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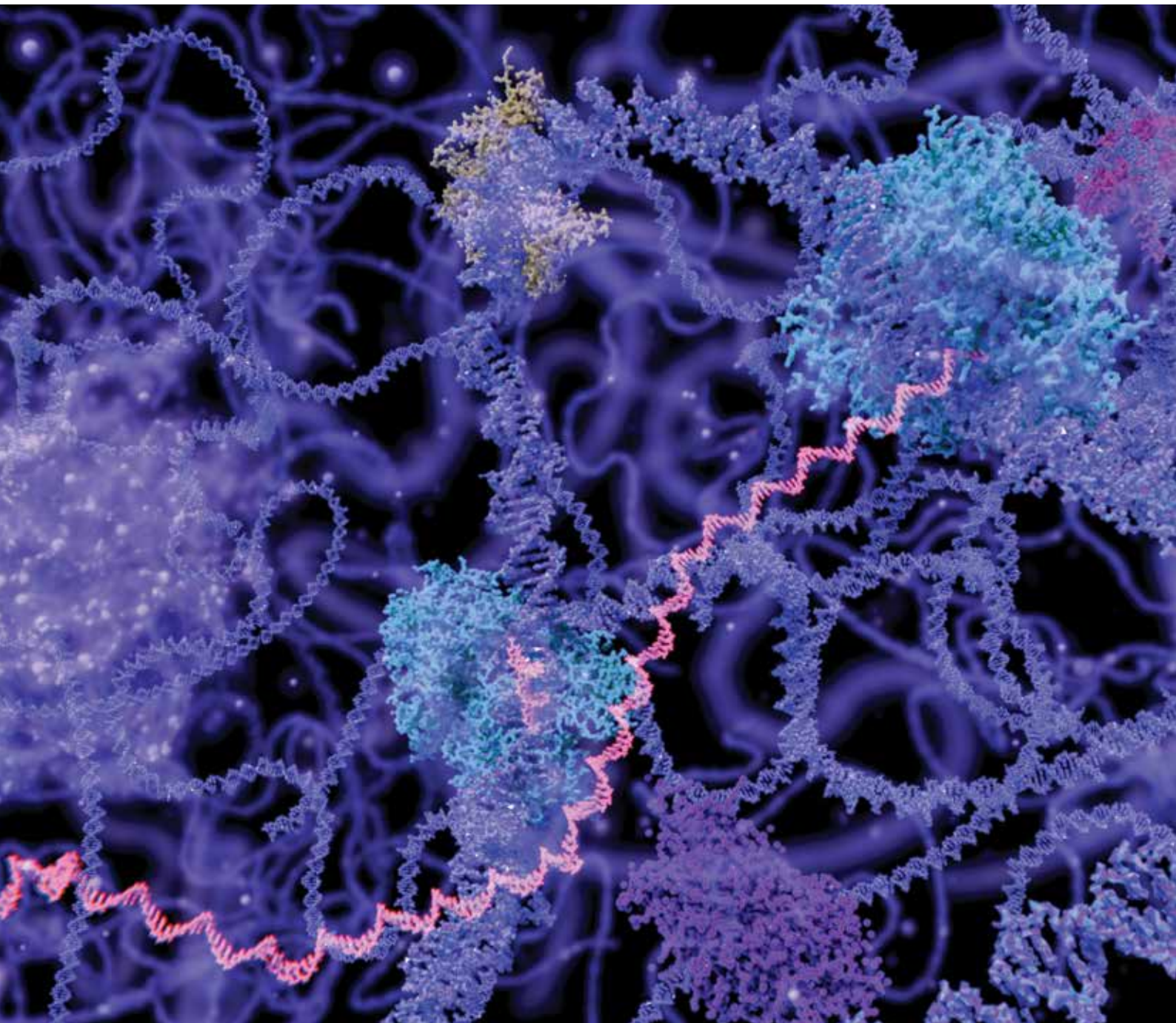
RNAi Application Guide 2016/17



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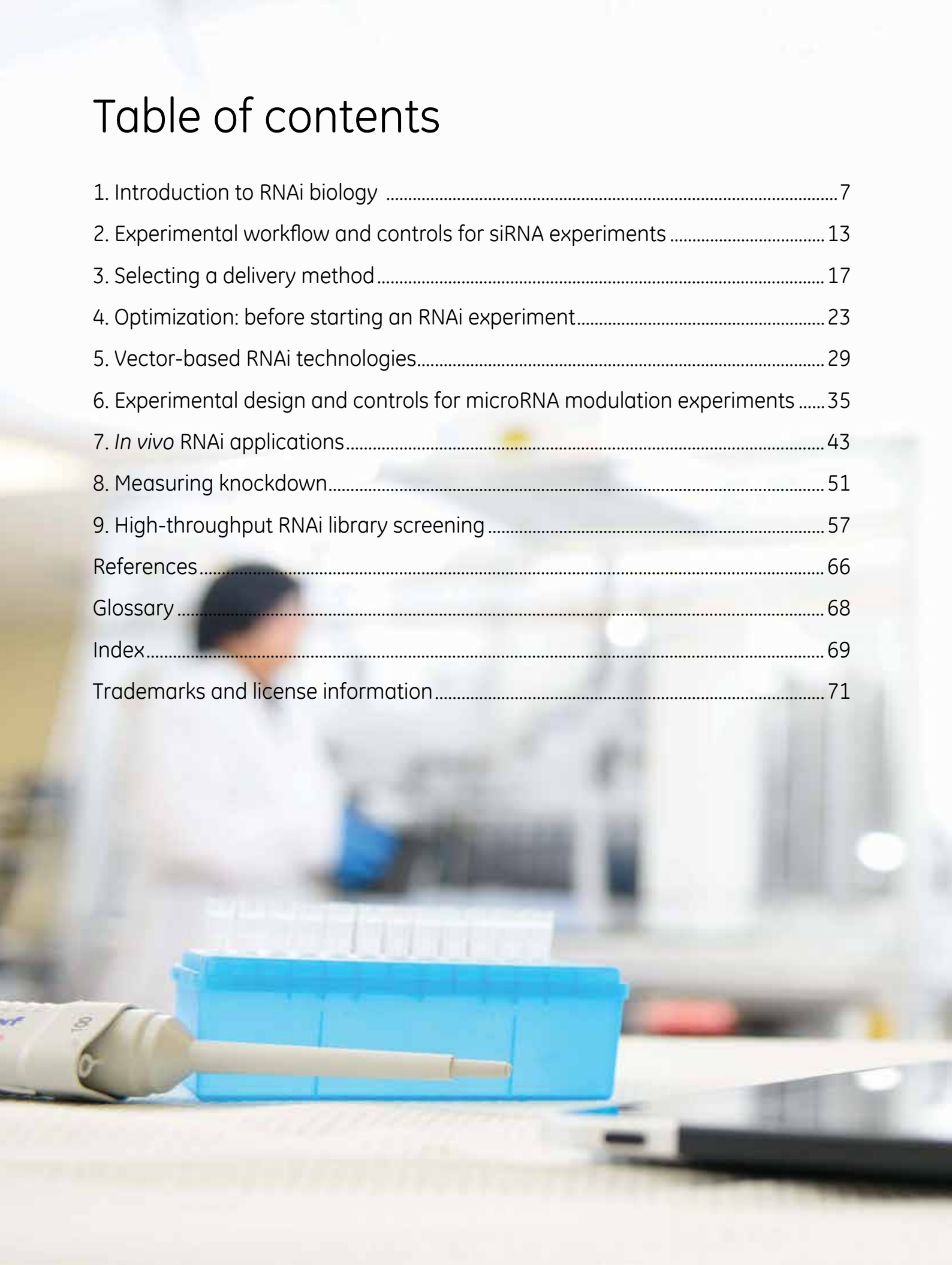
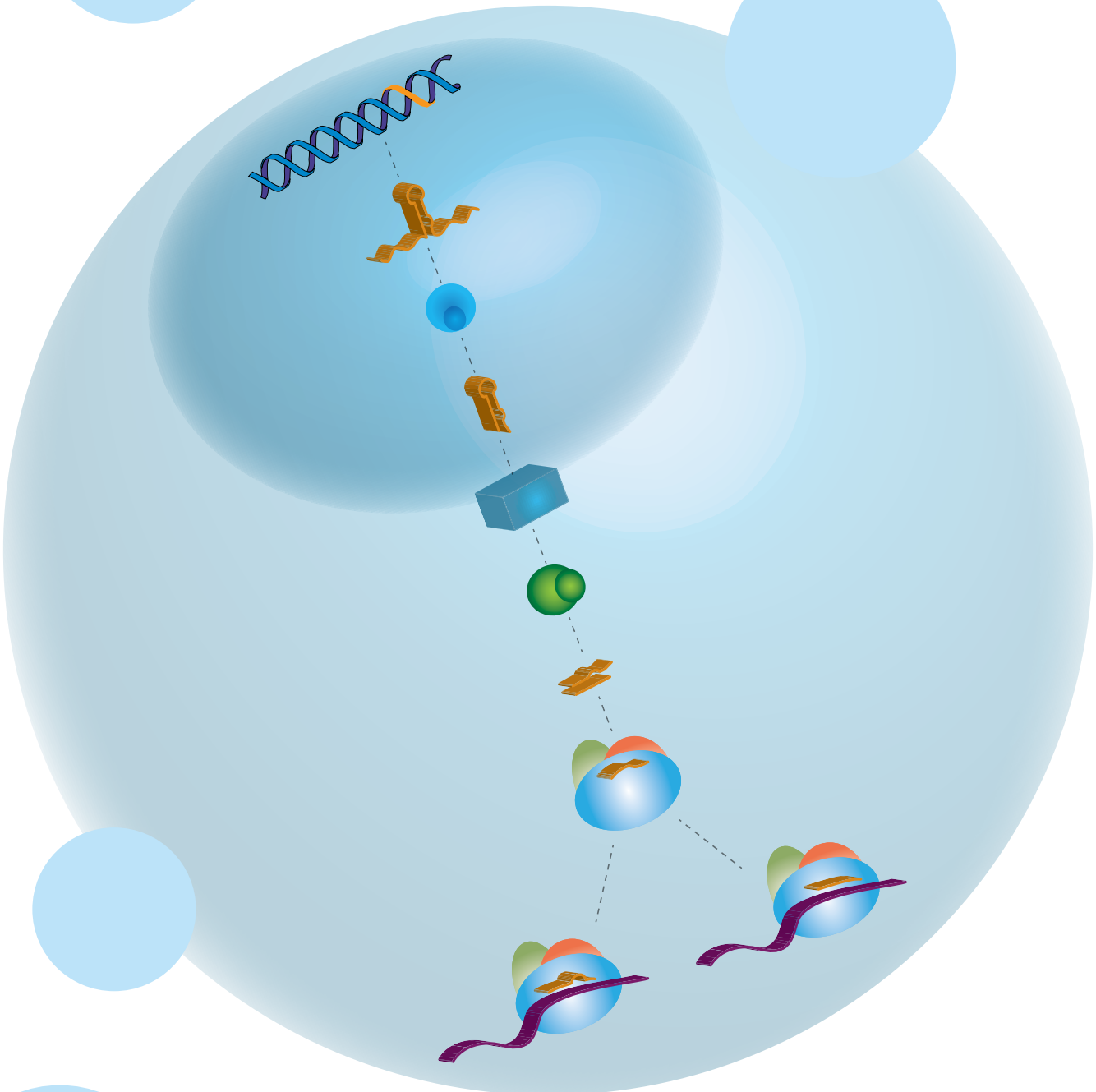




Chapter 1

Introduction to RNAi biology

Introduction to RNAi biology

RNA Interference (RNAi) has revolutionized the study of biology and offers numerous applications in basic biology as well as drug discovery research.

In 2001, two groups independently demonstrated that short, synthetic RNA duplexes (19–25 bp), siRNAs, mimicking the endogenous microRNA pathway, could be introduced into cultured mammalian cells to elicit potent, sequence-specific inhibition of target messenger RNA (mRNA) without induction of the interferon response.[1,2] Shortly thereafter, several groups published evidence that RNAi could also be effectively achieved in mammalian cells by transfection of plasmids encoding short hairpin RNAs (shRNAs), short RNA transcripts consisting of 19-29 base pair stems bridged by 4-9 nucleotide loops.[3-5] Researchers quickly recognized the utility of RNAi as a valuable tool for systematic gene functional analyses. The result has been a substantial shift towards implementing RNAi as a standard for discovery biology and target validation. The sequence complementarity of siRNA or shRNA-mediated RNAi permits knockdown of discrete genes, splice variants, or closely related family members, and is used to perform individual gene functional analyses, or survey entire genomes in a high throughput manner.

Harnessing RNAi

The discovery of the endogenous microRNA pathway has resulted in the development of increasingly specific RNAi tools for a variety of research applications.[9-11] The first and most commonly used tool generated to silence endogenous gene expression were synthetic or *in vitro* transcribed siRNAs, which allow for targeted short-term gene silencing. Experimentally, siRNAs or expression vectors encoding shRNAs are transfected into the cytoplasm of cells and loaded into the RNA-induced silencing complex (RISC) to cause target gene silencing. shRNA in viral particle format is able to integrate into the genome and is used for generation of stable cell lines, delivery into difficult-to-transfect cells as well as for *in vivo* RNAi applications. Endogenous microRNAs have also been shown to regulate gene expression through both translational attenuation and mRNA degradation.[12] To investigate the function of microRNAs, synthetic or expression vector-based microRNA mimics and inhibitors are used for transient and long-term expression. Experimentally, microRNA mimics and inhibitors are introduced to the cells in a similar manner to synthetic siRNAs or expression-based shRNAs. microRNA mimics and inhibitors are valuable tools for probing the function of target genes, and have helped reveal the role of microRNAs in development, differentiation, and disease.[13-17]

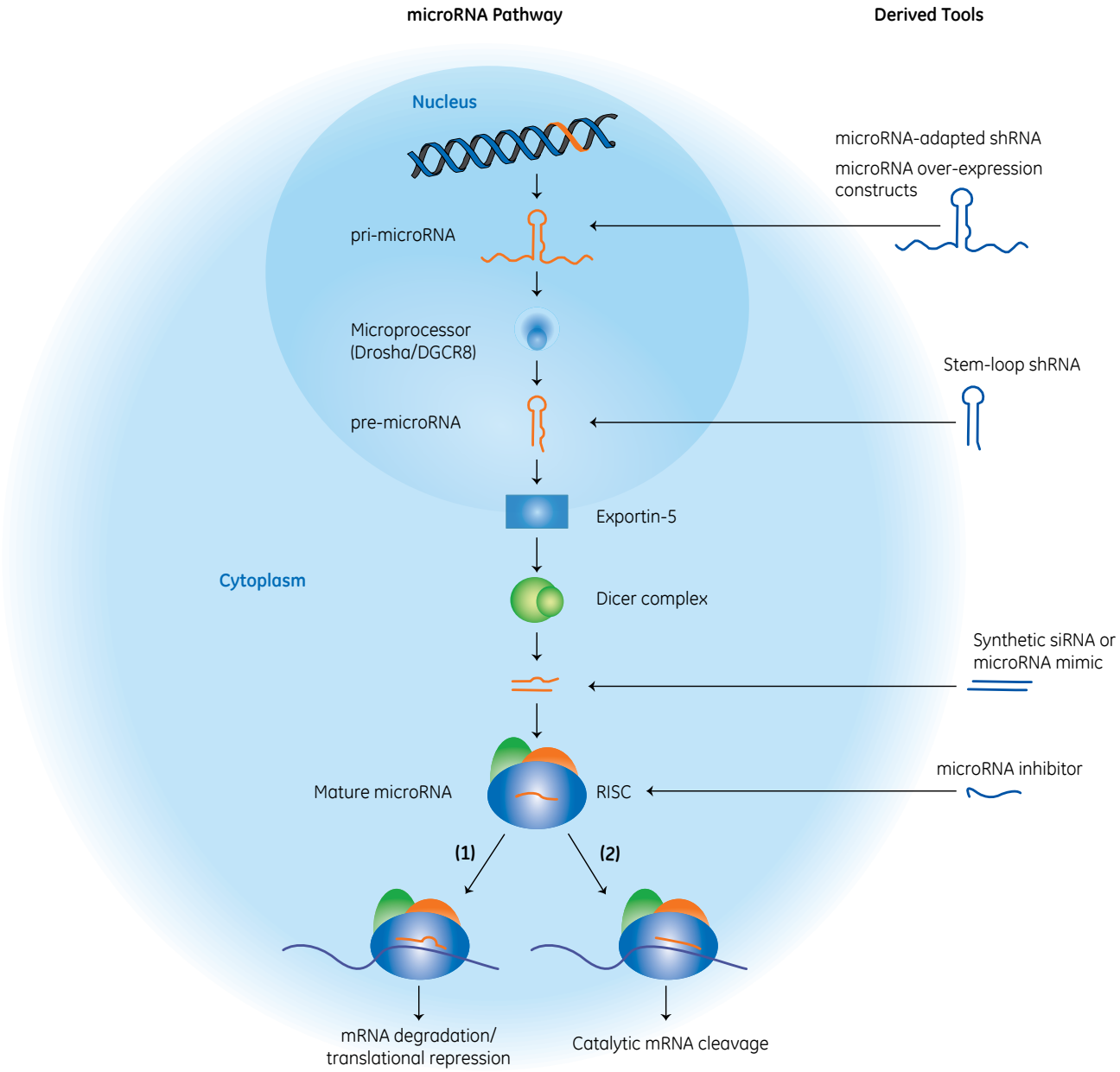


Figure 1. Mechanism of RNAi

The endogenous mammalian microRNA pathway (orange molecules) starts with transcription of the primary microRNA (pri-microRNA) in the nucleus and ends with gene silencing either by: (1) seed-mediated mRNA degradation or translational repression or (2) catalytic mRNA cleavage. The blue molecules to the right of the image illustrate RNAi tools that have been developed and where they enter the endogenous RNAi mechanism.

Development of effective and specific RNAi tools

One of the early challenges to the widespread adoption of RNAi as a tool for gene functional analysis was the design of siRNAs with a high degree of potency and specificity. To this end, large scale functional studies were performed, which led to the development of rules for the rational design of siRNAs.[18] The development of advanced algorithms using these rules greatly improved the frequency of identifying potent siRNA sequences. As research on specificity progressed, it was recognized that in addition to the gene of interest, unintended endogenous genes were also being silenced, a phenomenon called off-target effects.[19-22] The primary source of off-target effects in mammalian cell culture occurs through activation of a microRNA-like mechanism using the seed region.[23-25] To avoid seed-based off-targeting, siRNA and shRNA designs should take into account the content and avoiding sequence matches of the 6-8 nucleotides of the 5′ end of the antisense strand (seed region) to 3′ UTR of known microRNA targets. Therefore, it is important to take the seed region content into consideration when designing an siRNA or shRNA. Other sources of off-target effects include cellular responses to the delivery reagent, overall siRNA concentration, and incorrect strand loading into the RISC complex.[26-28] To minimize siRNA concentration dependent off-target effects, using the minimal concentration necessary for effective gene silencing is recommended. One strategy to address concentration issues is to pool several different siRNAs targeting an individual gene at unique locations, with each siRNA being used at a low concentration thereby reducing the likelihood of off-targeting and the frequency of off-target phenotypes.[29] This strategy is likely effective because the off-targets of each individual siRNA are diluted in a pool, whereas knockdown of the target gene remains as effective as for an individual siRNA. Additional approaches to reduce off-targets have included chemical modifications to the siRNA,[30] the exclusion of specific sequence motifs,[31] and the inclusion of filters that exclude seed sequences found in mammalian microRNAs.[32] As a result of these strategies, highly effective and specific pre-designed siRNAs are widely available for nearly all human, mouse, and rat genes.

See the siRNA section (p. 13).

Choosing an RNAi tool

With many options for generating gene silencing, it is important to choose the RNAi tool that will best fit the application or experimental need. Chemically synthesized siRNAs and microRNAs are the most efficient method for gene silencing and microRNA modulation, respectively, in standard cultured mammalian cell lines. These synthetic RNAi reagents are typically delivered to cells by means of lipid or polymer-based transfection reagents and have a silencing duration of 3-5 days. Viral vector systems, most often a lentivirus, offer an alternative delivery method for RNAi in many cell lines or cell types that are resistant to conventional transfection. Since these constructs can stably integrate into the host genome, the stable expression of shRNA can also support long-term silencing. Vector-based shRNA delivery systems typically contain a selectable marker, and often a fluorescent reporter to select the transduced cells. Vector-based shRNA can also permit for an inducible promoter system allowing control of when the silencing by the shRNA becomes active, which is beneficial for experiments involving silencing of essential genes.[33] RNAi can be utilized *in vivo* using specially modified siRNAs,[34] electroporation of plasmid shRNA,[35] or viral delivery systems.[36]

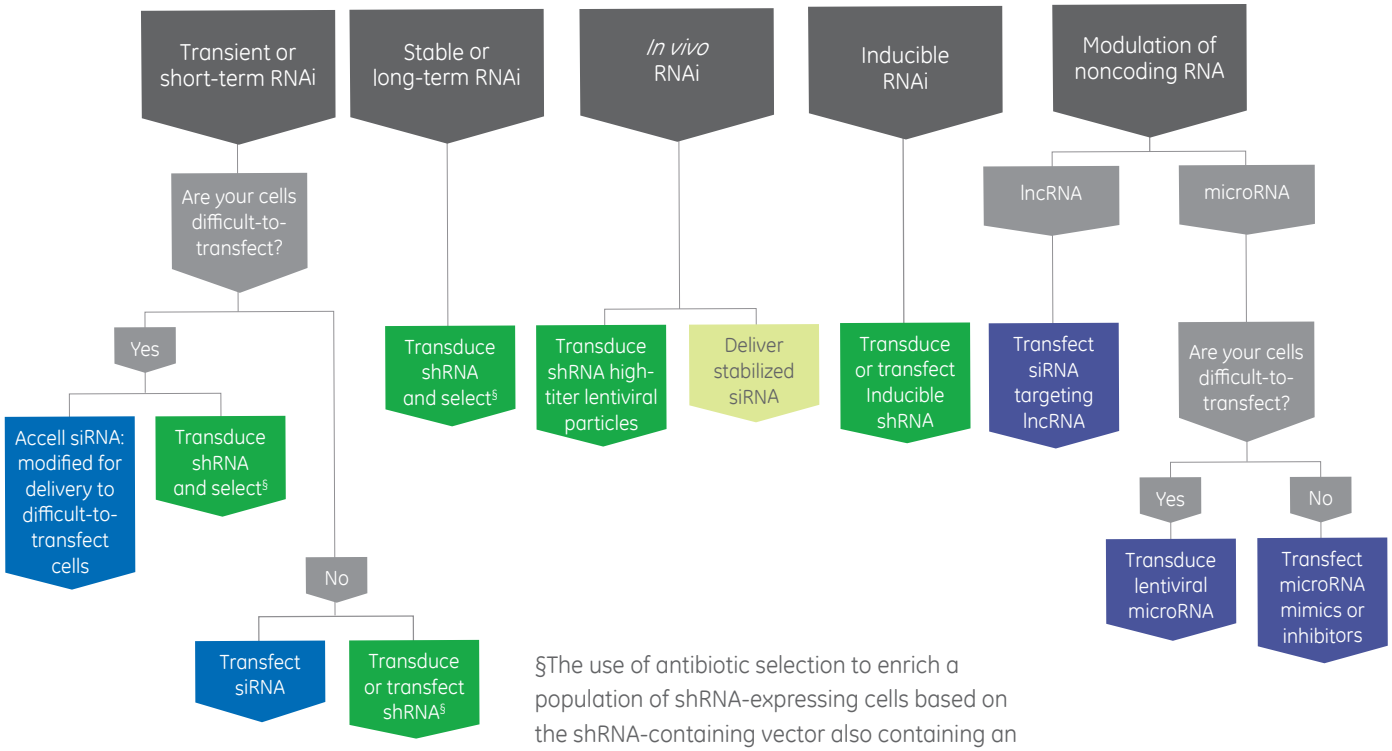
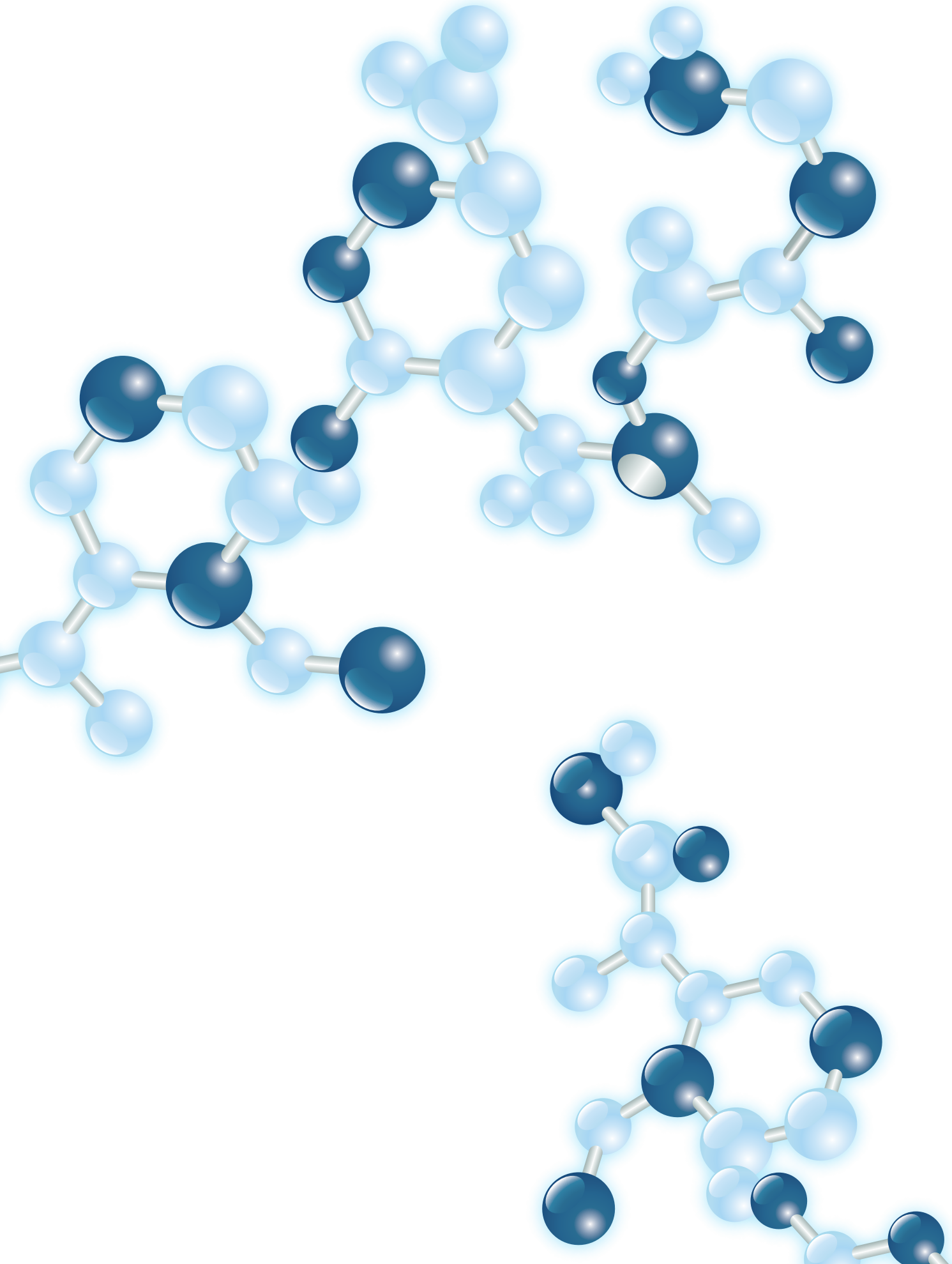
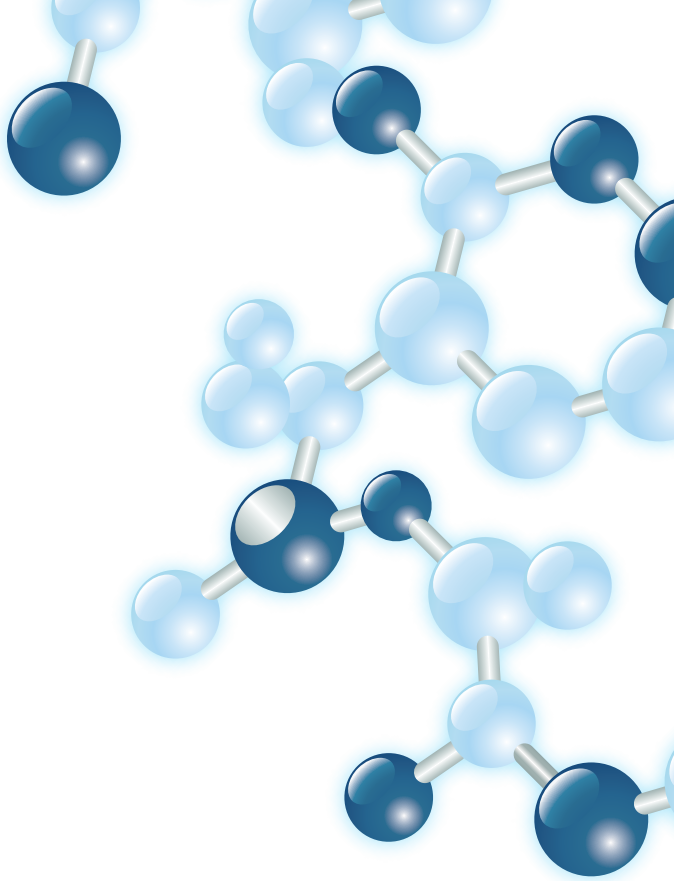


Figure 2. High level selection guide based on some common RNAi applications
Selecting the most effective RNAi tool application involves asking questions about the target gene to be knocked down as well as the target cell lines or model systems to be used. If the knockdown duration required is transient (2-4 days), and the cells are amenable to transfection reagents, traditional siRNAs can be used. However, if longer-term knockdown is needed (for example if the target protein has a long turnover rate) shRNA plasmid transfection or transduction might be required; generation of stable cell lines additionally requires a selection process. Modified siRNAs capable of entering cells without a separate delivery reagent, such as Accell™ siRNA, can be reapplied with minimal effects on the cell and is therefore also a viable option for extended silencing. For cells that are difficult-to-transfect (low transfection efficiency) and *in vivo* applications, both shRNA and modified siRNA can be used. When working with essential genes, it is recommended to use a shRNA vector system with an inducible promoter. When evaluating tools for miRNA based modulation the same principles apply.

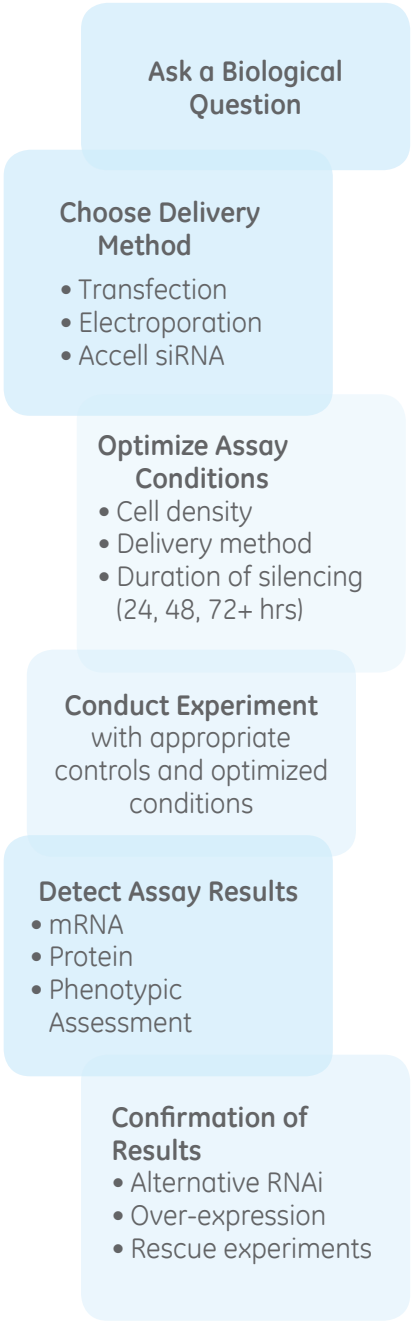




Chapter 2

Experimental workflow and controls for siRNA experiments

Experimental workflow and controls for siRNA experiments



Successful RNAi-based gene silencing experiments rely on good experimental planning and design.

A typical RNAi experimental workflow begins with a biological question and determination of the assay(s) needed to evaluate the biological pathway being investigated. Once the biological question of interest has been determined and selection of the appropriate assay has been decided, identification of the appropriate controls and delivery methods are chosen. As RNAi reagents specifically target the mRNA, quantitative measurements of gene expression at the mRNA level are essential, and should be carried out in combination with measurements of protein and phenotypic changes. The analysis of gene expression, being comparative by nature, requires appropriate controls for both the delivery aspect as well as the siRNA functionality aspect of each experiment. A carefully designed RNAi experiment should minimally include untreated cells, mock transfected cells, a negative or non-targeting control and a positive control.

Table 1. RNAi controls and their experimental functions

Control Type	Function
Untreated Cells	A baseline reference used to normalize cellular viability, target mRNA and protein levels, as well as phenotype in both control and experimental samples
Mock Transfection	To detect cellular effects (toxicity and gene expression changes) caused by the transfection reagent alone (no siRNA)
Negative or Non-targeting Control	1. To be used as a baseline reference for target-specific gene knockdown 2. Distinguishes sequence-specific silencing from non-specific effects
Positive Control	To optimize and monitor efficiency of delivery into cells between experimental replicates, across researchers, or on different days

Comparison against untreated cells

Untreated cells are the normal population against which all other samples, including those treated with positive and negative controls must be compared. Untreated cells determine the baseline level of cell viability, phenotype, and target mRNA gene expression levels.

Mock transfection

To ensure success in an RNAi experiment, it is imperative that the siRNA is delivered to the cell efficiently with minimal disruption of cellular function. Studies show that transfection (whether by chemical or physical means) disturbs gene expression.[26] This could be due to elements associated with the transfection reagent (*e.g.*, lipid) or to the transfection process itself (*e.g.*, electroporation).[37, 38] It is therefore important to test the delivery method of choice without siRNA to evaluate non-specific responses including the effects on cell viability following treatment.

Fluorescent transfection efficiency controls

Fluorescent-labeled RNAi controls are a common method of evaluating transfection efficiency into a cell, as they provide a rapid visual evaluation. The fluorescence may be detected by either fluorescence microscopy or flow cytometry. When using fluorescently labeled siRNA as a control it is important to recognize that visual confirmation of transfection efficiency using conventional fluorescent-labeled siRNA does not correlate quantitatively with siRNA-directed gene knockdown. This is due to several factors including the cleavage of the fluorescent label from the siRNA and rapid photo-bleaching of the fluorophore.

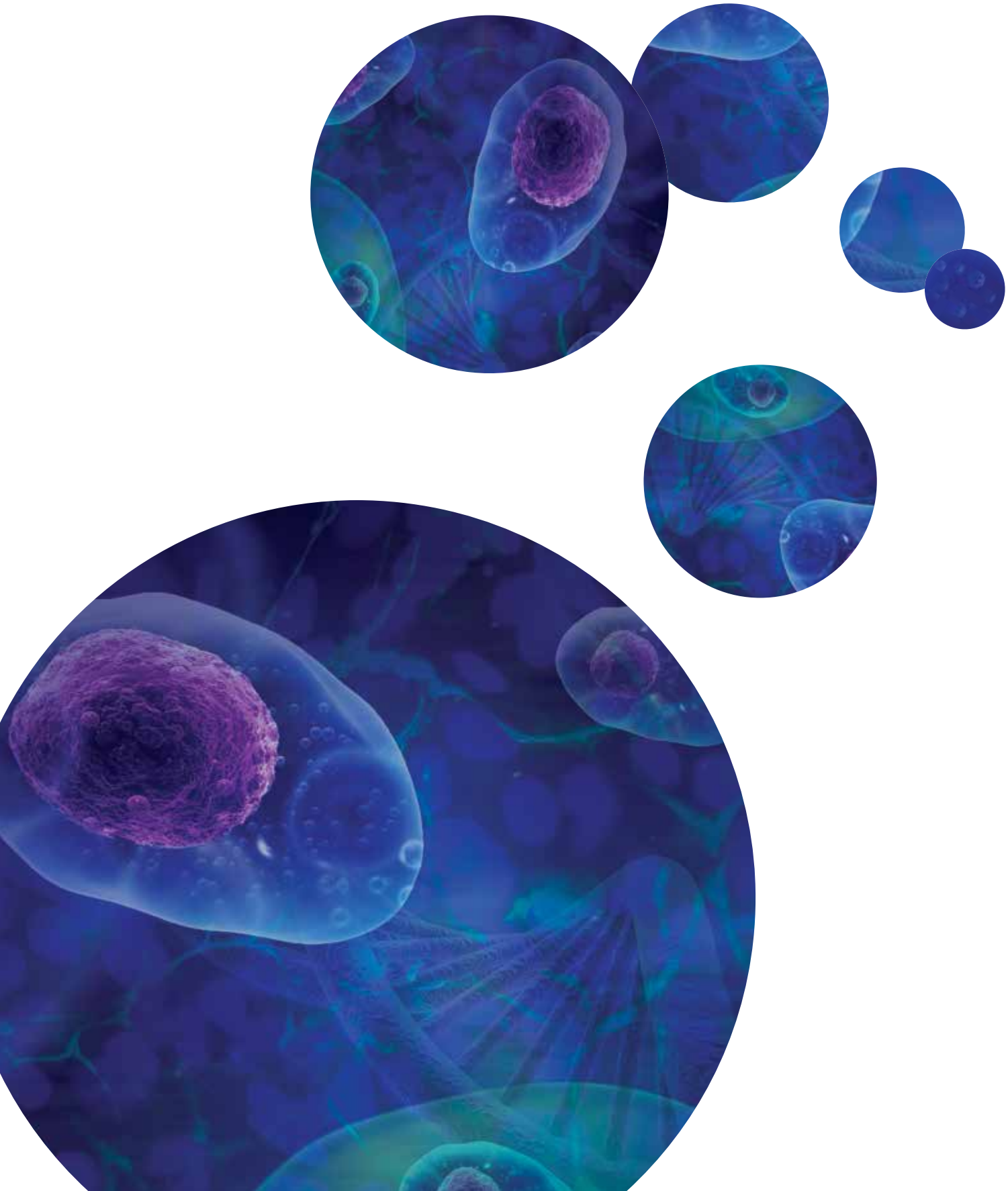
Negative or non-targeting controls

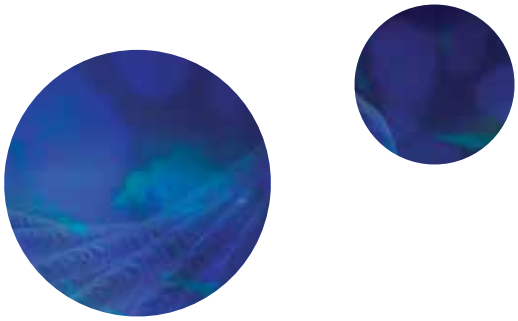
Negative controls are important for distinguishing non-specific off-target effects from sequence-specific knockdown in an RNAi experiment. Negative control siRNAs should be designed to have no known gene target in the cell line and species of interest, ideally with the same chemical modifications as the target siRNA to minimize non-specific effects. Negative controls should be used under the same conditions (concentration, delivery method, duration of knockdown, and detection methods) as targeted siRNA, and have no effect on cell viability, phenotype or target mRNA and protein levels. Because negative siRNA controls have similar biophysical properties such as charge density and molecular weight, these controls are used to compare with targeted siRNA when determining gene knockdown levels. When negative controls are used in this manner, it is assumed that knockdown by the targeted siRNA is specific, and any downstream effect to gene knockdown can be attributed to the specific knockdown of the target gene.

Positive control siRNAs

Positive control siRNAs are important indicators of transfection efficiency and overall assay robustness. As siRNA delivery efficiency may vary greatly from one experiment to another due to multiple factors (*e.g.*,variation in cell passage), it is important to incorporate an internal positive control to evaluate these variations. The best positive control target is an endogenous gene that is under normal transcriptional regulation, whose expression does not fluctuate with the cell cycle in the cell line being tested, and does not affect the cell phenotype or viability. Determining the appropriate control for each cell type is important. Housekeeping genes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Lamin and Cyclophilin B (PPIB), are often used as positive controls because they are abundantly expressed in multiple cell types and therefore are easy to evaluate at the mRNA and protein levels.

Once the appropriate negative and positive controls have been identified for the experimental system being used, the next steps are to optimize the experimental conditions. The key to a successful RNAi experiment is optimization of the delivery reagent, cell confluency, and concentration of the RNAi reagent being used.





Chapter 3

Selecting a delivery method

Selecting a delivery method

Of the many techniques for nucleic acid delivery, the most commonly used methods to deliver RNAi reagents are cationic lipid- or polymer-based transfection reagents (broadly termed "transfection reagents"), electroporation (or other instrument-based delivery) and viral-mediated transduction.

The primary considerations in selecting a delivery method for application of RNAi are the suitability of the method to the cells and the assay requirements for duration of silencing. The large diversity in cell types that exist means that no one delivery method will work for all cell types, as certain cells will be more resistant to one delivery method over another. In addition to the suitability of the delivery method with the cell type, the ultimate experimental goal should be considered, such as creating a stable cell line or the applicability to high throughput protocols for RNAi screening purposes. Optimization of the delivery method being used is critical to successful knockdown of gene expression, with the highest possible transfection efficiency (percentage of cells that receive the RNAi reagent being delivered) being the goal.

Transfection reagents

The most common delivery method, lipid or polymer based transfection reagents, is suitable for most cell types and can result in either short- or long-term silencing depending on whether a siRNA or shRNA molecule is delivered. Transfection reagents complex the siRNA duplex or shRNA expression plasmid to a cationic lipid or polymer producing a liposome that interacts with the cell membrane and results in endocytosis of the RNAi molecule. This process can have adverse effects on the cell causing cell death and disruption of pathways within the cell; therefore, it is important to find a transfection reagent that is the least disruptive for the chosen cell type. Different lipid formulations have been developed to introduce small double-stranded nucleic acids, such as siRNA and microRNAs, versus large circular plasmids. The goal is to efficiently introduce these nucleic acids into the cell with minimal disruption. Many transfection reagent vendors provide initial guidance on choosing the reagent most suited for your cell type. However, the amount of transfection reagent used in your particular assay should still be optimized to reduce cell toxicity and maximize efficiency.

Electroporation

The primary form of instrument-based RNAi delivery is electroporation. Mammalian cells are surrounded by a negatively charged lipid bilayer. Electroporation involves the application of an electrical current which temporarily alters the permeability of the cellular membrane, creating hydrophilic pores that drives delivery of negatively charged nucleic acids such as siRNA or plasmids to the cell.

Electroporation systems can range from single-cuvette to 96-well or 384-well format instruments for high-throughput *in vitro* experiments, and applications have the advantage over lipid-mediated delivery in that they avoid activation of immune response and can be used with difficult-to-transfect cells. For several of the better-known platforms, cell-specific protocols and reagents are available to reduce the user-required optimization time. However, even with very well-optimized delivery there can still be very high levels of cell death, and not all cell types and experimental paradigms are amenable to such systems.

Viral-mediated delivery

For many disease models, the cells of highest biological interest are not always amenable to transfection. For delivery of RNAi reagents to immune, neuronal, primary, or non-dividing cell types, as well as for *in vivo* applications, viral delivery is a powerful alternative to transfection. Viral-mediated RNAi delivery, or transduction, is the use of viral particles to deliver shRNA expression constructs into the cell. A variety of genetically engineered viral systems have been used for delivery of shRNA to target cells. Among the most commonly used systems are retrovirus, lentivirus, adenovirus, and adeno-associated virus (AAV). Each of these systems has its own set of advantages and limitations (Table 2). For example, retroviral delivery is limited to actively dividing cells, while lentivirus, adenovirus, and AAV have the ability to transduce dividing or non-dividing cells. Additionally, retrovirus, lentivirus and AAV can integrate into the host genome, while adenoviruses form an episome in the cellular nucleus. Importantly, the efficiency by which each of these viral vector systems transduces cells will vary by cell type. The choice of viral delivery method should be based on the cell type, whether stable integration is desired, and whether the cell is actively dividing or not. Secondary considerations may include what promoters are on the construct to drive the shRNA expression, whether simple hairpin or microRNA adapted scaffolds are desired, and whether a selection gene is required (*e.g.*,puromycin or GFP).

Viral-mediated delivery offers several advantages in that viruses provide targeted gene silencing in cells that are difficult-to-transfect, can be successfully applied *in vivo*, can result in development of cell lines that stably express the shRNA construct, and can be used to generate inducible systems for more advanced studies. However, delivery conditions must still be heavily optimized to ensure proper levels of knockdown and limit cellular toxicity due to the transduction event or overproduction of the hairpin.

Table 2. Characteristics of commonly used viral vector systems for RNAi

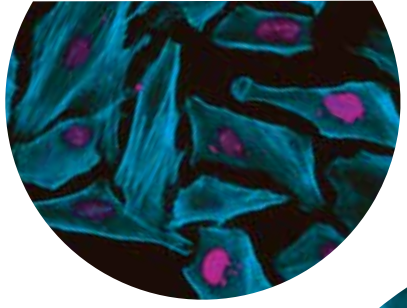
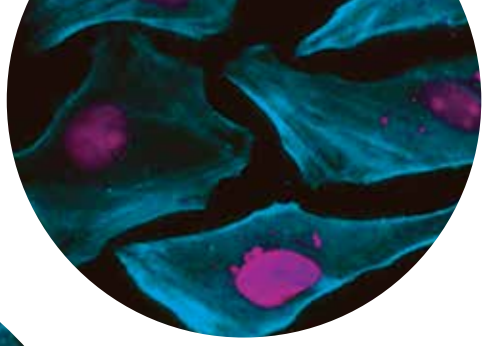
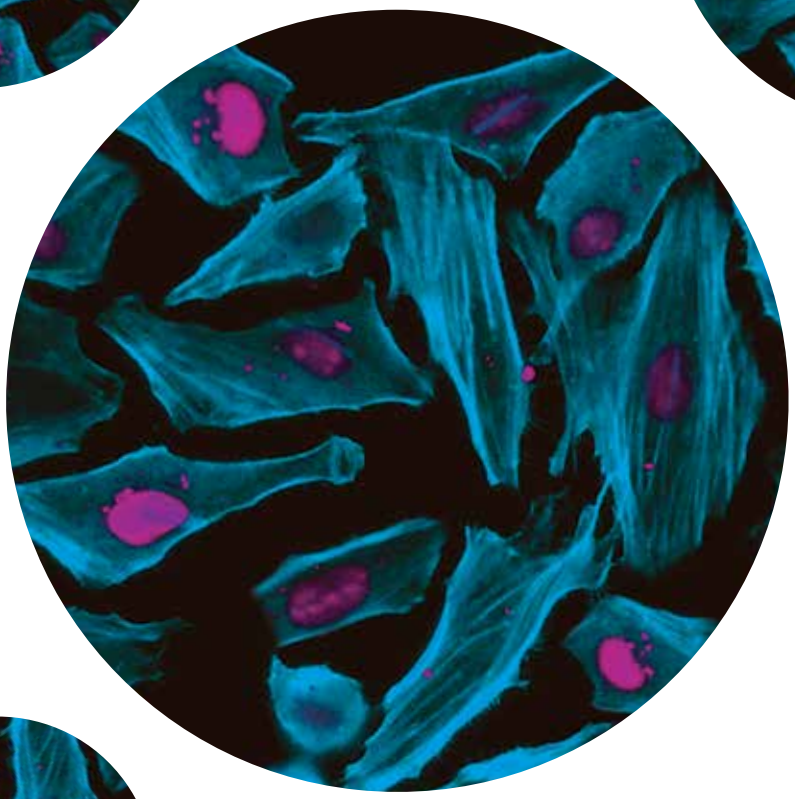
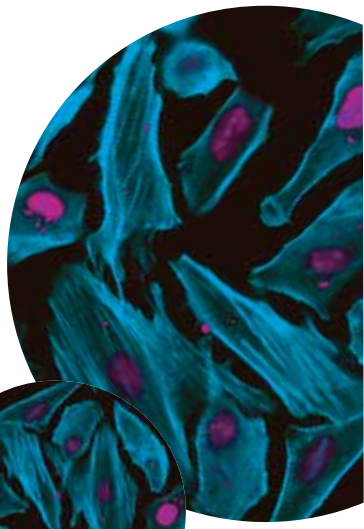
Virus Type	Delivery into dividing and non-dividing cells	Stable integration	Genome
Lentivirus	●	●	RNA
Retrovirus		●	RNA
Adenovirus	●		DNA
AAV	●	●	DNA

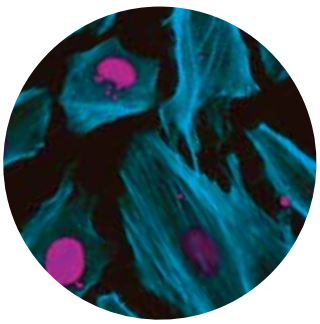
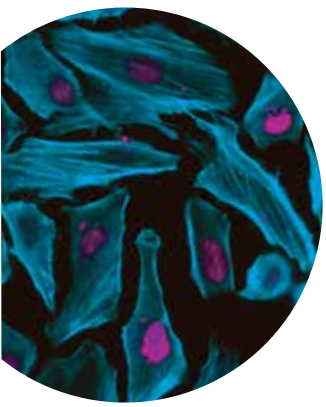
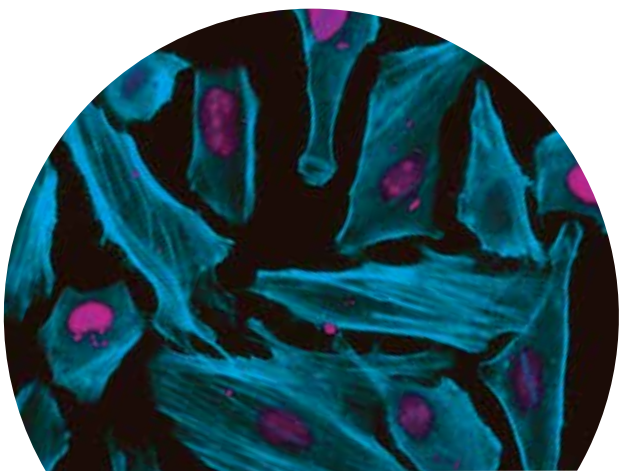
Modified siRNA

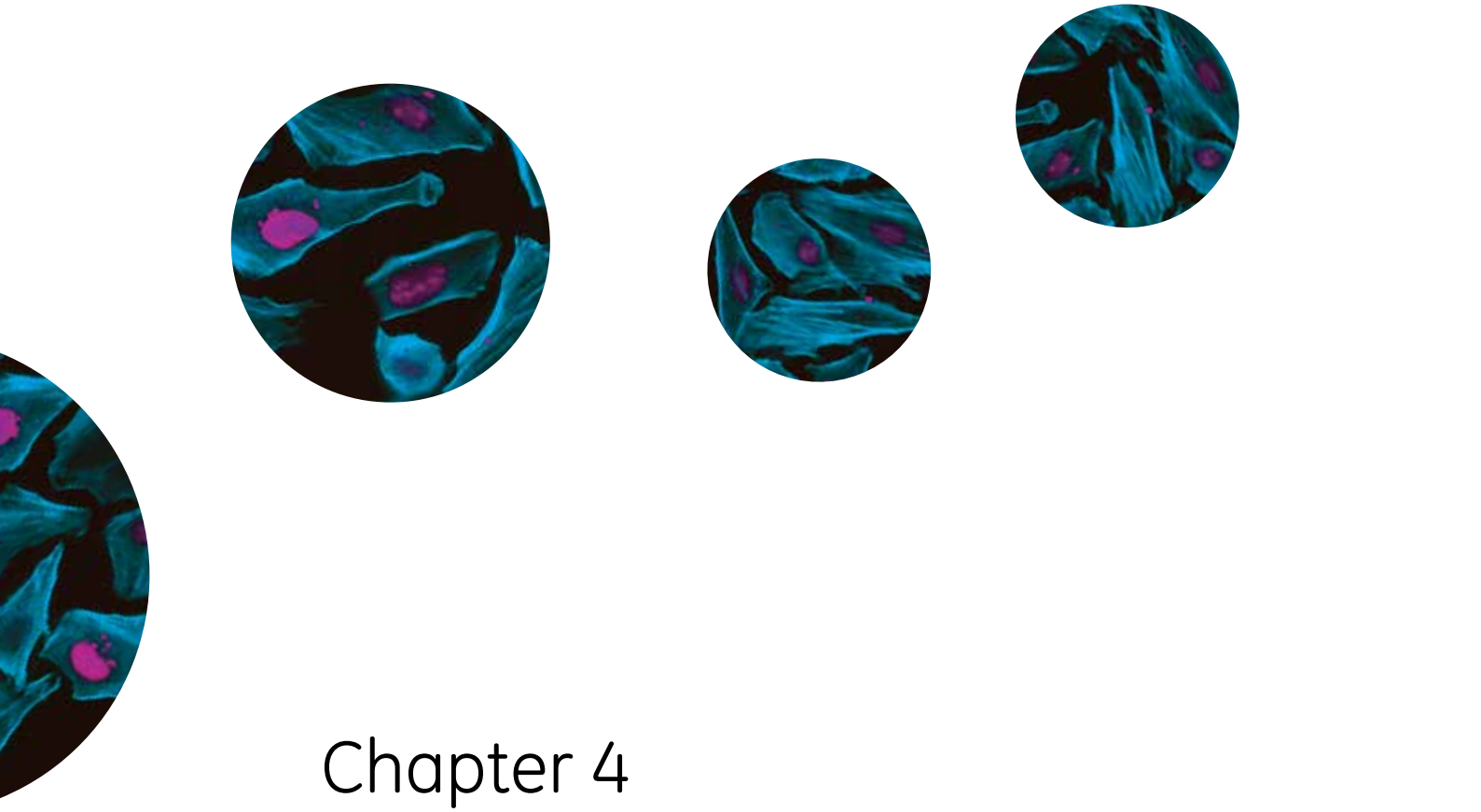
As an alternative to traditional viral-, lipid- or instrument-mediated delivery, siRNA molecules have been developed with modifications that promote self-delivery into cells that are otherwise difficult-to-transfect. These siRNAs are capable of self-delivery due to chemical modifications that allow passive transport across the cellular membrane. The primary example of this is Accell siRNA. The benefits of Accell siRNA are that it enables delivery of siRNA to difficult-to-transfect cell types, allows for repeated delivery for longer duration studies, and avoids the cytotoxic effects sometimes seen with lipid transfection reagents. The caveat is that Accell siRNA requires its own unique optimization, and should be approached in the manner of a new experimental paradigm, rather than a modified form of a traditional delivery technique. Considerations that must be taken into account: low serum requirements (≤ 3%), longer incubation times prior to silencing detection, and necessary siRNA concentrations to drive the delivery gradient. However, if the time is taken to work out the delivery conditions these modified siRNA molecules can provide significant returns by allowing experiments to be run in more medically relevant cell types and can also be successfully used for *in vivo*[39] and *ex vivo* applications.[40, 41]

Table 3. Advantages and disadvantages of different RNAi delivery techniques

Technique	Delivery Mode	Advantages	Disadvantages
Transfection	Cationic liposomes or polymer based	Delivery of siRNA, microRNAs, and shRNA plasmids into most cell types	Short-term silencing, not amenable to all cell types
Electroporation	Voltage pulse	Delivery into difficult-to-transfect cells	Short-term silencing, often high rate of cell death; high quantity of siRNA required
Viral-mediated delivery	Transduction by lentiviral, retroviral, or AAV particles	Delivery into difficult-to-transfect cells for use in stable cell line creation and *in vivo* applications	Requires virus handling knowledge and BSL2 level facilities
Accell siRNA	Modified siRNA for passive delivery	Short-term or extended-duration silencing in difficult-to-transfect cells and *in vivo* applications	No stable knockdown; high concentration of siRNA required



Chapter 4

Optimization: before starting an RNAi experiment

Optimization: before starting an RNAi experiment

Optimization of experimental conditions prior to conducting a target-specific experiment is critical for successful gene silencing.

Cell viability and culture conditions

Evaluation of cell health should be part of all experiments, as this gives an indication of toxicity levels for a particular cell type in relation to the transfection reagent and conditions being used. When choosing cells, it is generally accepted that the earliest passage possible is preferred, as older cells can exhibit genetic and morphological changes after prolonged growth and multiple passages. In addition, cells should be in an exponential growth phase and actively dividing at the time of transfection. The growth rate will determine optimal cell densities for plating and transfection. Cell densities that are too high should be avoided as overgrown cell populations limit access to transfection reagent complexes, and have a greater sensitivity to the toxic effects associated with transfection. Similarly, cell densities that are too low may also lead to toxicity due to excessive exposure to and uptake of transfection reagent complexes.

Optimization of delivery reagent

The most important considerations are the selection of the proper transfection reagent formulation and accompanying experimental conditions to result in the highest achievable transfection efficiency. The importance of transfection efficiency cannot be overstated. Transfection efficiency is a measurement of the percentage of cells that receive the siRNA or shRNA across all treated cells. It is particularly important for RNAi applications because non-transfected cells will continue to express normal levels of the gene being knocked down. This will contribute to background expression levels and inaccurate phenotypic analysis.

No single transfection reagent is appropriate for all cell types. Non-optimal formulations can result in poor transfection efficiency, high cytotoxicity, or high background effects in the assay. In addition to cell type characteristics, the composition and concentration of lipid plays a significant role in lipid-mediated transfection. Some reagents are incompatible with the presence of serum, and require serum-free conditions to ensure efficient transfer of siRNA:lipid complexes. Transfection conditions, such as total siRNA concentration and siRNA:lipid ratio are important parameters to consider when optimizing transfection procedures. Many transfection reagent vendors provide guidance concerning these parameters. However, the amount of transfection reagent used in a particular assay should still be optimized to reduce cell toxicity and maximize delivery efficiency.

To determine the most appropriate transfection reagent and concentration, an optimization grid should be run where RNAi reagent amount is held constant with a changing concentration of various lipids and cell densities, as depicted in Figure 3. The goal is to find the most effective knockdown with the least amount of toxicity.

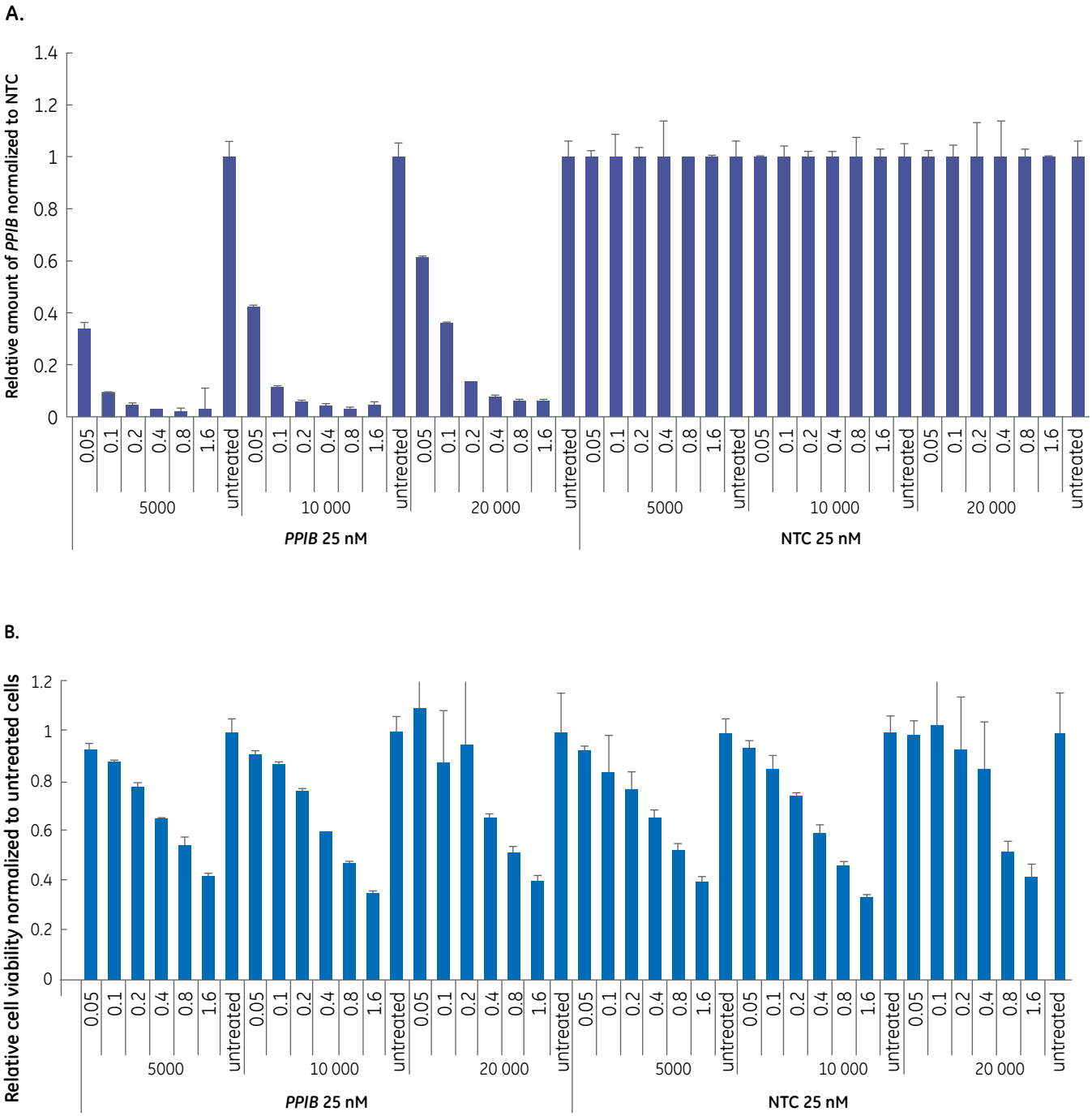


Figure 3. Optimization of delivery reagent for maximum knockdown and minimum toxicity

A. Delivery agent optimization. The concentration of the non-targeting control siRNA (NTC) and the positive control siRNA (PPIB) are kept constant at 25 nM, while the cell density per well and lipid transfection reagent amount is changed. Each cell density and transfection reagent amount (µL) is performed in triplicate. For each cell density tested, six different volumes of transfection reagent are used. Endogenous mRNA levels were evaluated by RT-qPCR, and normalized to NTC, to measure gene knockdown.

B. Corresponding viability graph. The same samples were assessed for cytotoxicity with Alamar Blue and normalized to untreated.

siRNA concentration

As part of the overall experimental design, a concentration-response analysis that measures the reduction in target mRNA levels as a function of siRNA concentration should be performed. The most important goal is to achieve optimal silencing with the least amount of siRNA reagent necessary to minimize off-target effects. Typically, concentration ranges from 5-50 nM are tested. The optimal time point for effective silencing should be determined by measuring the mRNA expression levels at 24 and 48 hours following delivery of siRNA to the cells. Protein levels are typically assessed starting at 72 hours post-transfection. Evaluation at multiple time points is important as the stability and turnover rate of target mRNA and protein is variable.

In screening experiments where many genes are being silenced in a high-throughput manner, this level of siRNA concentration optimization is not practical. It is recommended to screen at an siRNA concentration range of 25-50 nM. See Chapter 9 – High-throughput RNAi library screening for more details.

Optimization when using lentiviral delivery

In addition to the critical parameters described above, additional considerations are necessary when using lentiviral particles.

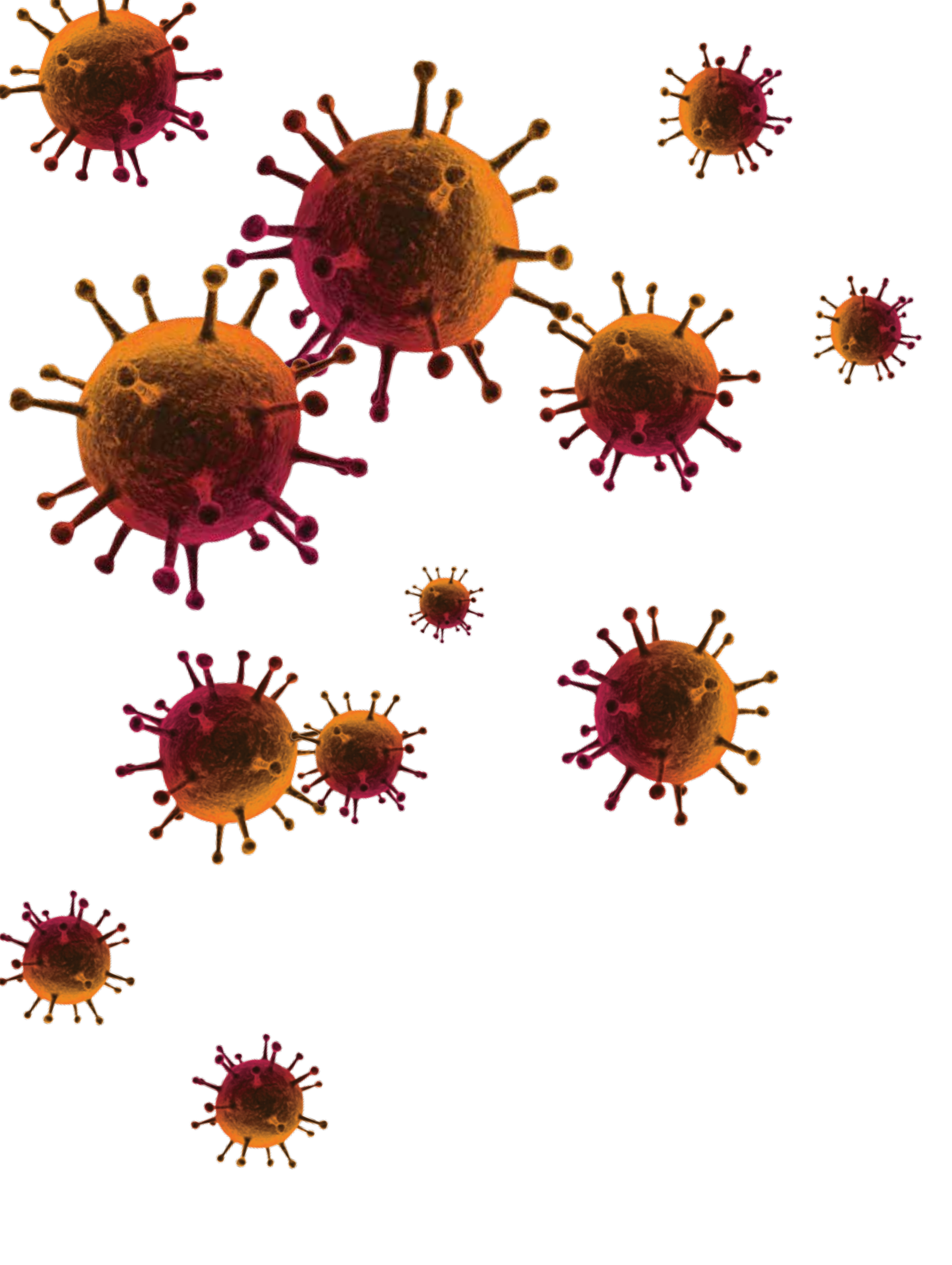
1. **Optimal conditions for lentiviral transduction:** While most lentiviral particles are suitable for a wide range of cells, the conditions for successful and efficient delivery can vary significantly. Therefore, it is essential to determine the optimal lentiviral transduction conditions for each cell type of interest, which can vary between batches and passages of cells.
2. **Relative transduction efficiency in cells of interest:** Because transduction efficiencies vary significantly between cell types it is necessary to assess the transduction efficiency of the lentiviral particles in each specific cell line or type to be used in a screen.
3. **Optimal conditions for puromycin selection:** Lentiviral vectors that contain the puromycin resistance gene allow antibiotic selection for cells which have integrated the shRNA construct. As with transduction, the optimal conditions for puromycin-based selection of shRNA-expressing cells can vary widely between different cell lines and types. Thus, these conditions must be established for cells of interest prior to starting your RNAi experiment.

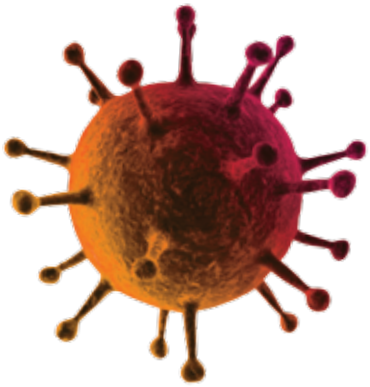
Key points for transfection optimization

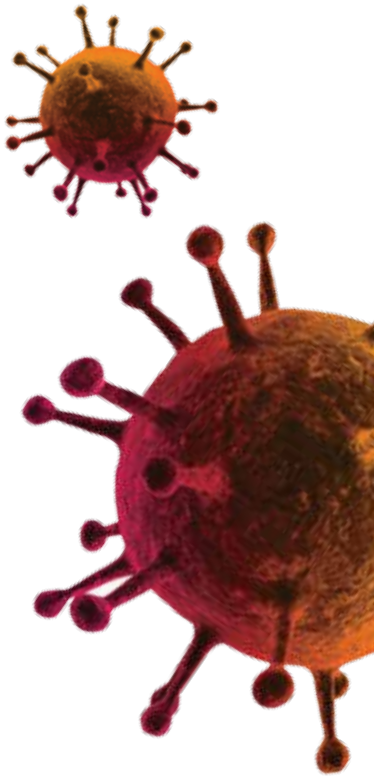
To obtain the highest transfection efficiency with minimal effects on cell viability, we recommend the following guidelines for optimizing transfection conditions in each cell line:

- The optimization experiment should include at least three cell densities and four transfection reagent volumes
- When selecting cell densities to assess, consider the assay and time-point requirements: lower cell densities for long-term assays and higher cell numbers for short-term experiments.
- Use positive and negative control siRNAs at 25 nM final concentration, as well as untreated cells to find conditions that show target mRNA knockdown of > 80% with the positive control siRNA and > 80% cell viability.
- Use these optimal conditions for your subsequent experiments with siRNAs targeting genes of interest.
- Since the siRNA amount for optimal silencing can vary due to intrinsic properties of the target gene, in smaller scale experiments we recommend performing a dose curve transfection with your test siRNA (using a range from 5 to 50 nM) to find the optimal siRNA concentration for your target-specific siRNA.

Note: For high-throughput siRNA screening in transfectable cells, we recommend using a reverse transfection protocol. The lipid volumes and siRNA amounts for reverse transfection often differ from the amounts needed for forward transfection. Therefore, a transfection optimization should be performed for the protocol that is going to be used for subsequent experiments. See Chapter 9 - High-throughput RNAi library screening for more details.







Chapter 5

Vector-based RNAi technologies

Vector-based RNAi technologies

Vector-based technologies are a valuable asset for RNAi based studies for multiple reasons.

While synthetic siRNAs provide the opportunity to induce gene knockdown in a wide variety of cell lines, the silencing that is achieved is transient; therefore experiments are limited to relatively short time frames. Conversely, as vector-based shRNAs are DNA based, they are able to integrate into the cellular genome, which allows for the generation of stable cell lines. Additionally, when used in combination with one of several viral delivery systems, they can be delivered into difficult-to-transfect primary cells and used for *in vivo* applications. Based on the delivery method and vector design, vector-based shRNAs can allow for long-term (or inducible) down-regulation of target genes. When planning an experiment using shRNA, there are many options to take into consideration based on the needs of the experiment. These include the shRNA design to be used, the vector features (*e.g.*,promoter), and the method of delivery.

shRNA design

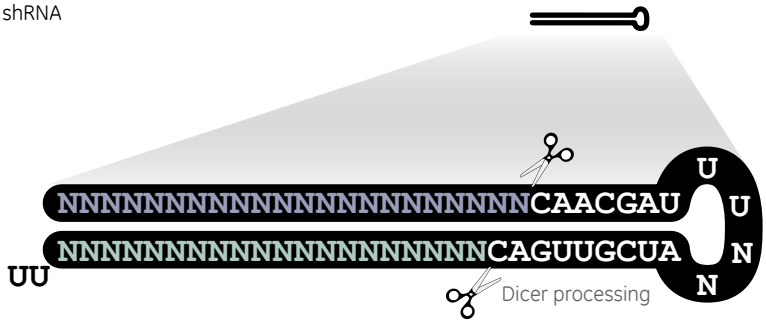
Simple stem-loop shRNA

Basic shRNAs are modeled on precursor microRNA (pre-miRNA), and are cloned into viral vectors where they are transcribed under the control of RNA Polymerase III (Pol III) promoters.[11, 42] shRNAs are produced as single-stranded molecules of 50–70 nucleotides in length, and form stem–loop structures consisting of a 19-29 base-pair region of double stranded RNA (the stem) bridged by a region of single-stranded RNA (the loop) and a short 3' overhang (Figure 4A).[43-45] Once transcribed, shRNAs exit the nucleus, are cleaved at the loop by the nuclease Dicer in the cytoplasm, and enter the RISC to direct cleavage and subsequent degradation of complementary mRNA.

microRNA-adapted shRNA

A microRNA-adapted shRNA consists of a shRNA stem structure with microRNA-like mismatches surrounded by the loop and flanking sequence of an endogenous microRNA (Figure 4B).[46] microRNA-adapted shRNAs are transcribed from RNA Polymerase ll (Pol ll) promoters, cleaved by the endogenous RNase III Drosha enzyme in the nucleus, and then exported to the cytoplasm where they are processed by Dicer and loaded into the RISC complex. Studies have suggested that the use of a microRNA scaffold, which is processed by both Drosha and Dicer, may promote more efficient processing and reduce toxicity for *in vivo* RNAi.[46-49]

A. Simple stem-loop shRNA



B. microRNA-adapted shRNA

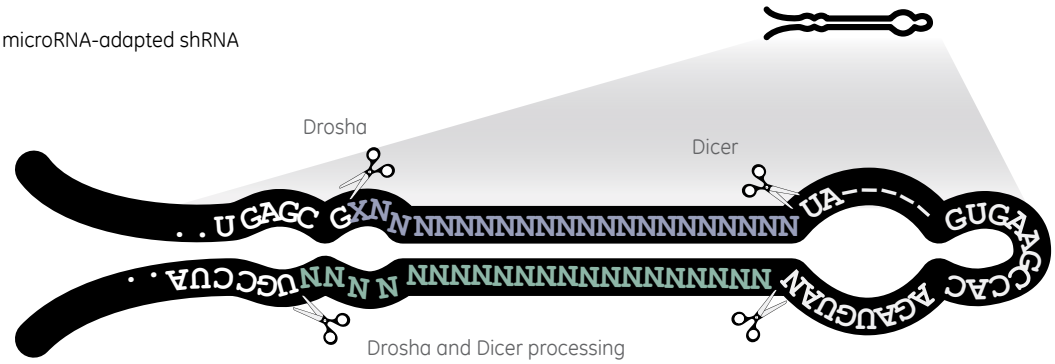


Figure 4. Structure of shRNA tools for gene knockdown.
A. A simple shRNA consists of a 19-29 bp stem, a loop of variable nucleotide length, and a two nucleotide 3' overhang. The antisense strand (green) is complementary to the gene target of interest.
B. The structure of microRNA-adapted shRNA mimics an endogenous microRNA. The primary sequence is first processed by Drosha to form a stem-loop shRNA structure. The antisense strand of the shRNA (green) is loaded into RISC following processing by Dicer.

Vector selection

Promoters

Careful consideration of vector features should be made before beginning a shRNA knockdown experiment. As discussed above, simple shRNA structures are typically expressed from Pol III promoters, while microRNA-adapted shRNA are expressed from Pol II promoters. Some of the most commonly used Pol II promoters include CMV and EF1a, while U6 and H1 are frequently used Pol III promoters. These promoters are popular because of the wide range of cell lines in which they are active. However, the activity of any promoter, and thus expression level of the transcribed shRNA, may vary substantially by cell type (Figure 5). Inducible promoters, such as those containing a tetracycline-responsive element, are beneficial for knockdown of genes essential for survival and other experimental setups where control of shRNA expression is required. Evaluating promoter activity in the cell line of interest through use of a fluorescent reporter is recommended prior to beginning a knockdown experiment.

Fluorescent reporters

Many commercially available vectors contain a fluorescent reporter gene to allow for visual tracking of transduced cells. For vectors that utilize Pol II promoters, the fluorescent reporter can be translated from the same transcript as the shRNA. This fluorescence serves as verification that the shRNA is being expressed. Because Pol III promoters only drive expression of short transcripts, fluorescent reporters must be expressed from a second promoter on the vector. For both Pol II and Pol III vector systems, it is important to understand that the expression level of the fluorescent reporter does not necessarily correlate with the potency of knockdown.

Selection markers

Mammalian selectable marker genes, such as Puromycin and Blasticidin, are often included on shRNA vectors. These markers allow for the selection of cells that express a shRNA of interest due to their resistance to treatment with the corresponding antibiotic. Selection may be applied to cells that have been transfected with plasmid DNA or transduced with viral particles. Selection of cells is essential for successful knockdown when using cells that have been transduced with a low MOI (discussed in the next section). Selection can also be applied to cells for the generation of stable cell lines. As with other variables of shRNA knock-down, the concentration of the antibiotic used for selection should be optimized in your cell line to ensure the highest probability of success.

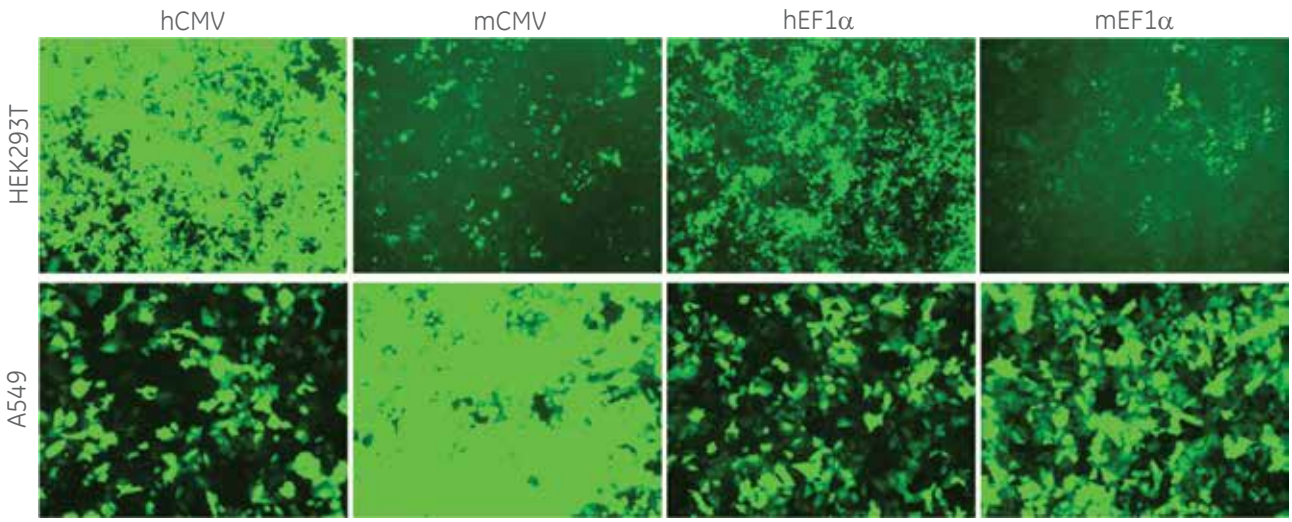


Figure 5. Promoter activity varies by cell type.
HEK293T (top panels) or A549 (bottom panels) cells were transduced with lentiviral vectors expressing TurboGFP from the human or murine CMV promoter, or human or murine EF1α promoter, at an MOI of 10. Cells were visualized by fluorescence microscopy 72 hours post-transduction.

Lentiviral delivery options

Viral shRNA-based constructs can be used in both transfection experiments as plasmid DNA, or in transduction experiments as packaged viral particles. Viral transduction is often used for long-term silencing experiments, as well as difficult to-transfect cells and *in vivo* applications. As discussed in the delivery section of Chapter 3, the most commonly used viral-mediated systems include retrovirus, lentivirus, adenovirus, and adeno-associated virus (AAV. Each of these systems has its own set of advantages and limitations (Table 2.

Some considerations when using lentiviral-mediated methods include identification of the appropriate biosafety level facilities, and whether pre-made particles will be purchased versus plasmid preparations that need to be packaged into viral particles. When preparing lentiviral particles from plasmid preparations, it is important to understand the vector elements being used, as they impact the type of viral packaging system employed. Packaging systems are often named by generation (first through fourth, to denote the number of plasmids encoding packaging components, and the necessity of the Tat gene. The Trans-Lentiviral packaging system available from Dharmacon will effectively package second, third, and fourth generation lentiviral vectors.

Once the shRNA plasmid of interest has been transfected along with the appropriate viral packaging plasmids, the cells will release lentiviral particles into the supernatant that can either be collected and used directly as supernatant (will include cellular debris, or used as concentrated lentiviral particles following high speed centrifugation of the supernatant. A typical packaging schematic is shown in Figure 6. Regardless of whether lentiviral particles are purchased or created, re-titering the virus in the target cell line to be used is recommended (see box on the right

Viral Titers and Multiplicity of Infection (MOI)

Viral titer is classically defined as the number of transducing units (TU) of virus within a given sample volume.

$$\text{Viral titer} = \frac{\text{Number of transducing units of virus}}{\text{mL}}$$

Whether produced or purchased from a vendor, it is important to understand the titer of your lentiviral particles before proceeding with an shRNA experiment. This is true in part because the multiplicity of infection (MOI), defined as the ratio of viral particles (or transducing units) to cells, may substantially influence the results seen in a knockdown experiment.

$$\text{MOI} = \frac{\text{Number of transducing units of virus}}{\text{cells}}$$

The MOI used for viral transduction will significantly influence the results of a gene knockdown experiment. At low MOIs (≤ 1), most transduced cells will contain only a single copy of the shRNA, while some cells will remain untransduced. At higher MOIs (≥ 10), most cells will likely have one or more copies of the shRNA and will therefore express the shRNA at a higher level. However, the use of high MOIs may lead to toxicity in some cell lines. Therefore, it is important to optimize the MOI for each cell line by assessing gene knockdown and cell toxicity within a range of MOIs (1-100), then selecting the MOI that provides the highest knockdown with the least amount of toxicity.

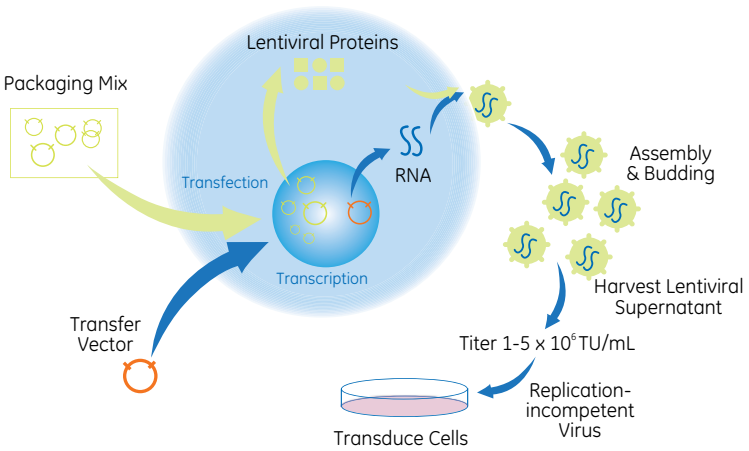
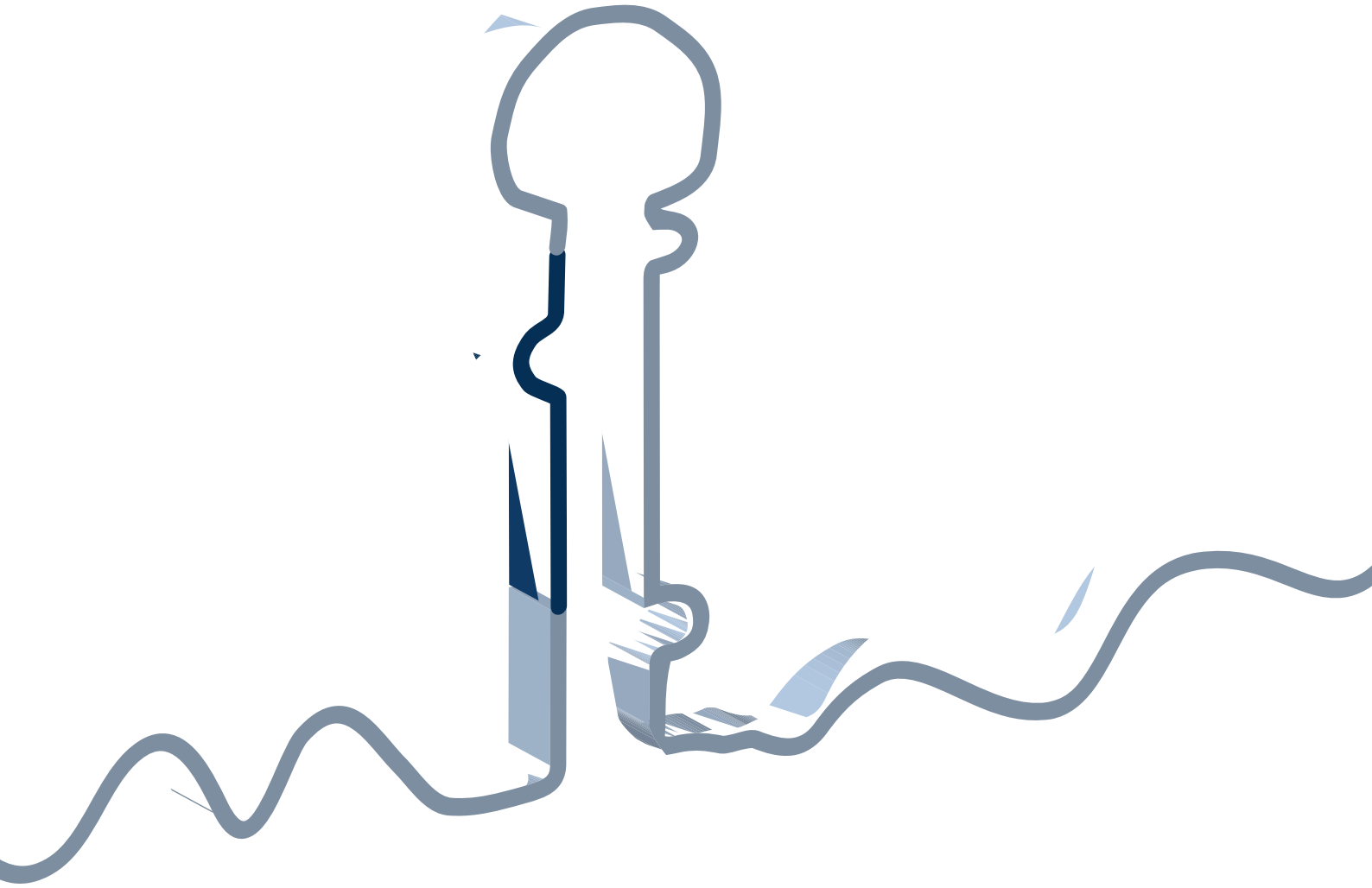


Figure 6.
Schematic showing production of lentiviral particles using the Trans-Lentiviral shRNA Packaging Kit. Co-transfection of the packaging plasmids and transfer vector into the packaging cell line, HEK293T, allows efficient production of lentiviral supernatant. The viral particles can then be transduced into a wide range of cell types, including both dividing and non-dividing mammalian cells.



Chapter 6

Experimental design and controls for microRNA modulation experiments

Experimental design and controls for microRNA modulation experiments

microRNAs are a naturally occurring class of small, noncoding RNAs that regulate gene expression post-transcriptionally – a process known as RNA interference (RNAi).

In concert with the RNA-induced silencing complex (RISC), microRNAs bind to targeted messenger RNAs (mRNAs) via imperfect sequence complementarity resulting largely in mRNA destabilization and translational attenuation (See Section I, Chapter I). microRNAs function within intricate regulatory networks that control diverse biological phenomena. Since microRNA targeting of transcripts is directed by partial sequence complementarity, each microRNA can potentially regulate hundreds of genes. Thus, a careful experimental design is critical to elucidate microRNA involvement in a biological system. Modulation of microRNA levels in both gain- and loss-of-function experiments are a fundamental way to understand not only microRNA function, but also the pathways in which they may be involved.

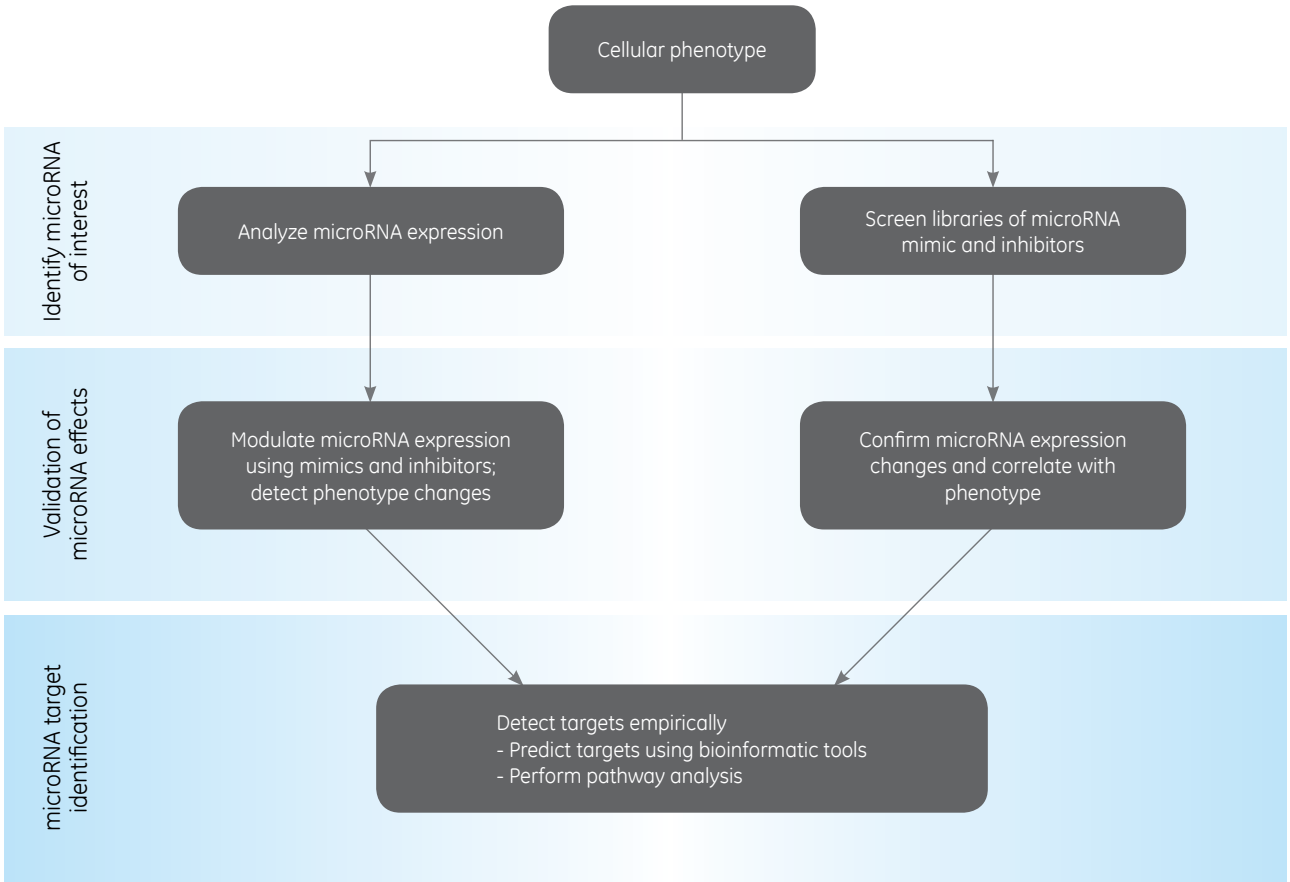


Figure 7. Workflow for identifying microRNA targets and function

Designing a microRNA modulation experiment

microRNA mimics

Two types of microRNA mimetics are used to achieve gain-of-function: synthetic mimics and over-expression constructs. Synthetic microRNA mimics are chemically synthesized double-stranded RNA molecules which are intended to mimic the endogenous duplex resulting from Dicer processing. One strand represents the mature microRNA as annotated in miRBase (www.mirbase.org). Like naturally occurring microRNAs, the two strands of a synthetic mimic are separated by RISC; the single-stranded mature microRNA is incorporated into RISC and thereby directs down-regulation of transcript levels. Synthetic microRNA mimics require delivery into cells using methods similar to those for siRNA (transfection reagents, electroporation, etc.). The gene expression down-regulation effect is transient, generally reaching maximum effects within 48 to 96 hours post-transfection.

Over-expression constructs are plasmids which encode native microRNA sequences to achieve exogenously introduced microRNA expression. Plasmid DNA can be directly transfected into the cell using chemical reagents such as transfection reagents or electroporation. Alternatively, plasmid vectors can be engineered to encode microRNA sequences in the context of a viral backbone for packaging the construct into viral particles suitable for the transduction of cells. Thus, by introducing these molecular mimics into a cell type of interest, one can enhance or supplement endogenous microRNA activity representing a gain-of-function assay.

microRNA inhibitors

Ideally, when studying the functional role of a microRNA of interest, one should seek to characterize microRNA effects by both gain-of-function and loss-of-function observations. As such, the introduction of microRNA inhibitors into a biological system results in a loss-of-function assay with a predicted decrease in endogenous microRNA function. Synthetic inhibitors, in general, are comprised of a non-hydrolyzable, single-stranded reverse complement to the mature microRNA. The mechanism of inhibition is likely mediated by irreversible binding of the inhibitor to mature microRNA, thus preventing interaction of the mature microRNA to its endogenous targets.[51, 52] Expressed inhibitors, (sometimes referred to as microRNA sponges), are typically constructs with multiple microRNA sites that prevent microRNAs from functioning on other targets.[53, 54] A combination of these approaches and tools in loss-of-function assays increases the likelihood of observing otherwise subtle phenotypes often associated with microRNA inhibition.

Optimization of experimental systems

The goal for optimization experiments is to identify a set of conditions that provide maximal delivery efficiency of mimics and/or inhibitors, allowing downstream gene regulation with minimal toxicity. Identifying such conditions can be achieved in a single multi-parameter optimization experiment with the appropriate positive and negative controls. It is strongly suggested that all experiments be performed as biological triplicates. Although it has been demonstrated that microRNAs are involved in many biological processes, the effects of microRNA on targets and resulting phenotypes are often subtle.

Negative controls

Negative controls are important for distinguishing between specific effects attributable to microRNA modulation, and non-specific effects, which may be due to mode of delivery. Because microRNAs exert regulatory influence primarily through a short 7 to 8 mer sequence near the 5′ end of the mature microRNA (the seed region), a scrambled sequence may inadvertently

cause unwanted down-regulation of genes. For this reason, it is not practical to use scrambled sequences as controls. A more appropriate negative control is a molecule which represents or targets a microRNA of an unrelated species (such as Dharmacon miRIDIAN negative controls, which represent and/or target *C. elegans* microRNAs). Even so, several negative controls should be tested in each assay to determine which is the "most negative" or, in other words, causes little to no identifiable effect on the pathway being studied.

Positive controls

Positive controls can facilitate data interpretation in both gain- or loss-of-function experiments. An ideal positive control induces a known effect robustly and reproducibly, across many different experimental systems. However, design of a universal positive control that applies to many types of experiments is challenging for several reasons. The identification of a suitable positive control for an assay or set of experiments requires prior knowledge of the microRNA expression profile for each cell line or type.

Natively, microRNAs are both spatially and temporally expressed. At any given point in time, stage of development, and in any given tissue or cell, less than one-third of all known microRNAs are expressed. However, as the field has progressed, many microRNAs have been linked to putative gene targets. These known microRNA-gene relationships can be tested through validation experiments to establish potential microRNA positive controls.

A known microRNA-mRNA target pair may serve as a positive control, but should be validated in each cell line or type of interest. A well-characterized example of this relationship is miR-122 regulation of the gene ALDOA.[105] In a gain-of-function or microRNA over-expression experiment, a positive control which mimics miR-122 may result in reduction of endogenous *ALDOA* mRNA levels, while delivery of an inhibitor, for a loss-of-function experiment, should result in an increase in *ALDOA* mRNA levels (Figure 8).

There are many microRNA-target gene relationships that have been characterized, and many remain unknown. Additionally, a suitable and robustly measured microRNA-target gene relationship may not be achievable in a particular cell line or type. Thus, it is also appropriate to use an siRNA or shRNA as a positive control for optimizing microRNA experimental conditions, so that novel microRNA and phenotypic effects can be discovered.

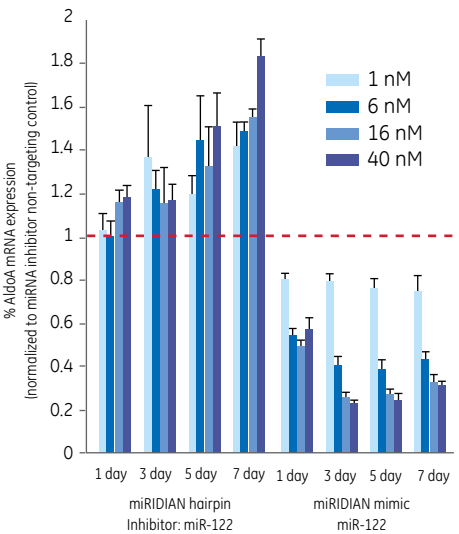


Figure 8. Modulation of *ALDOA* mRNA levels in Huh-7 cell line using miRNA Mimics and Inhibitors
Dashed red line indicates *ALDOA* expression level in untreated cells. *ALDOA* mRNA expression values
> 1 indicate inhibition of miR-122 and an up-regulation of *ALDOA*, while values
< 1 indicate additional down-regulation of *ALDOA* by miR-122.

Identifying microRNA targets

The observance of effects on gene expression and resulting phenotypes due to the modulation of microRNA levels is only one node in a broader microRNA experimental design strategy. While it is critical to establish an understanding of the functional roles of microRNAs within specific normal and disease physiology, it is also critical to propose putative gene targets that are upstream and/or downstream of microRNA regulation. Many recent publications build upon previous observations of microRNA regulatory events and are able to suggest genes that regulate microRNA expression, thus allowing larger networks involving feedback loops to be expanded upon.

The successful identification of putative gene targets of microRNA regulation requires a combination of multiple computational analyses, followed by carefully constructed series of phenotypic and biochemical assays to support the microRNA-gene target relationship. Most current target prediction tools rely heavily on microRNA seed sequences to generate lists of possible gene targets. This creates a challenge since the short length of seeds can match hundreds or thousands of potential genes, which cannot all be investigated. Thus it is necessary to combine multiple computational and empirical approaches.

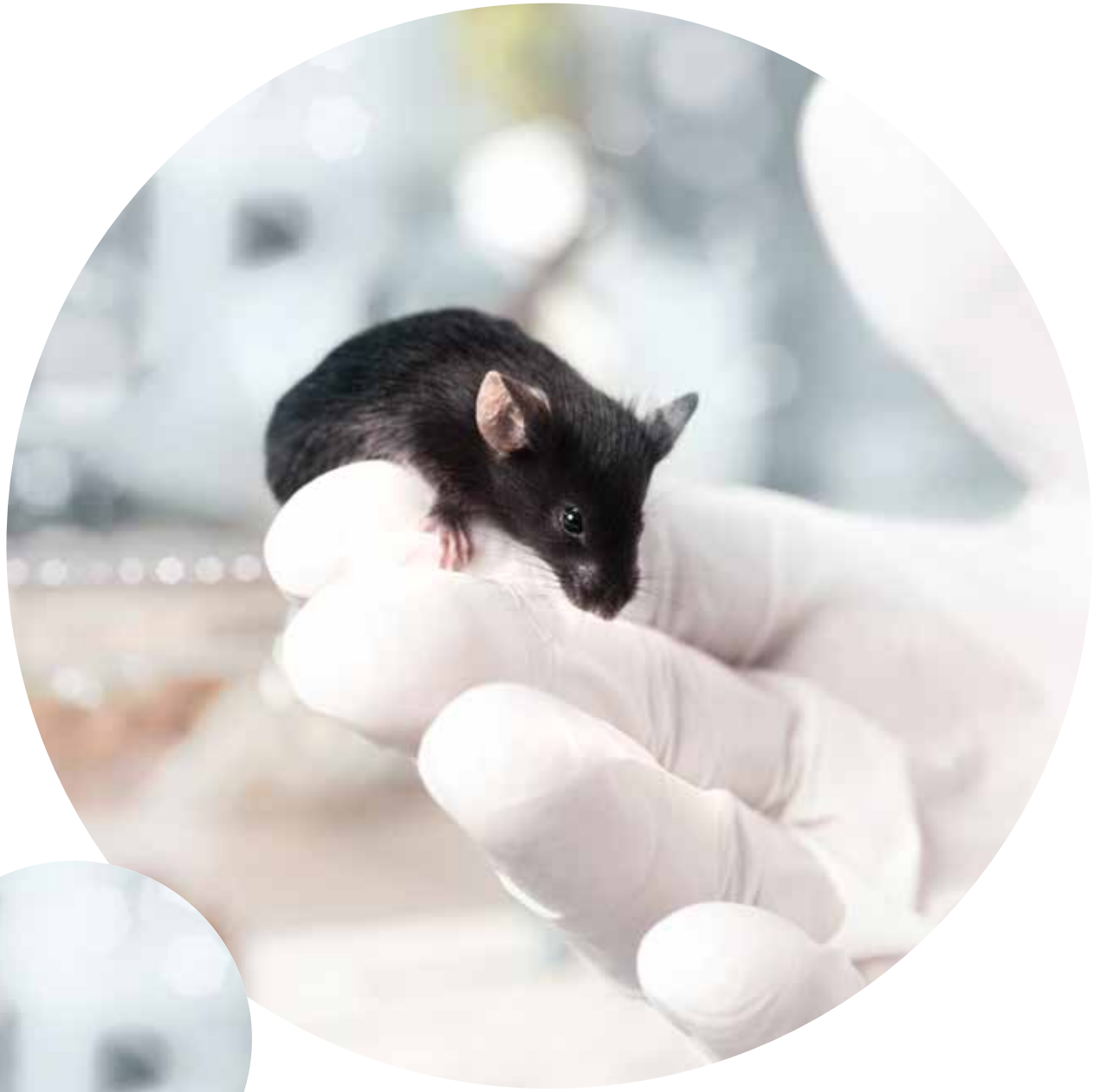
Experimental and analytical methods for microRNA target identification

1) Biochemical and phenotypic assays can be used as outputs of high-throughput microRNA library screening to quickly identify microRNAs of interest. The small numbers of molecules in complete microRNA mimic and inhibitor libraries allow for many screens to be run in a short period of time. Screening of libraries can be performed using high-content assays and equipment to yield information-rich multi-parametric data sets. Individual hits can be ranked according to magnitude of phenotypic changes, reproducibility, and statistical significance. Confirmation is performed using less high-throughput, but more biologically relevant, assays or cell types.
2) Microarray-based expression profiling and small RNA sequencing can be performed using samples from differing cell populations, such that differential expression analysis can identify fold-changes in microRNA levels. Those microRNAs with the most significant fold-changes in response to disease or developmental progression, drug treatment, or mutations can then be targeted for further characterization using modulation and computational prediction tools.
3) Computational target prediction programs, many of which are publicly available, can be a useful tool for researchers (see www.miRBase.org for several links). The main mode by which gene targets are proposed relies on the sequence alignment of a microRNA seed to the 3′ UTR of candidate genes. Additional filters exploit features such as degree of conservation, number and locations of 3′ UTR binding sites, and thermodynamic stability of the microRNA:mRNA complex.
4) Simple reporter assays are a commonly used to verify an interaction between a microRNA and a putative candidate mRNA. Gain- and loss-of-function experiments with modulation tools using phenotypic assays can provide clues as to whether specific microRNAs are involved in both normal and diseased biological processes of interest. These can be followed immediately by transcriptome profiling (whether by deep sequencing or microarray-based analysis) to identify genes which are up- and/or down-regulated in response to treatment with a mimic or inhibitor, respectively.

5) Biochemical methods designed to associate or capture the microRNA with its targeted transcript, such as immunoprecipitation of RISC or specific Argonaute proteins, and use of biotinylated microRNAs with streptavidin columns can narrow down a list of mRNA target candidates by focusing on an enriched population of microRNA targets.
6) Quantitative real-time PCR (qPCR) and Western blots can be used to validate the regulation of a target gene by candidate microRNA. Additionally, reporter assays can be used where the microRNA binding site is altered by a single point mutation in the seed region.
7) Finally, a truly comprehensive study would include over-expression and knockout experimentation *in vivo*. Both vector-based approaches as well as the use of chemically modified, highly stable synthetic oligonucleotides have been published in animal model systems.

Summary

microRNAs are noncoding RNAs that mediate post-transcriptional gene regulation through a mechanism that involves RISC-mediated binding to complementary sequences largely in the 3′ UTR of mammalian target genes. While microRNAs are clearly involved in critical steps of animal development, cellular differentiation, and disease, target identification remains challenging. Efforts to identify gene targets are confounded by the unique attribute of microRNA to regulate targets to which they display only partial complementarity. This creates the potential for modulation of hundreds of genes by a single microRNA, and the potential for combinatorial regulation of a single gene. Furthermore, microRNA-mediated regulation, and the resulting mRNA attenuation, often results in subtle phenotypic effects which can be challenging to detect or accurately quantify. The availability of microRNA-based mimics and inhibitors has provided researchers with the necessary tools to perturb specific microRNA activities and assess the phenotypic consequences in gain- or loss-of-function assays.



Chapter 7

In vivo RNAi applications

In vivo RNAi applications

The success observed in numerous peer-reviewed publications for siRNA- and shRNA-mediated gene silencing in cell culture naturally led to the next application of RNAi, the use of synthetic siRNA molecules or shRNA constructs for target-specific gene silencing in animal models.

In vivo RNAi has been used for target validation studies in animal disease models and has the potential to be used for therapeutic purposes where disease-causing genes could be selectively targeted and suppressed.[58, 59] Many different cancers, neuropathies, and immune-related disease or pathologies resulting from aberrant gene expression represent prime targets for RNAi-based methods. In these cases siRNAs or shRNAs can be used to uncover the underlying genetic pathways, thereby revealing potential drug targets, and may even serve as therapeutic molecules. The information offered in this chapter regarding *in vivo* considerations for RNAi represents a set of general guidelines; actual experimental protocols are the responsibility of the individual researcher and should adhere to regulations set down by the appropriate regulatory bodies prior to use.

In preparation for animal studies, it is critical to first evaluate the functionality of your siRNA or shRNA in an *in vitro* experimental system prior to evaluation *in vivo*. As with *in vitro* studies, reagent design, synthesis, and delivery of functional, stable siRNAs or shRNAs are factors that are paramount to experimental success. In contrast to *in vitro* studies, siRNAs for *in vivo* use may also require additional attributes that permit appropriate biodistribution and that result in the desired pharmacokinetic properties (ADMET: absorption, distribution, metabolism, excretion, toxicity) to achieve robust gene silencing.

Delivery: systemic versus localized

There are two main strategies for *in vivo* siRNA delivery: systemic or localized. These are both derived primarily from historical studies with antisense oligonucleotides and ribozymes. Systemic delivery, as the name implies, involves an injection into the systemic circulation system (usually through tail vein injection or intraperitoneal injection), and provides a widespread distribution of siRNA throughout the animal.[58, 60-62] Conversely, localized delivery involves an injection of siRNA directly in the target area, thus providing more limited biodistribution. Successful applications of localized delivery include intranasal,[63, 64] intrathecal,[65-67] intratesticular,[67] intraliver[68], intramuscular,[69, 70] intraretinal,[71, 72] and intratumoral.[73, 74]

Among the current strategies being employed for siRNA delivery, the greatest overall efficiency has been observed with rapid infusion or hydrodynamic injection of siRNA to a limited set of vascularized tissues. Several groups have reported the use of such a technique to successfully introduce siRNA or shRNA-expressing constructs into mice, achieving delivery and subsequent silencing of the target genes.[58, 59, 75] A key constraint to this method, and possibly to all systemic methods of delivery, is that delivery tends to be restricted to highly vascularized tissues such as the liver, spleen, or kidneys. In addition, this technique is not a viable method for delivery in human clinical studies.

Another potential caveat that should be considered for either systemic or localized delivery is the potential for initiating an immune response. A reactive immune system may make phenotypic observations difficult to attribute entirely to siRNA function, thereby complicating data analysis. It is always important to look for common indicators of this response such as enlarged or swollen lymph organs or inflammation at the site of injection.

Delivery reagents

Delivery reagents that facilitate efficient siRNA or shRNA plasmid entry into cells include conjugates, liposomes, and other complexes. Conjugates that have been employed include cholesterol and peptides that can be covalently linked to the siRNA molecule, aiding uptake into tissues.[61] Amino acid-based nanoparticles have also been complexed to siRNA for localized delivery to the arterial wall in an atherosclerotic rat model.[76] Furthermore, proprietary chemical modification patterns, such as those applied to Dharmacon Accell siRNAs, also confer a delivery advantage to the synthetic siRNA so that it may be administered or applied in the absence of any delivery reagent.[39, 41, 64, 77]

Cationic liposomes, dendrimers, and atelocollagen are examples of delivery reagents that form complex structures with siRNA or shRNA-expressing constructs to facilitate cellular uptake.[60, 67] Cationic liposomes (*e.g.*,transfection reagents) are used routinely, with success, in cell culture, although they require lengthy optimization of transfection conditions. The primary concern with such reagents *in vivo* is that the cationic nature of the lipids used in cell culture leads to aggregation when used in animals and results in rapid serum clearance and lung accumulation. However, there are reports citing success with lipid-mediated delivery of siRNAs and shRNA-expressing constructs in animal studies.[78, 79] Compared to cationic lipids, polymer-based dendrimers display greater transfection efficiency and less toxicity in many cell types, and thus may have a greater potential for *in vivo* application. Finally, atelocollagen, a naturally occurring protein that is low in immunogenicity, is used clinically for a wide range of purposes. Studies using atelocollagen for siRNA delivery indicate increased cellular uptake, nuclease resistance, and prolonged release of genes and oligonucleotides. Atelocollagen displays low-toxicity and low-immunogenicity when transplanted *in vivo*.[67]

In lieu of linking or coupling RNAi molecules to delivery reagents, alternative delivery methodologies have also been successful. Direct electroporation into tissue has been used for localized delivery to skin and muscle, as it is effective with nearly all cell and species type and may be performed with intact tissue.[69, 70, 72] However, several disadvantages of electroporation include cell damage, rupture, and cell death. Other more clinically relevant delivery approaches, such as intranasal delivery by inhalation of aerosolized siRNA or intradermal delivery by a dissolvable protrusion array device (PAD), have both shown promising results with animal models. [41, 64, 107]

Silencing reagents

Chemical modifications and processing for siRNA

Issues pertaining to the selection of functional silencing reagents have, in large part, been addressed with the widespread use of rational design procedures for siRNAs and shRNAs (*e.g.,* SMARTselection design algorithms, microRNA-adapted shRNA expression technologies, etc.), Historical issues related to purity and/or sequence integrity of *in vitro* transcribed RNA species have been addressed by the chemical synthesis of RNA, which is known to provide the best material for direct introduction or injection of siRNAs into living cells. Additionally, chemical synthesis is amenable to the application of chemical modifications to the ends of an siRNA, as well as the introduction of modified bases or a modified nucleic acid backbone. Since an shRNA is an expressed RNA product, chemical modification is not an option, and any functional manipulation is limited to the shRNA and shRNA vector sequences alone.

The physical stability of the chemically synthesized siRNA is an important consideration. Unmodified siRNAs have a half-life of less than 10 minutes in 100% human serum due to degradation by nucleases. Thus, nuclease resistance of siRNA is important for efficient target knockdown. This is especially true when siRNA is systemically delivered, as there is an increased transport time to the target cells. One approach to address this challenge includes modification strategies to the siRNA itself. Stabilizing modification patterns enhance the persistence of full-length duplexes in biological environments such as animal serum. Proprietary modification patterns such as Accell and siSTABLE have been carefully designed with stabilizing modifications to optimize siRNA endo- and exonuclease resistance. In addition, Accell siRNAs are further modified for passive delivery into cells without the need for a transfection reagent, and may be particularly useful for *in vivo* delivery. Both siSTABLE and Accell modification patterns have been applied to siRNAs used for successful *in vivo* experimentation.[39, 41, 64, 76, 77, 80-83, 106]

To minimize the potential side effects that may be associated with *in vivo* delivery of synthetic nucleic acids, special processing options and purification procedures have been developed for producing siRNAs suitable for *in vivo* protocols. These processes often include counter ion (Na^+) exchange, desalting, sterile filtration, and endotoxin testing. These procedures are collectively referred to as "*in vivo* processing." The addition of purification (often through HPLC) results in a greater percentage of full-length siRNA. This extra step can be important when modifications (such as dye conjugates) are added to the synthesized siRNA product, or when very large final amounts of product are necessary. In the latter case, different synthesis instrumentation is often required for production of hundreds of milligrams or gram amounts; these larger scale synthesis procedures may unavoidably introduce unwanted salts or other substances that would warrant purification for complete removal. Although *in vivo* siRNA experiments have been carried out using a variety of processing and purification options, it is widely accepted that extra precaution is justified when working with animal models.

Expressed shRNA

Current strategies employed for *in vivo* delivery of shRNA expression constructs primarily involve the use of viral-based vectors. Factors influencing the choice of vector system include the cell type or tissue targeted, the number of cells requiring transduction (degree of biodistribution), and the duration of shRNA expression desired.

For transient expression of shRNAs, non-integrating vectors derived from adenovirus or herpes simplex virus-1 (HSV-1) can be used. For stable, long-term knockdown, integrating vectors such as oncoretroviral-based and lentiviral-based vectors are preferred. Oncoretroviral vectors, which include those derived from murine leukemia virus (MMLV), cannot transduce non-dividing cells and are therefore only useful for a limited number of tissue or cell types. Conversely, lentiviral vectors derived from the human immunodeficiency virus type 1 (HIV-1) are capable of transducing both dividing and non-dividing cells and have therefore become a popular choice for *in vivo* gene delivery.

Adeno-associated virus (AAV) is another powerful and versatile vector of choice for shRNA delivery. AAV is a naturally defective virus that requires a helper virus for genome replication; it is therefore thought to confer superior biosafety for research purposes. Additionally, AAV is capable of infecting both dividing and non-dividing cells, and also has the potential for long-term expression.

Ultimately, the choice of viral vector will be highly dependent upon the tissue type targeted. For example, HSV-1 has a natural tropism for neuronal cells and is widely used for delivery to the central nervous system.[84] Some viral vectors can also be engineered to target a certain tissue or cell type.[85] Many custom or commercially available lentiviral vectors are pseudotyped with different envelope glycoproteins to alter tissue tropism, ultimately altering the infectivity of the virus in a tissue- or cell-specific manner. Furthermore, the development of AAV chimeric serotypes has produced a variety of vectors with the ability to target specific cells and tissues.[86]

Dosage amounts and regimens

In addition to delivery route, other critical issues to consider when planning your *in vivo* experiments include the concentration of siRNA or shRNA delivered and the dosing regimen. Currently, recommendations for these parameters are very broad, as the biodistribution and pharmacokinetics of siRNAs remain to be elucidated. In general, a higher dose of siRNA or shRNA is used for systemic injection compared to localized injection. Current publications report systemic doses that range from 0.01-80 mg/kg/day and localized doses that range from 0.01-4 mg/kg/day. Typically, when using bolus injections intravenously, the highest concentrations are achieved in heavily vascularized tissues such as the liver and kidneys. The least likely tissues to be affected are the eye and brain[107], suggesting that targeting siRNA to these tissues requires alternative, or more direct, modes of delivery. We strongly recommend that a dose range study be assessed in the context of the target tissue, whether delivered directly to a specific tissue or injected systemically.

Detection

Monitoring delivery

For the detection of siRNA or shRNA delivery, either a label attached to the siRNA molecule (such as fluorescence or radioactivity), or an expressed fluorescent protein in the case of shRNA, can be used to measure relative uptake. The fluorescent-labeled siRNA molecule may be easy to synthesize and use, but it is possible that the label will change the siRNA pharmacokinetics. In contrast, radioactivity uptake provides good biodistribution data, but has regulatory requirements and poses technical and handling challenges.

When measuring the expression of a fluorescent protein, such as GFP, in order to determine shRNA expression, it is imperative to consider the design of the expression vector. For instance, if the shRNA and fluorescent reporter are under the control of separate promoter elements, a discrepancy may be observed when attempting to equate shRNA and reporter expression due to the inherent differences between distinct promoters. In an effort to circumvent any uncertainty in this regard, viral constructs can be designed to include a bicistronic element for the shRNA and reporter, where both transcripts are driven by the same promoter. This approach provides the added assurance that cells which are identified to be expressing a fluorescent reporter will also be expressing the shRNA of interest.

Detection of gene knockdown

For the detection of the siRNA or shRNA activity, either the phenotype or target mRNA/protein levels can be analyzed. A phenotypic assay is usually quite simple but may be easily misinterpreted, as the phenotype may be caused by multiple factors, and is not a direct assessment of the mRNA knockdown. The detection of changes in mRNA and protein levels is the most reliable method for determining siRNA or shRNA functionality and biodistribution. However, the isolation of mRNA and protein *in vivo* is much more difficult than in cell culture, and may require additional steps in order to avoid mRNA and/or protein degradation during the isolation procedure.

In summary, there are numerous factors to consider that may either positively or negatively affect your *in vivo* experiments. These include:

- the biodistribution of the siRNA or shRNA
- the type, amount, and concentration of the silencing reagent
- the mode or mechanism of delivery
- the chemical modifications and processing options selected for siRNA
- the viral vector elements chosen for shRNA
- the detection of expression and knockdown
- accurate data analysis

Because many of the experimental obstacles encountered with *in vivo* studies are related to ADMET, it is imperative to begin all studies with a well-designed experimental plan that incorporates the highest quality silencing reagents. Although there are many considerations and challenges to performing meaningful *in vivo* RNAi experiments, significant advances have been made over the last decade to allow researchers to continue toward the establishment of standardized guidelines for *in vivo* experimentation.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Chapter 8

Measuring knockdown

Measuring knockdown

Current technologies allow for detection of RNAi-mediated gene knockdown at both the mRNA and protein levels.

RNAi-induced gene silencing is an mRNA-level event with downstream effects on the amount of protein, so it is recommended that both mRNA and protein levels for a gene of interest (GOI) are analyzed whenever possible. Elucidating the extent of mRNA knockdown confirms efficacy of the RNAi reagents and bolsters confidence that a subsequent decrease in the corresponding protein level is likely to be the cause of the observed phenotype. However, the level of mRNA knockdown does not necessarily correlate with the level of protein reduction at any one time, because the half lives of the mRNA and protein may be different. Together, detecting both mRNA and protein levels provides greater confidence in an observed phenotype, and facilitates biologically relevant conclusions.

mRNA detection

Common methods for measuring mRNA expression levels include reverse transcription-quantitative PCR (RT-qPCR),[87] RNA sequencing (RNA-seq),[88] gene expression microarrays,[89] branched DNA (bDNA).[90] Each method allows for detection of mRNA knockdown, but each also comes with certain advantages and limitations to consider, such as sensitivity (approximate mRNA copy number required), sample (number and amount), and throughput.

RT-qPCR

RT-qPCR is the most common method of mRNA detection due to its amenability to high throughput for both samples and GOIs, and high sensitivity (as few as 10 copies of mRNA are required for detection). In this method, RNA is isolated from samples and reverse transcribed by a reverse transcriptase enzyme to generate complementary DNA (cDNA). cDNA is then used as the template in the PCR step, which uses primer sets complementary to the GOI, and a DNA polymerase to exponentially amplify a sequence of interest so that it can be fluorescently detected. Fluorescence is measured during each amplification cycle on a real-time qPCR system, allowing qPCR products (amplicons) to be quantified using a standard curve method (absolute quantification) or by comparing the quantification cycle (Cq) values of the GOI to reference genes (Figure 9).

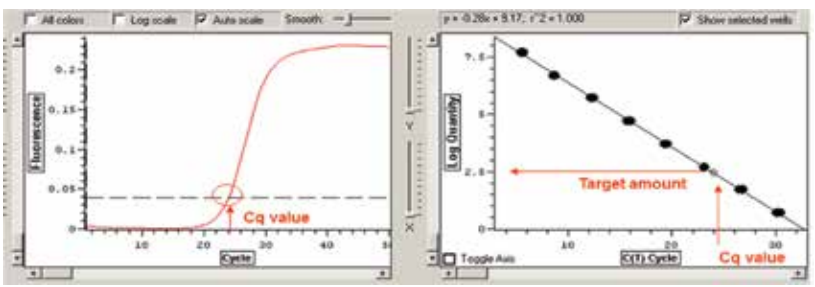


Figure 9. mRNA detection by RT-qPCR

Fluorescent detection of the amplified qPCR products can be accomplished with a double-stranded DNA binding dye (such as SYBR), or with fluorescent probes specific to the amplicons. Fluorescent probes contain a reporter dye and a quencher moiety, and work by two mechanisms: hybridization or hydrolysis. For hybridization probes, fluorescence is unquenched when the probe binds to the target sequence by separating the dye and the quencher spatially. Hydrolysis probes bind the target sequence of interest and the DNA polymerase subsequently hydrolyzes the probe, releasing the fluorescent dye from its proximity to the quencher.

A comparative quantification cycle (Cq) method (ΔΔCq) can be used to calculate relative gene expression, and therefore the percent knockdown, from Cq values obtained by qPCR analysis. In short, this method compares the number of cycles required for the GOI to reach the fluorescence threshold to a known REF. The ratio is then compared to treatment controls, yielding relative gene expression values that account for both experimental and non-experimental variation, reducing bias in the results.[92]

RNA-seq

RNA-seq uses next-generation sequencing (NGS) to reveal the presence and quantity of RNA in a biological sample at a given moment in time. This method is highly effective for detecting and quantifying mRNA, as well as other RNAs such as microRNA and long noncoding RNA, at the transcriptome level.[88] To generate an NGS library, RNA is isolated from control cells and experimental cells where siRNA has been delivered. The RNA is converted into cDNA and processed for analysis on a particular NGS system. Reads can be aligned to an annotated reference genome or assembled into overlapping sequence data (contigs). RNA-seq is more sensitive and less biased compared to gene expression microarray analysis, and increasingly more cost effective for the quality and amount of data obtained.

Gene expression microarrays

Microarrays can also be used to detect changes in relative gene expression. This format is similar to RT-qPCR in that it has comparable sensitivity and high GOI throughput (thousands of genes in a single experiment), but sample throughput is relatively low.[93] In the microarray workflow, RNA harvested from samples is reverse transcribed into cDNA, which is subsequently coupled to fluorophores. This labeled cDNA is then incubated on the microarray chip, allowing it to hybridize to probes immobilized on the chip. Each probe cluster is specific to one gene and has a known coordinate on the chip. Following vigorous washing to remove nonhybridized cDNA, fluorescence can be measured and relative gene expression and/or level of knockdown determined.

Branched DNA

Another method of mRNA detection is bDNA analysis, which is advantageous due to its ease of use and ability to process many samples. However, the limit of detection is approximately 3000 copies of mRNA, so is therefore not ideal for detecting genes with very low expression levels or when sample amount is limiting. This detection method requires no amplification of gene product and uses the concept of hybridization to detect the mRNA. bDNA utilizes capture and capture-extender oligonucleotides to hybridize the mRNA of interest to a solid support, typically the bottom of a 96-well plate. A bDNA probe, typically conjugated to an enzyme (such as alkaline phosphatase), and a label extender are then hybridized to the immobilized target mRNA. Washing steps are utilized to remove nonspecific binding, and then substrate is added to the well, which is cleaved by the conjugated enzyme, creating luminescence that can be detected using a plate reader. Gene expression is calculated by normalizing fluorescence levels of the GOI to REF(s) relative to controls.

Protein detection

The ultimate goal of RNAi gene knockdown experiments is not only to knockdown the mRNA of the GOI, but to also decrease the amount of subsequent protein to determine its biological role in a phenotype. For a thorough RNAi experiment it is essential to assess both mRNA and protein knockdown prior to drawing conclusions about an observed phenotype. While mRNA knockdown is typically detected within 24 hours post-transfection (depending on the half life of the mRNA), reduction in protein level may take a minimum of 24 to 96 hours, or possibly longer. Current methods for specific detection of proteins for relative quantification include western blotting[94] and Enzyme Linked Immunosorbant Assay (ELISA).[95]

Western blot analysis

One of the most established and commonly used methods for detecting changes in protein expression is Western blot analysis. Western blotting can be highly specific for the protein of interest, but it is both low throughput and requires a large amount of starting material for detection – approximately 20-30 µg of total protein or 10-100 ng of the protein of interest. Treated and control cells are lysed and proteins loaded into a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) denaturing gel for electrophoresis to separate protein by size. Separated proteins are then transferred to a membrane and treated with a primary antibody that binds specifically to an epitope on the protein of interest. The membrane is washed to reduce nonspecific binding, and an enzyme conjugated secondary antibody is added to bind specifically to the constant region on the primary antibody. Substrate is added and cleaved by the enzyme on the secondary antibody, allowing visualization of the relative protein abundance using an imaging system such as x-ray film. Western blotting is mainly a qualitative technique in which only large changes in protein abundance are detectable. Its application and success are dependent on the availability of high quality, specific antibodies. Regardless, Western blotting is still invaluable for detecting relative change in protein expression under varying cellular conditions.

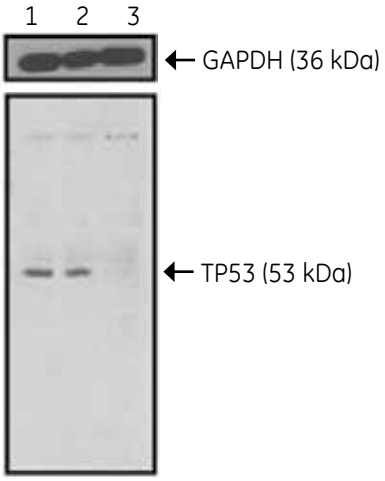


Figure 10. Western blot data demonstrating RNAi-mediated knockdown of TP53.
A549 cells were transfected with siRNA and then lysed after 72 hours. Cells were transfected with DharmaFECT transfection reagent alone (Lane 1), 50 nM ON-TARGET*plus* Non-targeting Pool (Lane 2), or 50 nM ON-TARGET*plus* SMARTpool siRNA reagent (Lane 3) targeting TP53. Western blot data for GAPDH is included as a control for equal protein loading.

ELISA

The ELISA allows high throughput protein quantification over a large dynamic range of starting material – typically ranging from approximately 50 to 5000 pg/mL. However, the amount of starting material and dynamic range of detection will vary depending on the assay and the protein of interest being detected. Most commonly, an antibody-sandwich ELISA is the method of choice. In short, a primary antibody, called the capture antibody, is immobilized to a solid surface such as the bottom of a 96-well plate and pre-incubated with a blocking reagent to prevent nonspecific binding in subsequent reactions. Wells containing the immobilized and blocked antibody are then incubated with cell lysate or extracts being tested for the protein of interest. In addition, wells are treated with samples containing known concentrations of the serially diluted protein of interest, creating a standard curve. Following incubation, the protein of interest is now captured by the primary antibody, and sandwiched with a secondary HRP-conjugated antibody. Finally, a chemiluminescent or colormetric substrate is subjected to HRP oxidation, creating light or a color change that can be detected by a plate reader. Unknown test wells are then compared to the known standard curve to quantify the protein concentration.

Chapter 9

High-throughput RNAi library screening

High-throughput RNAi Library Screening

High-throughput RNAi screening has become a key discovery methodology in many aspects of research, including disease onset and progression, drug target discovery, host-pathogen interactions, and pathway analysis.

In recent years, improvements in RNA chemistry and manufacturing processes have facilitated the cost-efficient production of genome-scale RNAi libraries. For example, in 2005, Dharmacon, now part of Horizon, produced the first library of siRNA reagents targeting each gene in the human genome, arrayed in microtiter plates. Such libraries permitted the rapid disruption of thousands of genes in parallel, and expanded the realm of functional genomics beyond gene expression microarrays. Today, high-throughput RNAi library screening, or the systematic RNAi-induced silencing of many genes in parallel, combined with sophisticated automation, data infrastructure, biological assays and bio-statistical analysis methods, is routinely employed in academic and pharmaceutical laboratories. Genome-scale RNAi libraries are utilized in conjunction with chemical compound screening and classical genetic tools to interrogate gene function, explore a diverse range of biological functions, and characterize pathways and networks.[96, 97]

Types and formats of RNAi libraries

Large libraries of knockdown reagents targeting mammalian genomes (predominantly for human and mouse) can be obtained as collections of siRNA or shRNA. Scientists must first consider which type of RNAi library will best meet the goals of the intended experiments. Both types of libraries have advantages over the other in specific aspects; for this reason, many advanced RNAi screening laboratories employ both siRNA and shRNA libraries.

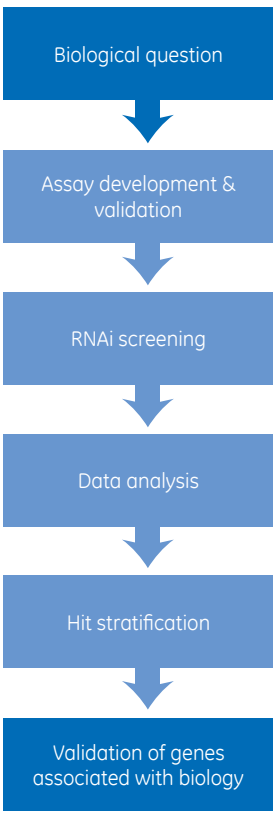
Genome-wide synthetic siRNA libraries can be produced with high quality and consistency, and are extremely amenable to automation and high-throughput methodology. In most cell types siRNAs can effectively be delivered using transfection reagents, and therefore only require resuspension prior to use. However, maximal silencing is generally achieved within 48-72 hours and disappears entirely within 5-7 days in actively dividing cells. Depending on the assay of interest, this duration of silencing might be insufficient.

Collections of shRNA libraries supplied in microtiter plates as plasmid-transformed *E. coli* glycerol stocks are readily available at the genome scale, and provide a renewable source of RNAi material. These are biological resources, and each clone must be grown and maintained for quality and viability. Plasmid preparation is required prior to use, which can be time consuming. Silencing efficacy is a function of cellular expression of the shRNA and is also transient, unless viral methods are employed.

The potential of shRNA is most fully realized when delivered using viral methods. shRNA vector constructs packaged into pseudotyped, non-replicating lentiviral particles are able to deliver their genetic payload into many cell types, including primary and non-dividing cells. However, production of lentiviral particles arrayed in 96-well plates is both expensive and technically challenging. It is difficult to control well-to-well and plate-to-plate titer variability; in addition, the optimal goal of using concentrated lentiviral particles requires expensive packaging of each individual construct and further manipulation to array into a suitable screening format. Thus, while lentiviral shRNA approaches are ideal for observing long-term, integrated silencing effects, technical limitations make this high-throughput genome-wide screening strategy more costly and labor intensive.

The desire to combine lentiviral delivery with high-throughput screening has led to the development of pooled lentiviral shRNA methods in which hundreds or thousands of unique shRNAs are pooled together and then packaged into lentiviral particles. In this manner, cells can be transduced and integrants expressing individual hairpins can be isolated and identified using several selection strategies. This innovative approach has yielded an increasing number of published functional genomic screens both *in vitro* and *in vivo*.[98-104]

General considerations and the high-throughput RNAi screening workflow



Today the availability and relative ease-of-use of siRNA, shRNA, and microRNA libraries permits routine high-throughput loss-of-function screening to dissect molecular mechanisms in both normal and disease physiology. Setting up a facility to perform large arrayed RNAi screens, requires, at a minimum: liquid handling, reagent dispensing, plate stacking, lidding/delidding and microplate washing instrumentation, data management systems, high-capacity incubators, and assay read-out infrastructure. Labs that plan to run many genome-scale arrayed screens often incorporate robotics, which greatly increases reproducibility and accuracy over manual execution of key screening steps. Therefore, many academic institutions have screening facilities that maintain all the equipment necessary for independent users.

Figure 11. General RNAi screening workflow.
Success of an RNAi screen relies heavily on rigorous planning and execution of each critical phase. Prior to the screen itself, cell culture, RNAi reagent delivery, and assay conditions must be carefully optimized, then validated using the automation, instrumentation, and data collection tools that will be utilized during screening. After primary screening, statistical methods that are appropriate for the type of data being collected are applied to identify positives (hits) that need to be verified through confirmatory screening and further, more focused assays.

RNAi screening typically can be carried out with fundamental cell-based assay instrumentation such as plate readers and automated fluorescent microscopes. Many homogenous colorimetric, fluorescent and luminescent end-point assays are commercially available as kits and are commonly used to assess cell proliferation, protein secretion, activity of engineered reporter genes, and induction of apoptosis. In recent years, high-throughput RNAi screening methods have been combined with sophisticated high-content imaging instrumentation to achieve spatial, temporal and kinetic multi-parametric data sets. High-content analysis permits the observation of loss-of-function phenotypes in a broadened biological context and may allow for increased sensitivity due to the ability to characterize several parameters in each well.

While the general experimental workflow and critical success factors are similar to those applied in single-gene experiments, there is a greater requirement for stringent assay development and optimization required prior to performing a screen.

Assay development and validation

All RNAi screens begin with development and optimization of key experimental parameters, including those of delivery methods and the phenotypic assay. Given that genome-scale screens generate enormous datasets, which must be analyzed and validated, careful assay development is required to minimize false positives and negatives.

Once the format of the screen has been chosen, based on the goals of the screening and the biology being explored, the conditions for efficient and consistent delivery of the RNAi trigger should be optimized. The goal is to achieve the best transfection, electroporation, or transduction conditions for effective gene silencing while minimizing cellular toxicity. Parameters which must be taken into consideration include not only the volume and concentration of siRNA-transfection reagent complex or viral particles, but also the cell density, the medium conditions, the length of incubation time, and the addition of any other additives to increase delivery efficiency. As such, optimization often begins with large, matrixed experiments, ideally carried out in biological triplicates, involving multiple reagent options, conditions and time points using positive and negative controls.

Good positive and negative controls are critical not only for determining optimal delivery conditions, but also for establishing the experimental window within which positive hits can accurately be identified and quantified. The rigorous testing of several negative and positive RNAi controls during delivery and assay optimization is the cornerstone of achieving high-quality data. A negative control ideally engages the endogenous RNAi machinery but does not target any gene of interest and results in no identifiable effect on the phenotype of interest. To ensure that a negative control is truly serving the intended purpose, multiple negative control candidates should be tested in each cell line and assay of interest and compared to untreated or mock transfected cells. In this manner, a negative control determines the baseline of the assay.

Appropriate positive and negative controls are required to define the magnitude of the potential phenotypic response, and thus, the dynamic range of the assay. Often the most challenging part of assay development is the identification of a suitable positive control gene target. The positive

control must reliably produce the phenotype, show a dose response, and the targeted gene must be expressed at a detectable level in the cell type(s) to be screened. Common approaches to find a suitable candidate can begin with literature searches or utilization of pathway analysis tools to find genes involved in the biology being studied and may require small RNAi screens to examine multiple gene candidates in the cells and assays of interest.

Once negative and positive controls are determined, these should be employed in establishing reproducibility across plates and replicates run on different days utilizing the software, instrumentation, and automation intended for the screen itself. Running multiple plate layouts of positive and negative controls allows the early identification of systematic issues such as row, column, or plate edge effects.

siRNA screening

Data quality, reproducibility, and overall assay performance should be monitored in real-time during high-throughput screening using appropriate quality metrics, such as calculating Z′ factors of all plates, to ensure sufficient maximum and minimum signals are consistently achieved. Running a pilot screen in which a small number of genes are silenced is useful for assessing reproducibility, sensitivity, and reliability of the assay and all experimental parameters and is also informative for considering what statistical method(s) of analysis may be applied for hit selection. For a comprehensive review of statistical methods suitable for RNAi screens, see Birmingham *et al.*[105]

Pooled lentiviral shRNA library screening

While many mammalian cell lines are suitable proxies for normal and disease biology models, the desire for screening in a more clinically relevant model may necessitate the use of cells which are difficult-to-transfect, such as primary cells, or hematopoietic, neuronal and immunological cell types. Alternatively, the phenotype being studied may take more than 4-5 days to fully develop, and thus require longer-term silencing than can be afforded by transfection of synthetic siRNAs and plasmid shRNA constructs. An alternative screening approach being utilized with growing frequency both *in vitro* and *in vivo* is transduction of lentiviral shRNA pools. Screening libraries are generated by combining shRNA constructs into pools of various sizes (ranging from 2,000 to an excess of 100,000 shRNA per pool) and generating batches of concentrated lentiviral particles. During a pooled screen, cells are simultaneously transduced with thousands of shRNAs, resulting in a mixed population of cells, each containing a single shRNA. This technique provides a high-throughput screening option without the need for extensive automation.

Pooled screening methods generally involve either positive or negative selection strategies. Both selection strategies begin by transducing cells at a low multiplicity of infection (MOI) with a lentiviral pool containing hundreds to thousands of unique shRNAs. Each cell in the resulting

transduced population will contain a unique shRNA integrated into its genome. Following transduction, a selective pressure is applied, such that shRNAs targeting genes involved in a biological response to the pressure may be identified.

Negative selection screens are performed to uncover changes in the abundance of integrated shRNAs as a result of the selective pressure, such as drug treatment. Genomic DNA (gDNA) is isolated from the initial transduced cell population (control cells) and from the transduced cell population that remains following the application of selective pressure. Primers are designed to amplify unique nucleotide sequences associated with each shRNA using PCR of gDNA isolated from the two cell populations. PCR-amplified gDNA can then be hybridized to microarrays or deep sequenced to assess differences in shRNA abundance between control and experimental cell populations.

Positive selection screens are performed to identify individual shRNAs which provide a particular advantage to cells under a given pressure. Following the application of selective pressure, gDNA is isolated from colonies of cells and PCR is used to amplify the shRNA. Following PCR-amplification of gDNA, either standard Sanger sequencing or deep sequencing can be employed to determine genes which may be involved in the observed phenotype.

To learn more about the critical parameters of successful pooled lentiviral shRNA screening, including the conditions necessary for maintaining a high shRNA fold-representation, please review the following publication: Ž. Strezoska, A. Licon, Optimized PCR Conditions and Increased shRNA Fold Representation Improve Reproducibility of Pooled shRNA Screens. *PLoS One* **7**, e42341 (2012).

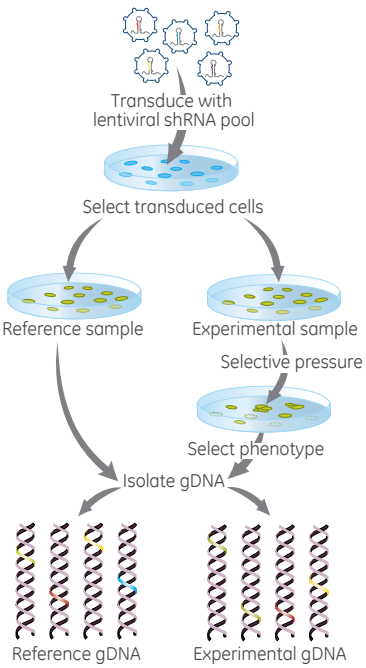


Figure 12. Overview of pooled screening workflow

Hit stratification

Hit lists created by statistical tools from the primary screen need to be stratified and validated. The primary screen is intended as an unbiased approach to the gene set, and cannot address questions about off-target effects, siRNA potency, mRNA knockdown levels, or biological pathway relevance of the gene. A confirmatory, repeat screen of the hits using the same reagents, assay and conditions is intended to reinforce the primary screen results and eliminate false positives that could have resulted from simple experimental variation. Secondary screening involves the use of additional assays, RNAi reagents, alternative cell types and reagent formats (arrayed or pooled), and serves to increase confidence in the hits prior to further, in-depth target validation.

Confirmation of results

In addition to the detection techniques described in the prior chapter, there are numerous other methods commonly employed following an RNAi screen to further stratify hits and filter out potential false positives, most often due to off-target effects.

Redundancy

The most common method to confirm experimental results is through the use of multiple RNAi reagents, including siRNA, shRNA, and microRNA mimics and inhibitors where appropriate. The purpose of using independent RNAi reagents comes back to the off-target effects discussed earlier, as each RNAi reagent has a different off-target signature. An RNAi reagent is considered unique when it utilizes a different target sequence and/or contains different modifications, such as an siRNA versus an shRNA, or siRNAs with different modification patterns. The goal is to silence the same target, but in the context of different off-target signatures. Higher confidence is placed on results when different reagents and their associated potential off-target effects results in similar mRNA knockdown and phenotypic results.

Rescue experiments

Functional rescue experiments are considered the gold standard for demonstrating the observed RNAi-mediated phenotype is not an off-target effect. In these experiments the endogenous target gene is silenced while an exogenous plasmid expresses an RNAi-resistant version of the gene. If the phenotype reverts, this is very strong evidence that silencing of the target gene was the cause of the phenotype. There are multiple ways to perform rescue experiments:

1. If the siRNA or shRNA has been designed to the 3' UTR: Transfect cells with an over-expression construct that contains only the open reading frame (ORF) of the gene of interest, or a plasmid that contains a heterologous 3'-UTR that is not responsive to the siRNA. In this case, the sequence that the siRNA targets will not be present in your rescue sequence.
2. If the siRNA or shRNA has been designed to the ORF: Transfect cells with an over-expression plasmid that contains silent mutations in the region targeted by the siRNAs. This will allow you to maintain the correct amino acid sequence of the protein, but the mRNA sequence will be different and thus not susceptible to siRNA cleavage.
3. Use an expression system with an mRNA sequence from a different species. There will need to be sufficient sequence differences between the two transcripts at the site of siRNA or shRNA binding.

CRISPR-Cas9 gene knockout

As a method for gene knockout that is independent of the RNAi pathway, CRISPR-Cas9 gene editing is an effective tool for orthogonal validation of gene knockdown by RNAi. While RNAi works at the level of the mRNA transcript, CRISPR-Cas9 permits cleavage and functional gene knockout at the DNA level, so the researcher has the benefit of observing a loss-of-function phenotype from two completely independent methods, and without overlap of potential off-targets. It should not be expected that the concordance of knockdown and knockout will always be identical, but when the two techniques are in agreement, it increases confidence that the observed phenotype is due to the loss of the intended protein target. As with siRNA, it is recommended to use 3-4 unique guide RNAs along the target gene to verify the result with multiple independent data points.

C911 controls

A proposed method for identification of false positives uses a control siRNA matched to an experimental siRNA which has successfully demonstrated a phenotype.[105] These control siRNAs, called C911 controls, are identical to the experimental siRNA but with mismatched bases at positions 9, 10, and 11 of the antisense strand (Figure 13). These mismatches are thought to disrupt cleavage of the original mRNA target, while cleavage while maintaining potential off-target effects, thereby controlling for phenotypes driven only by off-targets. Therefore, comparison of the original siRNA and the C911 control should distinguish phenotypes due to down-regulation of the intended target rather than off-target effects.

While this method is beneficial, such that it does not rely on additional assay development or technologies, it can be cost-prohibitive to generate custom siRNAs for tens or hundreds of siRNAs, as might be needed following an RNAi screen.

It is possible that some C911 controls may knockdown the original target gene to some degree. It is recommended that target knockdown be measured for both the experimental and C911 siRNAs to properly conclude whether on-target gene knockdown contributed to observed phenotypes.

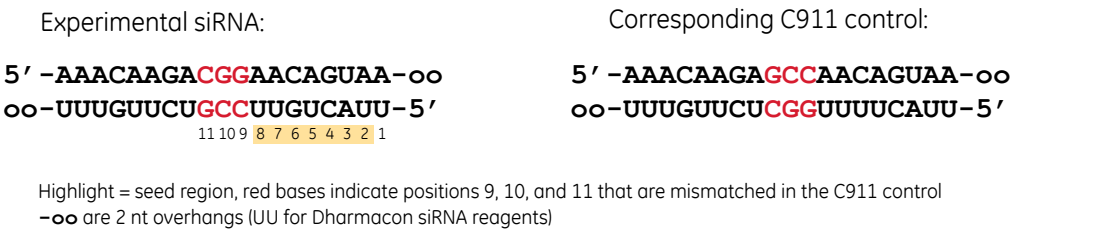


Figure 13. Illustration of an experimental siRNA and its corresponding C911 control

Highlight = seed region, red bases indicate positions 9, 10, and 11 that are mismatched in the C911 control
-oo are 2 nt overhangs (UU for Dharmacon siRNA reagents)

Seed sibling control siRNAs

A seed sibling is a siRNA that has no known target in a species of interest, but whose seed is a perfect match to an experimental siRNA that gives a phenotype (Figure 14). By running a validation study with experimental siRNAs side-by-side with 4-5 of its seed siblings, you can determine if the seed alone is active in initiating a phenotype (since it has no known targets) thereby indicating that the phenotype is likely due to a seed-mediated off-target effect. Alternatively, if the seed siblings do not generate a phenotype, there is good indication that the hit was due to knockdown of the intended target and not a seed-based off-target effect.

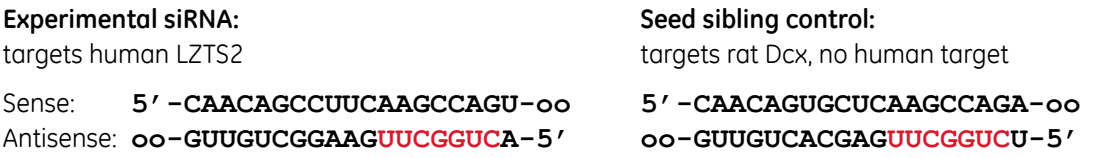


Figure 14. Example of an experimental siRNA and its seed sibling control siRNA

Highlight = Heptamer seed region **-oo** are 2 nt overhangs (UU for Dharmacon siRNA reagents

Obtaining seed sibling control siRNAs can be accomplished with a lookup tool as part of the Dharmacon Cherry-pick Plater (https://dharmacon.horizondiscovery.com/libraryimport/). When a researcher provides a list of experimental siRNAs to validate (either a Dharmacon catalog number or the sense/target sequence), the tool will return a list of catalog siRNAs that have the same seed region as the indicated experimental siRNA, but with no perfect match target in the species of interest. Since this approach uses existing Dharmacon catalog products, these reagents are immediately available as there is no custom synthesis required.

Additional considerations for successful screening

Many aspects of a screen need to be planned ahead of time in order to have confidence in the results generated. Here are some things to take into consideration before beginning a screen:

Banking

- Banking of sufficient cells for multiple screens
  - Changes in cell type during a screen or validation process can result in variable results
- Cells should all be the same passage
  - Cells react differently as they age or are cultured over time
- Protocols used for thawing cells should be identical
  - Changes in protocols during a procedure can have direct consequences on results
- Banking of any reagents used to maintain cells, such as medium, and serum
  - Serum batches in particular can have different levels of endotoxins, which are known to induce certain genes, therefore make sure all serum is from the same batch
- Banking of transfection reagents
  - Storage and handling of transfection reagents should be identical and preferentially all from the same lot number

Transfection

- Forward or reverse transfection?
  - Reverse transfection typically requires less transfection reagent, but some cells are sensitive to this method
- Which transfection reagent?
  - Need to optimize and determine which reagent works best for the cell line being used
- How much transfection reagent?
  - Needs to be optimized so the least amount of transfection reagent is used to reduce off-target effects and save on reagent costs

Incubation

- How long before phenotype interested in can be evaluated?
  - Important optimization parameter
- How many cells/well are needed?
  - Want cells to be able to survive length of screen

Assay

- What assay will be used?
- How much reagent is needed for this assay?
  - Bank any reagents necessary for assay
- What is the positive control necessary for this assay?
  - Cell type and assay specific
- Is automation needed
  - If yes, what facilities are available?

Assay data quality

- Meeting with statistician should be done before beginning screen
  - Recommended resource: Birmingham, A., L. M. *et al.* (2009). "Statistical methods for analysis of high-throughput RNA interference screens." *Nat Methods* **6**(8): 569-575.
- Determination of potential position effects needs to be performed
- Quality and robustness of assay should be measured (Z' factors), and cutoffs defined

Generate Hit List

- Raw Data Analysis
- What type of statistics will be performed?
  - Contact statistician
- Define hit rate

Validate Hits Identified

- Repeat assay for interested hits
  - To ensure true hits from initial screen
- Use different reagents to confirm phenotype
  - Unique RNAi reagents include reagents with different sequences as well as reagents with identical sequences but different modifications
- Use different assays and cellular systems
  - Confirmation from multiple systems gives further confidence in hits generated

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Glossary

PHRASE	EXPLANATION
Antisense strand	This represents the strand of an siRNA (or processed shRNA) that is complementary to the target mRNA; also referred to as the catalytic, targeting or guide strand.
Complementary DNA (cDNA)	DNA synthesized from a mature mRNA template in a reaction catalyzed by the enzyme Reverse Transcriptase. cDNA is used to clone eukaryotic genes for exogenous expression in prokaryotes or in other eukaryotic cells and for amplification in RT-PCR and RT-qPCR.
Clone	An exact copy of all or part of a macromolecule, for example DNA.
Coding sequence (CDS)	Section of a gene or mRNA that codes for a protein.
Dicer	A member of the RNase III family of nucleases present in the cytoplasm. It is involved in processing long dsRNA and microRNA into silencing intermediates that can interact with the RISC.
Drosha	A member of RNase III family of nucleases present in the nuclear compartment of a cell. It is the nuclease component of the Microprocessor complex and is responsible for the processing of naturally expressed primary (pri-) microRNA transcripts into precursor (pre-) microRNA hairpin structures. The products are then transported to the cytoplasm where they enter the RNAi pathway to regulate genes.
Exon	Segments of genomic DNA which will be incorporated into the final mature mRNA. Exons may include coding sequence, 5' untranslated region or the 3' untranslated region.
Expressed Sequence Tag (EST)	A partial sequence (~500-800 bp) derived from a cDNA. The data for the sequence information is limited typically to 3' or 5' end sequence. The EST is produced by one-shot sequencing of a cloned mRNA.
Gateway adapted	A cloning system that enables efficient transfer of DNA fragments between plasmids using a proprietary set of recombination sequences. It has effectively replaced the use of restriction endonucleases and ligases for some cloning applications. This process is patented by Life Technologies (formerly Invitrogen).
Knockdown	Reverse genetic technique for reducing or silencing the target mRNA or protein level as a result of RNAi.
Knockout	Genetic technique for deleting a gene from the genome of an organism to assess the gene function.
miRNA/microRNA	Short, ~17-25 mer endogenously expressed RNA molecules that function as gene expression modulators. They are transcribed from noncoding sequences in the nucleus, processed and transported to the cytoplasm where they interact with the RISC to effect gene modulation.
Multiplicity of infection (MOI)	The ratio of transducing viral particles to cells. An MOI of 10 indicates that there are ten transducing units (TU) for every cell in the well. It is important to note that different cell types require different MOIs for successful transduction and knockdown of the target gene.
Open Reading Frame (ORF)	The portion of an mRNA that contains a sequence encoding a protein. The 5' untranslated region and 3' untranslated region are typically included upstream and downstream of this sequence.
Phenotype	Observable features of cells or organisms, for example, shape, size, differentiation state, expression patterns.
RefSeq	Curated database of gene sequences that represents non-redundant, annotated consensus or reference sequences derived from entries to the International Nucleotide Sequence Database Collaboration (INSDC). This is the sequence database used by the Dharmacon research team to design silencing intermediates for RNAi (www.ncbi.nlm.nih.gov/RefSeq/).
RNA Induced Silencing Complex (RISC)	This multi-component complex of proteins incorprates short RNA strands from microRNA, siRNA or shRNA to form the activated RISC. Activated RISC surveys the mRNA population to identify its target and to effect sequence-specific gene silencing.
RNA interference (RNAi)	A cellular mechanism by which silencing intermediates (siRNA and microRNA) reduce the expression of a target gene through a sequence-specific mechanism.
Sense strand	The strand of an siRNA that is identical to the target mRNA region; also referred to as the passenger or non-catalytic strand.
short hairpin RNA (shRNA)	Small RNA sequences or transcripts consisting of 19 to 29 base pair stems bridged by 4 to 9 nucleotide loops. In the cell, it is cleaved by Dicer-containing complex into siRNA resulting in target gene silencing or knockdown.
small interfering RNA (siRNA)	A double-stranded RNA comprised of a 19 nucleotide core sequence with two nucleotide overhangs on the 3' end of each strand. One strand of the duplex is taken up by the RISC whereby the activated complex finds the corresponding message to silence or knockdown.
Transduction	The transfer of viral, bacterial or both bacterial and viral DNA from one cell to another using a viral vector.

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