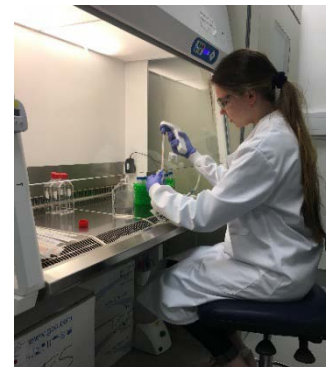
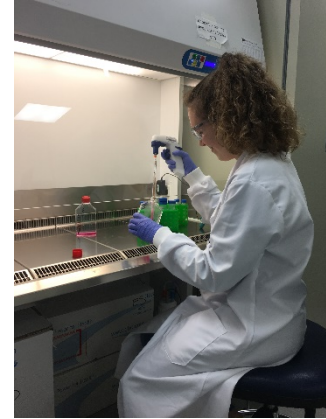
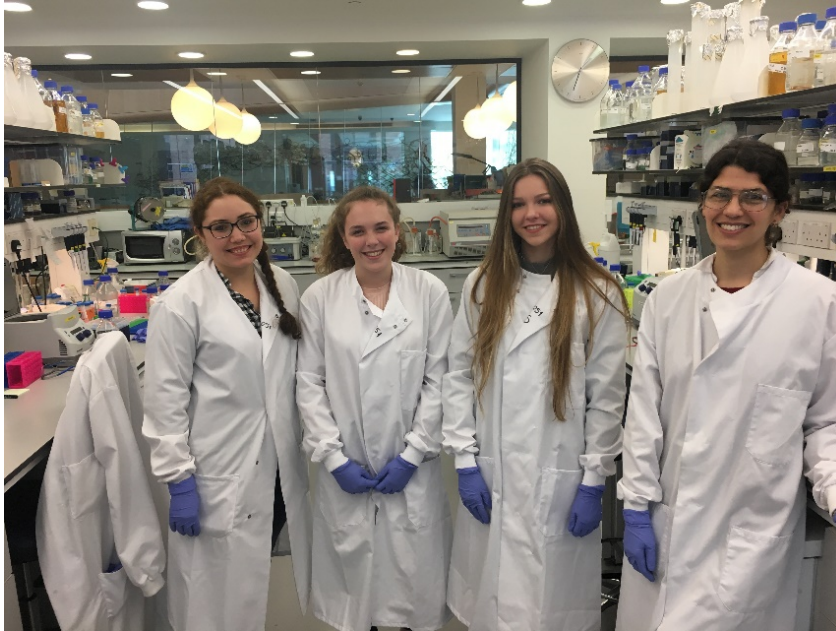


Lab Placement Report

Department of Biochemistry, University of Oxford, 22.03.18 - 27.03.18

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This report details an experiment carried out at the Oxford University Department of Biochemistry to investigate protein expression of cells transfected with plasmid DNA. Using protein gel and western blot techniques we obtained data proving that the desired proteins had been expressed.

Day One (22/03/2018)

Mini-prep DNA – Qiagen kit



In preparation for the experiment bacterial cultures were grown by inoculating the Lysogeny Broth medium (a nutrient rich yeast extract based formula) containing ampicillin, an antibiotic, with a bacteria strain (containing the plasmid with the desired gene – by cloning) over the course of one night.

These cultures were centrifuged at approximately 6000g to obtain the bacteria in pellet form. We disposed of the remaining liquid and disinfected it using Virkon tablets according to guidelines on the disposal of potentially hazardous waste, leaving the bacteria as pellets on the sides of the tubes. The pellet was then re-suspended in 250 μ l of a buffer containing the enzyme RNase which cleaves RNA. 250 μ l of a lysis buffer was added to lyse the cells, breaking down their plasma membranes with the detergent component of the buffer. This process also increased the pH, leading to 'alkaline lysis'. Release of the genomic DNA of

the bacteria causes the solution to become viscous. A pH indicator in the lysis buffer caused the solution to turn blue, providing an indication that the cells have been lysed.

Next, we added 350µl of buffer N3 to neutralise the solution and cause the protein and genomic DNA to precipitate out of the solution. The mixture thus becomes white and inhomogeneous. The mixture was centrifuged at 20,000g, the maximum capacity of the centrifuge, to separate the proteins from the plasmid DNA in the supernatant. The supernatant was pipetted into columns which contained a DNA-binding matrix and the mixture was centrifuged again at 12,000g, allowing the DNA to bind to the matrix. Components that do not bind the matrix flow through it and are discarded ('flow through'). A 70% ethanol buffer was added to wash the DNA, relying on the fact that DNA is not soluble in 70% ethanol. We used the centrifuge once again to help the 70% ethanol through the column where the DNA is located, allowing us to remove the flow-through.



The DNA in the matrix was centrifuged to dry for 2 minutes. To elute our DNA, the column was placed into a sterile (autoclaved) tube. 40µl of EB buffer (water around pH8) was added and the tube centrifuged in order to release the DNA into the flow through. Note that DNA is more stable in slightly basic conditions and soluble in water. It was important to keep everything clean and sterile at the point of elution, to ensure that the DNA did not become contaminated. This is because the mammalian cells used later in the experiment are extremely vulnerable to infection.

Finally, we measured the concentration of the DNA using a nanodrop spectrometer or *spectrophotometer*. In the nanodrop, a beam of light is shone through a defined amount of solution and the absorbance of light is used to measure the concentration of the DNA in the solution.

Tissue culture

Before arrival the culture medium was prepared by adding 50ml Foetal Bovine Serum (FBS), 5ml Non-Essential Amino Acids (NEAA) and 5ml Glutamine (L-Glut) to 500ml Dulbecco's modified Eagle medium (DMEM). This was left outside the fridge overnight in order to reach room temperature before use.

The flask containing a confluent layer of adherent Human Embryonic Kidney 293 cells (HEK-293) was removed from the incubator at 37 degrees and the media was removed from the side, avoiding the cells as they are easy to accidentally dislodge. We carefully rinsed the cells with 10ml of Phosphate Buffer Saline (PBS) in order to remove the medium which contains Trypsin inhibitor and calcium ions, again avoiding the cells. We added 2ml of Trypsin+EDTA (a metal ion chelator to sequester the calcium ions) to the side of the flask containing the cells, to digest the proteins with which the cells are attached and to remove the calcium ions which are required for these proteins to function.

The flask was incubated at body temperature (37degrees) for 4 minutes. During this time a new flask was prepared by adding 10ml culture medium. After 4 minutes all the cells had detached from the side. 8ml culture medium was added to the cells in the flask and pipetted gently up and down 10 times to create a monodisperse cell suspension. 2ml of the cell suspension was pipetted to the newly prepared flask, gently rocked and placed in the incubator, ultimately producing a 1 in 5 dilution of the cell suspension for cell maintenance.



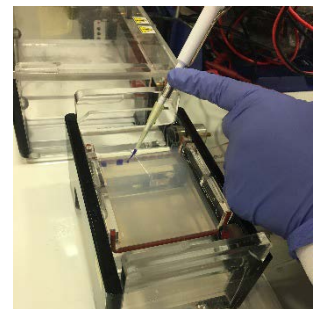
Using another 2ml of the cell suspension, to which was added 10ml of culture medium, 2ml of the resulting diluted solution was pipetted into each well of the 6-well plate. This was also placed into the incubator overnight.

These wells were set aside in preparation for an expression test that was to be carried out the following day.

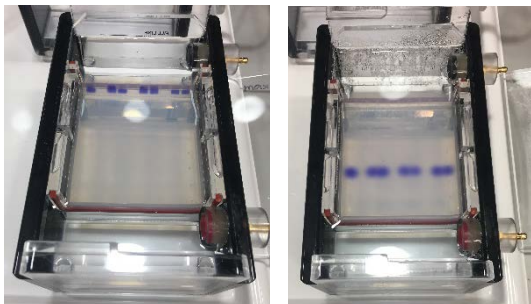
Day Two – 23/03/18

Running a DNA Gel

An Agarose gel containing SYBRsafe, a DNA binding dye which fluoresces when observed under blue or UV light, was pre-prepared 30 minutes prior to the experiment. The equivalent of 200 ng DNA from each mini-prep sample was mixed with a 6x concentrated Agarose gel loading buffer to run the DNA on a gel. This contained a blue stain called 'bromophenol blue' ($C_{19}H_{10}Br_4O_5S$) which is smaller than the DNA of interest and negatively charged and therefore runs faster than the DNA on the gel. This indicates when to stop applying current to the gel. In addition to the samples, a sample ladder containing pieces of DNA with known sizes was used as comparison.



The gel was in a running tank surrounded by running buffer which contained ions (specifically calcium and magnesium ions) to allow the current to run. The entirety of the 6 samples was loaded into pockets in the gel and run at 150V for 45 minutes.



We observed the DNA gel in two ways: initially we placed the DNA in an imager containing UV light which connected to a camera to allow us to photograph the fluorescing gel. We also observed the bands visually using a blue light box.

We were pleased with the results as we could easily see supercoiled and non-supercoiled plasmids, showing that our minipreps had been successful. The plasmid produced is particularly prone to supercoiling.

Lh vj004 – plasmid with an insert that codes for 'uncoordinated5'

M cz033 – plasmid with an insert that codes for an 'adhesion G-Protein Coupled Receptor'

Rh cz041- plasmid with an insert that codes for another 'adhesion G-Protein Coupled Receptor'



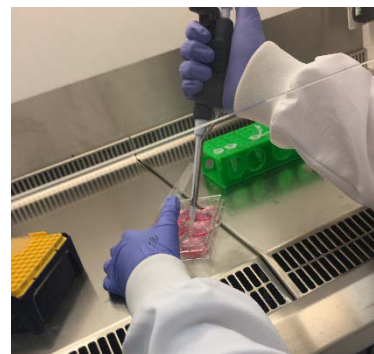
DNA Transfection

For each sample a transfection cocktail was made by combining 3µg mini-prep DNA, 100µl OptiMEM (a medium without serum to allow DNA and transfection reagent to bind efficiently) and 9µg Polyethamine Imine (PEI, transfection reagent) in an autoclaved 1.5ml Eppendorf tube. This was mixed vigorously and incubated for 10 minutes at room temperature to allow the DNA and PEI to combine and form complexes.

The 6 well plate of cells was removed from the incubator and examined under a microscope. By checking the medium was clear and the pH had not dropped due to rapid bacterial growth we confirmed that there was no infection.

The transfection cocktail containing one type of mini-prep DNA was added to the cells at the side of each well, whilst gently tilting the plate so the transfection cocktail and culture medium could mix.

The plates were left to incubate for 3 days at 37 degrees.



Day Three – 26/03/18

Protein Gel – SDS-PAGE (PolyAcrylamideGelElectrophoresis)

A protein gel was run to separate the proteins in our media samples by size. Initially we observed the 6 well plates containing cells covered by the now orange culture medium. The change in colour showed us that a pH drop had occurred as the cells had multiplied and metabolised, releasing acid into the medium.

2ml was transferred from each well to an Eppendorf tube and centrifuged to remove the dead cells. The supernatant was transferred to new tubes.

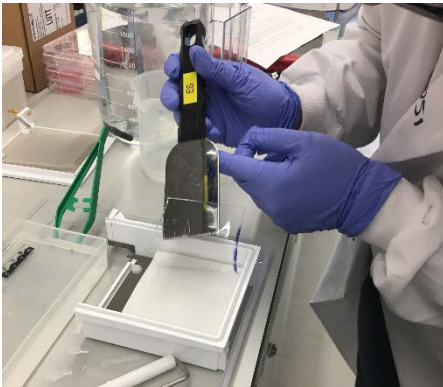
20µl of this supernatant was mixed with 10µl SDS containing loading buffer. SDS is Sodium Dodecyl Sulphate, a detergent that unfolds proteins and makes them negatively charged by binding to them. The tubes were placed in a heat block for 2 minutes to encourage the SDS to denature the protein.

20 µl of each sample was pipetted into each well of a pre-cast poly-acrylamide gel in the running tank and we added *MOPS* buffer to control the temperature and allow the current to run. As well as the samples, 10µl of a pre-stained protein ladder and 0.5µl of a protein ladder in which all proteins are Histidine tagged were pipetted into wells.

To run the gel we applied 180V for 55 minutes.



Protein Gel Transfer



To be able to use antibodies to visualise proteins efficiently they must first be transferred onto a membrane. First, the gel was released from the cassette and placed into a 'sandwich' as shown.

Filter paper was used to protect the sponges and ensure a smooth surface for the gel and membrane, and all aspects of the sandwich were soaked in a transfer buffer, which acts to facilitate the transfer of the proteins from the gel to the membrane. On assembling the sandwich each layer was individually rolled in order to avoid bubbles forming on the surface. This ensured that the protein could migrate from the gel onto the membrane in a homogenous fashion. To allow this, a current was applied

to the sandwich for 60 minutes at 30V.

A 10ml fat free milk solution of concentration 5% was used to block the membrane, thus avoiding unspecific binding later on; if no milk was added, as the membrane is 'sticky', the antibodies would attach non-specifically as well as to the tagged proteins. The blot was then overlaid with 10ml of 5% milk containing 10 μ l of the primary antibody which had been raised in a mouse against the poly-histidine tag of our proteins.

This was then left overnight to soak at four degrees Celsius.

In the afternoon we helped Maria in the transfection room with the day's Cell Maintenance as detailed under *Tissue Culture* on day 1.

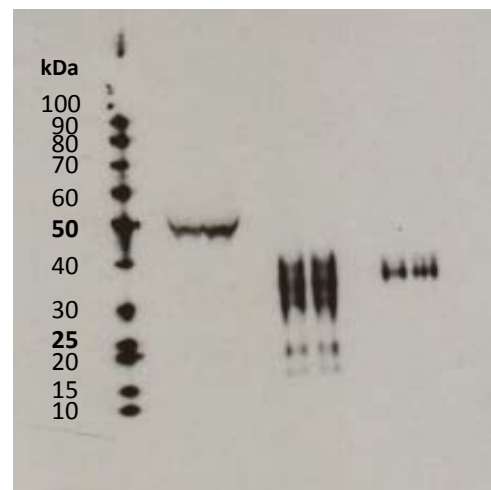
Day Four – 27/03/18

Initially, the primary antibody containing solution was removed from the blot and the blot rinsed with PBS containing 0.1% Tween-20 detergent. The membrane was then left to incubate in a milk solution containing the secondary antibody which is coupled to horseradish peroxidase (HRP) for an hour.

The membrane was washed twice for 5 minutes to remove excess secondary antibody to prevent background signals showing up on the film. 1ml each of the oxidising reagent and the luminol reagent were added together to oxidise the HRP protein and allow it to catalyse a reaction which releases energy in the form of light (chemiluminescence). The membrane was laid out on cling film, 2ml of the mixture added and the overlaid membrane was incubated for 2 mins.

The excess liquid was removed and the membrane placed upside down, enclosed in a new piece of cling film, ensuring there are no air-bubbles.

The covered membrane was placed in a metal cassette and transported to the dark room where the developer was located. The film was placed in the cassette for set periods of time find the optimal exposure time (which we discovered to be 10seconds). The films were then put through a developer to allow visualisation where the light produced by the HRP hit the film, allowing us to identify the sizes of our proteins.



A developer works by becoming oxidised which means that, in turn, it acts on exposed silver on the film to produce a black image. Where there is non-exposed silver a white image is formed. The developer itself contains hydroquinone (for high contrast) and metal/phenidone (for low contrast) and the solution is alkaline to maintain the optimum pH for the developer to work.

We were successful in our experiment, having been able to produce all three proteins, thus proving the success of all our mini-preps and ascertaining that these proteins are suitable for potential use in structural biology experiments as they could be scaled up and used in large amounts.

NB. – X-Ray Crystallography

Proteins are purified and crystallised under specific conditions. These crystals can be used in X-ray diffraction experiments to reveal the atomic structure of the protein. The light is diffracted because of the repeating units in the crystal, producing a diffraction pattern that can be captured on a detector. The resulting signal can be used to calculate the electron density map of the crystal, from which we can model the 3-dimensional structure of the protein to infer its functional properties.

Crystallography is used frequently in drug design in predicting what kind of drug will bind to a certain protein and what effect this will have.

Youtube video – Elspeth Garman on crystallography:

<https://www.youtube.com/watch?v=gLsC4wlrR2A>

<https://www.youtube.com/watch?v=Z7hXiKiZZPs>

Illustration of our Experiment:

