

FreeStyle 293-F cells

ThermoFisher Scientific, R79007

<https://www.thermofisher.com/order/catalog/product/R79007?SID=srch-srp-R79007#/R79007?SID=srch-srp-R79007>

<https://www.thermofisher.com/us/en/home/references/protocols/cell-culture/transfection-protocol/freestyle-293-expression-system.html#1>

General Information

Variant of the 293 cell line that has been adapted to suspension growth in FreeStyle 293 Expression Medium (ThermoFisher Scientific, 12338026). Media requires no supplements, but is extremely light sensitive- use and store media protected from light. Make aliquots of media to avoid repeated warming/cooling.

Grow cells in orbital shaker at 37°C, 120-125 rpm with 8% CO₂. Reduce speed to 90 rpm if growing culture volumes above 400 ml.

Doubling time is 22-28 hours. Subculture when cells reach 1-3 x 10⁶ cells/ml by seeding shaker flasks at ~0.2 or 0.35 x 10⁶ viable cells/ml in pre-warmed media (for subculture in 4 and 3 days, respectively). Do not dilute below 0.1 x 10⁶ viable cells/ml. Generally use 125 or 250 ml flasks and maintain a 1:3 or 1:4 culture volume to flask ratio (i.e. 30-40 ml cell suspension in 125 ml flask). Maintain cells in culture for no more than 30-35 passages.

**FreeStyle 293-F suspension cultures may grow as clusters of 2-10 cells initially. Vortex for 10-20 seconds when subculturing until they grow predominantly as single cells. Extensive cell clumping will reduce transfection efficiency.

Freeze cells in FreeStyle 293 Expression Medium (50:50 ratio of fresh to conditioned medium) with 10% DMSO at a density of 1 x 10⁷ viable cells/ml.

Rinse used flasks with 10% acetic acid followed by 10 flask volumes of dH₂O. After drying, cover with foil and autoclave for re-use. For vented caps, rinse with 70% EtOH (avoiding center) and leave in hood under UV light overnight to sterilize. Wrap in foil for storage.

Transfection of FreeStyle 293-F cells with Spike v1 and PEI (written for 250 ml transfection volume; scale as appropriate)

1. Subculture 3- 5 passages after thawing cells before use in transfection experiments.
2. Approximately 24 hours before transfection, passage cells at 0.6-0.75 x 10⁶ cells/ml. Place flask(s) on an orbital shaking platform at 125 rpm and 37°C, 8% CO₂.

3. On the day of transfection, cell density should be $\sim 1.2\text{-}1.5 \times 10^6$ cells/ml. Count cells and pellet 250×10^6 viable cells. Resuspend in 241 ml Expression Medium containing 0.1% Pluronic F-68 (Fisher Scientific, ICN2750049) to reduce shear forces during transfection. Transfer cells to a 1 L flask and return to incubator (final density will be 1×10^6 cells/ml).
 - a. Best to pellet cells and resuspend in fresh growth media. Gently resuspend cells in smaller volume and dilute to final density by adding additional growth media.
 - b. Viability must be $>90\%$.
4. Prepare DNA:PEI complexes
 - a. Dilute 250 ug DNA (1 ug per ml transfection volume, concentration 2 ug/ul) in 6.625 ml OptiMEM. Gently invert a few times to mix.
 - b. For a 1:9 DNA to PEI ratio, add 2.25 ml PEI Max (40 kDa at 1 mg/ml) and mix gently.
 - i. Total volume is 9.0 ml (3.6% of transfection volume).
 - c. Incubate 15 min at room temperature (no longer than 20 min).
 - d. Slowly add complexes to shaker flask, dropwise while swirling, and return flask to incubator.
5. Approximately 18-20 hours post-transfection, add 0.5 M valproic acid (Sigma, P4543) to a final concentration of 3.5 mM (VPA is an HDAC inhibitor that slows cell growth while maintaining cell viability, and can improve protein production).
6. Harvest 3-4 days post-transfection.

Optimization

1. Test 0.8-1.25 ug DNA per ml of transfection volume.
2. Volume of DNA-PEI-OptiMEM was kept at $\sim 3.5\text{-}4\%$ of total transfection volume.
3. Ratio of DNA to PEI should be tested for each construct. Suggest 1:2, 1:3, 1:4, 1:9, and maybe 1:12.
4. Maximal protein yield could be between 1-7 days post-transfection. Initial test should be much smaller scale (30 ml) and take daily samples to determine peak of protein production and monitor cell viability.
 - a. **Maximal yield may not correlate with protein quality/homogeneity.
5. VPA could range between 0.5 and 3.5 mM, with addition at 8-24 hours post-transfection (see You M et al., *Biosci Biotechnol Biochem* 2013).
6. Pluronic F-68 (Fisher Scientific, ICN2750049) can be added to reduce shear forces during transfection (and subculturing, if foam is generated). Add to a final concentration of 0.1% when resuspending cells for transfection.
7. Thermo recommends transfection with their proprietary reagent- FreeStyle MAX. Higher yields may be achieved relative to PEI, but have not tested. Based on experiments comparing Expifectamine and PEI transfection of Expi 293F cells, both cell viability and yield may be improved by using their transfection reagent.