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Protocol for enhancing *ex vivo* expansion of human haematopoietic stem cells by inhibiting ferroptosis

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We use this protocol and it's working

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Abstract

Efficient *ex vivo* expansion of human hematopoietic stem cells (HSCs) remains a major challenge for transplantation and genome-engineering applications, as standard culture systems lead to substantial HSC loss. This protocol describes a method to enhance HSC expansion by inhibiting ferroptosis, an iron-dependent form of regulated cell death. Treatment with ferroptosis inhibitors such as liproxstatin-1 (Lip-1) or ferrostatin-1 (Fer-1) significantly improves expansion of both cord blood (CB) and mobilized peripheral blood (mPB)–derived HSCs. Importantly, the expanded cells preserve phenotypic and molecular stem cell features and sustain long-term, multilineage engraftment in xenotransplantation assays. This step-by-step protocol outlines the optimized culture conditions and workflow for applying ferroptosis inhibition to improve *ex vivo* human HSC expansion and functional maintenance.

For complete details on the results and validation of this protocol, please refer to our work published in *Nature Cell Biology.*

Step-by-step protocol - SFEM II culture for human CD34⁺ HSPCs (with ferroptosis inhibition)

1 **Prepare reagents**

Warm the following media to room temperature (RT) or in a 37 °C water bath:

Thawing Medium: PBS + 1% FBS.

SFEM Culture Medium: StemSpan SFEM II (StemCell Technologies) supplemented with:

- 1% L-glutamine (Thermo Fisher Scientific)
- 1% penicillin/streptomycin (Life Technologies)
- 1×CC100 cytokine cocktail (FLT3L, SCF, IL3, IL6; StemCell Technologies)
- 100 ng/mL recombinant thrombopoietin (TPO; PeproTech)
- 35 nM UM171 (StemCell Technologies)

Ferroptosis inhibitors:

- Liproxstatin-1 (Lip-1; Cayman Chemical), prepare a 10 mM stock in DMSO; use at 10 µM final concentration.
- Ferrostatin-1 (Fer-1; MedChemExpress), prepare a 10 mM stock in DMSO; use at 5 µM final concentration.

2 **Thaw CB- or mPB-derived CD34+**

Thaw the cryovial in a 37 °C water bath until only a small ice crystal remains. Transfer the cell suspension dropwise into a 15 mL conical tube containing 10 mL of Thawing Medium and centrifuge at 300 × g for 10 min at RT.

3 **Cell culture**

Carefully aspirate, discard the supernatant, and resuspend the cell pellet in SFEM Culture Medium supplemented with either 10 µM Lip-1, or 5 µM Fer-1. Count viable cells and adjust the concentration to 5×10^5 cells/mL and plate cells in an appropriate culture vessel and incubate under standard culture conditions (37 °C, 5% CO_2).

4 **Culture maintenance**

Split the cultures every 2–3 days by diluting cells to a final concentration of $3\text{-}5\times10^5$ cells/mL in fresh SFEM Culture Medium, and continue expansion until the desired end point.

Replenish ferroptosis inhibitors by adding Lip-1 (10 µM) or Fer-1 (5 µM) at each medium change.

Step-by-step protocol - Cytokine-free expansion medium (CFEM) for human CD34⁺ HSPCs (with ferroptosis inhibition)

5 **Prepare reagents**

Prepare the medium and warm it to room temperature (RT) or in a 37 °C water bath:

CFEM Culture Medium:

- (Iscove's Modified Dulbecco's Medium-based (IMDM) (Life Technologies)
- 1% Insulin-Transferrin-Selenium-Ethanolamine (ITS-X; Life Technologies)
- 1% L-glutamine (Thermo Fisher Scientific)
- 1% Penicillin/Streptomycin (Life Technologies)
- 1 mg/mL Polyvinyl alcohol (PVA; Sigma-Aldrich)
- 1 µM 740Y-P (MedChemExpress)
- 0.1 µM Butyzamide (MedChemExpress)
- 70 nM UM171 (StemCell Technologies)

Ferroptosis inhibitors:

- Liproxstatin-1 (Lip-1; Cayman Chemical), prepare a 10 mM stock in DMSO; use at 10 µM final concentration.
- Ferrostatin-1 (Fer-1; MedChemExpress), prepare a 10 mM stock in DMSO; use at 5 µM final concentration.

6 **Cell isolation**

Isolate $CD34^+$ HSPCs from cord blood using the EasySep Human Cord Blood $CD34^+$ Positive Selection Kit (StemCell Technologies) following the manufacturer's instructions.

7 **Cell culture**

Count viable freshly isolated $CD34^+$ cells using trypan blue or an equivalent viability dye and adjust the final cell concentration to 7×10^4–1×10^5 cells/mL in CFEM Culture Medium supplemented with either 10 µM Lip-1 or 5 µM Fer-1. Seed $CD34^+$ HSPCs into CellBind plates, using 1 mL of medium per well for 24-well (Fisher Scientific, 08757216) plates or 5 mL of medium per well for 6-well plates (Corning, 3335).

8 **Culture maintenance**

Split cultures every 3–4 days to maintain a density of 7×10^4–1×10^5 cells/mL. Gently mix the culture to fully resuspend the cells, count them, and transfer an appropriate volume into fresh CFEM medium containing Lip-1 or Fer-1, replenishing the ferroptosis inhibitors with each medium change to maintain constant exposure.