VBCF – Protein Technologies	**Standard Operation Procedure** **HEK293 FreeStyle - PiggyBac stable cell lines**

1. **Aim:**
   Inducible recombinant expression in mammalian system in form of stable cell line (multiclonal) generation.
2. **Applicability:**
   MIDI-grade plasmid DNA of the gene of interest cloned into the vector containing the PiggyBac transposition elements
3. **Responsibilities:**
   Main Responsible: Jana Neuhold
   Substitute: Anita Lehner, Christian Aigner
4. **Required materials:**
   FreeStyle™ 293 Expression Medium (Thermo, 12338-026)
   Opti-MEM® I Reduced Serum Medium (Thermo, 31985-062)
   EX-CELL® 293 Serum-Free Medium (SigmaAldrich, 14571C-1000ML)
   Corning 125ml Flask vented cap (VWR, 734-1885)
   Corning 1000ml Flask vented cap (VWR, 734-1889)
   Sterile Erlenmeyer flask 250 ml (CarlRoth, PX74.1)
   Polyethylenimine (PEI 25K) (Polysciences, 23966-1)
   Doxycycline hyclate (Sigma, Cat No D9891)
   Puromycin (Invivogen, Cat No ant-pr-1)

5. **Procedure:**

A. Transfection of plasmid DNA for integration of the expression cassette

For transfection you will need a MIDI-grade plasmid DNA of the gene of interest cloned into the vector containing the PiggyBac transposition elements. The backbone carries also the rtTA transactivator fused via T2A to puromycin resistance under EF1a constitutive promoter and was derived from the plasmid 97421 from Addgene. This transfer plasmid has to be co-transfected with the HyTrs helper plasmid carrying the corresponding transposase.

If possible, use freshly-thawed stock of HEK293FreeStyle cells (but passaged at least 3x after thawing) in order to keep the passage number of the final cell line as low as possible.

Day 1

In the morning, dilute the cells to $1x10^6$ per ml in 30 ml FreeStyle™ 293 Expression Media and shake them for few hours as usual. In the afternoon, pre-warm OptiMEM media and an aliquot of PEI (1mg/ml). In tube A, mix vigorously 60 µl PEI (1 mg/ml) with 950 µl OptiMEM. Use a 2 ml tube to prepare DNA mix = tube B:

Target plasmid	20 µg	X µl
HyTrs	10 µg	X µl
OPTIMEM		950 µl

Mix the contents of tube A and B in the 2 ml Eppendorf tube, vortex for ~1 min, spin shortly and incubate at room temperature for 15 min. Then add the transfection mixture to the whirling cell suspension and grow as usual.

Day 3

Measure density/viability (usually varies between 1.5-3x10^6 per ml and 90-98% viability). Spin the cells at 60g for 15 minutes, transfer 3 ml of the conditioned media back into the flask and discard remaining supernatant. Resuspend the cells gently in 27 ml fresh FreeStyle™ 293 Expression Media, transfer them back into the culture flask and add 9 µl of puromycin (10 mg/ml). Grow as usual.

Day 5

Measure density/viability. The viability should have dropped considerably by now (~70-85%). Spin down volume of culture containing 6x10^7 cells (corre-sponds to 2x10^6 per ml when resuspended in 30 ml) for 15 min at 60g. Transfer 3 ml of the conditioned media into a new flask, then resuspend the cells gently in 27 ml fresh FreeStyle™ 293 Expression Media and transfer them into the new flask. Add 8.1 µl of puromycin (10 mg/ml) and grow as usual.

Day 8

Measure density/viability. The viability should be still pretty low (~50-60%). Spin the cells at 60g for 15 minutes, transfer 3 ml of the conditioned media back into the flask and discard remaining supernatant. Resuspend the cells gently in 27 ml fresh FreeStyle™ 293 Expression Media, transfer them back into the culture flask and add 8.1 µl of puromycin (10 mg/ml). Grow as usual.

Day 10

Measure density/viability. The viability should be still low, although possibly somewhat better than last time (~60-70%). Spin down volume of culture containing 4.5x10^7 cells (corresponds to 1.5x10^6 per ml when resuspended in 30 ml) for 15 min at 60g. Transfer 3 ml of the conditioned media into a new flask, then resuspend the cells gently in 27 ml fresh FreeStyle™ 293 Expression Media and transfer them into the new flask. Add 8.1 µl of puromycin (10 mg/ml) and grow as usual.

Day 12

Measure density/viability. The viability should be recovering now – if it is in the range between 85-95%, dilute the cells to the density of 0.5x10^6 per ml and add puromycin (0.3 µl per ml of fresh media). Grow as usual.

If the viability is lower, spin down volume of culture containing 3x10^7 cells (corresponds to 1x10^6 per ml when resuspended in 30 ml) for 15 min at 60g. Transfer 3 ml of the conditioned media into a new

flask, then resuspend the cells gently in 27 ml fresh FreeStyle™ 293 Expression Media and transfer them into the new flask. Add 8.1 µl of puromycin (10 mg/ml) and grow as usual.

Day 15*

Measure density/viability. The viability should be high again (above 90%). Dilute the cells to the density of $0.6x10^6$ per ml in 35 ml – use the same flask. Add puro-mycin (0.3 µl per ml of fresh media) and grow as usual.

Day 16*

Measure density/viability. Expand the cells to 80 ml in 250 ml flask at density of $0.5x10^6$.

Day 17*

Measure density/viability. The cells should be at density of ~$1x10^6$ and viability between 98-100%. Such culture can be now used for stock preparation.

Prepare 7 labelled 1.8 ml cryo-vials and one 15 ml falcon tube per construct. Pipet 800 µl sterile DMSO into the falcon tube and 3.6 ml fresh media. Then transfer volume of the cells corresponding to $7x10^7$ into 50 ml falcon tubes and spin down for 5 minutes at 100g. Pipet 3.6 ml of the supernatant (=conditioned media) into the 15 ml falcon tube (do not forget to label) and then carefully remove and discard the remaining media from the pelleted cells. Mix the content of the 15 ml falcon tube and use 7 ml of this freezing media to gently resuspend the cells and aliquot them by 1 ml into the cryovials. Transfer the vials into the freezing device (Mr. Frosty or the iso-propanol-free rigid foam container) kept at room temperature and place it over night into the -80°C freezer. It is advisable to process max 2 cell lines in parallel to minimize the time the cells are sitting around in the freezing media.

Use the remaining volume to keep a backup culture of 30 ml at $0.2x10^6$ per ml and do not forget to add puromycin (0.3 µl per ml of fresh media).

* Note: if the cells are not as viable as indicated, they will need to be kept under puromycin selection for longer before expanding for stock preparation.

Day 18

Transfer the stocks into the liquid nitrogen tank.

Day 19

Dilute the backup culture to $0.3x10^6$ per ml in 30 ml FreeStryle media + puromycin using the same flask.

Thaw one vial of the stock:

Prepare a 125 ml flask with 29 ml of prewarmed FreeStyle293 media.

Take out a frozen aliquot from the LN2 tank, swipe the outside with 70% ethanol and thaw the content in the laminar flow hood by gently adding and removing small amount of the prewarmed media. When the whole volume is transferred into the flask, shake the content for even distribution, take out a small sample and check the density + viability. If possible, wait few hours before adding 9 µl puromycin.

Day 22

Check the density and viability of the thawed stocks.

B. Expression test

Keep the cells in culture for 3 passages after thawing as usual, using puromycin in the media.

On the day of induction, spin down volume containing $3x10^7$ at 60g for 10 minutes – keep 3 ml of the media and resuspend cells in 27ml fresh+3ml conditioned media to obtain a culture of density $1x10^6$ per ml. Induce by addition of Doxycycline at final concentration of 1µg/ml (make sure the Doxycycline stock is not older than ~1-2 weeks). During the expression phase, omit puromycin.

24h after induction, feed the culture by adding 7.5 ml of Excell media. Monitor density/viability and take samples for Western blot every day up to 96h (or as required).

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