

# Isolation and *Ex Vivo* Expansion of Human Hematopoietic Stem Cells Derived from Umbilical Cord Blood

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## Abstract

Umbilical cord blood (UCB) is a current major source of hematopoietic stem cells (HSCs) for cell transplantation therapy. Cell transplantation with HSCs derived from UCB is advantageous over transplantation with HSCs from adult tissues. However, the low number of HSC derived from a single unit of UCB limits its application. Thus, *ex vivo* expansion is a good option to create more UCB HSCs for clinical application. The strategies for HSC expansion *in vitro* focus on mimicking the composition and structure of HSC natural niche by enhancing self-renewal and inhibiting lineage differentiation of HSCs. In the past decade, the mechanisms of the interaction between HSC and the natural niche have been deeply investigated. This great progress in basic research has led to advancements in UCB HSC *ex vivo* expansion. In addition, the biological characteristics of the originally isolated UCB HSCs correlate with outcome of subsequent *ex vivo* expansion. In this paper, we summarize the late progress achieved in isolation and *ex vivo* expansion of UCB HSCs. Importantly, we attempt to provide an impact and practicable procedure to expand UCB HSC *in vitro* from isolation of original HSCs to identification of expanded HSCs.

## Keywords

Hematopoietic Stem Cells, Isolation, *Ex Vivo* Expansion

## 1. Introduction

Transplantation of hematopoietic stem cells is an important curative therapy for hematologic malignancies and nonmalignant hematologic disorders [1]. UCB has been established as a major source of HSCs for cell transplantation-based therapies [2] [3]. Compared to HSCs from adult donors, UCB HSCs are more advantageous as they have: less stringent HLA matching requirements, lower risk of graft-versus-host disease (GVHD) [4], lower donor-recipient viral transmission risk, lower relapse rate, and higher survival rate. However, transplantation using HSCs derived from UCB has only been applied in pediatric patients due to the limited number of HSC obtained from a single unit of UCB [5] [6]. Attempts to expand UCB HSC in order to enable its applicability in all patients are still continuing.

A comprehensive procedure for UCB HSC expansion includes: UCB collection, HSC isolation, HSC *ex vivo* expansion and character identification of expanded HSC. The biological characteristics of the obtained HSCs are critical in determining the outcome characteristics of the *ex vivo* expanded HSCs [7] [8]. UCB collection and HSC isolation procedure affect the biological activity of unexpanded HSCs. UCB collection was suggested to carry out on delivery when the placenta is still unexpelled, the uterine paroxysmal contraction pressure increases volume of the collected cord blood [9]. The intermittent and gentle shaking of the blood bag during the collection process can prevent the formation of blood clots [9] [10]. Cyclosporine A (CSA), an inhibitor of reactive oxygen species (ROS), is used to maintain renewal property and inhibit lineage differentiation of the HSCs by ameliorating oxygen shock/stress (EPHOSS) [1].

Human HSCs have the capacity of massive self-renewal division *in vivo* [11] [12]. The robust *in vivo* expansion of HSC was demonstrated by HSC transplantation. However, HSC *ex vivo* expansion still encounters many challenges. HSC expansion *in vivo* is strictly controlled by HSC niche [11] [13]. The strategies for HSC *ex vivo* expansion aimed to mimic HSC niche *in vivo* [14] [15]. But the knowledge about composition and structure of HSC niche *in vivo* remains limited. The cell growth factors and cell development factors used in HSC *ex vivo* expansion were successfully identified from HSC niche cells [8] [16] [17] [18]. However, the cocktail of those protein factors was proclaimed to not support HSC self-renewal *ex vivo* [19]. This conclusion indicated that HSC self-renewal not only depends on the growth and development factors but also depends on the clues out of the protein factors. In the past ten years, chemical library screening based on HSC biological characteristics has identified some useful chemical compounds in HSC *ex vivo* expansion [20]. Among of those chemicals, SR-1 and UM171 have great potential in maintaining biological characters of expanded HSCs through their synergistic effects with the growth factor panel (SCF, TPO, Flt3-ligand) [19] [20]. The optimized recipe with combination of the two chemicals and the panel of growth factors was shown to expand HSC more than 30 folds in effective engraftment [20]. The expanded HSCs by SR-1 and

UM171 have entered clinical trials [21] [22]. For energy metabolism, HSCs prefer glycolysis over oxidative phosphorylation (OXPH) [23]. A low concentration of calcium was demonstrated to enhance HSC expansion in vitro through maintaining glycolysis in HSCs [24]. Growth factors and chemicals are mostly applied in HSC expansion procedures. Mathematical models were benefit for giving a meaning to guide the optimization of composition and concentration of the growth factors and chemicals. Here we summarize the updated procedures applied in the isolation and *ex vivo* expansion of UCB HSCs.

## 2. Methods

### 2.1. Isolation of Cord Blood-Derived Hematopoietic Stem Cells

#### 2.1.1. Collection of the Umbilical Cord Blood

##### **Materials**

70% Alcohol;

Iodine;

Disposable blood collection bag (200 mL, NIGALE);

Ice box (FYL-BW-11L, FuYi electric).

##### **Procedure**

An informed consent was sought from patients before procedure commencement.

1) Record obstetric and family medical history, exclude etiology and genetic anomalies.

2) Cut the cord while the placenta is still in utero upon delivery of the term baby. Use 70% alcohol and iodine to sterilize the umbilical cord, puncture the umbilical vein, then collect UCB by the intermittent contraction of the uterus [9]. Allow continuous gentle shaking of the blood collection bag until no more blood flows out of the cord. Close the blood collection bag and place it in the icebox.

3) Collect at least 60 mls of the cord blood. This contains more than  $10^8$  mononuclear cells [10]. Do not allow blood clots to form. Transport the cord blood in icebox immediately to the laboratory. HSC isolation should start within 2 hours.

#### 2.1.2. Isolation of Human CD34 Positive Cell

##### **Materials**

1) Phosphate buffered saline pH7.2 (PBS) (SH30256.01, HyClone).

2) IMDM Modified (SH30228.01, HyClone).

3) CSA (1101, R&D).

4) Ficoll-Paque PLUS (17-1440-02, GE).

5) EasySep Human Cord Blood CD34 positive selection kit II (17897, STEMCELL Technologies).

6) Biosafety cabinet.

7) Multichannel pipettes, Tips, Pasteur pipettes.

##### **Procedure**

- 1) Prepare Ficoll-Paque PLUS and umbilical cord blood at room temperature. Dilute the CSA to a final concentration of 50 µg/mL in PBS and label as solution A [1].
- 2) Dilute the umbilical cord blood in solution A at a ratio of 1:1.
- 3) Use a pipette to transfer 15 mL Ficoll to a sterile 50 mL centrifuge tube. Carefully layer the diluted blood sample (30 mL) on Ficoll-Paque PLUS.
- 4) Centrifuge the diluted blood sample at 400 g for 30 minutes at room temperature, the centrifuge needs to turn off the brake. Gently remove the sample and avoid mixing of the separated liquid layers.
- 5) Use a Pasteur pipette to transfer the lymphocyte layer to a sterile 50 mL centrifuge tube. Add at least 3 volumes of IMDM to the lymphocytes containing tube. Re-centrifuge at 500 g for 10 minutes at room temperature and remove the supernatant.
- 6) Suspend the lymphocytes in IMDM by gently drawing them in and out of the Pasteur pipette. Centrifuge again at 200 g for 10 minutes at room temperature, remove the supernatant, and re-suspend the cell pellet in IMDM. Then centrifuge at 400 g for 10 minutes at room temperature.
- 7) Isolate the stem cells using a CD34 positive human cord blood selection kit according to the manufacturer's instructions. All solutions for isolation contain CSA at a concentration of 50 µg/mL [1].

### 2.1.3. Phenotypic Characterization of Isolated CD34+ Cells

#### Flow Cytometry Analysis of Phenotype

##### *Materials*

- 1) Human hematopoietic stem cell.
- 2) PBS pH7.2.
- 3) Blocking buffer: 2% (v/v) mouse serum in PBS.
- 4) Dilution buffer: 2% (v/v) fetal bovine serum (FBS) in PBS.
- 5) PE-labeled anti-human CD34 (12-0349-42, eBioscience).
- 6) PE-Cy7 labelled anti-human CD45RA (25-0458-42, eBioscience).
- 7) PE-Cy5 labelled anti-human CD49f (551129, BD Biosciences).
- 8) APC labelled anti-human CD90 (559869, BD Biosciences).
- 9) FTIC labelled anti-human CD38 (11-0388-42, eBioscience).
- 10) BD FACSVerser flow cytometer, FlowJo software.

##### *Procedure*

- 1) Harvest the stem cells, centrifuge at 300 g for 5 minutes at room temperature, and keep the cell pellet.
- 2) Resuspend the cells in cold PBS, perform cell count using trypan blue and hemacytometer. The number of cells in each sample is approximately  $2 - 5 \times 10^5$ .
- 3) Add blocking buffer, incubate the cells at 4°C for 30 minutes. Then centrifuge at 300 g for 5 minutes at 4°C and discard the supernatant. Suspend the cells in a dilution buffer, 100 µL/sample.
- 4) Cells are stained with the following antibodies according to the manufacturer's instructions: PE-labelled anti-human CD34, PE-Cy7 labelled anti-human

CD45RA, PE-Cy5 labelled anti-human CD49f, APC labelled anti-human CD90, FTIC labelled anti-human CD38. Incubate the cells at 4°C for 30 minutes in dark [20].

5) Centrifuge at 300 g for 5 minutes at 4°C. Wash the cells with PBS for 2 - 3 times. Suspend the cells in PBS.

6) Put the cells on a shaker before reading at the flow cytometer. The human cord blood stem cells express CD34<sup>+</sup>CD38<sup>-</sup>CD45RA<sup>-</sup>CD90<sup>+</sup>CD49f<sup>+</sup> phenotype.

#### 2.1.4. Wright-Giemsa Stain

##### *Materials*

- 1) Wright-Giemsa solution (G1020, Solarbio).
- 2) Phosphate buffer pH6.4 [0.01 M].
- 3) Inverted microscope (Olympus).
- 4) Thin-layer Cell Preparation System (A78300003, ThermoFisher).

##### *Procedure*

1) Resuspend the cell pellet in 2% FBS in PBS per  $1 \times 10^5$  cells, to a final volume of 300  $\mu$ L.

2) Assemble the accessories of the thin-layer cell preparation system, clamp the slides under the specimen chamber. Immediately transfer cell suspension into the specimen chamber. Centrifuge at 1000 rpm for 5 minutes, dry in air.

3) Fix cells with 4% formaldehyde solution for 10 minutes and rinse the cells with distilled water to remove residual formaldehyde.

4) Stain cells with Wright-Giemsa solution for 1 - 2 minutes. Add Phosphate buffer pH6.4 [0.01 M] which equal volume to Wright-Giemsa solution, mix well, and stain for another 3 - 5 minutes.

5) Rinse cells three times with distilled water, use a filter paper to absorb the remaining liquid. Visualize the images under a light microscope.

## 2.2. Expansion of UCB HSC

### 2.2.1. Preparation of Basic Media

A low concentration of calcium (0.02 mM CaCl<sub>2</sub>) can enhance the function of hematopoietic stem cells and maintain the biological activity. StemSpan media (Stem cell biotechnology) which contains human growth factors and cytokines, is widely used in the expansion of human HSCs. These growth factors are supplemented in a basic media containing a high concentration of CaCl<sub>2</sub> (1.6 mM). The basic medium with a low concentration of calcium can be generated by the following procedure [24].

##### *Materials*

- 1) Ca<sup>2+</sup>-freeDMEM (21068028, Gibco).
- 2) StemSpan CD34+ Expansion Supplement (10X) (02691, STEMCELL technologies).
- 3) StemPro-34 SFM Kit (10639011, Thermo Fisher).
- 4) L-Glutamine (25030081, Gibco).
- 5) Sodium Pyruvate (11360070, Gibco).

6) HEPES (15630106, Gibco).

#### **Procedure**

1) Using Ca<sup>2+</sup>-free DMEM as the basic media reconstitute with 10 mM HEPES, 1 × Penicillin/Streptomycin Solution, 1 × L-Glutamine, 1 mM Sodium Pyruvate.

2) Supplement with 1x Nutrient supplement (StemPro™-34 SFM Kit) to provide serum-free components, and 1× StemSpan CD34+ Expansion Supplement to provide human cytokines [24].

3) Prepare a complete stock media containing 2 mM CaCl<sub>2</sub>, filter with 0.22 μm disposable filter, and store the filtrate at 4°C. Dilute the CaCl<sub>2</sub> stock solution to a final concentration of 0.02 mM to obtain a low concentration of calcium.

#### **2.2.2. Optimize the Concentration of Cytokines**

Many types of cytokines have been demonstrated to stimulate *in vitro* HSC expansion [22] [24]. However, the expansion effects with a combination of cytokines and the optimized cytokines' concentration have not yet been fully determined. Thus, optimization of combinations and concentration of cytokines is important for efficient HSC expansion. An efficient and reliable mathematical model can benefit for the optimization process.

Response Surface Methodology (RSM) [25] is often used to solve the multi-objective optimization problem in biomedicine [26] [27] [28] [29]. RSM is a collection of mathematical and statistical techniques that are useful for modeling and analysis of problems in which a response of interest is influenced by several quantifiable variables (or factors) [25] [30]. RSM is sequential, determining the significant factor variables, gradually eliminating the non-significant factors, and determining the optimal distance of the response value [25]. If the existing conditions are not optimal, a series of adjustments can give the optimal direction. The common method of RSM is the face-centered cube design (FC-CD) [25]. This model design only needs three levels of each factor: -1, +1, 0, the model design is cube [25] [31] [32].

We give an example for optimizing the concentrations of 3 growth factors using a face-centered cube design (FC-CD), the growth factors include stem cell factor (SCF), thrombopoietin (TPO) and FMS-like tyrosinekinase 3 ligand (Flt3-L).

#### **Materials**

1) Stemspan SFEM (09600, STEMCELL technologies).

2) Human recombinant SCF (78062, STEMCELL technologies).

3) Human recombinant TPO (78210, STEMCELL technologies).

4) Human recombinant Flt3/Flk-2 Ligand (78009, STEMCELL technologies).

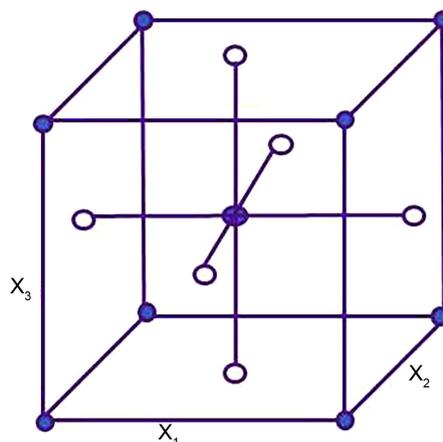
#### **Procedure**

1) Using StemSpan SFEM media culture HSC, and seed cells at the concentration of  $3 \times 10^4$ /mL.

2) Design for optimization of a cocktail of cytokines. SCF, Flt-3L and TPO which are tested either at low concentration (-1, 0 ng/mL), medium concentra-

tion (0, 50 ng/mL), or high concentration (+1, 100 ng/mL). The experimental design (**Figure 1**) has 8 factorial points, 6 axial points, 3 replicated center points (which improve the analysis accuracy), and a total of 17 runs as shown in **Table 1** [25] [30].

3) Carefully transfer HSC to a 37°C, 5% supplemented CO<sub>2</sub> incubator. On day 7, perform cell count and flow cytometry analysis of CD34 positive cells.



**Figure 1.** Experiments design model for 3 factors. The diagram was modified from the reference: Montgomery DC (2017).

**Table 1.** Design for optimization of the cytokines cocktail.

Runs	SCF	Flt3-L	TPO
1	-1	-1	1
2	-1	1	1
3	1	-1	1
4	1	1	1
5	1	1	-1
6	1	-1	-1
7	-1	1	-1
8	-1	-1	-1
9	0	0	1
10	0	0	-1
11	-1	0	0
12	1	0	0
13	0	1	0
14	0	-1	0
15	0	0	0
16	0	0	0
17	0	0	0

4) The expanded cells of each sample are cultured on methylcellulose media for colony-forming unit (CFU) assay.

5) Calculate and record every experiment FI (fold increase) total nucleated cells (TNC), FI CD34+, FI BFU-E, FI CFU-GM, FI CFU-GEMM, and FI CFU-mix as the expansion response. Perform statistical analysis using Design-Expert software and SAS. A p-value of all variables in the model of less than or equal to 0.05, is considered statistically significant. Finally, determine the optimal concentration range of SCF, TPO, Flt3-L and carry out the verification process [25].

### 2.2.3. Optimizing Concentration of the Chemical Compounds

SR-1 and UM171 are powerful chemical molecules screened from chemical library containing more than 5000 compounds [19] [20]. SR-1 is an antagonist of the aryl hydrocarbon receptor (AhR). It inhibits the AhR signaling pathway and promotes the proliferation of HSC by binding AhR to prevent its binding to AhR photoaffinity ligand (PAL) [19] [21]. Conversely, UM171 enhances the human HSC self-renewal machinery independently of AhR suppression [20], whereas induces a rheostatic regulation of inflammatory and anti-inflammation/detoxification programs [33]. UM171 greatly reduces the risk of long-term frequent immune complications (GVHD) [22]. But SR-1 or UM171 could not stimulate HSC proliferation alone [19] [20]. Meanwhile, the growth factor panel can prime HSC number increase but cannot inhibit HSC lineage differentiation [16] [20]. The combination of growth factors and chemical compounds achieved great progress in HSC *ex vivo* expansion [19] [20] [34]. Optimization of chemical concentrations used in the culture medium formula containing the growth factor panel is necessary. FC-CD model is a helpful tool to guide the procedure optimization.

#### **Materials**

- 1) StemSpan SFEM(09600, STEMCELL technologies).
- 2) Human recombinant SCF (78062, STEMCELL technologies).
- 3) Human recombinant TPO (78210, STEMCELL technologies).
- 4) Human recombinant Flt3/Flk-2 Ligand (78009, STEMCELL technologies)
- 5) SR-1 (72342, STEMCELL technologies).
- 6) UM171 (72914, STEMCELL technologies).

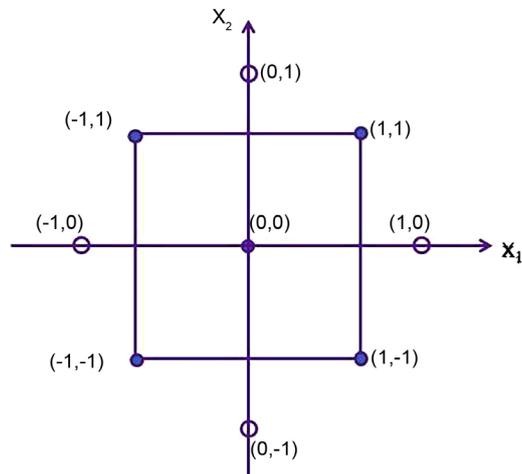
#### **Procedure**

1) Prepare StemSpan SFEM media with 50 ng/mL TPO, 100 ng/mL SCF, 100 ng/mL Flt3-L, 1 × Penicillin/Streptomycin Solution.

2) Seed cells at a concentration of  $3 \times 10^4$ /mL.

3) Design for optimization of the compounds. UM171 and SR-1 are tested either at a low level (-1, low concentration), middle level (0, average of low and high concentrations), high level (+1, high concentration). The experimental design as shown in **Figure 2** is composed of 4 factorial points, 4 axial points, 2 replicated center points with a total of 10 runs. [25]

4) Record every experiment FI TNC, FI CD34+, FI BFU-E, FI CFU-GM, FI CFU-GEMM, and FI CFU-mix.



**Figure 2.** Experiments design model for 2 factors. The diagram was modified from the reference: Montgomery DC (2017).

#### 2.2.4. HSC Expansion with a Bioreactor

A bioreactor system with fed-batch function can benefit for HSC *ex vivo* expansion by continuously providing with fresh medium and removing cell metabolites. The fed-batch culture system can keep HSC growth in a stable culture environment [20] [35]. The brands of cell culture bags approved by FDA have found a use in fed-batch culture [35]. The cell culture bag made of fluoroethyl polymer (FEP) is gas permeable and water impermeable. A bag loaded with the initial HSCs suspension is put on a shaker in cell culture incubator and connected to a pump-loaded syringe system (Figure 3). The pump is set up to continuously deliver the culture medium into the cell bag with a designed flow rate [20] [35].

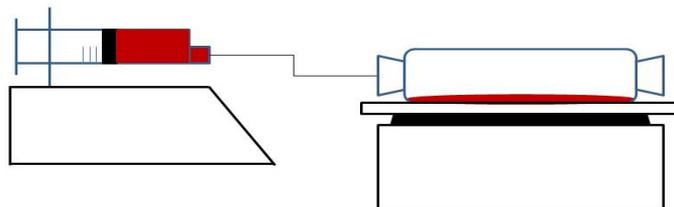
### 2.3. Function Analysis of Expanded HSC *in Vitro*-Clone Forming Unit (CFU) Assay

#### **Materials**

- 1) IMDM (Hyclone).
- 2) FBS (Gibco).
- 3) MethoCult H4034 Optimum (04034, STEMCELL technologies).

#### **Procedure**

- 1) Prepare medium, IMDM with 2% FBS.



**Figure 3.** Fed-batch culture system diagram. The diagram was modified from the reference: Fares I, J Chagraoui, Y Gareau, *et al.* (2014).

2) Harvest the stem cells, centrifuge at 300 g for 5 minutes, and remove the supernatant. Resuspend the cells in IMDM with 2% FBS, and count using Trypan blue.

3) Resuspend 500 cells in 100  $\mu$ l medium, add MethoCult H4034 Optimum 1 mL per well, transfer the mixture into 6 well plate.

4) Culture at 37°C, 5% CO<sub>2</sub> supplemented incubator for 14 - 16 days, count CFU-mix, CFU-GM, CFU-GEMM, BFU-E.

#### 2.4. HSC Functional Analysis *in Vivo*-Transplantation Study

The NSG mice at aging of 8 - 12 weeks are prepared for HSC transplantation by feeding with water containing antibiotics.  $1 \times 10^5$  of uncultured or expanded CD34+ cells are injected into the NSG mice sublethally irradiated (300 cGy) via tail vein. The mice were sacrificed 13 - 16 weeks after transplantation for assay of engraftment of human hematopoietic cells.

Blood cells and bone marrow (BM) cells from each mouse are analyzed for presence of cells expressing markers of human hematopoietic cells by flow cytometry. The markers usually include CD45, CD34, CD33, CD3, CD14, CD19, CD41 and CD71. For secondary transplantation,  $2 \times 10^6$  of bone marrow cells from the primary recipient NSG mouse are transplanted into sublethally irradiated NSG mouse. Blood cells and BM cells from mice 15 - 16 weeks after transplantation are collected to analyze the presence of human hematopoietic cells as above [1] [20].

The frequency of HSC in uncultured or expanded cells is analyzed by limiting dilution analysis (LDA). In each LDA experiment, mice are transplanted with 2000, 1000, 500 expanded cells. HSC frequency is calculated using L-Cal software (STEMCELL Technologies) and plotted using ELDA software (<http://bioinf.wehi.edu.au/software/ellda/>). Poisson statistics is used to calculate the p-value for all LDA analyses. Differences in HSC frequencies are analyzed by the chi-square test. \*P values  $\leq 0.05$  are considered statistically significant [1] [6] [34].

### 3. Conclusion

HSCs derived from umbilical cord blood have advantages inaccessible and hematopoiesis capacity over HSCs derived from adult tissues. The limited number of UCB HSCs impedes its clinical application. Expansion *ex vivo* of UCB HSCs could overcome the obstacles. In general, there are three different types of division for a single HSC: symmetric renewal, asymmetric renewal and symmetric commitment. The strategy for expansion *ex vivo* of HSC aims to maintain symmetric renewal and avoid symmetric commitment. However, the mechanism underlying determination of HSC division fate remains unknown. We currently have no good way to harness HSC division fate *in vitro*. Most chemicals and growth factors which were applied in HSC *ex vivo* expansion, were identified through screening chemical library or recombinant growth factors based on *in*

*vitro* and *in vivo* hematopoiesis capacity of the expanded HSC. The growth factors, including SCF, TPO and Flt-3L, are able to stimulate cell number increase by activating signaling pathways related to cell growth, but not able to maintain HSC stemness. Meanwhile, the chemical molecules, including SR-1, UM171, VPA, can maintain HSC stemness during HSC division by modifying metabolism or/and epigenetics pattern. Therefore, a few combinations of the identified growth factors with chemical molecules have been successfully applied in UCB HSC *ex vivo* expansion. But the process for optimization of the variable formula compositions of different growth factors or of different chemical molecules or of the combinations growth factor with chemical molecule is necessary and time costuming, the optional mathematics models can benefit for guiding the optimization process. In this paper, we integrate the updates in *ex vivo* expansion of UCB HSC and provide an impact and practicable procedure from the protocol of isolation of original UCB HSC to the protocol of character identification of expanded UCB HSC.

### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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