3.3 Lentivirus Production and Transduction

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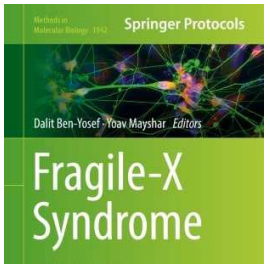

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Abstract

This is part 3.3 of the "**Induced Neurons for the Study of Neurodegenerative and Neurodevelopmental Disorders**" collection of protocols.

Collection Abstract: Patient-derived or genomically modified human induced pluripotent stem cells (iPSCs) offer the opportunity to study neurodevelopmental and neurodegenerative disorders. Overexpression of certain neurogenic transcription factors (TFs) in iPSCs can induce efficient differentiation into homogeneous populations of the disease-relevant neuronal cell types. Here we provide protocols for genomic manipulations of iPSCs by CRISPR/Cas9. We also introduce two methods, based on lentiviral delivery and the piggyBac transposon system, to stably integrate neurogenic TFs into human iPSCs. Furthermore, we describe the TF-mediated neuronal differentiation and maturation in combination with astrocyte cocultures.

Attachments

PDF

Sauter2019_Protocol_...

623KB

Materials

2. Materials

2.3 Lentivirus Production and Transduction

1. A highly transfectable cell line, such as 293T/17 cells (a gift from Didier Trono; ATCC® #CRL-11268).
2. DMEM with [M] 10 Mass Percent FBS : DMEM medium, [M] 10 Mass Percent FBS , [M] 1 Mass Percent penicillin–streptomycin . Store at 4 °C .
3. DMEM w/o FBS: DMEM medium, [M] 1 Mass Percent penicillin–streptomycin . Store at 4 °C .
4. [M] 1 Mass Percent PEI solution pH 7.1): adjust the pH with [M] 0.1 Mass Percent NaOH , sterile-filter (0.22 µm), aliquot, and store at -20 °C . After thawing, working aliquots can be stored at 4 °C .
5. Lentiviral vector containing the gene of interest under a doxycycline-inducible promoter (e.g., pLV_TRET_Ngn2-2A-Ngn1 (Addgene plasmid #61471) [4] and pLV_hEF1a_rtTA3 (Addgene plasmid #61472) [4] or pLIX403 (Addgene plasmid #41395)) (*see* **Note 3** and Fig. 3a, b). Store at -20 °C .
6. Viral packaging plasmid psPAX2 (a gift from Didier Trono, Addgene plasmid #12260). Store at -20 °C .
7. Viral envelope plasmid pMD2G (a gift from Didier Trono, Addgene plasmid #12259). Store at -20 °C .
8. [M] 50 Mass Percent PEG 6000 solution . Store at 4 °C .
9. [M] 4 Molarity (M) NaCl solution . Store at 4 °C .
10. PBS pH 7.2 without calcium and magnesium. Store at Room temperature .
11. Lenti-X™ GoStix™ (Clontech).
12. Antibiotic: If you would like to select the cells for the integrated lentiviral construct, use the appropriate antibiotic (such as blasticidin or puromycin). Store aliquots at -20 °C , after thawing store at 4 °C , protected from light.
13. DNA extraction kit (e.g., DNeasy® Blood and Tissue Kit (Qiagen)).
14. Albumin plasmid pAlbumin (a gift from Didier Trono, Addgene plasmid #22037). Store at -20 °C .
15. TaqMan® PCR master mix, such as TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific). Store at 4 °C .
16. TaqMan® primer and probes for WPRE and albumin detection (see Table 1). Dilute in ddH_2O to a concentration of [M] 10 micromolar (µM) and store at -20 °C .
17. Real time PCR system, such as StepOnePlus™Real-Time PCR System (Thermo Fisher Scientific).

	A	B	C
	Primer	Sequence	Fluorophore
	WPRE_forward	GGCACTGACAATTCCGTGGT	
	WPRE_reverse	AGGGACGTAGCAGAAGGACG	
	WPRE_probe	ACGTCCTTTCCATGGCTGCTCGC	FAM-BHQ
	Alb_forward	GCTGTCATCTCTTGTGGGCTGT	
	Alb_reverse	ACTCATGGGAGCTGCTGGTTC	
	Alb_probe	CCTGTCATGCCCACACAAATCTCTCC	FAM-BHQ

Table 1

TaqMan® primer and probes [8]

Safety warnings

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

Before start

NB Introduction, Notes, and References are in the **Collection Guidelines tab**

3.3 Lentivirus Production and Transduction

6d 1h 27m 35s

1 For the production and transduction of lentiviruses, titration and copy number determination, we follow the protocol from the Trono lab [8].

2 One day prior to transfection, seed 8,000,000 293T/17 cells in a 10 cm culture dish. The next day, replace the culture medium with 4 mL fresh DMEM with 10% FBS. The cells are transfected using 45 µg polyethylenimine (PEI) combined with 15 µg DNA containing the plasmid of interest (see Fig. 3a, b), the viral packaging (psPAX2) plasmid, and the viral envelope (pMD2G) plasmid in a 4:2:1 ratio. In detail, mix 45 µL 1 mg/ml PEI solution with 955 µL DMEM medium w/o FBS in a 1.5 ml tube.

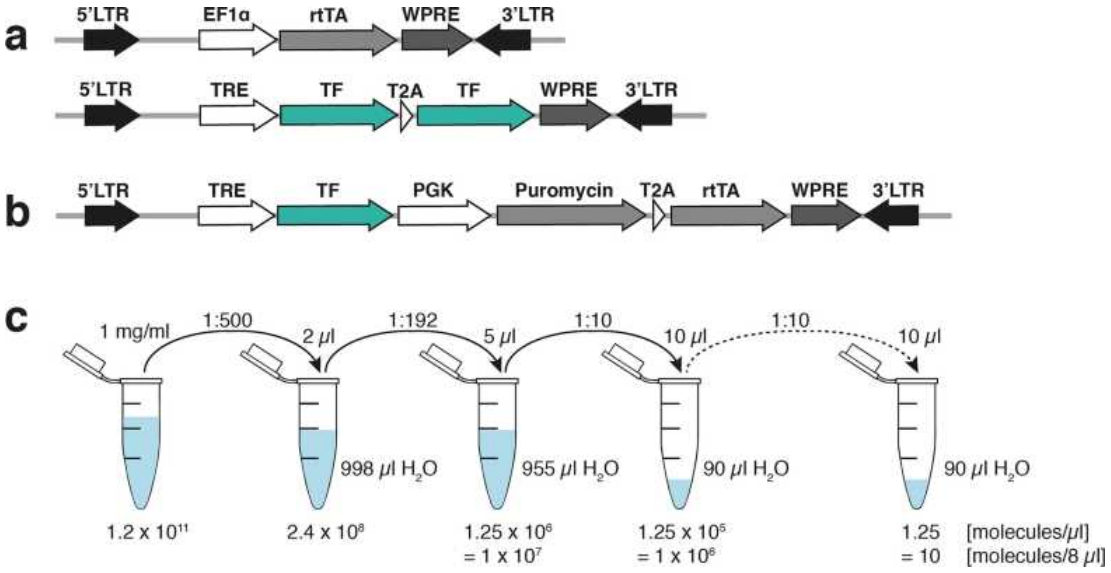

Fig. 3
Transduction of iPSCs with lentiviral vectors. (**a**) and (**b**) Schematic representation of the lentiviral constructs.
(**a**) The pLV system consists of two constructs: pLV_hEF1a_rtTA3 (top) expresses the rtTA transactivator under the control of the constitutively active EF1α promoter and pLV_TRET_Ngn2-2A-Ngn1 (bottom) expresses the transgenes under the control of the doxycycline-inducible TRE promotor [4]. The pLV plasmids depicted to here do not contain any selection markers. If selection is required, antibiotic resistance genes should be cloned into the plasmids.
(**b**) The pLIX403 system is an "all-in-one" doxycycline-inducible system. It expresses a puromycin resistance gene together with the rtTA transactivator under the control of the constitutively active PGK promotor and the transgene under the control of the doxycycline-inducible TRE promotor. 5' and 3' LTR—long terminal repeats, WPRE—woodchuck hepatitis virus posttranscriptional regulatory element.
(**c**) Schematic representation of the serial dilution of the pAlbumin plasmid for titration and copy number determination (modified from [8])

3 In a separate tube, mix 2.1 µg pMD2G, 4.2 µg psPAX2, and 8.4 µg vector of interest with 1 mL DMEM w/o FBS.

4 Combine PEI and DNA, mix, and incubate at Room temperature for 00:15:00 – 00:30:00. Subsequently, add the transfection mix dropwise to the 293T/17 cells and place the culture dish into the incubator (*see* **Note 18**). 45m

5 The next day, replace the medium with 7 mL DMEM with 10% FBS.

6 After 24:00:00, collect the supernatant, with the help of a syringe pass it through a 0.45 µm PES filter into a 50 ml Falcon tube and store at 4 °C. Add 7 mL fresh DMEM with 10% FBS and after another 24:00:00 collect the supernatant, filter and pool with the first collection. 2d

7 Mix 14 mL cell culture supernatant (48 h and 72 h harvests) with 6.6 mL diluted polyethyleneglycol (PEG) solution containing 3.5 mL 50% PEG 6000 solution, 1.5 mL 4 M NaCl solution, and 1.6 mL 1× PBS w/o Ca2+ and Mg2+, and keep at 4 °C Overnight or over the weekend. 30m

8 Centrifuge the tubes at 7000 x g, 4°C, 00:10:00 (*see* **Note 19**). After the centrifugation, a white pellet should be visible.

9 Carefully decant the supernatant and resuspend the pellet in 150 µL 1× PBS w/o Ca2+ and Mg2+ by pipetting up and down. Vigorously vortex the tubes for 00:00:20 – 00:00:30 to further resuspend the pellets. Lenti-X™GoStix™can be used to confirm the successful generation of viral particles. 50s

10 Transfer the virus suspension into 1.5 ml screw-cap tubes in aliquots of 50 µl, snap-freeze in crushed dry ice and store at -80 °C.

11 In order to transfect iPSCs with the viral particles, the cells should be approximately 40 ± 10% confluent. Wash the cells once with [M] 1 Mass Percent PBS w/o Ca2+ and Mg2+ and add mTeSR™1 medium to the well.

12 Add mTeSR™1 to the tube containing the virus suspension, mix gently and transfer dropwise to one or more wells (*see* **Note 20**).

13 Incubate the culture dish Overnight and change the medium on the next day (*see* **Note 21**). Starting 48 h after transduction, select for cells with an integrated construct with the appropriate antibiotic (*see* **Note 14**). 30s

14 For titration and copy number determination, we perform a qPCR on genomic DNA to count the number of integrated viral particles (WPRE) relative to the genome (albumin gene). Wait at least 96:00:00 before isolating genomic DNA using a genomicDNAextraction kit, such as the DNeasy® Blood and Tissue Kit (Qiagen), according to the manufacturer's instructions. The DNA should be placed On ice if used immediately or stored at -20 °C . 4d

15 Adjust the concentration of the pAlbumin plasmid used for normalization to [M] 1 Mass Percent , which corresponds to 1.2×10^{11} molecules/µl (*see* **Note 22**). Prepare a standard curve, with the first point being 1×10^{7} molecules in 8 µl (which corresponds to 1.25×10^{6} molecules/µl). Prepare the other points of the standard curve by serial tenfold dilutions until there are ten molecules in 8 µl (*see* Fig. 3c).

16 Prepare two master mixes of 8.5 µL TaqMan® Universal PCR Master Mix , 0.17 µL forward primer (10 µM) , 0.17 µL reverse primer (10 µM) , and 0.17 µL probe (10 µM) per sample including all samples and standards in duplicates (one master mix with albumin primers and probe for genomic DNA detection and one with WPRE primers and probe for lentivirus detection).

17 Aliquot 9 µL master mix per well of a 96-well plate and add 8 µL sample DNA (concentration between 50 ng and 100 ng DNA in 8 µL).

18 Seal the plate, carefully mix by vortexing and briefly spin down.

19 Run the qPCR with the settings for FAM fluorochromes and BHQ quenchers with the following program: 1 cycle of 00:10:00 at 95 °C followed by 50 cycles of 00:00:15 at 95 °C and 00:01:00 at 60 °C (*see* **Note 17**). 11m 15s

20 Plot the standard curve using the software of your qPCR machine or manually using other software such as Microsoft Excel, and calculate the quantity of albumin and WPRE for each sample using the equation of the standard curve.

21 Calculate the copy number for each sample as follows: Copy number = (quantity mean of WPRE sequence/quantity mean of Alb sequence) × 2.

22 Calculate the viral titer with the following formula: Titer (viral genome/ml) = (number of target cells counted at day 1 × number of copies per cell of the sample)/volume of supernatant (ml).