THE joy OF dna

COOKBOOK

The Simon Lab, UCONN

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Lab Organization and Data Checks

Everyone makes errors. At every step along the way in your procedures and analyses, it is important to have checks. It is also important to plan ahead and know what you are doing before you start.

Don't be over confident. Well-respected biologists have mixed-up the DNA's of well known organisms and even published bizarre conclusions and attempted to justify them. Some well-known studies were later found to contain chimeric DNA—mixed sequences from two different organisms—or misaligned data. To the embarrassment of these researchers, the mistakes were caught by peers after publication. If little-known organisms were to be mixed up—especially closely related species—the mistakes might never be discovered and incorrect conclusions would remain in the literature, possibly causing expensive problems for future researchers.

1) Before you begin the project, make an effort to contact all researchers world-wide who are currently working on your taxon. Exchange ideas and research plans.

2) In the specimen collecting stage:

a) Obtain collecting permits well before planned field trips. These can often take months to obtain. If working in a foreign country collaborate with local researchers. Be prepared for complications:

e.g. In Australia, "National Parks" are in reality state parks and permits must be obtained separately for each state.

Second Example: Brazil (Spring 2003) Paraphrasing F. R. Santos, Professor of Genetics and Evolution; Department of General Biology; Belo Horizonte, Brazil

There is a provisional law in Brazil that will become a definitive one perhaps this year (2003), regulating the access to Biodiversity. At present, foreign researchers cannot collect samples of Brazilian biodiversity unless collaborating with researchers from a Brazilian institution and with a proper license issued by a special commission. However, since October 2002, even Brazilian researchers were prohibited licenses to sample the biodiversity at any level regardless of their projects (whether or not genetic analysis is proposed).

The Santos lab in Brazil had problems even though they had a license to work with animals until February, 2003 (it was issued before October, 2002). They have been trying to get a formal license to work on biodiversity genetics since January without success. A license order must be sent to a National Committee that takes a long time to reply due to the number of issues they are discussing, the high number of projects to analyze and the change in the government. The Committee is named "CGEN -

Conselho de Gestco do Patrimtnio Genitico" and resolutions, as well as license issues can be followed at the site www.mma.gov.br

The Convention on Biological Diversity: www.biodiv.org/convention/articles.asp
"The objectives of this Convention are the conservation of biological diversity, the sustainable use of its components and the fair and equitable sharing of the benefits arising out of the utilization of genetic resources, including by appropriate access to genetic resources and by appropriate transfer of relevant technologies, taking into account all rights over those resources and to technologies, and by appropriate funding."

b) Investigate the ecology, geography, and climate of the areas to be visited (helps with packing as well as with collecting and data interpretation). Collect local field guides.

c) Create a check-list of field gear in your field notebook and use it to pack. Forgotten equipment can cause delays and data loss. Excessive gear can also cause problems. Consult with experienced colleagues for ideas. Bring sufficient collection supplies such as presses for plants, pinning boxes for insects, leak proof vials (and parafilm to seal them if necessary), and storage buffers/agents such as: dryrite (silicagel), the CTAB gel, FAA (for cytology and flower parts), and/paraldehyde buffer (for electron microscopy later). Be sure to take along a cooler to keep specimens cold on wet or dry ice; this is important even for alcohol specimens. Consider preserving some specimens in "RNA later," a buffer that preserves RNA for cDNA extractions (useful for hard-to-sequence nuclear genes that may have long introns; however, see notes by Jerry Regier in his 2005 web publication). Your field gear should include copies of any important taxonomic publications of the organisms under study (library search prior to the field trip).

d) Keep detailed field notes when collecting specimens including a daily log of activities, exact locations of specimens (GPS and a written description of how to get to the location using permanent markers). GPS units are getting cheaper! Take notes about the biology (behavior, natural history) of the organism and its habitat, temperature, weather conditions, etc. Do this on site not later in the day (although you could fill in details later if daylight time is short). Do not rely on memory!

e) Sample throughout a species range to determine species limits. What you think is one species could be two or three. (Examples from the NZ cicadas and Hawaiian damselflies).

f) Include standardized specimen labels with each sample showing country, province, exact location, full date and collectors. Include a species name if known.

g) Develop in advance a concise, preferably mnemonic, coding system for individual specimens or localities that can be carried through collection, extraction, cleaning, storage, PCR, sequencing, and databasing. Create a written description of this

procedure and keep it in the front or back of your field and lab notebooks. This system should be understandable to a naïve reader. Example from our laboratory: 04.NZ.OL.LHA.02 stands for: 2004, New Zealand, Otago Lakes District, State Highway 6 at Lake Hawea access rd., ca. 11km N. of Lake Hawea dam, 1km past Lake Hawea lookout, specimen number 2. This code is entered into the data base along with the species identity, exact date, method of preservation, latitude, longitude, and altitude plus any notes; this number is cross referenced in the field notebook.

h) Write all labels in pencil or permanent pen or printer ink (note: some permanent inks dissolve in alcohol and even if your specimen is not stored in alcohol now, it may be in the future or it may be stored near specimens in alcohol that may leak, especially during pressure changes in airplanes). Laser-printed or photocopied ink can lift off the paper eventually. Toasting labels in a microwave may help set the ink. Pencil is best. See notes on permanent labels.

i) Include a label inside a vial as well as on the outside. Outside labels are easily damaged especially when written on smooth plastic. Water-soluble labels on the outside of vials can disappear with condensation after removal from a freezer! Alcohol can leach from vials and remove permanent ink labels.

j) Specimens in the field can be stored in liquid nitrogen (very difficult to transport by air), dry ice (easier to transport by air), alcohol (cone lidded scintillation vials or cryovials with o-rings seem to hold well, otherwise use parafilm) on silica gel drying agent or in special high salt/high EDTA buffers (we've had these fail for us!). They should always be kept cold. The ancient DNA folks say that water and heat are the biggest enemies of DNA.

k) Collect voucher specimens at each locality that preserve all important characters including color (dry specimens as quickly as possible to retain colors; if using formalin to preserve color be sure to include a note on the label). Voucher specimens should be deposited in a museum at the conclusion of the study, paying attention to permit regulations regarding host country museums. Actual working specimens should also be preserved for future reference (i.e. use as little tissue as possible in your work so that future researchers can return to the same specimens); these are the best vouchers. A digital camera photo is a wonderful supplement to the voucher specimen. These are getting cheaper all the time. Photos can be downloaded in the field onto a portable laptop computer (well worth the investment) and used later in web keys and web sites as well as in journal publications.

l) Keep a running list at the front or back of your field notebook of all specimens collected. This will greatly facilitate databasing in the end. Collecting permits generally require a report soon after returning.

m) If possible database specimens in the field. If not, do this as soon as possible after returning. Note that if you have a laptop with you in the field, weather conditions are

often available on line. Barcodes can be used to link specimen vials to information in a database.

n) Back in the laboratory, develop an efficient scheme for keeping track of specimens in the freezer.

3) In the Extraction and PCR stage:

a) Aliquot various reaction components including extractions, primers, dNTP's, etc. Freeze/thaw cycles can cause degradation.
b) Label your tubes carefully.
c) Always do every step in the same order.
d) Use prepared blank PCR forms to record running conditions and amounts of the various components.
e) Always include positive controls (a specimen and primers you know to work) and negative controls (all PCR ingredients except DNA) with your PCR runs.
f) Know the size of piece to expect, i.e. know the locations of your primers (also know the degree of conservation of your primers so that you know whether to expect high probability of amplification.). Examine comparisons of primer sequences among species. Map your primers onto a diagram of the gene sequence; don't work blind. Name all newly created primers informatively!
g) Assemble PCR components on a lab bench where no amplified DNA has ever existed.
h) Dilute primers with special pipettors reserved for primer dilution only.
i) Pipette Taq with special pipettors reserved for Taq only.
j) Wear gloves.
k) IMPORTANT CONTROL: For population and species studies: extract more than one individual of each species and extract them on different days. Preferably, extract three or four individuals some from the same populations and others from the extremes of the range (cryptic species may exist). For deeper level studies, you may be able to substitute two species in the same genus for two specimens of the same species.

4) In the sequencing stage:

a) Sequence in both directions. As a last resort, if you can't sequence in both directions, use overlapping segments primed in the same direction to check. This is a less desirable alternative because there may be errors specific to one direction.
b) Sequence at least two individuals of each species and sequence them on different days.
c) Checking autosequencing results is an important step. The machines make predictable errors in calling that can be picked up by visual inspection. This is an essential step!

5) Look at your sequence data! And align with care.

a) Calculate the nucleotide bias of your DNA and check to see if it is typical of your organism. If you suspect errors, re-extract, re-PCR, and re-sequence. (the cow that was mistaken for a wasp would have certainly been found if this simple check had been done.)
b) Make sure that all sequences read the same in both directions (often one primer works better than the other). Check discrepancies by re-sequencing.
c) Align sequences from multiple individuals from the same population or multiple populations of the same species to identify errors. In a protein coding gene, all differences between sequences should be checked, especially second position mutations (all will change amino acids and this should be rare in closely related species). Remember that bases on the ends of a sequence are more like to be wrong so don't be greedy when chopping off the ends.
d) Align RNA and intron data using information from conserved motifs and secondary structure.
e) Fold the RNA into the secondary structure to make sure that bases in helical regions pair properly.
f) Check conserved motifs to see if they are conserved (substitutions do occur in these regions but they are rare).
g) Align protein genes using amino acid translations (in all three frames if necessary).
h) Look for frame shifts or deletions that are not multiples of threes (these usually indicate errors).
i) Alignments should not be done by ordering taxa according to presumed phylogenetic relationship to avoid subjective bias, but obvious taxa such as "plants" vs "animals" should be aligned in groups.

6) In the phylogenetic analysis stage:

a) Check for nucleotide bias among taxa. None of the regularly used methods can accommodate this.
b) If any information on your study organisms exist, use this to determine if any taxa are placed in strange positions on the tree. If so, check to see if any samples may have been mixed up; sequence another specimen and compare its sequence to the problematic specimen.
c) Know the assumptions of your phylogenetic analysis method. Compare the results of different models and different analyses to reveal potential biases in the data.

(Chris Simon, EEB 398 Fall 2005)

Specimen Labels

To summarize, most (but not all) agree that for semi permanent archival (up to 10ish years), laser printed on labels will be okay. Printing on bleach free paper or treating it with some sodium
thiosulphate should help mitigate the effects of halogens in the paper damaging the DNA. However, for very long term storage, much more sophisticated systems printing on polyester with a resin ink thermal printer are the best option. Most of the messages suggest that pencil labels are preferred and very permanent, but we have too many samples and record too much data on our labels for this to be practical. I think for the moment we will go with laser printed labels printed on bleach free paper, backed up with an external sticky labels referencing just the specimen code. (Vince Smith)

It is known that hypchlorite (bleach that may be used in paper manufacturing process) will damage DNA, but the extent of damage is a function of the time of exposure and the concentration of the bleach. If you are worried about bleach or other halogen damage, add a very small amount (<1%) of Sodium thiosulfate to the 70% ethanol or better yet, soak your labels in a solution with thiosulfate before you stick them in the vials? (John J. Peloquin)

Material in the Smithsonian entomology collections are only labeled with certain inks because laser printer “inks” do not stay attached over long periods of time. (Rick Wilkerson)

I would use pencil labels, on bleach-free paper on the inside, and printed labels on the outside of your tubes. (Graham Stone)

We label specimens with handwritten labels placed INSIDE the cryotube. The paper is archival linen stock, the inks are usually water/alcohol insoluble pigment-based inks. The preservative is 95% ethanol, and we store the tubes at -80°C or colder. We get good DNA out of these specimens. We do not put labels inside tubes containing DNA extracts or PCR reaction products. We are now printing EtOH vial labels on polyester stock with a resin ink ribbon on a "DataMax" thermal printer. This is supposed to be archival, permanent, and non-reactive. We are probably going to use the same printer to print sticky-backed barcode labels that will permanently go around the OUTSIDE of cryotubes containing DNA extracts. This number will reference records in a database containing all the label data. We are assured (by the supplier) that these sticky-backed labels are permanent even when immersed in liquid nitrogen. Research here at our museum has shown (or so I am told) that laser-printed labels are NOT permanent or archival in EtOH. Printed on archival (acid-free) linen stock, they are fine for pinned or other dry-label use, but can be expected to degrade over time in alcohol. (Ted Schultz)

(Compiled from BugNet)

DNA Storage

I have kept DNA samples from *Colias* butterflies, either as resuspended from ethanol precipitation into demineralized water, or as lightlybuffered Chelex extracts, in -70° to -80° C ultralow freezers for five years or more and had them amplify up in PCR as well as when they were first prepared. So my experience would suggest that DNA is not damaged by low temperature. However, repeated freeze- thaw cycles which would cause a lot of shearing.

I've had samples of DNA (phenol and cscl prepped) stored for years in TE in the freezer (-20° C) with no ill effects. Note however that these are samples from fish. I have seen problems with storage of invertebrate DNA in the freezer.

I use a Phenol/chloroform purification method on my bat DNA. I store the purified samples in TE/1 buffer as I was worried about the effect of EDTA on PCR reactions, but the DNA degrades if stored in water. I then store the samples at – 20°C and have not noticed any problems with the samples when I have used them subsequently.

DNA is stable when frozen. It is freeze-thaw cycles that can cause some shearing. But probably for PCR, even some freeze- thaw cycles are not very damaging. We have DNA stored at – 20° in TE that is
fine for at least 6 years.

We are keeping our phenol/chloroform purified DNA stocks in -70° C, they are in TE or H2O. Working stocks are kept at 4° C.

The best way to keep your DNA for long term storage is to precipitate it in ethanol. It will be good for ever. For working samples, 4° C is good enough for some time.

Cold doesn't kill DNA, but repeated cycles of freezing and thawing might. If this worries you, it might be best to keep your working stocks in small aliquots, and only thaw one at a time.

Low temperature is very good for the preservation of DNA. I have samples that are 10 years old, kept in water at -20°, have been thawed numerous times and are still very good for PCR. Keeping DNA at +4° is dangerous because it becomes degraded quickly.

For long-term storage the temperature of –20° freezers tend to fluctuate, causing ice crystals to tear DNA strands (especially long ones). Apparently this tearing does not happen with –80° or -160° degree freezers.

(Compiled from EvolDir)

Flowchart

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Basic Recipes

10X TAE

48.4g TRIS
11.42 ml glacial acetic acid
20 ml 0.5M EDTA
Dilute to 1000 ml
Adjust pH to ≈ 8.5

50X TAE

242g TRIS
57.1 ml glacial acetic acid
100ml 0.5M EDTA
Dilute to 1000 ml
Adjust pH to ≈ 8.5

10X TBE

Add 108g TRIS to:

800 ml ddH_2O
55g boric acid
40ml 0.5M EDTA (pH 8.8)

Dilute to 1000 ml

6X Dense Dye III

6ml Glycerol
50mg Bromphenol Blue
50mg Xylene cyanol
Dilute to 20ml

Ficoll loading buffer (from Chris Jiggins and Margarita Beltran)

Buffer is15% Ficoll + 0.03% Bromophenol Blue

Slowly add 3g Ficoll to 17 ml ddH_2O while stirring
Add 0.006g Bromophenol Blue

dDNTP Dilution 10mM Stock

To make 1 ml:

25 µl dGTP (100 mM)
25 µl dATP (100 mM)
25 µl dTTP (100 mM)
25 µl dCTP (100 mM)
900 µl ddH_20

Vortex

1 M EDTA:

1M EDTA = 372.24 g/l EDTA

1M $MgCl_2$ Stock Solution:

1M $MgCl_2$
400 ml dd H_20
101.65 g $MgCl_2$
Dilute to 500 ml in volumetric flask

1M NaCl: stock solution:

1M NaCl = 58.44 g/l NaCl

3M NaOAc:

123.045g NaOAc (anhydrous)/500 ml dH_20

3% SDS p:

15g SDS/ 485 ml dH_20

PCR Buffer (Paäbo**):**

3.5 ml of 1M Tris-HCl (pH 8.8)
100 µl BSA (0.1g/ml = 100mg/ml)

Strength	$MgCl_2$ (µl)	ddH_20 (ml)
1.0X	50	1.35
1.5X	75	1.325
2.0X	100	1.30
2.5X	125	1.275
3.0	150	1.250

pK Buffer:

Mix equal parts of 30mM TRIS, 30mM EDTA, 3% SDS.

1M Tris-HCl pH 8.8:

400 ml dd H_20
60.56 g TRIS
Adjust to pH 8.8 with 10M HCl
Dilute to 500 ml in volumetric flask

6% Acrylamide gel (500 ml)

WEAR GLOVES AND MASK

28.5g Acrylamide
1.5g Bis-Acrylamide
210g Urea
50ml 10X TBE Buffer
200ml ddH_2O

Warm to 37°-45°C Stir approximately 30-60 minutes. Let cool to 25°C. Bring volume to 500ml with ddH_2O. Filter Solution.

Cicada Tissue Preparation

A. Adult Tissue preparation:

1. Remove specimen from storage alcohol.
2. Obtain tissue
    A. Remove a front leg from specimen, split the leg open longitudinally, and place in a 1.5 ml microcentrifuge tube.
    B. Remove head from cicada, pull out a single thoracic muscle, and place in a 1.5 ml. microcentrifuge tube.
3. Add 1.0 ml sterile H_2O and allow to sit for 5 min.
4. Pipette off liquid and discard.
5. Place tibia or muscle on a clean tissue and dice with single-edge razor blade.
6. Put diced parts back into microcentrifuge tube and proceed to DNA extraction.

B. Eggs:

1. Cut away as much excess twig as possible.
2. Split the twig lengthwise and parallel with the slit of the eggnest using a single-edge razor blade or a #11 scalpel blade.
3. Place the twig into a glass petri dish filled with 70% ETOH and remove as much wood as necessary until the eggnest is clearly visible.
4. Carefully pluck or aspirate the eggs out of the nest and place them in a 1.0 ml microcentrifuge tube.
5. Add 1.0 ml sterile H_2O.
6. Centrifuge at 14,000 rpm and discard liquid.
7. Proceed to DNA extraction.

DNA Extraction Chemicals

Compiled by Steve Jordan. Information from a variety of sources including the Gibco BRL catalogue 1993-94, and Current Protocols in Molecular Biology, ed. Ausubel et al. Wiley Interscience, 1987.

Detergents. These shatter lipid membranes by inserting themselves into the lipid bilayer and disrupting the hydrophobic interactions that are responsible for the formation of the bilayer. This allows cell contents, including nuclear and mitochondrial contents, as well as nasty chemicals in the lysosymes, to get into solution.

> **SDS Sodium Dodecyl Sulfate (aka Sodium Lauryl Sulfate)**. This detergent functions as a protein denaturant. It also disrupts cell walls and dissociates nucleic acid protein complexes.
>
> **DTAB**. A powerful denaturing cationic detergent.

Chelating agents. These chemicals have an affinity for polyvalent metals ions that are cofactors of many enzymes, including those that destroy DNA.

> **EDTA**. Chelates heavy metals, including Mg++, a cofactor of most DNA destroying enzymes.
>
> **Chelex**. An ion exchange resin that has an unusually high preference for copper, iron, and other heavy metals over monovalent cations such as sodium and potassium (5,000 to 1 preference). Works even in high salt concentrations.

Protein control agents.

> **Proteinase K**. A nonspecific serine protease that is active over a wide pH range and in the presence of high concentrations of SDS, EDTA, and urea. This enzyme is stable at high temperatures. It rapidly inactivates nucleases from microbial and mammalian cells (and insect cells we presume).

Cleaners.

> **Phenol**. Denatures proteins and probably dissolves denatured proteins. The denatured proteins form a layer at the interface between the aqueous and organic phases and are thus isolated from the bulk of DNA in the aqueous phase.
>
> **Chloroform**. A useful protein denaturant that reduces the amount of aqueous solution in the organic phase to maximize the yield. Also stabilizes the boundary between the aqueous and organic phases.

DNA precipitating agents.

Ethanol. DNA precipitating agent that, in the presence of relatively high (0.1 to 0.5 M) concentrations of monovalent cations, induces a structural transition in nucleic acid molecules which causes them to aggregate and precipitate from solution.

CTAB. DNA-precipitating cationic detergent.

Others.

Isoamyl alcohol. prevents foaming as chloroform mixture is agitated

DNA Extraction Protocols

Clontech Kits:

A. Digestion.

1. Add 180 µl buffer T1 to the prepared tissue.
2. Add 25 µl proteinase K solution.
3. Using pipette tip, mash tissue pieces against side of microcentrifuge tube.
4. Incubate at 56°C for 12 hours (3 hours will work, and can go up to 20 hours). Shake during incubation, but do not vortex.
5. Briefly centrifuge tube to remove condensation from inner lid. A pulse will do it; do not pack the tissues in the bottom of the tube!
6. Add 200 µl buffer B3 to the sample and invert several (ca. 5- 10) times.
7. Incubate sample at 70°C for 10 minutes.
8. Briefly centrifuge tube to remove condensation from inner lid.
9. Add 200 µl chilled 100% ethanol and invert sample several (ca. 5- 10) times.

B. Binding to the column.

10. Pipet or carefully pour the solution into a labeled NucleoSpin mini column sitting in a new Clontech 2 ml collecting tube. It is OK to have tissue bits in the column. Do not wet the rim or outside of the column; this can lead to centrifuge contamination.
11. Centrifuge at 8,000-10,000 rpm (≥6000 x g) for 1 min. and discard collection tube and flow through.

C. Washing.

12. Place NucleoSpin column into a fresh Clontech 2 ml collection tube and add 500 µl wash buffer BW.
13. Centrifuge at 8,000-10,000 rpm (≥6000 x g) for 3 min. and discard collection tube and flow through.
14. Place NucleoSpin column into a fresh Clontech 2 ml collection tube and add 500 µl wash buffer B5.
15. Centrifuge at 8,000-10,000 rpm (≥6000 x g) for 1 min.
16. Carefully empty collection tube and place column back in collection tube.
17. Centrifuge at full speed for 1 min. and discard collection tube and flow through.

(Turn the page…..)

D. Elution.

18. Label 2 fresh 1.5 ml centrifuge tubes for each NucleoSpin column.
19. Place the NucleoSpin column into one of the tubes.
20. Add 100 µl ROOM TEMPERATURE elution buffer (or 200 µl ddH_2O; buffer has higher pH and may work better) to the NucleoSpin tube and allow to sit at room temperature for 1 minute.
21. Centrifuge at 8,000-10,000 rpm (≥6000 x g) for 1 min. This tube contains the first elution of DNA.
22. Place the NucleoSpin column into the other microcentrifuge tube.
23. Add 100 µl elution buffer (or 200 µl ddH_2O) to the NucleoSpin tube and centrifuge at 8,000-10,000 rpm (≥6000 x g) for 1 min. This tube contains the second elution of DNA.

The first elution will be more concentrated than the second. Depending on the final DNA concentration desired, you can combine the elutions or store and use them separately.

Qiagen Kits

A. Digestion.

1. Add 180 µl buffer ATL to the prepared tissue.
2. Add 20 µl proteinase K solution.
3. Using pipette tip, mash tissue pieces against side of microcentrifuge tube.
4. Incubate at 56°C for 3- 4 hours. Shake during incubation, but do not vortex.
5. Briefly centrifuge tube to remove condensation from inner lid. A pulse will do it; do not pack the tissues in the bottom of the tube!
6. Add 200 µl buffer AL to the sample and invert several (ca. 5- 10) times.
7. Incubate sample at 70°C for 10 minutes.
8. Briefly centrifuge tube to remove condensation from inner lid.
9. Add 200 µl chilled 100% ethanol and invert sample several (ca. 5- 10) times.

B. Binding to the column.

10. Pipet or carefully pour the solution into a labeled Qiagen mini column sitting in a new Qiagen 2 ml collecting tube. It is OK to have tissue bits in the column. Do not wet the rim or outside of the column; this can lead to centrifuge contamination.
11. Centrifuge at 8,000-10,000 rpm (≥6000 x g) for 1 min. and discard collection tube and flow through.

C. Washing.

12. Place Qiagen column into a fresh Qiagen 2 ml collection tube and add 500 µl wash buffer AW1.
13. Centrifuge at 8,000-10,000 rpm (≥6000 x g) for 3 min. and discard collection tube and flow through.
14. Place Qiagen column into a fresh Qiagen 2 ml collection tube and add 500 µl wash buffer AW2.
15. Centrifuge at 8,000-10,000 rpm (≥6000 x g) for 1 min.
16. Carefully empty collection tube and place column back in collection tube.
17. Centrifuge at full speed for 1 min. and discard collection tube and flow through.

D. Elution.

18. Label 2 fresh 1.5 ml centrifuge tubes for each Qiagen column.
19. Place the Qiagen column into one of the tubes.
20. Add 100 µl ROOM TEMPERATURE elution buffer (or 200 µl ddH_2O; buffer has higher pH and may work better) to the Qiagen tube and allow to sit at room temperature for 1 minute.
21. Centrifuge at 8,000-10,000 rpm (≥6000 x g) for 1 min. This tube contains the first elution of DNA.
22. Place the Qiagen column into the other microcentrifuge tube.
23. Add 100 µl elution buffer (or 200 µl ddH_2O) to the Quiagen tube and centrifuge at 8,000-10,000 rpm (≥6000 x g) for 1 min. This tube contains the second elution of DNA.

The first elution will be more concentrated than the second. Depending on the final DNA concentration desired, you can combine the elutions or store and use them separately.

Chelex

Uses Chelex 100 Resin, Biotechnology Grade (Bio-Rad cat. # 143-2832)

1. Make a 5% solution of Chelex 100 in water (see below) and aliquot - see below.

2. If Chelex solution is already made, simply obtain CLEAN 5% Chelex tubes from the rack in the freezer and turn on the heating block.

3. Place a TINY amount of tissue into a 1.5 ml microcentrifuge tube. One milligram of tissue is about the right amount. It is usually best to just stab a clean cotton-plugged pipette tip into the frozen tissue (1-3 ul of tissue is more than enough). 5-10 ul of tissue fluids is usually plenty. In general, the nastier the chemicals an organism makes, the less tissue that should be used.

4. Add 100 :l of the 5% chelex solution to the sample. [The Chelex resin beads must be distributed evenly in the solutions, so pipette the Chelex solution while it is stirring to keep it in suspension.] Pipette up and down several times to mix the chelex solution and the tissues. [Note you should use the same pipette tip for steps 2-4. The amount pipetted in step #2 is not really important - so just set your pipette to 100 ul , squirt some chelex solution into a new tube, stab the tip into the tissue, and then mix the tissue into the original chelex tube.]

5. Boil for 10 minutes in the heating block, or as long as it takes to do step 6.

6. Set up your PCR master mix.

7. Take your Chelex tubes off the heating block.

8. Distribute the PCR master mix into the PCR sample tubes.

9. Place 2 ul of Chelex solution (**Avoid the Beads**) into the sample tubes. You should physically stir by swirling the pipette tip in the upper aqueous portion of the tube, and pumping up and down. This will ensure that DNA in the tube is in the aqueous solution, but doesn't seem to stir up the beads too much. Remainder of extractions should be stored in the freezer.

10. Thermocycle with normal parameters. (You may find it necessary to add 3-5 cylcles to get equal amounts of product DNA because you start with very few copies.)

Preparation of Chelex 100 Master Mix and Aliquots

1. Obtain a new 50 mL polyethylene (Falcon) conical tube.

2. Sterilize a spatula and the tiniest magnetic stir-bead by soaking in a beaker of Chlorox for 10^{+} min. After soaking, rinse the stir-bead and spatula thoroughly with dH_2O. Note: the stir-bead can be held in place by using a larger stir-magnet on the outside of the beaker.

3. Place the stir-bead in the conical tube. Then, place the conical tube + stir-bead on the scale, and weigh 2.0 grams of Chelex 100 into the tube.

4. Fill the conical tube to the 40 mL mark with dH_20.

5. Place the conical tube on a stir plate and let the stir-bead do its thing. Ensure that the entire volume of Chelex + dH_20 is stirring. Note: You may wish to do this step and the next one in a Laminar Flow Hood.

6. Using the P1000 and filtered pipette tips, aliquot the stirring 5% Chelex into 1.5 mL micro-centrifuge tubes obtained from a freshly opened bag. It is usually best to make both 500 μL aliquots and 200 μL aliquots.

(From Travis Glenn, modified by Steve Jordan; Based on the protocol of: Walsh, S. P., D. A. Metzger, and R. Higuchi. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10(4): 506-513. See also Morin, P. A. and D. S. Woodruff. 1992. Paternity exclusion using multiple hypervariable microsatellite loci amplified from nuclear DNA of hair cells. Pages 63-81 in R. D. Martin, A. F. Dixon, and E. J. Wickings (eds). Paternity in primates: genetic tests and theories. Basel, Karger. Master mix and aliquot protocol Adapted from Museum of Vertebrate Zoology (MVZ) protocol.)

Salting Out

1. Use a small sliver of thorax muscle (few mm square).
2. Put the tissue into 1.5 ml eppendorf and crush in 10 μl Proteinase K (10 mg/ml).
3. Add 600 μl TNES (pH 7.5) buffer by letting it run down the crushing tool to wash as much as possible of the tissue into the eppendorf.
4. Mix and incubate at 37°C overnight (or 55°C for a few hours).
5. Add 190 μl 5M NaCl (saturated solution) and vortex until well mixed.
6. Centrifuge at 14,000 rpm (full speed) for 10 min.
7. Tip the liquid off into a new tube, making sure not to disturb the pellet.
8. Add 1 volume of cold 100% ethanol (800 μl). Gently invert tubes to precipitate DNA.
9. Spin at maximum rpm for 6 minutes to pellet DNA.
10. Tip off the supernatant and discard.
11. Gently wash pellet by pipetting 1 ml of 70% ethanol into the eppendorf and gently inverting several times.
12. Spin at maximum rpm for 6 minutes.
13. Tip off the ethanol.
14. Spin at maximum rpm for 2 minutes.
15. Carefully pipette off the last of the supernatant.
16. Air dry pellet with tube open, or place tube in 37°C block to dry.
17. Dissolve DNA in 100 μl of 1x TE Buffer.
18. Store DNA at –20°C.

TNES Buffer, pH 7.5
50mM Tris base pH 7.5 (with HCl)
400 mM NaCl
20 mM EDTA
0.5% SDS

(Sunnucks and Hales 1996: MBE 12: 510-524)

Sodium Acetate

Not recommended for long-term storage of DNA!!

1. Make a master mix of Proteinase K (Pk) in Pk buffer: approximately 0.4 mg/ml of the Pk enzyme in the Pk buffer (buffer is 10mM Tris (pH 8.0) with 10 mM EdTA (pH 8.0) and 1% sodium dodecyl sulfate (Sambrook et al. 1989 p. B 13).

2. Add 100 μl of the Pk in Pk buffer to the tissue and crush with a disposable pipette tip.

3. Incubate the tissue and Pk at 64° C for at least one hour. Do not vortex.

4. Centrifuge at 14,000 rpm for 3 minutes.

5. Transfer supernatant to new 0.5 ml microcentrifuge tubes.

6. Add 0.1 volumes of 3M sodium acetate (NaOAc) and mix by inversion. (for 100 μl this would be 10 μl NaOAc)

7. Add 0.6 volumes of isopropanol (for 100 μl this would be 60 μl isopropanol). Mix by inversion for at least 1 minute. Do not vortex.

8. Centrifuge at 14,000 rpm for 15 minutes.

9. Carefully dump the liquid onto a paper towel. Place the tube upside down on towel to get rid of as much liquid as possible.

10. Add 300μl 70% ethanol. "Snap" the tube several times to dislodge the pellet. Let stand a minimum of 2 hours; overnight is best.

11. Centrifuge at 14,000 rpm for 10 minutes.

12. Carefully dump the liquid onto a paper towel. Place the tube upside down on towel to get rid of as much liquid as possible.

13. Dry the DNA by placing open tubes in a rack and covering with a clean tissue. Dry until all liquid is gone; overnight is generally sufficient.

14. Dissolve the DNA in 100μl sterile H_2O overnight at room temperature. Adding a small amount of TRIS may stabilize the DNA.

15 Store the DNA at 4°C. DO NOT FREEZE.

(Modified from Woodin et al.)

Simple Phenol-Chloroform

In Hood:

1. Add 500 μl phenol to each sample or prepared tissue and mix
2. Centrifuge 5 min full speed
3. Transfer aqueous top layer into new labeled tubes
4. Add 500 ul phenol:chloroform:isoamyl alcohol (25:24:1) to each tube, mix
5. Spin 5 minutes, full speed
6. Transfer top layer to new labeled tube
7. Add 500 μl chloroform:isoamyl alcohol (24:1) to each tube. Mix.
8. Spin 2 minutes, full speed
9. Transfer top aqueous layer to new tube

Ethanol precipitation:

1. Add 1/10 volume 3M NaOAc pH 5.3
2. Mix and leave on ice 1- 3 min.
3. Add 2 volumes cold 100% ETOH
4. Mix by inversion
5. Remove supernatant
6. Wash with 500 μl 70% ETOH
7. Spin full speed 5 minutes to pellet DNA
8. Air dry and redissolve to suit.

Whiting Phenol-Chloroform protocol

1. If the specimen was stored in alcohol, dry the tissue for 20 minutes in the speedvac.

2. Do an initial dry grind using a drill of some type and a pestle. The specimen should turn to powder; if any liquid remains, place in the speedvac and dry again.

3. Add 100 µl of extraction buffer (100 mM EDTA, 10 mM tris – pH 7.5, autoclave). Grind until the tissue turns into a dark slurry. *Note: any more than 100 µl in the wet grind will spill out of the eppendorf.*

4. Add 800 µl more of extraction buffer.

5. Add 100 µl of 10% SDS solution (to a final concentration of 1%).

6. Add 5-20 µg of Proteinase K (predissolved in solution, stored at –20°C).

7. Cap, seal with parafilm, vortex slightly, and incubate with gentle rocking at 55°C overnight. *Note: shorter incubation times (e.g. 2-3 hours) do not seem to work well with this protocol.*

8. Working under a fume hood, with lab coat, gloves and eye protection, add 500 µl of equilibrated phenol, shake for 5 minutes at room temperature, cetrifuge for 5 min. at 12,000 g at room temperature, and transfer top layer (aqueous layer) to a new eppendorf. *Note: the phenol should be equilibrated and clear; yellow phenol gives poor extractions.*

9. Repeat step 8 at least twice more or until there is no more precipitate between the aqeous layers.

10. Under fume hood, add 500 µl of isoamyl alcohol:chloroform (1:24) and shake at room temperature for 1 minute. Spin at 12,000 g for 5 minutes and transfer to a new eppendorf tube.

11. Repeat step 10 at least once more or until there is no precipitate between the aqueous layers. Note: it is very important that the extractions be as clean as possible and that there are no more proteins or lipids remaining in solution.

12. If the initial tissue was abundant, add ~1 ml of 100% EtOH, mix well, and place at -80°C for 15 minutes. Centrifuge at 12,000 g for 15 minutes, decant, wash in 1 ml 70% EtOH, centrifuge for 5 minutes, decant, and dry pellet. Resuspend in 250-750 µl H20 and label tubes for long term storage.

(From Mike Whiting Lab)

CTAB-DTAB

1. Grind ~1g tissue in 300 µl 1X TE (pH8.8) using drill.

2. Immediately add 600 µl DTAB (a powerful denaturing cationic detergent), mix, and put in 68°C water bath for about 45 minutes. Invert solution every 15-30 minutes.

3. Add 600 µl chloroform, invert several times, spin for 15 minutes at 12,000 rpm at 0°C. Chloroform will cause an interface to form with DNA suspending in aqueous upper layer.

4. Transfer aqueous, upper layer to a new 1.5 ml eppendorf tube and repeat step 3. (You may want to repeat chloroform extraction several times.)

5. Step 5 is optional – only if you want to recover extra DNA. Do not discard tube with interface. Add 200 µl 1xTE (pH 8.8) to interface. Grind tubes with interface and 200 µl with drill. Repeat step 2.

6. Mix 900 µl ddH_2O and 100 µl CTAB (a DNA precipitating cationic detergent) in a new tube for each sample.

7. Transfer aqueous layer from step 4 to CTAB mixture from step 4 to CTAB mixture from 5, invert tube gently several times (DNA should precipitate out). Spin from 30 minutes at 12,000 rpm at 0°C. If pellet is not visible you may want to spin again.

8. Pipette supernatant off pellet into another 1.5 ml eppendorf, suspend DNA pellet in 300µl 1.2M NaCl (to exchange CTAB and helps precipitate DNA).

9. Add 750 µl 100% EtOH, invert tube gently several times to reprecipitate DNA, spin for 30 minutes at 12,000 rpm at 0°C.

10. Store pellet and supernatant tubes in –70°C freezer overnight.

11. Take tubes out of freezer. Spin from 30 minutes at 12,000 rpm at 0°C. If solid, wait till it thaws.

12. Pipette supernatant off pellet into new tube. Wash DNA pellets in 300 µl 70% EtOH, spin for 30 minutes at 12,000 rpm at 0°C.

13. Dry DNA pellet on lab bench or in speedvac.

14. Dissolve DNA pellet in 50-100 µl ddH_2O. It may be helpful to heat pellet in 68°C water bath after adding ddH_2O if pellet does not readily resuspend. Store at –20C.

DTAB:

8% dodecyltrimethylammonium bromide
1.5M NaCl
100mM Tris (pH 8.8)
50mM EDTA

CTAB:

5% hexadecyltrimethylammonium bromide
0.4M NaCl

(Gustincich, S., G. Manfioletti, G. Del Sal, C. Schneider, and P. Carninci. 1991. A fast method for extracting high quality genomic DNA extraction from whole human blood. Biotechniques 11:298-301.)

DNAzol

1. Obtain prepared tissue.

2. Remove storage ethanol from tissue.

3. Rehydrate tissue in 1.0 ml H_2O.

4. Add 70μl DNAzol. Agitate slightly.

5. When tissue appears dissolved, centrifuge briefly and remove tissue scraps.

6. Estimate volume, and add 1/2 volume absolute (100%) ETOH to precipitate DNA.

7. Centrifuge to sediment DNA. Discard supernatant.

8. Wash pellet in 1.0 ml 95% ETOH; centrifuge, discard supernatant. Do this step twice.

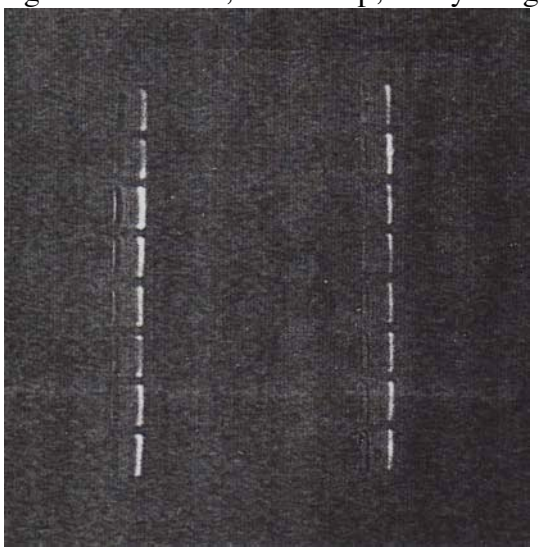
9. Remove All traces of ETOH and air dry sample.

10. Add 50 μl sterile H_2O.

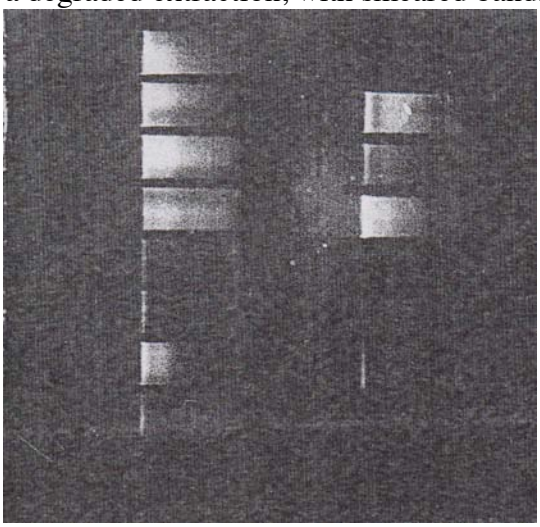
DNA Extraction Examples

These are images of DNA extracted from cicadas and run on ETBR-stained 1% agarose gels.

Below is an example of a good extraction, with sharp, heavy-weight bands:



Below is an example of a degraded extraction, with smeared bands:



Allozyme Protocols

Starch Gel Protocol:

1. Label a tube with cicada ID.

2. Pop the head off the cicada and pull out a thoracic muscle with a forceps. Place muscle in tube, and place forceps in bleach.

3. Add 50 µL dH_20 to tube and grind using hand drill. Centrifuge the tube in a refrigerated centrifuge at full speed for at least 10 minutes.

4. Make a sample wick by cutting a 5mm X 5mm square of filter paper.

5. Place sample wicks in wells of a 96-sample plate

6. Pipet 30-50 µl of supernatant onto wick, taking care to avoid debris at bottom of tube.

7. Cut off the end of the starch gel, and slide cut end back. Apply the wicks to the cut end and then replace the cut end.

8. Run the gel in a refrigerated cabinet. Don't allow the gel or buffers to freeze!

9. You may need to run the gel in the casting mold, and you may need to use a glass rod to keep the cut end of the gel from separating during running.

10. You may need to use a weight to keep the wicks in firm contact while running. A large block of ice cast in a glass dish works well—don't remove the ice from the dish, though!

11. When gel is finished running, trim all four edges before slicing it. Slice into sheets for staining.

12. Place slices in a staining dish, pour in the stain mixture, and put dish in a dark drawer.

13. You can use remaining homogenate for DNA extraction.

12% Starch gel preparation

Each gel requires 180 ml solution.

1. In a 1 L side-arm flask, mix the following:

    For LiOH gels: 18ml Solution A with 162ml Solution B.
    For Tris-Maleate gels: 18ml electrode buffer with 162 ml dH_20.

2. Weigh 21g starch into a 500ml flask.

3. Add approximately 1/3 of the buffer solution to this flask and agitate with a swirling motion.

4. Place the remaining buffer in the 1 L flask over a Bunsen burner until it just starts to boil. Do not allow the buffer to boil away.

5. While swirling both flasks, add the starch solution to the boiling buffer.

6. Continue to agitate over heat with a constant, vigorous swirling motion. Do not allow the starch solution to burn. Try to prevent large bubbles from forming. When the starch solution boils, it will turn clear.

7. De-gas the solution by applying vacuum to the flask and closing the side-arm valve.

8. When the bubbles have boiled out, release vacuum on the flask by slowly opening the side-arm valve.

9. Pour starch solution into center of gel mold. Solution should form a meniscus and be slightly higher than mold edges. Remove any bubbles.

10. Allow gel to cool for at least 30 minutes.

11. Slit gel near one end to make slot for inserting sample wicks.

12. When running gel, run with casting frame in place and insert glass rods to prevent shrinkage from breaking electrical continuity.

Extraction Buffer Recipe:

"Microbuffer" (Werth 1985)

Molarities of Components	Sigma	Amount in 100 ml
0.2 M Trizma base	T- 1 503	2.42 g
7 mM sodium borate, 10-hydrate, (= sodium tetraborate or borax)	B-9876	0.26 g
2 mM sodium bisulfite (= sodium metabisulfite)	S-9000	80 mg
50 mM ascorbic acid, sodium salt	A-7631	0.99 g
4 mM diethyldithiocarbamic acid	D-0632	68 mg
0.4 mM EDTA, disodium salt	ED2SS	13 mg

pH the solution to 7.5 with concentrated HCl
Add just prior to use:

0.17 % ß-mercaptoethanol	M-6250	0.17 ml
10 % (w/v) polyvinylpyrrolidone (PVP-40)	PVP-40T	10.0 g

Gel and Running Buffer Recipes for Starch Gels:

Lithium Hydroxide (Selander et al. 1971)
Solution A (used "straight" as electrode buffer)

(0.029 M lithium hydroxide, monohydrate; 0. 19 M boric acid; pH w/o adjustment ca. 8. 1)
LiOH-H_20 1.20 g/liter
Boric acid 11.89 g/liter

Solution B

(0.05 M Tris; 8.3 mM citrate; pH 8.4)
Citric acid (free acid) 1.6 g/liter
Tris (Trizma) base 6.2 g/liter

pH to 8.4 using 6.0 N NaOH. To make the gel buffer, mix 1 part of solution A with 9 parts of B. Run the gel at ca. 75 ma. Run times are ca. 4 - 5 hours.

Tris-Maleate (Selander et al. 1971)
Electrode Buffer
(0.1 M Tris, 0.1 M maleic acid, 0.01 M EDTA, 0.01 M $MgCl_2$)

Tris (Trizma) base 24.20 9/liter
Maleic acid 23.20 g/liter
EDTA (Na2 salt) 7.44 g/liter
$MgCl_2$-$6H_20$ 4.06 g/liter

Use 10 M NaOH 9 (ca. 25.9 ml) to pH to 7.4, bring vol. to 1.0 liter.

Gel Buffer: Use 1 part of electrode to 9 parts dH_20.

Run gel at ca. 75 - 100 ma. Running time is ca. 5 hours.

Running Buffer Recipes for Cellulose-acetate Gels:

Tris HCL pH 8.0

44.4g TRIZMA base
350ml 1M HCl
4 Liters H_20

TG Buffer

30g Trizma Base
144g Glycine
1 liter for stock, dilute 1:9 for use

CAEA

38.4 g citric acid
57.6 g CAEA
1 liter for stock; dilute 1:4 for use

Allozyme Stain Recipes:

β-EST:

Fast Blue RR Salt	dash
1% B-Napthyl acetate in acetone	5 drops
0.1M Phosphate Buffer pH 6.0	25ml

Run in LiOH Buffer. No agar overlay.

α-GPD:

α-DL-glycerophosphate	dash
NAD	dash
MTT	dash
PMS	dash
0.2M TRIS-HCl buffer pH 8.0	50ml

Run in LiOH Buffer. Agar overlay for staining.

PGM:

α-D-glucose-1-phosphate	dash
NAD	dash
MTT	dash
PMS	dash
G6PDH (10 units/ml)	10 drops
1 M $MgCl_2$	0.5ml
0.2M TRIS-HCl buffer pH 8.0	25ml

Run in TRIS-Maleate Buffer

Allozyme Stain Stock Solutions:

1% B-Napthyl acetate solution

B-Napthyl acetate	0.3g
Acetone	30ml

5% Glucose-1-phosphate (MUST be contaminated with G-1 6dP)

D glucose-1-phosphate	1g
dH_20	20ml

Agar overlay

Agar	4g
dH_20	250 µl

Allozyme Stain Buffer Recipes:

0.2M TRIS-HCl Buffer pH 8

Tris	24.2g
dH_20	1 liter

0.1M Phosphate Buffer pH 6

Solution A:

$NaH_2PO_4 \cdot H_20$	27.6g
dH_20	l liter

Solution B:

$NaHPO_4 \cdot 7H_20$	53.6g
dH_20	1 liter

Combine 1 part B with 5 parts A and 6 parts H_20 to yield a 0.1 M buffer of pH 6.0

PCR Protocols

PCR with X-Taq

Template	1-1.5μL
Primer A (10μM)	1.25μL
Primer B (10μM)	1.25μL
10x X-Taq buffer	2.5μL
dNTPs	2.5μL
H_2O	1.37μL
X-taq	0.13μL
Total	*10μL*

$MgCl_2$ may also be added

PCR with Titanium Taq:

Template	1μL
Primer A (10μM)	1μL
Primer B (10μM)	1μL
10x Titanium buffer	2.5μL
dNTPs	2.5μL
H_2O	1.37μL
X-taq	0.13μL
Total	*10μL*

PCR Annealing Temperatures:

12s 55°C
COI 53°C
COII 54°C
ef1α:

start (most conserved) (PAforwardcic-R752)	53°C
middle (f650-DVR1)	59°C
end (DVF1-R114)	58.5°C

PCR Temperature Regimes:

12s/COI/COII/ef1α start:

denature	45s
annealing	50s
extension	150s

30 cycles

ef1α middle and end:

denature	45s
annealing	120s
extension	150s

30-34 cycles

PCR Cleanup Protocols

Nucleospin PCR Cleanup Protocol

1. Adjust reaction volume with 200 µl Buffer NT2.

2. Insert NucleoSpin column into a collection tube.

3. Load PCR reaction into column.

4. Centrifuge at maximum RPM for 1 min. Discard flow-though.

5. Add 600µl Buffer NT3 to the column, Centrifuge at maximum RPM for 1 min. Discard flow-though.

6. Add 200µl Buffer NT3 to the column, Centrifuge at maximum RPM for 2 min. Discard flow-though.

7. Spin column again, for 1 minute, maximum RPM.

8. Place NucleoSpin column into a clean 1.5 ml microfuge tube.

9. Add 25-50µl (40 works well) warmed Buffer NE (or dH_20) to column and incubate at room temperature for 1 min.

10. Centrifuge at maximum RPM for 1 min.

Qiagen PCR Cleanup Protocol

1. Add 5 volumes buffer PB to sample (e.g. For a 25 μl reaction, with 5μl run out on an agarose gel, add 100 μl PB).

2. Insert Qiagen column into a collection tube.

3. Load PCR reaction into column.

4. Centrifuge at maximum RPM for 1 min. Discard flow-though.

5. Add 750μl Buffer PE to the column, Centrifuge at maximum RPM for 1 min. Discard flow-though.

6. Centrifuge at maximum RPM for 1 min. Discard flow-though.

7. Place Qiagen column into a clean 1.5 ml microfuge tube.

8. Add 25-50μl warmed dH20 to column and incubate at room temperature for 1 min.

9. Centrifuge at maximum RPM for 1 min.

Jiggins Lab PCR Cleanup Protocol

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DNA Visualization Techniques

Agarose Gels

Agarose gel concentration used to separate DNA

(From Chris Jiggins and Margarita Beltran)

MW of fragments (bp)	Agarose concentration (%)
10-100	4-6
100-500	3-4
500-1,000	2-3
500-10,000	1.0
800-12,000	0.7
1,000-30,000	0.5

Standard gel is1% agarose (by weight) in 1X buffer.

Tips:

Agarose can be made in bulk and stored.

1g agarose in 100 ml buffer is a convenient working size, and can be kept in a 250ml flask.

Add dry agarose to flask, add buffer, stir, and microwave. 1-2 minutes is sufficient, but watch carefully—agarose solution tends to boil over quickly.

If you put the flask in a large glass Petri dish when microwaving, you will contain any mess from boilovers.

Cover the flask and store at room temperature.

You can cast the DNA stain into the gel by adding it while the liquid is cooling.

Do not microwave agarose that is contaminated with ethidium bromide!

Stains such as SYBRsafe are light sensitive, and gels made with them must be run and visualized quickly.

To load the gel:

1. Put the gel in the rig, and fill with buffer to cover the wells.
2. Place a strip of parafilm, waxy side up, next to the gel rig.
3. Place small dots of loading dye on the parafilm, in a pattern matching the gel wells.
4. Pick up approximately 5 μl of your sample.
5. Deposit the sample onto one of the dots.
6. Pick up the sample by depressing the pipettor to the second (eject) stop*.
7. Load the dye and sample into the well.

**Note that this is not good for the pipettor. However, we have an old pipettor dedicated to routine gel loading for cases where exact amounts of sample and dye are not necessary. If you are trying to quantify DNA on a gel, or if you are band purifying DNA, then you will probably need to be much more accurate about the amounts of DNA and dye used.*

Using the Gel Imaging System

1. Open the light box door and place the gel on the transilluminator. Do not touch the door with gloves.
2. Close the door all the way.
3. Open the GeneSnap application
4. In the Illumination panel, choose “Upper White Light”
5. Move gel to center
6. Use focus tabs to focus camera
7. In the Illumination panel, choose “UV light”
8. Press the large red button to snap a picture
9. Print the picture by clicking the printer icon, or export the picture to disk.

Using the Polaroid Camera

The Polaroid camera is an old, cantankerous device that can quickly become an epicenter of frustration. There are a couple of tips that I have learned over the years that I thought I'd pass on, so that this knowledge is preserved.

It's my experience that most instances of "bad film" are actually problems with our equipment. I can't honestly say I've ever had an experience that pointed definitively to bad film.

Signs there is a problem:

The paper tab rips off the film when you try to pull the print from the camera
Your print has waves, streaks, or blank areas
Pictures are blurry, dark or too small

How to fix the problem:

The most important component of taking a successful picture is having clean equipment.

1. The Light Box. The surface of the light box must be kept clean. Please wipe it off after use, and do all your band cutting on the other light box.

2. The Orange filter. This filter can become very dirty. If it is, gently remove it from its holder and rinse it in warm water. Dry it gently with a Kimwipe.

3. The Camera. Polaroid photography requires that a developer/fixer paste be spread evenly over the print. In the Polaroid camera, a pair of stainless steel rollers spread this paste when you pull the print from the camera. If the rollers are not working right (e.g., dirty), they can jam or skid, and the paste won't be spread properly over the print-- and your picture will be ruined. Note when handling the pictures or the camera, that the paste is caustic, and that after developing, it likely contains silver or other heavy metals-- so wear gloves.

The camera is delicate-- and there is probably no opportunity to get repair parts, so whenever opening the camera, be extremely careful, and if you have questions, please come find me.

Whenever you change a film cartridge, make sure that the two rollers inside the camera look clean, and that there are no obvious whitish deposits on the rollers or the opening on the side of the camera. If there are, or if you get a bad picture, you must clean the camera. It is possible to remove a partially-loaded cartridge from the camera, and even though you lose some prints when doing so, it is preferable to shooting a whole cartridge of bad photos because the camera is dirty.

A quick cleaning job just involves wiping off the rollers with a Kimwipe. If the camera isn't too dirty, this might work.

A full clean involves disassembling the camera. To do so, remove the film carrier from the camera by sliding to the right-- you may have to lift up on the two wire tabs holding it. Take the carrier to a well-lighted area, AWAY from any sinks or places where small pieces could be lost. Open the carrier, and gently lift up on the grey plastic roller frame. It should just snap out—DON'T force anything. Set the carrier aside. Look at the roller frame, and note how everything is assembled. On the grey roller frame, note two shiny metal clips. These slide out-- away from the rollers. You will have to spread the fram slightly to release them. After doing so, you can lift up on the rollers--CAUTION-- two small plastic bushings-- one black and one white-- will also come out. It is important to note how these are oriented, becasue they go back in the same way. One side the of the frame is labeled "black" to facilitate assembly. Put all the pieces on a paper towel, and using 95% ETOH and kimwipes, gently clean all the deposits from the pieces. Let them air dry, and when they are fully dry, reassemble them. Remember, don't force anything.

Camera settings and using the camera:

If your images are blurry, dark, or too small, then the camera settings are not right. We use two sizes of gels, and the camera stand has specific settings for each. To move the camera up (larger gels, wider area on print), unlock the arm (lever immediately behind camera), and while turning the wheel on the right, lift up on the arm. To lower, do the opposite-- there is a black line at the appropriate height for minigels. Once you move the camera, you must focus it-- rotate the orange filter to the side, turn on the focusing light, and use the focusing knob (left side) to focus the camera (don't forget to rotate the orange filter back before taking your picture!). If you have faint bands or weak ETBR, you might consider a longer exposure--- but you don't necessarily have to change any settings-- just click the shutter twice. Finally, when pulling the print from the camera, pull on the white tab slowly and gently, and then pull the print out slowly.

Sequencing Protocols

Sequencing reactions:

	(1/4)
Reagent	**Quantity**
Big Dye v. 1.1	2.0 μl
Template	2.0 μl
Primer (2 μM)	1.5 μl
ddH_20	4.5 μl
Total	**10 μl**

Notes:

1. Vary template concentration if reaction fails. Too much template will give poor results. ¼ sequencing reactions are extremely sensitive to template concentration.
2. "Half Term" may be substituted for some of the ddH_20; typically 2μl of ddH_20 may be replaced by half term.
3. Total reaction volume can vary from 9-11μl, if template amount must be varied.
4. For ef1α, use 3μl of Big Dye reaction mix.

Half Term Recipe:

Sequencing temperature regime:

12s/COI/COII/ef1α start		
denature	30s	96°C
anneal	30s	50°C
extend	120s	60°C

ef1α middle and end		
denature	30s	96°C
anneal	30s	54.5°C
extend	120s	60°C

Notes: Typical annealing temperature is 50°C; however, it is extremely important to raise the annealing temperature to 54.5°C for ef1α middle and start.

Jiggins Lab Sequencing Protocol

Replace this page with .pdf document

Sephadex Column Preparation

A Sephadex slurry will bind to and remove unincorporated BigDye terminators and short sequence fragments. The object of this protocol is to run the sequencing reaction through a set length of slurry that will bind contaminants; the sequencing reaction will flow through.

1. Make a stock of 2g Sephadex in 32 ml dH_20. Place slurry on shaker table until well hydrated. Sephadex will NOT dissolve.

2. Add 800 µl Sephadex slurry to a spin column.

3. Spin 1 minute @ 500- 1000 rpm, drain fluid.

4. Spin 2-3 minutes @ 2000- 2800 rpm, drain fluid.

5. Place column in a 1.5 ml microfuge tube and add entire sequencing reaction.

6. Spin 3 minutes @ 2000- 2800 rpm

7. Bring volume to 10 µl or (optional) dry sample in speed vac and rehydrate to 10 µl.

8. Discard the Sephadex, rinse and dry the tube.

NOTE: The Sephadex columns have a "nub" on one side. Always orient this in the same direction when loading tube into centrifuge. NEVER exceed 4,000 rpm when spinning Sephadex tubes, as this will clog the cotton filter.

Instructions for using the 3100 Sequencer

1. Turn on the computer.

2. Turn on the sequencer

3. Open "3100 Data Collection"

4. Prepare the sequencer
    A. Check to see that the array is installed
    B. Check for bubbles in the tubes below the syringes. If bubbles are present, remove the "shot glass," place a kimwipe in the tray, and use the left syringe to expel the bubbles.
    C. If necessary, change the buffer in well #1 and in the "shot glass."
        Buffer recipe: 2.5 ml 10X stock in 22.5 ml ddH_2O

5. Prepare the samples
    A. Push tray button and remove tray from sequencer. Find clean slots on plate
    B. Add 10 µL formamide to dried sequencing samples or to 2 µl AFLP selective reactions.
    C. Transfer samples in order to wells A to H in first clean column
    D. If you have empty wells in a 16-well run, fill the empty ones with formamide.
    E. Place the tray back in the sequencer and close the door

6. Prepare a Plate File
    A. Select "new"
    B. Enter a plate name
    C. Click "Finish"
    D. A blank sample sheet will open

Sequence Data Preparation

1. Import sequence files from ABI 3100 to Sequencher on the Macintosh.
2. Convert files from DOS format using Applescript conversion utility
3. Open a Sequencher project
4. Import the sequence files
5. Trim the ends of the sequence files
6. Assemble contigs of each forward and reverse primer pair
7. Attempt to resolve discrepancies by viewing spectra
8. If sequence is poor, check signal strength
9. Export consensus of edited contig to text or nexus format.

7. Prepare a sample sheet.

For sequencing:

A. Enter sample name in first column. MAKE sure that you are assigning the samples to clean wells for the plate you will be using.
B. Choose Dye set (E)
C. Choose mobility File (DT3100POP6(BD)v.2.mob)
D. Set Project (3100_project1)
E. Choose Run Module (StdSeq.50_POP6)
F. Choose analysis module (BC3100_Seq.offFToff.saz)
G. Click "OK"
H. Sample sheet is now pending

For fragment analysis:

A. Enter sample name in first column. MAKE sure that you are assigning the samples to clean wells for the plate you will be using.
B. Choose Dye set (D)
C. Set Project (3100_project1)
D. Choose Run Module (GeneScan50Pop6)
E. Choose analysis module (GP400HDROX)
F. Click "OK"
G. Sample sheet is now pending

8. Link the tray file and sample sheet

A. Click on the name of the pending file
B. Click on the tray containing your samples
C. Press green run arrow

9. After the run, clean the tray by rinsing in distilled H_2O, placing tray in water filled beaker, and boiling in microwave for 5 minutes.

Generalized Restriction Digest

A. Extract DNA, If using a kit, combine the elutions.

B. Pipette 100 µl genomic DNA elution into an eppendorf tube and dry. Resuspend in 5.0 µl ddH_2O.

C. Make restriction master mix

D. Add 15.0 µl Restriction/ligation mix to 5.0 µl resuspended genomic DNA. Mix and centrifuge.

E. Incubate 5 hours at 37°C.

F. Run 5 µl of digestion product on a 2% agarose gel.

G. Store remaining product at –20°C.

AFLP Protocols

Genomic DNA isolation, digestion and Adaptor Ligation.

1. Extract DNA. If using a kit, combine the elutions.

2. Pipette 110 µl genomic DNA elution into an eppendorf tube and dry. Resuspend in 5.5 µl ddH_2O.

3. Mix both adaptors together.

4. Heat the mixed adaptors at 95°C for 5 minutes and allow to cool for 10 minutes.

5. Make restriction/Ligation master mix (per sample):

10X T4 Ligase buffer	1.1µl
0.5M. NaCl	1.1µl
BSA (1mg/ml)	0.5µl
Adapter Mix A	1.0µl
Adapter Mix B	1.0µl
Enzyme A	5 units
Enzyme B	5 units
T4 DNA ligase (3U/µl)	0.05µl
ddH_20	0.38µl

6. Add 5.5 µl Restriction/Ligation master mix to 5.5 µl resuspended genomic DNA. Mix and centrifuge.

7. Incubate overnight at 37°C.

8. Run 5 µl of digestion/ligation product on a 1% agarose gel.

9. Add 48 µl ddH_2O to remaining product and store at –20°C.

Preselective Amplification

1. Prepare Preselective amplification master mix (per sample):

dNTP mix (2.5 mM)	2.0µl
10X PCR buffer	2.0µl
Taq (5U/µl)	0.1µl
Primer A (2.75µM)	2.0µl
Primer B (2.75µM)	2.0µl
ddH_20	6.9µl

2. Combine 15 µl preselective master mix with 5µl dilute Restriction/Ligation product from step 1.

3. Amplify according to the following protocol. Increase to 30 cycles if template is weak.

1. 72°C	120s
2. 94°C	20s
3. 56°C	30s
4. 72°C	120s
5. Repeat steps 2-4 20 cycles	
6. 72°C	120s
7. 60°C	30 minutes
8. 4°C	hold

4. Run 5 µl of preselective product on 1% agarose gel.

5. Add 100 µl ddH_2O to remaining preselective product and store at –20°C

Selective Amplification

1. Prepare Selective amplification master mix (per sample):

dNTP mix (2.5 mM)	2µl
10X PCR buffer	2µl
Taq (5U/µl)	0.1µl
Primer A (2.75µM)	2µl
Primer B (2.75µM)	2µl
ddH_20	10.9µl

2. Combine 15 µl Selective master mix with 1µl dilute preselective amplification product from step 1.

3. Amplify according to the following protocol. Increase to 30 cycles if template is weak.

1. 94°C	120s
2. 94°C	20s
3. 66°C	30s
4. 72°C	120s
5. 94°C	20s
6. Decrease 1°/cycle	30s
7. 72°C	120s
8. Repeat steps 5-7 9 cycles	
9. 94°C	20s
10. 56°C	30s
11. 72°C	120s
12 Repeat steps 9-11 30 cycles	
13. 60°C	30 min
14. 4°C	hold

4. Run 5µl selective product on 1% agarose gel.

5. Load 2µl selective product and 10 µl GS-400 labeled Formamide into tray and run on ABI 3100.

AFLP Analysis

1. Find “Blue AFLP” template and open it into Genotyper
2. Add your files: (File menu: import --> From GeneScan File)
3. Select the blue lanes: (Edit menu: select --> Blue)
4. Mark the blue lanes for analysis: (Edit menu: mark)
5. Label the peaks: (Analysis menu: label peaks: the size in bp)
6. Label the samples: (View menu: show DyeLanes window; type sample names in sample information field)
7. Pre-normalization: Macro 1
8. Score for Editing: Macro 2
9. Final Table: Macro 3
10. Flip Table: Macro 5
11. Export table to an Excel file (Table: export…..)

Structure of AFLP primers and adapters

*Eco*RI adapters:
5’ CTC GTA GAC TGC GTA CC 3’
3’ CAT CTG ACG CAT GGT TAA 5’

*Eco*RI-0 primer:
5’ GAC TGC GTA CCA ATT C 3’

*Mse*I adapters:
5’ GAC GAT GAG TCC TGA G 3’
3’ TA CTC AGG ACT CAT 5’

*Mse*I-0 primer:
5’ GAT GAG TCC TGA GTA A 3’

*HinP1*I adapters:
5’ CAC GAT GAG TCC TGA A3’
3’ CTA CTC AGG ACT TGC5’

*HinP1*I-0 primer:
5’ GAT GAG TCC TGA ACG C 3’

*Mfe*I adapters:
5’ AAT TCC AAG AGC TCT CCA GTA C3’
3’ GG TTC TCG AGA GGT CAT GAT5’

*Mfe*I-0 primer:
5’ GAG AGC TCT TGG AAT TG 3’

*Bgl*II adapters:
5’ CGG ACT AGA GTA CAC TGT C3’
3’ C TGA TCT CAT GTG ACA GCT AG5’

*Bgl*II-0 primer:
5’ GAG TAC ACT GTC GAT CT 3’

Cloning Overview

Bacteria have circular genomes. Often, there are additional, circular pieces of DNA inside bacterial cells. These additional pieces are called plasmids, and they may contain genes that are expressed in the bacterial phenotype. Plasmids are replicated as bacterial cells grow.

Bacterial phenotypes may be altered by inserting plasmids containing genes for specific functions.

The Topo TA Cloning kit:

PCR products have overhanging, 3' terminal A residues.

The Topo TA cloning kit contains a linearized plasmid with terminal, overhanging T residues. This plasmid is called a vector, because it will be used to add DNA to bacterial cells. Bound to the vector is an enzyme (topoisomerase) that will insert PCR product into the linearized plasmid, producing a circular plasmid product.

The bacteria included in the cell are not antibiotic resistant, and they lack functional copies of the Lac-Za gene.

The vector has been carefully designed with the following features:

Flanking the insertion site are complementary sites for M13 primers.

Thus, the plasmid can be PCR amplified using standard M13 primers.

The vector contains a functional Lac-Za gene.

Thus, insertion of the plasmid into a bacterial cell adds a copy of the Lac-Za gene to that cell.

The insertion site is within the Lac-Za gene.

Thus, insertion of PCR product disrupts this gene, preventing production of the a-peptide of B-galactosidase

The plasmid contains a gene for antibiotic resistance.

Thus, insertion of the plasmid into a bacterial cell makes the cell resistant to antibiotics.

Therefore:

1. Transformed cells carry a copy of the PCR product
2. Transformed cells are antibiotic resistant
3. Transformed cells lack a functional Lac operon

Cells that have failed to incorporate a plasmid are not resistant to antibiotic
Cells that have taken up a defective plasmid lacking the PCR insert have a functional Lac operon.

How cloning works:

1. Preparing the plasmid:
   In this reaction, the linearized plasmid, covalently bound enzyme, and PCR product are combined. The product is a circularized plasmid ready for insertion.

2. Transforming the bacteria:
   In this step, the bacteria are caused to take up the plasmid. We use heat to shock the bacteria; heat changes the porosity of the bacterial cell wall/membrane, and allows plasmids to enter.

3. Growing the bacteria
   Following heat shock, all bacteria are grown in SOC broth.

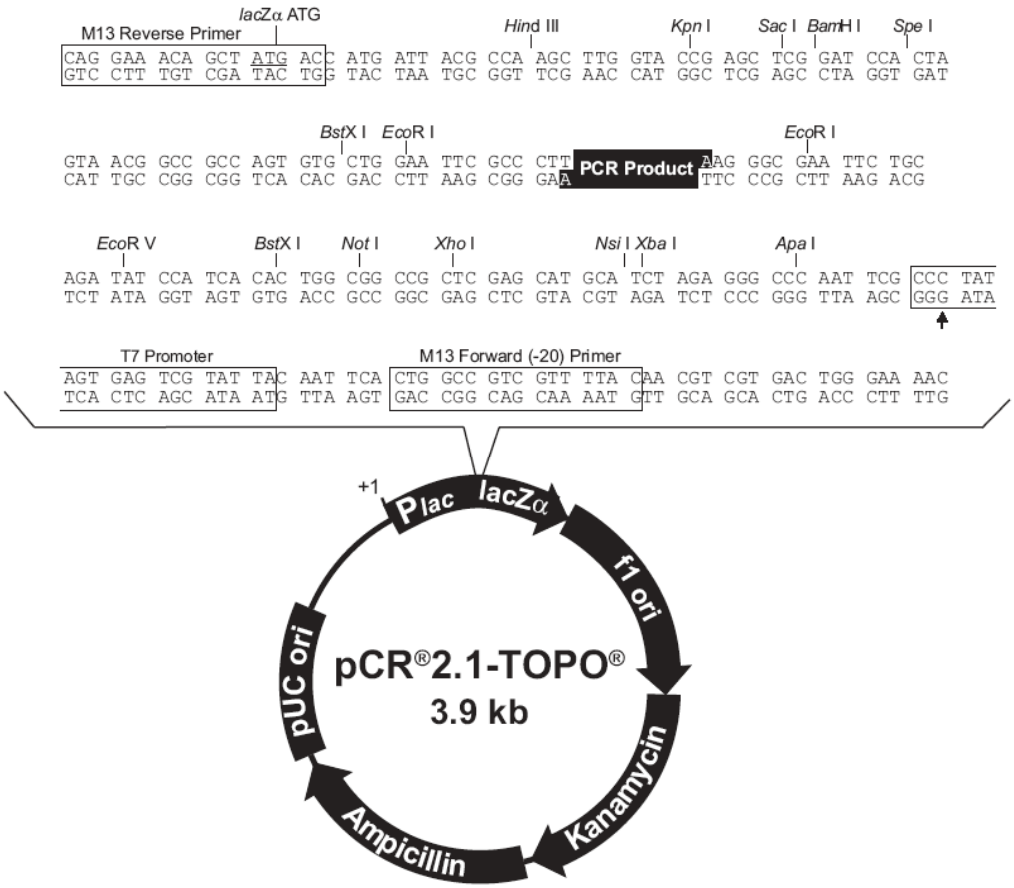
4. After the bacteria have been grown in SOC medium they are plated onto plates containing Kanamycin and X-Gal. Kanamycin kills untransformed colonies. X-Gal is an artificial substrate for the B-galactosidase. When metabolized by B-galactosidase, this substrate produces a blue color. As the plated bacteria grow and divide, they replicate their plasmids.

5. Pick the colonies.
   After the bacteria have grown on plates, white colonies are picked for PCR with M13 primers. White colonies have been transformed (because they grow on Kanamycin plates), and they have a plasmid PCR product insertion (because they cannot metabolize X-Gal). Blue colonies have been transformed, but since there is no PCR product insertion in the Lac-Za gene, they can metabolize X-Gal. PCR is extremely simple—set up a master mix, aliquot it into several tubes, and then grab a piece of colony with a yellow pipette tip; swirl the tip in the PCR tube with master mix, and PCR.

6. Confirm that you have some PCR product (the length of the product may be unknown) using an agarose gel.

7. Clean the PCR product and sequence using M13 primers.

8. Analysis: **BEWARE**: Your sequence should be clean and easy to align—but it will also contain vector sequence! You must *also* align the vector sequence to your sequence, so that the vector sequence may be removed. You can do this at the end—the vector sequence can actually be helpful during the alignment process; just be aware that you must make sure all vector sequence is removed from your finished sequences.



(compiled by John Cooley)

Cloning with a TOPO-TA kit

A. Preparation

1. Set water bath to exactly 42°C and incubator to exactly 37°C.

2. Place one LB/KAN+ plate for each PCR product in 37°C incubator for ~ I hour.

3. From the TOPO TA kit, thaw the salt solution and the SOC medium (stored at –20°C).

4. Place the TOPO Cloning vector on ice (stored in –20°C)

5. Place the competent *E. coli* cells on ice (stored in –80°C)

B. Preparing the plasmid

1. Prepare a cloning master mix:

Per cloning reaction:
0.5 µl salt solution
2.0 µl ddH_2O
0.5 µl cloning vector

2. To 2.0 µl PCR product, add 3 µl of master mix from Step B1.

3. Incubate on ice for 5 minutes.

C. Transforming the bacteria

NOTE: when transferring bacteria, cut the pointed tips off of yellow pipette tips

1. Add 2 µl reaction from step B3 to 25 µl of competent cells (1/2 vial).

2. Allow mixture to sit on ice 5-30 minutes (15 minutes works).

3. Heat shock cells @ 42°C for exactly 30 seconds and return cells to ice.

4. Add 125 µl SOC medium to cells and shake @ 37°C for one hour.

You may use this time to spread XGal onto plates—see LB/KAN recipe

5. For each tube of transformed cells, plate 50 µl of cells on one plate, and the remainder on another. Label plates—***not*** lids!

6. Parafilm the edges of the plates, invert, and incubate overnight @ 37°C.

7. Pick white colonies and add directly to PCR reaction using M13 primers.

LB Broth Plates for Cloning

	1 liter	***0.5 liter***	***0.25 liter***
H_2O	1000 ml	500 ml	250 ml
Tryptone	10g	5g	2.5g
Agar	15g	7.5g	3.75g
NaCl	10g	5g	2.5g
Yeast extract	5g	2.5g	1.25g

1 liter of solution makes about 40 plates
Use tap water, not distilled water

1. Use recipe above to make solution in a large Erlenmyer Flask.
2. Cover the top of the flask with foil and autoclave on liquid cycle.
3. Allow solution to cool below 55°C. You can use a water bath set to 45°-50°C to maintain the solution in a liquid state before pouring.
4. After the solution has cooled, per each liter of LB solution, add 1 ml of 50 μg/ml Kanamycin stock solution.
5. Pour the plates and allow them to solidify. You can place them in the refrigerator overnight.
6. On each plate, add and spread with a sterile glass rod 40μl of 40mg/ml Xgal stock solution

Xgal Stock solution:

1 g X-gal in 25ml Dimethyl Formamide
0.1g in 2.5 ml
0.5g in 12.5 ml
Aliquot into 1.5 ml tubes, wrap in foil, and store -20°C.

Dimethyl Formamide (DMF) is toxic. Use gloves and do not get on skin!

Kanamycin stock solution:

1 g Kanamycin powder per 20 ml dH_20
Aliquot into 1.5 ml tubes and store -20°C.

Cloning Primers and Vectors

M13 primers

M13-FOR:

5’ GTA AAA CGA CGG CC

M13-REV:

5’ AAC AGC TAT GAC CA

TOPO TA vectors (remember to excise the vector from your finished sequence!)

Vector sequence A:

ACAGCTATGACCATGATTACGCCAAGCTTGGTACCGAGCTCGGATCCACTAG
TAACGGCCGCCAGTGTGCTGGAATTCGCCCTT

Vector sequence B:

AAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCAT
CTAGAGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTC
GTTTTACAACGTCGTGACTGG

PGEM Cloning Protocol

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Electroporation Cloning Protocol

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