

# A simple and reliable procedure for cord blood banking, processing, and freezing: St Louis and Ohio Cord Blood Bank experiences

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## Background

*In UC blood banking, volume and RBC reduction of the collected UC blood allows more efficient long-term storage and decreases infusion-related hemolysis and DMSO toxicity. However, high cell yields are imperative. At the St Louis Cord Blood Bank, we have systematically addressed processing/freezing and have developed a simple processing/freezing procedure.*

## Method

*The methodology is a modification of the betastarch sedimentation and volume reduction approach of Rubinstein at the New York Placental Blood Program. Cord blood is mixed with a 1:5 v/v ratio of betastarch. The product is incubated for 45 min in an inverted position in a refrigerated centrifuge (4°C), and then is spun for 5 min at 50 g. RBC concentrate is drained from the bottom. The volume drained is calculated to remove 80% of RBC. The UC blood unit is then resuspended and spun for 13 min at 420 g. Plasma is expressed from the top.*

## Results

*A final product volume of 27 mL (range 16–58 mL) was obtained from an original 50–200 mL of UC blood collected. The average yield of total nucleated cells pre- and post-processing was 90% for the first 4055 UC blood units banked. Pre- and post-processing CFU and CD34 yields were tested in a cohort and were similarly conserved. With a processing time of 3 h for a single cord, this process is time efficient and lends itself well to processing several units at the same time. The technique has been exported to other laboratories with similar yields.*

## Discussion

*This simple methodology results in reliable yields and is well suited to larger scale banking.*

## Keywords

*Cord blood banking, stem cell processing.*

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## Introduction

With the development of UC blood (UCB) as an alternative source of hematopoietic stem cells for use in unrelated transplantation, a need has developed for banks of characterized cord blood units [1–3]. The clinical benefits of such a resource are many, and include the availability of a stem-cell product at a time that is optimal for the patient, and the ability to transplant across HLA barriers.

With the early development of UCB banking it became apparent that there were several novel issues that were unique in both blood banking and stem-cell processing. Importantly, the processing and freezing procedure must be simple and result in minimal stem-cell loss during processing and freezing. Volume reduction and RBC depletion of the collected UCB would allow more efficient long-term storage and decrease the issues of infusion-related

hemolysis or DMSO toxicity. Given that many UCB units may be processed at a given time, the procedures common to hematopoietic stem-cell processing were suboptimal.

With the development of the St Louis Cord Blood Bank, these issues were systematically addressed and a processing/freezing method was developed. The methodology is based on the procedure developed by Dr Pablo Rubinstein and colleagues at the New York Placental Blood Program [4]. Here we present our methods and the results of processing for our first 4000 UCB units banked. This technique has been shared with several other laboratories. Presented here are the results obtained by a second bank, Ross Cord Blood Bank, American Red Cross, Central Ohio Region, demonstrating the exportability of this procedure.

## Methods

### UCB processing

UCB was obtained as part of the First Gift Program of the St Louis Cord Blood Bank. In this program regional obstetricians/midwives and obstetrical units collected cord blood during third stage labor from volunteer families [5]. The program operates under institutional IRB approval and FDA IND.

Units were eligible for banking if the collected blood volume was  $> 50$  mL and TNC (total nucleated cell count)  $> 600 \times 10^6$  (from 1/96 to 1/98), or TNC  $> 800 \times 10^6$  (from 1/98 to 3/00). UCB units were routinely in the freezer within 36 h of collection

Cord blood was collected in 250 mL CPD bags (Baxter). At start of processing, a sampling-site coupler was added to the bag. Through this coupler pre-processing, samples were removed for cell count, HLA typing, ABO testing and back-up samples (4 mL). If minimum criteria were met for banking (cell dose, completed consents, appropriate labeling), a 150 or 300 mL transfer pack was attached to the second port. Importantly, this bag remained attached to the original collection bag through processing, limiting transfer errors. The UCB is drained into the transfer pack, where processing occurs.

Hetastarch (Hespan) was added to the product at a 1:5 v/v ratio of hetastarch to total volume of UCB and anti-coagulant (the working volume). The hetastarch is added through the collection-bag port, to rinse that bag, and is then drained into the transfer pack. Rinsing the collection bag with the hetastarch solution minimized cell loss. The UCB unit was placed in a refrigerated centrifuge (4°C) in

the inverted position. After a 45 min incubation, it was spun for 5 min at 50 g. It was important to support the unit vertically within the centrifuge bucket. This improved the interface and decreased cell loss. The sedimented RBC were then drained slowly by gravity. The amount of RBC concentrate to drain was calculated assuming that the sedimented RBC mass was 100% RBC. Weight of RBC by-product drained = UCB hematocrit  $\times$  UCB volume. This reliably removed 80% of RBC. The by-product RBC were drained back into the original collection bag, using a pan scale to monitor the amount drained. No attempt was made to standardize the final product volume.

Using a semi-quantitative calculation that incorporates the original UCB hematocrit and volume results in the ability to reliably remove a majority of the RBC without loss of the WBC component. The calculation is meant to supplement the need to observe the interface as RBC are being drained. We have found that the calculation makes the procedure much more readily exportable between laboratories and individual processors.

The UCB unit was then resuspended and centrifuged for 13 min at 420 g in the upright position. It was important to provide vertical support for the bag during centrifugation. Plasma was extracted from the top, using a plasma expressor, back into the original collection bag. It was important to observe the buffy coat carefully, avoiding disruption. The RBC- and plasma-depleted product was drawn into a syringe. Based on volume of the UCB, the required DMSO (Tera Pharmaceuticals) was calculated, to give a final DMSO concentration of 10%. Importantly, the original collection bag (with original labels) was attached to the product through processing and at this stage contains material that may be used for product testing (bacterial cultures, DNA-based testing).

At the completion of processing, UCB was tested for bacterial contamination and hematopoietic cell content (CFU and CD34). UCB product was infused into the final freezing bag. After the gradual addition of cold DMSO over 15–30 s, the product was ready for control-rate freezing.

### TNC measurement

Three automated hematology analyzers were used: the Coulter T890 (from 1/96 to 7/98), the Sysmex SE-9500 (from 7/98 to 2/99 and the Sysmex XE 2100 (from 2/99 to present). TNC was calculated by multiplying the

WBC ( $10^6/\text{mL}$ ) by the volume (mL). Absolute nucleated RBC counts were assessed using the SYSMEX XE 2100 and calculated by multiplying the nucleated RBC ( $10^6/\text{mL}$ ) by the product volume (mL).

### CD34<sup>+</sup> enumeration

UCB nucleated cells were double labeled with the anti-CD34 (HPCA-2) MoAb directly conjugated with PE (Immunotech) and anti-CD45 (Hle-1) MAb directly conjugated with FITC (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Isotype PE and FITC control were performed in parallel. Flow cytometric analysis was performed using the Cytoronabsolute (Ortho Diagnostics). Twenty thousand events were analyzed for each assay.

### CFU assays

Freshly isolated nucleated cells from the post-processed UCB were assessed in duplicate using a semi-solid clonogenic assay. The quantity  $2 \times 10^5$  cells was inoculated into 3.0 mL of Iscove's methylcellulose media (Methocult H4434, Stem Cell Technology Inc., Vancouver, Canada) and equally plated into two wells (6-well, Falcon). They were incubated at 37°C in 5% CO<sub>2</sub> humidified incubator. At 14 days, CFU-GM, CFU-GEMM and BFU-E were counted using an inverted microscope. The total CFU count was reported as total colonies per component.

### Statistics

Descriptive statistics were used to compare the percentage recovery of TNC, CD34 and CFU levels obtained at time of collection, post-processing and post-thaw for UCB. All statistical analyses were assessed with software from the Statistical Package for the Social Sciences (SPSS, Chicago).

## Results

### High yields of nucleated cells, CD34<sup>+</sup> cells and colony-forming units

As part of validation of this processing technique, pre- and post-processing samples were assayed for yields of TNC, CD34<sup>+</sup> cells and colony-forming units (CFUs). The median yields were 87%, 97% and 84% for TNC, CD34 and CFU respectively (Tables 1 and 2).

### A majority of nucleated RBC are saved with the UCB unit

We noted a variable number of nucleated RBCs in UCB units being banked (range 0.4–44% of pre-processing cell count) (Table 2). These nucleated RBC were included in the TNC. We evaluated nucleated RBC pre- and post-processing, and found that 69% of nucleated cells were collected with the final product — leaving a variable contribution to the final product total nucleated cells (TNC) (0.2 to 33%).

### Yields were consistent through routine UCB banking for 4 years

For the next 4055 UCB units processed between May 1996 and March 2000, TNC yields pre- and post-processing were followed. A mean of 90%  $\pm$  5.6% cell yield was obtained (Table 3).

### Processing was flexible for variable numbers of products processed daily

Between one and 12 UCB units were processed daily by a single technologist. This processing approach was amenable to the processing of multiple UCB units contemporaneously. In general, processing (from start of sampling to the start of freezing) was complete in 3 h for one cord blood unit and 8 h for 12 units.

**Table 1.** Yields pre- and post-processing for TNC, CD34, and nucleated RBC<sup>1</sup>

	TNC ( $\times 10^6$ cells)		CD34 ( $\times 10^6$ CD34 <sup>+</sup> cells)		Nucleated RBC ( $\times 10^6$ cells)	
	pre	post	pre	post	pre	post
Median	1116	946	4.4	4.4	121	67
Min.	684	660	1.0	1.0	21	17
Max.	2400	2278	19.3	15.8	362	217
Recovery (%)		87		97		69

<sup>1</sup> 130 paired samples.

**Table 2.** Yields pre- and post-processing for colony forming units (CFU)<sup>1</sup>

	Pre	Post
Median	9.8	8.3
Minimum	2.7	3.9
Maximum	109.4	73.6
Recovery (%)		84

<sup>1</sup> 41 paired samples were evaluated.

### Validation of processing approach

This UCB processing approach was shared with the team at Ross Cord Blood Bank, American Red Cross, Central Ohio Region. TNC recovery increased with use of this processing, from 58.5% to 92.1% for cords processed. The important modifications to conventional hetastarch depletion were 45 min incubation refrigerated in the inverted position, and the use of a hematocrit to estimate the RBC mass that could be drained off.

### Yields of progenitors are maintained when products are thawed for transplantation

Twenty-five UCB units from the time period evaluated for this study have been used in clinical transplantation at Cardinal Glennon Children's Hospital. Yields of nucleated cells (TNC) and CFUs from the completion of freezing to post-thaw are shown in Table 4. A median of 80% of frozen cells survive the thaw. Of note, UCB neutrophils, which were included in the frozen product, would not be expected to survive the freeze/thaw. Yields of CFUs were in the range of 75%. There was no difference in thaw characteristics between the CBUs processed

at the St Louis Cord Blood Bank and compared with 12 UCB units obtained from other banks and used in clinical transplantation (data not shown). For the 25 units used in transplantation at Cardinal Glennon Children's Hospital the mean time to recovery of an absolute neutrophil count > 500/ $\mu$ L was  $18 \pm 7$  days and platelet count >  $20 \times 10^9$ /L was  $60 \pm 28$  days.

### Discussion

Given that UCB units are being banked today for use many years in the future, it is critical that the highest of standards be applied to them. In addition to extensive infectious and genetic disease screening, UCB processing must be performed in a fashion that maximizes the preservation of hematopoietic stem cells, allows needed product testing, and is amenable to production of multiple products at the same time. Unique to UCB, when compared with other blood or hematopoietic stem-cell component manipulation, is that cell number is limiting, and it is critical to develop processes that preserve the hematopoietic potential of the product.

While many approaches to UCB processing have been explored, including freezing unmanipulated, gelatin separation and centrifugation [6–8], many laboratories are currently utilizing hetastarch sedimentation followed by volume reduction [9]. This technique, developed by Dr Rubinstein, works well because it is simple, takes a short time to perform, and fits well with stem-cell processing laboratory practice [4]. The modifications presented here have resulted in improved cell yield.

This processing has worked well for our banks, with multiple technologists, and now, for > 10 other UCB banks worldwide. Since it combines basic blood banking with established stem-cell banking skills, this processing

**Table 3.** Yield of TNC with processing (n = 4055)

	TNC ( $\times 10^6$ cells)		TNC (% recovery)	Volume (mL)		
	pre-	post-		pre-	post-	% reduction
Mean	1276	1141	90	109	28	74
Median	1155	1044	90	105	27	74
SD	479	399	5.6	23	5	4
Min.	403	295	55	56	16	43
Max.	5960	4700	100	246	62	85

<sup>1</sup> Yields obtained on routine UCB units consecutively processed from May 1996 to March 2000.

**Table 4.** Yield of TNC and CFU after thawing of the UCB units

	TNC ( $\times 10^6$ )		TNC	Viability	CFU ( $\times 10^5$ )		CFU
	pre-thaw	post-thaw	recovery (%)	(%)	pre-thaw	post-thaw	recovery (%)
Mean	1397	1132	81	88	10.1	7.2	76
SD	566	484	7	10	4.4	3.8	21
Median	1293	1104	79	92	10.2	6.5	76
Min.	528	396	67	60	1.7	2.8	32
Max.	2507	2161	97	96	18.9	15.0	92
n	25				11		

has been readily taught and applied. Importantly, UCB product yields are reliably high and there is minimal product manipulation — thus minimizing the time from start of processing to freezing.

We noted variable and often significant numbers of nucleated RBC in the UCB products prior to processing. One-third of these cells are lost in processing, contributing to the lower post-processing TNC. However, the remaining cells in the product, up to 33% of the product, are included in the TNC, which is used to estimate hematopoietic potential in the product selection process. Since these cells do not themselves contribute to hematopoiesis, it is important to include nucleated RBC contribution in the description of UCB units being considered for transplantation.

Central to UCB processing is the avoidance of labeling and product transfer errors. This processing procedure occurs with the original collection bag attached to the UCB product through to the final transfer into the freezing bags, minimizing opportunities for product misidentification.

We have evaluated the freezing procedure and have found no difference when the DMSO is added slowly over 15–30 s, compared with an infusion over 10–15 min. However, because the DMSO reaction is exothermic, it is important that all reagents are cold prior to addition.

UCB banking has opened up new opportunities and issues for the stem-cell laboratory. It is critical, with the rapid development of UCB transplantation, including stem-cell expansion technologies, that the banked products meet the highest of standards that can be attained. As new technologies are developed, it is important that that they are tested using engraftment and hematopoietic cell viability assays post-thaw as quality control endpoints.

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