The Expi293™ Expression System is a mammalian protein production system designed to allow large-scale transfection of 293 human embryonic kidney cells in suspension culture in a defined, serum-free medium. The pairing of a specialized medium with high-efficiency transfection reagent and enhancers produces up to ten times the level of protein produced with the FreeStyle™ 293 Expression System.

**Introduction**

The standardized protocol for the Expi293™ system provides a robust method that is optimized for cultures maintained in standard culture flasks. The increased yield of the system over previous suspension culture systems enables generation of useful levels of protein at sub-milliliter culture volumes, including in 96-well format, driving high-throughput applications. The Expi293™ Expression System demonstrates excellent scalability; however, the small diameter and volumes contained in 96-well plates pose unique challenges for culturing cells in suspension. Here we describe the optimization of culture conditions to maximize protein yield using 96-well microtiter plates.

**Materials**

- Expi293™ Expression System Kit
- Reporter genes: IgG, emGFP, IOH21059
- 96-square well microtiter block plates (2 mL capacity per well, V-shaped bottoms)
- PureLink® Air Porous Tape (microporous film) (Cat. No. 12262010)
- 37°C CO₂ incubator containing a plate shaker fitted with a plate clip (e.g., Eppendorf® MixMate® shaker)

**Methods**

1. **Prepare cells**
   
   Seed and maintain Expi293™ cells as directed in the system manual. To transfect cells on the following day (day 0), seed the cells at a density of $2.0 \times 10^6$ viable cells/mL.

2. **Determine the number of cells you will need for your experiment**
   
   On the day of transfection (day 0), determine cell viability using an automated cell counter or the trypan blue dye exclusion method. Important: Cells should not be clumping, and viability of cells must be >95% to proceed with transfection. Dilute cells to $2.8 \times 10^4$ cells/mL using Expi293™ Expression Medium. You will need 700 µL of cells per well in your 96-well block.

3. **Prepare transfection complexes**
   
   For each well to be transfected, prepare lipid–DNA complexes as follows:
   
   a. Dilute 0.7 µg plasmid DNA in 35 µL OptiMEM®-I and mix gently.
   
   b. Gently mix the ExpiFectamine™ 293 Reagent by pipetting it up and down, and dilute 1.9 µL in a separate tube containing 35 µL OptiMEM®-I.
   
   c. Add the diluted DNA to the diluted ExpiFectamine™ 293 Reagent and mix gently.
d. Incubate the mixture of DNA and ExpiFectamine™ 293 Reagent for 20 min at room temperature to allow the DNA–ExpiFectamine™ 293 Reagent complexes to form.

4. Transfect cells
   a. After the formation of DNA–ExpiFectamine™ 293 Reagent complexes is complete, add 70 µL of the complex to each well.
   b. Seal the plate with the microporous film and clip the block to the plate shaker.
   c. Shake the culture at 1,000–1,500 rpm inside a humidified 37°C tissue culture incubator with 8% CO2.

5. Add enhancers
   a. At 18–24 hours post-transfection, stop the shaker and remove the plate.
   b. In a tissue culture hood, prepare a cocktail of Enhancer 1 and 2 (3.5 µL of Enhancer 1 and 35 µL of Enhancer 2 per well to be treated).
   c. Peel back the film on the plate and add 38.5 µL of the enhancer cocktail to each well.
   d. Reseal the plate and return it to the shaker.
   e. Incubate with shaking for 3–7 days.

6. Determine optimal time of expression
   The time of optimal expression must be determined empirically for each protein; however, in most cases, we have observed expression in 4–5 days.

Results and discussion
Although the Expi293™ Expression System displays excellent scalability over a broad range of culture volumes and formats, certain parameters must be optimized in order to maintain a consistent protein yield per volume of culture in the 96-well microplate format.

While the same standard shakers used for Erlenmeyer- and Fernbach-style flasks can be used successfully with 24- and 48-well microplates (see the Expi293™ Expression System Manual for details), the small well diameter in 96-well plates requires more vigorous agitation to maintain cells in suspension. We expressed several proteins in 96-well format using the Expi293™ Expression System and observed almost no detectable expression in the samples incubated on standard shaker platforms, even at elevated speeds. Visual observation of the cultures revealed barely any movement of the meniscus of the medium, suggesting little to no mixing occurring within those cultures.

Expression was only seen in samples that were shaken more vigorously on a short (3 mm) throw shaker (Eppendorf® MixMate® shaker), where the shaking speed was sufficient to agitate the samples within the wells (Figure 1). On this faster shaker, no expression was seen at 700 rpm; optimal expression occurred at or above 1,000 rpm.

The geometry of the 96-well block also appears to impact time of optimal expression. A time course of expression with three different genes showed nearly maximal yield between day 3 and 5, with yield dropping significantly by day 7 (Figure 2). This contrasts with results with Erlenmeyer-style flasks where optimal yield was obtained by day 7, suggesting that further optimization with alternative well geometries might allow for increased yields at later time points. However, the IgG yield by day 5 (>500 µg/mL) indicates the system is capable of producing sufficient protein at this scale for most high-throughput applications.

![Figure 1. Shake speed optimization](image1)
![Figure 2. Time course optimization](image2)