population was eliminated with a specific ALDH inhibitor, DEAB (Figure 2F).

Plating and enumeration of postthaw CFU assay

An aliquot of cells from the CFU assay tube was diluted 1:10 in Cellpack solution (Sysmex America) and counted on an XE-1000i. The volume containing 1.0×10^5 cells was calculated and added to 2 mL of MethoCult (H4434; STEMCELL Technologies). The sample was mixed on a vortex mixer, allowed to sit for 2 minutes, and plated into 4 wells (0.4 mL each) in the middle of a 24-well plate. Sterile water was added to the outer wells for humidification. The plates were incubated at 37°C in a humidified incubator with 5% carbon dioxide for 14 days. CFUs, including erythroid burst-forming units, granulocyte-macrophage CFUs, and granulocyte-erythrocyte-macrophage-megakaryocyte CFUs were counted using a CKX41 inverted, phase-contrast microscope (Olympus America, Center Valley, PA) and a 40× objective, and reported as the mean colony count per 1.0×10^5 white blood cells. Total CFUs were calculated by adding the totals of the granulocyte-macrophage CFUs, granulocyte-erythrocyte-macrophage-megakaryocyte CFUs, and erythroid burst-forming units.

Validation of the potency assay

A validation plan for the ALDH potency assay was written according to the Harmonized Tripartite Guidelines Q2(R1)²⁰ set by the International Conference on Harmonization, in consultation with regulatory authorities. Acceptance criteria were set for accuracy (ALDH^{br} within 20% of the

expected value), repeatability ($\leq 20\%$ of the coefficients of variation [CVs] within a single operator on 3 replicates at 5 different concentrations), linearity (between the samples' target and tested values of $R^2 \geq 0.75$), intermediate precision (CVs between operators, samples, and instruments needed to be $\leq 20\%$), and specificity (the number of ALDH^{br} cells detected within a contaminating background of cells was required to be within 20% of the expected value).

Assessment of accuracy, repeatability, and linearity. Commercially available control standards for measuring ALDH^{br} cells are not available; therefore, reference standards were created in our laboratory. For validating accuracy, repeatability, and linearity, standardized control segments reflecting a specified range of concentrations of ALDH^{br} cells (0.05%, 0.1%, 0.5%, 1.0%, and 3.0%) were prepared from sorted CD34⁺/CD133⁺ cells to mimic the range of values obtained on segments from banked CBUs. Three segments were assayed at each concentration for the percentage of ALDH^{br} cells in the viable CD45⁺ gate. Accuracy was determined by comparing the predicted ALDH^{br} percentage, calculated based on the number of ALDH^{br} cells added to each control sample, with the actual ALDH^{br} percentages obtained by analysis for each sample. These data were used to assess repeatability by a single operator at each of the concentrations, using the CV. Linearity was assessed by linear regression using the R^2 statistic, comparing the target ALDH^{br} and actual values.

Assessment of intermediate precision. Intermediate precision was measured by 2 operators, each testing segments prepared from 5 CBUs on 2 Accuri C6 flow cytometers on 6 different days. All test samples consisted of segments prepared for the validation from randomly chosen CBUs. Segments were stored in liquid nitrogen to mirror storage of the actual segments on a CBU. Variability was assessed by comparing the ALDH^{br} (as a percentage of viable CD45) from each sample. The CVs were reported for segments from each CBU as a whole for both operators and instruments, and by assessing operators and instruments separately.

Assessment of specificity. ALDH^{br} cells make up $<1\%$ of total nucleated cells in a typical CBU. The primary impurities in the CBU segment are other cell types that are present in CBUs (eg, RBCs and monocytes that express low levels of ALDH^{br}). To evaluate the impact of these impurities, the ability to measure 5000 ALDH^{br} cells in increasing numbers of contaminating cells was evaluated. ALDH^{br} cells (20 000 within a population of CD34⁺/CD133⁺-selected cells) were spiked into 1×10^6 , 3×10^6 , 5×10^6 , and 10×10^6 nucleated cord blood cells taken from the CD34/CD133-negative fraction. Triplicate samples were run at each concentration. Results were reported as number of ALDH^{br} cells detected.

Assaying thawed or fresh units

Before cryopreservation, 100 μ L of fresh blood taken from a CBU was diluted in 4 mL of Aldecourt Lysis Buffer (Aldagen, Durham, NC). After a 15-minute incubation at 37°C, the sample was centrifuged at 250g for 5 minutes at 4°C, washed with 2 mL of Aldecourt Assay Buffer, and recentrifuged. The sample was then stained for ALDH^{br}, CD45, 7-AAD, and CD34.

Cryopreserved CBUs selected for assaying were thawed with the modified procedure used for preparation of units for transplant.¹² The washed cells were resuspended in dextran 40/5 albumin in a final volume of 50 mL. Aliquots (100 μ L) were diluted in 2 mL of Aldecourt Assay Buffer prior to centrifugation (250g for 5 minutes at 4°C), with staining as described earlier for segments.

Table 2. Data obtained of cell subsets as determined by flow cytometric analysis of the potency assay and CFUs

	Median	Minimum	Maximum
ALDH ^{br} as a percentage of viable CD45 ⁺ , %	0.37	0.00	2.30
CD34 ⁺ as a percentage of viable CD45 ⁺ , %	0.66	0.00	3.49
CD45 ⁺ viability, %	66.10	8.72	99.02
CD34 ⁺ viability, %	82.90	21.37	100
Mean total CFUs (per 1×10^5 white blood cells)	46.25	0	530

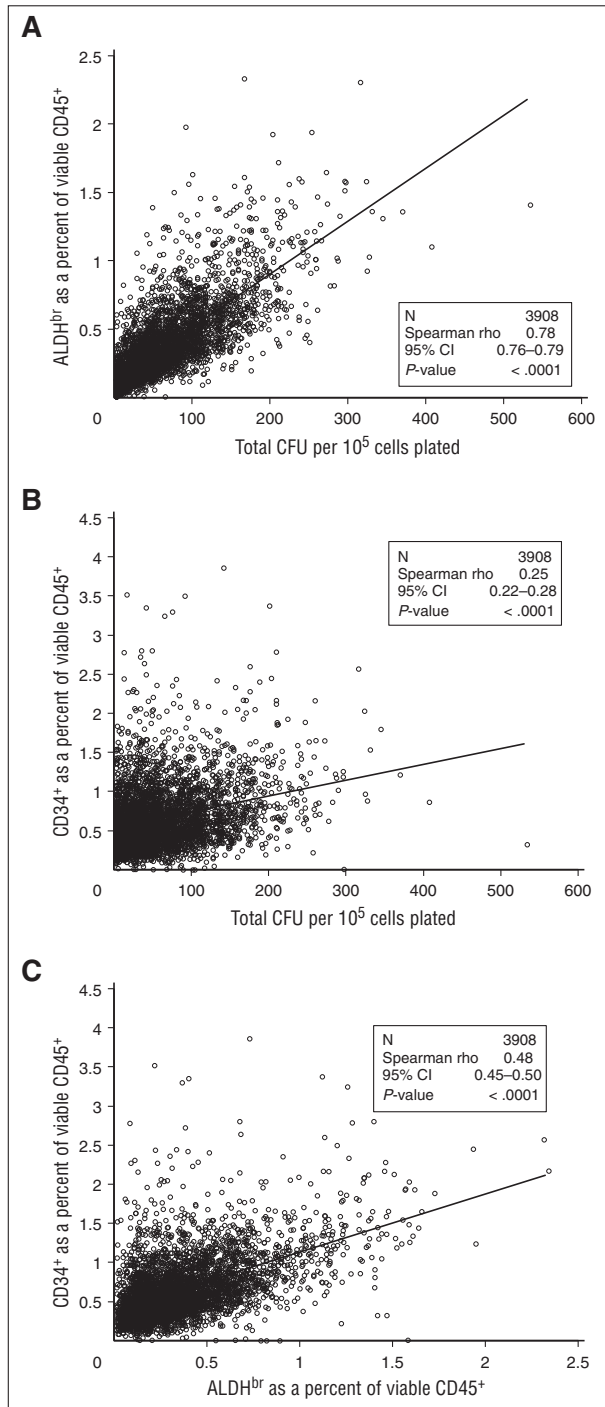


Figure 5. Comparison of cellular components of CT segments. (A) ALDH^{br} in viable CD45⁺ vs CFUs. (B) CD34⁺ in viable CD45⁺ vs CFUs. (C) ALDH^{br} in viable CD45⁺ vs CD34⁺ in viable CD45⁺. N = 3908.

Correlation of segment potency with engraftment

A search of our clinical database identified 78 single-cord transplants performed at Duke University Medical Center using single-CBU grafts that represented either the initial or the only transplant for that patient. Characteristics measured on segments from the transplanted units were examined to determine whether they were predictive of neutrophil engraftment (3 consecutive days of absolute neutrophil count >500/ μ L), using plots of the cumulative incidence function of each segment biomarker and differences determined by Gray's test. Death before engraftment (1 patient) and death before graft failure (4 patients) were treated as competing risks.

Results

We developed and validated a segment-based rapid assay to determine the potency of cryopreserved CBUs selected for consideration for HSCT before release from a US Food and Drug Administration–licensed public cord blood bank. The flow cytometric–based assay enumerates ALDH^{br}, CD34⁺, CD45⁺, and viable cells measured on a thawed segment attached to a CBU. A validation plan addressing accuracy, repeatability, linearity, intermediate precision, and specificity was created and executed.

Accuracy, repeatability, and linearity

Segments containing cord blood with 5 known percentages of ALDH^{br} (3 segments per percentage) were assayed and analyzed for accuracy, repeatability, and linearity. At each test concentration, ALDH^{br} measured by the assay fell within 20% of the expected value as required by our predefined validation specification for accuracy (Table 1). The CVs for repeatability, indicating a single operator's ability to achieve similar results on multiple replicates at each concentration, were well within specifications for acceptability, ranging from 1.2% to 7.5%, and there was no clear relationship between CV and concentration of ALDH^{br}. Results of the linear regression analysis showed excellent linearity between ALDH^{br} measured by the assay and the true concentration ($R^2 = 0.9878$).

Intermediate precision

The segments from 5 CBUs were assayed by 2 operators on 2 cytometers on 6 different days, analyzed separately, and collated. The data were reanalyzed with respect to overall sample precision across both operators and instruments (Figure 3; CVs, 13.1%–15.2%). In addition, intraoperator precision (data not shown; CVs, 10.2%–15.2%), and intrainstrument precision (data not shown; CVs, 7.5%–16.7%) were analyzed. All analyses met the $\leq 20\%$ CV acceptance criteria. Because the individual CBUs used in this experiment were chosen randomly, comparisons between the CBUs were not performed.

Specificity

We tested specificity by determining whether we could detect 5000 ALDH^{br} cells spiked into varying concentrations of cord blood mononuclear cells. Acceptance was scored by detecting between 4000 and 6000 ALDH^{br} cells in a background of 1 million to 10 million contaminating cells. This criterion was met at all levels of cell background, with CVs ranging from 0.6% to 9.0% (Figure 4). The largest variation (9.0%) was seen in the samples with the highest background contamination, in which the ALDH^{br} concentration was 100-fold less than that seen in routine clinical samples. In the range of typical CBU samples, the CVs were much tighter, ranging from 0.6% to 1.3%. RBC contamination was low in these samples due to the use of Ficoll.

Experience with the ALDH^{br} potency assay in a public cord blood bank: can the ALDH^{br} assay substitute for the CFU assay?

Since February 2010, we performed the potency assay on segments from CBUs requested for CT in the CBBB. Segments were detached from the CBU in vapor phase, thawed, and a small aliquot spotted onto

Table 3. Relationship of ALDH^{br} and viable CD34⁺ to CFUs with time in storage

	Spearman <i>r</i> (<i>P</i>)			
	All samples, N = 3908	Storage duration, y		
		<5, n = 3188	5-10, n = 579	>10, n = 141
ALDH ^{br} as a percentage of viable CD45 ⁺	0.78 (<.0001)	0.78 (<.0001)	0.78 (<.0001)	0.77 (<.0001)
CD34 ⁺ as a percentage of viable CD45 ⁺	0.25 (<.0001)	0.25 (<.0001)	0.25 (<.0001)	0.29 (.0004)

an FTA card and sent to the National Marrow Donor Program reference laboratory for CT. The remaining blood was used for the potency assay. Over a 5-year period, 3908 segments were tested for ALDH^{br}, CD45⁺, and CD34⁺ cells; viability; and CFUs. We detected a range of ALDH^{br} cells within the CD45⁺ viable population (0.00%-2.30%; median, 0.37%). CD45⁺ cells had a median cell viability of 66.10% (range, 8.72%-99.02%). Of the viable CD45⁺ cells, 0.66% (range, 0.00%-3.49%) coexpressed CD34 (Table 2).

We examined the correlation of ALDH^{br} cells to CFUs in the segments tested. ALDH^{br} (as a percentage of viable CD45⁺ cells) correlated strongly with total CFU (per 10⁵ cells) (Spearman *r* = 0.78; 95% confidence interval [CI], 0.76-0.79; Figure 5A). However, the correlation between CD34⁺ (as a percentage of viable CD45⁺ cells) and CFU was substantially weaker (*r* = 0.25; 95% CI, 0.22-0.28; Figure 5B). ALDH^{br} correlated more modestly with CD34⁺ (*r* = 0.48; 95% CI, 0.45-0.50; Figure 5C). The differences in correlation likely result from ALDH^{br} and CFU needing metabolically active cells, whereas CD34⁺ is an antibody-based test. These correlations with CFUs were consistent regardless of how long the cryopreserved CBUs were stored, suggesting that the potency of properly stored CBUs is stable over at least 15 years (Table 3).

Between July 2007 and August 2009, samples from fresh CBUs before cryopreservation were assayed for ALDH^{br}. As the CT was done, segments frozen with those CBUs were tested for ALDH^{br}. Comparison of the data from fresh CBUs and frozen segments (Figure 6A) revealed a weak correlation (Spearman *r* = 0.43), with segments tending to have a lower ALDH^{br} content. To determine the correlation of the potency assay between cryopreserved segments and bags, research units from the CCBB were randomly selected and the entire unit was thawed and then assayed along with the distal segment that would normally be assayed in for CT (Figure 6B). Although there was a strong overall correlation between segments and units (Spearman *r* = 0.88), segments still tended to underestimate the ALDH^{br} content of the unit.

ALDH^{br} impact on engraftment

We asked whether ALDH^{br}, CD34, and CFUs from the segment potency assay correlated with engraftment in a cohort of 78 patients transplanted at Duke University Medical Center with a single CBU. Ninety-four percent (73/78) of patients engrafted (18 malignant and 55 nonmalignant diagnoses). The median time to engraftment was 16 days (range, 10-38 days). The association between segment-based ALDH^{br} and CFUs in this subset of segments was similar to that observed in the entire sample of segments included in our study (*r* = 0.70; *P* < .001). We observed a trend toward faster engraftment with increased expression of ALDH^{br} (*P* = .03; Figure 7), although caution is warranted in interpretation of this result due to small subgroup sizes. CFUs and CD34 did not predict neutrophil engraftment in the entire cohort (*P* > .05 for all; data not shown).

Discussion

Our results demonstrate the successful development of a segment-based potency assay that meets the International Conference on Harmonization guidelines for accuracy, specificity, precision,

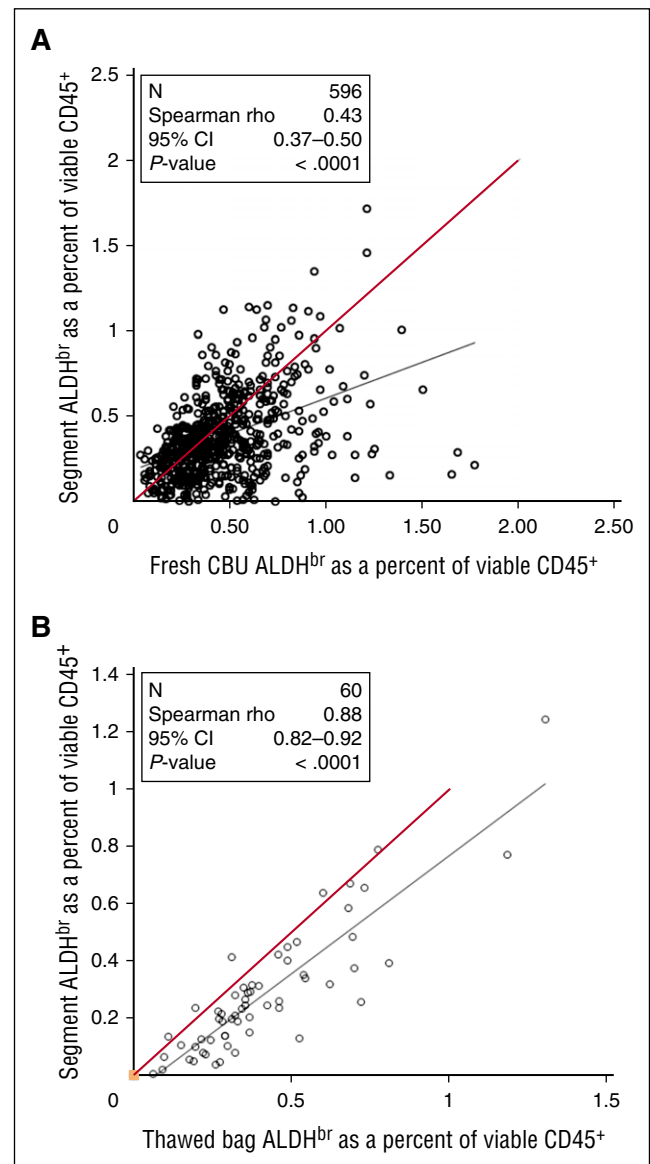


Figure 6. Comparison of CT segments in fresh or thawed CBUs. (A) Samples taken from fresh CBUs before cryopreservation between July 2007 and August 2009 were assayed for ALDH^{br} and compared with the results of the CT assay of segments from those units. N = 596. Red line indicates theoretical equality. (B) Research CBUs stored at the CCBB were selected. The bags were thawed with the segments and tested for ALDH^{br}. N = 60. Red line indicates theoretical equality.

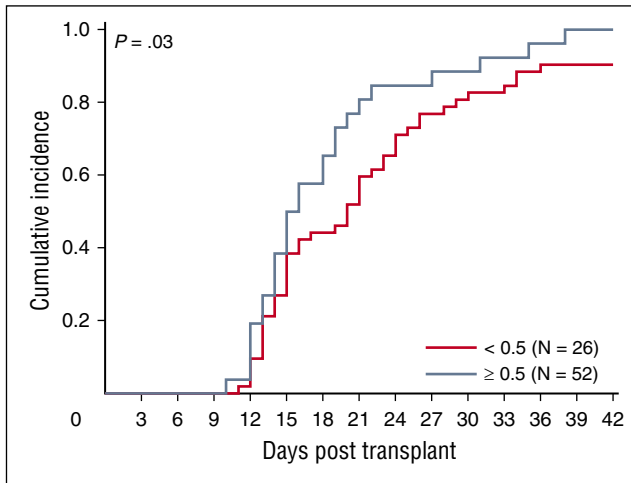


Figure 7. ALDH^{br} impact on engraftment. Impact of ALDH^{br} measured on segments during CT on engraftment of the corresponding unit. Probability plots are shown for the units with an ALDH^{br} >0.5% of viable CD45⁺ or the units with an ALDH^{br} <0.5% of viable CD45⁺ in reaching an absolute neutrophil count of 500/ μ L. N = 78.

repeatability, and linearity. Furthermore, we have demonstrated through a prospective evaluation of almost 4000 segments from cryopreserved CBUs that the assay (1) is highly correlated with CFUs, which have been previously shown to best predict neutrophil engraftment after umbilical cord blood transplant; and (2) can be efficiently integrated into routine cord blood banking release procedures during the CT process before final selection and release of the CBU to the TC.

Currently, CBUs are selected^{3,21-23} by a TC after review of the precryopreservation TNCC, HLA matching, and/or CD34⁺ cell content. These data fail to reflect insults to the CBU caused by freezing and thawing of the unit. Therefore, we focused on postthaw CBU characteristics or surrogates for these measures. We previously reported that postthaw CFU content best correlated with engraftment for both neutrophils and platelets.^{12,24} Although postthaw CFU content showed the strongest correlation with engraftment, it is not feasible to use this parameter for CBU selection because it is generally scored 14 days after plating the assay.

The established correlation of CFU and ALDH^{br} cells in fresh CBU and the availability of segments at the time of CBU selection led us to develop and optimize the ALDH^{br} assay on thawed CBU segments. This approach posed some technical challenges that had to be resolved for successful measurement of potency. To maintain cell viability and protect the cells from DMSO cytotoxicity during thawing, we followed a DA wash protocol based on a method by Rubinstein et al,²⁵ which emulates CBU washing for transplantation. We demonstrated that the temperature during this step was critical and that cells and DA needed to be at room temperature to avoid loss of detectable ALDH^{br} cells. This methodology is in contrast to protocols applied to CBUs that require washing the cells with ice-cold DA.

In these studies, we also observed that the ALDH^{br} content of the segments correlates well with the CFU content. This finding strongly suggests that measurement of ALDH^{br} can serve as a surrogate for the CFU assay. The stronger correlation between segments and frozen units, as opposed to fresh units, likely means that cryopreservation impacts both segments and units, with a possible greater impact on the segments. This means that the segment assay may disqualify some units with adequate potency from being used.

However, the segment assay is highly unlikely to identify low-potency units as high-potency units. Because the potency assay data are available at the time of final donor selection, it can be used as a prospective potency assay for selecting CBUs. Although confirmatory studies are needed, our data suggest that CBUs rich in ALDH^{br} cells are predictive of engraftment, which would be expected because this enzyme is characteristically highly expressed in the hematopoietic stem and progenitor cells from human umbilical cord blood,^{15,26} bone marrow,^{27,28} and mobilized peripheral blood.^{29,30}

Other methods to assess engraftment potential of CBUs have been reported in the literature. Higher postthaw CD34⁺ viability has been correlated with the dominant unit after double-CBU transplantation, and low CD34⁺ viability is associated with an increased risk of graft failure.^{31,32} Dominant-unit CD34⁺-infused cell dose has been found to predict engraftment after double-unit transplantation.³³ In addition, the use of the HALO adenosine triphosphate bioluminescence assay on segments and units indicates that potency assessment on the mononuclear cell fraction may be more robust and less variable than on the TNCC fraction.³⁴ Further refining of the CFU assay to decrease the length of the assay is also under investigation.^{35,36} The creation of the Cord Blood Apgar scoring system, which utilizes precryopreservation and postthaw graft variables (TNCC, mononuclear cell, CFU, CD34⁺, and volume), showed that a composite scoring system could be used to optimize selection of CBUs for transplantation.³⁷

The present studies demonstrated rapid, reliable measurement of ALDH^{br} cell content in thawed CBU segments by flow cytometry. The assay requires a small quantity of blood from a segment, and the integrity of the entire unit is unaffected. The assay is performed in less than 4 hours, providing an important advantage over the traditional CFU assay. Implementation of this complex assay in other cord blood banks will require focused technology transfer with targeted training and validation to ensure optimal performance of the assay. Additional studies to examine correlations with engraftment in clinical transplantation are under way to further validate this potency assay for use in selecting CBUs for transplantation.

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Authorship

Contribution: K.S. took the lead role in adapting the ALDH^{br} assay for use with segments during CT, performed much of the assays and data analysis, and wrote the first draft of the manuscript; P.N. performed many of the assays and helped with data analysis and editing of the manuscript; J.D.T. provided statistical analysis of the data and advice for the validation; L.C. and A.P. provided support

in the design, analysis, and interpretation of the data for the validation studies; K.P. contributed to the conception of the assay method, interpretation of the data for clinical use, and editing of the manuscript; T.G. and A.E.B. worked on developing the original ALDH^{br} assay, contributed to the design of the CT assay and the validation, and assisted in drafting and editing the manuscript; and J.K. was instrumental in the conception, design, and interpretation

of the studies presented here and in provided editing and critical revisions of the manuscript.

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References

- Wagner JE, Rosenthal J, Sweetman R, et al. Successful transplantation of HLA-matched and HLA-mismatched umbilical cord blood from unrelated donors: analysis of engraftment and acute graft-versus-host disease. *Blood*. 1996; 88(3):795-802.
- Rubinstein P, Carrier C, Scaradavou A, et al. Outcomes among 562 recipients of placental-blood transplants from unrelated donors. *N Engl J Med*. 1998;339(22):1565-1577.
- Gluckman E, Rocha V. Donor selection for unrelated cord blood transplants. *Curr Opin Immunol*. 2006;18(5):565-570.
- Cornetta K, Laughlin M, Carter S, et al. Umbilical cord blood transplantation in adults: results of the prospective Cord Blood Transplantation (COBLT). *Biol Blood Marrow Transplant*. 2005;11(2):149-160.
- Kurtzberg J, Laughlin M, Graham ML, et al. Placental blood as a source of hematopoietic stem cells for transplantation into unrelated recipients. *N Engl J Med*. 1996;335(3):157-166.
- Kurtzberg J, Prasad VK, Carter SL, et al; COBLT Steering Committee. Results of the Cord Blood Transplantation Study (COBLT): clinical outcomes of unrelated donor umbilical cord blood transplantation in pediatric patients with hematologic malignancies. *Blood*. 2008;112(10):4318-4327.
- Martin PL, Carter SL, Kernan NA, et al. Results of the cord blood transplantation study (COBLT): outcomes of unrelated donor umbilical cord blood transplantation in pediatric patients with lysosomal and peroxisomal storage diseases. *Biol Blood Marrow Transplant*. 2006;12(2):184-194.
- Wagner JE Jr, Eapen M, Carter S, et al; Blood and Marrow Transplant Clinical Trials Network. One-unit versus two-unit cord-blood transplantation for hematologic cancers. *N Engl J Med*. 2014; 371(18):1685-1694.
- Dahi PB, Ponce DM, Devlin S, et al. "No wash" albumin-dextran dilution for double-unit cord blood transplantation is safe with high rates of sustained donor engraftment. *Biol Blood Marrow Transplant*. 2014;20(4):490-494.
- McManus MP, Wang L, Calder C, et al. Comparison of pre-cryopreserved and post-thaw-and-wash-nucleated cell count on major outcomes following unrelated cord blood transplant in children. *Pediatr Transplant*. 2012; 16(5):438-442.
- Beksac M, Preffer F. Is it time to revisit our current hematopoietic progenitor cell quantification methods in the clinic? *Bone Marrow Transplant*. 2012;47(11):1391-1396.
- Page KM, Zhang L, Mendizabal A, et al. Total colony-forming units are a strong, independent predictor of neutrophil and platelet engraftment after unrelated umbilical cord blood transplantation: a single-center analysis of 435 cord blood transplants. *Biol Blood Marrow Transplant*. 2011;17(9):1362-1374.
- Storms RW, Green PD, Safford KM, et al. Distinct hematopoietic progenitor compartments are delineated by the expression of aldehyde dehydrogenase and CD34. *Blood*. 2005;106(1):95-102.
- Storms RW, Trujillo AP, Springer JB, et al. Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. *Proc Natl Acad Sci USA*. 1999;96(16):9118-9123.
- Hess DA, Meyerrose TE, Wirthlin L, et al. Functional characterization of highly purified human hematopoietic repopulating cells isolated according to aldehyde dehydrogenase activity. *Blood*. 2004;104(6):1648-1655.
- Kastan MB, Schlafler E, Russo JE, Colvin OM, Civin CI, Hilton J. Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells. *Blood*. 1990; 75(10):1947-1950.
- Lioznov MV, Freiburger P, Kröger N, Zander AR, Fehse B. Aldehyde dehydrogenase activity as a marker for the quality of hematopoietic stem cell transplants. *Bone Marrow Transplant*. 2005;35(9):909-914.
- Gentry T, Deibert E, Foster SJ, Haley R, Kurtzberg J, Balber AE. Isolation of early hematopoietic cells, including megakaryocyte progenitors, in the ALDH-bright cell population of cryopreserved, banked UC blood. *Cytotherapy*. 2007;9(6):569-576.
- Liu C, Chen BJ, Deoliveira D, Sempowski GD, Chao NJ, Storms RW. Progenitor cell dose determines the pace and completeness of engraftment in a xenograft model for cord blood transplantation. *Blood*. 2010;116(25):5518-5527.
- Baber N. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH). *Br J Clin Pharmacol*. 1994;37(5):401-404.
- Rubinstein P. Cord blood banking for clinical transplantation. *Bone Marrow Transplant*. 2009; 44(10):635-642.
- Gluckman E, Rocha V, Arcese W, et al; Eurocord Group. Factors associated with outcomes of unrelated cord blood transplant: guidelines for donor choice. *Exp Hematol*. 2004;32(4):397-407.
- Wall DA, Chan KW. Selection of cord blood unit(s) for transplantation. *Bone Marrow Transplant*. 2008;42(1):1-7.
- Migliaccio AR, Adamson JW, Stevens CE, Dobrila NL, Carrier CM, Rubinstein P. Cell dose and speed of engraftment in placental/umbilical cord blood transplantation: graft progenitor cell content is a better predictor than nucleated cell quantity. *Blood*. 2000;96(8):2717-2722.
- Rubinstein P, Dobrila L, Rosenfield RE, et al. Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution. *Proc Natl Acad Sci USA*. 1995; 92(22):10119-10122.
- Pearce DJ, Bonnet D. The combined use of Hoechst efflux ability and aldehyde dehydrogenase activity to identify murine and human hematopoietic stem cells. *Exp Hematol*. 2007;35(9):1437-1446.
- Gentry T, Foster S, Winstead L, Deibert E, Fiordalisi M, Balber A. Simultaneous isolation of human BM hematopoietic, endothelial and mesenchymal progenitor cells by flow sorting based on aldehyde dehydrogenase activity: implications for cell therapy. *Cytotherapy*. 2007; 9(3):259-274.
- Mirabelli P, Di Noto R, Lo Pardo C, et al. Extended flow cytometry characterization of normal bone marrow progenitor cells by simultaneous detection of aldehyde dehydrogenase and early hematopoietic antigens: implication for erythroid differentiation studies. *BMC Physiol*. 2008;8:13.
- Fallon P, Gentry T, Balber AE, et al. Mobilized peripheral blood SSCloALDHbr cells have the phenotypic and functional properties of primitive haematopoietic cells and their number correlates with engraftment following autologous transplantation. *Br J Haematol*. 2003;122(1):99-108.
- Sharma S, Shariatmadar S, Krishan A. Electronic volume, aldehyde dehydrogenase, and stem cell marker expression in cells from human peripheral blood apheresis samples. *Cytometry B Clin Cytom*. 2010;78(2):123-129.
- Avery S, Shi W, Lubin M, et al. Influence of infused cell dose and HLA match on engraftment after double-unit cord blood allografts. *Blood*. 2011;117(12):3277-3285, quiz 3478.
- Scaradavou A, Smith KM, Hawke R, et al. Cord blood units with low CD34+ cell viability have a low probability of engraftment after double unit transplantation. *Biol Blood Marrow Transplant*. 2010;16(4):500-508.
- Purtill D, Smith K, Devlin S, et al. Dominant unit CD34+ cell dose predicts engraftment after double-unit cord blood transplantation and is influenced by bank practice. *Blood*. 2014;124(19):2905-2912.
- Patterson J, Moore CH, Palser E, et al. Detecting primitive hematopoietic stem cells in total nucleated and mononuclear cell fractions from umbilical cord blood segments and units. *J Transl Med*. 2015;13:94.
- Radke TF, Barbosa D, Duggleby RC, Saccardi R, Querol S, Kogler G. The assessment of parameters affecting the quality of cord blood by the application of the annexin V staining method and correlation with CFU assays. *Stem Cells Int*. 2013;2013:823912.
- Wognum B, Yuan N, Lai B, Miller CL. Colony forming cell assays for human hematopoietic progenitor cells. *Methods Mol Biol*. 2013;946:267-283.
- Page KM, Zhang L, Mendizabal A, et al. The Cord Blood Apgar: a novel scoring system to optimize selection of banked cord blood grafts for transplantation (CME). *Transfusion*. 2012;52(2):272-283.