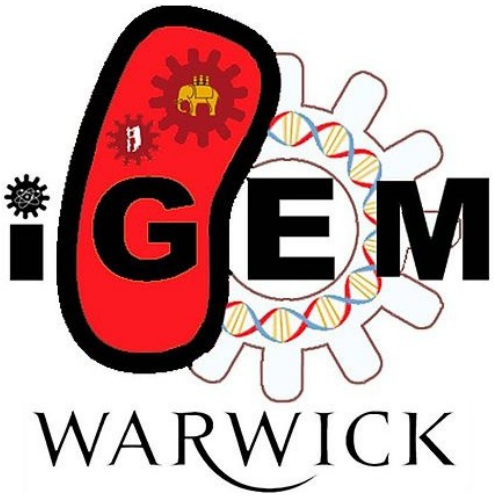
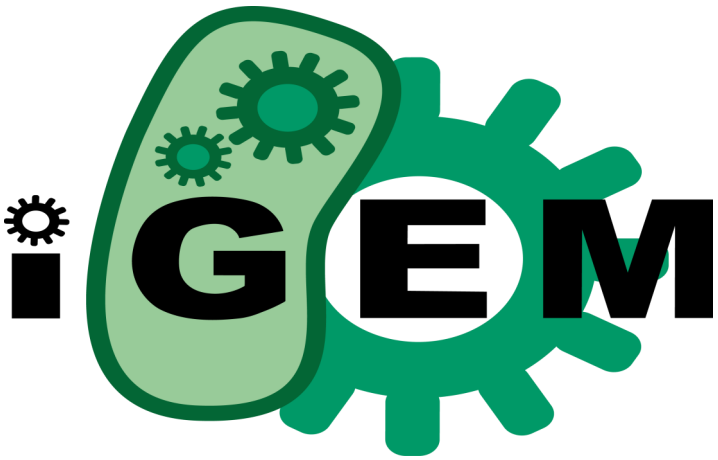
Protocols

IGEM 2014





Full part name	Abbreviations for labelling
Module 1	M1
Module 2	M2
Module 3	M3
Forwards aptazyme	FZ
siRNA target	DPP-iv
Neo a	NA
Neo B module	NB
EMCV IRES	EMCV
NKRF IRES	NKRF
Forwards GFP HUH	FG
Reverse GFP e.coli	RGE
Reverse GFP HUH	RGH
Reverse 5' UTR	R5
SLDel +8	SD8
B2(-)29G	B2
C2 (-) 26G	C2
SLC+8	SC8
C2 (-) 26G+Hair pin	C2/HP

Table of Contents

Making competent cells

Materials:	**Equipment:**
• 1 x starter culture	• 37°C shaking water bath
• 5ml of LB or SOC	• 1 or 2L sterilised conical flask
• 300ml LB broth	• Spectrometer
• 4ml TSS buffer	• 6 x 50ml falcon tube
	• Centrifuge
	• 1.5ml tubes
	• Pipettes
	• Ice bucket

1. Starter culture: Inoculate 5ml of LB or SOC broth with a single bacterial colony from an agar plate. Grow overnight in a **shaking** incubator at 37°C.
2. Transfer 300ml of LB broth into a pre-sterilised 1L (or 2L) conical flask, and add the starter culture.
3. Grow at 37°C until it reaches an optical density of 0.4-0.6 (*ideally should not let culture go beyond 0.6)* at wavelength 600nm (OD_{600}). Test the OD of the culture regularly against a blank of 1ml LB broth. (This should take between 1.5 and 3 hours).
4. Chill the culture on ice for 10 minutes. (*From this point on all equipment, tips, solutions, tubes etc. should be pre-chilled and all steps performed on ice or at 4°C.)*
5. Transfer culture to six 50ml falcon tubes.
6. Centrifuge at 3000rpm for 10minutes at 4°C.
7. Carefully pour off/pipette and discard the supernatant without disturbing the pellet.
8. Carefully and very **gently** re-suspend the pellet in 4ml of TSS buffer.
9. Transfer to 1.5ml tubes on ice in 200µl aliquots. Store at -80°C.

Rehydrating samples from the IGEM kit

Materials:

- Distilled water

Equipment:

- IGEM kit plates
- pipettes

1. With a pipette tip, punch a hole through the foil cover into the corresponding well of the part that you want. *(Make sure you have properly oriented the plate. Do not remove the foil cover, as it could lead to cross contamination between the wells.)*
2. Pipette 10µL of H_2O (distilled water) into the well. Pipette up and down a few times.
3. Let it sit for 5 minutes to make sure the dried DNA is fully re-suspended.
4. Transform 1 µl of the re-suspended DNA.

Transformation

Materials:

- 100µl Competent cells
- 1-5µl DNA
- 900µl LB broth

Equipment:

- Ice bucket
- 42°C water bath
- 37°C water bath
- timer
- Centrifuge
- 1.5ml tube
- pipettes

1. Thaw competent cells on ice.
2. Add 1µl DNA to 100µl cells (*can add up to 5µl of DNA if transformation does not work well or DNA concentration is very low).*
3. Rest on ice for 30minutes.
4. Heat-shock at 42°C for 45 seconds in water bath.
5. Rest on ice for 2 minutes.
6. Add LB Broth to a final volume of 1ml.
7. Incubate in water bath at 37°C for 1 hour.
8. Centrifuge at 13,000rpm for 1 minute.
9. Pipette out and discard 800µl of supernatant.
10. Re-suspend pellet in remaining supernatant with pipette tip.

Overnight culture

Materials:

- 5µl antibiotic
- 5ml of SOC media
- Transformed colony

Equipment:

- 37°C shaking water bath
- pipettes

1. Add 5 µl of appropriate antibiotic to 5ml of SOC media
2. Add a colony to the media using a pipette tip
3. Put in 37°C **shaker** overnight

Mini Prep

Materials:

- Overnight culture
- Reagents in *QIAGEN QIAprep spin mini prep kit.*

Equipment:

- *QIAGEN sample and assay technologies QIAprep spin mini prep kit.*
- Centrifuge
- Pipettes

1. Centrifuge at 13000rpm 1-5ml of overnight culture for 3 min at room temperature.
2. Pour off the supernatant.
3. Re-suspend the pellet in 250µl of buffer P1 and transfer to a micro centrifuge tube.
4. Add 250µl of buffer P2 and mix by inverting tube 4-6 times until solution becomes clear (*Do not allow the lysis reaction to proceed >5 min)*
5. Add 350µl buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
6. Centrifuge for 10 min at 13000rpm in a table top micro centrifuge.
7. Transfer the supernatant to the QIAprep spin column. Centrifuge for 30-60secs and discard the flow through.
8. Wash the QIAprep column in 500µl buffer PB. Centrifuge for 30-60 sec and discard flow through.
9. Wash the QIAprep sin column by adding 750µl buffer PE. Centrifuge for 30-60 sec and discard flow through.
10. Transfer the QIAprep spin column to the collection tube. Centrifuge for 1 min to remove residual buffer
11. Place the QIAprep spin column into a clean 1.5ml micro centrifuge tube. Add 50µl of EB buffer **to centre of the spin column** and allow to stand for 1 min.
12. Centrifuge for 1 min

Courtesy of QIAGEN sample and assay technologies QIAprep spin mini prep kit.

Plasmid digestion / linearization

Materials:

- 5µl of plasmid DNA
- 20µl of sterilized water
- 1µl of Xba1
- 1µl Spe1
- 3µl fast digest buffer
- 1µl SAP or CIP

Equipment:

- 37°C water bath
- Timer
- Agarose gel equipment
- Gel purification equipment
- 1.5ml tubes

1. To 5 µL plasmid DNA add:

   20 µL water

   1 µL Xba1

   1 µL Spe1

   3µL fast digest buffer

2. Incubate in 37°C water bath 10mins *(up to an hour)*
3. Add 1 µL SAP or CIP incubate at 37°C for 1hr
4. Make and run agarose gel
5. Gel purification resulting in tube of linearized plasmid
6. Check amount of DNA present using Nano drop machine

Making and running agarose gel

Materials:	**Equipment:**
• Agarose	• 37°C water bath
• 50ml TBE +extra to flood	• Timer
• 2-5µl Ethydium bromide	• microwave
• 5µl of plasmid	• Ethydium bromide flask
• 1µl DNA loading buffer	• Gel tank and comb
• 5µl ladder	• Fridge
	• Power pack
	• pipettes

1. Large fragments(>200bp) 0.8% agarose gel (0.4g of agarose in 50ml TBE). Small fragments 2.0% agarose (1g of agarose in 50ml) **Work quickly**
2. Microwave 30 sec at a time until all melted
3. Poor melted agarose into separate ethydium bromide flask leave to cool *(hold without burning hands)*
4. Add 2-5 µL ethydium bromide *(should be visible),* swirl to mix
5. Pour in gel tank and put in comb (*no bubbles)*
6. Set in fridge 10mins/ on side for 30mins
7. Add 5 µL PLASMID into fresh tube. Add 1 µL of DNA loading buffer mix with pipette tip.
8. Flood gel with TBE then remove comb
9. Add5 µL ladder to first well
10. Add DNA to well
11. Run gel 0.8% 30mins at 150V; 2% 45mins at 150V

Gel purification/extraction

Materials:

- Reagents from *QIAGEN sample and assay technologies QIAquick gel extraction kit.*

Equipment:

- scales
- Pipettes
- *QIAGEN sample and assay technologies QIAquick gel extraction kit.*

(for up to 10µl of DNA)

1. Add 96-100% ethanol to PE buffer
2. Excise desired DNA fragment from agarose gel with scalpel
3. Weigh gel slice (max =400mg)
4. If <2% agarose gel Add 3 volumes Buffer QG to 1 volume gel (100mg gel = 100µl). If >2% agarose gel Add 6 volumes Buffer QG to 1 volume gel
5. Incubate at 50°C for 10 minutes (*until gel slice completely dissolved)* Vortex every 2-3 minutes to aid in dissolution
6. When dissolved mixture should be yellow**.** [*If orange or violet add 10µl 3M sodium acetate, ph5.0, and mixture should return to yellow]*
7. Add 1 volume isopropanol (100%) and mix
8. Place a QIAquick spin column in 2ml collection tube and add sample.
9. Centrifuge for 1 minute. Discard flow through and place column back into same tube. **If sample was >800µl** repeat centrifugation.
10. Add 750µl Buffer PE *[if DNA will be used for salt sensitive applications e.g. sequencing, blunt ended ligation etc. then allow column to stand for 2-5 minutes after addition]*
11. Centrifuge for 1 minute. Discard flow through and replace in same tube.
12. Centrifuge for 1 minute to remove residual buffer.
13. Place QIAquick column into clean 1.5ml centrifuge tube. To extract DNA, add 50µl Buffer EB or water **to the centre of the membrane.** *(To increase yield of purified DNA incubate for 4 minutes after addition of EB)*
14. Centrifuge for 1 minute. *(if want increased DNA concentration: add 30µl Buffer EB to QIAquick membrane, let column stand for 1 minute and centrifuge 1 minute)*
15. Mix with pipette and add 1 volume of loading dye to 5 volumes of purified DNA to analyse on gel

Courtesy of QIAGEN sample and assay technologies QIAquick gel extraction kit.

G block primer amplification

Materials:

- 20µl TE
- Nuclease free water; 4µl GC buffer; 0.4µldNTPs; 1µl forwards primer; 1µl Reverse primer; 1µl Gblocks; 0.2µl DNA polymerase
- Agarose gel reagents
- *QIAGEN sample and assay technologies QIAprep spin mini prep kit reagents.*

Equipment:

- Timer
- Centrifuge/vortex
- Pipettes
- Thermocycler
- Gel doc
- QIAquick column Gel purification kit
- PCR tube

1. Centrifuge the tube for 3–5 sec at a minimum of 3000 x g to pellet the material to the bottom of the tube.
2. Add 20 µL TE to the tube for a final concentration of 10 ng/µL. Briefly vortex and centrifuge.
3. In a PCR tube add: Nuclease-free H2O 12.4 µL; 5X Phusion HF or GC Buffer 4 µL; 10 mM dNTPs 0.4 µL; 10 µM Forward Primer 1 µL; 10 µM Reverse Primer 1 µL; gBlocks® Gene Fragments 1 µL; DNA Polymerase 0.2 µL.Total volume 20 µL

Keep spare DNA clearly labelled in freezer

4. Gently mix the reaction and spin down in micro centrifuge. (~5 sec).
5. Carry out the amplification reaction in a thermocycler (PCR) with heated lid *(run samples of similar size).* Settings: 5 mins 95C (do once); (cycle 30 times) 1min 95C, 1min at Annealing temp ~55C, 1-2 min 58-72C elongation temperature; Final elongation (10 mins). Go to 4C to leave PCR in there.
6. Centrifuge to get all liquid in the bottom (few seconds)
7. Make and run agrose gel
8. Observe under gel doc *(is fragment in the right place, run for longer if ladder is scrunched)*

Run PCR through PCR purification column

1. Add 5 volumes Buffer PB to 1 volume PCR sample. Mix by pipetting.
2. Apply buffer + sample to QIAquick column (from gel purification kit) and centrifuge 13,000 rpm for 30-60s. Discard flow through liquid.
2. Add 0.75ml of Buffer PE to column and centrifuge for 30-60s. Discard flow through and centrifuge again for 1min.
3. Move column to a clean eppendorf. Add 30ul Buffer EB to the centre of the membrane (not sides) in the column.
4. Stand for 1min. Centrifuge for 1min.Purified pcr DNA is now in the eluted EB.

CLEARLY LABEL ALL AMPLIFIED DNA TUBES

Digestion

Materials:

- 20µl purified DNA
- 50ml TBE +extra to flood
- 5µl sterilized water
- 1µl Restriction enzyme 1
- 1µl Restriction enzyme 2
- 3µl fast digest buffer

Equipment:

- 37°C water bath
- Timer
- Pipettes
- 1.5ml tube

1. To the purified DNA tube (20 µL)add:

   1 µL restriction enzyme 1

   1 µL Restriction enzyme 2

   3µL fast digest buffer (*should = the amount of enzyme added)*

   5 µL water

2. Incubate in 37°C water bath 10mins *(up to an hour; can Freeze gBlock DNA/ heat inactivate)*

Ligation

Materials:	**Equipment:**
• purified DNA insert	• 37°C water bath
• Linearized vector	• Timer
• sterilised water	• Pipettes
• 1µl DNA ligase	• 1.5ml tube
• 2 µl DNA ligase buffer	
• 3µl fast digest buffer	

1. Into an Eppendorf add:
   1 µL DNA ligase
   2 µL DNA ligase buffer
   3:1 insert: vector OR 1:1 insert: vector
   Make up to 20 µL final volume with water
2. 37°C water bath 5 min
3. Leave on bench for 20 -60 min
4. Put spare ligation mixture in fridge overnight *(this can be used the following day if the first ligation didn't work)*

RNA transfection

Materials:

- Human cells
- K2 multiplier
- Serum free media
- mRNA
- K2 transfection reagents

Equipment:

- 96 well plate
- incubator
- pipettes

1. Add $0.15x10^5$ cells to each well in 100µl of media
2. Incubate for 24 hours
3. Add 1.2µl K2 multiplier to each well and leave for 2 hours

Mean while

Solution A: 5µl serum free media and 0.2µg mRNA per well (nano drop)

Solution B: 5µl serum free media and 0.4µl mRNA per well

Make master mix as appropriate add media first

4. Mix solutions A and B gently by pipetting leave at room temp for 15 mins.
5. Add 10.4µl and 1 volume of solution A to each well
6. Incubate for 24hours
7. Refresh media with 100µl of media
8. Load into Tecan plate reader

Maintaining human cell lines

Materials:

- 25ml DMEM media,
- 10ml PBS,
- 2ml Trypsin,
- (5ml Pen/strep, 50ml FBS).

Equipment:

- Pipettes
- Glass pipettes
- 1 tissue culture plate
- 15ml falcon tube
- Tissue hood
- 37°C water bath

Do every Mon/Wed/Fri to maintain cells.

Making fresh DMEM

1. defrost pen/strep and 50ml FBS in 37^{0}C waterbath (*1 hour before starting*).
2. Add 50ml FBS and 5ml pen/strep to DMEM media – label with name and date.

Maintenance

1. Warm DMEM, PBS and Trypsin in 37^{0}C waterbath at least 30min in advance of starting.
2. Check cell confluency with microscope
3. Aspirate off old media (with vacuum pump)
4. Wash cells with 10ml warm PBS and aspirate PBS off
5. Add 2ml Trypsin, move to incubator for 3-5min until cells begin to come off plate.
6. Check cells have detached from plate (microscope).
7. Add 5ml DMEM media, wash plate thoroughly to re-suspend all cells, move to 15ml tube.
8. Spin 4min at 1000rpm and room temp
9. Aspirate off media (do not disturb pellet)
10. Re-suspend cells in 1ml DMEM media, add a further 9ml DMEM. Mix thoroughly.
11. Plate 1ml culture and add 9ml DMEM (for a 1 in 10 dilution). Swirl plate side to side and up and down (not in a circular motion)
12. Grow 2 days at 37^{0}C and 5% CO-$_2$

Return DMEM and PBS to fridge, and pen/strep and trypsin to freezer.

Materials:

- 10g Bacto tryptone
- 5g Bacto-yeast extract
- 10g NaCl
- 1L ddH2O.
- NaOH
- Ppy strain
- Streptomycin; chlorophenicol
- 20% glycerol
- LB agar plates (10ug/ml streptomycin and 12.5ug/ml chloramphenicol

Equipment:

- scales
- autoclave
- 1l sterile bottle
- Sterile tube
- 37°C shaking incubator

Making lysogeny broth

1. Dissolve 10g Bacto tryptone, 5g Bacto-yeast extract, 10g NaCl in 900mL ddH2O. [Must be pH7.2 (adjust with NaOH)]
2. Make up to 1L with ddH2O Autoclave to sterilise and store at room temperature .

Maintaining PPY strain

1. Inoculate 1 single colony of PPY strain from LB agar plate in 5ml LB medium with 10µg/mL streptomycin and 12.5µg/mL chloramphenicol
2. Shake at 37°C and 225-338 rpm overnight.
3. In a sterile tube mix an equal volume of PPY culture with 20% autoclaved glycerol
4. Store at -80°C

Materials:

- Ppy strain
- Streptomycin, chlorophenicol
- Arabinose
- Cell lytic reagent
- 16g Bacto-tryptone,
- 10g Bacto-yeast extract,
- 5g NaCl
- NaOH
- 1L ddH_2O
- 100% glycerol
- LB agar plates (10ug/ml streptomycin and 12.5ug/ml chloramphenicol

Equipment:

- Pipettes
- Autoclave
- Sterile tubeProtein LoBind Tube 1.5ml (Eppendorf)_Protein Lo-Bind Tube 0.5ml (Eppendorf)
- 50ml centrifuge tube
- 250ml Nalgene Lab quality Wide -Mouth Bottles
- Spectrophotometer
- Scales

Preparing 2XYT medium

1. Dissolve 16g Bacto-tryptone, 10g Bacto-yeast extract, and 5g NaCl in 900mL ddH_2O. Must be pH 7.2 (adjust with NaOH)]
2. Then make up to 1L with ddH_2O
3. Autoclave to sterilise and store at room temperature

SLiCE

Preparing PPY SLiCE extract

1. Streak PPY glycerol stock or fresh culture on an LB agar plate.
2. Incubate at 37°C overnight
3. Inoculate 1 single colony into a 50ml centrifuge tube containing 25ml 2XYT with 10µg/ml streptomycin.
4. Shake at 37°C overnight at 330 rpm
5. Measure optical density$_{600}$ using spectrophotometer
6. Dilute overnight culture to 0.03 OD_{600} by inoculating appropriate volume of overnight culture into a 250ml Nalgene Lab Quality Wide-Mouth Bottle containing 50ml 2XYT medium with 10µg/ml streptomyicin
7. Shake at 37°C and 330rpm until the culture reaches OD_{600} 5.0-5.5
8. Add 0.2 %L-(+)-arabinose into the culture and continue shaking at 330 rpm for 2h at 37°C, to induce expression of λ prophage protein Red.
9. Remove 500µL from the culture to measure the actual OD_{600}
10. Transfer 48ml of bacterial culture into two 50ml centrifuge tubes (24ml each) Pellet the cells by centrifugation at (5000x*)* for 20 minutes at 4°C
11. Wash pellet with 50ml ddH_2O once
12. Pellet the cells by centrifugation at (5000x*g?????)* for 20 minutes at 4°C
13. Measure pellet weight
14. Resuspend pellet of about 0.23g of weight or from 24ml of original culture at OD_{600} ~ 6 in 300µl CellLytic B Cell Lysis Reagent
15. Transfer the resuspended cells into a low binding 1.5mL tube and incubate at room temperature for 10min
16. Centrifuge the cell lysate at 20,000 x g for 2 minutes at room temp. to pellet any insoluble material
17. Remove the resulting supernatant from the cell debris into a low binding 1.5ml tube
18. Mix cell extracts with equal volume of 100% glycerol and aliquot into 40-60 µl portions in low binding 0.5ml tubes labelles as PPY SLiCE extract
19. Store at -20°C for about 2 months or at -80°C for long term storage (no significant loss of activity)

SLiCE

Materials:

- PPY slice extract
- 10x SLiCE buffer: 500mM Tris-Cl (pH 7.5 at 25°C), 100mM $MgCl_2$, 10mM ATP, 10mM DTT and store at -20°C
- Primers
- DNA insert
- DNA vector

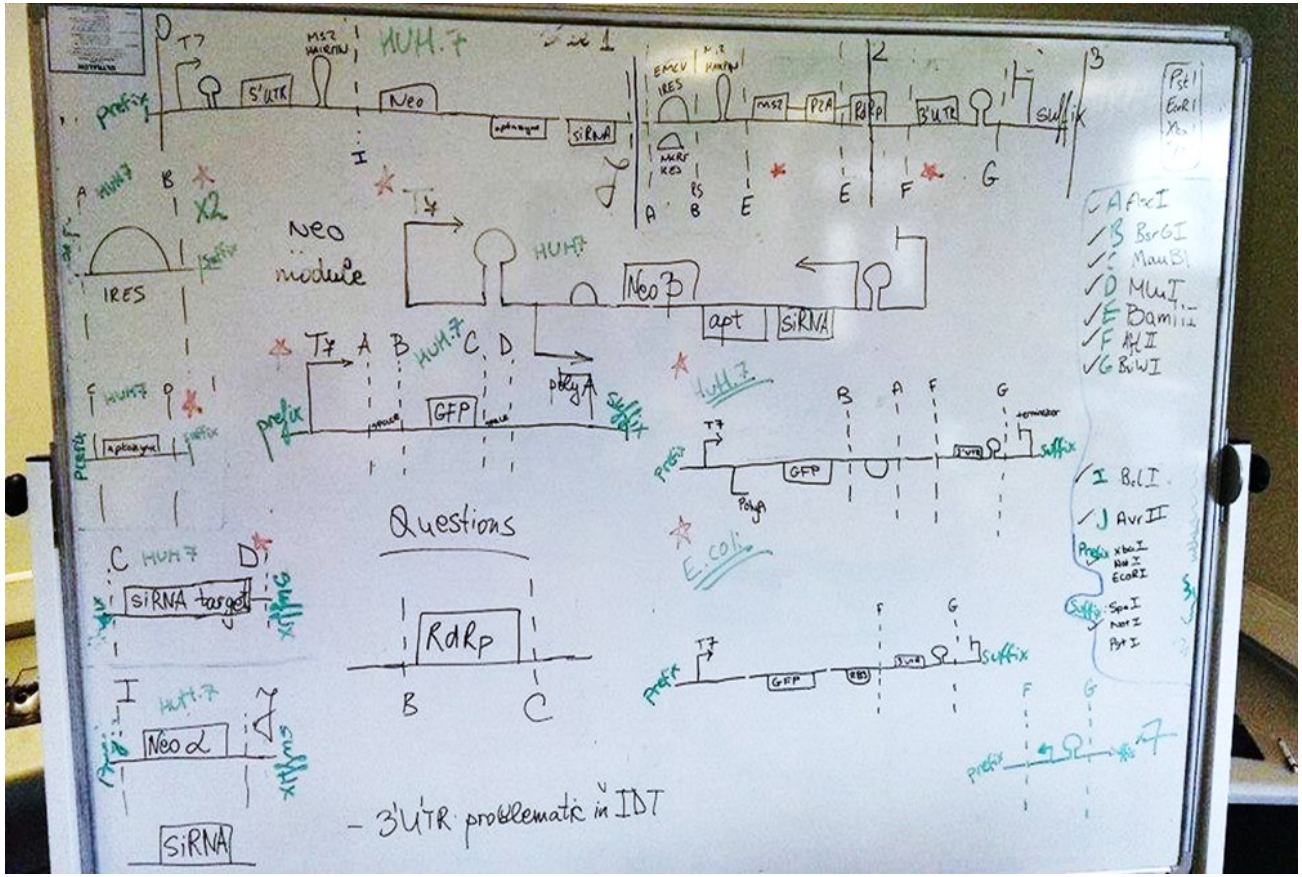
Equipment:

- Pipettes
- vortex
- Gel extraction kit
- 37°C water bath

Primers: the characteristics of SLiCE are **(a)** The 5'end of the primer contains 15-52 bases that are homologous to the sequence at one end of a DNA fragment for co-assembly or to other appropriate position within DNA fragment **(b)** The 3'-end of the primer contains 15-24 bases that are specific to the DNA fragment for amplification **(c)** desalted

SLiCE cloning

1. Prepare primers
2. Prepare DNA vector - linearise using restriction site
3. Prepare insert DNA – Amplify the cloning inserts using PCR with primers containing 5'-end homologies to the vector or to other inserts for co-assembly described as above.
4. Purify DNA by gel extraction and elute in EB
5. In 0.2ml micro-centrifuge tube add 50-200ng of purified vector DNA, insert DNA at a molar ratio of 1:1 -10:1 with vector, 1µl SLiCE buffer, 1µl PPY SLiCE extract and make up to 10µl with ddH_2O
6. Mix well by vortexing
7. Incubate at 37°C for 15min to 1hour (efficiency increased at 1hour) using PCR machine or water bath


HUH.7
Neo module
IRES
Neo 3
apt
siRNA
GFP
Questions
RdRp
siRNA target
Neo 2
siRNA
- 3'UTR problematic in IDT
E.coli