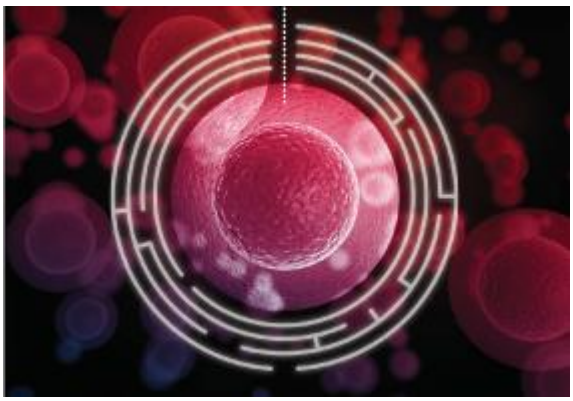
Transfection Techniques & Basics



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Transfection Techniques & Basics

INTRODUCTION TO TRANSFECTION

What is transfection?

Broadly defined, transfection is the process of artificially introducing nucleic acids (DNA or RNA) into cells, utilizing means other than viral infection. Such introductions of foreign nucleic acid using various chemical, biological, or physical methods can result in a change of the properties of the cell, allowing the study of gene function and protein expression in the context of the cell.

In transfection, the introduced nucleic acid may exist in the cells transiently, such that it is only expressed for a limited period of time and does not replicate, or it may be stable and integrate into the genome of the recipient, replicating when the host genome replicates. Types of Transfection

Transfection terminology

The terminology used for various gene delivery systems has evolved to keep pace with technological advances in the field and further refined to distinguish various methods and cell types.

Transfection

Transfection commonly refers to the introduction of nucleic acids into eukaryotic cells, or more specifically, into animal cells. Classically, the term transfection was used to denote the uptake of viral nucleic acid from a prokaryote-infecting virus or bacteriophage, resulting in an infection and the production of mature virus particles. However, the term has acquired its present meaning to include any artificial introduction of foreign nucleic acid into a cell.

Transformation

Transformation is often used to describe non-viral DNA transfer in bacteria, non-animal eukaryotic cells, and plant cells. However, transformation also refers to a particular event or a series of events that results in a permanent change in an animal cell's phenotype and implies genetic instability and a progression to a cancerous state. Although transformation in this sense can arise from infection with a transforming virus or from gene transfection, it can also arise spontaneously or following external stressors such as ionizing radiation or chemical mutagens. As such, the term should be avoided for animal cells when describing introduction of exogenous genetic material.

Transduction

Transduction is used to describe virus-mediated DNA transfer. However, the term transfection is also used to refer to infecting a cell specifically with viral nucleic acid that is isolated either from a eukaryote virus or from a bacteriophage.

APPLICATIONS

The two main purposes of transfection are to produce recombinant proteins, or to specifically enhance or inhibit gene expression in transfected cells. As such, transfection is a powerful analytical tool for the study of the function and regulation of genes or gene products, for the production of transgenic organisms, and as a method for gene therapy.

Gene expression

Transfection is most commonly performed to express a protein of interest in cultured cells (or an animal model) through the use of a plasmid vector or mRNA. Expression of the protein in eukaryotic cells allows the recombinant protein to be produced with proper folding and post-translational modifications required for its function. Further, introducing proteins with readily detectable markers and other modifications into cells allows the study of promoter and enhancer sequences or protein: protein interactions.

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In addition, transfection can be used in various forms of bioproduction depending upon the transfection strategy. For example, delivery of reprogramming transcription factors enables the generation of induced pluripotent stem cell (iPSC). Stable transfection, on the other hand, provides the means for the bioproduction of various therapeutic molecules.

GENE INHIBITION

Another frequent use of transfection is in inhibiting the expression of specific proteins through RNA interference (RNAi). In mammalian cells, RNAi occurs through endogenously expressed non-coding RNA in the form of microRNAs (miRNAs), which are derived from a double-stranded RNA (dsRNA) precursor. The precursor is processed to a mature miRNA that becomes part of a RNA-induced silencing complex (RISC), which acts to inhibit translation of complementary target mRNAs.

Vector-based systems express miRNA precursors or short hairpin RNA (shRNA) precursors that are processed by endogenous machinery to produce miRNAs or shRNAs, respectively, which then act to inhibit gene expression. These systems allow stable transfection of recombinant constructs and can permit inducible expression of precursor molecules.

Chemically synthesized short/small interfering RNAs (siRNAs) can also be incorporated into a RISC and induce gene silencing by targeting complementary mRNA for degradation. Modifications to siRNAs help to prevent off-target effects, and also to ensure that the active strand of the dsRNA is loaded into the RISC.

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Types of Transfection

Menu: Types of Transfection

There are a number of biological, chemical, and physical methods for introducing nucleic acids into cells. Not all of these methods can be applied to all types of cells and experimental applications, and there is a wide variation amongst them with respect to transfection efficiency, cell toxicity, effects on normal physiology, and level of gene expression. However, all of the transfection strategies can be broadly classified into two general types based on whether the introduced nucleic acid exists in the cell for a limited period of time (transient transfection) or whether it persists in the cells long-term and is passed to the progeny of the transfected cell (stable transfection).

Transient transfection

In transient transfection, the introduced nucleic acid exists in the cell only for a limited period of time and is not integrated into the genome. As such, transiently transfected genetic material is not passed from generation to generation during cell division, and it can be lost by environmental factors or diluted out during cell division. However, the high copy number of the transfected genetic material leads to high levels of expressed protein within the period that it exists in the cell.

Depending on the construct used, transiently expressed transgene can generally be detected for 1 to 7 days, but transiently transfected cells are typically harvested 24 to 96 hours post-transfection. Analysis of gene products may require isolation of RNA or protein for enzymatic activity assays or immunoassays. The optimal time interval depends on the cell type, research goals, and specific expression characteristics of the introduced gene, as well as the time it takes for the reporter to reach steady state. However, within a few days most of the foreign DNA is degraded by nucleases or diluted by cell division; after a week, its presence is no longer detected. Transient transfection is most efficient when supercoiled plasmid DNA is used, presumably due to its more efficient uptake by the cell. siRNAs, miRNAs, mRNAs, and even proteins can be also used for transient transfection, but as with plasmid DNA, these macromolecules need to be of high quality and also be relatively pure (see Factors Influencing Transfection Efficiency). While transfected DNA is translocated into the nucleus for transcription, transfected RNA remains in the cytosol, where it is expressed within minutes after transfection (mRNA) or bound to mRNA to silence the expression of a target gene (siRNA and miRNA) (see Guidelines for RNA Transfection).

Stable transfection

In stable transfection, foreign DNA is either integrated into the cellular genome or maintained as an episomal plasmid. Unlike transient transfection, stable transfection allows the long-term maintenance of the exogenous DNA in the transfected cell and its progeny. As such, stable transfection can provide persistent expression of the introduced gene through multiple generations, which can be useful for production of recombinant proteins and analysis of downstream or long-term effects of exogenous DNA expression. However, usually a single or a few copies of the exogenous DNA is integrated into the genome of the stably transfected cell. For this reason, the expression level of stably transfected genes tend to be lower than that of transiently transfected genes.

Because stable integration of foreign DNA into the genome is a relatively rare event, successful stable transfection requires both effective DNA delivery and a way to select cells that have acquired the DNA. One of the most reliable ways to select cells that stably express transfected DNA is to include a selectable marker in the DNA construct used for transfection and then apply the appropriate selective pressure to the cells after a short recovery period.

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Transfection Techniques & Basics

Frequently used selectable markers are genes that confer resistance to various selection drugs or genes that compensate for an essential gene that is defective in the cell line to be transfected. When cultured in selective medium, cells that were not transfected or were transiently transfected eventually die, and those that express the antibiotic resistance gene at sufficient levels or those that can compensate for the defect in the essential gene survive. Alternatively, phenotypical or morphological changes in the transfected cells can be used as a screenable trait in certain cases. For example, mouse CI127 cells transfected with vectors derived from bovine papilloma virus produce a morphological change (Sarver et al. 1981).

Although linear DNA results in lower DNA uptake by the cells relative to supercoiled DNA, it yields optimal integration of DNA into the host genome (see Factors Influencing Transfection Efficiency). As a rule, stable transfection is limited to DNA vectors, but siRNA and miRNA may be stably introduced into cells when they are delivered as short hairpin transcripts made from a selectable DNA vector (see Vector-mediated RNAi). However, RNA molecules by themselves cannot be used for stable transfection.

Transfection Techniques & Basics

Choosing a Transfection Strategy

Menu: Choosing a Transfection Strategy

Deciding whether you need transient or stable transfection depends on the time frame and ultimate goal of the experiment you wish to conduct. Transiently transfected cells are typically harvested 24–96 hours post-transfection and are often used for studying the effects of short-term expression of genes or gene products, performing RNA interference (RNAi)-mediated gene silencing, or rapidly producing recombinant proteins on a small scale. Transient transfection with mRNA can deliver even more rapid results; because mRNA is expressed in the cytosol without the need for translocation to the nucleus and the transcription process, it is possible for transfected mRNA to be expressed within minutes after transfection in some systems.

In contrast, stable transfection is more useful when long-term gene expression is required or when transfected cells need to be used over many experiments. Because integration of a DNA vector into the chromosome is a rare event, stable transfection of cells is a more laborious and challenging process, which requires selective screening and clonal isolation. As such, it is normally reserved for large-scale protein production, longer-term pharmacology studies, gene therapy, or research on the mechanisms of long-term genetic regulation.

Although transient transfection of mammalian cells has been employed for the production of recombinant proteins with proper folding and post-translational modifications (which are not available when expressing recombinant proteins in bacterial cells) since the invention of transfection reagents, the ability to express milligram-to-gram amounts of recombinant protein has relied mainly on the creation of stable cell lines. More recently, large volume transient transfection of HEK293 and CHO cells adapted to suspension culture has addressed the need to obtain high amounts of recombinant protein without having to resort to the laborious process of stable cell line development. Recombinant protein expression by transient transfection enables researchers to produce, starting from the vector of interest and suspension-adapted CHO or HEK293 cells, milligram-per-liter quantities of correctly folded and glycosylated recombinant proteins in three to seven days.

A major advancement in transient expression technology for rapid and ultra high-yield protein production in mammalian cells is the Expi293 Expression System, which is based on the high-density culture of Expi293Fcells in Expi293 Expression Medium and transfection using the cationic lipid-based ExpiFectamine 293 transfection reagent in combination with optimized transfection enhancers. All components work in concert to generate 2- to 10-fold higher protein yields than conventional culture systems such as the FreeStyle 293 Expression System, achieving expression levels of greater than 1 g/L for IgG and non-IgG proteins.

Clinical biotherapeutics are frequently generated using stable, high-expression transfectants, because they provide batch-to-batch consistency and low cost at extremely large-scales. However, in many drug discovery applications, it is beneficial to screen protein constructs quickly using transient transfection methods, which allow simultaneous evaluation of various candidate molecules in less than one week. In many instances, transient transfections are performed in parallel while more resource intensive stable cell lines are under development, which can take more than three months to accomplish.

Transfection Techniques & Basics

Transient Transfection	Stable Transfection
Transfected DNA is not integrated into the genome, but remains in the nucleus.	Transfected DNA integrates into the genome.
Transfected genetic material is not passed onto the progeny; genetic alteration is not permanent.	Transfected genetic material is carried stably from generation to generation; genetic alteration is permanent.
Does not require selection.	Requires selective screening for the isolation of stable transfectants.
Both DNA vectors and RNA can be used for transient transfection.	Only DNA vectors can be used for stable transfection; RNA by itself cannot be stably introduced into cells.
High copy number of transfected genetic material results in high level of protein expression.	Single or low copy number of stably integrated DNA results in lower level of protein expression.
Cells are typically harvested within 24–96 hours of transfection.	Requires 2–3 weeks of selection for the isolation of stably transfected colonies.
Generally not suitable for studies using vectors with inducible promoters.	Suitable for studies using vectors with inducible promoters.

Transfection Techniques & Basics

RNAi and Non-coding RNA Research

Menu: RNAi and Non-coding RNA Research

RNA interference (RNAi) is a very powerful tool for studying the basic biology of cells, allowing the knockdown of gene expression to study protein function in a wide range of cell types. Once viewed as a technique used only by select laboratories, RNAi is now considered essential for studying gene function. It has become a prominent tool for protein knockdown studies, phenotype analysis, function recovery, pathway analysis, in vivo knockdown, and drug target discovery.

Glossary of common RNAi terms

RNAi

Ribonucleic acid interference (first used by A. Fire and C. Mello et al., 1998).

siRNA

Short interfering RNA. siRNAs are 21–25 bp dsRNAs with dinucleotide 3’ overhangs and are processed from longer dsRNA by Dicer in the RNA interference pathway. Introduction of synthetic siRNAs can induce RNAi in mammalian cells. siRNAs can also originate from endogenous precursors.

shRNA

Short hairpin RNA; also short interfering hairpin. shRNAs are used in vector-based approaches for supplying siRNA to cells for stable gene silencing. A strong Pol III-type promoter is used to drive transcription of a target sequence designed to form hairpins and loops of variable length, which are processed by cellular siRNA machinery. Once in the cell, the shRNA can decrease the expression of a gene with complementary sequences by RNAi.

miR RNAi

Vectors that express microRNAs for RNAi. miRNAs are 19–23 nt single-stranded RNAs, originating from single-stranded precursor transcripts that are characterized by imperfectly base-paired hairpins. miRNAs function in a silencing complex that is similar, if not identical, to RISC (see below).

Chemically modified siRNA

siRNA molecules which have chemical modifications.

RISC

RNA-induced silencing complex (RISC). A nuclease complex composed of proteins and siRNA that targets and cleaves endogenous mRNAs complementary to the siRNA within the RISC complex.

Off-target effects

Effects that occur when one or a few genes not specifically targeted show loss of gene function following the introduction of an siRNA or d-siRNA pool. The effect may be mediated by the sense strand of an siRNA, which may initiate a loss-of-function response from an unrelated gene. Off-target effects can also occur as a secondary effect of the antisense strand of a specific siRNA, if it has sufficient homology to knock down the expression of a non-target gene.

Transfection Techniques & Basics

How RNAi works

Two types of small RNA molecules function in RNAi. The first are synthetic, short interfering RNA (siRNA) molecules that target mRNA cleavage, effectively knocking down the expression of a gene of interest. MicroRNA (miRNA) molecules, on the other hand, are naturally occurring single-stranded RNAs 19–22 nucleotides long, which regulate gene expression by binding to the 3' untranslated regions (UTRs) of target mRNAs and inhibiting their translation (Ambros, 2004).

Learn more about RNAi

siRNA analysis

There are several ways to induce RNAi: synthetic molecules, RNAi vectors, and in vitro dicing (Figure 1, below). In mammalian cells, short pieces of dsRNA—short interfering RNA— initiate the specific degradation of a targeted cellular mRNA. In this process, the antisense strand of siRNA becomes part of a multiprotein complex, or RNA-induced silencing complex (RISC), which then identifies the corresponding mRNA and cleaves it at a specific site. Next, this cleaved message is targeted for degradation, which ultimately results in the loss of protein expression.

Learn more about siRNA analysis

Transfection Techniques & Basics

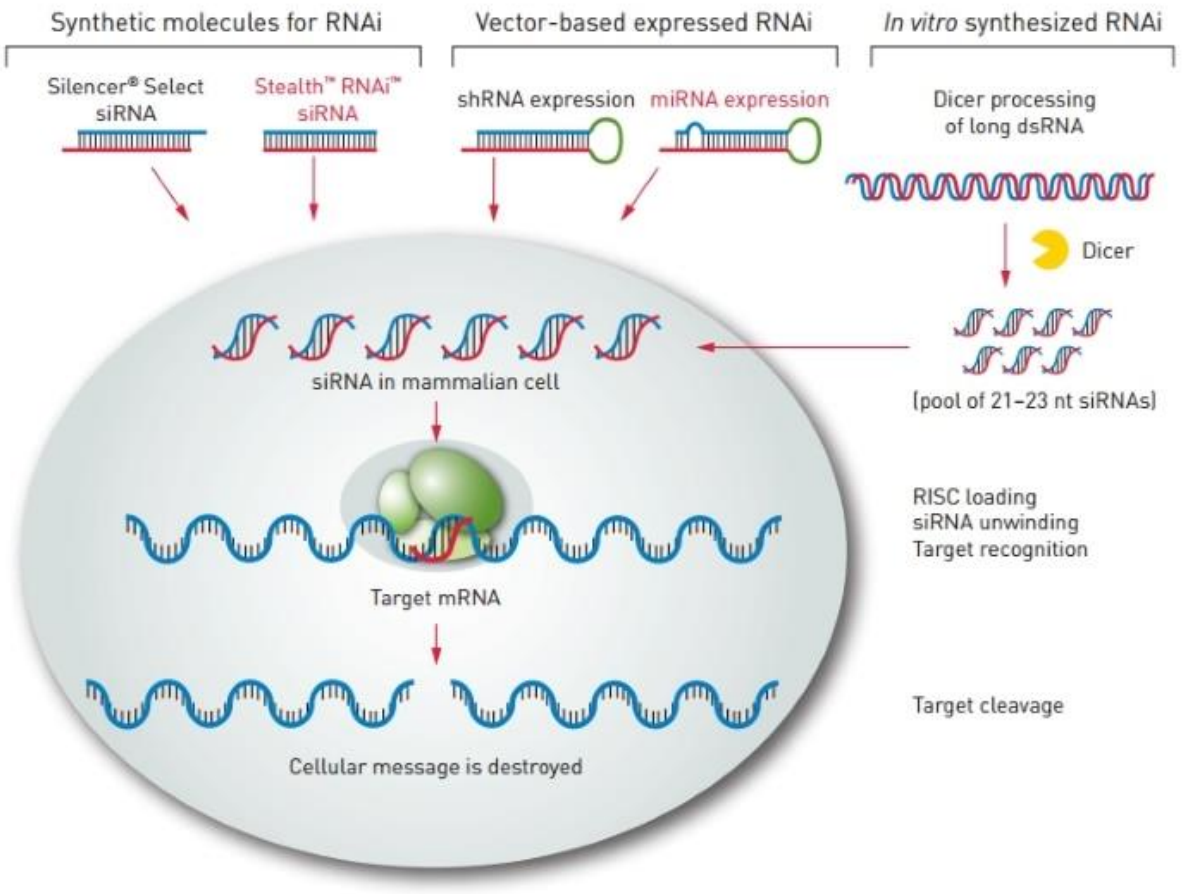

Figure 1: Methods of RNAi knockdown in mammalian cells.

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Transfection Techniques & Basics

miRNA analysis

Both RNA polymerase II and III transcribe miRNA-containing genes, generating long primary transcripts (pri-miRNAs) that are processed by the RNase III–type enzyme Drosha, yielding hairpin structures 70 to 90 bp in length (pre-miRNAs). Pre-miRNA hairpins are exported to the cytoplasm, where they are further processed by the RNase III protein Dicer into short double-stranded miRNA duplexes 19 to 22 nucleotides long. The miRNA duplex is recognized by the RNA-induced silencing complex (RISC), a multipleprotein nuclease complex, and one of the two strands, the guide strand, assists this protein complex in recognizing its cognate mRNA transcript. The RISC-miRNA complex often interacts with the 3' UTR of target mRNAs at regions exhibiting imperfect sequence homology, inhibiting protein synthesis by a mechanism that has yet to be fully elucidated (Figure 2, below).

Plant miRNAs can bind to sequences on target mRNAs by exact or near-exact complementary base pairing and thereby direct cleavage and destruction of the mRNA (Rhoades et al., 2002; Chen, 2005). Similar to the mechanism employed in RNA interference (RNAi), the cleavage of a single phosphodiester bond on the target mRNA occurs between bases 10 and 11 (Elbashir et al., 2001). In contrast, nearly all animal miRNAs studied so far do not exhibit perfect complementarity to their mRNA targets, and seem to inhibit protein synthesis while retaining the stability of the mRNA target (Ambros, 2004). It has been suggested that transcripts may be regulated by multiple miRNAs, and an individual miRNA may target numerous transcripts. Research suggests that as many as one-third of human genes may be regulated by miRNAs (Lim et al., 2003). Although hundreds of miRNAs have been discovered in a variety of organisms, little is known about their cellular function. Several unique physical attributes of miRNAs, including their small size, lack of polyadenylated tails, and tendency to bind their mRNA targets with imperfect sequence homology, have made them elusive and challenging to study.

Learn more about miRNA analysis

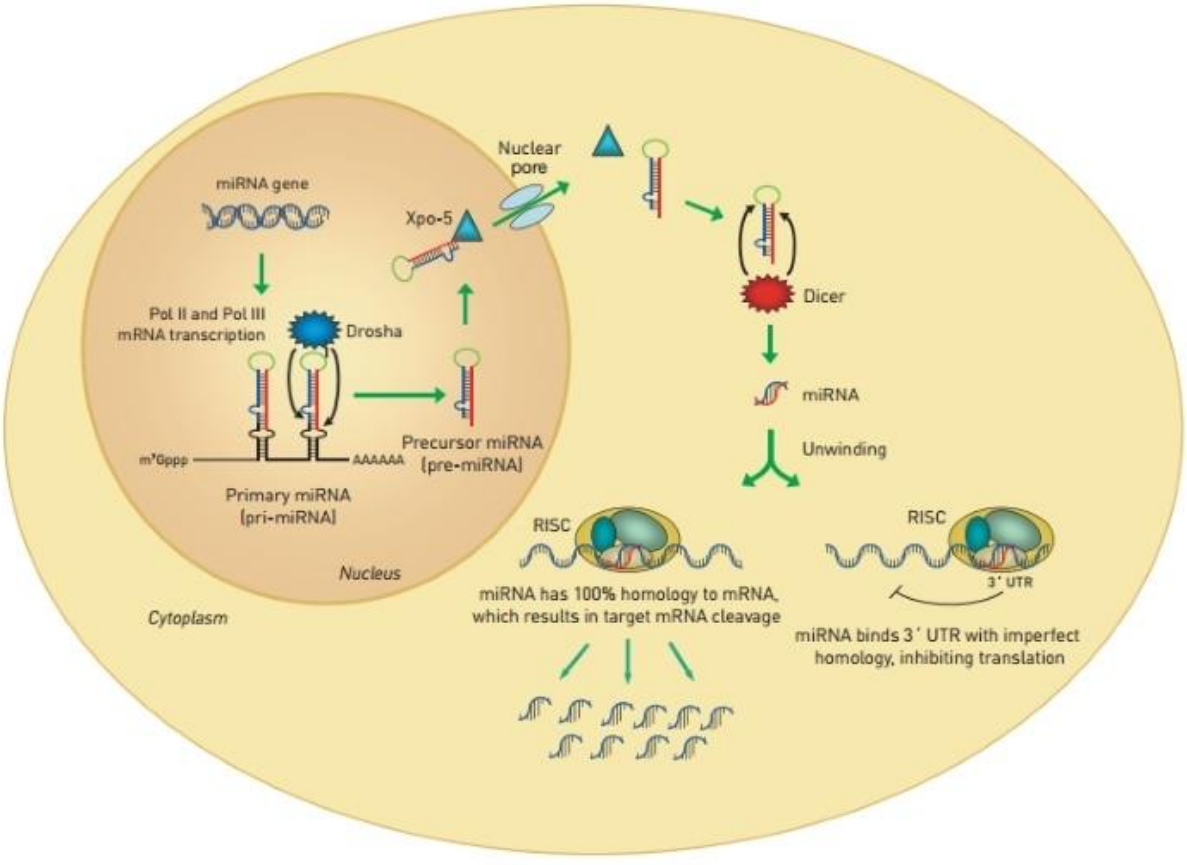

Figure 2: *Biogenesis and function of miRNA. MicroRNA transcripts, generated by RNA polymerases II and III, are processed by the RNase III enzymes Drosha (nuclear) and Dicer (cytoplasmic), yielding 19–22 nucleotide miRNA*

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duplexes. One of the two strands of the duplex is incorporated into the RISC complex, which regulates protein expression.

Choosing and RNAi approach

The process of RNAi (RNA interference) can be moderated by either siRNA or miRNA. Both are processed inside the cell by the enzyme called Dicer and incorporated into a complex called RISC (RNA-induced silencing complex). However, there are subtle differences between the two.

siRNA is an exogenous double-stranded RNA that can either be chemically synthesized and then directly transfected into cells, or generated inside the cell by introducing vectors that express short-hairpin RNA (shRNA), which are the precursors of siRNAs. miRNA, on the other hand, is single stranded and comes from endogenous non-coding RNA found within the introns of larger RNA molecules. However, the processing of shRNA into functional siRNA involves the same cellular RNAi machinery that naturally processes genome-encoded miRNAs, which are responsible for cellular regulation of gene expression by modulating mRNA stability, translation, and chromatin structures (Hutvagner and Zamore, 2002).

Another difference between siRNA and miRNA is that siRNA typically binds perfectly and specifically to its mRNA target in animals, while miRNA can inhibit translation of many different mRNA sequences because its pairing is imperfect. In plants, miRNA tends to have a more perfectly complimentary sequence, which induces mRNA cleavage as opposed to just repression of translation.

Both siRNA and miRNA can play a role in epigenetics through a process called RNA induced transcriptional silencing (RITS). Likewise, both are important targets for therapeutic use because of the roles they play in the controlling gene expression.

Transfection Techniques & Basics

	siRNA	miRNA
Occurrence	Occurs naturally in plants and lower animals. Whether or not they occur naturally in mammals is an unsettled question.	Occurs naturally in plants and animals.
Configuration	Double stranded	Single stranded
Length	21-22 nt	19-25 nt
Complementarity to target mRNA	100% perfect match; therefore, siRNAs knock down specific genes, with minor off-target exceptions.	Not exact; therefore, a single miRNA may target up to hundreds of mRNAs.
Biogenesis	Regulate the same genes that express them.	Expressed by genes whose purpose is to make miRNAs, but they regulate genes (mRNAs) other than the ones that expressed them.
Action	Cleave mRNA	Inhibit translation of mRNA
Function	Act as gene silencing guardians in plants and animals that do not have antibody-or cell-mediated immunity.	Regulators (inhibitors) of genes (mRNAs)
Uses	siRNAs are valuable laboratory tools used in nearly every molecular biology laboratory to knock down genes. Several siRNAs are in clinical trials as possible therapeutic agents.	Possible therapeutic uses either as drug targets or as drug agents themselves. Expression levels of miRNAs can be used as potential diagnostic and biomarker tools.

* Table adapted from Mack, 2007

Transfection Techniques & Basics

RNAi and Non-coding RNA Research

Menu: RNAi and Non-coding RNA Research

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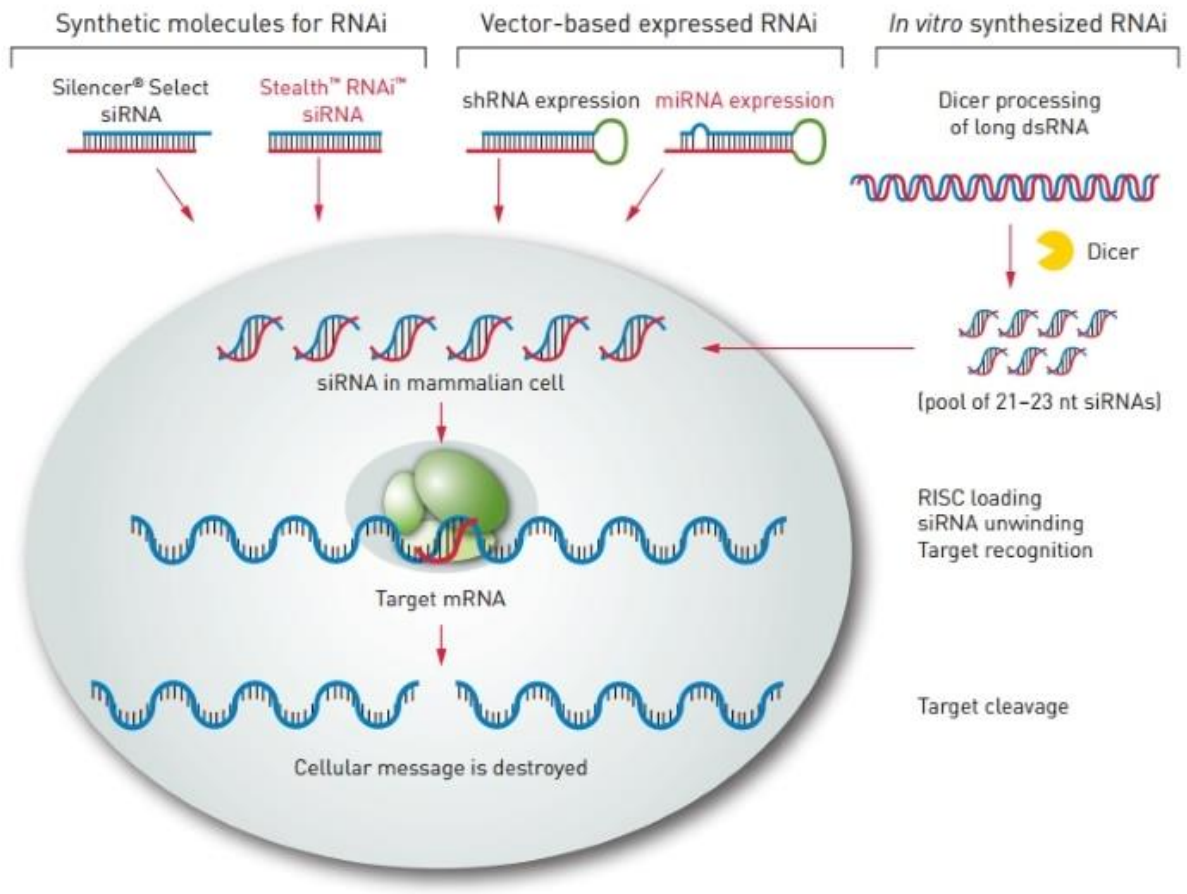

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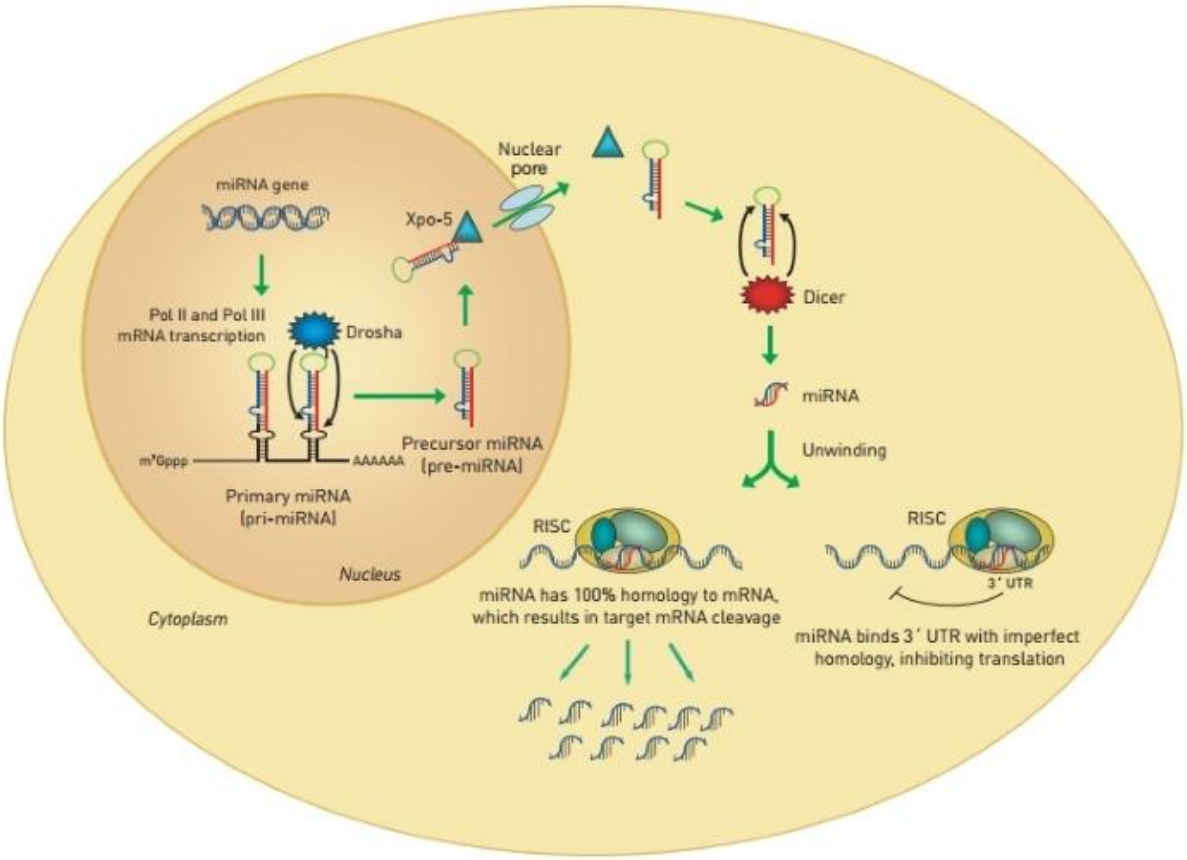

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Transfection Techniques & Basics

Transfection Reagents—DNA, RNA & Protein

Menu: Transfection Reagent Selection Guide

We offer transfection reagents for DNA, siRNA, RNA, and protein delivery, providing a range of options to best suit your transfection experiment:

- **DNA delivery**—transient and stable transfection used to study gene function and regulation, mutational analysis and biochemical characterization of gene products, effects of gene expression on the health and life cycle of cells, as well as for large scale production of proteins for purification and downstream applications
- **RNA delivery**—transient transfection allowing the knockdown of gene expression used in protein knockdown studies, phenotype analysis, function recovery, pathway analysis, in vivo knockdown, and drug target discovery
- **Protein delivery**—CRISPR-Cas9 transfection allows for genome editing in broad applications such as stem cell engineering, gene therapy, tissue and animal disease models, and engineering disease-resistant transgenic plants

When selecting a transfection method, consider the payload you wish to deliver (DNA, RNA, or protein) and the type of cells you want to transfect. Use the tables below to choose between our various cationic-lipid transfection reagents and our electroporation transfection system.

Invitrogen transfection reagent selection guide

Continuous cell lines

Continuous cell lines are capable of unlimited proliferative potential and are generally easier to work with than primary or finite cell cultures. However, because these cells have undergone genetic transformation to become immortalized, their behavior in culture may not necessarily reflect the in vivo situation.

Transfection Techniques & Basics

Reagent	DNA	mRNA	RNAi	Co-delivery	CRISPR-Cas9	Cell type(s)	Adherent or suspension
Lipofectamine 2000						Common and easy-to-transfect cell types	Adherent and suspension
Lipofectamine 3000						Workhorse (HeLa) through to hard-to-transfect (cancer cells)	Adherent
Lipofectamine LTX						CHO cells and some primary fibroblast, epithelial and neuronal cells (MEF, HMEC and E18 cells)	Adherent
Lipofectamine RNAiMAX						Established cell lines, hard-to-transfect cells, primary cells, stem cells	Adherent
Lipofectamine MessengerMAX						Neuronal cells, primary cells	Adherent
Lipofectamine CRISPRMAX						Tested in over 20 cell types including iPSC, mESC, N2A, CHO, A549, HCT116, HeLa, HEK293 and several others	Adherent
Neon Electroporation							Suspension
Invivofectamine 3.0						In vivo delivery to liver following tail vein injection	

Primary & stem cells

Primary cells are isolated directly from the tissue and proliferated under appropriate conditions. As such, they are morphologically and physiologically more similar to an in vivo state. However, they are usually more difficult to culture and transfect than continuous cell lines.

After the first subculture, the primary culture becomes known as a cell line. Cell lines derived from primary cultures have a limited life span (i.e., they are finite), and as they are passaged, cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population. Therefore, their phenotype is intermediate between primary cells and continuous cultures. The use of such cells is sometimes easier than the use of primary cells, especially for the generation of stably transfected clones.

Transfection Techniques & Basics

Reagent	DNA	mRNA	RNAi	Co-delivery	CRISPR-Cas9	Cell type(s)	Adherent or suspension
Neon Electroporation						Over 140 cell lines tested; excels in hard-to-transfect cells, primary and stem	Suspension
Lipofectamine 3000						Workhorse (Hela) through to hard-to-transfect (cancer cells)	Adherent
Lipofectamine Stem						Stem cells (expect HSCs)	Adherent and suspension
Lipofectamine LTX						CHO cells and some primary fibroblast, epithelial and neuronal cells (MEF, HMEC and E18 cells)	Adherent
Lipofectamine CRISPRMAX						Tested in over 20 cells tpes including iPSC, mESC, N2A, CHO, A549, HCT116, HeLa, HEK293 and several others	Adherent
Lipofectamine RNAiMAX						Established cell lines, hard-to-transfect cells, primary cells, stem cells	Adherent
Lipofectamine MessengerMAX						Neuronal cells, primary cells	Adherent

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Symbol	Explanation	Symbol	Explanation
	DNA for expression of protein, shRNA, and miRNA		mRNA for expression of protein
	Non-coding RNA for RNAi inhibition of gene expression		Co-delivery for cotransfection of RNAi vectors and siRNAs
	CRISPR-Cas9 for protein delivery		

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Transfection Techniques & Basics

Additional transfection reagents to consider

Transfection reagent	Key features & applications of reagent
293fectin Transfection Reagent	Used for transient protein production in combination with the Invitrogen FreeStyle 293 Expression System Optimized for suspension Invitrogen FreeStyle 293-F cells
Cellfectin II Transfection Reagent	Optimal transfection of insect cells, including S2, Sf9, Sf21 and Invitrogen High Five cells
DMRIE-C Transfection Reagent	Transfection of suspension cells, including CHO, lymphoid and Jurkat cell lines.
ExpiFectamine 293 Transfection Kit	Designed for transfection of high-density suspension cell culture, with matching transfection enhancers that boost transfection performance and protein expression Achieves protein yields 2- to 10-fold higher than other transfection reagents used on high density 293 cell cultures Provides robust and reproducible transfection results Scale transfections for culture volumes of less than 1 mL to greater than 10 liters, while maintaining equivalent volumetric protein yields
ExpiFectamine CHO Transfection Kit	Designed for transfection of high density suspension CHO cells, with matching transfection enhancer and culture feed to boost transfection performance and protein expression Achieves protein yields up to 3 grams/liter in high density Gibco ExpiCHO cell cultures Provides robust and reproducible transfection results Scale transfections for culture volumes of less than 1 mL to greater than 10 liters, while maintaining equivalent volumetric protein yields

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Transfection Techniques & Basics

FreeStyle MAX Transfection Reagent	Optimized for transient transfection in CHO suspension cells and also works for HEK-293 cells Used for a large-scale transient protein production with milligrams of protein yield
Lipofectamine 2000 CD Transfection Reagent	Same performance as Invitrogen Lipofectamine 2000, certified animal-origin free (“CD” = chemically defined)
Lipofectamine LTX & Plus Reagent	High transfection efficiencies and viabilities in common cell lines, particularly Chinese Hamster Ovary (CHO).
Lipofectamine Transfection Reagent	First generation reagent for plasmid DNA transfections In most cases, Lipofectamine 2000 provides better performance than Invitrogen Lipofectamine Transfection Reagent
Lipofectin Transfection Reagent	First generation reagent for plasmid DNA transfections In most cases, Lipofectamine 2000 provides better performance than Invitrogen Lipofectin Transfection Reagent
Oligofectamine Transfection Reagent	Transfection of antisense oligonucleotides
Optifect Transfection Reagent	Broad use reagent designed for low confluency applications (<70% confluent at the time of transfection) Useful for cell lines that are sensitive to transfection reagents

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Transfection Techniques & Basics

Selecting a Viral DNA Delivery System

Menu: Selecting a Viral DNA Delivery System

There are many options in selecting a viral delivery system matched to your specific needs. Life Technologies™ offers a variety of viral vector systems for delivering nucleic acids into mammalian and insect cells for protein expression and RNAi studies.

Learn more about the different types of Viral Vectors

Expression in mammalian cells

ViraPower™ Expression Systems from Life Technologies™ use replication-incompetent viral particles to ensure safe and highly efficient delivery of expression constructs for high-level constitutive or inducible expression in any mammalian cell type. A number of vectors available for use with the ViraPower™ systems offer various options for cloning method (TOPO® or Gateway® cloning, or GeneArt® genetic assembly) and promoter choice (constitutive or inducible), allowing the optimization of the experiment for each cell line or animal model.

- **ViraPower™** Lentiviral Expression System allows stable protein expression in dividing and non-dividing cells (e.g., stem cells, primary neuronal cells), and are ideal for analysis of long-term gene expression and functional analysis studies.

- **ViraPower™ HiPerform™** Lentiviral Expression System improves on the existing lentiviral systems by including the woodchuck posttranscriptional regulatory element (WPRE) and the central polypurine tract (cPPT) sequence from the HIV-1 integrase gene in the viral vectors for increased expression and increased lentiviral integration into the host genome, respectively. The ViraPower™ HiPerform™ kits have two versions: kits for high accuracy titer, allowing for precise control of copy number per cell, or kits for fast titering, which are ideal for high throughput screening studies.

- **ViraPower™ Lentiviral T-REx™** System combines the ViraPower™ HiPerform™ Lentiviral, T-REx™, and Gateway® technologies to facilitate easy recombinationbased cloning and lentiviral-based, regulated (Tetracycline-inducible), high-level expression of a target gene in dividing and non-dividing mammalian cells. This system is ideal expressing toxic proteins, because the inducible promoter allows the control of the timing of gene expression.

- **ViraPower™** Adenoviral Expression System is ideal for protein production and allows high-level transient gene expression in dividing and non-dividing mammalian cells from the CMV or another promoter of choice. The ViraPower™ Adenoviral System uses Gateway® Technology for fast, easy, and accurate cloning of the gene of interest.

Learn more about ViraPower™expression systems

Transfection Techniques & Basics

Viral system	Transient expression		Stable expression			
Cell state	Dividing cells	Non-dividing cells	Dividing cells	Neuronal cells	Growth - arrested cells	Contact - inhibited cells
Adenovirus	✔	✔				
Retrovirus	✔		✔			
Lentivirus	✔	✔	✔	✔	✔	✔

Expression in insect cells

Expression in insect cells offers significant advantages, including high expression levels, ease of scale-up, and simplified cell growth that is readily adapted to high-density suspension culture. Furthermore, because many of the posttranslational modification pathways present in mammalian systems are also utilized in insect cells, proteins produced in insect cells are antigenically, immunogenically, and functionally similar to native mammalian proteins. Life Technologies™ offers powerful and versatile baculovirus expression systems for high-level, recombinant protein expression in insect cells.

- BaculoDirect™ Baculovirus Expression System is a fast and easy method for generating recombinant baculovirus using recombinational Gateway® cloning. Baculovirus expression systems typically require bacterial transformation and isolation of a large bacmid or co-transfection of a transfer vector and linear baculovirus DNA into insect cells. The BaculoDirect™system eliminates these timeconsuming steps, allowing the isolation of purified virus within one week. The reduction of hand-on time for baculovirus generation makes the BaculoDirect™ system ideal for high-throughput expression.

- Bac-to-Bac® Baculovirus Expression System uses a unique bacmid shuttle vector that recombines by site-specific transposition to generate an expression bacmid in bacterial cells. The bacmid is then transfected into insect cells for the production of recombinant baculovirus particles. With easy blue/white screening of recombinant colonies, the Bac-to-Bac® Baculovirus Expression System is designed for fast, small scale production of recombinant baculovirus.

- Bac-to-Bac® HBM Baculovirus Expression System enables secreted protein expression via the honeybee melittin (HBM) secretion signal, which is ideal for proteins and glycoproteins that require a secretion signal to be glycosylated. In contrast to glycoproteins secreted from mammalian cells, glycoproteins secreted from baculovirus can be easily de-glycosylated in vitro, which is essential for crystallizing the proteins.

- Bac-N-Blue™ Baculovirus Expression System is the classic and trusted expression system for high-level recombinant protein production in insect cells. Recombinant viral DNA is generated by co-transfection of a transfer vector containing the gene of interest and the linear baculovirus DNA into insect cells. Recombinant baculovirus is isolated using a blue/white plaque visualization method, and then amplified in insect cells to generate a high-titer viral stock to initiate expression studies.

Lean more about baculoviral expression systems

Transfection Techniques & Basics

System	Host	Secretion signal	Fusion partner position	Fusion partner purif.	Fusion partner epitope	Promoter	Expression / inducer	Advantage
BaculoDirect™	Sf9, Sf21, or High Five™		N-term C-term	6xHis 6xHis	V5 V5	Polyhedrin	Infection	Fast and easy; ideal for high-throughput
Bac-to-Bac® or Bac-to-Bac® HBM	Sf9, Sf21, or High Five™	Honeybee melittin	GST N-term	6xHis	pFastBacHT pDEST10	Polyhedrin or P10	Infection production	Rapid baculovirus production; easy blue / white selection
Bac-N-Blue™	Sf9, Sf21, or High Five™	Honeybee melittin	C-term	6xHis	Xpress™ V5	Polyhedrin	Infection	High-level recombinant protein production
DES®	S2 cells	BIP	C-term	6xHis	V5	MT or Ac5	CuSO4 or constitutive	Constitutive or inducible expression; extremely high integration

Transfection Techniques & Basics

Transfection Methods

Menu: Transfection Methods

When you are trying out a new transfection method or simply looking for a recommendation this section covers common transfection methods and techniques.

Browse by Transfection Method

Cationic Lipid Transfection

Specially designed cationic lipids facilitate DNA & siRNA delivery into cells.

Electroporation

Mechanical transfection that electrical pulses to create temporary pores in cell membranes.

In Vivo Transfection

Effective & easy-to-use in vivo RNAi delivery reagents used to achieve phenotypic alternations in animals.

Cotransfection

Simultaneous transfection of 2 separate nucleic acid molecules.

RNAi & siRNA Transfection

Reverse transfect Invitrogen Stealth RNAi or siRNA into mammalian cells in a 24-well format

Transient Transfection

Rapid, scalable, high-yield protein production from transiently transfected suspension cultures.

Stable Transfection

Stable transfection introduces DNA into cells long-term and pass the introduced DNA to their progeny.

Calcium Phosphate Transfection

Reagents to enable the introduction of DNA into eukaryotic cells via calcium phosphate co-precipitation.

CRISPR transfection

We have optimized protocols to achieve high cleavage efficiency and ease of delivery.

Transfection Techniques & Basics

Gene Delivery Technologies

Menu: Gene Delivery Technologies

The cell membrane consists of a phospholipid bilayer with embedded proteins and carries a net negative charge. Thus, it presents an impenetrable barrier to large molecules that, like the phosphate backbones of DNA and RNA, are also negatively charged. To sneak nucleic acids through the cell membrane, researchers have developed a number of techniques each using a different approach—from using chemicals and carrier molecules that coat the nucleic acids to neutralize them to physical methods that create transient pores in the membrane to introduce the DNA directly into the cell.

Gene delivery technologies

Transfection technologies available today can be broadly classified into three groups: chemical, biological, and physical. No one method can be applied to all cells and all experiments. The ideal approach should be selected depending your cell type and experimental needs, should have high transfection efficiency, low cell toxicity, and minimal effects on normal physiology, and be easy to use and reproducible (**Kim and Eberwine, 2010**).

Chemical

Chemical methods that use carrier molecules to neutralize or impart a positive charge to the negatively charged nucleic acids and include:

- Cationic lipid transfection
- Calcium phosphate transfection
- DEAE-dextran transfection
- Delivery by other cationic polymers (e.g., polybrene, PEI, dendrimers)

Biological

Biological methods that rely on genetically engineered viruses to transfer non-viral genes into cells (also known as transduction) and include:

- Viral delivery

Physical

Physical methods directly deliver nucleic acids into the cytoplasm or the nucleus of the cell and include:

- Electroporation
- Biolistic particle delivery (particle bombardment)
- Direct microinjection
- Laser-mediated transfection (phototransfection)
- Compare chemical gene delivery methods

Transfection Techniques & Basics

Technology	Advantages	Disadvantages
Cationic lipid mediated delivery	Fast and easy protocols Commercially available with reproducible results High efficiency and expression performance Applicable to a broad range of cell lines and high-throughput screens Can be used for delivering DNA, RNA, and protein No size limitation on the packaged nucleic acid Applicable to both transient and stable protein production Can be used for in vivo delivery of nucleic acids	Optimization may be necessary—some cell lines are sensitive to cationic lipids Some cell lines are not readily transfected with cationic lipids Presence of serum may interfere with complex formation and lower transfection efficiency Absence of serum in the medium may increase cytotoxicity
Calcium phosphate co-precipitation	Inexpensive and easily available Applicable to both transient and stable protein production High efficiency (cell line dependent)	Requires careful preparation of reagents—CaPO4 solutions are sensitive to changes in pH, temperature, and buffer salt concentrations Reproducibility can be problematic Cytoxicity, especially in primary cells Does not work with RPMI due to high phosphate concentration of the medium Not suited for in vivo gene transfer to whole animals

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Transfection Techniques & Basics

DEAE-dextran	Relatively simple technique Reproducible results, inexpensive	Chemical cytoxicity in some cell types Limited to transient transfection Low transfection efficiency
Delivery by other cationic polymers (e.g., polybrene, PEI, dendrimers)	Typically stable in serum and not temperature sensitive High efficiency (cell line dependent), reproducible results	Cytoxicity in some cell types Non-biodegradable (dendrimers) Limited to transient transfection

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Transfection Techniques & Basics

Compare biological gene delivery methods

Technology	Advantages	Disadvantages
Viral delivery	Highest efficiency amongst gene delivery methods (80–90% transduction efficiency in primary cells) Works well with difficult to transfect cell types Can be used for in vivo delivery of nucleic acids Can be used for making stable cell lines (retroviral vectors) or for transient expression (adenoviral vectors)	Cell lines to transfect must contain viral receptors Limited insert size (~10 kb for most viral vectors versus ~100 kb for non-viral vectors) Technically challenging and time consuming to generate recombinant viruses Present biosafety issues (activation of latent disease, immunogenic reactions, cytotoxicity, insertional mutagenesis, malignant transformation of cells)

Transfection Techniques & Basics

Compare physical gene delivery methods

Technology	Advantages	Disadvantages
Electroporation	• Simple principle • Reproducible results after optimization • No need for vector • Less dependent on cell type and condition • Rapid transfection of large number of cells after optimization	• Requires special instrument • Optimization of electrical pulse and field strength parameters required • Significantly more manipulation of cells required • High toxicity levels may be observed • High mortality rate requires large number of cells • Can irreversibly damage the membrane and lyse the cells
Biolistic particle delivery (particle bombardment)	• Less dependent on cell type and condition • Can be used for *in vivo* delivery of nucleic acids • Straightforward method with reliable results • No limitation to the size and or number of genes that can be delivered • Primarily used for genetic vaccination and agricultural application	• Requires expensive instrument • Causes physical damage to samples • High mortality rate requires large number of cells • Preparation of microparticles is required • Relatively costly for research applications • Generally less efficient than electroporation or viral- or lipid-mediated delivery
Direct microinjection	• Less dependent on cell type and condition • Allows single-cell transfection • Straightforward method with reliable results • No limitation to the size and or number of genes that can be delivered • No need for vector	• Requires expensive instrument • Technically demanding and very labor-intensive (one cell at a time) • Often causes cells deaths

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Transfection Techniques & Basics

Laser-mediated transfection (phototransfection)	• Can be used for delivering DNA, RNA, proteins, ions, dextrans, small molecules, and semiconductor nanocrystals • Can be applied to very small cells Allows single-cell transfection or transfection of large number of cells at the same time • No need for vector • High efficiency • Applicable to a broad range of cell lines	• Requires expensive laser-microscope system • Requires cells to be attached • Technically demanding

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Transfection Techniques & Basics

Factors Influencing Transfection Efficiency

Menu: Factors Influencing Transfection Efficiency

Successful transfection is influenced by many factors—the choice of the transfection method, health and viability of the cell line, number of passages, degree of confluency, quality and quantity of the nucleic acid used, and the presence or absence of serum in the medium can all play a part in the outcome of your transfection experiment. While it is possible to optimize specific transfection conditions to achieve high transfection efficiencies, it is important to note that some cell death is inevitable regardless of the transfection method used.

Cell type

The choice of which cell type to use for a transfection experiment may seem obvious, but it is a critical factor that is often overlooked. Since each cell type is likely to respond differently to a given transfection reagent or method, choosing the appropriate cell type and proper experimental design are necessary to maximize results.

While established continuous cell lines are easier to work with in the laboratory, they may not be the best choice for modelling in vivo processes because of the multiple genetic changes that they have undergone. However, if the purpose of the transfection experiment is high-level production of recombinant proteins, it is not important that the cell line represents the in vivo situation as long as the cell line can express sufficient quantities of recombinant proteins with proper folding and post-translational modifications. For example, transient transfection of suspension-adapted Gibco Expi293F cells grown in Gibco Expi293 Expression Medium enables researchers to produce, starting from the vector of interest, greater than 1 g/L of correctly folded and glycosylated recombinant proteins.

Primary cultures, on the other hand, are often used because they more closely mimic natural tissues. However, they typically have a limited growth potential and life span and are more difficult to maintain in culture. When using primary cultures, it is important to maintain a largely homogeneous population of cells (for example, neuronal cultures should be enriched for neurons and suppressed with regard to glial cells) and use the cells as soon as practical.

In addition, biological properties of the cell type must be taken into consideration when designing transfection experiments. For example, some promoters function differently in different cell types and some cell types are not well suited to particular transfection technologies.

Transfection Techniques & Basics

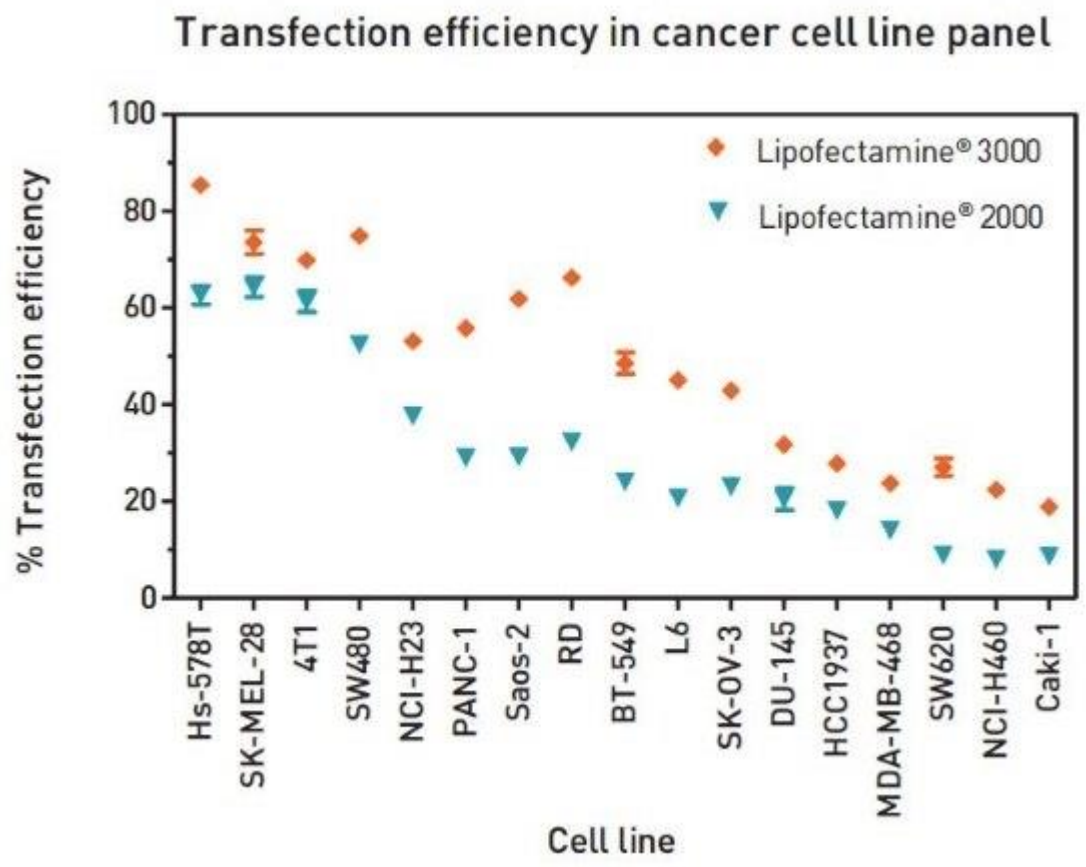

Figure 6.1 *Cell line-dependent differences in transfection efficiency. Invitrogen product line such as Lipofectamine 2000 reagent and Lipofectamine 3000 reagent were used to transfect 17 cell lines with a GFP-expressing plasmid in a 24-well plate format, using 0.5 µg plasmid/well and the recommended protocols for each reagent. GFP expression was analyzed 48 hours posttransfection. Each condition was tested in triplicate, and the data points show the mean transfection efficiency plus standard deviation.*

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Transfection Techniques & Basics

Cell health and viability

The viability and general health of cells prior to transfection is known to be an important source of variability from one transfection to another. In general, cells should be at least 90% viable prior to transfection and have had sufficient time to recover from passaging. We strongly recommend subculturing cells at least 24 hours before transfection to ensure that they recover from the subculture procedure and are in optimum physiological condition for transfection.

Cell cultures with immortalized cell lines evolve over months and years in the laboratory, resulting in changes in cell behavior with regard to transfection. Excessive passaging is likely to detrimentally affect transfection efficiency as well as total transgene expression level from the cell population as a whole. In general, we recommend using cells that have undergone less than 30 passages after thawing of a stock culture. Thawing a fresh vial of frozen cells and establishing low-passage cultures for transfection experiments allow the recovery of transfection activity. For optimal reproducibility, aliquots of cells of a low passage number can be stored frozen and thawed as needed. Allow 3 or 4 passages after thawing a new vial of cells.

Since contamination can drastically alter transfection results, cell cultures and media should be routinely tested for biological contamination (see Biological Contamination), and contaminated cultures and media should never be used for transfection. If cells have been contaminated or their health is compromised in any way, they should be discarded and the culture re-seeded from uncontaminated frozen stocks.

Confluency

For optimal transfection results, follow a routine subculturing procedure and passage cultures once or twice a week at a dilution that allows them to become nearly confluent before the next passage. Do not allow the cells to remain confluent for more than 24 hours.

The optimal cell density for transfection varies for different cell types, applications, and transfection technology, and should be determined for every new cell line to be transfected. Maintaining a standard seeding protocol from experiment to experiment ensures that optimal confluency at the time of transfection is reliably achieved. With cationic lipid-mediated transfection, generally 70–90% confluency for adherent cells or 5 × 105 to 2 × 106 cells/mL for suspension cells at the time of transfection provides good results.

Make sure that the cells are not confluent or in stationary phase at the time of transfection, because actively dividing cells take up foreign nucleic acid better than quiescent cells. Too high of a cell density can cause contact inhibition, resulting in poor uptake of nucleic acids and/or decreased expression of the transfected gene. However, too few cells in culture may result in poor growth without cell-to-cell contact. In such cases, increasing the number of cells in culture improves the transfection efficiency. Similarly, actively dividing cell lines are more efficiently transduced with viral vectors. When transducing a non-dividing cell type with viral constructs, the MOI (i.e., multiplicity of infection) may need to be increased to achieve optimal transduction efficiency and increased expression levels for your recombinant protein.

Media

Different cells or cell types have very specific medium, serum, and supplement requirements, and choosing the most suitable medium for the cell type and transfection method plays a very important role in transfection experiments. Information for selecting the appropriate medium for a given cell type and transfection method is usually available in published literature and may also be obtained from the source of the cells or cell banks. If there is no information available on the appropriate medium for your cell type, you must determine it empirically.

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Transfection Techniques & Basics

It is important to use fresh medium, especially if any of the components are unstable, because medium that is missing key components and necessary supplements may harm cell growth.

For cell culture media information, see Media recommendations for common cell lines. Some cell lines and primary cells may need special coating materials (e.g. poly-lysine, collagen, fibronectin etc.) to attach to the culture plates and get the optimal transfection results.

Serum

In general, the presence of serum in culture medium enhances transfection with DNA. However, when performing cationic lipid-mediated transfection, it is important to form DNA-lipid complexes in the absence of serum because some serum proteins interfere with complex formation. Note that the optimal amounts of cationic lipid reagent and DNA may change in the presence of serum; thus, transfection conditions should be optimized when using serum-containing transfection medium.

When transfecting cells with RNA, we recommend performing the transfection procedure in the absence of serum to avoid possible contamination with RNases. Most cells remain healthy for several hours in a serum-free medium.

The quality of serum can significantly affect cell growth and transfection result. Therefore, it is important to control for variability among different brands or even different lots of serum to obtain best results. After testing the serum on your cells, keep using the same serum to avoid variation in your result. All Gibco™ products, including sera, are tested for contamination and guaranteed for their quality, safety, consistency, and regulatory compliance.

Antibiotics

In general, antibiotics can be present in the medium for transient transfection. However, because cationic lipid reagents increase cell permeability, they may also increase the amount of antibiotics delivered into the cells, resulting in cytotoxicity and lower transfection efficiency. Therefore, we do not recommend adding antibiotics to the transfection medium. Avoiding antibiotics when plating cells for transfection also reduces the need for rinsing the cells before transfection.

For stable transfections, penicillin and streptomycin should not be used in selective medium, because these antibiotics are competitive inhibitors of the Gibco Geneticin selective antibiotic. When creating stable cell lines, allow 48 to 72 hours after the transfection procedure for cells to express the resistance gene before adding the selective antibiotic.

If using serum-free medium, use lower amounts of antibiotics than you would in serum-containing medium to maintain the health of the cells.

Type of molecule transfected

Plasmid DNA is the most commonly used vector for transfection. The topology (linear or supercoiled) and the size of the plasmid DNA vector influence the efficiency of transfection. Transient transfection is most efficient with supercoiled plasmid DNA. In stable transfection, linear DNA results in lower DNA uptake by the cells relative to supercoiled DNA but yields optimal integration of DNA into the host genome.

Although other macromolecules such as oligonucleotides, RNA, siRNA, and proteins can also be transfected into cells, conditions that work for plasmid DNA need to be optimized when using other macromolecules.

Transfection Techniques & Basics

Transfection method

There are a number of strategies for introducing nucleic acids into cells that use various biological, chemical, and physical methods. However, not all of these methods can be applied to all types of cells and experimental applications, and there is a wide variation with respect to transfection efficiency, cell toxicity, effects on normal physiology, level of gene expression etc. The ideal approach should be selected depending your cell type and experimental needs, and should have high transfection efficiency, low cell toxicity, minimal effects on normal physiology, and be easy to use and reproducible. For an overview and comparison of various transfection methods, see Gene Delivery Technologies.

Transfection Techniques & Basics

Guidelines for Plasmid DNA Transfection

Menu: Guidelines for Plasmid DNA Transfection

Classic transfection technologies have initially been developed for introducing plasmid DNA into cells, and plasmid DNA still remains the most common vector for transfection. DNA plasmids containing recombinant genes and regulatory elements can be transfected into cells to study gene function and regulation, mutational analysis and biochemical characterization of gene products, effects of gene expression on the health and life cycle of cells, as well as for large scale production of proteins for purification and downstream applications.

The topology (linear or supercoiled) and the size of the vector construct, the quality of the plasmid DNA, and the promoter choice are major factors that influence the efficiency of plasmid DNA transfection.

Learn more about optimizing Plasmid DNA transfection

Vector considerations

Transient transfections are more efficient with highly supercoiled DNA compared to linear DNA, presumably because circular DNA is not vulnerable to exonucleases, while linear DNA fragments are quickly degraded by these enzymes (McLenachan et al., 2007; von Groll et al., 2006). In addition, atomic force microscopy analysis shows very different complexation patterns between cationic lipid reagents and circular and linear DNA topologies: while compact spherical or cylindrical condensates are observed with circular DNA, linear plasmids show extended pearl necklace-like structures. Although the cationic lipid-mediated transfection of the more compact circular plasmids is likely to go through endocytosis, the pathway of entry of extended linearized DNA structures might be quite different and less efficient (von Groll et al., 2006).

Stable transfections are more efficient when using linear DNA due to its optimal integration into the host genome. Linear DNA with free ends is more recombinogenic and more likely to be integrated into the host chromosome to yield stable transformants, even though it is taken up by the cell less efficiently.

Despite similar uptake efficiencies in cationic lipid-mediated transfection, nuclear delivery of large plasmids is compromised compared with small plasmid molecules. This effect is observed using equivalent mass or molar concentrations of different-sized constructs, suggesting that nuclear delivery of plasmids may be limited by the rate of intracellular transit and that small plasmids evade degradation by rapid transit through the cytoplasm, rather than through the saturation of cellular defenses (Lukacs, et al., 2000; McLenachan et al., 2007).

Transfection Techniques & Basics

Quality of plasmid DNA

Purity and quality of the plasmid DNA is critical for a successful transfection. The best results are achieved with plasmid DNA of the highest purity that is free from phenol, sodium chloride, and endotoxins. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. Endotoxins, also known as lipopolysaccharides, are released during the lysis step of plasmid preparations and are often co-purified with plasmid DNA. Their presence sharply reduces transfection efficiency in primary and other sensitive cells. We recommend isolating plasmid DNA using PureLink HiPure Plasmid Kits (Mini, Midi, Maxi, Mega, and Giga) that provide highest quality DNA for transfections.

Although cesium chloride banding also yields highly purified DNA, it is a labor intensive and time consuming process. Excess vortexing of DNA-lipid complexes or DNA solutions may result in some shearing, especially with larger molecules, thereby reducing transfection efficiency. The concentration of EDTA in the diluted DNA should not exceed 0.3 mM.

Gene product and promoter

Promoter choice is dependent on the host cell line, the protein to be expressed, and the level of expression desired. Many researchers use the strong CMV (cytomegalovirus) promoter because it provides the highest expression activity in the broadest range of cell types. Another strong promoter for high-level protein expression in mammalian cells is the EF-1α (human elongation factor-1α). However, using too strong a promoter to drive the expression of a potentially toxic gene can cause problems in transient transfection of plasmid DNA. For the potentially toxic gene products, use of **weak promoters** are recommended.

Toxic gene products are also a problem for selection of stably transfected cells. Cells expressing a gene for antibiotic resistance lose their growth advantage when such gene expression is detrimental to the health of the transfected cell, which makes it impossible to obtain stably transfected clones with a constitutive promoter. In such cases, an **inducible promoter** can be used to control the timing of gene expression, which will allow for the selection of stable transfectants. Inducible promoters normally require the presence of an inducer molecule (e.g., a metal ion, metabolite, or hormone) to function, but some inducible promoters function in the opposite manner, that is, gene expression is induced in the absence of a specific molecule.

Cell-type specific promoters, such as the polyhedrin promoter for insect cell expression, are also common. Literature searches are the best tool to determine which promoter will work best for your cell line or application.

Controls

Regardless of the transfection method used, it is important to perform control transfections to check for cell health, to determine whether the reported assay is working properly, and to establish any insert-related problems. To check for optimal cell growth conditions, include a negative control (no DNA, no transfection reagent). To establish that the reporter assay is working properly, include a positive control (parallel transfection with established transfection method). To determine whether there are insert-related problems, transfect a plasmid without the gene of interest.

Transfection Techniques & Basics

Guidelines for RNA Transfection

Menu: Guidelines for RNA Transfection

Transfection of RNA is an offshoot of classic transfection technologies for introducing RNA into cells. The purpose of RNA transfection is similar to that of plasmid transfection. mRNA is introduced into cells to express the encoded protein, and study gene function and regulation. siRNA is used for RNAi studies that examine the effects of gene knockdown. One major difference between the two methods is that RNA can only be transiently transfected.

RNAi workflow

The diagram below depicts an RNAi experiment workflow following siRNA design and synthesis. When performing an RNAi experiment, make sure that you have the following on hand:

- Transfection/electroporation agent and protocol
- Assays to assess knockdown and other RNAi effect(s)
- Positive and negative control siRNAs
- Two or more siRNAs to gene of interest

Transfection Techniques & Basics

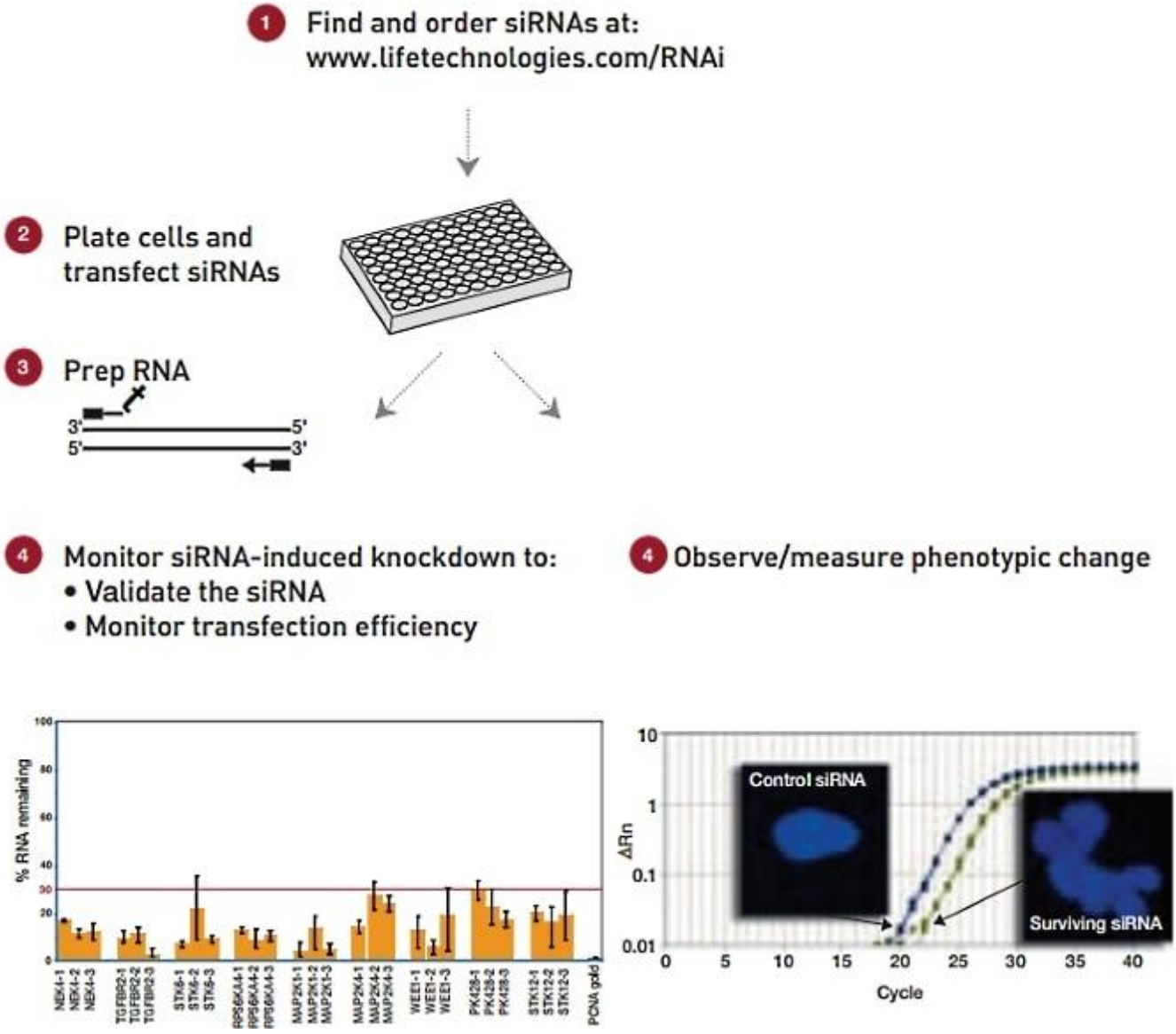

Figure 1: *RNAI workflow following siRNA design and synthesis*

https://www.thermofisher.com/au/en/home/references/gibco-cell-culture-basics/transfection-basics.html

Transfection Techniques & Basics

Handling RNA

RNA oligonucleotides are susceptible to degradation by exogenous ribonucleases introduced during handling.

- Wear gloves when handling RNA.
- Use RNase-free reagents, tubes, and barrier pipette tips for preparing RNA for transfection.
- Work areas should be wiped down with 70% ethanol or other RNase decontamination solution such as RNaseZap® RNase Decontamination Solution.

Transfection efficiency

The efficiency with which mammalian cells are transfected with siRNA will vary according to cell type and the transfection agent used. This means that the optimal concentration used for transfections should be determined empirically. The major variables that impact siRNA transfection efficiency are the following:

- Transfection reagent type and amount
- Number of cells plated in well
- Type of RNA or siRNA
- Concentration of RNA or siRNA

Positive controls

It is important to include a positive control in each experiment. The positive control should elicit a reproducible, easily measured response in the cells and assay used in your study. If you see maximal effect above/below a pre-determined threshold level with this control, you know that measurements from other experiments tested on the same day are reliable. Note that it is important to empirically determine the thresholds for each assay and control pair.

The degree of the response to a particular RNA or siRNA is directly linked to its transfection efficiency. To assess transfection efficiency, we recommend including the BLOCK-iT Fluorescent Oligo in every experiment. Using the BLOCK-iT Fluorescent Oligo in your transfection experiment allows you to easily assess oligomer uptake and transfection efficiency using any fluorescence microscope and a standard FITC filter set. Uptake of the fluorescent oligomer by at least 80% of cells correlates with high efficiency.

Negative controls

Negative controls are just as important as positive controls for obtaining meaningful data. Always include a set of transfections with an equimolar amount of at least one negative control to compare the effects of the target RNA or siRNA-treated and control treated cells. Data from these crucial controls serve as a baseline for evaluation of experimental target knockdown.

Non-transfected or cells-only negative controls are also very useful. By comparing expression of a housekeeping gene among cultures that were not transfected and cultures transfected with a non-targeting negative control, valuable information about the effects of transfection on cell viability can be obtained.

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Type of control	Recommended use	Recommended products
Transfection control	Calculate and monitor transfection efficiency with fluorescence	BLOCK-iT Alexa Fluor Red Fluorescent Control BLOCK-iT Fluorescent Oligo
Negative control	Nonspecific or scrambled controls used to measure knockdown levels vs. background	Silencer Select Negative Control siRNAs Stealth RNAi siRNA negative controls Silencer negative control siRNAs
Positive control	RNAi reagents known to achieve high levels of knockdown used to measure delivery and optimize experimental conditions	Silencer Select GAPDH Positive Control siRNAs Stealth RNAi siRNA positive controls Silencer positive control siRNAs
Untransfected control	Measure normal gene expression level and phenotype	
Multiple RNAi sequences to the same RNAi target	Use to verify phenotypic change, control for off-target effects for generating publication quality results	
Titration of RNAi	Use the lowest effective level to avoid altering the cells normal processes	
Rescue experiments	Turn off inducible RNAi or introduce a plasmid expressing the target mRNA that the RNAi sequence will not affect	BLOCK-iT Pol II miR RNAi or BLOCK-iT shRNA vectors with inducible promoters (CMV/TO and H1/TO respectively)

Co-transfection

Co-transfection is performed when the user wants to introduce both siRNA and a plasmid for expressing a protein into a cell. This protein can be part of the test system, or in most cases, it can be a reporter gene (luciferase, GFP, β-lactamase). In some cases, users may want to express a mutant protein along with the siRNA to block one pathway with the siRNA, and overexpress a mutant protein.

The presence of the plasmid may decrease transfection efficiency of all cargo (plasmid and siRNA) when a lipid transfection reagent is used, making transfection optimizations very important. Undesired and non-specific cell death can result with too much lipid, or too little knock-down or protein expression from the plasmid can occur if transfection conditions are not optimal.

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siRNA quality

The quality of siRNA can significantly influence RNAi experiments. siRNAs must be free of reagents carried over from synthesis, such as ethanol, salts, and proteins. Also, dsRNA contaminants longer than 30 bp are known to alter gene expression by activating the nonspecific interferon response and causing cytotoxicity (Stark et al., 1998). Therefore, we recommend using standard purity siRNAs that are greater than 80% full length.

siRNA storage

Store siRNAs at –20°C or –80°C, but do not use a frost-free freezer. Our data indicate that up to 50 freeze/thaw cycles are not detrimental to siRNAs in solution at 100 µM (as assessed by mass spectrometry and analytical HPLC). However, we recommend that siRNAs that have been resuspended in RNase-free water or buffer be stored in small aliquots to avoid potential contamination.

Nuclease resistance of siRNAs

Annealed, double-stranded siRNAs are much more nuclease resistant than single-stranded RNA. However, stringent RNase-free techniques should be used during all RNAi experiments.

Checking siRNA for degradation

If you suspect that a preparation of siRNA may be degraded, check the integrity of the siRNA by running ~2.5 µg on a non-denaturing 15–20% acrylamide gel. Visualize the RNA by staining with ethidium bromide and verify that it is the expected size and intensity. The siRNA should migrate as a tight band; smearing indicates degradation.

siRNA quantity

The optimal amount of siRNA and its capacity for gene silencing are influenced in part by properties of the target gene products, including the following: mRNA localization, stability, abundance, as well as target protein stability and abundance.

Although many siRNA experiments are still performed by transfecting cells with 100 nM siRNA, published results indicate that transfecting lower siRNA concentrations can reduce off-target effects exhibited by siRNAs (Jackson et al., 2003; Semizarov et al., 2003). For lipid-mediated reverse transfections, 10 nM of siRNA (range 1–30 nM) is usually sufficient. For siRNA delivery using electroporation, siRNA quantity has a less pronounced effect, but typically 1 µg/50 µL cells (1.5 µM) of siRNA (range 0.5–2.5 µg/50 µL cells or 0.75–3.75 µM) is sufficient.

Keep in mind that while too much siRNA may lead to off-target or cytotoxic effects, too little siRNA may not reduce target gene expression effectively. Because there are so many variables involved, it is important to optimize the siRNA amount for every cell line used. In addition, the amount of non-targeting negative control siRNA should be the same as the experimental siRNAs.

Volume of transfection reagent

The volume of transfection agent is a critical parameter to optimize because too little can limit transfection, and too much can be toxic. The overall transfection efficiency is influenced by the amount of transfection agent complexed to the siRNA. To optimize, titrate the transfection agent over a broad dilution range, and choose the most dilute concentration that still gives good gene knockdown. This critical volume should be determined empirically for each cell line.

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Cell density

While cell density is important for traditional, pre-plated transfection experiments, cell density is less critical and requires little to no optimization, when siRNAs are delivered by reverse transfection. However, if too many cells are used, and the amount of siRNA is not increased proportionally, the concentration of siRNA in the sample may be too low to effectively elicit gene silencing. When cell density is too low, cultures can become unstable. Instability can vary from well to well because culture conditions (e.g., pH, temperature) may not be uniform across a multiwell plate and can differentially influence unstable cultures.

Exposure to transfection agent/siRNA complexes

Although most transfection agents are designed to induce minimal cytotoxicity, exposing cells to excessive amounts of transfection agent or for an extended time can be detrimental to the overall health of the cell culture. Sensitive cells may begin to die from exposure to the transfection agent after a few hours. If transfection causes excessive cell death with your cells, remove the transfection mixture and replenish with fresh growth medium after 8–24 hours.

Presence of serum during transfection

Complex formation between transfection agents and siRNA should be performed in reduced-serum or serum-free medium, so that serum components will not interfere with the reaction. However, once complex formation has occurred, some transfection agents will permit transfection in serum-containing, normal growth medium (follow manufacturer's instructions). No culture medium addition or replacement is usually required following transfection but changing the media can be beneficial in some cases, even when serum compatible reagents are used. Be sure to check for serum compatibility before using a particular agent. Some transfection agents require serum free medium during the transfection and a change to complete growth media after an initial incubation with transfection complexes.

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Reporter Gene Assays

Menu: Reporter Gene Assays

Reporter genes are genes whose products can be readily assayed subsequent to transfection and can be used as markers for screening successfully transfected cells, for studying regulation of gene expression, or serve as controls for standardizing transfection efficiencies.

The ideal reporter gene should be absent from the cells used in the study or easily distinguishable from the native form of the gene, assayed conveniently, and have a broad linear detection range. It is also important that the presence of the reporter gene does not affect the normal physiology and general health of the transfected cells.

Reporter genes expression can either be constitutive or inducible, with an external intervention such as the introduction of IPTG in the β-galactosidase system. Generally, reporter gene assays are performed 1–3 days after transfection; however, the optimal time for the assay should be determined empirically.

Transfection assays

In contrast to selectable markers, which protect an organism from a selective agent that would normally kill it or prevent its growth, reporter genes used for screening transfectants make the cells containing the reporter gene visually identifiable. Reporter genes used in this way are normally expressed under their own promoter independent from that of the introduced gene of interest, allowing the screening of successfully transfected cells even when the gene of interest is only expressed under certain specific conditions or in tissues that are difficult to access.

Reporter genes can also serve as controls for transfection. For example, transfection efficiencies between different experiments can be normalized by comparing the expression levels of a reporter gene used in all of the experiments.

Gene regulation assays

Reporter gene assays are invaluable for studying regulation of gene expression, both by cis-acting factors (gene regulatory elements) and trans-acting factors (transcription factors or exogenous regulators). Furthermore, reporter gene systems enable the use of pathway-specific, tissue-specific, or developmentally regulated gene promoters as biomarkers for specific events processes.

In these assays, the detectable reporter gene acts as a surrogate for the coding region of the gene under study. The reporter gene construct contains one or more gene regulatory elements to be analyzed, the sequence for the reporter gene, and the sequences required for the transcription of functional mRNA. Upon introduction of the reporter construct into cells, expression levels of the reporter gene are monitored through a direct assay of the reporter proteins enzymatic activity.

Common reporter genes

Commonly used reporter genes that induce visually identifiable characteristics usually involve fluorescent and luminescent proteins.

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Green fluorescent protein (GFP)

Green fluorescent protein (GFP) causes cells that express it to glow green under UV light. A specialized microscope is required to see individual cells. Yellow and red versions are also available, allowing the investigation of multiple genes at once. It is commonly used to measure gene expression.

Luciferase

Luciferase as a laboratory reagent often refers to P. pyralis luciferase, although recombinant luciferases from several other species of fireflies are also commercially available. The luciferase enzyme catalyzes a reaction with its substrate (usually luciferin) to produce yellow-green or blue light, depending on the luciferase gene. Since light excitation is not needed for luciferase bioluminescence, there is minimal autofluorescence and thus virtually background-free fluorescence.

GUS assay

GUS assay (using β-glucuronidase) is an excellent method for detecting a single cell by staining it blue without using any complicated equipment. The drawback is that the cells are killed in the process. It is particularly common in plant science.

Blue-white screen

Blue-white screen is used in both bacteria and eukaryotic cells. The bacterial lacZ gene encodes a β-galactosidase enzyme. When media containing certain galactosides (e.g., X-gal) is added, cells expressing the gene convert the X-gal to a blue product and can be seen with the naked eye.