Miltenyi Biotec

IPC/QC of gene-engineered HSCs manufactured by the CliniMACS Prodigy®

Background

In-process and quality control (IPC/QC) are required for consistent manufacturing of gene-engineered hematopoietic stem cells (HSCs). The CliniMACS Prodigy HSC Engineering process allows for the manufacturing of such gene-engineered HSCs by viral transduction of human CD34$^+$ cells and enables control sampling at any time due to integrated sampling pouches within the CliniMACS® Tubing Sets. Viral transduction of CD34$^+$ cells is a promising approach for treating inherited disorders such as sickle cell disease, ß-thalassemia, or primary immunodeficiencies[1–5].

In this application note, the IPC/QC of gene-engineered HSCs manufactured by the CliniMACS Prodigy HSC Engineering process are described for reliable reproducibility and functionality of gene-engineered HSCs. For IPC, the cell viability over time and recovery were investigated. For QC, the functionality of the gene-engineered HSCs were analyzed by phenotypic analysis of CD34, CD90, and colony-forming unit (CFU) assays. Furthermore, the efficiency of the viral transduction was studied by the transduction efficiency and vector copy number (VCN).

Materials

Cellular starting material

CD34$^+$ cells were isolated from human leukapheresis from G-CSF mobilized donors using the LP-34 Enrichment process on the CliniMACS Prodigy.

Reagents

- HSC-Brew GMP Medium (# 170-076-310)
- MACS GMP Recombinant Human SCF 100 µg (# 170-076-133)
- MACS GMP Recombinant Human TPO 50 µg (# 170-076-134)
- MACS GMP Recombinant Human IL-3 25 µg (# 170-076-110)
- MACS GMP Recombinant Human FLT3-Ligand 100 µg (# 170-076-132)
- Human Serum Albumin (HSA) 25% (Octapharma # 6898264302)
- Iscove's Modified Dulbecco's Medium (IMDM)
- Fetal Bovine Serum (FBS)
- 0.9% NaCl
- Sterile water for injection
- CliniMACS PBS/EDTA Buffer (# 200-070-025)
- Bovine Serum Albumin (BSA) stock solution
- Lentigen GFP-coded lentiviral vector
- StemMACS HSC-CFU complete with Epo, human (# 130-091-280)
- MACS COPYcheck Kit, human (# 130-128-157)
- DNeasy Blood & Tissue Kit (Qiagen # 69504)
- Nuclease-free water
- Taqman™ Fast Advanced Master Mix (Thermo Fisher Scientific # 4444556)

Antibodies

Marker	Clone	Order no.
Propidium Iodide Solution	n.a.	130-093-233
CD34 Antibody, anti-human, PE-Vio 770	AC136	130-113-180
CD45 Antibody, anti-human, VioBlue	5B1	130-113-122
CD90 Antibody, anti-human, APC, REAfinity™	REA897	130-114-861
7-AAD Staining Solution	n.a.	130-111-568

Table 1: Marker panel for flow cytometry to determine cell count and phenotype.

Consumables

- CliniMACS Prodigy Tubing Set 520 (# 170-076-600)
- 3-way Tube Adapter (# 170-076-607)
- Luer/Spike Interconnector (# 130-122-744)
- 1 m Tube Extension (# 170-076-606)
- 150 mL Transfer Bags
- Syringes (10 mL, 30 mL and 60 mL)
- Tissue culture plates (24-well and 96-well)
- Falcon tubes
- Corning flasks 250 mL
- 0.22 µm filter
- 6-well plates
- 16 G blunt-end needles
- 96-well PCR plates for fast PCR protocols as recommended for the multicolor real-time PCR detection system by the respective supplier
- Adhesive sealing films

Equipment

- CliniMACS Prodigy (# 200-075-301)
- MACSQuant Analyzer 10 (# 130-096-343)
- TSCD-II Sterile Tubing Welder
- Centrifuge
- Incubator
- Laminar flow benches
- Cytation 3 Cell Imaging Multi-Mode Reader (BioTek)
- 7900HT Fast Real-Time PCR System with Fast 96-Well Block Module (Applied Biosystems)
- Block heater
- Vortex mixer
- 2× PCR workstations (DNA-free and for handling template DNA)

Methods

1. Manufacturing of gene-engineered HSCs

As cellular starting material of the CliniMACS Prodigy HSC Engineering process, $CD34^+$ cells were enriched from human mobilized leukapheresis (mLPs) by the LP-34 Enrichment process on the CliniMACS Prodigy. For the manufacturing of gene-engineered HSCs, the separated $CD34^+$ cells were transduced with GFP-coded lentiviral vector and harvested on day 2. Table 2 displays the experimental details.

Cellular starting material	
Material	Human $CD34^+$ cells enriched from mLP
Total cell number	7×10^7 – 1.4×10^8 cells
Culture conditions	
Cell concentration	1×10^6 cells/mL
Culture volume	50–140 mL
Medium	HSC-Brew GMP Medium with 2% HSA* and MACS GMP Recombinant Human Cytokines (Flt3-Ligand, SCF, TPO, and IL-3)**
Culture conditions	37 °C, 5% CO_2, Shaker type 2
Total culture duration	2 days
Culture duration before transduction	22 +/-2 hours
Transduction conditions	
Viral vector	Lentigen GFP-coded lentiviral vector
MOI (Multiplicity of infection)	30
Number of transduction rounds	1
Transduction duration	16 +/-1 hour
Final product	
Final product	Gene-engineered $CD34^+$ cells
Volume	100 mL
Formulation solution	Physiologic sodium chloride with 0.5% HSA

Table 2: Experimental details of manufacturing gene-engineered HSCs by the CliniMACS Prodigy HSC Engineering process.
* HSC-Brew GMP Medium was prepared as described in the data sheet and protected from light.
** The MACS GMP Recombinant Human Cytokines were reconstituted as described in the data sheet. The final activity of each cytokine in the culture medium is listed in table 3 below.

Cytokine	Size [µg]	Final activity in culture medium [U/mL]
SCF	100	270
Flt3-Ligand	100	300
TPO	50	1600
IL-3	25	120

Table 3: Overview of the required cytokines during culture and their final activities in culture medium.

Detailed information on the required material, process specification, and the CliniMACS Prodigy HSC Engineering process are provided in the application sheet "Generation of gene-engineered hematopoietic stem cells" (# 130-126-605) and in the CliniMACS Prodigy Hematopoietic Stem Cell Engineering System User Manual (# 38029/01).

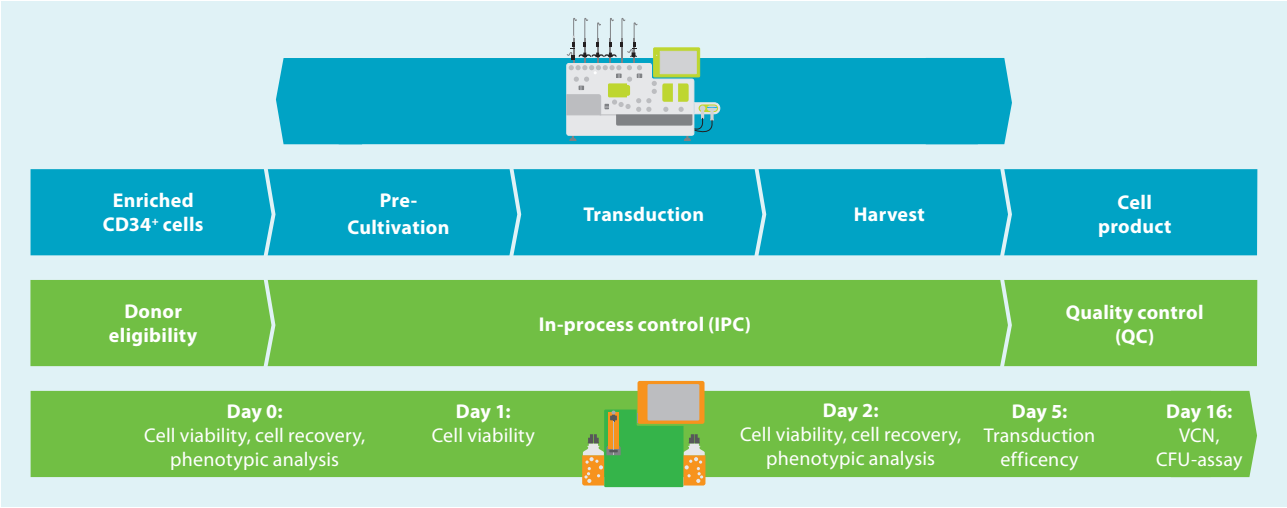

Figure 1: Overview of the IPC/QC timeline of gene-engineered HSCs.

2. IPC/QC of gene-engineered HSCs

For IPC/QC, the integrated sampling pouches on the CliniMACS Tubing Sets allowed for controls to be collected throughout the running of the cell manufacturing process. The samples in the HSC Engineering process (fig. 1) showed cell viability on days 0, 1, and 2. Cell recovery and phenotypic analysis for CD34 and CD90 were determined on days 0 and 2. The harvested cells from day 2 were further cultured manually to analyze the transduction efficiency by flow cytometry on day 5 and VCN on day 16. Furthermore, CFU assays were performed with the harvested cells from day 2 and analyzed on day 16. Individual analysis methods are described below.

2.1 Cell viability and recovery

To analyze cell viability and recovery, cell samples were taken on days 0 and 2 via the integrated sampling pouches on the CliniMACS Tubing Sets. To analyze cell viability over time, an additional sample was taken on day 1. Shortly before measurement with the MACSQuant® Analyzer 10, Propidium Iodide (PI) Solution was added in a 1:100 ratio to the taken samples to exclude dead cells.

MACSQuant Analyzer 10 parameters

- Uptake volume: 40 µL
- Sample volume: 200 µL
- Flow rate: Low
- Mix sample: Medium
- Automated PI addition (1:100)

Setting gates for analysis

To set the correct gating for all samples, the following steps were performed.

- A forward scatter (FSC-A) versus side scatter (SSC-A) plot was created to set a gate that excluded debris and included all conducted cells (fig. 2A).
- The events of gate A were opened in a new plot and displayed FSC-A versus FSC-H. The single cells were then gated (fig. 2B).
- The single cells of gate B were displayed in a new plot with PE-A versus PI-A. Dead cells were PI+ and thus could be excluded from the gate (fig. 2C).

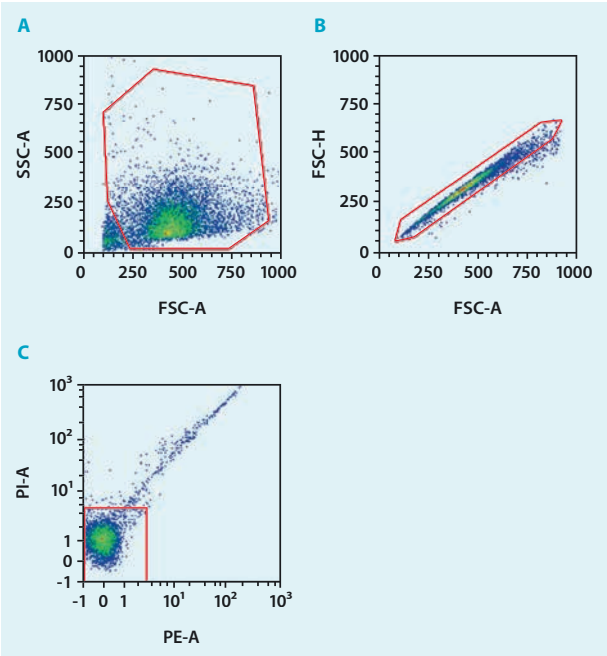

Figure 2: Gating strategy to determine cell viability and recovery. All cells gated with forward scatter (FSC-A) versus side scatter (SSC-A) (A), single cells visualized by FSC-A versus FSC-H (B), and viable cells visualized by PE-A versus PI-A (C).

2.2 Phenotypic analysis

Cell samples from days 0 and 2 were analyzed for CD34 and CD90 expression by staining with 7-AAD, CD34-PE Vio®770, and CD90-APC antibodies, to determine $CD34^+CD90^+$ cells with the use of the MACSQuant Analyzer 10.

MACSQuant Analyzer 10 parameters

- Uptake volume: 170 µL
- Sample volume: 200 µL
- Flow rate: 2000 events/s
- Mix sample: Medium

Setting gates for analysis
To set the correct gating for all samples, the following steps were performed.

- A FSC-A versus SSC-A plot was used to set a gate that excluded debris and included all cells (fig. 3A).
- The events of gate A were opened in a new plot and displayed FSC-A versus FSC-H. The single cells were then gated (fig. 3B).
- The single cells of gate B were displayed in a new plot and displayed 7-AAD-A versus SSC-A. Dead cells were 7-AAD$^+$ and thus were excluded from the gate (fig. 3C).
- The cells from gate C were used to create a new plot displaying CD90-APC-A versus CD34-PE-Vio770-A. CD34$^+$CD90$^+$ cells were then chosen for density gating (fig. 3D).

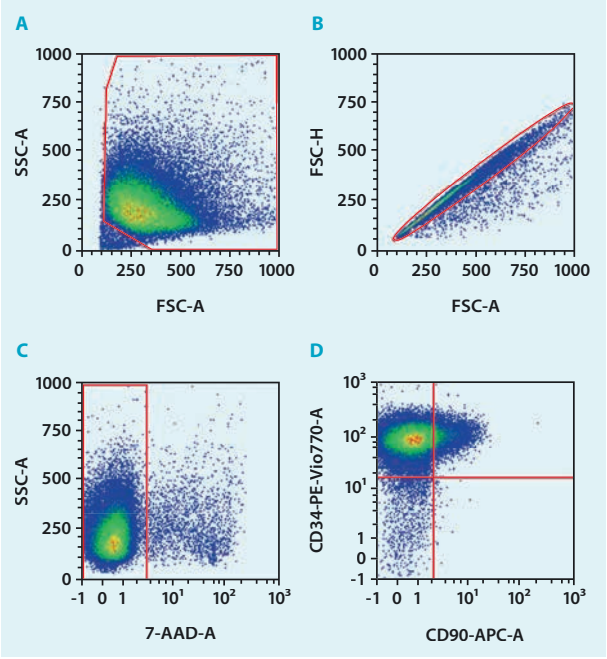

Figure 3: Gating strategy in order to determine CD34$^+$ and CD90$^+$ cells. All cells gated with FSC-A versus SSC-A (A), single cells visualized by FSC-A versus FSC-H (B), viable cells visualized by 7-AAD-A versus SSC-A (C), and CD34$^+$CD90$^+$ cells visualized by CD90-APC-A versus CD34-PE-Vio770-A (D).

2.3 Transduction efficiency by flow cytometry
A sample of harvested cells from day 2 was further cultured in 96-well round bottom tissue culture plates to determine the transduction efficiency on day 5. For this, 5000 cells were plated in each well and cultured at 37 °C and 5% CO_2.
On day 5, the cells were stained with 7-AAD, CD45-VioBlue® and CD34-PE-Vio770 antibodies in order to determine CD45$^+$CD34$^+$GFP$^+$ cells.

MACSQuant parameters
- Uptake volume: 170 µL
- Sample volume: 200 µL
- Flow rate: 2000 events/s
- Mix sample: Medium

Setting gates for analysis
To set the correct gating for all samples, the following steps were performed.

- A FSC-A versus a SSC-A plot was created to set a gate that excluded debris and included all cells (fig. 4A).
- The events of gate A were opened in a new plot and displayed FSC-A versus FSC-H. The single cells were then gated (fig. 4B).
- The single cells of gate B were used to create a new plot displaying CD45-VioBlue-A versus SSC-A. The CD45$^+$ cells were chosen (fig. 4C).
- The events of gate C were opened to create a new plot displaying CD34-PE-Vio770-A versus SSC-A. The CD34$^+$ cells were chosen (fig. 4D).
- The CD45$^+$CD34$^+$ cells of gate D were displayed in a new plot and displayed 7-AAD-A versus SSC-A. Dead cells were 7-AAD positive and thus could be excluded from the gate (fig. 4E).
- The viable CD45$^+$CD34$^+$ cells of gate E were used to create a new plot displaying CD34-PE-Vio770-A versus GFP-A. The viable CD45$^+$CD34$^+$GFP$^+$ cells were chosen for the determination of the transduction efficiency (fig. 4F).

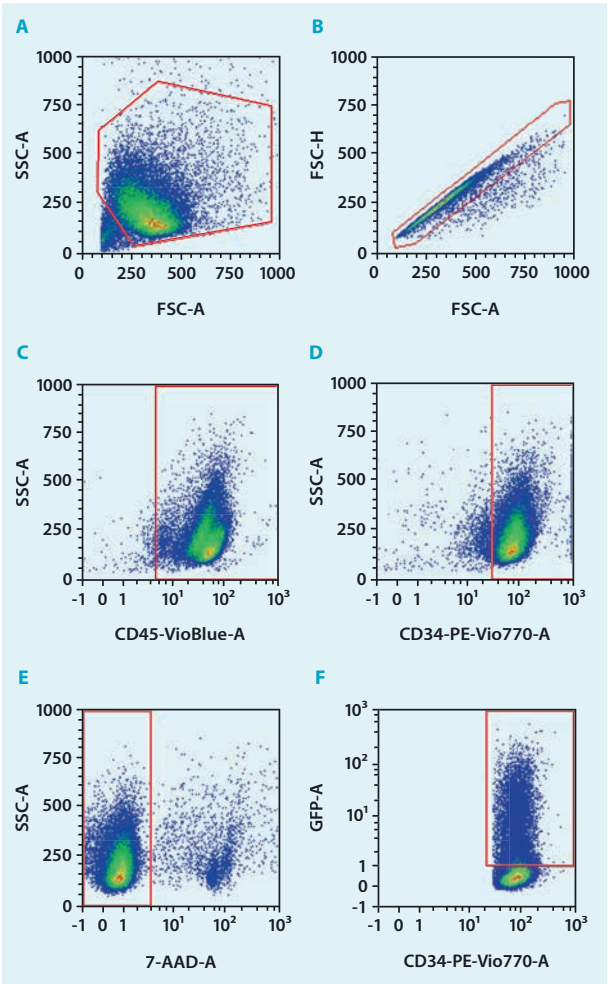

Figure 4: Gating strategy in order to determine the viable CD45$^+$CD34$^+$GFP$^+$ cells. All cells gated with FSC-A versus SSC-A (A), single cells visualized by FSC-A versus FSC-H (B), CD45$^+$ cells visualized by CD45-VioBlue-A versus SSC-A (C), CD45$^+$CD34$^+$ cells visualized by CD34-PE-Vio770-A versus SSC-A (D), viable CD45$^+$CD34$^+$ cells visualized by 7-AAD-A versus SSC-A (E), and viable CD45$^+$CD34$^+$GFP$^+$ cells visualized by CD34-PE-Vio770-A versus GFP-A (F).

2.4 Transduction efficiency by CFU assays

In order to determine the transduction efficiency, and furthermore the proliferation and differentiation potential of the transduced CD34+ cells, HSC-CFU assays were performed with cells harvested on day 2. The cells were diluted in IMDM medium before being cultivated in methylcellulose-containing medium. The HSC-CFU assays were set up in 6-well plates, with duplicates for each sample, and a cell concentration of 250 live cells per well. Detailed information was provided in the datasheet of the StemMACS™ HSC-CFU Media, human (# 130-091-280).

After 14 days of culture (16 days since the sample processing began), in a humified incubator (37 °C, 5% CO_2), the generated CFUs were analyzed for colony type (data not shown) and total colony number using a conventional light microscope. The transduction efficiency for the harvested cells from day 2, measured as the frequency of GFP+ cells, was determined using the Cytation 3 Cell Imaging Multi-Mode Reader.

2.5 Vector copy number (VCN) analysis

To determine the average number of integrated lentiviral vectors per cell genome after *ex vivo* transduction, VCN analysis was performed.

The cells harvested on day 2 were expanded in IMDM supplemented with 10% FBS, 300 ng/mL SCF, 60 ng/mL IL-3, and 60 ng/mL IL-6 to achieve a sufficient cell number for VCN analysis. After 14 days of culture (16 days since the sample processing began), the cells were harvested, and cell number and transduction frequency (i.e. the frequency of GFP+ cells) were determined using the MACSQuant Analyzer 10. Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen) according to the manual. The VCN analysis was performed using the MACS®COPYcheck Kit, human (# 130-128-157) as described in the datasheet. The copies of the lentiviral gag gene were determined in relation to the human reference gene, PTBP2, by quantitative PCR. After quantification of gag and PTBP2 amplicons, the VCN per transduced cell was calculated using the following formula.

$$\text{VCN per transduced cell} = \frac{\text{qPCR copy no./µL (gag)}}{\text{qPCR copy no./µL (PTBP2)}} \div \frac{\text{transduction freq. (\%)}}{100\%} \times 2$$

Results & Discussion

As shown in figure 1, cell samples were taken at several time points to perform IPC/QC analysis on day 0 (before process start), day 1 (during process), and day 2 (after harvest of gene-engineered HSCs). The following results show the comparison of gene-engineered HSCs by the CliniMACS Prodigy HSC Engineering process against "classic" manual transduction executed with open handling steps. The manual transduction of CD34+ cells was performed in parallel to the automated manufacturing of gene-engineered HSCs with the CliniMACS Prodigy Instrument. The experimental details were the same as for the automated manufacturing by the CliniMACS Prodigy HSC Engineering process (table 2).

The cell viability of the CD34+ cell samples were analyzed over time from day 0 to day 2 (table 4). Cell viability was continuously over 90% and confirmed highly viable cells during the complete viral transduction procedure. As a control, CD34+ cells were cultured without performing transduction (untransduced control) and showed a comparable cell viability of over 90% on day 2. This confirmed that performing transduction with either the CliniMACS Prodigy or a manual approach (manually transduced) does not have an influence on cell viability. Moreover, no difference in cell viability on day 2 was observed for the harvested gene-engineered CD34+ cells on the CliniMACS Prodigy HSC Engineering process or with manual transduction.

In order to determine cell recovery, the number of viable cells on day 0 was set to 100%. The related values measured on day 2 were in most cases over 100% and showed comparable values for the untransduced control, the manually transduced CD34+ cells, and the CD34+ cells processed on the CliniMACS Prodigy (fig. 5). The data of the CliniMACS Prodigy experiments showed a much lower standard deviation (SD) of 9.74% and thus higher robustness than the manual untransduced control (SD 27.13%) and manually transduced control (SD 27.96%).

Cell viability	Day 0	Day 1	Day 2		
	CD34+ cells*	CliniMACS Prodigy	Untransduced control	Manually transduced	CliniMACS Prodigy
Mean	94.56%	94.09%	95.26%	95.55%	96.31%
Standard deviation	3.32%	2.02%	1.67%	1.40%	1.46%

Table 4: Measured cell viability of CD34+ cells by determination of PI negative frequency on day 0, day 1, and day 2 (n=8).
* Human CD34+ cells were the cellular starting material for further culture (untransduced control, manually transduced, CliniMACS Prodigy).

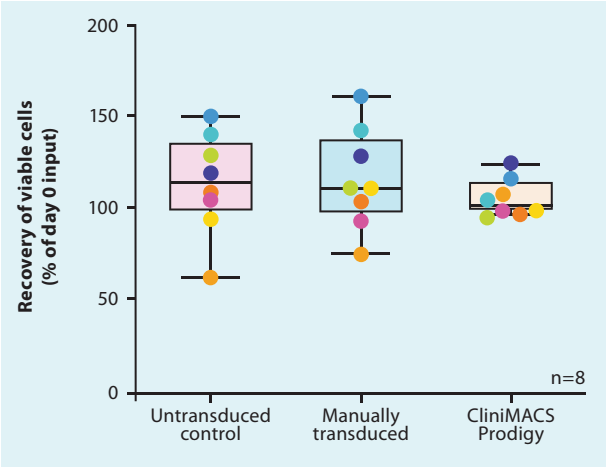

Figure 5: Cell recovery of viable cells on day 2 related to the number of viable cells measured on day 0 as set to 100%.

The phenotypic analysis on day 0 and 2 revealed a viable CD34⁺CD90⁺ population (fig. 6). The detected values were between 5% and 10% and were comparable between day 0 and day 2, similar results were seen between the untransduced control, the manually transduced CD34⁺ cells, and the CD34⁺ cells processed on the CliniMACS Prodigy.

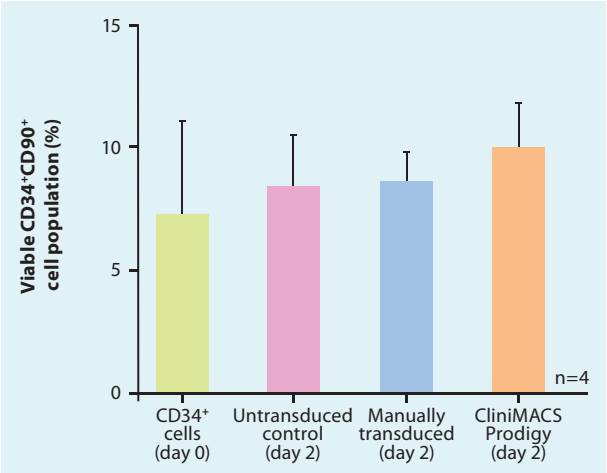

Figure 6: Frequency of viable CD34⁺CD90⁺ cells on day 0 and day 2 (n=4).

After harvest on day 2, the gene-engineered CD34⁺ cells were further cultured manually to analyze the transduction efficiency by flow cytometry on day 5. Furthermore, CFU assays and VCN analysis were performed.

The transduction efficiency by flow cytometry was determined by the measurement of viable CD34⁺CD45⁺GFP⁺ cells on day 5. The results are shown in figure 7. The cells processed on the CliniMACS Prodigy showed a significantly higher transduction efficiency compared to the manually transduced cells (p=0.0349, n=8, paired t-test).

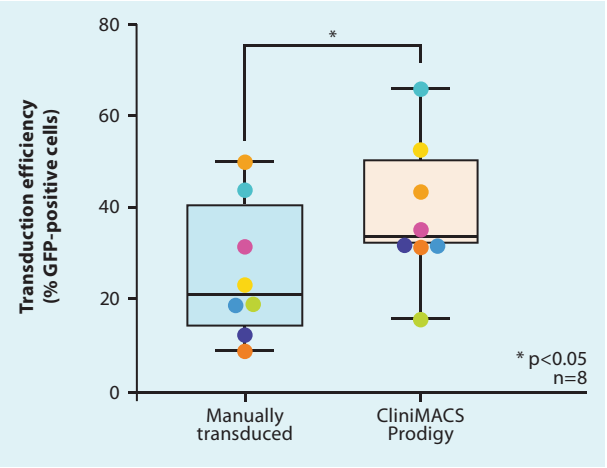

Figure 7: Transduction efficiency of viable CD34⁺CD45⁺ cells detected on day 5 by measurement of GFP⁺ cells.

Furthermore, transduction efficiency was measured by CFU assays on day 16, after culture for 14 days in StemMACS HSC-CFU Medium. Figure 8 shows exemplary microscopic images of the generated colonies on day 16 for transduced cells by the CliniMACS Prodigy HSC Engineering process (fig. 8A and B) and manual transduction (fig. 8C and D). Figure 9 exemplifies the quantified transduction efficiency that is visualized according to the microscopic analysis. Cells transduced by the CliniMACS Prodigy led to higher GFP⁺ colonies compared to manually transduced cells. Figure 10 shows a comparable distribution of the total number of colonies between untransduced and transduced CD34⁺ cells either manually or via the CliniMACS Prodigy Instrument. Thus, the proliferation and differentiation potential of the used CD34⁺ cells were maintained during transduction.

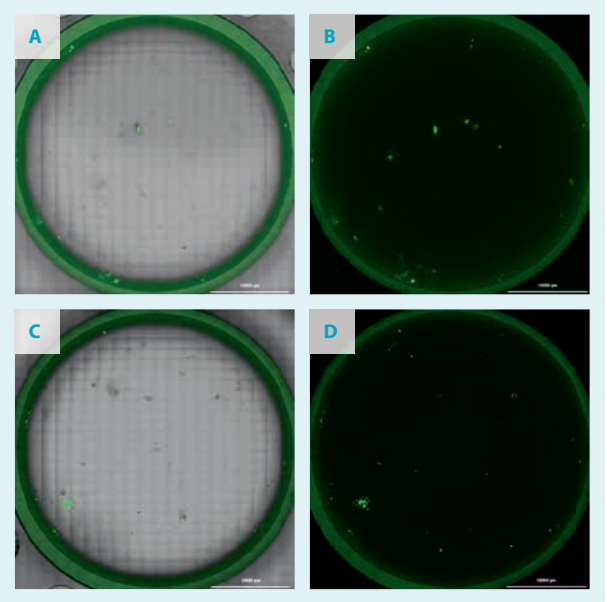

Figure 8: Transduction efficiency of colonies derived from transduced and differentiated CD34⁺ cells on day 16 as measured on the Cytation 3 (n=3). The generated colonies by transduced cells with the CliniMACS Prodigy HSC Engineering process (A and B) and manual transduction process (C and D) were visualized by microscopic brightfield and fluorescent images to detect the GFP⁺ colonies.

On day 16, the VCN of the cells processed on the CliniMACS Prodigy were comparable to the manual control (fig. 11). All measured VCNs per transduced cell were between 1.5 and 3.

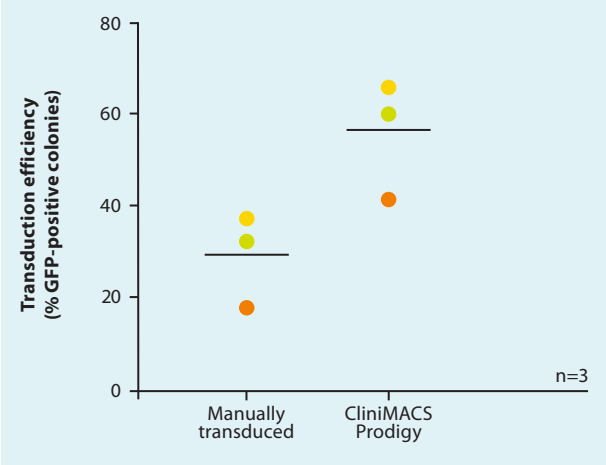

Figure 9: Quantified transduction efficiency by CFU assays on day 16 according to microscopic analysis using Cytation 3 (n=3).

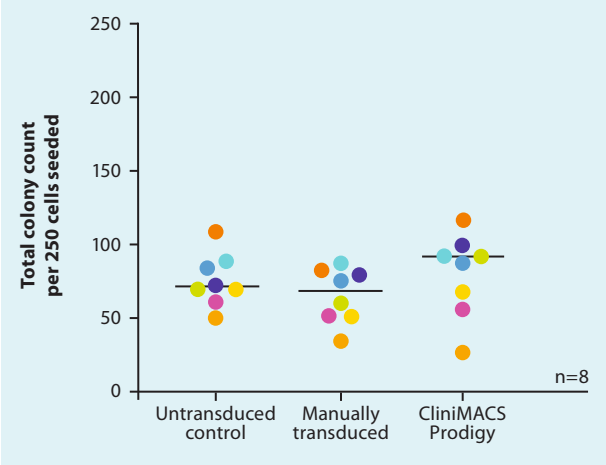

Figure 10: Total number of colonies on day 16 per 250 cells seeded (n=8).

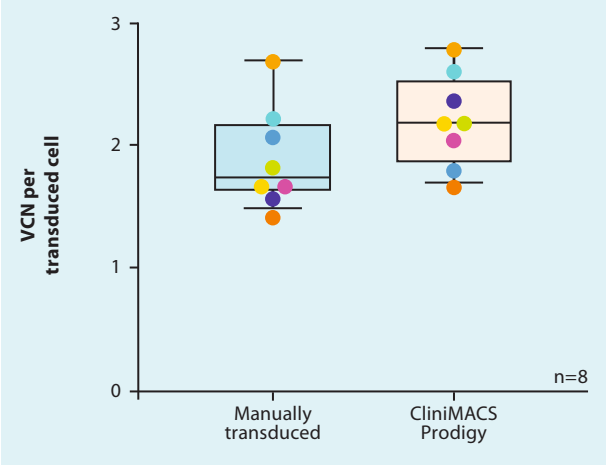

Figure 11: VCN per transduced cell determined by qPCR on day 16 (n=8).

Conclusions

- Automated cell processing and genetic manipulation of HSCs can be performed efficiently in a closed system with minimal user interaction by the CliniMACS Prodigy HSC Engineering process.
- To ensure reliable and reproducible cell manufacturing, IPC/QC of gene-engineered HSCs has to be investigated at several time points. The integrated sampling pouches on the CliniMACS Tubing Sets allow for controls to be collected at any time during the cell manufacturing process.
- With the recovery of $CD34^+$ cells after transduction, the CliniMACS Prodigy HSC Engineering process resulted in a significantly lower variability compared to manual processing, generating higher transduction rates at low, non-saturating MOIs for the viral transduction of human $CD34^+$ cells with GFP vector.
- The IPC/QC results at different process time points confirmed the functionality of gene-engineered HSCs, either manually transduced or via the CliniMACS Prodigy HSC Engineering process.

References

1. Papanikolaou, E. *et al.* (2021) The promise and the hope of gene therapy. Front Genome Ed. 3:618346. doi:10.3389/fgeed.2021.618346.
2. de Dreuzy, E. *et al.* (2016) Current and future alternative therapies for beta-thalassemia major. Biomed J. 39(1):24-38. doi: 10.1016/j.bj.2015.10.001.
3. Papanikolaou, E. *et al.* (2010) Major challenges for gene therapy of thalassemia and sickle cell disease. Curr. Gene Ther. 210(5): 494-12. doi: 10.2174/156652310793180724.
4. Lebensburger, J. *et al.* (2008) Progress toward safe and effective gene therapy for beta-thalassemia and sickle cell disease. Curr. Opin. Drug Discov. Devel. 11(2):255-32.
5. Ferrari, G. *et al.* (2021) Gene therapy using haematopoietic stem and progenitor cells. Nat.Rev. Genet. 22(4):216-234. doi: 10.1038/s41576-020-00298-5.

Miltenyi Biotec

Miltenyi Biotec B.V. & Co. KG | Phone +49 2204 8306-0 | Fax +49 2204 85197 | macsde@miltenyi.com | **www.miltenyibiotec.com**
Miltenyi Biotec provides products and services worldwide. Visit **www.miltenyibiotec.com/local** to find your nearest Miltenyi Biotec contact.

130-130-772