Notes on home-based PCR for barcoding fungi

Jerry Cooper, 2021

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Morphological mycological taxonomy

For two centuries the classification of fungi has been dominated by studies based on morphology. The professional mycologist needing to confirm the identify of a collection would compare the morphology with a comprehensive verified published description, or better still direct comparison with verified material in a Fungarium (dried collections of fungi), or ultimately with the *Type* specimen on which a name is based. However, not everybody has access to those kinds of resources. For most of us fungal identification is a hit and miss affair using what resources we can lay our hands on. Some mushrooms are so distinct a photo is enough to name them with certainty. Many require microscopy to be certain. Some people with a lot of experience (or a natural talent) can see, but not always describe the subtle differences that allow them to make good decisions from photos. I am not one of those people. And then there are those who flick through guidebooks or the internet and make a guess based on similarity.

You may think the professional making a comparison with a type collection is guaranteed a correct answer, but that is not so. Frequently the dried material is old, fragmented and it is difficult to find and revive the critical characters. Often the original descriptions had no accompanying photographs or illustrations and were usually too vague to pin things down adequately. Coupled to that problem is the amazing plasticity of fungi. Within a single species the fruitbodies can vary enormously in size, colour and other features. Most of our fungi in New Zealand have not been described and the available descriptions for the small fraction of named species are often imprecise. The descriptions were written by those who did not have access to lots of collections of the same species and did not see the full range of variation. As we improve our data, we realise those earlier accounts (pre 2000) were often over-confident in describing species and made incorrect assumptions about reliable characters and variability. Correct identification of fungi is a challenge, and an interesting one in my view, but it always comes with a degree of uncertainty that must be admitted. The upshot of all this uncertainty is that many identifications are wrong, stretching all the way back for 200 years. Our fungaria have a very significant numbers of incorrect identifications and nobody has the resource to tackle that legacy, but we can make a difference in the future. We can now do that by taking advantage of modern technologies like the vast amount of information on the web (but treated with the caution that necessitates), the social media networks that instantly communicate what people have found (like iNaturalist), and more than any of these, the use of gene sequencing.

The Molecular era

Around the year 1990 we began to see the introduction of affordable **Sanger DNA sequencing** (see the glossary for terms in bold). We also identified of short stretches of DNA that could be used as **barcodes regions**, and we had the technique of PCR (Polymerase Chain Reaction) which, when combined with so-called **primers**, could selectively amplify these barcode DNA regions from tiny samples. Along with this relatively cheap wet chemistry a plethora of free digital resources became available to deal with the strings of ACGTs coming from the sequencing machines. Foremost among these is the formidable **GenBank** database of sequences which continues to grow exponentially. Today we are fortunate to have GenBank data that can be analysed by many free computer programs for comparing sequences and hypothesising the evolutionary relationships between sequences. The taxonomy of all organisms underwent a rapid revolution around the turn of the century, and no more so than in mycology. The relative degree of objectivity provided by sequence data supported some pre-DNA views, but it also over-turned vast areas of fungal taxonomy where we were wrong in our assumptions about relationships. Morphological similarities between extant species often tell us nothing about their ancestors. That revolution in fungal taxonomy continues today. One of the realizations provided by sequence data was the existence of **cryptic species**.

Collections that look the same from different parts of the world are usually different species. Often, we see small but distinct phylogenetic differences in identical looking taxa from one biogeographic region to next. Sometimes we uncover similar looking species, even from the same region, that are not even closely related according to phylogenetics. The modern working practice of a taxonomist is turned on its head, or at least it should be in my opinion. We should now rely on the sequence data to tell us what species are out there in the environment, and then use that sequence information to explore the variability in morphology, distribution, and ecology for these species. Only once we have this baseline data can we establish robust species boundaries, and from that deduce the stable morphological characters that can be used to formally describe new species and revise existing ones. Then we can create the revisions, guides and keys based on observable characters allowing species to be identified more easily - if indeed such characters exist. At least that is my approach to modern taxonomy. It is a so-called polyphasic approach, using multiple lines of evidence, but with the genetic data providing the basic framework. I have learnt to let go of my old pre-conceptions about the identification of fungi and I let the sequence data (a lot of it) keep me on the right path. The approach requires the acceptance that sometimes it is simply not possible to identify things at species level based on morphology or ecology alone. This is not to say the sequence-based approach to taxonomy is somehow fool proof. It does not generate the degree of objectivity that many assume. For a start we must come to grips with what we mean by the term *species*. For fungi that has always been a slippery concept. The phylogenetic species concept we have all adopted since 1990 is not especially objective, immutable, or always comparable with our older traditional species concepts (of which there are many definitions). In addition, the way we analyse the sequence data and make hypotheses about relationships remains a mixture of art and science, although most practitioners would defend *their* analyses as robust science. The methods we use for analysing the large amount of sequence data are highly dependent on what taxa we include and exclude and what genes we include. The variety of algorithms have a range of hugely adjustable fiddle factors, and at their heart all phylogenetic analyses involve mathematical approximations and simplifications to address computationally intractable problems (until we all have quantum computing laptops). We have replaced a historical over-confidence in morphological-based relationships and species concepts, with a current over-confidence in phylogenetic trees and what they mean. As usual with all areas of science it is necessary to critically evaluate all the evidence presented and to draw your own conclusions. For those not trained in modern scientific methods it is getting harder to make sense of things, especially for those who survive on a diet of fuzzy information from social media and who have never acquired a significant capacity for critical evaluation of information (and I'm not talking specifically about mycology!).

Role of non-professionals

Throughout the history of mycology non-professionals (by which I simply mean those not paid to do mycology as a job) have made very significant contributions by observing, collecting, and describing species. The seriously committed individual could invest in the technology (microscopes!) and the relevant books/journals and they could make ground-breaking contributions. However, since the advent of the molecular age I feel there is a growing sense of disenfranchisement amongst some groups. Non-professionals can longer contribute at a fundamental level in the way they once could because they don't have access to labs, technicians, sequencers, super-computers etc. This situation contributes to the perception of an *us and them* split between professionals and non-professionals. But is sequencing really the barrier it seems to be? There are a growing number of labs where private citizens can pay reasonable sums to send samples and get back sequences (e.g., Alvalab), and even a follow-up phylogenetic analysis. However, it is much more fun, rewarding and revealing to do it yourself. It is possible to do this stuff without too much difficulty. All that is required is some

perseverance and a bit of financial outlay, but certainly no more so than that required to buy a microscope and a few mycology books. It is not as hard or complicated as some think, although it can be very time-consuming. You just need to snap on the rubber gloves and do basic cookery with some exotic utensils and ingredients. It is immensely rewarding – when it works.

I was an early investor in the BentoLab Kickstarter project designed specifically to democratize DNA based analysis, to make it accessible, affordable and bring it out of the specialist laboratories. The 2020 relatively brief pandemic lockdown period in New Zealand allowed me to invest some time in getting a home-lab setup based on the BentoLab and other bits of kit acquired online. These notes are a compilation of what it took to get that home laboratory up and running. Of course, I'm not really a typical user, because through my work I do have access to labs, equipment, and technicians, and I have had to 'cheat' occasionally to get things working. I am especially grateful to Duckchul Park of Manaaki Whenua – Landcare Research (EcoGene) for answering many questions based on his long experience.

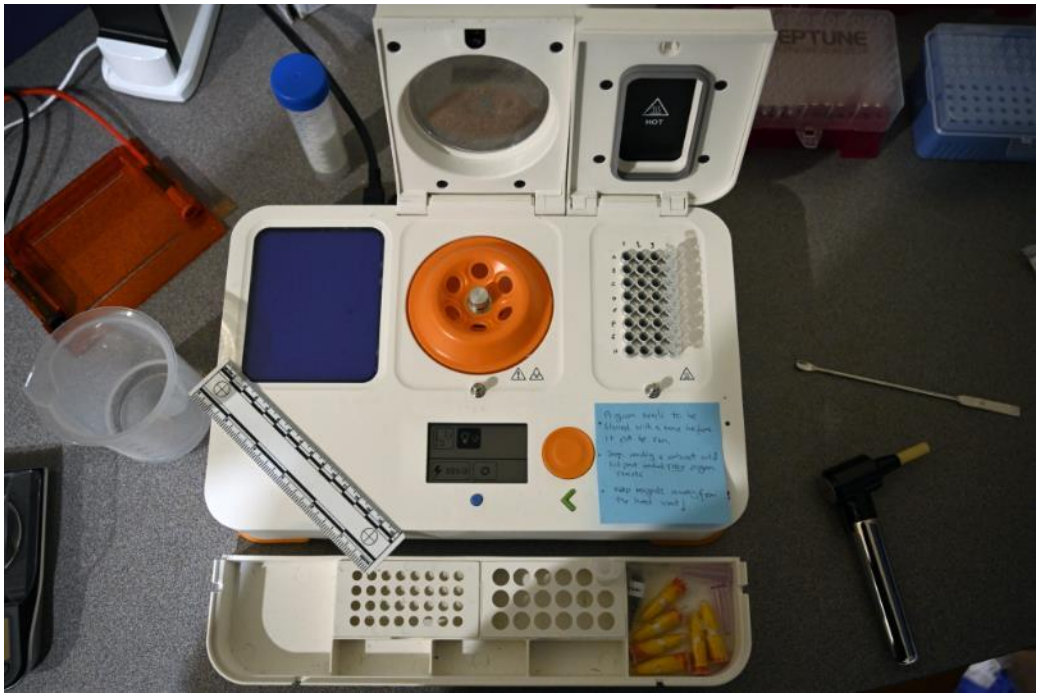
The basic toolkit for DIY PCR

You can pick up all the equipment you need second hand on eBay but it still isn't cheap and can take up a lot of space, and in New Zealand at least it can cost a fortune just to items shipped from the US.

Used 96-well thermocycler and eBay-purchased UV transilluminator (both relatively big)

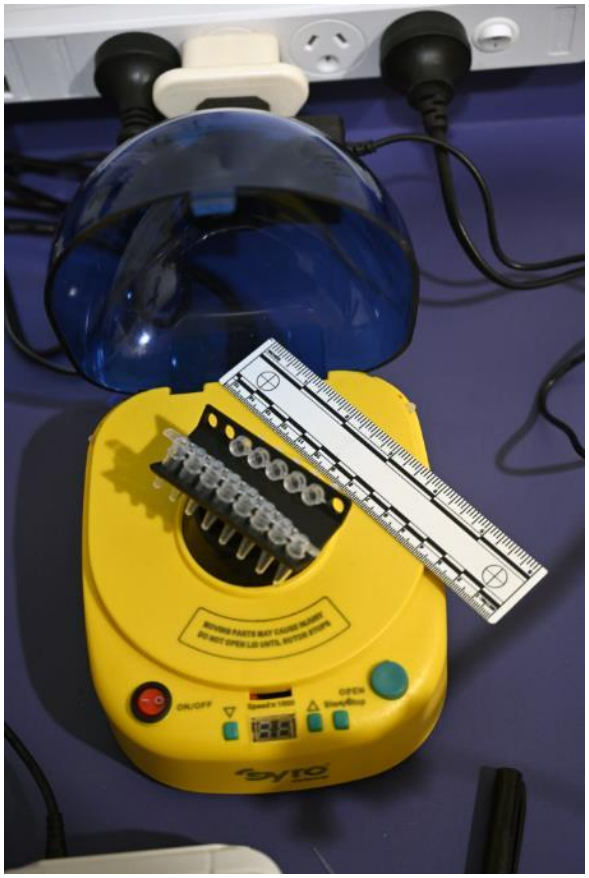
Currently it is probably more cost effective to buy the small new units designed specifically for the non-professional market. Two of the leaders are *BentoLab* in the UK and *miniPCR* in the USA. The advantage of the BentoLab is that everything you need is in a single box.

The BentoLab – electrophoresis, centrifuge, thermocycler



The compact unit contains a blue-light transilluminator/electrophoresis unit, 6-well centrifuge and a 32-well PCR thermocycler. MiniPCR supply separate units for each, with a 16-well thermocycler and a slightly slower but more configurable centrifuge and a separate blue-light electrophoresis unit.

The miniPCR centrifuge with 8-PCR tube strip holders fitted



The current cost of the BentoLab is $3,145 NZ and for the miniPCR separate units $1,911 NZ. I am not generally enthusiastic about the blue-light transilluminator/electrophoresis units in both the BentoLab and the miniPCR offering and both centrifuges are a bit limited. Apart from these tools as a bare minimum you will need at least a couple of pipettes 2-20ul and a 20-200ul. To start you will also need at least a couple of hundred disposable pipette tips in the relevant sizes, together with a couple of hundred 1.5ml centrifuge tubes, and 0.2ml PCR tubes. Do not underestimate the volume of consumables you will get through.

Rack of pipettes and boxes of sterile disposable pipette tips

Start-up chemicals needed (using the BentoLab/MiniPCR kit) include concentrated TBE buffer, agarose powder or tablets, GelGreen DNA stain, 100bp DNA ladder, a PCR Taq *ReadyMix* (Hot start Taq recommended), ddH20 (see glossary for what all these things are, and what they do). You will also need DNA lysis/extraction buffer (BentoLab sell one that will work for some fungi), and you will need standard ITS primers (e.g., ITS1/ITS4F). This last item is harder to get and would need ordering from a specialist supplier (or a lab contact), but the rest are easy to buy and both BentoLab and miniPCR sell them.

Those are the basic tools and ingredients you will need, and there are some straightforward recipes you should easily find, including in these notes. You may not bake the perfect DNA cake first time in your DIY DNA kitchen, but you will bake something vaguely edible (by a sequencer) eventually. You need to experiment and find what works for you with your equipment and your fungi. This is not an exact science – at least not with the equipment available to most of us. It is worth remembering that your home laboratory will never compete with the efficiency and quality of a professional molecular laboratory staffed with experienced lab techs and very expensive equipment.

The most basic protocol (Quick start)

This what you need to do …

Extract DNA (cell lysis) -> Amplify barcode region using PCR and primers -> Run gel electrophoresis to see if it worked -> Send good PCR products to sequencing facility.

Sounds easy- right?

Most of this article is about how DNA extraction works and how to optimise PCR using basic equipment. But before that it is appropriate to present a minimal protocol for amplifying the ITS

(Internal Transcribed Spacer) barcode region. You can give it a go, and it might work, but I can almost guarantee you will eventually want to know more. I will outline the protocol for a single PCR reaction. For multiple samples you need to scale up – particularly by creating a Master Mix with all the ingredients added to a 1.5ml Centrifuge tube, except your DNA **template**. In this protocol I will assume the use of the BentoLab and the consumables they can supply.

I recommend first practising pipetting, especially small quantities like 1uL. It is also worthwhile watching this set of You Tube videos based on the miniPCR equipment …

[Fungal PCR at home, Part 1: Setting up your lab - YouTube]

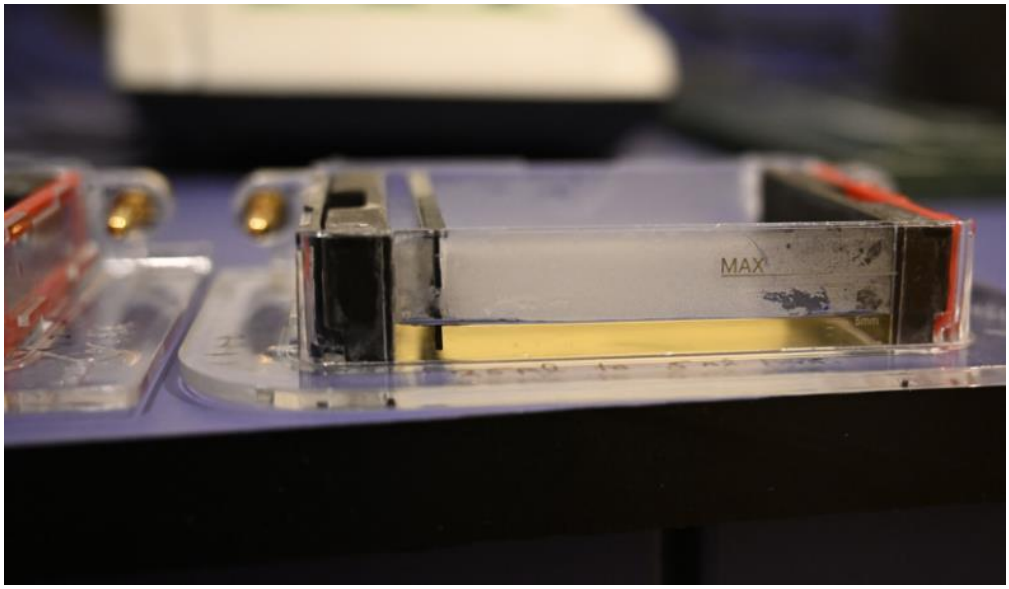
Don't forget to discard pipette tips each time you add a new reagent or touch the target PCR tube, and wear nitrile gloves to keep things uncontaminated and to protect yourself from the chemicals. Add 75 µL of Alkaline Lysis Solution (HotSHOT DNA extraction) to 1-2 mm^3 of a sample in a 0.2 mL PCR tube. Grind the sample using the technique suggestion in the video (converted pipette tips) or some suitable sterile implement. I have used sterile stainless-steel rivets that fit into PCR tubes nicely! Use the thermocycler and incubate at 95C for 30 minutes. Allow to cool and then add 75 µL of Neutralising Buffer. This is your source of genomic DNA (gDNA) but it has lots of other stuff as well. Now create your PCR combined Mastermix in 1.5ml centrifuge tube. For each reaction (total volume 20uL) you will need 4ul of 5X Hot Firepol BentoLab Mastermix, 1uL of 5uM Forward ITS primer and 1uL of 5uM Reverse ITS primer, and finally 11uL of PCR grade water. If you are doing multiple reactions, then multiply up accordingly and add an extra reaction to consider pipetting error. Close the lid to the combined Mastermix and tap the tube a few times to make sure everything is mixed up. Use a **vortexer** if you have one.

My Vortexer (available on Alibaba for mixing nail varnish!)

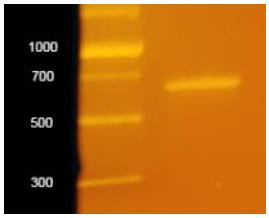
Now pipette 17uL into each PCR tube. Finally add 3uL of your gDNA template to a tube, close the lid and tap to mix. The thermocycler will need to be programmed for PCR mix containing hot-start Taq. Initial denaturing temperature 95C for 15 minutes. Then 35 cycles of … denaturing (separate double-stranded DNA) at 94C for 30S, annealing (allow primers to find/stick to single strands) at 55C for 30S, extension (Taq extends primers) at 72C for 60S (for the length of ITS). Then a final extension at 72C for 5 minutes and then the reaction can be left at room temperature. Label and put the tubes in the freezer (-20C) as soon as practical for long term storage.

Now you need to find out if it worked. Make a 1% agarose gel by adding 0.5g of **agarose** powder (or tablet) to a beaker, add 50ml of **TBE** buffer and heat in a microwave for a about a minute (quantities depending on the size of your gel tank – the BentoLab tank uses about 25ml. Keep an eye on it because it needs to just boil just very slightly. Then give it a swirl to mix and let it cool for a few minutes to 60C (just bearable to touch) and add 5uL of **gelGreen** DNA stain to the mix and swirl. Put the comb and rubber dams into the BentoLab electrophoresis unit. I find it useful to lightly grease the rubber dams with petroleum jelly. Then pour in the molten agarose mix and let it cool and solidify.

The BentoLab gel tank with molten gel to 5mm



Now carefully remove the dams and the comb, avoiding the delicate platinum wire electrodes. Now add more TBE buffer to cover the electrodes and the gel with a depth above the gel of 2-3mm. Now pipette 5ul of a 100KB DNA ladder into one of the wells and pipette 5ul of your PCR products into other wells. It takes some practice and a steady hand to get this right. Be sure not to pierce the bottom of the gel. You do not need to add loading dye/buffer as seen in the YouTube video because it is already included in the BentoLab MasterMix. Now close the electrophoresis unit and connect at 70V for about 40 minutes. Switch on the blue light illuminator after 10 mins. The visible dyes in Readymix (blue and orange) should be visible as broad bands on the gel and show the DNA is migrating. In a 1% agarose gel the blue dye (Bromophenol) migrates equivalent to 500bp and so your ITS band will be slightly behind it. Do not let the blue band run off the end of the gel. You will need to turn on the transilluminator to see the PCR product fluorescing in the green and you should certainly see the bands in the ladder start to separate. ITS is about 700bp long, so after 30 mins you should have a single bright band around 700bp according to the ladder calibration.



If you have a success, then you need to put 5uL of the PCR product in a PCR tube and send it to a sequencing facility according to their requirements. Don't forget if you are using a ReadyMix containing normal Taq, not hot-start, then the initial thermocycler temperature/time will be quite

different. Store PCR product tubes in a 0.2 mL tube box or small ziplock bag, clearly labelled. Refrigerate for the short term or freeze at –20 °C for longer term storage.

That's all you need to do 😜

What I want to do now is to provide some context about this process, more about the ingredients you can use, and how to fine-tune things. Sometimes protocols do not work, and it helps to know what is happening. I need to admit up-front that I am not an expert and I rely on very skilled lab technicians to do this stuff for my work. These notes are my self-help guide and written as I tried various protocols and had successes and failures with my home laboratory.

Professional laboratories usually use off-the-shelf kits for extraction/amplification, but they are expensive so these notes are about cheaper alternatives. The emphasis is given to easier protocols requiring less sophisticated equipment, and to chemicals that are relatively cheap and easy to obtain, although some necessary chemicals will be a problem e.g., pure ethanol.

The following assumes some basic knowledge of chemistry. I have tried to make the biochemical explanations relatively simple, and I hope this is not too technical, boring, wrong, or confusing. In reading the literature I have noted a surprising degree of vagueness, but then that's biology for you (I was originally trained in Physics).

Where is the DNA?

The first task is to extract the DNA from your fungal tissue. Before we get to that part, I think it is important to build a mental model of how things are arranged inside fungal cells and how the components behave. In each fungal cell/hyphae the DNA is contained within a nucleus. The nucleus is bound by a double membrane made of lipids (fatty molecules), but the nucleus is itself wrapped in various structures, for example the endoplasmic reticulum made of proteins, and ultimately all the various bits of molecular machinery are encased in a sturdy outer cell wall. In fungi the cell wall is made of chitin and in this respect, fungi are closer to animals in having cells walls made of chitin rather than cellulose/lignin in plants. Chitin is a biopolymer, specifically a polysaccharide (made of sugar components), and so too is lignin. The long strands of polymer molecules are bound to each other through hydrogen bonding, which has consequences for trying to open the structure to get at the DNA. When the cell is living the DNA is protected by these various layers and by the aqueous chemical soup inside the cell. DNA is a very robust molecule under the right conditions. It needs to be robust, or life based on DNA would not exist. For example, DNA can survive surprisingly high temperatures – over 130C. It does unravel and separate into single strands from its normal double-stranded form, but it does not irreversibly degrade into its component parts. The PCR process makes use of this robustness and ability to unravel (denature) and re-form the double strand.

Properties of DNA

We should now have a quick review of the DNA molecule and its chemical properties. The structure of DNA comes from a string of simple sugar groups (Fig. 1) and phosphate groups (Fig. 2) bound to each other by covalent bonding (shared electrons) to form a polymer chain. The sugar groups in the backbone are the deoxyribose of deoxyribose nucleic acid.

Figure 1. 2-deoxy-d-ribose sugar

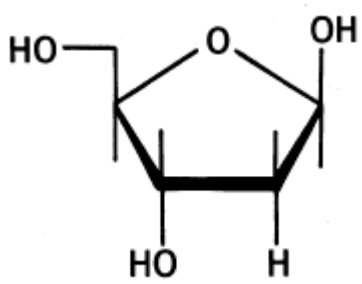
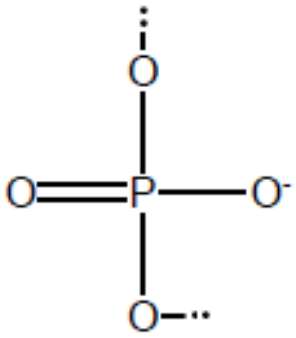


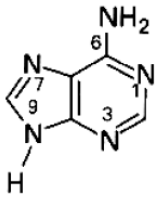
Figure 2. Phosphate linking group



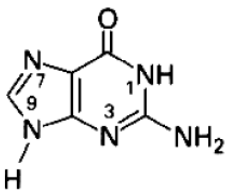
Each sugar component along the backbone *binds sideways* to one of the four nitrogen containing bases (the AGCT of sequence data). These 4 bases come in two structural forms. Adenine and guanine are classed as purines, whereas cytosine and thymine are classed as pyrimidines (Fig. 3).

Figure 3. The 4 DNA Bases

Purine:

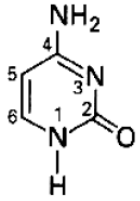


adenine

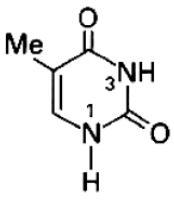


guanine

Pyrimidine:



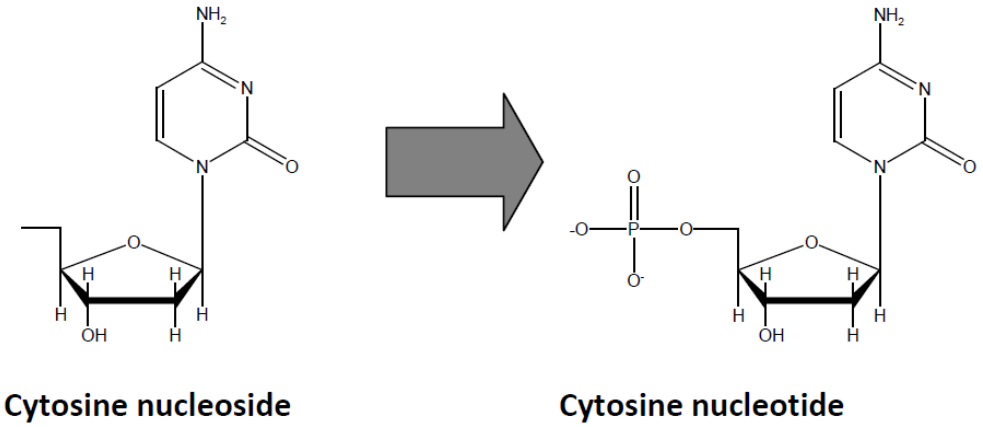
cytosine



thymine

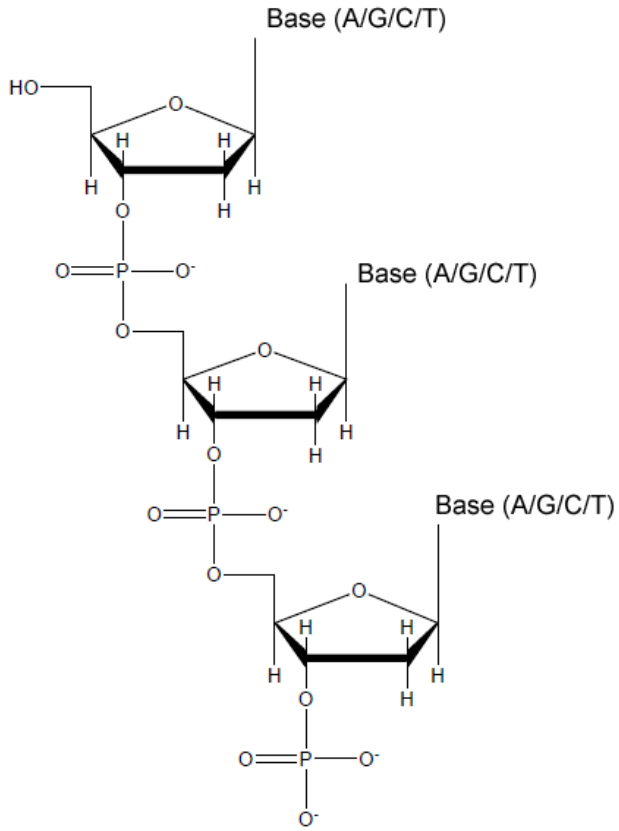
These bases link to sugar groups and the combination called a nucleoside. When a nucleoside links to the phosphate backbone it is termed a nucleotide (Fig. 4.).

Figure 4. Nucleoside & Nucleotide for Cytosine



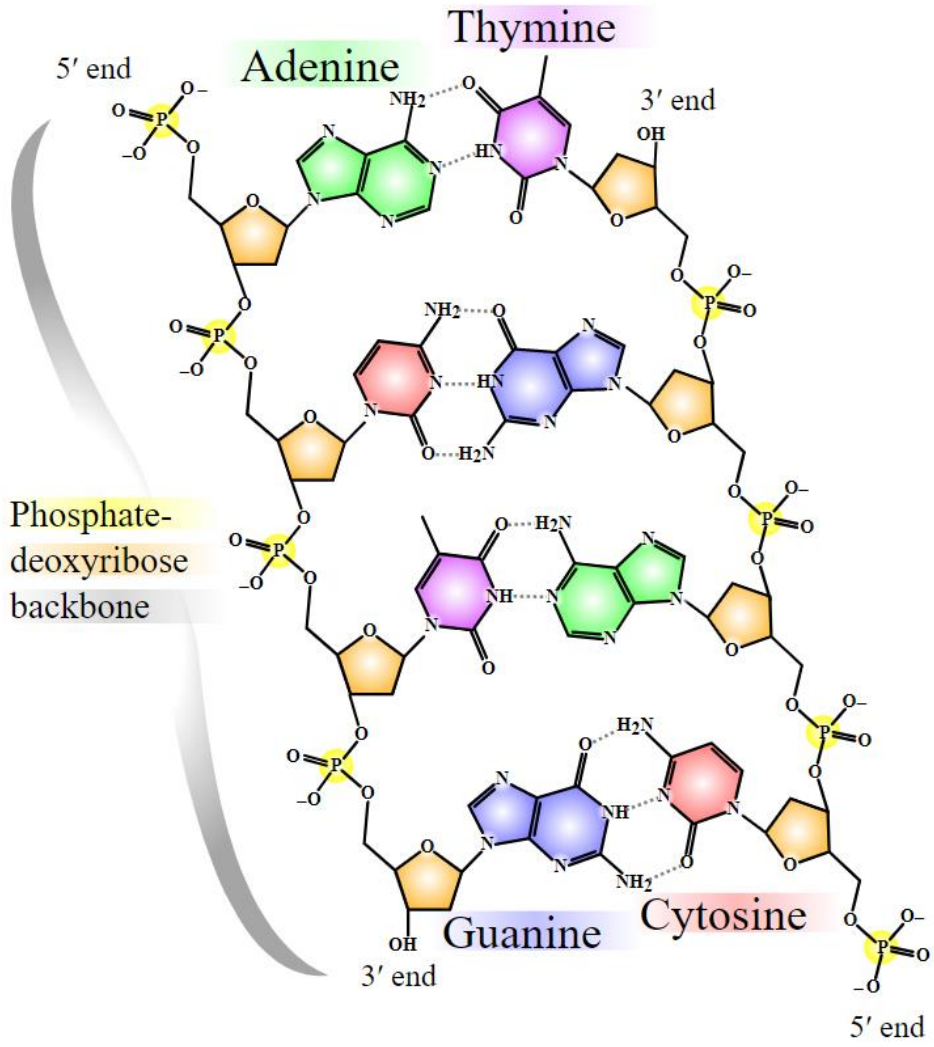
This all links together to form the repeating pattern of a single stranded DNA polymer chain (Fig. 5).

Figure 5. Single stranded DNA



Double stranded DNA consists of **forward** and **reverse** strands of nucleotides with complementary bases. These strands have **lined-up** their phosphate-sugar backbones and with complementary bases from each strand next to each other. The purine A (adenine) on one strand links to the complementary pyrimidine T (thymine) on other, and G (guanine) link to C (cytosine) (Fig. 6).

Figure 6. Double stranded DNA (Wikipedia, Madeleine Price Ball, CC BY-SA 3.0)



These base-pairs between nucleotides on each strand are attracted to each other through hydrogen bonding. This kind of bond forms when certain *electrically strong* atoms, like oxygen when bound to hydrogen, grab more than a fair share of time with the shared cloud of dancing electrons. The overall charge distribution between the atoms is then skewed leaving parts of a molecule negatively charged and other parts positively charged. This *charge dipole* attracts other dipoles in a north pole - south pole magnet kind-of-fashion. Hydrogen bonding is relatively weak compared with covalent bonding which in turn is weaker than the ionic bonding of inorganic chemistry. The weak hydrogen bonding is why increasing the temperature of double-stranded DNA melts (denatures) the hydrogen bonds between the complementary bases gluing two strands together but leaves the strong covalently bonded components of the single nucleotide strands intact. The same kind of hydrogen bonding interaction, but even weaker, occurs between the sugar-phosphate backbone and surrounding water molecules. For that reason, DNA is hydrophilic and dissolves in pure water. However, the hydrophobic nature of the bases on the inside results a twist in the molecule to minimise the tension, thus creating the familiar spiral of double-stranded DNA.

Basic Chemistry

Now for another brief detour into acids, bases and pH. In a single strand of DNA the bonding of a base to a phosphate-sugar group to form a nucleoside (Fig. 4) is the same as the simple chemistry class where you added a base to an acid to form a salt. In this covalent bonding the phosphate group is an *electron acceptor* (acidic) and the base is an *electron donor* (basic). Another way of looking at the same picture is to say an acid is a proton donor and a base is a proton acceptor. Acids have a strong tendency to give away their protons (and grab electrons) leaving them with a low proton density, i.e., a low pH value. When the DNA is doubled-up in a complementary fashion the base components of the nucleosides are inside the helical structure and the phosphate backbone exposed to the surrounding environment. The exposed phosphate backbone therefore displays an overall negative charge, because of the orientation of the hydrogen bond dipoles. It is therefore polarized, and acts as an acid (hence the A-Acid in DNA). That is the same as saying it is a *polar anionic molecule*, i.e., negatively charged and thus attracted to a positive electrode (anode), which is useful in the electrophoresis step. This superficial external charge on the DNA molecule affects the way it interacts with water molecules, as described above. It also affects the way it interacts with any chemicals in solution, especially in relation to pH and the presence of charged ions, which in turn affects the stability of DNA. DNA is happiest at around neutral pH 7 to alkali pH9. Acids are especially bad news for DNA because they break the phosphate-sugar bonds, although that is useful if you want to clean DNA residues from equipment (with Hydrochloric or Phosphoric acid).

One of the most common words you will hear in molecular biology is **buffer**. A buffer is a solution that able to maintain the pH within a certain range even when acids or bases are added to a solution (although the term is used rather loosely). Buffers of various kinds are extremely important for stabilising DNA as it goes through the various cookery processes.

Salts when dissolved in water break up into independent charged bits of molecules termed ions. The negatively charged sugar-phosphate backbone attracts a cloud of positive ions around it and the charge on the DNA is effectively neutralised. The consequence is that DNA stops interacting with other molecules in solution, and with a bit more of a push (by adding alcohol) it stops being soluble (it is no longer hydrophilic) and is precipitated out of high concentration salt solutions. That can be a useful trick when purifying DNA, and it can be precipitated by various methods. It is always critically important to subsequently remove any salt/alcohol contamination which can affect PCR.

OK after that background detour we can back to the problem of lysis and extracting our DNA from cells.

Cell Lysis & DNA Extraction

Lysis is the term used to describe the disruption of the cell wall and the cellular contents to release the genomic DNA into solution (the lysate). This lysate can either be used in a **direct PCR** reaction where the lysate is used directly as a DNA template in the PCR reaction, and/or the DNA in the lysate can be extracted and cleaned up for subsequent storage and use. The various protocols for lysis are always a balance between competing factors. The harshness of chemical needed to disrupt the cells can damage/fragment the DNA. Another source of problems is the Deoxyribonuclease (DNase) enzyme present in living cells and involved in cell repair and replication. Normally its activity is tightly controlled. However once cell lysis starts it is released into solution and quickly starts degrading the DNA. Its activity needs to be minimised by doing extractions at ice-cool temperatures, or it needs to be deactivated quickly (95c), or with **EDTA** to mop-up the necessary catalytic metal ions.

Some of the chemicals needed to thoroughly extract the DNA can poison a subsequent PCR process in a direct PCR. If the extracted DNA goes through a cleaning step then those poisons can be removed, but often the simplest/quickest/cheapest method is to use the raw cell lysate directly as a template for PCR and not bother with a DNA cleaning step. Some fungi will be associated with specific problematic chemicals, like lignin and melanin (polyphenols), and residue proteins and enzymes. All of these can severely interfere with the subsequent PCR process unless they are minimised or removed. If your lysate looks strongly pigmented (brown, black, red, yellow) then expect problems with your PCRs.

From a home-PCR perspective the ideal approach is to find a methodology that uses direct PCR from the gDNA lysate without the need for time-consuming DNA cleanup. It is the quickest and easiest approach and if you find the right conditions should work well for amplifying multi-copy loci like ITS and LSU and even some multi-copy protein-encoding genes. However, some loci, especially those with low copy number (like RPB2) will probably require relatively large amounts of clean amplified DNA.

So how do we break down the cell walls and liberate the DNA from complex stuff inside? With some protocols you don't need to break up the cells totally and you can rely primarily on an alkali (or other chaotropes) to open-up holes in the cell-wall biopolymers and the nuclear membrane to release at least some DNA. However mechanical disruption of the cells walls is commonly used if you want larger amounts of DNA. You need to start with a very small amount of dried or fresh fungal material – a crumb just big enough to sit in the bottom of a 1.5ml centrifuge tube. I find working with 0.2ml PCR tubes forces you to minimise the material and often works just as well. One of the most common mistakes that beginners make is to start with too much material. It is tempting to think more material gives more DNA means greater chance of success. That is incorrect because more material means more nasties that can derail the PCR process. Less is more in this situation. If your material is bone dry then try disrupting it initially by grinding in the tube, and then again after adding a small amount (50-100ul) of extraction/lysis buffer (more on that in a moment). Beware of powdery material which can get everywhere – or may find that every sequence comes out as the one puffball specimen you ground up. The type of fungal tissue you choose is important. Obviously, it should be as clean as possible. For larger mushrooms I recommend pulling them apart and using some of the internal softer tissue. For a long time, I assumed gill tissue would be good because of all the spores, but spores are very difficult to break open during lysis (they have evolved to be super-tough) so softer body tissue is better. Special plastic pestles can be bought that fit inside the centrifuge tubes, and they are attached to a small vibrator/rotator.

Vibrator and plastic pestle designed for grinding in 1.5ml centrifuge tubes

Or small sterilised stainless-steel ball bearings can be added to a tube and vigorously vibrated using a *bead-beater.* I tried making my own bead-beater out of an old multi-tool, but that was too vigorous, and tubes disintegrated! I have had success using improvised pestles which are stainless steel rivets from Mitre10 chosen to fit into the bottom of the tube, and which can be inserted into a piece of plastic tube as a handle. They need to be sterilised first.

You will find variable advice about how vigorous mechanical disruption needs to be. If you are trying to optimise a direct PCR protocol it may be best to avoid any mechanical disruption to minimise the release of cell residues that poison PCR. It is common to use an alkali (as a chaotrope) to open pores in cell membranes to leak DNA and any residual PCR poisons mopped up by **BSA** in the PCR Mastermix. But if you need larger amounts of DNA then mechanical disruption is needed. Too much mechanical disruption can shear the DNA strands, i.e., it breaks them up into smaller bits. We are using PCR to amplify small fragments so shearing shouldn't be an issue, however, smaller DNA fragments are less able to precipitate out of solution, which is an essential step if you want to clean your DNA. So, if you intend to do any DNA clean-up then probably the grinding should be less vigorous. A freeze-thaw cycle can be useful for disrupting cell walls. Advice on all these aspects in the literature is variable. Incidentally if you see any recipes that say *mix by pipetting* rather than vortexing, then it is intended to be gentle to avoid shearing, which may be relevant in some situations, but not generally in gDNA extraction for ITS PCR.

So, in summary, the role of the grinding and the extraction solution/lysis buffer is to help freeing the DNA from inside the nuclear membrane and other internal cell constituents. The specific role of a buffer is to keep the DNA in the extract at a comfortable pH. However, lysis solutions will also contain reagents to dissolve cellular components and they may contain other ingredients that inactivate or precipitate compounds that may poison the downstream PCR, such as polyphenols and proteins.

You will find many recipes for lysis extraction, but they usually have various strengths of some common reagents. An alkali-based lysis is a two-step process with initial alkali extraction and then the addition of a stabilising buffer. Or it can be a one-step process without initial alkali and using another chaotrope and/or surfactant. I have provided a glossary with many of the chemical reagents

used, and their roles and pitfalls. A one-step lysis buffer generally contains these chemicals: **Tris** buffer to maintain pH; **EDTA** to mop-up poisons such as metal ions that promote enzyme degradation; a surfactant such as the anionic **SDS** to break up any lipids and help loosen the polysaccharide cell walls; usually **salt** to neutralize the DNA charge so it doesn't stick to nasty things; **PVP** to bind to polyphenols and remove them; sometimes **Beta-Mercaptoethanol** as a reducing agent to deal with polyphenols and protein, and a **Proteinase-K** step to digest proteins. From these reagents **Tris+EDTA+SDS** are normal/essential, and the rest are nice-to-have extras. The molar concentrations for each of these components you will see in various recipes vary enormously, so they aren't too critical, just as long as you don't over-poison the solution, especially if you are considering direct PCR without a DNA clean-up step. SDS especially is a PCR-killer.

If you do start mixing your own reagents from the ingredients …

My chemical cupboard needs to be bigger

… then you should also buy a set of digital scales capable of weighing milligram quantities. You will probably need one anyway for making gels.

Digital Scales

Note that **CTAB** surfactant extractions are different to those using SDS. CTAB is a cationic surfactant and is combined with high salt and its use requires an extra DNA extraction/washing step.

It also worth noting a possible interaction when using alkali (chaotrope) lysis followed by neutralisation using a buffer containing SDS. The SDS surfactant is useful for assisting in the opening-up of membranes and for binding to proteins. However, any unzipped double-stranded DNA and unravelled proteins can stick to each other. If you added SDS then it may bind to the DNA-protein mix. Then, if you precipitate the SDS to remove proteins (e.g., with Potassium acetate) you will likely take the bonded gDNA out of solution along with it. Smaller fragments of DNA will be less affected by this mechanism, so shearing (shredding) in this case is a good thing.

A significant issue for direct PCR using the lysate as a template is that some of components you use for extraction (e.g., SDS and EDTA) are also inhibitors of PCR enzyme activity. You can have a thorough lysis step and get a lot of DNA, but then you need to separate the DNA before you can use it as a template, not just to get rid of residue cellular material, but also to get rid of the chemicals you used to extract it! On other hand PCR only needs tiny amounts (of the right) DNA because the PCR amplification is so strong, and so gentler handling of smaller amounts of tissue at this stage means a better chance of using the extract to work directly as a template. This advantage is enhanced if you add BSA to the Mastermix (but BSA will not negate the effect of EDTA and SDS) and/or use Hot-start Taq (see the glossary).

Now you should start to understand the tricky balancing act of DNA extraction protocols. A DNA clean-up step will generally give you consistent results, and you can be harsh with the lysis to be sure of getting enough DNA, but it is a time-consuming step, and you will lose DNA during cleaning (a problem if you start with tiny amounts anyway). If your fungus has relatively soft pale tissue, then an alkali/chaotrope extraction/neutralisation or a gentle one-step lysis process followed by direct PCR should work. If you are dealing with woody brackets or fungi with strong pigmentation, then direct PCR may not work because of the poisoning effect of polyphenols. You can try diluting your

template by a factor of 1-20 (start with 4) and hope that you dilute the effect of poisons and still have enough DNA, or you may need a DNA clean-up step. Quite often a 10x dilution will give reaction products, although often contaminants are preferentially, or co-amplified and need separting out with gel extractions.

Note that I generally find it easier to extract DNA from dried tissue if you have tough and/or gelatinous fruitbodies. Otherwise, fresh tissue will probably give a slightly higher success rate. If you get over 70% success with any direct-PCR protocol, then you are doing very well.

Your extracted gDNA should not be left at room temperature for any length of time. Keep it in the fridge prior to PCR, it can tolerate a few days, but in the longer-term store at -20C in the freezer.

DNA Extraction Protocols

Google also "*DNA Extraction from Herbarium Specimens Lenka Záveská Drábková*" for a good overview of various extraction protocols.

Strong alkali Lysis

Firstly, I will list the basics of some standard protocols taken straight from the literature and then add some observations of my own. In this process alkali is acting as a chaotrope denaturing biopolymers.

[S. Jakob. You Tube] Using PCR tubes. 30uL of 0.5M NaOH to each tube. Macerate with pestle. Allow to stand for 10 mins. Add 150uL of 100mM TRIS-HCL 10mM EDTA buffer to each tube. Incubate for 10 mins at 95C. Centrifuge at 1000 for 5 mins. [5:1 dilution]

[Wang 1993] Place a few milligrams tissue into a 1.5 ml tube and, to every mg of tissue, add 10 ul 0.5 M NaOH. Grind until no large pieces of tissue are left. Transfer 5 ul quickly to a new tube containing 495 ul 100 mM Tris pH 8.0, mix well and use 1 ul directly in PCR. [100:1 dilution]

[Dovana et al, 2017] 20 mg of dry sample were homogenized in 250 µl of 0.5M NaOH with a pestle. After 10 minutes to allow for sedimentation, 5 µl of the extract was removed and diluted in 195 µl of 100mM Tris-HCl at pH 8.0, and 1 µl of the dilution was used as template DNA [40:1 dilution]

[*Osmundsen et al 2013]* For NaOH extraction, 200 µL 0.5 M NaOH was added to ~ 75 mg of dried tissue, ground with a micropestle, centrifuged at 14000 RPM for 2 min, and 5 µL of the resulting supernatant added to 495 µL 100 mM Tris-HCl buffered with NaOH to pH 8.5–8.9 (Tris-HCl-DNA extraction solution) [100:1 dilution].

My observations: The key aspect of these alkali extractions is degree of dilution with neutralising buffer added after treatment with NaOH. With this strong alkali then only a small portion of the lysate is diluted in a much larger volume of buffer and taken forward to direct PCR. The neutralising/dilution buffer will bring down the pH, although it shouldn't need bringing down too much to be compatible with the highly alkaline PCR reaction mixture. The neutralising step will certainly improve long term storage of the gDNA. The process would be different if a DNA clean-up step is included. The remaining DNA extract from these protocols can be frozen (immediately) for future use. I would strongly recommend the 95C heating step because, apart from improving extraction, it will neutralise the effects of DNase. In the literature there also references to the use of 0.2M NaOH + 0.1M urea as a lysis reagent which is supposed to enhance the degradation of chitin. However, urea is known to be a strong PCR inhibitor so unsuitable for subsequent direct PCR. There are also many references to a cold extraction step. So, after macerating (or directly after adding the extraction fluid if you don't macerate), rather than standing for 10 mins at room temperature, the

solution is put in the freezer overnight (-20C). Cell disruption and enhanced extraction occurs, without significantly activating DNase. Note that these kinds of strong alkali and heat lysis protocols can possibly fragment DNA to the point that PCR fails.

My recommendation: 20mg of dry sample homogenised in 250ul of 0.5M NaOH with a centrifuge pestle – vigorously to deliberately shear DNA. Quickly freeze the result and leave overnight (optional step – needs testing). Heat rapidly to 95C for 10 mins. Centrifuge at 14k g for 2 mins. Transfer 5uL of supernatant to PCR tube with 195uL of 100mM Tris-HCl at pH 8.0. This becomes the stock DNA and store at -20C (but note possible degradation over time). Use 1-3uL of this as a direct PCR template.

My musings: It would be useful to experiment with no grinding. This may work with direct PCR – considering that Readymix is also high pH so strong (ish) alkali makes no difference. It might also be worth experimenting with some more neutralising steps to get rid of non-target stuff. For example, to the bulk lysis extract (which may need buffering with Tris as in the protocols) add 2M Ammonium acetate + 0.2% SDS + 2% PVP (+ Proteinase-K + Guanidine hydrochloride), incubate 50C for 30 mins, vortex, then add 5M Potassium acetate (all this to precipitate crud), then centrifuge and transfer lysate. But noting that long chain DNA may have precipitated out bound to the SDS/protein mix. Addition of Tween 20 will negate the inhibitory effects of SDS. This part needs testing but if it works it should lead to much cleaner DNA. You may need to centrifuge as fast as possible for 5 minutes or more.

Weak alkali Lysis

[BentoLab protocol] This is a variation on the so-called HotSHOT protocol (hot Sodium hydroxide and Tris). Here the lysis Solution: 25 mM NaOH, pH 12. Neutralising Buffer: 100 mM Tris-HCl, 0.5 mM EDTA, pH 8. Place 75 µL of Alkaline Lysis Solution in a 0.2 mL PCR tube per sample. Subsample 1–2 mm^3 of sample tissue and transfer into each tube, making sure it is immersed in the Alkaline Lysis Solution. Adding excess sample is likely to cause the extraction and/or PCR to fail. Grind or not. Place the tubes in a thermocycler and incubate at 95 °C for 30 minutes using the Heat Block programme of the thermocycler. Add 75 µL of Neutralising Buffer to each 0.2 mL tube. Use 2–4 µL of extract as a DNA template source in a PCR reaction.

My observations: Here we are using alkali that is 20 times weaker than the strong alkali extraction process and so not as harsh on DNA integrity, but also not as effective at lysing. Note in this protocol EDTA is included and the gDNA stock will be more viable long term. Simple and cheap when it works.

Moderate EDTA/SDS

Here you are not using an alkali and just relaying on surfactants to soften/open cell walls. You will need a lot of grinding with these non-chaeotrope protocols. This approach should be used only if you intend to do a post-extraction DNA clean-up because the high levels of EDTA and SDS will both poison direct PCR.

Material ground with 600 ul of 50 mM Tris-HCl (pH 8), 50 mM EDTA, 3% SDS, 0.15M NaCl. Grind tissue and incubate at 65C of 60 minutes. Add Proteinase K 50 mg/L and further incubated at 35 C for 1 h (optional step). Follow by DNA precipitation and wash.

Weak EDTA/SDS

Use this for direct PCR.

Material ground with 20mM Tris (pH8), 5mM EDTA, [0.5% SDS], 25mM NaCl, 3% PVP (optional), Proteinase-k 50 mg/L added at end (optional), incubate 5-60C 30 mins. Final heat at 90C for 10 mins. to inactivate enzymes. Add 5% Tween to inactivate SDS.

This buffer should allow direct PCR on most fungi.

My musings: see under Strong Alkali lysis. I think some of the same optimisations might be applied if they work. It would also be worth experimenting with 0.2% Tween-20 to replace SDS, although not as effective (non-ionic surfactant) it won't poison direct PCR. The Tween-20 could be added after to negate SDS but 0.5% is a high amount to negate. 0.05% might be more appropriate, followed by Tween-20 negation. There are papers suggesting a simple TE buffer extraction Tris (10mM Tris, 1mM EDTA) with no SDS is effective for direct PCR (on single chasmothecia of powdery mildews!).

CTAB

A CTAB extraction with high salt has a high ionic concentration to bind/precipitate the polyphenols as well as acting as a (cationic) surfactant, but the DNA will require further extraction/clean-up and should not be used for direct PCR. As usual there are numerous variants on the recipe. CTAB recipe: 2% CTAB, 100 mM Tris-HCl (pH 8), 20mM EDTA (pH 8), 3M Sodium Chloride, 1% PVP, with 0.2% Mercaptoethanol (optional, added just before use).

Add 0.5 mL of pre-warmed extraction buffer to the ground material. Mix the tubes by inverting several times and then heated in a water bath for 60 min at 65 °C. Contents mixed by tube inversion every 10 minutes. Cool to room temp. Then a DNA clean up (chloroform method or paramagnetic beads).

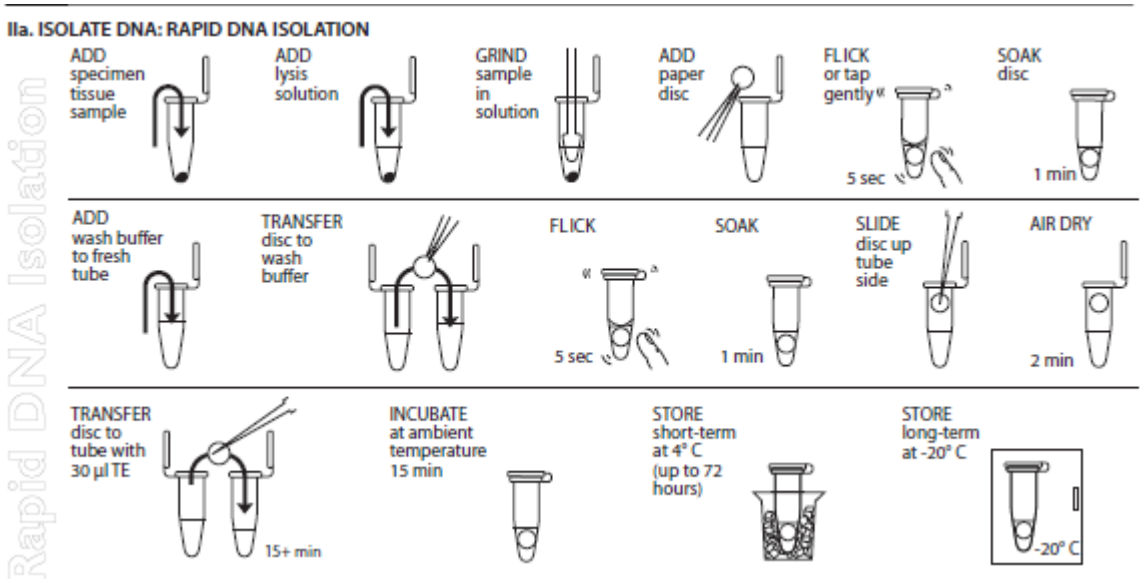
My observation: I have not used any CTAB protocol so can't comment.

Guanidine hydrochloride extraction

This lysis protocol from the Cold Harbour Spring group is worth presenting because it sems to be a relatively simple way of both extracting and cleaning a DNA sample. The process is well summarised by a graphic I have borrowed from their manual (which is worth reading as an introduction to barcoding) …

https://dnabarcoding101.org/files/using-dna-barcodes.pdf

Rather than lysis with surfactants and/or alkali this method relies on a mechanical disruption of cells and the strong chaotrope Guanidine hydrochloride to denature biomolecules with an immediate binding/elution step (somewhat like the 'dipstick method' – see later). It combines a lysis/clean up relatively efficiently.



In addition, in the stated form It avoids the need for heating or a centrifuge, although I suspect a heating step using a thermocycler and a centrifuge step might be included with some benefit. I have inserted some proposed modifications in brackets.

Reagents:

Lysis solution recipe (can be store at room temperature (for 6 months))
Dissolve 57.32 g of Guanidine hydrochloride (MW = 95.53) in 50 mL of deionized or distilled water.
Add deionized or distilled water to make a total volume of 100 mL of solution.

[ie. 6M GHCL]

Cellulose disks.
Use a dedicated, clean single hole paper punch to create disks from Whatman's No 1 filter paper.

Wash buffer
Store at -20°C (indefinitely).
234 mL distilled water
1 M Tris (pH 7.4), 10 mL
5 M NaCl, 5 mL
0.5 M EDTA, 1 mL
100% Ethanol, 250 mL

[i.e. Tris 20mM, NaCl 50mM, EDTA 1mM, EtOH 50%]

TE Buffer
In a 200-mL beaker mix the following:
99 mL of deionized or distilled water
1 mL of 1 M Tris pH 8.0
200 µL of 0.5 M EDTA

[ie. Tris 10mM, EDTA 1mM]

Protocol:

Add 50 µL of lysis solution to each tube.
Grind the tissue for at least 2 minutes. Use a clean pestle for each sample.

[possibly useful is a step of 10 mins at 65C followed by centrifuge at full speed for 1 minute and transfer of lysate to a fresh tube. Also consider the benefit of proteinase-K and SDS at this stage, with subsequent addition of 5% Tween 20 to the wash]

For each sample, use a separate sterile tweezer to add one 3-mm diameter disc of Whatman No. 1 Chromatography paper to the lysed extract. Tap or flick the tube gently to ensure the disc is fully submerged in the extract. Allow the disc to soak in the extract for 1 minute.
While the disc is soaking, add 200 µL of wash buffer to a clean 1.5-mL tube
Remove the disc from the extract using a sterile tweezer or pipette tip and transfer the disc into the fresh tube containing wash buffer. Tap for 5 seconds, then allow the disc to sit in the wash buffer for 1 minute.

[note that ethanol DNA precipitation/wash buffers are generally 70-75% ethanol, not 50% and .5M NaCl, not 0.05M NaCl]

Use a sterile pipette tip to gently drag the disc out of the wash buffer and up the tube wall to dry at the top of the tube. Ensure that little to no debris is attached to the disk. Allow the disc to air dry for 2 minutes to evaporate the ethanol on the disc.
While the disc is air-drying, add 30 µL of TE to a clean 1.5-mL tube
Once dry, carefully transfer the disc using a sterile tweezer or pipette tip into the fresh tube containing 30 µL of TE. Allow the disc to soak for a minimum of 15 minutes at ambient temperature (soaking the disc overnight at 4° C is optimal). Use 2ul as a template.
The Cold Harbour Spring manual also includes a modified version using silica powder that is reported to be slightly superior and relatively simple.

My observations: I have not used this protocol, but it looks promising and worth trying. The benefits of extra heating/centrifuging steps need investigation, as does the addition of other reagents such as Proteinase-K and SDS, both said to enhance impact of Guanidine hydrochloride considerably, but SDS will need negating by the subsequent addition of 5% Tween-20 in the wash buffer.

Sigma extraction/dilution reagents

Highly recommended if money is no problem and as part of the optimised REDExtract-N-Amp system (Sigma Aldrich) - See the glossary entry.

DNA Clean-up Protocols

If direct PCR does not work for you (even after diluting the extract 1:4 with water) then it will be necessary to clean up the DNA. This is about separating the DNA from all the other cellular residue and usually requires multiple steps. These are processes for precipitating out/binding the DNA, separating it, washing, and then putting back into solution. This generally requires the use of a (very fast) centrifuge and/or filter and pipetting to separate the solid/organic/aqueous phases. Alternatively, for a moderate financial outlay you can use magnetic bead separation which is straightforward and does not need a fast centrifuge or time-consuming filtration/precipitation steps. You could also try electrophoresis and gel extraction of the gDNA (see *Bands*).

The DNA can be precipitated directly from the raw lysate using salt (which may already be present) plus ethanol or Isopropanol, and then the separated DNA subsequently washed and re-dissolved Note here that isopropanol is relatively easy to obtain, although pure alcohol is not. This is relatively

quick and easy approach but there is the chance your precipitated DNA is still bound to protein/SDS complexes and may contain salt, all of which will interfere with PCR.

Alternatively, you can separate out some of the non-DNA stuff into an immiscible organic solvent prior to precipitation of the cleaner DNA in the aqueous phase. This is usually done using a chloroform or chloroform/phenol mix, followed by careful separation of the organic and aqueous phases. Phenol and chloroform are nasty, not easy to obtain, and can also easily poison downstream PCR, especially the presence of phenol. Phenol can be excluded from the mix.

Finally, there are techniques for binding the DNA to a solid surface, washing it, and then eluting back into solution.

Below are more details on some of the standard methods.

Magnetic bead separation

This is perhaps the easiest and quickest method of cleaning up DNA (from gDNA extractions, gel extractions and PCR products prior to sequencing) but does require some outlay. The process uses paramagnetic beads that bind DNA to their surface. The beads are tiny fragments of a paramagnetic material – that is they react to a magnetic field but do not themselves become magnetised. These beads are surface coated with something that binds to the chemical you want to concentrate, and in our case that is a silica coating that binds to DNA. You mix your solution of beads with the lysate and a binding buffer and then place it in a high magnetic field generated using rare-earth permanent magnets. I recommend buying a rack that supports a single 8 PCR tube strip. After a few seconds the DNA binds to the beads which are attracted to the wall (or bottom) of the tube and form a clump. You then carefully pipette off the liquid containing residues. The magnet is removed and you add a wash solution and disperse the beads, then re-apply the magnetic field and remove the liquid. Several cycles of washing with isopropanol and 80% ethanol will remove any contaminants. On the final cycle you allow the clump to sit for a few minutes to evaporate off the alcohol, and then remove the magnet and elute the DNA from the beads using a standard Tris+EDTA buffer. Again after a few seconds you use the magnets to separate the beads and now pipette off your solution of cleaned DNA. The beads can be reused to process more of the same extract if needed, but otherwise should be discarded. The common protocols use either hydroxyl-silica coated beads and with a binding buffer containing a strong chaotrope (e.g, Guanidine thiocyanate or G. hydrochloride) and ethanol/isopropanol, or carboxyl-silica coated beads and a binding buffer containing PEG (6000 polyethylene glycol) and salt. The history of these combinations of hydroxyl+chaotrope and carboxyl+PEG pre-date magnetic beads. They arise from standard methods from precipitating DNA onto a silica substrate. The pH of the binding buffer should be around 8.5. A traditional lysis protocol can be followed by adding binding buffer and beads, or the lysis protocol can be modified to match a bead step, for example by using a lysis buffer consisting of Guanidine thiocyanate and Sarkosyl (or more concentrated Guanidine hydrochloride and SDS). In practice the conditions for binding seem rather flexible. In addition, the binding conditions, such as the ratio of beads to buffer, can be configured to partition DNA according to size. A smaller ratio of binding buffer to lysate will restrict binding to smaller fragments, useful for cleaning up PCR products. In practice magnetic bead purification is straightforward and robust and it surprises me anybody bothers with chloroform/phenol, spin columns, high speed centrifuges etc. Unfortunately, magnetic beads can be expensive, although they shouldn't be because the process of making them in large quantities is not complex and does not use expensive reagents. Suppliers seem to be profiteering, and the same is true of most of the 'kits' sold for extraction and clean-up, but I suppose they do come with guarantees around purity and efficacy.

Chloroform/Isoamyl alcohol/phenol

In this process the hydrophobic lipids in the raw lysate (after grinding, centrifuging, separating) will partition into a lower organic phase of a mix of water/solvent, and the proteins will remain at the interphase between the two phases, while the nucleic acids (as well as other residual contaminants such as salts, sugars, etc.) remain in the upper aqueous phase. The upper aqueous phase can then be pipetted off. Care must be taken to avoid pipetting any of the organic phase or material at the interface. This procedure is often performed multiple times to increase the purity of the DNA.

The addition of phenol to chloroform mix increases the density of the organic layer and so improves separation, especially if you don't have access to a high G centrifuge but note the dangers of phenol (see glossary). A protocol without phenol is described below. Some ratios quoted in papers often appear to be inconsistent with the original protocol (Lee and Taylor 1995). The ratio is phenol:chloroform:isoamyl alcohol (24:24:1), i.e. there should be equal amounts of chloroform and phenol, with isoamyl alcohol at a much lower ratio. Raw Phenol is usually in crystalline form. The phenol used in the recipe is a saturated solution in TE buffer (10 mM Tris-HCl (pH 8), 1 mM EDTA The DNA is precipitated from the aqueous layer and washed, as described below.

Chloroform/Isoamyl alcohol – Isopropanol precipitation (CIA)

This depends on high salt being present in the lysate (3M – see CTAB protocol). A volume of chloroform:isoamyl alcohol (24:1 v/v; CIA), approximately equal to the lysis buffer, added to the sample tubes, vortex for 10 seconds. Centrifuge at 2.5 – 5k G for ten minutes at room temperature. The upper aqueous phase carefully transferred to a new tube by pipetting, avoiding disturbance of the debris between phases. Following extraction and centrifugation of the lysates, the aqueous phase is usually colourless or light tan. If not colourless then repeat the CIA extraction with centrifugation at up to 13k G for 10 minutes and recover the upper phase in a fresh tube.

[The following is effectively the Isopropanol precipitation step outlined below but the high salt is already present in the CTAB extraction fluid. Note also this protocol taken from the literature recommends low temperature isopropanol, which does not seem to be necessary or effective.]

DNA is precipitated from the recovered upper phase by the addition of 0.1 times its volume of 3M sodium acetate pH 5.2 and 0.66 times its volume of cold isopropanol (stored at -20˚C). Tubes were mixed by inversion and kept at -20˚C for one hour. DNA was pelleted by centrifugation at 13k G(!) for 10 minutes at room. The supernatant is carefully decanted off and tubes drained by resting inverted on paper towels. Remaining DNA pellets are washed by the addition of 0.7–1 ml of 70% ethanol and tubes centrifuged for 10 minutes, as before. Supernatants were carefully removed by aspiration to avoid loss of the nucleic acid pellet and tubes left to dry open at room temperature for approximately one hour or vacuum dried at room temperature for 10 minutes. DNA pellets following isopropanol precipitation were usually small, compact, translucent, and only rarely brownish, indicating that the prewash effectively removes polysaccharides and polyphenols. Pellets were then suspended in 100 µl TE containing 0.1 mg ml-1 DNase-free RNase A and incubated at 37˚C for 30 minutes. The extracted DNA can be stored at -20˚C until required.

More on Isopropanol/Ethanol precipitation

The idea is that you add a mixture of salt and alcohol to your lysate and the DNA precipitates out. Recall I said that salt goes into solution as ions and the positive Na+ ions surround the negatively charged phosphate backbone and neutralise the charge, which means the molecule loses its ionic character and ability to interact with other ionic chemicals in solution. But this mechanism has a limitation for some subtle reasons the Na+ ions aren't very good at getting close to the phosphate backbone and they aren't good at pushing the surround water molecules away because the

hydrogen bonding mechanism in water is overpowering. The addition of alcohol brings the sodium closer and pushes the water away, and the DNA then precipitates (and an appropriate concentration of Guanidine hydrochloride has the same effect). In this process it is common to use either ethanol or isopropanol and there are advantages/disadvantages to both.

Ethanol precipitation - 75% ethanol and 0.5M NaCl chilled to -20C. Add 0.7- to 1 times the volume of the lysate. Freeze at -20 for at least an hour and preferably longer (overnight). Centrifuge as high as possible to pellet the DNA at the bottom of the tube (20k G!). The DNA may or may not be visible as a tiny translucent blob. Beware a white blob because that would be problematic proteins or salt. Decant off the alcohol (keep your eye on the side of the tube where the pellet should be).

Isopropanol precipitation - 35% isopropanol and 0.5M NaCl at room temperature. Add 2-2.5 times the vol of the lysate and leave for a while before centrifuging and decanting. No need to cool, and in this situation, cooling is not advised, even though you will see it mentioned a lot in protocols. Some procedures mention the use of 3M Sodium acetate in place of NaCl as it is more soluble and less likely to precipitate with the DNA.

In both cases the pellet needs to be cooled and washed in cold 70% ethanol and then dried (preferably under vacuum at 40C for 10 mins) before redissolving in water or buffer. It may take a while to go back into solution (helped by 37C for 30mins). See also the post-CTAB isopropanol precipitation step outlined below.

The isopropanol method needs no freezing and the DNA precipitates out more easily, but unfortunately any residual salt also tends to precipitate, which if you don't remove by subsequent washing may cause problems downstream (20mM in the Readymix will stall PCR).

It is preferable to redissolve the DNA in a weak EDTA/Tris buffer because even distilled water can have a slightly low pH which is bad for the DNA, and metal ions can accumulate.

Cellulose paper bind/elution

Cellulose paper (specifically Whatman's paper no 1) has been found in several studies to bind to DNA quite strongly, and it seems the effect is due to tiny microfibres sticking out of the surface of the main fibres. The exact binding mechanism is unclear (to me anyway) because the surface of cellulose is naturally negatively charged and so should repel DNA. A similar process occurs in commercial clean-up kits which use silica as a binding surface (see entry for silica), which is also negatively charged (although reports differ). Binding is increased by adding salt, thus negating like-charge repulsion, but that does not explain the underlying binding property. When using a silica substrate (or cellulose) in a spin-column, the recipes often include a chaotropic salt (e.g. Guanidine hydrochloride) which weakens hydrogen bonding and can denature proteins and DNA. In the literature it mentions this process allows ions to form 'salt bridges' linking the DNA and the substrate, presumably a semi-stable layer of dispersed positive ions that attract the DNA on one side and the silica/cellulose surface on the other. But surely a chaotrope that relaxes the complementary bonding inside the DNA would expose, at least to some extent, the positively charged inner surface of the DNA and so provide an attractive binding surface for the cellulose/silica, but maybe that's too simplistic. Whatever the exact mechanism the binding property does seem to work quite efficiently. Cellulose can be used to clean-up DNA in a couple of simple ways. One method is known as the *dipstick method*. Strips of filter paper are cut 2-3cm x 2 mm (to fit into PCR tubes) and sterilised (and you could try roughing them with fine emery paper first). In the original proposal the filter-paper is dipped into paraffin wax half-way before cutting. That provides impervious semi-rigid handles to each strip. I am not recommending that process because paraffin wax is reported to be a strong PCR

inhibitor, and from my attempts I don't think handles are needed. A strip is dipped 3-times into the lysate, then 3-times into a wash buffer, and then 3-times directly into 20uL of PCR Readymix in a PCR tube. Enough cleaned DNA is eluted into the PCR mix to provide an adequate template. The wash buffer is 10 mM Tris pH 8.0, 0.1% Tween-20. And incidentally the Tween-20 is known to counteract the negative effects of any residual SDS.

Guanidine hydrochloride probably improves the process, as a denaturing chaotrope, but then again it might also poison the subsequent PCR. It is worth recording here the exact formula of the lysis solution and ash buffers used in the papers describing this technique.

Surfactant Lysis:
20 mM Tris pH 8.0, 25 mM NaCl, 2.5 mM EDTA, 0.05% SDS. Note the very low concentration of SDS but even that may supress PCR.

Chaotrope Lysis:
800 mM Guanidine hydrochloride, 50 mM Tris (pH 8), 0.5% (vol/vol) Triton X-100, 1% (vol/vol) Tween 20 [but note reported increase effectiveness with additions – see Guanidine entry, and possible protein ppt/centrifuge step and 95C enzyme deactivation]

Wash buffer (for both):
10 mM Tris (pH 8.0), 0.1% (vol/vol) Tween 20

Another approach with cellulose is to use it exactly like filter paper in a funnel but using a centrifuge to force the lysate through the paper. To do that you need a *spin column*, i.e., a mini funnel. A spin column can be improvised from a pipette tip or a re-purposed commercial spin column from a DNA purification kit. Note also that these expensive commercial silica spin columns can also be cleaned and re-used. I won't explore the mechanics of making or re-purposing spin columns further. The reference is … https://doi.org/10.1371/journal.pone.0203011 The described protocol is of interest because of the relatively strong reagent strengths compared with most protocols and the high spin necessary to separate materials.

Add 100mg material to 400ul of lysis buffer (0.5M SDS, 8% PVP, 250mM NaCl, 25mM EDTA, 200mM Tris-HCl (pH7.5)). Homogenise with pestle. Incubate at 65C for 10 mins, inverting 2 or 3 times. Add 130ul of protein precipitation buffer (5M Potassium acetate pH 6.5). Centrifuge at top speed (> 16k G) for 10 mins. If lysate not clear then you have a problem, especially if you don't have a high-speed centrifuge. Transfer lysate to new tube with 1.5 x volume of binding buffer (2M Guanidine hydrochloride, 75% ethanol) and mixed by pipetting. Transfer to spin column (recycled silica or 2-filter paper layer) and centrifuge 6k G for one minute. Discard flow through. Use 500ul wash buffer (10mM NaCl, 10mM Tris-HCl (pH 6.5), 80% ethanol) and centrifuge at 6k G for 1 minute. Second wash with 500ul of 95% ethanol and centrifuge at 6k g for 1 minute. Then centrifuge at top speed (>16 k G) for at least 2 mins and let it dry for 10 minutes in air. It is critical that you remove all traces of alcohol from the bound DNA. If you don't have a high-speed centrifuge for this step then you then you will need to make sure you have evaporated the alcohol, preferably under vacuum. Insert spin column into new tube and let dry for 10 mins. Add 100ul of elution buffer (10mM Tris-HCl pH 8.5) and let stand for 5 mins. Centrifuge at 6k G for 1 min to collect DNA solution.

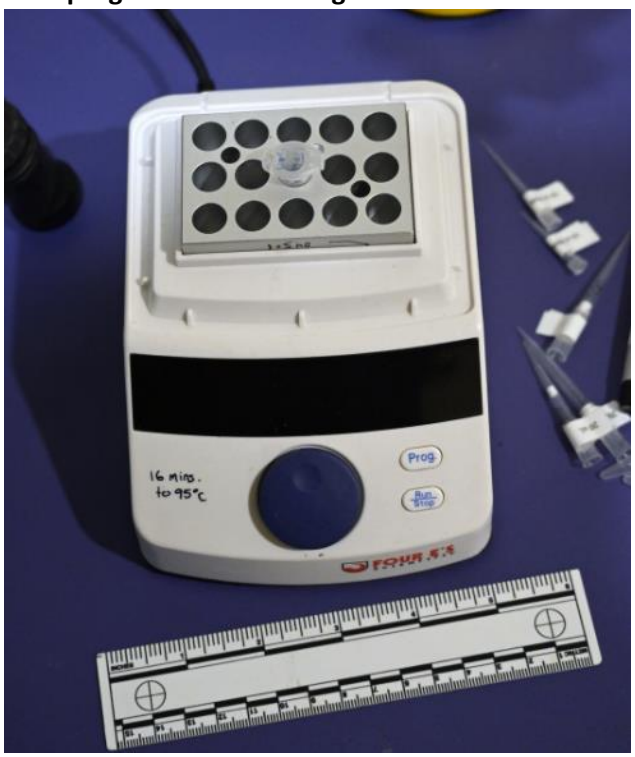
See also the glossary entry for silica powder.

Tools and techniques

Tube heating/cooling methods

Many of the protocols call for heating the sample to 60 or 95C. If you are working with small samples in PCR tubes, then it is easy to program the thermocycler to do this. However, if you want to work with larger volumes in Centrifuge tubes then another solution is needed. I purchased a relatively cheap laboratory mini **dry-heating block** from Hong Kong (via Alibaba).

Mini programmable heating unit with 1.5ml tube block



These units can be programmed to reach temperatures for defined times. I also bought replacement blocks that fit into the heating unit. One of these carries 15 centrifuge tubes and the other can take 40 smaller 0.2ml PCR tubes. These blocks serve a double purpose because I can put them in the freezer and they become a convenient way of processing sample at freezing temperatures, especially preparing the PCR Mastermix (with non hot-start Taq) and the PCR reaction tubes. They stay cold for quite a while.

Centrifuging

Spinning samples to generate a centrifugal force is critical for separating components in many protocols. You will often see references to RPM (revolutions per minute) and G (gravitational force equivalent – also called RFC relative centrifugal force). RPM and G are related and dependent on the radius from the rotor centre to the tube mid/end position. G Force (RCF) = $(RPM)^2 \times 1.118 \times 10^{-5} \times r(cm)$. The G force is the important value, the RPM meaningless without a known radius. A professional laboratory centrifuge can provide around 20,000 G and often much higher but these instruments can cost tens of thousands of dollars and are the size of a small fridge, especially versions that are temperature adjustable. Reasonably priced minicentrifuges for home use cannot reach very high speeds, have shorter radius rotors, operate at room temperature, and few and closely spaced wells (unskirted for 1.5ml centrifuge tubes). The BentoLab has a centrifuge radius of about 4cm (to the tube bottom) and can spin at 14,000 RPM generating a G of about 8,000. The miniPCR centrifuge can offer about 6,900 G. This limitation is an important consideration for some of

the protocols listed here. Any process that requires sedimentation under very high centrifugal force is going to be a problem, but you can just try leaving things on maximum spin for longer. Apart from sedimentation there is the specific case of centrifuging to remove the alcohol wash from a spin column (see protocol above) where very high g is needed and where alternative evaporation might be used. It is critical to remove any trace of alcohol/isopropanol prior to PCR. The miniPCR centrifuge currently has the advantage over the BentoLab because the rotor head can be replaced with one that carries 2 x 8-pcr tube strips which are convenient to use. Both centrifuges have the disadvantage that the short radius means they don't have much room for getting 1.5ml tubes in and out easily, although the BentoLab has slightly better access (tip – insert tubes with cap hinge to rotor centre). Neither unit can accommodate 6 x 2ml tubes with spin-columns inserted because there simply isn't enough room for them side by side, although BentoLab recently introduced a new version that does support this configuration. These minicentrifuges slow down the workflow when using complex protocols on multiple samples. If you can get hold of a second-hand larger lab centrifuge at 16,000 G (and you have room for it) then I would recommend that, although they are still likely to be well over $1000 even second hand, and with high shipping costs (from the US). I bought a broken unit cheaply and fixed a known problem on the control circuit board using a 2$ capacitor.

Mixing defined molarity solutions

A one molar solution has the molecular weight of the compound in grams dissolved in 1 litre of water. Diluting stock solutions should follow the formula V1C1=V2C2 rearranged, as necessary. V are volumes, and C are molarity.

e.g. let's say you have a stock 100uM solution of primer and you want to create 1.5ml of working solution at 5uM. (it is 1/20 obviously but following the equation …) V1C1 = V2C2, V1=V2C2/C1 = (1.5mL x 5uM)/100uM = 0.075mL = 75uL. Most of the time the stuff you add will be relatively small and won't make much difference to bulk volumes, but strictly here you would need 1.5ml-75uL of water added to 75uL of 100x stock in order to have a final volume of exactly 1.5ml.

Mixtures of compounds are treated as independent components according to the same formula and then topped up with the missing volume of water.

For example, let's say we have stock solutions 5M NaCl and 1M Tris. We want to end up with 250ml of 0.1M NaCl + 0.5M Tris

For NaCl component …
V1 = (C2V2)/C1
V2 = 250ml, C2=0.1, C1=5, so V1 = 5ml

For Tris component …
V2=250ml, C2= 0.5, C1= 1, so V1=125

Combination volume is 130ml, but we wanted 250ml so need to add 120ml of H2O

Another example, say we had stock solutions Tris 100mM, EDTA 10mM, NaCl 100mM. We want 250ml of 20mM Tris + 2.5mM EDTA + 25mM NaCl

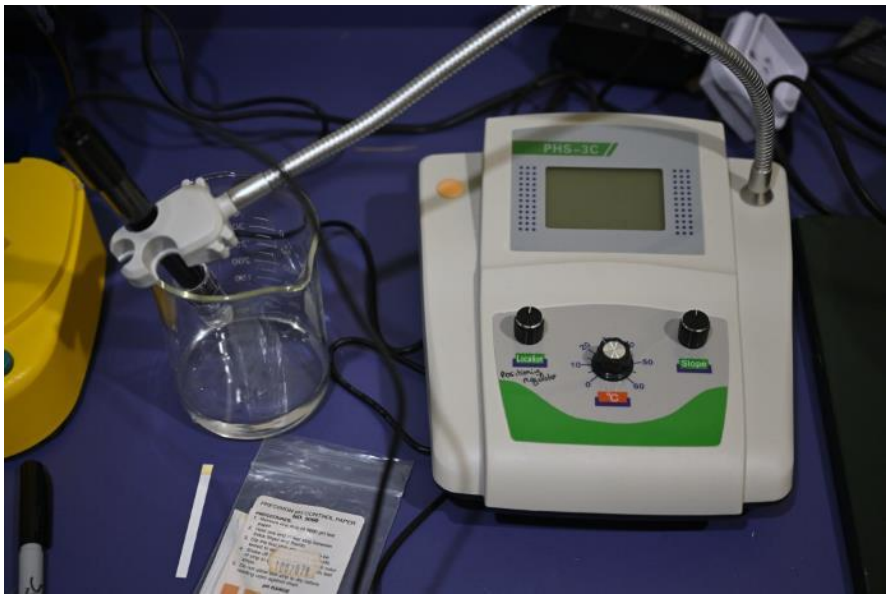
Tris, V1 = (250 x 20mM)/100mM = 50 ml
EDTA, V1 = (250 x 2.5mM)/10mM = 62.5ml
NaCl , V1 = (250 x 25mM)/100mM = 62.5

Combined = 175ml. so add 75ml H2O

Adjusting pH of solutions

You may have noted that many of the solutions will say things like Tris-HCL pH 8. When you make stock solutions you will almost certainly need to adjust the pH up or down by adding Sodium hydroxide (NaOH) pellets, or Hydrochloric acid (HCl). Sometimes significant amounts are needed – showing that the buffer is doing its job. You will need to test the pH as you add reagents. You can do this by periodically checking using a piece of indicator-strip (new strip each time). If you want to be more precise you will need to purchase a laboratory pH meter.

pH meter with glass electrode



Look for something with a small glass electrode designed to work in strong acid/alkali environments. Don't buy a soil/fish tank model! It is worth remembering that during this titration process you are creating salt ions. For example, adding NaOH to a solution of Tris-HCl is creating NaCl ions in the solution, and so if your protocol requires NaCl then you will already have some present (in an unknown molarity) and it is a PCR poison at high enough concentrations.

Mixing PCR components and the PCR run

You need to look at the recommendations for the Taq/Readymix you are using. You are probably aiming for about 20uL of reaction mix per tube. You should multiply-up all the volumes you need and mix into one MasterMix tube (without the template obviously) and with a bit to spare of each ingredient. It is generally appropriate to add BSA to the initial MasterMix volume (see glossary). The multiplied volume of primers should be added last. This MasterMix should be vortexed briefly and distributed into each tube, then finally 1-3ul of template per tube, but noting that too much template can be just as problematic as too little template. This process is preferably done at ice-cold temperature if you are not using a modified or hot-start Taq.

The settings for the PCR thermocycler are considered elsewhere (my quick-start protocol). In general, they won't change much except for the initial temperature to initiate hot-start Taq, and the extension time, which will be dependent on the length of the region you are amplifying. Thermocyclers take time to shift between temperatures due to thermal inertia. A good design (expensive system) will be quick but still not step-like, and with some hysteresis (over-shoot). Cheaper thermocyclers will probably wobble around temperature set-points quite a lot. You may need to play with values to optimise settings for your system. The PCR finishes with an extended

time (5-10mins) around 72C to ensure all remaining dNTPs are used up. It is worth noting that you will often find references to a final 'storage' temperature of 4C for the PCR run, i.e., that when the reaction has finished the product is cooled to 4C until you switch the machine off. This is totally unnecessary. The PCR product is stable for many days at room temperature (but you should transfer to a -20C freezer as soon as practical). In fact, reducing the final temperature to 4C is likely to significantly shorten the life of your thermocycler – it is not intended to be a fridge. If you are really concerned then use 10C, but it really isn't necessary and is just another of the many myths that have emerged around successful PCR.

I like to use 8-tube PCR strips for carrying out PCR. The easiest to use are those with individual caps. I have noted that my BentoLab unit often results in popped tubes and consequent evaporation loss, even though it has a spring-loaded heated lid. I found a bit of improvisation is needed by adding a weight to the lid.

Electrophoresis

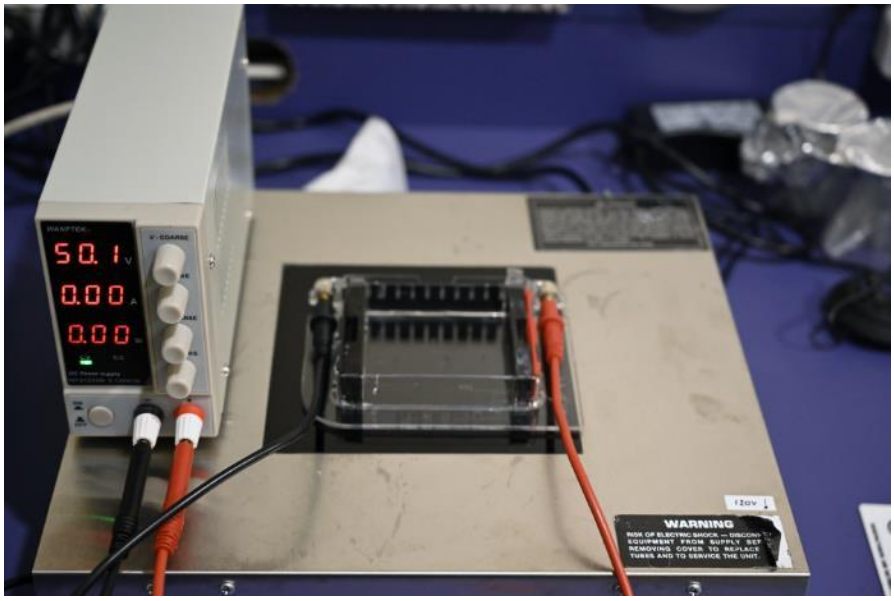
To check if your PCR product is sensible you need to *run a gel* by pipetting a sample into a well in an agarose gel immersed in buffer in an electrophoresis tank (see glossary entries for Agarose, Bands, DNA ladder, TAE/TBE Buffer, Eithidium bromide, Loading Dye, GelGreen/GelRed). Electrophoresis units are surprisingly expensive for what they are – a plastic tank with a couple of electrodes. If you buy a unit then make sure it has platinum electrodes. Some cheaper units available online have nichrome electrodes and they won't last long before the buffer/electrolysis erodes them. You insert a comb into the tank and then pour in molten agarose gel (plus DNA binding dye). After the gel has set you remove the comb, which leaves wells in the gel. The BentoLab unit takes about 25ml of gel to produce a 5mm thick gel.

You then add buffer to the tank to immerse the electrodes and above the gel for about 2mm depth. The BentoLab fine comb produces well 3mm wide. You then pipette your PCR products or DNA ladder and for 3mm well I use 3ul of product/ladder. A minimum number of wells (teeth on the combs to insert into the gel) are 9/10 or 13/14 teeth to provide 8 or 12 PCR product lanes and 1 or 2 DNA ladders. For Units made of perspex/plexiglass will transmit the blue light of the built-in BentoLab or miniPCR transilluminators. The blue light makes the bands fluoresce green, although orange plastic lids are needed to filter out the blue light, so the fluorescence stands out. You get better visualisation with a UV transilluminator, and no need for an orange filter, but most plastics (except methacrylate) are only partially transparent to UV and so best fluorescence will be seen if the finished gel is lifted out of the tank and placed directly on the transilluminator surface. However, there is usually enough transmission through plastic to see if the bands are forming whilst the gel is running.

The DNA fragments are negatively charged and so are attracted to a positively charged electrode. The result is that fragments move towards a positive electrode at a speed according to their size. The electrophoresis tank has electrodes at each end and a DC voltage is applied. Your wells will be near the negative electrode and the DNA will migrate through the gel. As soon as you switch the voltage on you should see small bubbles forming around the electrodes (electrolysis), and the tank will eventually heat up. Do not be tempted to reuse gel buffer. The solution is electrolysed, and its properties change. Reusing buffer will slow down band migration significantly. The voltage needed is 5-10V per cm depending on the strength of the gel/buffer, the size of the fragments and your patience waiting for the bands to form. In the BentoLab unit the electrodes are 10cm apart and with a 1% TBA agarose gel you will need about 80V applied for about 45 minutes. One problem I found with the BentoLab arrangement is the need to have the electrophoresis until encased with the

orange filter lid during electrophoresis. This arrangement is for safety reasons, so you can't poke your fingers around the DC supply. The problem is that the gel always heats up and buffer evaporates and fogs the lid of the unit. Two slits are cut into the unit as a vent, but it doesn't significantly decrease fogging and lets sufficient blue light to escape that seeing the bands during electrophoresis is difficult. I have not tried the miniPCR all-in-one unit, but it may have the same issues. If you run your gels at a higher voltage then the DNA moves faster, but the gel will heat up more ad often distorts, and the bands aren't as crisp.

Gel tank and constant voltage supply



I also prefer electrophoresis units where the gel sits on a cradle and can be easily lifted out of the tank to place directly on a transilluminator. I also had an initial problem with the BentoLab because the voltage would cut-out complaining that the gel tank resistance was wrong. I traced that back to an incorrect agarose concentration. I also use a separate gel tank on a UV transilluminator, and attached to a variable voltage supply with a voltage/current limiter. These power supplies are relatively cheap (0-120V, 3A). You need to ensure the voltage cannot be applied whilst there is (finger/hand) access to the gel/buffer/electrodes. A 50V DC supply may not sound dangerous (the old, fixed phone lines ran at 48V DC) but under the right circumstances the current induced by 50V DC can be fatal, so treat any electrical equipment with caution and respect. After running the gel to separate the bands I take a photograph and annotate it for my records. See the glossary 'Bands' entry for more information on interpreting them.

Sequencing

You probably won't be doing your own sequencing and you will need to send a sample of your PCR product (perhaps 5uL) to a sequencing facility. Many are likely to want some indication of the DNA concentration, which you won't know unless you have access to a quantitative DNA fluorescence unit (like a NanoDrop). You can make a rough estimate by comparing the brightness of your gel band to a quantified DNA ladder band (see entry for DNA ladder). Usually you will need 20-100ng of DNA. You may also need to purify your PCR product to remove unused primers, dNTPs and any Primer-Dimer, or pay for the PCR product to be cleaned by the sequencing facility. Often you can get away without PCR product purification.

The most common and cheapest sequencing technology is Sanger, and it has remained unchanged for years. In Sanger sequencing the amplified DNA fragment undergoes a final round of PCR using a

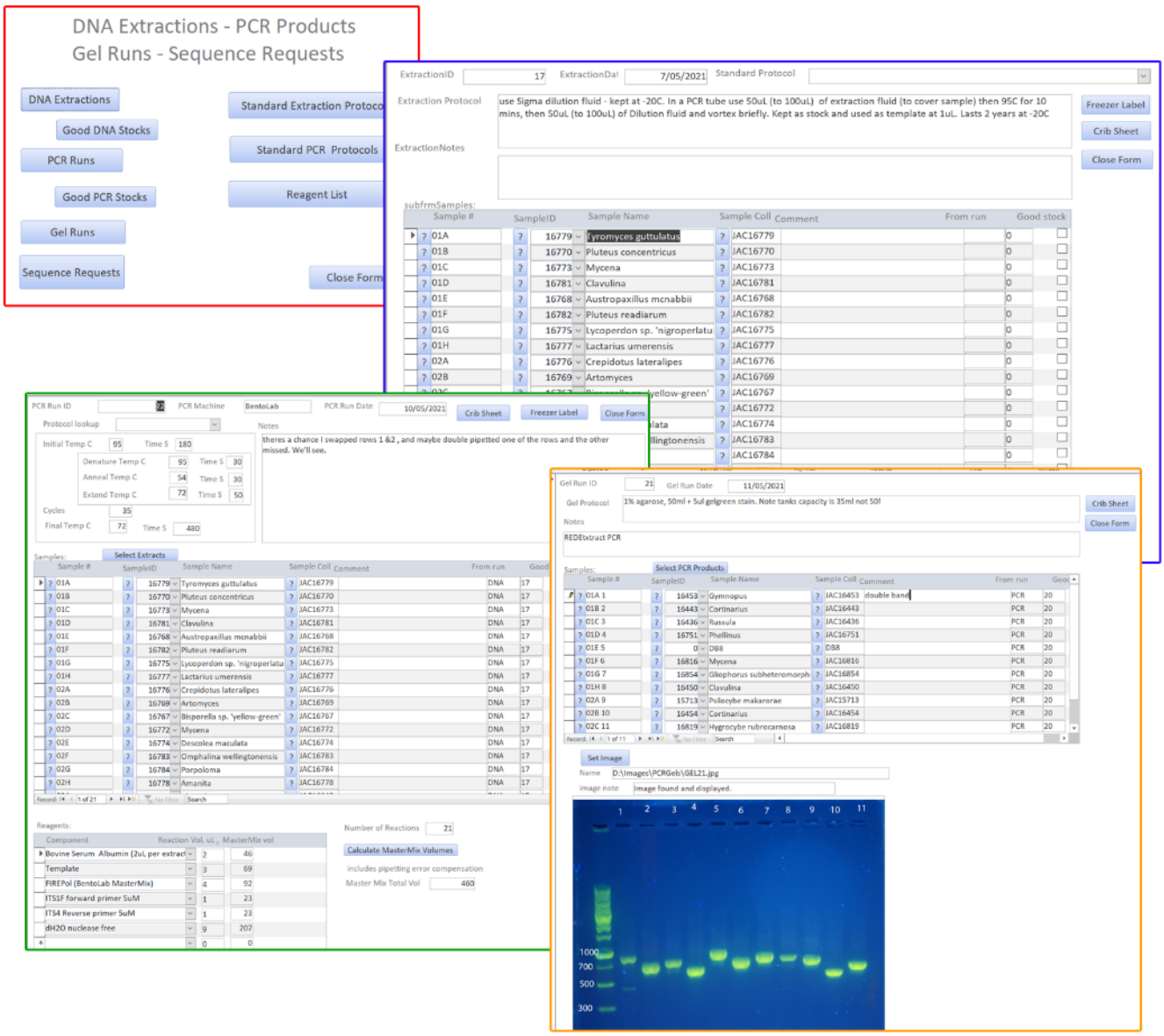
proportion of modified versions of the dNTPs. Each of these modified forms is added as a single base to a DNA strand by Taq. The modification then halts the extension of that strand (chain-terminating dNTPs). In addition, each of the 4 modified dNTPs carries a different fluorescent marker. When a single primer (forward or reverse) is added to a mix with a *proportion* of these modified dNTPs the PCR cycles will generate lots of separate strands of every possible length, from the initial primer sequence all the way through to the end of the amplified fragment (3' to 5' direction starting with the forward primer). The final product is a mix of lots of strands of every possible length. And in addition, each specific length carries on the end the same fluorescence marker corresponding to the nucleotide present at that point. This mix is then separated by size in a special electrophoresis unit. The sequence of fluorescent bands is a read-out by a laser to generate a chromatogram which corresponds to the sequence of nucleotides. Usually, this modified PCR is done for both the forward primer and the reverse primer to generate two chromatograms which can then be *assembled* into a single sequence, with some measure of the quality of the signal at each position. It is these chromatograms you should get back from the sequencing facility, and you should ask for both forward and reverse reads. The sequencing facility will need to know what forward/reverse primer sequences you used, or you will need to supply them. The fact the forward sequence is read by adding just the forward primer tells you why the PCR product needs to be first cleaned to remove all residual primers from your PCR. If both primers are present during the Sanger PCR cycle, then forward and reverse will be amplified simultaneously, and the chromatogram will be just a noisy mess of reads in both directions.

Once you have your chromatogram the real work can begin – but that requires a different 'beginners guide' document.

Keeping records

Every sample you try will generate at least one DNA extraction you probably want to keep. Every DNA extraction will be part of at least one PCR reaction with certain primers and PCR settings. Every PCR reaction will be associated with an electrophoresis gel where the PCR sample will be in one of the wells. Lots of tubes need to be labelled and kept in fridges/freezers. Some of the successful samples will be sent off for sequencing. The consequence of all these steps is that you will rapidly generate lots of tubes and associated data. You need to keep track of tube contents, what you did, and what the results were, and where you put them. You need to have good record-keeping and (freezer) filing system. This is the kind of problem I solve by writing a database management system, which is what I did. It works for me. If you like spreadsheets then use that.

My data management system – screen shots



You need to find your own solution to this data management problem. Do not underestimate this book-keeping task, or the family kickback about taking over the freezer compartment of your kitchen fridge.

Chemicals/Glossary

Agarose – a pure form of one of the main ingredients of agar. Agarose is used in electrophoresis instead of agar because the impurities and other components of agar can be electrically charged and interfere with DNA migration under and electric field. The pure extract agarose is electrically neutral. The agarose needs to be dissolved in a warm buffer solution and allowed to solidify and cool. The concentration of the agarose affects the speed with which DNA fragments move through the gel, with lower concentrations for large fragments (up to including whole gDNA). I generally use a 1% solution for ITS PCR products. Dissolving the agarose in a buffer rather than water is necessary for two reasons. First the buffer provides ions which carry charge through the gel and DNA feels the effect of the local electric field from these charge carriers and is dragged along. There needs to be a dominance of uniform charge carriers provided by the buffer. If you use pure water rather than a buffer stuffed with electrolyte then the DNA will still migrate but you will see a trailing smear as DNA fragments are pulled differently by different charge carriers. Secondly the buffer does its job and keeps the pH constant which would otherwise change because of electrolysis, and once again affect the dominance of uniform charge carriers. TBE buffer is recommended for separating smaller fragments like ITS. When using 0.5x TBE buffer with ITS products a recommended gel concentration is 1%. A DNA stain is either added to the molten solution when it has cooled to 60C and before pouring, or to the PCR products. See Bands.

Ammonium acetate (MW 77) – sometimes used in a DNA wash cycle at 0.1M + 70% ethanol, or at 2-2.5M to precipitate proteins, or at 5M in the presence of alcohol or isopropanol to precipitate DNA (and residual proteins).

Bands - PCR products migrate through the agarose gel during electrophoresis to form bands visible under excitation from blue/UV light of a transilluminator. The size and brightness of the band tells you if you amplified something, and how much PCR product you created. See DNA ladder entry for estimating DNA concentration. A good product should produce a sharp single band at the right length (e.g., around 750bp for ITS). If the gel warps then check you don't have the voltage too high, or perhaps the agarose was not uniformly mixed, or the buffer was not uniformly mixed, or the concentration/composition of the buffer in the gel, and immersing the gel, were different. If you have a smeared band that corresponds to the extension time x 1000 (bp per second taq activity), then one primer failed, or you forgot to add it (been there – done that). The gel may also show a fuzzier band of much shorter length, indicating unused primers, and/or at a slightly longer length it indicates primer-dimer formation. Sometimes a lane will have multiple bands, or a smear and a band. A smear can have several causes. If it occurs in all lanes, then it is likely a problem with the gel mixture (agarose & dissolving buffer), or too much product in the wells, or all your PCR reactions failed due to faulty setting/reagents/primers and the smear is the unamplified template gDNA and/or partial amplification products. If the smear appears in discreet lanes then it indicates your template contained contaminating proteins, salt, or the primers did not bind correctly, or there was too much template etc. Frequently PCR will fail due to inhibitors or insufficient DNA and there will be no bands. Most often the problem will be PCR inhibitors and diluting the template (x10) and re-running the PCR may allow the PCR to work, or you may need to cleanup the gDNA. Multiple bands indicate the template gDNA was mixed. If this happens you can extract the product from the *right band*. You may need to use TAE buffer for gel extractions because the Boron in TBE will supress Taq enzyme activity, although after dilution the effect seems to be minimal. The gel is sliced using a clean/sterile scalpel blade and minimise the amount of gel that isn't fluorescing. Unfortunately, you can't just use a piece of the gel and use it in a new PCR reaction because the gel stuffs things up. In addition, UV from a transilluminator will very quickly destroy free DNA – in a matter of seconds. If

you intend to cut out gel slices, then use a blue-light transilluminator (and orange goggles) and not a UV transilluminator (unless you are quick). Extracting the free product from the slice can be done in several ways. Gel extraction kits (expensive) dissolve the gel in a strong chaotrope (5M Guanidine isothiocyanate or Sodium iodide) or use the enzyme Agarase. However, there is an alternative simple and fast technique. Cut off the tip-end of 100 or 200ul pipette tip with wire cutters (cutting edge flamed) so it fits inside a 1.5ml centrifuge tube with the lid closed. You will need to use tips that have a filter plug. Then load your small gel slice (with flamed tweezers) into the top of the tip above the filter and insert the modified filter into a 1.5ml centrifuge tube and spin for 30s-60s (not too long). A little liquid with the product is squeezed from the gel through the filter and into the centrifuge tube. The gel stays stuck to the filter. Sometimes it may be possible to extract enough good product to directly sequence (you can try running a thick gel with a large comb and 20ul in the well to extract a lot). Usually, it is easier to get a tiny amount of squeezed extract and use it as a template in a repeat PCR. If the original bands were strong you will need to dilute your extract at least 100 fold by serial dilution (1/10 followed by 1/10) and 10 fold if they were weak. You will also need to reduce the number of PCR cycles (try 30, or even 20 rather than 35). If you don't dilute and reduce cycles you will just get a blurry band buried in a smear of random length amplicons – and sometimes those products will sequence ok and sometimes not. Apart from separating multiple PCR products a gel-extraction can be useful for separating a product from unused dNTPs, dyes and any PD. For information the residual DNA fluorescent binding dye doesn't supress PCR because it binds to the groove in double stranded DNA and so does not interfere with annealing or the elongation process. If you don't get PCR bands and diluting the template (10 or 100) does not work then you will need to clean the gDNA, if indeed you have any gDNA. You can test that by running a 1% gel with stain with 3uL of template +loading dye at around 80v for 40 minutes. The large fragments of any gDNA should have migrated out of the well in that time. If you have no fluorescence then there is no gDNA and you will need a better extraction method (or your DNA extract process really chewed things up).

Barcode region - For fungi the primary barcode region is the ITS (Internal Transcribed Spacer). It is usually quite good at discriminating mushroom species, but much less reliable for many ascomycetes, especially plant pathogens. Differences between species will show up as at least a 2% sequence difference. There are lots of caveats to this. For some species, especially recently diverged species, there may be strong ecological/morphological differences but no difference in ITS. Conversely ITS is present in fungi in multiple copies, and in additional basidiomycetes are dikaryotic, carrying separate nuclei from parent strains. The result is that some fungi can carry multiple slightly different copies of ITS, and which one (or more) gets amplified through PCR may differ. And finally, the sequence quality may be poor, resulting in assemblies of forward/reverse reads with ambiguous and incorrect bases. So, you can get different ITS sequences for the same species.

The GenBank Blast utility is often used to identify sequences, but this is also subject to many problems. GenBank contains many incorrectly identified sequences and in addition the Blast algorithm does not provide an adequate representation of phylogenetic differences. The correct approach to analysing sequences is an entirely separate article. I just want to note that Blast does not tell you everything you need to know, and you should not rely on it.

If you want to know about phylogenetic relationships (and not just species identity) then the ITS barcode does not usually provide adequate resolution. It will need to be combined with other loci. LSU (large subunit) is often sequenced, but of critical importance are protein encoding regions, especially RPB2, TeF, and BTUB (Google them). These genes are trickier to amplify because they are present only in low copy number and relatively large quantities of clean DNA are needed.

BSA - Bovine Serum Albumin – a small stable non-reactive protein. It generally binds to enzymes and so blocks their action (useful in buffers) but in a PCR reaction it stops Taq binding to the walls and binds to melanin/polyphenols which would otherwise poison PCR and also to any problematic residual enzymes DNase, Proteinase-k etc. It has no effect on the inhibitory effects of SDS, NaCl or Triton (surfactant). The literature often indicates it should be in combination an organic solvent like DMSO or formamide (1-5%) under high GC conditions but noting that DMSO can reduce Taq activity. High GC (>60%) indicates well-bonded double strands, but most fungi (all loci) will be < 55%. The recommended concentration of BSA in a PCR mix varies between 0.01 µg/µl to 0.5 µg/µl. I would add 2µl of a 2mg/ml stock solution to each 20µl PCR reaction (i.e., 0.2 µg/µl). Lyophilized BSA powder should be added slowly to ddH2O at cold (fridge) temperatures. It doesn't like dissolving (quickly) and can clump, or foam if vortexed. Don't let it foam. Store at -20C. Because it is animal-derived with the potential to contain viruses there are restrictions on importation into New Zealand.

Chaotrope – a substance that reduces hydrogen bonding. See Guanidine hydrochloride.

Chloroform – a non-polar organic solvent. Generally used in a CTAB extraction in a 25:24:1 ratio (phenol:chloroform:isoamyl alcohol). It is classed as a hazardous substance, and so along with phenol, is not recommended or DNA extractions.

CTAB (MW 365g/mol) – Cetrimonium bromide. This is a cationic surfactant and thus should not be mixed with anionic surfactants like SDS. The way it interacts with cellular components changes according to the concentration. At high salt concentrations it binds to polysaccharides, although I'm not sure why it doesn't also precipitate DNA at the same concentration. It seems this does happen below 15C but not at normal room temperature, and the DNA remains in solution.

ddH20 - double distilled water (PCR grade water). Anything that goes into the PCR reaction tube needs to be ultrapure and especially free from organics and enzymes. The purity of the water you use is critical. Any water stored in a container over time, and with air above it, will decrease in pH as absorbs Carbon dioxide, so the pH may not be neutral.

DNA ladder - a mix of DNA fragments of known length. A sample is pipetted into one of the wells in your gel. Under electrophoresis they separate out to form a visible ladder of bands. The ladder allows to estimate the size of PCR products appearing in each lane on the gel, when fluorescing under the transilluminator. The DNA ladder serves another purpose. They are usually calibrated so certain bands have known DNA quantities. You can compare the brightness of your band to an equivalent in the ladder and read-off the amount of DNA in your sample – roughly. For example, the current MiniPCR 1kb ladder has a 100bp band that contains 41ng/10ul. The BigDye Terminator (BDT) sequencing needs 5-20ng for ITS, i.e. 2ul of your PCR product if it is as bright as the 100bp band.

dNTPs – deoxyribose nucleoside triphosphate, where the nucleoside is one of the 4 AGCT nucleotides. They are the building blocks the Taq enzyme uses to extend the DNA strand during each PCR cycle. They are generally added to the PCR mix at 0.2mM. High levels of Mg++ can bind to dNTPs and at high concentration they inhibit PCR.

EDTA (MW 292) Ethylenediaminetetraacetic acid - binds to metal ions such as Mg++ and Ca++ but does not bind to Na+, K+. It is useful for inactivating DNase enzyme present in the cell which would degrade the DNA (and incidentally DNase is also irreversibly inactivated at 65C). These enzymes need metal ions to work and so anything binding to them stops their catalytic action. It is also useful in capturing ionic cations that stabilise the nuclear membrane lipids. But EDTA may also hinder the downstream PCR because it will bind to the Mg++ present in the Readymix. Mg++ is added as a cofactor to Readymix to make the Taq enzyme operate. However, unless there are high

concentrations of EDTA then the amount of MgCl++ in a master-mix should overcome andy residual EDTA (or it can be increased to overcome the effect of EDTA). For a lysis step it isn't clear to me why it isn't simply sufficient to quickly heat a sample to 65C in buffer to deactivate DNase and so not require the addition of EDTA, although understandably longer-term maintenance of DNA integrity will be supported if metal ions are removed.

Ethanol (MW 46) – I suspect getting hold of high quality and high concentration ethanol could be a major problem if you don't have access to a lab. It is difficult to ship without heaps of safety measures and paperwork, and obviously its use is closely monitored. However, for most DNA clean-up protocols it really is needed, and I have not come across a replacement. It is essential in any DNA clean-up/wash steps and the dilution needs to be critically controlled. Old ethanol may have taken up water vapour from the atmosphere and reduced the effective concentration – and thus DNA is likely to be washed away and not cleaned or precipitated. Ethanol and isopropanol are PCR inhibitors and must be removed by high-g centrifuge or evaporation before any material is used as a template.

Ethidium bromide – In the olden days this was used to visualise DNA in electrophoresis gels. It is a fluorescent compound that intercalates between the nucleotides of the double stranded DNA. It has a reputation as being carcinogenic because of this binding action but in fact the danger seems to have been overstated. The molecule generally cannot enter cells or get near *living* DNA to cause damage. It has been used in veterinary medicine for decades to treat cattle at 1000 times the concentration used in molecular biology. Because of the reputation it has generally been replaced by so-called safer substitutes with larger molecules and less chance of entering cells. However, some of these have been demonstrated to be more carcinogenic, and considerably more expensive, although very small quantities are used to stain gels. Ethidium bromide fluoresces in the red with UV absorption peaks at 210 and 285 nm. See GelGreen and GelRed as replacements.

Fridge/Freezer - It is critical to keep some reagents/buffers/stocks at room, fridge or freezer temperatures and it sometimes difficult to work out what should go where. Usually when you buy reagents it will tell you on the packet. Anything containing enzymes in solution should be kept in the freezer. In fact, nearly everything to do with the PCR reaction should be kept in the freezer. Most standard kitchen freezer compartments operate between -15 and -20C. Enzymes should be fine for at least a year at this temperature, but of course you need to plan-ahead and take them out to thaw before you use them. Try not to put them through too many thaw/freeze cycles. Larger volumes of reagents should be aliquoted into smaller volumes, so you aren't freezing/thawing large volumes every time. Some reagents, such as loading dye, contain glycerol which microbes can use to grow and so they should be kept in the fridge. Some reagents are light sensitive (DNA binding dyes) and they should be kept in the dark (and a fridge is dark). Some reagents are used ice-cold (e.g. ethanol/isopropanol) and so it makes sense to keep those in the fridge/freezer (they won't freeze). Some commercial lysis buffers (e.g. Sigma) need to be kept frozen. Most buffers however should be kept at room temperature. Reducing their temperature may result in components precipitating out of solution – and if they do then they need to be redissolved by warming the bottle under water from the hot tap.

Gels - see band

GelGreen/GelRed (SYBR) – so-called safe replacements for Ethidium bromide. GelRed emits in the red and is only excited by UV. GelRed seems to produce a lightly cleaner signal than GelGreen with good PCR products. For GelGreen the absorption bands are in blue light 460-480nm (blue) or 254nm (hard UV) and it emits at 535nm (green) when excited by blue light, and green and/or 635nm (red)

when excited by UV. I've noticed red emission when using GelGreen and UV excitation seems to be associated with impurities in gDNA mix – that will cause PCR to fail. The safe blue light excitation is used in some electrophoresis/transilluminator combinations (BentoLab/miniPCR). An orange plastic filter is needed to block the blue illumination so the green fluorescence can be seen. Blue led illuminators are a safe alternative to UV, although, similar to Ethidium bromide, the danger seems to have been a bit overstated. UV illumination generates a cleaner/stronger fluorescence (in my opinion). These dyes are added to the gel, or to the PCR products, or post-stained. Generally, it is easiest to add it to the gel. I've never tried adding it to the mastermix although DNA dyes are central to the real-time PCR methodology so some kind of formulation must work.

GenBank & Blast … an article itself

Guanidine hydrochloride (MW 96 - GHCL) – is a very strong chaotropic agent with a relatively high ionic potential (pH <7). That means it disrupts hydrogen bonding. The effect is a weakening of any hydrophobic effects in solutions, increasing solubility of non-polar components, but most importantly it unravels proteins, opens-up biopolymers, can solubilise the components, and can significantly reduce the denaturing temperature of DNA. It is known to enhance the binding of DNA to cellulose and silica. The mechanism seems to be unclear to me, and supposedly associated once again with reducing the protective hydrogen-bonded coat of water molecules, but I suspect it is also connected to the exposure of positively charged DNA nucleotides to the negatively charged binding surfaces. Guanidine isothiocyanate (GITC) is a stronger chaotrope but can produce deadly cyanide gas if it contacts bleach. Less potent chaotropic agents include Ethanol, Magnesium chloride, Sodium iodide, SDS and Urea. Antichaotropic agents, such as Ammonium sulphate have the opposite effects. Guanidine hydrochloride can be used as a 1-step lysis reagent. Strong chaotrope solutions and gentle heat are also capable of disrupting agarose gel to release DNA in gel extractions, although the enzyme agarase is gentler. Note that high concentrations solutions of GHCL and GITC (3-6M) used for lysis and gel extraction are very caustic liquids and, like all reagents used in molecular biology, should be handled with care wearing nitrile gloves and eye protection (from accidental splashes).

Hydrochloric acid (HCL) (MW 37) – at 1M will degrade DNA. Useful for washing/recycling spin columns, pipette tips etc. Equipment needs multiple washes in distilled water, sterilisation, and evaporation. Can be bought at hardware stores as *spirit of salt* at adequate concentration and is probably good enough for most applications. Also used for *re-charging* the silica-based spin columns in commercial kits. 1M Phosphoric acid is apparently 10 times more effective, although harder to get.

Isoamyl alcohol (MW 88) – an aliphatic non-polar solvent used together with Chloroform and Phenol in a CTAB extraction. The combination phenol/chloroform/isoamyl alcohol creates a strong polar solvent that cleanly dissolves non-polar compounds and separates from water.

Isopropanol (MW 60) – used like alcohol and added to a lysate containing salt or Ammonium acetate or Sodium acetate to precipitate DNA. Salt is less soluble in isopropanol than alcohol and so may precipitate out with the DNA. Subsequent washing with 70% ethanol is needed to remove salt. Note that both ethanol and isopropanol are strong PCR inhibitors and must be removed by high-g centrifuge, evaporation etc.

Loading buffer/dye - A dense glycerol-based solution that needs to be in the PCR product pipetted into the gel wells. This loading buffer causes the aliquots to sink so the PCR product doesn't immediately diffuse into the buffer solution above the wells. Usually, this loading buffer also has a couple of visible dyes added. An orange dye that trails behind your DNA fragments and a blue dye

that moves in advance of the DNA fragments. They are useful for tracking how the run is progressing if you can't visualise the fluorescence of the DNA itself using a transilluminator. These tracking dyes are next to useless in the blue-light fluorescence units with orange plastic lids. Loading dye generally contains EDTA to stop any enzyme reactions. Some Readymix formulations have these compounds added, except the EDTA, and they don't seem to interfere with PCR. If your Readymix doesn't have them, you will need to add loading dye to each PCR product/gel sample (or risk wasting your time). Spotting appropriate volumes onto aluminium foil, parafilm or plastic petri dish surface and then mixing with each PCR gel aliquot by pipetting seems to be the accepted protocol. If you have lots of PCR tube strips and spare time, then the loading dye/PCR product can be mixed in tubes. You could add the dye to the entire PCR product but I'm uncertain if that interferes with subsequent sequencing.

Magnesium chloride MgCl (MW 95) – a required cofactor for Taq and added at 1-4mM. However excess Mg affects Taq fidelity. Often the concentration needs to be tuned by experiment. Non target products show up at low MW in gels.

Magnetic beads – the use of magnetic beads for DNA extraction, gel extraction, and PCR cleanup is attractive because it generally requires less processing and does not require the use of a high RCF centrifuge, although it is generally less efficient than silica-column based methods. The process uses superparamagnetic particles (microscopic beads) which react strongly to a magnetic field but are not themselves permanently magnetic (ferromagnetic), so they don't (shouldn't) clump to each other once an external magnetic field is removed. These particles are coated in a layer that makes the paramagnetic core non-reactive, such a polymer, and a DNA binding layer of silica. These particles can also be further coated with a layer that binds specifically to molecules of interest. For DNA binding either bare silica or carboxyl/hydroxyl coated beads are appropriate, and each requires a different binding buffer solution. Much more sophisticated binding mechanism are available (e.g., streptavidin) that can be highly selective and super-concentrate only the molecules of interest. A mix is created of these particles, the impure lysis fluid, and a binding buffer. The DNA binds to the particles, exactly as it does in a silica column cleanup. The mix is then placed near a powerful permanent magnet, usually made of various rare-earth metal alloys. The paramagnetic particles with their surface-attached DNA molecules stick to the side of a tube and can be washed clean with successive high ethanol solution before being eluted back into clean, low salt buffer, and finally separated from the particles using the magnet. For DNA the process uses beads of various sizes, either to extract gDNA or separate PCR products from dimer, primer, dNTPs etc. The size is important and for gDNA extract particles around 1-3um (at a concentration of 25mg/ml) are needed. Note that some shearing of the gDNA is beneficial so long DNA strands don't bind the beads to each other. For bare silica/hydroxyl beads the binding buffer is a chaotropic salt solution (as in silica column cleanup), and for carboxyl coated beads a PEG (polyethylene glycol) based buffer is needed. Combinations of bead size, bead concentration and ratio of binding buffer can be used to selectively absorb DNA by the length of the fragments.

MasterMix - This is the term I am using for the totality of ingredients per PCR reaction, including primers. *ReadyMix* is the term I am using to refer to the pre-manufactured combination of Taq, buffers, MgCl++ etc. These terms are used variously in the literature.

2-**Mercaptoethanol**. A strong reducing agent which cleaves the disulphide bonds in nuclease enzymes, thus stopping their degradation of DNA and RNA in lysates. Known to be toxic.

Micelle – a bunch of molecules grouped into a globular structure in a fluid (as a colloidal suspension) where the centre consists of hydrophobic (non-polar) units drawn together, with hydrophilic tails

sticking out. These structures are important in mopping up non-polar compounds in a mixture. They are pulled into the micelles and the units are hydrophilic. It's the way detergents work to free grease when washing.

Phenol – a non-polar organic solvent not miscible with water. It is a highly hazardous substance, considered to be carcinogenic and a *watched* chemical (used in illicit drug manufacturing), and so its use is not recommended, even if it could be obtained outside a laboratory environment, which I doubt. When mixed with cell contents the non-polar compounds (like proteins and polysaccharides with non-polar regions) will dissolve in the phenol leaving the polar compounds (like anionic DNA) in the water. A phenol extraction needs to be alkaline. If the mixture goes pink it has become acid and the DNA will have oxidised.

Plates and numbering – Most molecular lab equipment is designed to deal with 1.5ml centrifuge tubes, or 200ul PCR tubes arranged in sets of eight adjacent tubes (strips). Twelve of these strips of eight tubes are bulked-up into plates containing ninety-six wells/tubes. By (silly) convention the wells are labelled as the 8 letters A-H, and 12 numbers.

A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
C01	C02	…									

It is usual to keep records in a spreadsheet or database table as a single column. The problem is that you need to maintain the critical order of tubes in each strip, but the sorting function in Excel and most databases will mangle the strip order to give you A01, A02, A03. It is not easy to manage data labelled this way. I cheat by reversing the coordinates …

01A	02A	03A	04A	05A	06A	07A	08A	09A	10A	11A	12A
01B	02B	03B	04B	05B	06B	07B	08B	09B	10B	11B	12B
01C	02C	…									

Then you can order sensibly by strips and then columns but remember to translocate back to the (silly) convention before you send tubes/plates away for sequencing.

Potassium acetate (MW 98) – is used (at 3-5M) to precipitate SDS and SDS-bound proteins from solution (any residual potassium present in reagents will affects SDS solubility). In the Qiagen protocol this step is done on ice. If any trace of alcohol is present, then DNA will also precipitate. Also, if a prior alkali cell lysis was carried out then long chain DNA/protein complexes will precipitate. Residual potassium ions, if the concentration isn't too high, may not affect PCR. Small amounts have been shown to improve primer annealing.

Proteinase-K - Degrades proteins and so aids the release of DNA, supresses the agglomeration of long protein fragments to which DNA can bind, and is a DNase inhibitor. A useful addition to a lysis mix (at 1.25ug/ml) to degrade proteins (at 50-60C for 15-50 mins) and can be heat deactivated at > 80C after the job is done. It can operate between pH 4 and 12.5 with an optimum at pH8. It is not affected by Calcium the presence of EDTA in buffers does not impact on activity. 800mM Guanidine hydrochloride will double or treble the effectiveness and 0.5-1% SDS also enhances action but noting the PCR inhibitory action of SDS. Salt concentration does need to be relatively low for proteinase-K to operate. If lysis is done using strong chaotropes then Proteinase-k should be used in an initial 2-step process with a lower concentration.

Primers – Primers are short unique sequences that identify the start and end of the region you want to amplify through PCR. They are chosen such that they are relatively stable across the species you want sequence and don't stick to each other. That is so the primers will just bind to the correct positions on the forward and reverse single DNA strands. The match doesn't have to be perfect, but it does have to be close. Primers are relatively cheap, and for quantities that will last a long time, but they do need ordering from specialist suppliers. They are generally shipped as dry powder (~400 ug in tubes) and stable at room temperature in this form. They should be diluted with PCR grade water to create a stock solution at 100uM concentration (in ~500-600uL ddH2O). That can be kept at -20C and diluted further x20 to give a final 5uM working solution (e.g. 1ml of working stock from 50uL of stock). So 1 tube of powder will result in 10mL of final working 5uM stock. 1uL of this stock will contain 5 pico moles – enough for a 20ul reaction tube. Each tube of dry primer is enough for about 10,000 sequence runs! Too high a concentration of primer and Primer-Dimer production is enhanced (visible as a low MW smear in gels). It is best to create a pre-mix of primers and PCR grade water (the total volume of primers plus the volume needed to bring the per reaction volume up to 20ul).

Primer-dimer (PD) – caused during PCR when two primers have stretches that are complementary and they stick to each other, especially at room temperature in the Readymix. The PCR then preferentially amplifies the PD and all free dNTPs are rapidly used up. On a gel it can appear as a diffuse band < 50bp (the length of each primer added together an often a bit larger), and it is sometimes is co-present with a normal PCR product band. This situation is often signalled in the final sequencing run by a rapid extinction of the signal. Just the short dimer has been sequenced because that's dominant, even though it may not look that way on a gel. Hot start Taq can alleviate the issue, and perhaps also ensure the template is added to the Readymix **before** the primers. Cooling all the PCR reagents reduces the chance of premature Taq activity. Paramagnetic beads provide a relatively easy way to cleanup PCR products, including the removal of PD if the conditions are right, or a gel-extract (maybe needing subsequent concentration with beads) can be used to separate the product from the PD and residual dNTPs.

PVP - Polyvinylpyrrolidone - binds to polyphenols and is added to the lysis buffer between 1% and 3% with higher amounts for high polyphenol tissue, like wood decay fungi.

Readymix – the name I am giving to the cocktail of ingredients used for PCR minus the specific primers and the template. It is easiest to run your PCRs using purchased pre-formulated Readymix. It generally consists of Taq DNA polymerase, 5X reaction buffer (0.4 M Tris-HCl, 0.1 M (NH4)2SO4, 0.1% w/v Tween-20), 7.5 mM MgCl2 (in 1X PCR solution: 1.5 mM MgCl2), 1 mM each dNTP (in 1X PCR solution: 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 200 µM dTTP), Blue dye with migration rate equivalent to 3.5-4.5 kb DNA fragment, Yellow dye with migration rate in excess of primers in 1% agarose gel, Compound that increases sample density for direct gel loading. Note the pH of Readymix is often quite high, pH9.5. Readymix will survive for a while at room temperature but not for too long. Longer term it needs to be stored at -20C. Really it needs delivered using a cold-chain. If your PCRs don't work then make sure your shipped enzymes weren't hanging around a hot warehouse for days before you received them (my BentoLab enzymes once got stuck in Abu Dhabi). See also the PCR entry.

Sanger Sequencing – see main entry for sequencing.

SDS - Sodium dodecyl sulphate (MW 288) – an anionic surfactant and critical for disrupting nuclear membranes and softening the chitin cell walls. The activity is a consequence of the long aliphatic chain connected to a polar head-group, and thus bind to both polar and non-polar components. It is one of the molecules that can form micelles, and it has chaotropic activity. SDS has a very strong

inhibiting effect on PCR so concentrations must be kept relatively low for direct PCR or removed in a subsequent clean-up step. The effect of SDS can be negated by adding a 5% Tween20 solution and is perhaps the easiest way of supressing inhibition in direct PCR. The addition of Potassium acetate will precipitate SDS (and any Potassium from any source will impact SDS solubility) but beware this might also remove long chain DNA bound to the proteins. In native form SDS is a waxy solid and will only dissolve in higher pH solutions. It also tends to precipitate out of solution and solubility affected by temperature. Do not keep SDS solutions in the fridge. Precipitates should be re-dissolved by heating the buffer to 60C for a while (under a hot tap will do).

Sigma extraction/dilution reagents - These are expensive but effective, especially as part of the Sigma REDExtract-N-Amp system. Keep reagents at -20C. The dilution reagent especially needs to be kept cold long term. The extraction solution is an alkali. Add 50ul to fragment, vortex briefly, heat to 95C 10 mins, add equal amount of dilution reagent and vortex to mix. No grinding required, and probably detrimental. The Sigma protocol says 1uL of this as template and with 1.5ul each of extraction & dilution buffer added to PCR Readymix but there are differences between the recommended protocol (for plants) and our use for fungi. Note this solution is intended for use with the REDExtract-N-Amp ReadyMix, which uses a hot start Taq. If dilution is required, then again it is done with an equal mixture of extraction/dilution fluids. The alkali plus 95C is sufficient to open-up the cells walls to allow DNA out into solution but keeps the cellular components intact so they can't inhibit PCR, and any residual components get bound by BSA. The dilution reagent is said to contain BSA.

Silica powder - Silica is frequently use as part of commercial DNA clean up kits, usually as part of a spin column. It can be used with a chaotrope in simple DNA clean up protocols (see Cold Harbour Spring barcoding manual, mentioned under Guanidine hydrochloride extraction). Note that commercial silica spin columns (e.g., Qiagen) use two washes once the DNA has been bound to the silica (at low pH). The first wash will often include a low concentration of chaotropic salts to remove residual proteins and pigments. This is always followed with an ethanol wash to remove the salts. This ethanol absolutely must be removed (by very high-speed centrifugation or evaporation) or the DNA will be eluted from the silica by subsequent Tris buffer.

Sodium chloride NaCl (MW 58) – salt is ionic in solution and protects DNA by providing a positive shell of ions around the negative phosphate backbone. This neutralises the hydrophilic nature of DNA and reduces its interaction with other chemicals in solution. Salt is often use in procedures to precipitate DNA but on its own salt cannot do that. Despite the protective layer of positive ions around DNA in solution the strength of bonding to water molecules remains due to hydrogen bonding. The effect of those water molecules is drastically reduced by adding an alcohol which effectively pushes the water away from the DNA (It is acting as a chaotrope). The DNA, no longer bound at all to water, will precipitate from the solution. See also Ammonium acetate. Salt is detrimental to subsequent PCR, for all the same reasons, and a concentration higher than 20mM in the PCR mix it will stall PCR. Try to use AnalaR NaCl, not table salt! In any process using salt there must be a subsequent wash step to remove it.

Sodium hydroxide NaOH (MW 40) - As a basic *proton stealer* NaOH disrupts hydrogen bonding (see also chaotrope). It destabilises polysaccharide cells walls, and unzips double-stranded DNA. In a lysing solution this can be used at high molarity (0.5-1M, sometimes with 2% SDS), then heated at 95C for 10 mins, and then needs a good quantity of dilution buffer. Or a gentler but less effective molarity of NaOH can be used (25mM + 0.2mM EDTA) and then relying on a high molarity Tris buffer (100mM) to stabilise the pH. A suitable neutralising solution is 40mM -100 Tris-HCl (pH 5-8). Note that an unbuffered solution of NaOH at pH 9 corresponds to 0.01mM, most alkali lysis solutions are

way higher than pH9. Note you should not be tempted to substitute KOH for NaOH in lysis because it may react and precipitate any SDS present.

Taq/Hot-start Taq – All organisms contain enzymes called polymerase that synthesise a complementary DNA strand to an existing single strand by incrementally adding complementary free nucleotides. Taq is a version of this enzyme that is stable at relatively high temperatures and was isolated from a bacterium adapted to hot volcanic springs (*Thermus aquaticus*). The temperature stability means that it survives temperatures that denature free DNA into single strands (but is not active), and then when the temperature is reduced the enzyme gets to work on the single strands and builds complementary strands starting with an annealed primer fragment. It is this ability to survive DNA denaturing whilst inactive and re-activation when cooled that forms the basis of the PCR reaction and the successive doubling of DNA strands in a heating-cooling cycle. However, Taq is not indestructible, and PCR relies on a balance between the melting temperature necessary to split the double-stranded DNA and the survival of Taq at this temperature. Standard Taq's optimum temperature for activity is 75–80 °C and in a PCR reaction it can generally extend by adding around 1000 bases per minute. It has a half-life of greater than 2 hours at 92.5 °C, 40 minutes at 95 °C and 9 minutes at 97.5 °C. There are modified versions of Taq with greater thermal stability. The melting temperature of double-stranded DNA depends on GC content but PCR protocols usually recommend 94C. Your thermocycler needs to do what it says on the box, and it is worth checking the calibration regularly with a thermocouple/data logger. Any temperature overshoot will likely kill your PCR reaction. At 94C any overshoot needs to be < 2C after 30S. This balance between melting temperature and Taq degrading is critical. 35 cycles of 30S at 94C amounts to 17minutes and that means your Taq will be seriously reduced/gone after a few cycles if the temperature is just a bit higher than it says. A PCR cycle typically consists of a period (30S) at this high temperature of around 94C to denature the template double stranded DNA. The subsequent annealing and extension temperatures are less critically sensitive.

After denaturing the temperature is reduced to around 50C, which is too cold for Taq to operate efficiently, but it does allow the forward and reverse primers to stick/anneal to their matching positions on the single strands. During the annealing phase then decreasing the temperature to around 50C means the rate of nucleotide addition by Taq is only about 1.5 nucleotides per second. At this temperature complementary strands can anneal together to form double stranded DNA.

The temperature is then raised to around 70C. Taq is at its optimal temperature and adds free nucleotides starting at the end of a primer and continues to build a complementary strand until it terminates at the end of that fragment. The cycle starts again with the temperature raised to 94C to denature the newly formed double strands. During each cycle the number of strands doubles and their length quickly becomes equal to the length between the forward and reverse primer and these long fragments dominate. After 35 cycles theoretically there will be 2 to the power 35 replicas which is the staggeringly large multiplier of 3.4×10^{10}.

To get some ideas of numbers let us consider some average values. The yeast genome is about 10^8 Daltons. One Dalton equals the mass of a single hydrogen atom = 1.67×10^{-24} grams. Therefore, a single yeast genome weighs 1.67×10^{-16} grams. 1 ng of yeast DNA will therefore contain 6 million double-strands of DNA. The average mass of a 700 bp ITS single strand is 3.8×10^{-19} grams (1 single-stranded DNA unit = 325 Daltons). 35 cycles of PCR would theoretically generate 13 ng of ITS strands. If we started with 6 million gDNA molecules, then we would expect to generate 22 mg of ITS strands. PCR tubes will contain around 20uL of PCR mixture, which if it were all water would weigh 200mg. I.e about 10% of the mixture would end up as amplified ITS DNA. These are all rough figures, but it gives you a feel for the numbers of DNA molecules and why around 35 PCR cycles are required

to generate a decent amount of product from a sensible (although very small) amount of starting material. For ITS the effective amplification is greater because it exists in multiple copies throughout the genome. ITS copy number per genome for dikaryotic basidiomycete fungi averages about 100 (and about 50 for ascomycetes) but it can be very much more, or less, and there can be different variants present in the same genome.

One of the problems with standard PCR is that at room temperature, whilst you are preparing the PCR reaction mixture, the Taq polymerase is not totally inactive. The small amount of activity can lead to problems in amplifying a spurious primer-dimer rather than your ITS region. The additional presence of other residues from the DNA extraction process (if you are doing direct-PCR) can also cause problems. For that reason, it is often recommended the assembly of the components should be done at ice-cold temperatures, and that certainly once the primers are added the mixture should not be kept standing at room temperature for too long. To some extent these problems are reduced in recent Taq formulations (e.g. FIREPol), but the issue can be substantially resolved by using so-called hot-start Taq. This version of the enzyme contains antibodies that render the Taq totally inert until it is heated to a high temperature for some time. If you are intending to do direct PCR then I recommend you use hot start Taq. The thermocycler programming will need an initial step at high temperature to activate the hot-start Taq.

TBE/TAE buffer - These buffers are generally used for making the agarose gels and to cover the gel in the electrophoresis unit. 50x TAE buffer is 2M Tris base, 1 M Acetic acid, 50mM EDTA, pH8 and 10x TBE buffer is 1 M Tris base, 1 M Boric acid, 20mM EDTA. TBE buffer has better buffering capacity than TAE, so if you are doing extended long runs or repeated runs in the same buffer, you should use TBE buffer. TAE buffer has better conductivity than TBE, so DNA fragments will migrate faster in TAE buffer than TBE. TBE buffer supports better agarose cross-linkage, so you'll get better resolution of large DNA fragments in TBE buffer and better resolution of smaller DNA fragments in TAE buffer. The borate in TBE buffer inhibits many common enzymes used in molecular biology, so if you want to extract DNA for further PCR you should use TAE buffer. And finally, TBE buffer costs more to make than TAE buffer.

Template – The term used for the solution containing your gDNA which acts as a template for PCR amplification. 3-30ng of gDNA is needed for a PCR. But without some means of estimating DNA quantity (UV fluorescence instrument – e.g., Nanodrop) it isn't possible to say how much you may have. Generally, start with 1-3uL of template of your lysate or re-suspended, cleaned DNA. If the resulting band is faint, then the number of cycles can be increased but then non-target products can be preferentially amplified, or you can try a re-amplification of the PCR product (with 30 cycles). Too much template can lead to primer-dimer formation.

Transilluminator – The name given to the UV/blue light source used to illuminate the gel with DNA stain. I am using a 120W UV unit capable of stimulating the 320nm absorption band of typical safe gel stains, as well as those used in blue-light fluorescence. This is about the same output as a sunbed and it should be treated with the same degree of caution. It won't make you blind, burn or give you skin cancer if you use it normally (but may give you a Vitamin D boost). The risk from exposure to transilluminator radiation is not zero but it is overstated. Molecular biologists seem to have a somewhat over-cautious approach to things like UV and Ethidium bromide, but that is no bad thing.

Tris (Tris base MW 121; Tris-HCL MW 158) – The disruption of the cell contents can shift pH and a buffer is needed for that reason. Tris acts as a strong buffer between pH 7 and 9 and stocks should be adjusted to pH8 with HCl/NaOH. It can be purchased as either Tris-base or Tris-HCl. Tris-HCl, the former requiring less titration – and note any titration process will automatically create ionic NaCl in

the solution so the total molarity of NaCl may be higher than recipes suggest (and it causes problems for PCR). Tris, along with EDTA, is almost ubiquitous in molecular biology.

Tween-20 (polysorbate) – a non-ionic surfactant. Used for membrane dissolution in a lysis buffer (0.2%) and post-precipitation washing (0.1%), without binding. As a wash it has the useful effect of counteracting the action of SDS as a PCR inhibitor and is sometimes added to ReadyMix. In a lysis mix it won't be as effective in denaturing proteins as SDS but won't poison PCR if direct PCR is used.

Vortexer - For vibrating/mixing tube contents. I bought a relatively cheap vortexer from eBay intended for mixing nail varnish! It works well.