Khurana Lab BRIGHAM HEALTH BRIGHAM AND WOMEN'S HOSPITAL HARVARD MEDICAL SCHOOL	SOP-VK	**PiggyBac Transfection iPSC**	Author: Aazam Approved: Asia	Revision: Asia	Issued 04-19-2020 Revised 08-04-20

1. **Purpose**
   Generation of stably transfected iPSC with piggybac constructs.

2. **Scope**
   This procedure is used to introduce exogenous NGN2 gene into iPSCs. The established cell lines are used for trans-differentiation into neurons by doxycycline induction.

3. **Materials**
   6-well plate
   Cell culture hood
   Pipets (10, 20, 1000p)
   1.5mL autoclaved Eppendorf tubes
   Cell counter

4. **Reagents**
   TransIT-LT1 Transfection Reagent (Mirus, MIR 2304)
   Opti-MEM Reduced Serum Medium (Gibco, 31985062)
   Matrigel growth factor reduced (Corning, 354230)
   DPBS without Ca and Mg, 500mL (Corning, 21-031-CV)
   Accutase (Gibco, 00-4555-56)
   StemFlex medium (Gibco, A3349401)
   ROCK inhibitor (Peprotech, 1293823)
   Transposase pEf1α-hyPBase
   Piggybac plasmid construct: pEXP-piB-BsD-Tet-NGN2-Puro-IRES-SNAP-PGKtk; Plasmid #1022 in Khurana's Plasmid logs

5. **Procedure**
   6.1. Day before transfection: Re-plating the cells
      6.1.1. Aspirate the medium from one confluent well of 6-well plate, and wash once with PBS.
      6.1.2. Add 1 mL accutase and leave in the incubator for 2 min.
      6.1.3. Add 1 mL growth medium or hES medium with a 1000p, and pipet up and down to dissociate the cells and break them into single cells.
      6.1.4. Transfer the cell suspension to a 15mL conical tube. Centrifuge at 800 rpm for 3 min. Remove the supernatant.
      6.1.5. Add 1 mL StemFlex to resuspend and count the cells with cell counter.

6.1.6. Take out 6-well plate coated with Matrigel from 37℃ incubator after at least one-hour incubation.
6.1.7. Aspirate the Matrigel carefully without scratching the surface. Wash once with PBS to remove residual Matrigel.
6.1.8. Plate 1.5 X 10^6 cells (when slow growers) in 2 mL StemFlex supplemented with 10 µM ROCK inhibitor in one well of Matrigel-coated 6-well plate.
6.1.9. You may seed another well with fewer cells at 1.0 X 10^6 to compare the wells on the day of transfection to choose one.
6.1.10. Cells should be about 70% confluent the next day.

6.2. Day of transfection

6.2.1. Prepare the transfection mix in 1.5mL Eppendorf tubes under cell culture hood.
6.2.2. Add 200 µL Opti-MEM medium to the 1.5mL tube for each transfection.
6.2.3. Add 2 µg of the piggybac plasmid to the tube.
6.2.4. Add 1.5 µg of the transposase plasmid to the mix.
6.2.5. Add 10.5 µL TransIT-LT1 Transfection Reagent and mix well (ratio 1:3 DNA:Reagent).
6.2.6. Leave under the hood at room temperature for 20 min.
6.2.7. Remove the cell culture plate from day before and choose the well that is about 70-80% confluent in single layer cells.
6.2.8. Remove the medium and feed with 2 mL StemFlex supplemented with 10 µM ROCK inhibitor (Y).
6.2.9. Add the 200 µL transfection mix evenly and drop-wise on the culture plate.
6.2.10. Shake the plate very gently to make sure it is dispersed evenly.
6.2.11. Leave at 37°C incubator for 6 hours without disturbing it.
6.2.12. Aspirate the medium and feed with 2 mL StemFlex + Y.
6.2.13. Change the medium for every day with StemFlex + Y.
6.2.14. Two days after transfection add 5 µg/mL blasticidin (BsD) if the backbone of the piggybac plasmid has the selection gene for blasticidin resistance. If cells look unhealthy after the transfection, do 2.5 µg/mL blasticidin (BsD).
6.2.15. Continue selection for 5 days (max). Changing the medium every day with StemFlex + 10 µM Y + 5 µg/mL BsD.

6.2.16. If you see more than 80% of cells die before 5 days, stop selection and feed the cells with StemFlex without BsD until it becomes confluent for expansion.

6.2.17. You do not need to supplement StemFlex with ROCK inhibitor after day 7 of transfection, but do use RI at the following passage.

6.2.18. Expand the cell line (use RI during the passaging) and freeze a large number of tubes. When passaging the cells for the 1st time, add RI to the media.

7. Important QC tests on the newly generated ***polyclonal*** iPSC lines:

7.1. Depending on the experiment, you may choose to keep the line as ***polyclonal*** or ***monoclonal*** by subcloning it and select for single clones to use moving forward (2-3 clones for QC and you can pool or keep separate).

7.1.1. Expand a polyclonal line upfront at the lowest passage possible into a large batch.

7.1.2. Keep the passage as low as possible. Preferred to not use the line more than 5 passages after the Piggybac transfection. If the line is stable and has good karyotype, if may be reconsidered to use the line at later passages.

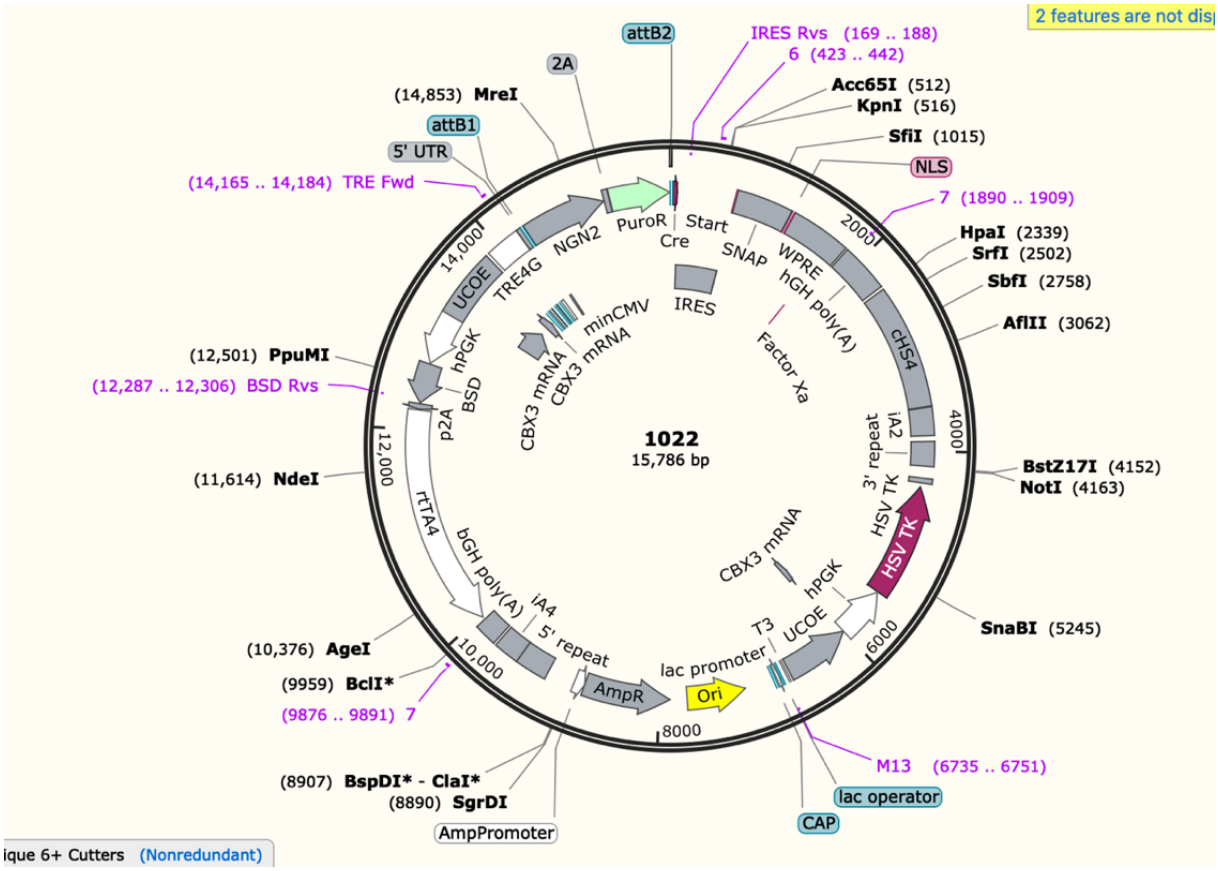
7.2. **Karyotype** the newly generated line to determine any abnormal chromosomal events.

7.2.1. For most purposes karyotyping the ***polyclonal*** line on 50 cells to get a sense of the karyotypically unstable population (if any) is fine. Karyotype the lowest passage available.

7.2.2. For a ***monoclonal*** culture of your key cell line you may perform the standard 20 count cell karyotyping.

7.3. Perform the **Splinkerette PCR assay** to locate and map the exact location of each transposition event in the genome of the cells that are stably transfected with piggybac construct. It also shows the copy number for the number of transposon integrations (protocol available at the Khurana Lab/Aazam).

NGN2-Puro-PB Construct Map:


2 features are not dis
attB2
IRES Rvs (169 .. 188)
6 (423 .. 442)
2A
Acc65I (512)
KpnI (516)
(14,853) MreI
attB1
SfiI (1015)
5' UTR
NLS
(14,165 .. 14,184) TRE Fwd
7 (1890 .. 1909)
HpaI (2339)
SrfI (2502)
SbfI (2758)
AflII (3062)
(12,501) PpuMI
(12,287 .. 12,306) BSD Rvs
(11,614) NdeI
BstZ17I (4152)
NotI (4163)
SnaBI (5245)
(10,376) AgeI
(9959) BclI*
(9876 .. 9891) 7
(8907) BspDI* - ClaI*
(8890) SgrDI
AmpPromoter
M13 (6735 .. 6751)
lac operator
CAP
1022
15,786 bp
PuroR
Start
Cre
NGN2
TRE4G
UCOE
SNAP
WPRE
hGH poly(A)
cHS4
IRES
minCMV
CBX3 mRNA
Factor Xa
hPGK
BSD
p2A
rtTA4
bGH poly(A)
lA4
5' repeat
AmpR
Ori
lac promoter
T3
UCOE
hPGK
CBX3 mRNA
HSV TK
3' repeat
lA2
14,000
2000
4000
6000
8000
10,000
12,000
ique 6+ Cutters (Nonredundant)