Transient Transfection of HEK-293F Suspension Cultures using PEI

Cells are grown in suspension on a platform shaker in a humidified 37°C CO_2 incubator with rotation at ~150 rpm. Maintain cultures for 5-7 passages prior to performing transfections to ensure stable growth patterns. When using suspension-adapted HEK293 cells (Freestyle™ 293-F cells, Life Technologies, Cat. No. R790-07) grow the cultures in Freestyle™ 293 Expression Medium (Life Technologies, Cat. No. 12338026). Cells should be maintained between 4 x 10^5 and 3 x 10^6 cells/ml in a volume not to exceed 20% of the total volume of the culture flask. Optimum rotation rate should be determined for each flask type and culture volume. Freestyle™ 293-F cells from Life Technologies are prone to clumping at higher densities and volumes.

Polyethylenimine (PEI) (25 kDa linear PEI, Polysciences, Inc., cat. No. 23966) is prepared as a stock solution at a concentration of 1 mg/ml in a buffer containing 25mM HEPES and 150 mM NaCl (pH 7.5). The PEI is added to the buffer and vortexed until completely dissolved (*this can take MANY minutes of vortexing*). Once fully dissolved PEI can be sterile filtered using a 0.22 μM syringe filter, aliquoted, and frozen at -20°C until needed.

For best transfection efficiency, cells should have a viability of >95% at the time of transfection. Twenty four hours prior to transfection, split cells to a density of ~1 x 10^6 cells/ml and culture overnight in the CO_2 incubator with shaking at 37°C. Cell density should be ~2 x 10^6 at the time of transfection.

Transfections should be performed at a cell density of between 2.5 x 10^6 and 3.0 x 10^6 cells/ml. Count cells using a hemocytometer and spin down a sufficient volume to resuspend the cells at a density of 2.5 x 10^6 cells/ml in fresh 293F Freestyle Media in the desired volume for transfection. ***Resuspension in fresh media prior to transfection is critical. Conditioned media contains metabolites that inhibit transfection.*** Cells should be spun at 1200 rpm for 10 min at room temperature. Transfections will be diluted 1:1, 24 hours after transfection, so the initial volume transfected is one half of the final volume desired.

> Example: culture density of the suspension culture stock = 2.8 x 10^6 cells/ml.
> For a final transfection volume of 100 ml, you will need 50 ml of cells at a density of 2.5 x 10^6 cells/ml.
> $V_1 \cdot 2.8 \times 10^6$ cells/ml = 50 ml $\cdot$ 2.5 x10^6 cells/ml
> V_1 = 44.6 ml
> Spin 44.6 ml at 1200 rpm for 10 min and remove media from cell pellet.
> Resuspend cells in 50 ml of fresh pre-warmed Freestyle™ 293 medium.
> Return the culture to the CO_2 incubator while preparing DNA and PEI mixtures for transfection. Reserve an aliquot of the culture for cell counting to confirm cell density.

HEK293 cell transfection procedure:

For transfection, add the expression vector plasmid DNA to the cells at a final concentration of 3 μg/ml and PEI to a final concentration of 9 μg/ml of transfection volume. (DNA concentration may be varied from 1-6 μg/ml to optimize expression, while PEI is maintained at 9 μg/ml). A dilution is prepared from the plasmid preparation DNA stock to a final concentration of 0.5 μg DNA/μl in Freestyle™ 293 Medium.

> Example: Expression plasmid prep stock DNA concentration = 2.5 μg/μl (in sterile H_2O)
> For 50 ml transfection (final volume = 100 ml) you need 3 μg/ml DNA x 50 ml = 150 μg DNA
> 150 μg DNA/2.5 μg/μl DNA stock = 60 μl DNA stock (+10% for pipetting error) = 66 μl DNA stock
> 150 μg DNA/0.5 μg/μl = 300 μl total volume (+10% for pipetting error) = 330 μl final volume
> Therefore, dilute 66 μl of the 2.5 μg/μl DNA stock into 264 μl of pre-warmed medium for a final volume of 330μl of DNA/Freestyle mixture at 0.5 μg/μl (300 μl +10%).
> Briefly vortex the mixture and spin to return the solution to the bottom of the tube.
> Add 300 μl of DNA/Freestyle media mix to the cells.
> Swirl the culture to mix the DNA and return the flask to shaker platform in the incubator for 5 minutes.

A dilution of the 1 mg/ml PEI stock is prepared to a final concentration of 0.5 μg/μl in Freestyle™ 293 Medium.

> Example: PEI stock concentration = 1 mg/ml
> For 50 ml transfection (final volume = 100 ml) you need 450 μg PEI at a concentration of 0.5 μg/μl.
> Dilute 495 μl of PEI into 495 μl of pre-warmed medium for 990 μl of PEI (900 μl + 10%)
> Briefly vortex the mixture and spin to return the solution to the bottom of the tube.
> Add 900 μl of PEI/medium mix to the cells.
> Swirl the culture to mix the PEI, return the flask to shaker platform in the incubator.

After 24 hr, dilute the cells 1:1 with pre-warmed Freestyle™ 293 Medium supplemented valproic acid (VPA) (Sigma cat. No. P4543-100G) to a final concentration of 2.2 mM. A stock solution of 220 mM VPA in water allows for the addition of 10 μl of VPA per 1 ml of final transfection volume. No further media supplementation is required for the duration of the transfection. Return the culture to the CO_2 incubator with shaking at ~150 rpm on the orbital shaker.

Harvest the transfected cells 3-6 days after transfection. Protein production can be monitored by taking small samples (~100 μl) at 24 hr intervals, clarifying the media by centrifugation and analysis by SDS-PAGE and/or fluorescence measurement to determine the optimal time for harvest.

Vessel	Initial Volume	Final Volume	DNA	PEI	VPA
500 ml bottle	50 ml	100ml	300 μl	900 μl	1 ml

Quick Reference

Materials:
Freestyle™ 293-F cells from Life Technologies (Cat. No. R790-07)
Freestyle™ 293 Expression Medium from Life Technologies (Cat. No. 12338026)
Polyethylenimine (PEI) Polysciences, Inc. (Cat. No. 23966)
VPA (Sigma Cat. No. P4543)

Procedure:
Culture cells between 4 x 10^5 and 3 x 10^6 cells/ml.
Split cells to 1 x 10^6 24 hours prior to transfection.
Collect cells by centrifugation and resuspend the cell pellet in fresh medium for transfection at a density between 2.5 x 10^6 and 3 x 10^6 cells/ml.
Add 3 μg of DNA per ml of transfection volume from a 0.5 μg/μl dilution of DNA in medium.
Return cells to incubator for 5 minutes to shake.
Add 9 μg of PEI per ml of transfection volume from a 0.5 μg/μl dilution of PEI in medium.
Dilute the transfection 1:1, 24 hours post transfection and supplement with 2.2 mM VPA.

Variations: High mannose-type glycosylation

Recombinant glycoprotein production to generate high mannose glycans can be achieved using mutant HEK293 cells lines deficient in glycoprotein maturation (HEK293S GnTI- cell line, Reeves, PJ, et al (2002) 99, 13419-24). This cell line has been adapted for growth in suspension culture in serum-free culture medium similar to the Freestyle™ 293-F cell line.

Materials:

HEK293S GnTI- cell line (ATCC cat. no. CRL-3022)
Ex-Cell® 293 Serum-Free Medium (Sigma cat. no. 14571C)
ESF serum free medium (Expression Systems cat. no. 98-001)

Suspension adapted HEK293S GnTI- cells grow well in Ex-Cell 293 media or in a 50:50 mix of Ex-Cell and Freestyle™ 293 medium, but do not grow well on Freestyle™ 293 medium alone. However, Ex-Cell and ESF media do not support efficient transfection of HEK293 cells (presumably there is a component in the media that inhibits DNA/PEI transfection). As a result, the transfection procedure is identical to the transfection of Freestyle™ 293-F cells with the exception that cells are propagated and maintained in the 50:50 mix of Ex-Cell/Freestyle media until the time of transfection. The cells are then collected by centrifugation and resuspended in Freestyle™ 293 Medium alone at a 2.5 x 10^6 cells/ml. DNA and PEI addition is identical to transfections of Freestyle™ 293-F cells and the cells are maintained in the Freestyle™ 293 medium for 24 hours post-transfection. After 24 hours the cultures are diluted 1:1 with Ex-Cell or ESF medium with added VPA to 2.2 mM. The cultures are then maintained for recombinant production for 3-6 days on a platform shaker in a CO_2 incubator. Expression levels are monitored and cultures harvested identical to the Freestyle™ 293-F cells. ESF medium has considerably lower intrinsic fluorescence than Ex-Cell medium and is preferable for culture dilution when expression of GFP-fusions will be monitored in the conditioned medium. Overall expression is moderately higher when the cultures are diluted with ESF or Ex-Cell medium by comparison to when cultures are diluted with Freestyle™ 293 medium.