

Webinar Q&A Report

Optimizing CD34⁺ Cell Genome Editing for Efficiency and HSPC Maintenance



Questions in this Q&A Report were submitted during the live webinar, [Optimizing CD34⁺ Cell Genome Editing for Efficiency and HSPC Maintenance](#).

Can you speak to the difference between mobilized CD34⁺ cells and those from bone marrow if the desired end product is differentiated erythroid cells?

Dr. Marta Walasek: When appropriate culture conditions and expansion medium are used, differentiation of either mobilized peripheral blood (mPB) or bone marrow (BM) to the erythroid lineage results in a highly pure population of differentiated erythroid cells. These can be identified by expression of CD71 and GlyA cell surface markers and by expression of mainly adult-type (beta-) hemoglobin. The cell yield is donor dependent. Additionally, there may be differences in HSPCs isolated from BM and mPB as suggested in the paper below by Mende et al.

Mende N et al. (2022) [Unique molecular and functional features of extramedullary hematopoietic stem and progenitor cell reservoirs in humans](#). Blood 139(23): 3387–3401.

Can this culture system for CD34⁺ expansion be used for hPSC-derived CD34⁺ cells?

Dr. Marta Walasek: [StemSpan™ CD34⁺ Expansion Supplement](#) (Catalog #02691) has been developed and optimized to promote expansion of primary CD34⁺ cells derived from cord blood (CB) or BM. The supplement has not been tested for the expansion of human pluripotent stem cell (hPSC)-derived CD34⁺ cells, which may have different growth factor requirements than primary cells. CD34⁺ expansion may also depend on the method used to generate the CD34⁺ cells from hPSCs.

What is the usual CD34 purity using the EasySep™ system?

Dr. Marta Walasek: The purity of CD34⁺ cells isolated with the EasySep™ system depends on whether the cells were isolated using positive or negative selection methods. Additionally, the purity and number of obtained CD34⁺ cells is dependent on quality, age, and size of the start sample. On average, for 36 – 48 hours-old cord blood samples, EasySep™ positive selection

results in 87% ($\pm 12\%$) pure CD34⁺ cell population when using [EasySep™ Human Cord Blood CD34 Positive Selection Kit III](#) (Catalog #17897) or 93.5% ($\pm 1.1\%$) when using [EasySep™ Human CD34 Positive Selection Kit II](#) (Catalog #17856). In contrast, the purity of CD34⁺ cells obtained via negative selection with [EasySep™ Human Progenitor Cell Enrichment Kit II](#) (Catalog #17936) is on average 78% ($\pm 16\%$).

How does the marker expression of CD34/CD90/CD45RA typically change over the first 4 - 7 days in culture?

Dr. Marta Walasek: HSPCs in non-cultured samples can be identified based on the CD34⁺CD38⁻CD45RA⁻CD90⁺CD49f⁺ phenotype, as shown by Fares and coworkers (see reference below). However, the cellular phenotype changes during cell culture, reflecting the progression through the differentiation process. For example, cells may acquire CD45RA and lose CD90 marker expression, and eventually lose CD34 expression. The kinetics of the changes in CD34, CD45RA, and CD90 marker expression will depend on culture conditions and the duration of culture. In general, shorter culture is recommended when maintenance of stem and progenitor cell phenotype and function is prioritized over cell yields. In other words, the frequency of stem cells may be higher with shorter culture, but at the cost of low cell numbers. Additionally, antigen expression on cultured cells may not be as predictive for determining non-differentiated status or lineage potential when compared to antigen expression on CD34⁺ cells that have not been cultured. For example, primary CD34⁺ cells with low or undetectable CD38 expression (CD34⁺CD38⁻ phenotype) are highly enriched for hematopoietic stem cells and primitive progenitor cells, but the CD34⁺CD38⁻ phenotype of cultured cells may not be as primitive. In recent years, several markers, such as endothelial protein C receptor (EPCR), have been shown to enrich HSPCs following cell culture.

Fares I et al. (2017) [EPCR expression marks UM171-expanded CD34⁺ cord blood stem cells](#). Blood 129(25): 3344–51.

Why is UM171 not available anymore?

Selena Hallahan (Product Manager, Hematology): UM171 was discontinued for sale by STEMCELL in October 2019 per the wishes of the patent holder.

Regarding the culture protocol outlined in the webinar, similar results are expected when using [UM729](#) (Catalog #72332) prepared to a final concentration of 10X the concentration of UM171 (i.e. 1 μ M). The equivalency of these molecules in expanding human cord blood cells has been shown by Fares and coworkers and confirmed by STEMCELL.

Please note that STEMCELL is the only authorized retailer of UM729. All other vendors are selling these products without the express permission of the patent holder.

Fares I et al. (2014) [Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal](#). Science. 345(6203): 1509-12

Is UM171 used in place of StemSpan™ CD34⁺ Expansion Supplement or in addition to it?

Selena Hallahan: UM171 is the small molecule that was used in addition to [StemSpan™ CD34⁺ Expansion Supplement](#) (Catalog #02691).

Please note that UM171 is no longer available for sale by STEMCELL; however, similar results are expected when using [UM729](#) (Catalog #72332) prepared to a final concentration of 1 μM.

Will the expansion media components interfere with HSPC differentiation?

Selena Hallahan: Culture conditions may impact the differentiation of HSPCs. The choice of growth factors and supplements added to the culture media affects whether the cell is stimulated to expand or differentiate. Early-acting cytokines and compounds that prevent differentiation can be used to expand HSPCs, whereas lineage-specific differentiation is achieved with the use of lineage-specific growth factors, such as erythropoietin (EPO) to stimulate erythroid differentiation.

StemSpan™ serum-free [media](#) have been developed to support the expansion and differentiation culture of hematopoietic cells and do not contain hematopoietic growth factors. This allows users the flexibility to supplement the medium with growth factors and/or other stimuli of choice to promote expansion or lineage-specific differentiation of HSPCs.

StemSpan™ expansion [supplements](#) are pre-mixed cocktails of recombinant human cytokines and other additives. Each has been formulated to selectively promote the expansion of CD34⁺ stem and progenitor cells, or to stimulate their differentiation into erythroid, myeloid (granulocyte or monocyte), or megakaryocyte progenitor cells, when added to StemSpan™ media.

Together, StemSpan™ media and supplements support greater expansion of CD34⁺ cells and differentiation of erythroid cells, granulocytes, monocytes, and megakaryocytes, than other media tested.

What was the medium used when plating the pre-editing culture?

Dr. Marta Walasek: We tested various StemSpan™ media as well as alternative commercial media to identify optimal culture conditions for genome editing. While all StemSpan™ media and supplements were compatible with the genome editing workflow, we found that [StemSpan™ SFEM II](#) (Catalog #09605) supplemented with [StemSpan™ CD34⁺ Expansion Supplement](#) (Catalog #02691) and the small molecule, [UM729](#) (Catalog #72332), for 2 days prior to CRISPR-Cas9 editing best supports maintenance of CD34 expression and expansion of primitive HSPC subsets during genome editing.

More information on the culture conditions tested can be found in this [technical bulletin](#).

Have you ever coupled lentivirus infection of an sgRNA with electroporation of Cas9? If so, how do you recommend we do it?

Danielle Nguyen Truong: We have developed a protocol for genome editing using CRISPR-Cas9 with electroporation of the RNP system into CD34⁺ cells. We have not coupled lentivirus infection of an sgRNA with electroporation of Cas9. It has been shown that lentiviral transduction of Cas9 is less efficient in sensitive cell types such as HSPCs. More information about lentivirus infection of sgRNA with electroporation of Cas9 has been described by Yudovich and coworkers.

Yudovich D et al. (2020) [Combined lentiviral- and RNA-mediated CRISPR/Cas9 delivery for efficient and traceable gene editing in human hematopoietic stem and progenitor cells](#). Sci Rep 10: 22393.

Different types of donor templates can induce significantly different levels of cytotoxicity. Do you have any suggestions about electroporation conditions during AAV delivery?

Danielle Nguyen Truong: Homology-directed repair (HDR) efficiency is usually higher with short single-stranded oligodeoxynucleotide (ssODN) donor templates, which are often used to correct a single base change, or short insertions or deletions. HDR is less efficient when double-stranded (dsDNA) templates are introduced, and a high concentration of dsDNA can cause cytotoxicity in cells. Shy et al. developed a novel system that takes advantage of lower toxicity with single-stranded DNA (ssDNA), in which the hybrid ssDNA HDR templates were incorporated with Cas9 target sequences (CTS) to improve the knock-in efficiency and reduce toxicity in cells.

Shy et al. (2021) [Hybrid ssDNA repair templates enable high yield genome engineering in primary cells for disease modeling and cell therapy manufacturing](#). bioRxiv

What is the delivery efficiency of the lentiviral system in CD34⁺ cells? What is the best viral vector for CD34⁺ cell transduction?

Danielle Nguyen Truong: Lentiviral systems generally have high editing efficiency and are effective in a variety of cell lines, primary cells, and stem cells. Yudovich and coworkers used Cas9 pLentiCRISPRv2 (pLCv2) to deliver sgRNA, followed by the transient expression of Cas9 using electroporation; the knockout efficiency of CD45 and CD44 was about 90% in edited HSPCs. However, the biggest concern of the lentivirus system is the random integration into the genome, which may disrupt vital genes or alter the regulation of gene expression. This suggests that non-integrating lentiviral vectors should be used for delivery of CRISPR-Cas9 components if enhancing the safety of gene editing is a concern. A paper by Uchida et al. describes an editing process that uses non-integrating lentiviral vectors (NILVs) to correct the SCD mutation by up to 42% at the protein level.

Yudovich D et al. (2020) [Combined lentiviral- and RNA-mediated CRISPR/Cas9 delivery for efficient and traceable gene editing in human hematopoietic stem and progenitor cells](#). Sci Rep 10: 22393.

Uchida N et al. (2021) [Cas9 protein delivery non-integrating lentiviral vectors for gene correction in sickle cell disease](#). Mol. Ther. Methods Clin. 21: 121–32

Do you ever isolate more purified HSPCs (i.e. additional markers to CD34) and perform RNP electroporation on a more purified population?

Danielle Nguyen Truong: The CRISPR gene-editing protocol presented in our [webinar](#) has been optimized using bulk CD34⁺ cell editing; it has not been tested on sorted and purified HSPC subsets. However, gene-editing efficiency of HSPC subsets during bulk CD34⁺ cell editing has been evaluated and the data showed efficient editing and maintenance of the CD34⁺CD45RA⁻CD90⁺ HSPC subset. Therefore, the presented genome editing protocol should be applicable when starting with purified HSPC subsets.

Gene editing of more purified HSPCs has been described in the literature. For example, in the paper below, Zonari et al. genome edited sorted CD34⁺CD38⁻ cells and suggested that such an approach may allow for better tailored gene transfer to LT-HSPCs.

Zonari E et al. (2017) [Efficient ex vivo engineering and expansion of highly purified human hematopoietic stem and progenitor cell populations for gene therapy](#). Stem Cell Rep: 8(4): 977–90.

Are the pre-editing CRISPR parameters the same as for lentivirus transduction?

Danielle Nguyen Truong: The culture conditions and cell pre-activation of the pre-editing CRISPR is similar as for lentivirus transduction. Please refer to our [technical bulletin](#) for instructions on how to culture CD34⁺ cells before transfection.

Do both alleles get edited with the RNP electroporation?

Danielle Nguyen Truong: Both alleles do not necessarily get edited with the RNP electroporation. As non-homologous end joining (NHEJ) is an error-prone DNA repair process, insertions and deletions (indels) are often randomly introduced into the gene, resulting in frameshifts or loss of gene function. It is necessary to determine if there are indels in one or both alleles by subcloning the edited population and sequencing these clones to determine the exact genotype of the edited clones.

If you'd like to confirm successful genome editing prior to transplantation of the cells, can you keep the cells in culture for 2 - 3 days before transplanting? Or is that too long to wait?

Danielle Nguyen Truong: Cells can be grown in culture for an additional 2 or 3 days before transplanting of the cells without any impact to editing efficiency. However, the cells need to be passaged if they are over-confluent to avoid overgrown cultures.

While longer cell culture may be required to confirm successful genome editing, the engraftment potential of the cells decreases with extended cell culture. It has been demonstrated that CD34⁺ cells retain engraftment potential following 6 – 7 days culture in optimal conditions and medium.

Have you used this genome editing system to generate knock-in (KI) cell lines?

Danielle Nguyen Truong: We have demonstrated genetic knock-in with the [ArciTect™ CRISPR-Cas9 Genome Editing System](#), through green fluorescent protein (GFP) to blue fluorescent protein (BFP) conversion, by targeting the enhanced green fluorescent protein (eGFP) locus in eGFP-tagged WLS-1C iPS and H1 ES cell lines. For more details, explore our [technical bulletin](#).

Contact Information

If you have additional questions for the STEMCELL team, contact them at:
<https://www.stemcell.com/contact-us>.



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