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Genetic Modification of Human Hematopoietic Cells: Preclinical Optimization of Oncoretroviral-mediated Gene Transfer for Clinical Trials

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Summary

This chapter provides information about the oncoretroviral transduction of human hematopoietic stem/progenitor cells under clinically applicable conditions. We describe in detail a short –60 h transduction protocol which consistently yields transduction efficiencies in the range of 30–50% with five different oncoretroviral vectors. We discuss a number of parameters that affect transduction efficiency, including the oncoretroviral vector characteristics, the vector stock collection, the source of CD34+ cells and transduction conditions.

Keywords

Retroviral gene transfer; CD34+ cells; Hematopoietic stem cells; Transduction; Oncoretroviral vector; Vector production; RetroNectin; Tissue culture bags; Preclinical optimization

1. Introduction

Human hematopoietic stem cells (HSCs) can be genetically modified with oncoretroviral vectors to express therapeutic genes for the treatment of either inherited (1–12) or acquired disorders (1–4, 6, 7, 11). Oncoretroviral vectors have the ability to integrate permanently into the chromosomes of mammalian cells. HSCs have both self-renewal and multilineage differentiation capacities. Thus, stable engraftment of genetically modified HSCs potentially leads to the stable expression of transgene in stem and/or progenitor cells, depending on the promoter/enhancer combination that controls transgene expression.

Active target cell cycling is required for integration of oncoretroviral vectors into the host cell's chromosomal DNA (12). Primitive HSCs which provide long-term engraftment are mostly quiescent (9) and thus transduced at a lower frequency than cycling progenitors. Prestimulation with early acting cytokines is therefore required for efficient gene transfer into HSCs with oncoretroviral vectors. Compared with several cytokine combinations that contain IL-3, and/or IL-6 (5, 10), a mix of stem cell factor, FLT3 ligand, and thrombopoietin induces better, more synchronous ex vivo CD133+ (8) or CD34+/Thy.1+ (13) cell expansion, yields higher transgene marking (14), improves survival (13), and maintains the multipotential engraftment ability of HSCs in NOD/SCID mice (5). Ex vivo stimulation with hematopoietic growth factors increases the expression of the envelope receptors on HSCs

(15–19) and the number of cells that are actively cycling, and also maintains cell viability by inhibiting HSC apoptosis (20). Prolonged ex vivo manipulation of HSCs may result in either differentiation of HSCs and/or loss of their engraftment potential (21–24). Pre-stimulation must therefore allow the quiescent stem cells to enter the cell cycle while preserving their engraftment potential.

GaLV and amphotropic envelope receptors, Pit-1 and Pit-2, respectively, are inducible phosphate receptors. Their density on the target cells defines the rate of transduction efficiency (19, 25). Human CD34+ cells enter active cycling after 36–48 h of cytokine stimulation (26), while the expression of Pit-1 is concomitantly induced (15, 25). The length of prestimulation, the number of transduction cycles, and time interval between two transductions varies among various published retroviral gene transfer protocols (14, 26–34), so that total ex vivo cell manipulation time fluctuates between 60 and 120 h. In this chapter, we describe in detail our short transduction protocol of 60 h (27) which consistently yields transduction efficiencies in the range of 30–50% with five different oncoretroviral vectors, i.e., SFG^{mpsv}-eGFP, SFG^{mpsv}-NTP4, SFG^{mpsv}-DHFR/CD, SFG^{mpsv}-huTyr, and SFG^{mpsv}-huTyr-ires-eGFP produced under serum-free conditions. We have previously demonstrated that this protocol does not alter the engraftment potential of the CD34+ transduced cells and that the transgene expression in the progeny of the repopulating CD34+ cells in NOD/SCID mice in vivo is at least as good as that of cells transduced in the presence of serum or using longer, more conventional transduction protocols (27).

Physical parameters such as the number of viral particles per cell (multiplicity of infection-MOI) and the virus concentration also affect retroviral transduction efficiency (27). Increased human CD34+ cell transduction has been demonstrated by colocalizing the vector particles with the target cells, using the RetroNectin[®]-CH-296 domain of fibronectin to coat tissue culture vessels (29, 35–38), adding polycations into the transduction media (39–43), or spinoculation (44–50). We and others have reported that polycations (27, 35), i.e., polybrene or protamine sulfate, and centrifugation (14, 27) are not required for efficient transduction of HSCs in the presence of RetroNectin[®]. Vector preloading might improve gene transfer efficiency by both increasing viral particle-cell interaction and decreasing exposure to inhibitory factors contained in the vector stocks (51). Systematic prescreening of vector stocks titers on human CD34+ cells might eliminate producer cell clones that secrete inhibitory factors. In our hands, preloading with prescreened vector stocks does not result in higher CD34+ cell transduction efficiency (27).

Keeping the CD34+ transduction efficiency around 30–50% is also advisable to reduce the probability of multiple integrations in single cells (52) and limit the risk of insertional mutagenesis previously observed in four patients who developed T-cell leukemia after integration of a Mo-MuLV-derived, LTR-driven vector. Indeed, it has been shown that oncoretroviruses preferentially integrate in open chromatin regions at transcriptionally active sites in the vicinity of cellular promoters. High vector copy numbers per cell increases the risk of insertional mutagenesis (53), and there is a quantitative correlation between overall gene transfer rate and integration frequency in single cells (52). There is usually less than one vector copy per cell when gene transfer rates are less than 30%, and an average of three vector copy per cell for gene transfer rates around 60% (52). Evaluation of retroviral

insertion sites with linear amplification PCR (LAM-PCR) has been considered for monitoring clonal integration sites in patient cells. LAM-PCR is an informative test more than a diagnostic one, but data accumulated through systematic monitoring of integration sites might help to further understand the mechanism of this potential adverse effect.

Retroviral transduction protocol for human CD34⁺ cells under clinically relevant serum-free conditions is depicted in Fig. 1. Briefly, isolated human CD34⁺ cells are prestimulated at a cell density of $2-8 \times 10^5$ cells/mL for 36 h in X-Vivo ten serum-free media supplemented with recombinant human stem cell factor, thrombopoietin, and FLT3-ligand. Following prestimulation cells are washed and transferred into RetroNectin[®]-coated bags with serum-free vector stock supplemented with fresh cytokines. Approximately 12 h after, cells are washed and resuspended in a new batch of vector stock with fresh cytokines. Cytokines are maintained throughout ex vivo transduction at a concentration of 100 ng/mL for each cytokine. Twelve hours following the end of the second transduction, cells are then wash and concentrated in a transfusion bag. Samples are collected for FACS or colony-forming assays, or to transplant into NOD/SCID mice. Additionally an aliquot of transduced cells, and DNA and RNA isolated from genetically modified cells are archived.

Serum-free ex vivo cell manipulations do not compromise either the transduction efficiency or the engraftment potential of human CD34⁺ cells. Prestimulation for 36 h (27, 35) and two cycles of transduction 12 h apart are sufficient to maximize the transduction efficiency. Adding a third transduction cycle or performing transduction cycles 24 h apart does not result in better gene transfer. Coating of tissue culture vessels with Retro-Nectin[®] improves both human CD34⁺ cell transduction by eight to tenfold and increases cell viability by protecting the cells from apoptosis. These effects are observed with doses of RetroNectin[®] as low as $2 \mu\text{g}/\text{cm}^2$ (27) (Fig. 2).

The important factors to achieve adequate levels of gene transfer are the vector titer, the physical proximity of the target cells and viral particles, the cell cycle status of the cells, and the level of expression and density of the envelope receptor on the target cells. The vector concentration becomes limiting when it is lower than $4-5 \times 10^5$ tu/mL, and this limitation cannot be compensated by using higher transduction volumes to increase the multiplicity of infection (MOI) (27). Spinoculation might increase gene transfer in human CD34⁺ cells (44–50) ; however, under serum-free conditions combining polycations with spin-oculation significantly decreases cell viability without any gain in gene transfer (27).

2. Materials

2.1. Maintenance of Vector-Producing Cell Line and Serum-Free Vector Production

1. PG13 producer cell line (*see* Note 1).
2. Dulbecco's Modified Eagle Medium (DMEM) high glucose (1×), liquid, with L-glutamine and sodium pyruvate, but no phenol red (DMEM-HG), 500 mL.
3. Fetal Bovine Serum (FBS), prescreened for producer cell growth and vector production, 500 mL.

4. Gentamicin 50 mg/mL, 10 mL storage at room temperature. Final concentration in media, 50 µg/mL.
5. Trypsin-EDTA.
6. Dulbecco's Phosphate-Buffered Saline (D-PBS), without calcium and magnesium, 500 mL.
7. X-Vivo 10 Media without phenol red, 1 L (BioWhittaker Cambrex Bioscience Walkersville, Inc.) (*see* Note 1).
8. T75, T150 Vented Tissue Culture Flasks.
9. Ten tray-Cell Factories, cell culture surface area: 6,320 cm² (Nalgene Nunc, Inc.).
10. Cell Factory Accessories; CF HDPE Connectors, White Tyvek Cover caps, Blue sealing caps, Bacterial Air Vent filter, silicone tubing, and a tubing clamp. (Nalgene Nunc, Inc.).
11. Kimax Aspirator bottle, 2 L (Kimble Glass, Inc.).
12. Sterile 2- and 4-Spike to Membrane Port Adapter Sets (Baxter Healthcare).
13. Sterile Plasma Transfer Sets (Baxter Healthcare).
14. Autoclavable Septum Closure (Nalgene Nunc, Inc.).
15. Sterile Square Media Bottles, 125 mL, 250 mL, 500 mL, 1 L, and 2 L (Nalgene Nunc, Inc.).
16. Dual inline filter (No. 4C8030, Baxter Healthcare).
17. 20 µm filter (No. 4C7704, Baxter Healthcare).
18. Sepa-cell filter (No. 4C2481, Baxter Healthcare).
19. Vented Spike Adapter (Nalgene Nunc, Inc.).
20. Bioprocessing Bag 5, 10 L (TC Tech).
21. Cryocyte Freezing Containers, 250, 500, 1,000 mL (Baxter Healthcare Corporation).
22. Cryocyte Cassettes (Custom Biogenics).

¹The PG13 parental packaging cell line (ATCC Catalog number; CRL-10686) is derived from the murine NIH 3T3 TK⁻ embryo fibroblasts (57) and contains a bipartite retroviral packaging system. The Moloney murine leukemia virus gag-pol expression construct was introduced by cotransfection using the herpes simplex virus thymidine kinase gene, and the gibbon ape leukemia virus (GaLV) envelope was introduced by cotransfection with a mutant methotrexate dihydrofolate reductase gene (DHFR*). These selection markers confer resistance to amethopterin. Selection against loss of the plasmid DNAs conferring the packaging functions can be performed by growing the cells in medium containing dialyzed FBS and 100 nM amethopterin for 5 days followed by cultivation in medium containing HAT and untreated FBS for an additional 5 days. Resting the cells in hypoxanthine-containing media for at least 2 days is required for eliminating amethopterin from the environment (*see* ATCC recommendations). Cross-infection of the packaging cells with vector stocks originally obtained by transfection increases the number of high-titer packaging clones relative to direct transfection (43, 58). It is possible to generate stable high-titer PG13 clones by cross-infection with vector stocks derived from either Phoenix-Eco (<http://www.stanford.edu/group/nolan/publications/publications.html>), or VSV-G pseudotyped 293GPG cells (59). Generation and selection of high-titer PG13 producer cell lines are summarized elsewhere (60).

2.2. Cell Preparation and Prestimulation

1. Human bone marrow (BM), mobilized peripheral blood (MPB), or umbilical cord blood (UCB)-derived human CD34+ cells, either fresh or frozen, will be used for gene transfer. Purification of human CD34+ cells is beyond the scope of this chapter.
2. Human Serum Albumin Solution 5%, 250 mL (Buminate; Baxter Healthcare Corporation).
3. 10% Dextran 40 in 0.9% Sodium Chloride IV, 500 mL (Gentran Viaflex, Baxter Healthcare Corporation).
4. Transfer Packs with Spike, 300 mL (Baxter Healthcare Corporation).
5. VueLife FEP bag, 300–500 mL (American Fluoroseal Corporation). The average fill volume limits the solution height approximately to 1 cm allowing optimal gas transfer.
6. Interlink System Blood Bag Injection Site (Baxter Healthcare Corporation).
7. X-Vivo 10 Media without phenol red, 1 L, storage at +4°C.
8. Gentamicin 50 mg/mL, 10 mL storage at room temperature. Final concentration in media, 50 µg/mL.
9. Sterile 2- and 4-Spike to Membrane Port Adapter Sets.
10. Sterile Plasma Transfer Sets.
11. Autoclavable Septum Closure.
12. Recombinant Human Stem Cell Factor (SCF), lyophilized 10 µg vial (PeproTech, Inc.) Both the lyophilized protein and the reconstituted solution are stored at –20°C. The lyophilized protein is diluted in 1 mL injectable dH₂O and filtered to generate a stock solution at 100 ng/µL; 1 µL should be added for each mL of media for a final concentration of 100 ng/mL.
13. Recombinant Human Fms-related tyrosine kinase 3 ligand (FLT3L), lyophilized 10 µg vial (PeproTech, Inc.) Both the lyophilized protein and the reconstituted solution are stored at –20°C. The lyophilized protein is diluted in 1 mL injectable dH₂O and filtered, to generate a stock solution at 100 ng/µL; 1 µL should be added for each mL of media for a final concentration of 100 ng/mL.
14. Recombinant Human Thrombopoietin (TPO), lyophilized 10-µg vial (PeproTech, Inc.) Both the lyophilized protein and the reconstituted solution are stored at –20°C. The lyophilized protein is diluted in 1 mL injectable dH₂O and filtered to generate a stock solution at 100 ng/µL; 1 µL should be added for each mL of media for a final concentration of 100 ng/mL.
15. Sterile Water Injection, USP, 10-mL vial.
16. Millex-GV PVDF 0.2-µm, syringe-driven filter unit (Millipore Corporation).

2.3. Coating of Cell Culture Bags with RetroNectin®

1. RetroNectin, lyophilized 2.5 mg protein (Takara Bio Inc.). The lyophilized protein should be stored at +4°C and the reconstituted solution (1 mg protein/mL) should be stored in aliquots at –20°C. For reconstitution, add sterilized 2.5 mL distilled water to obtain a 1-mg protein/mL solution, filtrate through 0.22-µm filter and store in aliquots at –20°C. To keep the protein integrity, avoid vigorous mixing (no vortex) and repeated freeze-thaw cycles.
2. Human Serum Albumin Solution 5%, 250 mL.
3. 0.9% Sodium Chloride Injection, USP, 250 mL.
4. Transfer Packs with Spike, 300 mL (Baxter Healthcare Corporation).
5. VueLife FEP bag, Average fill 225 or 255 mL, and surface areas are 500 and 561 cm², respectively (American Fluoroseal Corporation). For RetroNectin® coating, bags with improved cell adherence should be used.
6. Interlink System Blood Bag Injection Site.
7. Sterile Water Injection, USP, 10 mL vial.
8. Millex-GV PVDF 0.2 µm, syringe-driven filter unit.

2. 4. Oncoretroviral Transduction in RetroNectin®-Coated Bags (Fig. 1b)

1. Frozen vector stock in Cryobags from **Subheading 4.2.1.**
2. Prestimulated human CD34+ cells in VueLife FEP bag from **Subheading 4.2.2.**
3. RetroNectin®-coated adherence improved VueLife FEP bag from **Subheading 4.2.3.**
4. 0.9% Sodium Chloride Injection, USP, 250 mL.
5. Transfer Packs with Spike, 300 mL.
6. Interlink System Blood Bag Injection Site.
7. X-Vivo 10 Media without phenol red, 1 L, storage at +4°C.
8. Gentamicin 50 mg/mL, 10 mL, storage at room temperature. Final concentration in media, 50 µg/mL.
9. Sterile 2- and 4-Spike to Membrane Port Adapter Sets.
10. Sterile Plasma Transfer Sets.
11. Autoclavable Septum Closure.
12. Recombinant Human SCF, stock concentration 100 ng/µL, 1 µL should be added for each mL of media, final concentration 100 ng/mL (*see Subheading 4.2.2.*).
13. Recombinant Human FLT3 L, stock concentration 100 ng/µL, 1 µL should be added for each mL of media, final concentration 100 ng/mL (*see Subheading 4.2.2.*).

14. Recombinant Human TPO, stock concentration 100 ng/ μ L, 1 μ L should be added for each mL of media, final concentration 100 ng/mL (*see Subheading 4.2.2*).
15. Sterile Water Injection, USP, 10 mL vial.

2.5. Single-Colony PCR for Determining Transduction Efficiency

1. Colony-forming units that are growing in methylcellulose semisolid media.
2. DNA Lysis Buffer- Tris 5 mM, 0.45% Tween 20 at pH 8.0. Store at room temperature.
3. Proteinase K, Solution (Roche) in 10 mM Tris-HCL, pH 7.5, 15.1 mg/mL. Store at 2–8°C.
4. Dulbecco's Phosphate-buffered Saline (D-PBS), without calcium and magnesium, 500 mL.
5. Phenol/ CHCl_3 /isoamyl alcohol (25:24:1) mixture.
6. Glycogen, 20 mg/mL solution (Boehringer-Mannheim).
7. Ammonium Acetate, 7.5 M Solution.
8. Absolute Ethanol.
9. AmpliTaq Gold-DNA Polymerase (Applied Biosystems).
10. GeneAmp 10 $\times$ PCR Buffer II (Applied Biosystems).
11. 25 mM MgCl_2 solution (Applied Biosystems).
12. dNTP GeneAmp Blend, 10 mM (Applied Biosystems).
13. β -Actin forward and reverse primers.
14. Transgene specific forward and reverse primers.

3. Methods

3.1. Maintenance of Vector-Producing Cell Line and Serum-Free Vector Production

1. After thawing the vial(s) at 37°C, dilute the cells at 1:10 (v:v) ratio with complete DMEM media supplemented with 10% FBS containing 50 μ g/mL Gentamicin and pellet the cells by centrifugation. Aspirate the DMSO-containing supernatant. Plate the retroviral packaging cells in complete media in T150 flasks and expand the cells into four (4) 10-tray Cell Factories (Note 2).
2. Once the cells are plated in ten tray-Cell Factories in 1,000 mL complete media, let them grow for approximately 24 h at 37°C and 5% CO_2 . The following day, wash the cells two to three times with D-PBS, and replenish the cell factories with 625 mL of X-Vivo 10 media. After incubation at 32°C (or 37°C) and 5% CO_2 for 24 h, collect the vector stocks from cell factories in a 5-L bioprocessing bag by constructing a four-way manifold system using sterile 4-spike to Membrane Port Adapter sets and a peristaltic pump, at a flow rate of 500 mL/min. Each harvest

should be step-filtered on the day of collection through a serially connected filtration system using a dual inline filter, a 20- μ filter, and a Sepacell filter and transferred into a second sterile bioprocessing bag (54, 55). Two additional harvests can be performed on each of the following 2 days without significant vector titer loss (27, 54, 56).

3. Store the first two harvests at 4°C until pooling. After filtration of the third harvest, pool the three filtered harvests into a sterile 10-L bioprocessing bag, and aliquot as needed into sets of eight sterile Cryocyte Freezing Containers using a peristaltic pump (54). After filling, load the vector stocks aliquots into Cryocyte cassettes and store at -80°C (*see* Note 3).
4. Biosafety testing and vector titration (*see* Note 4) should be performed on each batch of clinical-grade vector stocks.

²Production of vector stocks under serum-free conditions increases the biosafety of retroviral transduction of human CD34+ cells for clinical applications. The risk of transmitting spongiform encephalitis by exposure of target cells to poorly defined bovine products, the demonstration that better cell expansion can be obtained in the absence of serum (61), and the fact that some fetal bovine serum proteins have been shown to be immunogenic (62, 63) are prompting the development of gene transfer protocols under serum-free conditions. Although the PG13 packaging cells are serum-dependent for proliferation, they adapt to short-term culture in serum-free medium, allowing serum-free vector stock collection (64). Vector stocks produced under serum-free conditions display similar (65) or lower (27, 66) titers on indicator Hela cells. However, the transduction efficiency in human CD34+ cells is comparable, the differentiation of CD34+ cells is less (27) (Fig. 2 i), and in vivo gene marking levels in NOD/SCID mouse are at least as good under serum-free conditions (27) in comparison to those obtained in presence of serum. Among the tested serum-free media (X-Vivo 10, X-Vivo 15, Stem-Pro 34 SFM, IMDM, QBSF60), X-Vivo 10 media provided the highest titers after either 16 or 24 h of incubation (27, 56).

The stability of Mo-MuLV-derived retroviral vectors can be augmented by increasing the medium osmotic pressure from 335 up to 410–450 mOsm/kg, which decreases the cholesterol content of both virus particles and producer cells (67). The vector stocks produced under high osmolar conditions have been shown to yield three to fourfold higher vector titers. However, these conditions have not been tested yet on large-scale vector stocks production (67).

Adding sodium butyrate to the media during vector production has been shown to enhance expression of the vector and packaging construct, leading to a 10–1,000-fold increase in viral production (68). However, in our experience, adding sodium butyrate to X-Vivo 10 media did not increase vector titers (unpublished data).

Vector stocks with titers below 5×10^5 tu/mL may be concentrated, but the type of envelope should be considered. The vector stocks produced by the packaging cell lines that encode either Eco-, Amphi-, or GaLV-envelope proteins cannot be concentrated by ultracentrifugation. Those envelope proteins are composed of two domains: an extracellular domain and a transmembrane domain which are linked by disulfide bonds only. The stress of centrifugation and filtration often causes the surface domain to be shed which results in soluble, free-floating surface domain peptides that can block infection by saturating receptors on the target cells. On the other hand, VSV-G pseudotyped viral vectors infect cells via membrane fusion, and, are therefore not dependent on receptor recognition. VSV-G is a strong glycoprotein that can withstand ultracentrifugation at $50,000 \times g$ for 90 min at 4°C and allows viral particle concentration up to 100–200 fold. Sheer-force sensitive viral particles pseudotyped with Eco-, Amphi-, or GaLV- envelopes can only be concentrated up to tenfold by either low-speed centrifugation (9,500 rpm in Beckman rotor JA-14 at 4°C for 12 h) (41), or centrifugation and filtering for 35 min at $3,000 \times g$, 15°C through centrifugal filter devices with a 100,000 molecular weight cut-off (27).

When the cells reach confluence, each T150 flask contains approximately $4\text{--}5 \times 10^7$ cells. Six T150 flasks 70–90% confluent are used to seed a single 10-tray Cell Factory which is itself used, once it reaches 70–90% confluence, to seed four (4) 10-tray-Cell Factories (27). By reducing the volume of harvest medium to 0.1 mL per cm^2 , titers could be increased up to fourfold (66), and repetitive harvests of vector stocks are feasible over three to four consecutive days (27, 54, 56, 66) after an incubation period of 16 or 24 h at 5%CO₂ as previously published (56, 69, 70). The optimal incubation temperature for retroviral vector production is somewhat controversial. Some studies show greater retroviral vector inactivation and/or lower vector titers at 37°C when compared to 32°C (27, 54, 70, 71). On the other hand, the viral particles produced at 37°C are less rigid than those produced at 32°C. They are therefore more stable and thus provide higher transduction efficiency (56, 72). At this time, we recommend to compare side by side the titers of vector stocks harvested at 32°C and 37°C for the selected high-titer packaging cell clone.

³Frozen vector stocks collected in serum-free conditions and stored at -80°C in Cryocyte bags are stable over a period of at least 12 months, and adding either human serum albumin or FBS does not change the stability of the frozen vector stocks (27). The half-life of frozen vector stocks is suggested to be biphasic. Following 25–30% loss of vector titer upon the first freeze/thaw cycle, the decay of viral particles is slow, and half-life of this stage varies from 18 to 41 months (54, 73). Interestingly, testing the frozen vector stocks only on target/indicator cell lines may be misleading, as transduction efficiency in primary cells, i.e., T lymphocytes or CD34+ cells may not show the same decrease (27, 54). This different outcome between cell types may be due to differential cell surface receptor saturation of envelope receptors on indicator cell lines such as Hela and primary cells.

It is a common research practice to filter vector stocks through a 0.45- μ m filter to remove cellular debris. Do not use filters that contain detergents, as wetting agents will affect the integrity of the viral particles as well as the viability of the target cells (unpublished observation).

3.2. Cell Preparation and Prestimulation (see Note 5) (Fig. 1a)

1. Transfer 125 mL of Dextran 40 in 0.9% Sodium Chloride and 125 mL of 5% Human Serum Albumin solution to a transfer bag by using the fluid pump mode of the Cytomate™ Cell Washer (*see* Note 6). This solution should be kept at +4°C with an expiration time of 24 h.
2. Transfer X-Vivo 10 Media to a transfer bag through a septum closure using a 2-spike transfer set. Add gentamicin and cytokines to the bag through an Interlink System Blood Bag Injection Site. The volume of prestimulation media should be calculated according to the total viable cell number using a final cell concentration of $2\text{--}8 \times 10^5$ cells/mL.
3. Remove the cryopreserved CD34+ cells from the vapor phase of liquid nitrogen, place into a sterile zipper-locked bag, and thaw the cells at 37°C in a waterbath until total conversion to liquid phase.

⁴Vector particle counts can be determined either by direct particle count using an electron microscope, or by indirect methods such as quantitative real-time PCR (74, 75) or transduction of target/indicator cells. Vector stocks contain infectious viral particles as well as noninfectious particles. The vector titers defined by transduction of target cells depend in part on the level of expression of the envelope receptors such as PiT-1, PiT-2, or ecotropic receptor (19, 76) which varies among target cells (27). When serial dilutions of the vector stocks are used to transduce CEM, HeLa, and human CD34+ cells, the transduction rates obtained on CD34+ cells are comparable to the transduction rates obtained on HeLa cells (27). We recommend to initially test the transduction efficiency on both the type of primary cells that will be used for clinical application and various target/indicator cell lines, and for subsequent vector titrations, to select the cell line that gives transduction rates comparable to that obtained on primary cells. The methods for determining vector stock titers are beyond the scope of this chapter.

⁵The source of hematopoietic stem cells (HSCs) used for retroviral transduction has been shown to affect the transduction efficiency and engraftment potential of the cells. UCB-derived CD34+ cells display the highest transduction efficiency and engraftment potential (77–79) as UCB contains larger numbers of primitive cells that are capable of forming secondary multipotential colonies upon replating in vitro (80–82), NOD/SCID repopulation potential of UCB-derived CD34+ cells in G0 or G1 phase are similar (83–85), while MPB-derived CD34+ cells SCID repopulating cells (SRCs) mainly reside in the G0 population (22). Adult HSCs are more dormant and less responsive to cytokine stimulation when compared with UCB. They require prolonged cytokine stimulation to enter the cell cycle, to upregulate envelope receptor expression, and to be efficiently infected by oncoretroviral vectors (35, 86). Higher levels of envelope receptor mRNA are present in UCB-derived HSCs when compared to HSCs derived from bone marrow (17, 18). The expression of the amphotropic receptor on MPB-derived CD34+ cells, as assessed by indirect immunofluorescence assay, was approximately one log higher than that of steady-state BM or PB CD34+ cells (87). G-CSF-mobilized peripheral blood CD34+ cells are the favored source of autologous HSCs in clinical transplantation; however, G-CSF + stem cell factor (SCF)-mobilized peripheral blood CD34+ cells have been shown to engraft better than G-CSF-mobilized peripheral blood or steady-state bone marrow-derived CD34+ cells in nonhuman primates (88–90). Additionally, engraftment after transplantation using GzSCF/SCF primed BM cells is more rapid than that using steady-state bone marrow in nonhuman primates (88). Similarly in a dog model, G-CSF + SCF primed marrow provided the highest in vivo gene marking, in comparison to steady-state marrow, and MPB (91). Resting the transduced cells for an additional 2 days in presence of SCF on RetroNectin® coated support was suggested to decrease active cycling and proliferation, and consequently resulted in a significantly higher in vivo engraftment when compared to nonrested cells in non-human primates (92–94).

In planning a clinical trial, the source of the human CD34+ cells should be carefully considered. Though it is possible to immunoselect CD34+ cells from frozen samples, the variability of the resulting CD34+ purity and yield (95, 96) makes it nearly impossible to reliably predict how many cells should be collected and stored for transduction. CD34+ cells from pooled leukapheresis MPB products or fresh bone marrow samples can be selected within 24–48 h upon collection. Both negative and positive fractions should subsequently be frozen for future use. Thawing the frozen purified human CD34+ cells in Dextran/Albumin solution increases the post-thaw viability (97). This method can be applied to a closed system with a reasonable cell viability and recovery by using an automated cell washer (98). Frozen/thawed UCB-derived CD34+ cells have been shown to express higher levels of amphotropic receptor mRNA, but not of GaLV receptor (Pit 1) mRNA (16). The ability of purified CD34+ cells to respond to cytokine stimulation does not change upon freezing and thawing (99).

⁶The fully automated cell washer system, Cytomate™ (Baxter Healthcare, Deerfield, IL), is a versatile instrument that can be programmed step by step for fluid transfer, media/solution preparation, cell washing and concentration. The spinning membrane is connected to a filtered wash bag, a buffer bag, and a waste bag. Four weight scales and probes monitor the fluid transfer between bags. The sterile disposable sets that are used for either fluid transfer or cell wash create closed fluid path, and allow cell processing under current Good Manufacturing Practice (cGMP). Cryobags, transfer bags, etc. can be docked to sterile disposable sets either using spikes or sterile connecting device, so that closed system requirements can be established throughout all stages of the ex vivo retroviral transduction process.

According to our experience, the mean recovery of viable CD34+ cells using the closed system cell washer is usually in the range of $97 \pm 5\%$, with a mean cell viability of $87 \pm 6.5\%$ (our unpublished data). In our hands, this constitutes a better recovery rate than conventional open system cell washing with centrifugation. The cell wash process takes approximately 1 h.

4. Wash the cells on the Cytomate™. Set up the Cytomate disposable cell wash set on the Cytomate™ as per the manufacturer's instructions. Connect two bags to the wash/buffer line with a Y connector through spikes. The first wash buffer contains 500 mL D-PBS with 2% HSA, and the second bag should have ice-cold Dextran/Albumin solution. Close the line on the first wash buffer with Roberts clamp. Attach the bag containing the CD34+ cells to the cell source line on the Cytomate disposable cell wash set; dilute the cells twice with the Dextran/Albumin solution (v:v) at the rate of 20 mL/min, and then with the same volume at the rate of 60 mL/min. Total volume of Dextran/Albumin solution transferred to the source bag will be at least equal to the volume of source bag and should not be in excess of two volumes of the source bag. Gently shake the bags during dilution steps in order to avoid cell sedimentation. Close the Roberts clamp on the line of the Dextran/Albumin solution and open the clamp to D-PBS with 2% HSA solution. The CD34+ cells are washed and concentrated by flowing through the spinning membrane, and subsequently transferred into a collection bag. Rinse and wash the bag containing the CD34+ cells and tubing with an additional 100 mL of D-PBS with 2% HSA solution in order to minimize the cell loss.
5. Detach the cell collection bag and with an Interlink System Blood Bag Injection Site take a sample for cell count, viability and flow cytometry analysis. Calculate the amount of prestimulation media needed and store in a transfer bag as described in **Subheading 4.2.2**.

Example. plating density – 5×10^5 cells/mL, total number of cells – 150×10^6 cells

Volume of Prestimulation Media = Total number of cells/Plating density

Volume of Prestimulation Media = 150×10^6 cells / 5×10^5 cells/mL = 300 mL

An additional 10% volume should be accounted for loss in tubing; therefore, 330 mL (300 + 30 mL) of X-Vivo 10 media supplemented with cytokines (330 µL from the main 100 ng/µL stock) and Gentamicin (330 µL from the main 50 mg/mL stock) should be prepared.

An additional bag containing only X-Vivo 10 without cytokines and without Gentamicin should be transferred to a transfer bag. The volume should be equal to two volumes of cell suspension.

Install a new cell washer set on the Cytomate™ as per the manufacturer's instructions. Connect the bag containing the prestimulation media and the bag containing the X-Vivo 10 wash media through a Y-connection and attach to the wash buffer line. The cell collection bag containing the washed CD34+ cells from **step 5** will now be the source bag. Close the Roberts clamp on the prestimulation media until the cells are washed and concentrated through the spinning chamber with the X-Vivo 10 wash buffer. Subsequently transfer the CD34+ cells to a Vuelife bag in the prestimulation media. Clamp the line to X-Vivo wash media and unclamp the Roberts clamp on the prestimulation media line.

6. Detach the VueLife bag from the set and incubate at 37°C, in 5% CO₂ incubator for 36 h.

3.3. Coating of Culture Bags with RetroNectin®

1. Calculate the amount of RetroNectin® according to the surface of the bags; the surface area for a 225-mL volume VueLife FEP bag is 500 cm², and the bags will be coated with RetroNectin® at a dose of 2 µg/cm².

Total amount of RetroNectin® = Surface Area × 2 µg/cm² = 1,000 µg = 1 mg

Dilute 1 mL of 1 mg/mL stock solution (*see Subheading 4.2.3.1*) in 49 mL of either 0.9% saline or D-PBS (final concentration of 20 µg/mL; for effective coating RetroNectin® concentration should be kept between 20 and 100 µg/mL) and transfer into VueLife bag through interlink injection site. Incubate either at room temperature for 2 h or overnight at +4°C.

2. Prepare 100 mL blocking solution in a transfer bag as 2% Human Serum Albumin (40 mL 5% human serum albumin solution) and 0.9% NaCl (60 mL). After draining the RetroNectin®-containing solution to a waste bag through a 2-spike connector, transfer the blocking solution to the VueLife bag.
3. After 1/2 h incubation at room temperature drain the solution to the waste bag and gently wash the bag with another 100 mL of normal saline. If the RetroNectin®-coated bag is not used immediately, it can be kept at +4°C for approximately 1 week.
4. Depending on the target CD34+ cell dose, it may be necessary to coat two or more bags with RetroNectin® (*see Note 7*).

3. 4. Retroviral Transduction in RetroNectin®-Coated Bags (Fig. 1b)

1. Install a new cell washer set on the Cytomate™ as per the manufacturer's instructions. Connect the bag containing the prestimulated cells to the source line, the VueLife FEP–RetroNectin®-coated bag/s to the wash/collection line. Connect two bags to the wash/buffer line with a Y connector through spikes. The first wash

⁷For prestimulation, CD34+ cells can be plated at cell densities of 1×10^5 – 1×10^6 cells/mL without altering their viability during a 36-h incubation period. The plating of human CD34+ cells at different cell concentrations – 2×10^5 , 5×10^5 , 8×10^5 cells/mL – does not influence the ex vivo cell expansion rate – 1.7 ± 0.2 , 2.1 ± 0.27 , 1.85 ± 0.17 fold, respectively (unpublished data). At low cell density (2×10^5 cells/mL), the percentage of CD34+/CD38- cells is statistically higher than the percentage obtained at higher cell densities (Fig. 2 iii), but increasing the cell density from 2×10^5 , 5×10^5 to 1×10^6 (MOI values range from 0.5 to 2) do not change the transduction efficiencies (45.1 ± 13 , 54 ± 10.2 , $49.3 \pm 8.6\%$, respectively) ($p > 0.05$) (unpublished data). The culture of cells at low density requires more cytokines and bags. To our knowledge, the RC3 centrifuge (Sorvall) is the only centrifuge suitable for spinoculation. It can accommodate two bags with a maximum volume of 2×250 mL per cycle, which limits the volume of transduction to 500 mL per run. Starting with at least 2×10^6 viable CD34+ cells/kg for a 70-kg adult requires a total of at least 140×10^6 viable cells to be transduced. Using these calculations, we recommend a cell plating density in the range of 3 – 5×10^5 cells/mL. We recently observed that spinoculation at $1,000 \times g$ for 2 h at 10°C increases the transduction rate of the human CD34+ cells approximately twofold ($28.5 \pm 9.7\%$ vs. $55 \pm 7.8\%$, $p < 0.05$), while the viability of the cells was similar among the groups (unpublished validation data).

After prestimulation our hypothetical 140×10^6 viable cells become approximately 210×10^6 viable cells with a mean 1.5 fold expansion. Transduction volume will be around 420 mL when the cells are plated at a density of 5×10^5 cells/mL. The only available centrifuge option that can hold the bags horizontally is so far Sorvall RC3, and the custom-made holder/rotor system can only hold two bags of 240 mL at the same centrifugation cycle. The maximum number of the cells that can be spinoculated at once is 240×10^6 viable cells (adequate for an 80-kg adult patient using a cell dose of 3×10^6 cells/kg). We prefer not to exceed 5×10^5 cells/mL ex vivo culture cell density because of the significant loss of CD34+/CD38- population in high-density cultures.

buffer contains 300 mL D-PBS with 2% HSA or 0.9% NaCl, and the second bag will contain the transduction media including the vector stocks, the fresh cytokines, i.e., SCF, FL3L, TPO, and gentamicin. During the cell wash process, the Roberts clamp on the second bag line should be closed.

2. Transfer the cells washed with the first wash buffer back to the source bag in 50 mL volume, then give a pause interval and through an Interlink Injection Site collect a sample. Determine the cell count and viability. The viable cell count defines the amount of vector stocks that need to be thawed.
3. Thaw the cryobag(s) containing the frozen vector stocks quickly by placing the bag in an overwrap pouch in a 37°C water bath until disappearance of the ice crystals. For vector stocks with high concentration (above 5×10^5 tu/mL), vector titer should be adjusted by adding X-Vivo 10 Media with Gentamicin to prevent multiple vector copy integration per cell. Cytokines, SCF, FLT3L, TPO, should be added to the transduction media at a final dose of 100 ng/mL through an Interlink Injection Site. While attached to the cell washer for fluid transfer, the transduction media is roughly at room temperature.
4. For the second “cell wash step” on the Cytomate™, use the transduction media prepared in **step 3** and transfer the cells and transduction media to the RetroNectin®-coated bag(s). By using a Y connector, two RetroNectin®-coated bags can be connected to the wash/collection line.
5. Detach the RetroNectin®-Coated bags from the wash set and spinoculate at $1,000 \times g$ for 2 h at 10°C, then incubate at 37°C, in a 5% CO₂ incubator for 10 h.
6. For the second transduction cycle, repeat **steps 1–5** again.
7. At the end of the second transduction cycle (2 h spinoculation and 10 h of incubation), wash and resuspend the cells in 2% HSA-containing normal saline. Concentrate the cells at a final volume of 50 mL. Take samples for cell count and viability, flow cytometry analysis for CD34 and other relevant markers,

hematopoietic progenitor colony assay (*see* Notes 8 and ⁹), sterility, Mycoplasma, Endotoxin, RCR, and for archives, i.e., DNA/RNA and frozen cell samples.

8. Infuse the CD34+ transduced cells or freeze as needed. Freezing will likely be required if complete biosafety testing and vector copy number are required prior to infusion.

3.5. Single-Colony PCR for Determining Transduction Efficiency (*see* Note 9)

1. Fill microcentrifuge tubes with 1 mL D-PBS. Aspirate single colonies from the methylcellulose medium in a volume of 20–50 μ L with individual plugged pipette tips (P200), under visualization with a phase-contrast inverted microscope and add to the D-PBS-containing tube. Place each colony into an individual tube.
2. Leave the pipette tip in the tube for about an hour at room temperature to allow the methylcellulose to dissolve. Then pipette D-PBS in and out to thoroughly resuspend the cells in D-PBS.
3. Centrifuge the tubes at 2,000 rpm for 10 min in a microcentrifuge. Aspirate carefully the supernatant using individual pipette tips without disturbing the tiny cell pellet. It is acceptable to leave up to 10 μ L D-PBS in the tube to avoid cell loss.
4. Prepare the appropriate volume of DNA lysis buffer as per **Subheading 4.2.5** for the number of picked colonies (Total volume = Number of colonies picked $\times$ 50 μ L + 10% of volume). Add proteinase K to DNA lysis buffer so that the final concentration is 0.1 mg/mL.
5. Transfer 50 μ L of proteinase K-containing lysis buffer to each tube and resuspend cell pellet by pipetting in and out.
6. Incubate the samples at 56°C for 90 min.
7. Inactivate proteinase K at 95°C for 5 min.
8. Add 150 μ L of D-PBS to each tube.

⁸One can always produce one's own methylcellulose media for colony assay, but the variability of the ingredients from batch to batch necessitates the use of commercially available products. We utilize serum-free MethoCult (Stem Cell Technologies, Vancouver, Canada), and plating 500–750 cells per mL. Each sample should be plated at least in triplicate and well mixed. Following 14 days of growth, colony-forming units should be enumerated. Enumeration of the colonies requires appropriate training as there can be substantial variability among operators. It is worth testing the proficiency of the operators annually. The details of plating and enumerating hematopoietic colonies in semisolid media are beyond the scope of this chapter.

⁹There are actually no golden standard for determining the transduction rate of long-term hematopoietic stem cells. Calculating SCID-repopulating potential of transduced cells in NOD/SCID mice is an indirect estimation. This approach is useful when comparing different protocols, vector backbones, cell sources, etc. but highly impractical and costly when measuring gene transfer efficiency in patient cells enrolled in a clinical trial. It is more customary to report the transduction efficiency using single-colony PCR to detect the transgene, which actually defines the transduction rate in the hematopoietic progenitor pool. In this protocol, the DNA is extracted from individual hematopoietic colonies. The colonies growing in semisolid media usually contain less than 200 cells, and any DNA isolation method needs at least a couple of thousand cells to start with. The amount of DNA extracted varies from picograms to a couple of nanograms. When the isolated material is used to detect the transgene of interest by PCR, a second control PCR using β -actin primers has to be run to prove the presence of DNA in the sample. The failure rate of isolating DNA from the samples depends on the size of the colony but is usually less than 15%.

The number of colonies to pick depends on the expected transduction efficiency. There should be at least one colony among the picked colonies that will give a positive PCR product. Example: If the transduction efficiency in the hematopoietic progenitors is around 2%, you need to pick at least 58 colonies (1 out of 50 will be positive, and there can be a 15% failure in extracting DNA, so $50 + 15\% \times 50 = 58$).

9. Mix 200 μ L phenol/ CHCl_3 /isoamylalcohol with DNA-containing solution from **steps 7**. Leave at room temperature for 10 min and centrifuge at 14,000 rpm on microcentrifuge for 10 min.
10. Collect the aqueous upper phase (approximately 150–180 μ L) and precipitate by adding 2 μ g glycogen, 18 μ L of 7.5 M Ammonium Acetate, 500 μ L absolute ethanol. Incubate overnight at -20°C .
11. Centrifuge the DNA at 14,000 rpm on microcentrifuge for 10 min. Wash the pellet with 70% ethanol. Dry the DNA pellet on the bench top; do not use vacuum drying.
12. Resuspend the DNA in 50 μ L of either TE Buffer or dH_2O . 3–5 μ L of this solution should be adequate to detect the presence of most transgene by PCR.
13. Run the PCR reaction with the transgene specific primers. A PCR reaction with β -Actin primers should also be run to confirm the presence of DNA in the sample.

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Fig. 1. Closed system retroviral transduction of human CD34+ cells; **(a)** Prestimulation, and **(b)** Transduction in RetroNectin-Coated Bags.

Fig. 2.

(i) Expression of human CD34 and eGFP after transduction of CD34⁺ cells transduced under three different conditions; (a) prestimulation in serum-free media and vector stock for transduction in serum-free X-Vivo 10 media (b) prestimulation in serum-free X-Vivo 10 media, and transduction with serum-free vector stock supplemented with 10% FBS, (c) all steps are in serum-containing conditions. (ii) Influence of RetroNectin dose on both transduction efficiency and cell apoptosis; (a–f) RetroNectin-coating increases the transduction, but this effect is independent of the amount of RetroNectin (between 2 and 20

$\mu\text{g}/\text{cm}^2$). (g–l) Apoptotic cells are stained with Annexin V-APC and 7-AAD staining. The percentage of Annexin-V+7-AAD- is higher in the absence of Retronectin and lower in presence of Retronectin at all concentrations. (iii) Cell density during the transduction protocol might influence CD34+/CD38-cell population intensity. After 60 h of ex vivo manipulation, cultures plated at starting cell densities of 2×10^5 , 5×10^5 , and 8×10^5 CD34+ cells/mL contain CD34+/CD38- populations of $18.9 \pm 1.7\%$, $12 \pm 0.6\%$, and $7.5 \pm 0.6\%$, respectively. Under low cell density conditions CD34+/CD38- immature hematopoietic progenitor/stem cell population is preserved better than high-density cultures.