

feature article

Improved *Ex Vivo* Expansion of Functional CD34⁺ Cells Using Stemline™ II Hematopoietic Stem Cell Expansion Medium

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Introduction

Hematopoietic stem cells (HSC) have the ability to repopulate the hematopoietic system by differentiating into all of the necessary erythroid, lymphoid, and myeloid lineages. Due to this rare ability, HSCs are used as therapeutic agents in the treatment of malignant and benign diseases of the blood forming and immune systems. There have been many advances in the area of clinical HSC research, but the availability of suitable cells for transplantation still remains a major limiting factor.^{1,2}

HSCs can be isolated from three different sources: umbilical cord blood (CB), bone marrow, and mobilized peripheral blood. CB is currently the preferred source because it has been shown to have a lower risk of graft versus host disease (GVHD), presumably due to its immunological naïveté. However, because the volume of CB is limited, each umbilical cord generally has only enough cells to successfully transplant a small child. In order to transplant an adult, the HSCs from CB typically must be expanded *ex vivo*. It is critical that the expansion be performed in a manner to ensure that the HSCs not only differentiate along appropriate hematopoietic lineages, but also self-renew, leaving undifferentiated stem cells in the expanded culture. The differentiated cells will allow for short-term engraftment that will reduce the effects of neutropenia and thrombocytopenia in the patient. The undifferentiated cells will allow for long-term engraftment that will establish a new, permanent hematopoietic system for the patient. In order to expand these very specific cell types in the absence of potentially adventitious agents, which may be present in typical medium components, such as fetal bovine serum, an optimized serum-free medium is needed.

With these parameters in mind, we have developed Stemline™ II Hematopoietic Stem Cell Expansion Medium

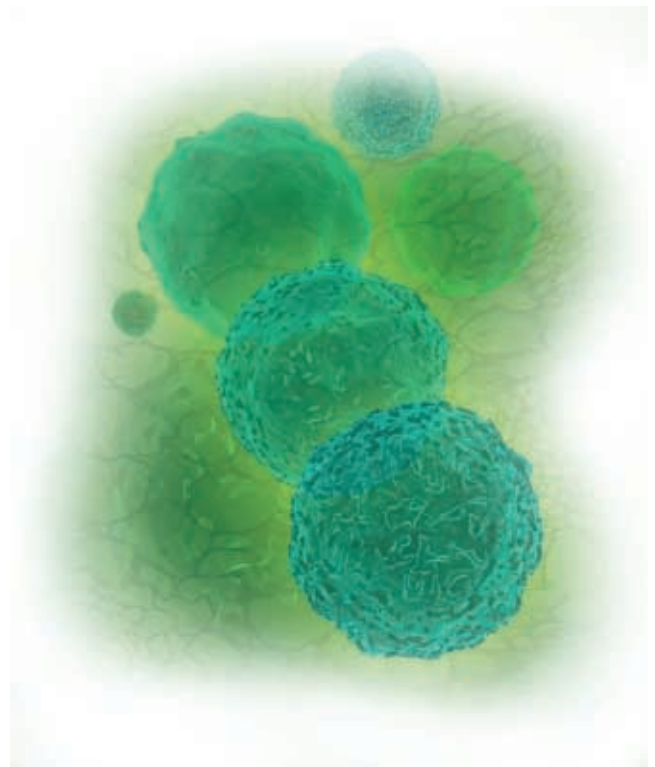
(Product Code [S 0192](#)). This formulation has led to a significant increase in the number of cells expanded from cord blood CD34⁺ cells, as well as CD34⁺ cells from mobilized peripheral blood and bone marrow. Flow cytometry shows the surface antigen profiles to be consistent with previously reported profiles for *ex vivo* expanded cells. The expanded cells form all of the appropriate hematopoietic lineages in a colony-forming unit assay (CFU) and demonstrate long-term engraftment (primary and secondary recipient) in NOD/SCID mouse models. This serves as an indication that the expanded cells are representative of all of the proper lineages required for a successful transplant. Clinical studies are currently being designed to evaluate the engraftment potential of cells expanded in Stemline II Hematopoietic Stem Cell Expansion Medium.

Materials and methods

All materials were supplied by Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated.

Cell preparation

For all experiments, cryopreserved, human CD34⁺ cells were obtained from independent suppliers (Stemgenix, Amherst, NY; AllCells, LLC, Berkeley, CA) and were handled in a manner consistent with the manufacturers' instructions with regard to storage and reconstitution. Cells were counted using either a hemocytometer or Guava Personal Cytometer (Guava Technologies, Hayward, CA) to determine cell density and viability.



Serum-free expansion medium preparation and bench-scale expansion

Stemline Hematopoietic Stem Cell Expansion Media, IMDM, X-VIVO 15 (Cambrex, Walkersville, MD), HPGM (Cambrex, Walkersville, MD), QBSF-60 (Quality Biological, Gaithersburg, MD), StemPro-34 (Invitrogen, Carlsbad, CA), and StemSpan H3000 (StemCell Technologies, Vancouver, BC) were purchased fresh, aliquoted and stored according to the manufacturers' recommendations. For each experiment, a 10-ml volume of each expansion medium was warmed to 25 °C. One ml of each medium was pipetted in triplicate in 24-well culture plates (Corning/Costar, Corning, NY) to which thrombopoietin (TPO, Product Code [T 1568](#)), stem cell factor (SCF, Product Code [S 7901](#)), and granulocyte colony-stimulating factor (G-CSF, Product Code [G 0407](#)) were added to a final concentration of 100 ng/ml each. Sterile PBS was added to unused wells to maintain humidity. Plates were incubated at 37 °C and 5% CO₂ for 15 minutes prior to the addition of the revived CD34⁺ cells. Viable recovered CD34⁺ cells were added to each well at 1.0 x 10⁴ cells/ml and allowed to proliferate in a humidified incubator at 37 °C and 5% CO₂ for 10 days. Following the incubation period, the expanded total nucleated cells were counted.

Flow cytometry

The direct determination of the absolute count of CD38⁺ and CD38⁻ cells was assessed utilizing the Immunotech Stem Kit CD34⁺ Hematopoietic Progenitor Cell (HPC) Enumeration Kit (Beckman-Coulter, Fullerton, CA), CD38-PE, CD34-ECD, and CD45-FITC Antibodies. The processed samples were identified and enumerated using Beckman-Coulter's flow cytometer (EPICS XL-MCL™).

Clinical-scale expansion

A 2-step, clinical-scale assay³ using Teflon® culture bags (American Fluoroseal, Inc., Gaithersburg, MD) was set up for a comparison study between Stemline Medium (Product Code [S 0189](#)) and Stemline II Medium. For clinical-scale studies, CB CD34⁺ cells were cultured for 7 days in 100-ml Teflon culture bags containing 50 ml of each culture medium plus cytokine concentrations as previously described. Cells were harvested from these bags and a 10-ml aliquot was transferred to a second 100-ml Teflon bag containing 90 ml of each selected medium plus cytokines and cultured for an additional 7-day culture period. At the end of the culture protocol, cells were harvested, counted by hemocytometer, viability tested, and assayed for functional hematopoietic activity *in vivo* and *in vitro*.

NOD/SCID studies

Immunodeficient NOD/SCID mice were used as recipients of transplanted human cord blood cells expanded at clinical-scale as previously described. NOD/SCID mice were

lightly irradiated (3.0 Gy) 3-6 hours prior to infusion of human cells by injection intravenously into the retro-orbital plexus.⁴ The mice, prior to and throughout the treatment period, were maintained under pathogen barrier conditions and their drinking water was supplemented with antibiotics. Depending on the experiment, between 500,000 and 6 million cells were transplanted. At various times after transplant, blood was obtained from each recipient by retro-orbital blood sampling. Human cells were distinguished by immunofluorescence and flow cytometry using an antibody to CD45 (Becton-Dickinson, Franklin Lakes, NJ). Presence of CD34 was used to distinguish progenitor cells and representation of human cells in the lymphocyte lineage was determined using CD19 and CD20; human myeloid cells were identified by CD15 and CD66b. The same panel of antibodies was used in the analysis of bone marrow at the termination of the experiment, or in preparation for injection into secondary recipients to verify the presence of self-renewing human stem cells in the primary recipients.

Results and discussion

To test the ability of Stemline II Hematopoietic Stem Cell Expansion Medium to expand CD34⁺ hematopoietic stem cells, we designed a bench-scale expansion assay. Cells were seeded into the wells of 24-well tissue culture plates. One milliliter of medium was added to each well with the appropriate cytokines to stimulate growth (100 ng/ml each of TPO, SCF, and G-CSF). Each condition was performed in triplicate and seeded with 10,000 cells per ml in each well. This was the standard assay used to evaluate the expansion of CD34⁺ cells from cord blood, bone marrow, or mobilized peripheral blood.

Due to the clinical importance and the donor-to-donor variability typical to the expansion of umbilical cord blood-derived cells, we elected to test 15 donors for expansion and surface antigen expression. The cells were counted on day 10 and the fold increase was determined by $\frac{\text{cells}_{\text{final}}}{\text{cells}_{\text{initial}}}$. In cord blood, Stemline and Stemline II outperformed the other serum-free HSC media (Figure 1A). While Stemline already performed better than or equal to the other HSC media, Stemline II exhibited a significant increase in expansion compared with the other products ($p < 0.00001$). Figure 1B represents the fold increase for each medium in all 15 donors. This also shows that Stemline II consistently provides the maximum number of total nucleated cells (TNC). The third graph in this series shows the percentage of CD34⁺ cells/ μl expanded from the initial CD34⁺ cord blood cells, normalized to the number expanded in Stemline (Figure 1C). This represents an average \pm S.E.M. for 3 donors. This data is further broken down into CD38⁻ and CD38⁺ progenitors as an indication of their degree of differentiation.

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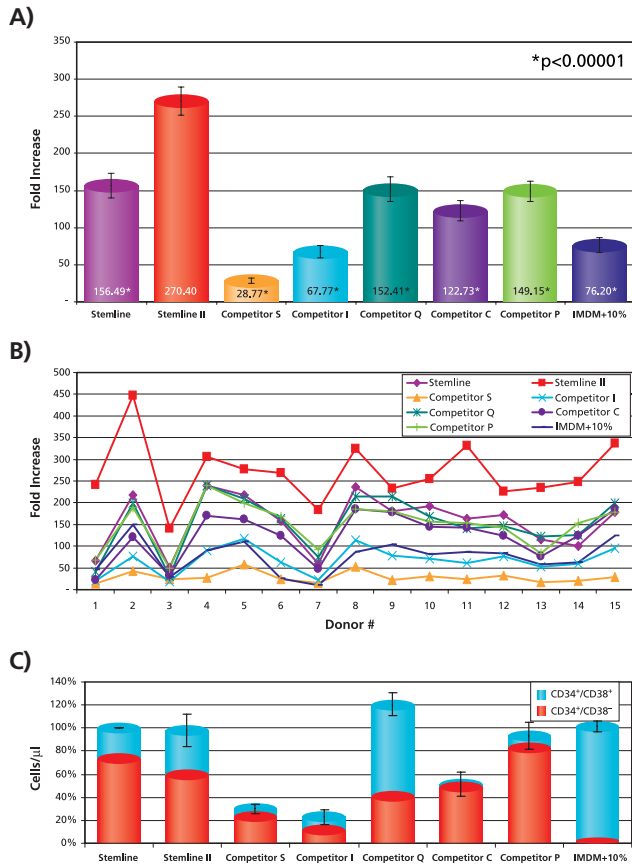


Figure 1. Expansion of CD34⁺ cells from cord blood in Stemline™ II compared to other commercially available, serum-free, HSC media. A. In cells purified from cord blood, Stemline™ II outperforms the other serum-free HSC media, exhibiting a significant increase in expansion ($p < 0.00001$). B. The second graph depicts the fold increase for each medium in all 15 donors. This also shows that Stemline II consistently provides the maximum number of TNC. C. The third graph in this series shows the percentage of CD34⁺ cells/µl expanded from the initial CD34⁺ cord blood cells, normalized to the number expanded in Stemline™. This represents an average \pm S.E.M. for 3 donors. This data is further broken down into CD38⁺ and CD38⁻ progenitors as an indication of their degree of differentiation.

Using the same bench-scale assays, counted on day 14, for the expansion of bone marrow CD34⁺ cells, Stemline performed as well as, or better than, the other competitors (Figure 2A). However, Stemline II was vastly superior to the other commercially available serum-free HSC media, giving approximately 5-fold more total nucleated cells ($n = 5$ donors; $p < 0.05$).

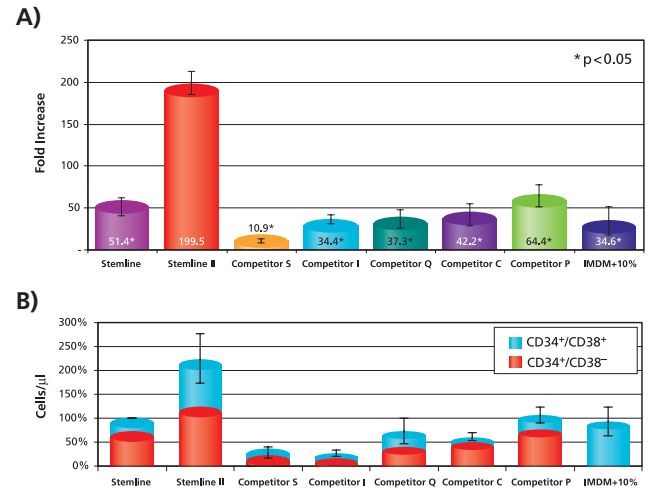


Figure 2. Stemline™ II bench-scale expansion of CD34⁺ cells from bone marrow compared to other HSC media. A. For the expansion of bone marrow CD34⁺ cells, Stemline™ II was vastly superior to the other commercially available serum-free HSC media ($n = 5$ donors; $p < 0.05$). B. Flow cytometry on the expanded cells reveals that Stemline™ II also expanded a greater number of CD34⁺ stem cells (both CD38⁺ and CD38⁻). Cells/µl were normalized to the average number of cells/µl in Stemline™ \pm S.E.M. ($n = 3$ bone marrow donors).

Counted on day 14, cells derived from mobilized peripheral blood also consistently exhibited high levels of expansion of total nucleated cells when expanded in both Stemline products ($n = 7$; $p < 0.05$; Figure 3A). Flow cytometry on the expanded cells reveals that Stemline II also expanded a large number of CD34⁺ stem cells (Figure 3B; both CD38⁺ and CD38⁻). Cells/µl were normalized to the average number of cells/µl in Stemline \pm S.E.M. ($n = 2$ mobilized peripheral blood donors).

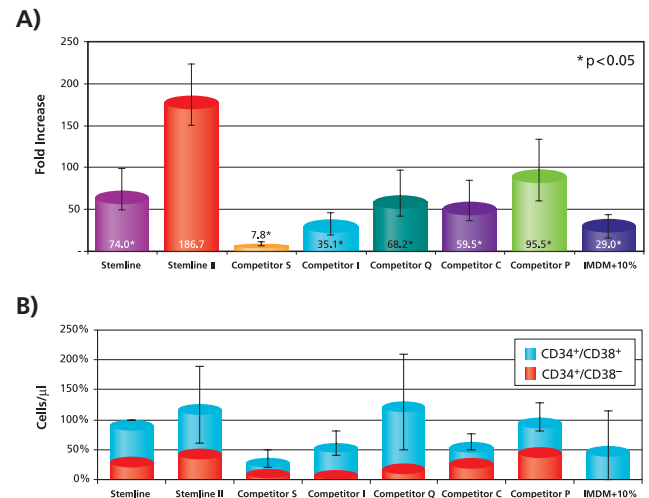


Figure 3. Bench-scale expansion of CD34⁺ cells from mobilized peripheral blood in Stemline™ II. A. In mobilized peripheral blood, both Stemline™ products consistently exhibit high levels of expansion of total nucleated cells ($n = 7$; $p < 0.05$). B. Flow cytometry on the expanded cells revealed that Stemline™ II also expands a large number of CD34⁺ stem cells (both CD38⁺ and CD38⁻). Cells/µl were normalized to the average number of cells/µl in Stemline™ \pm S.E.M. ($n = 2$ mobilized peripheral blood donors).

Overall, Stemline II Hematopoietic Stem Cell Expansion Medium provides for increased expansion of total nucleated cells from all three major cell sources. It also provides increased numbers of both early and late progenitors, as determined by the presence of CD38 surface antigens. These expansions also demonstrated another benefit of Stemline II Hematopoietic Stem Cell Expansion Medium. We often see donors, from all 3 sources, that do not generate enough cells to use for transplant. In these donors, Stemline II Hematopoietic Stem Cell Expansion Medium seems to rescue the proliferative capacity of the cells, in many cases allowing up to ten times more expansion of TNC than any of the other commercially available products (data not shown).

In order to prove that expansion of these cells was reproducible in a more clinically relevant format and that the cells retained their engraftment potential, we replicated a clinical-scale experiment to test both Stemline products. A two-step clinical-scale expansion was performed to compare cell growth in Stemline and Stemline II. Briefly, the cells were seeded into 100-ml bags and incubated for 7 days. On day 7, a portion of the expanded cells was inoculated into a fresh 100-ml bag for an additional 7 days. Both media demonstrated increased potential for expanding TNC from cord blood (Figure 4), supporting excellent growth and high viability (>80%). The expanded cells were analyzed by flow cytometry for expression of CD34 and CD38. The majority of the CD34⁺ cells expanded in Stemline remained undifferentiated, early progenitors (CD34⁺/CD38⁻), while cells expanded in Stemline II contained both early (CD34⁺/CD38⁻) and late progenitor (CD34⁺/CD38⁺) phenotypes (Figure 5). Both media expand high levels of early progenitors, which is important for long-term engraftment. Stemline II also expands high levels of the late progenitors required for early engraftment and amelioration of the post-transplant nadir in mature myeloid cells.

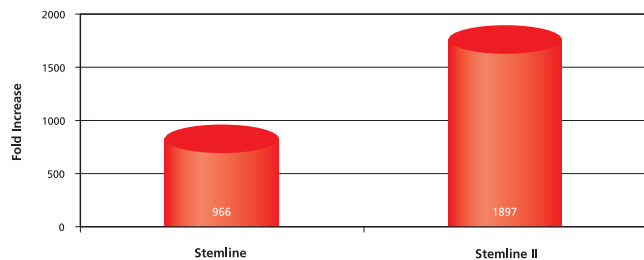


Figure 4. Comparison of Stemline™ II to Stemline™ in a clinical-scale expansion of CD34⁺ cells from cord blood. A two-step clinical-scale expansion was performed to compare cell growth in Stemline™ and Stemline™ II. Both media demonstrate increased potential for expanding CD34⁺ cells from cord blood, supporting excellent growth and high viability (>80%).

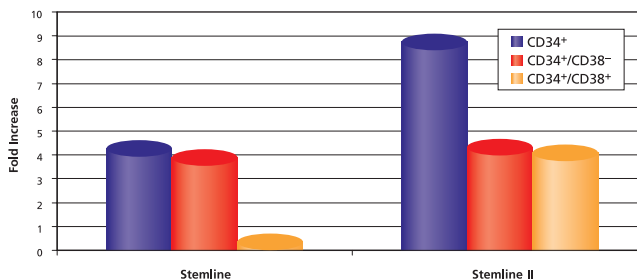


Figure 5. Flow cytometric analysis on CD34⁺ cord blood cells from clinical-scale expansion. The majority of the CD34⁺ cells expanded in Stemline™ remained undifferentiated, early progenitors (CD34⁺/CD38⁻), while cells expanded in Stemline™ II contained both early (CD34⁺/CD38⁻) and late progenitor (CD34⁺/CD38⁺) phenotypes.

After the two-step expansions were complete, a sample of cells from the Stemline and Stemline II media cultures were prepared for transplantation into NOD/SCID mice. Three different doses of cells were chosen for injection into the mice. A high percentage of the mice survived transplantation with cells expanded from both media (higher with Stemline II), all of which contained CD45⁺ human cells as proof of engraftment (a smaller number of which were also CD34⁺; Table 1). Both media expanded enough functional, early progenitors to achieve long-term engraftment. The higher survival rate in Stemline II may be explained by the higher levels of the late progenitors required for early engraftment and amelioration of the post-transplant nadir in mature myeloid cells. Further studies with secondary recipients are in process, with survival in excess of 9 weeks.

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Table 1. Summary data from the transplantation of expanded cells in the NOD/SCID mouse model.

Injected Cells	Stemline			Stemline II		
	600,000	1,800,000	5,400,000	600,000	1,800,000	5,400,000
Survival Rate	5/10 50%	3/10 30%	3/7 43%	7/10 70%	6/10 60%	6/7 86%
Average % CD45 ⁺	0.064 ± 0.061	0.017 ± 0.006	0.143 ± 0.081	0.036 ± 0.013	0.018 ± 0.019	0.108 ± 0.162
Average % CD45 ⁺ /CD34 ⁺	0.000 ± 0.000	0.003 ± 0.006	0.007 ± 0.006	0.011 ± 0.009	0.002 ± 0.004	0.010 ± 0.000

Both media expanded enough functional, early progenitors to achieve long-term engraftment. Further studies with secondary recipients are in process, with survival in excess of 9 weeks.

Summary

In bench-scale expansions, Stemline and Stemline II media are capable of expanding CD34⁺ cells from umbilical cord blood, adult bone marrow, and mobilized peripheral blood. Both Stemline media expand CD34⁺ cells from all three sources better than other serum-free commercially available media. In clinical-scale expansions, both Stemline media were able to expand CD34⁺ cells from cord blood. Flow cytometric analysis of the clinical-scale expansions revealed that Stemline and Stemline II expanded comparable numbers of early progenitor cells (CD34⁺/CD38⁻). Stemline II also demonstrated the additional benefit of a higher capacity for the expansion of the CD34⁺/CD38⁺ late progenitors required for short-term engraftment. Finally, cells expanded in both Stemline and Stemline II were capable of repopulating NOD/SCID mice using serial passage, demonstrating self-renewal of expanded cells, a critical functional test. Overall, the increased expansion along with the ability to produce the medium in a state-of-the-art cGMP facility makes Stemline II the best choice for clinicians seeking a serum-free product for their clinical hematopoietic stem cell applications.

Acknowledgements

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References

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Ordering Information

Product	Description	Unit
S 0192	Stemline™ II Hematopoietic Stem Cell Expansion Medium	500 ml 6 x 500 ml
S 0189	Stemline Hematopoietic Stem Cell Expansion Medium	500 ml 6 x 500 ml
T 1568	Thrombopoietin	5 µg
S 7901	Stem Cell Factor	10 µg
G 0407	Granulocyte Colony-Stimulating Factor from Human	5 µg