

Protocol

Transfection of HEK293-EBNA1 Cells in Suspension with Linear PEI for Production of Recombinant Proteins

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This protocol was adapted from "Transient Expression in HEK293-EBNA1 Cells," Chapter 12, in *Expression Systems* (eds. Dyson and Durocher). Scion Publishing Ltd., Oxfordshire, UK, 2007.

INTRODUCTION

Fast and efficient production of recombinant proteins (r-proteins) remains a major challenge for the academic and biopharmaceutical communities. Pure r-proteins are often required in large amounts (hundreds of milligrams to gram quantities) when being developed as biotherapeutics, or in smaller quantities (milligrams) for high-throughput screening campaigns and structural or functional studies. Mammalian cells are often preferred over prokaryotic systems when expressing cDNAs of mammalian origin due to their superior capability to conduct elaborate post-translational modifications. Large-scale transfection of mammalian cells is now establishing itself as a "must-have" technology in the scientific community, as it allows the production of milligram to gram quantities of r-proteins within a few days after cDNA cloning into the appropriate expression vector. Although calcium-mediated large-scale transfection is very effective, it is usually achieved in serum-containing medium under tightly controlled conditions that are difficult to achieve on a large scale. In contrast, polyethylenimine (PEI) is much easier to use: It binds to and precipitates DNA efficiently and the resulting DNA-PEI complexes are suitable for efficient transfection of mammalian cells. PEI has been used successfully on a large scale in serum-containing and serum-free cultures. In particular, the linear isoform of PEI is more effective for transfecting cells in suspension. This protocol describes the steps needed for successful transfection of HEK293 cells adapted to serum-supplemented or serum-free medium in suspension culture using linear PEI.

RELATED INFORMATION

A number of protocols are available for large-scale transfection of mammalian cells for production of milligram to gram quantities of r-proteins (e.g., Jordan et al. 1998; Schlaeger and Christensen 1999; Durocher et al. 2002; Baldi et al. 2005). Additional protocols can be found in this issue, including **Transfection of Adherent HEK293-EBNA1 Cells in a Six-Well Plate with Branched PEI for Production of Recombinant Proteins** and **Transfection of HEK293-EBNA1 Cells in Suspension with 293fectin for Production of Recombinant Proteins**. For a method to purify His-tagged transfected proteins, see **Purification of His-Tagged Proteins Using Fractogel-Cobalt** (this issue).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <I>, and recipes for reagents marked with <R>.

Reagents

Cell lines, 293E or 293-6E (NRC-BRI), maintenance cells

See *Culture of HEK293-EBNA1 Cells for Production of Recombinant Proteins (this issue)* for preparation. The presence of G418 does not interfere with transfection.

Use appropriate culture medium listed below, depending on cells used.

Culture medium for 293E cells (e.g., LC-SFM medium [Invitrogen])

Supplement with 1% (v/v) heat-inactivated serum and 0.1% (w/v) Pluronic F-68 prior to use.

Culture medium for 293-6E cells (e.g., F17 culture medium [Invitrogen] or HyQ SFM4Transf-293 [HyClone])

Add 10 mL of Pluronic F-68 stock solution per liter of culture medium to a final concentration of 0.1% (w/v) prior to use.

<R>Phosphate-buffered saline (PBS)

Plasmid DNA of interest, purified

Prepare using CSH Protocols articles *Preparation of Plasmid DNA by Alkaline Lysis with SDS: Maxiprep* or *Preparation of Plasmid DNA by Large-Scale Boiling Lysis*.

Plasmid, fluorescent protein-expressing (optional; see Step 6)

Adding a plasmid encoding a fluorescent protein (e.g., green fluorescent protein [GFP]) to 5% in the transfection mixture does not significantly alter expression of the gene of interest and allows visual (or quantitative, if using flow cytometry) confirmation of transfection efficiency. A few GFP-positive cells can be detected as early as 3–4 h post-transfection using a fluorescence microscope.

Pluronic F-68, 10% stock solution (w/v) (Invitrogen)

<R>Polyethylenimine (PEI), linear (1 mg/mL)

TN1 medium, prewarmed (e.g., FreeStyle medium or LC-SFM containing 20% [w/v] Tryptone N1 [Organotechnie]) (optional; see Step 10)

Supplement with 0.1% (w/v) Pluronic F-68 and filter-sterilize prior to use.

Equipment

Containers, plastic (e.g., Tupperware)

Flasks, Erlenmeyer, shake, plastic, disposable (Corning)

Hemocytometer

Incubator preset to 37°C, humidified, 5% CO₂

Microscope, fluorescence (optional; see Step 6)

Paper towels

Plates, six-well, disposable (Nunc)

Shaker, orbital

Tubes, 1.5- or 15-mL

Vortex mixer

METHOD

Small-Scale Transfection in Six-Well Plates or 125-mL Shake Flasks

While the protocol described here uses a DNA:PEI ratio of 1:2, the ratio selected for production should be determined by testing various ratios in six-well plate experiments. These small-scale tests must include regular samplings to determine the optimal harvest periods. The flask procedure is scalable to 650 mL in a 2-L shake flask.

1. One or two days prior to transfection, dilute the cells to 2.5×10^5 cells/mL (for 48 h prior) or 5×10^5 cells/mL (for 24 h prior) with the appropriate cell culture medium.
Optimal cell density should be reached on the day of transfection. This avoids having to centrifuge the cells, which can have a detrimental effect on transfection efficiency if performed shortly prior to transfection.
2. On the day of transfection, warm the PBS to 25°–37°C. Thaw the DNA and PEI.
3. From a maintenance flask, determine the cell density and viability using a hemocytometer.

Cell density at transfection should range from 8×10^5 to 1.2×10^6 cells/mL, provided the doubling time is 24 h and viability is greater than 95%.

4. Add 1.8 mL of cells to each of the six wells (or 18 mL to a new 125-mL shake flask). Transfer to an incubator.

To minimize medium evaporation, put the plates in a plastic container lined with a wet paper towel to preserve humidity. "Trap" some CO₂ in the container before closing the lid.

5. Add 200 μ L of PBS to six 1.5-mL tubes (or 2.0 mL to a 15-mL tube for the 125-mL shake flask).
6. Add 2 μ g of DNA to each tube (or 20 μ g to the 15-mL tube for the 125-mL shake flask). Vortex gently.

To evaluate transfection efficiency, add a GFP expression plasmid at 5% of the final DNA content used. A reasonable indicator of successful transfection is when the percentage of GFP-positive cells is between 30% and 50% by 48-72 h post-transfection.

7. Add 4 μ L of PEI to each tube (or 40 μ L to the 15-mL tube for the shake flask) containing DNA solution. Vortex immediately (three times, 3 sec each) after PEI addition.

Compare different DNA:PEI ratios (1:2 vs. 1:3, etc.) to see whether different lots and/or production batches affect transfection efficiency and productivity. Each new PEI preparation must be tested.

8. Incubate the mixture for 15 min at room temperature.
9. Remove the culture (from Step 4) from the incubator. Add the DNA/PEI mixture(s) and swirl. Return the culture to the incubator.
10. For secreted protein production, add 50 μ L of prewarmed TN1 medium at 24-48 h post-transfection to each of the six wells (or 500 μ L for the 125-mL shaker flask). Return the culture to the incubator.

The final TN1 concentration in the culture(s) should be 0.5% (w/v). TN1 supplementation is not necessary for intracellular protein production.

11. Harvest the r-proteins:

For intracellular r-proteins

- i. Harvest the cells at 48-72 h post-transfection.

Establish the best harvesting time by performing an expression kinetics study.

For secreted r-proteins

- ii. Harvest the supernatants between 96 and 168 h post-transfection as long as the culture viability is high and the product titer is increasing.

Since culture viability decreases with time, it is important to harvest when the viability is relatively high (i.e., greater than 75%) to protect the r-protein from proteolytic degradation.

See Troubleshooting.

TROUBLESHOOTING

Problem: The transfection does not work.

[Step 11]

Solution: Consider the following:

1. Make sure the cells are in exponential growth phase for transfections. If the culture grows to higher-than-prescribed transfection densities, medium nutrient depletion and metabolic by-products will compromise the transfection.
2. Anti-clumping agents such as dextran sulfate can inhibit transfection.
3. DNA plasmid quality may be inadequate. Check for contaminants by A_{260}/A_{280} ratio readings; acceptable values range between 1.80 and 1.95. In addition, the DNA plasmid can be digested and run on an agarose gel to verify its integrity (it should be mostly in a supercoiled form) and/or to detect RNA contamination.
4. Monitor cell line productivity occasionally by transfecting the cells with an easily measured protein such as secreted alkaline phosphatase or GFP. Low transfection efficiencies are readily indicated

when the percentage of GFP-positive cells is below 30% by 48 h post-transfection. Also, for each new medium or reagent lot, monitor transfection efficiencies using six-well plate assays.

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