FEEDER-FREE LENTIVIRAL-BASED REPROGRAMMING

By Rick I. Cohen, 2014© Stemcellcourse.org. Any use of this publication must reference the Stem Cell Course Manual from Stemcellcourse.org.

1.1 Introduction

The protocol described herein uses pLRC-V1, a Self-Inactivating Lentivirus encoding a polycistronic gene comprised of Oct4-2A-Sox2-2A-KLF4-2A-L-Myc-2A-mRFP/Blast fusion to deliver a gene into fibroblastic cells for purposes of cellular reprogramming into a pluripotent state. This plasmid is available from stemcellcourse.org at nominal charge.

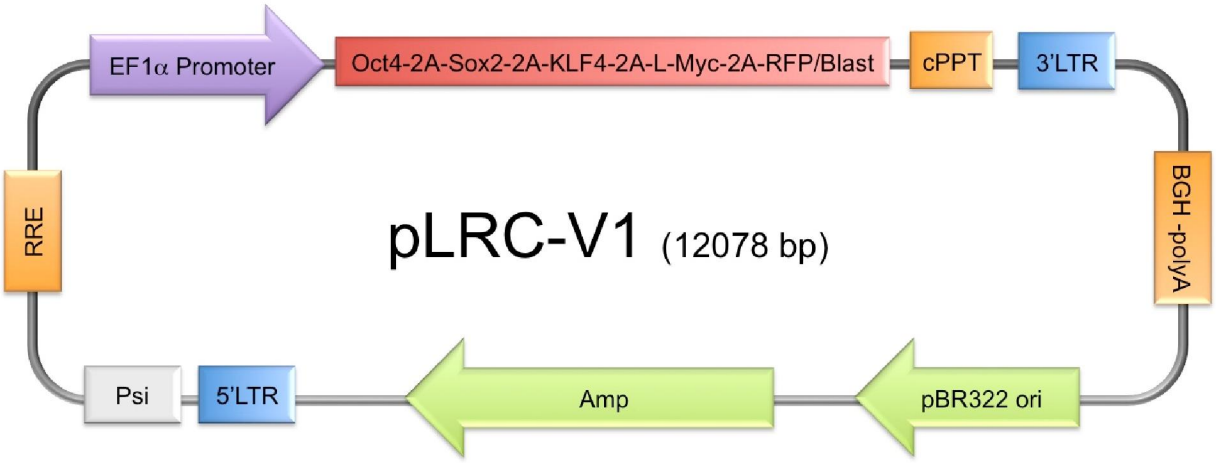
1.2 Plasmid information

pLRC-V1, is a highly engineered polycistronic lentiviral expression vector that can be used in combination with a Helper and Viral Coat plasmid to package Self-Inactivating Lentivirus particles using HEK293 cells. This plasmid employs a strong EF1-alpha promoter to drive a single polycistronic gene containing four 2A type self-splicing motifs. The result of viral transduction yield a target cell permanently modified to express one mRNA that is translated into 5 individual proteins.

Two other options are included in this protocol using a 4 or 2 virus combination. In all lentiviral options one virus contains a fusion of mRFP fused together with Blasticidin-S-Deaminase.

A - 4 lentivirus combination	B - 2 lentivirus combination	C - Single lentivirus
Oct4-IRES-RFP/Blast Sox2-IRES-Puro KLF4-IRES-Puro L-Myc-IRES-Puro	Oct4-2A-Sox2-2A-KLF4-2A-RFP/Blast L-Myc-IRES-Puro	Oct4-2A-Sox2-2A-KLF4-2A-L-Myc-2A-RFP/Blast

When using (A) or (B) choices, c-Myc or Glis can substitute for L-Myc. The use of c-Myc increases the numbers of apparently transformed colonies, whereas Glis1 alters the kinetics, slows reprogramming, and produces many colonies with small-granulated non-IPSC like cells.



1.3 Lentiviral Packaging and Transduction information

Transfecting a combination of pLRC-V1 with Helper (Addgene psPAX2) and Viral coat (Addgene pMD2.G) plasmids into HEK293 cells allows for packaging of the 1, 2, and 4 virus combination paradigm. While the single gene plasmids package into viral particles more efficiently, the pLRC-V1 plasmid yields functional viral particles despite the large insert size. We recommend using 2 to 4 mM Caffeine (as 25-50X concentrate) to boost the titer of all lentiviral packaging, and make it somewhat mandatory if using large inserts in transfer vector backbones. Concentrating lentiviral supernatant by ultra-centrifugation or PEG precipitation allows for more efficient storage of virus,. However, we routinely store filtered unfractionated virus at -80C in 2 mL aliquots which are sufficient to infect 4 wells of a 4 well dish plated with 25 to 50 thousand cells/well. One to two rounds of viral transduction is recommended, however, selection with Blasticidin (5 to 10 ug/mL final) for up to 5 days allows for efficient selection of transduced cells which go on to efficiently for proto-IPS colonies within 14 days.

1.4 Cell Culture Information

It is highly recommend that fibroblasts be derived and/or cultured in FibroLife medium (Life Line Cell Technologies) with 2% Heat-Inactivated high quality or stem cell qualified FBS (or appropriate substitute), however any medium can be used as long as low passage cells are available (less than p4). Fully supplemented FibroLife medium contains FGF, ascorbic acid, and hydrocortisone and appears to support the long-term growth of primary cultures (derived from tissue) with characteristics of skin-derived precursors (SKiPS).

1.5 Small Molecule Information

Most small molecules listed below are readily soluble in DMSO, can be further diluted in ethanol, or dissolved directly in ethanol. All of these are to be filtered using 0.22m Nylon syringe filters (Corning) together with an all-plastic Luer-Lok syringe (National Scientific). Do not use syringes with rubber or lubricated with silicone oil. Likewise, aqueous stocks can be filtered using same type of filtration material or other pore size equivalent material however we recommend using SFCA (surfactant free cellulose acetate). Alternatively the compounds can be stored unfiltered and once diluted in aqueous medium, be filtered with standard laboratory vacuum filtration devices (PES, or SFCA). We recommend storing the compounds as per manufacturers instructions, however we routinely store ethanolic ($\pm$ 10-20% DMSO) at -20C, as they remain in liquid form and easily used for dilutions.

1.6 Summary

The combination of fibroblastic cultures, media systems, single virus system, efficient selection with Blasticidin, stem cell media, and small molecules imparts a streamlined and highly successful method to reprogram human somatic cells.

1.7 Laboratory Equipment

1.7.1 Laminar flow biosafety level 2 hood.
1.7.2 Micropipettes (1000, 100, and 10 uL sizes)
1.7.3 Motorized serological pipettor.
1.7.4 Benchtop centrifuge (for 15 and 50 mL tubes).
1.7.5 Standard inverted microscope or EVOS-XL (Core) system
1.7.6 Hemocytometer or other cell counting device.

1.8 Consumable Supplies

1.8.1 70% Ethanol
1.8.2 Wipers (L30 WYPALL – Fisher Scientific 18-892-194)
1.8.3 Miscellaneous sized gloves (latex and or nitrile)
1.8.4 Low DNA binding microtubes (1.5 mL).
1.8.5 Screw top microtubes (0.5, and 2.0 mL sizes).
1.8.6 15 mL conical tubes
1.8.7 50 mL conical tubes,
1.8.8 Variety of sized plastic serological pipets (aspirating, 2 5, 10, 25, and 50 mL).
1.8.9 5 mL glass serological pipets.
1.8.10 Micropipette tips (10, 100, and 1000 uL sizes)
1.8.11 Sterile plastic transfer pipette (3 mL size).
1.8.12 Luer-Lok syringe, all plastic (National Scientific, S7515-3 = 3 mL, S7515-5 = 5 mL, S7515-10 = 10 mL, and S7515-20 = 20 mL).
1.8.13 0.2 µm Syringe Filters – 25 mM sterile, NY Membrane, (Corning, 431224).
1.8.14 0.45 µm Syringe Filters – 28 mM sterile, SFCA Membrane, (Corning, 431220).
1.8.15 Steriflip 40 µm filter (Millipore SCNY00040).
1.8.16 Luer-Lok syringe, all plastic (National Scientific, S7515 series -3 = 3 mL, -5 = 5 mL, -10 = 10 mL, and -20 = 20 mL). Do not use syringes with rubber or lubricated with silicone oil.

1.9 Plasmid and Cell Line Supplies

1.9.1 Human fibroblastic cultures (Life Line Cell Technology, FC-0001, or xeno-free version = FC-0037).
1.9.2 HEK293FT cells (Life Technologies, R700-07 or equivalent)
1.9.3 psPax2 (Addgene 12260)
1.9.4 pMD2.G (Addgene 12259)

1.10 Media, Components and Buffers

1.10.1 PEI or other relevant transfection reagent for HEK cells.
1.10.2 Hexadimethrine bromide/Polybrene (Sigma, 107689, make 10 ug/mL stock)
1.10.3 Bovine Serum Albumin (BSA IgG Free, Fatty Acid Free, Endotoxin Free, Sigma A8806)
1.10.4 Ascorbic Acid (2-phosphate sesqui-magnesium salt hydrate form A8960). Prepare 100 mg/mL stock solution, sterilize using 0.2 um syringe filter, and freeze in 2 mL aliquots at -20C for up to 6 months.
1.10.5 Caffeine (Sigma C0750). Dissolve into media or PBS containing 100 ug/mL Ascorbic Acid to make 100 mM solution = 25X, sterilize using 0.2 um syringe filter, and freeze in 2 mL aliquots at -20C for up to 6 months.

1.10.6 100X ITS Mix (Life Technologies 41400-045)

1.10.7 D-PBS (Life Technologies, 14190-250 or equivalent)

1.10.8 0.5 M EDTA (Life Technologies, 15575-020, or equivalent).

1.10.9 D-PBS with 0.5 mM EDTA. Dilute EDTA 1:1000 into D-PBS.

1.10.10 1 M Hepes, pH 7.4 (Life Technologies, 15630080)

1.10.11 NEAA (Life Technologies, 11140050)

1.10.12 TrypLE (Life Technologies, 12563029. Use as 1X or can be diluted 1:10 using PBS/0.5 mM EDTA).

1.10.13 Penicillin/Streptomycin 10kU/mL (Life Technologies, 15140122).

1.10.14 Blasticidin S Hydrochloride (Life Technologies, R21001, 5 mg/mL stock = 500 – 1000x).

1.10.15 Matrigel™, hESC Qualified (Corning, 354277).

1.10.16 2-Mercapto-Ethanol (Sigma, M3148; 10,000x = 1M. First dilute 14.3 M stock to 1M and sterile filter using Nylon 0.22 micron filter).

1.10.17 FBS, Defined (Hyclone, SH30070.03). User may heat-inactivate if so desired.

1.10.18 Knock-Out Serum Replacer - KOSR (Life Technologies, 10828028) or equivalent.

1.10.19 Knock-Out DMEM/F12+Glutamax (Life Technologies, 12660012) or equivalent Low Osmolarity media.

1.10.20 DMEM/F12 + Glutamax (Life Technologies, 10565018 or equivalent).

1.10.21 Fibroblast Media; FibroLife (Life Line Cell Technology LL-0001, or Xeno-free version = LL-0048).

1.10.22 **Stem Cell Media**; PeproGrow-hESC (Peprotech, BM-hESC)

1.11 Media Recipes

1.11.1 **Fibroblast Media**: FibroLife media (Life Line Cell Technologies) containing 2% Heat-Inactivated FBS without or with 1 x Pen/Strep.

1.11.2 **Recovery Media**: FibroLife media containing 2% FBS, and 3 small molecules (No antibiotics).

1.11.3 **K3-Reprogramming Media:** All formulae are made as 50 – 100 mL aliquots and supplemented with 20 ng/mL of FGF-2, along with 3 small molecules.

Component/100 mL	[Stock]	[Final]	Volume
Knock-out DMEM/F12	1X	1X	79 ml
20% V/V KOSR	100%	20%	20 ml
NEAA	100x	1x	1 ml
2-mercaptoethanol	1 M	0.1 mM	10 ul
FGF-2	100 ug/mL	20 ng/mL	20 ul
Pen/Strep 10KU/mL (optional)	100x	1x	1 ml
PS48	2000x	1x	50 ul
Sodium Butyrate	2000x	1x	50 ul
A-83-01	2000x	1x	50 ul

1.12 Small Molecules and Growth Factors

1.12.1 PS48 (Tocris, 4087; 2000x = 10 mM ETOH stock for 10 mg size use 3.4874 mL).

1.12.2 Sodium Butyrate (Fisher Scientific (Alfa Aesar), AAA1107936; 2000x = 500 mM aqueous stock 100 mg use 1.817 mL).

1.12.3 A-83-01 (Tocris, 2939; 2000x = 1 mM stock. First dissolve in DMSO at 10 mM, and dilute using ETOH to 1 mM).

1.12.4 FGF2 (PeproTech, AF-100-18B; 1000x = 100 ug/mL. Dissolve 100 ug into 1 mL of 5 mM Tris pH 7.5-7.6, aliquot and store at -20C, use as needed).

1.12.5 **Serum-Free Transfection Media**: can use standard serum free media for HEK293FT cells or the following:

a) DMEM/F12 + Glutamax
b) 0.3% BSA
c) 20 mM Hepes
d) NEAA (1X)
e) 10 ug/mL Ascorbic Acid
f) 20 ng/mL FGF2
g) 1x ITS Mix (Life Technologies)

1.13 Preparation of HEK293FT packaging cell line

1.13.1 Seed HEK293FT cells at $9x10^6$ cells per 15 cm culture dish from actively growing cultures in HEK Media and incubate overnight at 37°C, 5% CO_2 in a humidified incubator. Ensure these cultures are high quality and mycoplasma free. We routinely passage using TrypLE, and make large volume of low passage stocks for Lentiviral packaging.

1.14 Lentivirus production using PEI based Transfection (15 cm dish)

NOTE: you can start protocol in the AM and have time to add serum back to the cultures after a 6 hour incubation period. Alternatively you can begin in the evening and let the transfection protocol continue overnight. Both protocols work, however, the 293 cells may shrink slightly with the overnight choice.

1.14.1 One hour before transfection, change media on HEK293FT cells to transfection media. Optional: wash cultures once with warm DMEM/F12 to remove dead cells, antibiotics and serum.

1.14.2 Transfer 1.4 ml of DMEM/F12 into a 2 ml tube (low DNA binding recommended)

1.14.3 Add the following into the center of the liquid

- 24 ug of the pLRC-V1 vector
- 16 ug of PsPax2
- 8 ug of VSV-G.

1.14.4 Mix, and centrifuge briefly to bring down liquid from walls and top of tube.

1.14.5 Add 168 uL of PEI (user determined) and vortex for 10 sec, or mix vigorously.

NOTE: *The optimal ratio we use here is 3.5 volume PEI to 1 ug equivalents, but this will be empirically determined when making your own batch of PEI. If using commercial source, refer to product insert for volume to ug equivalents as they will differ from company to company. Alternatively any other HEK compatible reagent can be used but you should determine suitability on smaller*

scale prior to scaling to a 15 cm dish.

1.14.6 Centrifuge briefly to bring down fluid from walls and top of tube.

1.14.7 Wait 10-15 min.

1.14.8 Add DNA/PEI mixture dropwise while swirling media.

1.14.9 **Optional**: Incubate for 6 hours and then add serum.

1.14.10 Place back in incubator overnight at 37°C, 5% CO2.

1.14.11 The following day aspirate the transfection reagent-containing medium, add 20 ml of fresh HEK medium, and return the cells to the incubator. If you wish to use straight viral supernatant without concentration, replace medium with your fibroblast medium of choice. We highly recommend using FibroLife (Life Line Cell Technology), supplemented with 1-2% HI-FBS.

1.14.12 **Optional:** at this step one may boost titer by the addition of 1/25th volume of freshly made 100 mM caffeine (in transfection media or any media containing 10-100 ug/mL Ascorbic acid). Please note if the HEK cells are not well adhered to the dish, the addition of caffeine may cause cell detachment. If cells detach, virus will still be produced, but when collecting the supernatant one must centrifuge the cells, and remove supernatant for later filtering steps. We keep the floating HEK cells and replate to collect round 2 of virus.

1.15 Lentivirus Harvest

1.15.1 After 24 hours in fresh media (48 hours post transfection), collect the medium, and repeat a day later.

1.15.2 Spin supernatant down at 1200-1500 RPM in table top centrifuge, and filter using surfactant free Cellulose acetate or PES 0.45 micron filter (Corning or Millipore).

1.15.3 Use virus as is (1:2-1:4 dilution with 8 ug/mL of polybrene pre-added to supernatant), or concentrate using standard methods, here we recommend Ultra-centrifugation at 50,000x G for 2 hours at 4C. Other methods using Polyethylene Glycol (8000 MW at final of 8.5% in solution with adjusted 300 mM NaCl, also use PEG-IT from Systems Biosciences). For single all-in-one virus we have observed loss of activity following concentration.

1.15.4 If you've chosen to ultracentrifuge or PEG precipitate the viral supernatant, then, resuspend virus in 1/100th volume in sterile PBS and use or freeze at -80C. Avoid freeze thaw cycles. Here we use a freshly filtered stock of PBS to ensure sterility.

1.16 Preparation of Human Fibroblastic Cells

1.16.1 Count the number of cells, and seed human somatic cells (for example, human foreskin fibroblasts) at 25,000 – 100,000 cells per well in a 6 well dish. Incubate the dish overnight at 37°C, 5% CO2. We only use FibroLife based media with 2% Heat-Inactivated hESC qualified Serum (Hyclone, and please note we use small batch heat inactivation in 50 mL tubes to ensure even Heat-Inactivation at 56C for 30 min).

1.17 Lentivirus Transduction of Human Fibroblastic Cells

1.17.1 Prior to the application of virus, change FibroLife media to serum and antibiotic-free base media, and supplement in 8 ug/mL of polybrene (Sigma). We routinely wait 1 hour while we finish preparing fresh viral stocks, or thaw frozen stocks.

1.17.2 For transduction;

1.17.3 **4 Virus Protocol**: apply equal volume of each viral concentrate (7-10 uL per well in a 6 well dish) or supernatants (300 uL x 4 per 2.4 mL final). One can also opt to use 2 times the amount of Oct4.

1.17.4 **2 Virus Protocol**: apply OSK-2ARB + partner (c-Myc, L-Myc, or Glis1) in 3:1 ratio. While we recommend L-Myc, others function. The kinectics when using c-Myc are faster, however many false positive colonies appear. One can also titer the amount of partner up or down to ensure proper reprogramming. For 6 well (1 mL) we routinely add 15-20 uL of OSK-2ARB and 5-10 uL of partner. If you decide to titer the virus, then add 3:1 equivalents.

1.17.5 **For Single Virus Protocol:** apply unfractionated virus in a 1:1 to 3:1 dilution in a final volume of 1.2 mL. Concentrated virus (1:100) can be used at 12 to 24 uL per well of a 6 well dish.

1.18 Generation of iPS cells (d1 – d21)

1.18.1 Change medium the following day (+1d) to Reprogramming Media of choice.

1.18.2 The following day (d2) the cultures can be passaged onto Matrigel coated dishes using TrypLE (or Accutase) with K3 with 2 uM Y-27632 and 5 - 10 ug/mL Blasticidin. The selection procedure is essential. This step kills off non-transduced cells, which could potentially, in over-abundance, inhibit the successful reprogramming of the transduced cells. You may continue Blasticidin treatment for up to 5 days (d7).

1.18.3 Media is changed every other day until colonies begin to form, then change media daily.

1.18.4 When colonies become large enough to be picked, there are several ways to subclone;

1.18.4.1 Mechanical passaging using “Pick-to-save” procedures in the presence of 2 uM Y-27632 without or with limited Dispase treatment. This option allows for purification of IPSCs away from feeder layers.

1.18.4.2 Bulk passaging using PBS/EDTA. This option allows for expansion of both self-feeders and newly disaggregated IPSC colonies.

1.18.5 Expand the cells using methods for hESCs and after 1-3 passages the small molecules mixture can be removed and expand in a relevant hESC/IPSC media like PeproGrow-hESC.

1.19 References:

1.19.1 Saiyong Z., et al. (2010) Reprogramming of Human Primary Somatic Cells by OCT4 and Chemical Compounds. Cell Stem Cell 7, pp 651-655.

1.19.2 Lin., T, et al. (2009) A chemical platform for improved induction of human iPSCs. Nature Methods, 6, pp 805-808 plus on-line methods.