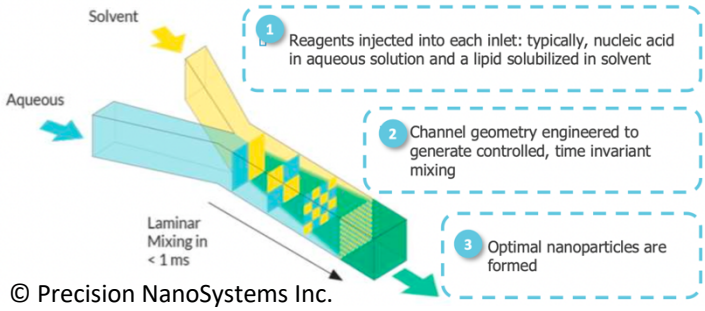
Laboratory 5 – Lipid Nano Particles

Liposomes made using a mixture of neutral, ionizable cationic lipids, cholesterol, and other modified lipids (e.g. PEGylated lipids) are commonly used as carriers for nucleic acids. The Pfizer/BioNTech and Moderna COVID-19 vaccines are well known examples of this.



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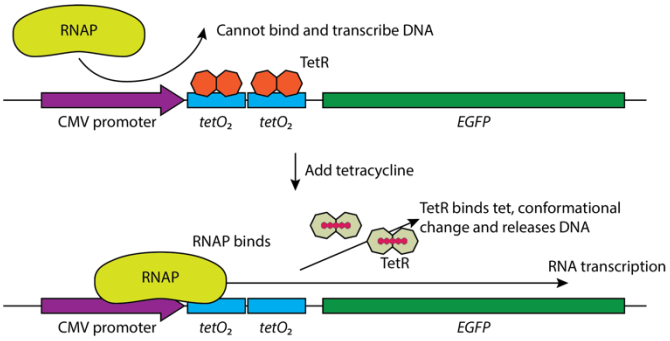
Although extrusion can be used to produce liposomes, liposomes used to encapsulate RNA are typically produced using microfluidics. Extrusion based methods are often harsh (especially for sensitive components like RNA), give variable results, and can be difficult to scale. Microfluidic manufacturing methods use micro-scale channels engraved onto chips to allow for carefully controlled mixing of lipids and aqueous solutions. This process gives highly reproducible results, is easy to scale, and provide much gentler conditions that prevent degradation of sensitive RNA molecules.

Many types of nucleic acids can be encapsulated using liposomes, however, the larger the payload, the more difficult it is to encapsulate. It is for this reason that most labs currently work with small interfering RNA (siRNA), other small RNAs, or mRNA as their payload when developing liposomal formulations.

In this lab, we will use the Precision NanoSystems Spark NanoAssembler and Hepato9 RNA encapsulation kits to produce lipid nanoparticles that encapsulate siRNA that target green fluorescent protein (GFP). We have a human cell line that have been stably transfected to contain the GFP gene (Flp-In T-Rex 293-GFP). This gene is under the control of the strong CMV promoter; however, transcription is blocked due to the presence of two *tetO*$_2$ operator sequences immediately following the TATA box. These sequences each bind a TetR repressor homodimer, and block transcription from proceeding. The TetR repressor is part of the *E. coli* tetracycline resistance operon, and has been engineered into the cell line to control protein expression. When tetracycline is added to the culture media, it will enter the cell, and bind the TetR repressor protein causing a conformational change resulting in the protein releasing the DNA. This allows the RNA polymerase complex to bind the promoter and produce the full-length mRNA. Generally, within 24-hours of the additional of tetracycline, you will see a high level of protein expression, however the amounts of tetracycline and the time to maximal expression must be optimized for each protein being produced.



This can be important if dealing with a potentially toxic protein, or a protein that might inhibit growth if expressed in high levels. By controlling when the protein is expressed, you can ensure enough cells have survived to produce a large amount of the desired protein upon induction, even if the cells die afterwards or are no longer able to replicate.

If the cells are treated with anti-GFP siRNA, at the same time as they are induced, the mRNA produced by the cells will be targeted for destruction by RISC, preventing the majority of GFP expression compared to untreated cells, or cells treated with a control construct that does not target GFP.

1. Production of liposome encapsulated DsiRNA by microfluidics

Background

In today's lab, we will be creating lipid nanoparticles (LNPs) that encapsulate DICER-substrate short interfering RNAs (DsiRNAs), 27mer duplex RNAs optimized for DICER processing, to knock down expression of our gene of interest, in this case EGFP, which is produced by our stably transfected Flp In T-REx 293-EGFP cell line upon induction by tetracycline. Mammalian cells do not normally take up DNA or RNA from the surrounding environment, so we must use a delivery vehicle to get our nucleic acids in. Traditionally, cells are stimulated to uptake DNA through the use of Lipofectamine, which is a 3:1 mixture of the cationic lipid DOSPA (2,3-dioleoyloxy-N- [2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propaniminium trifluoroacetate), and the neutral lipid DOPE (l-α-1,2-dioleyl-*sn*-glycerophosphoethanolamine). These lipids form a complex with the negatively charged DNA, masking the negative charge and allowing the complex to approach the negatively charged cell membrane. Once there, the complex is endocytosed and either fuses with the endosome (aided by the presence of the fusogenic DOPE), releasing the DNA to the cytoplasm, or is eventually degraded in the lysosome. These DNA-lipid complexes are large (~400-600 nm), have high variability in size and shape, and often form multilamellar structures. Due to this, only a portion of the DNA is ever released into the cells, with even less making its way into the nucleus for transcription. Although RNA can also be delivered using these methods, it is less efficient, and other (often more expensive) lipid mixtures must be used to effectively deliver these molecules, in part due to the need to protect the RNA from endogenous RNases in the cell media. Additionally, lipofectamine, and other similar transfection reagents, tend to result in high levels of cellular mortality, possibly due to activation of cellular stress response genes (Fiszer-Kierzkowska et al., 2011).

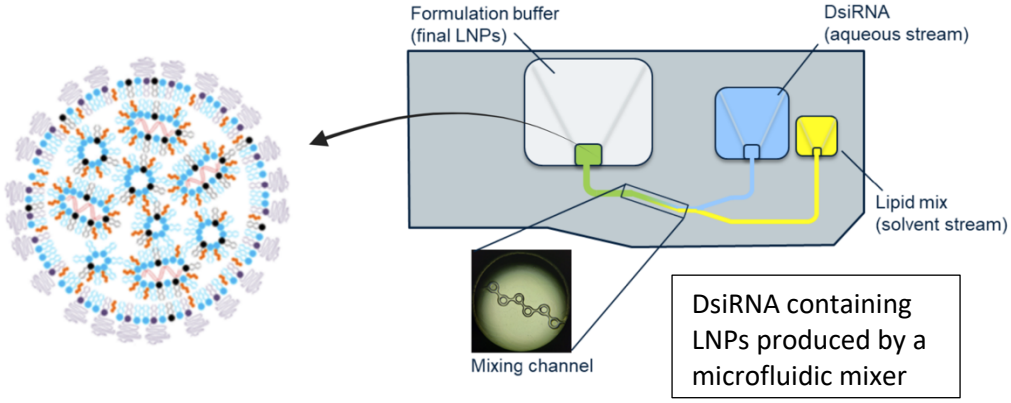
The use of LNPs to encapsulate RNA is becoming increasingly popular. These particles have a number of advantages over traditional transfection methods including enhanced protection from nucleases, better delivery and release of the payload into the cytoplasm, decreased cellular toxicity seen using both chemical (lipofectamine) and physical (electroporation) based transfection methods, and there are no safety concerns as are seen with the use of viral vectors (which are also very labour intensive and time consuming to prepare). Finally, the use of LNPs allows for a single approach for RNA delivery to cells for all stages of research and drug development, and with appropriate optimizations, could be directly developed into a marketable therapeutic.

The LNPs that we will be making are a proprietary mixture of lipids (including neutral-, ionizable-, and PEGylated lipids, as well as cholesterol) which will complex with our DsiRNA in a process called microfluidic mixing. Microfluidic mixing is different from the extrusion-based method we used previously in that the particle formation takes place inside a microscale mixing channel that rapidly mixes two liquid streams (an aqueous and organic phase) to promote nanoparticle self-assembly. For this method we will use the NanoAssemblr Spark system from Precision NanoSystems.

For the microfluidic mixing, the nucleic acid payload (our DsiRNA that will be loaded into LNPs) is prepared in an aqueous buffer, while the lipid components of the LNPs are dissolved in an organic solvent (typically ethanol). Both solutions are then loaded into separate compartments (or wells) of the microfluidic chip (or microfluidic mixer) as described below. These two liquids are then injected separately into the microfluidic mixer at a constant flow rate and combined in the beginning of the mixing channel (at a Y-shaped junction). As both steams are passing through the channel, they undergo rapid mixing (<1ms) which is enabled by a special

channel geometry (ring structures in flow path) that generates a controlled laminar mixing for a uniform particle production.

The formation of LNPs in the mixer is driven by the rapid increase of polarity of the solvent stream when it is diluted with the aqueous stream. As the solution gets increasingly more polar, lipids become less soluble and self-assemble into lipid nanoparticles (Zhigaltsev et al., 2012). During this procedure cationic (positively charged) lipids in the mixture associate with the negatively charged oligonucleotides to form inverted micellar structures that serve as nucleation sites for particle formation. As these inverted micelles aggregate other lipid components subsequently associate around them and a single layer of PEGylated lipids among other lipid components eventually coats the final LNP (Leung et al., 2015).



DsiRNA containing LNPs produced by a microfluidic mixer

We will be encapsulating two different DICER-substrate short interfering RNA (DsiRNA) RNA duplexes, one for the silencing of EGFP, and the other is a negative control which is an RNA sequence that does not target any gene in the human, mouse or rat transcriptome. These have the sequences shown below, written 5'-3', with the lower case 'r' indicating ribonucleotides. When duplexed, the dsRNA has a 2 nucleotide 3' unpaired overhanging end on the antisense strand to improve DICER recognition, additionally, the final 2 nucleotides at the 3' end of the sense strands are deoxyribonucleotides in order to improve stability.

EGFP-S1 DsiRNA:

sense: 5'-phos/rArCrC rCrUrG rArArG rUrUrC rArUrC rUrGrC rArCrC rArC CG-3' (MW: 7924.763 g/mol)

antisense: 5'-rCrGrG rUrGrG rUrGrC rArGrA rUrGrA rArCrU rUrCrA rGrGrG rUrCrA-3' (MW: 8728.277 g/mol)

eGFP-S1 DsiRNA

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    5'-rArCrCrCrUrGrArArGrUrUrCrArUrCrUrGrCrArCrCrArCdCdG-3'
3'-rArCrUrGrGrGrArCrUrUrCrArArGrUrArGrArCrGrUrGrGrUrGrC-5'
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Non-targeting DsiRNA:

sense: 5'-phos/rCrGrU rUrArA rUrCrG rCrGrU rArUrA rArUrA rCrGrC rGrU AT-3' (MW: 7925.8 g/mol)

antisense: 5'-rArUrA rCrGrC rGrUrA rUrUrA rUrArC rGrCrG rArUrU rArArC rGrArC-3' (MW: 8601.2 g/mol)

Negative control DsiRNA

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    5'-rCrGrUrUrArArUrCrGrCrGrUrArUrArArUrArCrGrCrGrUdAdT-3'
3'-rCrArGrCrArArUrUrArGrCrGrCrArUrArUrUrArUrGrCrGrCrArUrA-5'
```

DsiRNA resuspension (done for you)

Our DsiRNA is provided as a lyophilized poweder and must be resuspended before it can be used. We will make a concentrated stock solution at 20 mg/mL in sterile water, which we will further dilute to 930 µg/mL in the kit

aqueous formulation buffer. **It is important that all steps of nanoparticle creation be performed in the biological safety cabinet as they will be used to treat cultured cells later, and any contamination could ruin the experiment.** *The instructor and TAs have already made the master diluted stocks.*

1. Calculate the mass of each DsiRNA that have been supplied at 5 nmol. Using the given MWs, we can calculate the mass of DsiRNA in each tube using the formula n = m/MW.
    a. GFP
        i. sense: 5*10^-9 M = m/(7924.763 g/mol), m = 39.6238 µg
        ii. Antisense: 5*10^-9 M = m/(8728.277 g/mol), m = 43.6414 µg
        iii. Total mass of dsRNA = 39.6238 µg + 43.6414 µg = 83.2652 µg

    b. Negative Control
        i. sense: 5*10^-9 M = m/(7925.8 g/mol), m = 39.629 µg
        ii. Antisense: 5*10^-9 M = m/(8601.2 g/mol), m = 43.006 µg
        iii. Total mass of dsRNA = 39.6238 µg + 43.6414 µg = 82.635 µg

2. Dilute the DsiRNA to 20 mg/mL:
    a. To the tube of GFP-S1 DsiRNA, add 4.16 µL sterile mQH_2O.
    b. To the tube of Non-targeting DsiRNA add 4.13 µL sterile mQH_2O.
    c. Gently pipet up and down to mix. Store any unused RNA at -80°C.

Production of encapsulated DsiRNA

Preparation of siRNA in Formulation Buffer 1

Maintain sterile conditions. Conduct all steps in a sterile biosafety cabinet.

1. Add **1.02 µL** of each siRNA (20 mg/mL) to **21 µL** of **Formulation Buffer 1** in separate RNAse-free microcentrifuge tubes to obtain RNA solutions with a final concentration of 930 µg/mL. Pipet the solution up and down >3 times to ensure mixing of siRNA in the buffer.

Loading of SPARK cartridge

Note: As we need a very small volume of particles for our tests, we will be making the smallest possible batch of particles to save on reagents. Due to this, loading the chip becomes substantially more difficult. This will also be completed by a TA or lab instructor in the biosafety cabinet.

1. Place **48 µL** of siRNA Spark **Formulation Buffer 2** to a RNase free microcentrifuge tube.
2. Fill the largest well (**Well 1**) of the Spark Cartridge with **24 µL** of Spark **Formulation Buffer 2**.
    a. NB: Place the pipette tip just above the hole at the bottom of the well and slowly dispense the solution into the well, avoiding bubbles during the process.
3. Fill the middle well (**Well 2**) of the Cartridge with **18 µL** of either the EGFP DsiRNA-Formulation Buffer 1 solution, or the Negative control DsiRNA-Formulation Buffer 1 solution.
4. Fill the smallest well (**Well 3**) of the Cartridge with **6 µL** of **Nanoparticle Mix**.

Creation of the DsiRNA-LNPs

1. Turn the Spark NanoAssembler on, and purge the air inside twice to ensure that it is sterile.
2. Set the NanoAssembler to setting 3. This will allow for complete mixing of all our samples.
3. Place the cartridge cap over the base of the cartridge. Align the holes on the cap with the posts on the cartridge and insert into the instrument.
4. Press the green-lit “Start” button on the base of the instrument.
5. Once the instrument completes its process, remove the cartridge from the instrument and lift the cap off carefully.

6. Using a micropipette, remove the entire contents from the largest well (**Well 1**) of the cartridge and add it to the microcentrifuge tube containing **48 µL** of the Formulation Buffer 2 prepared beforehand. Label this as siRNA nanoparticles at a concentration of 130 µg/mL of siRNA.
    a. *Note: The concentration of 130 µg/mL of siRNA is an estimation.*
7. *The nanoparticles will remain stable for approximately 14 days if stored at 4°C. After this point, the RNA may start to degrade resulting in lower efficacy if used in an experiment. This stable time period will vary depending on what has been encapsulated.*

2. Examination of RNA encapsulation efficiency

Background

Just like with the liposomes we produced using extrusion, it is possible to determine the loading efficiency of the RNA containing liposomes we produce using microfluidics. However, to do this, we must use another method to determine the concentration of the RNA present, as it is not inherently fluorescent like our Nile blue dye.

RiboGreen is a proprietary fluorescent nucleic acid binding dye that only exhibits fluorescence in the presence of DNA and RNA. Unlike earlier dyes that efficiently bound dsDNA, RiboGreen is able to efficiently bind RNA as well, with high detection sensitivity, and a broad linear response range.

The optimal excitation and emission values for this dye are approximately 500 nm and 520 nm, however we commonly read the plates using 485 nm and 528 nm wavelengths, similar to the wavelengths at which fluorescein, another commonly used fluorescent dye, shows a good response. This is because many plate readers operate using specific filters, and thus can only emit and detect certain wavelengths, so it is important to maximize the range at which a dye can be detected. The SpectraMax iD3 plate reader we have in lab uses diffraction gratings to alter the wavelengths emitted and detected, so we would be able to set our machine to any wavelength desired (in 1 nm increments). However, to maintain consistency with published protocols, we will use the settings recommended by Precision NanoSystems, the company that makes the microfluidics device we are using to generate our particles.

Caution is required when designing and preparing quantification tests using RiboGreen, as it is **highly sensitive to contamination by various salts and other nucleic acids**. It is possible to reduce the signal by as much as 15% with the presence of as little as 20 mM NaCl, and 2% chloroform can cause a 15% increase in signal. So, if your standard curve and samples have different buffer compositions, you may drastically under- or over-estimate your sample concentration. Additionally, the presence of DNA in your sample will also confound your results as RiboGreen will also bind DNA. If you have a sample that contains a mixture of DNA and RNA, you frequently need to perform an additional step to remove the DNA, using DNAses, before continuing with the assay. As we will have pure RNA suspended in a mostly salt-free buffer, this should not be of particular concern to us.

Preparation of RiboGreen working reagent

1. To a 5 mL tube make a 1:100 dilution of the Ribogreen reagent, making enough for 26 wells + 4 to account for sample error.
    a. **30 µL** of Ribogreen Reagent
    b. **2970 µL** of 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 7.5)
2. Vortex the RiboGreen Solution for ~10 seconds to mix.
3. Wrap the tube in aluminium foil and store in the dark until it is time to use it.

Preparation of RNA standard curve

Note: This assay is most accurate when you use the same RNA for your standard curve as what you encapsulated. Due to different lengths, nucleotide composition, and strandedness, there can be variability in dye binding, and it is best to reduce this as much as possible. *Due to the limited stocks of our DsiRNA the instructor and/or TAs will prepare the standard curve.*

1. Prepare a working stock of EGFP-DsiRNA at **20 µg/mL** using our pure EGFP-DsiRNA that was diluted in formulation buffer 1 (at ~930 µg/mL)
    a. Combine **2 µL** of the 930 µg/mL stock with **91 µL** of 1X TE buffer.

2. Setup a standard curve in duplicate using the EGFP-DsiRNA directly in the wells of a black fluorescent plate. The table below shows the amounts of each reagent needed for one well of each point.

Final RNA (µg/mL)	20 µg/ml DsiRNA Stock (µL)	1X TE Buffer (µL)	2% Triton X-100 Buffer (µL)	Total Volume per Well (µL)
2.5	25	25	50	100
1	10	40	50	100
0.5	5	45	50	100
0.25	2.5	47.5	50	100
0.1	1	49	50	100

Preparation of samples to determine encapsulation efficiency

To lyse our mRNA liposomes, we will use a final concentration of 1% Triton X-100, a common non-ionic surfactant that will gently disrupt the liposome without destroying our RNA payload.

Load order diagram:

	1	2	3	4	5	6
A	DsiRNA EGFP Std 2.5 µg/ml	DsiRNA EGFP Std 2.5 µg/ml	EGFP TE-1	EGFP TE-2	EGFP TX-1	EGFP TX-2
B	DsiRNA EGFP Std 1.0 µg/ml	DsiRNA EGFP Std 1.0 µg/ml	NC TE-1	NC TE-2	NC TX-1	NC TX-2
C	DsiRNA EGFP Std 0.5 µg/ml	DsiRNA EGFP Std 0.5 µg/ml				
D	DsiRNA EGFP Std 0.25 µg/ml	DsiRNA EGFP Std 0.25 µg/ml				
E	DsiRNA EGFP Std 0.1 µg/ml	DsiRNA EGFP Std 0.1 µg/ml				
F	TX-blank	TX-blank				
G	TE-blank	TE-blank				

1. Prepare diluted samples of nanoparticles in separate tubes:
    a. Combine 18 µL of EGFP-DsiRNA-LNP with 282 µL of 1X TE buffer in a clean 1.7 mL microfuge tube.
    b. Combine 18 µL of Negative control-DsiRNA-LNP with 282 µL of 1X TE buffer in a clean 1.7 mL microfuge tube

   c. Combine 18 µL of PBS with 282 µL 1X TE to make our dilution blank.

2. Add 50 µL of 1X TE buffer to the six TE wells (blue shading; A3-4, B3-4, G1-2).

3. Add 50 µL of 2% Triton X-100 buffer to each of the six TX wells (green shading; A5-6, B5-6, F1-2).

4. Add 50 µL of the diluted EGFP-DsiRNA-LNP to each of the **EGFP** TE and TX wells (A3-5).

5. Add 50 µL of the diluted Negative control-DsiRNA-LNP to each of the **NC** TE and TX wells (B3-5).

6. Add 50 µL of the diluted PBS dilution blank to each of the TE-blank and TX-blank wells (F1-2, G1-2).

7. Incubate the plate at 37°C for 10 minutes to lyse the RNA-LNPs.

Determination of encapsulation efficiency

1. Remove the 96-well plate from 37°C incubator.
2. Add 100 µL of Ribogreen solution to each well, mixing gently with a pipette, ensuring that no bubbles are formed.
3. Place the plate in the plate reader and perform a fluorescent reading with excitation at 485 nm, and emission at 528 nm.
4. Save the resulting file, and copy the data into the previously prepared quantification workbook to automatically calculate the concentrations and encapsulation efficiencies of each sample.

3. Nanoparticle sizing

Background

Our DsiRNA containing liposomes can be sized the same way as we sized the Nile blue containing liposomes we produced via extrusions – by dynamic light scattering (DLS).

Measure liposome size and PDI using the Zetasizer

1. Open the Malvern Instrument software and ensure that it is set to the correct settings for measuring our particles
   a. Material: lipid polymer
   b. Dispersant: water
   c. Cell: 4 mL polystyrene cuvette
   d. Measurement: 173° backscatter, manual duration: 11 runs, 10 sec, 3 measurements

2. Prepare your diluted liposome sample for measurement
   a. To a 4 mL cuvette, add 980 µL of mQH_2O and 20 µL of your diluted EGFP-S1 DsiRNA containing liposomes. Mix well by pipetting.
   b. Make sure to label the cuvette on the side, as close to the top as possible so that you can identify your sample while not impeding the light-path.

3. Place the cuvette into the machine, ensuring that the triangular shape embossed in the plastic is facing forwards.

4. Place the cuvette cover on the cuvette, and close the lid of the machine.

5. Start the measurement process. This will take approximately 5 minutes, and the machine will display intermediate results on screen as it progresses.

6. Record the size and PDI of your particles.

7. Repeat this process using a new cuvette and your negative control DsiRNA containing liposomes. Record the results.

4. Gene knockdown mediated by liposome encapsulated DsiRNA

Background

RNA interference (RNAi) allows the reversible silencing genes in eukaryotic cells, mediated by specific RNAs, including micro-RNA (miRNA) and short interfering RNA (siRNA). The discovery of RNAi enabled the creation of an entirely new class of therapeutics that can target and "silence" disease-causing genes which are otherwise not druggable. This includes novel therapeutics such as ONPATTRO® (patisiran) and GIVLAARI™ (givosiran) for the treatment of hereditary amyloidogenic transthyretin amyloidosis with polyneuropathy and acute hepatic porphyria respectively. It is through the use of LNPs that this technology has truly managed to shine, and there are currently a massive number of new siRNA drugs being developed for the treatment of cancers, rare genetic diseases, immunotherapies, and might revolutionize the way we can treat these diseases.

RNAi takes place in cells after transcription of DNA into its respective messenger RNA (mRNA). MicroRNAs (miRNA) are encoded in the genome and transcribed into ~1000 nt long single stranded primary transcripts (the pri-miRNA) which contains an ~70 by long duplex region. The double stranded hairpin region is cleaved from the pri-RNA by the double-strand-specific ribonuclease, Drosha. The resulting ~70 bp long pre-miRNA is exported from the nucleus, and cleaved into mature miRNA by an enzyme complex called DICER. The miRNA sequence then binds to the *RNA-induced silencing complex* (RISC). The siRNA sequence in RISC acts as a guide sequence that directs the complex to a complementary mRNA sequence where it binds and cleaves the mRNA, effectively silencing the gene by preventing translation from occurring.

We can co-opt the naturally occurring RNAi machinery by artificially introducing dsRNA into a cell to silence a gene of interest. Once in the cytoplasm of the cell, the dsRNA is cleaved into smaller 20-25bp long fragments called "siRNA" by DICER. Like miRNAs, these siRNA segments are incorporated into RISC where they act as guide sequences for the enzyme complex. Recent advances have shown that providing slightly longer 27-mer duplexes, known as DICER-substrate short interfering RNAs (DsiRNAs), allows for better DICER processing and more effective gene silencing compared to the 21-mer dsRNA fragments that were initially used.

We will use the two Dsi-RNA-LNPs that we created earlier to transfect our eGFP expressing cell line and simultaneously knock down *EGFP* gene expression when the cells are induced to start transcribing the mRNA.

Induction of eGFP expression and dosing of cells with LNPs

We will treat our cells using 0.1 nM, 1 nM, 10 nM, and 100 nM DsiRNA encapsulated within our LNPs to cause gene silencing. Assuming that we produce particles at 130 μg/mL, and that the approximate molecular weight of the EGFP DsiRNA duplex is 16653.04 g/mol, we can calculate our concentration using CV = m/MW, getting a

final concentration of approximately 7.81 µM, and 7.87 µM for our Negative control DsiRNA (MW: 16527.0 g/mol).

Our cells will only express GFP after being induced by the presence of tetracycline. This can be done by supplementing our media with tetracycline HCl so that it has a final concentration of 2 µg/mL.

As with all cell culture work, this has been done by the lab instructor and/or technician using the biosafety cabinet. Due to the fact that it takes several days for the proteins to be produced and silencing to take effect, this has been done in advance of the lab for you.

Treat cells with appropriate media

We will have 10 treatments in total: EGFP DsiRNA-LNP at 100, 10, 1, and 0.1 nM, Negative control DsiRNA-LNP at 100, 10, 1, and 0.1 nM, an induced (positive) control, and a non-induced (negative) control. **The induced control should express EGFP as it has been exposed to tetracycline in the absence of any silencing. The non-induced control should not express any significant amount of EGFP as these cells have not been stimulated to produce the protein.** These wells will provide examples of what our treatments may look like under a best-case scenario.

1. The day before treatment, split cells and plate onto a 48-well plate such that there are ~8 000 cells per well.
2. The next day, remove and discard media from all wells of plate.
3. Replace the media with 200 µL of media containing 100 nM, 10 nM, 1 nM, or 0.1 nM eGFP or NC dsiRNA LNPs as well as 2 µg/mL tetracyline in triplicate.
    a. Non-induced cells will be treated with unaltered complete media.

Loading chart:

	1	2	3	4	5	6	7	8
A	100 nM egfp	100 nM egfp	100 nM egfp	100 nM negative	100 nM negative	100 nM negative	PBS	PBS
B	10 nM egfp	10 nM egfp	10 nM egfp	10 nM negative	10 nM negative	10 nM negative	PBS	PBS
C	1 nM egfp	1 nM egfp	1 nM egfp	1 nM negative	1 nM negative	1 nM negative	PBS	PBS
D	0.1 nM egfp	0.1 nM egfp	0.1 nM egfp	0.1 nM negative	0.1 nM negative	0.1 nM negative	PBS	PBS
E	Induced ctrl	Induced ctrl	Induced ctrl	Non-induced	Non-induced	Non-induced	PBS	PBS
F	Induced ctrl	Induced ctrl	Induced ctrl	Non-induced	Non-induced	Non-induced	PBS	PBS

Examination of fluorescence via microscopy

Using the inverted microscope, it is possible to examine the living cells in real-time, comparing treated and untreated cells while they continue to grow and divide.

1. Place plate of cells on the stage of the inverted microscope.
2. Examine each well using brightfield imaging to compare the living cells.
3. Turn on the fluorescent laser light source, and select the EGFP filter on the microscope with the assistance of your instructor or TA.
4. Turn out the lights in the cell culture room and allow your eyes to adjust to the dark
5. Examine each well under the scope for the appearance of fluorescence. Start with the induced, but untreated negative control wells that should produce high levels of EGFP.
6. Record your observations.

Examination of fluorescence via plate reader

The enhanced Green Fluorescent Protein (EGFP) that we are examining today has an excitation maximum of 488 nm and an emission maximum of 509 nm. The fluorescence of our cells can be quantified using the plate reader, however due to the fact that the cells are grown in clear plates, we will need to harvest and lyse the cells to properly quantify our protein levels.

1. Remove media from each well of 96-well plate, and gently rinse cells with 100 µL of DPBS. Discard waste into a labelled liquid biohazardous waste beaker.
2. To each well of the 96-well plate add 50 µL of 0.25% Trypsin, and place in incubator for 5 minutes.
3. Add 50 µL of complete media to each well to inactivate Trypsin, mix well
4. Transfer cells and media to 1.7 mL microfuge tubes
5. Centrifuge the cells at 1000 x g for 5 minutes to pellet the cells away from the media.
6. Remove and discard supernatant into liquid biohazardous waste beaker
7. Resuspend pellet in 100 µL of 1% Triton X-100 buffer, mix well by vortexing.
8. Incubate at 37°C for 10 minutes to allow cells to lyse
9. Transfer 100 µL of lysed cells into the wells of a black 96-well plate, recording the load order.
10. Place the plate in the plate reader and perform a fluorescent reading with excitation at 475 nm, and emission at 520 nm.
11. Export the values and create a graph showing the relative fluorescence of each treatment.

<u>Mammalian cell culture</u>

The following information is provided for your information, but due to the time and extensive training needed to do cell culture, we will not be doing this during the lab itself. Instead, the instructor and technician will have prepared these materials for you prior to lab.

Background

The Flp-In T-REx-GFP stable cell line we are using was produced by the lab of Dr. Colin Ross, using a cell line donated by Dr. Abby Collier. Production of a stable cell line can be a long and laborious process, even when using modern, commercial, site-specific recombination methods such as the Flp-In T-REx system. Details about the process of making a recombinant cell line can be found in the links in the references section below.

Mammalian cell culture is very sensitive to contamination, and many precautions must be taken when working with these cell lines, including the use of aseptic technique within a Cass II Biological Safety Cabinet (see Appendix II for details on the use of this equipment). *Due to the sensitivity, biohazardous nature of the work, and amount of time required, the Instructor and TAs have done all of the preparatory cell culture for you, and will do all cell treatments.*

Cell culture and splitting

The Flp-In T-Rex 293-GFP cells are cultured using high-glucose (4.5g/L) DMEM, 10% FBS, 2mM L-glutamine, and 1% Pen-Strep. Media that is referred to as complete contains FBS and all needed components. This cell line is typically cultured with Hygromycin B in the culture media to ensure that the gene of interest remains integrated, however we will do without this year due to the short period of time for which we are doing our culturing, and because we will not be keeping this cell line going past this lab. The culturing example below uses T75 flasks, however you can easily modify this protocol for larger or smaller flasks or multi-well plates by increasing or decreasing the volumes of reagents used.
Your Instructor and TAs have and will do all cell culture for you, this protocol is supplied as an example only.

1. Sterilize the biosafety cabinet and all materials according to the procedures in Appendix II
2. Pre-warm your media, DPBS, and 0.25% trypsin at 37°C for ~20 minutes until warm.
3. Remove your cells from the incubator and examine them using the inverted microscope
    a. You are checking to confirm they are confluent (spread across the bottom of the dish with minimal space between cells). If the cells are not confluent you will have poor growth of the daughter cells.
4. Decant old media from flask and discard
5. Gently wash cells with 10 mL of DPBS
6. Add 4 ml of Trypsin to flask and incubate at 37°C for 5 minutes
7. Inactivate trypsin using 3-4x the volume of complete media
8. Gently pipet to mix and use this liquid to wash down bottom of flask to collect all cells
9. Transfer the cells and media to a 15 ml centrifuge tube and centrifuge at 100 x g for 5 minutes
10. Decant and discard supernatant, and resuspend cells in 5 ml of fresh complete media
11. Add 9 ml of fresh complete media to 5 new T75 flasks
12. Add 1 ml of resuspended cells to each flask (this is a 1:5 split)
    a. If these cells are split between a 1:5 and 1:10 dilution, they should reach 80-90% confluency within 3-4 days, though this varies with the gene(s) inserted.
    b. Splitting at a lower dilution (e.g. 1:3) will allow cells to reach confluency sooner.
13. Place cells in 37°C incubator with 5.0% CO_2 and allow to grow 1-2 days, checking cells daily.
14. Repeat this process whenever cells are >~90% confluent.
15. Clean up according to the protocols in Appendix II

Cell counting

It is often required that you plate a specific number of cells in a dish or well so that you can obtain reproducible results – having different numbers of cells in the well or dish between experiments could lead to dramatically different results. We count cells using a device called a haemocytometer, essentially a microscopic grid that allows us to count a known volume of cells.
The instructor has split and counted the cells one to two days before the lab, and has plated our dishes so that we have ~45 000 cells per well of a 48-well plate. This is denser than normal plating so that we can be sure to have sufficient cells for our experiment.

1. Complete the standard cell culturing method until step 10.
2. Take 100 µL of these cells, combine with 400 µL of Trypan blue dye, and gently pipet to mix.
3. Place the cover slip atop the haemocytometer, and slowly add enough cell suspension to fill the chamber.

4. Using the inverted microscope, count the number of cells present in each of the 9 sections of the grid (each 1 mm^2)
5. Calculate Your number of cells – white cells are living, and blue cells are dead. Count both, keeping separate tallies.
    a. Determine the average number of cells per grid
        i. Note: if there are too many cells to count, dilute your initial cell sample further and start over
    b. Correct for the volume of each square (0.1 µL) by multiplying by 10 000
    c. Correct for you trypan blue dilution by multiplying by 5
        i. This gives you the concentration of cells in your initial cell suspension (i.e. 10 mL volume)
6. Calculate the percent viability of your cells:
    a. Living cells / total (living + dead) cells * 100 = % viable
7. The haemocytometer can be cleaned with water, then 70% ethanol, with all materials that touch cells being disposed of in the biohazardous waste bin.

Cell culture – plating for RNAi treatment

The optimal number of cells to plate for a given experiment vary depending on numerous factors, and generally must be determined empirically. The size of the well or flask, the cell line, the experiment being performed, the antibiotics used, and genes being expressed will all affect optimal plating densities and cell growth.

As a general rule of thumb, 30 000 cells per well work well for a 48-well dish (1.1 cm^2 per well), but this is only a guideline as cell type used, and the time frame in which the cells will be needed, and for what purpose, will all cause these to change! Due to the fact that we need to let our cells grow for multiple days after seeding, we use a much lower cell density in the lab (~8 000 cells per well).

You typically seed your plates with cells ~24 hours prior to the start of your experiment to ensure that you have fresh, healthy, growing cells.

5. **References**

https://www.thermofisher.com/ca/en/home/references/protocols/proteins-expression-isolation-and-analysis/protein-expression-protocol/inducible-protein-expression-using-the-trex-system.html

Precision NanoSystems (2017) SiRNA Spark Transfection kit – 2nmol Protocol. Document ID: PNI-SOP-SP-012-EXT.

Fiszer-Kierzkowska A, Vydra N, Wysocka-Wycisk A, Kronekova Z, Jarząb M, Lisowska KM, and Krawczyk Z (2011) Liposome-based DNA carriers may induce cellular stress response and change gene expression pattern in transfected cells. *BMC Mol Biol* **12**:27.

Jones LJ, Yue ST, Cheung C-Y, and Singer VL (1998) RNA Quantitation by Fluorescence-Based Solution Assay: RiboGreen Reagent Characterization. *Analytical Biochemistry* **265**:368–374.

Leung AKK, Tam YYC, Chen S, Hafez IM, and Cullis PR (2015) Microfluidic Mixing: A General Method for Encapsulating Macromolecules in Lipid Nanoparticle Systems. *J Phys Chem B* **119:**8698–8706.

Zhigaltsev IV, Belliveau N, Hafez I, Leung AKK, Huft J, Hansen C, and Cullis PR (2012) Bottom-Up Design and Synthesis of Limit Size Lipid Nanoparticle Systems with Aqueous and Triglyceride Cores Using Millisecond Microfluidic Mixing. *Langmuir* **28**:3633–3640.

PHAR 203 – Laboratory 5 Worksheet Name:

PHAR 203 – Laboratory 5 – Lipid Nano Particles Worksheet [28 points total]
Due: In lecture, day after lab.

1. What did the various RNA and lipid solutions look like prior to formation of the nanoparticles? [1]

2. What did the DsiRNA-LNPs look like once they had been made? [1]

3. Compared to making the liposomes via extrusion, was this process easier and faster? What is a downside with using this method to create LNPs? [2]

4. Why do the final LNPs need to be stored at 4°C and not at -80°C? [1]

5. What were the values obtained for our RNA concentration testing?
   a) EGFP [3]:
      i. Total DsiRNA concentration: _____
      ii. Encapsulated DsiRNA concentration: _____
      iii. Encapsulation efficiency: _____
   b) Negative control [3]:
      i. Total DsiRNA concentration: _____
      ii. Encapsulated DsiRNA concentration: _____
      iii. Encapsulation efficiency: _____

6. How much did the observed amount of encapsulated DsiRNA differ from the expected (130 μg/ml) amount? [1]

7. What is the average particle size and PDI for our EGFP and Negative control DsiRNA-LNPs? [2]
   a) EGFP DsiRNA-LNP
   b) Negative control DsiRNA-LNP

PHAR 203 – Laboratory 5 Worksheet Name:

8. What is the actual molar concentration (in µM) of our EGFP and Negative control DsiRNA present within our nanoparticles? Assume that we produce the full 96 µL expected volume for each LNP. How much of this, in µL, would be needed to make 2 mL of a 100 nM stock solution?
   a) EGFP DsiRNA [2]

   b) Negative control DsiRNA [2]

9. What passage number are the cells we are examining at? _____ [1]

10. What did the cells look like under the microscope using brightfield imaging? [1]

11. How did treating the cells with our DsiRNAs affect GFP expression? What there a difference between the 0.1 nM, 1 nM, 10 nM, and 100 nM treatments? [4]

12. Using Excel or another spreadsheet program, create a bar graph of the results of our quantified fluorescence. Include error bars of the calculated standard deviation for each treatment, labelled axes, and a title. Does this data match up with what you observed under the microscope? [4]