Lab Techniques and Skills: A Guide

A quick summarized guide intended to demonstrate some of the wet-lab skills and protocols I have mastered over the span of my academic career. Each experiment I performed on my own and have complete mastery. Further results and examples are available upon request.

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Nanoflow Cytometry

Key words: Flow Cytometry, antibodies, plasma, HLA-G, PLAP, obstetrics, oncology, diagnostics

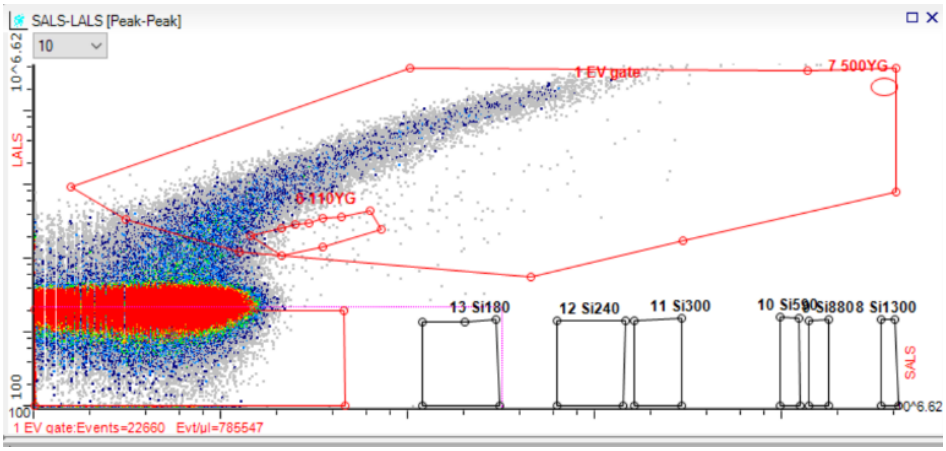

Figure 1. Example output of total events captured in 5 µL of human plasma from a normal pregnancy patient. The x-axis denotes SALS while the y-axis denotes LALS. The gate '1 EV gate' is used to collect the 'Total events' captured within this sample for further downstream analyses.

Background: Flow cytometry is a technique used to detect and analyse the chemical and physical characteristics of cells or particulates. Nanoflow cytometry (nFC) follows the same principle, but on a nano sized scale. Typically, a sample is suspended in fluid and focused so one cell or particulate is measured at a single time through a laser beam so the light scatter can be measured. Two main measurements include Large Angle Light Scatter (LALS) and Side Angle Light Scatter (SALS), measuring size and complexity, respectively. Fluorescent antibodies are used to detect the presence of specific markers, and to help separate populations.

Result: In the image above, each dot represents a particulate detected by the machine, labelled as an event. Samples have 'noise' which can correspond to contaminants, fragmented cells, the machinery, etc. To avoid capturing noise in downstream analyses, a gating strategy is used to remove background noise, labelled as '1 EV gate,' representing the total events in this sample.

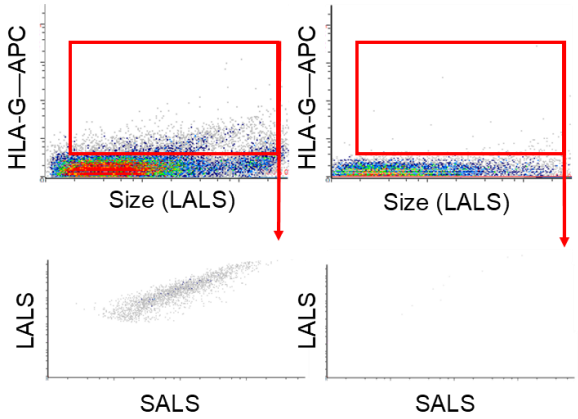

Figure 2. Example output of HLA-G events captured in 5 µL of human plasma from a normal pregnancy patient. The graphs on the left show the positively tagged cellular fragments using the antibody, while the right graphs show the control to visualize the baselevel noise of the antibody to determine what is true 'signal.'

The fluorescent tagged antibodies can be separated from the 'total events' population based on the channel or colour spectra they are binded to. For HLA-G, the antibody can be individually analyzed in the APC or red colour channel (left boxes), where again, a gating strategy is used to remove antibody noise by comparing it to the isotype antibody (right boxes). This method is advantageous as more than one antibody can be analyzed at a given time, and two antibodies can be compared to one another to determine if those populations share any particulates, as seen in the image below.

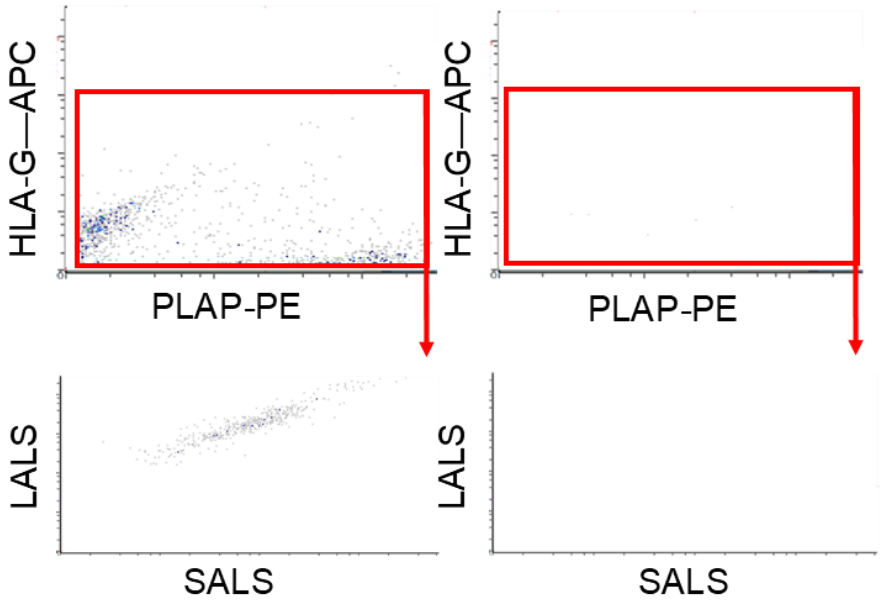

Figure 3. Example output of HLA-G and PLAP events captured in 5 µL of human plasma from a normal pregnancy patient. The graphs on the left show the positively tagged cellular fragments using the antibody, while the right graphs show the control to visualize the baselevel noise of the antibody to determine what is true 'signal.'

Importance: My study, "Towards a Nanoscale Flow Cytometry Assay of Circulating Extracellular Vesicles for Maternal Health Trajectories" investigated the role of extracellular vesicles (EVs) found in maternal blood to assess pregnancy progression. Using nFC, blood samples were analyzed for biomarkers HLA-G or PLAP bearing EVs, and EVs that present both biomarkers. To summarize the findings, HLA-G EVs and HLA-G and PLAP bearing EVs showed significant differences in patients with pregnancy disorders such as preeclampsia, gestational diabetes, and patients undergoing amniocentesis, compared to normal healthy pregnancies. **Using this technique, a novel diagnostic tool was developed to potentially detect pregnancy disorders in real time within pregnant patients to improve maternal health trajectories.**

In situ Hybridization

Keywords: whole mount embryo ISH protocol, optimization, RNA probes, heat-shock, alkaline phosphatase

Background: *In situ* hybridization (ISH) is a technique used to detect the localization of a specific nucleic acid sequence within a cell, referred to as a probe. It can be used in tissue sections, but also in *Drosophila* embryos, with the method becoming whole mount ISH. This powerful technique is useful in visualizing the localization of specific nucleic acid sequences within an embryo, and the organization,

regulation, and function of such genes. Typically, an RNA probe is used in whole mount ISH protocols where probes can be attached to a fluorophore and becoming a slightly different protocol called Fluorescent DNA ISH (FISH).

In brief, the *Drosophila* embryos protocol begins with collecting embryos at specific age points and transferring them to a 1:1 bleach solution to be dechorionated. Washed bleached embryos are placed in stress treatments (ex. Heat shock at 37 degrees Celsius) if needed. Embryos are then fixed and washed with heptane and methanol for devitalization. The fixed embryos can now be rehydrated and postfixed, followed by a proteinase K incubation. This treatment allows for the probe to permeate the embryo. To remove the proteinase K, glycine washes are performed, followed by another round of postfixing. The embryos are prepped in hybridization solution and incubated with the specific DIG-labelled RNA probes. The washed probes embryos are blocked with anti-DIG antibody coupled to alkaline phosphatase. Visualization occurs by adding a developing solution which reacts with the alkaline phosphatase a deposits pigment that can be viewed under the microscope.

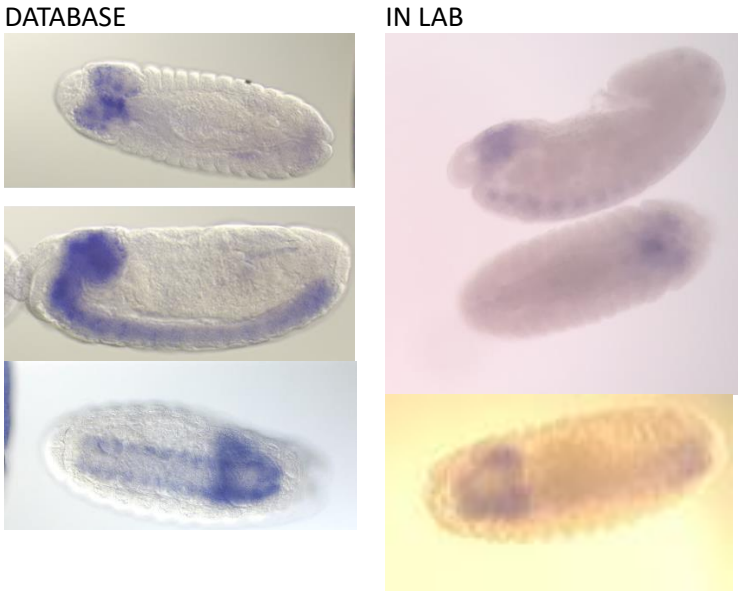

Figure 4. Examples of staining using in-house made RNA probes (left) to those found in the Berkely Drosophila Genome Project (BDGP) using ISH staining protocols.

Results: Following the whole mount ISH protocol with DIG-labelled probes, the staining pattern can be compared to those in a database, to assess how successful the protocol was, as well as how specific the probes are for that specific gene. The database images are shown in the image above on the left, while those collected in lab are on the right, for a BRAT gene RNA probe. Based on these images, the protocol was not only successful but highly specific to the gene where the specific expression patterns are very comparable to those done by the database.

Importance: The protocol used to generate that staining pattern presented in the images above comes from an optimized protocol for large collections and for high throughput probing. To develop such a protocol involved creative experimental design, dedication, and ingenuity to ensure that it could still produce a highly specific signal and requires extensive optimization. For example, the dechorionation, devitalization, fixing, proteinase K, blocking treatment, and AP visualization steps all required optimization. As RNA probes were made in house, that protocol also needed optimization to strengthen

the signal in the embryo and to ensure it was specific in what it was targeting and binding too. **Optimizing my whole mount ISH protocol for large collections with in house probes was difficult and tedious but proved to be worth the effort as multiple probes could be tested at the same time and produce clean signal results in the embryo.**

Gel Electrophoresis

Keywords: gel electrophoresis, CRISPR-Cas9, single nucleotide mutation, restriction digest, PCR, Sanger Sequencing, DNA extraction, *Drosophila*, fly husbandry

Introduction: Gel electrophoresis is a separation method used to separate DNA, RNA, or protein based on molecular size. This occurs due to an electrical field which pushes negatively charged molecules through a gel matrix which contains small pores to restrict the size of what molecules can pass through. This can be adjusted based on the percent gel that is prepared. A ladder is run with the samples to determine what sizes the molecules are.

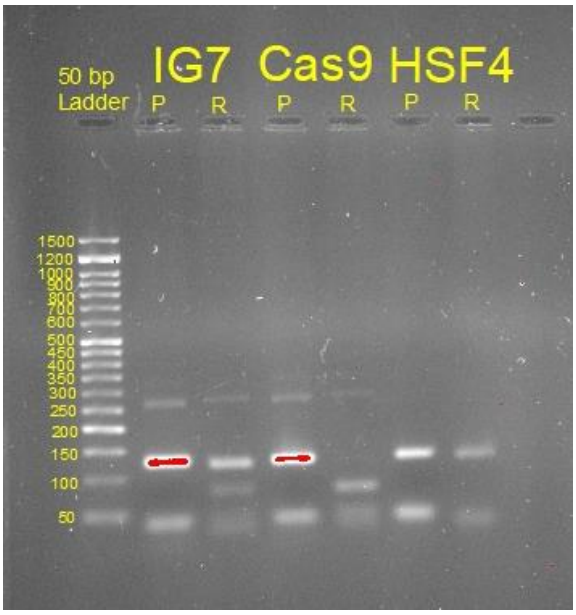

Figure 5. PCR amplification and restriction digest of potential *hsf*^{4} mutant fly. Male IG7 flies proceeded through a PCR amplification and restriction digest protocol with the controls of the HSF4 plasmid DNA and the Cas9 insert fly line. The PCR amplification samples were prepared followed the *2X Taq FroggaMix* protocol where it was amplified at a 65 °C annealing temperature and run on the *Thermo Fisher Veriti 96-well Thermo Cylcer*. The restriction digest used the EcoRII restriction enzyme. For each sample, the PCR amplification and restriction digest ran on a 2% agarose gel (denoted as P and R, respectively) along with a 50 bp Ladder. The gel was run at 120 V for 45 minutes. The gel was imaged using *ImageLab* and was observed for banding at the 127 bp mark.

Results: The image above shows an example of gel electrophoresis whereby drosophila lines with mutations (IG7, CAS9, HSF4) were PCR amplified (P) and treated with a restriction digest (R). The ladder helps to establish the sizes of the bands that are seen in each column. This specific gel was a 2% agarose gel run at 120V.

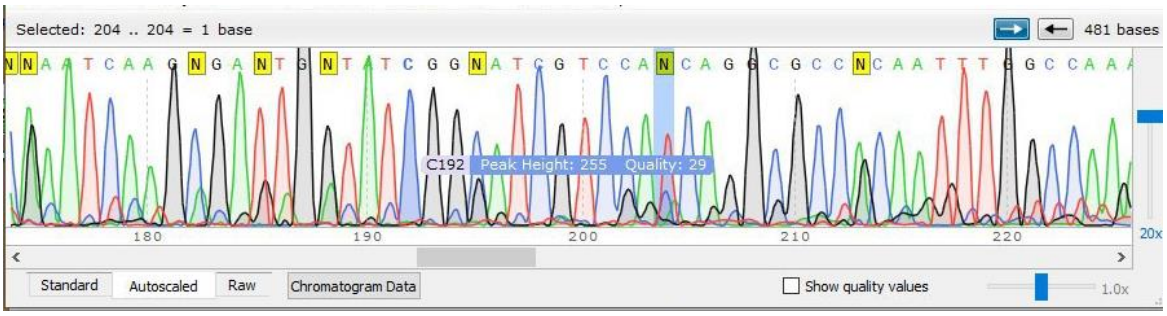

Figure 6. Trace sequencing of the CRISPR-edited male fly *hsf* gene at the *hsf*^{4} mutation site. Purified genomic DNA was PCR amplified using a Phusion High-Fidelity DNA Polymerase protocol with an annealing temperature of 72 °C in the *Thermo Fisher Veriti 96-well Thermo Cylcer*. The highlighted region indicated the single-base pair mutation within the DNA-binding domain of the HSE where the N corresponds to either pyrimidines bases of thymine and cytosine.

Importance: This experiment was to test if a mutant line with a single nucleotide change, IG7, was homozygous for the Hsf4 mutation. This fly line was generated using a CRISPR-Cas9 system where flies which had the Cas9 driver were given the mutation via CRISPR editing, generating the IG7 fly line. Thus, as a control, the Cas9 flies which would be considered as a wildtype and the HSF4 flies which have the mutation were used. Each sample successfully underwent PCR amplification (as indicated by the 'P' column). The restriction digest was used to detect if the mutation was present as there would be no cleavage of the band (HSF4); wildtype would cut as there is no mutation (Cas9) impeding the cut site, resulting in a 127 bp band. The IG7 line was heterozygous as it had both the cut band and the homozygous 127 bp band.

To confirm, purified extracted DNA was Sanger sequenced to asses if the mutation was present. Highlighted by the grey column, the sequencing results showed that some wildtype signal of the cytosine trace at the mutation site, however the stronger peak is that of the mutation nucleotide trade of thymine. **This experiment was important to show that the mutant line still had some wildtype traces which may be due to a CRISPR event where recombination may have inserted the *hsf*^{4} sequence at other sites, or that the wildtype *hsf* that was cut out may have been inserted at another site instead. Although, the later is unlikely due to the specificity of the cuts and binding that occurs during a CRISPR event and no other residual C trace was in any surrounding nucleotide position.**

Immunohistochemistry (IHC)

Key words: Immunostaining, human placental tissues, antibodies, HLA-G, tissue sectioning

Background: Immunohistochemistry (IHC) is a common method of immunostaining, whereby exploiting the antigen-antibody binding to a specific, localized antigen or proteins present on tissues and cells. The presence of specific antigens on tissues or cells can be visualized using a method called chromogenic IHC, where a enzyme conjugated to an antibody reacts with peroxidase to induce a colour changing chemical reaction. Dissected tissue samples are paraffin embedded and sectioned and mounted to slides. Processes of deparaffinization, rehydration, and a series of ethanol and PBS washes prepare the tissue for antigen retrieval. A peroxidase solution incubation is followed with blocking and the antibody incubation. Visualization occurs with a peroxidase kit and hematoxylin stain, followed by a series of ethanol washes and xylene, and finally mounted with cytoseal and a coverslip to be imaged.

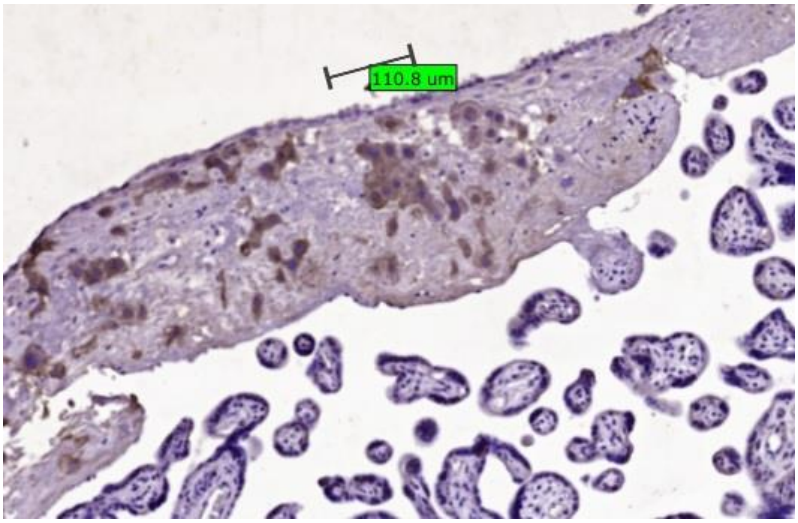

Figure 7. IHC stain on fetal-side placental tissue section with HLAG antibody. Positive signal is indicated by the colour brown while negative staining is in blue.

Result: When analyzing IHC stains, the colours presented on the image indicate where the target protein of interest is localized. Positive staining is visualized in brown, while negative staining is visualized in blue, resulting from the hematoxylin counter-stain. In the image above, a fetal-side placental tissue section underwent IHC with a HLA-G antibody, where positive staining in brown demonstrates the presence of HLA-G in the tissue, and not in the villi structures below it (the circular structures).

Importance: To understand where HLA-G EVs that are detected in maternal blood come from during pregnancy, placental sections were treated with HLA-G to find those EV origins. Based on the staining results, HLA-G is present in high concentrations on the fetal-side of the placenta. **This suggests that the HLA-G EVs may originate directly from the fetus, and that the changes in HLA-G EV concentrations observed in pregnancy disorders may indicate the health of the fetus as well.**

Western Blot

Key words: western blot, CRISPR-Cas9 Knockout, AML, SDS-PAGE

Background: Western blotting is a technique used in the detection of a specific protein, where it is separated to identify proteins at specific sizes within a given sample. The process follows the key steps of separating the proteins by size, followed by a transfer so the target protein can be visualized using a primary and secondary antibody. The separation of proteins is completed via gel electrophoresis on denatured proteins, using the procedure of Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SGS-PAGE). Once separated, the gel is transferred to a membrane (generally nitrocellulose) whereafter it is visualized using Ponceau S to ensure transfer was successful, and then blocked prior to incubation with the primary antibody. Last, the secondary antibody is applied to target the primary antibody and the target protein is visualized using chemiluminescent detection methods.

Figure 8. Western blot of CRISPR-edited K562 cells for SUV3 with 5 guide RNAs and a control.

Result: For this experiment, lymphoblast cells isolated from a myelogenous leukemia patient called K562 cells were used in the knockout of SUV3, a component of the mitochondrial degradosome that mediated RNA turnover, using CRISPR-Cas9 system. The efficacy of the knockout was tested with 5 guide RNas and visualized using western blots where actin and control guide RNA K562 cells were used.

Importance: The RNA guides 1 and 1-4 (sgSUV3-1 and sgSUV3-1-4) show a successful knockdown of SU3 protein in the K562 cells. As actin is present in the sample, the protein extraction and SGS-PAGE was run successfully and the lack of SUV3 protein is due to the knockout. Also, the control (sgControl) produces a band while the RNA guides 1 and 1-4 do not. **Therefore, the RNA guides 1 and 1-4 (sgSUV3-1 and sgSV3-1-4) demonstrate a successful CRISPR knockdown of SUV3 and those cell lines should be used for further experiments.**

Cell Proliferation Assay:

Key words: cell proliferation assay, microscopy, AML

Background: Cell proliferation or cell viability assays are used to monitor the growth rate of cell populations. Cell populations are counted using the 'real-time' method of eye counting using a grid and looking at proliferation-positive cells under a microscope at x10-x20 objective. Cell counts are tracked across time.

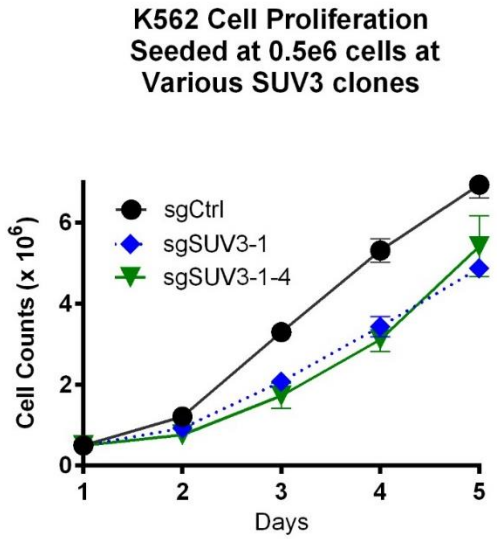

Figure 9. Cell proliferation assay graph comparing 2 cell lines developed from CRISPR-editing K562 cells for SUV3 knockout using two guide RNAs against the control. Measurements were taken at the same time everyday for the duration of 5 days.

Result: For this experiment, lymphoblast cells isolated from a myelogenous leukemia patient called K562 cells were used in the knockout of SUV3, a component of the mitochondrial degradosome that mediated RNA turnover, using CRISPR-Cas9 system. The efficacy of the knockout was tested, and two RNA guides sgSUV3-1 and sgSUV3-1-4 showed successful knockout of SUV3. These cell lines were expanded and compared to control K562 cells across 5 days.

Importance: Compared to the normal, SUV3 knockout cells in both cell lines proliferate less than control Both knockout cell lines perform very similarly as well. This suggests that the role that SUV3 had in regulating RNA turnover has a direct impact on the survivability of the cell. **As these are myelogenous leukemia cells, this suggests that SUV3 can be a potential target for therapeutics and aid in the treatment of AML.**