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Cell Culture Basics Handbook

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1. Introduction

Purpose of the Handbook

Cell Culture Basics Companion Handbook is a supplement to the Cell Culture Basics instructional videos available online at **www.lifetechnologies.com/cellculturebasics**.

The handbook and videos are intended as an introduction to cell culture basics. The first four chapters of the handbook focus on cell culture, covering topics such as getting familiar with the requirements of a laboratory dedicated to cell culture experiments, laboratory safety, aseptic technique, and microbial contamination of cell cultures, as well as providing basic methods for passaging, freezing, and thawing cultured cells. The subsequent two chapters of the handbook focus on various transfection technologies and provide general guidelines for the selection of the appropriate transfection method, the transfection of cells with plasmid DNA, oligonucleotides, and RNA, as well as culture preparation for *in vitro* and *in vivo* transfection and selection of the transfected cells.

The information and guidelines presented in the handbook and the instructional videos focus on cell lines (finite or continuous) and omit experiments and techniques concerning primary cultures and stem cells, such as isolating and disaggregating tissues, reprogramming cells into pluripotent stem cells, or differentiating stem cells into various lineages.

Note that while the basics of cell culture experiments share certain similarities, cell culture conditions vary widely for each cell type. Deviating from the culture conditions required for a particular cell type can result in different phenotypes being expressed; we therefore recommend that you familiarize yourself with your cell line of interest, and closely follow the instructions provided with each product you are using in your experiments.

Introduction to Cell Culture

What is cell culture?

Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a favorible artifical environment. The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been established.

Primary culture

Primary culture refers to the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate (i.e., reach **confluence**). At this stage, the cells have to be **subcultured** (i.e., passaged) by transferring them to a new vessel with fresh growth medium to provide more room for continued growth.

Cell line

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**; see below), and as they are passaged, cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population.

Cell strain

If a subpopulation of a cell line is positively selected from the culture by cloning or some other method, this cell line becomes a **cell strain**. A cell strain often acquires additional genetic changes subsequent to the initiation of the parent line.

Finite vs. continuous cell line

Normal cells usually divide only a limited number of times before losing their ability to proliferate, which is a genetically determined event known as **senescence**; these cell lines are known as **finite**. However, some cell lines become immortal through a process called **transformation**, which can occur spontaneously or can be chemically or virally induced. When a finite cell line undergoes transformation and acquires the ability to divide indefinitely, it becomes a **continuous cell line**.

Culture conditions

Culture conditions vary widely for each cell type, but the artifical environment in which the cells are cultured invariably consists of a suitable vessel containing a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, and gases (O_2, CO_2), and regulates the physico-chemical milieu (pH, osmotic pressure, temperature). Most cells are **anchorage-dependent** and must be cultured while attached to a solid or semi-solid substrate (**adherent** or **monolayer culture**), while others can be grown floating in the culture medium (**suspension culture**).

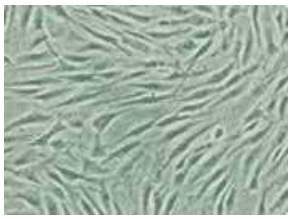
Cryopreservation

If a surplus of cells are available from subculturing, they should be treated with the appropriate protective agent (e.g., DMSO or glycerol) and stored at temperatures below –130°C (**cryopreservation**) until they are needed. For more information on subculturing and cryopreserving cells, refer to the **Guidelines for Maintaining Cultured Cells**, page 26.

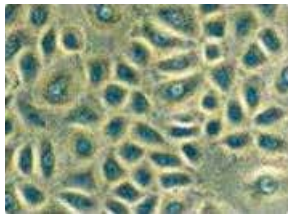
Morphology of cells in culture

Cells in culture can be divided in to three basic categories based on their shape and appearance (i.e., **morphology**).

- **Fibroblastic** (or fibroblast-like) cells are bipolar or multipolar, have elongated shapes, and grow attched to a substrate.



- **Epithelial-like** cells are polygonal in shape with more regular dimensions, and grow attached to a substrate in discrete patches.



- **Lymphoblast-like** cells are spherical in shape and usually grown in suspension without attaching to a surface.

Applications of cell culture

Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (e.g., metabolic studies, aging), the effects of drugs and toxic compounds on the cells, and mutagenesis and carcinogenesis. It is also used in drug screening and development, and large scale manufacturing of biological compounds (e.g., vaccines, therapeutic proteins). The major advantage of using cell culture for any of the these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells.

2. Cell Culture Laboratory

Safety

In addition to the safety risks common to most everyday work places, such as electrical and fire hazards, a cell culture laboratory has a number of specific hazards associated with handling and manipulating human or animal cells and tissues, as well as toxic, corrosive, or mutagenic solvents and reagents. The most common of these hazards are accidental inoculations with syringe needles or other contaminated sharps, spills and splashes onto skin and mucous membranes, ingestion through mouth pipetting, animal bites and scratches, and inhalation exposures to infectious aerosols.

The fundamental objective of any biosafety program is to reduce or eliminate exposure of laboratory workers and the outside environment to potentially harmful biological agents. **The most important element of safety in a cell culture laboratory is the strict adherence to standard microbiological practices and techniques.**

Biosafety levels

The regulations and recommendations for biosafety in the United States are contained in the document *Biosafety in Microbiological and Biomedical Laboratories*, prepared by the Centers for Disease Control (CDC) and the National Institutes of Health (NIH), and published by the U.S. Department of Health and Human Services. The document defines four ascending levels of containment, referred to as biosafety levels 1 through 4, and describes the microbiological practices, safety equipment, and facility safeguards for the corresponding level of risk associated with handling a particular agent.

Biosafety Level 1 (BSL-1)

BSL-1 is the basic level of protection common to most research and clinical laboratories, and is appropriate for agents that are not known to cause disease in normal, healthy humans.

Biosafety Level 2 (BSL-2)

BSL-2 is appropriate for moderate-risk agents known to cause human disease of varying severity by ingestion or through percutaneous or mucous membrane exposure. Most cell culture labs should be at least BSL-2, but the exact requirements depend upon the cell line used and the type of work conducted.

Biosafety Level 3 (BSL-3)

BSL-3 is appropriate for indigenous or exotic agents with a known potential for aerosol transmission, and for agents that may cause serious and potentially lethal infections.

Biosafety Level 4 (BSL-4)

BSL-4 is appropriate for exotic agents that pose a high individual risk of life-threatening disease by infectious aerosols and for which no treatment is available. These agents are restricted to high containment laboratories.

For more information about the biosafety level guidelines, refer to *Biosafety in Microbiological and Biomedical Laboratories, 5th Edition*, which is available for downloading at **www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm**.

SDS

Safety Data Sheet (SDS) is a form containing information regarding the properties of a particular substance, including physical data such as melting point, boiling point, and flash point, as well as information on its toxicity, reactivity, health effects, storage, disposal, recommended protective equipment, and handling spills.

SDSs for all Life Technologies products are available at **www.lifetechnologies.com/sds**.

Safety equipment

Safety equipment in a cell culture laboratory includes **primary barriers** such as biosafety cabinets, enclosed containers, and other engineering controls designed to remove or minimize exposure to hazardous materials, as well as **personal protective equipment (PPE)** that is often used in conjuction with the primary barriers. The **biosafety cabinet** (i.e., cell culture hood) is the most important equipment to provide containment of infectious splashes or aerosols generated by many microbiological procedures. For more information, see **Cell Culture Hood**, page 7.

Personal protective equipment (PPE)

Personal protective equipment (PPE) form an immediate barrier between the personnel and the hazardous agent, and they include items for personal protection such as gloves, laboratory coats and gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. They are often used in combination with biosafety cabinets and other devices that contain the agents, animals, or materials being handled. We recommend that you consult your institution's guidelines for the appropriate use of PPE in your laboratory.

Safe laboratory practices

The following recommendations are simply guidelines for safe laboratory practices, and they should not be interpreted as a complete code of practice. Consult your institution's safety committee and follow local rules and regulations pertaining to laboratory safety.

For more information on standard microbiological practices and for specific biosafety level guidelines, refer to *Biosafety in Microbiological and Biomedical Laboratories, 5th Edition* at **www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm**.

- Always wear appropriate personal protective equipment. Change gloves when contaminated, and dispose of used gloves with other contaminated laboratory waste.
- Wash your hands after working with potentially hazardous materials and before leaving the laboratory.
- Do not eat, drink, smoke, handle contact lenses, apply cosmetics, or store food for human consumption in the laboratory.
- Follow the institutional policies regarding safe handling of sharps (i.e., needles, scalpels, pipettes, and broken glassware).
- Take care to minimize the creation of aerosols and/or splashes.
- Decontaminate all work surfaces before and after your experiments, and immediately after any spill or splash of potentially infectious material with an appropriate disinfectant. Clean laboratory equipment routinely, even if it is not contaminated.
- Decontaminate all cultures, stocks, and other potentially infectious materials before disposal.
- Report any incidents that may result in exposure to infectious materials to appropriate personnel (e.g., laboratory supervisor, safety officer).

Cell Culture Equipment

The specific requirements of a cell culture laboratory depend mainly on the type of research conducted; for example, the needs of mammalian cell culture laboratory specilizing in cancer research is quite different from that of an insect cell culture laboratory that focuses on protein expression. However, all cell culture laboratories have the common requirement of being free from pathogenic microorganisms (i.e., asepsis), and share some of the same basic equipment that is essential for culturing cells.

This section lists the equipment and supplies common to most cell culture laboratories, as well as beneficial equipment that allows the work to be performed more efficiently or accurately, or permits wider range of assays and analyses. Note that this list is not all inclusive; the requirements for any cell culture laboratory depend the type of work conducted.

Basic equipment

- Cell culture hood (i.e., laminar-flow hood or biosafety cabinet)
- Incubator (humid CO_2 incubator recommended)
- Water bath
- Centrifuge
- Refrigerator and freezer (–20°C)
- Cell counter (e.g., Countess® II Automated Cell Counter or hemocytometer)
- Inverted microscope
- Liquid nitrogen (N_2) freezer or cryostorage container
- Sterilizer (i.e., autoclave)

Expanded equipment

- Aspiration pump (peristaltic or vacuum)
- pH meter
- Roller racks (for scaling up monolayer cultures)
- Confocal microscope
- Flow cytometer
- EG bioreactors
- Cell cubes

Additional supplies

- Cell culture vessels (e.g., flasks, Petri dishes, roller bottles, multiwell plates)
- Pipettes and pipettors
- Syringes and needles
- Waste containers
- Media, sera, and reagents
- Cells

Cell Culture Laboratory

Aseptic work area

The major requirement of a cell culture laboratory is the need to maintain an aseptic work area that is restricted to cell culture work. Although a separate tissue culture room is preferred, a designated cell culture area within a larger laboratory can still be used fort sterile handling, incubation, and storage of cell cultures, reagents, and media. The simplest and most economical way to provide aseptic conditions is to use a **cell culture hood** (i.e., biosafety cabinet).

Cell culture hood

The cell culture hood provides an aseptic work area while allowing the containment of infectious splashes or aerosols generated by many microbiological procedures. Three kinds of cell culture hoods, designated as Class I, II and III, have been developed to meet varying research and clinical needs.

Classes of cell culture hoods

Class I cell culture hoods offer significant levels of protection to laboratory personnel and to the environment when used with good microbiological techniques, but they do not provide cultures protection from contamination. They are similar in design and air flow characteristics to chemical fume hoods.

Class II cell culture hoods are designed for work involving BSL-1, 2, and 3 materials, and they also provide an aseptic environment necessary for cell culture experiments. A Class II biosafety cabinet should be used for handling potentially hazardous materials (e.g., primate-derived cultures, virally infected cultures, radioisotopes, carcinogenic or toxic reagents).

Class III biosafety cabinets are gas-tight, and they provide the highest attainable level of protection to personnel and the environment. A Class III biosafety cabinet is required for work involving known human pathogens and other BSL-4 materials.

Air-flow characteristics of cell culture hoods

Cell culture hoods protect the working enviroment from dust and other airborn contaminants by maintaining a constant, unidirectional flow of **HEPA-filtered air** over the work area. The flow can be **horizontal**, blowing parallel to the work surface, or it can be **vertical**, blowing from the top of the cabinet onto the work surface.

Depending on its design, a **horizontal flow hood** provides protection to the culture (if the air flowing towards the user) or to the user (if the air is drawn in through the front of the cabinet by negative air pressure inside). **Vertical flow hoods**, on the other hand, provide significant protection to the user and the cell culture.

Clean benches

Horizontal laminar flow or vertical laminar flow "clean benches" are **not** biosafety cabinets; these pieces of equipment discharge HEPA-filtered air from the back of the cabinet across the work surface toward the user, and they may expose the user to potentially hazardous materials. These devices only provide product protection. Clean benches can be used for certain clean activities, such as the dust-free assembly of sterile equipment or electronic devices, and they should never be used when handling cell culture materials or drug formulations, or when manipulating potentially infectious materials.

For more information on the selection, installation, and use of biosafety cabinets, refer to to *Biosafety in Microbiological and Biomedical Laboratories, 5th Edition*, which is available for downloading at **www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm**.

Cell culture hood layout

A cell culture hood should be large enough to be used by one person at a time, be easily cleanable inside and outside, have adequate lighting, and be comfortable to use without requiring awkward positions. Keep the work space in the cell culture hood clean and uncluttered, and keep everything in direct line of sight. Disinfect each item placed in the cell culture hood by spraying them with 70% ethanol and wiping clean.

The arrangement of items within the cell culture hood usually adheres to the following right-handed convention, which can be modified to include additional items used in specific applications.

- A wide, clear work space in the center with your cell culture vessels
- Pipettor in the front right and glass pipettes in the left, where they can be reached easily
- Reagents and media in the rear right to allow easy pipetting
- Small container in the rear middle to hold liquid waste

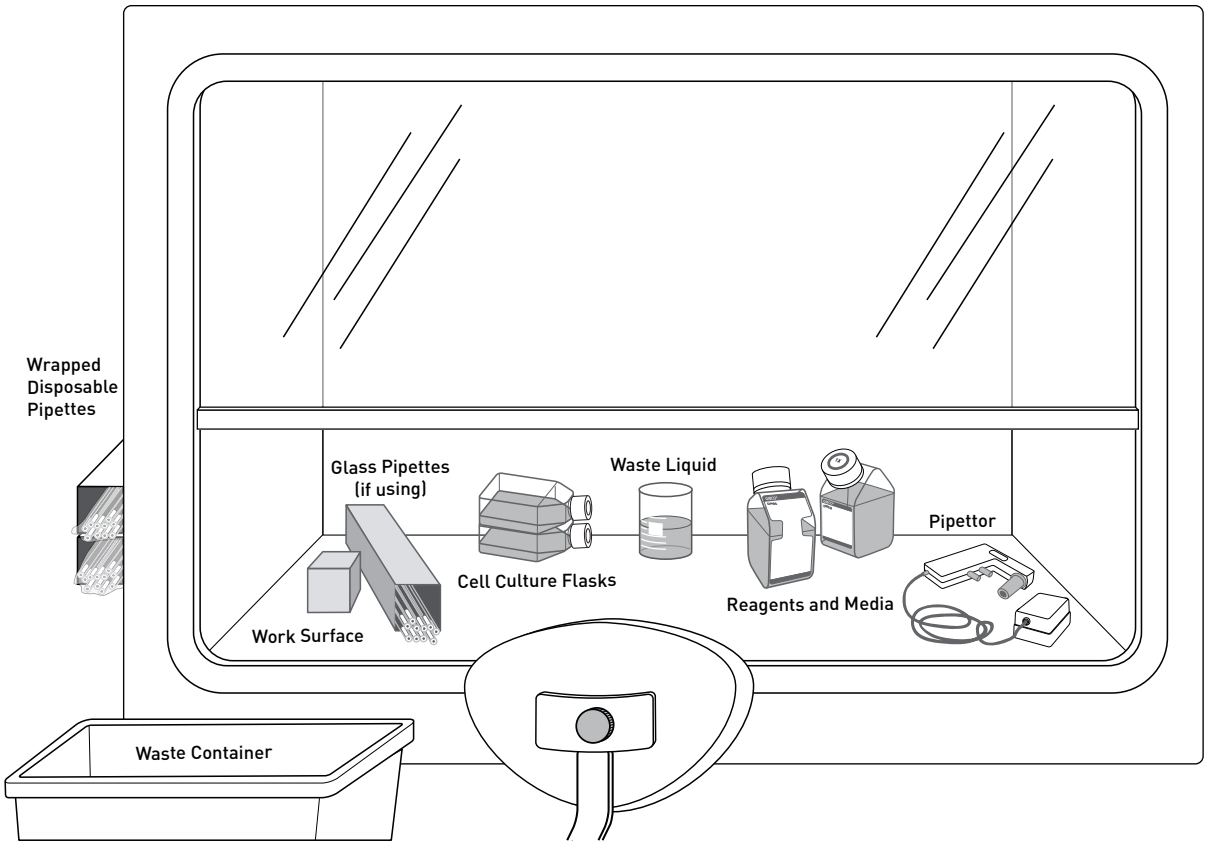


Figure 2.1 The basic layout of a cell culture hood for right-handed workers. Left-handed workers may switch the positions of the items laid out on the work surface.

Incubator

The purpose of the incubator is to provide the appropriate environment for cell growth. The incubator should be large enough, have forced-air circulation, and should have temperature control to within ±0.2°C. Stainless steel incubators allow easy cleaning and provide corrosion protection, especially if humid air is required for incubation. Although the requirement for aseptic conditions in a cell culture incubator is not as stringent as that in a cell culture hood, frequent cleaning of the incubator is essential to avoid contamination of cell cultures.

Types of incubators

There are two basic types of incubators, dry incubators and humid CO_2 incubators. **Dry incubators** are more economical, but require the cell cultures to be incubated in sealed flasks to prevent evaporation. Placing a water dish in a dry incubator can provide some humidity, but they do not allow precise control of atmospheric conditions in the incubator. **Humid CO_2 incubators** are more expensive, but allow superior control of culture conditions. They can be used to incubate cells cultured in Petri dishes or multiwell plates, which require a controlled atmosphere of high humidity and increased CO_2 tension.

Storage

A cell culture laboratory should have storage areas for liquids such as media and reagents, for chemicals such as drugs and antibiotics, for consumables such as disposable pipettes, culture vessels, and gloves, for glassware such as media bottles and glass pipettes, for specialized equipment, and for tissues and cells.

Glassware, plastics, and specilized equipment can be stored at ambient temperature on shelves and in drawers; however, it is important to **store all media, reagents, and chemicals according to the instructions on the label**.

Some media, reagents, and chemicals are sensitive to light; while their normal laboratory use under lighted conditions is tolerated, they should be stored in the dark or wrapped in aluminum foil when not in use.

Refrigerators

For small cell culture laboratories, a domestic refrigerator (preferably one without a autodefrost freezer) is an adequate and inexpensive piece of equipment for storing reagents and media at 2–8°C. For larger laboratories, a cold room restricted to cell culture is more appropriate. Make sure that the refrigerator or the cold room is cleaned regularly to avoid contamination.

Freezers

Most cell culture reagents can be stored at –5°C to –20°C; therefore an ultradeep freezer (i.e., a –80°C freezer) is optional for storing most reagents. A domestic freezer is a cheaper alternative to a laboratory freezer. While most reagents can withstand temperature oscillations in an autodefrost (i.e., self-thawing) freezer, some reagents such as antibiotics and enzymes should be stored in a freezer that does not auto-defrost.

Cryogenic storage

Cell lines in continuous culture are likely to suffer from genetic instability as their passage number increases; therefore, it is essential to prepare working stocks of the cells and preserve them in cryogenic storage (for more information, see **Freezing Cells**, page 37). Do **not** store cells in –20°C or –80°C freezers, because their viability quicky decreases when they are stored at these temperatures.

There are two main types of liquid-nitrogen storage systems, vapor phase and liquid phase, which come as wide-necked or narrow-necked storage containers. **Vapor phase** systems minimize the risk of explosion with cryostorage tubes, and are required for storing biohazardous materials, while the **liquid phase** systems usually have longer static holding times, and are therefore more economical.

Narrow-necked containers have a slower nitrogen evaporation rate and are more economical, but **wide-necked** containers allow easier access and have a larger storage capacity.

Cell counter

A cell counter is essential for quantitative growth kinetics, and a great advantage when more than two or three cell lines are cultured in the laboratory.

The Countess® II Automated Cell Counter is a benchtop instrument designed to measure cell count and viability (live, dead, and total cells) accurately and precisely in less than a minute per sample, using the standard Trypan Blue uptake technique. Using the same amount of sample that you currently use with the hemocytometer, the Countess® II Automated Cell Counter takes less than a minute per sample for a typical cell count and is compatible with a wide variety of eukaryotic cells.

Aseptic Technique

Introduction

Successful cell culture depends heavily on keeping the cells free from contamination by microorganisms such as bacterial, fungi, and viruses. Nonsterile supplies, media, and reagents, airborne particles laden with microorganisms, unclean incubators, and dirty work surfaces are all sources of biological contamination.

Aseptic technique, designed to provide a barrier between the microrganisms in the environment and the sterile cell culture, depends upon a set of procedures to reduce the probability of contamination from these sources. The elements of aseptic technique are a sterile work area, good personal hygiene, sterile reagents and media, and sterile handling.

Sterile work area

The simplest and most economical way to reduce contamination from airborne particles and aerosols (e.g., dust, spores, shed skin, sneezing) is to use a cell culture hood.

- The cell culture hood should be properly set up, and be located in an area that is restricted to cell culture that is free from drafts from doors, windows, and other equipment, and with no through traffic.
- The work surface should be uncluttered and contain only items required for a particular procedure; it should not be used as a storage area.
- Before and after use, the work surface should be disinfected thoroughly, and the surrounding areas and equipment should be cleaned routinely.
- For routine cleaning, wipe the work surface with 70% ethanol before and during work, especially after any spillage.
- Using a Bunsen burner for flaming is not necessary nor recommended in a cell culture hood.
- Leave the cell culture hood running at all times, turning them off only when they will not be used for extended periods of time.

Good personal hygiene

Wash your hands before and after working with cell cultures. In addition to protecting you from hazardous materials, wearing personal protective equipment also reduces the probability of contamination from shed skin as well as dirt and dust from your clothes.

Sterile reagents and media

Commercial reagents and media undergo strict quality control to ensure their sterility, but they can become contaminated while handling. Follow the guidelines below for sterile handling to avoid contaminating them. Always sterilize any reagents, media, or solutions prepared in the laboratory using the appropriate sterilization procedure (e.g., autoclave, sterile filter).

Sterile handling

- Always wipe your hands and your work area with 70% ethanol.
- Wipe the outside of the containers, flasks, plates, and dishes with 70% ethanol before placing them in the cell culture hood.
- Avoid pouring media and reagents directly from bottles or flasks.
- Use sterile glass or disposable plastic pipettes and a pipettor to work with liquids, and use each pipette only once to avoid cross contamination. Do not unwrap sterile pipettes until they are to be used. Keep your pipettes at your work area.
- Always cap the bottles and flasks after use and seal multi-well plates with tape or place them in resealable bags to prevent microorganisms and airborn contaminants from gaining entry.
- Never uncover a sterile flask, bottle, petri dish, etc. until the instant you are ready to use it and never leave it open to the environment. Return the cover as soon as you are finished.
- If you remove a cap or cover, and have to put it down on the work surface, place the cap with opening facing down.
- Use only sterile glassware and other equipment.
- Be careful not to talk, sing, or whistle when you are performing sterile procedures.
- Perform your experiments as rapidly as possible to minimize contamination

Aseptic Technique Checklist

The following checklist provides a concise list of suggestions and procedures to guide you to achieve a solid aseptic technique. For an in-depth review of aseptic technique, refer to *Culture of Animal Cells: A Manual of Basic Technique* (Freshney, 2000).

Work Area	
Is the cell culture hood properly set up?	
Is the cell culture hood in an area free from drafts and through traffic?	
Is the work surface uncluttered, and does it contain only items required for your experiment?	
Did you wipe the work surface with 70% ethanol before work?	
Are you routinely cleaning and sterilizing your incubators, refrigerators, freezers, and other laboratory equipment?	
Personal Hygiene	
Did you wash your hands?	
Are you wearing personal protective equipment?	
If you have long hair, is it tied in the back?	
Are you using a pipettor to work with liquids?	
Reagents and Media	
Have you sterilized any reagents, media, and solutions you have prepared in the laboratory using the appropriate procedure?	
Did you wipe the outside of the bottles, flasks, and plates with 70% ethanol before placing them on your work surface?	
Are all your bottles, flasks, and other containers capped when not in use?	
Are all your plates stored in sterile re-sealeable bags?	
Does any of your reagents look cloudy? Contaminated? Do they contain floating paticles? Have foul smell? Unusual color? If yes, did you decontaminate and discard them?	
Handling	
Are you working slowly and deliberately, mindful of aseptic technique?	
Did you wipe the surfaces of all the items, including pipettor, bottles, flasks with 70% ethanol before placing them in the cell culture hood?	
Are you placing the caps or covers face down on the work area?	
Are you using sterile glass pipettes or sterile disposable plastic pipettes to manipulate all liquids?	
Are you using a sterile pipette only once to abvoid cross contamination?	
Are you careful **not** to touch the pipette tip to anything nonsterile?	
Did you mop up any spillage immediately, and wiped the area with 70% ethanol?	

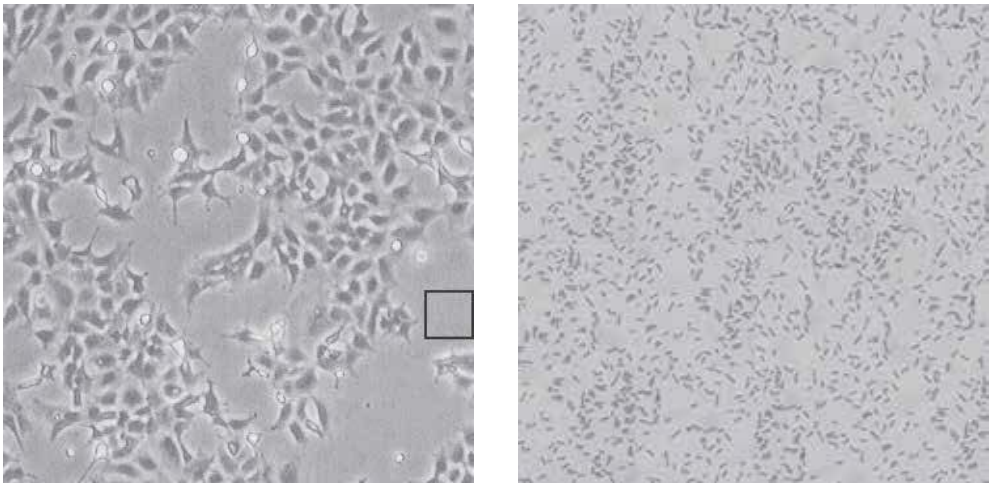
Biological Contamination

Introduction

Contamination of cell cultures is easily the most common problem encountered in cell culture laboratories, sometimes with very serious consequences. Cell culture contaminants can be divided into two main categories, **chemical contaminants** such as impurities in media, sera, and water, endotoxins, plasticizers, and detergents, and **biological contaminants** such as bacteria, molds, yeasts, viruses, mycoplasma, as well as cross contamination by other cell lines. While it is impossible to eliminate contamination entirely, it is possible to reduce its frequency and seriousness by gaining a thorough understanding of their sources and by following good aseptic technique. This section provides an overview of major types of biological contamination.

Bacteria

Bacteria are a large and ubiquitious group of unicellular microorganisms. They are typically a few micrometers in diameters, and can have a variety of shapes, ranging from spheres to rods and spirals. Because of their ubiquity, size, and fast growth rates, bacteria, along with yeasts and molds, are the most commonly encountered biological contaminants in cell culture. Bacterial contamination is easily detected by visual inspection of the culture within a few days of it becoming infected; infected cultures usually appear cloudy, sometimes with a thin film on the surface. Sudden drops in the pH of the culture medium is also a frequently encountered. Under a low-power microscope, the bacteria appear as tiny granules between the cells, and observation under a high-power microscope can resolve the shapes of individual bacteria. The simulated images below show an adherent 293 cell culture contaminated with *E. coli*.



A B

Figure 2.2 Simulated phase contrast images of adherent 293 cells contaminated with *E. coli*. The spaces between the adherent cells show tiny, shimmering granules under low power microscopy, but the individual bacteria are not easily distinguishable (panel A). Further magnification of the area enclosed by the black square resolves the individual *E. coli* cells, which are typically rod-shaped and are about 2 µm long and 0.5 µm in diameter. Each side of the black square in panel A is 100 µm.

Yeasts

Yeasts are unicellular eukaryotic microorganisms in the kingdom of Fungi, ranging in size from a few micrometers (typically) up to 40 micrometers (rarely). Like bacterial contamination, cultures contaminated with yeasts become turbid, especially if the contamination is in an advanced stage. There is very little change in the pH of the culture contaminated by yeasts until the contamination becomes heavy, at which stage the pH usually increases. Under microscopy, yeast appear as individual ovoid or spherical particles, that may bud off smaller particles. The simulated image below shows adherent 293 cell culture 24 hours after plating that is infected with yeast.

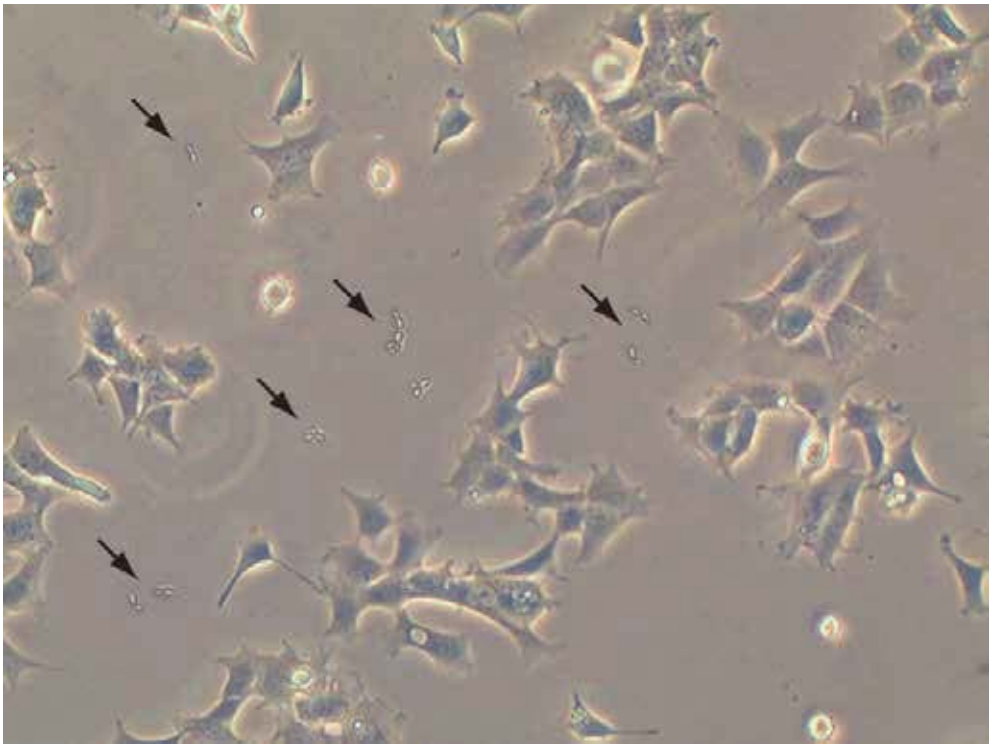


Figure 2.3 Simulated phase contrast images of 293 cells in adherent culture that is contaminated with yeast. The contaminating yeast cells appear as ovoid particles, budding off smaller particles as they replicate.

Molds

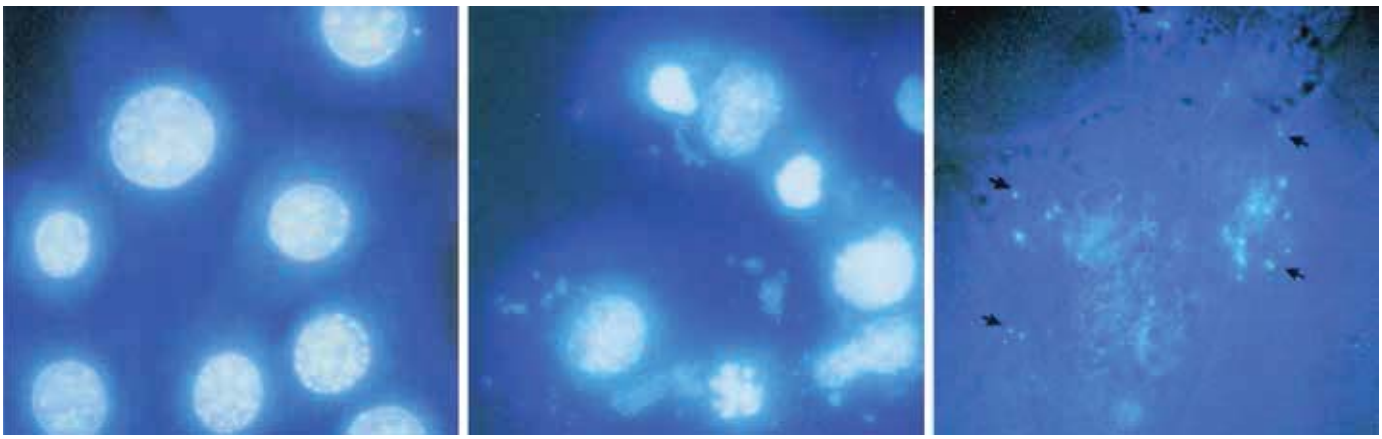
Molds are eukaryotic microorganisms in the kingdom of fungi that grow as multicellular filaments called hyphae. A connected network of these multicellular filaments contain genetically identical nuclei, and are referred to as a colony or mycelium. Similar to yeast contamination, the pH of the culture remains stable in the initial stages of contamination, then rapidly increases as the culture become more heavily infected and becomes turbid. Under microscopy, the mycelia usually appear as thin, wisp-like filaments, and sometimes as denser clumps of spores. Spores of many mold species can survive extremely harsh and inhospitable environments in their dormant stage, only to become activated when they encounter suitable growth conditions.

Viruses

Viruses are microscopic infectious agents that take over the host cells machinery to reproduce. Their extremely small size makes them very difficult to detect in culture, and to remove them from reagents used in cell culture laboratories. Because most viruses have very stringent requirements for their host, they usually do not adversely effect cell cultures from species other than their host. However, using virally infected cell cultures can present a serious health hazard to the laboratory personnel, especially if human or primate cells are cultured in the laboratory. Viral infection of cell cultures can be detected by electron microscopy, immunostaining with a panel of antibodies, ELISA assays, or PCR with appropriate viral primers.

Mycoplasma

Mycoplasma are simple bacteria that lack a cell wall, and they are considered the smallest self-replicating organism. Because of their extremely small size (typically less than one micrometer), mycoplasma are very difficult to detect until they achieve extremely high densities and cause the cell culture to deteriorate; until then, there are often no visible signs of infection. Some slow growing mycoplasma may persists in culture without causing cell death, but they can alter the behavior and metabolism of the host cells in the culture. Chronic mycoplasma infections might manifest themselves with decreased rate of cell proliferation, reduced saturation density, and agglutination in suspension cultures; however, the only assured way of detecting mycoplasma contamination is by testing the cultures periodically using fluorescent staining (e.g., Hoechst 33258), ELISA, PCR, immunostaining, autoradiography, or microbiological assays.



A B C

Figure 2.4 Photomicrographs of mycoplasma-free cultured cells (panel A) and cells infected with mycoplasma (panels B and C). The cultures were tested using the MycoFluor™ Mycoplasma Detection Kit, following the kit protocols. In fixed cells, the MycoFLuor™ reagent has access to the cell nuclei, which are intesensely stained with the reagent, but the absence of fluorescent extranuclear objects indicates that the culture is free from mycoplasma contamination (panel A**)**. In fixed cells infected with mycoplasma, the MycoFluor™ reagent stains both the nuclei and the mycoplasma, but the intense relative fluorescence of the nuclei obscure the mycoplasma on or near the nuclei. However, the mycoplasma separated from the bright nuclei are readily visible (panel B). In live cells, the MycoFluor™ reagent does not have access to the nuclei, but readily stains the mycoplasma associated with the outside of cells (panel C). The emission spectra of the MORFS are designed to have a homogeneous intensity that closely matches that of mycoplasma stained according to the MycoFluor™ mycoplasma detection protocol, allowing the researchers to discriminate between stained mycoplasma and other forms of background luminescence, including viruses, bacteria and cellular autofluorescence. The images were obtained using 365 nm excitation and a 100/1.3 Plan Neofluar objective lens coupled with a 450 ± 30 nm bandpass filter.

Cross-contamination

While not as common as microbial contamination, extensive cross-contamination of many cell lines with HeLa and other fast growing cell lines is a clearly-established problem with serious consequences. Obtaining cell lines from reputable cell banks, periodically checking the characteristics of the cell lines, and practicing good aseptic technique are practices that will help you avoid cross-contamination. DNA fingerprinting, karyotype analysis, and isotype analysis can confirm the presence or absence of cross-contamination in your cell cultures.

Using antibiotics

Antibiotics should never be used routinely in cell culture, because their continuous use encourages the development of antibiotic resistant strains and allows low-level contamination to persist, which can develop into full-scale contamination once the antibiotic is removed from media, and may hide mycoplasma infections and other cryptic contaminants. Further, some antibiotics might cross react with the cells and interfere with the cellular processes under investigation.

Antibiotics should only be used as a last resort and only for short term applications, and they should be removed from the culture as soon as possible. If they are used in the long term, antibiotic-free cultures should be maintained in parallel as a control for cryptic infections.

3. Cell Culture Basics

This section provides information on the fundamentals of cell culture, including the selection of the appropriate cell line for your experiments, media requirements for cell culture, adherent versus suspension culture, and morphologies of continuous cell lines available from Life Technologies™.

Note that the following information is an introduction to the basics of cell culture, and it is intented as a starting point in your investigations. For more in-depth information, we recommend that you consult published literature and books, as well as the manuals and product information sheets provided with the products you are using.

Cell Lines

Selecting the appropriate cell line

Consider the following criteria for selecting the appropriate cell line for your experiments:

- **Species:** Non-human and non-primate cell lines usually have fewer biosafety restrictions, but ultimately your experiments will dictate whether to use species-specific cultures or not.
- **Functional characteristics:** What is the purpose of your experiments? For example, liver- and kidney-derived cell lines may be more suitable for toxicity testing.
- **Finite or continuous:** While choosing from finite cell lines may give you more options to express the correct functions, continous cell lines are often easier to clone and maintain.
- **Normal or transformed:** Transformed cell lines usually have an increased growth rate and higher plating efficiency, are continuous, and require less serum in media, but they have undergone a permanent change in their phenotype through a genetic transformation.
- **Growth conditions and characteristics:** What are your requirements with respect to growth rate, saturation density, cloning efficiency, and the ability to grow in suspension? For example, to express a recombinant protein in high yields, you might want to choose a cell line with a fast growth rate and an ability to grow in suspension.
- **Other criteria:** If you are using a finite cell line, are there sufficient stocks available? Is the cell line well characterized, or do you have the perform the validation yourself? If you are using an abnormal cell line, do you have an equivalent normal cell line that you can use as a control? Is the cell line stable? If not, how easy it is to clone it and generate sufficient frozen stocks for your experiements?

Acquiring cell lines

You may establish your own culture from primary cells, or you may choose to buy established cell cultures from commercial or non-profit suppliers (i.e., cell banks). Reputable suppliers provide high quality cell lines that are carefully tested for their integrity and to ensure that the culture is free from contaminants. We advise against borrowing cultures from other laboratories because they carry a high risk of contamination. Regardless of their source, makes sure that all new cell lines are tested for mycoplasm contamination before you begin to use them.

Life Technologies™ offers a variety of primary cultures and established cell lines, reagents, media, sera, and growth factors for your cell culture experiments. The **Appendix** section contains a list of the more commonly used cell lines available from Life Technologies™ (see page 97). For more information on Life Technologies™ and Gibco® products, refer to **www.lifetechnologies.com**.

Culture Environment

One of the major advantages of cell culture is the ability to manipulate the **physiochemical** (i.e., temperature, pH, osmotic pressure, O_2 and CO_2 tension) and the **physiological environment** (i.e., hormone and nutrient concentrations) in which the cells propagate. With the exception of temperature, the culture environment is controlled by the growth media.

While the physiological environment of the culture is not as well defined as its physiochemical environment, a better understanding of the components of serum, the identification of the growth factors necessary for proliferation, and a better appreciation of the microenvironment of cells in culture (i.e., cell-cell interactions, diffusion of gases, interactions with the matrix) now allow the culture of certain cell lines in serum-free media.

Adherent vs. suspension culture

There are two basic systems for growing cells in culture, as monolayers on an artificial substrate (i.e., **adherent culture**) or free-floating in the culture medium (**suspension culture**). The majority of the cells derived from vertebrates, with the exception of hemopoietic cell lines and a few others, are anchorage-dependent and have to be cultured on a suitable substrate that is specifically treated to allow cell adhesion and spreading (i.e., **tissue-culture treated**). However, many cell lines can also be adapted for suspension culture. Similarly, most of the commercially available insect cell lines grow well in monolayer or suspension culture. Cells that are cultured in suspension can be maintained in culture flasks that are not tissue-culture treated, but as the culture volume to surface area is increased beyond which adequate gas exchange is hindered (usually 0.2–0.5 mL/cm^2), the medium requires agitation. This agitation is usually achieved with a magnetic stirrer or rotating spinner flasks.

Adherent Culture	Suspension Culture
Appropriate for most cell types, including primary cultures.	Appropriate for cells adapted to suspension culture and a few other cell lines that are nonadhesive (e.g., hematopoietic).
Requires periodic passaging, but allows easy visual inspection under inverted microscope.	Easier to passage, but requires daily cell counts and viability determination to follow growth patterns; culture can be diluted to stimulate growth.
Cells are dissociated enzymatically (e.g., TrypLE™ Express, trypsin) or mechanically.	Does not require enzymatic or mechanical dissocation.
Growth is limited by surface area, which may limit product yields.	Growth is limited by concentration of cells in the medium, which allows easy scale-up.
Requires tissue-culture treated vessel.	Can be maintained in culture vessels that are not tissue-culture treated, but requires agitation (i.e., shaking or stirring) for adequate gas exhange.
Used for cytology, harvesting products continuously, and many research applications.	Used for bulk production, batch harvesting, and many research applications.

Media

The culture medium is the most important component of the culture environment, because it provides the necessary nutrients, growth factors, and hormones for cell growth, as well as regulating the pH and the osmotic pressure of the culture.

Although initial cell culture experiements were performed using natural media obtained from tissue extracts and body fluids, the need for standardization and media quality, as well as an increased demand led to the development of chemically defined media. The three basic classes of media are **basal media**, **reduced-serum media**, and **serum-free media**, which differ in their requirement for supplementation with **serum**.

Serum is vitally important as a source of growth and adhesion factors, hormones, lipids and minerals for the culture of cells in basal media. In addition, serum also regulates cell membrane permeability and serves as a carrier for lipids, enzymes, micronutrients and trace elements into the cell. However, using serum in media has a number of disadvantages including high cost, problems with standardization, specificity, and variability, and unwanted effects such as stimulation or inhibition of growth and/or cellular function on certain cell cultures. If the serum is not obtained from reputable source, contamination can also pose a serious threat to successful cell culture experiments. All Life Technologies™ and Gibco® products, including sera, are tested for contamination and guaranteed for their quality, safety, consistency, and regulatory compliance.

Basal media

The majority of cell lines grow well in basal media, which contain amino acids, vitamins, inorganic salts, and a carbon source such as glucose, but these basal media formulations must be further supplemented with serum.

Reduced-serum media

Another strategy to reduce the undesired effects of serum in cell culture experiments is to use reduced-serum media. Reduced-serum media are basal media formulations enriched with nutrients and animal-derived factors, which reduce the amount of serum that is needed.

Serum-free media

Serum-free media (SFM) circumvents issues with using animal sera by replacing the serum with appropriate nutritional and hormonal formulations. Serum-free media formulations exist for many primary cultures and cell lines, including recombinant protein producing lines of Chinese Hamster Ovary (CHO), various hybridoma cell lines, the insect lines Sf9 and Sf21 (*Spodoptera frugiperda*), and for cell lines that act as hosts for viral production, such as 293, VERO, MDCK, MDBK, and others. One of the major advantages of using serum-media is the ability to make the medium selective for specific cell types by choosing the appropriate combination of growth factors. The table below lists the advantages and disadvantages of serum-free media.

Advantages	Disadvantages
• Increased definition • More consistent performance • Easier purification and downstream processing • Precise evaluation of cellular functions • Increased productivity • Better control over physiological response • Enhanced detection of cellular mediators	• Requirement for cell type-specific media formulations • Need for higher degree of reagent purity • Slower growth

Life Technologies™ offers a wide range of classical basal media, reduced-serum media, and serum-free media, as well as sera, growth factors, supplements, antibiotics, and reagents for your cell culture experiments. The **Appendix** section contains a list of the more commonly used cell culture products available from Life Technologies™. For more information on Life Technologies™ and Gibco® cell culture products, refer to **www.lifetechnologies.com**.

pH

Most normal **mammalian cell lines** grow well at pH 7.4, and there is very little variability among different cell strains. However, some transformed cell lines have been shown to grow better at slightly more acidic environments (pH 7.0–7.4), and some normal fibroblast cell lines prefer slightly more basic environments (pH 7.4–7.7). **Insect cell lines** such as Sf9 and Sf21 grow optimally at pH 6.2.

CO_2

The growth medium controls the pH of the culture and buffers the cells in culture against changes in the pH. Usually, this buffering is achieved by including an organic (e.g., HEPES) or CO_2-bicarbonate based buffer. Because the pH of the medium is dependent on the delicate balance of dissolved carbondioxide (CO_2) and bicarbonate (HCO_3^-), changes in the atmospheric CO_2 can alter the pH of the medium. Therefore, it is necessary to use exogeneous CO_2 when using media buffered with a CO_2-bicarbonate based buffer, especially if the cells are cultured in open dishes or transformed cell lines are cultured at high concentrations. While most researchers usually use 5–7% CO_2 in air, 4–10% CO_2 is common for most cell culture experiments. However, each medium has a recommended CO_2 tension and bicarbonate concentration to achieve the correct pH and osmolality; refer to the media manufacturer's instructions for more information.

Temperature

The optimal temperature for cell culture largely depends on the body temperature of the host from which the cells were isolated, and to a lesser degree on the anatomical variation in temperature (e.g., temperature of the skin may be lower than the temperature of skeletal muscle). Overheating is a more serious problem than underheating for cell cultures; therefore, often the temperture in the incubator is set slightly lower than the optimal temperature.

- Most **human and mammalian cell lines** are maintained at 36°C to 37°C for optimal growth.
- **Insect cells** are cultured at 27°C for optimal growth; they grow more slowly at lower temperatures and at temperatures between 27°C and 30°C. Above 30°C, the viability of insect cells decreases, and the cells do not recover even after they are returned to 27°C.
- **Avian cell lines** require 38.5°C for maximum growth. Although these cells can also be maintained at 37°C, they will grow more slowly.
- Cell lines derived from **cold-blooded animals** (e.g., amphibians, cold-water fish) tolerate a wide temperature range between 15°C and 26°C.

Note that cell culture conditions vary for each cell type. The consequences of deviating from the culture conditions required for a particular cell type can range from the expression of aberrant phenotypes to a complete failure of the cell culture. We therefore recommend that you familiarize yourself with your cell line of interest, and closely follow the instructions provided with each product you are using in your experiments.

Cell Morphology

Regularly examining the **morphology** of the cells in culture (i.e., their shape and appearance) is essential for successful cell culture experiments. In addition to confirming the healthy status of your cells, inspecting the cells by eye and a microscope each time they are handled will allow you to detect any signs of contamination early on and to contain it before it spreads to other cultures around the laboratory.

Signs of deterioration of cells include granularity around the nucleus, detachment of the cells from the substrate, and cytoplasmic vacuolation. Signs of deterioriation may be caused by a variety of reasons, including contamination of the culture, senescence of the cell line, or the presence of toxic substances in the medium, or they may simply imply that the culture needs a medium change. Allowing the deterioration to progress too far will make it irreversible.

Mammalian Cells

Variations in mammalian cell morphology

Most mammalian cells in culture can be divided in to three basic categories based on their morphology.

- **Fibroblastic** (or fibroblast-like) cells are bipolar or multipolar and have elongated shapes. They grow attached to a substrate.
- **Epithelial-like** cells are polygonal in shape with more regular dimensions, and grow attached to a substrate in discrete patches.
- **Lymphoblast-like** cells are spherical in shape and they are usually grown in suspension without attaching to a surface.

In addition to the basic categories listed above, certain cells display morphological characteristics specific to their specialized role in host.

- **Neuronal cells** exist in different shapes and sizes, but they can roughly be divided into two basic morphological categories, **type I** with long axons used to move signals over long distances and **type II** without axons. A typical neuron projects cellular extensions with many branches from the cell body, which is referred to as a dendritic tree. Neuronal cells can be unipolar or pseudounipolar with the dendrite and axon emerging from same process, bipolar with the axon and single dendrite on opposite ends of the soma (the central part of the cell containing the nucleus), or multipolar with more than two dendrites.

Morphology of 293 cells

The 293 cell line is a permanent line established from primary embryonic human kidney, which was transformed with sheared human adenovirus type 5 DNA. The adenoviral genes expressed in this cell line allow the cells to produce very high levels of recombinant proteins. Life Technologies™ offers several variants of the 293 cell line, including those adapted for high-density suspension culture in serum-free media. For more information, visit our mammalian cell culture pages on our website.

The phase contrast images below show the morphology of healthy 293 cells in adherent culture at 80% confluency (Figure 3.1) and in suspension culture (Figure 3.2). Note that adherent mammalian cultures should be passaged when they are in the log phase, before they reach confluence (see **When to subculture**, page 27).

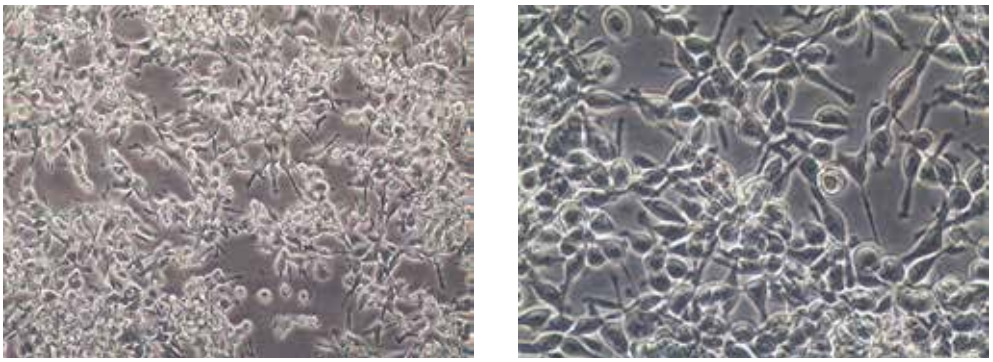


Figure 3.1 Phase contrast images of healthy 293 cells in adherent culture. The cells were plated at a seeding density of 5×10^4 viable cells/cm^2 in 293 SFM II medium and grown as a monolayer in a 37°C incubator with a humidified atmosphere of 5% CO^2 in air. The images were obtained using 10X and 20X objectives (panels A and B, respectively) 4 days after plating.

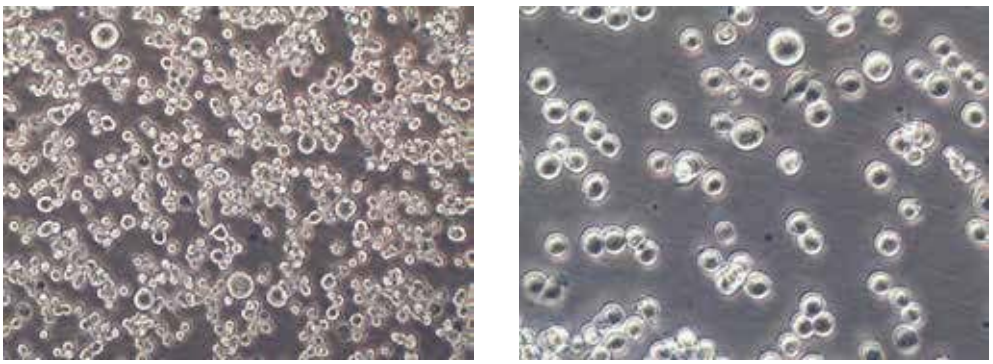


Figure 3.2 Phase contrast images of healthy 293F cells grown is suspension. The culture was started in a shake flask at a seeding density of 2×10^5 viable cells/mL in 293 SFM II medium and grown in a 37°C incubator with a humidified atmosphere of 5% CO^2 in air. 4 days after seeding, the cells were diluted 1:3, and the images were obtained using 10X and 20X objectives (panels A and B, respectively).

Insect Cells

Morphology of Sf21 cells

Sf21 cells (IPLB-Sf21-AE) are ovarian cells isolated from *Spodoptera frugiperda* (Fall Armyworm). They are spherical in shape with unequal sizes, and have a somewhat granular appearance. Sf21 cells can be thawed and used directly in suspension culture for rapid expansion of cell stocks, propagation of baculovirus stocks, and production of recombinant proteins. Because Sf21 cells attach firmly to surfaces, they can be used as a monolayer for transfection or plaque assay applications.

The images below show the morphology of healthy Sf21 insect cells in suspension culture (Figure 3.3) and in adherent culture at confluency (Figure 3.4). Note that insect cells should be subcultured when they reach confluency (see **When to Subculture**, 27).

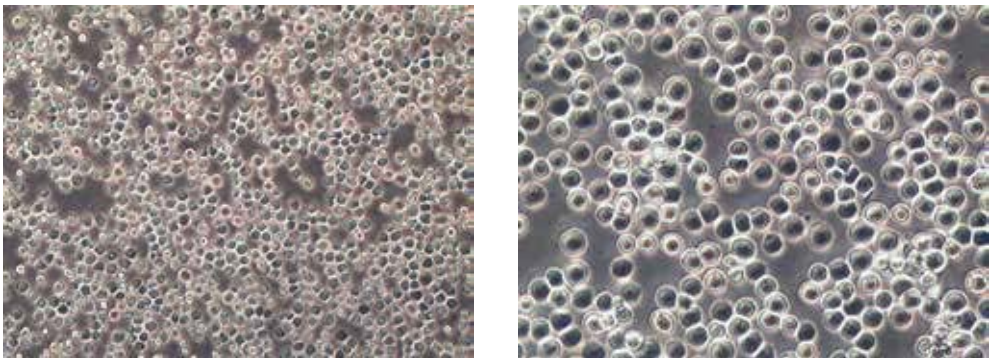


Figure 3.3 Phase contrast images of healthy Sf21 insect cells grown is suspension. The culture was started in a shake flask at a seeding density of 3×10^5 viable cells/mL in Sf-900 II SFM medium and it was maintained in a 28°C, non-humidified, ambient air-regulated incubator. The images were obtained using 10X and 20X objectives (panels A and B, respectively) 3 days after seeding.

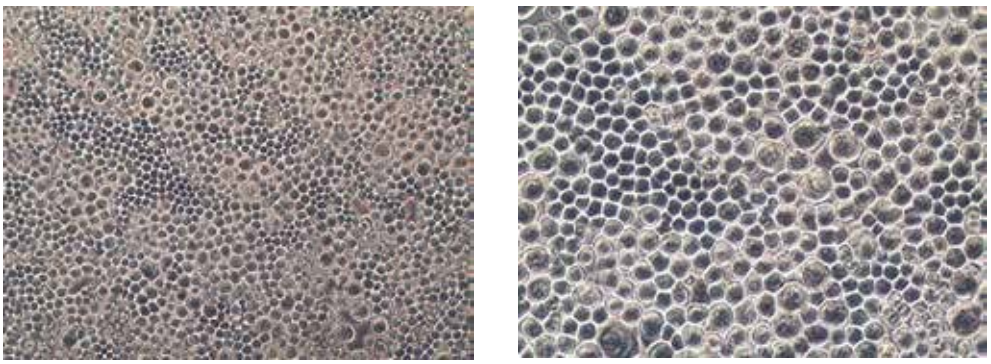
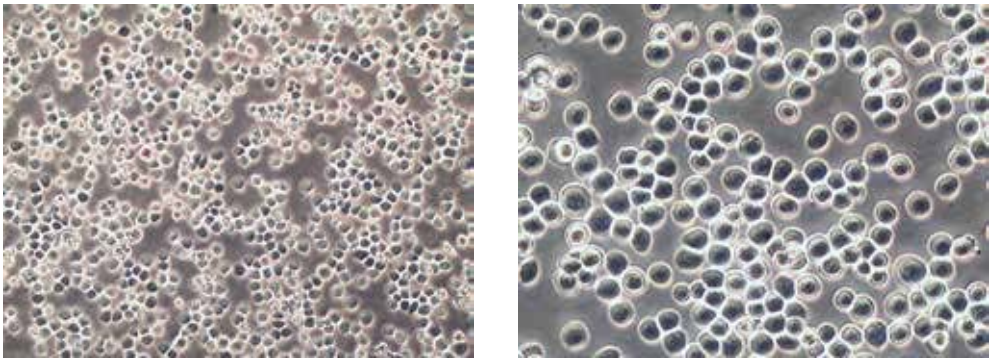


Figure 3.4 Phase contrast images of Sf21 insect cells grown as an adherent monolayer in 293 SFM II medium. The cells were plated at a seeding density of 5×10^4 viable cells/cm^2 in a T-25 flask and grown as monolayers in a 28°C, non-humidified, ambient air-regulated incubator. The images were obtained using 10X and 20X objectives (panels A and B, respectively) 7 days after seeding, when the culture had reached confluency.

Morphology of Sf9 cells

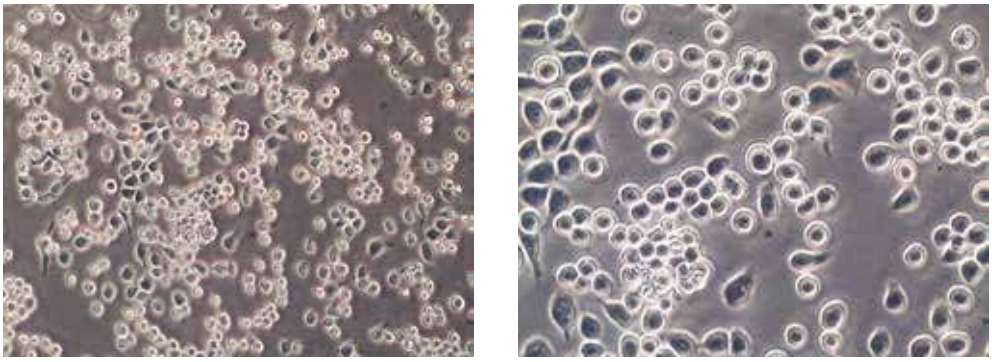
The Sf9 insect cell line is a clonal isolate derived from the parental *Spodoptera frugiperda* cell line IPLB-Sf-21-AE, and it is a suitable host for expression of recombinant proteins from baculovirus expression systems (e.g., Life Technologies™, Bac-to-Bac® and Bac-N-Blue™ Expression Systems). Although insect cells have been historically cultured in stationary systems utilizing T-flasks and serum-supplemented basal medium, insect cells are generally not anchorage dependent and can easily be maintained in suspension culture.

The images below show the morphology of healthy Sf9 insect cells in suspension and adherent cultures. Sf9 cells attach firmly to surfaces, and their small, regular size makes them exceptional for the formation of monolayers and plaques.



A B

Figure 3.5 Phase contrast images of healthy Sf9 insect cells grown is suspension. The culture was started in a shake flask at a seeding density of 3×10^5 viable cells/mL in Sf-900 II SFM medium and it was maintained in a 28°C, non-humidified, ambient air-regulated incubator. The images were obtained using 10X and 20X objectives (panels A and B, respectively) 3 days after seeding.



A B

Figure 3.6 Phase contrast images of healthy Sf9 insect cells grown is suspension. The culture was started in a shake flask at a seeding density of 3×10^5 viable cells/mL in Sf-900 II SFM medium and it was maintained in a 28°C, non-humidified, ambient air-regulated incubator. The images were obtained using 10X and 20X objectives (panels A and B, respectively) 3 days after seeding.

4. Cell Culture Methods

This section provides guidelines and general procedures for routine subculturing, thawing, and freezing of cells in culture. Note that cell culture conditions vary for each cell type. The consequences of deviating from the culture conditions required for a particular cell type can range from the expression of aberrant phenotypes to a complete failure of the cell culture. We therefore recommend that you familiarize yourself with your cell line of interest, and closely follow the instructions provided with each product you are using in your experiments.

Guidelines for Maintaining Cultured Cells

What is subculture?

Subculturing, also referred to as **passaging**, is the removal of the medium and transfer of cells from a previous culture into fresh growth medium, a procedure that enables the further propagation of the cell line or cell strain.

The growth of cells in culture proceeds from the **lag phase** following seeding to the **log phase**, where the cells proliferate exponentially. When the cells in adherent cultures occupy all the available substrate and have no room left for expansion, or when the cells in suspension cultures exceed the capacity of the medium to support further growth, cell proliferation is greatly reduced or ceases entirely (see Figure 4.1, below). To keep the culture at an optimal density for continued cell growth and to stimulate further proliferation, the culture has to be divided and fresh medium supplied.

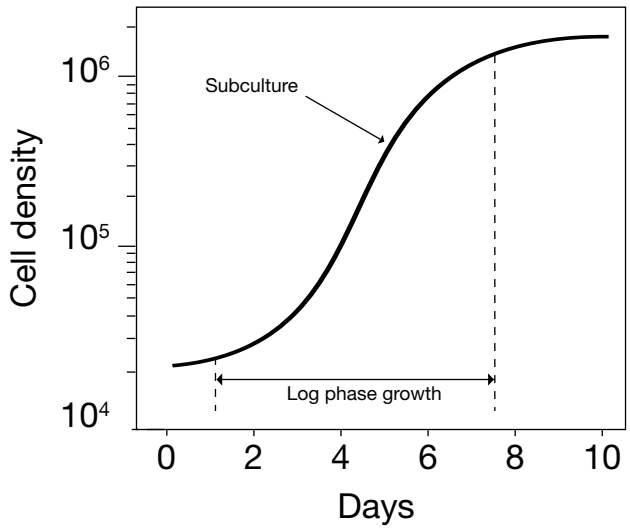


Figure 4.1 Characteristic growth pattern of cultured cells. The semi-logarithmic plot shows the cell density versus the time spent in culture. Cells in culture usually proliferate following a standard growth pattern. The first phase of growth after the culture is seeded is the lag phase, which is a period of slow growth when the cells are adapting to the culture environment and preparing for fast growth. The lag phase is followed by the log phase (i.e., "logarithmic" phase), a period where the cells proliferate exponentially and consume the nutrients in the growth medium. When all the growth medium is spent (i.e., one or more of the nutrients is depleted) or when the cells occupy all of the available substrate, the cells enter the stationary phase (i.e., plateau phase), where the proliferation is greatly reduced or ceases entirely.

When to subculture?

The criteria for determining the need for subculture are similar in adherent and suspension cultures; however, there are some differences between mammalian and insect cell lines.

Cell density

- **Mammalian cells:** Adherent cultures should be passaged when they are in the log phase, before they reach confluence. Normal cells stop growing when they reach confluence (**contact inhibition**), and it takes them longer to recover when reseeded. Transformed cells can continue proliferating even after they reach confluence, but they usually deteriorate after about two doublings. Similarly, cells in suspension should be passaged when they are in log-phase growth before they reach confluency. When they reach confluency, cells in suspension clump together and the medium appears turbid when the culture flask is swirled.
- **Insect cells:** Insect cells should be subcultured when they are in the log phase, before they reach confluency. While tightly adherent insect cells can be passaged at confluency, which allows for easier detachment from the culture vessel, insect cells that are repeatedly passaged at densities past confluency display decreased doubling times, decreased viabilities, and a decreased ability to attach. On the other hand, passaging insect cells in adherent culture before they reach confluency requires more mechanical force to dislodge them from the monolayer. When repeatedly subcultured before confluency, these cells also display decreased doubling times and decreased viabilities, and are considered unhealthy.

Exhaustion of medium

- **Mammalian cells:** A drop in the pH of the growth medium usually indicates a build up of lactic acid, which is a by-product of cellular metabolism. Lactic acid can be toxic to the cells, and the decreased pH can be sub-optimal for cell growth. The rate of change of pH is generally dependent on the cell concentration in that cultures at a high cell concentration exhaust medium faster than cells lower concentrations. You should subculture your cells if you observe a rapid drop in pH (> 0.1–0.2 pH units) with an increase in cell concentration.
- **Insect cells:** Insect cells are cultured in growth media that are usually more acidic that those used for mammalian cells. For example, TNM-FH and Grace's medium used for culturing Sf9 cells has a pH of 6.2. Unlike mammalian cell cultures, the pH rises gradually as the insect cells grow, but usually does not exceed pH 6.4. However, as with mammalian cells, the pH of the growth medium will start falling when insect cells reach higher densities.

Subculture schedule

Passaging your cells according to a strict schedule ensures reproducible behavior and allows you to monitor their health status. Vary the seeding density of your cultures until you achieve consistent growth rate and yield appropriate for your cell type from a given seeding density. Deviations from the growth patterns thus established usually indicate that the culture is unhealthy (e.g., deterioration, contamination) or a component of your culture system is not functioning properly (e.g., temperature is not optimal, culture medium too old). We strongly recommend that you keep a detailed **cell culture log**, listing the feeding and subculture schedules, types of media used, the dissociation procedure followed, split ratios, morphological observations, seeding concentrations, yields, and any anti-biotic use.

It is best to perform experiments and other non-routine procedures (e.g., changing type of media) according to your subculture schedule. If your experimental schedule does not fit the routine subculture schedule, make sure that you do not passage your cells while they are still in the lag period or when they have reached confluency and ceased growing.

Media recommendations for common cell lines

Many continuous mammalian cell lines can be maintained on a relatively simple medium such as MEM supplemented with serum, and a culture grown in MEM can probably be just as easily grown in DMEM or Medium 199. However, when a specialized function is expressed, a more complex medium may be required. Information for selecting the appropriate medium for a given cell type 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, choose the growth medium and serum empirically or test several different media for best results. In general, a good place to start is MEM for adherent cells and RPMI-1640 for suspension cells. The conditions listed below can be used as a guide line when setting up a new mammalian cell culture.

Insect cells are cultured in growth media that are usually more acidic that those used for mammalian cells such as TNM-FH and Grace's medium

Mammalian Cell Culture

Cell Line	Cell Type	Species	Tissue	Medium*
293	fibroblast	human	embryonic kidney	MEM and 10% FBS
3T6	fibroblast	mouse	embryo	DMEM, 10% FBS
A549	epithelial	human	lung carcinoma	F-12K, 10% FBS
A9	fibroblast	mouse	connective tissue	DMEM, 10% FBS
AtT-20	epithelial	mouse	pituitary tumor	F-10, 15% horse serum, and 2.5% FBS
BALB/3T3	fibroblast	mouse	embryo	DMEM, 10% FBS
BHK-21	fibroblast	hamster	kidney	GMEM, 10% FBS, or MEM, 10% FBS, and NEAA
BHL-100	epitheliall	human	breast	McCoy'5A, 10% FBS
BT	fibroblast	bovine	turbinate cells	MEM, 10% FBS, and NEAA
Caco-2	epithelial	human	colon adeno carcinoma	MEM, 20% FBS, and NEAA
Chang	epithelial	human	liver	BME, 10% calf serum
CHO-K1	epithelial	hamster	ovary	F-12, 10% FBS
Clone 9	epithelial	rat	liver	F-12K, 10% FBS
Clone M-3	epithelial	mouse	melanoma	F-10, 15% horse serum, and 2.5% FBS
COS-1, COS-3, COS-7	fibroblast	monkey	kidney	DMEM, 10% FBS
CRFK	epithelial	cat	kidney	MEM, 10% FBS, and NEAA
CV-1	fibroblast	monkey	kidney	MEM, 10% FBS
D-17	epithelial	dog	osteosarcoma	MEM, 10% FBS, and NEAA
Daudi	lymphoblast	human	blood from a lymphoma patient	RPMI-1640, 10% FBS
GH1, GH3	epithelial	rat	pituitary tumor	F-10, 15% horse serum, and 2.5% FBS

*** BME**: Basal Medium Eagle; **DMEM**: Dulbecco's Modified Eagle Medium; **FBS**: Fetal Bovine Serum; **GMEM**: Glasgow Minimum Essential Medium; **IMDM**: Iscove's Modified Dulbecco's Medium; **MEM**: Minimum Essential Medium; **NEAA**: Non-Essential Amino Acids Solution.

Mammalian Cell Culture, continued				
Cell Line	**Cell Type**	**Species**	**Tissue**	**Medium***
H9	lymphoblast	human	T-cell lymphoma	RPMI-1640, 20% FBS
HaK	epithelial	hamster	kidney	BME, 10% calf serum
HCT-15	epithelial	human	colorectal adenocarcinoma	RPMI-1640, 10% FBS
HeLa	epithelial	human	cervix carcinoma	MEM, 10% FBS, and NEAA (in suspension, S-MEM)
HEp-2	epithelial	human	larynx carcinoma	MEM, 10% FBS
HL-60	lymphoblast	human	promyeolocytic leukemia	RPMI-1640, 20% FBS
HT-1080	epithelial	human	fibrosarcoma	MEM, 10% HI FBS, and NEAA
HT-29	epithelial	human	colon adenocarcinoma	McCoy's 5A, 10% FBS
HUVEC	endothelial	human	umbilical cord	F-12K, 10% FBS, and 100 µg/mL heparin
I-10	epithelial	mouse	testicular tumor	F-10, 15% horse serum, and 2.5% FBS
IM-9	lymphoblast	human	marrow from myeloma patient	RPMI-1640, 10% FBS
JEG-2	epithelial	human	choriocarcinoma	MEM, 10% FBS
Jensen	fibroblast	rat	sarcoma	McCoy's 5A, 5% FBS
Jurkat	lyphoblast	human	lymphoma	RPMI-1640, 10% FBS
K-562	lymphoblast	human	myelogenous leukemia	RPMI-1640, 10% FBS
KB	epithelial	human	oral carcinoma	MEM, 10% FBS, and NEAA
KG-1	myeloblast	human	marrow from erythroleukemia patient	IMDM, 20% FBS
L2	epithelial	rat	lung	F-12K, 10%FBS
LLC-WRC 256	epithelial	rat	carcinoma	Medium 199, 5% horse serum
McCoy	fibroblast	mouse	unknown	MEM, 10% FBS
MCF7	epithelial	human	breast adenocarcinoma	MEM, 10% FBS, NEAA, and 10 µg/mL insulin
WI-38	epithelial	human	embryonic lung	BME, 10% FBS
WISH	epithelial	human	amnion	BME, 10% FBS
XC	epithelial	rat	sarcoma	MEM, 10% FBS, and NEAA
Y-1	epithelial	mouse	tumor of adrenal	F-10, 15% horse serum, and 2.5% FBS
Insect Cell Culture				
Sf9, Sf21	fall army worm *(Spodoptera frugiperda)*		pupal ovary	TNM-FH and 10% FBS, or Sf-900 II SFM (serum-free), or Sf-900™ III SFM (serum-free)
High Five™ (BTI-TN-5B1-4)	cabbage looper *(Trichoplusia ni)*		ovary	TNM-FH and 10% FBS, or Express Five® SFM (serum-free)
Schneider 2 (S2), D.Mel-2	fruit fly *(Drosophila melanogaster)*			Schneider's *Drosophila* medium and 10% heat-inactivated FBS

*** BME**: Basal Medium Eagle; **DMEM**: Dulbecco's Modified Eagle Medium; **FBS**: Fetal Bovine Serum; **GMEM**: Glasgow Minimum Essential Medium; **IMDM**: Iscove's Modified Dulbecco's Medium; **MEM**: Minimum Essential Medium; **NEAA**: Non-Essential Amino Acids Solution; **TNM-FH:** *Trichoplusia ni* Medium-Formulation Hink (i.e., Grace's Insect Medium, Supplemented).

Dissociating adherent cells

The first step in subculturing adherent cells is to detach them from the surface of the culture vessel by enzymatic or mechanical means. The table below lists the various cell dissociation procedures.

Procedure	Dissociation Agent	Applications
Shake-off	Gentle shaking or rocking of culture vessel, or vigorous pipetting.	Loosely adherent cells, mitotic cells
Scraping	Cell scraper	Cell lines sensitive to proteases; may damage some cells
Enzymatic dissociation	Trypsin	Strongly adherent cells
	Trypsin + collagenase	High density cultures, cultures that have formed muliple layers, especially fibroblasts
	Dispase	Detaching epidermal cells as confluent, intact sheets from the surface of culture dishes without dissociating the cells
	TrypLE™ dissociation enzyme	Strongly adherent cells; direct substitute for trypsin; applications that require animal origin-free reagents

TrypLE™ dissociation enzymes

TrypLE™ Express and TrypLE™ Select are microbially produced cell dissociation enzymes with similar kinetics and cleavage specificities to trypsin. Although TrypLE™ enzymes can directly substitute trypsin in dissociation procedures without a need for protocol changes, we recommend that you initially optimize the incubation time for dissociation for best results. Because TrypLE™ enzymes are recombinant fungal trypsin-like proteases, they are ideal for applications that require animal origin-free reagents. The table below compares TrypLE™ Express and TrypLE™ Select to trypsin.

TrypLE™ Express and TrypLE™ Select	Trypsin
Completely free of animal- and human-derived components	Porcine- or bovine-derived
Stable at room temperature for at least six months.	Not stable at room temperature.
Not inhibited by serum	Inhibited by serum
Does not require trypsin inactivators	Requires trypsin inactivators

Subculturing Adherent Cells

The following protocol describes a **general procedure for subculturing adherent mammalian cells in culture**. Note that the procedure for passaging insect cells differs from that for mammalian cells on several crucial steps. For more information, refer to **Notes on Subculturing Insect Cells**, next page.

For passaging your own cell line, we recommend that you closely follow the instructions provided with each product you are using in your experiments. The consequences of deviating from the culture conditions required for a particular cell type can range from the expression of aberrant phenotypes to a complete failure of the cell culture.

Materials needed

- Culture vessels containing your adherent cells
- Tissue-culture treated flasks, plates or dishes
- Complete growth medium, pre-warmed to 37°C
- Disposable, sterile 15-mL tubes
- 37°C incubator with humidified atmosphere of 5% CO_2
- Balanced salt solution such as Dulbecco's Phosphate Buffered Saline (DPBS), containing no calcium, magnesium, or phenol red
- Dissociation reagent such as trypsin or TrypLE™ Express, without phenol red
- Reagents and equipment to determine viable and total cell counts (e.g., Countess® II Automated Cell Counter)

Protocol for passaging adherent cells

All solutions and equipment that come in contact with the cells must be sterile. Always use proper sterile technique and work in a laminar flow hood.

1. Remove and discard the spent cell culture media from the culture vessel.
2. Wash cells using a balanced salt solution without calcium and magnesium (approximately 2 mL per 10 cm^2 culture surface area). Gently add wash solution to the side of the vessel opposite the attached cell layer to avoid disturbing the cell layer, and rock the vessel back and forth several times.

   Note: The wash step removes any traces of serum, calcium, and magnesium that would inhibit the action of the dissociation reagent.

3. Remove and discard the wash solution from the culture vessel
4. Add the pre-warmed dissociation reagent such as trypsin or TrypLE™ to the side of the flask; use enough reagent to cover the cell layer (approximately 0.5 mL per 10 cm^2). Gently rock the container to get complete coverage of the cell layer.
5. Incubate the culture vessel at room temperature for approximately 2 minutes. Note that the actual incubation time varies with the cell line used.

6. Observe the cells under the microscope for detachment. If cells are less than 90% detached, increase the incubation time a few more minutes, checking for dissociation every 30 seconds. You may also tap the vessel to expedite cell detachment.

7. When ≥ 90% of the cells have detached, tilt the vessel for a minimal length of time to allow the cells to drain. Add the equivalent of 2 volumes (twice the volume used for the dissociation reagent) of pre-warmed complete growth medium. Disperse the medium by pipetting over the cell layer surface several times.

8. Transfer the cells to a 15-mL conical tube and centrifuge then at 200 × *g* for 5 to 10 minutes. Note that the centrifuge speed and time vary based on the cell type.

9. Resuspend the cell pellet in a minimal volume of pre-warmed complete growth medium and remove a sample for counting.

10. Determine the total number of cells and percent viability using a hemocytometer, cell counter and Trypan Blue exclusion, or the Countess® II Automated Cell Counter. If necessary, add growth media to the cells to achieve the desired cell concentration and recount the cells.

11. Dilute cell suspension to the seeding density recommended for the cell line, and pipet the appropriate volume into new cell culture vessels, and return the cells to the incubator.

    Note: If using culture flasks, loosen the caps before placing them in the incubator to allow proper gas exchange unless you are using vented flasks with gas-permeable caps.

Notes on subculturing adherent insect cells

While the general procedure for subculturing insect cells follows the same steps as mammalian cells, some key requirements of these culture systems are different. For best results, always follow the instructions provided with each product you are using in your experiments.

- Passage insect cells at log phase. However, if your insect cells are strongly adherent, you may passage them at confluency or slightly after when they are starting to pull away from the bottom of the flask. Cells will be easier to dislodge.
- Densities lower than 20% confluency inhibit growth. The healthiest cells are those taken from log phase cultures.
- CO_2 exchange is not recommended for insect cell culture.
- Maintain insect cells at 27°C in a non-humidified environment. Cells can be maintained at room temperature on the bench top if protected from light or in a drawer. However, a 27°C controlled environment is recommended.
- Use media specifically formulated for insect cell growth.
- Insect cells attach very tightly to substrates under serum-free conditions and require additional effort to detach. To dislodge the cells, you may need to give the flask **one** quick shake using a wrist-snapping motion. To avoid contamination, always tighten the cap before this procedure.

  Caution: We do not recommend shaking the flask vigorously, because it may result in damage to the cells.

Subculturing Suspension Cells

The following protocols describe **general procedures for subculturing mammalian cells in suspension culture**. Note that the procedure for passaging insect cells differs from that for mammalian cells on several crucial steps. For more information, refer to **Notes on Subculturing Insect Cells**, page 36.

For passaging your own cell line, we recommend that you closely follow the instructions provided with each product you are using in your experiments. The consequences of deviating from the culture conditions required for a particular cell type can range from the expression of aberrant phenotypes to a complete failure of the cell culture.

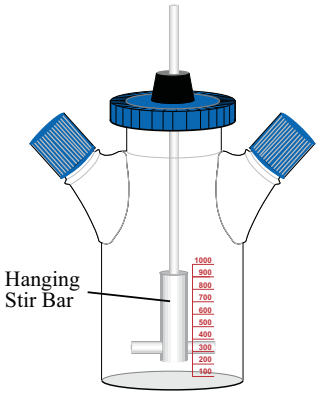
Passaging suspension cultures

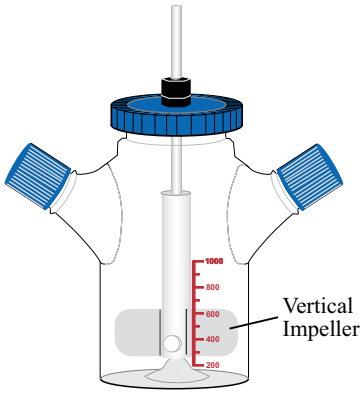
Subculturing suspension cells is somewhat less complicated than passaging adherent cells. Because the cells are already suspended in growth medium, there is no need to treat them enzymatically to detach them from the surface of the culture vessel, and the whole process is faster and less traumatic for the cells. Replacement of growth medium is not carried out in suspension cultures; instead, the cells are maintained by feeding them every 2 to 3 days until they reach confluency. This can be done by directly diluting the cells in the culture flask and continue expanding them, or by withdrawing a portion of the cells from the culture flask and diluting the remaining cells down to a seeding density appropriate for the cell line. Usually, the lag period following the passaging is shorter than that observed with adherent cultures.

Suspension culture vessels

Suspension cultures can be maintained in sterile culture flasks that are not tissue-culture treated; however, **spinner flasks** (i.e., stirrer bottles) specifically designed for suspension cell culture allow for superior gas exchange and permit higher volumes of cells to be cultured. Roller bottles rotating on a rack may also be used to agitate suspension cultures.

Spinner flasks have two basic designs; the medium is agitated (i.e., stirred) by a hanging stir-bar assembly or with a vertical impeller. The vertical impeller provides better aeration. The total culture volume in a spinner flask should not exceed half of the indicated volume of the spinner for proper aeration (e.g., a 500 mL spinner should never contain more than 250 mL of culture).





Materials needed

- Culture vessels containing your suspension cells
- Shaker flasks without baffles or spinner bottles (see **Suspension Culture Vessels**, previous page)
- Complete growth medium, pre-warmed to 37°C
- 37°C incubator with humidified atmosphere of 5% CO_2
- Magnetic stir plate (if using spinner flasks), roller rack (if using roller bottles), or shaking platform (if using conventional culture flasks or petri dishes)
- Reagents and equipment to determine viable and total cell counts (e.g., Countess® II Automated Cell Counter)

Protocol for passaging suspension cells

All solutions and equipment that come in contact with the cells must be sterile. Always use proper sterile technique and work in a laminar flow hood. Subculture cells when they are in log-phase growth before they reach confluency. When they reach confluency, cells in suspension clump together and the medium appears turbid when the culture flask is swirled. The maximum recommended cell density before passaging varies with cell lines; refer to the cell-specific product insert or manual for details.

Cells grown in shaker flasks

The following protocol describes a **general procedure** for passaging mammalian cells grown in suspension culture using shaker flasks in a shaking incubator. **For detailed protocols, always refer to the cell-specific product insert.**

Note: Make sure that the shaker flask does **not** have baffles (i.e., the indents at the bottom of the flask designed to provide agitation), because they ruin the shaking rhythm.

1. When the cells are ready for passaging (i.e., log-phase growth before they reach confluency), remove the flask from the shaking incubator, and take a small sample from the culture flask using a sterile pipette. If cells have settled down before taking the sample, swirl the flask to evenly distribute the cells in the medium.

2. From the sample, determine the total number of cells and percent viability using the Countess® II Automated Cell Counter or a hemocytometer, cell counter, and Trypan Blue exclusion.

3. Calculate the volume of media that you need to add to dilute the culture down to the recommended seeding density.

4. Aseptically add the appropriate volume of pre-warmed growth medium into the culture flask. You may split the culture to multiple flasks if needed.

5. Loosen the caps of the culture flasks one full turn to allow for proper gas exchange (or use a gas-permeable cap), and return the flasks to the shaking incubator. The shaking speed depends on the cell line.

   Note: To minimize the accumulation of cell debris and metabolic waste by-products in shaker cultures, gently centrifuge the cell suspension at 100 × *g* for 5 to 10 minutes, and resuspend the cell pellet in fresh growth medium once every three weeks (or as needed).

Cells grown in spinner flasks

The following protocol describes a **general procedure** for passaging mammalian cells in suspension grown using spinner flasks. **For detailed protocols, always refer to the cell-specific product insert.**

Note that cells are sensitive to physical shearing. Ensure that impeller mechanisms rotate freely and do not contact vessel walls or the base. The top of the paddles should be slightly above the medium to ensure adequate aeration to the culture. Adjust the spinner mechanism so that paddles clear the sides and the bottom of the vessel. The table below lists the minimum volumes of media needed for different spinner flask sizes.

Size of Spinner Flask	Minimum Media Volume
100 mL	30 mL
250 mL	80 mL
500 mL	200 mL

We do not recommend initiating a spinner culture into a spinner flask larger than 500 mL. We suggest scaling up from smaller spinners that have already been established.

1. When the cells are ready for passaging (i.e., log-phase growth before they reach confluency), remove the flask from the shaking incubator, and take a small sample from the culture flask using a sterile pipette. If cells have settled down before taking the sample, swirl the to evenly distribute the cells in the medium.

2. From the sample, determine the total number of cells and percent viability using the Countess® II Automated Cell Counter or a hemocytometer, cell counter and Trypan Blue exclusion.

3. Calculate the volume of media that you need to add to dilute the culture down to the recommended seeding density.

4. Aseptically add the appropriate volume of pre-warmed growth medium into the culture flask. You may split the culture to multiple flasks if needed.

5. Loosen the side arm caps of the spinner flasks one full turn to allow for proper gas exchange, and return the flasks to the incubator. The spinner speed depends on the cell line and the impeller type. Make sure that the spinner speed is kept within the recommended values to avoid damage to the cells from shear stress.

   Note: To minimize the accumulation of cell debris and metabolic waste by-products in spinner cultures, gently centrifuge the cell suspension at 100 × *g* for 5 to 10 minutes, and resuspend the cell pellet in fresh growth medium once every three weeks (or as needed).

Notes on subculturing suspension insect cells

While the general procedure for subculturing insect cells follows the same steps as mammalian cells, some key requirements of these culture systems are different. **For best results, always follow the instructions provided with the insect cell lines you are using in your experiments.**

- It is not necessary to change medium when you are culturing cells in suspension. Regular subculturing requires the removal of cell suspension and the addition of medium sufficient to dilute culture to the appropriate density (refer to the cell-specific product insert). Adding fresh medium is sufficient to replenish cell nutrients.
- CO_2 exchange is not recommended for insect cell culture.
- Maintain insect cells at 27°C in a non-humidified environment. Cells can be maintained at room temperature on the bench top or in a drawer, however, a 27°C controlled environment is recommended.
- Use media specifically formulated for insect cell growth.
- Use a surfactant to decrease shearing. 0.1% Pluronic® F-68 is recommended for spinner insect cultures. Pluronic® F-68 is a surfactant that decreases cell membrane shearing due to impeller forces.
  Note: Sf-900 II SFM and Express Five® SFM already contain surfactants.
- Certain insect cell lines may require adaptation to suspension culture. For more information, refer to the cell-line specific product insert or manual.

Freezing Cells

Cryopreservation

Cell lines in continuous culture are prone to genetic drift, finite cell lines are fated for senescence, all cell cultures are susceptible to microbial contamination, and even the best-run laboratories can experience equipment failure. Because an established cell line is a valuable resource and its replacement is expensive and time consuming, it is vitally important that they are frozen down and preserved for long-term storage.

As soon as a small surplus of cells becomes available from subculturing, they should be frozen as a **seed stock**, protected, and not be made available for general laboratory use. **Working stocks** can be prepared and replenished from frozen seed stocks. If the seed stocks become depleted, cryopreserved working stocks can then serve as a source for preparing a fresh seed stock with a minimum increase in generation number from the initial freezing.

The best method for cryopreserving cultured cells is storing them in liquid nitrogen in complete medium in the presence of a cryoprotective agent such as dimethylsulfoxide (DMSO). Cryoprotective agents reduce the freezing point of the medium and also allow a slower cooling rate, greatly reducing the risk of ice crystal formation, which can damage cells and cause cell death.

Note: DMSO is known to facilitate the entry of organic molecules into tissues. Handle reagents containing DMSO using equipment and practices appropriate for the hazards posed by such materials. Dispose of the reagents in compliance with local regulations.

Guidelines for cryopreservation

Following the guidelines below is essential for cryopreserving your cell lines for future use. **As with other cell culture procedures, we recommend that you closely follow the instructions provided with your cell line for best results.**

- Freeze your cultured cells at a high concentration and at as low a passage number as possible. Make sure that the cells are at least 90% viable before freezing. Note that the optimal freezing conditions depend on the cell line in use.
- Freeze the cells slowly by reducing the temperature at approximately 1°C per minute using a controlled rate cryo-freezer or a cryo-freezing container (e.g., "Mr. Frosty," available from NALGENE® labware)
- Always use the recommended freezing medium. The freezing medium should contain a cryoprotective agent such as DMSO or glycerol (see **What is Subculture?**, page 26).
- Store the frozen cell below –70°C; frozen cells begin to deteriorate above –50°C.
- Always use sterile cryovials for storing frozen cells. Cryovials containing the frozen cells may be stored immersed in liquid nitrogen or in the gas phase above the liquid nitrogen (see **Safety Note**, page 38).
- Always wear personal protective equipment.
- All solutions and equipment that come in contact with the cells must be sterile. Always use proper sterile technique and work in a laminar flow hood.

Safety note

Biohazardous materials **must** be stored in the gas phase above the liquid nitrogen. Storing the sealed cryovials in the gas phase eliminates the risk of explosion. If you are using liquid-phase storage, be aware of the explosion hazard with both glass and plastic cryovials, and always wear a face shield or goggles.

Freezing medium

Always use the recommended freezing medium for cryopreserving your cells. The freezing medium should contain a cryoprotective agent such as DMSO or glycerol. You may also use a specially formulated complete cryopreservation medium such as Recovery™ Cell Culture Freezing Medium or Synth-a-Freeze® Cryopreservation Medium.

Recovery™ Cell Culture Freezing Medium is a ready-to-use complete cryopreservation medium for mammalian cell cultures, containing an optimized ratio of fetal bovine serum to bovine serum for improved cell viability and cell recovery after thawing.

Synth-a-Freeze® Cryopreservation Medium is a chemically defined, protein-free, sterile cryopreservation medium containing 10% DMSO that is suitable for the cryopreservation of many stem and primary cell types, with the exception of melanocytes.

Materials needed

- Culture vessels containing cultured cells in log-phase of growth
- Complete growth medium
- Cryoprotective agent such as DMSO (use a bottle set aside for cell culture; open only in a laminar flow hood) or a freezing medium such as Synth-a-Freeze® Cryopreservation Medium or Recovery™ Cell Culture Freezing Medium
- Disposable, sterile 15-mL or 50-mL conical tubes
- Reagents and equipment to determine viable and total cell counts (e.g., Countess® II Automated Cell Counter)
- Sterile cryogenic storage vials (i.e., cryovials)
- Controlled rate freezing apparatus or isopropanol chamber
- Liquid nitrogen storage container

For freezing adherent cells, in addition to the above materials, you need:

- Balanced salt solution such as Dulbecco's Phosphate Buffered Saline (DPBS), containing no calcium, magnesium, or phenol red
- Dissociation reagent such as trypsin or TrypLE™ Express, without phenol red

Cryopreserving cultured cells

The following protocol describes a **general procedure** for cryopreserving cultured cells. **For detailed protocols, always refer to the cell-specific product insert.**

1. Prepare freezing medium and store at 2°C to 8°C until use. Note that the appropriate freezing medium depends on the cell line.

2. For adherent cells, gently detach cells from the tissue culture vessel following the procedure used during the subculture. Resuspend the cells in complete medium required for that cell type.

3. Determine the total number of cells and percent viability using a hemocytometer, cell counter and Trypan Blue exclusion, or the Countess® II Automated Cell Counter. According to the desired viable cell density, calculate the required volume of freezing medium.

4. Centrifuge the cell suspension at approximately 100–200 × *g* for 5 to 10 minutes Aseptically decant supernatant without disturbing the cell pellet.

   Note: Centrifugation speed and duration varies depending on the cell type.

5. Resuspend the cell pellet in cold freezing medium at the recommended viable cell density for the specific cell type.

6. Dispense aliquots of the cell suspension into cryogenic storage vials. As you aliquot them, frequently and gently mix the cells to maintain a homogeneous cell suspension.

7. Freeze the cells in a controlled rate freezing apparatus, decreasing the temperature approximately 1°C per minute. Alternatively, place the cyrovials containing the cells in an isopropanol chamber and store them at –80°C overnight.

8. Transfer frozen cells to liquid nitrogen, and store them in the gas phase above the liquid nitrogen.

Thawing Frozen Cells

Guidelines for thawing

The thawing procedure is stressful to frozen cells, and using good technique and working quickly ensures that a high proportion of the cells survive the procedure. As with other cell culture procedures, we recommend that you closely follow the instructions provided with your cells and other reagents for best results.

- Thaw frozen cells rapidly (< 1 minute) in a 37°C water bath.
- Dilute the thawed cells slowly, using pre-warmed growth medium.
- Plate thawed cells at high density to optimize recovery.
- Always use proper aseptic technique and work in a laminar flow hood.
- Always wear personal protective equipment, including a face mask or goggles. Cryovials stored in liquid-phase present a risk of explosion when thawed.
- Some freezing media contain DMSO, which is known to facilitate the entry of organic molecules into tissues. Handle reagents containing DMSO using equipment and practices appropriate for the hazards posed by such materials.

Materials needed

- Cryovial containing frozen cells
- Complete growth medium, pre-warmed to 37°C
- Disposable, sterile centrifuge tubes
- Water bath at 37°C
- 70% ethanol
- Tissue-culture treated flasks, plates, or dishes

Thawing frozen cells

The following protocol describes a **general procedure** for thawing cryopreserved cells. **For detailed protocols, always refer to the cell-specific product insert.**

1. Remove the cryovial containing the frozen cells from liquid nitrogen storage and **immediately** place it into a 37°C water bath.
2. Quickly thaw the cells (< 1 minute) by gently swirling the vial in the 37°C water bath until there is just a small bit of ice left in the vial.
3. Transfer the vial it into a laminar flow hood. Before opening, wipe the outside of the vial with 70% ethanol.
4. Transfer the thawed cells **dropwise** into the centrifuge tube containing the desired amount of pre-warmed complete growth medium appropriate for your cell line.
5. Centrifuge the cell suspension at approximately 200 × *g* for 5–10 minutes. The actual centrifugation speed and duration varies depending on the cell type.
6. After the centrifugation, check the clarity of supernatant and visibility of a complete pellet. Aseptically decant the supernatant without disturbing the cell pellet.
7. Gently resuspend the cells in complete growth medium, and transfer them into the appropriate culture vessel and into the recommended culture environment.

   Note: The appropriate flask size depends on the number of cells frozen in the cryovial, and the culture environment varies based on the cell and media type.

5. Transfection Basics

This chapter provides an overview of transfection, including general information on various transfection technologies and selecting the appropriate transfection method for your cell line and experimental needs. Guidelines for transfection of cells with DNA and RNA, considerations for successful transfection experiments, and experimental workflows are provided in the **Transfection Methods** chapter, starting on page 69.

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 (see **Types of Transfection**, page 43).

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.

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.

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 of high quality and relatively pure (see **Factors Influencing Transfection Efficiency**, page 69). 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**, page 90).

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 (see **Selective Screening**, page 62).

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**, page 69). 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**, page 89). However, RNA molecules by themselves cannot be used for stable transfection.

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 Expi293F™ cells 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. For more information on the Expi293™ Expression System, go to **www.lifetechnologies.com/expi293**.

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.

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.

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.

Transfection technologies available today can be broadly classified into three groups: **chemical methods** that use carrier molecules to neutralize or impart a positive charge to the negatively charged nucleic acids, **biological methods** that rely on genetically engineered viruses to transfer non-viral genes into cells (also known as transduction), and **physical methods** that directly deliver nucleic acids into the cytoplasm or the nucleus of the cell. However, 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 gene delivery methods

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 proteins • 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 interefere 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—$CaPO_4$ 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
DEAE-dextran	• Relatively simple technique • Reproducible results • Inexpensive	• Chemical cytoxicity in some cell types • Limited to transient transfection • Low transfection efficiency, especially in primary cells
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

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 expresssion (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)
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 applications	• 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
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

Cationic lipid-mediated delivery

Cationic lipid-mediated transfection is one of the most popular methods for introducing foreign genetic material into cells. Although first generation of lipid-based transfection reagents relied on artificial liposomes that could envelop nucleic acids and then fuse with the cell membrane to deposit their cargo inside (Fraley *et al.*, 1980), newer cationic lipid-based reagents spontaneously form condensed nucleic acid-cationic lipid reagent complexes via electrostatic interactions between the negatively charged nucleic acid and the positively charged head group of the synthetic lipid reagent. These complexes are believed to be taken up by the cell through endocytosis and then released in the cytoplasm. Once in the cell, transfected DNA is translocated to the nucleus to be expressed by a yet unknown mechanism, while RNA or antisense oligonucleotides skip the translocation step and remain in the cytoplasm (see **Cationic Lipid-Mediated Transfection**, page 58).

The advantages of cationic lipid-mediated transfection are the ability to transfect a broad range of cell lines with high efficiency, its applicability to high-throughput screens, and the ability to deliver DNA of all sizes, as well as RNA and proteins. In addition, this method can be applied to both stable and transient expression, and unlike other chemical methods, it can be used for *in vivo* transfer of DNA and RNA to animals and humans. The main drawback of cationic lipid-mediated transfection is the dependence of transfection efficiency on the cell type and culture conditions, requiring the optimization of transfection conditions for each cell type and transfection reagent (see **Considerations for Cationic Lipid-Mediated Transfection**, page 80).

Life Technologies™ offers a wide range of cationic lipid-mediated transfection reagents for efficiently introducing DNA, RNA, siRNA, or oligonucleotides into a broad range of cell types, including the Lipofectamine® 3000 reagent. The Lipofectamine® 3000 reagent leverages the most advanced lipid nanoparticle technology to enable superior transfection efficiency and reproducible results in a broad spectrum of difficult-to-transfect cell types with improved viability (**www.lifetechnologies.com/3000**). For more information on selecting the appropriate transfection reagent for your application, see **Cationic lipid transfection reagents**, page 56.

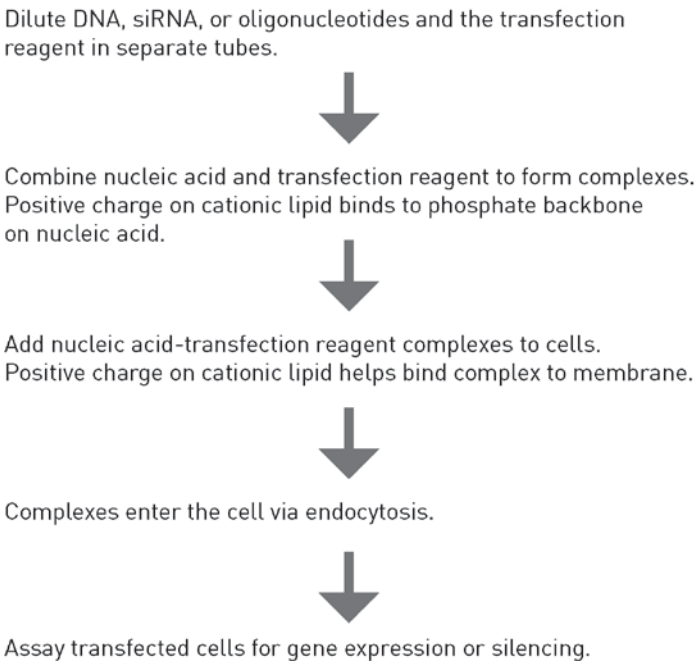


Figure 5.1 Cationic lipid-mediated transfection workflow.

Calcium phosphate co-precipitation

Calcium phosphate co-precipitation has been a popular transfection method since its introduction in the early 1970s (Graham and van der Eb, 1973) because the components it requires are easily available and inexpensive. Furthermore, the technique is easy to master, it is effective with many types of cultured cells, and it can be used for both transient and stable transfection of a variety of cultured cell types. However, calcium phosphate co-precipitation is prone to variability due to its sensitivity to slight changes in pH, temperature, and buffer salt concentrations, and can be cytotoxic to many types of cell cultures, especially of primary cells. In addition, it is unsuitable for *in vivo* transfer of nucleic acids to whole animals, and it shows relatively poor transfection efficiency compared to other chemical transfection methods such as lipid-mediated transfection.

The principle of calcium phosphate co-precipitation involves mixing DNA with calcium chloride in a buffered saline/phosphate solution to generate a calcium-phosphate–DNA co-precipitate, which is then dispersed onto cultured cells. Calcium phosphate facilitates the binding of the condensed DNA in the co-precipitate to the cell surface, and the DNA enters the cell by endocytosis. Aeration of the phosphate buffer while adding the DNA-calcium chloride solution helps to ensure that the precipitate that forms is as fine as possible, which is important because clumped DNA will not adhere to or enter the cell as efficiently.

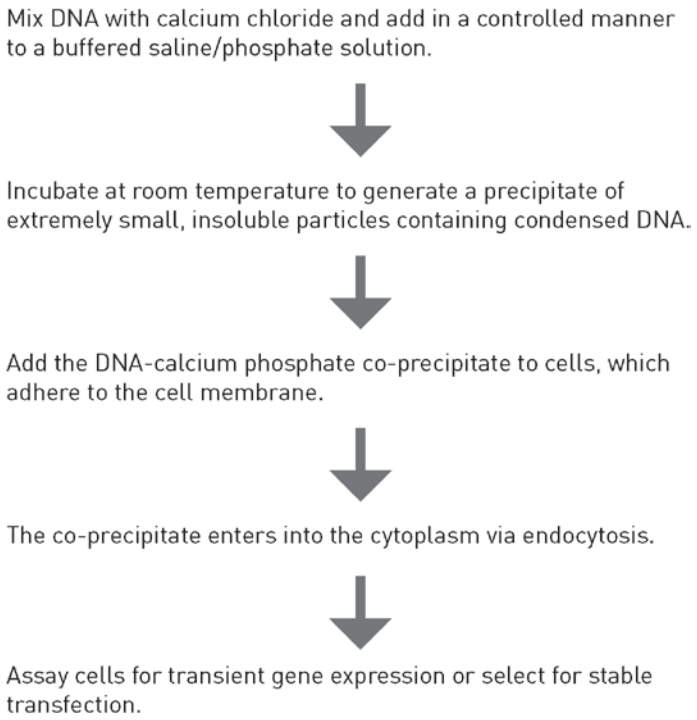


Figure 5.2 Calcium-phosphate co-precipitation workflow.

DEAE-Dextran-mediated delivery

Diethylaminoethyl (DEAE)-dextran is a polycationic derivative of the carbohydrate polymer dextran, and it is one of the first chemical reagents used to transfer nucleic acids into cultured mammalian cells (Vaheri and Pagano, 1965). The cationic DEAE-dextran molecule tightly associates with the negatively charged backbone of the nucleic acid, and the net positive charge of the resulting nucleic acid-DEAE-dextran complex allows it to adhere to the cell membrane and enter into the cytoplasm via endocytosis or osmotic shock induced by DMSO or glycerol.

The advantages of DEAE-dextran method are its relative simplicity, reproducibility, and low cost, while its disadvantages include cytotoxicity and low transfection efficiency for a range of cell types (typically less than 10% in primary cells), as well as the requirement for reduced serum media during the transfection procedure. In addition, this method is limited to transient transfections, and is not suitable for generating stable cell lines.

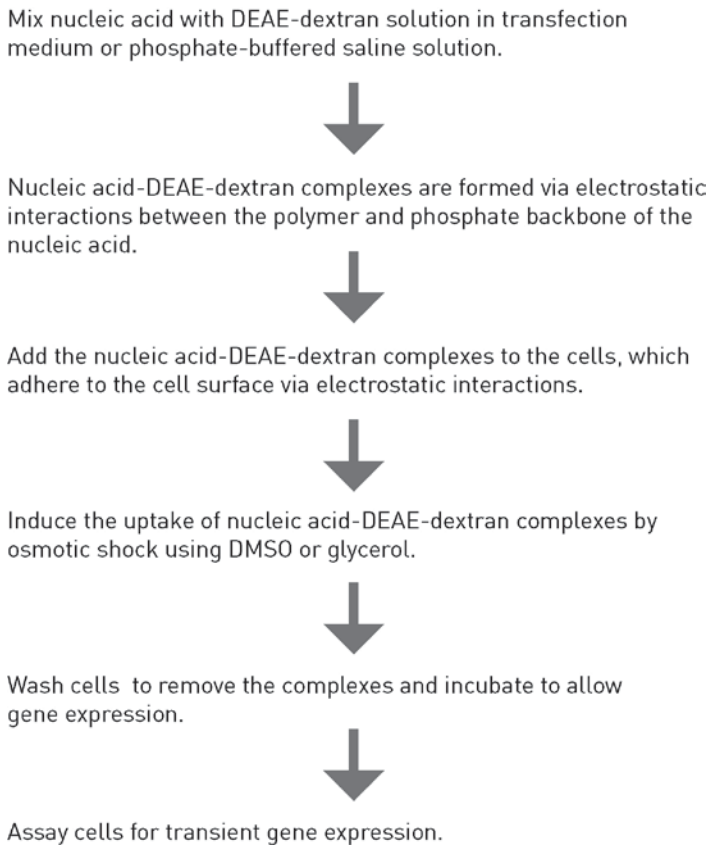


Figure 5.3 DEAE-dextran-mediated transfection workflow.

Delivery by other cationic polymers

Other cationic polymers used for gene delivery include **cationic peptides** and their derivatives (e.g., polylysine, polyornithine), linear or branched **synthetic polymers** (e.g., polybrene, polyethyleneimine), **polysaccharide-based delivery molecules** (e.g., cyclodextrin, chitosan), **natural polymers** (e.g., histone, collagen), and activated and non-activated **dendrimers**.

Cationic polymers differ from cationic lipids in that they do not contain a hydrophobic moiety and are completely soluble in water. Although they differ dramatically in their degree of transfection efficiency and cytotoxicity, all cationic polymers work in a similar fashion by allowing the formation of nucleic acid-polymer complexes, which adhere to the cell membrane through electrostatic interactions and are taken up by the cell via endocytosis. The efficiency of uptake can be improved by conjugating cell-targeting ligands or nuclear localization signals onto the polymer.

While cationic polymers can offer increased complex stability, more reproducible results, and higher transfection efficiencies when compared to DEAE-dextran, their main limitations continue to be cytotoxicity and their limitation to transient transfection studies. While higher molecular weight (MW) cationic polymers tend to be non-biodegradable and more cytotoxic than lower MW polymers, they show higher transfection efficiencies due to their increased polymer-to nucleic acid-charge ratio. However, the higher toxicity of larger MW polymers can be reduced by biodegradable cross-linking of small polymers into larger polymeric structures.

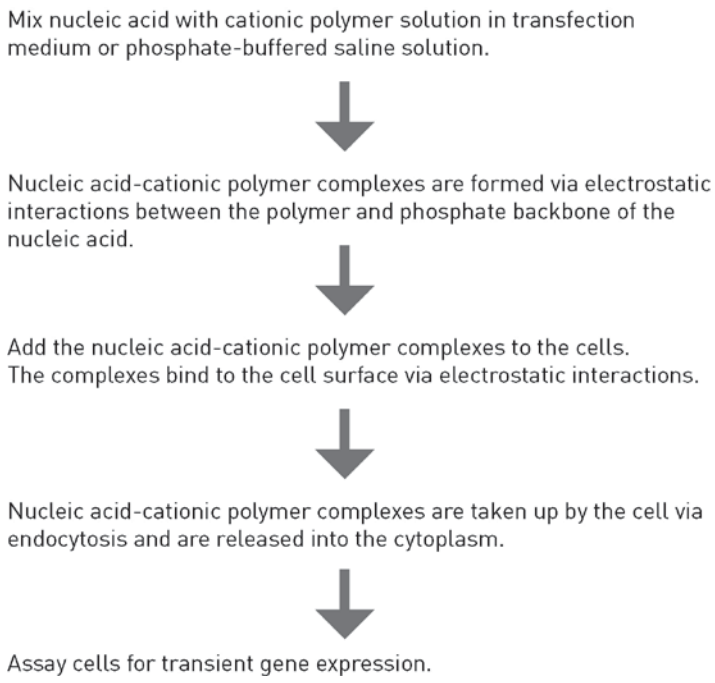


Figure 5.4 Cationic polymer-mediated transfection workflow.

Viral delivery

For cell types not amenable to lipid-mediated transfection, viral vectors are often employed. Virus-mediated transfection, also known as transduction, offers a means to reach hard-to-transfect cell types for protein overexpression or knockdown, and it is the most commonly used method in clinical research (Glover *et al*., 2005; Pfeifer and Verma, 2001). Adenoviral, oncoretroviral, and lentiviral vectors have been used extensively for gene delivery in mammalian cell culture and *in vivo*. Other well-known examples for viral gene transfer include baculovirus and vaccinia virus-based vectors. For the more information on various viral delivery systems, see **Virus-Mediated Gene Transfer**, page 58.

While viruses are the preferred system for gene delivery in clinical trials owing to their high *in vivo* transfection efficiency and sustained gene expression due to their integration into the host genome, they have a number of drawbacks including their immunogenicity and cytotoxicity, technically challenging and laborious production procedures for vectors, high costs due to biosafety requirements, low packaging capacity (~10 kb for most viral vectors compared to ~100 kb for non-viral vectors), and variability in the infectivity of viral vector preparations (Glover *et al*., 2005; Kim and Eberwine, 2010; Vorburger and Hunt, 2002).

A typical transduction protocol involves engineering of the recombinant virus carrying the transgene, amplification of recombinant viral particles in a packaging cell line, purification and titration of amplified viral particles, and subsequent infection of the cells of interest. While the achieved transduction efficiencies in primary cells and cell lines are quite high (~90–100%), only cells carrying the viral-specific receptor can be infected by the virus. It is also important to note that the packaging cell line used for viral amplification needs to be transfected with a non-viral transfection method.

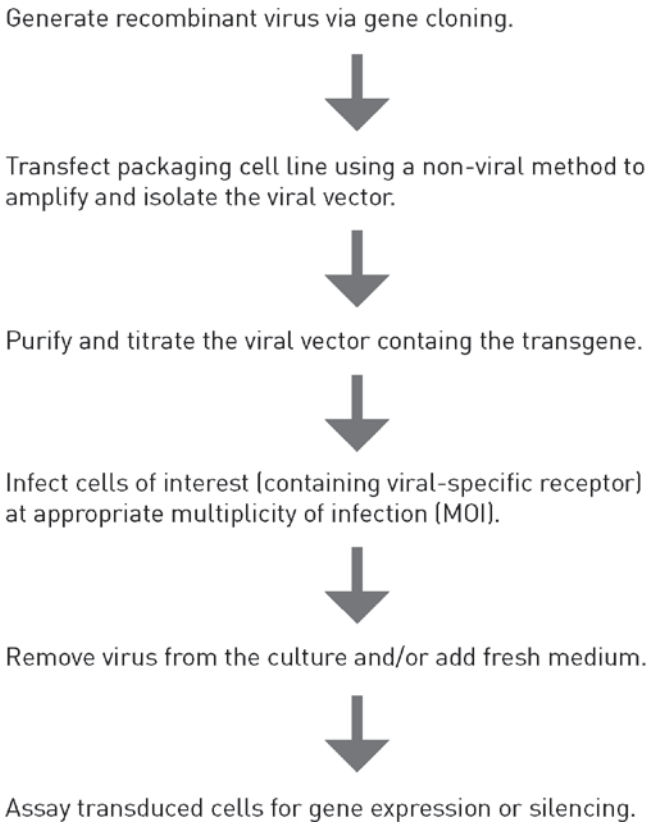


Figure 5.5 Viral delivery workflow.

Electroporation

Electroporation is a physical transfection method that uses an electrical pulse to create temporary pores in cell membranes through which substances like nucleic acids can pass into cells. It is a highly efficient strategy for the introduction of foreign nucleic acids into many cell types, including bacteria and mammalian cells.

Electroporation is based on a simple process. Host cells and selected molecules are suspended in a conductive solution, and an electrical circuit is closed around the mixture. An electrical pulse at an optimized voltage and only lasting a few microseconds to a millisecond is discharged through the cell suspension. This disturbs the phospholipid bilayer of the membrane and results in the formation of temporary pores. The electric potential across the cell membrane simultaneously rises to allow charged molecules like DNA to be driven across the membrane through the pores in a manner similar to electrophoresis (Shigekawa and Dower, 1988).

The main advantage of electroporation is its applicability for transient and stable transfection of all cell types. Furthermore, because electroporation is easy and rapid, it is able to transfect a large number of cells in a short time once optimum electroporation conditions are determined. The major drawback of electroporation is substantial cell death caused by high voltage pulses and only partially successful membrane repair, requiring the use of greater quantities of cells compared to chemical transfection methods. While more modern instrumentation, such as the Neon® Transfection System offered by Life Technologies™, overcome high cell mortality by distributing the electrical pulse equally among the cells and maintaining a stable pH throughout the electroporation chamber, optimization of pulse and field strength parameters is still required to balance the electroporation efficiency and cell viability (see **Neon® Transfection System**, page 60).

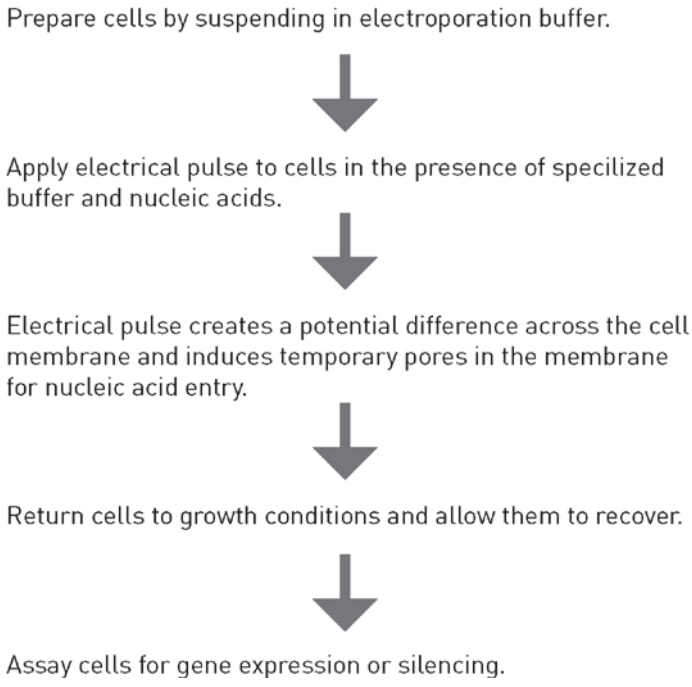


Figure 5.6 Electroporation workflow.

Other physical delivery methods

Physical gene delivery methods other than electroporation include **biolistic particle delivery**, **direct microinjection**, and **laser-mediated transfection**. Although these physical methods differ in the tools they employ, they all enable the direct transfer of nucleic acids into the cytoplasm or the nucleus by membrane penetration without using chemicals or viruses.

In brief, **biolistic particle delivery**, also known as particle bombardment, involves projecting microscopic heavy-metal particles (often gold or tungsten) coated with nucleic acids into recipient cells at high velocity using a ballistic device (i.e., "gene gun"). Biolistic particle delivery can be used to transiently transfect dividing and non-dividing cells in culture as well as cells *in vivo*, and it is often used for genetic vaccination and agriculture applications (Klein *et al*., 1992; Ye *et al*., 1990; Burkholder *et al*., 1993). While this technique is reliable and fast, it requires costly equipment, causes physical damage to the samples, and necessitates high cell numbers due to high mortality.

Direct microinjection delivers nucleic acids into the cytoplasm or the nucleus one cell at a time by means of a fine needle; therefore, this method is limited to *ex vivo* applications such as the transfer of genes into oocytes to engineer transgenic animals or the delivery of artificial chromosomes (Cappechi, 1980; Cappechi, 1989; Telenius *et al*., 1999). Although direct microinjection is nearly 100% efficient, it demands considerable technical skill, is extremely labor-intensive, and often causes cell death. As such, this method is not appropriate for studies that require the transfection of large number of cells.

Laser-mediated transfection, also known as phototransfection, laserfection, or optoporation, uses a laser pulse to transiently permeabilize the cell membrane (Shirahata *et al*., 2001; Schneckenburger *et al*., 2002). When the laser induces a pore in the membrane, the osmotic difference between the medium and the cytosol facilitates the entry of nucleic acids or other desired substances in the medium (ions, small molecules, proteins, semiconductor nanocrystals, etc.) into the cell. Advantages of laser-mediated transfection include high transfection efficiency and the ability to make pores at any location on the cell. However, the method requires an expensive laser-microscope system and the cells to be attached to a substrate.

In addition to the methods mentioned above, other physical delivery technologies use hydrodynamic pressure, ultrasound, or magnetic field to drive naked nucleic acids or nucleic acid-particle complexes into recipient cells.

Cationic Lipid-Mediated Transfection

Mechanism

Specially designed cationic lipids, such as the Lipofectamine® Transfection Reagents, facilitate DNA and siRNA delivery into cells (Chesnoy and Huang, 2000; Hirko *et al.*, 2003; Liu *et al.*, 2003). The basic structure of cationic lipids consists of a positively charged head group and one or two hydrocarbon chains. The charged head group governs the interaction between the lipid and the phosphate backbone of the nucleic acid, and facilitates DNA condensation. Often, cationic lipids are formulated with a neutral co-lipid or helper lipid, followed by extrusion or microfluidization, which results in a unilamellar liposomal structure with a positive surface charge when in water.

The positive surface charge of the liposomes mediates the interaction of the nucleic acid and the cell membrane, allowing for fusion of the liposome/nucleic acid **transfection complex** with the negatively charged cell membrane. The transfection complex is thought to enter the cell through endocytosis. Endocytosis is the process where a localized region of the cellular membrane uptakes the DNA:liposome complex by forming a membrane bound/intracellular vesicle. Once inside the cell, the complex must escape the endosomal pathway, diffuse through the cytoplasm, and enter the nucleus for gene expression. Cationic lipids are thought to facilitate transfection during the early steps of the process by mediating DNA condensation and DNA/cellular interactions.

The principle of delivery using cationic lipid reagents thus differs from prior attempts to use neutral liposomes for transfections. With cationic lipid reagents, the DNA solution is not deliberately encapsulated within the liposomes; rather, the negatively charged DNA binds spontaneously to the positively charged liposomes, forming DNA-cationic lipid reagent complexes.

Some of the problems associated with traditional transfection methods like calcium phosphate co-precipitation, DEAE-dextran, polybrene, and electroporation include low efficiency of DNA delivery, poor reproducibility, cell toxicity, and inconvenience. In contrast, cationic lipid reagent-mediated transfection yields high and previously unattainable transfection efficiencies in a wide variety of eukaryotic cells. It is simple to perform, and ensures consistently reproducible results. Moreover, a number of cell lines normally resistant to transfection by other methods transfect successfully with cationic lipid reagents.

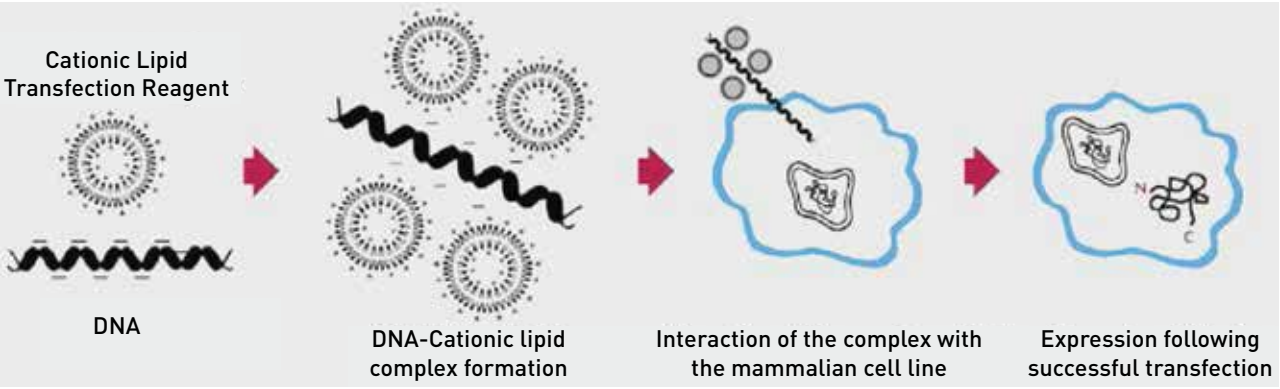


Figure 5.7 Mechanism of cationic lipid-mediated delivery.

Cationic lipid transfection reagents

Cationic lipid-mediated delivery is a fast, simple, and reproducible means for easily introducing DNA, RNA, siRNA, or oligonucleotides into eukaryotic cells. It allows the highly efficient transfection of a broad range of cell types, including adherent, suspension, and insect cells, as well as primary cultures. When selecting a transfection reagent, you must consider the **payload** you wish to deliver (DNA, RNA, or protein) and the **type of cells** you want to transfect, because the choice of the transfection reagent strongly influences transfections results.

The table below lists the key features and applications of various cationic-lipid transfection reagents available from Life Technologies™. For more information on each transfection reagent and for optimized transfection protocols for a wide range of cell lines, go to **www.lifetechnologies.com/transfection**.

Transfection Reagent	Payload	Key Features and Applications
Broad-Spectrum Reagents		
Lipofectamine® 3000		• Highest efficiency and expression results for plasmid and RNAi cotransfections • High transfection efficiency even at low doses • Works effectively with a wide range of cell types, both adherent and suspension • No need for wash steps before or after transfection • Complexes can be added to cells growing in serum-containing media
Lipofectamine® 2000		• High efficiency and expression results for plasmid or RNAi transfections • Works effectively with a wide range of cell types, both adherent and suspension • For robust cells • No need for wash steps before or after transfection • Complexes can be added to cells growing in serum-containing media • Ideal at >90% confluency at the time of transfection • Recommended for delivery of Stealth RNAi® and Silencer® Select siRNAs, dicer-generated siRNA pools and plasmids containing shRNA cassettes • High-throughput
Lipofectamine® 2000 CD		• Same performance as Lipofectamine® 2000, certified animal-origin free ("CD" = chemically defined)
Lipofectamine® LTX with PLUS™ Reagent		• Highest transfection efficiency in Chinese Hamster Ovary (CHO) cells • High transfection efficiency and significantly lower toxicity for a wide range of cell lines • Very gentle to cells • Wide range of cell lines, including primary and disease-related cells • High protein expression • Delivery of shRNA and miR RNAi vectors • Significantly improved transfection performance in a number of primary and hard-to-transfect cell lines • Optimized protocols for over 30 different cell lines are available, so much less time is needed for evaluation and optimization
Lipofectamine®		• First generation reagent for plasmid DNA transfections
Lipofectin®		• First generation reagent for plasmid DNA transfections

Transfection Reagent	Payload	Key Features and Applications
RNA Reagents		
Lipofectamine® RNAiMAX		• Recommended for transient delivery of Stealth RNAi® and Silencer® Select siRNAs, dicer-generated siRNA pools, mirVana™ miRNA Mimics and Inhibitors, mRNA, and snRNA • Requires lower RNAi concentrations leading to more effective gene knockdown with minimal non-specific effects • Minimal cytotoxicity across a 10-fold concentration range of transfection reagent • Compatibility with a broad range of cell types • High-throughput • Highest knockdown with less RNAi • *in vitro* transfection
Lipofectamine® MessengerMAX		• Highest transfection efficiency in neurons (> 2-fold) and a broad spectrum of primary cell types • Enhanced gene editing outcomes using mRNA CRISPRs • Faster protein expression with no risk of genomic integration
Invivofectamine® 2.0		• Efficiently delivers siRNA *in vivo* through systemic delivery • Highly effective mRNA, protein, and functional knockdown • Low toxicity profile and easy to use
Oligofectamine®		• Transfection of antisense oligonucleotides • High-throughput • Highly-specific, non-toxic • Ideal for low confluency (30–50% confluent at the time of transfection)
Protein Production Reagents		
ExpiFectamine® 293		• Transfection of high density 293 suspension cell culture for bioproduction • Transfection enhancers boost performance and protein expression with yields 2- to 10-fold higher than other transfection reagents used on high density 293 cell cultures • 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
FreeStyle™ MAX		• Used for rapid large-scale transient mammalian protein expression for bioproduction • High-yield production with milligrams of protein yield • Optimized for transient transfection in CHO suspension cells and also works for HEK-293 cells
293fectin™		• Used for transient protein bioproduction in combination with the FreeStyle™ 293 Expression System • Optimized for suspension FreeStyle™ 293-F cells
Optifect™		• Ideal for low confluency (<70% confluent at the time of transfection) • For cell lines that are sensitive to transfection reagents
Cellfectin®		• Optimal transfection of insect cells, including S2, Sf9, Sf21 and High Five™ cells
DMRIE-C Reagent		• Transfection of suspension cells, including CHO, lymphoid and Jurkat cell lines

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

Virus-Mediated Gene Transfer

For cell types not amenable to lipid-mediated transfection, viral vectors are often employed. Virus-mediated transfection, also known as transduction, offers a means to reach hard-to-transfect cell types for protein overexpression or knockdown, and it is the most commonly used method in clinical research (Glover *et al*., 2005; Pfeifer and Verma, 2001). One of the main advantages of viral delivery is that the process can be performed inside a living organism (*in vivo*) or in cell culture (*in vitro*) with gene delivery efficiencies approaching 95–100%.

Key properties of viral vectors

Viral vectors are tailored to their specific applications, but must generally share a few key properties.

- **Safety:** Although viral vectors are occasionally created from pathogenic viruses, they are modified in such a way as to minimize the risk of handling them. This usually involves the deletion of a part of the viral genome critical for viral replication, allowing the virus to efficiently infect cells and deliver the viral payload, but preventing the production of new virions in the absence of a helper virus that provides the missing critical proteins. However, an ongoing safety concern with the use of viral vectors is **insertional mutagenesis**, in which the ectopic chromosomal integration of viral DNA either disrupts the expression of a tumor-suppressor gene or activates an oncogene, leading to the malignant transformation of cells (Glover *et al*., 2005).
- **Low toxicity:** The viral vector should have a minimal effect on the physiology of the cell it infects. This is especially important in studies requiring gene delivery *in vivo*, because the organism will develop an immune response if the vector is seen as a foreign invader (Nayak and Herzog, 2009).
- **Stability:** Some viruses are genetically unstable and can rapidly rearrange their genomes. This is detrimental to predictability and reproducibility of the work conducted using a viral vector. Therefore, unstable vectors are usually avoided.
- **Cell type specificity:** Most viral vectors are engineered to infect as wide a range of cell types as possible. However, sometimes the opposite is preferred. The viral receptor can be modified to target the virus to a specific kind of cell. Viruses modified in this manner are said to be **pseudotyped**.
- **Selection:** Viral vectors should contain selectable markers, such as resistance to a certain antibiotic, so that the cells that have taken up the viral vector can be isolated.

Common viral vectors

Adenoviruses are DNA viruses with broad cell tropism that can transiently transduce nearly any mammalian cell type. The adenovirus enters target cells by binding to the Coxsackie/Adenovirus receptor (CAR) (Bergelson *et al*.,1997). After binding to the CAR, the adenovirus is internalized via integrin-mediated endocytosis followed by active transport to the nucleus, where its DNA is expressed episomally (Hirata and Russell, 2000). Although adenoviral vectors work well for transient delivery in many cell types, for some difficult cell lines such as non-dividing cells and for stable expression, lentiviral vectors are preferred. The packaging capacity of adenoviruses is 7–8 kb.

Retroviruses are positive-strand RNA viruses that stably integrate their genomes into host cell chromosomes. When pseudotyped with an envelope that has broad tropism, such as vesicular stomatitis virus glycoprotein (VSV-G), these viruses can enter virtually any mammalian cell type. However, most retroviruses depend upon the breakdown of nuclear membrane during cell division to infect cells and are thus limited by the requirement of replicating cells for transduction. Other disadvantages of retroviruses include the possibility of insertional mutagenesis and the potential for the activation of latent disease. Like adenoviruses, retroviruses can carry foreign genes of around 8 kb.

Lentiviruses are a subgroup of the retrovirus family; as such, they can integrate into the host cell genome to allow stable, long-term expression (Anson, 2004). In contrast to other retroviruses, lentiviruses are more versatile tools as they use an active nuclear import pathway to transduce non-dividing, terminally differentiated cell populations such as neuronal and hematopoietic cells.

Adeno-associated viruses are capable of transducing a broad range of dividing and non-dividing cells types, but they require coinfection with a helper virus like adenovirus or herpes virus to produce recombinant virions in packaging cells. This causes difficulties in obtaining high quality viral stocks that are free of helper viruses. Furthermore, adeno-associated viruses have only limited packaging capacity of up to 4.9 kb. On the other hand, adeno-associated viruses show low immunogenicity in most cell types, and they have the ability to integrate into a specific region of the human chromosome, thereby avoiding insertional mutagenesis.

Other viral vector systems that can be used for overexpression of proteins include vectors based on **baculovirus**, **vaccinia virus,** and **herpes simplex virus**. While baculoviruses normally infect insect cells, recombinant baculoviruses can serve as gene-transfer vehicles for transient expression of recombinant proteins in a wide range of mammalian cell types. Furthermore, by including a dominant selectable marker in the baculoviral vector, cell lines can be derived that stably express recombinant genes (Condreay *et al*., 1999). Vectors based on vaccinia virus can be used for introducing large DNA fragments into a wide range of mammalian cells. However, cells infected with vaccinia virus die within one or two days, limiting this system to transient protein production. Herpex simplex viruses are a class of double-stranded DNA viruses that infect neurons.

Viral system	Size	DNA insert size	Maximum titer (particles/mL)	Infection	Expression	Drawbacks
Adenovirus	36 kb (dsDNA)	8 kb	1×10^{13}	Dividing and non-dividing cells	Transient	Elicits strong antiviral immune response
Retrovirus	7–11 kb (ssRNA)	8 kb	1×10^{9}	Dividing cells	Stable	Insertional mutagenesis potential
Lentivirus	8 kb (ssRNA)	9 kb	1×10^{9}	Dividing and non-dividing cells	Stable	Insertional mutagenesis potential
Adeno-associated virus	8.5 kb (ssDNA)	5 kb	1×10^{11}	Dividing and non-dividing cells	Stable; site-specific integration	Requires helper virus for replication; difficult to produce pure viral stocks
Baculovirus	80–180 kb (dsDNA)	no known upper limit	2×10^{8}	Dividing and non-dividing cells	Transient or stable	Limited mammalian host range
Vaccinia virus	190 kb (dsDNA)	25 kb	3×10^{9}	Dividing cells	Transient	Potential cytopathic effects
Herpex simplex virus	150 kb (dsDNA)	30–40 kb	1×10^{9}	Dividing and non-dividing cells	Transient	No gene expression during latent infection

Neon® Transfection System

The Neon® Transfection System, a second-generation benchtop electroporation device offered by Life Technologies™, uses an electronic pipette tip as an electroporation chamber to efficiently transfect mammalian cells including primary and immortalized hematopoietic cells, stem cells, and primary cells. The design of the electroporation chamber distributes the current equally among the cells and maintains a stable pH throughout the chamber, resulting in less ion formation and negligible heat generation for increased cell viability and transfection efficiency compared to traditional cuvette-based electroporation systems.

The Neon® Transfection System efficiently delivers nucleic acids, proteins, and siRNA into all mammalian cell types, including primary and stem cells, with a high cell survival rate. The transfection is performed using as few as 1×10^4 or as many as 5×10^6 cells per reaction using a sample volume of 10 µL or 100 µL in a variety of cell culture formats (60-mm, 6-well, 48-well, and 24-well).

Because the Neon® Transfection System uses a single transfection kit (Neon® Kit) that is compatible with various mammalian cell types including primary and stem cells, the need to determine an optimal buffer for each cell type is avoided. Furthermore, the Neon® device is pre-programmed with a 24-well optimization protocol to optimize conditions for nucleic acid/siRNA and cell type, and allows the programming and storage of up to 50 cell-specific protocols in the Neon® device database. Optimized protocols can also be conveniently downloaded from **www.lifetechnologies.com/neon** to maximize transfection efficiencies for many commonly used cell types.

Cell types successfully transfected with the Neon® Transfection System*					
Blood/Immune Cells					
Human BC-1 Cells	Human Jiyoye Cells	Human Raji Cells	Human SKW6.4 Cells	Human HL-60 Cells	Human Macrophage Cells
Human BJAB Cells	Human K-562 Cells	Human Ramos Cells	Human RPMI8226 Cells	Human NAMALWA Cells	Human KG-1 Cells
Human IM-9 Cells	Human LCL Cells	Human Dendritic Cells	Human RS4-11 Cells	Human PBMC Cells	Human CCRF-CEM Cells
Human SCID.adh Cells	Human U-937 Cells	Human Jurkat Cells	Mouse RAW 264.7 Cells	Mouse MPC-11 Cells	Mouse Ramos Cells
Mouse RBL-2H3 Cells	Mouse BW5147 (T200-A)5.2 Cells	Mouse M1 Cells	Mouse EL4 Cells	Mouse P815 Cells	
Connective Tissue					
Human HOS Cells	Human MH7A Cells	Human BJ Cells	Human HT-1080 Cells	Human U-2 OS Cells	Human IMR-90 Cells
Human Saos-2 Cells	Human Neonatal Dermal Fibroblast Cells Gibco®	Human WI-38 Cells	Mouse Embryonic Fibroblast Cells (MEF)	Mouse NIH-3T3 Cells	Mouse PA317 Cells
Mouse L-929 Cells	Mouse 3T3-L1 Cells	Monkey COS-7 Cells	Monkey Vero Cells	Horse Embryonic Dermal Fibroblast Cells (NBL-6)	
* For optimized Neon® transfection protocols using the cell types listed here, go to **www.lifetechnologies.com/neon**.					

Cell types successfully transfected with the Neon® Transfection System, continued*					
Epithelial Cells					
Human T24 Cells	Human ChangX-31 Cells	Human HEK 293 Cells	Human ARPE-19 Cells	Human COLO 201 Cells	Human HCT 116 Cells
Human 253J Cells	Human HT-29 Cells	Human HCT15 Cells	Human RKO Cells	Human SW480 Cells	Human WiDr Cells
Human 293A Cells	Human J82 Cells	Human RT4 Cells	Human Hep G2 Cells	Human Hep3B Cells	Human BT-20 Cells
Human Mammary Epithelial Cells Gibco®	Human SK-HEP-1 Cells	Human SNU-387 Cells	Human HCC1937 Cells	Human Hs-578T Cells	Human MCF7 Cells
Human MDA-MB-231 Cells	Human SK-BR-3 Cells	Human T-47D Cells	Human SK-OV-3 Cells	Human DU 145 Cells	Human MCF-ADR Cells
Human LNCaP Cells	Human A549 Cells	Human PANC-1 Cells	Human BxPC-3 Cells	Human NCI-H23 Cells	Human PC-3 Cells
Human TSU-Pr1 Cells	Human BEAS-2B Cells	Human NCI-H69 Cells	Human HN3 Cells	Human G-361 Cells	Human ARO Cells
Human HeLa Cells (ATCC)	Human FRO Cells	Human Calu-3 Cells	Human MEWO Cells	Human NPA Cells	Human A-431 Cells
Human C-33 A Cells	Mouse P19 Cells	Rat GH3 Cells	Rat NRK Cells	Rat PC-12 Cells	Rat H-4-II-E Cells
Chinese Hamster CHO-K1 Cells	Chinese Hamster CHO DG44 Cells	Hamster BHK-21 Cells	Canine MDCK Cells		
Endothelial Cells					
Human Endothelial Cells Gibco®	Human HUVEC Cells	Mouse b-END.3 Cells			
Muscle Cells					
Human Aortic Smooth Muscle Cells Gibco®	Mouse C2C12 Cells	Rat L6 Cells	Rat Cardiomyocyte Cells		
Neural/Glial Cells					
Human T98G Cells	Human U-87 MG Cells	Human SK-N-MC Cells	Human SH-SY5Y Cells	Mouse GT1-1 Cells	Mouse GT1-7 Cells
Mouse Glial Cells	Rat Cortical Astrocyte Cells Gibco®	Rat Primary Cortical Neuron Cells Gibco®	Rat Astrocyte Cells	Rat Glial Precursor Cells Gibco®	Rat Primary Hippocampal Neuron Cells Gibco®
Rat HiB5 Cells	Rat C6 Glial Cells	Rat SCN2.2 Cells	Rat F-11 Cells		
Secretory Cells					
Human SW-13 Cells	Human SV40 MES 13 Cells				
Stem Cells					
Human Mesenchymal Stem Cells (hMSC)	Human BGO1V Embryonic Stem Cells	Human H9 Embryonic Stem Cells	Human Neural Stem Cells Gibco®	Human Adipose-derived Stem Cells (ADSC)	Mouse Embryonic Stem Cells
Rat Neural Stem Cells Gibco®					

* For optimized Neon® transfection protocols using the cell types listed here, go to **www.lifetechnologies.com/neon**.

Selection of Stable Transfectants

Successful stable transfection requires both effective DNA delivery and a way to select cells that have acquired the DNA. Approximately one in 10^4 transfected cells will stably integrate DNA, although the efficiency varies with cell type and whether linear or circular DNA is used. Integration is most efficient when linear DNA is used.

One of the most reliable ways to select cells that stably express transfected DNA is to include a selectable marker on the DNA construct used for transfection or on a separate vector that is co-transfected into the cell, and then apply the appropriate selective pressure to the cells after a short recovery period. When the selectable marker is expressed from the co-transfected vector, the molar ratio of the vector carrying the gene of interest to the vector carrying the selectable marker should be in the range of 5:1 to 10:1 to ensure that any cell that contains the selectable marker also contains the gene of interest.

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 will die, and those that express the antibiotic resistance gene at sufficient levels or those that can compensate for the defect in the essential gene will survive.

Selection antibiotics for eukaryotic cells

Life Technologies™ offers high-quality selection reagents to complement its wide variety of selectable eukaryotic expression vectors. Geneticin® (G418 sulfate), Zeocin™, hygromycin B, puromycin, and blasticidin antibiotics are the most commonly used selection antibiotics for stable cell transfection. These antibiotics provide unique solutions for your research needs, such as dual selection and rapid, stable cell line establishment.

Geneticin® Selection Antibiotic

Geneticin® reagent, also known as G418 sulfate, is commonly used for the selection of mammalian, plant, or yeast cells. The higher purity of Geneticin® reagent available from Life Technologies™ means that 15–30% lower concentrations are required compared to other G418 products; therefore, surviving clonal colonies may arise faster, and cells appear healthier.

Zeocin™ Selection Antibiotic

Zeocin™ reagent is effective in mammalian cell lines, yeast, insect cells, and bacteria. Resistance to Zeocin™ reagent is conferred by the *She ble* gene, which prevents the binding of Zeocin™ reagent and cleavage of cellular DNA in cells expressing the protein. The concentration required for selection ranges from 50 to 2,000 µg/mL (typically 300 µg/mL), depending on the cell type.

Hygromycin B Selection Antibiotic

Hygromycin B is an aminoglycosidic antibiotic that inhibits protein synthesis by disrupting translocation and promoting mistranslation of the 80S ribosome. Because its mode of action is different from Geneticin® or Zeocin™ reagents, hygromycin B can be used in dual-selection experiments. Resistance to hygromycin B is conferred by the *E. coli* hygromycin resistance gene (*hyg* or *hph*). The concentration for selection ranges from 100 to 1,000 µg/mL (typically 200 µg/mL), and should be optimized for each cell line.

Puromycin Dihydrochloride Selection Antibiotic

Puromycin, a translation inhibitor in both prokaryotic and eukaryotic cells, is an aminonucleoside antibiotic from *Streptomyces alboniger*. Resistance is conferred by the puromycin N-acetyltransferase gene (*pac*) from *Streptomyces*. Puromycin has a fast mode action, causing rapid cell death even at low antibiotic concentrations, allowing the generation of puromycin-resistant stable cell lines in less than one week. Adherent mammalian cells are sensitive to concentrations of 2–5 µg/mL, while cells in suspension are sensitive to concentrations as low as 0.5–2 µg/mL.

Blasticidin S HCl Selection Antibiotic

Blasticidin, a potent translational inhibitor in both prokaryotic and eukaryotic cells, is a nucleoside antibiotic from *Streptomyces griseochromogenes*. Resistance is conferred by the *bsd* gene product from *Aspergillus terreus*. *E. coli* strains are generally sensitive to concentrations of 50 µg/mL, while mammalian cells are sensitive to concentrations as low as 2–10 µg/mL. Cell death occurs rapidly in cells sensitive to blasticidin, and blasticidin-resistant, stable mammalian cell lines can be generated in less than one week at low antibiotic concentrations.

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 can be expressed constitutively or inducably 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.

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 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 (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 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.

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.

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). For more information on RNAi, go to **www.lifetechnologies.com/rnai**.

siRNA analysis

There are several ways to induce RNAi: synthetic molecules, RNAi vectors, and *in vitro* dicing (Figure 5.8, 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. For more information on siRNA analysis, go to **www.lifetechnologies.com/sirna**.

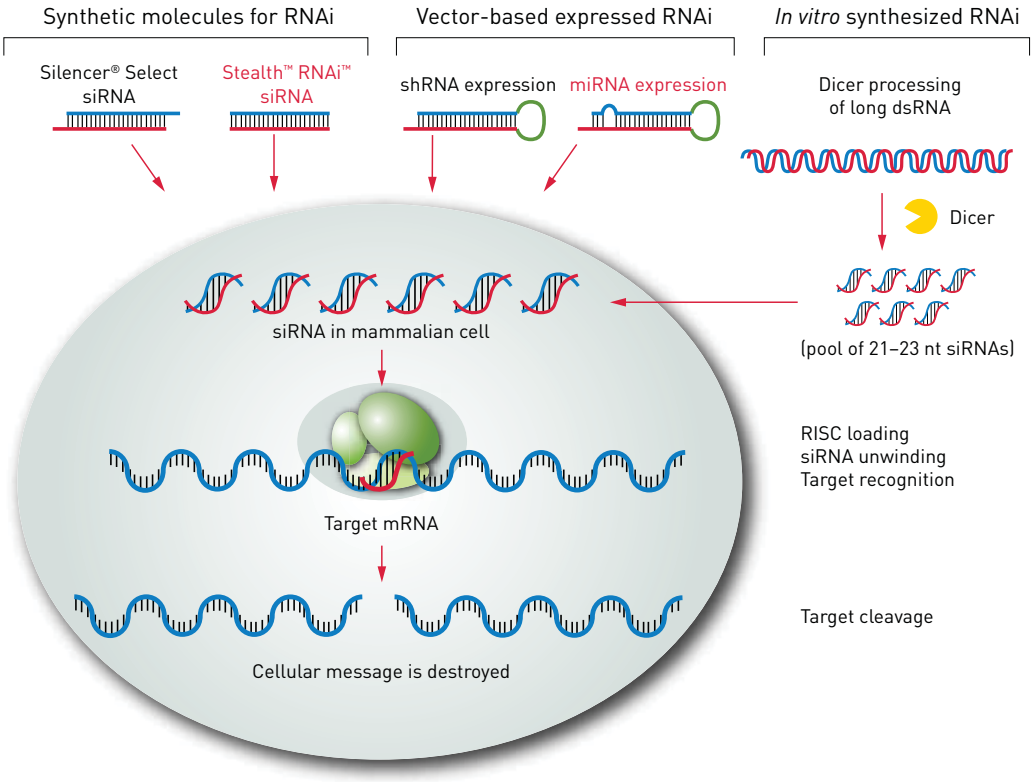


Figure 5.8 Methods of RNAi knockdown in mammalian cells.

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 multiple-protein 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 5.9, 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. For more information on miRNA analysis, go to **www.lifetechnologies.com/mirna**.

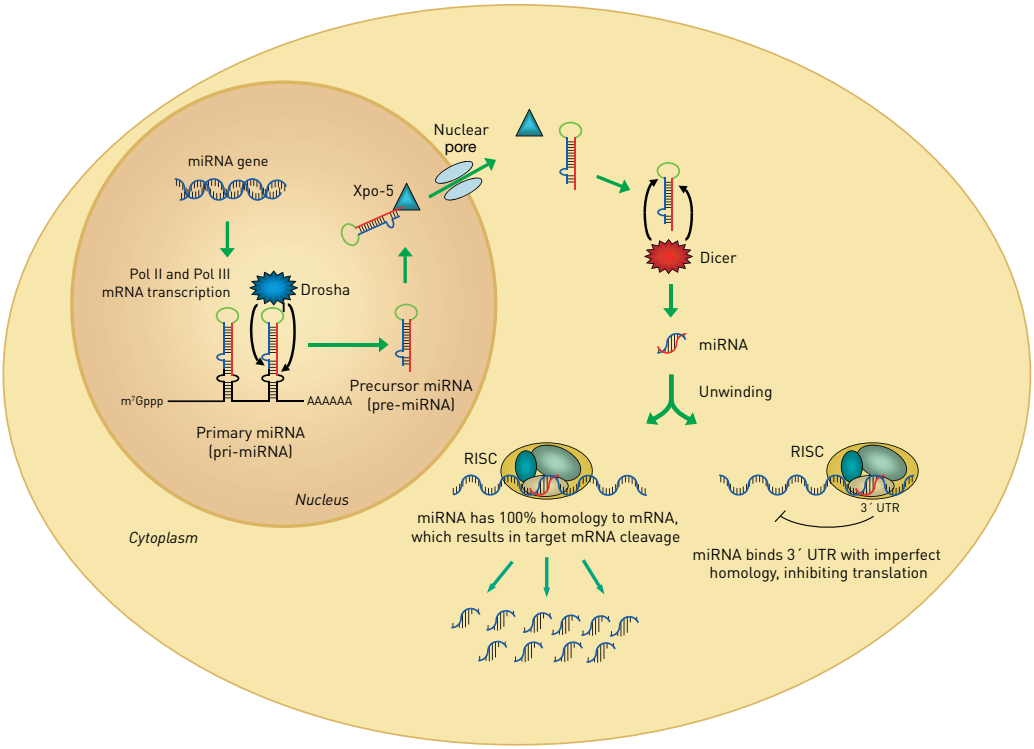


Figure 5.9 Biogenesis and function of miRNA. MicroRNA transcripts, generated by RNA polymerases II and III, are processed by the RNase III enzymes Drosha (nuclear) and Dicer (cytoplasmic), yielding 19–22 nucleotide miRNA duplexes. One of the two strands of the duplex is incorporated into the RISC complex,which regulates protein expression.

Choosing an 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.

	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.**

6. Transfection Methods

This section provides useful information and general guidelines for the transfection of cells with plasmid DNA, oligonucleotides, and RNA, preparation of cultures for *in vitro* and *in vivo* transfection, and selection of transfected cells.

Note that while the basics of transfection experiments share certain similarities, conditions vary widely depending upon the cell type used for transfection. Therefore, we recommend that you familiarize yourself with the cell line of interest and the appropriate transfection method, and closely follow the instructions provided with each product you are using in your experiments.

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 modeling *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 Expi293F™ cells grown in 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.

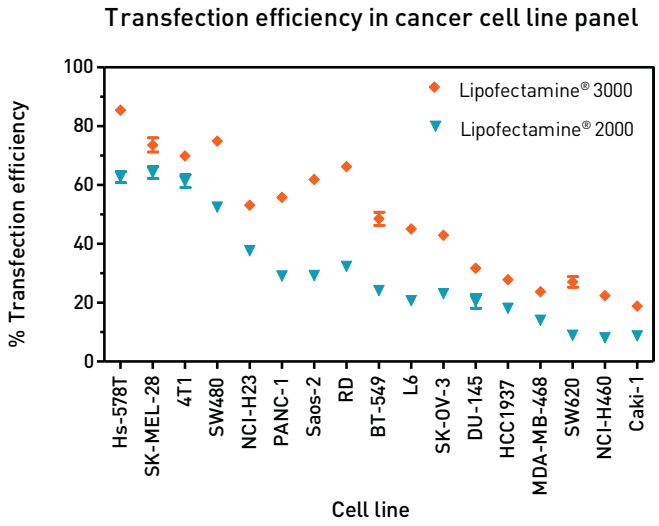


Figure 6.1 Cell line-dependent differences in transfection efficiency. Lipofectamine® 2000 reagent and Lipofectamine® 3000 reagent were used to transfect 17 cell lines with a GFP-expressing plasmid in a 24-well plate format, using 0.5 µg plasmid/well and the recommended protocols for each reagent. GFP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

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**, page 14), 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 5×10^5 to 2×10^6 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.

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**, page 28, or refer to our website (**www.lifetechnologies.com**). 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 Life Technologies™ and 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 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 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**, page 46.

Selecting a Transfection Method (non-viral)

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 the various cationic-lipid transfection reagents and the Neon® Transfection System available from Life Technologies™. For more information on each transfection method, as well as optimized protocols for the transfection of wide range of cell lines, go to **www.lifetechnologies.com/transfection**.

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 method	Payload	Adherent cells		Suspension cells
		easy-to-transfect	hard-to-transfect	
Lipofectamine® 3000 reagent		• • • •	• • • •	• •
Lipofectamine® 2000 reagent		• • •	• •	•
Lipofectamine® RNAiMAX reagent		• • • •	• • • •	• •
Lipofectamine® LTX with PLUS™ reagent		• • • •	• • •	•
Invivofectamine® 2.0 reagent		N/A	N/A	*in vivo*
Neon® electroporation		• • • •	• • • •	• • • •

Symbol	Explanation	Symbol	Explanation	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

Primary cells and finite cultures

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 method	Payload	Neurons	Stem cells	Blood cells	Others
Lipofectamine® 3000 reagent		• • •	• • •	• •	• • •
Lipofectamine® 2000 reagent		• • •	• •	•	• •
Lipofectamine® RNAiMAX reagent		• • •	• • •	• •	• • •
Lipofectamine® LTX with PLUS™ reagent		• •	• •	•	• • •
Invivofectamine® 2.0 reagent		N/A	N/A	*in vivo*	
Neon® electroporation		• • •	• • •	• • •	• • •

Symbol	Explanation	Symbol	Explanation	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

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.

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 recombination-based 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.

For more information on ViraPower™ expression systems as well as on other expression systems not discussed here, refer to **www.lifetechnologies.com/proteinexpression**.

Viral system	Transient expression		Stable expression			
	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 time-consuming 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.

For more information on baculoviral expression systems as well as on other expression systems not discussed here, refer to **www.lifetechnologies.com/proteinexpression**.

System	Host	Secretion signal	Fusion partner			Promoter	Expression/ inducer	Advantage
			Position	Purif.	Epitope			
BaculoDirect™	Sf9, Sf21, or High Five™	—	N-term C-term	6×His 6×His	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	6×His	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	6×His	Xpress™ V5	Polyhedrin	Infection	High-level recombinant protein production
DES®	S2 cells	BIP	C-term	6×His	V5	MT or Ac5	$CuSO_4$ or constitutive	Constitutive or inducible expression; extremely high integration

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.

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).

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 DNA using PureLink® HiPure Plasmid Kits (Mini, Midi, Maxi, Mega, and Giga), available from Life Technologies™, that provide highest quality DNA for transfections. For more information, refer to **www.lifetechnologies.com/nap**. 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-1□). 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.

Optimization of Plasmid DNA Transfection

With any transfection procedure, a critical first step is to optimize the transfection conditions. Every cell type and transfection procedure has a characteristic set of requirements for optimal introduction of foreign DNA, and these conditions have a large degree of variability even among cell types that are very similar to one another.

The single most important factor in optimizing transfection efficiency is selecting the proper transfection protocol for the cell type. Once the appropriate transfection method is selected, a transient reporter assay system can be used to optimize the procedure by transfecting a reporter gene under a variety of conditions, and monitoring the transfection efficiency by assaying for the reporter gene product.

This section provides general guidelines for optimizing calcium phosphate–mediated gene transfer, electroporation using the Neon® Transfection System, and cationic lipid-mediated transfection.

Considerations for calcium phosphate co-precipitation

The primary factors that influence the efficiency of calcium phosphate transfection are the amount of DNA in the calcium-phosphate–DNA co-precipitate, the length of time the cell is incubated with the co-precipitate, and the use and duration of glycerol or DMSO shock.

Total DNA amount used in calcium phosphate transfection is usually 10–50 µg in 450 µL sterile water and 50 µL of 2.5 M $CaCl_2$ per 10-cm dish, but varies widely among plasmid preparations as well as with different cells and media. While with some cell lines 10–15 µg of DNA added to a 10-cm dish results in excessive cell death and very little uptake of DNA, other cell lines, especially primary cells, much higher concentrations of DNA is required. Each new plasmid preparation and each new cell line being transfected should be tested for optimum DNA concentration.

The optimal length of time that the cells are incubated with co-precipitate also varies with cell type. Some hardy cell types, such as HeLa, NIH 3T3, and BALB/c 3T3, are efficiently transfected by leaving the co-precipitate on for up to 16 hours, which might kill some more sensitive cells.

A pilot experiment varying the amount of DNA, incubation time, and exposure to glycerol or DMSO shock will indicate whether the cell type is tolerant to long exposure to a calcium phosphate precipitate and whether glycerol shock should be used. Once the results of the pilot experiment are obtained, further optimization can be performed by adjusting the experimental variables even finer. For instance, if shocking the cells with 10% glycerol for 3 minutes as shown in the example below enhances transfection efficiency, an experiment varying the time of glycerol shock or using 10–20% DMSO shock might also be tried.

Dish (10-cm)	Reporter plasmid (µg)	Incubation (hour)	Glycerol shock (minutes)
1	5	6	—
2	10	6	—
3	15	6	—
4	20	16	—
5	25	16	—
6	30	16	—
7	5	6	3
8	10	6	3
9	15	6	3
10	20	16	3
11	25	16	3
12	30	16	3

Figure 6.2 Pilot experiment example for the optimization of transfection by calcium phosphate co-precipitation.

Considerations for cationic lipid-mediated delivery

Four primary parameters affect the success of DNA transfection by cationic liposomes: the amount of DNA, the ratio of transfection reagent to DNA, incubation time of the lipid-DNA complex, and the cell density at the time of complex addition. These factors should be systematically examined for every cell type and vector combination, and once optimized, kept constant in all future experiments to help ensure reproducible results.

For best results, follow the optimization protocols provided by the manufacturers of the reagent. Life Technologies™ provides optimization protocols for all of its transfection reagents. For more information, refer to **www.lifetechnologies.com/transfection**.

Amount of DNA

The optimal amount of DNA varies depending on the characteristics of the transfected plasmid (e.g., promoter, size of plasmid, origin of replication), number of cells to be transfected, size of the culture dish, and the target cell line used. In many of the cell types tested, relatively small amounts of DNA are effectively taken up and expressed. In fact, higher levels of DNA can be inhibitory in some cell types with certain cationic lipid preparations. In addition, cytotoxicity may result if a plasmid encoding a toxic protein or too much plasmid with a high expression rate is used.

Ratio of transfection reagent to DNA

The overall charge of the transfection complexes is determined by the ratio of transfection reagent to DNA. The negative charge contributed by phosphates within the DNA backbone needs to be offset by the positive charge contributed from the transfection reagent for both good complex formation and for neutralizing the electrostatic repulsion imparted on the DNA by the negatively charged cell membrane.

The optimal ratio of transfection reagent to DNA is highly cell type-dependent. As a starting point, the amount of transfection reagent should be varied while keeping a constant plasmid DNA concentration (for example, 1:1, 3:1 and 5:1 ratios of volume to mass). Additional benefits may be derived by maintaining the ratio and increasing the amount of plasmid added.

Incubation time

The optimal incubation period of cells with the transfection complexes depends on the cell line and transfection reagent used. In general, transfection efficiency increases with time of exposure to the lipid reagent-DNA complex, although toxic conditions can develop with prolonged exposure to certain lipid reagents, requiring removal by centrifugation or dilution with fresh medium after a given incubation period to minimize cytotoxic effects. However, newer and gentler transfection reagents such as the Lipofectamine® 3000 reagent do not necessitate complex removal or dilution after transfection (see **www.lifetechnologies.com/3000** for more information).

When using cationic lipid reagents that require adding or replacing the medium, vary the incubation time after complex addition (e.g., 30 minutes to 4 hours, or even overnight) and monitor cell morphology during the this interval, particularly if the cells are maintained in serum-free medium as some cell lines lose viability under these conditions.

Cell density

Cell density also affects overall transfection efficiency. To achieve transcription and ultimately protein production, nuclear deposition of DNA is required, which is largely dependent on membrane dissolution and reformation during mitosis, requiring that the cells have to be actively dividing.

For adherent cells, the best efficiency is often attained at a confluency of 80%, but protocol recommendations may range from 40–90%. For suspension cells, we recommend splitting the cells the day prior to transfection to ensure that the cells will be in optimal physiological condition for the transfection procedure. The optimal density is highly dependent on cell type and reagent-specific toxicity, and should be determined empirically.

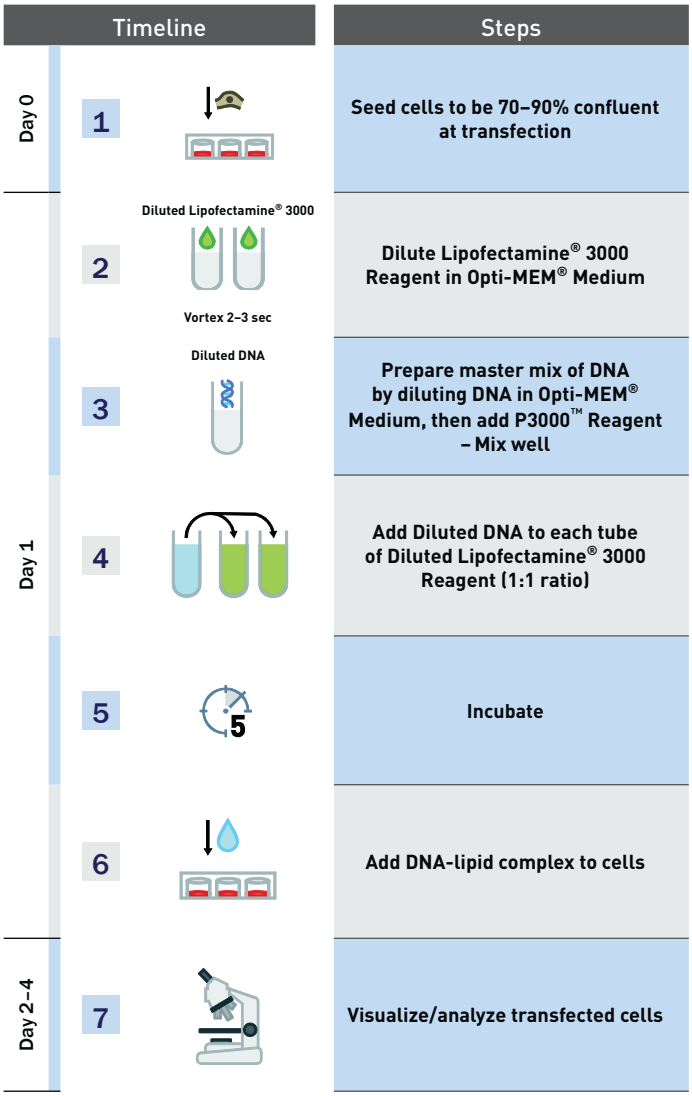


Figure 6.3 Example transfection workflow using the Lipofectamine® 3000 transfection reagent.

Considerations for electroporation

Electroporation is mainly dependent on the combination of three electric parameters: the pulse voltage, pulse width, and pulse number. Perhaps because it is not a chemically based protocol, electroporation is less affected by DNA concentration; however, it requires almost five-fold more cells and DNA compared to calcium phosphate-mediated transfection. Generally, 1–5 µg of DNA per 10^7 cells is sufficient, and there is a good linear correlation between the amount of DNA present and the amount taken up.

The objective in optimizing electroporation parameters is to find a pulse that maintains 40–80% survival of the cells. The pulse width is determined by the capacitance of the power source and the extent to which this can be varied depends on the electronics of the power supply generating the pulse. If excessive cell death occurs, the length of the pulse can be lowered by lowering the capacitance.

Keeping cells on ice often improves cell viability and results in higher effective transfection frequency, especially at high power which can lead to heating (Potter *et al*., 1984). However, some cell lines electroporate with higher efficiency at room temperature under low voltage/high capacitance conditions (Chu *et al*., 1991).

The Neon® Transfection System, available from Life Technologies™, is pre-programmed with 18- and 24-well optimization protocols that allow quick optimization of electric parameters for many adherent and suspension cell lines within days. Cell line-specific optimized protocols for the Neon® Transfection System can also be conveniently downloaded from **www.lifetechnologies.com/neon** to maximize transfection efficiencies for many commonly used cell types.

Selection of Stable Transfectants

Selection of stably transfected cells begins with successful transient transfection with a plasmid containing a selectable marker, such as an antibiotic resistance gene. As a negative control, cells should be transfected using DNA that does not contain the selectable marker.

Before starting

- Ensure that the cell line you are using can produce colonies from isolated cells as some cells require contact with one another to grow. For such cells, adapted or conditioned medium may be beneficial.
- Choose an appropriate selectable marker (see **Selection Antibiotics for Eukaryotic Cells**, page 62).
- Select a transfection procedure suitable for your cell type.
- Determine the selective conditions for your cell type by establishing a dose-response curve (**kill curve**) (Ausubel *et al*. 1995).

Kill curve

A kill curve should be established for each cell type and each time a new lot of the selective antibiotic is used.

1. Split a confluent dish of cells at approximately 1:5 to 1:10 (depending on the cell type and cell density post-transfection) into medium containing various concentrations of the antibiotic.
2. Incubate the cells for 10 days replacing selective medium every 4 days (or as needed).
3. Examine the dishes for viable cells using the desired method (e.g., Countess® II Automated Cell Counter, hemocytometer with trypan blue staining).
4. Plot the number of viable cells versus antibiotic concentration to establish a kill curve to determine the most appropriate selective drug concentration required to kill untransfected cells.

Selection workflow

1. Transfect the cells using the desired transfection method. If the selectable marker is on a separate vector, use a 5:1 to 10:1 molar ratio of plasmid containing the gene of interest to plasmid containing the selectable marker.

   Note: Perform control transfections with a vector containing the selectable marker but not the gene of interest. If colonies are obtained from cells transfected with the control plasmid but not from cells transfected with plasmid containing the gene of interest, indicating that the gene of interest may be toxic. It is also important to perform replicate transfections in case the transfection fails or the cultures become contaminated.

2. Forty-eight hours after transfection, passage the cells at several different dilutions (e.g., 1:100, 1:500) in medium containing the appropriate selection drug. For effective selection, cells should be subconfluent, because confluent, non-growing cells are resistant to the effects of antibiotics like Geneticin®. Suspension cells can be selected in soft agar or in 96-well plates for single-cell cloning.

3. For the next two weeks, replace the drug-containing medium every 3 to 4 days (or as needed).

   Note: High cell densities in suspension cultures require frequent medium changes that may deplete critical soluble growth factors, thereby reducing cell viability and the efficiency of the system.

4. During the second week, monitor cells for distinct "islands" of surviving cells. Depending on the cell type, drug-resistant clones will appear in 2–5 weeks. Cell death should occur after 3–9 days in cultures transfected with the negative control plasmid.

5. Isolate large (500–1,000 cells), healthy colonies using cloning cylinders or sterile toothpicks, and continue to maintain cultures in medium containing the appropriate drug (for the isolation of clones in suspension culture, see Freshney, 1993).

6. Transfer single cells from resistant colonies into the wells of 96-well plates to confirm that they can yield antibiotic-resistant colonies. Ensure that only one cell is present per well after the transfer.

Selecting a RNAi Strategy

The two common approaches for RNAi delivery are lipid-mediated transfection and viral-mediated transduction. Determining which one of these approaches to use depends on the cell type being studied and whether transient or stable knockdown is desired. The most popular application, transient transfection of *Silencer*® Select siRNAs or Stealth RNAi™ siRNA duplexes, uses cationic lipid–based reagents because they are suitable for delivering molecules across a diverse range of commonly used cell lines (see **Non-vector siRNA technologies**, page 86)

For cell types that are not amenable to lipid-mediated transfection, viral vectors are often employed (see **Vector-mediated RNAi**, page 89). Adenoviral vectors work well for transient delivery in many cell types. However, when stable RNAi expression is desired, or for difficult cell lines, such as nondividing cells, lentiviral vectors are the best delivery method. Another approach for determining the most favorable RNAi delivery conditions is to use Life Technologies™ delivery optimization service—a scientific resource with extensive knowledge and expertise in viral vectors and non-viral delivery reagents for testing a matrix of delivery parameters.

siRNA vs. vector approaches

Both siRNA and vector-based RNAi can be extremely effective at producing loss of function phenotypes. In general, most researchers choose siRNA because they can start quickly and there are no special preparations needed other than basic cell culture techniques. However, there are a number of reasons why a researcher might choose either siRNA or a vector-based RNAi.

Typically, researchers strive to achieve the highest levels of transfection efficiency possible. This objective is particularly important for RNAi applications because non-transfected cells will continue to express the gene targeted for knockdown, thus contributing to background expression levels.

For many disease models, the most desirable cell types are primary cultures. However, these cannot be transfected adequately with commercially available cationic lipid-mediated transfection reagents. A powerful alternative is viral delivery of vectors expressing RNAi sequences. This option is recommended for delivery to hard-to-transfect, primary, and nondividing cells. Viral delivery can also be used to create stable cell lines with inducible RNAi expression or to express RNAi sequences with tissue-specific promoters.

Cell type	Transient expression		Stable expression
	(<7 days)	(>7 days)	
Fast-growing adherent cells (A549, HeLa)	Lipid transfection of *Silencer*® Select siRNA or Stealth RNAi™ siRNA	Lipid transfection of RNAi vectors or adenoviral delivery	Lipid transfection of RNAi vectors or lentiviral delivery
Fast-growing suspension cells (THP-1)	Lipid transfection or electroporation of *Silencer*® Select siRNA or Stealth RNAi™ siRNA	Lipid transfection of RNAi vectors or adenoviral delivery	Lipid transfection or electroporation of RNAi vectors or lentiviral delivery
Primary cells			Lentiviral delivery
Nondividing cells			Lentiviral delivery

Non-vector siRNA technologies

For transient knockdown experiments, synthetic, non-vector approaches offer significant advantages over vector-based methods for RNAi delivery. In particular, nonvector experiments are typically easier to design and perform and can result in higher levels of transient knockdown. In addition, recent improvements in RNAi design have increased the likelihood of achieving high-level knockdown after testing only a few RNAi molecules. Consequently, using synthetically generated RNA duplexes is the most popular method for conducting RNAi experiments.

Synthetic siRNAs

Traditional RNAi methods for gene knockdown in mammalian cells involved the use of synthetic RNA duplexes consisting of two unmodified 21-mer oligonucleotides annealed together to form short/small interfering RNAs (siRNAs). Life Technologies™, *Silencer*® Select siRNA and Stealth RNAi™ siRNA improve upon these traditional duplexes by using proprietary chemical modifications to ensure better RNAi results. For more information, see **www.lifetechnologies.com/sirna**.

- ***Silencer*® siRNAs** are Ambion®-designed siRNAs available for all human, mouse, and rat gene targets in the RefSeq database. These siRNAs are designed for maximum potency and specificity using a highly effective and extensively tested algorithm. Each siRNA is synthesized to the highest quality standards and is provided with full sequence information.
- **Stealth RNAi™ siRNA** molecules are chemically modified, blunt-ended, 25-mer double-stranded duplexes that are recognized by the RNA-induced silencing complex (RISC) to mediate inhibition of a target gene. Proprietary chemical modifications allow Stealth RNAi™ siRNA to overcome many *in vivo*–specific obstacles, allowing effectiveness and stability in *in vivo* applications.
- ***Silencer*® Select siRNAs** are the best-performing siRNAs for *in vitro* studies, and are available in a variety of formats including preplated collections and custom libraries to simplify screening experiments. They are up to 100-fold more potent than other siRNAs (modified and unmodified), allowing a higher percentage of "on-target" phenotypes.

	Silencer® siRNA	Stealth RNAi™ siRNA	*Silencer*® Select siRNA
Potency	100 nM recommended concentration	20 nM recommended concentration	5 nM recommended concentration
Target specificity	Moderate	High	Highest
Innate immune response	Low	Minimized through chemical modifications	Minimized through chemical modifications
Molecular format	Unmodified 21-bp duplex with overhangs	Modified 25-bp duplex with no overhangs	LNA modified 21-bp duplex with overhangs
Coverage	Coding RNA	Coding RNA	Coding and non-coding RNA
Target species	Human, mouse, rat (Other species: use custom tool)	Human, mouse, rat (Other species: use custom tool)	Human, mouse, rat (Other species: use custom tool)
Custom design tool	**Custom *Silencer*® siRNA**	**Custom Stealth RNAi™ siRNA**	**Custom *Silencer*® Select siRNA**
Notes	Cost-effective siRNA	Good knock-down, low off-target effects	Highest knock-down, lowest off-target effects

miRNA mimics and inhibitors

Analyses of miRNA function are performed using strategies that are similar to those used for protein-encoding genes. Transfecting cultured cells with miRNA mimics can help identify gain-of-function phenotypes; down-regulation or inhibition experiments using miRNA inhibitors can be conducted to identify loss-of-function phenotypes. The combination of up-regulation and down-regulation can be used to identify genes and cellular processes that are regulated by specific miRNAs.

- **Ambion® Pre-miR™ miRNA Precursors** are small, chemically-modified double-stranded RNA molecules that are similar, but not identical, to siRNAs, and are designed to mimic endogenous mature miRNAs.
- **mirVana™ miRNA Mimics** are small, chemically modified double-stranded RNAs that mimic endogenous miRNAs and enable miRNA functional analysis by up-regulation of miRNA activity. These molecules are more specific than their predecessors due to inactivation of the star strand by proprietary chemical modifications. mirVana™ miRNA Mimics are available individually or as libraries
- **Ambion® Anti-miR™ miRNA Inhibitors** are chemically modified, single-stranded nucleic acids designed to specifically bind to and inhibit endogenous miRNAs.
- **mirVana™ miRNA Inhibitors** are small, chemically modified single-stranded RNA molecules designed to specifically bind to and inhibit endogenous miRNA molecules and enable miRNA functional analysis by down-regulation of miRNA activity. They have the highest potency *in vitro* inhibition at the lowest miRNA inhibitor concentration of available miRNA mimics. mirVana™ miRNA Inhibitors are available individually or as libraries.

Due to their small size, these synthetic molecules are easier to transfect than vectors, and can be delivered using conditions identical to those used for siRNAs. In contrast to miRNA expression vectors, they can also be used in dose response studies.

Note: Pre-miR miRNA Precursors are not hairpin constructs and should not be confused with pre-miRNAs.

	Ambion® Pre-miR™ Precursors	mirVana™ miRNA Mimics	Ambion® Anti-miR™ Inhibitors	mirVana™ miRNA Inhibitors
Function	Mimic endogenous miRNAs	Mimic endogenous miRNAs	Inhibit endogenous miRNAs	Inhibit endogenous miRNAs
Experimental objective	Gain of function (*in vivo* or *in vitro*)	Gain of function (*in vivo* or *in vitro*)	Loss of function (*in vivo* or *in vitro*)	Loss of function (*in vivo* or *in vitro*)
Content database	100% Coverage of miRBase v 15*	100% Coverage of miRBase v 19†	100% Coverage of miRBase v 15*	100% Coverage of miRBase v 19†
Model system	*in vitro*	*in vitro* & *in vivo*	*in vitro*	*in vitro* & *in vivo*
Species covered	Human	Human, mouse, rat (Other species: use custom tool)	Human	Human, mouse, rat (Other species: use custom tool)
Custom design tool	GeneAssist™ miRNA Workflow Builder	GeneAssist™ miRNA Workflow Builder	GeneAssist™ miRNA Workflow Builder	GeneAssist™ miRNA Workflow Builder
miRNA libraries	Pre-Designed or Custom Libraries	Pre-Designed or Custom Libraries	Pre-Designed or Custom Libraries	Pre-Designed or Custom Libraries
Notes	Novel design to minimize off-target effects	Next generation chemistries for lowest off-target effects	Chemically modified for good efficacy	Next generation chemistries for highest efficacy

* 1090 distinct human sequences; † 2019 distinct human sequences.

siRNA transfection

siRNAs are easily introduced into cells with a siRNA transfection reagent . Soon after being inserted in the mammalian cell, the siRNA molecules become a part of the RNA-induced silencing complex (RISC). Guided by the antisense strand of the siRNA, RISC degrades the targeted mRNA inhibiting its translation. Assays are then performed to detect the RNAi activity. Controls are normally set up so RNAi results can be properly compared.

The success of RNAi is dependant on correct delivery of siRNA in appropriate amount at a time when it will brings about the maximum expected response. Such precision can be tricky. Off-targeting by siRNAs proves lethal and poses analytical issues at times. Researchers are looking for better ways of designing and delivering siRNA

Transfection method	Payload	Transfection efficiency	Cell viability	Notes
Lipofectamine® 3000 reagent		Superior	Superior	Most efficient versatile reagent for the widest range of cell types including difficult-to-transfect cells.
Lipofectamine® 2000 reagent		High	High	High efficiency versatile reagent for a wide range of common cell types.
Lipofectamine® RNAiMAX reagent		Superior	Superior	Most efficient reagent for siRNA/miRNA delivery. Efficient gene knock-down.
Neon® electroporation		Maximal	Good	High-efficiency electroporation for all cell lines.

Symbol	Explanation	Symbol	Explanation	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

Vector-mediated RNAi

For cell types not amenable to lipid-mediated transfection, such as hard-to-transfect, primary, and non-dividing cells, viral vectors containing RNAi cassettes are often employed. Viral delivery can also be used to create stable cell lines with inducible RNAi or to express RNAi sequences with tissue-specific promoters. Adenoviral vectors work well for transient delivery in many cell types, while lentiviral vectors are best for stable delivery in dividing and non-dividing cells, lentiviral vectors are best.

- **BLOCK-iT™ Adenoviral RNAi Expression System** facilitates the creation and delivery of a replication-incompetent adenovirus to transiently express shRNA in most dividing or non-dividing mammalian cell types and animal models for RNAi analysis. The key advantage of the BLOCK-iT™ Adenoviral RNAi Expression System is Gateway® recombination technology, which simplifies the cloning and generation of an adenoviral vector, eliminating the tedious and time-consuming manipulations, screening, and multiple transformations that other adenoviral systems require
- **BLOCK-iT™ Lentiviral RNAi Expression System** enables the creation and delivery of engineered shRNAs and miRNAs into dividing and non-dividing mammalian cells, including primary and hard-to-transfect cells. The system can be used without selection for transient RNAi analysis or, with appropriate antibiotic selection, to generate a stable cell line for long-term knockdown studies.
- **BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System** combines BLOCK-iT™ Pol II miR RNAi and ViraPower™ Lentiviral technologies to facilitate the creation and stable delivery of engineered miRNAs into nondividing, primary, and hard-to-transfect cells. The Pol II promoter in the expression vector enables co-cistronic expression of multiple miRNAs, allowing knockdown of multiple targets from a single construct, a process is ideal for knockdown of more than one pathway component or splice variant, or for using knockdown to create synthetic phenotypes.
- **BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System with EmGFP** provides all the components and benefits of the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System listed above, plus the easy expression tracking with co-cistronic EmGFP. The HiPerform™ version of the expression vector contains an mRNA-stabilizing sequence (WPRE) and a nuclear import sequence (cPPT) that can generate up to 5-fold higher virus titers.
- **BLOCK-iT™ Inducible H1 Lentiviral RNAi System** is a complete lentiviral system for long-term inducible or constitutive shRNA expression in any cell type. Regulation of the RNAi response via the tetracycline operator ($TetO_2$) sequence permits the study of changes over time and loss-of-function experiments even with essential genes, and provides an excellent control system to measure phenotypic changes during recovery of gene function.

Viral system	When to use
Adenoviral RNAi delivery	• High-level transient shRNA expression • Effective delivery to a wide range of human cell types • Studies in animal models
Lentiviral RNAi delivery	• Stable expression of RNAi in any cell line, even in non-dividing cells including stem cells, lymphocytes, and neurons • Inducible or constitutive shRNA or miR RNAi expression • Studies in animal models

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

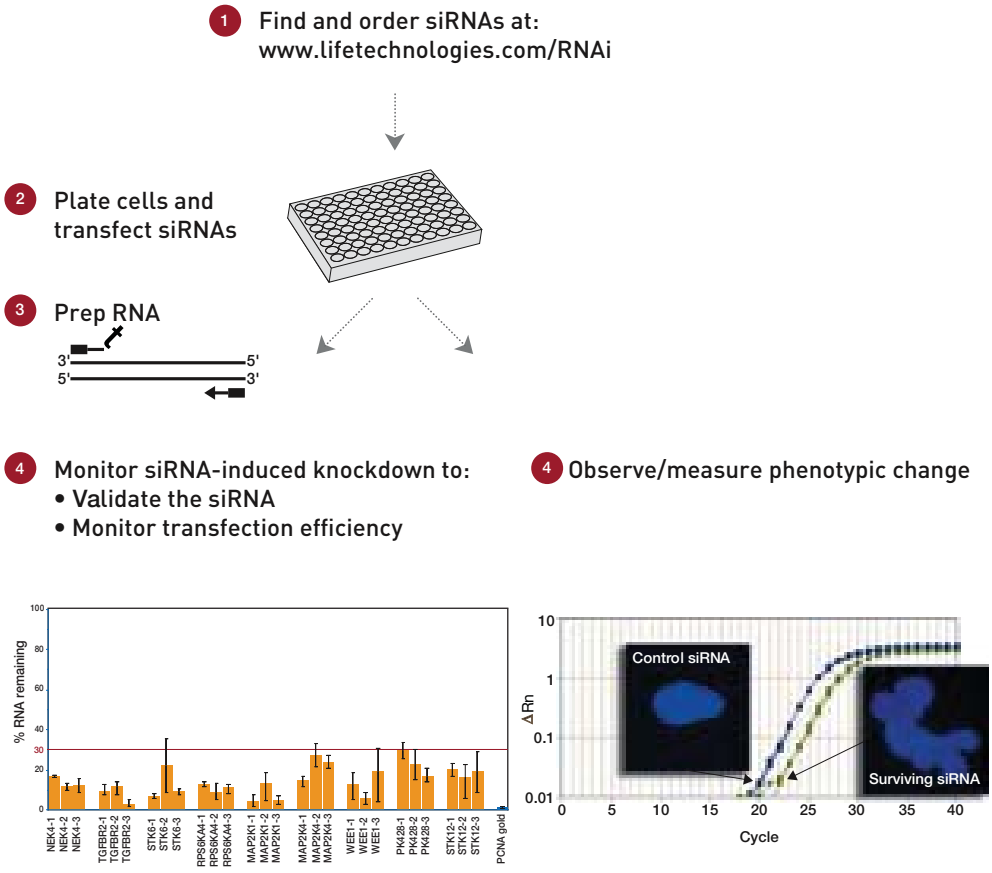


Figure 6.4 RNAi workflow following siRNA design and synthesis.

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.

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 controls	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 controls	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 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.

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.

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.

Tips for a successful siRNA experiment

1. **Design and test two to four siRNA sequences per gene.** Do not attempt to design siRNAs on your own. Go to **www.lifetechnologies.com/rnai** and utilize the best-in-class design algorithms to design your siRNAs.

2. **Avoid RNases!** Trace amounts of ribonucleases can sabotage siRNA experiments. Since RNases are present throughout the laboratory environment on your skin, in the air, on anything touched by bare hands or on anything left open to the air, it is important to take steps to prevent and eliminate RNase contamination. Life Technologies™ offers a complete line of products designed to detect and eliminate RNases.

3. **Maintain healthy cell cultures and strict protocols for good transfection reproducibility.** In general, healthy cells are transfected at higher efficiency than poorly maintained cells. Routinely subculturing cells at a low passage number ensures that there will be minimal instability in continuous cell lines from one experiment to the next. When performing optimization experiments we recommend transfecting cells within 50 passages, since transfection efficiency drops over time.

4. **Avoid antibiotic use.** Avoid the use of antibiotics during plating and up to 72 hours after transfection. Antibiotics have been shown to accumulate to toxic levels in permeabilized cells. Additionally, some cells and transfection reagents require serum-free conditions for optimal siRNA delivery. We suggest you perform a pilot transfection experiment in both normal growth media and serum-free media to determine the best condition for each transfection.

5. **Transfect siRNAs using optimized reagents.** Use an optimized siRNA transfection reagent and protocol for your cell type. The choice of transfection reagent is critical for success in siRNA experiments. It is essential to use transfection reagents formulated to deliver small RNAs (most commercially available transfection reagents were designed for large plasmid DNA, not small RNA molecules). Also, some reagents have been developed for the transfection of specific cell lines while others have broader specificity. For help selecting the appropriate transfection reagent, see **siRNA transfection**, page 88.

6. **Use an appropriate positive control to optimize transfection and assay conditions.** Housekeeping genes are suitable positive controls for most cell types. To optimize conditions, transfect target cells with several concentrations of an siRNA specific to your chosen positive control and to your experimental target siRNA. Measure the reduction in the control protein or mRNA level compared to untransfected cells 48 hours after transfection. Too much siRNA can lead to cell toxicity and death. For maximum convenience, Life Technologies™ offers positive control siRNAs against a variety of gene targets (see page 92).

7. **Use a negative control siRNA to distinguish non-specific effects** (see page 92). Negative controls should be designed by scrambling the nucleotide sequence of the most active siRNA. However, be sure to perform a homology search to ensure that your negative control sequence lacks homology to the genome of the organism being studied.

8. **Use labeled siRNAs for protocol optimization.** Fluorescently labeled siRNA can be used to analyze siRNA stability and transfection efficiency. Labeled siRNA is also useful to study siRNA subcellular localization and in double label experiments (with a labeled antibody) to visualize cells that receive siRNA during transfection and to correlate transfection with down-regulation of the target protein.

Optimization of siRNA Transfection

Factors affecting siRNA transfection efficiency

Maximizing transfection efficiency while minimizing cytotoxicity are crucial for optimal gene silencing. Similar to balancing siRNA-induced knockdown and cell viability, there may also be a balance between siRNA delivery and downstream phenotypic assay conditions. It may be necessary to re-optimize siRNA delivery conditions for different downstream assays that are used in siRNA screening passes. The best transfection efficiencies are achieved for each cell type by identifying the following factors (in order of importance):

1. Choice of transfection reagent
2. Volume of transfection agent
3. Amount of siRNA
4. Cell density at the time of transfection
5. Length of exposure of cells to transfection agent/siRNA complexes
6. Transfection method: traditional transfection where cells are pre-plated or reverse transfection where cells are transfected as they adhere to the plate
7. Presence or absence of serum

Once the conditions for maximal gene silencing are determined, keep them constant among experiments with a given cell type.

Appendix

Troubleshooting

The table below lists some potential problems and possible solutions that may help you troubleshoot your cell culture experiments. Note that the list below includes only the most commonly encountered problems in cell culture, and provides guidelines to solutions only. To help evaluate your results more successfully, we recommend that you consult the manuals and product information sheets provided with the products you are using as well as the published literature and books on the subject.

Problem	Reason	Solution
No viable cells after thawing stock	Cells were stored incorrectly	Obtain new stock and store in liquid nitrogen. Keep the cells in liquid nitrogen until thawing.
	Home made freezer stock is not viable	Freeze cells at a density recommended by the supplier.
		Use low-passage cells to make your own freezer stocks.
		Follow procedures for freezing cells **exactly** as recommeded by the supplier. Note that the freezing procedure recommended in this handbook is a general procedure provided as a guideline only.
		Obtain new stock.
	Cells were thawed incorrectly	Follow procedures for thawing cells **exactly** as recommeded by the supplier. Note that the thawing procedure recommended in this handbook is a general procedure provided as a guideline only.
		Make sure that you thaw the frozen cells quickly, but dilute them slowly using pre-warmed growth medium before plating.
	Thawing medium is not correct	Use the medium recommended by the supplier. Make sure the medium is pre-warmed.
	Cells are too dilute	Plate thawed cells at high density as recommended by the supplier to optimize recovery.
	Cells not handled gently	Freezing and thawing procedures are stressful to most cells. Do **not** vortex, bang the flasks to dislodge the cells (except when culturing insect cells), or centrifuge the cells at high speeds.
	Glycerol used in the freezing medium was stored in light (if applicable)	If stored in light, glycerol is gets converted to acrolein, which toxic to cells. Obtain new stock.
Cells grow slowly	Growth medium is not correct	Use pre-warmed growth medium as recommended by the supplier.
	Serum in the growth medium is of poor quality	Use serum from a different lot.
	Cells have been passaged too many times	Use healthy, low passage-number cells.
	Cells were allowed to grow beyond confluency	Passage mammalian cells when they are in the log-phase before they reach confluence.
	Culture is contaminated with mycoplasma	Discard cells, media, and reagents. Obtain new stock of cells, and use them with fresh media and reagents.

Cell Culture and Transfection Products

Life Technologies™ offers a variety of primary cultures and established cell lines, as well as reagents, media, sera, and growth factors for your cell culture experiments. The tables below contain lists of the more commonly used cell lines and other cell culture products available from Life Technologies™. For more information on Life Technologies™ and Gibco® products, refer to **www.lifetechnologies.com/cellculture.**

Cell lines

In addition to the mammalian and insect cell lines listed below, Life Technologies™ offers primary mammalian cells and complete cell culture systems, including keratinocyte, fibroblast, melanocyte, hepatocyte, corneal and mammary epithelial, large vessel and microvascular endothelial, smooth muscle, and neuronal cell culture systems. For a comprehensive list of cells, technical resources and related technologies, visit **www.lifetechnologies.com/cellculture**.

Product	Quantity	Cat. no.
Mammalian Cell Lines		
293-F Cells, SFM adapted (7.5×10^6 cells)	1.5 mL	11625-019
293-H Cells, SFM adapted (7.5×10^6 cells)	1.5 mL	11631-017
293FT Cell Line	3×10^6 cells	R700-07
293A Cell Line	3×10^6 cells	R705-07
GripTite™ 293 MSR Cell line	1 kit	R795-07
CHO DG44 Cells (cGMP banked) and Media Kit	1 kit	A11000-01
Insect Cell Lines		
Sf9 Cells (SFM adapted) (1.5×10^7 cells)	1.5 mL	11496-015
Sf21 Cells (SFM adapted) (1.5×10^7 cells)	1.5 mL	11497-013
Mimic™ Sf9 Insect Cells (1×10^7 cells)	1 mL	12552-014
Sf9 Cells adapted in Sf-900™ III SFM (1.5×10^7 cells)	1 vial	12659-017
Sf21 Cells adapted in Sf-900™ III SFM (1.5×10^7 cells)	1 vial	12682-019
Sf9 Frozen Cells (Grace's media) (1×10^7 cells)	1 mL	B825-01
Sf21 Frozen Cells (Grace's media) (1×10^7 cells)	1 mL	B821-01
High Five™ Cells, adapted to Express Five® SFM (3×10^6 cells)	1 mL	B855-02

Media for mammalian cell culture

Life Technologies™ provides you with all of your cell culture needs through its Gibco® Cell Culture Media, and offers products to support the growth of a range of mammalian cell lines. All cell culture media products available from Life Technologies™ are tested for contamination, and guaranteed for their quality, safety, consistency, and regulatory compliance. In addition to the media listed below, Life Technologies™ offers a large selection of serum-free and specilized media for culturing primary cells, established cell lines, and stem cells, as well as for virus production, protein expression, stem cell differentiation, and cytogenetics. For more information and a complete list of cell culture media, visit **www.lifetechnologies.com/cellculture**.

Product*	Quantity†	Cat. no.
D-MEM (1X), liquid (high glucose with no glutamine)	10 × 500 mL	35053-036
D-MEM (1X), liquid (low glucose with no glutamine)	500 mL	11054-020
D-MEM (1X), liquid (high glucose with GlutaMAX™-I)	10 × 500 mL	10564-029
D-MEM (1X), liquid (low glucose with GlutaMAX™-I)	10 × 500 mL	10567-022
Advanced D-MEM (1X), liquid (high glucose with no glutamine)	10 × 500 mL	12491-023
D-MEM/F-12, liquid, 1:1 (with GlutaMAX™-I)	10 × 500 mL	10565-042
D-MEM/F-12, liquid, 1:1 (with L-glutamine)	10 × 500 mL	11320-082
Advanced D-MEM/F-12, liquid, 1:1 (with no glutamine)	10 × 500 mL	12634-028
Minimum Essential Medium (MEM) (1X), liquid (with no glutamine)	10 × 500 mL	11090-099
Minimum Essential Medium (MEM) (1X), liquid (with GlutaMAX™-I)	10 × 500 mL	41090-101
Advanced MEM (Minimum Essential Medium) (1X), liquid (with no glutamine)	10 × 500 mL	12492-021
RPMI Medium 1640 (1X), liquid (with no glutamine)	1000 mL	21870-084
RPMI Medium 1640 (1X), liquid (with GlutaMAX™-I)	10 × 500 mL	61870-127
Advanced RPMI Medium 1640 (1X), liquid (with no glutamine)	10 × 500 mL	12633-020
293 SFM II, liquid	1,000 mL	11686-029
CD 293 Medium, liquid	1,000 mL	11913-019
Gibco® Freestyle™ 293 Expression Medium	1,000 mL	12338-018
CD CHO Medium (1X), liquid	1,000 mL	10743-029
CHO-S-SFM II	1,000 mL	12052-098
Gibco® Freestyle™ CHO Expression Medium	1,000 mL	12651-014
CD OptiCHO™ Medium (1X), liquid	1,000 mL	12681-011
Recovery™ Cell Culture Freezing Medium, liquid	50 mL	12648-010
Synth-a-Freeze® Cryopreservation Medium	50 mL	R-005-50

*Most of the media listed in this table are available with L-glutamine, GlutaMAX™-I, or no glutamine, with or without phenol red, as well as in powder and liquid formulations. †Also available in different quantities and packaging sizes.

Media for insect cell culture

Insect cell culture is a common choice for heterologous protein expression. For large scale production or basic research, insect cells are able to express large quantities of protein with complex post-translational modifications. Gibco® insect media from Life Technologies™ have been formulated for maximum growth and protein yields. For more information, visit **www.lifetechnologies.com**.

Product*	Quantity†	Cat. no.
Grace's Insect Cell Medium, Unsupplemented (1X), liquid	500 mL	11595-030
Grace's Insect Cell Medium, Supplemented (1X), liquid	500 mL	11605-094
Sf-900 II SFM (1X), liquid	500 mL	10902-096
Sf-900™ III SFM (1X), liquid	500 mL	12658-019
Schneider's *Drosophila* Medium (1X), liquid	10 × 500 mL	11720-067
IPL-41 Insect Medium (1X), liquid	1,000 mL	11405-081
Express Five® SFM (1X), liquid	1,000 mL	10486-025

*Most of the media listed in this table are available in powder and liquid formulations. †Also available in different quantities and packaging sizes.

Serum products for cell culture

Life Technologies™ supplies a wide range of Gibco® animal sera, both bovine and nonbovine, for cell culture applications, the most widely used being fetal bovine serum (FBS). The table below lists a small selection of sera available from Life Technologies™. For a complete list and more information on the use, sources, traceability, collection, and bottling of serum, refer to **www.lifetechnologies.com**.

Product*	Quantity†	Cat. no.
Fetal Bovine Serum (FBS), Certified, Heat-Inactivated	500 mL	10082-147
Fetal Bovine Serum (FBS), Qualified, Heat-Inactivated	500 mL	10100-147
Fetal Bovine Serum (FBS), Certified	1,000 mL	16000-069
Fetal Bovine Serum (FBS), Qualified	1,000 mL	10099-158
OneShot™ Fetal Bovine Serum, Certified	50 mL	16000-077
OneShot™ Fetal Bovine Serum, Qualified	50 mL	26140-111
Bovine Serum	500 mL	16170-078
Bovine Serum, Heat-Inactivated	500 mL	26170-043
Horse Serum	500 mL	16050-122
Horse Serum, Heat-Inactivated	500 mL	26050-088
Porcine Serum	500 mL	26250-084
Goat Serum	500 mL	16210-072
Rabbit Serum	500 mL	16120-099

*To ensure supply, Life Technologies™ sources FBS from the United States, New Zealand, Australia, and other countries meeting USDA importation requirements (i.e., USDA-Approved). All other serum products are sourced from New Zealand with the exception of rabbit serum, which is sourced from the United States. †Also available in different quantities and packaging sizes.

Laboratory reagents for cell culture

The table below lists a small selection of laboratory reagents for cell culture that are available from Life Technologies™. For more information and a complete list, refer to **www.lifetechnologies.com**.

Product	Quantity*	Cat. no.
Balanced Salt Solutions: D-PBS†, EBSS, HBSS‡, PBS		
Dulbecco's Phosphate Buffered Saline (D-PBS) (1X), liquid	1,000 mL	14040-117
Earle's Balanced Salt Solution (EBSS) (1X), liquid	500 mL	14155-063
Hank's Balanced Salt Solution (HBSS) (1X), liquid	1,000 mL	14025-076
Phosphate-Buffered Saline (PBS) pH 7.4 (1X), liquid	500 mL	10010-023
Phosphate-Buffered Saline (PBS) pH 7.2 (1X), liquid	500 mL	70013-032
Buffers and Chemicals		
HEPES Buffer Solution (1M)	20 × 100 mL	15630-130
Sodium Bicarbonate Solution, 7.5% (w/v)	100 mL	25080-094
Cell Dissociation Reagents		
TrypLE™ Express Dissociation Reagent with Phenol Red	500 mL	12605-028
TrypLE™ Express Dissociation Reagent without Phenol Red	100 mL	12604-013
TrypLE™ Select Dissociation Reagent	500 mL	12563-029
Trypsin, 0.5% (10X), liquid, with EDTA 4Na, without Phenol Red	100 mL	15400-054
Trypsin, 0.25% (10X), liquid, without EDTA, with Phenol Red	500 mL	15050-057
Collagenase Type I	1 g	17100-017
Collagenase Type II	1 g	17101-015
Dispase	5 g	17105-041
Trypsin Inhibitor, soybean	1 g	17075-029
Supplements		
L-Glutamine†, 200 mM (100X), liquid	100 mL	25030-081
GlutaMAX™-I Supplement	100 mL	35050-061
D-Glucose (Dextrose)	1 kg	15023-021
Pluronic® F-68, 10% (100X)	100 mL	24040-032
MEM Amino Acids Solution (50X), liquid	100 mL	11130-051
MEM Non-Essential Amino Acids Solution 10 mM (100X), liquid	100 mL	11140-050
MEM Sodium Pyruvate Solution 10 mM (100X), liquid	100 mL	11360-070
MEM Vitamin Solution (100X), liquid	100 mL	11120-052
2-Mercaptoethanol (1,000X), liquid	50 mL	21985-023
CHO CD EfficientFeed™ Kit	1 kit	A10241-01
FoamAway™ Irradiated AOF Anti-Foaming Agent	500 mL	A1036902

*Products are also available in different quantities and packaging sizes. †Product is available in liquid or powder formats. ‡HBSS is available with or without magnesium and calcium, and with or without phenol red.

Antibiotics and antimycotics

Antibiotics are used to protect the integrity of your cell culture as well for selection and establishmeny of cell lines; Life Technologies™ offers a wide selection of antibiotics, antimycotics and detection kits. For more information, refer to **www.lifetechnologies.com**.

Product	Quantity*	Cat. no.
Antibiotics and Antimycotics		
Antibiotic-Antimycotic (100X), liquid	100 mL	15240-062
Fungizone® Antimycotic, liquid	20 mL	15290-018
Gentamycin Reagent Solution (10 mg/mL), liquid	10 mL	15710-064
Gentamycin Reagent Solution (50 mg/mL), liquid	10 mL	15750-060
Gentamycin/Amphotericin Solution	10 × 1 mL	R-015-10
Neomycin Sulfate, powder	100 g	21810-031
Penicillin-Streptomycin, liquid	100 mL	15140-122
Penicillin-Streptomycin-Neomycin (PSN) Antibiotic Mixture	100 mL	15640-055
Selection Antibiotics		
Geneticin® Selective Antibiotic, liquid	20 mL	10131-027
Geneticin® Selective Antibiotic, powder	1 g	11811-023
Hygromycin B	20 mL	10687-010
Puromycin Dihydrochloride, Selection Antibiotic, liquid	10 × 1 mL	A11138-03
Blasticidin S HCl, Selection Antibiotic, liquid	20 mL	A11139-02
Blasticidin S HCl, powder	50 mg	R210-01
Zeocin™ Selection Reagent, powder	1 g	R250-01
Contamination Detection Kits		
Cell Culture Contamination Detection Kit - 200 assays	1 kit	C7028
MycoFluor™ Mycoplasma Detection Kit	1 kit	M7006
*Products are also available in different quantities and packaging sizes.		

Growth factors and purified proteins

Life Technologies™ offers an array of highly-potent and highly-pure growth factors, chemokines, cytokines, and other proteins and protein inhibitors validated for use in cell culture. These products have been validated in live cell bioassays using Gibco® media. For more information and a complete list, refer to **www.lifetechnologies.com/cellculture**.

Accessory products for cell culture

The table below lists a small selection of accessory products for cell culture that are available from Life Technologies™. For more information and a complete list, refer to **www.lifetechnologies.com**.

Product	Quantity*	Cat. no.
Attachment Factors and Matrices		
Geltrex® Reduced Growth Factor Basement Membrane Matrix	5 mL	12760-021
AlgiMatrix™ 3D culture System 6-well plates	1 plate	A10982-01
AlgiMatrix™ 3D culture System 24-well plates	1 plate	12684-023
AlgiMatrix™ 3D culture System 96-well plates	1 plate	12684-015
Human Plasma Fibronectin	5 mg	33016-015
Natural Mouse Laminin	1 mg	23017-015
Instruments		
Countess® II Automated Cell Counter	1 unit	AMQAX1000
Countess® II FL Automated Cell Counter	1 unit	AMQAF1000
Mallassez Hemocytometer	1 unit	99503
Qubit® 3.0 Fluorometer	1 unit	Q33217
*Some of the products listed in the table are also available in different quantities and packaging sizes.		

Transfection reagents

Life Technologies™ offers the most complete collection of cationic lipid-based transfection reagents with exceptional performance that can be used for delivery of DNA, siRNA, oligonucleotide, and RNA. The table below lists a small selection of cationic-lipid transfection reagents that are available from Life Technologies™. For more information and a complete list, refer to **www.lifetechnologies.com/transfection**.

Product	Quantity*	Cat. no.
Lipofectamine® 3000 Transfection Reagent	1.5 mL	L3000-015
Lipofectamine® 2000 Transfection Reagent	1.5 mL	11668-019
Lipofectamine® 2000 CD (Chemically Defined) Transfection Reagent	1 mL	12566-014
Lipofectamine® RNAiMAX Transfection Reagent	1.5 mL	13778-150
Lipofectamine® LTX with Plus™ Reagent	1 mL	15338-100
Lipofectamine® MessengerMAX	1.5 mL	LMRNA015
Invivofectamine® 2.0 Reagent	1 mL	13775-01
ExpiFectamine™ 293 Transfection Kit	1 × 1 L culture	A14524
FreeStyle™ MAX Reagent	1 mL	16447-100
Cellfectin® II Reagent	1 mL	10362-100
*Products are also available in different quantities and packaging sizes.		

Neon® Transfection System

The Neon® Transfection System efficiently delivers nucleic acids, proteins, and siRNA into all mammalian cell types, including primary and immortalized hematopoietic cells, stem cells, and primary cells, with a high cell survival rate. For more information on the Neon® Transfection System and optimized Neon® transfection protocols for many commonly used cell types, refer to **www.lifetechnologies.com/neon**.

Product	Quantity	Cat. no.
Neon® Transfection System 100 µL Kit	192 reactions	MPK10096
Neon® Transfection System 10 µL Kit	192 reactions	MPK1096
Neon® Transfection System	1 each	MPK5000
Neon® Transfection System Starter Pack	1 pack	MPK5000S
Neon® Transfection System Pipette	1 each	MPP100
Neon® Transfection System Pipette Station	1 each	MPS100
Neon® Transfection Tubes	1 pack	MPT100

RNA interference

RNAi is a specific, potent, and highly successful approach for loss-of-function studies in virtually all eukaryotic organisms. Life Technologies™ has developed two types of small RNA molecules that function in RNAi, short interfering RNA (siRNA) molecules and microRNAs (miRNA), and offers a variety of products for RNAi analysis *in vitro* and *in vivo*, including libraries for high-throughput applications. Your choice of tool depends on your model system, the length of time you require knockdown, and other experimental parameters.

In addition, Life Technologies™ offers the most complete collection of cationic lipid-based transfection reagents with exceptional performance that can be used for delivery of assorted RNAi reagents, including shRNA and miR RNAi vectors and synthetic molecules such as siRNA, Stealth RNAi™ siRNA, and Dicer-generated siRNAi pools. Further, cell specific RNAi transfection protocols have been developed using these transfection reagents for many popular cell lines.

For more information and a complete list of RNAi products available from Life Technologies™, refer to **www.lifetechnologies.com/rnai**.

Additional Resources

Mammalian and insect cell cultures

For more information on mammalian and insect cell culture, cell type specific protocols, and additional cell culture products, refer to **Mammalian Cell Culture** and **Insect Cell Culture** portals on our website.

Cell and tissue analysis

Understanding the structural and functional relationships of cells and tissues is critical to advancements in key research disciplines, including molecular biology, genetics, reproductive function, immunology, cancer and neurobiology. Key components of cell and tissue analysis are cell viability and proliferation, cell signaling pathways, cell cycle analysis, and cell structure. Life Technologies™ has a broad portfolio of reagents and kits for cell and tissue analysis, including Molecular Probes® Fluorescence Products and technologies as well as Dynal® Bead-based Solutions for cell isolation and expansion. From antibodies and stem cell research products to benchtop instruments like the Countess® II Automated Cell Counter and the Qubit® Fluorometer, Life Technologies™ has the tools essential for cellular analysis research. For more information, refer to the **Cell & Tissue Analysis** portal on our website.

Safety data sheets

Safety Data Sheets (SDS) are available at **www.lifetechnologies.com/sds**.

Certificate of analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

Technical support

For more information or technical assistance, call, write, fax, or email our award winning Technical Support.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

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