Transfection Optimization and Induction Protocols for Adherent Cell Lines

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Introduction to Transfection

Why Optimize?

Compared to Prokaryotic transformations, Eukaryotic transfection is more complicated and touchy. Variations between cell lines, transfection technique, reagent choice and plasmid quantity can drastically influence the success of your transfection. Due to this, we recommend conducting a series of preliminary tests to experimentally determine the optimal conditions for your experiment, measured in terms of percent transfection efficiency.* The results and recommendations below were optimized for a Lipofectamine 3000 transfection system but are applicable for most other chemical methods of transfection as well.

Transfection of a single constitutive fluorescent plasmid (ie. pMAX-GFP or mCherry) conveys quick results that can be obtained through UV imaging or flow cytometry, and can be used as an initial experimental model to optimize the process within your cell line and gain experience before beginning transfection of a more complex, possibly multi-plasmid, system. We have outlined the optimal transfection settings for the three cell lines used in our 2019 iGEM project (NIH-3T3, CHO-DG44, and AML-12) but the same tests can be applied to any adherent cell line.

*transfection efficiency = number of transfected cells/number of total cells

Background: A Review of Common Techniques

Transfection is the process of inserting genetic material into eukaryotic cells, and can either be transient or Stable. *Transient Transfections* occur when the construct remains as a plasmid, unable to replicate alongside cell division in mammalian cells. *Stable Transfections* occur when the DNA inserted is uptaken into the cell's genome; a less consistent occurrence usually achieved via microinjection or viral transduction.

A variety of transfection methods exist for different cell lines and experimental conditions; current common methods fall into the following main categories:

1. **Physical Transfection** - agitation of the cells causes temporary degradation of the membrane, increasing the cell's ability to uptake DNA in the environment. Some examples include;
    a. Electroporation:[1] The formation of small, temporary pores in the phospholipid bilayer through short-term exposure to strong electrical pulse
    b. Sonoporation:[2] The formation of small, temporary pores in the phospholipid bilayer through exposure to high-intensity ultrasound
    c. Impalefection:[3] DNA is bound to nanotubes which are then used to puncture the cell membrane and transfer genetic material into the cells

   d. Microinjection:[4] injection of DNA into cell cytoplasm or nucleus through fine, glass microcapillary pipette. Frequently used for mammalian oocytes and early embryos and significantly more expensive than other methods.

2. **Particle Bombardment Transfection** - DNA is coupled to small particles which then penetrate the cell
   a. Gene gun:[4] DNA is coupled to nanoparticles of inert metal (usually gold) and ballistically shot into a plate of cells. Most commonly used for plant cell transfections due to presence of cell wall
   b. Magnetofection:[5] DNA is coupled to magnetic nanoparticles which is then directed towards the cells using magnetic force

3. **Viral Transduction** [6]- use of a retrovirus, lentivirus, adenovirus, adeno-associated virus, or herpes simplex virus to insert DNA into cells. Only possible with small fragments of DNA

4. **Chemical Transfection** - use of chemical solutions to adhere and transport the DNA across the membrane by disrupting membrane stability
   a. Calcium Phosphate:[7] one of the oldest and least expensive methods, phosphate ions in HEBES buffered saline solution (HeBS) interact with a calcium chloride and DNA solution, forming a precipitate which also contains the genetic material. Cells will uptake the DNA alongside the precipitate upon exposure.
   b. Lipofection:[8] DNA to be transfected is coated in cationic which is then able to interact directly with the outer cell membrane then the nuclear envelope once inside, bringing the DNA into the nucleus.

Our experiments were conducted using mainly Lipofectamine 3000 and a single experiment of Calcium Phosphate based Transfection, so protocols and experimental data were made for these protocols. While lipofectamine may favor different conditions than other techniques, the same steps can be taken to optimize most known chemical transfections.

Preliminary Tests

Optimizing your Technique: Tests to Consider

1. Seeding Density
2. Adherence
3. Wash
4. Lipofectamine Concentration
5. DNA Concentration
6. Plasmid Ratios (for 2+)

Seeding Density

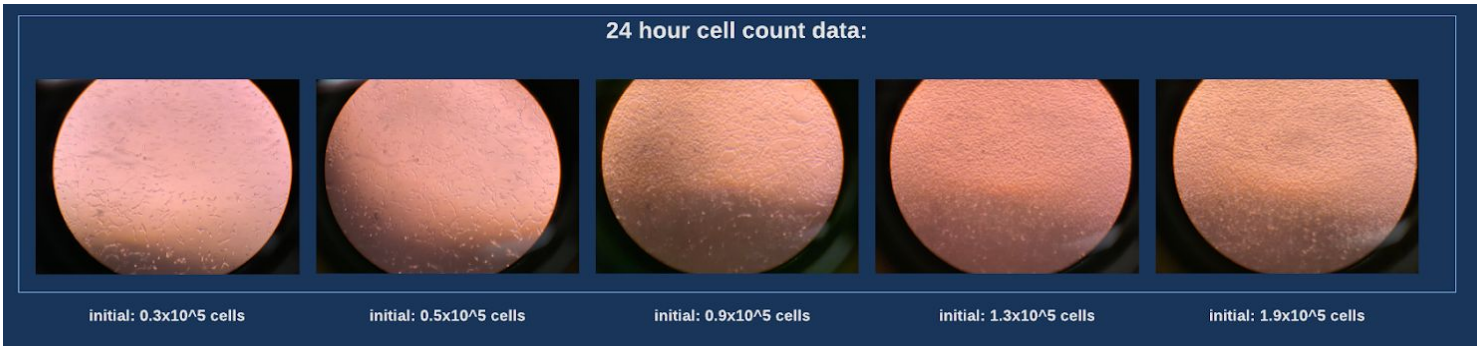
Depending on the surface area of the plate wells, total incubation time, and adhered cells themselves, the optimal number of cells per well will vary. At the time of transfection it is reccomended to have approximately 70-90% confluent growth, so we recommend experimental determination. Reference the spec sheets pertaining to your cells (ie. from ATCC) or this reference guide by Thermo Fisher about the optimal seeding density for your plate dimensions, then seed wells at these densities as well as a few above and below to obtain an accurate picture.

As the transfection process itself can greatly influence final cell counts, especially if the transfection method of choice is chemically or physically disruptive, it is not uncommon for the final cell count to be lower in transfected wells vs untransfected wells. Additionally, if your experiments involve further disruption, such as light or temperature variations, that may affect cell count as well.

Seeding Densities: Quick-Check Reference Chart

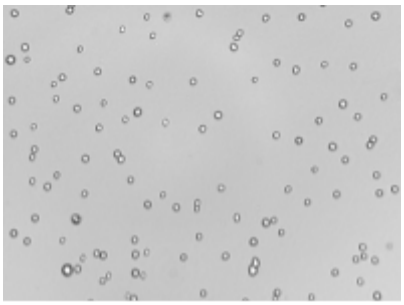
Cell line	Preferred seeding concentration	For T75 flask ($75cm^2$)	For 24 well-plate ($1.9cm^2$ per well)
CHO-DG44	$2x10^4$ cells/cm^2	$1.5x10^6$ cells in 14ml media total	$0.5x10^5$ cells in 0.5-1ml media per well
AML-12	$2x10^4$ cells/cm^2	$1.5x10^6$ cells in 14ml media total	$0.5x10^5$ cells in 0.5-1ml media per well
NIH-3T3	$1.5x10^4$ cells/cm^2	$1x10^6$ cells in 14ml media total	$0.5x10^5$ cells in 0.5-1ml media per well

*24 well plate seeding densities were determined experimentally, and do not correspond with the normally recommended scaling proportions

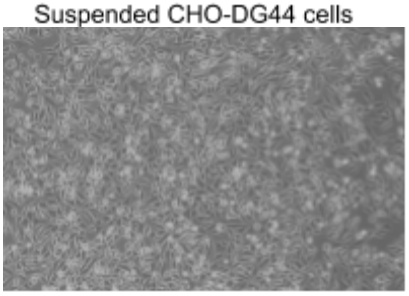


For more information on determination of seeding density, see our seeding test on the Zorya Wiki design page

Adhered vs Non-Adhered



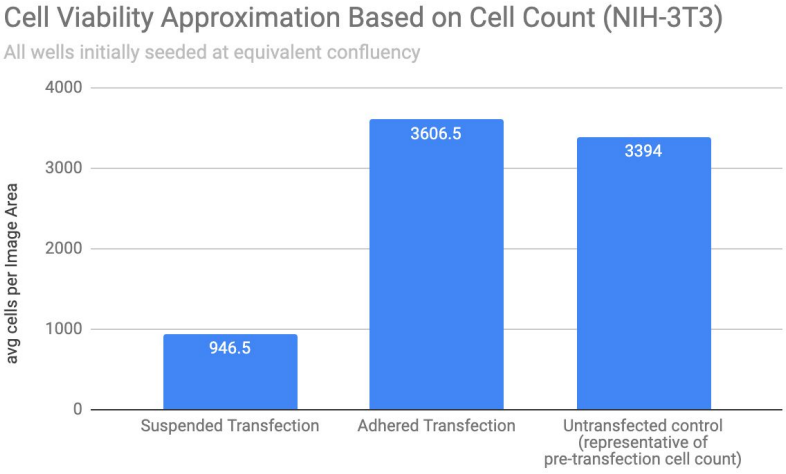
Suspended CHO-DG44 cells



Adhered CHO-DG44

Some cell lines “flatten” more than others once they have been given the time to adhere to the surface of the plate- for example NIH-3T3 have a larger adhered surface area, at 18μm[9] than CHO-DG44, at 14-15μm[10]. This variation in percent exposed cell-membrane in combination with possible cell overlapping can limit a cell’s exposure to the liquid transfection solution.

Transfecting cells directly after seeding the new plate and before the cells get a chance to adhere maximizes available surface area and increases contact to the transfection solution[11]. However, cells in suspension are less durable than adhered and transfection in this state may lead to high cell fatality rates for more sensitive cell lines(see collected data below).



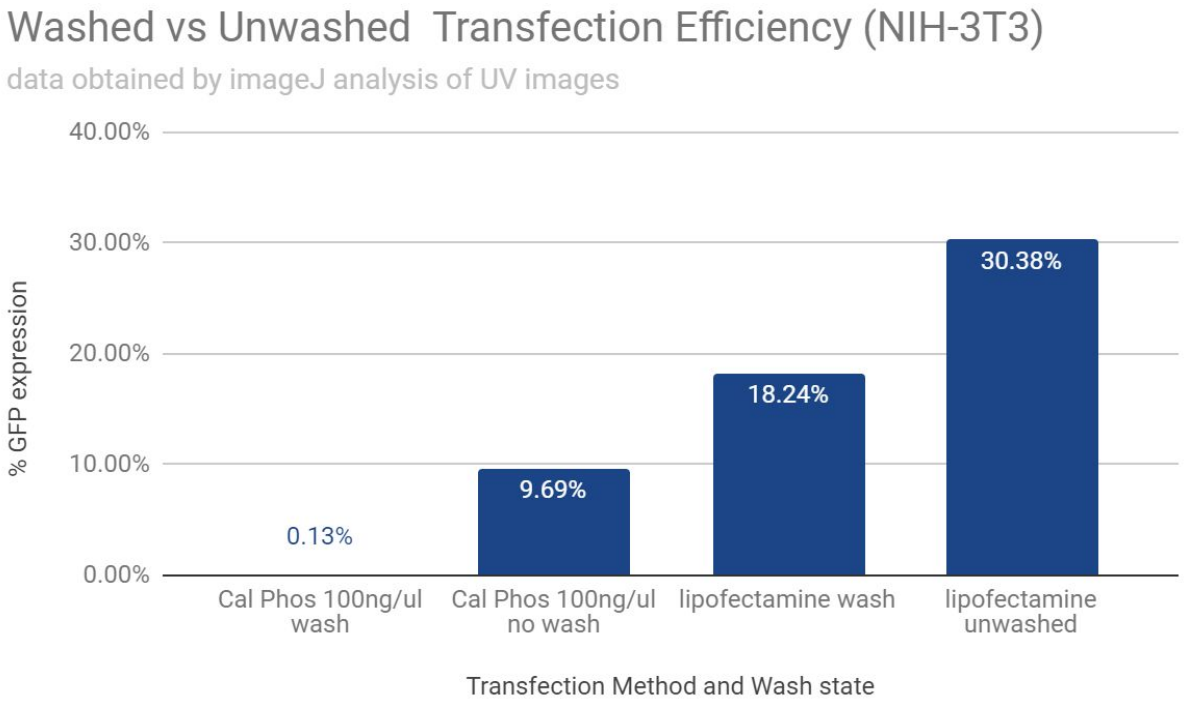
We transfected all three cell lines, some samples directly after seeding the plate, ensuring cells were still in suspension (suspended transfection) and some samples were incubated overnight prior to transfection, ensuring cells had time to adhere to the plate surface[12] (Adhered Transfection). UV imaging of GFP expression 24 hours later revealed that while adhered transfection resulted in lower cell mortality, the transfection efficiency was 5x higher for all suspended transfections. (see Zorya Wiki for experimental data)

Wash vs No Wash

Many chemical-based transfection solutions contain reagents highly toxic to cells upon prolonged exposure. To counteract these effects and promote cell survival post-transfection, it is possible to aspirate the media containing the chemical solution, wash adhered cells with PBS, then replace with fresh media and allow to continue growing for the duration of the desired incubation period.

Washes were performed on samples two hours post-transfection for all three cell lines. We observed that the wash step resulted in a decrease of transfection efficiency by removing many transfected but not adhered cells along with the media. In the case of lipofectamine and calcium phosphate, the wash step is not encouraged but in the case of other more chemically

strenuous transfection methods the wash step can increase both cell survival and transfection efficiency. (see Zorya Wiki for more information)



Lipofectamine Concentration

Invitrogen[13] recommends the use of two different concentrations of lipofectamine based on the plate specifications during initial testing. Higher concentrations of lipofectamine may increase transfection rates but may also be detrimental to total cell count due to its toxicity. More sensitive cell lines as well as those that are more receptive to transfection can use the lowest recommended volume (or even lower) to conserve resources.

An additional method we found to influence final transfection efficiency was to artificially increase the Lipofectamine concentration. When plating your cells (preferably during suspended transfection) only add half the final amount of media, then transfect. This will double the contact the lipofectamine-DNA solution has with your cells and increase transfection efficiency. Allow the cells to transfect for 2-3 hours then add the remainder of the desired media for overnight growth before data collection.

DNA Concentration

While many of the protocols available in literature state that DNA is preferred at a concentration of 1ug/ul, this would require using a speedvac to further concentrate our plasmid mini- and midi-preps. We concentrated a sample of DNA as the protocol dictated and compared the results against 1ug of unconcentrated vector straight from the midiprep and found an insignificant difference between the two. (see Zorya Wiki) We concluded that the final mass of DNA is the deciding factor rather than the volume.

This conclusion may not apply to all transfection conditions, especially if using a chemical method other than Lipofectamine 3000, and should be re-tested for your cell line.

Plasmid Ratios

Transfecting a system that requires multiple plasmids of differing sizes vastly increases the complexity of the process. By determining which plasmid is limiting, which can either be assumed based on size[14] or experimentally determined using flow cytometry and plasmid dye[15], you can vary the plasmid ratios to optimize dual transfection rates.

If your system does not naturally express fluorescence as an indicator (for example ours is a CRISPR based targeting system that upregulates endogenous genes) we recommend generating an altered version that can be detected by visual analysis or flow cytometry for the optimization process.

The three concentrations we tested were 1ug of each of our 3 plasmids, 0.33ug of each plasmid adding up to 1ug total, and a weight-based ratio of 0.5ug, 0.25ug, 0.25ug with the 5ug vector being the largest to better account for size and number.

Protocols

Lipofectamine3000 Transfection of Adherent Mammalian Cells

To transfect our cells, we used Lipofectamine 3000 according to the protocol by Thermo Fisher. These protocols have been designed for use in both 96-well and 24-well plates, see protocol for variations.

Overview:

1. Calculate
2. Create solutions
3. Seed plate
4. Transfect

Procedure

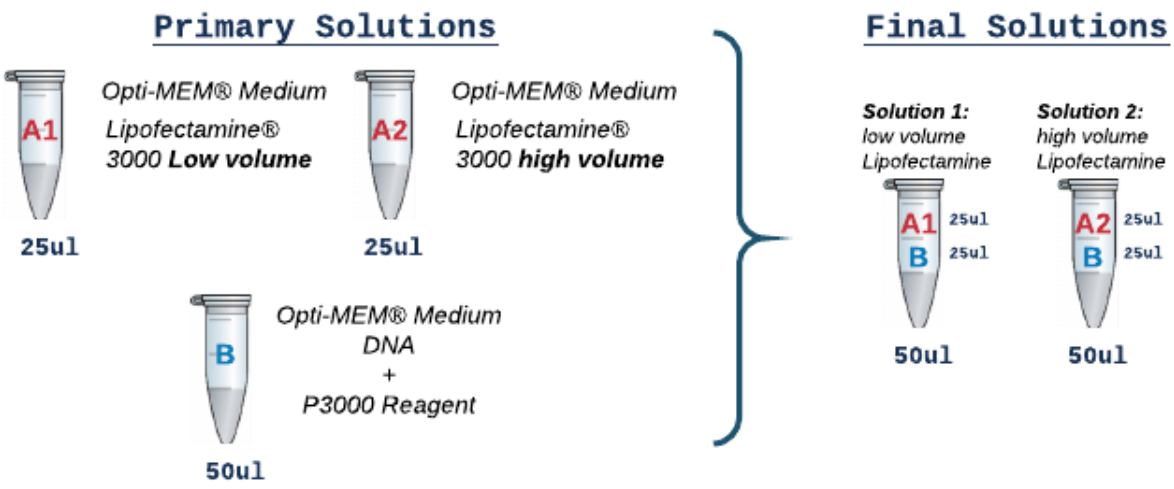
1. Grow cells until they are between 80-90% confluent before harvesting
2. Prepare your biosafety cabinet by leaving the UV light on for 10 minutes prior to experimentation, spray with ethanol and wipe down. Place complete media and PBS in a 37°C water bath for 20 minutes.
3. If you are doing a suspended transfection, skip this step and proceed to 4. If doing an adhered transfection trypsinize cells from growth flask and seed in testing plate at a confluency that will result in 70-90% confluency at the time of transfection (12-24hrs later) Incubate before proceeding.
4. On the benchtop, prepare two versions of **solution A** (for each of the different lipofectamine concentrations) by combining the following in 1.5ml microcentrifuge tubes (all volumes are provided per well) then vortex for 2-3 seconds.

Solution A Reagent	24-well plate	96-well plate
Opti-MEM® Medium	25ul in both A1 and A2	5ul in both A1 and A2
Lipofectamine® 3000 Reagent	1.5ul in A1 and 0.75ul in A2	0.15ul in A1 and 0.3ul in A2

5. Prepare one tube of solution B by combining the following per well. Dilute DNA with *Opti-MEM* then add P3000 last. Mix by pipetting up and down.

Solution B Reagent	24-well plate	96-well plate
Opti-MEM® Medium	50ul	10ul
DNA (0.5–5 µg/µL)	1ug	0.2ug
P3000™ Reagent (2 µL/µg DNA)	2ul	0.4ul

6. Incubate both solutions A and B at room temperature for 15 minutes before combining in 1:1 ratio of 25ul of each A and B. Mix by pipetting and incubate AB mixture for another 15 minutes.



7. If doing a suspended transfection, prepare cells as solutions incubate.
    a. Trypsonize a flask of 80-90% confluent mammalian cells and seed them at a confluency of 80-90% per well in half the total desired volume of complete media (ie. 0.5ml rather than 1ml in 24 well plates) to increase Lipofectamine concentration in contact with the cells.
    b. Let seeded cells sit in 37ºC incubator for 10 minutes prior to transfection.
8. To each well, add 50ul (24-well) or 10ul (96-well) of your final transfection solution, dropwise moving around the well for even coverage.
9. Incubate cells for 2-3 hours, then add second half of media. Incubate 12 or 24 hours total prior to imaging, flow, or qPCR.

Optimal Conditions

Experimentally determined optimal conditions for 96-well plates (Team Cenozoic 2018) and 24-well plates (Team Zorya 2019)

Optimal Conditions as determined by UCD iGEM 2018 ***(96-well plate)***

Variable Condition	CHO-DG44	AML-12
Seeding Density	$0.3x10^5$	$0.3x10^5$
Lipofectamine Concentration	0.3ul	0.3ul
DNA Concentration	100ng	100ng

All numbers determined through transfection of constitutive pcDNA-EGFP under CMV promoter control- see Team Cenozoic Wiki for details

Optimal Conditions as determined by UCD iGEM 2018 ***(96-well plate)***

Variable Condition	CHO-DG44	AML-12	NIH-3T3
Seeding Density	$0.5x10^5$	$0.5x10^5$	$0.5x10^5$
Lipofectamine Concentration	No statistical difference between 1.5ul and 0.75ul , went with 0.75 to conserve resources	0.75ul	1.5ul
DNA Concentration	1ug total	1ug total	1ug total
Adherence at point of transfection	Suspended transfection	Suspended transfection	Suspended transfection
Wash with PBS	No wash	No wash	No wash
Plasmid ratios	1ug divided evenly among 3 plasmids (0.33ng:0.33ng:0.33ng)	1ug divided evenly among 3 plasmids (0.33ng:0.33ng:0.33ng)	1ug divided evenly among 3 plasmids (0.33ng:0.33ng:0.33ng)

All numbers were determined both by single plasmid constitutive pMAX transfection as well as transfection with a two plasmid CRISPR-dCas9 targeting system with sgRNAs designed for an eGFP target plasmid (3 total)

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