Cresko Laboratory Procedures and Protocols

Cresko Laboratory

Friday, March 22, 2024

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How to use this book

This is a Quarto book that contains all of the Procedures and Protocols for the Cresko Laboratory in the Institute of Ecology and Evolution at the University of Oregon.

The book is organized into major section that contain

- General Laboratory Protocols or the lab
- More detailed Laboratory Protocols
- Husbandry protocols for vertebrate animals primarily stickleback and pipefish, but also zebrafish
- Husbandry protocols for *Daphnia*
- Bioinformatic protocols including how to get on to **Talapas**

You can scroll through the book using the index on the left, but also use the search field to find all relevant protocols.

There are also useful appendices at the end, as well as a section for the references cited throughout the book.

This book was written in Markdown using Quarto. To learn more about Quarto books visit https://quarto.org/docs/books.

Part I

General Laboratory Protocols

This section of the book contains general protocols for working in the laboratory.

1 Contact Information

Col1	Col2	Col3
Bill Cresko	541-285-5446	Cell
Mark Currey	541-505-0006	Cell
Susie Bassham	xxxx	Cell

2 Cresko Lab Safety Highlights

2.1 Introduction

- **Purpose**: This procedure describes important rules to heed in the lab for your safety and for the safety of others.
- **Procedure Type**: General Lab
- **Author**: xxx
- **Date Created**: xxx

2.2 Materials

- xxx
- xxx
- xxx

2.3 Solutions

- xxx
- xxx
- xxx

2.4 Procedure

> **!** IMPORTANT
>
> EMERGENCY CONTACT: dial 911 first, AND 6-2919 (EHS). Mark Cell 541-505-0006

1. **Safety Shower, Eyewash, Fire Extinguishers.**

   Eyewashes must be flushed weekly. *Undergraduate research assistants are responsible for flushing the safety showers each week. Each lab member is responsible for knowing the locations of safety showers and fire extinguishers in the lab.* Safety showers and fire extinguishers are tested annually by EHS.

2. **Wear a lab coat and closed-toed shoes when working with the following chemicals**:

   1. Organics (e.g. phenol/chloroform, Trizol, DNAzol, formaldehyde, formamide, methanol)
   2. Strong acids and bases

3. **Wear eye protection when working with**:

   1. UV light (UV opaque glasses/face shield).
   2. Phenol/chloroform, strong acids/bases, and any splash hazard with anything hazardous in it.

4. **Wear safety gloves when working with ANY of the reagents above**.

   Heed the "one glove rule": remove one glove when moving between rooms to avoid touching doorknobs with a contaminated glove. Note that glove materials differ in their permeability to different reagents. Standard nitrile gloves are adequate for our lab's standard procedures. However, if you are planning experiments that involve more dangerous reagents, consult with Luke Sitts at EHS to select appropriate gloves.

5. **Disposal of common hazardous reagents (EHS DISPOSAL: 6-3192)**

   1. E. coli plates and recombinant materials: autoclave buckets or EHS biohazard incineration boxes.
   2. E. coli flasks/liquids: bleach, rinse, drain.
   3. Used alcohols, formaldehyde, and kit waste: waste containers under the thermocyclers.

   4. Organic solvents: waste bottles in hood.

6. **Storage of Hazardous Liquids**

   1. Store flammables and strong acids in a latched METAL SAFETY CABINET UNDER THE HOOD.

7. **Heating Liquids in the Microwave Oven**

   Triple check that the cap is very loose or (better) remove it entirely. Re-melting of gels with DNA binding dyes is forbidden.

8. **Bunsen Burners**

    1. Triple check that the gas is shut completely off before you leave the bench/hood.
    2. Keep burners far away from any flammable liquids.

9. **Liquid Nitrogen and Dry Ice**

    1. Use only in well ventilated spaces to avoid asphyxiation.

    2. Never store in sealed containers to avoid explosions.
    3. Wear lab coat, gloves, goggles. In case of frostbite or burn, soak affected part in tepid water, seek medical attention.

2.5 Associated Papers

- xxx
- xxx
- xxx

3 Cleaning PCR Mats

3.1 Introduction

- **Purpose**: This procedure describes how to clean PCR mats.
- **Procedure Type**: General Lab
- **Author**: N. Earp
- **Date Created**:

3.2 Materials

- PCR mats to clean

3.3 Solutions

- 5% bleach solution
- Tap water
- DI water

3.4 Procedure

1. Cover used PCR mats in 5% bleach solution.
2. Let sit in 5% bleach solution for 15 minutes.
3. Rinse PCR mats with tap water, then DI water.
4. Lay PCR mats to dry.

3.5 Associated Papers

- xxx
- xxx
- xxx

4 Cleaning Pestles

4.1 Introduction

- **Purpose**: This procedure describes how to clean pestles.
- **Procedure Type**: General Lab
- **Author**: xxx
- **Date Created**: xxx

4.2 Materials

- Large beaker (or similar vessel in which to soak pestles)
- Aluminum foil
- Autoclave tape
- Autocalve

4.3 Solutions

- 10% bleach solution
- xxx
- xxx

4.4 Procedure

1. Rinse dirty pestles with tap water.
2. Place pestles into a large beaker and fill the beaker with 10% bleach solution. Let the pestles sit for 10 minutes.
3. Drain the bleach solution and rinse the pestles with DI water.
4. Section the pestles into 5-10 pestle bunches, wrap each bunch in tin foil, and use a section of autoclave tape to close foil.
5. Autoclave foil-wrapped pestles using the 25 minute Gravity program

4.5 Associated Papers

- xxx
- xxx
- xxx

5 Disposing of Hazardous Waste

5.1 Introduction

- **Purpose**: This procedure provides a guideline of how to store and get rid of various forms of hazardous waste (HW) that are produced in the Cresko Lab.
- **Procedure Type**: General Lab
- **Author**: Mark Currey
- **Date Created**: July 30, 2022

5.2 Materials

- xxx
- xxx
- xxx
- xxx

5.3 Solutions

- xxx
- xxx
- xxx

5.4 Procedure

> **!** IMPORTANT
>
> ALL CONTAINERS USED TO HOUSE HAZARDOUS WASTE NEED TO BE LABELED WITH CONTENTS, DATE, AND OWNER.

1. **Common streams of HW**: Paraformaldehyde (PFA), alcohol (ETOH, methanol, isopropanol, etc), and common kit waste.

    1. Procedure: Collect these waste streams separately. They can be collected locally (e.g. small containers on lab bench) and then put into the common large carboy for pickup.
    2. Pick up: Every two weeks a lab assistant will assess if common collections need to be picked up. If so, they will initiate a pick up via EHS. https://safety.uoregon.edu/hazardous-materials

2. **Exotic or nasty HW**: Trizol, phenol, chloroform, formamide, etc…

    1. Procedure: Researchers are responsible for communicating with the lab assistant as to how these materials will be temporarily stored and the periodicity of disposal. As an example, if DNA extractions are being done with Trizol, solid (e.g. tips and tubes) that come into contact with this reagent and used reagent will be collected in the hood.
    2. Pick up: Once the extractions are done or after two weeks (whichever is first) a "pick up" will be initiated with EHS. https://safety.uoregon.edu/hazardous-materials

5.5 Associated Papers

- xxx
- xxx
- xxx

6 Bacterial Waste Cleaning and Disposal

6.1 Introduction

- **Purpose**: This procedure describes how to clean and dispose of bacterial waste.
- **Procedure Type**: General Lab Maintanence
- **Species**:
- **Author**: Mark Currey
- **Date Created**: March 8, 2024
- **Date Updated**: xxx

> ⚠ NOTES
>
> xxxx

6.2 Materials

- xxx
- xxx
- xxx
- xxx

6.3 Solutions

- 30% Bleach solution
- xxx
- xxx
- xxx

6.4 Procedure

1. Pour approximately 10-15 mL of 30% bleach solution into each tube containing bacteria
2. Let the bleach solution sit for 3-5 hours, or until the solution becomes clear again
3. Pour the bleach and bacterial waste into the large carboy labeled 'Bacterial Waste' r
4. Rinse the tubes with DI water and place them on the shelving units below the kitchen

Note - we should add how to deal with the bacterial carboy to this protocol.

6.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

7 Cleaning Loading Boats

7.1 Introduction

- **Purpose**: This procedure describes how to clean loading boats.
- **Procedure Type**: General Lab Maintanence
- **Species**:
- **Author**: Mark Currey
- **Date Created**: March 8, 2024
- **Date Updated**: xxx

> ⚠ NOTES
>
> xxxx

7.2 Materials

- Aluminum Foil
- Autoclave Tape

7.3 Solutions

- xxx
- xxx
- xxx

7.4 Procedure

1. Rinse dirty loading boats with tap water and DI water and let dry

> **NOTE**
>
> If extremely dirty, you may rinse the loading boats in a 10% bleach solution

2. Cover the large (~2 in x 5 in) boat with aluminum foil and use the autoclave tape to attach the foil to the loading boat.
3. Autoclave foil-wrapped loading boats using the 25-minute Gravity program

7.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

8 Changing Spill Paper

8.1 Introduction

- **Purpose**: This procedure describes how to change spill paper in common areas.
- **Procedure Type**: General Lab Maintanence
- **Species**:
- **Author**: Mark Currey
- **Date Created**: March 8, 2024
- **Date Updated**: xxx

> ⚠ NOTES
>
> xxxx

8.2 Materials

- Tape
- Spill Paper
- Scissors

8.3 Solutions

- xxx
- xxx
- xxx

8.4 Procedure

1. Remove old spill paper and place in trash (bring to solid waste if contaminated)
2. Wipe where the spill paper used to be if there is any remaining residue and dispose of any contaminated substances
3. Cut out appropriate-sized spill paper and set it flat on surface with the plastic surface of the spill paper facing down
4. Tape the edges of the spill paper to the surface it lies on (try to keep the spill paper flat)

8.5 Associated Papers

- xxx
- xxx
- xxx
- xxx

9 Refilling Nanopure Water

9.1 Introduction

- **Purpose**: This procedure describes how to refill the 20L Nalgene jug with nanopure water.
- **Procedure Type**: General Lab
- **Author**: Micah Woods
- **Date Created**: March 11, 2024

9.2 Materials

- 20L Nalgene Jug
- Barnstead NANOpure infinity machine

9.3 Solutions

- xxx
- xxx
- xxx

9.4 Procedure

1. Take 20L Carboy to Pac 314.
2. Using the Barnstead NANOpure infinity machine, turn the top dial to "On."
3. Press "Standby" and then press "Start/Stop" button.
4. Ensure that the water is continually running into the Nalgene, and wait approximately 20 minutes for it to fill with water.
5. When Nalgene is full, press "Start/Stop" button.
6. Push "Standby" button."

7. Turn top dial to “Off.”

9.5 Associated Papers

- xxx
- xxx
- xxx

10 Refilling Pipette Tips

10.1 Introduction

- **Purpose**: This procedure describes how to refill pipette tips.
- **Procedure Type**: General Lab
- **Author**: xxx
- **Date Created**: xxx

10.2 Materials

- Used pipette tip boxes
- Autoclave
- Autoclave tape
- Pipette tip refills

10.3 Solutions

- xxx
- xxx
- xxx

10.4 Procedure

1. Remove all tape from used pipette tip box and recycle inner cartridge (200 µL will only have a thin removable portion).
2. Refill non-filtered tip boxes prior to autoclave, for directions see following steps, and tape down lids with autoclave tape (only tape the front side for 20 µL and 1000 µL boxes, but tape front and back side of the 200 µL boxes.)
3. Take all used boxes to the autoclave in room PAC 306D and run Gravity for 25 minutes (for use, 1=OK and 4=back).

> **!** IMPORTANT
>
> DO NOT place filter tips in the autoclave.

4. Let the boxes cool until there is no visible condensation, then replace tips.
5. Replace tips

    - Replacing 20 µL and 1000 µL tips:

        1. Place blackened autoclave tape on top of lid and label “filter”, then remove lid.
        2. Remove paper bottom of new tips and place on top of clean tip box.
        3. Slightly push inward on the new tips (to make sure tabs fit inside filter tip box) and push down on one side of tips.
        4. Once this side is in, repeat this for the other side.
        5. Remove plastic covering and place the lid back on the box.

    - Replacing 200 µL tips:

        1. Place blackened autoclave tape on top of lid and label “filter”, then remove lid.
        2. Remove paper bottom of new tips and place on top of clean tip box.
        3. Push down.
        4. Remove plastic covering and place the lid back on the box.

10.5 Associated Papers

- xxx
- xxx
- xxx

Part II

Molecular Protocols

This section of the book contains common protocols used for molecular biology and genomics in the laboratory. These include standard protocols such as setting up creating reagents, setting up PCRs and running gels, as well as advanced protocols such as creating constructs.

11 PCR with Phusion

11.1 Introduction

- **Purpose**: This procedure describes how to set up a basic PCR with Phusion polymerase
- **Procedure Type**: Molecular
- **Species**: N/A

The following guidelines are provided to ensure successful PCR using Phusion® DNA Polymerase. These guidelines cover routine PCR. Amplification of templates with high GC content, high secondary structure, low template concentrations or long amplicons may require further optimization.

We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed and centrifuged prior to use. It is important to add Phusion DNA Polymerase last in order to prevent any primer degradation caused by the $3' \rightarrow 5'$ exonuclease activity.

Phusion DNA Polymerase may be diluted in 1X HF or GC Buffer just prior to use in order to reduce pipetting errors. Please note that protocols with Phusion DNA Polymerase may differ from protocols with other standard polymerases. As such, conditions recommended below should be used for optimal performance.

11.2 Materials:

- PCR tubes, plates or strip tubes (1 per reaction & 1 for positive control)
- Forward and reverse primers
- npH2O
- Phusion DNA polymerase
- 5X Phusion HF or GC Buffer
- 10 mM dNTPs
- DMSo (optional)

11.3 Solutions:

NONE

11.4 Procedure:

Prepare PCR reactions

Table 11.1: Table 1. Mixtures for PCR Reaction

	Component	20ul Reaction	50ul Reaction	Final Concentration
1	npH20	to 20 ul	to 50 ul	
2	5x Phusion Buffer	4 ul	10 ul	1X
3	10mM dNTPs	0.4ul	1.0 ul	200 uM
4	10 uM forward Primer	1.0 ul	2.5 ul	0.5 uM
5	10 uM reverse Primer	1.0 ul	2.5 ul	0.5 uM
6	Template DNA	variable	variable	<250 ng
7	DMSO (optional)	(0.6 ul)	(1.5 ul)	3%
8	Phusion polymerase	0.2 ul	0.5 ul	0.4 units/ 20 ul rxn

Mix together reagents in a 1500 ul tube for the number of reactions needed, but add an extra reaction for pipeting error and one for a negative control. For example, if a PCR will be run on 23 samples and one control, mix enough for 25 reactions.

⚠ Keep reactions cold

- Set up PCR reactions on ice
- Gently mix the reaction
- Collect all liquid to the bottom of the tube by a quick spin if necessary
- Overlay the sample with mineral oil if using a PCR machine without a heated lid

PCR Conditions

Table 11.2: Table 2. Thermocycler regime

	Step	Temperature	Duration	Number cycles
1	Initial Denaturation	98 C	30 seconds	1 cycle
2		98 C	30 seconds	
3	Amplification	Primer Anneal Temp	30 seconds	25-30 cycles
4		72 C	15-30 seconds/kb	
5	Final Extension	72 C	5 minutes	1 cycle
6	Hold	10 C	indefinite	1 cycle
7				
8				

Anneal temperature and length of amplification step

- The annealing temperature and length of time for amplification will depend upon the primers and the length of the fragment to be amplified.
- 15 seconds/kb works for most reactions. 30 seconds/kb can be used for more complex reactions.

12 cDNA basic

12.1 Introduction

- **Purpose**: This procedure describes how to synthesis cDNA for use with PCR.
- **Procedure Type**: Molecular
- **Species**: N/A

12.2 Materials:

- 2 µl Oligo d(T)23 VN (50 µM, NEB; anchored-dT primer)*
- X µl up to 5 µg total RNA
- 1 µl 10 mM dNTP
- water
- 2 µl 10x RT buffer (Invitrogen)
- 4 µl 25 mM MgCl2
- 2 µl 0.1 mM DTT – Invitrogen
- 1 µl RNase inhibitor – e.g., RNAseOUT (Invitrogen)
- 1 µl Superscript III reverse transcriptase (200 u/µl – Invitrogen)

12.3 Solutions:

NONE

12.4 Procedure:

First strand synthesis

Combine:

- 2 µl Oligo d(T)23 VN (50 µM, NEB; anchored-dT primer)*
- X µl up to 5 µg total RNA

- 1 µl 10 mM dNTP mix
- Water (if necessary) to bring total to 10 µl

Heat to 65°C for 5 min., then ice

Collect contents at bottom of tube by brief centrifugation.

Add:

- 2 µl 10x RT buffer (Invitrogen)
- 4 µl 25 mM MgCl2
- 2 µl 0.1 mM DTT – Invitrogen
- 1 µl RNase inhibitor – e.g., RNAseOUT (Invitrogen)
- 1 µl Superscript III reverse transcriptase (200 u/µl – Invitrogen)

Mix by gentle aspiration

- 25°C for 5 min.

Reaction can be scaled up to accommodate more starting RNA

Synthesis: Incubate at 50°C for 50 min.

Inactivation: 85°C for 5 min. Chill on ice, collect contents to bottom by short spin.

Destroy RNA template: 1 µl RNase H (2 u/µl), incubate at 37°C for 20 min.

Proceed to PCR. Depending on expression level, may be able to use a dilution of cDNA as template – try 1:50 dilution in EB, use 2 µl as template in a 20 µl reaction. Don't dilute your entire amount of cDNA, as some products may require a higher concentration of template.

13 cDNA Synthesis for PCR

13.1 Introduction

- **Purpose**: This procedure describes how to synthesize cDNA for use with PCR.
- **Procedure Type**: Molecular
- **Author**: Susie Bassham
- **Date Created**: July 2011

13.2 Materials

- xxx
- xxx
- xxx

13.3 Solutions

- 2 µl Oligo d(T)23 VN (50 µM, NEB; anchored-dT primer)*
- X µl up to 5 µg total RNA
- 1 µl 10 mM dNTP
- water
- 2 µl 10x RT buffer (Invitrogen)
- 4 µl 25 mM MgCl2
- 2 µl 0.1 mM DTT – Invitrogen
- 1 µl RNase inhibitor – e.g., RNAseOUT (Invitrogen)
- 1 µl Superscript III reverse transcriptase (200 u/µl – Invitrogen)

13.4 Procedure

1. **First strand synthesis**

    1. Combine:

        1. 2 µl Oligo d(T)23 VN (50 µM, NEB; anchored-dT primer)*
        2. X µl up to 5 µg total RNA
        3. 1 µl 10 mM dNTP mix
        4. Water (if necessary) to bring total to 10 µl

        > **!** NOTE
        >
        > *Recommend using Oligo d(T)23 VN if starting with total RNA to bias cDNA to messenger. Random Primer Mix will do a better job of covering 5' ends if starting with mRNA. If the RNA is degraded or if very long sequences are required, may instead use 2 µl Random Primer Mix (60 µM, NEB; a mix of ran-dom hexamers, anchored-dT primers, and 1 mM dNTPs)

2. Heat to 65 ˚ C for 5 min., then ice
3. Collect contents at bottom of tube by brief centrifugation.
4. Add:

    1. 2 µl 10x RT buffer (Invitrogen)
    2. 4 µl 25 mM MgCl2
    3. 2 µl 0.1 mM DTT – Invitrogen
    4. 1 µl RNase inhibitor – e.g., RNAseOUT (Invitrogen)
    5. 1 µl Superscript III reverse transcriptase (200 u/µl – Invitrogen)

    > **!** NOTE
    >
    > Reaction can be scaled up to accommodate more starting RNA

5. Mix by gentle aspiration
6. 25 ˚ C for 5 min.
7. *Synthesis*: Incubate at 50 ˚ C for 50 min.
8. *Inactivation*: 85 ˚ C for 5 min. Chill on ice, collect contents to bottom by short spin.
9. *Destroy RNA template*: 1 µl RNase H (2 u/µl), incubate at 37 ˚ C for 20 min.

10. Proceed to PCR. Depending on expression level, may be able to use a dilution of cDNA as template – try 1:50 dilution in EB, use 2 µl as template in a 20 µl reaction. Don't dilute your entire amount of cDNA, as some products may require a higher concentration of template.

13.5 Associated Papers

- xxx
- xxx
- xxx

14 Double Stranded cDNA Synthesis

14.1 Introduction

- **Purpose**: This procedure describes how to synthesize double stranded cDNA.
- **Procedure Type**: Molecular
- **Author**: Susie Bassham
- **Date Created**: July, 2011

14.2 Materials

- Thermomixer or incubator
- Ice
- Centrifuge
- Zymo DNA column

14.3 Solutions

- Random Primer Mix
- 10 mM dNTP mix
- mRNA
- Water
- 5x First-Strand Buffer (Invitrogen)
- 0.1 mM DTT (Invitrogen)
- 1 µl RNase inhibitor – e.g., RNAseOUT (Invitrogen)
- 1 µl Superscript III reverse transcriptase (200 u/µl – Invitrogen)
- 15 µl 10x Second-Strand Synthesis Reaction Buffer (B6117S, NEB)
- 1 µl E. coli ligase (10 u/µl)
- 4 µl E. coli DNA Polymerase I (10 u/µl)
- 0.4 µl E. coli RNase H (5 u/µl)
- T4 DNA polymerase
- 0.5 M EDTA

14.4 Procedure

1. **First strand synthesis**
    1. Combine:
        1. 2 µl Random Primer Mix (60 µM, NEB; a mix of random hexamers, anchored-dT primers, and 1 mM dNTPs)*
        2. 0.8 µl 10 mM dNTP mix
        3. X µl up to 0.5 µg mRNA
        4. Water (if necessary) to bring total to 12 µl

        > **!** NOTE
        >
        > *Random Primer Mix should do a better job of covering 5' and 3' ends than Oligo d(T) or random hexamer primers alone.

    2. Heat to 65 ˚C for 5 min., then ice
    3. Collect contents at bottom of tube by brief centrifugation.
    4. Add:
        1. 4 µl 5x First-Strand Buffer (Invitrogen)
        2. 1 µl 0.1 mM DTT (Invitrogen)
        3. 1 µl RNase inhibitor – e.g., RNAseOUT (Invitrogen)
        4. 1 µl Superscript III reverse transcriptase (200 u/µl – Invitrogen)

        > **!** NOTE
        >
        > Reaction can be scaled up to accommodate more starting RNA

    5. Mix by gentle aspiration
    6. 25 ˚C for 10 min.
    7. *Synthesis*: Incubate at 50 ˚C for 50 min.
    8. *Inactivation*: 85 ˚C for 5 min. Chill on ice, collect contents to bottom by short spin.
2. **Second strand synthesis**:
    1. On ice, add:
        1. 106.6 µl water
        2. 15 µl 10x Second-Strand Synthesis Reaction Buffer (B6117S, NEB)
        3. 3 µl 10 mM dNTPs

4. 1 µl E. coli ligase (10 u/µl)
5. 4 µl E. coli DNA Polymerase I (10 u/µl)
6. 0.4 µl E. coli RNase H (5 u/µl)

2. Mix by aspiration on ice.

3. *Synthesis*: incubate at 16 ˚C for 2 hours (do not allow to warm above 16 ˚C).

3. Optional (for cloning cDNAs, have to blunt ends):

1. Add 3.3 µl (10 u) T4 DNA polymerase (3 u/µl, NEB). Incubate 5 min. at 16 ˚C.]
2. Add 10 µl 0.5 M EDTA to stop reaction.

4. Purify with Zymo DNA column, e.g.:

1. Elute two times with 50 µl EB and proceed to shearing and adaptor ligation for EST building or expression profiling via Illumina sequencing, OR,
2. Elute two times with 20 µl EB and proceed to restriction digestion for eRAD.

14.5 Associated Papers

- xxx
- xxx
- xxx

15 2x Turbo

15.1 Introduction

- **Purpose**: This procedure describes how to create 2x Turbo PCR mix.
- **Procedure Type**: Molecular
- **Species**: N/A

15.2 Materials:

- 33,000 µl npH2O
- 2000 µl MgSO4 (100mM)
- 1600 µl 1M Tris-HCl (pH 8.6)
- 800 µl 1M KCl
- 800 µl 1M (NH4)2SO4
- 800 µl Triton-X 100 (10%)
- 400 µl DMSO (100 %)
- 120 µl dATP (100mM)
- 120 µl dGTP (100mM)
- 120 µl dTTP (100mM)
- 120 µl dCTP (100mM)
- 80 µl 100mg/ml BSA

Total = 40 ml of buffer

15.3 Solutions:

NONE

15.4 Procedure:

- Mix above reagents together
- Place in 1.5 ml ependorph tubes
- Store at -20C

16 Paraformaldehyde

16.1 Introduction

- **Purpose**: This procedure describes how to make 8% paraformaldehyde. This protocol is the one I have used and makes use of pH to get the PFA into solution relatively quickly - then you readjust the pH. It's for 8% - then you can add 1:1 2x PBS.
- **Procedure Type**: Molecular
- **Species**: N/A

16.2 Materials:

- xxx

Total = 40 ml of buffer

16.3 Solutions:

NONE

16.4 Procedure:

> ⚠ HUMAN HEALTH WARNGING
>
> Paraformaldehyde can be hazardous to your health - make sure you prepare in the fume hood.

- Add 40 g Paraformaldehyde to 450 ml distilled water (or scale for desired final volume).
- Add 1 ul of 10 N NaOH per ml of water (i.e. 500 ul for 500 ml).
- Apply medium heat while stirring at medium speed to dissolve - approx 15-20 min.
- Solution should not go above 60º C.

- Eventually, granules will fully dissolve and the solution will become translucent.

> **DO NOT LET THE SOLUTION STIR BEYOND THIS POINT**
>
> It will form a fuzzy precipitate that reduces the solution strength after filtering.

- Once the granules have dissolved and the solution clears, turn off the heat and equilibrate to pH 7.4 with approx 1.5 ml of 20% HCl (or scale, depending on target volume).
- Bring volume to 500 ml (or scaled volume) with distilled water.
- Filter while still warm to 0.45 um (or 0.2 um). Aliquot and store at -20^{o} C.

17 Alizarin Staining

17.1 Introduction

- **Purpose**: Alizarin staining of fixed adult fish.
- **Procedure Type**: Molecular
- **Species**:
  - Threespine stickleback, *Gasterosteus aculeatus*
  - Gulf pipefish (*Syngnathus scovelli*)
  - and others
- **Author**:
- **Date Created**:

17.2 Materials:

- Alizarin red S
- KOH
- 6% or 30% H_2O_2
- NaOH
- containers for solutions
- shaker

17.3 Solutions:

- **0.5% Alizarin red S Stock**:
  - To make 50 mls add 0.25g alizarin red S powder to 50 ml water.
- **0.025% Alizarin Stain**
  - To make 100 mls: Add 500µl 0.5% alizarin red S (stock) to 99.5ml 1% KOH
  - 1 Liter: Add 5ml 0.5% alizarin red S (stock) to 9950ml (1 liter) 1%KOH

- **3% H202/0.5%KOH**: Mix and keep at 4C; Before using, bring to room temperature to hold down introducing bubbles under the skin: 0.5ml 6%H202 & 0.5ml 1%KOH.
- **Euthanasia strength Mesab** {#sec-husb-adutl_sb_euthanasia}
- **8% PFA**: {#sec-molec-para}
- **2X PBS**
  - 1.6% NaCl
  - 0.04% KCl
  - 0.04 M PO4 pH 7.0- 7.3
- **Isopropanol**

> ⚠ Chemical Warning
>
> Please use appropriate PPE when handling chemicals.

17.4 Procedure:

1. Fix in 4% PFA 2h to overnight depending on the size of the fish.

> ℹ Note
>
> We've found that adult fish should be fixed overnight.

2. Wash with 1% KoH 1 hour or longe on shaker.

> ℹ Note
>
> If found that a longer KoH wash results in better staining.

3. Bleach with 3% H202 : 1% KoH solution for 1-4 hours until the eyes and skin lose most of the pigment.

> ℹ Warning
>
> This step produces a gas. Do not put lids on containers while bleaching.

4. Wash with 1% KoH 2 hour on shaker.
5. Stain with 0.025% Alizarin Red solution for 2 h or longer.

::: {.callout-note title="Note"} Check for the strenght of staining often. I prefer to slightly over stain as slight non-specific (muscles and etc) will clear in the following steps. ::

6. Wash with 1% KoH 2 hour or longer on shaker depending on clearing of non-specific tissues.
7. Store in 50% Isopropanol.

17.5 Papers:

- xxx
- xxx
- xxx

18 Pouring and Running a Gel

18.1 Introduction

- **Purpose**: This procedure describes how to pour and run a gel to visualize DNA.
- **Procedure Type**: Molecular
- **Author**: Micah Woods
- **Date Created**: February 14, 2024

18.2 Background

Gel electrophoresis is a technique used to separate mixtures of DNA, RNA, or proteins based on their molecular size. During gel electrophoresis, an electrical field is used to help molecules travel through the pores of an agarose gel matrix. At one end of the gel, furthest away from where the samples are loaded, is a positive charge, and at the other end is a negative charge. Since DNA and RNA are both negatively charged molecules, they are pulled through the gel matrix towards the positive charge. Since proteins do not have a negative charge, researchers must first mix them with a sodium dodecyl sulfate detergent which gives them the negative charge needed to migrate towards the positive end of the gel. The speed at which molecules travel through the gel is inversely related to their molecular size. For example, small DNA molecules have a low molecular weight, enabling them to travel further through the gel matrix than a larger DNA molecule. When a gel is stained with DNA/RNA/protein-binding dye, these molecules can be seen as bands, whose location along the length of the gel represents the molecule?s size in terms of number of base pairs. When dye-stained DNA/RNA/protein is run beside a ladder with known fragment size, one can estimate the size of the assayed molecule. After the DNA/RNA/protein molecules have been separated using gel electrophoresis, the gel is imaged under UV light, visualizing the DNA/RNA/proteins present across the length of the gel.

18.3 Materials

- 500 ml Corning glass bottle labeled “Agarose Gels”
- Scale

- Weigh paper
- Microwave
- Gel comb
- Gel tray
- Pipets
- Pipet tips
- Parafilm or PCR strip tubes for mixing DNA samples and Loading Dye
- Gel box with enough buffer inside to cover gel
- Electrodes attached to power supply (Bio-Rad PowerPac)
- Gel transferring tray
- Device for visualizing gel
  - Azure Biosystems c200, located in Pacific 314
- Gel image printer
- Kimwipes

18.4 Solutions

- 0.5X TBE
- Safe View
- Molecular Biology Grade Agarose
- 6X Loading Dye
- Ladder of choice
- EB Buffer

18.5 Procedure

> **!** This is important
>
> Before beginning this procedure, read the section "Electrophoresis Tips" at the bottom of this procedure for more background information and to understand and anticipate the mistakes commonly associated with this protocol.

1. Weigh desired amount of Molecular Biology Grade Agarose - scale, weigh paper, and Agarose located on the common use bench on the north end of the laboratory - and pour it into the 500 ml Corning glass bottle labeled "Agarose Gels" (located on top of or inside the microwave on the south end of the laboratory).
   a. 1-1.2% Agarose (1 g-1.2 g) is a good starting amount of Agarose for DNA samples, though the amount of Agarose can vary depending on your particular samples and

goals. The concentration of Agarose in a gel depends on the sizes of DNA fragments to be separated, with most gels ranging between 0.5%-2% Agarose.

2. Fill bottle with 0.5X TBE buffer, located in the 20-liter Nalgene located at the south sink. The volume of TBE buffer used will be based on the size of the gel mold. For example, for a 14"x10" gel tray, use 100 ml of 0.5X TBE buffer. 3. Put bottle in microwave and microwave for one minute.
3. Using the heat mittens located on top of the microwave, remove the bottle from the microwave, swirl the mixture around, and put it back in the microwave for 30 seconds. By the end of this step, the Agarose should be melted into the TBE and there should be no remaining solid Agarose particles.
4. Using the heat mittens, remove the bottle from the microwave and carry it to the fume hood.
5. Select a gel tray from the drawer under the gel boxes. Put tape around the two longest sides of the gel tray, pressing tape on firmly so it is air-tight with the tray. The distance between the top of the tape and the bottom of the tray should be at least half-an-inch tall so that it can hold all of the liquid from the Agarose Gel bottle without it spilling over the sides or leaking through any air gaps in the tape.
6. Select a gel comb from the drawer under the gel boxes. Different combs make different sized wells that can hold different volumes, so select a comb based on your specific experiment. Place the gel comb in the notches of the gel tray. The combs should not touch the bottom of the tray but be slightly above it.
7. Let the agarose solution cool for a few minutes. Using the heat mittens, swirl the Agarose Gel bottle again. Add 5 ul of Safe View (located in the Styrofoam box in the fume hood) to the Agarose Gel bottle. Gently swirl the bottle to mix in the Safe View; try to avoid creating bubbles while swirling.
8. Slowly pour the Agarose Gel mixture into the gel tray with gel comb. Be careful not to create bubbles in the gel mixture while pouring. If there are bubbles, pop or move them to the bottom of the tray, away from the comb. Slowly lift the comb up and down a few times to ensure there are no bubbles around the comb.
9. Let the gel mixture sit in the tray under the hood until it solidifies (usually takes around 20 minutes).
10. While you are waiting for the gel to solidify, clean the Agarose Gel bottle. Fill the bottle halfway with water, swirl the water, microwave for 30 seconds, swirl again and microwave for another 30 seconds. Pour out the water and set the bottle out to dry next to the microwave.
11. Once the agarose mixture has solidified, carefully remove the tape from the sides of the tray.

> **!** This is important
>
> Tape should be removed from the tray while held under the fume hood. Tape should be removed slowly to prevent any splattering of residual liquid on the top of the solidified gel.

12. Carefully remove the comb from the gel matrix by slowly pulling it straight up.
13. Carry the gel in the gel plate to the gel boxes, remove gel box lid, and slowly lower the gel and plate into gel boxes.
14. Load ladder of choice into the left-most well of the gel. The volume of ladder you load will vary depending on your experiment.

> **!** This is important
>
> When expelling the ladder into the well, only pipet to the first stop. Pipetting to the second stop risks expelling bubbles into the well which could interfere with the experiment.

> **!** NOTES
>
> *The ladder is made of DNA and needs a gel loading dye. When selecting a ladder to use, make sure that the ladder is not clear but has been mixed with a loading dye (should be a bright color like blue, orange, green, or purple).

15. Prepare DNA samples to be loaded into the wells of the gel. Pipet out 10 ul of DNA from each sample onto a piece of parafilm or into a PCR strip tube, using a fresh pipet tip for each sample to avoid cross contamination.

> **!** NOTES
>
> *The volume of DNA you mix load into each well depends on the concentration of DNA and how much of DNA of your total you can afford to use for the gel, though a generally good concentration range is 10-100 ng/ul to ensure the DNA can be visualized on the gel. If you are aiming to load a specific concentration of DNA into each well, calculate the concentration of your DNA sample and find that the corresponding volume is less than 10 ul, you can add the appropriate volume of EB Buffer to bring the sample's volume up to 10 ul.

16. Add 2 ul of 6X Loading Dye to each DNA sample. Thoroughly mix the dye and DNA sample by aspiration, but mix slowly to avoid creating bubbles. Be sure to use a fresh pipet tip in between DNA samples.

> **!** NOTES
>
> *The exact volume of loading dye you add to each sample can change depending on the amount of DNA you add, but the final dilution should be 1 part 6X Loading Dye to 5 parts DNA sample.

17. Load 10 ul of the DNA sample/dye mixture into the next available well in the gel.

> **!** This is important
>
> *When expelling the DNA sample/dye mixture into the well, only pipet to the first stop. Pipetting to the second stop risks expelling bubbles into the well which could interfere with the experiment.

18. Put gel box cover on. Attach the electrodes to the metal posts at the top and bottom of gel box; the black electrode (-) attaches to the metal post on the top of the box and the red electrode (+) attaches to the metal post on the bottom of the box.

> **!** NOTES
>
> Since DNA is a negatively charged molecule, it will run through the gel matrix towards a positive charge. A helpful way to remember electrode placement, then, is "Run to red," since the red electrode has a positive charge and should be placed furthest away from the DNA samples.

19. Turn on the Bio-Rad PowerPac and set it to your desired voltage. Press "Run." You can check to see if an electric current is running through the gel box by looking for a stream of small bubbles rising up the back of the gel box.

20. Run samples for desired time.

21. Press the "Stop" button on the Bio-Rad PowerPac and turn off the power. Remove the electrodes from either end of the gel box, loosely wind them up and place them above or next to the Bio-Rad PowerPac.

22. Remove the lid from the gel box. Carefully lift the gel tray out of the gel box, ensuring that the gel does not slide off the tray. Place the gel tray and gel onto the gel transferring tray (located behind the faucet on the south sink). Carry the gel to the gel imaging station (Azure Biosystems c200) in the Phillips Microfluidics Laboratory (Pacific 314).

23. To image the gel,

    a. Open the door of the c200 Azure Biosystems gel imaging station, and carefully slide the gel inside. The long side of the gel should be facing the opening of the imaging station. Position the gel so that it is centered inside the imaging platform, and the

short sides of the gel are parallel to the sides of the imaging platform. Close the door.
   b. Select the "cSeries Capture Software" on the screen.
   c. Select the "GEL" option in the top left corner, to the right of the "GALLERY" option.
   d. Select the "BRIGHT BANDS" option in the bottom right corner.
   e. Select "CAPTURE" to take an image of the gel.
   f. Once your image has been captured, you may rotate, flip, crop, or zoom in/out of the image.
   g. To print the image, select "PRINT" and choose the number of copies you would like to print.

24. Open the door of the c200 Azure Biosystems gel imaging station and carefully remove the gel. Wipe down the gel platform using a Kimwipe and close the door.

25. Carry your gel and printed images back to the lab. You may either dispose of your gel in the trash or store the gel in a plastic bag in the refrigerator for future use.

26. Rise off the gel transferring tray and set it to dry behind the south sink faucets. Rinse off the gel tray and the gel comb, ensuring that there is no remaining agarose between the tines of the gel comb. Set the comb and the tray to dry on the drying rack hanging above the south sink.

18.6 Electrophoresis Tips

Background

The effect of electrophoresis is to separate DNA fragments by size in an agarose matrix and buffer using an electrical current. DNA is negatively charged and will migrate toward the positive pole. Many parameters can affect how the DNA moves through the gel: buffer composition, voltage, length of the gel, percentage of agarose in the gel (in other words, the density of the gel matrix), presence of salt in the DNA, protein bound to DNA, and other factors. Some of these conditions also affect which size ranges of DNA will effectively be "resolved" (i.e., separated enough from one another so you can see them as distinct fragment sizes). "Safeview" is a dye we use in the gel that fluoresces under UV or blue light when bound to DNA, allowing us to see and photograph it when viewed through a special orange filter to cut out the background light. The research goals of using electrophoresis might include:

- measuring the size of fragments in a DNA sample such as the products of PCR or assessing the intactness of the purified DNA from a tissue extraction
- separating fragments from one another so that a particular amplicon or size range of DNA can be purified out of the gel for other downstream applications (such as for cloning a

PCR fragment for making probes or for Sanger sequencing, or for size selecting a smear of fragments for making a RAD library) while excluding the other fragment sizes

- checking for the presence or absence of a particular product of PCR as in a screen for orientation of a cloned fragment in a plasmid present in different bacterial colonies, a screen for an insertion in a transgenic animal, a screen to determine the genetic sex or the mitotype of a fish.

Common Mistakes

- Boiling over the agarose when making a gel. Result: failure to monitor the agarose when you are trying to dissolve it in the microwave can easily cause it to boil over because powdered substances create a lot of "nodes of nucleation" for bubbles to form. This is especially easy to accidentally do with higher percentages of agarose. It wastes expensive agarose and creates a mess in the microwave. What remains in the bottle will be of an indeterminate percentage/gel stiffness.
- Not making sure the tape is adhering well to the mold before casting a gel. Result: liquid agarose will leak out causing a mess and reagent waste. Make sure the mold is dry beforehand, and use the back edge of a comb, e.g., to run across the tape and make sure it has firm contact.
- Positioning the comb teeth too close to one side or to the bottom of the mold when setting up for pouring a gel. Result: the bottoms (or that side) of wells may be torn when the comb is pulled out, causing the loaded samples to leak out into the buffer during gel loading.

  > **!** NOTE
  >
  > Even when the comb teeth are not too close to the bottom, low percentage gels are soft and will benefit from having the comb pulled while submerged in the tank - otherwise the suction created by surface tension between the comb plastic and the agarose as you pull up on the comb can tear the bottoms out of the wells, causing loss of samples during loading.

- Not using the right % of agarose for your DNA size range of interest. Result: bands you care about won't resolve optimally (i.e., you might not be able to accurately measure the sizes of your bands and might not be able to tell if something is one band or multiple bands). Pour a higher percentage gel to resolve large fragments (e.g. less than 1%) or a lower percentage gel (i.e. between 1 and 4%) to resolve small to very small ones, depending on your needs/expectations for what size bands you will see, how many of them, or how important it is to accurately estimate their sizes.
- Not homogeneously mixing the Safeview into the agarose before pouring. Result: DNA might not be visible in all parts of the gel.

- Over-cooling the agarose before pouring. Result: Safeview will not be evenly distributed in the gel, and/or the gel will have lumps and not be of uniform thickness because some of the agarose will have already started polymerizing.
- Running a gel with a lot of bubbles in it. Result: DNA migration can be impeded/distorted by bubbles in the gel. Usually as a gel is cooling, bubbles will migrate to the top where (usually) they will be out of the path of the DNA unless the DNA volume fills the wells to the top. But bubbles can sometimes occur deeper in the gel – particularly in a very high % agarose gel. Try to nudge bubbles out of the way before the gel solidifies, either by raking them with a comb that is not on a holder or nudging individual bubbles with a pipette tip before the gel congeals. If the gel is already solid, avoid loading in lanes that will run across a bubble.
- Running a gel that wasn't mixed homogeneously before pouring in the mold. Result: there will be lumps in the gel of more dense agarose that will cause distortion of how the DNA migrates.
- Accidentally contacting the agarose with a pipette tip. Result: the bottom or side of the well can become perforated, causing the sample to leak out the bottom or into the next well. If the tip is pressed against the agarose, the sample can be forcefully and suddenly expelled and blast out of the well.
- Overloading the wells with too much sample volume. Result: samples can become cross-contaminated by DNA from adjacent wells.
- Overloading the lane with too much DNA. Result: DNA can become retarded during running and the apparent size will not be accurately gauged by the ladder.
- Forgetting to load a DNA ladder. Result: you won't know if your DNA bands are the right size when you look at and photograph your gel.
- Not adjusting the amount of ladder for the comb width. Result: if you use a wider comb, you need to increase the amount of ladder or it will appear dimmer (because ladder DNA is more dilute across the span of the lane).
- Not using gel loading mix in the DNA or using ladder that isn't premixed with gel loading mix. The DNA or ladder (which is also DNA) will be lost mostly during loading without the gel loading mix that makes it sink to the bottom of the wells in the gel and stay there while you are loading. Both your samples and the ladder are DNA in an aqueous solution that is about the same density as the buffer (usually) – therefore they both should have gel loading mix in them in order to be denser than the tank buffer. The gel loading mix also includes convenient, charged dyes that migrate at different rates to help you see that your gel is running and how far it has progressed. The gel loading mix types only really matter if one type happens to have a dye that migrates exactly like your band of interest - in that case, you may want to switch to a different mix so that the dye doesn't block the fluorescence of your band during visualization.

- Not thoroughly mixing gel loading mix into your DNA before loading. Result: you could lose part of your DNA to floating out of the well during loading. (This can also happen if there are other reasons your DNA is not very dense, such as if there is residual ethanol in it from preceding processing. In that case, you will see your DNA rapidly floating up to the surface as soon as you start expelling it from the tip).
- Adding premixed DNA ladder to your samples instead of gel loading mix. Result: ladder will appear in every lane, obscuring your bands and wasting expensive ladder.
- Forgetting to start the current on your loaded gel. Result: DNA will diffuse both out of the wells and into the gel in all directions causing loss of some of the DNA and blurriness of the remaining DNA once the gel is run. Diffusion can also happen if it takes a long time to load the gel. If there are a great number of samples to load – such as in a two- or three-comb gel, it is often best to load one tier and run the gel for 5 minutes so the DNA enters the gel and diffuses more slowly before moving on to load the next tier. Diffusion can happen more quickly if the buffer is warm from a previous run. If the buffer is warm, replace it before trying to load another gel in the same box.
- Not keeping track of the order of sample loading. Result: you won't know which lane corresponds to which sample. This can happen, for example, when loading samples from a strip of PCR tubes, where their order can accidentally be rotated 180 degrees, or when loading from a PCR plate, where the plate orientation can be rotated 180 or 90 degrees (e.g., wells are loaded with respect to rows versus columns).
- Running your gel backwards by accidentally reversing the positive and negative electrodes. Result, your DNA will migrate out of the end of the gel nearest the wells (the "top" of the gel) and be lost into the tank buffer. Always check that your electrodes are hooked up to the correct leads relative to the terminals at the power source, that your gel is oriented so that the DNA will run toward the positive pole. If you have already loaded your gel in the wrong orientation, do not lift the gel out of the tank, but just reverse the leads (red to black) to correct the current. Remember that DNA is negatively charged and will run to the positive (red) pole. When you start the current, the negative electrode (anode) at the well end of the gel should be making noticeably more bubbles than the positive electrode (cathode) at the bottom end of the gel. That's because electrolysis of water (H2O) will produce twice as much free hydrogen at the anode than the cathode makes free oxygen. Making a habit of checking that tells you two things: that current is really flowing and that you do have the right orientation of poles.
- Running a gel at too low a voltage. Result: low molecular weight bands may look fuzzy and faint because they will be diffusing in random directions as they migrate.
- Running DNA that is too salty - e.g., DNA in Phusion buffer or in NEB restriction buffer 3 (or 3.1) etc. Result: a "salt front" will form where DNA in those salty buffers will be slowed ("retarded") relative to the ladder, making the ladder a useless measure of the actual size of the DNA. Bands will be compressed at a "front" and will be a weird shape like a smile or a frown rather than a straight band. If you know your DNA is in a

salty buffer, you can remove this problem by cleaning the DNA first (i.e., via a cleanup column or with paramagnetic beads), or you can mitigate the problem by diluting only a few microliters (e.g. 5 µl) of your DNA into water and gel loading mix before loading (assuming you have a high enough concentration of DNA that it can still be seen if you load only a small fraction of it).

- Running a gel made up in a different buffer (or different concentration of buffer) than the buffer in the gel tank; this includes using old buffer that has been evaporating in the tank through multiple runs or over time. Result: bands will not migrate as expected. "Fronts" may form where the leading edge of the migrating DNA is compressed.
- Losing your gel off the end of the gel mold/gel sliding off the end of the gel mold when you are taking it out of the gel box. Result: the gel could shatter and be unsalvageable. Solution: transport gels in a dish and be especially careful when moving flabby, low percentage agarose gels (under 1%).

Potentially Dangerous and/or Destructive Mistakes

- Heating a bottle with a lid on it. Result: possible explosion. Just leave the lid off when heating agarose.
- Adding Safeview to molten agarose that is too hot. Result: much of the Safeview will be degraded by heat, causing your DNA to be hard to see or image. **The plastic gel mold could be permanently warped if the agarose is too hot**. The tape may fail, causing a mess in the fume hood and reagent wastage. A swirled bottle should be just comfortable (70°C or a bit less).
- Running a gel at too high a voltage. Result: **buffer could overheat during running and permanently warp the plastic of the gel box**. Bands might look smear and not be well resolved.
- Dissolving agarose in water instead of electrophoresis buffer or putting water in the electrophoresis tank instead of buffer. Result - total failure for DNA to migrate into the gel, loss of samples. **If there is only water and not buffer in the tank, it could overheat and permanently warp the plastic of the gel box.**
- Over-running your gel. Result: the DNA might run off the bottom of the gel and be lost. If you had more than one tier of wells in the gel, the DNA in the top tier will run into the zone of the next tier down, causing distortion and blurriness of the bands as they cross the wells of the lower tier and creating confusion in interpretation because very small fragments from the top tier will be overlapping very large fragments of the lower tier. If the gel runs for a long time, the **buffer could overheat and permanently warp the plastic of the gel box.**

Conscientiousness

Please really clean the gel molds and combs after use. The next person shouldn't have to fish another lab mate's combs and molds out of the sink and clean and dry them before they can pour their gel. After pulling combs from a polymerized gel, make sure to really rub them under a flow of water; a skin of polymerized agarose left on the combs (higher percentages of agarose are especially prone to this) will mean the next person has to clean this off before they can pour a gel. Likewise, make sure the gel melting bottle is rinsed immediately after you pour your gel. If residual agarose is allowed to dry in the bottle, it can create an almost insoluble blob in the gel of the next person to use the bottle. If you see that some of your agarose has polymerized in the bottle, add water to the bottle and microwave it for a few minutes to dissolve and discard the residue.

18.7 Associated Papers

- Lee PY, Costumbrado J, Hsu CY, Kim YH. Agarose gel electrophoresis for the separation of DNA fragments. J Vis Exp. 2012 Apr 20;(62):3923. doi: 10.3791/3923. PMID: 22546956; PMCID: PMC4846332.
- Gel electrophoresis. Scitable by Nature Education. https://www.nature.com/scitable/definition/gel-electrophoresis-286/#:~:text=Gel%20electrophoresis%20is%20a%20laboratory,gel%20that%20contains%2

19 Alizarin Staining

19.1 Introduction

- **Purpose**: Alizarin staining of fixed adult fish.
- **Procedure Type**: Molecular
- **Species**:
  - Threespine stickleback, *Gasterosteus aculeatus*
  - Gulf pipefish (*Syngnathus scovelli*)
  - and others
- **Author**:
- **Date Created**:

19.2 Materials:

- Alizarin red S
- KOH
- 6% or 30% H_2O_2
- NaOH
- containers for solutions
- shaker

19.3 Solutions:

- **0.5% Alizarin red S Stock**:
  - To make 50 mls add 0.25g alizarin red S powder to 50 ml water.
- **0.025% Alizarin Stain**
  - To make 100 mls: Add 500µl 0.5% alizarin red S (stock) to 99.5ml 1% KOH
  - 1 Liter: Add 5ml 0.5% alizarin red S (stock) to 9950ml (1 liter) 1%KOH

- **3% H202/0.5%KOH**: Mix and keep at 4C; Before using, bring to room temperature to hold down introducing bubbles under the skin: 0.5ml 6%H202 & 0.5ml 1%KOH.
- **Euthanasia strength Mesab** {#sec-husb-adutl_sb_euthanasia}
- **8% PFA**: {#sec-molec-para}
- **2X PBS**
  - 1.6% NaCl
  - 0.04% KCl
  - 0.04 M PO4 pH 7.0- 7.3
- **Isopropanol**

> ⚠ Chemical Warning
>
> Please use appropriate PPE when handling chemicals.

19.4 Procedure:

1. Fix in 4% PFA 2h to overnight depending on the size of the fish.

> ℹ Note
>
> We've found that adult fish should be fixed overnight.

2. Wash with 1% KoH 1 hour or longe on shaker.

> ℹ Note
>
> If found that a longer KoH wash results in better staining.

3. Bleach with 3% H202 : 1% KoH solution for 1-4 hours until the eyes and skin lose most of the pigment.

> ℹ Warning
>
> This step produces a gas. Do not put lids on containers while bleaching.

4. Wash with 1% KoH 2 hour on shaker.
5. Stain with 0.025% Alizarin Red solution for 2 h or longer.

::: {.callout-note title="Note"} Check for the strenght of staining often. I prefer to slightly over stain as slight non-specific (muscles and etc) will clear in the following steps. ::

6. Wash with 1% KoH 2 hour or longer on shaker depending on clearing of non-specific tissues.
7. Store in 50% Isopropanol.

19.5 Papers:

- xxx
- xxx
- xxx

20 cDNA for eRAD

20.1 Introduction

- **Purpose**: This procedure describes how to synthesis cDNA for use with eRAD.
- **Procedure Type**: Molecular
- **Species**:
- **Author**: xxx
- **Date Created**: xxx

> ⚠ NOTES
>
> xxxx

20.2 Materials

- 2 µl Random Primer Mix (60 µM, NEB; a mix of random hexamers, anchored-dT primers, and 1 mM dNTPs)*
- 0.8 µl 10 mM dNTP mix
- X µl up to 0.5 µg mRNA
- Water (if necessary)
- 4 µl 5x First-Strand Buffer (Invitrogen)
- 1 µl 0.1 mM DTT – Invitrogen
- 1 µl RNase inhibitor – e.g., RNAseOUT (Invitrogen)
- 1 µl Superscript III reverse transcriptase (200 u/µl – Invitrogen)
- 15 µl 10x Second-Strand Synthesis Reaction Buffer (B6117S, NEB)
- 3 µl 10 mM dNTPs
- 1 µl E. coli ligase (10 u/µl)
- 4 µl E. coli DNA Polymerase I (10 u/µl)
- 0.4 µl E. coli RNase H (5 u/µl)
- 3.3 µl (10 u) T4 DNA polymerase (3 u/µl, NEB).
- 10 µl 0.5 M EDTA
- Zymo DNA column

20.3 Procedure

First strand synthesis

Combine:

- 2 µl Random Primer Mix (60 µM, NEB; a mix of random hexamers, anchored-dT primers, and 1 mM dNTPs)*
- 0.8 µl 10 mM dNTP mix
- X µl up to 0.5 µg mRNA
- Water (if necessary) to bring total to 12 µl

Heat to 65 ˚ C for 5 min., then ice

Collect contents at bottom of tube by brief centrifugation.

Add:

- 4 µl 5x First-Strand Buffer (Invitrogen)
- 1 µl 0.1 mM DTT – Invitrogen
- 1 µl RNase inhibitor – e.g., RNAseOUT (Invitrogen)
- 1 µl Superscript III reverse transcriptase (200 u/µl – Invitrogen)

Mix by gentle aspiration

- 25°C for 10 min.

> ⚠ NOTES
>
> Reaction can be scaled up to accommodate more starting RNA

Synthesis: Incubate at 50°C for 50 min.

Inactivation: 85°C for 5 min. Chill on ice, collect contents to bottom by short spin.

21 Cleaning/concentrating DNA with magnetic beads

21.1 Introduction

- **Purpose**: This procedure describes how to concentrate DNA or to clean DNA using magnetic beads. You might use this to elute the DNA from a larger volume to a smaller one, or to remove oligo DNA such as primers, remove very short fragments of DNA, remove salts and other soluble impurities, or to change the buffer type the DNA is dissolved in (e.g., TE, AE, or EB).
- **Procedure Type**: Molecular
- **Species**:
- **Author**: Susan Bassham
- **Date Created**: November 27, 2023
- **Date Updated**: xxx

> ⚠ NOTES
>
> xxxx

21.2 Materials

- DNAse-free tubes, such as microcentrifuge tubes, PCR strip tubes, or plates
- Magnet (for 1.5 ml tubes, use Invitrogen DynaMag-Spin Magnetic Particle Concentrator; for PCR plate or strip tubes, use DynaMag-96 Slide magnet or Catalyst 96 Slotted Ring Magnet Plate)

  > ❗ This is important
  >
  > do not use magnets if you have a pacemaker. Do not use these powerful magnets near electronic devices like laptops or cell phones.

- Pipettes
- Pipette tips

21.3 Solutions

- DNA in solution
- Omega Tech paramagnetic beads (we buy through GC3F, our sequencing Core facility)
- 80% ethanol
- Elution buffer of choice for re-elution

21.4 Procedure

1. Determine the volume of beads you need to add based on starting volume of DNA.
    a. For genomic and higher molecular weight DNA, calculate 60% of the starting volume of DNA; this is the volume of beads that will be added to each tube (e.g., if the starting volume of DNA in buffer is 100 uL, then 60 uL of beads will be added to that sample in step 4). For purifying lower molecular weight DNA, you might need a higher proportion of beads. Consult the attached figure provided by UO GC3F or consult manufacturer's instructions (Omega Tech; Total Pure NGS beads).
2. Make up 80% ethanol solution.
3. Bring beads to **room temperature. Fully homogenize beads** via vortexing.
4. Add appropriate volume of homogenized room temperature beads calculated in step 1 to each DNA sample. Mix by aspiration using a pipette set at a volume at least double the volume of beads added to allow for adequate mixing.
5. Cover the plate or close the tubes and let samples incubate for 10 minutes at room temperature.
6. Put samples on magnet. Wait until mix clears and beads condense to the magnet.
7. Pull off supernatant, careful not to disturb the beads. Discard supernatant.
8. Wash the beads with **200 ul 80% ethanol two times (3 minutes/wash)**. During each wash, let the beads condense to the magnet before removing the supernatant. Dispersing the beads with each wash isn't necessary.
9. Discard the supernatant, trying to get as much as you can. You might want to first use a larger tip size and then switch to a smaller tip size to collect all of the ethanol.
10. Allow the beads to dry for 5 minutes. If you weren't able to get all the visible liquid out after the second wash, you might have to go longer. You should not see any liquid after drying but do not let the beads dry completely (if they are starting to get lighter in color around the edges, that is long enough; if the middle of the bead pellet is turning light or if the pellet is starting to crack, that is too long for high molecular weight DNA, which has a harder time going back into solution after being dried).
11. Remove plate or tubes from magnet and add desired volume of elution buffer of choice to each sample. Make sure there are no beads out of the solution. Pipet the buffer onto the beads to wash them down.
12. Incubate at room temperature for 10 minutes.