Fungal DNA BARCODING PROTOCOL

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A blend of the highly recommended Counter Culture Lab protocol, Damon Tighe's quick + dirty protocol, a protocol I found on ResearchGate and the protocol Alan Rockefeller uses here and here (which has a great section on analyzing results at the end). In addition to this written protocol I've also created a series of YouTube videos that show these steps (though some of them are out of date, unlike this protocol).

Also check out this very comprehensive lecture by Alan Rockefeller that includes not only the PCR process but sequence analysis, Genbank upload and tree building. Another great resource are Everymanbio's YouTube videos on at-home sequencing, sending your samples to the lab and interpreting the results. Harte Singer also recently posted some great videos: editing a trace file, analyzing that sequence with BLAST, building a contig from forward and reverse reads using Seqtrace, and saving your contig and checking it with BLAST.

Danny Miller has created a number of great resources: a video explaining DNA sequencing; a video and accompanying document describing how to make genetic trees and a video and accompanying document describing how to analyze DNA sequences. They can all be found here.

Here is my guide to bulk uploading sequences to Genbank.

UPDATE: I've made changes to this protocol based on updated practices by Alan Rockefeller and Stephen Russell - all credit goes to them

I've found this protocol to work decently well for fresh and dried gilled fungi, jelly fungi, softer polypores, mycelium, soft and hard ascomycetes/pyrenomycetes.

SUPPLIES FOR EXTRACTION AND AMPLIFICATION

(For summary of equipment, supplies and cost see page 8 onward)

HARDWARE (see also page 7 for options and cost)

- Thermal cycler (I use a miniPCR 16 well thermal cycler - but consider using a used industrial thermal cycler like a used Geneamp 9700 thermal cycler or a used Biorad My Cycler, which are cheape and have greater capacity (see page 9 for more info)
- Centrifuge (I use the Gyro centrifuge that came with my miniPCR kit that can be adapted to take 6 1.7ml or up to 16 0.2ml tubes)
- Gel electrophoresis setup (I use the miniPCR one that came with my kit)

Cheaper hardware can be found used on eBay. Some people don't use a centrifuge and do OK.

- 3 micropipettes: 1-10 µl, 2-20 µl, 20-200 µl (came with my miniPCR kit). Here is a video on how to use a pipette.
- Scale for very small amounts to measure out agar powder
- Regular kitchen scale for weighing TBE buffer which you'll use to make a gel block

MISC

- Fine tipped permanent marker (to label tubes)
- Pointed tip tweezers WITHOUT RIDGES to cleanly rip small piece of tissue from mushroom
- Waste container (any plastic container)
- Tube rack (many available on eBay or Amazon) - or improvise one
- Large-ish glass cup for boiling agarose and measuring out buffer
- ***If you're not using mastermix that already has dye in it*:** strips of aluminum foil for mixing loading dye and fungal DNA before loading into gel

For eight samples/one strip:

CONSUMABLES

- Lots of 10µl and 200µl pipette tips, loose or in boxes (buy 1000 loose in bulk on eBay to refill boxes)
- Two 0.2 ml tubes per sample, as eight-tube strips (MC Lab wants their samples in a strip, GeneWiz doesn't)
- One loose 0.2 ml tube to pre-mix mastermix, primers and distilled water
- A pair of disposable non-powdered gloves (box of 100)

EXTRACTION REAGENTS

- Distilled water (drug store) or molecular grade water
- 0.5M NaOH - buy as crystals and dissolve. You only have to do this once - this will last you forever. To make 0.5M NaOH, measure out 5g of sodium hydroxide and dissolve it in 250ml of distilled water (or whatever quantity you want, as long as the ratio is 1:50)
- Tris 8.0ph buffer - this needs to be diluted 1:10. Do prepare this freshly ever so often. Bacteria can grow in older reagents.

NEW OPTIONS

- Alan points out that IBI Scientific offers a solution (X-Amp 50ml) that turns extraction into a single step and can be kept at room temperature. Upfront cost is high ($128), but actual cost per sample is under $0.10. Worth it if you plan to extract a lot of samples.

- MiniPCR's DNA single-step extraction buffer does away with the need to grind, wait and centrifuge. All it requires is a 10 minute heat block. This has worked some of the time for me, but but not all of the time.

Everymanbio just alerted me to this new reagent:

- 6x Green Fluorescent DNA Loading Dye:
  https://www.bioland-sci.com/index.php?main_page=product_info&products_id=950
  This one is cool because it combines the gel dye and loading dye into one. With this product, you won't need to add a separate dye to your gel. Just add your loading dye like normal to your pcr sample and it will dye the dna so you can see it on the gel.

AMPLIFICATION REAGENTS

- Mastermix (the ODIN if you need very small quantities or suppliers like New England Bio Labs). I use mastermix that already has loading dye in it (Empirical Bioscience - 12.5ml gets you 1000+ reactions).

- ITS1 and ITS4 primers You can order ITS1 and ITS4 fungal primers from ODIN if you want to get started with a small quantity. I prefer to order from IDT or GeneWiz where it's much cheaper and you get much larger quantities and they're guaranteed to be fresh. I recommend ordering them in dried form, as follows: For IDT, go to this page. Give your primer a name, choose 25 nanomole oligo, add the primer sequence you want in the sequence field (see page 12 for primer sequences), formulation: none. They'll send you a seemingly empty tube that has a tiny flake in it at the bottom and you will resuspend it which means you will add distilled or molecular grade water (1940 µl for ITS1 for a standard 10µl dilution, 3670 µl for ITS4 for a standard 10µl dilution and 2380 µl for ITS1-KYO for a standard 10µl dilution. You will need a larger tube (2ml) tube to store them, frozen at -20C. When adding water to the flake you want to shake the tube to make sure it dissolves. The primers are about $9 per primer plus shipping and last for hundreds and hundreds of reactions.

- There are other primers, eg ITS4-B for basidiomycetes only (good if there might be mold contamination), but you'll need to get them custom-mixed (GeneWiz or IDTDNA). I've recently been using ITS1-F_KYO2 instead of straight ITS1 (TAGAGGAAGTAAAAGTCGTAA, custom order by GeneWiz or IDT).

  Optional: (tip from Damon Tighe): DMSO which can make for longer sequences, 1µm added to every tube, along with mastermix, primers and water.

GEL ELECTROPHORESIS REAGENTS

- Agarose powder
- TBE buffer (miniPCR, came with kit but available elsewhere) - concentrate can stay at room temperature. I've been buying buffer in powdered form, with a higher upfront cost but it lasts for many hundreds of rounds of gel electrophoresis
- Gel stain (miniPCR, came with kit, but available elsewhere) - can stay at room temperature
- Loading dye (the ODIN) - keep in freezer **(not needed if you use mastermix with dye)**
- Small glass of isopropyl alcohol to sterilize hands, tweezers etc throughout (drugstore)

Storing your reagents

A regular household freezer compartment is enough. Note however that many modern household freezers occasionally raise their temperatures to prevent freezer burn. That's not good for reagents. I highly recommend storing your reagents in an insulated shipping container or some other kind of insulated container with a few small cold packs.

Reagents also don't like being thawed and frozen multiple times. I notice that reagents sometimes stop working after a few cycles of freezing and thawing. To help with that I 'aliquot' (parcel out) reagents into smaller quantities, into smaller tubes so that I'm not freezing and thawing a large tube of mastermix or primer many times over, but only thaw and freeze a smaller tube a few times. I usually aliquot about 24 reactions worth of primerss in one PCR tube, and do several strips at a time so I only have to do this once in a long while. I aliquot enough mastermix for eight reactions in one PCR tube.

PREPARATION

1. Decide which fungi to process
2. Enter fungi into log/spreadsheet to keep track of them
3. (If not using X-Amp) Make dilutions of the NaOH (1:50 from granules) and the TBE buffer (1:10 from stock mixture) - *you only need to do this once in a while since this will create enough for many, many sessions*
4. Set up centrifuge
5. Set up thermal cycler, connect thermal cycler to computer or download miniPCR app and pair via Bluetooth, create programs for amplification and 10 minute heat block *(you'll only need to do this once) - see later on how to do this*
6. Clean area
7. Lay fungi out on a plate in the order of the spreadsheet (I order them by iNat number)
8. Put on gloves
9. Have small glass or sprayer with isopropyl alcohol handy to disinfect hands ever so often
10. Label tubes (for eight samples you will need two eight-tube strips)

EXTRACTION

- Rip off a bit of tissue from the specimen with tweezers (smaller than a grain of rice) and place each in an 0.2ml labeled tube. Smaller is always better. If specimen is not clean, break it open and take from inside. Make sure to sterilize your tweezers between each specimen by wiping them down with a paper towel with isopropyl alcohol on it.

The traditional way

- Add 30µl 0.5M NaOH to each tube (no need to switch pipette tips between tubes if you don't touch the fungal tissue)
- Grind and macerate each sample with a pipette tip for a few seconds - **one fresh tip per sample**
- Allow to stand for 10 mins (put dried fungi back in bags while you're waiting)
- Add 150µl of diluted Tris 8.0ph buffer to each tube (no need to switch pipette tips between samples as long as you don't touch the liquid in the tubes with your pipette tip)
- Heat at 95˚ Celsius in your thermal cycler for 10 minutes (so-called "heat block")

The X-Amp way (courtesy of Alan Rockefeller and Stephen Russell)

- Drop tiny pieces of tissue into separate tubes, wiping tweezers with isopropyl alcohol between samples
- Add 35µl of X-Amp to each tube (careful, it's drippy)
- Put into thermal cycler for 15 minutes at 80C

For both methods

- Centrifuge for 5 mins at 5,000 rpm or higher. Make sure the centrifuge is balanced ie same number of samples on each side, centered. If you're doing eight samples this means cutting the strip in half. **Make sure the tops are properly closed!**

AMPLIFICATION

Combine in a loose 0.2ml tube the following reagents **for eight samples**. Do this step quickly. Reagents will start reacting with each other the moment you mix them, and you'll want to get them into the thermal cycler quickly.

IF YOU'RE DOING A FORWARD READ ONLY

- 95µl of mastermix (for eight samples)
- 5µl of forward primer (for eight samples)
- 5µl of reverse primer (for eight samples)
- 85µl of distilled or molecular grade water (for eight samples)

IF YOU'RE DOING A FORWARD AND REVERSE READ

- 110µl of mastermix (for 8 samples)
- 7µl of forward primer (ITS1) (for 8 samples)
- 7µl of reverse primer (ITS4) (for 8 samples)
- 100µl of distilled or molecular grade water or distilled water (for 8 samples)

Optional: If you're using DMSO to make your sequences longer, for every tube replace 1µm of water with 1µm of DMSO (make sure not to get it onto your hands)

Mix the mixture by aspirating it up the pipette when you add the water.

Pipette 20µl (if you're only doing a forward read) or 26µl (if you're doing a forward and reverse read) of the mix into each of the tubes of the strip.

Add 1µl of the mushroom liquid you've prepared in the extraction step to each of these tubes (2µl if you have used X-Amp), using a new pipette tip for every sample. This is a very small amount. Make sure you've actually picked up liquid by visually inspecting the pipette tip.

Pipette up and down a bit to mix. Close tops **and flick each tube** to ensure it's all mixed well

Put tubes into thermal cycler

Updated run times per updated Alan Rockefeller/CounterCulture Lab protocol

If you haven't already programmed your thermal cycler program it now:

Initial denaturation: 2 minutes at 95C
30 cycles of the following:

- Denaturation: 30s at 95C
- Annealing: 30s at 54C for regular ITS1, 52C for ITS1-KYO2
- Extension 55s at 72C

Final extension 120s at 72C

Let it run until the program is done. The samples can sit at room temperature for a while.

GEL ELECTROPHORESIS

EQUIPMENT

Scale for very small amounts with mini container and scoop, for weighing out agarose powder
Regular kitchen scale to weigh TBE buffer to make gel, and to pour over gel

Microwaveable glass cup for boiling agar and TBE buffer

REAGENTS
I used reagents from miniPCR - other suppliers might have different dilution Ratios
Agarose
TBE buffer Diluted 1:20 from stock liquid TBE buffer or from buffer in powdered form
Gel stain regular or SeeGreen gel stain
Loading dye (the ODIN) unless you're using a mastermix that already has loading dye in it

MiniPCR have created a video about how to cast a gel

CASTING A GEL

1. Place the gel tray inside the casting platform. Place on a level surface to ensure uniform gel thickness.

2. Determine the percentage gel to make:

Size of DNA to separate	Gel percentage (%)	Agarose (g)	1x TBE* (ml)
600bp to 12kb	0.8	0.16	20
500bp to 10kb	1	0.2	20
400bp to 7kb	1.5	0.3	20
200 bp to 5kb	2	0.4	20
60bp to 2kb	3	0.6	20

Note: if using two rows of wells (two combs) resolution will be reduced due to shorter separation distance.

** Warning: blueGel™ is designed to work best with 0.5 to 1.0X TBE (Tris Borate EDTA) buffer. Use of other buffers such as TAE or SB may result in impaired performance.*

3. Weigh the desired amount of agarose according to the chart above and add it to a 100ml size flask (or larger) containing 20ml of 1x TBE electrophoresis buffer. Mix well by swirling.

I usually mix for a 1% gel percentage, ie 0.2 grams of agarose and 20ml of TBE

Tip: If pouring more than one gel, agarose and buffer quantities can be multiplied by the number of gels to be poured. Increase heating time by ~15 sec per additional gel and use a larger flask

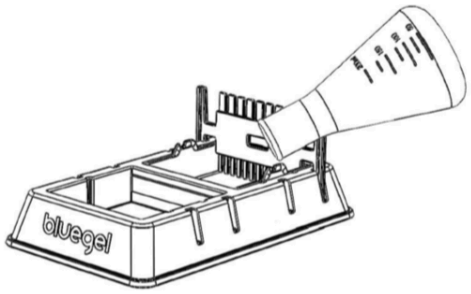
4. Place flask in microwave (~30 seconds) or on a hot plate until all the agarose is dissolved. The agarose/buffer mix is ready when no agarose particles are visible upon swirling.

CAUTION: liquid may bubble over the mouth of the flask and cause burns. Handle with care using protective equipment.

5. Let agarose/buffer mix cool for ~2-3 minutes and add 2µl of Gel Green™ DNA stain 10,000X stock (1µl per 10ml of TBE). Swirl well to mix. See Appendix B for additional DNA staining dyes that work with blueGel™.

If you're using SeeGreen gel stain you only need 1µl

6. Place the comb in the top slot and pour all the agarose/buffer mix into the gel tray. To double the well capacity add a second comb in the middle of the gel tray. Each comb will form either 9 or 13 wells. Remove any air bubbles using a disposable micropipette tip.



7. Let gel stand for ~10 minutes until completely set. For faster set time place the casting platform with the gel in a refrigerator. Do not disturb the gel during this time.

8. After the gel has solidified, remove comb/s gently by pulling straight upward.

9. Remove the gel tray from the casting platform. If a small amount of gel has formed underneath the gel tray, wipe it off and discard it.

RUNNING A GEL

1. Place the gel tray containing a gel in the buffer chamber and place the buffer chamber inside the blueGel™ base. The wells should be closest to the (-) end.

2. Add 30 ml of 1x TBE buffer in the buffer chamber. The buffer should just cover the agarose gel.

CAUTION: Do not overfill the gel chamber as it may overflow when the cover is placed over the gel.

3. Remove air bubbles (if any) trapped between the gel and the gel tray, or between the gel tray and the buffer chamber

If you're using loading dye, ie if you're <u>not</u> using a mastermix that already has loading dye in it
Set a small sheet of foil in front of the gel box and pipette 3µL of loading dye onto the foil for each of the samples you want to put through the gel

Add 4µL of your PCR mix to each drop for each sample, <u>using a new pipette tip for each one</u>

If you're using mastermix that has loading dye in it, simply place 5µm of each sample in a separate well.

4. Load the DNA samples in the wells using a micropipette.

5. Place the orange cover on the blueGel™ base. The cover contains the electrodes and will only fit in one direction, with the (+) electrode positioned to attract the negatively charged DNA.

6. Press the power button to start the run. The green LED indicator located next to the power button should light up. Small bubbles will form near the electrodes

Wait a few minutes

Put black cover over the orange cover. Switch on blue light (left button). You should see a band on each lane after a few minutes. Photographing through the hole with your smartphone can help you see better. If you don't see a band even after waiting for a few minutes the PCR didn't work for that sample.

Once you know you have a good sample, pipette your PCR mix into numbered tubes in a tube strip and prepare for shipment. You can store it in the fridge for a day or two.

TROUBLESHOOTING AT THE GEL ELECTROPHORESIS STAGE

No bands across the board?
You made a mistake mixing the reagents, perhaps forgetting a primer, or adding the same primer twice instead of one each; you used a much smaller quantity of primer by mistake
You forgot to add loading dye
Your thermal cycler didn't work
You forgot to add the sample to the mastermix and primers
You forgot to add a stain to the agarose
One of your reagents is too old

Very weak bands across the board?
You didn't add enough stain to the agarose
You forgot to flick the tubes before putting them in the thermal cycler

No bands in some lanes?
The DNA didn't amplify, eg because the specimen had issues
You didn't flick the tubes before putting them in the thermal cycler
You didn't grind the sample enough (eg hard fruitbodies)

WHICH LAB TO USE FOR SANGER SEQUENCING?

As a non-professional your options are limited. **GeneWiz** for example wants you to be affiliated with an institution. You could try and make that institution your local mushroom club, if you're a member. That worked for me, though it took a couple of phone calls with customer service. They're more expensive but they'll re-run sequences for you and you can call them to troubleshoot.

MCLab
Molecular Cloning Lab, or **MCLab**, the lab that I use, put up no such barriers. They're a lot cheaper and they're very quick. I've never used their customer service but I assume it's bare bones. They're based in South San Francisco. If I mail my samples priority mail on a Monday, they'll often arrive on Wednesday, and results are posted early in the morning on Thursday.

Label as per MC Lab specifications: A01, B01, C01 etc. They want at least 10 microliters for a forward read, and an additional 10 microliters for reverse read - if you are doing a reverse read.

[UPDATE: they apparently now let you submit a single tube for forward and reverse reads)

I usually just do a forward read.

If you're using ITS1 and or ITS 4, you no longer need to send a primer sample. They have them in stock. Just specify the primer you want used on your order form. If you're using other primers they will still want a sample of it in addition to your regular samples, 3.3 microliters of primer with 6.7 microliters of distilled water.

Email me at sigridjakob@gmail.com if you need help with their order form - it's not very newbie-friendly.

MCLab pricing: $1.99 for a forward read + $1 for cleanup (recommended); double that if you want a forward and reverse read.

GeneWiz
You want "Sanger Sequencing PCR Product - Un-Purified" They have primers so you don't need to submit yours. They'll want a picture of your gel as part of the ordering process.

GeneWiz pricing: $9 per sample. They'll automatically do a forward and reverse read and cleanup.

NOTE:

Even if gel electrophoresis signaled that DNA was amplified does not guarantee a good sequence from the lab. Expect some sequences to fail at this stage.

Many things can go wrong. The most common is a contaminated specimen which will result in two sequences on top of each other.

Another common cause of failure is using old reagents, or reagents that have been thawed and frozen too many times.

HOW MUCH WILL THE EQUIPMENT AND REAGENTS COST?

I have not researched new equipment beyond miniPCR, so you will have to do your own research.

eBay is a good source for everything, whether used or new. The Odin sometimes has refurbished equipment, plus some new equipment

	NEW	USED/CHEAP
Thermal cycler	miniPCR 8 well thermal cycler currently $650 plus shipping miniPCR 16 well thermal cycler $795 (has Bluetooth). There is an advantage to buying used thermals cyclers because they	$300+ on eBay but buyer beware, buy from reputable sellers. GeneAmp PCR System 9700 is popular as is Biorad My Cycler for running PCR. They are both very reliable brands

	have lot more capacity than new consumer-targeted products like miniPCR	that can run up to 96 reactions at a time, and you can find a Geneamp for $100-$250 or a Biorad for $250-$400. . This is a place that has been recommended for purchasing used equipment
Microcentrifuge	Gyro $160 to $250. Cheap centrifuges can be had for $80+	You could get away with not using a centrifuge
Gel electrophoresis setup	miniPCR setup currently $350 plus shipping, no transilluminator needed	$75+ plus shipping, but you will also need a transilluminator
Transilluminator		$80 plus shipping
3 micropipettes: 1-10 µl, 2-20 µl, 20-200 µl	miniPCR, $50 each plus shipping	$50 per pipette seems to be the general price; you could get away with using just a 1-10µl and a 20-200µl pipette
Lots of 10µl and 200µl pipette tips, loose or in boxes	$20 for a bag of 1000 (to refill a box)	No cheaper options
Loose 0.2µl tubes and 0.2µl tubes in a strip	$58+ for 125 strips (1000 tubes), $25 for 1000 loose tubes	No cheaper options
1.7ml tubes	From $10 plus shipping	No cheaper options
Disposable gloves	$8 to $12 for 100 plus shipping	You could wash your hands a lot
Kitchen scale	$5 to $15 plus shipping	Measuring cup for small amounts
Scale for very small amounts	$17 plus shipping	You could try and get away with using a regular kitchen scale
Permanent marker	$1 - $2	No cheaper options
Tweezers with sharp tips	$4 - $5	Use regular tweezers (but harder to grab tissue)
Tube rack	$5+ plus shipping	Use pipette tip racks
Glass cup for boiling agarose and buffer	$6	Any heat resistant cup
Distilled water	$3	$3
0.5M NaOH - sodium hydroxide	$15 for a lifetime's supply plus	No cheaper options

crystals	shipping	
Tris pH8 (1:10 dilution)	$28 plus shipping	No cheaper options
Mastermix	$30 plus shipping	No cheaper options
ITS1 and ITS4 primers	$10 for both, together plus shipping	IDI custom mixes are sold as flakes which you can reconstitute; will last a long time
Optional: DMSO	$10 for a 2oz bottle which will last forever	You'll be fine without it
Agarose	$20 plus shipping	No cheaper options
Isopropyl alcohol	$4	No cheaper options
TBE buffer	$18 plus shipping	No cheaper options
Gel stain	$40 plus shipping	No cheaper options
Loading dye	$5 plus shipping	No cheaper options

ACTUAL COST PER REACTION (as of end of 2021)

So what does it cost to do your own PCR, not factoring in the cost of the hardware?

I've calculated the cost of reagents, 'consumables' (ie pipette tips and tubes), shipping and Sanger sequencing for one sample, forward read only, using MCLab, assuming 16 samples are shipped at any one time. It comes out at **$4.74 per sample including first class shipping**

	Cost	**Number**	**Cost per item**	**Needed per reaction**	**Cost per reaction**
Tris 8.0ph buffer	$26.00	2500	$0.01	1	$0.01
Gloves	$13.00	100	$0..13	2	$0.26
TBE Buffer	$15.00	800	$0.02	1	$0.02
*Loading dye**	*$5.00*	*150*	*$0.03*	*1*	*$0.03*
Agarose	$40.00	2000	$0.02	8	$0.16
Gel stain	$48.00	1600	$0.03	1	$0.03
Large pipette tips	$18.95	1000	$0.02	4	$0.08
ITS1 primer	$5.00	50	$0.10	1	$0.10
ITS4 primer	$5.00	50	$0.10	1	$0.10

Small pipette tips	$21.20	1000	$0.03	5	$0.11
Mastermix**	$30.00	60	$0.50	1	$0.50
Tubes on a strip	$58.00	1000	$0.06	2	$0.12
Processing (MC Lab forward read only inc cleaning)					$2.99
First class postage	$4.30	8	$0.26		$0.54
Cost for forward only assuming 8 reactions					**$5.05**

*If you use loading dye

**I buy my master mix in bulk, and it only costs me $0.20 per reaction, but I realize not everyone will spend $200 for 1000 reactions

Primer sequences

Primer name	**Sequence**	**Tm**
Primer ITS_KYO	TAGAGGAAGTAAAAGTCGTAA	50.0˚
Primer ITS1	TCCGTAGGTGAACCTGCGG	59.5˚
Primer ITS1-F	CTTGGTCATTTAGAGGAAGTAA	52.2˚
Primer ITS2	GCTGCGTTCTTCATCGATGC	57.0˚
Primer ITS3	GCATCGATGAAGAACGCAGC	57.0˚
Primer ITS4	TCCTCCGCTTATTGATATGC	58.0˚
Primer ITS4-B	CAGGAGACTTGTACACGGTCCAG	59.0˚
Primer ITS4 Cantharelllus	TCCTCCGCTTATTGATTGC	52.6˚
Primer ITS5	GGAAGTAAAAGTCGTAACAAGG	51.3˚
Primer LR0R	ACCCGCTGAACTTAAGC	52.4˚
Primer LR7	TACTACCACCAAGATCT	45.6˚
Primer RBP2-b6F	TGGGGYATGGTNTGYCCYGC	62.7˚
Primer RPB2-b7R	GAYTGRTTRTGRTCRGGGAAVGG	57.9˚

Primer EF1-983F	GCYCCYGGHCAYCGTGAYTTYAT	61.2°
Primer EF1-1567R	ACHGTRCCRATACCACCSATCTT	58.8°

RPB2	
RBP2-b6F / RPB2-b7R	
Initial denaturation at 95 °C for 300 seconds	
Denaturation at 95 °C for 30s	40 cycles
Annealing at 55 °C for 45s	
Extension at 72 °C for 45s	
Final extension at 72 °C for 420 seconds	
LSU	
LR0R/LR7	
Initial denaturation at 98 for 300 seconds	
Denaturation 98C 30 seconds	39 cycles
Annealing 47.2C for 30 seconds	
Extension 72C for 30 seconds	
Final extension at 72C for 60 seconds	
TEF1	
EF1-983F / EF1-1567R and EF1-983F / EF1-2218R and EF1-1018F / EF1-1620R	
Initial denaturation at 95C for 600 seconds	
30 cycles	
Denaturation 95C 60 seconds	30 cycles
Annealing 62C for 60 seconds (decreasing 1 °C every 3 cycles)	
Extension 72C for 90 seconds	
Final extension at 72C for 420 seconds	

Other extraction protocols
PROTOCOLS

Osmundson protocol
Add 100 µL of 0.5 M NaOH to dried tissue
Grind
Heat to 95C for 10 mins
Centrifuge at 14000 RPM for 2 min
Add 2 µL of the supernatant to 200 µL of 100 mM Tris-HCl, buffered with NaOH to pH 8.5–8.9

Cooper protocol
Vigorously homogenize 20mg of dry sample in 100µm of 0.5M NaOH with a pestle
Heat to 95C for 10 mins
Centrifuge at 14000 rpm for 2 mins
Add 5µl of supernatant to 195uL of 100mM Tris-HCl at pH 8.0

DSMO addition
For every tube replace 1µm of water with 1µm of DMSO (make sure not to get it onto your hands)

Counterculture labs protocol (Alan Rockefeller)

Extraction Solution (ES)
Add 10 ml of 1 M Tris stock (pH=8.0) into clean 100 ml vessel
Add 1.86 g KCl
Add 0.37 g EDTA
Add 80 ml DI or ultrapure H2O and shake until solutes dissolve
Titrate with 1 M NaOH to pH ~ 9.5-10.0
Top up to 100 ml with DI H2O
Filter sterilize into sterile 2 ml Eppendorf tubes

Dilution Solution (BSA 3%)
Add 3 g of BSA into clean vessel
Top up to 100 ml with DI H2O
Shake
Filter sterilize into Eppendorf tubes

Procedure
Pipette out 20 µl of Extraction Solution into 8-strip tubes
Place tissue sample into Extraction Solution. Submerge sample and grind
Incubate at room temp for 10+ minutes then incubate for 10 minutes at 95˚ C.
Add an equal volume of Dilution Solution to each tube

Any questions at all, just email me - sigridjakob@gmail.com