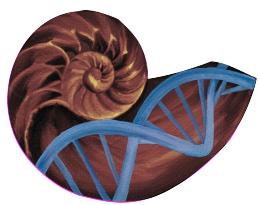


Lotterhos Laboratory Handbook for Molecular Techniques 2015



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1 Preface

This handbook is a compilation of protocols followed by the Lotterhos Laboratory members, and it is meant to transfer information among members of the Lotterhos lab. The original source of the protocols can be found below the title of each protocol. This handbook is not meant to replace any safety documentations needed to work safely in a lab.

2 Introduction to the Lab

Welcome to the Lotterhos Lab. We work with multidisciplinary protocols that require expertise in fields such as bioinformatics, molecular science and marine biology. This manual has a list of the lab rules, reagent recipes and protocols used at the lab. Be respectful of the lab and follow the University safety rules to maintain the lab a safe shared environment. For example, keep computer areas free of chemicals. People using the computers may not use the same level of protection that they would use if they were to use a bench meant for chemicals.

This handbook should not be a limitation to the techniques that you can use in this lab. To the contrary, we expect this handbook to be a basic introduction to the techniques that you could use in your research. Adding or taking steps to some parts of the protocols may make some of the protocols more adequate to your research. As the field advances, some of these protocols may also become obsolete. It would be an advantage to the lab if you update this handbook with additional protocols, notes and improved protocol versions.

Review your expectations and results with your lab mates and Dr. Lotterhos whenever possible. Also human error may occur, the cause of errors in research may come from malfunctioning machines, expired reagents, and other incidental causes. To maintain the optimal flow of a molecular lab, it is crucial that you and your lab-mates report any malfunctioning machines and follow the laboratory rules.

Your time in the lab should end with you making sure that everything is stored appropriately and that your research is documented. If you have any questions of the manual or of your role in the lab please ask Dr. Lotterhos.

If you are a new member to the lab, regardless of your experience, you have to get trained in lab safety. You should check for approval from Dr. Lotterhos to start your lab work after you have gone through the safety training of the University, gotten familiarized with the SOP for the chemicals that you are planning to use, and you have gone through the checklist of Health and Safety information.

2.1 Lab Rules

- Lab training is mandatory for all members of the lab. No work should be done in the lab without training and the approval of Dr. Lotterhos to initiate work.
- Your work in the lab should be limited to the work that you have been trained and approved to do by Dr. Lotterhos. Additional approval and training should be sought for any additional work outside the prior approved work.
- Food and drink should be limited to the approved areas (outside the lab).
- Use shoes that cover your feet (closed-toed shoes). No open-toed shoes allowed.
- Use the protective gear required for your work.
- Plan ahead and inform the lab members of any changes to a function of a bench.
- Clean your working area before and after use. If working with DNA and RNA, use 10% of bleach to clean the bench and then 70% ethanol.
- Gloves are meant to be used for a specific task and care should be taken not to spread contamination with gloves around the lab. GLOVES SHOULD NEVER BE USED TO OPEN DOORS (except the fridges).
- The main stocks are meant to be used to make aliquots for everyone's project. You should take all of the precautions to maintain the main stock in optimal conditions
- Aliquots are meant for personal use. After the use of an aliquot, the aliquot should be stored in a personal box labeled with your personal information. EVERYONE HAS THEIR OWN ALIQUOT.
- Make sure to label all of the tubes with at least the sample #, the date and your initials.
- Use sterile techniques to remove supplies from a shared area.
- If possible, use a positive and negative control in your experiment. A positive control is a group that has been previously tested to work. A negative control is a group that does not

have the variable being tested. These control groups are helpful because it helps you compartmentalize where a source of error may come from. For example, in a PCR reaction a positive control would include a DNA sample that has previously worked and the negative control would not have any DNA at all. The presence of a band in the negative control would indicate a contamination problem, and the lack of a band in the positive control could indicate a bad reagent or PCR mix problem.

- Use filter tips for RNA work, when training and when working with volumes of types of chemicals that may find a way to contaminate the pipette.
- Prevent contamination by closing containers as soon as you are done using them.
- Plan in advance and order or replenish any supply before we are out of the supply. PLEASE PUT ORDER REQUESTS ON QUARTZY.
- Plan to acquire all of the permits required to work with animals prior to your work. PLAN 6-8 MONTHS FOR PERMIT AND ANIMAL CARE APPROVAL.
- Chemicals and their waste should be discarded as mandated by their MSDS and the University rules.
- Avoid working directly with the main stock of supplies unless you are going to make aliquots. Aliquots should be made with the minimal amount of reagent needed for a few reactions. Aliquots maintain your reagents fresh and reduce contamination problems. When you make chemicals, please label with date and your initials.
- Put autoclave tape on everything that has been in the autoclave.
- Pay attention to glass thermometers - there are 3 levels of immersion (35mm to full immersion), and IT MATTERS
- ITEMS FALLEN TO THE FLOOR: The floor is contaminated with PCR product transferred on the shoes of individuals coming from the post-PCR area; therefore, anything falling to the floor must be treated as contaminated. Disposable items that have fallen to the floor, such as empty tubes, pipette tips, gloves, lab coat hangers, must be discarded.

Non-disposable items that have fallen to the floor, such as a pipette or an important sample container, must be immediately and thoroughly cleaned with a 0.5% Sodium Hypochlorite (10% Bleach) solution to remove PCR product contamination. Clean any lab surface that has come in contact with the contaminated item. Individuals handling anything that has fallen to the floor, disposable or non- disposable, must discard their lab gloves and put on a new pair.

2.2 Lab Expectations

- THAT YOU DO NOT FABRICATE DATA. Above all else, this is my most basic expectation.
- THAT YOU HAVE FUN, ARE POSITIVE, AND BE PART OF THE TEAM! Science is fun when we work as a team. When one project does not turn out as expected, I expect everyone to reflect internally on how they could have helped the team prepare better. Although it is impossible to foresee all possible outcomes of an experiment, I expect everyone to have a positive attitude and be persistent in the face of difficulty. There is always a reason when something isn't working.
- That you communicate when you do not fully understand something, or when you make a mistake. It is easier to forgive an honest mistake than a one who pretends to understand.
- That you read the literature relevant to your project, and you do not completely rely on other's expertise.
- That you pick up and clean up after yourself before you leave for the day.
- That you maintain a meticulous lab notebook AND online open notebook documenting your activities related to the lab. Starting in Fall 2014, all lab notebooks are to be kept in the lab. Your lab notebook contains original data that is necessary to preserve in the interest of repeatability in science. I prefer that you make copies if you need information in the notebook, and only check the notebook out when you need to add to it.
- That you read the Material Safety Data Sheets for all chemicals that you will be using in the lab, BEFORE you use them. I expect that you will make yourself aware of how to handle and dispose of hazardous chemicals, and what to do in the event of a chemical spill.
- That you use a pipette correctly and you do not contaminate them. This includes knowing how to use the first stop on the pipette correctly, and how to check the tip and make sure there is no air bubbles in it. That you autoclave pipettes on a regular basis.
- That you put gloves on before you touch anything that is autoclaved.

- That you put gloves on before you handle any hazardous chemicals.
- That you are familiar with the locations of eye wash, safety shower, and fire extinguisher. I also expect that you will be familiar with Environmental Health and Safety standards, and receive appropriate training when necessary.
- That you do not touch door handles or walk around the building with gloves on. If you need to carry something hazardous, please wear a glove in one hand and use the ungloved hand to open doors.
- That when you are using an expensive and sensitive piece of equipment (i.e., the CO₂ analyzer, the mass flow controllers, the pipettes, the scales, the pH meter, etc.), you make yourself aware of the cost of that equipment, and are careful not to exceed the parameter range for that equipment. For example, flow to the CO₂ analyzer cannot exceed a certain amount. Likewise, overloading a sensitive scale can cause harm.
- That when working with seawater, you understand that EVERYTHING needs to be rinsed in freshwater before you pack up for the day. This is especially true for knives, needles, pliers, forceps, and anything else that is steel (even stainless steel will start to rust). Plastic will get brittle after prolonged use in seawater.
- That with working with spawning invertebrates, you rinse your hand after you touch a male. This is important to prevent the contamination of pesky sperm to other males and females.
- Finally, I expect that you think through what you are (or the team is) going to do and be prepared before you start. The exemplary team member is not the one that simply follows directions-it is the one that has thought through every step, researched multiple avenues, and is prepared.

3 What You Should Be Keeping Track of In the Lab And Your Lab Notebook

1. Everything you do
2. How many boxes of tips you use
3. How many uL, mL, or mg of expensive reagents. If you are unsure of reagent cost, please check online or on Quartzy.
4. How many items in a kit. E.G. Qiagen DNAeasy, cassettes of the Pippin, Bioanalyzer chips, Kapa Illumina adapter kits, etc...
5. Whenever you use a piece of equipment to do something, note the model number in your lab notes and open notebook posts.

4 Lab Calendar

1. AUGUST, ANNUALLY: pipette calibration
2. AUGUST, ANNUALLY: freezer defrost
3. LAST WEEK OF EVERY MONTH: CLEAN UP. This clean up is mainly to reduce any contamination. I hit the common used areas with bleach and alcohol, and I try to improve any situations that may lead to contamination (sterilize shared containers, re-organized areas that are being problematic, etc). Acid wash all of the plastic containers that are autoclavable and are used to carry RNA or DNA. Use a 10% Hydrochloric acid (and wear all of the protection needed). Autoclave pipettes. Wash glassware in detergent and rinse thoroughly.
4. FIRST WEEK OF EVERY MONTH: REMIND Dr. L TO DO THE MONTHLY BUDGET. A copy of all receipts from the previous month should be sent to Heather.
5. WEEKLY: backup and update computers
6. WEEKLY: chart of freezer temperatures

7. DAILY: CLEANING: Note: To prevent sample or reagent degradation, make sure that all vapors from the cleaning solution have fully dissipated before beginning any processes. A daily cleaning of the area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps to eliminate PCR product that has entered the pre-PCR area, and reduces the amount of PCR product in the post-PCR area. Identify areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution before beginning any pre-PCR processes. High-risk pre-PCR areas might include, but are not limited to: Bench tops, Door handles, Refrigerator/freezer door handles, Computer mouse, Keyboards. High-risk post-PCR areas include those above and: Thermal cyclers, Bench space used to process amplified DNA.

5 Lab Online Accounts

1. beckman coulter (ampure beads) -
2. agilent - k.lotterhos, lab password
3. qiagen -
4. sigma aldrich - k.lotterhos, lab password
5. VWR - lab password
6. bioexpress (k.lotterhos@neu.edu, lab password) (always have trouble with paying credit card on their site)
7. Kroll - identity protection - k.lotterhos, biennium
8. Zymo Research Account (username: k.lotterhos lab password)
9. airgas - k.lotterhos@neu.edu, lab password
10. Fisher NLSU pin: 4956 (expires March 1, 2016)
11. Eppendorf, k.lotterhos, lab password, Currently have \$4000 to spend in ecredits

6 Lab Manager (RA) Responsibilities

1. Order supplies, be aware of deals and when they expire.
2. Manage QUARTZY web inventory and keep it up to date.
3. Manage lab safety and compliance programs including mandatory training of all lab members
4. Oversee management of the laboratory collections of protocols & procedures
5. Train students and lab members in general in a variety of laboratory procedures
6. Maintain a lab calendar with equipment needs of inspection or calibration
7. Keep lab documents organized
8. Write meticulous directions for new protocols
9. Keep samples organized in the freezer
10. Once per week check on equipment, if anything is broke take steps to check on warranty and contact reps.
11. Sterilize tips, tubes, and other consumables for all lab users.
12. Daily cleaning

7 Prevent PCR Product Contamination

Note that our lab isn't really set up to do this. But these are important considerations.

The PCR process is commonly used in the laboratory to amplify specific DNA sequences. Unless proper laboratory hygiene is used, PCR products can contaminate reagents, instrumentation, and genomic DNA samples, causing inaccurate and unreliable results. PCR product contamination can shut down lab processes and significantly delay normal operations. Make sure that the lab is set up appropriately to reduce the risk of PCR product contamination:

7.1 Physically Separate Pre-PCR and Post-PCR Areas

- Physically separate laboratory space where pre-PCR processes are performed (DNA extraction, quantification, and normalization) from the laboratory space where PCR products are made and processed during the (post-PCR processes).
- Never use the same sink to wash pre-PCR and post-PCR troughs.
- Never share the same water purification system for pre-PCR and post-PCR processes.
- Store all supplies used in the protocols in the pre-PCR area, and transfer to the post-PCR area as needed.

7.2 Use Dedicated Equipment and Supplies

- Dedicate separate full sets of equipment and supplies (pipettes, centrifuges, oven, heat block, etc.) to pre-PCR and post-PCR lab processes, and never share between processes.
- Dedicate separate storage areas (freezers and refrigerators) to pre-PCR and post-PCR consumables.

Because the pre- and post-amplification reagents are shipped together, it is important to unpack the reagents in the pre-PCR lab area, and then move the post-amplification reagents to the proper post-PCR storage area.

8 Health and Safety Information

Checklist

Please make sure to follow the steps noted below before you start your lab work:

1. Complete the following safety trainings online: list here
2. Read the SOP for all chemicals you use but especially the following chemicals: -
Formaldehyde (corrosive) - Mercuric Chloride. -EtBr
3. Get a Research Laboratory Training of the Lotterhos Lab.
4. Get approval from Dr. Lotterhos.

9 Resources for the Molecular Biologist

1. The Simple Fool's Guide to PCR (Palumbi) (in this folder)
2. Molecular Zoology: Advances, Strategies and Protocols (Ferraris and Palumbi)
3. Roche PCR guide (in this folder)

10 Lab Equipment Information/Tips

10.1 pH Meter Operation Tips

(adapted from the BITC2441 Lab Manual Fall 2011)

Small errors in the pH adjustment of a buffer can have large effects on sensitive reagents used in the molecular lab. There are many things that you can do to improve the performance of a pH meter.

1. Never assume that a pH meter is in calibration. Even when properly maintained and cared for, a pH meter undergoes considerable drift in a matter of hours after calibration. Follow the appropriate SOP to determine whether your pH meter is in calibration and to bring it into calibration. The standard buffers used to calibrate a meter should bracket the pH of the sample to be measured. Use standard solutions to calibrate the pH meter. Note that the pH of the buffer depends on temperature. So after calibration, sometimes the pH meter reads a value that is different than the buffer because of temperature (i.e. 7.04 instead of 7.00). You may need to check a chart to see if the reading on your buffer is correct for the temperature of the liquid.
2. Verify that the standard buffers that you are using have not expired. This is especially important for pH 10 buffer, where CO₂ dissolved from the air will cause the pH to go down over time.
3. Avoid direct contact of solids or surfaces on the bulb of the pH electrode as it has a very fragile membrane. The electrode should not be wiped dry because static discharge can build up on the electrode.
4. Follow the manufacturer instructions for the proper care and use of an electrode. MOST electrodes, such as gel-filled electrodes, should be stored in pH storage solution (this is typically pH 4.0 buffer), and will be ruined if stored dry.

5. The best indicators of the electrode condition are the slope of the calibration curve and response time required to obtain a stable pH reading. As any electrode ages, the slope decreases from 100%. The recommended operating range varies by manufacturer but is usually 92 - 100%. The response time will become longer as the sample components coat the sensing glass bulb with continued usage. This can often be remedied with cleaning and/or replacing the filling solution, following the manufacturer directions.

11 Reagents

11.1 Reagent Preparation Tips

(adapted from the BITC2441 Lab Manual Fall 2011)

The ability to make reagents is an essential skill. The accuracy of calculation and of measurement is critical to the outcome of any experiment, whether it be one you do yourself or one in which you prep for someone else. There are several critical aspects to making solutions that should be followed at all times.

- Check and recheck each calculation. It is best if two people make a calculation independently and then cross check their answers.
- Read each reagent bottle twice, once before using and once afterwards. This helps ensure that the right reagent is used.
- Write down the solution prep for every reagent that you make. Ideally this should include the formula, the supplier and catalog number if available, as well as the concentration, the expiration date of the chemical, when it was received, how it was stored upon receipt, and the amount weighed out for each reagent. If the pH is adjusted or the solution is sterilized, information about these procedures should be documented. Some solution prep forms will also have space to include the balance number, pH meter number and other pieces of important information. The storage conditions for the solution that was prepared should also be recorded here.
- Label each solution bottle before filling. Write down the name of the solution, its concentration, its pH if it is a buffer, your initials, the control # assigned to the solution (if any), and the date. Some industries have special blank labels to be used for each reagent. Others use tape and a permanent marker. There are labeling software programs and systems for labeling and making electronic records for solutions prepared in laboratories.
- Record any changes observed in materials during solution preparations, no matter how trivial they might seem. This includes the formation of gas bubbles and any change in color.

This record can be used to trace back a problem to its source quickly and easily or to confirm that a problem does not lie in the reagents or their preparation.

11.2 Aliquots

Aliquots are smaller portions of the reagents to be used that are divided into smaller containers.

Aliquots act as a safety net in your molecular work by preventing contamination and to minimize the number of times your reagents are frozen-thawed.

The volume of the aliquots should be enough for a few samples / reactions. Aliquots of small volumes will keep you changing your aliquots and therefore minimizing your chances of contamination and of exposing your reagent to repetitive freeze-thawing. An additional good practice would be to not use the same pipette tip between an old and a new reagent.

The use of aliquots also creates an environment that is friendly for beginners. Aliquots allow everyone to start with high quality reagents by preventing contamination from shared use. If you feel like you may have made an error that could have caused a contamination, or things are not working well (maybe one reagent went bad because of high temperatures) - then throw your aliquots away and start with fresh ones!

You can make aliquots of your samples too! Aliquots of your samples are often called working stocks. For example: I have 30ul of DNA from an extraction. DNA extractions often are of high concentrations ($> 300 \text{ ng}/\mu\text{l}$), so I make aliquots of 1:5 or 1:10 DNA dilutions. This allows me to have at least one main stock of uncontaminated samples for my future use or for the lab use.

Aliquots should:

- have a label with name, concentration and date
- be made from an original source that is clean and of optimal quality
- be made in a clean environment. Try to think about your work, your labmates work, and of potential future work. For example: working with barcodes genes would be a pain if your aliquots already come with fungi spores.
- whenever possible try to take additional steps to guarantee the quality of the aliquots. For example: starting with high water quality may not be enough once you have exposed the aliquot to the environment - You can always pass the water aliquots through UV light

11.3 Reagent Recipes and Ordering Information

7.5M ammonium acetate

ammonium acetate (Fisher #A637500) 57.81g

water (to a final volume of 100ml)

*If needed, sterilize by filtration in 0.2um filter.

*final pH will be 5.5

Bleach 10%

Bleach 10ml

DI Water 90ml

*solution is good for up to 7 days

0.5M EDTA (pH - 8)

Disodium ethylenediamine tetraacetate (Fisher #BP120500) 186.1g

Water 80ml

NaOH (Fisher #BP359-212) 18g

*Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0. Sterilize by autoclaving

1mM EDTA (pH - 8) for storing extracted RNA EDTA should help stabilize and protect the RNA. Use sterile 25mL pipettes to measure RNA free water and put into a 100mL glassware (autoclaved, wiped clean with RNAaseZap or RNAase Away, and rinsed with RNAase-free water). Clean magnetic spinbar with RNAaseZap. Calibrate pH meter and wipe outside with RNAase zap. Use pasteur pipette to add HCl for pH adjustment, but wipe outside with RNAase-Zap.

Disodium ethylenediamine tetraacetate (Fisher #BP120500) 0.372 g

Water 80ml

NaOH (Fisher #BP359-212) 0.036 g

*Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0. Sterilize by autoclaving

Ethidium Bromide (10mg/ml)

CAUTION: Ethidium bromide is a mutagen and is toxic. Wear gloves when working with ethidium bromide solutions and a mask when weighting the powder.

Ethidium bromide (Fisher #BP1025) 1g

Water 100ml

*stir on magnetic stirrer for several hours to ensure that the dye has dissolved

*light sensitive, wrap the container in aluminum foil or transfer to a dark bottle

*store at 4C

CTAB

4M NaCl	(Fisher #S271-1)	35ml
0.5M EDTA pH 8.0	(Fisher #BP120500)	4ml
1M Tris-HCl pH 8.0		10ml
CTAB (Cetyl Trimethyl-Ammonium Bromide) (Fisher #AC22716100)		2g

*Mix ingredients together in a clean beaker. Stir on hot plate with stir bar. Heat gently until CTAB is dissolved. Pour into graduated cylinder and bring volume up to 100ml using nanopure H₂O. Pour into a bottle with a cap. Autoclave before using. Store at room temperature. Discard after 1 year.

dNTPs (10mM)

To make 48 aliquots of 25ul each of 10mM dNTPs

All of the items below come in a Fisher package #FERR01811

	12X	1X
dTTP (100mM)	120ul	10ul
dATP (100mM)	120ul	10ul
dGTP (100mM)	120ul	10ul
dCTP (100mM)	120ul	10ul
ddH ₂ O	720ul	60ul

5M NaCl 500ml

NaCl (Fisher #S271-1) 146.1 g

H₂O ~350 ml

*Dissolve, then bring up to volume with H₂O

*Sterilize by autoclaving (15 minutes)

NaOH 10N

NaOH (Fisher #BP359-212) 40g

H₂O 80ml

*continue adding water to 100ml

Proteinase K (Fisher #BP1700100)

NOTE THAT QIAGEN DNAeasy BLOOD AND TISSUE KITS COME WITH PROTEINASE K. Dissolve powder ordered from Invitrogen (or Gibco, or Sigma, whatever company) in STERILE nanopure H₂O to a final concentration of 25mg/ml. Generally, I order a 100mg bottle of Proteinase K but only use 25mg at one time. Proteinase K has a tendency to degrade over time after H₂O has been added to it. But, when it remains in its powder form, it will be fine. After adding H₂O to the powder, mix well, label centrifuge tube, and store in the -20C freezer. (I usually use a 1.5ml centrifuge tube and label it as PK and the date).

RNA Loading Buffer (may not be needed)

Formamide	(Fisher #BP227500)	50ul
Formaldehyde	(Fisher #F77P-4)	20ul
10X MOPS	(Fisher #BP2900500)	10ul
Ethidium Bromide		2ul

*use 8ul of master mix for 2ul of sample ~200ng RNA

*This buffer may not be needed. Denaturing RNA before running the RNA through the gel may be the only thing needed.

10% Tween-20

100% Tween-20	(Fisher #97062-332)	1ml
Add distilled H ₂ O to make final volume of 10ml		

TBE 10X (stock solution)

Tris base	(Fisher #BP15210)	108g
Boric acid	(Fisher #A73 1)	55g

*dissolve in Milli-Q water

0.5M EDTA (pH 8.0)	40ml
--------------------	------

*increase the final volume to 1 liter

*store at room temperature or at 4C

*might be too concentrated for long term storage, ok if you are planning to do a lot of work

TBE 5X (stock solution)

Tris base (Fisher #BP15210) 54g

Boric acid (Fisher #A73 1) 27.5g

*dissolve in Milli-Q water

0.5M EDTA (pH 8.0) 20ml

*increase the final volume to 1 liter

*store at room temperature or at 4C

*(1X TBE = 200ml of 5X TBE + 800ml)

TE Jon makes a low TE buffer that is 0.1mM EDTA. This is better for DNA that is destined for sequencing, because the EDTA interferes with enzymatic reactions.

Supplies needed:

Tris-HCl (pH 8.0) 10mM

EDTA (pH 8.0) 1mM

- Start with a small volume of nanopure H₂O, about half of your final volume, in a beaker with a stirbar.
- Measure out the amount of Tris-Hcl needed for a 10mM solution
- Add to H₂O
- Measure out the amount of EDTA needed for a 1mM solution
- Add to H₂O and Tris
- While stirring solution, carefully insert calibrated pH probe
- Adjust pH as needed with either HCl or NaOH
- Note: EDTA will not fully dissolve until pH is near 8.0 and will cause pH to fluctuate as it dissolves, so be prepared to make many small adjustments in either direction (it is best to work with diluted acids and bases).
- Also: if you overshoot 8.0, it is okay to readjust in the other direction (even if you wildly overshoot the mark) and you DO NOT need to throw the solution out and start again

- After pH is adjusted, transfer solution to graduated cylinder and add H₂O to bring to final concentration.
 - Some people think TE buffer should not be autoclaved, but I usually autoclave it. Tris breaks down easily after autoclaving and will precipitate out more easily, so you may have to make the solution up more frequently. In my opinion, that is better than chancing a solution with DNase.
 - Store at room temperature and discard if sediment is forming on the bottom of the container or if it starts growing or if it gets cloudy.
 - For 100ml:
 - 1M Tris-HCl (pH 8.0) 1ml
 - 0.5M EDTA (pH 8.0) 200ul
 - Bring to a final volume of 100ml with nanopure H₂O

1M Tris

Tris-base (Fisher #BP15210) 121.1g

*Bring volume up to 1 liter with ddH₂O

1M Tris-HCL

Tris base (Fisher #BP15210) 60.56g

ddH₂O 400ml

*Adjust pH to 8.0 using HCL and bring final volume to 500ml

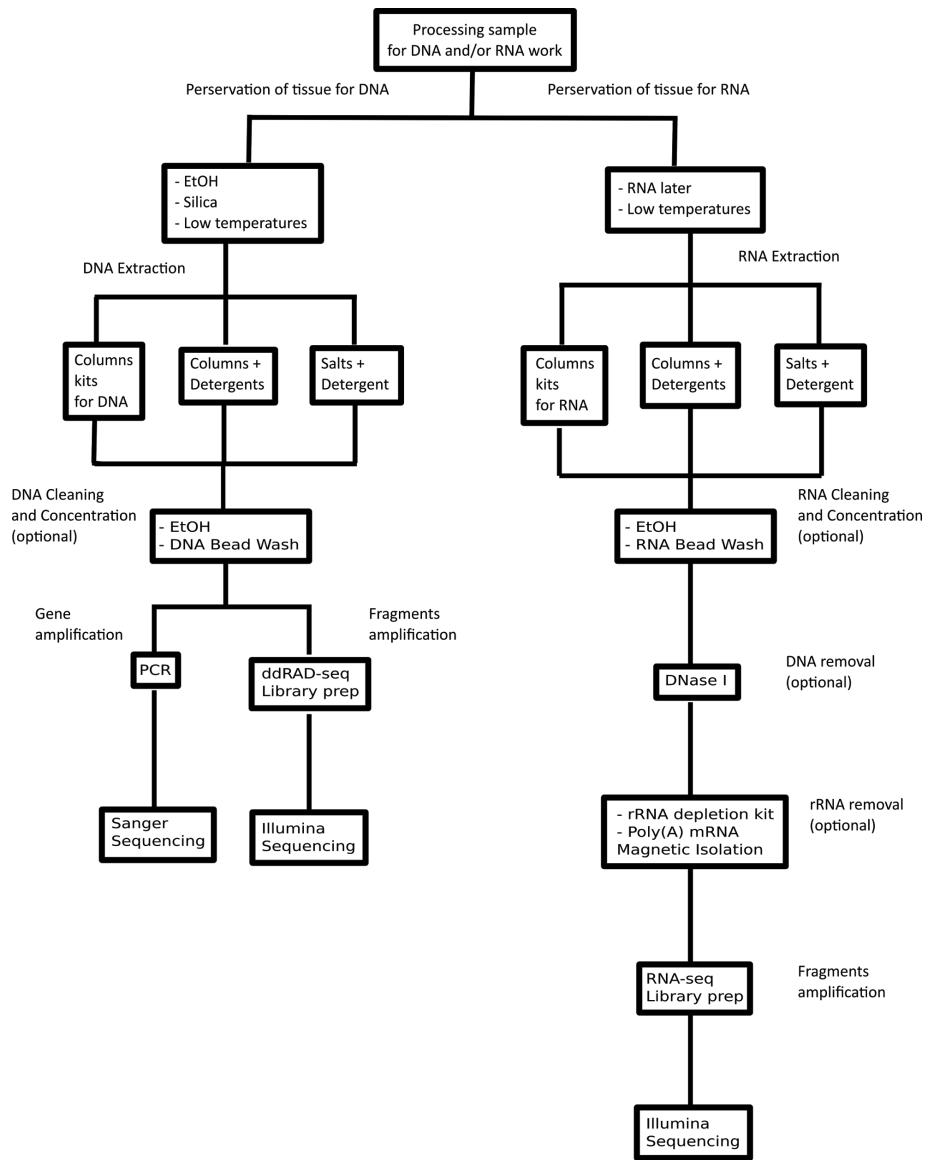
12 How do I perform a DNA precipitation to concentrate my sample?

From Qiagen website

- Add 1/10 volume of 3 M Na-Acetate pH 5.2 (Thermo Scientific R1181), and 2 to 2.5 volumes of ice-cold 100% ethanol to the DNA sample
- Mix, and store at -20°C for at least 1 hour to precipitate the DNA
- Recover the precipitated DNA by centrifugation at full speed in a microcentrifuge for 15-20 minutes
- Pour off the ethanol and wash the pellet twice with room-temperature 70% ethanol
- Allow the DNA pellet to air-dry
- resuspend the DNA in a suitable volume of sterile TE buffer or distilled water

13 DNA and RNA Isolation

13.1 Flow Chart - does this make sense to everyone - need to update



13.2 Collection of samples for DNA Extraction

With next-generation sequencing, we have to try to reduce contamination of our samples with bacteria and other microorganisms, as well as from other individuals. Some guidelines:

Materials

- Scalpels with replaceable, sterile blades
- Autoclaved glass pyrex dish for cutting sample on and a flame to torch it; alternatively can use weigh boats that are RNAase/DNAase free

- Autoclaved tweezers, probes, etc. Ideally use tweezers without the ridges, between which tissue gets stuck.
- Ethanol in an autoclaved beaker for rinsing dissecting tools
- A small flame for sterilizing dissecting tools between samples
- Prepared, Autoclaved and labeled (with EtOH-proof pen) microcentrifuge tube(s), filled with 95% or higher EtOH. When filling tubes do so in a sterile manner (i.e. use a squirt bottle that has been autoclaved or a pipette with autoclaved tips)

Routine

- Dissect tissue on glass plate and place in microcentrifuge tube
- Place the remaining part of the organism in -80°C and the sample in EtOH in the freezer
- Clean glass plate with ethanol and kimwipe, use blade to scrape off any tissue. Ideally flame the glass plate if a torch is available.
- Clean dissecting tools in ethanol, use kimwipe if necessary to remove any tissue stuck to them.
- Pass dissecting tools over flame

13.3 Tissue Prep for DNA Extraction

1. Materials

- small beaker with DI water
- small beaker with EtOH
- autoclaved tweezers
- weigh boats (enough for each sample)
- flame
- lighter
- sterile scalpel blades (enough for each sample)
- kimwipes
- Sterile, labeled microcentrifuge tubes (enough for each sample)
- Styrofoam box with ice
- Bleach
- 70% EtOH for cleaning

2. Procedure

- 2.1. Wash work counter with Bleach first and 70% EtOH second
- 2.2. Collect 10 samples from freezer and place in ice box. By using only 10 samples at a time, you limit the potential exposure of tissues to temperatures above -20°C
- 2.3. Place fresh weigh boat on balance and tare it
- 2.4. Remove weigh boat and place first tissue sample into boat
- 2.5. Cut desired amount of tissue using scalpel blade and tweezers and write down final weight in lab notebook
- 2.6. Place tissue into labelled microcentrifuge tube, put directly on ice and put any remaining tissue back into original vial
- 2.7. Throw away scalpel blade in sharps container and throw weigh boat in trash
- 2.8. Clean tweezers:

- Rinse in DI water
- Wipe down with a fresh kimwipe
- Rinse in EtOH and place directly over flame

2.9. Repeat above steps for remaining 9 tissues

2.10. Put all 10 original containers and new weighed out samples into -20°C freezer and collect next 10

2.11. Repeat all above steps until all tissues have been prepped

13.4 DNA Isolation Review

(adapted from the BITC2441 Lab Manual Fall 2011)

The fundamental steps of DNA purification are sample lysis and purification of the DNA from contaminants. There are a myriad of protocols available for isolating DNA from organisms in the molecular lab. The more classical methods have remained essentially unchanged for decades, and the more modern methods that involve kits that are commercially available. Basically, the best method for any particular application depends on these fundamental considerations:

- Where the DNA is isolated from will determine the cell lysis techniques used.
- The purity requirements of the intended use of the DNA being isolated will determine how many purification steps will be involved.
- The type of DNA being isolated: genomic DNA has different physical properties from those of plasmid DNA.

The successful isolation of DNA requires methods that prevent nuclease degradation of the DNA. Some buffer constituents used to promote lysis and denaturation of nucleases include:

- Detergents - used to solubilize cell membranes: Popular choices are SDS (sodium dodecyl sulfate, aka SLS, sodium lauryl sulfate), Triton X-100, and CTAB (cetyltrimethyl ammonium bromide)
- Proteinase K - sometimes added to cleave glycoproteins and to help the detergents to inactivate DNases.
- Denaturants such as urea, guanidinium salts, and other chaotropes are sometimes applied to inactivate enzymes. Heat is often applied to enhance the lysis of cells and the denaturation of proteins.
- For microbial sources of DNA, enzymes must be added to break down cell walls in order to make the cells susceptible to lysis.
- RNases are often added to a lysis buffer to remove contaminating RNAs, which can interfere with the intended use of the DNA being isolated.

In selecting a lysis method for a particular application, top priority should be given to choosing a method that has simplicity and speed (least number of steps and solutions required). Remember that every constituent added to cells during the lysis procedure could become a culprit by sabotaging the activity of an enzyme later on. You will want to remove anything added to your DNA at the beginning of DNA isolation sometime later, so try to keep the number of constituents in your lysis buffer to those that are absolutely needed. The number of steps in a cell lysis protocol should also be kept to a minimum, since any delays during this part of the DNA isolation procedure runs the risk of DNA degradation by nucleases in the cells. DNA will not be safely stabilized until it has been purified from all protein contaminants. In general, animal tissues are easily lysed, due to the fact that they have no cell wall, and a gently detergent treatment usually is sufficient to break open cells. Yeast and microbial cells, on the other hand, have rigid cell walls that must be weakened enzymatically before the cell will release its DNA. In the case of bacteria, lysozyme enzyme is added, while in the case of yeast a more complex mixture of enzymes must be used to degrade cell wall polymers. Plant cell walls are generally abraded mechanically by grinding frozen plant tissue, often with glass beads or sand and a mortar and pestle.

The second phase of DNA isolation protocols is the purification of the DNA released from the cell from other components of the cell and the lysis buffer. The method you select for your application depends on the size and source of the DNA to be isolated. When plasmid DNA is being isolated from bacteria such as Escherichia coli (E. coli), an alkaline solution of SDS is sufficient to release plasmid DNA, leaving behind the genomic DNA still associated with the cellular debris. The genomic DNA is then conveniently removed from the plasmid DNA by a quick centrifugation step. Genomic DNA can frequently be rendered insoluble and quickly spooled from the lysed cells by addition of alcohol to the mixture. The spooled DNA can be transferred to a fresh buffer to redissolve the genomic DNA.

For some applications, this low level of DNA purity will suffice. Often, though, there are proteins or polysaccharides (especially in plant sources of DNA) that coprecipitate with the DNA and interfere with subsequent enzymatic treatments. Classically, the further purification of DNA involves the removal of proteins by aqueous phenol solutions, followed by numerous alcoholic precipitation steps to remove traces of phenol from the isolated DNA. Alcohol precipitations of DNA also serve to concentrate the DNA into a smaller volume, and to purify the DNA from any

water-soluble contaminants. The phenol extraction is an inefficient method of purification and suffers from a poor yield of DNA. Also, phenol reagents are unstable, and fresh solutions must be used or the quality of the reagent must be monitored, generally by observed changes in pH. This, along with safety concerns in the use of phenolic solutions, is a serious drawback in this method of DNA purification.

An alternative procedure is the use of so-called spin columns, which are small chromatography columns that purify the DNA from other solutes. While this procedure is more expensive than phenol extractions and alcohol precipitations, the purification and yields of product by spin columns are improved. Also, the reagents used are more stable, so provide a more reliable, or robust, method. The final DNA prepared with spin columns is free of protein and salt contaminants and can be used directly in restriction digests, Southern blotting, and PCR applications. All components of this system are stable at room temperature for one year. Another advantage of purchased kits for plasmid preparation is that the quality of reagents can be tested and assured the vendors. For these reasons, many biotechnology labs routinely use kits for their plasmid preparations.

Binding and elution from silica beads has become the method of choice for isolation of genomic DNA from animal tissues. A high concentration of chaotropes serves to bind nucleic acids to silica surfaces. The adsorption step to bind DNA to the silica particles is followed by wash steps, usually with salt/ethanol solutions which will not interfere with the strong binding of nucleic acids but will wash away remaining impurities and excess chaotrope. Elution of DNA from silica columns requires the use of nuclease-free water or low ionic strength buffers such as TE. This is an advantage since it means that the isolated DNA can be used directly in further manipulations without further cleanup.

A potential problem with the use of silica columns for the binding of DNA is the possibility of overloading the column with DNA, resulting in a wash-through of non-adsorbed DNA and reducing the overall yield of DNA. There is also some loss of material that does not elute from the silica resin. The smaller the DNA size is, the tighter is its interaction with silica surfaces. Although size is not a problem with isolations of genomic DNA, loading the silica resin with too little DNA can also lead to a low overall yield of DNA eluted from a silica column.

Protocols for the use of spin columns are unique to each vendor, and so the vendor's protocol

should be followed when they are used. Some examples of some more general protocols are found below, along with a discussion of how they work.

NOTE THAT FOR NEXT GENERATION SEQUENCING, SOME METHODS OF DNA EXTRACTION ARE NOT RECOMMENDED. THIS INCLUDES CTAB EXTRACTION AND PHENOL-CHLOROFORM.

13.5 CTAB DNA Extraction

(adapted from Levitan's protocol) NOTE THAT FOR NEXT GENERATION SEQUENCING, SOME METHODS OF DNA EXTRACTION ARE NOT RECOMMENDED. THIS INCLUDES CTAB EXTRACTION AND PHENOL-CHLOROFORM.

1. Make sure you have all solutions (see solutions sheet for directions).
 - 1.1. CTAB
 - 1.2. Proteinase K
 - 1.3. 100% Ethanol
2. Turn on water bath to begin heating to 65°C, make sure it has enough water to cover 3/4 of a 1.5 mL microcentrifuge tube.
3. Set up and label individual 1.5 mL microcentrifuge tubes.
4. Add 500 microliters (uL) of CTAB solution to each tube.
5. Next add 10 uL of proteinase K solution to each tube, make sure to change tips. If you are processing more than 18 samples at once, you should do sets (or stages) of samples so they do not sit for too long.
6. Remove a small amount of the sample preserved in EtOH
7. Blot the sample on kimwipe, and place in microcentrifuge tube.
8. Close Tube
9. Return forceps to 100% EtOH to re-sterilize
10. Repeat steps 4-8 for remaining samples
11. Place samples in holder (floating) in waterbath. Do not worry too much at this point if the temperature is not quite at 65C
12. After 30 minutes, mix the samples by inverting gently.
13. At this point the temperature should be at 65C, and should be stabilized here.. continue to monitor temperature.

14. Leave samples in waterbath overnight (at least 14hours, I have left samples in the waterbath for 48 hours which yielded high DNA concentrations). Samples should be digested when you no longer see any gelatinous/stringy material when inverting them.
15. Clean your samples by doing a DNA Beadwash (see DNA Beadwash protocol in Appendix)

13.6 Cleaning your Sample before DNA extraction- Optional

This protocol was used for copepod parasites, but could be used for other samples as well.

Copepods have a small amount of DNA that can easily be contaminated with other organisms/detritus/plankton/etc present on the outside of the body. To avoid contamination, you can add a cleaning step to wash your copepods.

1. Prepare small slides of Nylon (6/6) woven mesh sheet (you can cut a 12" width, 12" length, 10 microns mesh size, 2% open area). Cut the mesh to a convenient size that can hold your sample and that will fit in the tube use for digestion with proteinase K. Autoclave the mesh.
2. Set the mesh and the copepod over an empty clean tube or kimwipe, and add molecular grade ethanol (<75%) to the sample until you don't see any debris or you have given the copepod a few washes.
3. Let the copepod dry in a control environment where the copepod won't fly away (you can set the copepod inside a 1.5 ml microcentrifuge tube to dry and use the same tube for the following steps.

** Please note that your sample may degrade if you leave it at room temperature for too long. You want to leave your sample to dry for the minimum amount of time - avoid overnight drying situations!

13.7 DNA Extraction for Animal Tissues

(adapted from DNeasy Blood & Tissue (cat#69504) Handbook Introduction)

In general, follow the directions supplied with the kit. Always cross-check the instructions below with the kit instructions as they may change through time

Safety Information

- When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs).

CAUTION: DO NOT add bleach or acidic solutions directly to sample-preparation waste.

- Buffer AL and Buffer AW1 contain guanidine hydrochloride, which can form highly reactive compounds when combined with bleach. If liquid containing this buffer is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.
- Buffer AL and Buffer AW1 (concentrate) Contains guanidine hydrochloride: harmful, irritant. Risk and safety phrases: * R22- 36/38, S13-26-36-46
- Proteinase K: sensitizer, irritant. Risk and safety phrases: * R36/37/38- 42/43, S23-24-26-36/37

Consumable Supplies and Equipment

(Qiagen Kit #69504 contains all of the reagents except molecular grade ethanol)

- Buffer ATL and Buffer AL. Buffers may form precipitates upon storage. If necessary, warm to 56°C until the precipitates have fully dissolved.
- Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96- 100%) as indicated on the bottle to obtain a working solution.

- Preheat a thermomixer, shaking water bath, or rocking platform to 56°C
- If using frozen tissue, equilibrate the sample to room temperature. Avoid repeated thawing and freezing of samples since this will lead to reduced DNA size.
- Vortexer
- Molecular grade (100%) ethanol
- Optional: RNase A (100mg/ml; cat. No. 19101)
- Microcentrifuges tubes (1.5ml or 2ml) (any type)
- Water bath
- Centrifuge (that can reach 20000g, room temperature)
- Collection tube (provided in the kit)
- Columns (provided in the kit)

Things to do Before Starting

Preheat the water bath to 56°C

Equilibrate the sample to room temperature

Make sure appropriate rotors are placed in the centrifuge

Protocol

Basically the protocol below is the same as that which comes with the kit. Some tips:

1. If the tissue suffered freezer burn, then ice may permeate the tissue and may add to weight.
Suggest thawing small pieces in ethanol to "suck up" water in tissue.
2. Sterilize blade and tweezers between tissues by rinsing in ethanol, wiping with kimwipe, then rinsing again and burning off ethanol over an open flame. Use a sterilized blade to chop up tissue (stored with dissecting tools below large centrifuge).
3. If tissue was preserved in ethanol, set up two weigh boats (one on scale). Pour out tissue and ethanol into weigh boat not on scale. Blot tissue on kimwipe until completely dry before weighing. Weigh 25mg. Take weigh boat off scale and chop tissue. Store on ice until add buffer ATL and proteinase K.

4. For tissue digestion lysis step (first step), use Eppendorf thermomixer with 500 rotations per minute. Also vortex at high speed a few times.
5. After lysis step and before adding 200ul buffer AL, be sure to vortex for 15 seconds at high speed.
6. After the protocol below, we elute the DNA in water instead of AE; it might be worse for long-term storage but it should be better for NGS.
 1. Use up to 25 mg tissue (up to 10mg spleen). If tissue was preserved in ethanol, then wipe the ethanol of the tissue with kimwipes. Cut up the tissue into small pieces, and place in a 1.5 ml microcentrifuge tube.
 2. Add 180ul Buffer ATL.
 3. Add 20ul proteinase K. Mix thoroughly by vortexing, and incubate at 56C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample. Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1-3 hours but if it is more convenient, samples can be lysed overnight; this will not affect them adversely.
 4. Vortex the lysed sample for 15 seconds. Add 200ul Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200ul molecular grade ethanol (100%), and mix again thoroughly by vortexing. It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples. Some tissues type may form a gelatinous lysate after addition of Buffer AL and ethanol. In this case, vigorously shaking or vortexing the preparation is recommended.
 5. Transfer the mixture from step 4 (including any precipitate) into the the DNeasy Mini spin column placed in a 2ml collection tube. Centrifuge at >6000 x g for 1 min. Discard flow-through and collection tube.
 6. Place the DNeasy Mini spin column in a new 2ml collection tube (provided), add 500ul Buffer AW1, and centrifuge for 1 min at >6000 x g. Discard flow-through and collection

tube.

7. Place the DNeasy Mini spin column in a new 2ml collection tube (provided), add 500ul Buffer AW2, and centrifuge for 3 min at 20000 x g to dry the DNeasy membrane. Discard flow-through and collection tube. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20000 x g.
8. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube, and pipette 200 ul Buffer AE or water (water is advised for ddRAD-seq libraries) directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at >6000 x g to elute.
9. Elution with 100ul (instead of 200ul) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield.
10. For maximum DNA yield, repeat elution once. A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate.

13.8 DNA Extraction with QIAamp DNA Micro Kit

For purification of genomic DNA from small amounts of tissues or samples (larvae, etc)

(Adapted from the QIAamp DNA Micro Handbook of Qiagen Cat# 56304)

Safety Information

- When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs).

CAUTION: DO NOT add bleach or acidic solutions directly to sample-preparation waste.

- Buffer AL and Buffer AW1 contain guanidine hydrochloride, which can form highly reactive compounds when combined with bleach. If liquid containing this buffer is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.
- Buffer AL and Buffer AW1 (concentrate) Contains guanidine hydrochloride: harmful, irritant. Risk and safety phrases:^{*} R22- 36/38, S13-26-36-46
- Proteinase K: sensitizer, irritant. Risk and safety phrases:^{*} R36/37/38- 42/43, S23-24-26-36/37

Consumable Supplies and Equipment

(reagents provided with QIAamp DNA Micro Kit (Qiagen #56304))

- Buffer AL. Check whether precipitate has formed in the buffer. If necessary, dissolve by heating to 70°C with gentle agitation.
- Buffer AW1. Before using, make sure that 25ml molecular grade ethanol has been added to the bottle; this needs to be done 1 time only.
- Buffer AW2. Before using, make sure that 30ml of molecular grade ethanol has been added to the bottle; this needs to be done 1 time only.

- Carrier RNA. Add 310ul Buffer AE to the tube containing 310 ug lyophilized carrier RNA to obtain a solution of 1ug/ul. Dissolve the carrier RNA thoroughly, divide it into conveniently sized aliquots, and store at -20°C. Do not freeze-thaw the aliquots of carrier RNA more than 3 times
- 1.5 ml microcentrifuge tubes (any kind)
- Mesh (optional) - autoclave before use (cat# CMN-0010-C)
- Columns (provided with kit)

Protocol

1. Transfer a tissue sample of less than 10 mg in weight to a 1.5 ml microcentrifuge tube. If you are working with larvae or small organism, dump the sample in a small autoclaved mesh. Then flush the sample with ethanol (the mesh will hold your sample) to get rid of any debris. The sample can be transferred with the mesh to the microcentrifuge.
2. Immediately add 180 ul Buffer ATL, and equilibrate to room temperature (15-25°C).
3. Add 20 ul proteinase K and mix by pulse-vortexing for 15 s.
4. Place the 1.5 ml tube in a thermomixer or heated orbital incubator, and incubate at 56°C overnight until the sample is completely lysed. For small amounts of tissue, lysis is complete in 4-6 h, but best results are achieved after overnight lysis.
5. Add 200 ul Buffer AL, close the lid, and mix by pulse-vortexing for 15 s. To ensure efficient lysis, it is essential that the sample and Buffer AL are thoroughly mixed to yield a homogenous solution. Note: If you are working with very small samples, add 1ug dissolved carrier RNA to the each sample. If working with small larvae, add 8ug dissolved carrier RNA to each sample.
6. Add 200 ul ethanol (96-100%), close the lid, and mix thoroughly by pulse-vortexing for 15 s. Incubate for 5 min at room temperature (15-25°C). Note: If room temperature exceeds 25°C, cool the ethanol on ice before adding to the tube.

7. Briefly centrifuge the 1.5 ml tube to remove drops from inside the lid.
8. Carefully transfer the entire lysate from step 7 to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim. Close the lid, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through. If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute column is empty.
9. Carefully open the QIAamp MinElute column and add 500 ul Buffer AW1 without wetting the rim. Close the lid, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow- through.
10. Carefully open the QIAamp MinElute column and add 500 ul Buffer AW2 without wetting the rim. Close the lid, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow- through. Contact between the QIAamp MinElute column and the flow-through should be avoided. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains ethanol, coming into contact with the QIAamp MinElute column. Take care when removing the QIAamp MinElute column and collection tube from the rotor, so that flow-through does not come into contact with the QIAamp MinElute column.
11. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min to dry the membrane completely. This step is necessary, since ethanol carryover into the eluate may interfere with some downstream applications.
12. Place the QIAamp MinElute column in a clean 1.5 ml microcentrifuge tube and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute column and apply 20=100 ul Buffer AE or distilled water to the center of the membrane. If high pH or EDTA affects sensitive downstream applications, use water for elution. Important: Ensure that Buffer AE or distilled water is equilibrated to room

temperature (15-25°C). If using small elution volumes (<50 ul), dispense Buffer AE or distilled water onto the center of the membrane to ensure complete elution of bound DNA. QIAamp MinElute columns provide flexibility in the choice of elution volume. Choose a volume according to the requirements of the downstream application. Remember that the volume of eluate will be up to 5 ul less than the volume of the solution applied to the column.

13. Close the lid and incubate at room temperature (15-25°C) for 1 min. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 1 min. Incubating the QIAamp MinElute column loaded with Buffer AE or water for 5 - 30 min at room temperature before centrifugation generally increases DNA yield.

13.9 RNA Isolation Review

(adapted from the BITC2441 Lab Manual Fall 2011 and Vomelova et al. 2009)

RNA may be used in the molecular lab to make cDNA in order to clone genes, to study gene regulation, to determine the size and structure of specific messages, to identify gene products, to determine rates of specific mRNA synthesis or degradation, to name a few. There are many procedures for isolating RNA, depending on the type of RNA to be isolated, the type of tissue that the RNA is being isolated from, and the intended use of the isolated RNA. The successful isolation of intact RNA by any procedure requires that four important steps be performed:

1. effective disruption of cells or tissue
2. denaturation of nucleoprotein complexes
3. inactivation of endogenous ribonuclease (RNase) activity
4. purification of RNA away from contaminating DNA and protein

The most important of these is the immediate inactivation of endogenous RNase activity which is released from membrane-bound organelles upon cell disruption. RNases are very stable and generally require no cofactors such as magnesium and other divalent cations, so they cannot be inhibited or inactivated by adding chelators such as EDTA.

Tissues to be used for the isolation of RNA should be properly stored immediately after collection to prevent RNA degradation from the activity of RNases. To do so, the tissue should be preserved under -70 C temperatures (flash freezing with liquid nitrogen preferred) or the tissues should be preserved in a preservative agent that inactivates RNases (such as RNA later). The quality of the RNA to be isolated would depend greatly in reducing the amount of elapse time from killing an organism and preserving the tissue.

Prior to the isolation of RNA, the tissues have to go through an enzymatic or mechanical process. Potent RNase inactivating ingredients that denature proteins (including RNases) are added to the lysing buffer. The most effective and most commonly used denaturant is guanidinium isothiocyanate (usually abbreviated as GITC or GuSCN). Another strong protein denaturant is phenol (which is also very caustic!), and historically, phenol or mixtures of phenol and chloroform

were used in RNA isolation. Detergents are also able to denature proteins and most lysis solutions contain detergents. These denaturing agents also disrupt cell membranes and aid in solubilization of the tissue.

RNA isolation methods fall in three approaches 1) procedures relying on the different solubility of cellular components in organic solvents, such as phenol, ethanol or isopropanol; 2) methods based on RNA adsorption to specific surfaces in the presence of chaotropic salts; and 3) protocols exploiting RNA separation on isopycnic gradient centrifugation.

The choice of an RNA isolation method depends upon a number of factors, such as:

1. the source of RNA - i.e., tissues with high fat content have different isolation requirements
2. the type of RNA to be purified
3. the relative abundance of the RNA
4. the sample size

Once RNA has been isolated, it is usually resuspended in water or water with trace amount of salts or EDTA. At this point the RNA is unprotected and highly vulnerable to degradation. Therefore it is extremely important that surfaces and solutions, which come into contact with the RNA preparation, are completely free of active RNase.

Because of the stringent requirements for high-quality reagents, many if not most labs prefer to purchase RNA isolation kits from commercial sources where the quality of the materials have been validated and spin columns help to speed the extraction process.

TECHNIQUE TIPS:

1. Observe all the guidelines for creating an RNase-free working environment in your workspace and lab.
2. Keep reagent bottles closed if not in use.
3. Keep tissues, reagents, and RNA samples on ice to prevent degradation by RNases.

13.10 Creating a RNase-FREE Environment

(adapted from the BITC2441 Lab Manual Fall 2011 and Vomelova et al. 2009)

RNases are ubiquitous and notoriously difficult to inactivate. The following notes are good things to consider in setting up for an RNA isolation:

1. Two of the most common sources of RNase contamination are the researcher's hands and bacteria or molds that may be present on airborne dust particles or laboratory equipment and supplies. To prevent contamination from these sources, sterile technique should be employed when handling any of the reagents used for RNA isolation or analysis. Gloves should be worn at all times and exchanged often.
2. To avoid RNA degradation, all solutions, glassware, and plasticware that may contain RNase should be treated to remove RNases. Diethylpyrocarbonate (DEPC) inactivates RNases by reacting with histidine residues, found at the active site and so is the usual method for producing RNase-free equipment. To render water RNase-free, it is treated as follows:
 - Measure water into RNase-free glass bottles.
 - Add diethylpyrocarbonate (DEPC) to make a 0.1% (v/v) solution. (ie add 0.1 mL DEPC for each 100 mL of water).
 - Shake vigorously to mix
 - Autoclave for 15 minutes at 121°C on liquid cycle, OR incubate at least 12 hours at 37°C and then heat to 100°C for 15 minutes.

NOTE: Tris buffers cannot be treated with DEPC. DEPC is a suspected carcinogen and should be handled with care. Always use gloves and open under a fume hood.

3. Disposable plasticware such as pipette tips and microcentrifuge tubes is generally RNase free if used straight out of the package and the package is kept closed and gloves are worn when items are touched and removed. It is best to pour tubes from an unopened bag onto an RNase-free environment (such as plastic wrap).
4. Chemicals and equipment for use in RNA isolation and analysis would be best reserved

separately from other uses. Wear gloves when handling this equipment, and use only baked spatulas and untouched weigh boats or weigh paper.

5. Use filter-tips for micropipetting when isolated RNA is to be used in amplification procedures.
6. Use heat-blocks and not water baths. Dirty water baths are a source of RNases.
7. Clean counter with bleach, EtOH, and RNAase away. Once the surface is clean, don't put anything on it that hasn't been treated. Make sure if you move something to another counter and move it back, to treat the bottom.
8. After extraction, RNA can be stored in RNA-ase free TE or RNA-ase free 1mM EDTA.
9. There are guidelines online for RNAase free plasticware.

13.11 Qiagen RNA extraction

(adapted from RNeasy Mini Kit Handbook, RNeasy Plus Universal Handbook and Fuxjager Lab)

For purification of total RNA from animal tissues. RNeasy Mini Kit Protocol (Qiagen #73404).

RNeasy Plus University Mini Kit combines the use of Qiazol with the kit - we should probably switch to this kit (Qiagen #73404).

Introduction

This kit purifies all RNA molecules longer than 200 nucleotides from up to 50 mg of tissue (or up to 100 mg of brain or adipose tissue) per RNeasy Mini spin column.

Safety Information

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste

Trizol Lysis Reagent and Buffer RW1 contain guanidine thiocyanate. Guanidine salts can form highly reactive compounds when combined with bleach. If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water.

Use a double pair of nitrile gloves when handling Trizol.

The solid waste of anything that touches the bugger and/ or reagents of the RNeasy Mini Kit, Trizol, or chloroform should be discarded in the Organic Solvent Carboy, and the solids that touch these solutions should be discarded in the Research Solid Waste.

Consumable supplies

- RNeasy Mini Kit (Qiagen #74104)
- 1.5 microcentrifuges tubes (4 per sample)
- Culture test tubes (1 per sample plus 4 tubes for washing)
- 75% ethanol (to clean counter)
- RNase Away (VWR #72830-022)

- Bleach
- Kimwipes
- Chloroform (try to use chloroform without ethanol if possible)
- Trizol
- 70% ethanol (molecular grade and made with DEPC treated water)
- DEPC treated water
- Nitrile Gloves (why Nitrile) (Use of Trizol and Phenol which are highly toxic and corrosive requires a thicker, more durable glove than latex)

Equipment

- Tissue homogenizer (9001271) (alternative homogenizer with exchangeable probes (probes Cat#02-070-MGXL-12) (homogenizer - any PRO homogenizer model from pro scientific).
- Centrifuge 5430 R (Eppendorf)

Things to do before

- Make sure RPE buffer has 4 volumes of ethanol (100%)
- Prepare your 70% diluted ethanol with DEPC treated water
- Set centrifuge to 4°C
- Label 4 microcentrifuge tubes per sample (1 will be used for the final storage of RNA, and 1 for the working stock to use for quantity and quality assessment)
- Label 1 RNeasy Mini spin column per sample
- Get one 2ml additional collection tube per sample from the RNA kit.
- Clean your working area (hood) and supplies with Bleach, then Ethanol (75%), then RNase Away, then Ethanol (75%)
- Wash the homogenizer with Trizol (*Trizol source should be kept in ice or put back in the 4C refrigerator when not in use).

- Label a culture tube for each of the four steps needed to clean the homogenizer (2 water steps, 1 ethanol step and 1 Trizol step).
- Get ice (to transfer reagents and RNA) (ice code is 6907)

Protocol

*these steps are to be done in the hood unless noted otherwise

1. Sterilize your tools for handling tissue and minimizing contamination among samples **This step and the weighing of the tissue can be done outside the hood
2. Add 1ml volume of Trizol Lysis reagent to the culture test tubes (prepare a tube per sample and one additional trizol tube for washing)
3. ***Gloves: If accidental contact occurs, remove and discard contaminated gloves immediately (The breakthrough time for a 4 mm nitrile glove is approximately 3 minutes for chloroform)
4. * Put the Trizol back on ice or in the 4C refrigerator
5. Add 1ml volume of DEPC treated water to two culture tubes for washing the homogenizer (be sure to label tubes to not confuse with ethanol wash tube)
6. Add 1ml volume of 70% ethanol molecular grade to a culture tube for washing the homogenizer
7. Clean homogenizer before the first sample and between each homogenization of the samples following these steps:
 - clean the probe with a tissue and rinse in sterile DEPC treated water
 - dry the probe with a tissue and rinse in 70% ethanol (molecular grade and made with DEPC treated water)
 - dry the probe with a tissue and rinse in sterile DEPC treated water
 - dry the probe with a tissue and rinse in trizol
8. Get your samples out from the -20 (once you have everything set up and ready to work with the samples)

9. Weigh and transfer your tissue to a culture tube with Trizol for homogenization: 100 mg of brain tissue, 30 mg of adipose, liver, spleen, or thymus tissue, or 50mg tissue for other tissues
10. * When weighing tissue stabilized in RNAlater, remove any crystals that may have formed (with sterilized forceps)
11. * Tissues not treated with RNAlater should not be allowed to thaw and the tissue should be cut into smaller parts in dry ice
12. Homogenize the lysate using the Tissue Rupture for 20-40 seconds depending on the toughness and size of the sample
13. Place the tip of the probe half the distance from the bottom of the tube and against the side of the tube. This will minimize foaming. Half the speed will sufficiently disrupt the tissue without producing foam
14. When homogenization is complete, decrease the speed of the probe to low and gently tap the probe against the side of the tube and remove from the solution in order to minimize the amount of sample remaining in the probe
15. Wash homogenizer between samples using the steps explained above
16. * Foaming may occur during homogenization. Let the homogenate stand at room temperature for 2-3 min until the foam subsides before continuing with the procedure
17. Transfer the lysate to a labeled 1.5 mL epi tube
18. Place the tube containing the homogenate on the benchtop at room temperature (15-25C) for 5 min
19. Change your gloves
20. Add 200 ul chloroform (use chloroform without ethanol if available). Securely cap the tube containing the homogenate, and shake it vigorously for 15s
21. Place the tube containing the homogenate on the benchtop at room temperature for 2-3min
22. Centrifuge at 13,000 x g for 15 min at 4C
23. After centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. For tissues with an

especially high fat content, an additional, clear phase may be visible below the red, organic phase. The volume of the aqueous phase should be approximately 600 uL

24. After centrifugation, heat the centrifuge to room temperature (15-25C)
25. To warm up the Centrifuge 5430R, set the centrifuge to 25C and press the fast button
26. Transfer ~80% of the upper aqueous phase to a new 1.5 mL epi-tube, be careful not to take any of the middle phase
27. With a 200ul pipette, remove 200ul at a time. Ideally, only remove 400ul total from the upper aqueous phase
28. Discard the middle and bottom phase
29. Add 1 volume (usually 400-500 ul) of 70% ethanol to the recovered aqueous phase, and mix thoroughly by pipetting up and down
30. Precipitates may be visible after addition of ethanol. Resuspend precipitates completely by vigorous shaking, and proceed immediately to step
31. Transfer up to 700 ul of the sample to an RNeasy Mini spin column placed in a 2ml collection tube. Close the lid gently, and centrifuge for 15s at 13000 x g at room temperature (15-25C). Discard the flow-through. Repeat the step with any remainder of the sample using the same collection tube
32. Add 700 ul Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15s at 13000 x g to wash the membrane. Discard the flow-through. Reuse the collection tube in the next step
33. After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow- through. Be sure to empty the collection tube completely
34. Add 500 ul Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15s at 13000 x g to wash the membrane. Discard the flow through. Reuse the collection tube for the next step
35. Add 500 ul Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at 13000 x g to wash the membrane. Discard the flow through

36. After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow- through. Otherwise, carryover of ethanol will occur
37. Place the RNeasy spin column in a new 2ml collection tube, and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at 18000 x g for 1 min (to remove carryover of Buffer RPE)
38. Place the RNeasy spin column in a new labeled 1.5ml epi tube. Add 30-50 ul RNase-free water directly to the spin column membrane. Close the lid gently. To elute the RNA, centrifuge for 2:30 min at 13000 x g
39. * for higher RNA recovery, incubate the RNeasy spin column on the benchtop for 1-2 min with RNase-free water before centrifuging.
40. Place the recovered RNA on ice right away and then transfer to the -20
41. Repeat the above step using another volume of RNase-free water, or using the eluate from step 15 (if high RNA concentration is required). Reuse the collection tube from prior step
** reusing the eluate will be 15-30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher
42. Place the recovered RNA on ice
43. Transfer 5 uL of eluted RNA into second 1.5 mL epi tube as a working stock: 1 uL will be used for Nanodrop quality assessment, 1 uL will be used for Qubit quality assessment, 2 uL will be used for Agarose gel quality assessment

****Make note of phenol solving problem in black rockfish breakdown**

13.12 DNA-Free RNA Kit

(Now Called RNA Clean & Concentrator-5 from Zymo Research)

Cleans up to ~5ug of RNA product from DNA and some impurities. Although the last RNeasy Mini Kit contains some DNase1, most protocols recommend doing an additional cleaning step with the DNA-Free RNA Kit.

Consumable supplies

- RNA Clean & Concentrator -5 Kit (Zymo Research #R1013)
- 100% molecular grade ethanol
- 1.5 mL epi tubes (3 per sample)

Things to do before

- Make sure the RNA wash buffer has the appropriate amount of ethanol added.
- If using a new kit before starting, add 48 ml 100% ethanol (52 ml 95% ethanol) to the 12 ml RNA Wash Buffer concentrate (R1013) or 96 ml 100% ethanol (104 ml 95% ethanol) to the 24 ml RNA Wash Buffer concentrate (R1014).
- Label 3 RNase-free 1.5 mL epi tubes per sample.
- 1 epi tube to be used to transfer the amount of RNA needed for the DNase I mix.
- 1 epi tube to be used for the elution of your RNA.
- 1 epi tube to be used as a working stock of your eluted RNA for quality. and quantity assessment.
- Label 1 column collection tube per sample
- Bring 100% molecular grade ethanol.
- *** Follow RNA working procedures to assure the procedure is performed in an RNase-free environment (work in the hood, clean everything well, keep your samples in ice - when they are not in columns, etc).

- *** Some of the reagent contains Chaotropic reagents. Irritant. Please discard the waste of this procedure appropriately and use proper safety precautions with the reagents.

Protocol

(for small RNA elimination for total RNA of > 200 nt)

** Please look at the original protocol from Zymo if you want to get RNA smaller than 200 nt.

1. Digest RNA samples with DNase I.

- Transfer 20 ul of RNA sample ((<5ug) in water or TE Buffer) to a 1.5 mL epi tube
- Add the following mix to each sample RNA sample:

Reagent	Number of samples 1X (uL)
10x DNase I Buffer	5 uL
RNase-Free DNase I	2 uL
RNase-Free Water	23 uL

2. Mix 1 volume of RNA Binding Buffer with 1 volume ethanol (95-100%) (e.g., 50 ul buffer and 50 ul ethanol).
3. Add 2 volumes of the adjusted buffer from step 2 to 1 volume of RNA sample mix from step 1 (e.g., 100 ul adjusted buffer and 50 ul sample) and mix well.
4. Transfer the mixture to the Zymo-Spin IC Column in a Collection Tube and centrifuge at $\geq 12,000 \times g$ for 1 minute. Discard the flow- through.
5. Add 400 ul RNA Prep Buffer to the column and centrifuge at $\geq 12,000 \times g$ for 1 minute. Discard the flow-through.
6. Add 800 ul RNA Wash Buffer to the column and centrifuge at $\geq 12,000 \times g$ for 30 seconds. Discard the flow-through. Repeat the wash step with 400 ul RNA Wash Buffer.
7. Centrifuge the Zymo-Spin IC Column in an emptied Collection Tube at $\geq 12,000 \times g$ for 2 minutes. Remove the Zymo-Spin IC Column carefully from the Collection Tube and transfer it into an epi tube.

8. Add \geq 6 ul of DNase/RNase-Free Water directly to the column matrix and let stand for 1 minute at room temperature. Centrifuge at 10,000 x g for 30 seconds. The eluted RNA can be used immediately or stored at -70°C.

** Waiting for 1 to 2 minutes after adding the RNase-free water to the column matrix may increase RNA yield. Also, the yield may be increased by performing a second elution.

9. Remove 5 ul of the RNA, if you have enough RNA for your work, and transfer it to a new 1.5 mL epi tube to be used for your quality and quantity assessments (this will protect your original RNA from thawing and additional exposure to the environment).

- 1 uL will be used for Nanodrop quality/quantity assessment
- 1 uL will be used for Qubit quality/quantity assessment
- 2 uL will be used for Agarose gel quality assessment

14 ddRAD-seq

Amplified restriction fragments for genomic enrichment **NOTE THIS SECTION IS UNDER CONSTRUCTION**

14.1 DNA Extraction

Follow instructions for Qiagen DNA Blood & Tissue Kit (page 28). We don't use RNA removal, although if we were going to do it we would add RNase A (cat # 19101) while extracting the DNA with the Qiagen DNA Blood & Tissue Kit. At the end of the extraction, store the DNA in autoclaved Milli-Q water.

Should we recommend beadwash after DNA Extraction?

14.2 Overview of ddRAD Protocol

Genomic DNA is first digested with two restriction enzymes. This results in a pool of fragments that have the sticky- ended restriction cut sites on either end, and these ends provide a template for ligation of the customized adaptor sequences. The adaptor sequences contain the Illumina adaptors and primer sequences for multiplexing sequencing (and hence provide a binding site for the Illumina PCR primers), and the first barcodes of 5-8 base pairs are incorporated to the left adaptor. The bases at one of the end of each adaptor oligo correspond to the ligation site; that is, they match the restriction cut site. NOTE THAT WE MAKE SURE OUR BARCODE DESIGN DOES NOT RE-CREATE THE RESTRICTION SITE. This can be important when using MspI, which is not inactivated by heat. The ligation reaction is pooled and concentrated with a bead wash in preparation for the size selection step. Size selection allows the fragments that are too small or oversized to be removed. The fragments of the appropriate size are amplified through a Polymerase Chain Reaction where the fragments with the left and right adapters get amplified (fragments that carry the wrong combination of adapters won't amplify due to the selectiveness of the right adapter). The amplified products now have 2 barcodes, and they can be pooled and concentrated with a bead wash. The final library is good for paired-end sequencing.

In January 2016 we ordered new adapters and made some modifications. The first modification was to use the Peterson protocol, instead of a mix of different protocols. The second modification was the adapters. Both the p5 and p7 adapters were designed to be offset by a 0-2 bp insertion. For both reads, this will offset the sequencer at the restriction site so that it is not blinded by the laser. The p7 adapter (right) was designed to also contain a 4-bp degenerate base sequence that we can use to identify PCR duplicates. This sequence was accompanied by another 4-bp that were designed to make sure the adapters anneal together well.

FOR DDRAD, YOU SHOULD CHOOSE ENZYMES THAT ARE NOT SENSITIVE TO DNA METHYLATION.

A) Sphi-HF and MspI/Hpall

5' GCATG^CNNNNNN-NNNNNNNC^CGG 3'
 3' C^GTACGNNNNNN-NNNNNNNGGC^C 5'

Fragment after digestion:

5' CNNNNNN-NNNNNNNC 3'
 3' GTACGNNNNNN-NNNNNNNGGC 5'

Ligation (P5 on left and P7 on right):

5' ACACTTTCCCTACACGAGCTTCCGATCTBARCOINSATG CNNN-NNNNC CGINCCIINNNNAGATCGGAGAGCGAGAACAA 3'
 3' TGTGAGAAAGGGATGTCGAGAAGGCTAGAOCRABDSNI GTACGNNN-NNNNNGGC NIIGMMHNNNNCTAGCCTTCTCGTGTGACACTTGAGGTCACTG 5'

The insertion site (0-3bp) functions to offset and increase sequence variability to preserve the lasers of the sequencer from blinding by the identical bases in the restriction sites.

- (M) A or C
- (H) A, C, or T
- (N) A, C, T, or G
- (I) 2'-deoxyinosine (bonds C, A, T, then G)

For P5: Make sure barcode or insertion site (whichever is last) does not end with a "G" or restriction site will be re-created.
 For P7: Make sure barcode or insertion site (whichever is last) does not begin with "G" or restriction site will be re-created.

BARCODE is the 6bp barcode

NNNNNIIICC (from Schwenen paper) On the top strand five Ns are located next to three 2'-deoxyinosine (I), which can pair with any of the four DNA bases, preferring, in decreasing order, C, A, and T (Watkins and SantaLucia, 2005), followed by two C-residues: 5' NNNNNIIICC 3'. The design of this fragment is expected to minimize mispairings of the top and the bottom strands during hybridization. Hybridization of both strands was performed according to Peterson et al. (2012), with the exception of the temperature profile (97 °C for 5 min followed by a decrease in temperature of 2 °C per min in a 1.5-ml Eppendorf tube in a thermal block.

PCR:

5'AATGATAACGGCACCACCGAGATCTACACTTTCCCTACACGACGCTTCCGATCT 3'>>
 5' ACACTTTCCCTACACGACGCTTCCGATCTBARCOINSATG CNNN-NNNNC CGINCCIINNNNAGATCGGAGAGCGAGAACAA 3' DIVERGENT Y'
 3' TGTGAGAAAGGGATGTCGAGAAGGCTAGAOCRABINS GTACGNNN-NNNNNGGC NIIGMMHNNNNCTAGCCTTCTCGTGTGACACTTGAGGTCACTG 5'
 <<<3' CGTGTGCAGACTTGAGGTCACTGXEDNIAAGAGCATACGGCAGAGACGAAC 5'

Illum. Read-1 5' ACACTTTCCCTACACGACGCTTCCGATCT 3' >>
 5' GATCGGAAGAGCACACGCTGAACTCCAGTCAC >> Illumina index read after read 1
 5' AATGATAACGGCACCACCGAGATCTACACTTTCCCTACACGACGCTTCCGATCTBARCOINSATG CNNN-NNNCCGINCCIINNNNAGATCGGAGAGCACACGCTGAACTCCAGTCACINDEXXTCTCGTATGCCGTCTCTGCTTG 3'
 3' TTATATGCCGCTGGCTCTAGATGTGAGAAAGGGATGTCGAGAAGGCTAGAOCRABINS GTACGNNN-NNNNGCNIGGMHNNNNCTAGCCTTCTCGTGTGAGACTTGAGGTCACTGXEDNIAAGAGCATACGGCAGAGACGAAC 5'
 <<<3' TCTAGCCTTCTCGTGTGAGACTTGAGGTCACTG 5' Illumina Read 2

OLIGOS
 P7 PCR primers

Peterson
 Generic CAAGCAGAAAGACGGCATAACGAGATINDEXXGTGACTGGAGTTAGACCTGTG*C

12 of these

P7 adapters

After annealing separately, these adapters will be applied to each individual at random.

CCCCIIINNNNAGATCGGAAGAGCGAGAACAA
 CCCCCIIINNNNCAGATCGGAAGAGCGAGAACAA
 CCCCCIIINNNNATAGATCGGAAGAGCGAGAACAA

GTGACTGGAGTTACACGTTGCTTCCGATCTNNNNHHMGG
 GTGACTGGAGTTACACGTTGCTTCCGATCTNNNNHHMGG
 GTGACTGGAGTTACACGTTGCTTCCGATCTNNNNHHMGG

- (M) A or C
- (H) A, C, or T
- (N) A, C, T, or G
- (I) 2'-deoxyinosine

P5 primer

P5_primer_PCR1 AATGATAACGGCACCACCGAGATCTACACTTTCCCTACACGACGCTTCCGATCT

1 of these

P5 adapters

Generic
 Generic reverse complement
 INSOCRABAGATCGGAAGAGCGTGTGAGGGAAAGAGTGT

16 of these

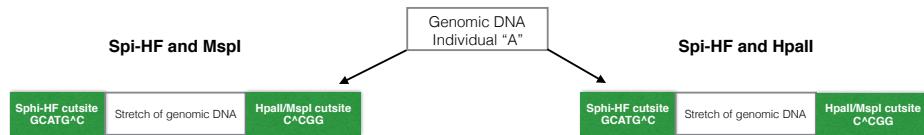
Figure 1: ddRAD and methylRAD adapter design

14.3 Overview of methylRAD Protocol

The methylRAD protocol follows the ddRAD protocol, except first the DNA from each individual is split in half. Each half is digested with a pair of restriction enzymes: R1-MspI or R1-HpaII. HpaII will not cut at a methylated site, so we predict that we can compare the proportion of reads at the cutsite to infer whether that site is methylated. Here is the conceptual idea:

Preparation of an EpiRAD genomic library

1) Split genomic DNA in half and digest with a different pair of enzymes



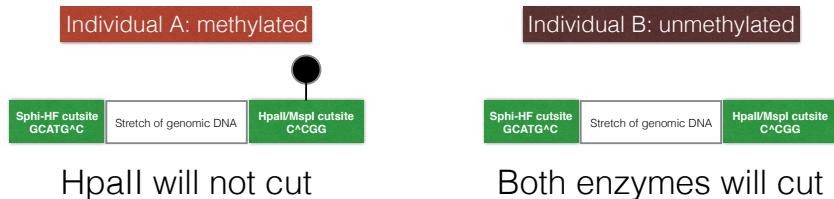
2) Ligate individual barcodes and adapters for PCR to each half



3) Add indexes during PCR (specific to enzyme pair) and Illumina® sequencing primers



Figure 2: Overview of methylRAD library prep



After library prep, sequencing, and alignment:

BarcodeA	cutsite	Read 1	Read 2	cutsite	MspI Index
BarcodeA	cutsite	Read 1	Read 2	cutsite	MspI Index
BarcodeA	cutsite	Read 1	Read 2	cutsite	MspI Index
BarcodeA	cutsite	Read 1	Read 2	cutsite	MspI Index
BarcodeA	cutsite	Read 1	Read 2	cutsite	MspI Index
BarcodeA	cutsite	Read 1	Read 2	cutsite	MspI Index

100% reads have MspI Index

BarcodeB	cutlet	Read 1	Read 2	cutsite	HpaII Index
BarcodeB	cutsite	Read 1	Read 2	cutsite	HpaII Index
BarcodeB	cutlet	Read 1	Read 2	cutsite	HpaII Index
BarcodeB	cutlet	Read 1	Read 2	cutsite	MspI Index
BarcodeB	cutlet	Read 1	Read 2	cutsite	MspI Index
BarcodeB	cutsite	Read 1	Read 2	cutsite	MspI Index

50% reads have MspI Index

Figure 3: Overview of methylRAD bioinformatics

14.4 Before you start: testing restriction enzymes with your DNA

TO DO

14.5 Before you start: order oligos and anneal adapters

We can order oligos through Eurofins Genomics through Fisher on Marketplace. The PCR primers and oligos should be designed in a Word document according to the template outlined above. The PCR primers should be universal. You can modify the Rscript MakeddRADoligos_SphI_MspI.R for adapters for your specific restriction enzymes. BE CAREFUL - ordering oligos is a large expense and it is easy to make mistakes. I also suggest using CostAnalysis_ddRAD.R to think about optimal cost-effective design for your study. With very large sample sizes, it is worthwhile to invest in more barcodes.

When you receive them, you should resuspend the primers which is described in section [16.7](#). Then you organize the oligos in PCR strip-tubes or on a PCR plate so that it is easy to keep them in order. Then you should anneal the adapters together, which is described next and make aliquots, which is described in section [ADD REF](#).

14.6 Preparing working stocks of Oligos for ddRAD-seq (Annealing and Hybridization of Adapters)

Adapted from the Amplified restriction fragments for genomic enrichment protocol version 2.6

This protocol is meant to create working stocks of oligos to be used for ddRAD-seq. All of the adaptors and the primers have a barcode, so it is important to make sure that all of the adaptors and primers combinations have matching barcodes.

These adaptor sequences come in pairs and after annealing become one double-stranded adaptor. The barcodes are chosen from the Peterson protocol but may also be created using python scripts described in (Meyer & Kircher, 2010). Further information on the python scripts can be found at (<http://bioinf.eva.mpg.de/multiplex/>). A second barcode will be attached on left side after PCR amplification (index). The second barcodes comes from the Peterson et al. 2012 flex adapters.

14.6.1 Peterson protocol

For the annealing buffer, might want to try NEBuffer 4 (B7004S).

1. To create Adapter P1 (P5 or "left"), combine each oligo 1.1 with its complementary oligo 1.2 in a 1:1 ratio in working strength annealing buffer (final buffer concentration 1x) for a total annealed adapter concentration of 40uM (for example, if purchased oligos are resuspended to an initial concentration of 100uM, use 40ul oligo 1.1, 40ul oligo 1.2, 10ul 10x annealing buffer and 10ul nuclease-free water). Do the same for oligos 2.1 and 2.2 to create the common adapter P2 (P7 or "right"). $(100\mu M * 40\mu L) / (100\mu L \text{ final volume}) = 40\mu M$
2. Combine in strip tubes or covered PCR plate:
 - 40 uL P1.1 or P2.1 "top" Adapter (stock concentration 100 uM)
 - 40 uL P1.2 or P2.2 "Bottom" Adapter (stock concentration 100 uM)
 - 10 uL 10x Annealing Buffer or nuclease free water (buffer comes with T4 ligase from NEB)
 - 10 uL H2O
3. In a thermocycler, incubate at 97.5C for 2.5 minutes, and then cool at a rate of not greater than 3C per minute until the solution reaches a temperature of 21C. Hold at 4C.
4. Setup of annealed primer stock in the freezer with each individual annealed adapter in a single tube setup in 96 well format. TREAT THESE LIKE GOLD- please take aliquots.

14.6.2 Schweyen protocol for P2/P7/"right" adapters with degenerate sequence

Hybridization of both strands was performed according to Peterson et al. (2012), with the exception of the temperature profile (97C for 5 min followed by a decrease in temperature of 2C per min in a 1.5-ml Eppendorf tube in a thermal block).

14.7 Before you start: choose barcodes and indexes

You should choose the barcodes and indexes you plan to use to tag and identify your individuals. The "left" barcode is ligated on when the adapters are ligated; the "right" index is

added in PCR. Thus, ligation must be performed separately, but individuals with different "left" barcodes can be pooled together for size selection and PCR. This can save \$ - another reason to think about your sampling design before starting.

For the indexes, illumina uses a green laser to sequence G/T and a red laser to sequence A/C. At each cycle at least one of two nucleotides for each color channel need to be read to ensure proper registration. It is important to maintain color balance for each base of the index read being sequenced, otherwise index read sequencing could fail due to registration failure.

502	C T C T C T A T
502	C T C T C T A T
503	T A T C C T C T
503	T A T C C T C T
	✓ ✓ ✓ ✓ X X X X

Figure 4: An overview of the low-plexity problem with Illumina

PCR2_01	_ATCACG	CAAGCAGAAGACGGCATACGAGA	T	C	G	T	G	A	T		GTGACTGGAGTTCAAGACGTGTG*C
PCR2_02	_CGATGT	CAAGCAGAAGACGGCATACGAGA	T	A	C	A	T	C	G		GTGACTGGAGTTCAAGACGTGTG*C
PCR2_03	_TTAGGC	CAAGCAGAAGACGGCATACGAGA	T	G	C	C	T	A	A		GTGACTGGAGTTCAAGACGTGTG*C
PCR2_04	_TGACCA	CAAGCAGAAGACGGCATACGAGA	T	T	G	G	T	C	A		GTGACTGGAGTTCAAGACGTGTG*C
PCR2_05	_ACAGTG	CAAGCAGAAGACGGCATACGAGA	T	C	A	C	T	G	T		GTGACTGGAGTTCAAGACGTGTG*C
PCR2_06	_GCCATT	CAAGCAGAAGACGGCATACGAGA	T	A	T	T	G	G	C		GTGACTGGAGTTCAAGACGTGTG*C
PCR2_07	_CAGATC	CAAGCAGAAGACGGCATACGAGA	T	G	A	T	C	T	G		GTGACTGGAGTTCAAGACGTGTG*C
PCR2_08	_ACTTGA	CAAGCAGAAGACGGCATACGAGA	T	T	C	A	A	G	T		GTGACTGGAGTTCAAGACGTGTG*C
PCR2_09	_GATCTG	CAAGCAGAAGACGGCATACGAGA	T	C	T	G	A	T	C		GTGACTGGAGTTCAAGACGTGTG*C
PCR2_10	_TAGCTT	CAAGCAGAAGACGGCATACGAGA	T	A	A	G	C	T	A		GTGACTGGAGTTCAAGACGTGTG*C
PCR2_11	_GGCTAC	CAAGCAGAAGACGGCATACGAGA	T	G	T	A	G	C	C		GTGACTGGAGTTCAAGACGTGTG*C
PCR2_12	_CTTGTG	CAAGCAGAAGACGGCATACGAGA	T	T	A	C	A	A	G		GTGACTGGAGTTCAAGACGTGTG*C

Figure 5: The Illumina indexes, by color code (Alan Downey-Wall).

Illumina has a tool you can use to check your indexes. You can download the Experiment Manager from the Illumina website at <http://www.illumina.com>. Go to the Nextera DNA Library Preparation support page and click Downloads. A MyIllumina account is required. IEM will notify you if improper index combinations are used when creating a sample sheet for use with CASAVA, so it is highly recommended to create your sample sheet prior to performing library prep/ pooling. The IEM tool can be run on any Windows platform. Or, you can just do it by hand.

Table 1: Example of ddRAD LOW PLEXITY experimental barcoding setup

Individual	"left" barcode	"right" index MspI	"right" index HpaII
I1	SphI_02	PCR2_09	PCR2_12
I2	SphI_04	PCR2_09	PCR2_12
I3	SphI_07	PCR2_09	PCR2_12

Table 2: Example of ddRAD experimental barcoding setup

Individual	"left" barcode	"right" adapter
I1	Sph01	Index01
I2	Sph02	Index01
I3	Sph03	Index01
I...	Sph...	Index01
I16	Sph16	Index01
I17	Sph01	Index02
I18	Sph02	Index02
I...	Sph...	Index02
I32	Sph16	Index02
I33	Sph01	Index03
I34	Sph02	Index03
I...	Sph...	Index03
I48	Sph16	Index03

14.7.1 Note on low plexity pooling

For the SphI adapters and PCR2 indexes ordered on 201601, the following can be used for low plexity:

14.7.2 Example of ddRAD experimental barcoding setup

14.7.3 Example of methylRAD experimental barcoding setup

Table 3: Another example with $3 \times 24 = 72$ different barcode combinations (L is the left barcode and R is the right barcode)

L1 R1	L9 R1	L17 R1	L1 R2	L9 R2	L17 R2	L1 R3	L9 R3	L17 R3
L2 R1	L10 R1	L18 R1	L2 R2	L10 R2	L18 R2	L2 R3	L10 R3	L18 R3
L3 R1	L11 R1	L19 R1	L3 R2	L11 R2	L19 R2	L3 R3	L11 R3	L19 R3
L4 R1	L12 R1	L20 R1	L4 R2	L12 R2	L20 R2	L4 R3	L12 R3	L20 R3
L5 R1	L13 R1	L21 R1	L5 R2	L13 R2	L21 R2	L5 R3	L13 R3	L21 R3
L6 R1	L14 R1	L22 R1	L6 R2	L14 R2	L22 R2	L6 R3	L14 R3	L22 R3
L7 R1	L15 R1	L23 R1	L7 R2	L15 R2	L23 R2	L7 R3	L15 R3	L23 R3
L8 R1	L16 R1	L24 R1	L8 R2	L16 R2	L24 R2	L8 R3	L16 R3	L24 R3

Table 4: Example of methylRAD experimental barcoding setup

Individual	"left" barcode	"right" adapter MspI	"left" barcode	"right" adapter HpaII
I1	Sph01	Index01	Sph01	Index02
I2	Sph02	Index01	Sph02	Index02
I3	Sph03	Index01	Sph03	Index02
I...	Sph...	Index01	Sph...	Index02
I16	Sph16	Index01	Sph16	Index02
I17	Sph01	Index03	Sph01	Index04
I18	Sph02	Index03	Sph02	Index04
I...	Sph...	Index03	Sph...	Index04
I32	Sph16	Index03	Sph16	Index04

14.8 Reagents and Equipment

- Note that we chose these enzymes because they were not sensitive to methylation.
- Restriction Enzyme 1: (depends) SphI-HF (NEB, 20,000 units/ml) R3182S
- Restriction Enzyme 2: (depends) MspI (R0106S), HpaII (R0171S) or (MluCI NEB, 10,000 units/mL R0538S)
- 10x Concentrate Enzyme Buffer (part of the enzyme package)
- T4 DNA Ligase (NEB, 400,000 units/mL) M0202L
- 10x T4 buffer (part of the Ligase package)
- BioRad Iproof High Fidelity DNA polymerase Cat # 172-5301 (424 for 250 uL [500 units])
- DMSO (part of the package of the Iproof polymerase)
- MgCl₂ (part of the package of the Iproof polymerase)
- 1 mg/mL BSA (BSA may not be needed, it is now incorporated in the enzyme buffer, but we keep it in anyway) Cat # BP675-1 Fisher
- 1 M NaCl (follow reagent recipe)
- dNTP Cat # R0181 Thermo Scientific
- SphI-HF adaptors (add page number for ordering adaptors)
- MluCI adaptors (add page number for ordering adaptors)
- Milli-Q Water
- Thermal Cyclers
- 96 well plates and strip caps or PCR strip tubes Cat # AB-0600 Thermo Scientific
- 1.5 microcentrifuge tubes for PCR mixes (any 1.5 microcentrifuge would work)
- Full plate centrifuge (if using a full plate)

- Qubit (we used the Qubit 3.0 Cat #Q33216) and special tubes Qubit Cat # Q32856 or VWR Cat # PCR-05-C
- Note that the concentrations used in our protocol are from a Qubit, which is more accurate than a Nanodrop (the Nanodrop estimates concentrations 2-10x higher than the Qubit). Agarose and gel electrophoresis materials (see Section Quality assessment of DNA and RNA with gel electrophoresis)
- 0.1X TE
- Homemade DNA beads (please see the Home made bead protocol if you need to make them) or
- EtOH molecular grade
- QIAquick Gel Extraction Kit (#28704)

14.9 Things to do before starting

- Make sure the adaptors are annealed, and also make sure the adaptors and the primer mix are easily accessible in plate format. Please see the section above.
- Be familiar with the Pippin Prep and how to use it.
- Know what size range you want to select - see details in size selection section. To do this, you should have a plan for sequencing.
- Make sure all of the supplies needed for the protocol are present and are sufficient for the number of samples that you will be processing. This protocol is long and it has few stopping steps.
- Make sure the enzyme combination is the best for your organisms. The types of enzymes and the digestion length should be checked at least one time prior this protocol.

14.10 Protocol

14.10.1 1. Restriction Double Digest (~ overnight)

From NEB website: There are several key factors to consider when setting up a restriction endonuclease digestion. Using the proper amounts of DNA, enzyme and buffer components in the correct reaction volume will allow you to achieve optimal digestion. By definition, 1 unit of restriction enzyme will completely digest 1 μg of substrate DNA in a 50 μl reaction in 60 minutes. This enzyme : DNA : reaction volume ratio can be used as a guide when designing reactions. However, most researchers follow the "typical" reaction conditions listed, where a 5–10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity. NEB offers the following tips to help you to achieve maximal success in your restriction endonuclease reactions. NEB recommends 10 units restriction enzyme, 1 μg DNA, 1X NEB Buffer (5 μL), and 1 hour incubation time.

DNA (measured with the Qubit) should ideally be at a minimum total amount of 1.5 μg and no more than 2.5 μg . For example, you could use 10 μL of 150-250 ng/ μL of DNA. Keep on ice. Note that your DNA needs to be concentrated at least 125 ng/ μL for this protocol (2,500 ng desired/125ng/ μL = 20 μL + may need 5-7 μL master mix depending on enzymes).

For each sample prepare a master mix I (Table 1), mix by vortexing and then centrifuge. For these and all other reactions make sure to prepare an excess of mix to accommodate multiple rounds of pipetting, particularly if you are working with whole plates. Because the enzymes are stored in glycerol and other viscous solutions, a substantial volume is lost through adhesion to the outside of pipette tips. We suggest making more than what you think you will need (this may be overkill, if you have fewer columns than just do one extra per column).

Reagent	Number of samples 1X (μL)
20 units of Enzyme 1	(Calc)
20 units of Enzyme 2	(Calc)
(10x Concentrate Enzyme Buffer)	3 ul
Genomic DNA (1.5-2.5 μg)	(Calc)
Molecular grade H ₂ O to make a final volume of 30 μL	(Calc)

Table 1: Reagents and volumes for Restriction Digest master mix I (30 μL prepared per sample).

Note: The original protocol listed 10xT4 Buffer instead of the 10X Concentrate Enzyme Buffer. We think this may have been a typo. 10xT4 Buffer is designed to work with the Ligase (we use Ligase in the following section) and the 10x Concentrate Enzyme Buffer is designed to optimize the enzyme. See NEB website.

1. Incubate samples at 37C overnight. NOTE THAT MspI CANNOT BE KILLED BY HEAT.
SUGGEST PROCEEDING IMMEDIATELY TO BEAD WASH.
2. After incubation, let reaction cool to room temperature during this time also remove the AMPURE XP beads from the fridge and let them warm to room temperature
3. Add 45 uL of AMPURE XP to each reaction, mix with pipetting 10 times, and then let incubate for 5 mins on the bench
4. Place plate onto the magnet plate and let the beads separate for 5 mins
5. Carefully remove the supernatant from each well and discard
6. Note that you will not be able to remove all of the supernatant without disturbing the beads. It's best to leave about 5 uL remaining in each well
7. Leaving the plate in the magnet, wash the reaction by adding 200 uL of FRESH 70% ethanol **70% ethanol should be mixed that day!**
8. Remove and discard ethanol and repeat with a second ethanol wash. (From Gold Lab Protocol: Puritz prefers to use only 150 uL of ethanol for second wash. Hollenbeck prefers to use only 1 wash step)
9. After final wash, let plates air dry for 5-10 mins. All ethanol needs to be evaporated, but beads should not be overdried.
10. Remove plate from magnet and add 30 uL of molecular grade H2O to each well and incubate for 5 mins. Make sure that water comes into contact with beads by mixing well with pipet
11. Place plate back on magnet and let it separate for 2 minutes, then remove supernatant and transfer to clean plate
12. If doing a second set of digestion, repeat steps 4-11.

14.10.2 Digest Quantification

Check quantification of DNA on Qubit. For extra accuracy, reread standards every 48 samples.

These numbers will be used in next step.

Typically get between 10ng/uL and 100ng/uL.

Optional sanity check: Run your restriction digest product on a gel and compare to genomic DNA.

14.10.3 Adaptor Ligation (~2-3 hours) - proceed directly to pooling after

1. Thaw adaptors (or the L and R adaptors). Have these adaptors annealed and easily accessible in plate format.
2. For each sample, calculate the amount of template needed for 100 ng of DNA and calculate the amount of H₂O to add to this amount by using the formula 22.2-(ul of Template)= uL of H₂O (total final volume should be 22.2 uL). If samples are of low DNA concentration, this can be adjusted to 31.2-(ul of Template)= uL of H₂O for all samples.
3. Place the correct amount of H₂O and then DNA into ligation plate A. This will take some time. Take out T4 ligase buffer to thaw during this time.
4. Transfer 2 uL of “left” adapter to each reaction. PAY ATTENTION TO YOUR DESIGN.
5. Transfer 2 uL of “right” adapter to each reaction. PAY ATTENTION TO YOUR DESIGN.
If the right adapter is universal, it can be incorporated into master mix.
6. Total ligation mix as follows:

Reagent	Number of samples 1X (uL)
UNIQUE DNA template	22.2
UNIQUE “left” universal adapter	2 uL
UNIQUE “right” universal adapter	2 uL
MASTER (NEB Buffer #4 10X) ligation buffer	3 uL
MASTER T4 ligase (Add this last)	0.8 uL

7. Add 3.8 uL of master mix to each reaction, as quickly as possible

8. Incubate on bench top for one hour, then place in thermocycler and heat-kill at 65C for 10 min. After the heat-kill, cool the solution at 2C per 90 seconds until it reaches room temperature. Hold at 4C or place in fridge.
9. Optional sanity check: you should have 100ng/30ul=3.33 ng/uL of DNA. Use Qubit HS dsDNA kit to double check.

14.11 Pooling and Beadwash

Set up your pools for size selection on the Pippin Prep. You should have experience using the Pippin before you perform this step.

1. After ligation, it is safe to pool samples within one index. Collect all 30 uL from each well and place it into a single 1.5 mL tube (Use a 2.0 mL tube if using 40 uL ligations). If you are using 16 barcodes within an index, this is $16 \times 30 = 480$ uL.
2. Mix each pool with pipetting or gentle vortexing. If it is a large volume split each into equal aliquots according to the number of replicate size selections. (These replicates will be combined in last step. It doesn't affect the total amount of beads used, and you can choose to do fewer if you don't have a lot of product).
3. Add 1.5X uL of AMPURE XP to each aliquot, mix with pipetting 10 times, and then let incubate for 5 mins on the bench. For instance, if you did two replicate size selections of a pool with 16 barcodes, each one would be $480\text{uL}/4 = 120$ uL of template and 180 uL of beads.
4. Place tube onto the magnet bar and let the beads separate for 5 mins
5. Carefully remove the supernatant from each tube and discard
6. Sometimes pulling tube upwards and tilting bottom towards the magnet allows for complete supernatant removal. If not, leave a few uL.
7. Leaving the tubes in the magnet, wash the reaction by adding 500 uL of FRESH 70% ethanol **70% ethanol should be mixed that day!**. Volume is approximate but should cover all beads
8. Remove ethanol, let tubes air dry for 2 mins. All excess ethanol needs to be evaporated, but shiny beads are OK and beads should not be overdried. Pippin is sensitive to EtOH so err on side of more dry.
9. Remove tubes from magnet and add 30 uL of TE buffer to each tube and incubate for 5 mins. Make sure that water comes into contact with beads by mixing well with pipet or gentle vortexing

10. IF DOING REPLICATES: Remove tubes from magnet and add 30 uL of molecular grade H₂O to each tube and incubate for 5 mins. Make sure that water comes into contact with beads by mixing well with pipet or gentle vortexing
11. IF NOT DOING REPLICATES: Remove tubes from magnet and add 30 uL of TE to each tube and incubate for 5 mins. Make sure that water comes into contact with beads by mixing well with pipet or gentle vortexing. (We use TE here because it helps the DNA stay in the well of the Pippin Prep).
12. Place tubes back on magnet and let beads separate for 2 minutes, then remove supernatant and transfer to a clean 1.5 mL tube. If you used replicates, pool within each index. This means going from multiple aliquots to one tube for each index.
13. IF YOU USED REPLICATES, DO THE FOLLOWING STEPS TO POOL. IF NOT, YOU ARE DONE. If you used replicates, add 1.5X uL of AMPURE XP to each tube, mix with pipetting 10 times, and then let incubate for 5 mins on the bench.
14. Place tubes onto the magnet bar and let the beads separate for 5 mins
15. Carefully remove the supernatant from each tube and discard. Sometimes pulling tube upwards and tilting bottom towards the magnet allows for complete supernatant removal. If not, leave a few uL.
16. Leaving the tubes in the magnet, wash the reaction by adding 500 uL of FRESH 70% ethanol. Volume is approximate but should cover all beads
17. Remove and discard ethanol, and let plates air dry for 2 mins
18. All ethanol needs to be evaporated, but beads should not be overdried. Pippin is sensitive to EtOH so err on side of more dry.
19. Remove tubes from magnet and add 20 uL of TE buffer to each tube and incubate for 5 mins A. Make sure that water comes into contact with beads by mixing well with pipet or gentle vortexing

20. Remove tubes from magnet and add 20 uL of TE to each tube and incubate for 5 mins A.
Make sure that water comes into contact with beads by mixing well with pipet or gentle vortexing. We use TE here because it helps the DNA stay in the well for the pippin.

21. Place tubes back on magnet and let beads separate for 2 minutes, then remove supernatant and transfer to a clean 1.5 mL tube

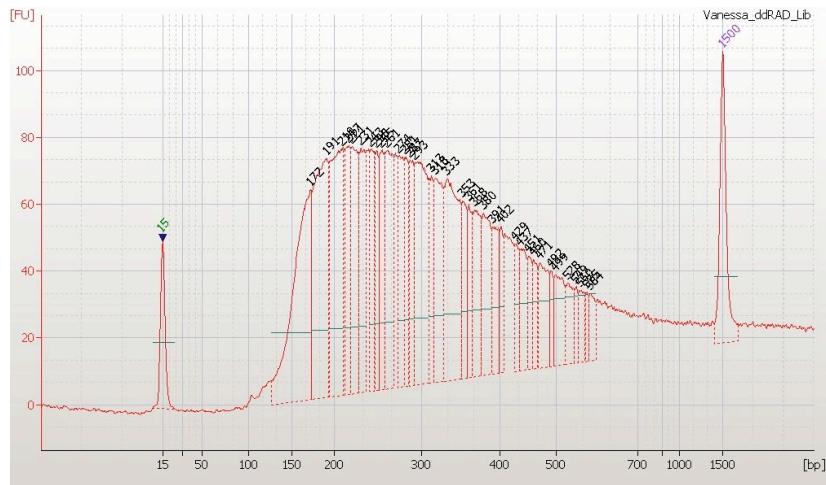


Figure 6: SAFE STOP POINT. Store at 4C for a month, or -20C for longer. It is important to save this, as all of this product is not used for the PCR, and if the results of the PCR do not look good, you can start again from this step.

14.12 Size selection (~ 2 hours)

We use a Pippin Prep to do size selection. Pippin works in a similar way to typical gel electrophoresis, but uses standardized gel cassettes and automated size selection, rather than a hand-poured gel and manual excision of fragments from a gel. It is necessary to specify a size range of fragments that you would like represented in the final genomic library for sequencing. The target size range can be adjusted to modify the number of fragments expected for sequencing. Because techniques such as this typically result in a negative relationship between fragment size and number, selecting larger fragments should decrease the final number of fragments in the template and increase the coverage depth of these regions after sequencing. Similarly, decreasing the size interval selected will also reduce the number of fragments and increase coverage. The number of fragments produced is also in part a function of genome size, so awareness of your organism's genome size is helpful in making an appropriate choice for size selection (see Fig. 3 of Alex's protocol, and Figure 1 in SCHWEYEN 2014). Most people use a 100-150 bp range somewhere between 250 and 500 bp in total fragment length (meaning that we might select, as an example, fragments that are 300 - 400 bp in length). With 8 samples per index, we found a 200 bp range gave better results because more DNA was left after size selection (300-500 bp). With more samples per index, a 150 bp range would probably be OK.

Dr Lotterhos has an R script that can be used to do an in silico digest of a genome. It is called ddRADoysterdigestion.R (for *C. gigas* oyster) but can be applied to any genome. The basic code is in a post on her blog. You should calculate your size range based on the the number of fragments in your selected size range, and your plan for sequencing (the number of paired-end reads and their read length and the total number of bases captured).



NOTE: We tried and tested gel size selection and decided that this approach should be only used if Pippin is not a possibility. After cutting fragments from the gel, purifying them, and re-running them on another gel, we found that the gel had a tails outside the targeted fragments size (Figure #). Nothing we tried could get rid of these artifacts. We tried to add a denaturing step and a bead wash to remove artifacts or contaminants (Figure # and #), but we continued to have tails outside the desire fragment range. This may be due to differences on the movement of fragments.

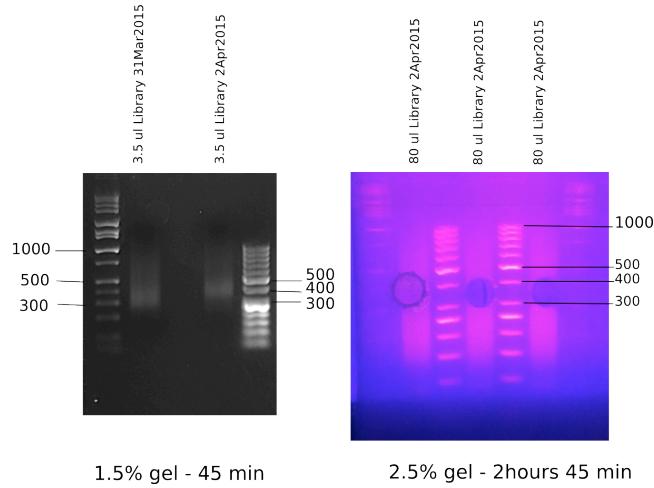


Figure 9: Gel size selection (right) 2.5% gel ran for 2 hours and 45 minutes. The fragments in the range of 300-400bp were removed using a pipette tip. (left) Gel picture of the PCR products after the gel size selection.

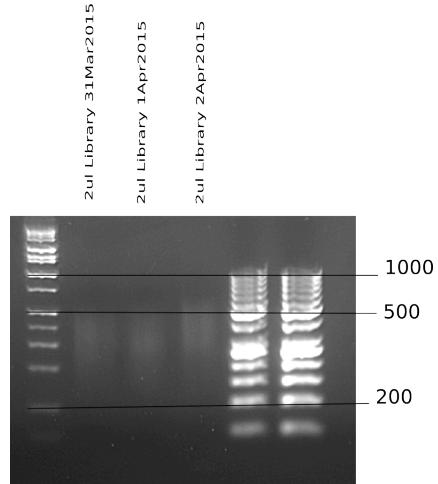


Figure 10: Gel picture of the libraries after amplification (PCR), gel size selection and a denaturing step.

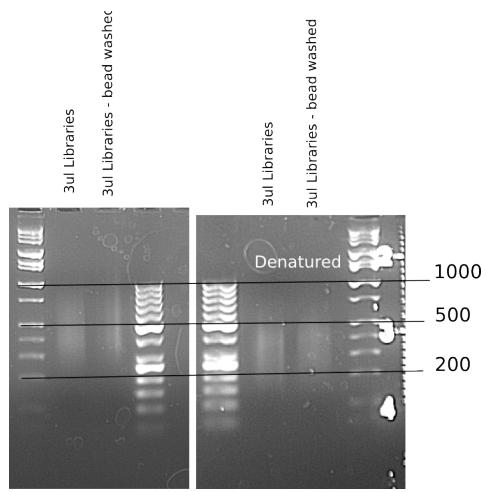


Figure 11: Gel picture of libraries that were denatured before size selection and ran in a final gel (left). An additional denaturing step prior to the final gel picture was done for comparison (right).

14.12.1 Targeted Index PCR Amplification (~8 hours including checking on gel and final beadwash)

Following Ligation, pooled groups of samples with individual left adapters are amplified with a unique right adapter. The right adapter would add a unique barcode to the right side that would allow all of the pools to be pooled in one lane for sequencing. To ameliorate stochastic differences in PCR production of fragments in reactions, we run 3-8 replicate reactions per right barcode group, and later combine them. NOTE THAT WE USE IPROOF HF-TAQ, which is cheaper than the Phusion Taq that most people are using. There are alternative polymerases for ddRAD-seq libraries that may be equally or more effective than Iproof Taq. NEB has good alternatives.

1. For 8 individuals per index after Pippin, we've measured 1000-2000 ng/mL (1-2 ng/uL) and this works well with Vanessa's protocol.
2. Prepare the following Master Mix. Prepare an individual master mix for each individual right adapter according to the number of replicate PCRs that you want to run. WE SUGGEST 6 REPLICATES. You will notice two protocols: Vanessa protocol and Peterson protocol. Vanessa protocol works better with 4-8ng of DNA total, we haven't yet tested larger amounts of DNA. The Peterson protocol is included as a reference.

VANESSA PROTOCOL	Number of samples 1X (uL)
ddH ₂ O	10.4 (or calc)
5x Iproof buffer	4
dNTP (10 mM)	0.4
MgCl ₂ (50 mM)	0.4
PCR Primer 1 (10 uM) universal	0.35
PCR Primer 2 (10 uM) index specific	0.35
Iproof Taq	0.2
DMSO	0.15
DNA pooled template (after size selection)	4 (or calc)

3. Thermocycler profile for this PCR: 98C for 1min; 12 cycles of: 98C for 10s, 62C for 30s, 72C for 30s; final extension at 72C for 10 min. Hold at 4C.
4. Check if the amplification of the PCR product has gone well by running 2uL of the PCR product in a 2% agarose gel (you can use the same gel to run the final pooled PCR product

PETERSON PROTOCOL	Number of samples 1X (uL)
ddH2O	1.75 (or calc)
5x Iproof buffer	4
dNTP (10 mM)	0.4
MgCl2 (50 mM)	0.5
PCR Primer 1 (10 uM) universal	2
PCR Primer 2 (10 uM) index specific	2
Iproof Taq	0.2
DMSO	0.15
DNA pooled template (after size selection)	5 (or calc)

cleaned made in the following step). We have found that if there is a lot of primer-dimer, you can get a higher qubit reading, even though the PCR worked poorly.

14.12.2 Final beadwash and pooling of PCR products

1. Combine the replicate PCR reactions into a single 1.5 mL tube ($6 * 16\text{uL} = 96 \text{ uL}$)
2. Add 144 uL ($96 * 1.5 \text{ uL}$) of AMPURE XP to each aliquot, mix with pipetting 10 times, and then let incubate for 5 mins on the bench.
3. Place tube onto the magnet bar and let the beads separate for 5 mins
4. Carefully remove the supernatant from each tube and discard. Sometimes pulling tube upwards and tilting bottom towards the magnet allows for complete supernatant removal. If not, leave a few uL.
5. Leaving the tubes in the magnet, wash the reaction by adding 500 uL of FRESH 70% ethanol A. 70% ethanol should be mixed that day! Volume is approximate but should cover all beads
6. Remove ethanol and repeat wash
7. Remove ethanol, let tubes air dry for 5-10 mins. All ethanol needs to be evaporated, but beads should not be overdried
8. Remove tubes from magnet and add 30 uL of molecular grade H2O to each tube and incubate for 5 mins

9. Make sure that water comes into contact with beads by mixing well with pipet or gentle vortexing
10. Place tubes back on magnet and let beads separate for 2 minutes, then remove supernatant and transfer each index to a clean 1.5 mL tube.
11. These four tubes are your final library
12. Prepare working solution for the Qubit by combining 199 uL of stock solution with 1 uL of dye for every reaction.
13. Collect 0.5 ml sample tubes, one for every reaction and two for standards
14. Prepare standards by combining 190 uL of working stock with 10 uL of standard (stored in refrigerator)
15. Turn on Qubit by touching screen and press button to read new standards. Follow prompts on screen.
16. Prepare 6 samples for quantification by putting 199 uL of working stock with 1 uL of sample in a 0.5ml tube
17. Use Qubit to measure DNA content, remembering to press button to calculate stock concentration and to press button to save data before switching tube and pressing “read new sample”
18. If each index is between 5-100 ng/uL, celebrate. You are finished.

14.12.3 Check libraries on Bioanalyzer

- Bioanalyzer is in Vollmer Lab. When we get a key, we will keep it in the drawer with the pens.
- The password for the computer is under the keyboard.
- Have ready 350 uL RNAase free water, and pipettes and tips. Plan time to let reagents get to room temperature.

- Clean the electrodes: (1) Fill one well of the electrode with 350 uL RNAase-free water. (2) Place the electrode cleaner in the bioanalyzer. (3) Close the lid and let sit for 5 mins. (4) Afterwards, leave the lid open for at least 30 sec to allow for evaporation.
- There are DNA chips and RNA chips. They are actually the same thing, but there are some differences in the calculations done on the machine. The RNA chips are slightly cheaper. You can only use 12 wells on a chip at a time, for example if you only use 3 wells then that chip is done.
- Follow instructions that come with the chip.
- The gel-dye mix is only good for 4 weeks, so don't make too much at a time.
- When you close the chip priming station, MAKE SURE IT CLICKS.
- The chip vortexer is kind of freaky. The chip will squish into it. Make sure to test it with the electrode cleaner first so you can see how it works.

14.12.4 Quantify adapters with Kapa Kit

TO DO

15 RNA Library Prep

(Adapted from the NEBNext rRNA Depletion Kit manual, NEBNext Ultra RNA Library Prep Kit for Illumina manual, and the NEBNext Multiplex Oligos for Illumina manual)

15.1 Introduction

This protocol is designed to build barcoded cDNA libraries from DNA-free RNA. The protocol uses three kits: NEBNext rRNA Depletion Kit, NEBNext Ultra RNA Library Prep Kit for Illumina, and the NEBNext Multiplex Oligos for Illumina kit. Extracting total RNA results in a product made up of a high proportion of ribosomal RNA (rRNA). When building a cDNA library, we are interested in the mRNA and non-coding intronic and intergenic RNA that make up only a small proportion of our total RNA extraction. Therefore, to begin making a cDNA library, we first need to remove as much of the rRNA as we can. This is done in the first section of the protocol with the NEBNext rRNA Depletion Kit. This kit reduces the amount of rRNA that is found in the sample. NOTE: your initial concentration of RNA should be between 100 ng - 1 ug in 12 uL. We recommend the higher side .75 - 1 ug due to how much will be depleted in this step of the protocol (~10% of starting product will be left i.e. start with 750 ng after depletion you should have 75 ng).

Once the rRNA has been depleted, we can move on to the building of our cDNA libraries, which is done using the NEBNext Ultra RNA Library Prep Kit (if interested in having strand specific information this kit would be substituted with the NEBNext Ultra Directional RNA Library Prep Kit #E7420S). This kit creates cDNA libraries that will be ready for next-generation sequencing.

Finally, the NEBNext Multiplex Oligos for Illumina provide Index primers 1-12 that will be used to provide unique identifiers for samples being sequenced in the same lane on the sequencer. By doing this, you can sequence many samples within a single lane, which reduces sequencing costs. However, you do not want to have too many samples in a single lane because this can reduce the overall sequencing coverage of each sample i.e. Illumina HiSeq 2000 produces 100 - 200 million reads per lane and Illumina HiSeq 2500 produces 100-150 million reads per lane. Based on how many reads you want per sample, you can use this information to determine how many samples to

put into each lane.

If this is your first time building a cDNA library or using this protocol in particular, it is important to go through the protocol with just one sample to ensure you know the steps well. Each step is temperature sensitive and needs to be carried out quickly. Therefore, walking through the protocol once will allow you to learn the steps before using too many samples.

Once you know the protocol, it is also advisable to make no more than 5 libraries at a time. As discussed above, each step is temperature sensitive and you want to make sure you are moving fast enough to not expose your samples to temperatures that may reduce the quality/quantity of your product. By making a maximum of 5 libraries at a time, you can move through each step without much delay. If you are seeing a reduced quality/ quantity in your first samples because of the time it takes you to finish the last samples, reduce the amount of libraries you make at a time.

15.2 Consumable Supplies

- NEBNext Ultra RNA Library Prep Kit (NEB #E7530S)
- NEBNext rRNA Depletion Kit (NEB #E6310L)
- NEBNext Multiplex Oligos for Illumina kit (NEB #E7335S)
- DNase Free Water from DNA-Free RNA Kit (to dilute samples to the recommended concentration)
- 5 PCR tubes per sample (for example, for 8 samples get 5 PCR strip tubes of 8 wells)
- Magnetic Rack for PCR plates
- Agencourt AMPure XP Beads for DNA (Beckman Coulter, Inc. #A63881)
- Agencourt RNAClean XP beads (Beckman Coulter, Inc. #A63987)
- 0.1 X TE pH 8
- 10mM Tris-HCl pH 7.5-8
- 2.5 uL, 10 uL, 20 uL, and 200 uL pipettes (RNA pipettes for day 1 - RNA work) and matching tips (filtered tips for day 1 - RNA work)
- KimWipes

- Gloves
- Marker
- 96-well tube tray from tip box to hold PCR strip tubes
- RNase Away
- 10% Bleach
- 70% ethanol for cleaning the bench

15.3 Equipment

- Qubit 2.0 Fluorometer (Life Technologies #Q32866)
- tubes (Life Technologies #Q32856),
- Qubit RNA HS Assay kit (Life Technologies #Q32852)
- Qubit dsDNA HS Assay kit (Life Technologies #Q32851)
- Tabletop centrifuge for quick spindown
- Vortexer
- Bioanalyzer

15.4 Things to do before

- Prepare 80% diluted ethanol. The ethanol has to be made fresh for every day of use.
- Get two small trays of ice (to transfer reagents and RNA).
- Make sure you have all of the reagents needed for the protocol. Only bring the reagents that you will be immediately using. This is a long protocol and you should plan accordingly to prevent exposing the reagents to warm temperatures. Set up the reagents in the appropriate carrying medium (ice, -20 gel box, etc).
- Clean your working area. Wipe down counter with Bleach, then Ethanol, and lastly RNase Away if you are working with RNA. Wipe down tip boxes, pipettes and all other supplies with Ethanol and if working with RNA, RNase Away as well.

- Get DNase-Free RNA samples from -80. Studies have shown that -20 may still cause RNA degradation, so make sure RNA is stored at -80. Before beginning cDNA library prep, your samples should be run on a biolalyzer to ensure that the RIN > 9 meaning you have high quality RNA and to get a precise measurement of the quantity to be used to determine dilution of samples before beginning the protocol.

15.5 Symbols

 **SAFE STOP** This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.

 This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the type of RNA input.

 Colored bullets indicate the cap color of the reagent to be added. The protocol has been optimized using high quality Universal Human Reference Total RNA.

15.6 Protocol

Starting Material: 100 ng - 1 ug total RNA in a 12 ul total volume.

Hybridize the Probes to the RNA (NEBNext rRNA Depletion Kit) (work in the hood) (prepare on ice)

NOTE: I did not do any master mixes when I did this protocol. I made sure to do 5 or fewer libraries at a time to ensure each step did not take too long and I could pipette exact amounts into each sample. If you decide to do more than 5 libraries at a time, you can make a master mix for each step.

1. Prepare a RNA/Probe master mix as follows:

Reagent	Number of samples 1X (uL)
NEBNext rRNA Depletion Solution	1 uL
Probe Hybridization Buffer	2 uL
Total Volume	3 uL

2. Add 3 ul of the above mix to 12 ul total RNA sample.
3. Mix by pipetting up and down.
4. Spin down briefly in a tabletop centrifuge, and immediately proceed to the next step.
5. Place samples in a thermocycler, and run the following program, which will take approximately 25 minutes to complete: 2 minutes at 95°C, 0.1°C/sec at 95-22°C, 5 minutes hold at 22°C.
6. Spin down the samples in a tabletop centrifuge, and place on ice. Proceed immediately to the next step to prevent non-specific degradation of RNA.

RNase H Digestion (NEBNext rRNA Depletion Kit) (work in the hood) (keep in ice)

1. On ice, prepare a master mix according to the following, and mix by pipetting up and down; use immediately.

Reagent	Number of samples 1X (uL)
RNase H	2 uL
RNase H Reaction Buffer	2 uL
Nuclease-free Water	1 uL
Total Volume	5 uL

2. Add 5 ul of the above mix to the RNA sample from Step 6 in Section 1
3. Mix by pipetting up and down
4. Place samples in a thermocycler (with lid at 40°C) and incubate at 37°C for 30 minutes.
5. Spin down the samples in a tabletop centrifuge, and place on ice. Proceed immediately to the next step to prevent non-specific degradation of RNA.

DNase I Digestion (NEBNext rRNA Depletion Kit) (work in the hood) (keep in ice)

1. On ice, prepare a DNase I Digestion Master Mix according to the following table, and mix by pipetting up and down; use immediately

Reagent	Number of samples 1X (uL)
DNase I Reaction Buffer	5 uL
DNase I (RNase-free)	2.5 uL
Nuclease-free Water	22.5 uL
Total Volume	30 uL

2. Add 30 ul of the above mix to the RNA sample from Step 5 in Section 2.2, and mix by pipetting up and down.
3. Place samples in a thermocycler (with lid at 40°C) and incubate at 37°C for 30 minutes.
4. Spin down the samples in a tabletop centrifuge, and place on ice. Proceed immediately to the next step to prevent non-specific degradation of RNA.

RNA Purification after rRNA Depletion Using Agencourt RNAClean XP (work in the hood) (this step does not need to be in ice)

1. Add 2.2X (110 ul) Agencourt RNAClean XP Beads to the RNA sample from the previous section and mix by pipetting up and down.
2. Incubate samples on ice for 15 minutes. (Prepare solutions for Qubit see recipe below)
3. Place the tube on an appropriate magnetic rack to separate beads from the supernatant.
4. When the solution is clear (about 5 minutes), discard the supernatant. Remove ~90% of supernatant to ensure you do not remove any beads. Go in first with a 20 uL pipette tip to remove most of the supernatant, and then use 10 uL pipette to take off remaining.
5. Add 200 uL of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. It is ok if a couple of uL stay behind after this first wash.

6. Repeat Step 5 once for a total of 2 washes
7. Briefly spin the tube, and put the tube back in the magnetic rack.
8. Completely remove the residual ethanol. Use 20 uL pipette to take off most of the liquid and then switch to the 10 uL pipette to remove any small amounts of ethanol left to ensure you do not remove any beads. Air dry the beads, but don't let the beads dry too much, a little of shininess in the beads from the ethanol is ok (~3 min).
9. Remove the tube from the magnetic rack. Elute RNA from the beads with 8 uL nuclease-free water.
10. Mix well by pipetting up and down, and put the tube in the magnetic rack until the solution is clear.
11. Transfer 6 uL of the supernatant to a clean PCR tube. (use extra 1 uL to do Qubit)
12. Place the sample on ice and proceed to next section.

Quantify the RNA with Qubit

At this step, we still have RNA so the quantification on the Qubit should use the RNA HS Assay Kit. Below is the recipe for one sample (1x), but vary Working Solution quantities for number of samples that you will be analyzing (i.e. for 3 samples plus the 2 standards you will want to make enough for 6 reactions (6x) that will give you the five you need plus one extra = 1194 uL buffer and 6 uL reagent).

1. Make Working Solution:

Qubit RNA HS Buffer: 199 uL

Qubit RNA HS Reagent: 1 uL

2. Make Standards:

Working Solution: 190 uL

Standard: 10 uL

3. Make Samples:

Working Solution: 199 uL

RNA: 1 uL

If you started with 750 ng, you should expect to have about 75 ng left after depletion. Having a range from 40-90 ng/uL is ideal. However, having as low as 30 ng/uL should be good to move on to day 2.

RNA Fragmentation, Priming and First Strand cDNA Synthesis (NEBNext Ultra RNA Library Prep Kit) (work in the hood) (keep in ice)

 RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in step 2 below.

1. Set up the following reaction and mix by gentle pipetting:

Reagent	Number of samples 1X (uL)
Ribosomal depleted RNA	5 uL
• (pink) NEBNext First Strand Synthesis Reaction Buffer (5X)	4 uL
• (pink) Random Primers	1 uL
Total Volume	10 uL

2.  Incubate the sample at 94°C following the recommendations in Table below for fragments sizes ~200 nt

Table 4.1 Suggested fragmentation times based on RIN number of RNA input.

RNA TYPE	RIN	FRAG TIME
Intact RNA	>7	15 min. at 94°C
Partially Degraded RNA	2-6	7-8 min. at 94°C

Refer to Appendix B for fragmentation conditions if you are preparing libraries with large inserts (>200 bp). Conditions in Appendix B only apply for intact RNA.

3. Transfer the tube to ice.

First Strand cDNA Synthesis (NEBNext Ultra RNA Library Prep Kit) (work in the hood) (keep in ice)

- To the fragmented and primed mRNA add the following components and mix by gentle pipetting:

Reagent	Number of samples 1X (uL)
● (pink) Murine RNase Inhibitor	0.5 uL
● (pink) ProtoScript II Reverse Transcriptase	1 uL
Nuclease free water	8.5 uL
Total Volume	20 uL

NOTE: If you are following recommendations in Appendix B (for intact and longer RNA), increase the incubation at 42°C from 15 minutes to 50 minutes in Step 5.

- ⚠️ Incubate the sample in a preheated thermal cycler as follows: 10 minutes at 25°C, 15 minutes at 42°C, 15 minutes at 70°C, Hold at 4°C
- Proceed directly to Second Strand cDNA Synthesis.

Perform Second Strand cDNA Synthesis (NEBNext Ultra RNA Library Prep Kit) (work in the hood) (keep in ice)

- Add the following reagents to the First Strand Synthesis reaction (20 uL):

Reagent	Number of samples 1X (uL)
● (orange) Second Strand Synthesis Reaction Buffer (10x)	8 uL
● (orange) Second Strand Synthesis Enzyme Mix	4 uL
Nuclease free water	48 uL
Total Volume	20 uL

- Mix thoroughly by gentle pipetting.
- Incubate in a thermal cycler for 1 hour at 16°C, with heated lid set at $\leq 40^\circ\text{C}$.

Purify the Double-stranded cDNA Using 1.8X Agencourt DNA AMPure XP Beads (from this point on, the work can be done outside the hood)

- Vortex DNA AMPure XP Beads to resuspend.

2. Add 144 ul (1.8X) of resuspended DNA AMPure XP Beads to the second strand synthesis reaction (~80 ul). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube in a microcentrifuge to collect any sample on the sides of the tube.
Place the tube on an appropriate magnetic rack to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. Only remove ~90% of supernatant to ensure you do not remove any beads.
5. Add 200 ul of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once for a total of 2 washing steps.
7. Completely remove the residual ethanol. Use 20 uL pipette to remove most of the ethanol and then use the 10 ul pipette to remove any small amounts of ethanol left. Do not remove any beads. Air dry the beads for 5 minutes while the tube is on the magnetic rack with lid open. Air dry the beads, but don't let the beads dry too much, a little of shininess in the beads from the ethanol is ok (~3 min).
8. Remove the tube from the magnet. Elute the DNA target from the beads into 60 ul 0.1X TE Buffer or 10 mM Tris-HCl pH 8.0. We have used the 0.1X TE Buffer for all libraries in the past. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.
9. Remove 55.5 ul of the supernatant and transfer to a clean nuclease free PCR tube.

 **SAFE STOP Note:** If you need to stop at this point in the protocol samples can be stored at -20°C.

Perform End Prep of cDNA Library (NEBNext Ultra RNA Library Prep Kit) (keep in ice)

1. Mix the following components in a sterile nuclease free tube:

Reagent	Number of samples 1X (uL)
Purified double-stranded cDNA	55.5 uL
● (green) NEBNext End Repair Reaction Buffer (10X)	6.5 uL
● (green) NEBNext End Prep Enzyme Mix	3 uL
Total Volume	65 uL

2. Incubate the sample in a thermal cycler as follows: 30 minutes at 20°C, 30 minutes at 65°C,
Hold at 4°C
3. Proceed immediately to Adaptor Ligation.

Perform Adaptor Ligation (NEBNext Ultra RNA Library Prep Kit) (NEBNext Multiplex Kit) (keep in ice)

 Dilute the ● NEBNext adaptor* prior to setting up the ligation reaction using the RNA quantities from INITIAL dilution (which should be between 750 ng to 1 ug). Takes a long time to thaw so remove from freezer well in advance of when you need it.

INPUT RNA	DILUTION REQUIRED
100 ng	30 fold dilution in 10 mM Tris-HCl pH 7.5 or 10 mM Tris-HCl with 10 mM NaCl pH 7.5
> 100 ng up to 1 ug	10 fold dilution in 10 mM Tris-HCl pH 7.5 or 10 mM Tris-HCl with 10 mM NaCl pH 7.5

1. Add the following components directly to the End Prep Reaction (Caution: Do not mix the components to prevent adaptor-dimer formation):

Reagent	Number of samples 1X (uL)
End Prep Reaction	65 uL
● (red) Blunt/TA Ligase Master Mix	15 uL
● (red) Diluted NEBNext Adaptor *	1 uL
Nuclease-free Water	2.5 uL
Total Volume	83.5 uL

*The adaptor is provided in NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E7600) Oligos for Illumina.

NOTE: the only time you would use the NEBNext Singleplex kit is when you only have one sample per lane and do not need to uniquely identify multiple samples within a sequencing lane. See discussion of Multiplexing below in PCR Library Enrichment Section.

2. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.
3. Incubate 15 minutes at 20°C in a thermal cycler.
4. Add 3 ul of • (red) USER Enzyme to the ligation mixture from Step 3. Mix well and incubate at 37°C for 15 minutes.

NOTE: This step is only required for use with NEBNext Adaptors. USER Enzyme can be found in the NEBNext SinglePlex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7600) Oligos for Illumina.

Purify the Ligation Reaction Using DNA AMPure XP Beads

 Note: If you are selecting for larger size fragments (> 200 nt) follow the size selection recommendations in Appendix B.

1. Add nuclease-free water to the ligation reaction to bring the reaction volume to 100 ul. It is important to ensure the final volume is 100 ul prior to adding DNA AMPure XP Beads.
Note: X refers to the original sample volume of 100 ul from the above step.
2. Add 100 ul (1.0X) resuspended DNA AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contain unwanted fragments (Caution: do not discard the beads). Only remove ~90% of supernatant to ensure you do not remove any beads.

5. Add 200 ul of freshly prepared 80% ethanol to the tube while in the magnetic rack.
Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. It is ok to leave a little of ethanol behind.
6. Repeat Step 5 once for a total of 2 washing steps.
7. Briefly spin the tube, and put the tube back in the magnetic rack.
8. Completely remove the residual ethanol. Use 20 uL pipette to remove most of the ethanol and then use the 10 uL pipette to remove any small amounts of ethanol left. Do not remove any beads. Air dry the beads for 5 minutes while the tube is on the magnetic rack with lid open. Air dry the beads, but don't let the beads dry too much, a little of shininess in the beads from the ethanol is ok (~3 min). Caution: Do not over dry the beads. This may result in lower recovery of DNA target.
9. Remove the tube from the magnet. Elute DNA target from the beads with 52 ul nuclease-free water. Mix well on a vortex mixer or by pipetting up and down, incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.
10. Transfer the 50 ul supernatant to a clean PCR tube. Discard beads.
11. To the 50 ul supernatant, add 50 ul (1.0X) of the resuspended DNA AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
12. Incubate for 5 minutes at room temperature.
13. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contains unwanted fragments (Caution: do not discard the beads). Only remove ~90% of supernatant to ensure you do not remove any beads.
14. Add 200 ul of freshly prepared 80% ethanol to the tube while in the magnetic rack.
Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. It is okay to leave behind a few ul of ethanol.
15. Repeat Step 14 once for a total of 2 washing steps.

16. Briefly spin the tube, and put the tube back in the magnetic rack.
17. Completely remove the residual ethanol. Use 20 uL pipette to remove most of the ethanol and then use the 10ul pipette to remove any small amounts of ethanol left. Do not remove any beads. Air dry the beads for 5 minutes while the tube is on the magnetic rack with lid open. Air dry the beads, but don't let the beads dry too much, a little of shininess in the beads from the ethanol is ok (~3 min). Caution: Do not over dry the beads. This may result in lower recovery of DNA target.
18. Remove the tube from the magnet. Elute DNA target from the beads with 25 ul nuclease-free water. Mix well on a vortex mixer or by pipetting up and down, incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.
19. Without disturbing the bead pellet, transfer 20 ul of the supernatant to a clean PCR tube and proceed to PCR enrichment. Note: Be sure not to transfer any beads. Trace amounts of bead carry over may affect the optimal performance of the polymerase used in the NEBNext High-Fidelity 2X PCR Master Mix in the subsequent PCR step.
20. Proceed to the next step (Perform PCR Library Enrichment).

**Perform PCR Library Enrichment (NEBNext Ultra RNA Library Prep Kit)
(NEBNext Multiplex Kit) (keep in ice)**

1. To the cDNA (20 ul) add the following components and mix by gentle pipetting:

Reagent	Number of samples 1X (uL)
● (blue) NEBNext High-Fidelity PCR Master Mix, 2X	25 uL
● (blue) Universal PCR Primer (10uM)	2.5ul (if using the lot made in 2015) or 1ul if using the lot made in 2014
Total Volume	50 uL

2. Add 2.5 ul (if using the lot made in 2015) or 1ul (if using the lot made in 2014) of one of the 12 ● (blue) Index Primers (10uM) to one of the cDNA mixes. Each Index (X) primer has an independent barcode.

NOTE: It is VERY important to write down which index goes with which sample.

When multiplexing, all the reads will be mixed from each lane and your indexes will tell you which reads came from which samples. If you will be multiplexing, make sure to tell the sequencing facilities and be sure to do paired-end sequencing.

3. PCR Cycling Conditions

CYCLE STEP	TEMPERATURE	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	
Annealing	65°C	30 seconds	12-15*,**
Extension	72°C	30 seconds	
Final Extension	72°C	5 minutes	1
Hold	4°C		

* The number of PCR cycles should be adjusted based on RNA input. If 100 ng total RNA is the starting input, it is recommended to perform 15 cycles of PCR.

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, larger molecular weight products (> 500 bp) will appear on the bioanalyzer trace.

All past libraries have been made using 15 cycles of PCR. Tests were done to see if less PCR cycles (13) would give higher product yield of the size of interest, but it resulted in too little product at the end and could not be used for sequencing.

4. Proceed to Purify the PCR Reaction using DNA Agencourt AMPure Beads

Purify the PCR Reaction using DNA Agencourt AMPure XP Beads

Note: X refers to the original sample volume from the above step.

1. Vortex Agencourt AMPure XP Beads to resuspend.
2. Add 45 uL (0.9X) of resuspended Agencourt AMPure XP Beads to the PCR reaction (~50 ul). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.

4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. Only remove ~90% of supernatant to ensure you do not remove any beads.
5. Add 200 uL of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. It is okay to leave behind a little of ethanol between washes.
6. Repeat Step 5 once for a total of 2 washing steps.
7. Completely remove the residual ethanol. Use 20 uL pipette to remove most of the ethanol and then use the 10 uL pipette to remove any small amounts of ethanol left. Do not remove any beads. Air dry the beads, but don't let the beads dry too much, a little of shininess in the beads from the ethanol is ok (~3 min).
8. Remove the tube from the magnetic rack. Elute the DNA target from the beads into 23 uL 10 mM Tris-HCl or 0.1X TE. Mix well on a vortex mixer or by pipetting up and down, quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.
9. Transfer 20 uL of the supernatant to a clean PCR tube, and store at -20°C.

Assess Library Quality on a Qubit first and if high enough product yield send to Bioanalyzer (Agilent High Sensitivity Chip).

For Qubit, follow the recipe from earlier in the protocol. However, this time you have cDNA so you should be using the Qubit dsDNA HS Assay Kit to prepare solutions. Ideally, you should have quantities that are all within a similar range above 20 ng/uL. However, the minimum requirement for Illumina sequencing is 10 ng/uL. If you have this much and when you run a gel it is of the size of interest (~300-400 bp), then it is okay to send to Bioanalyzer. If the Bioanalyzer comes back with RIN > 9, then it is okay for sequencing.

See Bioanalyzer results under Quantification in the following link for an example of a good trace:

<http://dnasusequencing.org/nextgen/> pipel

16 Appendix

16.1 Quality assessment of DNA and RNA with gel electrophoresis

Adapted from the protocols of the Levitan Lab, Cipriano Lab and the California Academy of Science molecular Lab

Safety Note

Note that this protocol involves the use of EtBr, which is a carcinogen. All glassware, materials, and the microwave should be dedicated to using with this chemical and not used for anything else. Bench space should be clearly marked. Nothing that touches EtBr should be thrown in regular garbage. Special containers should be here for tips and reagents. (Need info on health and safety contact that will clean up containers). TBE can be use up to ~5 times before the pH changes. The TBE should be dispose in an EtBr friendly container.

Supplies

- EtBr (Fisher #BP1025)
- Ladders (pick a ladder that meets your needs)
- Agarose (pick an agarose that meets your needs)
- Parafilm (Fisher #1337410)

General Information

- The smaller the fragment to be analyzed, the thicker the gel needed to size-separate
- The slower the gel is run, the more precise the sizing and the better-separated the bands

Protocol

1. Casting the Gel

- 1.1. Choose the % of gel needed for the DNA size that you are targeting

% Gel	Optimum Resolution for Linear DNA (kb)
0.5	30 to 1
0.7	12 to 0.8
1	10 to 0.5
1.2	7 to 0.4
1.5	3 to 0.2

- 1.2. Figure out how many samples you need to run. The size of the gel box and tray to be used is based on the quantity of samples to be run. The more samples you have, the more likely you are to need a bigger tray. There are different size trays than can also be cut in half if there is no need for the full tray. Each row in a comb is a space for a sample.
- 1.3. Assembly your gel casting tray. To do so, moisten the rubber ends of the gel tray. Proceed by putting the gel casting tray in the middle of the electrophoresis box. The rubber ends of the casting gel should go against the wall of the electrophoresis box. Alternative, you can use a gel tape in the casting tray to hold the melted gel in place.
- 1.4. With the % of agarose gel chosen and the agarose gel casting tray chosen, you can now calculate the amount of agarose needed. You can use the following calculation:
- $$(C_i) (M_i) = (C_f) (M_f)$$
- Where C = concentration, M = mass, i = initial, f = final
- The formula would look as follow for a 2% gel in a 35ml tray
- $$(100\%) (M_i) = (2\%) (35g)$$
- $M_i = 0.7 \text{ g agarose powder}$

Note: once you calculate the amount of agarose needed, you can post it in a shared environment so you skip doing the calculation again in the future for a similar set up.

- 1.5. Add 1X TBE or another buffer of choice (not previously used) in a 250ml Erlenmeyer Flask labeled for gel work. Swirl the flask a few times.

- 1.6. Microwave the gel until no agarose particles are visible (~1:15). Note: The agarose is melted when the solution is completely clear.
- 1.7. Let the gel cool to lukewarm temperature (3-4 minutes).
- 1.8. Add 0.1% of the final agarose volume worth of EtBr to the boiled gel. For example, add 0.35 uL of EtBr check on EtBr of the lab and add it here to the 35 mL agarose made in the example of step number 3.
- 1.9. Pour the gel into the gel casting tray.
- 1.10. Immediately scrub and rinse your flask with warm H2O.
- 1.11. Leave your gel to set for 20-30 min.

2. Loading your gel

- 2.1. Remove the combs of the gel and proceed to the Electrophoresis step or store the gel in the refrigerator at 4°C.
- 2.2. Clean the combs with water and let them dry.
- 2.3. Remove gel tray from cast and place tray inside gel box by aligning the center groove of the tray with the notch in the gel box.
- 2.4. Add enough 1X TBE running buffer to the box such that your gel is submerged.
- 2.5. Cut a piece of parafilm and, using the multichannel pipette, aliquot out 1 uL of loading dye (either blue or purple is available) for each sample. Blue dye (6x): Use for fragments between 500 - 1000 bps. It will separate out into a purple band (at about 500bps) and a blue band (at about 4 kb). Purple dye (6x): Use for fragments smaller than 500 bps. It has one orange band that migrates at about 100 bp. Always use Purple dye when loading DNA extracts and PCR products for gel excision.
- 2.6. Add 3 - 5 uL of PCR product to your loading dye. Use clean tips!
- 2.7. Load 2 uL of ladder to the first well (use the appropriate ladder based on your fragment size of interest).
- 2.8. Load your samples into the gel wells. Green Tip: Use a tip wash reservoir with H2O or buffer in it to rinse and reuse tips for loading samples to gels. But don't forget to always use a clean tip for loading your negative control.

- 2.9. Cover your gel box with a lid.
- 2.10. Using a dry hand, carefully plug in your power supply.
- 2.11. Set your voltage at 100 V and run gel for approximately 25 minutes (for a small gel) or 40 minutes (for a large gel). For DNA extract gels, run at 135 V for 20 minutes. Note: The slower and longer you run your gel, the better the resolution will be between bands.
- 2.12. Store remaining PCR product in the refrigerator or freezer. Be sure your products are labeled well.
- 2.13. Take a picture of the gel
- 2.14. Wash the PCR box with water. Make sure the electrodes do not touch the water. And dry the box upside down.
- 2.15. Clean the bench with Bleach.

RNA (need to add details for RNA gel)

1. Put a gel in the electrophoresis box and add 1X TBE buffer until the buffer covers half of the electrodes height (well above the height of the gel)
2. Pull the RNA working stock samples from the refrigerator and put them over ice.
3. Mix the following reagents in a PCR strip (keep the mix chill in ice)

Reagents	Volume (uL)
RNA Buffer	8
RNA Sample	2

4. Denature mix at 65°C for 10 min. Put the sample immediately in ice for 3 min.
5. Add 2 uL of blue loading dye to each sample, mix and load to gel
6. Load up 3 uL of ladder to a gel well

16.2 Gel extraction with QIAquick Gel Extraction Kit using Microcentrifuge

Modified from the QIAquick Gel Extraction protocol

Introduction

This protocol is designed to extract and purify DNA of 70bp to 10kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column.

Supplies and Reagents

- Molecular grade ethanol 100%
- 1.5 epi tubes
- QIAquick Gel Extraction Kit (28704)

Before you start

Make sure to add ethanol before starting; this should only be done once.

Procedure

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg \sim 100 uL). For example, add 300 uL of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 min during the incubation. IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 ?l of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow. The adsorption of DNA to the QIAquick membrane is efficient only at pH ?7.5.

Buffer QG contains a pH Indicator which is yellow at pH \leq 7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. Add 1 gel volume of isopropanol to the sample and mix. For example, if the agarose gel slice is 100 mg, add 100 uL isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage
6. Place a QIAquick spin column in a provided 2 mL collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min. The maximum volume of the column reservoir is 800 uL. For sample volumes of more than 800 ?l, simply load and spin again.
8. Discard flow-through and place QIAquick column back in the same collection tube.
Collection tubes are reused to reduce plastic waste.
9. Recommended: Add 0.5 mL of BufferQG to QIAquick column and centrifuge for 1 min. This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription, or microinjection.
10. To wash, add 0.75 mL of Buffer PE to QIAquick column and centrifuge for 1 min. Note: If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2-5 min after addition of Buffer PE, before centrifuging.
11. Discard the flow-through and centrifuge the QIAquick column for an additional 1min at 17,900 x g (13,000 rpm). IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12. Place QIAquick column into a clean 1.5 mL microcentrifuge tube.
13. To elute DNA, add 50 uL of Buffer EB (10mMTris-Cl, pH 8.5) or water (pH 7.0-8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 uL elution buffer to the center of the QIAquick

membrane, let the column stand for 1 min, and then centrifuge for 1 min. After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA. IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 uL from 50 uL elution buffer volume, and 28 uL from 30 uL. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at -20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

14. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

16.3 Quality and quantity assessment of DNA and RNA with Nanodrop

Adapted from the California Academy of Science Protocol and the Assessment of Nucleic Acid Purity article from Thermo Scientific

NanoDrop 2000c is a spectrophotometer machine that can give you an estimate of the DNA and RNA concentrate of your sample and detect contaminants by analyzing the absorbance of uv-visible light of your sample. The measurements of the samples will be a result of the total absorbance of the dsDNA/ssDNA/RNA present in the sample. The sample measurements are fast.

The NanoDrop 2000c results should be consider to be a ball park of what the real results are. A Fluorescence machine such as Qubit or a Bionalayzer should be used to get more accurate DNA or RNA concentrations for protocols such as ddRAD-seq or RNA-seq

Protocol

1. Open the Nanodrop program
2. Click on the option for NUCLEIC ACID.
3. Click on DNA or RNA (depending on the type of sample that you have).
4. Open the arm of the NanoDrop. Clean the nanodrop pedestal with a kimwipte moisten with water.
5. Add 2 uL of your blank solution to the pedestal. Your blank solution would be the elution solution that you use to Resuspend your DNA or RNA product. If you eluted DNA in water, then your blank solution would be water.
6. Lower the arm and click Blank in the screen.
7. Wipe down pedestal with dry kimwipe.
8. Type your SAMPLE ID for your first sample in the top right corner.
9. Gently mix your sample first for accurate concentration reading.
10. Raise the arm of the Nanodrop instrument and load 2 uL of your sample onto the pedestal. Lower the arm and click MEASURE in the top left corner of the screen.

11. You will be asked to name the Nanodrop workbook you are creating. Navigate to your Molecular folder, edit filename, and save workbook (leave as .twbk file). Your DNA concentration value will then appear in the top right corner of the screen with the units ng/uL. (add information of what we expect to look at in good DNA and RNA)
12. IMPORTANT: Once you have finished reading all of your samples, wipe down the Nanodrop pedestal with a damp kimwipe to clean it and return the arm of the instrument to the lowered position.

Concentration Results

The concentration of the sample will be given in ng/uL in a box labeled ng/uL. The concentration results may be heavily skewed by contaminants or the presence of other non-targeted nucleotide.

Contaminants Results

Assessing the curve and ratios of your results would allow you to see if the concentrations results are skewed and if there are contaminants present in your samples.

- 260/230 ratio - ratios above or below 2.0 - 2.2 may be the result of a contaminant absorbing at 230nm or less. Some contaminants have characteristic profile, e.g. phenol, however many contaminants present similar characteristics.
 - A low 260/230 ratio
 - * Carbohydrate carryover (often a problem with plants)
 - * Residual phenol from nucleic acid extraction
 - * Residual guanidine (often used in column based kits)
 - * Glycogen used for precipitation.
 - A high 260/230 ratio
 - * Making a Blank measurement on a dirty pedestal
 - * Using an inappropriate solution for the Blank measurement. The blank solution should be the same pH and of similar ionic strength as the sample solution.
- 260/280 ratio - a ratio above or below 1.8 for DNA or 2.0 for RNA may be the result of a contaminant absorbing at 280 nm or less.

- A low 260/280 ratio
 - * Residual phenol or other reagent associated with the extraction protocol
 - * A very low concentration (>10 ng/uL) of nucleic acid.
- A high 260/280 ratio is not indicative of an issue

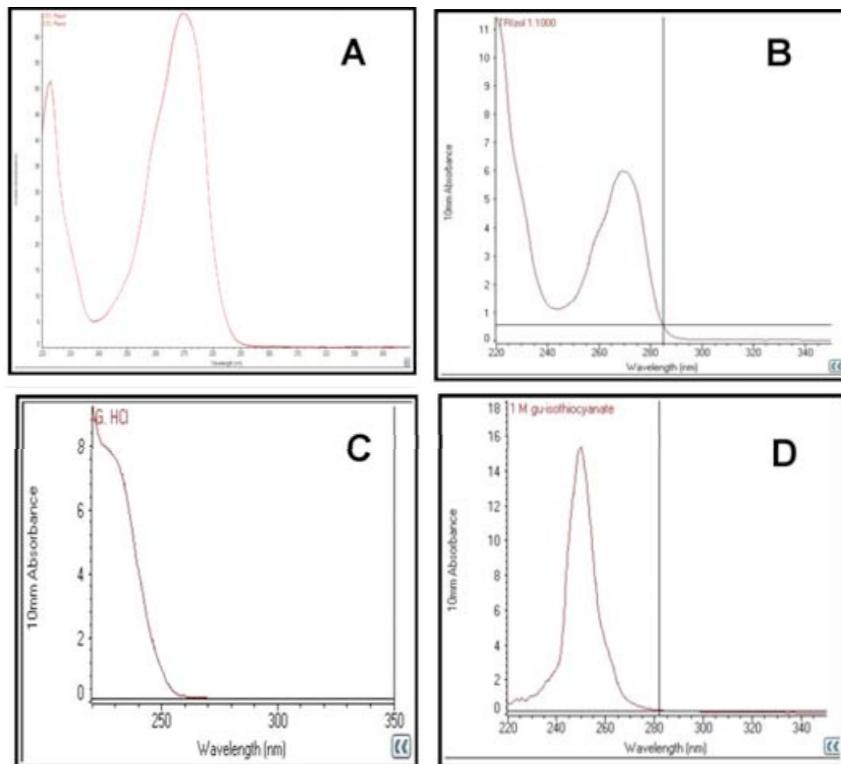


Figure 12: Spectra of reagents used in the isolation of nucleic acids. A) Trizol, B) Phenol, C) Guanidine HCL and D) Guanidinium isocyanate.

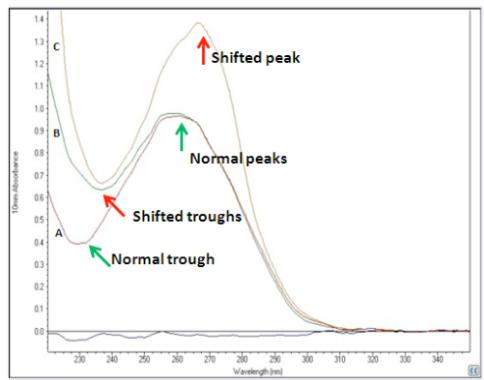


Figure 13: Spectra of purified DNA without contamination (A), and of the same DNA sample contaminated with guanidine (B) and phenol (C).

16.4 Quality and quantity assessment of DNA and RNA with Qubit

No data are currently available addressing the mutagenicity or toxicity of the Qubit BR reagents. This reagent is known to bind nucleic acid. Treat the Qubit BR reagent with the same safety precautions as all other potential mutagens and dispose of the dye in accordance with local regulations.

The Qubit has several kits based on the sample needs. Use the appropriate kit for your sample. Each kit has its own buffer, reagents, and standards; so follow the protocol below using the appropriate buffer, reagents, and standards for your sample.

If you have RNA and your samples are expected to give you

- a yield of 20 - 1000 ng, use the RNA BR Assay Kit.
- a yield of 0.2 - 100 ng, use the RNA HS Assay Kit.

If you have DNA and your samples are expected to give you

- a yield of 20 - 1000 ng, use the DNA BR Assay Kit.
- a yield of 0.2 - 100 ng, use the DNA HS Assay Kit.

Use filter tips for this procedure.

To do before you start

- Remove the standards from the 4°C refrigerator and let them warm to room temperature for 15-30 min. Qubit reagents are sensitive to light, so keep the reagents in the dark.
- Get an epi tube or falcon tube (depending on the quantity of samples that you will be working with) for mixing your working solution.
- Label your Qubit assay 0.5 ml tubes: 2 tubes for the standards and 1 tube per sample
- *** Use only thin-wall, clear 0.5 mL optical-grade real-time PCR tubes. Acceptable tubes include Qubit assay tubes (500 tubes, Invitrogen Cat. no. Q32856) or Axygen PCR-05-C tubes (VWR, part number 10011-830).

Protocol

1. Get standards from refrigerator. Let them warm to room temperature
2. Make the working solution for the samples and 2 standards by diluting the reagent and the BR buffer in 1:200 ratio. Use a clean plastic tube each time you make working solution. Do not mix the working solution in a glass container. For example, for 8 samples, prepare enough working solution for the samples and 2 standards: ~200 uL per tube in 10 tubes yields 2 mL of working solution (10 uL of reagent plus 1990 uL of buffer).

Reagent	Number of samples 1X (uL)
Reagent (RNA BR, or DNA BR, or DNA HS	1 uL
BR buffer (RNA BR, or DNA BR. or DNA HS)	199 uL

3. Prepare standard 1 and standard 2 by mixing 190 uL working solution and 10 uK of standard 1 or standard 2 in 0.5 mL tubes. Vortex 2-3 seconds, being careful not to create bubbles.

Reagent	Number of samples 1X (uL)
Working Solution (RNA BR, or DNA BR, or DNA HS	190 uL
Standard 1 (RNA BR, or DNA BR. or DNA HS)	10 uL

Reagent	Number of samples 1X (uL)
Working Solution (RNA BR, or DNA BR, or DNA HS	190 uL
Standard 2 (RNA BR, or DNA BR. or DNA HS)	10 uL

Note: Careful pipetting is critical to ensure that exactly 10 uL of each standard is added to the 190 uL of working solution.

4. Prepare the samples by mixing 199 uL of the working solution with 1uL of sample (RNA or DNA) in 0.5 mL tubes and mix by vortexing 2-3 seconds. Repeat this step for every sample that you have.

Reagent	Number of samples 1X (uL)
Working Solution (RNA BR, or DNA BR, or DNA HS	199 uL
Sample (RNA or DNA)	1 uL

Note: If samples are suspected to be low in concentration, then use up to 20 μ L of sample and adjust the working solution volume so the overall volume of the mix remains 200 μ L.

16.5 Homemade Magnetic Beads (SpeedBeads)

B.Faircloth & T.Glenn et al. 2011

<https://ethanomics.files.wordpress.com/2012/08/serapure_v2-2.pdf>

Reagents

- Sera-mag SpeedBeads (Fisher# 09-981-123)
- PEG-8000 (Amresco 0159)
- 0.5 M EDTA pH 8.0 (Amresco E177)
- 1.0 M Tris, pH 8.0 (Amresco E199)
- Tween 20 (Amresco 0777)
- 5 M NaCL
- Fermentas ladder(s) (Ultra low range: Fisher # FERSM1211, 50bp: FERSM0371)
- Rare earth magnet stand (Ambion AM10055 or NEB S1506S)
- TE

Protocol part 1:

1. Transfer 500 uL SpeedBeads to microfuge tube (Sera-Mag SpeedBeads #65152105050250)
2. Collect beads in magnetic stand. Wash the beads two times with 500 uL TE. Remove the microcentrifuge from the magnetic stand to do the wash and the bring back the tube to the magnetic stand to remove the wash.
3. Resuspend in 500 uL TE *do not add the resuspended beads until the last part of the part 2 protocol

Protocol part 2:

1. Add to a 50 mL tube:
 - 4.5g PEG-8000 (Amresco #97061-102)

- 5 mL 5M NaCl
 - 250 uL 1M Tris pH 8
 - 50 uL 0.5M EDTA
 - add sterile water to ~24ml
 - mix until PEG dissolves (vortex vigorously for a few minutes)
2. add 137.5 uL 10% Tween-20 or add 13.75 uL of Tween 20 to conical and mix gently
 3. Add 500 uL of the washed SpeedBeads from part 1
 4. Add TE or water to 50 mL

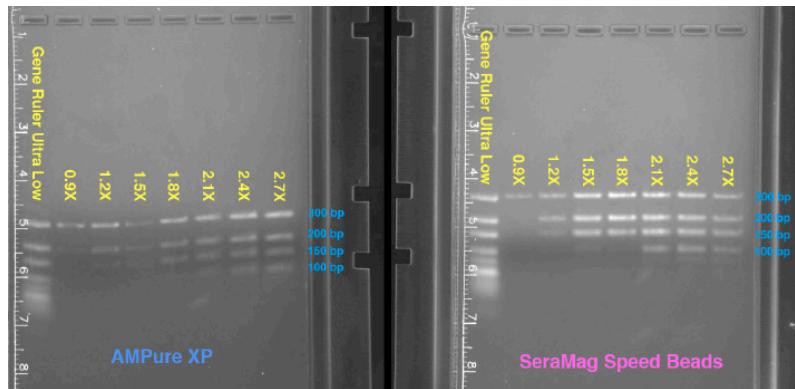
Protocol part 3:

You should test the Serapure mixture to ensure that it is working as expected. You can do this using DNA ladder (Fermentas GeneRuler - NEB ladders may cause problems):

1. Prep fresh aliquots of 70% EtOH
2. Mix 2 uL GeneRuler with 18 uL dH2O
3. Add 20 uL GeneRuler mixture to a volume of Serapure and/or AMPure (the specific volume depends on whether you are trying to exclude small fragments or not).
4. Incubate mixture 5 min. at room temperature.
5. Place on magnet stand
6. Remove supernatant
7. Add 500 uL 70% EtOH
8. Incubate on stand for 1 min
9. Remove supernatant.
10. Add 500 uL 70% EtOH
11. Incubate on stand for 1 min
12. Remove supernatant

13. Place beads on 37°C heat block for 3-4 min until dry
14. Rehydrate with 20 uL H2O
15. Place on magnet stand
16. Transfer supernatant to new tube
17. Mix supernatant with 1 uL loading dye.
18. Electrophoresis in 1.5% agarose for 60 min at 100V

The following image from the Faircloth protocol compares the results of "purifying" a mix of 2 uL Fermentas Ultra Low Range Ladder + 18 uL dH2O using several different amounts of AMPure or Serapure solution to DNA solution. AMPure is on the left, "Serapure" is on the right. After preparing 20 uL of ladder + water mix, we combined that with the volumes of AMPure or Serapure listed below and then purified using the standard protocol:



As you can see, the volume of AMPure or SeraPure controls the size of fragments recovered. More specifically, it is the ratio of PEG solution used to the volume of the DNA in solution which makes the difference, not the count of beads in solution (provided they are above the minimum level). This is what makes it possible to do "double-SPRI" size selection.

16.6 DNA Bead Wash with pre-made Speed Beads

Adapted from Agencourt AMPure XP PCR Purification protocol

The homemade beads for this protocol should be prepared prior the procedure detail in this protocol. We will refer to the aliquots of pre-made Speed Beads as DNA beads. Please look in section 10.3 for a protocol on how to prepare Speed Beads. This protocol can be used for samples in plate or in individual epitubes.

Protocol

1. Shake the aliquots of DNA beads (pre-made Speed Beads) to Resuspend any magnetic particles that may have settled.
2. Add 1.8X volume of DNA beads to the sample. For example add 180 uL of DNA beads to a sample of a 100 uL volume.
3. Mix reagent and sample thoroughly by pipette mixing 10 times. Let the mixed samples incubate 5 minutes at room temperature for maximum recovery.
4. Place the reaction plate onto a magnetic plate for 2 minutes to separate beads from solution. Wait for the solution to clear before proceeding to the next step.
5. While the reaction tube or plate are situated in the magnet plate, aspirate the cleared solution from the reaction plate and discard. Leave 5 uL of supernatant behind, otherwise beads are drawn out with the supernatant.
6. While the reaction tube or plate are situated in the magnet plate, dispense 200 uL of 70% ethanol to each well of the reaction plate and incubate for 30 seconds at room temperature. Aspirate the ethanol and discard. If the total volume of sample plus reagent exceeds 200 uL, then use a wash volume of at least the volume of sample plus reagent.
7. Remove the reaction plate from the magnet plate, and then add 40 uL of elution buffer to each well of the reaction plate and pipette mix 10 times. Incubate for 2 minutes. The liquid level will be high enough to contact the magnetic beads at a 40 uL elution volume. A greater volume of elution buffer can be used, but using less than 40 uL will require extra mixing (to ensure the liquid comes into contact with the beads, and may not be sufficient to

elute the entire PCR product. If you are wishing for a higher DNA concentration use a lower elution volume than the initial sample volume.

8. Place the reaction plate/tube back in the magnetic plate for 1 minute to separate beads from the solution.
9. Transfer the eluate to a new plate/tube.

Note: Bead carryover into the final plate is usually not a cause for concern. The samples can be stored in the freezer with beads and the beads are inert in downstream enzymatic reactions. If bead carryover must be limited for any reason, 2 uL - 5 uL of eluate can be left behind the original plate. In addition, a second transfer away from the beads is optional. To do so, place the final plate containing beads and eluate onto the magnet for 1 minute to separate the beads. Transfer the eluate into another clean plate.

16.7 Resuspend Primer Stocks

Adapted from the California Academy of Science Protocol

Primer resuspension

1. Briefly vortex and centrifuge primer stocks when they arrive. The dry pellet may have become dislodged from the bottom of the tube.
2. Label the top of the lids of the primer vial. It will help in future efforts of working with the primer.
3. Oligos can be resuspended in Milli-Q water or 1X TE buffer. TE buffer would keep the quality of the oligos for longer. Calculate the amount of TE buffer or water needed to be added to bring the primer to a 100uM concentration. You can multiply the nmol quantity amount, noted in the primer data sheet, by 10. This primer is now your main stock, and you should only use the main stock to make working stocks of the primer.
4. For example, if your primer's nmol quantity is 36, you should add 360 uL of TE buffer to the tube. This will give you a final stock concentration of 100uM.
5. Vortex the primer at low speed a couple of times and let the primer sit for at least 5 minutes before use.
6. Do a quick spin to the primer tube sand store the primers in the -20 freezer or make working stocks for future use.

Preparing work stocks from primers main stock 10 uM

1. . PCR reactions tend to require 10 uM oligos stock. Label a 0.5 mL or 1.5 mL tubes per primer or primer mix. It is better to make a few working stocks at once.
2. To each tube add 90 uL of Milli-Q water
3. Add 10 uL of primer stock. Quick vortex and quick spin. The 10 uM primers now can be stored in the freezer.