1 Practical Day One

Part 1: Seeding Cells
Late Morning Monday 18th September 2017
Aim: Prepare four wells of 293T cells for transfection.

Tip 1: 293T cells are robust cells and can be handled roughly (in comparison to other cell lines and iPSCs), they adhere to the surface of treated plates in monolayers but they do not bind tightly (pipetting will dislodge the cells if not careful).

1.1 Go to the Cell Culture Laboratory
1.2 Wear Proper PPE (Lab coat and gloves at a minimum)
1.3 Take Complete Media and 1x Trypsin-EDTA aliquots from the refrigerator and place in the waterbath at 37°C
1.4 Turn on culture hood and clean thoroughly with 70% Ethanol

Note: Each culture hood will be split between two groups (5 people total); each hood should share pipette tips, pipettes, pipetteboys, and waste bottles.

1.5 Take 1x 10cm culture plate from Incubator

Note: Check under the microscope

1.6 Pipette off the media
1.7 Carefully add 5ml of PBS

Note: Do not add directly to the monolayer as the cells will come away

1.8 Add 0.5 ml 1x Trypsin-EDTA
1.9 Tilt the plate backwards and forwards until the cells start to disaggregate from the surface
1.10 Take a 5ml stripette and pipette 4.5ml Complete Media onto the plate
1.11 Completely disaggregate the cell suspension by pipetting onto the surface of the plate using the 5ml stripette
1.12 Transfer the monodispersed cell suspension into a 15ml tube
1.13 Count the cells using a haemocytometer

Note: Use a 0.4% Trypan Blue Solution at a ratio of 1:1 to 1:9 (cells:trypan); count only the viable cells (white) and ignore the dead cells (blue).

1.14 Take 6-well treated cell culture plate
1.15 Add 2ml PBS to two wells
1.16 Add 1.5 ml Complete Media to four wells
1.17 Inoculate the four wells with 0.4M cells ($4x10^5$)
1.18 Tilt the plate backwards and forwards to disperse the cells evenly across the surface of each well
1.19 Put plate in the incubator (we will leave this for >4 hours).
1.20 Last thing, take out the DMEM (medium without supplements) and PEI aliquots and leave at room temperature (RT).
1.21 Tidy up

293T Complete Media Composition		
Component	Volume	Notes
Dulbecco's Modified Eagle's Medium (DMEM)	500 ml	Basic media for cell growth
Heat-inactivated Fetal Bovine Serum (FBS)	56 ml	Support growth of the cells
100x Penicillin(100U/ml)-Streptomycin (100mg/ml)	5.6 ml	Antibiotics against gram +ve and -ve bacteria
100x Non-Essential Amino Acids (10mM each)	5.6 ml	Glycine, Alanine, Asparagine, Aspartic Acid, Glutamic Acid, Proline and Serine
Tylosin (8mg/ml)	560 µl	Antibiotic against *Mycoplasma*

Lunch Time

Part 2: Transfection
Afternoon Monday 18th September 2017
Aim: Transfect the four wells with the following constructs: negative control (no construct), Cas9-GFP-only (one construct), Guide 1 + Cas9-GFP (two constructs), and Guide 1 + Guide 2 + Cas9-GFP (three constructs).

1.22 Go to the Cell Culture Laboratory
1.23 Wear Proper PPE
1.24 Turn on culture hood and clean thoroughly with 70% Ethanol
1.25 Take 4x 0.5ml/1.5ml tubes
1.26 Label the four tubes
1.27 Calculate the amount of each vector that should be added

Note: Each transfection should contain 5µg of DNA total (with the exception of the negative control); for more than one vector, the total should be equally divided (e.g. 2 vectors = 2x 2.5µg).
Note: The concentration of each vector is as follows: Cas9-GFP = 300 ηg/µl, Guide 1 = 700 ηg/µl, Guide 2 = 900 ηg/µl.

1.28 Add 250 µl DMEM (medium without supplements) to each tube
1.29 Add 18.75 µl to each tube
1.30 Immediately vortex each tube (at maximum speed) for 15s
1.31 Incubate each tube at RT for 20-30 min
1.32 Take the 6-well plate from the incubator
1.33 Label the plate
1.34 Add each PEI-DNA-DMEM solution to each well by dropping evenly across the top of the well surface
1.35 Put plate in the incubator
1.36 Tidy up

Further information: Polyethylenimine (PEI) transfection
Transient Mammalian Cell Transfection with Polyethylenimine (PEI)
Longo, P. A., Kavran, J. M., [...], Leahy, D. J. Methods Enzymology 529:227-40 (2013)
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4012321/

End of Day

2 Practical Day Two

Part 1: Media Change
Morning Tuesday 19th September 2017
Aim: Change the media for the transfected cells.

2.1 Go to the Cell Culture Laboratory
2.2 Wear Proper PPE
2.3 Take Complete Media from the refrigerator and place in the waterbath at 37°C
2.4 Turn on culture hood and clean thoroughly with 70% Ethanol

Note: Again each culture hood will be split between two groups (5 people total); each hood should share pipette tips, pipettes, pipetteboys, and waste bottles.

2.5 Take the 6-well plate from the incubator
2.6 Carefully aspirate and discard all the media
2.7 Add 1.5ml of warmed Complete Media
2.8 Return the 6-well plate to the incubator and incubate overnight
2.9 Tidy up.

Talks / Coffee Break

3 Practical Day Three

Part 1: Visualise and Harvest
Morning Wednesday 20th September 2017
Aim: Gauge transfection efficiency by GFP expression and harvest adherent (live) cells

3.1 Go to the Cell Culture Laboratory
3.2 Wear Proper PPE
3.3 Take 1x Trypsin-EDTA and place in the waterbath at 37°C
3.4 Turn on culture hood and clean thoroughly with 70% Ethanol
3.5 Take 6-well plate from the incubator
3.6 Use a microscope with a GFP filter to judge transfection efficiency (in a different laboratory)

Note: this method is to judge success of the experiment but will not give a transfection percentage; if this is desired (and is recommended) then a fraction of the cells can be tested by flow cytometry using the FL1 detector to judge the percentage of GFP positive cells (thus the transfection efficiency).

3.7 Return to the Cell Culture Laboratory
3.8 Aspirate all media from each well.
3.9 Carefully wash each well with 1 ml PBS

Note: Do not add directly to the monolayer as the cells will come away

3.10 Add 100 µl 1x Trypsin-EDTA
3.11 Tilt the plate backwards and forwards until the cells start to disaggregate from the surface
3.12 Using a P1000 pipette, add 900 µl PBS to each well and disaggregate the cells at the same time
3.13 Transfer the disaggregated cells into labelled 1.5 ml tubes
3.14 Tidy up

Part 2: Extract gDNA
Morning Wednesday 20th September 2017
Aim: Pellet recovered cells and then gDNA extract using the Qiagen DNeasy Blood and Tissue Kit

3.15 Go to the Molecular Biology Laboratory with the four 1.5 ml tubes and PBS stock
3.16 Wear proper PPE
3.17 Centrifuge the cell suspensions at 1,000xg for 5 min
3.18 Aspirate the media
3.19 Extra gDNA from samples using the Qiagen DNeasy Blood and Tissue Extraction Kit (See Manufacturers Protocol)

Tip 1: Optional RNase A can be included between steps 1d and 2; add 4 µl of RNase A to each sample and incubate at RT for 2 min.
Tip 2: Do not elute the gDNA in Buffer AE, use H_2O instead; it is recommended to do a double elution using 25 µl of H_2O each time for optimal recovery (final volume 50 µl).
Tip 3: Remember to elute each sample in a labelled 1.5 ml tube

3.20 Put the recovered gDNA samples on ice.
3.21 Tidy up (a bit)

Coffee / Lunch Break

Part 3: Quantify and Amplify
Late Morning Wednesday 20th September 2017
Aim: Quantify the gDNA using a Qubit and amplify the region of interest by PCR and site specific oligonucleotides

3.22 Go to the Molecular Biology Laboratory
3.23 Wear Proper PPE
3.24 Remember to retain the gDNA samples on ice
3.25 Quantify the gDNA samples using the Qubit (See Protocol)

Tip 1: Each person should make their own standards
Tip 2: Prepare samples using 1 µl of each gDNA
Tip 3: Be careful with the units and the dilution factor.

3.26 Set-up PCRs, in 0.2 ml PCR tubes, on each of the samples (See Manufacturers Protocol, but read all **Tips** before proceeding)

Tip 1: The only "active" ingredient in each PCR is the Polymerase (notably has 3'=> 5' exonuclease activity), so add this last and work quickly after that – you can work slowly and methodically before this.
Tip 2: Each group of 10 has to coordinate, we will only be using one PCR machine – therefore do not add the Polymerase until everyone is ready.
Tip 3: Include a "no template control"; therefore, 5 reactions in total each
Tip 4: 100 ng of gDNA in each PCR sample (except the control)
Tip 5: Use 5x HF Phusion Buffer
Tip 6: No need for additional Mg^{2+} or DMSO in this scenario
Tip 7: dNTPs stock at 10mM
Tip 8: Use 30x cycles
Tip 9: It is easier to do 50 µl reactions for each
Tip 10: Primer concentration is 25 µM (not 10 µM like in the protocol)
Tip 11: Primer sequences are: F = CTGCCTGAGCCAGTCAGATG and R = CAGAGGCAGAGTGGATGGTG ; we recommend the annealing temperature of 65°C (as determined to be optimal by Gradient PCR), but can use the NEB site to determine the temperature if desired (http://tmcalculator.neb.com/)
Tip 12: The region of interest is 927 bp (use this to determine your elongation duration); this can be accurately determined by using a primer blast site (e.g. https://www.ncbi.nlm.nih.gov/tools/primer-blast/) [the same site is very good at designing primers from regions of interest – just remember to set your template (e.g. mRNA, gDNA, *Homo sapiens*, *Mus musculus* etc) up correctly]
Tip 13: We have limited PCR machines, so everyone will be working in a group – so the group will have to decide the thermocycling conditions and set-up the reactions at the same time.
Tip 14: The only "active" ingredient in each reaction is the Polymerase (notably has 3'=> 5' exonuclease activity), so add this last and work quickly after that – you can work slowly and methodically before this.
Tip 15: The group has to agree on the thermocycling conditions!

3.27 Once PCR samples are running, store the gDNA at -20°C
3.28 Tidy up (a bit)

Lunch / Coffee Break

Part 4: Pour Gel (Ignore This Section – the gel will be poured prior to the practical)
Afternoon Wednesday 20th September 2017
Aim (Parts 4-8): Test 10% of the PCR samples by gel electrophoresis and purify 90% using Zymo clean-up columns.

3.29 Go to the Molecular Biology Laboratory
3.30 Wear Proper PPE
3.31 If PCR thermocycling has finished, then remove samples and place on ice.
3.32 Set-up a 2% $^{w}/_{v}$ agarose gel (See Protocol (or the manufacturer of the rig), but read all **Tips** before proceeding)

Tip 1: We will be working in teams, so plan accordingly
Tip 2: It is mandatory to include one well for a DNA Ladder; however, it is recommended to include two wells for this, one each side of the loaded samples.
Tip 3: Use 1x TBE instead of 1x TAE
Tip 4: Use Ethidium Bromide Solution.

3.33 Once the gel is poured, place at 2-8°C if there is space (or leave at RT)

Part 5: Load Gel (We need a brave soul to load all the samples and DNA Ladders with the multichannel pipette)

3.34 From the PCR samples, transfer 5 µl of each into fresh PCR tubes
3.35 Add 2 µl 6x Loading Dye to each of the five test samples
3.36 Check agarose gel is solidified and place in rig
3.37 Ensure the gel is submerged before removing the comb(s)
3.38 Load all samples (the entire group) and 1kb DNA Ladder to the wells (read all **Tips** first and then decide as a group)

Tip 1: Use multichannel pipette to speed up the process.
Tip 2: Absolutely minimum of 2 wells with the 1kb DNA ladder; however, many Ladder wells can be included and can be used to divide groups of samples as desired.
Tip 3: 5-10 µl (?) of the 1kb DNA Ladder for each well

3.39 Run the gel between 90-150(?) volts

Note: The amperage should be set high (500mA) to avoid it limiting the current.

3.40 The gel should be run until the lower of the dyes is about ½ to ⅔ down
3.41 Whilst running the gel proceed to Part 4

Part 6: Recover DNA

3.42 Recover the DNA from the remaining (45 µl) of each sample using Zymo Clean and Concentrate Columns (See Attached Manufacturers Protocol)

Tip 1: You only need to purify the 3 main samples: Cas9-only, Guide 1, Guide 1 +2 (the "no template" PCR control can be discarded and "no construct" transfection can be discarded)
Tip 2: Do not elute the gDNA in DNA Elution Buffer, use H_2O instead; it is recommended to do a double elution using 10 µl of H_2O each time for optimal recovery (final volume 20 µl).
Tip 3: Remember to elute each sample in a labelled 1.5 ml tube

3.43 Put the four recover DNA samples on ice

Part 7: Quantify DNA

3.44 Quantify the recovered DNA samples using the Qubit (See Manufacturers Protocol)

Tip 1: Each person should make their own standards
Tip 2: Prepare samples using 1 µl of each gDNA
Tip 3: Be careful with the units and the dilution factor

3.45 Write the concentration on the side of each sample and store them at -20°C

Note: if the concentration of each sample is >12 ηg/µl, then the original gDNA samples can be binned.

Part 8: Image Gel

3.46 Once the agarose gel had run sufficiently, take to the UV Gel Imager to visualise and take a picture (if possible, get a jpeg of the image – use USB Flash Drive or online storage)
3.47 Discard the gel and study the image for differences

Note: Before looking at the gel image in detail, think about what you expect…

3.48 Tidy up

Coffee Break (or End of Day)

4 Practical Day Four

Part 1: Hybridization
Aim (Parts 1-2): To denature all the PCR amplified DNA and then rehybridize slowly to promote imperfect annealing.
Morning Friday 22th September 2017

4.1 Go to the Molecular Biology Laboratory
4.2 Wear Proper PPE
4.3 Transfer 200 ηg of each DNA (from PCR) to a PCR tube
4.4 Make the total volume to 17 µl with H2O
4.5 Add 2 µl NEB Buffer 2
4.6 Mix by inversion
4.7 Pulse centrifuge and place on ice
4.8 Run the samples in the thermocycler as follows:

Temperature	Ramp Rate	Time
95°C	Maximum	5 min
95-85°C	-2°C/s	1 min
85-25°C	-0.1°C/s	1 min
4°C	Maximum	…

Note: We will use one PCR thermocycler for this, so the whole group will have to coordinate; as there are no "active" ingredients per se then samples can be prepared and then can leave them on ice until everyone is ready.

Part 2: Pour Gel (Ignore This Section – the gel will be poured prior to the practical)
4.9 Set-up a 2% $^{w}/_{v}$ agarose gel (See Addgenes Protocol (or the manufacturer of the rig), but read all **Tips** before proceeding)
Tip 1: You will be working in pairs, so plan accordingly (you will eventually have 8 samples each, so 16 in total – not including DNA Ladders)
Tip 2: It is mandatory to include one well for a DNA Ladder; however, it is recommended to include two wells for this, one each side of the loaded samples.
Tip 3: Use 1x TBE instead of 1x TAE
Tip 4: Use SYBR Safe DNA Stain instead of Ethidium Bromide Solution.
4.10 Once the gel is poured, place at 2-8°C if there is space (or leave at RT)

Coffee Break

Part 3: Digestion
Aim (Parts 3-5): To digest, and compare, any imperfect annealed DNA at loops generated by Cas9-mediated indels, using a T7 Endonuclease I
Morning Friday 22th September 2017

4.11 Take the samples from the thermocycler and immediately place on ice
Note: it is important to not warm the samples up beyond 37°C
4.12 In new PCR tubes, divide each sample into two (9.5 µl in each)
Note: This will be 6 in total

4.13 Label each pair as "No Enzyme" and "T7 Endonuclease I"
4.14 Then add 0.5 µl H_2O to each "No Enzyme"
4.15 Then add 0.5 µl (5 Units) T7 Endonuclese I to each "T7 Endonuclease I"
4.16 Flick the tubes to mix
4.17 Pulse centrifuge
4.18 Incubate in a waterbath at 37°C for 15 min
4.19 Immediately add 0.75 µl 0.25M EDTA
4.20 Place the samples on ice

Part 4: Load Gel

4.21 Add 3 µl 6x Loading Dye to each sample
4.22 Check agarose gel is solidified and place in rig
4.23 Ensure the gel is submerged before removing the comb(s)
4.24 Load all samples (the entire group) and 1kb DNA Ladder to the wells (read all **Tips** first and then decide as a group)

Tip 1: Use multichannel pipette to speed up the process.
Tip 2: Absolutely minimum of 2 wells with the 1kb DNA ladder; however, many Ladder wells can be included and can be used to divide groups of samples as desired.
Tip 3: 5-10 µl (?) of the 1kb DNA Ladder for each well

4.25 Run the gel between 90-150(?) volts

Note: The amperage should be set high (500mA) to avoid it limiting the current.

4.26 The gel should be run until the lower of the dyes is about ½ to ⅔ down

Lunch Time

Part 5: Image Gel

4.27 Once the agarose gel had run sufficiently, take to the UV Gel Imager to visualise and take a picture (if possible, get a jpeg of the image – use USB Flash Drive or online storage)
4.28 Discard the gel and study the image for differences

Note: Before looking at the gel image in detail, think about what you expect…

4.29 Tidy up (everything this time)

End of Practicals!
Coffee Break