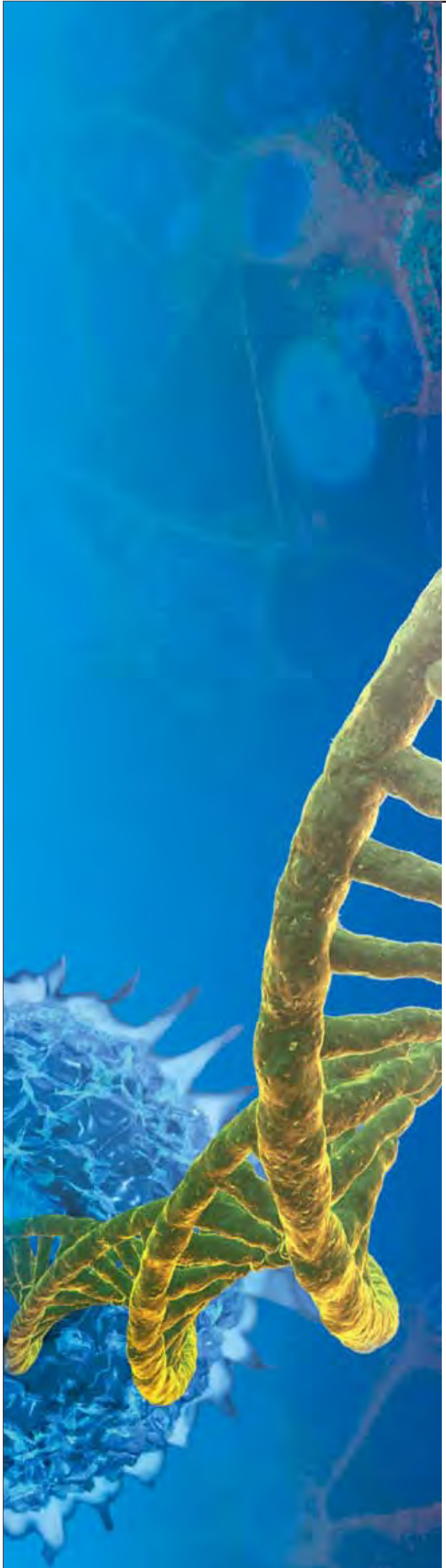


Technical Tip

Transfection

Content

Transfection Methods

The term transfection refers to a technique of delivering nucleic acids like DNA or RNA and even protein into mammalian cells.

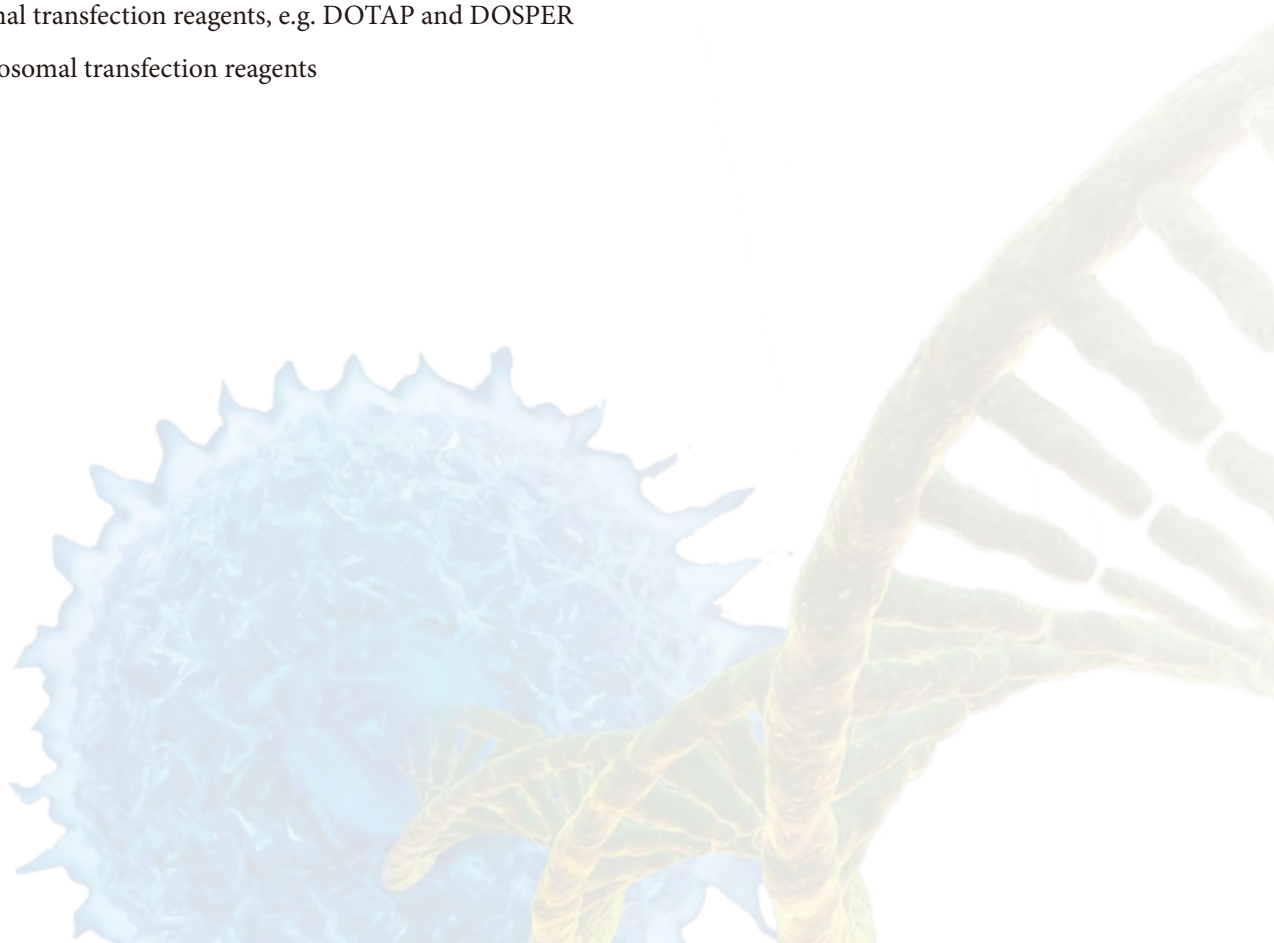
Under normal conditions, mammalian cells take up and express externally applied DNA with very low efficiency. This is mostly due to the lipid bilayer of the eukaryotic cell membrane, which acts as a barrier to the entry of charged molecules into a cell. Several transfection methods have been developed to overcome this problem. With these methods, the study of gene expression in cultured cells using DNA or RNA transfection has become routine.

A distinction is made between transient and stable transfection. Transfected DNA is only rarely integrated into the cellular genome, whereas RNA is not integrated at all. Therefore, the information is gradually lost during mitosis (transient transfection). However, when a gene conveying resistance to a toxin is cotransfected, cells that have integrated the foreign DNA will be selected by adding the respective toxin. This procedure is called stable transfection.

Goals for transfection include the study of gene regulation, as well as protein expression and function.

There are a number of methods available to successfully introduce foreign nucleic acids into cells:

- Carrier molecules, e.g. DEAE-dextran
- Chemical methods, e.g. Calcium phosphate
- Particle-based transfection
- Physical methods, such as electroporation or microinjection
- Receptor-mediated transfection
- Virus-mediated methods, referred to as transduction
- Liposomal transfection reagents, e.g. DOTAP and DOSPER
- Non-liposomal transfection reagents



Transfection Methods

Not all transfection methods can be applied to all types of cells or experiments. The different methods vary greatly with respect to the level of gene expression that can be achieved.

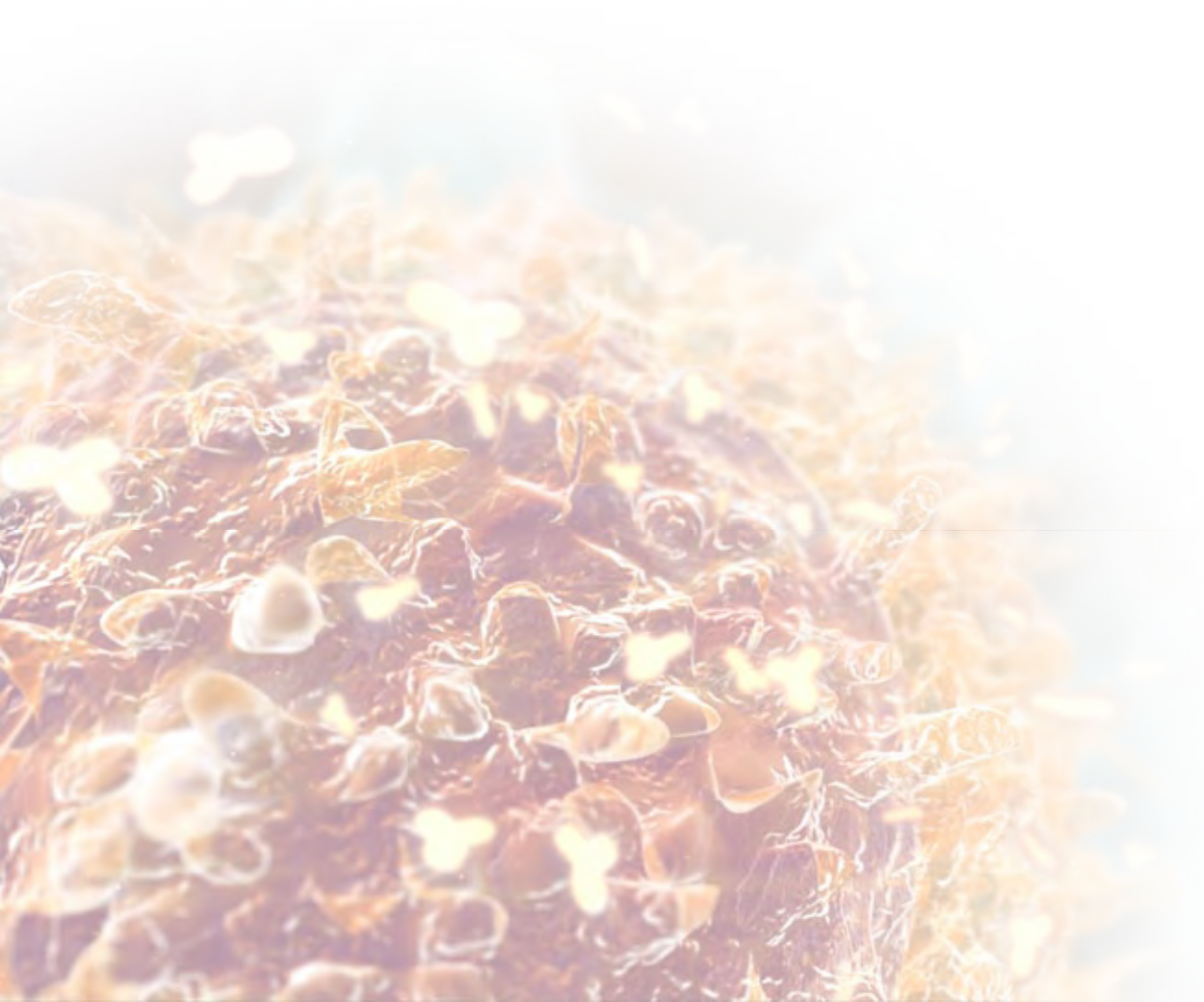
Moreover, no single technique is suitable for the multitude of different cellular systems used for transfection experiments. Each transfer method may have advantages or disadvantages, depending on, for example, the cell type to be transfected (especially true for difficult cell lines), the molecule to be transfected (DNA, RNA, oligonucleotides, proteins), or even the demands of high-throughput applications.
In all cases, however, the success of transfection depends on transfection efficiency, low cytotoxicity, and reproducibility.

To ensure a highly efficient transfection, you need to choose a reliable transfection technology and the reagents that work optimally under your cell culture conditions.

Many transfection methods are harsh to the cells, especially physical methods but also methods based on chemicals or liposomal reagents, resulting in low cell survival, highly stressed cells and dramatic changes in gene expression patterns (Jacobsen et al. 2009).

Therefore reagents were developed that achieve high transfection efficiency while exhibiting low toxicity to cells. One of the mildest and at the same time most efficient methods is the use of non-liposomal reagents.

There are non-liposomal reagents on the market that can transfect a broad range of cell lines. However, transfection remains a complex process and several parameters directly affect the result of a transfection experiment.



Transfection efficiency and toxicity of the transfection reagent

One of the criteria used to judge the result of a transfection experiment is the efficiency. Several methods can be used to measure this; one of the most common is the use of reporter gene assays, like beta-Gal or GFP to determine the ratio of transfected versus non-transfected cells.

However, this method does not take into account those cells that die due to the toxic effect of the transfection reagent used. Often the absolute number of cells will be dramatically reduced or damaged, which sometimes can even be observed in morphological changes.

Therefore, a better way to estimate the transfection efficiency is to look at numbers of transfected cells in relation to the cell viability after transfection (Figure 1 and 2).

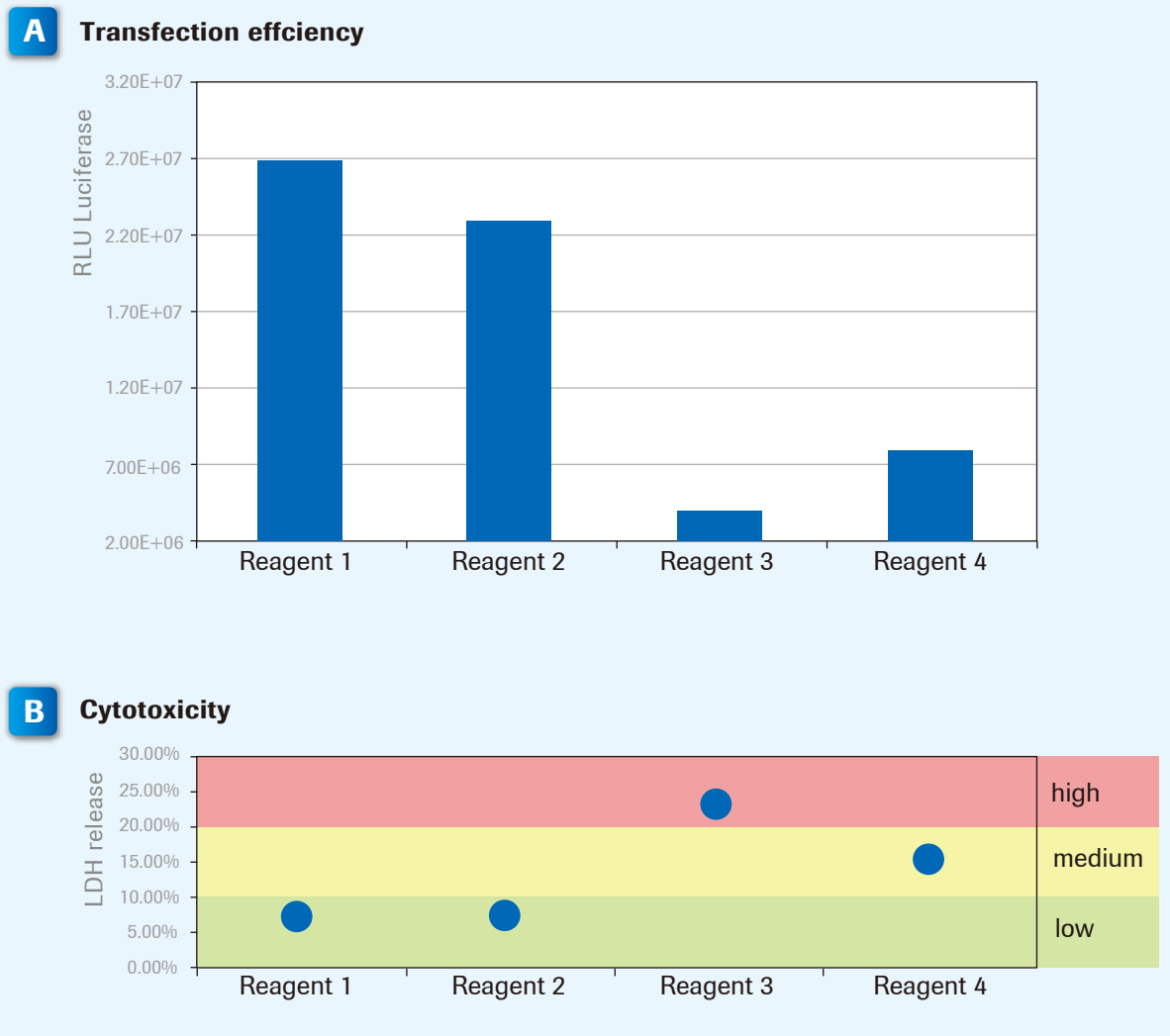


Figure 1: HeLa cells were transfected using 4 different reagents at the reagent/DNA ratio recommended in the supplier's manual. Reagents 1 and 2 show the ideal balance of high efficiency and low cytotoxicity.

Transfection efficiency and toxicity of the transfection reagent

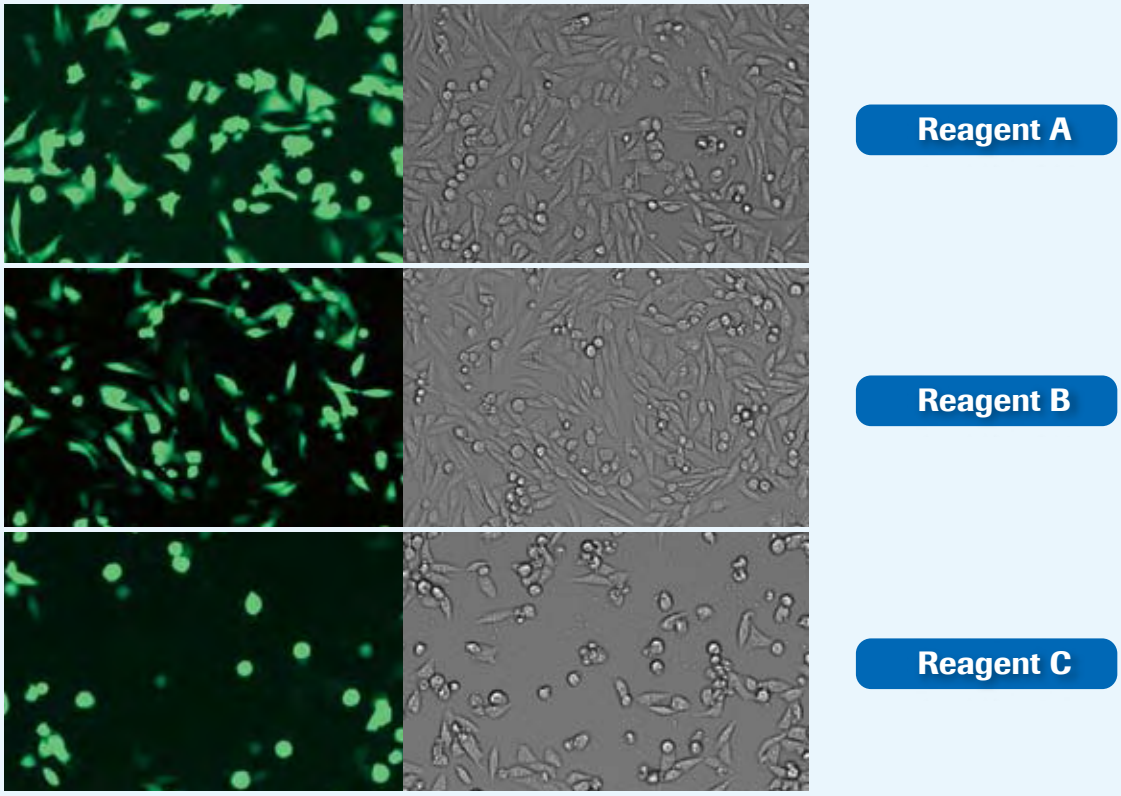


Figure 2: CHO-K1cells were transfected with a GFP expression plasmid using 3 different reagents at the reagent/DNA ratio recommended in the supplier's manual. Fluorescent signals after transfection (left) and the corresponding bright field pictures (right) are shown. After transfection with reagent D, fluorescent signals are visible, but the number of viable cells is strongly reduced. Reagents A and B show high efficiency and at the same time low cytotoxicity.

However a reagent's toxicity is not only important for cell survival, but also for the final scientific result itself, or in other words, its effect on gene expression patterns, physiological processes or morphology. Transfection reagents with high toxicity will probably show high relative transfection efficiency but at the same time high, so-called off-target effects. Off-target effects often lead to misinterpretation of results and misleading conclusions.

An ideal transfection reagent, therefore, shows a high relative transfection efficiency and at the same time low toxicity (Figure 3).

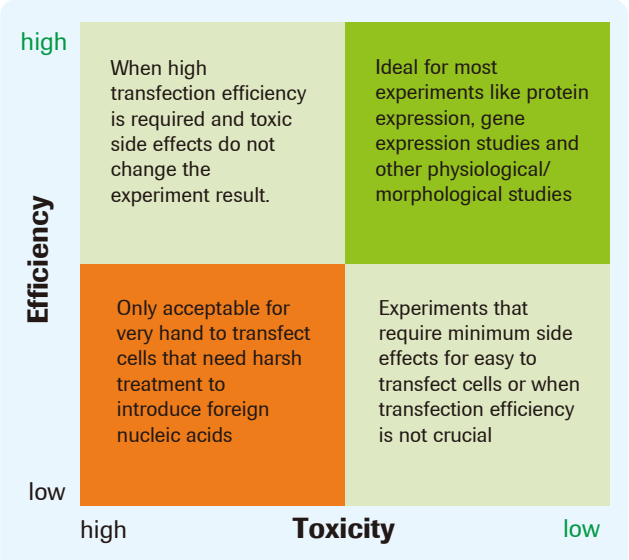


Figure 3: Selection of transfection reagents depending on their toxicity and efficiency.

Quality of the cells

The quality and vitality of the cells is a key factor not only for efficiency but also for the reproducibility of transfection experiment results. In practice, it is very often the source of highest variability. Therefore, the first step is always to develop a suitable protocol for cultivation and to determine the optimal cell density for transfection of the nucleic acid of interest.

Generally speaking, the cells should be viable and, at the timepoint of transfection, in the growth phase. However, the optimal cell density has to be adjusted for each cell line and transfection reagent used. For example, when using cells that show contact inhibition such as NIH/3T3 cells, the cell density has to be lower.

Another factor that can strongly influence the viability and characteristics of cell lines is the number of passages. The effects regularly observed for different passage numbers are very complex and depend on different parameters like cell type, tissue source and culture conditions.

TIP

To avoid this use quality-controlled cells from reputable sources, like the ones offered by the American Type Culture Collection (ATCC). www.lgcstandards-atcc.org

Contamination with mycobacteria, bacteria, viruses, molds and yeast is a concern for most cell culture labs. Sometimes even cross contamination with other cell-types are observed. Contaminants adversely affect experimental results and, therefore, the quality of scientific research; they cannot be fully avoided, but they can be reduced.

TIP

We do not recommend the regular use of antibiotics in cell culture, since mycoplasma will not be affected and the regular addition of antibiotics can easily lead to the development of resistances and slowly growing contaminating organisms that are hard to detect and can manifest in cell cultures over a long period of time without obvious changes of morphology or growth rates. However, they can influence experimental results and by the time the effects are detected, a lot of time and money might have been lost.

Mycoplasma

Mycoplasma, the most common contaminants observed in cell culture, are present in 5- 30% of all cell cultures worldwide and in some countries, the infection rate can be as high as 80%. First discovered as the etiological agent of contagious bovine pleuropneumonia in 1898 and classified in the mid 1950s, mycoplasma were found to have a broad range of hosts - humans, animals, insects and plants.

With diameters ranging from 0.2 to 0.8 µm, mycoplasma are macroscopically not visible and are resistant to antibiotics commonly used in cell culture. They may induce cellular changes by influencing metabolic activity, growth rates, morphology and other host cell processes, and can even induce chromosomal aberrations and damage.

Importantly, mycoplasma infections can alter the results of transfection experiments and lower protein expression levels, as well as transfection efficiency.

The source of contamination is generally other infected cultures, but can also be contaminated reagents, such as serum, or infection by personnel.

Guidelines for an efficient quality control program for the prevention of mycoplasma infection of cell cultures (Kotani et al., 1985) should be closely followed. The publication from Roche Applied Science "Basic techniques for culturing and monitoring of animal cells" provides an excellent overview.

In case of contamination, cell cultures should be discarded and replaced by a new culture from a trusted source. However, when the infected culture is irreplaceable, treatment with specific antibiotics, such as BM-Cyclin, is an option (see technical tip in "Basic techniques for culturing and monitoring of animal cells").

Before introducing cell lines into your cell culture, always test for mycoplasma (Drexler and Uphoff, 2002). Such tests should also be an obligatory component of quality control in every tissue culture laboratory on a regular basis for all cultured cell lines.

- **Direct staining methods** show low sensitivity but have the advantage of being rapid and cheap. A common reagent is DAPI (4',6-diamidino-2-phenylindole).
- **ELISA methods** are fast and show medium sensitivity levels. However, the range of species detection is often limited.
- **PCR-based methods** are the method of choice due to their high sensitivity and broad range of species detected. There are several options available to detect mycoplasma by PCR:
  - Conventional PCR
  - PCR ELISA assays, such as the Mycoplasma PCR ELISA.
  - Real-time PCR assays

Mycoplasma

If a real-time PCR instrument is available in your laboratory, a real-time assay for the detection of mycoplasma can be a good option. Commercial qPCR tests are widely available.

Alternatively, an in-house qPCR assay can be established. A range of primers are published to detect mycoplasma in cell culture. Most published primers target highly conserved regions to detect a broad range of Mycoplasma species. Dussurget et al., 1995 provides a good overview on available PCR-based methods. However, proper optimization under local conditions is essential to produce reliable and reproducible results. Furthermore, the following controls should be included in each instance:

- A negative control (sterile water or growth medium) and
- A positive control, such as a sample from a contaminated cell line or a standard preparation of known mycoplasma species.

A PCR ELISA assay combines the advantages of a PCR and an ELISA test, does not require qPCR instrumentation and offers quick and sensitive detection. In a comparison of different methods, this method was shown to reliably detect the highest number of species in cell cultures (http://www.ibdl.ca/Application%20Notes/FeiapplicationNote.pdf).

Depending on the mycoplasma species, the Roche Mycoplasma PCR ELISA detects 1 to 10 fg of DNA contained in the amplification vial (corresponding to about 1 to 20 gene copies) per reaction. At least 10^3 colony forming units (cfu) of mycoplasma per ml of cell culture medium are detected. The primers in the kit hybridize to conserved regions of the genome and detect all of the mycoplasma and acholeplasma species found in cell cultures (see Table 1) using one standard protocol. In addition, eukaryotic DNA is not detected by the Mycoplasma PCR ELISA.

Mycoplasma/ Acholeplasma species	Cell line	Amount of mycoplasma inoculated (cfu/ml)	Mycoplasma PCR ELISA ($\triangle A_{450nm}$-A_{690nm})	Culture method Without prior enrichment		Culture method After prior enrichment	
				Medium A	Medium B	Medium A	Medium B
M. arginini	Vero	10^6	0.35	+++	+++	++++	++++
	Vero	10^3	0.35	++	++	++++	++++
	NSO	10^6	0.36	++++	++++	++++	++++
	NSO	10^3	0.35	++	++	++++	++++
M. lermentans	Vero	10^6	1.56	++++	++	++++	++
	Vero	10^3	1.77	+++	++	++++	+
	NSO	10^6	1.82	++++	++	++++	++
	NSO	10^3	1.70	++	+	++++	+++
M. hominis	Vero	10^6	1.54	++++	++++	+++	+++
	Vero	10^3	1.69	++	++	+++	++++
	NSO	10^6	1.76	+	+++	+	++++
	NSO	10^3	1.75	+	+	+	++++
M. hyorhinis	Vero	10^6	1.68	+++	++	+++	+++
	Vero	10^3	1.85	++	+	+++	+++
	NSO	10^6	1.83	+++	++	+++	++
	NSO	10^3	1.78	+	+	++	+
M. orale	Vero	10^6	0.49	+++	++++	+++	+++
	Vero	10^3	0.24	++	+	++	++++
	NSO	10^6	0.37	++++	+++	+	+++
	NSO	10^3	0.13	+	+	+	++++
A. laidlawii	Vero	10^6	1.82	++++	++++	++++	++++
	Vero	10^3	1.74	+++	+++	++++	++++
	NSO	10^6	1.64	++++	++++	++++	++++
	NSO	10^3	0.64	+++	+++	++++	++++

Table 1: Detection of various mycoplasma species and Acholeplasma laidlawii using the Mycoplasma PCR ELISA or the culture method, respectively.

Adherent cells (Vero), or cells growing in suspension (NSO) were artificially contaminated with mycoplasma corresponding to 10^3 and 10^6 cfu (colony-forming units)/ml, respectively and incubated for 4 h at 37 °C. Detection of mycoplasma infection by the culture method was carried out using pig (medium A) or horse serum (medium B) supplemented media, with or without prior enrichment of mycoplasma in broth (++++: 104 cfu/ml, +++: $>10^3$-10^4 cfu/ml, ++: 10^2-10^3 cfu/ml, +: 10-10^2 cfu/ml). Mycoplasma PCR ELISA was performed as described below without prior enrichment. Samples producing absorbance values (A) >0.2 A_{450nm}-A_{690nm} units are regarded as being positive.

Quality and Sequence of the DNA

In most cases, the plasmid to be transfected is propagated in bacteria and purified by affinity chromatography. This eliminates contaminations, such as endotoxins, in the plasmid preparation. DNA should be handled carefully to avoid strand breaks. For long-term storage, we recommend to aliquot the DNA and store it at –20°C. Avoid repeated freeze-thaw cycles because this can result in DNA damage and reduced transfection efficiency.

Ratio of DNA and Transfection Reagent

All transfection reagents are most efficient at a certain reagent/DNA ratio. The optimal ratio of DNA and transfection reagent varies between different reagents and cell lines. The time it takes to find the optimal ratio is time well spent. Not only is the ratio important, but the amount of complex added is often critical. Also the range of recommended DNA/transfection reagent ratios differ from reagent to reagent. When determining the optimal ratio, the full range recommended by the manufacturer should be tested.

TIP

To save time and costs during the experimental set up, choose a reagent with a narrow recommended range of DNA/transfection reagent ratio.

Controls

Depending on the objective of an application, different controls have to be set up.

If the transfection is done to express and purify protein, only a few controls might be necessary. However, in gene expression or RNA-interference studies, proper controls should be set up, especially to be able to distinguish off-target effects from specific effects caused by the transfected nucleic acid.

TIP

Recommended controls are:

- Non-transfected cells
- Cells + nucleic acid alone (without transfection reagent)
- Cells + transfection reagent (without nucleic acid) and/or
  cells + plain vector + transfection reagent (also called mock transfection) or
  cells + non-specific siRNA + transfection reagent
  For siRNA experiments, a positive control is recommended, e.g. siRNAs targeting GAPDH or several siRNAs targeting the gene of interest.

Timeline and Planning

TIP

Set up a suitable timeline for your transfection experiment to ensure a successful end result.

Start of Transfection

Twenty-four hours before transfection, split the cells into culture plates. At the start of transfection, the culture should be approximately 50-80% confluent so they will be near confluency by the end of the experiment.

For some transfection reagents, the medium has to be changed or serum has to be added a few hours before or after the addition of the transfection complex. The reason is the incompatibility of the reagents with serum or their high cytotoxicity. If serum is taken away during transfection, the cells may arrest for a while.

The uptake of the transfection complex by the cells is generally complete within 0.5-6 hours. After this time, there is no detectable increase in transfection efficiency. If the complex is left on the cells until the time of assay, transfection efficiency increases for some cell lines, while in other cells, there is no further increase after the first few hours.

Although it is usually not necessary to remove the transfection reagent/DNA complex following the transfection step, it is necessary to feed your cells with fresh media during extended growth periods. This is especially important if the transfected cells are allowed to grow for 3-7 days.

TIP

If possible, use a transfection reagent that does not require a change of medium and is not affected by serum. It will reduce handling steps and time and, in addition, increase the reproducibility of the experiment.

Incubation time

The cultivation time after transfection depends on the type and design of the experiment, the cell line, cell density, gene of interest and protein to be expressed. The post-transfection time is crucial for the experimental results.

Harvesting cells too early can result in suboptimal results in some cases, for example when the effect of the transfected gene reaches a maximum only some days after transfection; in others, cultivating cells too long can have a negative effect as well. For example, in protein production, the cultivation time often ranges between two days to one week. However, if the expressed protein is toxic, cells may die when too much protein is expressed, resulting in decreased yields.

TIP

Recommended post-transfection incubation times.
The optimal time point has to be determined individually for each experiment.

- Qualitative protein expression/gene expression studies: 4 hours – 5 days (mostly after 24 hours)
- Quantitative protein expression: 24 hours – 7 days
- Gene knock down via siRNA transfection: 24 hours – 3 days with one transfection or when the protein is very stable, up to 7 days with multiple siRNA transfections.

Protein Expression

Previously, to produce high levels of protein, cells had to be transfected, then stable cells had to be selected for expansion and accumulation of proteins. Selection of such cell lines often took weeks or months, due either to the cytotoxicity of the reagent or inefficient transfection.

If the transfection reagent was cytotoxic, then fewer cells survived and the protein yield was consequently low. If only a few cells were transfected, then untransfected cells rapidly outgrew the transfected cells, and little protein was produced.

Now, to produce high levels of protein, we recommend using a gentle non-liposomal reagent for transient transfection and protein expression.

However, some proteins are produced at higher levels than other proteins. Thus, critical variables for high levels of protein expression, additional to those already discussed, include:

- Cell line (some cell types are higher producers than others)
- Plasmid back bone (e.g., enhancers, promoters, transcriptional regulatory elements)
- Protein produced (some proteins simply are not well produced)
- cDNA sequence of protein (e.g. codon optimization)
- Medium (nutrition, wastes, inhibitors of transfection)

At the time of harvest it is a good idea to include protease inhibitors, such as cOmplete Ultra Protease Inhibitor Tablets in the medium to prevent degradation or PhosSTOP to prevent dephosphorylation.

Stable Transfection

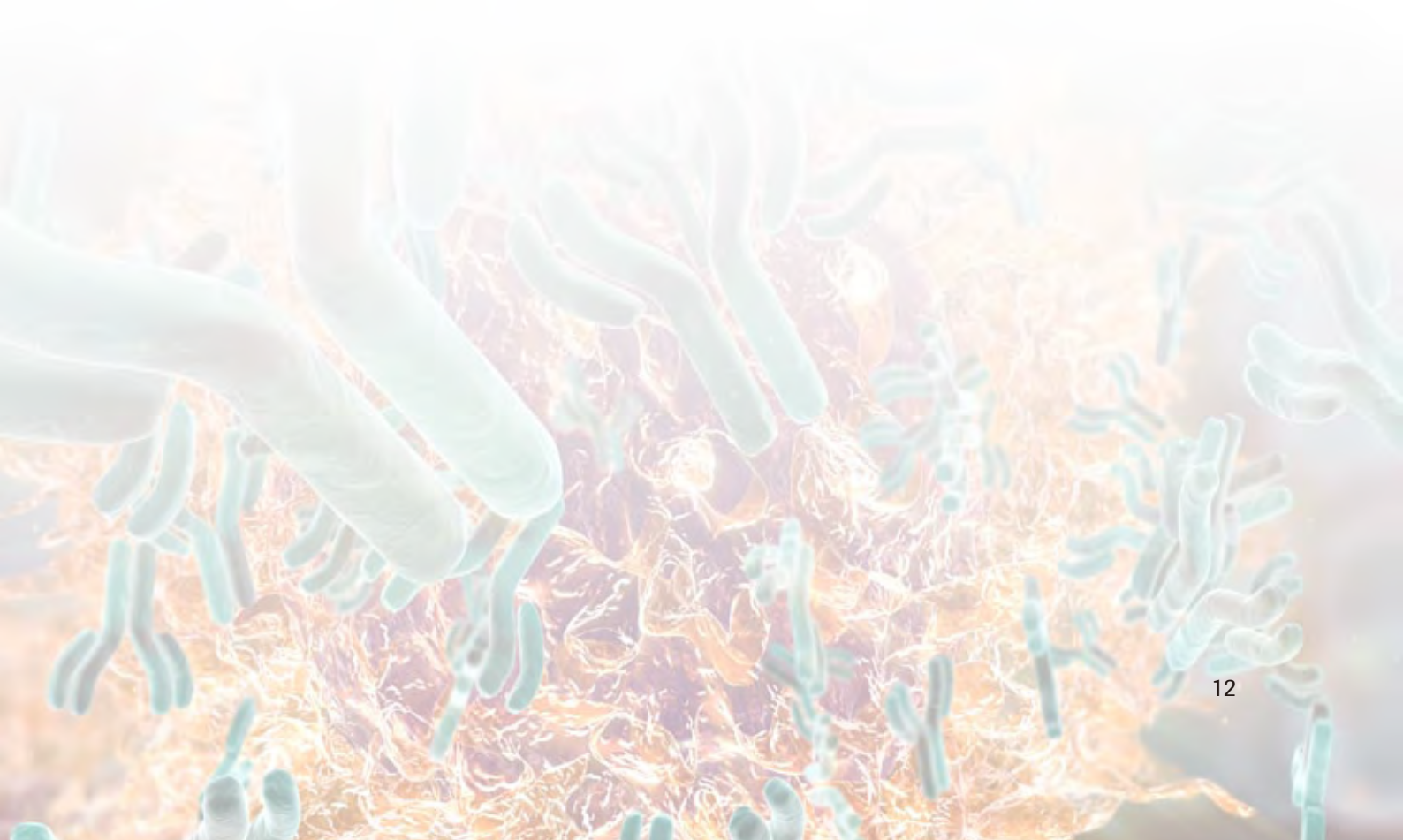
The first step in preparing a stable cell line is choosing a vector that contains both a selectable marker and the gene of interest. The selectable marker is usually one that produces a protein or enzyme that allows cells to grow in the presence of an otherwise toxic chemical.

Once the plasmid vector is chosen, the transfection is done the same way for either transient or stable expression. The transfected cells are allowed to grow, at least overnight in standard medium, before the selective agent is added. This gives the cells time to express sufficient quantities of the protein that allows them to overcome the selection agent.

The cells are then grown in the presence of the selection agent. Cells that do not have the plasmid are killed; only transfected cells are able to grow. In some instances, the selection agent is continuously added to the culture medium to maintain selective pressure on the cells.

TIP

For a stable transfection, incubate the cells after transfection without changing the medium until the next passage. This ensures high expression rates of the selection markers. Afterwards, the respective toxin has to be added to select cells that have integrated the foreign DNA.



Further Reading

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