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

By
Kevin W Shoulars¹, Pamela Noldner¹, Jesse D Troy¹, Lynn Cheatham¹, Amanda Parrish¹, Kristin Page², Tracy Gentry¹, Andrew E Balber¹, and Joanne Kurtzberg¹,²,³

¹Duke Translational Research Institute, Duke University Medical Center, Durham, North Carolina
²Pediatric Blood and Marrow Transplant Program, Duke University Medical Center, Durham, North Carolina
³Carolinas Cord Blood Bank, Duke University Medical Center, Durham, North Carolina

Running Title: ALDHbr potency assay for umbilical cord blood

Abstract Word Length: 245

Text Word Length: 4000

Category: Hematopoiesis and Stem Cells
Key Points

Cord Blood content of ALDH<sup>br</sup> cells correlate well with colony forming units and may act as a surrogate potency assay for cord blood units.

ALDH<sup>br</sup> cells in segments are assayed rapidly and allow potency results to be used for release of the unit from a public cord blood bank.

Abstract

Banked, unrelated umbilical cord blood (UCB) provides access to hematopoietic stem cell transplantation for patients lacking matched bone marrow donors, yet 10-15% 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 pre-cryopreservation neglecting changes in potency occurring during freezing and thawing. Colony forming units (CFU) have been previously shown to predict CBU potency defined as the ability to engraft in patients by day 42 post-transplant. However, the CFU assay is difficult to standardize and requires 2 weeks to perform. Consequently, we have developed a rapid multiparameter flow cytometric CBU potency assay that enumerates cells expressing high levels of the enzyme aldehyde dehydrogenase (ALDH<sup>br</sup>) along with viable CD45<sup>+</sup>, CD34<sup>+</sup> cell content. These measurements are made on a segment that was attached to a cryopreserved CBU. We validated the assay with pre-specified criteria testing Accuracy, Specificity, Repeatability, Intermediate Precision, and Linearity. We then prospectively examined the correlations between ALDH<sup>br</sup>, CD34<sup>+</sup>, and CFU content of N=3,908 segments over a 5-year period. ALDH<sup>br</sup> (r=0.78, 95% CI: 0.76-0.79), but not CD34<sup>+</sup> (r=0.25, 95% CI: 0.22-0.27).
0.22-0.28) was strongly correlated with CFU content as well as ALDH<sup>br</sup> content of the CBU. These results suggest the ALDH<sup>br</sup> segment assay is a reliable assessment of potency that allows rapid selection and release of CBUs based on unit characteristics measured before release from the cord blood bank to the transplant center for transplantation.
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 human leukocyte antigen (HLA) matching\(^1\)\(^-\)\(^5\). Despite these benefits, delays in engraftment or primary graft failure are observed in 10-15% of patients after transplantation\(^6\)\(^-\)\(^8\). 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 (TC) 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 (CFU) and CD34\(^{+}\) cell content, often adjusted for cell dose. Freezing, storage and thawing of CBUs reduce the overall potency. Pre-cryopreservation measures 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 CFUs measured on thawed CBUs are predictive of neutrophil engraftment and survival post-transplant\(^12\). However, CFU assays are typically scored two 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.

ALDH\(^{br}\) is highly expressed in hematopoietic stem and progenitor cells\(^13\)\(^-\)\(^17\). Flow-sorted ALDH\(^{br}\) CBU cells co-express CD34 and CD133 antigens that are associated with
primitive repopulating and progenitor cell functions\textsuperscript{15}. Furthermore, ALDH\textsuperscript{br} populations isolated from CBU are highly enriched for CFUs, while ALDH\textsuperscript{dim} cells possess little colony forming ability\textsuperscript{18}. Transplantation of purified ALDH\textsuperscript{br} human cord blood progenitors into immunodeficient, interleukin-2 receptor-gamma null (NS\textgamma) mice results in lymphoid and myeloid reconstitution\textsuperscript{19}. Accordingly, we developed a potency assay based on ALDH\textsuperscript{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 the cryobag was filled as a series of blood-containing segments remain attached to the unit. These segments can be removed individually without compromising the integrity of the CBU and are used for confirmatory typing (CT) of the HLA match prior to final selection of a CBU for transplantation. As 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 \textit{in vitro} flow cytometric assay measuring ALDH\textsuperscript{br} cell content of a CBU-segment for evaluating potency of a CBU during CT. We consider the CFU assay as the best available laboratory surrogate for clinical engraftment and therefore prospectively compared the correlation of CFU content of 3,908 segments from a public cord blood bank with several flow parameters. Our results suggest that the ALDH\textsuperscript{br} cell content of segments is the parameter that is most highly correlated with CFU. Therefore, ALDH\textsuperscript{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 serve as assay controls. Hetastarch (6%, Hospira, Lake Forest, IL) was steriley added to the CB collection bag at a 1:5 ratio, the CB bag docked to a Sepax cell separation kit (CS-530.1, Biosafe Systems, Houston, TX) and processed on a Sepax-1 system using the program for cord blood volume reduction and 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 dimethyl sulfoxide (Akron Biotech, Boca Raton, FL) (DMSO, 55% in 5% Dextran 40) was added (1mL/min) using a Fusion One Syringe Infusion Pump (Chemyx, Inc., Stafford, TX).

Segments with ALDH<sup>br</sup> cells, purified by magnetic cell separation (MACS) according to 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 FcR 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 (Miltenyi Biotec) added to 1450 mL of autoMACS Rinsing solution (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 percent of ALDH<sup>br</sup> cells in each fraction was determined by flow cytometry using Aldecount and cell counts obtained with a XE-1000i Automated Hematology System (Sysmex America, Lincolnshire, Illinois). The
eluate was spiked into the pass-through to create segments with specific ALDH\textsuperscript{br} values and cryopreserved for validation studies.

Segments were prepared using ethylene vinyl acetate tubing (provided by Medsep, Pall, Port Washington, NY); the tubing used in CBU cryopreservation bags. Four-inch lengths of tubing were heat-sealed at one end. Processed CB was dispensed into the tubing using a syringe and a 16-gauge blunt needle (BD Biosciences, San Jose, CA) and segments created by heat-sealing the tubing in thirds. The segments were inserted in cryotubes, placed into a Cryo 1\textdegree C Freezing Container (Nalgene, Rochester, NY) and placed in a -80\textdegree C freezer. The resulting freezing rate of 1\textdegree C per minute simulated control rate freezers. The following day, the segments were transferred to long-term storage in the vapor phase of liquid nitrogen (-196\textdegree 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 thawed. A segment was thawed by holding it between the fingers, then 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 \textmu L). DA was prepared by adding human serum albumin (25\% HSA, Octapharma, Hoboken, NJ) to dextran 40 (10\% in 0.9\% sodium chloride, Hospira) for a final concentration of 5\% HSA. All segments were processed before proceeding to the next steps. The blood and DA was mixed by shaking the tube and incubated at room temperature for 10 min followed by step-wise DA additions, mixing and incubations: 200 \textmu L of DA for 3 min, 400 \textmu L of DA for 3 minutes and 200 \textmu L 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 2000 X g for 30 minutes at 4˚C, the cell pellet washed in 2 mL Aldecount® Assay Buffer (STEMCELL Technologies, Vancouver, BC, Canada), re-centrifuged at 250 X g for 5 minutes at 4˚C. The cell pellet was resuspended in 0.5 mL of Aldecount buffer for ALDH staining (Figure 1), which was done according to manufacturer’s instructions. Sample was added to an activated Aldecount Reagent tube (STEMCELL Technologies). Then, 0.5 mL of sample was transferred to the 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 Aldecount Reagent tubes were stained with phenotyping antibodies, anti-CD45-phycoerythrin (PE, mouse, clone 5B1, Miltenyi Biotec), anti-glycophorin-A-PE-Cy5 (Gly-A, 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 (250 X g for 5 minutes at 4˚C). The cell pellet in each tube was resuspended in Aldecount 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 nm and 635 nm lasers with appropriate color 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 was analyzed using FCS Express (De Novo Software, Glendale, CA). All dot plots were gated off R1 in a side versus forward light scatter cytogram (SSC/FSC, Figure 2A). Lysis of RBC 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 RBC with GlyA-PE-Cy5 and creating a dump gate. Detection of Gly-A and the viability stain in the same channel (Figure 2B) allowed simultaneous exclusion of RBCs and non-viable 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. ALDHbr cells were detected as a well-separated low SSC and BAA 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 Post Thaw Colony Forming Assay**

An aliquot of cells from the CFU assay tube was diluted 1:10 in Cellpack solution (Sysmex America) and counted on a XE-1000i. The volume containing 1.0 X 10^5 cells was calculated and added to 2 mL of MethoCult (#H4434, STEMCELL Technologies). The sample was vortexed, allowed to sit for two minutes and plated into four 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% CO₂ for 14 days. CFU colonies, including erythroid burst forming units (BFU-E), granulocyte macrophage (CFU-GM) and granulocyte, erythrocyte, macrophage, and megakaryocyte (CFU-GEMM) colony forming units were counted using a CKX41 inverted, phase-contrast microscope (Olympus America, Center Valley, PA) and a 40x objective and reported as the mean colony count per 1.0 ×10⁵ WBC. Total CFUs were calculated by adding the totals of the CFU-GM, CFU-GEMM and BFU-E.

Validation of the Potency Assay

A validation plan for the ALDH potency assay was written according to guidelines set by the International Conference on Harmonisation (ICH), Harmonised Tripartite Guidelines Q2(R1)²⁰ in consultation with regulatory authorities. Acceptance criteria were set for accuracy (ALDHbr within 20% of the expected value), repeatability (≤20% CVs within a single operator on three replicates at five different concentrations), linearity (between the samples’ target and tested values of R-squared ≥0.75), intermediate precision (CVs between operators, samples and instruments needed to be ≤20%) and specificity (the number of ALDHbr cells detected within a contaminating background of cells were required to be within 20% of the expected value).

Assessment of Accuracy, Repeatability, and Linearity

There were no commercially available control standards for measuring ALDHbr cells, reference standards were created in our laboratory. For validating accuracy,
repeatability and linearity, standardized control segments reflecting a specified range of concentrations of ALDH\textsuperscript{br} cells (0.05\%, 0.1\%, 0.5\%, 1.0\% and 3.0\%) were prepared from sorted CD34\textsuperscript{+}CD133\textsuperscript{+} 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\textsuperscript{br} cells in the viable CD45\textsuperscript{+} gate. Accuracy was determined by comparing predicted ALDH\textsuperscript{br} percentage, calculated based on the number of ALDH\textsuperscript{br} cells added to each control sample, with the actual ALDH\textsuperscript{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-squared (R\textsuperscript{2}) statistic, comparing the target ALDH\textsuperscript{br} and actual values.

**Assessment of Intermediate Precision**

Intermediate precision was measured by two operators, each testing segments prepared from five CBU on two Accuri C6 flow cytometers on six different days. All test samples consisted of segments prepared for the validation from randomly chosen cord blood units. Segments were stored in LN\textsubscript{2} to mirror storage of the actual segments on a CBU. Variability was assessed by comparing the ALDH\textsuperscript{br} (as a percent of viable CD45\textsuperscript{+}) from each sample. The CV was reported as segments from each CBU as a whole for both operators and instruments, and by assessing operators and instruments separately.

**Assessment of Specificity**
ALDH\textsuperscript{br} cells comprise less than 1\% of total nucleated cells in a typical CBU. The primary impurities in the CBU segment are other cell types that are present in CBU (e.g. RBCs and monocytes that express low levels of ALDH\textsuperscript{br}). To evaluate the impact of these impurities, the ability to measure 5,000 ALDH\textsuperscript{br} cells in increasing numbers of contaminating cells was evaluated. ALDH\textsuperscript{br} cells (20,000 within a population of CD34\textsuperscript{+}/CD133\textsuperscript{+} selected cells) were spiked into 1\times10^6, 3\times10^6, 5\times10^6 and 10\times10^6 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\textsuperscript{br} cells detected.

**Assaying Thawed or Fresh Units**

Prior to cryopreservation, 100 \mu L of fresh blood taken from a CBU was diluted in 4 mL of Aldecount Lysis Buffer (Aldagen Inc., Durham, NC). Following a 15 minutes incubation at 37\(^\circ\)C, the sample was centrifuged at (250 X g for 5 minutes at 4\(^\circ\)C), washed with 2 mL of Aldecount Assay Buffer and recentrifuged. The sample was then stained for ALDH\textsuperscript{br}, CD45, 7-AAD and CD34.

Cryopreserved CBUs selected for assaying were thawed with the modified procedure used for preparation of units for transplantation\textsuperscript{12}. The washed cells were resuspended in dextran 40/5 albumin in a final volume of 50 mL. Aliquots (100 \mu L) were diluted in 2 mL of Aldecount Assay Buffer prior to centrifugation (250 X g for 5 minutes at 4\(^\circ\)C) with staining as described above for segments.
Correlation of Segment Potency with Engraftment

A search of our clinical database identified 78 single-cord transplants performed at Duke using single cord blood unit 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 predict of neutrophil engraftment (3 consecutive days of ANC > 500/µL) using plots of the cumulative incidence function of each segment biomarker with differences determined by Gray’s test. Death prior to engraftment (1 patient) and graft failure (4 patients) were treated as competing risks.

Results

We developed and validated a segment-based rapid assay to determine potency of cryopreserved CBUs selected for consideration for HSCT prior to release from a FDA licensed public cord blood bank. The flow cytometric based assay enumerates ALDHbr, 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 five known percentages of ALDHbr (three segments per percentage) were assayed and analyzed for accuracy, repeatability and linearity. At each test concentration, ALDHbr measured by the assay fell within 20% of the expected value as required by our pre-defined 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\textsuperscript{br}. Results of the linear regression analysis showed excellent linearity between ALDH\textsuperscript{br} measured by the assay and the true concentration ($R^2=0.9878$).

**Intermediate Precision**

The segments from five CBUs were assayed by two operators on two cytometers on six different days, analyzed separately and collated. The data was reanalyzed with respect to overall sample precision across both operators and instruments (Figure 3, CVs: 13.1-15.2%). In addition, intra-operator precision (not shown, CVs: 10.2-15.2%), and intra-instrument precision (not shown, CVs 7.5-16.7%) were analyzed. All analyses met the $\leq20\%$ CV acceptance criteria. As 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 5,000 ALDH\textsuperscript{br} cells spiked into varying concentrations of cord blood mononuclear cells. Acceptance was scored by detecting between 4,000 and 6,000 ALDH\textsuperscript{br} cells in a background of 1-10 million contaminating cells. This was met at all levels of cell background with CVs ranging from 0.6-9.0\% (Figure 4). The largest variation (9.0\%) was seen in the samples with the highest background contamination, in which the ALDH\textsuperscript{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 0.6-1.3%. RBC contamination was low in these samples due to the use of Ficoll.

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

Since February of 2010, we performed the potency assay on segments from CBUs requested for CT in the CCBB. Segments were detached from the CBU in vapor phase, thawed and a small aliquot spotted onto an FTA card and sent to the NMDP 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 0.00-2.30% ALDH$^{br}$ cells within the CD45$^+$ viable population (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%) co-expressed CD34 (Table 2).

We examined the correlation of ALDH$^{br}$ cells to CFUs in the segments tested. ALDH$^{br}$ (as a percent of viable CD45$^+$ cells) correlated strongly with total CFU (per 10$^5$ cells) (Spearman r=0.78, 95% CI: 0.76-0.79; Figure 5A). However, the correlation between CD34$^+$ (as a percent 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 the correlation likely results from ALDH$^{br}$ and CFU needing metabolically active cells while CD34$^+$ is an antibody-based test. These correlations with CFUs were consistent regardless of how long the
cryopreserved CBUs were stored suggesting that potency of properly stored CBUs is stable over at least 15 years (Table 3).

Between July 2007 and August 2009, samples from fresh CBUs prior to cryopreservation were assayed for ALDH\textsuperscript{br}. As the CT testing was done, segments frozen with those CBUs were tested for ALDH\textsuperscript{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\textsuperscript{br} content. To determine the correlation of the potency assay between segments and bags and cryopreservation, research units from the CCBB were randomly selected, the entire unit thawed and assayed along with the distal segment that would normally be assayed in for CT (Figure 6B). While there was a strong overall correlation between segments and units (Spearman \(r=0.88\)), segments still tended to underestimate the ALDH\textsuperscript{br} content of the unit.

**ALDH\textsuperscript{br} Impact on Engraftment**

We asked whether ALDH\textsuperscript{br}, CD34 and CFU from the segment potency assay correlates with engraftment in a cohort of 78 patients transplanted at Duke with a single CBU. Ninety-four percent (73/78) of patients engrafted (18 malignant and 55 non-malignant diagnoses). The median time to engraftment was 16 days (range: 10-38 days). The association between segment-based ALDH\textsuperscript{br} and CFU in this subset of segments was similar to that observed in the entire sample of segments included in our study (\(r=0.70\), \(P < 0.001\)). We observed a trend towards faster engraftment with increased expression of ALDH\textsuperscript{br} (\(P=0.03\), Figure 7), although caution is warranted in
interpretation of this result due to small subgroup sizes. CFU and CD34 did not predict neutrophil engraftment in the entire cohort ($P > 0.05$ for all; data not shown).

**Discussion**

Our results demonstrate the successful development of a segment-based potency assay that meets ICH guidelines for Accuracy, Specificity, Precision, Repeatability, and Linearity. Furthermore, we have demonstrated through a prospective evaluation of almost 4,000 segments from cryopreserved CBU that the assay: (1) is highly correlated with CFUs, which have been previously shown to best predict neutrophil engraftment after UCBT; 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 transplant center after review of the pre-cryopreservation 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 post-thaw CBU characteristics or surrogates for these measures. We previously reported that post-thaw CFU content best correlated with engraftment for both neutrophils and platelets\(^12,24\). While post-thaw CFU content showed the strongest correlation with engraftment, it is not feasible to use this parameter for CBU selection since 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. In order 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\textsuperscript{25} which emulates CBU washing for transplantation. We demonstrated that the temperature during this step was critical and cells and DA needed to be at room temperature to avoid loss of detectable ALDH\textsuperscript{br} cells. This contrasts protocols applied to CBUs that requires washing the cells with ice cold DA.

In these studies, we also observed that ALDH\textsuperscript{br} content of the segments correlates well with the CFU content. This finding strongly suggests that measurement of ALDH\textsuperscript{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 possible greater impact on the segments. This means that the segment assay may disqualify some adequate potency units from being used. However, the segment assay is highly unlikely to identify low potency units as high potency units. As the potency assay data is available at the time of final donor selection, it can be used as a prospective potency assay for selecting CBUs. Our data suggest that CBUs rich in ALDH\textsuperscript{br} cells are predictive of engraftment but confirmatory studies are needed. This would be expected as this enzyme is characteristically highly expressed in the hematopoietic stem and progenitor cells from human umbilical cord blood\textsuperscript{15,26}, bone marrow\textsuperscript{27,28} and mobilized peripheral blood\textsuperscript{29,30}.

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

These studies demonstrated rapid, reliable measurement of ALDH\textsuperscript{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 four 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 cord blood units for transplantation.
Acknowledgments

This project was partially supported by Grant Number HHSH-234007002C 07-N234-5754 from Health Resources and Services Administration National Cord Blood Inventory and Grant Number K23HL104575 from the National Heart, Lung, And Blood Institute. Additional support was provided by the Robertson Foundation and the National Marrow Donor Program. The authors would to thank the staff of the Carolinas Cord Blood Bank and the Duke Stem Cell Laboratory for providing and testing cord blood units used in the experiments described in this manuscript.

Authorship Contributions

Kevin Shoulars took the lead role in adapting the ALDH$^{br}$ assay for use with segments during confirmatory typing, performed much of the assays and data analysis and wrote the first draft of the manuscript. Pamela Noldner performed many of the assays, helped with data analysis and editing of the manuscript. Jesse Troy provided statistical analysis of the data and advice for the validation. Lynn Cheatham and Amanda Parrish provided support in the design, analysis and interpretation of the data for the validation studies. Kristin Page contributed to the conception of the assay method, interpretation of the data for clinical use and editing of the manuscript. Tracy Gentry and Andrew Balber worked on developing the original ALDH$^{br}$ assay, contributed design of the CT assay and validation and assisted in drafting and editing the manuscript. Joanne Kurtzberg was instrumental in the conception, design and interpretation of the studies presented here and provided editing and critical revisions of the manuscript.
Conflict of Interest Disclosures

The authors declare no competing financial interests.

References

## Table 1: Statistics for segments used for Accuracy, Linearity and Repeatability.

<table>
<thead>
<tr>
<th>Expected Test Concentration (%)</th>
<th>Actual Segment #1 (%)</th>
<th>Actual Segment #2 (%)</th>
<th>Actual Segment #3 (%)</th>
<th>Mean</th>
<th>Standard Deviation</th>
<th>CV</th>
<th>Meets Specifications?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>0.052</td>
<td>0.054</td>
<td>0.049</td>
<td>0.051</td>
<td>0.002</td>
<td>3.3</td>
<td>Yes</td>
</tr>
<tr>
<td>0.10</td>
<td>0.108</td>
<td>0.108</td>
<td>0.105</td>
<td>0.106</td>
<td>0.001</td>
<td>1.2</td>
<td>Yes</td>
</tr>
<tr>
<td>0.50</td>
<td>0.524</td>
<td>0.535</td>
<td>0.481</td>
<td>0.513</td>
<td>0.023</td>
<td>4.5</td>
<td>Yes</td>
</tr>
<tr>
<td>1.00</td>
<td>0.974</td>
<td>1.101</td>
<td>1.133</td>
<td>1.069</td>
<td>0.069</td>
<td>6.4</td>
<td>Yes</td>
</tr>
<tr>
<td>3.00</td>
<td>2.692</td>
<td>3.084</td>
<td>2.596</td>
<td>2.791</td>
<td>0.211</td>
<td>7.5</td>
<td>Yes</td>
</tr>
</tbody>
</table>
### Table 2: Data obtained of cell subsets as determined by flow cytometric analysis of the potency assay and CFU.

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALDH&lt;sup&gt;br&lt;/sup&gt; as a percent of viable CD45&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.37%</td>
<td>0.00%</td>
<td>2.30%</td>
</tr>
<tr>
<td>CD34&lt;sup&gt;+&lt;/sup&gt; as a percent of viable CD45&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.66%</td>
<td>0.00%</td>
<td>3.49%</td>
</tr>
<tr>
<td>CD45&lt;sup&gt;+&lt;/sup&gt; viability</td>
<td>66.10%</td>
<td>8.72%</td>
<td>99.02%</td>
</tr>
<tr>
<td>CD34&lt;sup&gt;+&lt;/sup&gt; viability</td>
<td>82.90%</td>
<td>21.37%</td>
<td>100%</td>
</tr>
<tr>
<td>Total CFU (per 1X 10&lt;sup&gt;5&lt;/sup&gt; WBC)</td>
<td>46.25</td>
<td>0</td>
<td>530</td>
</tr>
<tr>
<td></td>
<td>All Samples (n=3908)</td>
<td>0 to 5 (n=3188)</td>
<td>5 to 10 (n=579)</td>
</tr>
<tr>
<td>--------------------------</td>
<td>----------------------</td>
<td>-----------------</td>
<td>----------------</td>
</tr>
<tr>
<td><strong>ALDH&lt;sup&gt;br&lt;/sup&gt; in Viable CD4&lt;sup&gt;5+&lt;/sup&gt; (%)</strong></td>
<td>0.78 (&lt;.0001)</td>
<td>0.78 (&lt;.0001)</td>
<td>0.78 (&lt;.0001)</td>
</tr>
<tr>
<td><strong>CD34&lt;sup&gt;+&lt;/sup&gt; in Viable CD4&lt;sup&gt;5+&lt;/sup&gt; (%)</strong></td>
<td>0.25 (&lt;.0001)</td>
<td>0.25 (&lt;.0001)</td>
<td>0.25 (&lt;.0001)</td>
</tr>
</tbody>
</table>

**Table 3: Relationship of ALDH<sup>br</sup> and vCD34<sup>+</sup> to CFU with time in storage.**
Figure Legends

Figure 1: Flow chart of the aldehyde dehydrogenase potency assay performed on attached segments of CBUs requested for confirmatory typing for donor selection.

Figure 2: Potency Assay Gating. (Panel A) Debris-free gate. (Panel B) Gating of the nonviable (7-AAD+) and RBC (PE-Cy5-Gly-A+) cells. (Panel C) Gating of the PE-CD45+ cells. (Panel D) Gating of APC-CD34+ cells. (Panel E) Gating of ALDHbr cells. (Panel F) Gating of cells stained with the Aldecount reagent in the presence of the DEAB inhibitor.

Figure 3: Intermediate Precision Assessment. Coefficient of Variation of segments from five CBU analyzed over 6 days using two operators (Op1 and Op2) and two Instruments (In1 and In2). The target specification was a CV ≤ 20%.

Figure 4: Validation-Specificity. 20,000 purified ALDHbr cells were spiked into varying amounts of CBU cells (1X10^6, 3X10^6, 5X10^6, 10X10^6), segments were created and cryopreserved. The segments were subsequently thawed and analyzed with potency assay with a target of collecting 5,000 ALDHbr cells. The target of 4-5,000 was met at all concentrations.

Figure 5: Comparison of cellular components of confirmatory typing segments. (Panel A) ALDHbr in Viable CD45+ vs. CFU. (Panel B) CD34+ in viable CD45+ vs. CFU. (Panel C) ALDHbr in Viable CD45+ vs. CD34+ in viable CD45+. N=3,908.

Figure 6: Comparison of confirmatory typing segments to fresh or thawed cord blood units. (Panel A) Samples taken from fresh CBUs prior to cryopreservation between July 2007 and August 2009 were assayed for ALDHbr and compared with the results of the CT testing of segments from those units. N=596. Red line indicates theoretical equality. (Panel B) Research CBUs stored at the CCBB were selected. The bags were thawed with the segments and tested for ALDHbr. N=60. Red line indicates theoretical equality.

Figure 7: ALDHbr Impact on Engraftment. Impact of ALDHbr measured on segments during CT on engraftment of the corresponding unit. Probability plots are shown for the units with an ALDHbr > 0.5% of viable CD45+ or units with an ALDHbr < 0.5% of viable CD45+ in reaching an absolute neutrophil count of 500 per µL. N=78
Figure 1

Spot 2-3 drops of blood on FTA card. Ship overnight to HLA typing lab.

Thaw segment and remove blood with a needle

Dilute with Dextran/Albumin

Wash with HSA/PBS

Centrifugation (2000 g X 30’)

Centrifugation (1200 g X 5’)

30 minute incubation at 37°C
with Aldecound Reagent
15 minute incubation on ice
for phenotyping

CD45, CD34 and Gly A
Antibodies

Activated Aldecound Reagent

T-AAD

Fluorescent Aldecound Reagent

Perform flow cytometry and analyze

ALDH^+ cells

CD45^+ cells

RBC and Nonviable cells for exclusion

CD34^+ cells
Figure 2
Figure 4

ALDH$^{br}$ Cells Detected

Total Cell Count prior to addition of 20,000 ALDH$^{br}$ cells

CV=1.3  CV=0.6  CV=6.0  CV=9.0
Figure 5
Figure 6

A.

N 596
Spearman Rho 0.43
95% CI 0.37-0.50
P-Value <.0001

B.

N 60
Spearman Rho 0.88
95% CI 0.82-0.92
P-Value <.0001
Figure 7
Development and validation of a rapid, aldehyde dehydrogenase bright-based, cord blood potency assay

Kevin W. Shoulars, Pamela Noldner, Jesse D. Troy, Lynn Cheatham, Amanda Parrish, Kristin Page, Tracy Gentry, Andrew E. Balber and Joanne Kurtzberg