Adventures in PCR:
The Impact of Alcian Blue Staining on Danio rerio Embryos.

Brian G. Saucedo and Andrea James
School of Biological Sciences
University of Northern Colorado, Greeley, CO
Contact: andrea.james@unco.edu

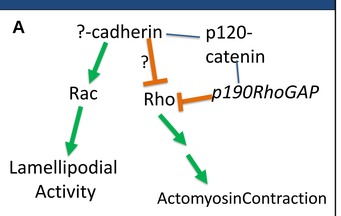
Abstract

p190RhoGAP(p190) is a member of the Rho family GTPase activating proteins, that have been shown to play a role in cytokinesis and play roles in cell proliferation. Studies have shown that *p190* plays various roles in nervous system development and defects are similar to defects seen in cell adhesion disorders. In conjunction with another research project examining the role of *p190RHoGAP* in jaw development via Alcian blue staining, this study set out to optimize genotypic verification of *p190* in zebrafish embryos. We used polymerase chain reaction (PCR) to amplify the *p190* genetic contributions in individual embryos followed by Restriction Fragment Length polymorphism (RFLP) analysis to distinguish between different genotypes of the *p190* gene; wild type(250 and 350b.p), mutant (600b.p), heterozygous (600, 350 and 250 b.p). Currently the common methods include single cell extraction at the 32-cell stage or head vs. tail dissections which are both technically challenging. While head vs tails extractions have been previously utilized in our lab, we sought to streamline this process to exclude embryo dissections. During the procedure, PCR optimization and trouble shooting had to be done prior to, determining if DNA samples would be viable for PCR when DNA extractions were done on previously dyed embryos . As a control we compare previously dyed embryos to tailed embryos that were non-dyed tail extractions. Our results indicate the Alcian blue staining did not have a notable impact on our genotypic verification when comparing the stained and non-stained embryos. Our data highlight the delicate nature of working with PCR on different embryo treatments which can halt forward progress in genotypic analysis of mutant embryos that may have subtle or late developing phenotypes.

p190RhoGAP and PCR genotyping

p190RhoGAP

Rho Gaps are a protein domain of GTPase activating proteins which can alter actin arrangements when responding to extracellular signals. *p190RhoGAP* is a Rho regulator that can play a mirid of roles in development, from the nervous system to playing a role in eye development. Deficiencies in Rho Gaps have been shown to have terrible morphogenetic results similar to those described in cases of cell adhesion mediators.



Adapted from Yamada, *et al*, 2007

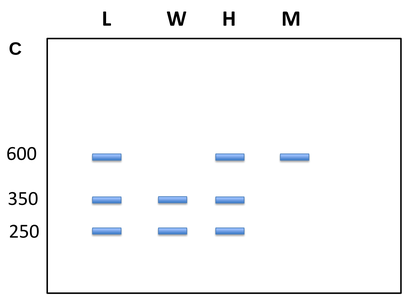
p190RhoGAP Talen

When looking at p190RhoGAP, fish with a *p190* Talen cut site were used. Talen (Transcription activatior- like effector nucleases). Talen enzymes can be engineered by using TAL effector DNA binding domain to a nuclease that can cut DNA strands. This can be used to find specific proteins or genes and make cuts into specific strands. Zebrafish were then engineered to have a *p190* cut site and breed with non *p190* Talen fish to have the Heterozygous offspring.

James, A., *unpublish data*

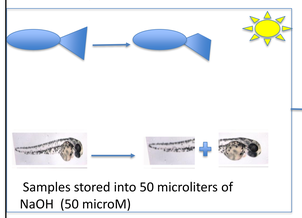
Wild type vs. heterozygous vs. mutant.

When using gel electrophoresis to visualize the genotyping, there are three possible outcomes. First is wild type where there is a cut in the band making two bands at 350 and 250 b.p long. The mutant is left uncut leaving a band 600 b.p long. The heterozygous genotype is categorized with having cuts and non-cuts resulting with all three bands.
L is the ladder, W is expected wild type, H is expected heterozygous, and M is mutant.



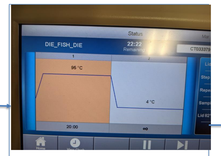
Methods- PCR , RFLP, Gel Electrophoresis

1. Sample collection



Samples stored into 50 microliters of NaOH (50 microM)

2.Preparation



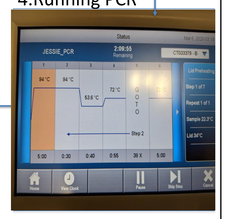
PCR used to Explode cells, and Tris HCl for pH

3. PCR set up

1µl Forward primer*
1µl Reverse primer*
5µl Taq buffer
2 µl $MgCl_2$
1 µl dNTP
.5 µl Taq polymerase.
34.5/35.5 µl H_2O

This is the formula that is added to the 5µl of genomic DNA.

4.Running PCR

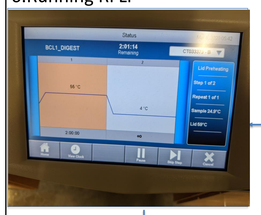


5.RFLP set up

5 µl buffer 3.1/2.1
.5 µl Bcl I
4.5 µl H_2O

This is the formula that is added to 40 µl of the PCR product.

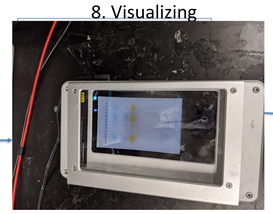
6.Running RFLP



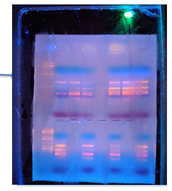
7.Running gel

We run the gel using Ethidium Bromide and using 1-1.5% agarose gel

8. Visualizing



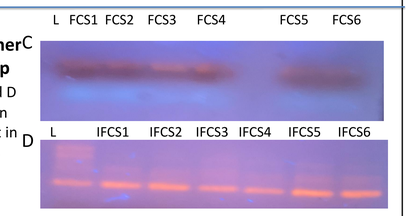
Results



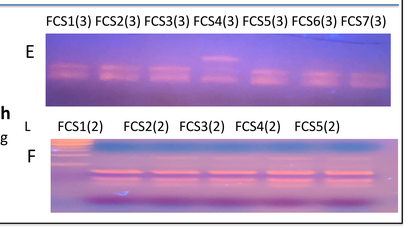
Optimizing PCR and genotyping

. **One way to combat this we tried increasing the water concentration from 34.5 to 35.5 resulting in figure A. GSK is genomic sample (kaede). FCS is fin clip sample** Figures A and B show examples of roadblocks we encountered. B shows some of the failures signaling that primer dimers

Next trials were to test primer optimization through primer dilutions and evaluating primer pair sets. FSC is fin clip samples, IFCS is fin clip samples ran with the Itgb primer. Figures C and D are results of primer evaluation. Figure C is the test run where the primers where diluted. While improvement in results, there was still primer dimerization. Figure D is results of different primers set (Itgb1) being run with same protocol to test if our *p190* primers set.

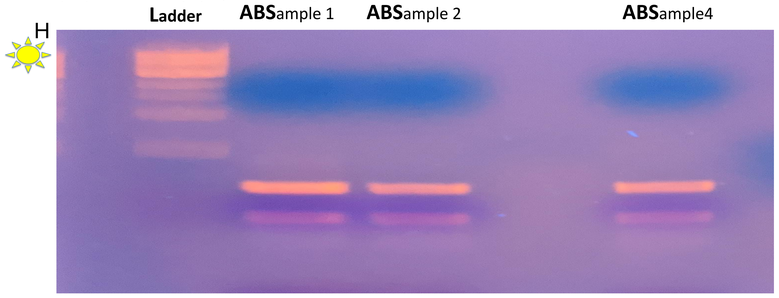


To. make sure that there was not going to be any difference between the 3.1 buffer and the 2.1 buffer for the RFLP procedure, we tested the two buffers. FCS(3) is fin clip ran with 3.1 buffer, FCS(2) is fin clip ran with 2.1 buffer Figure E is the successful run after using the corrected primers with the 3.1 buffer. Figure F was a trial run with 2.1 buffer for RFLP.



p190RhoGAP and Alcian Blue Results

This timeline is a frame of possible times to collect or prepare our samples. The green star is the time where we prepare the samples as outlined in the methods. The first method doesn't require PFA as it is the embryo split method. Important to note that the embryos require preparation 5 days pdf, either being split or preservation in PFA.



ABS stands for Alcian Blue Sample and was worked on using Kaede embryos. The preliminary results shows that the Alcian blue staining process doesn't seem to alter or effect the DNA if at all. It is recommended to use more RFLP product in the gel agarose(it is found that compared to 10 µl, 20 µl is better to see in gels.) When compared to the current method where we would split the embryo in two and run tests on each component the results were similar with no distinct difference. Sample preparation was kept the same such as using thermocycler to blow up cells and using Tris HCl for pH. PCR and RFLP set ups were kept the same. As of now most of the samples used are using old staining protocol.

Conclusions

- PCR has shown to have many steps that be considered when optimizing. We recommend evaluating the primers first, examining if it is the correct sequence and that it is diluted.
- The lab also found that there is little difference between the two water concentrations once primers where corrected. That said the higher dosages was helpful step in right direction and seemed to help with showing some results.
- The two buffers seem to work identically and didn't seem to have much difference.
- We were able to find that the Alcian blue staining did not imped or effect the samples. Going forward rather than having to harvest cells from a cell in the initial hours, or having to split embryos, we could just go forward with our staining and still use the samples and check their genotyping.

Future Directions

- Going forward we would use these protocols to help provide genotyping data in other studies to help provide data for consideration.
- We would also like to test and see if it is possible to use this protocol for other procedures, such as whole mount immunofluorescence, to streamline and optimize genotyping of samples by removing harvesting at the initial step of experiments.

Acknowledgements

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