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Terminology
Terminology

**Transfection:** Introduction of foreign DNA into the nucleus of eukaryotic cells. Cells that have incorporated the foreign DNA are called transfectants.

**Stable transfectants:** Cells that have integrated foreign DNA in their genome.

**Transient transfectants:** Foreign DNA does not integrate in the genome but genes are expressed for a limited time (24–96 hours).
Factors Affecting Transfection

Host Cell
- Cell health
- Cell culture

Genetic Material
- DNA quality and quantity
Cell Health

- Cells should be grown in appropriate medium with all necessary factors
- Cultures must be free of contamination
- Fresh medium must be used if it contains chemically unstable components, such as thiamine
- Cells should be incubated at 37°C with CO₂ supplied at the correct percentage (5–10%) and 100% relative humidity
- Cells should be maintained in log phase growth
Cell Culture

Confluency and Growth Phase
- Cells should be transfected at 40–80% confluency (cell type dependent)
  - Too few cells cause cell cultures to grow poorly without cell-to-cell contact
  - Too many cells result in contact inhibition, making cells resistant to uptake of DNA
    and other macromolecules
- Actively dividing cells take up DNA better than quiescent cells (breakdown and perforation
  of the nuclear membrane during mitosis enable nuclear delivery)

Number of Passages for Primary Cells
- The number of passages should be low (<50)
- The number of passages for cells used in a variety of experiments should be consistent
- Cell characteristics can change over time with immortalized cell lines and cells may not
  respond to the same transfection conditions
- Cells may not respond to the same transfection conditions after repeated passages
DNA Quality and Quantity

- Use high-quality plasmid DNA that is free of proteins, RNA, and chemicals for transfections; endotoxin removal should be part of the preparation procedure.
- Typically, DNA is suspended in sterile water or TE buffer to a final concentration of 0.2–1 mg/ml.
- The optimal amount of DNA to use in the transfection will vary widely depending upon the type of DNA, transfection reagent/method, target cell line, and number of cells.
Transfection Workflow
Transfection Workflow

1. **Tissue culture**
   - Count cells

2. **Resuspend cells in electroporation buffer**

3. **Add nucleic acid**

4. **Transfect cells**

5. **Plate cells**

6. **Analysis**
   - **Protein expression**
     - Western blot analysis
   - **Gene expression**
     - Reporter gene activity
     - Microscopy
     - Flow cytometry
     - Real-time qPCR
Transfection Methods

Reagent-Based Methods
- Lipids
- Calcium phosphate
- Cationic polymers
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Instrument-Based Methods
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Virus-Based Methods
Cationic Lipids: Liposomes/Lipoplexes

- Cationic lipids are amphiphilic molecules that have a positively charged polar head group linked, via an anchor, to an apolar hydrophobic domain generally comprising two alkyl chains.
- Structural variations in the hydrophobic domain of cationic lipids include the length and the degree of non-saturation of the alkyl chains.
- Electrostatic interactions between the positive charges of the cationic lipid head groups and the negatively charged phosphates of the DNA backbone are the main forces that allow DNA to spontaneously associate with cationic lipids.
Lipid-Mediated Gene Delivery

Lipid-mediated gene delivery is also referred to as lipofection, or liposome-based gene transfection. It uses lipids to cause a cell to absorb exogenous DNA.

Transfer of genetic material into the cell takes place via liposomes, which are vesicles that can merge with the cell membrane since they are both made of a phospholipid bilayer.
Transfection Methods

Reagent-Based Methods

Lipids

Method Overview

2–4 µl Transfectin™ lipid reagent per 50 µl serum-free medium

0.25–1 µg plasmid DNA per 50 µl serum-free medium

Mix equal volumes of TransFectin and DNA solutions

Incubate 20 min to form DNA-liposome complexes

Mix; add DNA-liposome complexes directly to cells (100 µl/24-well plate)

Plated cells

Aspirate medium from cells

Incubate overnight and assay
Pros and Cons

**Advantages of Lipids**
- Deliver nucleic acids to cells in a culture dish with high efficiency
- Easy to use, minimal steps required; adaptable to high-throughput systems
- Using a highly active lipid will reduce the cost of lipid and nucleic acid, and achieve effective results

**Disadvantage of Lipids**
- Not applicable to all cell types
Calcium Phosphate

The protocol involves mixing DNA with calcium chloride, adding this in a controlled manner to a buffered saline/phosphate solution, and allowing the mixture to incubate at room temperature.

This step generates a precipitate that is dispersed onto the cultured cells. The precipitate is taken up by the cells via endocytosis or phagocytosis.
Method Overview

**Solution A:** DNA in calcium solution

**Solution B:** 2x Hanks buffered saline solution

1. Add solution A to solution B while vortexing.

2. Incubate 20–30 min. Apply the solution to a subconfluent cell culture.

3. Incubate 2–12 hr. Replace the solution with complete growth medium.

4. Assay for transient gene expression or begin selection for stable transformation time.
Pros and Cons

Advantages of Calcium Phosphate
- Inexpensive
- High efficiency (cell type dependent)
- Can be applied to a wide range of cell types
- Can be used for transient and stable transfection

Disadvantages of Calcium Phosphate
- Reagent consistency is critical for reproducibility
- Small pH changes (±0.1) can compromise transformation efficiency
- Size and quality of the precipitate are crucial to the success of transfection
- Calcium phosphate precipitation does not work in RPMI, due to the high concentration of phosphate within the medium
Cationic Polymers

Cationic polymers differ from cationic lipids in that they do not contain a hydrophobic moiety and are completely soluble in water. Given their polymeric nature, cationic polymers can be synthesized in different lengths, with different geometry (linear versus branched). The most striking difference between cationic lipids and cationic polymers is the ability of the cationic polymers to more efficiently condense DNA.

There are three general types of cationic polymers used in transfections:

- Linear (histone, spermine, and polylysine)
- Branched
- Spherical

Cationic polymers include polyethylenimine (PEI) and dendrimers.
DEAE-Dextran

DEAE-dextran is a cationic polymer that tightly associates with negatively charged nucleic acids. The positively charged DNA:polymer complex comes into close association with the negatively charged cell membrane. DNA:polymer complex uptake into the cell is presumed to occur via endocytosis or macropinocytosis.
Method Overview

**Solution A:** DNA (~1–5 µg/ml) diluted into 2 ml of growth medium with serum containing chloroquine

**Solution B:** DEAE-dextran solution (~50–500 µg/ml)

**Solution C:** ~5 ml of DMSO

**Solution D:** Complete growth medium

1. Add solution A to solution B, then mix gently.

2. Aspirate cell medium and apply the mixed A and B solutions to the subconfluent cell culture. Incubate the DNA mixture for ~4 hr.

3. Aspirate supernatant.

4. Add solution C to induce DNA uptake.

5. Remove DMSO and replace with complete growth medium; assay for transient gene expression.
Pros and Cons

**Advantages of DEAE-Dextran**
- Inexpensive
- Easy to perform and quick
- Can be applied to a wide range of cell types

**Disadvantages of DEAE-Dextran**
- High concentrations of DEAE-dextran can be toxic to cells
- Transfection efficiencies will vary with cell type
- Can be used only for transient transfection
- Typically produces less than 10% delivery in primary cells
Activated Dendrimers Structure

Positively charged amino groups (termini) on the surface of the dendrimer molecule interact with the negatively charged phosphate groups of the DNA molecule to form a DNA-dendrimer complex.

The DNA-dendrimer complex has an overall positive net charge and can bind to negatively charged surface molecules on the membrane of eukaryotic cells. Complexes bound to the cell surface are taken into the cell by nonspecific endocytosis. Once inside the cell, the complexes are transported to the endosomes.

DNA is protected from degradation by endosomal nucleases by being highly condensed within the DNA-dendrimer complex.

Amino groups on the dendrimers that are unprotonated at neutral pH can become protonated in the acidic environment of the endosome. This leads to buffering of the endosome, which inhibits pH-dependent endosomal nucleases.
Magnet-Mediated Transfection

Magnet-mediated transfection uses magnetic force to deliver DNA into target cells.

Nucleic acids are first associated with magnetic nanoparticles. Then, application of magnetic force drives the nucleic acid–particle complexes toward and into the target cells, where the cargo is released.
Method Overview

1. Dilute nucleic acid in medium.
2. Add magnetic nanoparticle.
3. Incubate 10–20 min.
4. Add medium to adherent cells (2–4 x 10^5 cells).
5. Add nucleic acid/nanoparticle solution.
6. Place culture plate on magnet plate.
7. Incubate 15 min.
8. Remove magnet plate.
Pros and Cons

**Advantages of Magnetic Beads**
- Rapid
- Increased transfection efficiency by the directed transport, especially for low amounts of nucleic acids
- High transfection rates for adherent mammalian cell lines and primary cell cultures (suspension cells and cells from other organisms also successfully transfected but need to be immobilized)
- Mild treatment of cells
- Can also be performed in the presence of serum

**Disadvantages of Magnetic Beads**
- Relatively new method
- Requires adherent cells; suspension cells need to be immobilized or centrifuged
How Electroporation Works

1. Electroporation exposes a cell to a high-intensity electric field that temporarily destabilizes the membrane.

2. During this time the membrane is highly permeable to exogenous molecules present in the surrounding media.

3. DNA then moves into the cell through these holes.

4. When the field is turned off, the pores in the membrane reseal, enclosing the DNA inside.
Type of Electrical Pulse — the Waveform

The most common electrical fields are exponential and square waveforms. The waveform has a significant effect on the transfection efficiency for different cell types. Both exponential-decay and square-wave pulses have been used very effectively for electroporation.

**Exponential Waveform**
- Capacitors charged to a set voltage
- Set voltage is released from the selected capacitor and decays rapidly (exponentially) over time

**Square Waveform**
- Determined by pulse duration and/or number of pulses
- Several short pulses may be more beneficial than one long pulse
Pros and Cons

**Advantages of Electroporation**
- Nonchemical method that doesn’t seem to alter the biological structure or function of the target cells
- Easy to perform
- High efficiency
- Can be applied to a wide range of cell types

**Disadvantage of Electroporation**
- Cell mortality (if using suboptimal conditions)
Biolistic Particle Delivery

Biolistic transformation is the delivery of nucleic acids into cells via high velocity nucleic acid–coated microparticles.

Helios® Gene Gun
- For in situ, in vivo, and in vitro transformations
- Applications for animals, plants, cell culture, nematodes, yeast, and bacteria
- Pressure range 100–600 psi enables fine-tuning of penetration
- Highly portable — can be used in the field
- Small target area for accurate targeting

PDS-1000/He™ Biolistic Particle Delivery System
- For in vitro, ex vivo, and in vivo (for some plants and microbes) transformations
- Applications for animal cell and organ cultures, plant cell cultures and explants, pollen, insects, algae, fungi, and bacteria
- Pressure range 450–2,200 psi gives flexibility and penetration — ideal for plant applications
- Large target area — more cells can be transformed
Helios® Gene Gun — Process Overview

1. Precipitate DNA onto gold particles.
2. Load DNA/gold into tubing.
3. Rotate tubing to coat DNA gold over inside surface.
4. Cut tubing into cartridges.
5. Load cartridges into gene gun.
6. Deliver DNA into target cells.
### PDS-1000/He™ System — Process Overview

1. DNA-coated gold particles (microcarrier) are spread over the central area of a thin plastic disk (macrocarrier).
2. Disk loaded with the DNA-gold particles is placed into a holder inside the PDS-1000/He system.
3. The system uses high-pressure helium, released by a rupture disk, and partial vacuum to propel the macrocarrier loaded with microcarrier toward the target cells.
4. Macrocarrier is stopped after a short distance by a stopping screen.
5. DNA-coated gold particles continue travelling toward the target to penetrate the cells.
6. Sample chamber is subjected to partial vacuum, from 15 to 29 inches of mercury, depending on the target cells.

![Diagram of PDS-1000/He™ System process]

- **Gas acceleration tube**
- **Rupture disk**
- **Macrocarrier**
- **Stopping screen**
- **Microcarrier launch assembly**
- **DNA-coated microcarriers**
- **Target cells**
- **Target shelf**
## Helios® Gene Gun vs. PDS-1000/He™ System

### Factors Affecting Transformation

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<thead>
<tr>
<th></th>
<th>Helios Gene Gun System</th>
<th>PDS-1000/He System</th>
<th>PDS-1000/He™ System With Hepta™ Adaptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental conditions</td>
<td>In situ, in vitro, in vivo, ex vivo</td>
<td>In vitro, ex vivo, in vivo (plants)</td>
<td>In vitro, ex vivo, in vivo (plants)</td>
</tr>
<tr>
<td>Target area</td>
<td>Small (2 cm²)</td>
<td>Large (40 cm²)</td>
<td>Largest (~75 cm²)</td>
</tr>
<tr>
<td>Pressure range</td>
<td>100–600 psi</td>
<td>450–2,200 psi</td>
<td>450–2,200 psi, reduced by 7-way spread of helium</td>
</tr>
<tr>
<td>Target type</td>
<td><strong>Animals:</strong> Any tissue exposed to barrel (skin, organs); cell, explant, and organ culture <strong>Plants:</strong> Field and greenhouse use, plant cell culture, explants <strong>Yeast, bacteria, other microbes</strong></td>
<td><strong>Animals:</strong> Cell and organ culture <strong>Plants:</strong> Small intact plants, plant cell culture, explants <strong>Yeast, bacteria, other microbes</strong> <strong>Organelles (chloroplasts, mitochondria, etc.)</strong></td>
<td><strong>Animals:</strong> Cell and organ culture <strong>Plants:</strong> Cells with thin cell walls <strong>Yeast, bacteria, other microbes</strong></td>
</tr>
</tbody>
</table>
Pros and Cons

**Advantages of Biolistic Technology**
- Simple, rapid, versatile technique
- Targeted intracellular gene delivery
- Cell type independent
- Uses small amounts of DNA
- Delivers single or multiple genes
- No carrier DNA needed
- Can deliver large DNA fragments
- No extraneous genes or proteins delivered
- Requires little manipulation of cells
- High reproducibility

**Disadvantages of Biolistic Technology**
- Generally lower efficiency compared to electroporation or viral- or lipid-mediated transfection
- Limited bacterial transfection data
- Preparation of microparticles
- Instrument cost
- Requires purchase agreement
Microinjection

- Direct injection of naked DNA
- Laborious (one cell at a time)
- Technically demanding and costly
- Can be used for many animals

Fertilized egg, 100 µm

Holding pipet

Injecting pipet

Transgene
Laserfection/Optoinjection

- This procedure uses laser light to transiently permeabilize a large number of cells in a very short time.
- Various substances can be efficiently optoinjected, including ions, small molecules, dextrans, short interfering RNAs (siRNAs), plasmids, proteins, and semiconductor nanocrystals, into numerous cell types.
- Advantages: very efficient; works with many cell types; fewer cell manipulations needed.
- Disadvantages: requires the cells to be attached; expensive laser-based equipment needed.
Viral Vectors

**Retroviruses**
- Murine leukemia virus (MuLV)
- Human immunodeficiency virus (HIV)
- Human T-cell lymphotropic virus (HTLV)

**DNA Viruses**
- Adenovirus
- Adeno-associated virus (AAV)
- Herpes simplex virus (HSV)

**Retroviruses** — a class of viruses that can create double-stranded DNA copies of their RNA genomes; these copies can be integrated into the chromosomes of host cells. HIV is a retrovirus.

**Adenoviruses** — a class of viruses with double-stranded DNA genomes that cause respiratory, intestinal, and eye infections in humans. The virus that causes the common cold is an adenovirus.

**Adeno-associated viruses** — a class of small, single-stranded DNA viruses that can insert their genetic material at a specific site on chromosome 19.

**Herpes simplex viruses** — a class of double-stranded DNA viruses that infect a particular cell type, neurons. Herpes simplex virus type 1 is a common human pathogen that causes cold sores.
## Viral Attributes

<table>
<thead>
<tr>
<th>Viral Vector</th>
<th>DNA Insert Size</th>
<th>Maximum Titer</th>
<th>Cell Type</th>
<th>Expression</th>
<th>Pitfalls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retroviral</td>
<td>8 kb</td>
<td>$1 \times 10^9$</td>
<td>Dividing cells</td>
<td>Stable</td>
<td>Random insertion site</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>9 kb</td>
<td>$1 \times 10^9$</td>
<td>Dividing cells</td>
<td>Stable</td>
<td>Random insertion site</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>8 kb</td>
<td>$1 \times 10^{13}$</td>
<td>Dividing cells</td>
<td>Transient</td>
<td>Highly immunogenic</td>
</tr>
<tr>
<td>Adeno-associated virus (AAV)</td>
<td>5 kb</td>
<td>$1 \times 10^{11}$</td>
<td>Dividing cells</td>
<td>Stable, site-specific location</td>
<td>Requires helper virus to grow; difficult to remove helper virus</td>
</tr>
<tr>
<td>Herpes simplex virus</td>
<td>30–40 kb</td>
<td>$1 \times 10^9$</td>
<td>Dividing cells</td>
<td>Transient</td>
<td>No gene expression during latent infection</td>
</tr>
<tr>
<td>Vaccinia virus</td>
<td>25 kb</td>
<td>$3 \times 10^9$</td>
<td>Dividing cells</td>
<td>Transient</td>
<td>Potential cytopathic effects</td>
</tr>
</tbody>
</table>
Viral Workflow

Transfection Methods

Virus-Based Methods

Virus Preparation

Transfect a 293 producer cell line with the gene of interest inserted into the expression vector

Expression vector

Transfect 8–10 hr

Target Cell Infection

Plate target cells

Add the viral supernatant to the target cells to produce a stably expressed cell line

Harvest supernatant

48–72 hr

Collect virus

Infect with diluted virus 18 hr

Determine viral titer (1 week)

2–3 days

Select cells and analyze

Determine the titer of virus stock

Virus Preparation

Transfect a 293 producer cell line with the gene of interest inserted into the expression vector

Common Transfection Methods
Pros and Cons

**Advantages of Virus-Based Methods**
- Very high gene delivery efficiency, 95–100%
- Simplicity of infection

**Disadvantages of Virus-Based Methods**
- Labor intensive
- Best for introducing a single cloned gene that is to be highly expressed
- P2 containment required for most viruses
  - Institutional regulation and review boards required
  - Viral transfer of regulatory genes or oncogenes is inherently dangerous and should be carefully monitored
  - Host range specificity may not be adequate
- Many viruses are lytic
- Need for packaging cell lines