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MO BIO Laboratories, Inc.

20TH ANNIVERSARY

Tips and Tricks

for isolation of DNA & RNA from challenging samples

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SOIL

DIFFICULT SAMPLES

DNA & RNA Isolation from

MOLECULAR BIOLOGY OF SOIL: AN INTRODUCTION

One of the most difficult sample types we work with in our labs is soil. When developing products and methods for isolation of microbial DNA and RNA from soil, we have to take into account the wide diversity of soils in regards to their organic content, texture, pH, and where the soil was collected. These factors and more impact the microbial load and therefore, the yields of DNA and RNA that can be obtained.

Humic substances:

A major factor that impacts DNA and RNA isolation from soil is the level of humic substances (humic acids, fulvic acids, and humins). Humics are formed by the degradation of organic matter (plant and microbial material) and results in the dark color of soil (due in part to the quinone structure of the molecule) (1). They are large stable macromolecules that can vary in size and have both phenolic and carboxylic groups. Humics will chelate multivalent cations, making them readily available for microbes and plants that need them (2). A great overview of humic substances can be found here.

Because humic and fulvic acids are large water-soluble anionic polymers like DNA and RNA, they will co-purify in nucleic acid extraction protocols (3). For this reason, the humic substances are best removed before the final purification.

Inhibitor Removal Technology (IRT):

Inhibitor removal technology is the MO BIO patented method for removal of humic substances as well as polyphenolics and polysaccharides from samples. The system works by using changes in pH to solubilize and release charged molecules followed by removal of protein and then dropping the pH to precipitate the insoluble large macromolecules. The nucleic acids do not precipitate and are cleared of inhibitors. This also works for molecules like heme in blood and fecal samples, melanin in skin, and dyes from clothing from forensic samples such as blood stained clothing.

Lysis of microorganisms:

Removal of inhibitors is a major issue but the second most important issue is achieving strong lysis of the microbial species in the soil so that there is an accurate representation of the microbial community. Mechanical lysis provides the fastest and most efficient method for lysing bacteria and fungus in soil. At MO BIO Labs, we prefer to use the Vortex Genie 2 because it offers many advantages over the high-powered bead beating instruments.

Doing metagenomics?

A major advantage of using the vortex is that is provides higher quality (molecular weight) DNA because it uses less force over longer time (10 minutes) to pulverize cells. The lower force means that the sample doesn't heat up excessively during lysis and reduces damage due to over-heating of DNA and RNA. The combination of the MO BIO lysis solutions for soil with the vortex

MOLECULAR BIOLOGY OF SOIL: DNA ISOLATION PART I

MO BIO Laboratories has several products for DNA isolation from soil. For the purposes of this article, we will focus on the PowerSoil DNA Isolation Kit because it is our most popular product for this purpose. It uses our patented Inhibitor Removal Technology® (IRT) for the removal of humic substances and polysaccharides and is performed using a mini spin filter and a microcentrifuge.

Important notes before starting…

Something to keep in mind is that all soils vary in microbial load and organic content so DNA yield among different soils can vary. Yield is not based on the amount of material processed alone. Even soils collected from the same core but at different depths within the ground will have variable load and organic makeup.

For better consistency…

Consistency in yields between preps is difficult to achieve with soil because each scoop can contain different amounts of organic material, such as plant leaves or debris, insects, pebbles or sand. At MO BIO Laboratories, we sieve the soil for the best consistency so that the texture is uniform and the large particles are removed. If uniform yield among your preps is important to you, sieve first.

More is not always better….

It is important to note, processing more soil does not always yield more DNA. This is because the lysis buffer will be absorbed by the bead solution making sample homogenization inefficient. Scale up of soil is possible but is soil-type dependant. The

PowerSoil DNA Kit is meant for small scale preps. If you need to process more soil than 0.25 grams, MO BIO offers alternative kits such as the PowerMax® Soil DNA Kit for 10 grams of sample and the RNA PowerSoil® Kit with DNA Elution Accessory Kit for starting with 2 grams of soil.

Now let's go over the DNA Isolation protocol step by step. We will talk first about lysis and specifically about the mechanical aspects of lysis.

Step One: Lysis

High yields of high quality intact DNA requires a strong lysis. The lysis needs to be strong enough to break open microbes and fungus without severely shearing the DNA. There has to be a balance between the types of beads used and the amount of time used for mechanical homogenization. Temperature can also be used to boost lysis of tough organsisms or spores in combination with bead beating.

Bead types:

There are many bead choices for the lysis of microbes in soil. The type of bead, shape, and size will all impact the DNA yield and integrity.

MO BIO prefers to use a garnet rock type of bead that varies in size and has sharp edges. Because of the size variation, the rocks can help break down both large clumps of soil and grind microorganisms that shake loose. Garnet is soft so will break down into smaller pieces when used in a high powered bead beating instrument. This works fine as many of our customers use them in the FastPrep or Precellys when they want to increase the lysis power for isolation of DNA from fungus (references below).

Other beads may be used, such as 0.5 mm glass or 0.1 mm glass if bead beating in a high powered instrument for longer periods of time is desired. These beads will cause more DNA damage but for very tough organisms, such as spores, it can be helpful. You can even mix the glass and garnet together if you need a combination of large and small

The homogenization equipment:

As described in the previous article, the vortex homogenization method allows for the best integrity DNA and is also the least expensive method. The time for vortex is ten minutes and this gives optimal results for lysis of bacterial cells in 0.25 grams of soil in our lysis buffer. Longer vortex times do not seem to increase the yield and will cause more DNA shearing.

The use of a Precellys or FastPrep is an option if you have one and want stronger lysis. Most customers use these for only 45

seconds to 1 minute for isolation of bacterial and fungal DNA at a setting of 5 on the FastPrep. A setting of 5 m/s on the FastPrep is equal to about 5200 rpm on the Precellys. Some customers prefer using a FastPrep setting of 4 (Precellys setting of 5000 rpm) for 15-30 second intervals and 3 or 4 pulses per sample. As you can see, using a high powered bead beater will require some evaluation on your part to determine the best setting for your sample. A list of references where the FastPrep was used in combination with the MO BIO UltraClean Soil or PowerSoil Kits is at the end of this article.

The lysis buffer:

The other key ingredient in a strong lysis is the solution used to pop the cells. This buffer needs to fulfill several functions when it comes to soil. First it needs to disrupt cell membranes in combination with the mechanical homogenization. Second, it needs to be gentle enough to not denature the DNA, and third, it needs to work regardless of the pH of the soil. Soils that are acidic need to be neutralized for optimal DNA yields since the acidic conditions are harmful to the DNA. The lysis buffer in combination with the Solution C1 or S1 (in PowerSoil and UltraClean Soil kits, respectively) provides the optimal conditions for microorganism lysis from any soil type.

What about heating?

For those cases where a stronger lysis is desired, besides trying a high powered beating method and glass beads, it can be helpful to heat the sample before beating. An incubation of the soil in the lysis buffer at 65°C-70°C for 10-15 minutes will help to weaken the cell walls before homogenization. This treatment has been effective for spores and fungus.

Another method is to perform freeze/thaw cycles (3) with the soil, alternating between -20°C or -80°C and 37°C. This can enhance cell breakage as well, although might be less convenient than simply heating as described above.

Summary:

To summarize, the lysis step is the area where the most optimization is possible and depending on what you want to do with your DNA, you can go as easy or hard as you need. It is the combination of the beads + the equipment + the buffer that works together to provide you optimal yields and integrity of DNA. Really, this applies to any sample you are lysing whether it is animal tissues for RNA, bacterial cultures for DNA, or biofilm for RNA or DNA.

Fortunately, MO BIO labs R&D scientists are spending a lot of time working out these optimal conditions for a host of environmental samples, saving you time for your experiments.

But we can't work with every sample type so we would love to hear from you and how you optimized the lysis of your sample type for the best result. Let us know what you do; bead type, time and homogenization method, and which MO BIO Kit you use to get optimal yields of DNA or RNA.

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Removal of Inhibitors:

We are now ready to remove the PCR inhibiting substances from the soil homogenate. The humic acids are what give the sample the brown color. Present will also be polysaccharide if your soil sample had plant material or even some biolfilm content. Removal of inhibitors is what makes MO BIO and the PowerSoil kits stand out. MO BIO Labs developed a patented method, called inhibitor removal technology (IRT) to precipitate out the humics, phenolics, and polysaccharides from lysates. IRT involves a two-step process where-by the proteins and debris are removed first followed by flocculation of large insoluble macromolecules. After using inhibitor removal solution (IRS), samples typically look clear. The PowerSoil and PowerWater protocols are all optimized for the amount of IRS needed to clear even the most problematic samples, however, more can be used if inhibitors are still present (as determined by PCR). Repeat flocculations with IRS are ok to do as necessary.

The IRT steps are performed using cold temperatures to enhance the flocculation but for the second step (IRS), we recommend not to extend the time significantly over five minutes. Lower DNA yields may result from prolonged incubation in IRS.

Stopping points?

If you need a stopping point in the PowerSoil protocol, the best place to pause is after the IRS step and before

adding the binding solution. The lysate can be frozen at -20°C and used for binding to silica spin filters the next day.

Binding to Silica Filter Membranes:

At this point, the DNA is ready for purification on a silica membrane. The lysate should look clear (can be slightly yellowish if the soil was heavy in organics). For the DNA to be captured on silica membranes, it requires the presence of a high level of chaotropic salts. The ratio of the binding solution (Solution C4) to the lysate is critical for good yields. If too much is used, recovery of degraded RNA will result. If too little is used, a portion of the high molecular weight genomic DNA is lost. For this reason, we instruct you to take up to 750 µl of your lysate into this step so that the entire lysate will fit in the 2 ml collection tube once the 1.2 ml of binding salts are added.

If you need to take more than 750 µl, you will need to increase the binding solution as well. A good ratio is two volumes of binding solution C4 per sample volume. You will need to split the sample into two 2 ml collection tubes or a larger tube (5 ml or 15 ml) to make sure everything is well mixed.

Vacuum Manifold Option:

Normally, if you followed the standard protocol, binding to the spin filter requires three loadings of the column. One way to speed this process up is to try the PowerVac Manifold System. It is very fast and easy and results in less handling. If you have a vacuum manifold already, then all you need are the PowerVac Mini Spin Filter Adapters. In our lab, we regularly use this method to speed up processing. If you decided to use more of the lysate than recommended and increased the amount of binding salts, using the vacuum manifold will be the best way to reduce the time required for loading the column 4 or 5 times.

Washing the DNA:

Because of IRT, most of the soil related contaminants are removed so the column will not need a heavy salt wash like with other kits. The washing step here is needed to remove the chaotropic salts from the column. If any salt is left behind on the column membrane, the DNA will not elute efficiently and the DNA that does elute will be contaminated with guanidine. To remove salts from the column, the wash buffer contains ethanol which solubilizes and rinses away salt. One wash typically does the trick. However, if you are having problems with low 260/230 readings (as observed by high 230 absorbance on a Nanodrop), then a second wash may be performed. If you run out of wash buffer, 100% ethanol can also be used to wash the membrane as well. We use 100% ethanol on the vacuum manifold protocol and this can be used manually in the event you run out of wash solution and need more.

Don't forget to spin dry the column before elution so the DNA can be eluted efficiently. Left over ethanol on the column will make the DNA release from the membrane inefficient.

Elution:

The final step is releasing your DNA from the membrane into a 10 mM Tris pH 8.0 buffer. DNA dissolves faster in a neutral to slightly basic pH. You may use water to elute but because water tends to have a low pH (usually around 4-5), the efficiency could be reduced. One hint for an increased yield during elution is to allow the buffer to incubate on the membrane a few minutes at room temperature before centrifugation. Incubation from 1-5 minutes will help resolubilize the DNA in a smaller volume. Don't elute in less than 50 µl or you will leave too much DNA behind.

Your DNA is now ready to use in PCR or for gel electrophoresis!

FAQs:

How much DNA is typically in soil?

After all of this discussion, you may be wondering how much DNA can I expect from soil? The answer is that it varies. The moisture content, organic content, and where collected will all play a role.

In our labs using "normal" soils or temperate soils, such as garden soil, the yields can range from 2-5 ug of DNA per 0.25 gram (a prep). We have worked with some agricultural soils, such as soil from the Strawberry Fields in Carlsbad, and these yields are far lower- around 0.25 ug per 0.25 gram of soil. Sandy and clay soils tend to have lower yields and very low organic content.

What can I do to increase yields in clay and sandy soils?

One current theory with sandy soils and clay soils is that the released nucleic acids are tightly binding to the soil itself. There are several references looking at ways to pre-block soils to prevent loss of the microbial DNA, including the use of skim milk (1). Some evidence suggests that divalent cations are playing a role in DNA binding to the surface of soils (2). For this reason, some of our customers have found success by adding EDTA into the bead tube during the homogenization step at a final concentration of 50 mM.

SUMMARY:

To summarize, soils vary widely in their characteristics and microbial load so expect the yields to vary when extracting different samples. Two key steps for obtaining high yields and integroty of DNA are the homogenization step and the binding step. If your yields are lower than expected, optimization is usually done at these steps. And remember, using more soil will not result in more DNA.

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10 Tips
for the Isolation of High Quality RNA from Soil

Working with DNA is far less stressful compared to working with RNA. Yields are always higher and there is no worry about degradation.

RNA however…

RNA Isolation from soil is one of the most difficult applications we perform in environmental molecular biology. RNA purification is always an arduous task and from soil it becomes a bigger challenge. One of the biggest problems is the yield of RNA from soil. Because typical yields of RNA are so much lower than for DNA, usually between 10-20% of the yield of DNA, starting with a larger amount of sample is desired. This requires a method that uses larger tubes (15 ml) and a bigger centrifuge.

The other major issue is, of course, the humic acid and inhibitor content of soil co-contaminating the RNA. Purity for RNA applications is even more important because dilution of the RNA for reverse transcription is not desired when looking for low copy genes. The RNA needs to be concentrated when added to the reaction and inhibitors cannot be present.

Isolation of RNA from soil has special requirements

For these reasons, MO BIO developed a completely different process to purify RNA from soil that does not use silica spin filters. Today we are going to talk about the RNA PowerSoil Kit and how to achieve the best possible results.

The protocol is a combination of methods. It uses IRT for inhibitor removal, phenol-chloroform extraction for complete microbial lysis, and anion-exchange for high quality purification. The end result is the isolation of very clean RNA in the volume desired allowing for maximal use in RT-PCR.

Today I would like to share with you ***some tips and tricks*** for using this method to minimize the amount of troubleshooting or optimization you need to do. Because every soil is different in texture, moisture, and microbial load, soils can behave differently during extraction. Let's go through the protocol and discuss the key steps where problems may occur and where changes can be made to improve the results. Let's get started!

1 Starting sample (step 1): For most soils, 2 grams of soil should be the maximum amount used. However, for sediments, the wet weight results in much less actual soil in the prep and reduced yields from samples that already have low microbial load. With sediment samples, I have used up to 5 grams wet weight of sample. If there is significant water sitting on top of the soil, you can centrifuge the sample briefly after adding it to the bead tube and remove the excess water.

2 Phenol: Chloroform: Isoamyl alcohol (PCI) type (step 5): It is important to use the correct PCI and we give some recommendations in the manual. The phenol should be a 25:24:1 ratio of PCI and the pH should be between 6.7 and 8 and stored under TE buffer pH 8.0. Many people want to use an acidic phenol for the prep because low pH phenol is sometimes used for RNA preps for other samples such as animal tissues and cells. For soil, we do not recommend this. Stick with the neutral pH phenol for best results.

3 Isopropanol precipitation optimization (step 12): After PCI extraction and the addition of Solution SR3, the next step is an isopropanol precipitation to isolate the total nucleic acids. If you started with sediments, you may have more than 5 ml of sample after adding SR3. Increase the amount of SR4 (isopropanol) to equal the volume of sample at step 11 to ensure a complete precipitation.

4 Isopropanol precipitation temperature (step 12): The standard protocol recommends freezing the samples at -20oC. For samples with high salinity perform the precipitation at room temperature. The freezing temperature will cause the salt to precipitate and change the binding conditions to the anion-exchange column in the purification. You will know if the sample precipitated salt by the way the pellet looks. It should be flat and glossy, like a normal RNA pellet. If it is large and crusty, you have some salt in there. Sediment samples, because of the excess water, tend to be salty, even from freshwater lakes.

a. Stopping Point: I have extended the incubation at step 12 for longer than 30 minutes and even overnight and the RNA was fine. I wouldn't recommend it for every sample and you may want to test it for your soils. In an emergency, you can delay or stop here.

5 Anion-exchange column flow issues (step 15): The columns used for the final purification of the RNA are a packed resin that flows using gravity to drip through the column. Sometimes these can move slowly because of packing down of the resin. To help increase the flow of the buffers and sample through the column, we will sometimes use positive pressure to gently motivate the buffers through the resin. If the column is still having difficulty with the flow rate, we will use the syringe and barrel from a 5 ml syringe to apply light pressure to the column to enhance the flow. To do this, hold the barrel of the syringe flush against the opening of the column. Push the syringe plunger through the syringe, holding

the barrel so that the air does not escape around the top of the column. Very gently apply the pressure. Do not exceed a flow rate of 1 drop per second.

6 Shake, shake, shake Solutions SR5 and SR6 (step 15): Give your solutions SR5 and SR6 a good shake before use to ensure the components are well mixed. Sometimes solutions containing isopropanol can separate while sitting on the shelf and are not homogenous unless mixed first. A few good shakes will do the trick.

7 Elution time-saving tip (step 19-20): I sometimes elute directly into my 2 ml collection tube instead of into the 15 ml tube to save a transfer step and some plastic. Make sure the gravity column is balanced on the collection tube in a rack in a way that it can't fall over. This tip is for the technically savvy. Don't try this if you are using the kit for the first time.

8 Final isopropanol precipitation (step 20): After elution from the gravity flow column, the final precipitation is done using the isopropanol again (Solution SR4). This is incubated at -20oC. Do perform this step at -20oC (vs. room temperature). Extended time at this step is ok.

a. Stopping point: If you can't finish the prep, this is an ok place to stop for the night. The sample is frozen at -20oC and the RNA is stable.

9 The RNA pellet (step 22): After centrifugation to collect the RNA from the isopropanol, the normal pellet will be small and glassy. Make sure to orient the tubes in the centrifuge the same way so you can quickly identify where the pellet is in all of the tubes when you decant the isopropanol. When drying the pellet, to make the process go faster, we like to place the tubes inverted onto a kemwipe placed on the air flow intake of the tissue culture hood while it's on.

10 RNA resuspension (step 23): Now that you have a nice dry pellet, resuspend the RNA in a volume based on what you need for reverse transcription. In our lab, if the soil has a high yield of microbes, we'll resuspend in 50-100 of water (usually the final concentration is ~100-200 ng/ul). For sediments and dry soils with low microbial biomass, we'll use 25 ul so the RNA is more concentrated for use. This step is flexible and you can use the amount of water to resuspend the pellet that is best for you.

Bonus Tip

You can isolate DNA from the column also since most of it stays behind after elution of the RNA in Solution SR6. To get the DNA out, we have Solution SR8 (from theDNA Elution Accessory Kit) that has a higher salt concentration and will elute the genomic DNA. And since the isolation procedure is very gentle, the DNA molecular weight is very high. An additional benefit of the anion-exchange column method is the ability to get the RNA and DNA from the same sample and eluted in two different tubes.

Double Bonus Tip

RNA STABILIZATION AND STORAGE IN SOIL:

We are often asked about the stabilization of RNA in soils upon collection and the use of RNALater for soils. RNALater is not compatible with soil. We have performed time-courses of soil

stored in RNALater at various temperatures and found that RNALater results in excessive humic acid release and co-purification with the RNA that cannot be removed with anion-exchange. The longer the storage, the darker the sample becomes.

To help those researchers that need to stabilize soils upon collection and want to ensure that the microbial profile remains constant during transport, we use LifeGuard Soil Preservation Solution. The composition of this solution results in stasis of the microbial content and isolation of intact RNA regardless of the length of time in storage or temperature. The ratio of LifeGuard to soil can vary based on the content and the microbial load (wet soils and sediments should use more and for normal soils we use 2.5 ml per gram of soil). More information including data can be found here.

Summary:

The most challenging sample extraction is RNA from soil. No other extraction procedure requires isolation of highly labile low abundance RNA in the presence of so many inhibitors and microbial RNases. But, high yields of clean RNA are possible. If you have some of your own short cuts, tips and tricks, and advice, let us know. We love to hear how researchers make changes to get the results they need.

PLANTS

Get to the Root of Plant DNA and RNA Isolation

If you've figured out how to extract high quality DNA from an elephant, chances are that without too much trouble, you'd be able to do the same from a moose, a mouse or even a meerkat. However, if you've figured out how to extract DNA from an Arabidopsis plant, well that might be about all you've figured out. That's because plants have developed something akin to chemical warfare in order to survive a variety of climactic extremes, pathogens and predators, without the luxury of being mobile. As a result, plants harbor an enormous variety of organic compounds, some with antifungal and antimicrobial properties and some which make them taste bad to herbivores. Other structures are complex networks of polymers that store water and nutrients for both feast and famine.

It's all well and good for the plants and seeds, but many of these substances muck up DNA extractions. Some, such as polyphenols, bind almost irreversibly to DNA, interfering with downstream enzymatic applications. Others, like polysaccharides, also bind to DNA and in addition can form a gelatinous mess during the extraction, making the DNA concoction akin to alien slime. Complicating matters, plants in the same family, genera or even species can contain radically different varieties and amounts of these substances, making it problematic to generalize techniques that work with one plant to work with another. It's enough to give plant molecular biologists nightmares.

No wonder we get a lot of technical phone calls from weary plant scientists, skeptical that we have anything off the shelf that can be of use to them. And while our PowerPlant Pro DNA and PowerPlant RNA Isolation Kits may not be the end all and be all for every plant out there, they are a step in the right direction towards botanical bliss. Our PowerPlant kits can help you isolate high quality DNA and RNA from a wide variety of specimens while avoiding some

of the cumbersome methods that have traditionally plagued plant extractions such as liquid nitrogen, CTAB, phenol, and chloroform treatment.

How to isolate DNA from plants and seeds

For any DNA extraction, the first step is to break open the cells so that the DNA is accessible. Plants of course are no exception. Unlike animal tissue, however, plant cell walls are tough and hearty against osmotic pressure. So to get at their DNA, you've got to get tough. Traditional methods use liquid nitrogen and a mortar and pestle to grind up the frozen tissue or the blades of a blender to slash the smithereens out of it. These work but they can be either time consuming or risk sample cross-contamination. In MO BIO's plant kits we use a method of mechanical lysis called bead beating. With this technique a small amount of sample tissue is placed inside a tube with beads and some lysis buffer, and is shaken at high velocity either on a vortex with a vortex adaptor or on a specialized high powered bead beating instrument. The beauty of this method is each sample is homogenized inside its own sterile tube. For optimal homogenization of plant and seed tissue we've found that a few steel and ceramic beads between 2-3 mm in diameter are very effective at breaking down the cells.

Removal of polyphenols and polysaccharides

Once the plant cells are broken apart then you've got to deal with the issue that your DNA is free to mix with all those complex plant molecules mentioned earlier. Polyphenols like flavonoids, anthocyanins, lignans, and tannins may be great for lowering your cancer risk, but they are nasty for DNA extractions. And because of all the positive press that polyphenols have recently received, scientists are really focusing on studying those plants with the highest levels of these compounds. In the past six months we've received calls regarding plant such as soybeans, chocolate, coffee, strawberries, orange peels, sunflower seeds, and corn, all with very high levels of polyphenols.

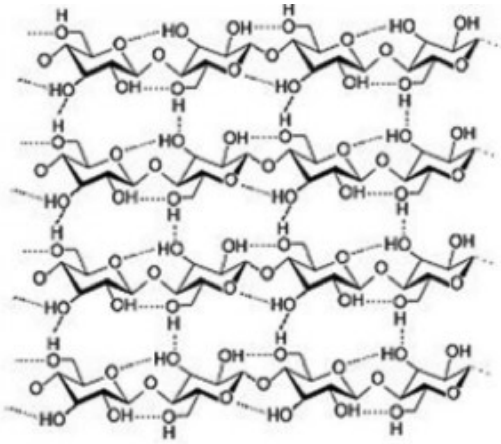
Tannic acid, one of the most common plant phenols

When plant material is macerated in order to release the DNA, polyphenols become exposed to oxygen and react with enzymes, most importantly polyphenol oxidases (PPO). These are the same enzymes that turn apples and potatoes brown. It is these polyphenol oxidation products that can covalently bind to nucleic acids, making them virtually impossible to remove. So, it's better to try to prevent the two from associating at the get-go. Common methods involve using detergents (CTAB & SDS), antioxidants (bME, Ascorbic acid, & DTT) or certain polymers (polyvinylpyrrolidone (PVP) & polyvinylpolypyrrolidone (PVPP). Detergents help by solubilizing lipids and enzymes that complex with DNA making them easier to remove. Antioxidants work by denaturing and suppressing the activity of the PPO enzymes slowing down their breakdown of polyphenols. PVPP and PVP work by binding up the polyphenols and preventing them from reacting with the DNA.

In both our PowerPlant Pro DNA and PowerPlant RNA Isolation Kits we've included a specially formulated Phenolic Separation Solution (PSS) that can be added to the bead tube before homogenization. It is very effective at keeping phenolics at bay. We have observed that the affect is variable, however. For some samples it greatly improves the nucleic acid yield and in other cases it has no effect. It's part of the variability of plants. So it's best to try a test run with and without the PSS to see how your sample type will respond.

Polysaccharides, used for food storage in plants, are the other great offenders in plant DNA extractions. Plant polysaccharides can be enormous and complex. DNA can get all bound up in them, often adding a visible viscosity to the DNA slurry. People who study the effects of polysaccharides on downstream enzymatic reactions have found it useful to categorize them as either neutral or acidic. Acidic polysaccharides inhibit the enzymes involved in PCR and restriction digests, while neutral polysaccharides don't. Some common examples of acidic polysaccharides are pectin, xylan, and carrageenan. Some neutral polysaccharides are dextran, gum locust bean, starch, and inulin.

Acidic polysaccharides can be removed from DNA during the prep under high salt conditions. The DNA can be out-competed with a cationic detergent such as cetyltrimethyl ammonium bromide (CTAB). The CTAB:polysaccharide complex can then



Cellulose is a very common plant polysaccharide

be preferentially precipitated out. A few disadvantages of the technique are that it is time consuming, expensive, and it is difficult to keep CTAB in solution while it's hanging out in the lab.

Our PowerPlant Pro DNA and PowerPlant RNA Isolation Kits avoid the use of CTAB with the use of our Inhibitor Removal Technology (IRT). They use a combination of chemistry in the lysis buffer and in the subsequent step after bead beating that is very effective at removing polysaccharides. For samples that are very high in polysaccharides, however, it might be necessary to use less starting material, since large amounts of polysaccharides might overwhelm the chemistry. When polysaccharides are combined

with alcohol they can precipitate into a gelatinous blob making it difficult to work with, for example when loading it onto the spin column.

Each plant and seed has a unique combination of inhibitors One last thing to keep in mind with plant DNA extractions is that levels of polyphenols and polysaccharides will vary in different parts of a plant and even in the same plant at different times in its development. For some plants the levels of polyphenols may be very high in the leaves but low in the roots. For others, the stem might contain a lot of stored sugars but have little in the leaves. So if one part doesn't yield good results you may need to try another. It's all par for the course in the plant world. Usually, younger plants have the least amount of offending substances so these are often easier to work with. But, of course it's not always possible to be choosy. If you want to study RNA expressed in a certain part of the plant or some embedded fungal DNA, you may not have that luxury. In that case you will need to depend on the power of chemistry to give you the best results in your nucleic acid isolation.

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Molecular Biology of BIOFILM

In many ways, biofilms are similar to soils in that they are mixed microbial communities, with varying degrees of cell densities, moisture content, chemical composition and inhibitors. Much like soils they can contain humic substances, metals, and salts, not to mention the polysaccharides, all of which can impact isolation and purification of nucleic acids. However, the basic structural components of soils and biofilms are vastly different and require different approaches for optimized recovery of DNA and RNA. Here we describe some of the things to consider when working with biofilms.

Collection

Collecting biofilms can be as easy as scraping the side of a very slimy rock or cutting a sample from a microbial mat or it can require the taking of both the biofilm and substrate it is attached too and separating the two by extensive mixing, homogenization, sonication, or chemical/enzymatic treatment. Basically, the bigger and thicker the biofilm the easier it is to take a sample. When biofilms grow thinly on substrates such as rocks, showers, or teeth, sample collection becomes more difficult. The most commonly employed methods for separating the microbial community from the

and scraping. Chemical and enzymatic treatments can be more specific and so may not work effectively on different types of extracellular compounds that may be present.

EPS

The extrapolymeric substances that bacteria secrete are important not only structurally but it can protect the bacteria from environmental stressors including application of antibiotics and cell lysis buffers. The older and more developed the biofilm the more EPS and rigidity there is. Thicker biofilms may also have more sediment, salt and mineral deposits. For the best lysis conditions, using less sample is key. It is also worthwhile to take samples from more than one location due to potential differences in microenvironment that would influence the community composition. For thinner biofilms, using more sample may be better as there may be fewer organisms within the community. Additionally, if the substrate is small enough it may be possible to lyse the microbes directly from it by adding both the substrate and associated biofilm directly to the bead beating tube.

Lysis

Ensuring complete lysis is probably one of the most difficult things when it comes to biofilms. A high powered bead beater can be used successfully, especially with microbial mats, but the more EPS that is present in the sample the more viscous the lysate will become if the bead beating time is too long. As a result, as time increases the amount of lysate that can be removed and processed declines. This will ultimately reduce overall nucleic acid yields. Excessive homogenization is not a problem when using the vortex for lysis. When combined with the PowerBiofilm™ lysis buffers which contain an EPS treatment and lysis enhancer, ten minutes of vortex lysing is sufficient and will result in yields similar to what can be obtained with high powered bead beating.

Inhibitors

Even with sufficient lysis, carryover of the degraded polysaccharide, humic substances, and other organic/inorganic compounds can occur. One method for removing the degraded polysaccharide after the lysis step is Inhibitor Removal Technology (IRT). Depending on the color and viscosity of the lysate (an indicator of the level of inhibitors) the IRT steps can be modified. For relatively clear samples or samples that are known to have less EPS (and therefore, less viscosity), less inhibitor removal can be used. For viscous samples or samples that have a lot of humic related brown color to them, more inhibitor removal can be used. Two different amounts are suggested because in the absence of inhibitors some nucleic acid removal may occur. Therefore, using the right amount of inhibitor removal will help to optimize overall yields.

SUMMARY

For the best inhibitor free nucleic acid yields, it is important to balance EPS content with cell densities. This is done by selecting the right amount of sample to process and not bead beating for too long. Selecting the right amount of inhibitor removal can also help to optimize yields.

For more information of biofilms and microbial mats visit these sites:

http://www.biofilmsonline.com/cgi-bin/biofilmsonline/index.html://www.biofilmbook.com

6 Tips
for the Isolation of High Quality DNA & RNA from BioFilm

Here are some very important tips for isolation of DNA or RNA from biofilm samples. After working with numerous different biofilms and biomats, these recommendations are based on our experience and the experiences of the scientists we worked with while developing the PowerBiofilm kit.

Here is our current list of Do's and Don'ts for working with biofilm:

1 DON'T use too much sample. When working in a mini-prep format with 2 ml bead tubes, the recommended sample size range is 0.05 to 0.2 g. While some researchers have successfully used more (0.25 – 0.3 g) this was optimized within their own laboratories. Using more than the recommended sample volume can and often will result in no yield (see also point 3). This sample range is provided not as a guideline but as a range in which the lysis chemistry is optimized. Using more than the recommended sample size will prevent optimal matrix treatment and cell lysis.

2 DO use the bead tubes provided in the kits. The PowerBiofilm Bead Tubes have been specially formulated to work with the lysis chemistry. It's not just a simple bead mixture. The tube itself is a tough tube so it can be used on the vortex and a high powered bead beater without risk of breaking. Resist the temptation to transfer the beads to a different tube. This may result in components

being left behind and incomplete removal of polysaccharide. We know that some people haven't used opaque bead tubes before, but transferring the homogenate is easy because the debris packs down after lysis. Try it and if you have any problems or concerns, just call us.

3 DON'T homogenize for too long. Using your laboratory's standard bead beating settings may not be ideal- it actually may be too much! In our experience, beating biofilm samples longer or harder does not improve yield. The longer and harder you homogenize, the finer the polysaccharides and other organic/inorganic material becomes, causing a thickening of the lysate. Much of this material is not soluble and traps nucleic acid, resulting in its loss. If you are removing less than 400 µl of lysate after bead beating using the Powerbiofilm kit, then you may have bead beat for too long. Beating for 30 seconds at a high setting is a good starting point.

4 DO elute in the proper volume. This rule applies to the silica spin filters used for purification. The optimal elution volume is 100 µl. This enables the maximum amount of nucleic acid to be released from the spin column membrane. The minimum amount is 50 µl. If applied evenly to the membrane then you can still obtain your nucleic acid with high efficiency, however, 100 µl ensures a complete recovery. Eluting in less than 50 µl will seriously impact your yield. Remember you can always concentrate your sample after elution if you need a smaller volume. If you need help or a protocol, contact us.

5 DON'T assume that all biofilms and biomats are the same. Some biofilms are more matter and less microbe so the yields may not be as high as you expect. If you don't see measureable DNA or RNA on a Nanodrop after elution, and you were careful not to use too much and not to over-homogenize, it may still be present in a very low concentration. You should give the PCR a try (see point 6). When in doubt about your biofilm sample and expected yield, contact our Technical Services (technical@mobio.com), where we can likely provide additional optimization steps. For more information on typical yields from different biofilms and biomats, click here.

6 DO evaluate your nucleic acid on a gel or by PCR. When measuring yield using UV, a number of things can influence readings. Humic substances and co-eluting RNA can inflate A260 values significantly. Additionally, sheared DNA will give higher readings than intact DNA. It's always a good idea to look at your nucleic acid on a gel to make sure that yields as measured on a spec are really accurate. Because the PowerBiofilm method uses Inhibitor Removal Technology (IRT), it is very pure and so the readings, while low, are most likely accurate compared to methods that do not sufficiently clean the DNA or RNA. But if your yields are too small to see on a gel, then try PCR. The incorporation of IRT in the protocol will enable amplification out of biofilm samples that would fail using other methods.

Summary

We hope this list of technical tips for working with biofilms is a help to all of you struggling to get molecular information from these precious samples. We know how much time and effort (and money) goes into the field trips for collecting biofilm and biomat and we want you to be successful. More technical tips will be posted over time. We welcome you to share with us some of your tips and tricks for biofilm work.

Fungus

Tips for the Isolation of Fungal DNA

No two environmental samples are ever quite alike. And when working with microbes, they exist anywhere and everywhere, so the substrate often complicates the matter. That's why choosing a method for DNA isolation can be confusing. Luckily, here at MO BIO, we've seen and heard it all and we know what to do.
Take this question, for example…..

Hi MO BIO Technical Support,
I'm considering using one of your PowerSoil kits for an experiment I'm doing. I'm not actually extracting from soil, however. I'm extracting fungal DNA from wood and paper that I've allowed to be colonized by fungi in the environment.
It sounds like combining the Powersoil kit with the Powerlyzer bead-beating would take care of fungal cell disruption and humics from the wood & paper.
However, I'm concerned about polysaccharides and polyphenolics, which may potentially be present in high concentrations in these samples. Would "inhibitor removal technology" be able to remove these inhibitors? Could you fill me in as to whether this kit would be good to use on substrates

that might contain high amounts of polysaccharides and polyphenolics? If so, are any changes to the protocol provided for soil necessary?

Thanks for your help.

PowerSoil and PowerLyzer PowerSoil DNA Isolation Kits are often used for samples involving plant roots and rhizosphere studies because of its ability to lyse microbial and fungal cells while minimizing the release of plant cell DNA, using small beads that are efficient for microbial lysis. For fungal cell lysis, we are recommending the PowerLyzer PowerSoil Kit because it has the 0.1 mm glass beads which are highly effective using high powered bead beating when you need a harder lysis.

In this question, the sample is a type of wood that is high in phenolics, and paper, which is basically cellulose pulp. These substrates can introduce additional inhibitors into the DNA prep, besides any soil and debris might be present on the sample from collection. The PowerLyzer PowerSoil Kit is definitely worth a try.

However, another option which may be more effective for this sample type is the PowerPlant Pro DNA Isolation Kit. PowerPlant Pro is a new, faster version of the PowerPlant Kit. PowerPlant Pro has IRT like the PowerSoil Kit but also has an additional inhibitor removing component, called Phenolic Separation Solution (PSS). Using this additive, we have found that yields of DNA are greatly increased (over not using it) for samples containing high levels of phenolics generated from plants, in this case, lignins found in wood and paper. Using PSS, nucleic acids are separated from the sticky phenolics, keeping them soluble during the IRT step. The phenolics are removed and the DNA is not, resulting in high yields of clean DNA.

For a sample like wood and paper, the PSS may give the additional benefits of higher yields of fungal DNA, so we recommended they try the PowerPlant Pro Kit.

Now, the PowerPlant Pro Kit has a different bead tube; 2.38 mm metal beads. This bead type is very effective for plant sample homogenization. It will have the benefit of breaking down a hard sample like wood to release microbes that may be hiding inside the bark. But it will also release more of the plant DNA. If the microbes you want are not inside the tissue, but on or close to the surface, you could use a 0.1 mm or 0.5 mm glass bead tube instead to minimize plant DNA release. The choice of bead tube will be sample dependent so it's a good idea to try a few things and see which gives the best results.

Remember, we sample everything, including bead tubes so you can try a combination of things and customize your own protocol. We highly encourage that!

Your sample is unique and sometimes mixing and matching different beads and kit chemistry will lead to the perfect result. And we're here to help!

More on Extracting Nucleic Acids from Fungi

Fungi are a funny breed of microbe. What other species can range in size from a single cell to the largest known organism on earth encompassing almost 4 square miles of soil (in the Blue Mountains of Oregon)? That's right: Fungus. Fungus are everywhere, many beneficial, some tasty, and others deadly. No matter what you think of them, there is no denying that these are the toughest of microbes.

What makes them so tough?

What makes them so resilient to lysis? Why can they resist the same forces of heat and bead beating that would send most other microbes into a soupy mix of protons and neutrons? The composition of their cell walls is the key to their longevity. Fungi employ a combination of glucans with chitin to protect their cell membrane. Chitin is the same compound that forms exoskeletons in insects and when combined with calcium carbonate, makes up the shell of crustaceans. Pretty much, biological cement.

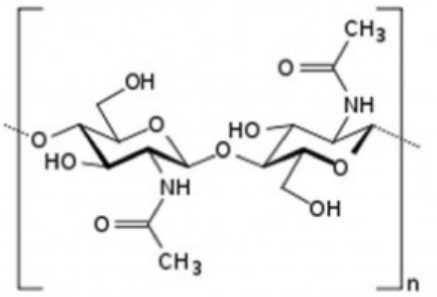
The composition of chitin is repeating units of modified nitrogen containing polysaccharides held together by covalent β-1,4 linkages. The structure allows for increased hydrogen bonding between polymers, generating chitin's bionic strength. (see figure left)

Clearly this cell wall has been instrumental for the long term survival of fungi. A quick search on the evolution of fungi brings up much interesting reading, but to summarize in one sentence, the earliest appearance of fungi in fossils appears around the Proterozoic eon, 1,430 million years ago. Even fungi prove the earth is greater than 9000 years old!

On to the purpose of this post: to tell you how to get the most DNA and RNA from fungi. There are several approaches using MO BIO Kits you can take depending on the sample matrix.

DNA and RNA Isolation from a Pure Culture

For DNA isolation from a pure culture of fungus, we recommend using the glass bead tubes that come in the PowerLyzer UltraClean Microbial DNA Isolation Kit for mechanical homogenization.



Structure of the chitin molecule, showing two of the N- acetylglucosamine units that repeat to form long chains in β-1,4 linkage.

Ideally, a high powered bead beater should be used as well. The vortex will work but we usually recommend a heating step before the vortex step to enhance breakage. Try warming the cells in the bead tube containing lysis buffers at 65-70°C for 15 minutes. If you have a high powered bead beater, such as the PowerLyzer or similar type instrument, you can skip the heating step but you'll want to optimize for the best settings to not over homogenize.

For RNA isolation from a pure culture, try the UltraClean Microbial RNA Isolation Kit. This kit uses a silica carbide bead that has sharp edges which does a good job of not only lysing cells but shearing genomic DNA.

Some of our customers are using the PowerBiofilm DNA or RNA Kit with excellent results. Because of the rich polysaccharide nature of the chitinous cell wall, the chemistry in the PowerBiofilm Kit can help dissolve the glucans and make the cell wall easier to break. And the bead tubes in the PowerBiofilm kits have a combination of bead sizes which may help break down more of the cells, especially in a culture with mycelium. And of course environmental samples containing inhibitors will be removed with the PowerBiofilm Kits.

Fungal DNA and RNA Isolation from Soil

When it comes to soil and fungal microbiomes, we would recommend using thePowerLyzer PowerSoil DNA Isolation Kit because of the glass bead tube. The glass will work better in the high powered bead beaters. Read more on the comparison of bead types and soil DNA isolation here.

For RNA from soil, the best product to use is the RNA PowerSoil Isolation Kit. This kit combines the silica carbide beads from the Microbial RNA Kit with a phenol based lysis to ensure that all microbes have no chance of staying intact. And since this product starts with 2 grams of soil, you'll get enough RNA from even low biomass samples. For tips and tricks using this kit, check here.

Water Filter Membranes

And of course, if you are working with water samples and 47 mm filter membranes, then the PowerWater DNA and RNA Kits are the best choices.

As you can see, the matrix is equally as important as the target organism.

Other Sample Types:

We could go on and on with sample types: saliva, body fluids, blood cultures, swabs, stool…. etc. The basic answer is, if you have a biological sample and need fungal RNA or DNA from it, we have a recommended method for it.

There are so many interesting and vitally important fungi in need of further study, and MO BIO Labs has the tools to do it!

Tips for Working with Water Filters for Isolation of DNA

We speak with many scientists who work with filtered water for isolating microbial DNA and RNA. Water samples can be difficult because of their typically low biomass (depending on the water source) and because these samples are often from precious and unique sources.

Why is molecular research on microbes in water difficult?

For some people, getting back to the original source of water may not be possible for months or even years. For example, we talk to scientists collecting samples at hydrothermal vents in the middle of the ocean, in the Antarctic, and in the Baltic Sea. For some researchers, water samples may have been collected after a certain event, such as a flood or heavy rain and so the conditions of the water will not be the same in a week or even after a day. They need to get answers from every sample collected and they need it to accurately reflect the current microbial content.

Choosing a Filter:

People who want to determine the microbial communities of collected water will filter them onto filter membranes. The typical size is a 47 mm membrane. This is large enough to have a good flow but small enough to work for DNA or RNA extraction. If the membrane is too small (25 mm), it may clog if the water contains higher levels of debris and if it is too big (142 mm), it will need to be sliced up in order to fit in standard 5 ml and 15 ml tubes. Ideally, the less handling and manipulations going on with the

water filter, the more microbial DNA and RNA can be recovered. To help make sure that the 47 mm filter membranes are extracted the most efficiently without needing to be sliced into small pieces, MO BIO Labs uses a 5 ml screw cap tube (see picture right). This tube allows for full access of the microbial side of the filter to be homogenized with the garnet grinding resin. We have found after thorough testing that this tube allows for maximal recovery of DNA from all types of filter membranes.

Another question we hear from customers is how to choose a type of membrane. There are many choices from polyethersulfone (PES) to mixed cellulose esther, MCE (cellulose acetate and cellulose nitrate) to polycarbonate to aluminum oxide. Each of these membrane types handle a bit differently and will give slightly different results after extraction. It is important to remember that the different characteristics of a membrane also reflect its use for other applications such as direct culturing (PES, MCE) or light and electron microscopy (polycarbonate, aluminum oxide). Overall selection of a membrane for DNA and RNA isolation is more dependent on pore size, sample volume, and retention of inhibitors such as pesticides. In other words, more than one membrane type may work for your application.

In our experience here is what we found:

- **Polyethersulfone:** Are one of the toughest membranes and can be handled more than the others. They dry quickly under vacuum making them easy to fold without tearing. Both 0.45 and 0.22 micron pore sizes can be used but a 0.22 micron pore size is best when you want to filter large volumes of water with low microbial biomass because they can handle the longer harder pressure of the vacuum. For nucleic acid extraction, we can get yields equivalent to the mixed cellulose esther with the PowerWater® DNA and RNA Isolation Kits.
- **Mixed cellulose esther (cellulose acetate and cellulose nitrate):** Are best for when a 0.45 micron pore size is needed. We recommend the use 0.45 micron pore size if your water has a lot of debris and tends to clog or filter very slowly with 0.22 micron pore sized membrane. Cellulose membranes tend to retain water making them a little more difficult to handle. The video below will demonstrate how we handle them in our lab. There are several published studies demonstrating that pesticides and herbicides can bind to cellulose acetate and cellulose nitrate so if you are using water that may contain pesticides and herbicides, avoid using cellulose membranes.
- **Polycarbonate:** This type of filter can be more difficult to work with due to its thinness and the ease at which it can wrinkle. A 0.45 micron pore size is commonly used to prevent clogging. Unlike the PES and MCE membranes, microbes in your water sample will sit on top of the membrane rather then inside. This leads to clogging faster but also retention of smaller particles that would have been able to pass through. We have found that for isolating DNA, less extreme bead beating will give you higher molecular weight DNA. If your sample is used for PCR only, then the stronger bead methods should be fine although expect a lot of shearing.

Aluminum Oxide: This type of filter is also known as an Anodisc™ filter membrane (Whatman). It handles like a thin sheet of glass and will break up easily in any bead tube. Most labs are not using these due to the difficulty in transferring them to storage tubes. These are used with samples containing very low biomass such as ocean water. They come in both 0.45 and 0.22 micron sizes. Similar to the polycarbonate, microbes are retained more on top rather than within the filter, leading to easy extraction of DNA and RNA but also increased shearing with bead beating.

Tips for Isolating Virus from Water Samples

If you want to isolate microbial DNA from environmental water samples, you need to first separate the microbes from the water. And since size exclusion filtering is one of the easiest methods to isolate microbes from large volumes of water, this is usually the preferred method. With this in mind the MO BIO PowerWater DNA and RNA Isolation kits were designed for the isolation of nucleic acids from microbes captured on water filters. These kits contain 5 ml bead tubes that are large enough so that a standard 47 mm water filter can be rolled up and easily slid into the tube. Virtually any 0.2 or 0.4 micron size exclusion membrane filter will work, with one caveat. Bacteria, fungi and protists will be captured on the membrane. However, virus won't. Extracting virus from environmental water samples is a bit trickier.

We recently received the following technical question regarding virus in water. It's a common question we get here at MO BIO technical support.

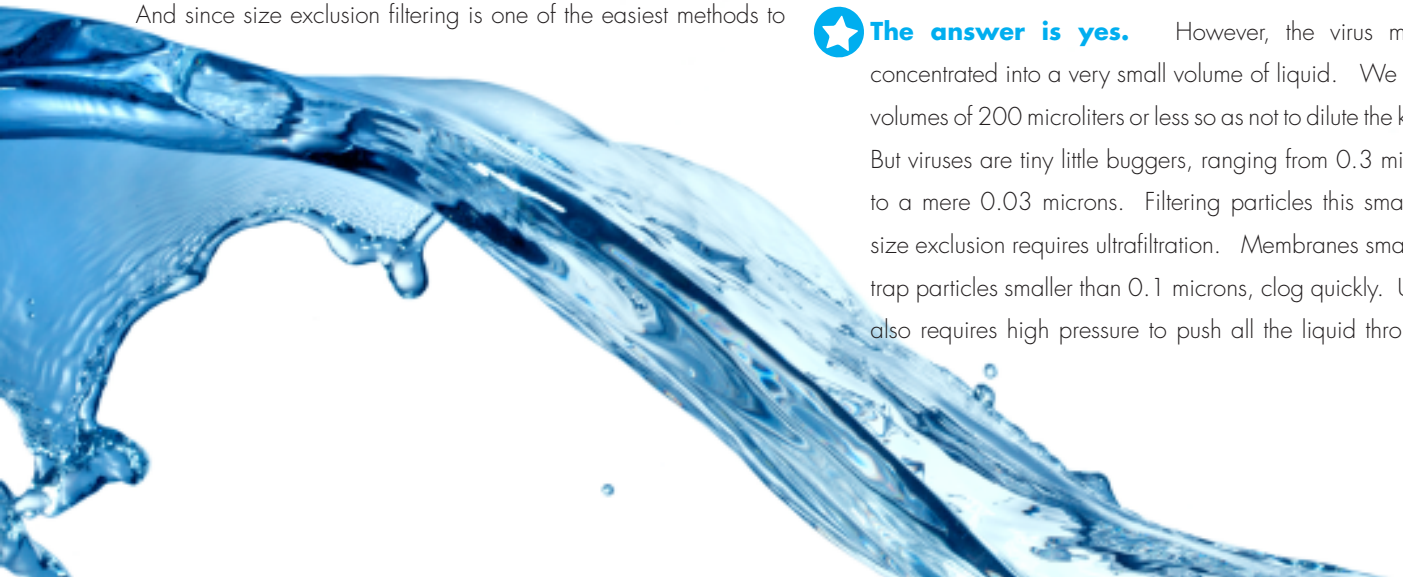
Dear Technical Support,

We are interested in using the MO BIO PowerWater DNA Isolation Kit to isolate DNA from virus in water. However, we think the virus is too small to be captured by a 0.2 micron water filter. Is it still possible to use the kit?

Thank you and best regards,

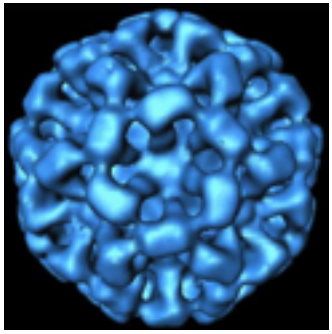
M

The answer is yes. However, the virus must first be concentrated into a very small volume of liquid. We recommend volumes of 200 microliters or less so as not to dilute the kit chemistry. But viruses are tiny little buggers, ranging from 0.3 microns down to a mere 0.03 microns. Filtering particles this small based on size exclusion requires ultrafiltration. Membranes small enough to trap particles smaller than 0.1 microns, clog quickly. Ultrafiltration also requires high pressure to push all the liquid through the tiny



little holes in the filter. All this makes the method costly and rather impractical for field work or for large volumes of water.

Centrifugation, another standard method for isolating microbes from liquid, is also trickier with virus. While bacteria are heavy enough so that they can be pelleted from water using low force (<3,000 x g) in just a matter of minutes, pelleting out virus requires forces greater than 100,000 x g for hours. (Ikner et. Al., 2012) Ultracentrifuge machines are costly and generally can only handle small volumes of liquid, less than 50 ml. So again, it's not very practical. Unless you have water samples that are already very high in virus (like sewage) it's not a very viable option.



Isolating virus from environmental samples requires an entirely different technique. Instead of depending on virus size or mass it utilizes viral charge. Most viruses are negatively charged under conditions of neutral pH or above. By passing these negatively charged viruses over both a positively charged and highly adsorptive media, the viruses can be captured. (Wommack et. al., 2009) First, bacterial and other large cells are pre-filtered out of the water with a standard 0.2 micron filter. Then the water sample is passed through a charged matrix, usually a micro-porous membrane, cartridge or glass wool. As the viruses pass over the positively charged material they are electrostatically "grabbed" and stick inside the matrix.

Some common commercial examples of these positively charged filter media are the 1MDS Virozorb filters and the NanoCeram Virus Sampler cartridges. (Luisa A. Ikner et. Al. Appl. Environ. Microbiol. 2012) Large volumes of water ~20 liters can be run through these charged matrices. The viruses can then be eluted from the matrix by adding either high salt or protein to knock off the virus. At this point the volumes of liquid are small enough to where either centrifugation or size exclusion can be used to concentrate the virus further down into the range of hundreds of microliters. Volumes in this range can be used for DNA or RNA extraction.

Considering that viruses are not only the most abundant biological entities in water but the cause of most host infections, as well, (Suttle; 2007) it's no wonder so many of our customers are looking for ways to get at their hands on their tiny genetic material. If you have any questions regarding the extraction of virus from any other sample types, whether it be blood, bananas or backwash please contact us at: technical@mobio.com

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Homogenization Tips:
Choosing a Bead Tube

When it comes to isolating DNA and RNA from all kinds of samples, the fastest and most thorough approach is high speed bead beating. Whether you have microbes, mouse tissues, plant seeds and leaves, or difficult soils, homogenization using beads will break open cell walls and membranes and release the desired DNA and RNA so it can be isolated and purified.

Here we will summarize the characteristics of all of the bead matrices we use at MO BIO Labs and what types of samples we recommend for them.

Vortex

We'll start with bead tubes typically used on the vortex. The vortex is excellent for homogenization of samples for DNA work because it is gentle and allows for higher integrity DNA.

0.15 mm Garnet: This is used for extracting DNA from microbial cultures, including pure cultures or bacteria pelleted out of water, urine, or blood sepsis cultures. This fine sand-like matrix has rough edges for excellent cell wall shearing, but is soft and does not damage DNA under vortex homogenization.

Kits used: We use this bead type in our UltraClean Microbial DNA Kit, PowerFood DNA Kit (in a 0.5 ml tube) and Bacteremia DNA Kit (in a 2.0 ml tube).

0.7 mm Garnet: This bead type looks more like different shaped rocks. They are very irregular in shape and a mixture of sizes from large to small. They are sharp and bulky and can break down any semi-solid matrix or chunky samples.

Kits used: We use this bead type for breaking down animal tissues on a vortex in the UltraClean Tissue and Cells DNA Isolation Kit and for soil DNA extraction in the UltraClean Soil and PowerSoil DNA Isolation Kits and the UltraClean Fecal DNA Isolation Kit, in a 2 ml tube. It also is provided in 15 ml tubes in the RNA PowerSoil Isolation Kit and UltraClean Water DNA Isolation Kit and in 50 ml tubes in the PowerMax Soil DNA Isolation Kit.

0.15mm/0.7 mm Garnet: A mixture of these bead types is used in the Powerwater DNA Isolation Kits and RapidWater DNA Isolation Kits in a special 5 ml bead tube. We had to change the tube so that a complete 47 mm water filter membrane could fit inside the tube without bending. We give more advice on how to choose a filter membrane and how to transfer it to the bead tube here. The combination of grinding beads allows for complete release of microbes from within and on top of the membrane surface for lysis in the bead tube.

0.25 mm Silica Carbide: This bead type has a sharp flake like consistency. These are fine slivers of silica carbide that effectively tear open microbial cells and help shear genomic DNA. We use these for RNA preparations.

Kits used: We use this bead type for RNA purification from microbes in a 0.5 ml tube in the UltraClean Microbial RNA Isolation Kit. We also use this bead in the Biostic Stabilized Blood RNA Isolation Kit to shear genomic DNA before binding RNA to a spin filter. It is extremely effective for reducing viscosity in RNA preps.

Vortex or High Powered Bead Beating

Glass bead tubes can be used on both the vortex or the high powered bead beater. However, glass beads are the preferred choice for high powered bead beating, because they do not crush under high velocity.

0.1 mm Glass: The smallest glass bead is 0.1 mm glass. These are spherical in shape and because they are extremely hard they won't break under high forces. We find these to be the best choice for certain soils, such as clay, sediment or sand, and when extracting DNA from spores or fungus. We have found this type of bead to work well on both the vortex or PowerLyzer.

Kits used: We use these beads in the PowerLyzer UltraClean Microbial DNA Isolation Kit in a 0.5 ml tube and the PowerLyzer PowerSoil DNA Isolation Kit in a 2 ml tube. More data showing their effectiveness for clay soils is here and coming soon in a new MO BIO Labs study we will publish online.

0.5 mm Glass: The next size up are the 0.5 mm glass eads which are spherical and also hard enough to be used in high powered bead beating. Although we do not employ these into any kits, they may be useful for isolation of DNA from fungus or samples where you need a slightly bigger bead to break down a tough matrix. We offer these in a 2 ml tube.

Bead Tubes for High Powered Bead Beating

The following beads are large in size and used for breaking the toughest sample types or for RNA extraction. For this reason, a vortex typically does not have the force required for a complete extraction.

2.38 mm Metal Bead Tubes: This bead tube is used for plant DNA extraction in our lab. The metal beads are tough and can even be used on a vortex for soft plant tissue, such as leaf. For most plant samples, a high powered bead beating will release more DNA. The metal works well for plant DNA because the lysis buffer does not contain high concentrations of chaotropic salts that cause discoloration of the metal beads. And these beads come in our Tough Tube which will not break under extreme forces.

Kits used: We utilize this bead type in the PowerPlant DNA Isolation Kit.

The PowerLyzer™ 24 Bench Top Bead-Based Homogenizer is a bead beating instrument uniquely designed for the most efficient and complete lysis and homogenization of nucleic acids from even the toughest biological samples.

PowerLyzer™ 24 Bench Top Bead-Based Homogenizer, (110/220V) - **Cat #13155**

1.4 mm Ceramic Bead Tubes: The small ceramic beads are useful for RNA extraction in some applications where the sample is too small to be efficienctly broken by the 2.8 mm beads. We do not use the 1.4 mm ceramic in kits because we find that the larger 2.8 mm ceramic bead does the best job for all of the plants and animal tissues we've tested. However, these ceramic beads are available for custom applications. Sometimes ceramic beads are called zirconium by other suppliers. Zirconium is a ceramic material.

2.8 mm Ceramic Bead Tubes: We use these for most applications involving the PowerLyzer. The 2.8 mm Ceramic Beads are great for isolation of RNA from all kinds of tissues and also from plant stem, leaves, and roots. The ceramic bead will not rust or change color in the presence of the strong lysis buffers used for RNA isolation. These also come pre-loaded in Tough Tubes.

Kits used: We use these in 2 ml bead tubes in the PowerLyzer UltraClean Tissue & Cells RNA Kit and the PowerLyzer UltraClean Plant RNA Kit.

All of these bead tubes are available as stand alone items in case you want to develop your own protocols and try your own combinations. We now offer most of the beads in bulk so you can save money and make your own bead tubes. Tough Tubes can be purchased separately as well.

If you have an application that does not fall into any of these descriptions and want help choosing beads for your sample, just let us know and we are happy to help. We are at technical@mobio.com.

Homogenization and Bead Tube Methods for RNA Work

One of the most efficient ways to extract nucleic acids from a sample is by smashing it against a hard surface repeatedly under high speed until cell walls and membranes crush from the pressure and release their internal contents. In other words: bead beating. Bead beating is a great way to do what enzymes take hours to accomplish and sometimes never fully succeed in, which is cell lysis to release DNA or RNA for isolation. While enzymes can be successful for DNA isolation from a limited number of sample types, results are achieved a lot faster if you break down the matrix first. And RNA cannot be isolated in a timely fashion without the use of some kind of mechanical maceration.

The questions inevitably arise though, how hard do I need to beat to lyse my sample and how do I know what bead type to use? The answers depend on a great number of variables, so to avoid beating your head against the wall to sort through them all, I have written this two-part blog series offering advice on the methods that we have used at MO BIO and found to work best for us.

This first article will focus on guidelines for RNA from tissues and plants. There is so much to discuss about soil and microbes, so we'll keep that for the second blog article, which will include data from our own research.

The Old Way to Isolate RNA- Liquid Nitrogen

RNA from tissues always requires serious pulverization. In the past, the most common method was using liquid nitrogen to freeze the sample and a mortar and pestle to grind the tissue to a powder. Although this approach works well, it is not complete. Once the sample is powdered and resuspended in a chaotropic lysis buffer, the genomic DNA is still high molecular weight and will add viscosity to the sample that can clog spin filters. To overcome this, the next step is to shear the genomic DNA with a needle and syringe which improves the efficiency of removing the genomic DNA from the column.

Drawbacks of Liquid Nitrogen Processing and Rotor Stator Homogenizers

Now, however, it's the year 2010 and liquid nitrogen/ mortar and pestles are not the preferred method. Using these outdated methods, you need to either clean tools between samples, or you need to have a lot of them on hand and ready for use. The same is true for hand held rotor-stator homogenizers. This method is excellent for breaking a tissue down quickly and thoroughly so that the RNA is isolated with minimal degradation. However, the probe also needs to be cleaned between samples and there is always risk of cross-contamination. If you can use disposable probes on your rotor-stator, it is a better way to go. The drawback, however, is that you still can only process one sample at a time.

High Velocity Bead Beating- More Samples, No Cross-Contamination

This is where the high powered bead beaters come in and supersede the abilities of one-at-a-time methods. At MO BIO, we've developed a new instrument called a PowerLyzer™ bench top homogenizer. After working with and testing many models, we custom designed a machine with features that had everything we wanted for our own use, knowing that everyone else using bead beaters would desire the same changes. Basically, it's a lot quieter and doesn't vibrate your entire bench top. And, the homogenization time needed for best results is also shortened (resulting in less heat and damage to the nucleic acids) because the horizontal positioning of the bead tube, (similar to the vortex adapter), is more efficient at grinding. Less time means better RNA integrity.

What Bead Tubes do I Use?

For RNA from plant and animal tissues, we use the 2.8 mm ceramic bead tubes for two reasons. First, the 2.38 mm bead size is perfect for a 10-20 mg piece of animal tissue or 50 mg of plant tissue. We tested the 1.4 mm beads for tissues and the small size does not give as good a result with short homogenization times. The longer time needed to liquify the sample increase degradation of RNA.

Using a single bead type (vs. a bead mix) allows for better consistency and homogeneity from prep to prep, since the larger more effective beads have complete access to the tissue and are not blocked by smaller less effective beads. The second reason for using the 2.8 mm ceramic bead tubes is because they are pre-loaded in a "Tough Tube" which is a specially made plastic bead tube that can withstand high force without breaking.

What is the Best Speed and Number of Cycles for Homogenization

This will vary based on the instrument used. For animal tissues, we tested a wide range of speeds from 3500 to 5000 RPM, and while they all worked, the optimal RNA recovery was observed using 3500 RPM for 2 x 45 seconds using a 30 second rest between cycles. This speed is about equivalent to a setting of 5.5 on the FastPrep. However, unlike the FastPrep, the PowerLyzer allows you to program any number of cycles and rest time in between cycles, so you can save the protocol and program others and keep them handy for next time.

For Plant RNA, we found the optimal setting to be 4200 RPM for 2 x 45 second cycles with a 30 second rest in between cycles. When 3500, 4200, and 5000 RPM were compared, 4200 gave the highest yield RNA from a variety of samples that included leaf, stem, roots, and seeds.

Summary

High powered bead beaters have many advantages over methods that process only one sample at a time. But for many people, this means re-optimizing current protocols. Fortunately, we've done a lot of the optimization already so you can get up and running right at the start. And RNA yields and integrity are going to be better when you can homogenize everything quickly and at once, and not have long lag times with your samples on ice while you move through all your preps.

If you are interested in ready-to-use kits for the PowerLyzer™ or other high powered bead beaters that are complete with the validated and optimal bead tube, you can find more information at these links.

For RNA from tissues go to the PowerLyzer UltraClean Tissue & Cells RNA Isolation Kit page and for RNA from plants, go to the PowerPlant RNA Isolation Kit page.

The PowerPlant DNA Isolation Kit is ready-to-use on high powered bead beaters, with tough tubes containing 2.38 mm stainless steel beads.

Determining the Best Homogenization Protocol for Any Soil

As part of our research on the best practices for soil microbial DNA extraction, we collect a wide variety of samples for product development. So when we were developing the protocols for the PowerLyzer, we wanted a protocol that worked for most of the samples tested. Our work on homogenization and bead

tubes previously showed that depending on the soil, sometimes a different bead type could give you an increased yield of DNA. We decided to do a similar study using the PowerLyzer to ask the question: what is the difference in DNA yields and integrity using high powered bead beating between two different soils using the same protocols? It is not uncommon for people to simply adopt a protocol from a paper for their soil type without doing any optimization. But, does one protocol really work best for every soil?

We wanted to compare the results of DNA yields and integrity from two different soil types; one with high clay content and one with high carbon content using high powered bead beating (the PowerLyzer) and comparing two different bead types; 0.1 mm glass beads (cat# 13118) vs. 0.7 mm garnet beads (cat# 13123). The results were very surprising.

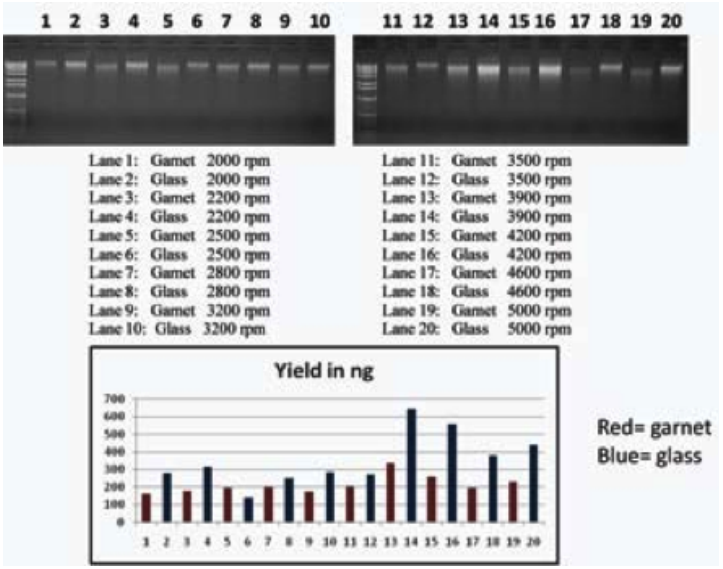
Keep reading below….

Methods:

We received a variety of soils from the California Polytechnic State University Soil Science Center. We chose two soils for this study that were similar in clay content but different in carbon content. One of the soils was 45% clay and low in carbon (2.5%) and the other was 40% clay but higher in carbon (5%). The carbon content results in a difference in microbial biomass. The high carbon content soils have the highest biomass and therefore higher yields of DNA.

For the comparison, we compared our 0.7 mm garnet bead tubes which come in the PowerSoil DNA Isolation Kit with the 0.1 mm glass bead tubes which come with the PowerLyzer PowerSoil DNA Isolation kit. We performed DNA isolations on soils starting at a speed of 2000 RPM on the PowerLyzer and increasing all the way to 5000 RPM, the highest setting on the PowerLyzer, and all preps were run for 45 seconds.

DNA was isolated using the PowerSoil Kit and the DNA quantified on a Nanodrop and run on gels to check for integrity. We plotted the yields on bar graphs with the data represented by glass beads in blue and the garnet in red.



Results:

High Clay, Low Carbon Soil

This first figure shows the results of the higher clay soil on the PowerLyzer.

We see that the glass beads in general extract more DNA from this soil and this peaks at a speed of 3900-4200 RPM (lanes 14 and 16). What is interesting is that when you use speeds higher than 4200 RPM, yields actually go down. This demonstrates that you can beat a soil too much or too hard and lose DNA. There may be an optimal speed or time for your soil that you would not

want to go past to get the best yields.

We also see that at the lower speeds, slower than 3900 RPM, the glass and garnet beads were extracting equal yields. There really was not a big difference in yields between the two. Why could this be?

One theory is that perhaps the soils have two predominat types of communities: easy to lyse and hard to lyse. It may be that the easy to lyse organisms break open with either bead type at the lower speed and then the boost at 3900 with the glass beads breaks the second subset of microorganisms. It may be that this second subset are the fungus and spores.

However, bead too much, and perhaps the DNA from the easy to lyse organisms is destroyed and this results in the low yields.

Lower Clay, High Carbon Soil

In this second panel are the results of the same experiment for a similar soil but with a high carbon content. The clay is still high (40%), but not as high as the first soil (45%) but the results are very different.

Here the yields of DNA from both garnet and glass beads start out even and then the garnet beads outperform the glass beads from speeds 3200-3500 RPM. However we also see more shearing in the gel. This sheared DNA will be detected by the Nanodrop (and picogreen) as more DNA, so it can sometimes give a false sense of higher yields. This is why we always recommend checking the DNA on a gel and not just going by a spec readings.

There are many other reasons why a spec reading for DNA may not match the actual yield. I wrote about this in our very popular article Popular Misconceptions about DNA Isolation and Quantification.

For this particular soil, the overall yields are higher and we see that the Garnet beads in this case give a very efficient extraction of DNA across a wide range of speeds that also peaks at 4200 RPM, after which yields do not continue to rise. This data also supports the idea that a maximum speed or time is reached where no further DNA will be extracted, and in fact, DNA may be lost.

Summary:

1. These data show us how diverse and individual each and every soil is. A sample of beach sand is not going to extract like forest soil and clay soils will respond differently even between each other. The consistency of the soil, the level of biomass, and the organic content are going to influence how much DNA you have and the best way to homogenize.

2. It is important to always run an agarose gel to go with spec readings of the soil. Using the PowerSoil DNA Isolation Kit, only genomic DNA is isolated, not RNA. Other methods will isolate the total nucleic acid content including RNA, which will drive up spec readings and give a false sense of a good extraction. And for checking integrity, a gel picture will let you know if you are beating too hard or not hard enough.

3. Because every soil is different, it is always a good idea to do a preliminary test of your soil under a range of speeds or time and maybe even with two different bead types. As a starting point for the PowerSoil Kit, we recommend 4000 RPM for 45 seconds, since this was in the optimal range for most of the soil types tested with both types of beads. However, you may find that you want to turn the speed down, or up, depending on whether your samples is high in spores, or is low in biomass. A few test runs at some different speeds will let you know that you are getting maximal yields of high quality DNA.

As part of this study we collaborated with the Chris Kitts lab at Cal Polytechnic State University and his undergraduate students performed t-RFLP analysis on the DNA extracted from 5 soils using

either the vortex or the PowerLyzer and for garnet beads vs. glass beads. The results are in this poster if you want to see more.

In summary, we recommend that when starting a new project, do a speed-course study with your bead beater of choice to see if the same settings you used for your last soil still apply or if you need something optmized for the new soil. Since many of these samples are irreplaceable, a little extra time at the beginning may be worth the valuable data you will obtain later.

Which Bead Beating Instruments Work with the PowerSoil DNA Isolation Kit?

A. Vortex
B. PowerLyzer
C. FastPrep
D. Precellys
E. All of the Above……

The answer is E! MO BIO Kits work with everything!

Many people ask us about the adaptability of our kits with all the various bead beaters. Everything we make is compatible with any bead beater on the market.

> ***Hello,***
>
> ***I am interested to use the PowerSoil® DNA Isolation Kit. I want to use it with a FastPrep. Is that a problem? Are your tubes adapted for this machine as well?***
>
> ***Best regards,***
>
> ***PhD student***

We have many references for the PowerSoil DNA Isolation Kit and the FastPrep. We included a short list of papers at the bottom of this article that span the last couple years. The typical protocol using the FastPrep is a 45 second pulse at a setting of between 5 and 6. Just like we recommend with any new soil, it's always a good idea to determine the best homogenization method for the soil, to see what setting gives the best yields with the least amount of sheared DNA.

In 2010 we launched a new version of the PowerSoil Kit with a glass bead tube. This was called the PowerLyzer PowerSoil DNA Isolation Kit. The original PowerSoil Kit and the PowerLyzer PowerSoil Kit are identical in their chemistry and protocol. The only difference is the bead tube. The glass bead tube in the PowerLyzer version of the kit contains 0.1 mm glass beads, ideal for lysis of microorganisms in soil using high powered bead beaters. The glass stays intact under the stronger forces and will increase the yield of DNA, depending on the soil type, as we saw in the article on homogenization mentioned above.

Both bead types work and can be used on high powered bead beaters. However, the PowerLyzer PowerSoil Kit was designed for this purpose, hence the name.

An in-depth study looking at the differences in microbial profiles

between 6 different soil types homogenized on the vortex vs. the PowerLyzer, with garnet vs. glass beads can be found here. The final conclusion was that the differences seen between the methods was more related to the soil texture and microbial load and less due to the method itself.

In summary, all of our 2 ml bead tubes supplied in the DNA and RNA kits can be used on other bead beaters. A overview of the different bead types we offer and their uses can be found in this article on Choosing a Bead Tube. And for RNA isolation, here is an article discussing Homogenization and Bead Tube Methods for RNA Work.

At MO BIO, we specialize in breaking down walls….bacterial and fungal cell walls that is!

If you're working with difficult to lyse samples and want help optimizing the best way to break them open and maximize DNA yields, give us a call!

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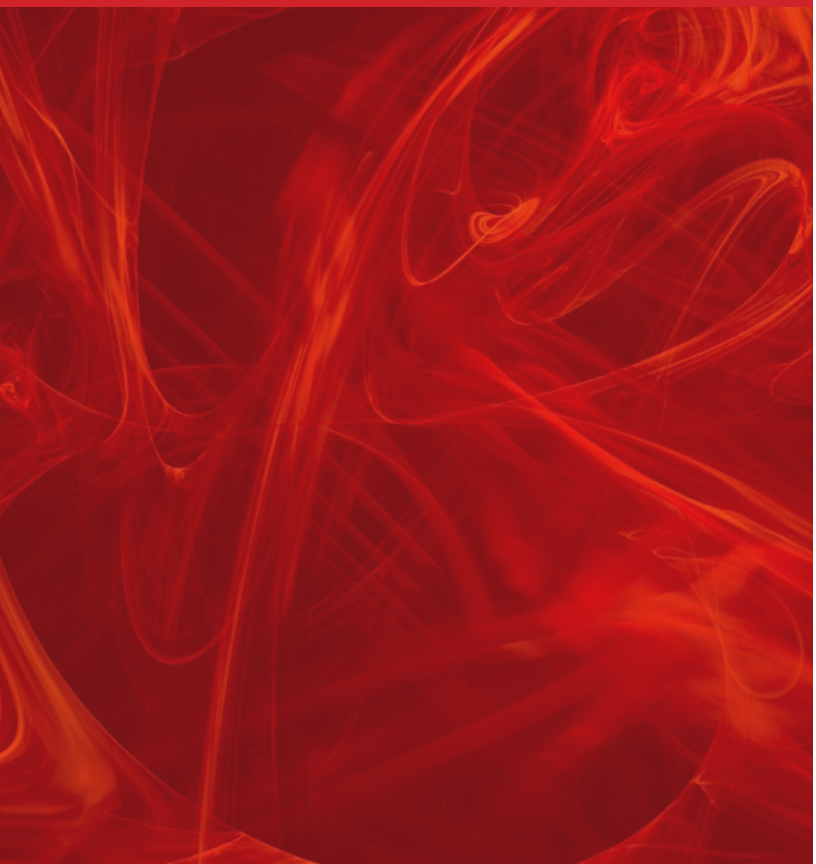
Tips for Working with Blood



Blood, human or animal, is different from anything else you'll work with. It is a complicated matrix of cells, plasma, and protein. Human erythrocytes number around 5 x 109 cells per ml of blood, but because they do not have a nucleus, they contain no DNA (avian erythrocytes are nucleated and do indeed contain DNA, hence the need to start with 10 fold less blood for DNA extractions). And hemoglobin levels average around 150 mg/ml of blood. This high level of protein is a major issue in DNA and RNA contamination and PCR inhibition. The combination of cellular debris and protein make this sample as heavenly as candy corn for blood-sucking vampires, but not so for molecular biologists.

Anticoagulants

One key point about working with blood is that the sample should always be collected in anticoagulant to prevent clotting. Isolation of DNA from clotted blood is not efficient and most of the cells will be lost in the clot. Proteinase K digest of the blood clots will not work to release the DNA. The best you can do is remove the clot and start with any remaining liquid sample left in the tube.

The best anticoagulants to use are EDTA and Citrate. These do not interfere with downstream genetic analysis. Heparin is another anticoagulant sometimes used but is not advisable. Heparin is highly negatively charged and will co-extract with the DNA inhibiting PCR (1).

DNA from Blood

Getting DNA from blood is a lot easier than getting RNA. Blood can be fresh or frozen since DNA levels are much higher than RNA and DNase is much more labile than RNase. However, repeated freeze thawing of blood should be avoided or DNA

integrity will decrease with each new thaw.

The best way to store blood if you can't extract DNA right away is at 4oC for the short term (up to 2 weeks should be ok) and -20oC or -80oC for long term storage. Use -80oC for archival storage.

The easiest method for isolating DNA from blood is a simple blood spin kit such as MO BIO's UltraClean Blood Spin DNA Kit. With this method, 200 ul of blood is digested with proteinase K and a highly denaturing buffer for only 10 minutes. The DNA is next purified over a silica membrane and typical yields are around 4-6 ug per 200 ul. If you need to start with more than 200 ul, then another fast and easy method is the non-spin kits that perform a basic lysis and isopropanol precipitation of the DNA. MO BIO offers these ready-to-go methods in a UltraClean Blood DNA Isolation Kit (300 ul blood) size, and then in kits that can process up to 1,000 ml of blood in any size volumes you wish.

Blood DNA is, of course, considered a delicacy by vampires, so these kits are perfect for preparing your next dinner party. Blood DNA can be spiced with almond essence, crushed chili or oyster extract, depending on your tastes. But do NOT use garlic.

RNA from Blood

For RNA extraction from blood, your choices are much more limited. As every vampire knows, blood must be fresh to get the best result. RNases are tough scavengers and even frozen they will have activity. A freeze/thaw cycle only makes it easier for them to have access to their prey. Plus, lysis of WBCs during the thaw will mean loss of the RNA during the red blood cell (RBC) lysis step. To get the best quality RNA from blood, starting working on it immediately. We have stored blood at 4oC for up to an hour before processing and obtained good quality RNA.

The first step in most RNA from blood extraction protocols is RBC lysis using a hypotonic lysis buffer. The result is a nice white blood cell pellet (WBC) that is easy to extract RNA from. If you can't finish the RNA prep in the same day, your best bet is to get to this step and lyse the WBC pellet in a guanidine lysis buffer containing beta-mercaptoethanol (such as the one provided in the BiOstic Blood RNA Isolation Kit- details below) and store the pellets at -20oC or -80oC. Once you are ready to extract, warm up the sample completely to dissolve the salts and then proceed with the ethanol addition step. Always add the ethanol, (or red wine for vampires), fresh.

The BiOstic Blood RNA Isolation Kit

When we developed the BiOstic Blood RNA Isolation Kit we kept in mind the steps where the most damage can occur to the RNA and reduced them. Every minute the cell pellet is not stabilized in the lysis buffer is time the RNA may be suffering from the RNA leeches. To combat this, we reduced the time and number of RBC lysis steps. This results in higher recovery of total WBCs and higher integrity RNA from the cells. We find that yields average between 0.10 µg and 0.5 µg/ 100 µl of human blood.

What about blood stabilized in PAXgene Blood RNA Tubes?

For processing blood that comes from another location, many labs are using the PAXgene Blood RNA Tube for collection and storage of samples. This reagent allows for collection of 2.5 ml of blood per tube and the entire amount is processed on a single spin column. Now that you know how rich in protein blood is, you can see what a tricky sample this would be.

Because the PAXgene collected sample contains a high level of WBCs, RBCs, and hemoglobin, specialized protocols are used for extracting RNA from this sample. For a normal person, 2.5 ml of blood contains around 60-80 ug of genomic DNA and between 4-12 ug of RNA. This is a lot of nucleic acids for a small spin column.

To extract RNA from PAXgene Blood RNA Tube samples, MO BIO Labs developed their own protocol in the BiOstic Stabilized Blood RNA Isolation Kit for homogenizing and releasing high levels of RNA from this very viscous pellet. We found that by using a unique DNA shearing column containing our silica shearing beads, we could release more RNA from the debris compared to the Qiagen version of this kit.

PAXgene Blood RNA Tubes have also proven popular amongst vampire moms, since they can hold just enough tasty blood to keep the kids satisfied when you're out and about. Why not give them a try?

Finally…

Whether you work with DNA, RNA, or just drink the stuff straight, working with blood requires care. Fortunately, there are many great commercial products available that make your life easier. Don't let a little blood scare you away from getting the results you need. Have any questions? Looking for more tricks or have a problem? Contact us at technicalcare@mobio.com.

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Formalin Fixed Paraffin Embedded Tissue DNA Isolation:

The Basics

Formalin fixed, paraffin embedded (FFPE) samples are derived from tissues (usually suspected tumor samples) that are fixed with formalin to preserve the cytoskeletal and protein structure and then embedded in a type of paraffin wax so the tissue can be sliced on a microtome, an instrument used to prepare very fine slices, 5-10 microns thick. Formalin irreversibly cross-links proteins via the amino groups, preserving the structural integrity of the cells so they can be stained with dyes used to analyze for abnormalities in the tissue that indicate cancer. However, the effect of these cross-linking fixatives on the nucleic acids is detrimental. Isolation of nucleic acids is impaired by both the paraffin wax and the cross-links that block DNA polymerases and inhibit PCR if they are not

removed.

The outlook for getting DNA from these samples sounds pretty grim. Fortunately, we've overcome both of these major obstacles and can isolate pure, high quality DNA from FFPE tissues with no problem.

How do I remove paraffin wax from FFPE samples?

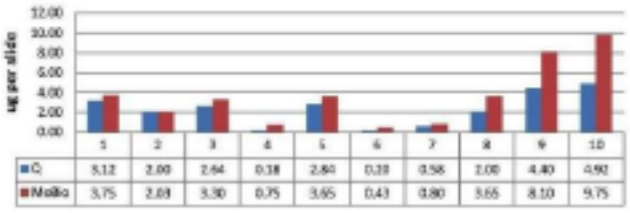
The traditional method for removing the wax has always been to use xylene, a highly flammable organic solvent. The tissue is washed several times in xylene to dissolve the wax and then the xylene is removed by performing multiple washes with ethanol before DNA isolation. This results in many extra handling steps where the tissue is repeatedly washed, and each time some of the tissue may become dispersed with the wax and removed with the solvent.

Another approach is the non-toxic BiOstic Paraffin Removal Reagent. This works very similarly to xylene but is safe and biodegradable.

If I skip wax removal, can I get higher yields of DNA?

Yes you can. It isn't necessary to remove the wax if you have a method for increasing the activity of proteinase K to allow for digestion right through the wax. This reduces handling time and loss of tissue.

To achieve this effect, we've come up with a combination of solutions that results in a strong denaturing environment and promotes proteinase K activity at elevated temperatures resulting in complete digestion of the tissue while the wax is melting. If you've used the BiOstic FFPE Tissue DNA Isolation Kit, you know these as Solutions FP1 and FP2. The synergy between these two solutions surpasses the level of activity achieved using standard proteinase K digestion buffers, resulting in higher yields of DNA as demonstrated in Figure 1 below. In this example (provided by a customer) using 10 micron thick single slices removed from histology slides, the samples in blue were pre-processed with xylene and DNA isolated following the a protocol from manufacturer Q, and the MO BIO samples (red) were extracted directly without wax removal steps and DNA was purified using the BiOstic FFPE Tissue DNA Isolation Kit. Yields were quantified on the NanoDrop. As you can see, yields can range from 0.5 ug up to 10 ug per



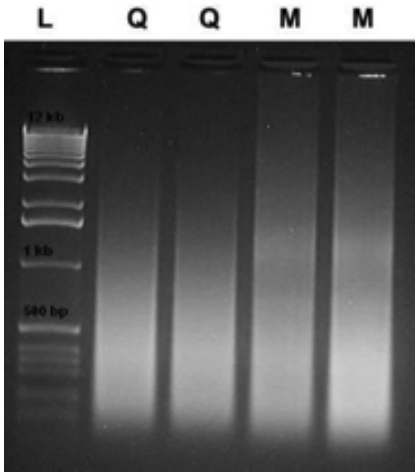
slice with an average of 1-2 ug for these samples. Not all slices are alike, but the BiOstic FFPE Tissue DNA Isolation Kit provided higher DNA yields in all cases.

Figure 1. DNA Yield of samples isolated using two methods.

Molecular weight of DNA isolated from FFPE tissue samples

The size of DNA isolated will be mostly influenced by the fixation process and how long it was fixed. Size can also be affected by the age of the tissue sample. Typical sizes are generally small, around 100-500 bp (see Figure 2 for example). Here, samples

were isolated using the kit from manufacturer Q, as described above (lanes labeled Q), or the BiOstic FFPE Tissue DNA Isolation Kit (lanes labeled M). Using the BiOstic FFPE Tissue DNA Isolation Kit, which avoids solvents and extra handling, may prevent further DNA damage and loss (note the higher molecular weight DNA in the "M" lanes, processed without xylene). You really don't know



what your sample will look like until you're done, therefore we always recommend using a qPCR assay designed for the smallest possible amplicon.

Figure 2. Agarose gel analysis of DNA isolated using two methods.

RNA Isolation from FFPE Tissue

Obviously, degradation is pretty severe for these sample types. They aren't handled in an RNase-free environment and the tissues can be many years old, sitting on slides in drawers at room temperature. But people are isolating RNA from FFPE tissues and generating informative data, so it can be done.

RNA isolation is possible using the BiOstic FFPE Tissue DNA Isolation Kit as well, with some minor protocol changes. The FP1 and FP2 combination is too harsh for RNA so we switched this out for another buffer with RNA protective qualities and a neutral pH (called BF2). Additionally, the temperature for removing the protein cross-links can't be performed at 90 degrees Celsius like it is for DNA. In the paper by Masuda et. al., Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples, Nucleic Acids Research, 1999, vol. 27, No. 22, pages 4436-4443, a temperature gradient was used to determine which temperature and for how long can the RNA be incubated to remove the protein cross-links and result in successful RT-PCR. They found that 30 minutes at 70 degrees Celsius was sufficient to do the job.

Contact MO BIO technical support by emailing technical@mobio.com to enquire about our modified protocol for RNA isolation from FFPE tissue. You can request a free sample of the BiOstic FFPE Tissue DNA Isolation Kit here.

Isolation of nucleic acids from FFPE tissues made easy!

FFPE Tissues are a unique sample type with a lot of challenges, but when it comes to DNA isolation, we've made that part easy. Samples are available and can be ordered on the web at no charge or upon request if you call technical support or customer service. We're waiting to help you out!

Getting DNA from Swabs

A frequent question to our technical support team is how to isolate DNA from buccal swabs or swabbed material. Here are our recommendations for performing an extraction of DNA from swabs based on feedback from our customers. Whether or not to use bead beating depends on whether you are trying to isolate DNA from microbes or human (or host animal) cells.

The eukaryotic cells of the host will lyse easily with guanidine containing lysis buffers and proteinase K. These do not need to be mechanically broken open. However, microbial DNA isolation does need the force of mechanical lysis to obtain the optimal yields. So for this reason, we have several recommendations for working with swabs, depending on your sample type and how dirty it may be.

For human (host) DNA isolation from swabs or easy to lyse bacteria:

The Blood Spin DNA Kit may be used:

1. With this kit, take the swab (of your choice) and brush the wall of the cheek up and down (8-10 times). Buccal swabs may be stored and shipped dry and at room temperature until ready for processing.
2. Place the swab in 200 ul of 10 mM Tris, 1 mM EDTA. If that volume does not cover your swab, increase it to 400 ul.
3. Rotate the swab in the buffer to release the cells into the solution- give it a minute.
4. Some swabs have a head that can be snapped off and left in the tube. If you have this type of swab, go ahead and break it off into the buffer. If you do not have this type of swab, remove it as described in step 7 below and continue to step 5.
5. Add an equal volume of the Solution B1 (400 ul if you used 400 ul of TE buffer) from the blood spin kit and the proteinase K (10 ul).
6. Let the sample digest for 30 minutes at 50-60C.
7. Remove the swab by gently squeezing it against the wall of the tube to remove as much of the solution as possible. Then discard.
8. Add an equal volume (400 ul) of 100% ethanol (Solution B2) and then bind the DNA to the column in two spins with 600 ul per spin.
9. Proceed with the protocol as directed.

For human or bacterial cells:

Another option for human cells and bacterial cells that uses bead beating is the UltraClean Tissue and Cells DNA Isolation Kit. With this kit, we use our large garnet beads to lyse cells in the presence of a strong lysis buffer. This protocol also employs the proteinase K digest step to increase yields.

1. With this kit, after taking the swab from the cheek cells (as described above) or environment, place it into a tube containing 1 ml of the Solution TD1 lysis buffer. If you are looking for microbial DNA, place the swab into the bead tube provided in the kit. Rotate the swab in the buffer to shake the cells into the solution. If possible, snap the head of the swab into the tube and let it incubate during digestion.
2. Add the proteinase K provided and allow the sample to digest for 30 minutes at 55-60C.
3. Remove the swab by squeezing off the excess liquid onto the side of the tube and remove as much of the liquid as you can. Discard the swab.
4. Proceed with the vortex step in the protocol if you are looking for microbial cells and if not, you may proceed with the DNA binding to the Spin Filter step and continue as directed.

For swabs collected from samples with PCR inhibitors:

Some of our customers are working with samples that contain a lot of PCR inhibitors. For example, some labs use rectal swabs from animals or babies to analyse the microbiota of the gut. Swabs taken from the environmental may contain dust that will cause PCR inhibition. So for these sample types, the BiOstic Bacteremia DNA Isolation Kit becomes the preferred choice. The Bacteremia Kit has

a 2 ml bead tube that can accommodate a swab tip and contains our 0.15 mm garnet beads for optimal lysis of bacterial cells. The protocol uses a strong lysis buffer and employs our IRT method for removal of PCR inhibitors.

1. Place the swab in the bead tube and add 450 ul of Solution CB1 (the lysis buffer in the kit).
2. Rotate the swab in the buffer to release the cells into the solution- give it a minute.
3. Snap the head off and leave in the tube if you have this type of swab otherwise, squeeze out as much liquid from the swab as you can and remove.
4. Perform the protocol as described, heating the sample at 70C for 15 minutes to help break the tougher microbes in the vortex step.
5. Proceed with the protocol as directed.

The Bacteremia Kit is best for microbial samples because of the strong lysis and bead beating, however, if you have a sample with inhibitors but want human DNA (for example, a swab taken from a person who forgot to avoid eating or drinking before taking the sample), then you can use this kit too.

Do not use the bead beating tube and instead place the swab into a standard microcentrifuge tube containing Solution CB1 lysis buffer (450 ul) and let soak. Remove the swab after a few minutes and discard. Perform the heating step to lyse the cells and 10 ul of proteinase K may be used here during the incubation. Then proceed with the rest of the protocol starting with Solution CB2 which removes the inhibiting substances.

As you can see, we have a lot of options for swabbed samples depending on where it came from and the cell type of interest. If you have any questions, or have an unusual sample and need advice, we'd love to help you with it. Just drop us an email at ***technical@mobio.com.***

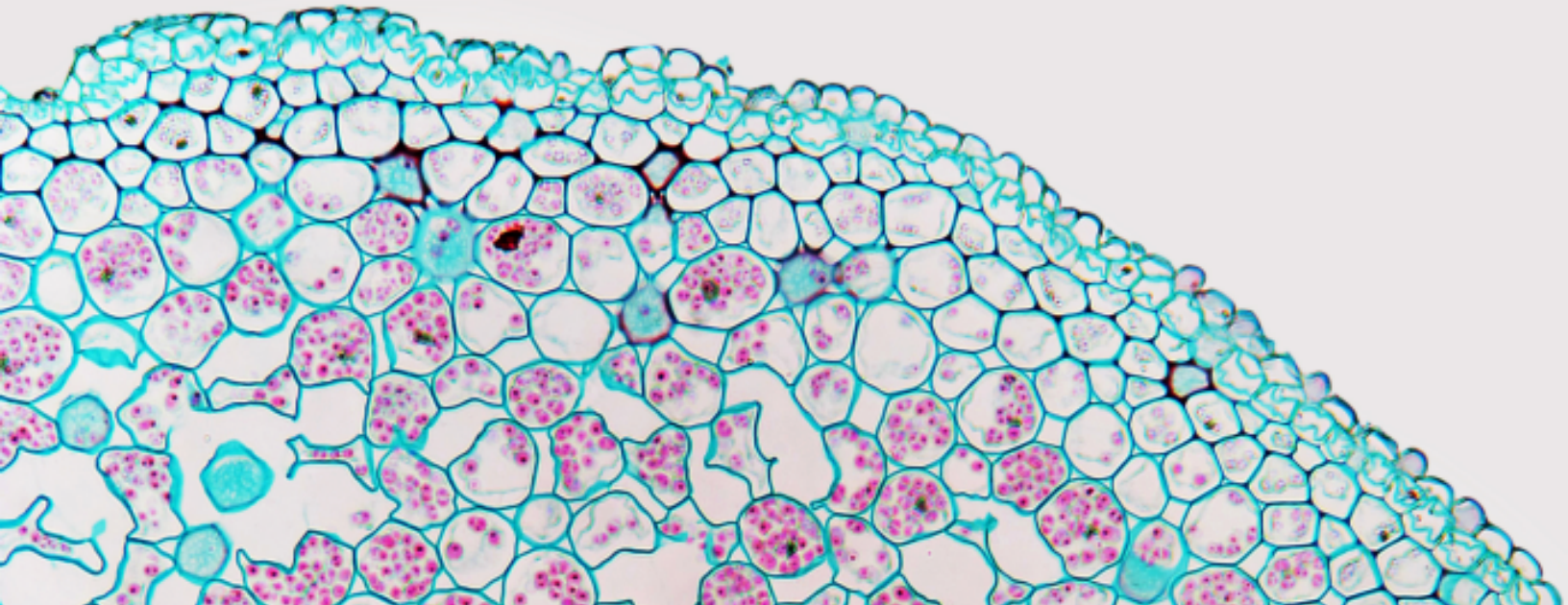
microRNA from Fresh Tissue and FFPE Samples Using Modified Protocols

We frequently get requests for isolation of microRNA (miRNA) from tissue samples. MicroRNAs are very short 22 base sequences that play a role in regulating gene expression of mRNA by binding to sequences in the 3' untranslated region of transcripts, usually resulting in silencing. A single miRNA can repress hundreds of mRNAs (1) and mistakes in expression of miRNAs are linked to disease. Because of their important role in down regulation of gene expression, a current research focus for miRNA is their use in treating cancer (2).

miRNA purification is not that complicated

The small size of miRNAs means that some adjustments need to be made to enhance binding of the short fragments to a silica spin column. Current formulations that focus on capture of messenger RNA promote the binding of larger species and removal of the tRNA and small RNAs that can make detection of low copy transcripts more difficult. Since the 5s and tRNA can account for 20% of the total RNA, removal means enrichment of the messenger RNA.

Fortunately, capturing the small RNA is easy to do. When it comes to silica technology, binding to the membrane is influenced by two factors: guanidine salts and ethanol concentration. By modifying the amount of ethanol used in the binding step, we can bind more of the small fragments of RNA that would normally wash through. Why spend more money on another kit when the change is so



simple?

The protocol

Here is our protocol for using the UltraClean Tissue and Cells RNA Isolation Kit for purification of the miRNA along with the mRNA.

1. Follow the protocol in step 1 of the protocol for homogenization and clearing of the lysate.
2. Add 1.5 volumes of 100% ethanol to the lysate.
3. Continue as directed.

Very simply, if you started with 300 ul of Solution TR1 (lysis buffer), add 450 ul of 100% ethanol. If you started with 600 ul of Solution TR1, add 900 ul of 100% ethanol.

Normally, an equal volume of 70% ethanol is added to the lysate at this step. This concentration of ethanol allows for binding of the mRNA and rRNA and removal of the small RNA (<200 bases). This is desired when you need to find a transcript. But if you are working with degraded RNA and want to capture all the small pieces or if you want the miRNA, an increase in ethanol will work.

RNA from FFPE Tissues

Talking about degraded RNA brings me to another subject, which is RNA from formalin-fixed paraffin embedded (FFPE) samples. FFPE samples will contain mostly small species of RNA. If you use a kit for this procedure now, you'll notice that you are using a high volume of ethanol for binding.

MO BIO Labs also has a protocol for isolation of RNA from FFPE samples using a modification of the UltraClean FFPE Tissue DNA Isolation Kit. This protocol requires four main changes.

- The first is a different lysis buffer for digestion of the RNA. This new lysis buffer protects the RNA during the heating steps that are needed to digest the tissue through the wax (no paraffin removal required!) and to de-crosslink the proteins from the DNA.
- The temperature used to de-crosslink the protein and RNA is adjusted to 70oC for 30 minutes (instead of 90oC for an hour like for DNA) (3).
- The ethanol is increased to 2 volumes per volume of lysate to ensure that all of RNA binds the membrane under the salt conditions used in this kit.
- And the last is the incorporation of the On-Spin Column DNase Kit to remove the genomic DNA from the membrane before elution.

The rest of the FFPE DNA Kit components are the same.

If you like our FFPE DNA Isolation Kit and want to try the RNA protocol, we'll send you the RNA digestion buffer free of charge.

Summary

With so many kits available for RNA, it becomes difficult to compare the protocols and to choose what to evaluate. The methods are really not that complicated, so why buy two different kits for isolating RNA when all you need is one? And why pay more for miRNA vs. total RNA when the chemistry is basically the same?

If you work with miRNA and want to try using the MO BIO Labs kits with our modifications above, just send us an email (technical@mobio.com) and we'll send you samples to get you started.

Tips and Tricks for Protecting Your RNA

Isolation of RNA, no matter what the source, is nerve wracking, but especially when samples are limited or irreplaceable. Because RNA is so labile, working quickly but carefully is the key. There are ways to protect your RNA during the procedure so that you can work at a relaxed pace and without so much anxiety. Here are some tips and tricks for isolation of RNA that will help you work smarter, faster and increase your overall success.

1. Chemical Protection

The protection of the RNA begins at the very first step, and this is the homogenization step. You may have noticed that many isolation protocols have you add beta mercaptoethanol (BME) to the lysis buffer. BME is a reducing agent that permanently denatures RNases. So while the smell might keep the sales people out of your lab for the duration of the protocol, you don't want to skip adding this unless you are working with a low complexity sample, such as tissue culture cells. Tissue samples can have a much higher level of nuclease activity so it is best to add the BME. If your extraction is taking place in the presence of phenol, then the BME can be skipped because the phenol will do the job of nuclease inactivation.

2. Fast Homogenization!

The very next thing you want to do is add the BME containing lysis buffer (or phenol based lysis reagent) to your sample for homogenization. We've discussed lysis and homogenization before, including a thorough overview of bead tubes and sample types. Bead beating is a great way to homogenize a lot of

samples quickly and under the exact same conditions, eliminating a source of variability between preps.

The important point to remember to protect your RNA is that you want to move through this step fast. The tissue sample should go right from the freezer to the bead tube or vessel as quickly as possible. Once the sample is removed from the freezer and begins to thaw, the countdown has begun for RNase activation.

Before I take my tissues out of the freezer, I set up my bead tubes with lysis buffer and put them in a Stratacooler or a similar chill block and place it by the scale, keeping my tubes cold. The lysis buffer does have a high concentration of guanidine thiocyanate salt which does precipitate when chilled. However, once the sample goes into the bead beater, the heat generated dissolves the salts quickly and the RNA integrity is higher when the bead tubes start out cold.

For my tissue samples, my approach is to freeze aliquots of tissue in RNALater in ~50 mg pieces. I typically use 25 mg per prep so when I thaw one tube of tissue, I know I have two preps worth of sample and I can quickly slice the tissue in half and drop them into pre-chilled bead tubes. My sample goes from the freezer to the scale and then quickly into a -20C chilled bead tube. Once I have all my samples in bead tubes, I place them in the PowerLyzer where they are homogenized for two cycles at 45 seconds. Any frozen salts quickly dissolve.

The reason fast homogenization is so important is because you want to expose every cell to the guanidine lysis buffer and the BME. If you have any cell clumps in the lysate, no matter how small, those pieces will have active RNases that can cause low RIN values and degraded looking RNA in your final gel. Complete homogenization is important.

A hand held polytron or rotor-stator homogenizer may be used for RNA extractions also, and this is especially handy for large scale preps. I can usually get the tissue broken down in about 30 seconds. But all of the other samples are sitting and waiting as one prep at a time is homogenized. Keeping the lysis buffer cold can help but it can't be frozen before homogenization.

3. Removal of DNA

The rest of the protocol is easy, and as long as the sample was high quality when you started, the RNA will be high quality when it is eluted. So what else is there that could cause degradation or loss of the RNA? That's right, the DNase step.

DNase digestion is frequently performed on the spin column and this is a great way to save some time on the post extraction processing. However, some samples have so much DNA (for example, spleen and thymus, even some soils) that the on-column DNase is just not efficient for complete removal. In this case, DNase digestion is solution is necessary.

Room Temperature Stable DNase and Removal Resin

The typical protocols for DNase involve inactivation of the enzyme using EDTA and heat. Both of these things can cause problems in RT-PCR. EDTA can inhibit the RT-PCR enzymes and heating the RNA can cause a reduction in integrity. And most DNase enzymes are stored frozen and need to be aliquoted to avoid freeze/thaw cycles that can reduce enzyme efficiency.

We came up with a better system that protects the RNA all the way to the final step. RTS DNase is a liquid room temperature stable DNase with very high activity (1 ul of enzyme can digest 30 ug of DNA in 20 minutes). The best part is the clean up step. The DNase comes with a removal resin that binds the enzyme and cations and pulls them out of the RNA sample making it ready to use in qRT-PCR without any inhibitory additives or heat steps. The resin is so efficient that even with 10 units of enzyme in the reaction, it is completely removed (figure below, lanes 3-4) compared to an

alternative resin method where only 2 units of DNase enzyme is used (lanes 1-2).

This means that you can protect your precious RNA as well as hours of work and get better accuracy in gene expression assays.

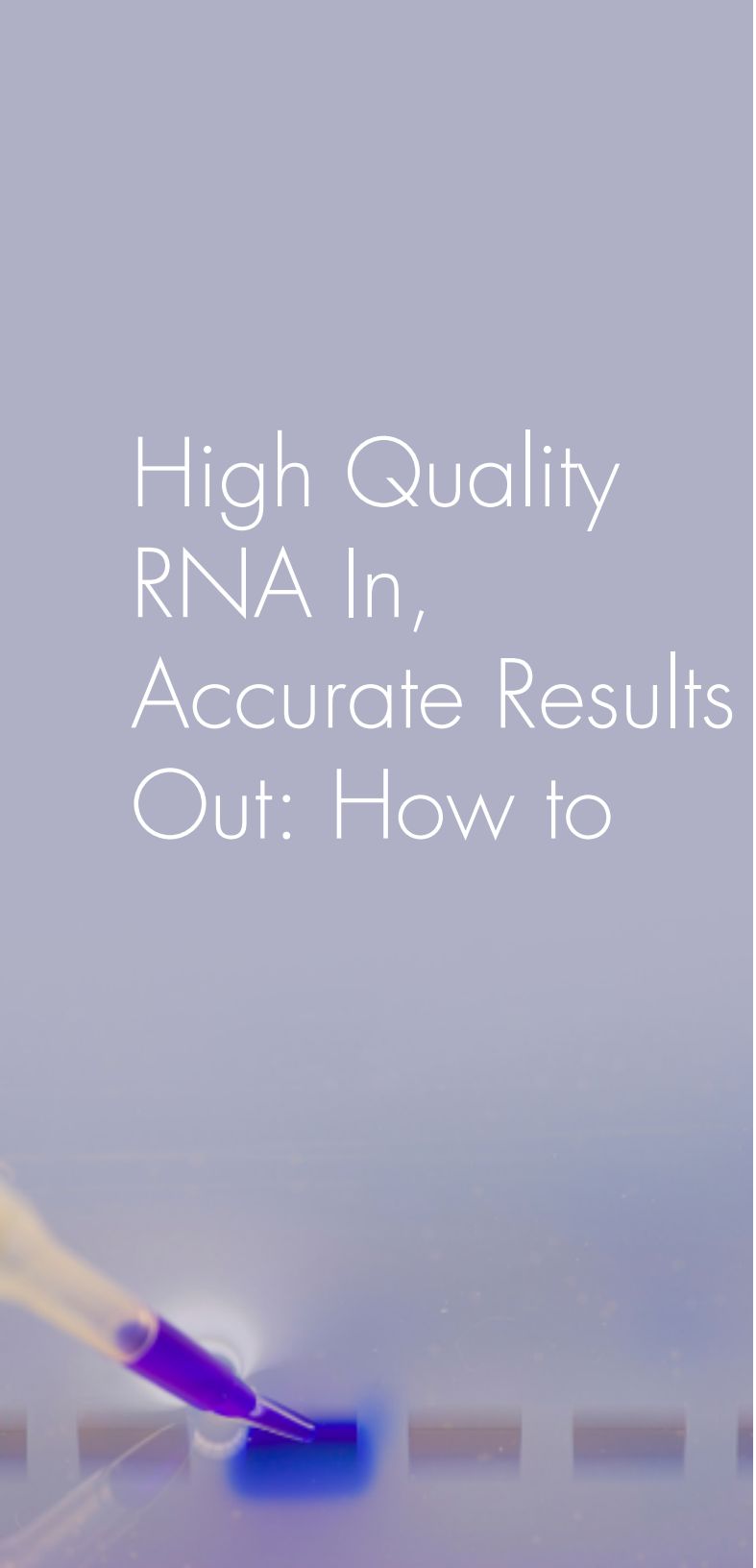
RTS DNase Removal Resin completely removes DNase. Samples were subjected to DNase treatment and enzyme removal using the RTS DNase™ Kit or a competitor's kit, and then analyzed for residual DNase activity using the MO BIO DNase-free certification assay. Lane 5 is the negative control and did not receive DNase. Samples were incubated for 1 hour at 37oC, followed by inactivation for 5 minutes at 65oC. Results are shown on a 1% agarose gel. The RTS DNase Removal Resin successfully removed the DNase (lanes 3-4), while the competitor's resin failed to remove all of the DNase from the samples (lanes 1-2).

Summary

Working with RNA is easy when you know how to protect against the sources of trouble. Fast homogenization in a protective lysis buffer is critical and then gentle DNase treatment of the RNA at the end is the icing on the cake.

Yes, certified RNase-free gloves are a great extra to have as well as UltraClean Lab Cleaner for removal of nucleases from the bench and equipment. We use these routinely in our labs. But the essential requirement for high quality RNA preps involve the very chemicals and plastics or beads that are going to come in contact with your sample and the RNA. The use of BME, consistent and fast homogenization, and RNase-free DNase with removal resin will be your ticket to success in every prep no matter what the sample.

High Quality RNA In, Accurate Results Out: How to



Accurate analysis of your RNA is critical for ensuring repeatable results in the next steps. Consistency begins at the very start.

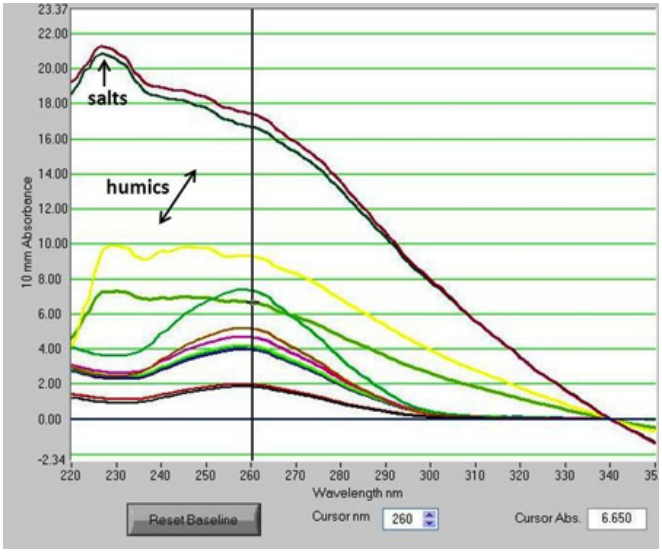
Measuring RNA Yields:

There are several methods for quantifying your RNA. The most frequently used method and the least expensive is using a Nanodrop or similar type UV absorbance measuring instrument. The Nanodrop has the advantage of providing you information on RNA purity. You'll get your 260/280 ratios, which measures the level of protein contamination in the sample, and the 260/230 ratio which tells you whether contaminants from the prep are in the sample (guanidine salts). For RNA, the ideal 260/280 ratio is between 1.8-2.1 and the 260/230 ratio ideally is above 1.5. Below is an example of the type of information provided by the Nanodrop.

Sample ID	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 rfac
MB kidney	770.22	15.405	8.504	2.17	2.60	40.00	230	5.687	0.022
MB lung	295.82	5.915	2.590	2.14	2.68	40.00	230	3.079	0.001
D kidney	583.57	14.089	8.678	2.11	2.18	40.00	230	8.471	0.057
D lung	182.51	4.563	2.231	2.06	1.75	40.00	230	3.643	0.008

Keep in mind that if RNA yields fall below a certain level (20 ng/ul), the Nanodrop readings are not quite accurate. We find that the RNA peak at 260 is not significantly high enough to balance the 280 or 230 readings, resulting in ratios that may not look right. In this case, take a look at the wavelength plot data on the Nanodrop. Make sure you are not seeing a peak at 220-230 or at 280. If the only bump in the curve is at 260, your RNA is ok. To give you an example of what I mean, here are some wavelength plots from a soil RNA experiment. In this experiment you see the two curves that are peaking high at 230 and then coming down

like a ski slope. This is not good. The high peak at 230 usually indicates salt contamination which typically comes from guanidine thiocyanate or other similar compounds used in RNA preps to lyse



cells and inhibit RNases. If not washed out completely, these will affect the entire spectrum through the wavelengths and lead to false high 260 readings.

The yellow and green curves are example of samples with a lot of both salt and humic acid inhibition. Humic acids will also absorb at 230 and continue throughout the spectrum of wavelengths, peaking at 320. All of these first four samples were brown at the end of the isolation.

The correct looking plots are the ones shown below the green and yellow curves. Here we see a nice downslope at the 230 reading followed by the peak at 260 and then the curve goes down again at 280. If we look at the very bottom two samples, in red and black, you can see what would happen if you had very pure RNA but a low concentration. Now the 230 and 280 readings are low such that there is no longer a 2:1 ratio between the 260/230 or 260/280. This doesn't mean the RNA is not good quality. As long as we don't see a ski slope or the curve going straight across, this RNA is ok to use.

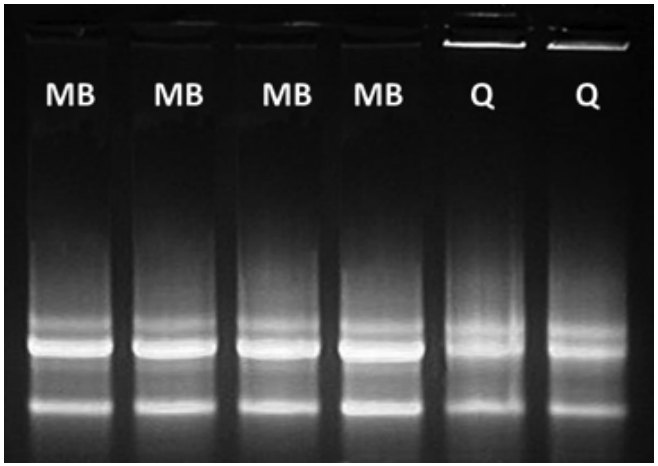
However there are other ways to measure RNA yields with greater sensitivity at low concentrations. We sometimes use the Ribogreen kit with our Qubit instrument. The advantage of Ribogreen dye for quantification is that it measures the RNA content only. While this is very convenient, it does not give you any information about RNA integrity or purity. We've covered the difference between UV absorbance vs fluorescent dye in quantifying DNA in a previous article using plasmid preps as an example. For more comparison data on this, check out the article linked above.

Measuring Integrity:

Besides knowing your yields of RNA, the other key factor for determining if your RNA prep worked well is looking at integrity. Integrity means, how intact and undegraded is the RNA. Traditionally, we determine this by looking at the intensity of the rRNA bands on an agarose gel. In eukaryotic cells, the 28S should be double the intensity of the 18S band an in bacterial cells, we look at the 23S in relation to the16S rRNA bands. To check RNA integrity, we simply run 5-10 ul of the sample on a standard 1% DNA agarose gel in 1X TAE buffer. This works very well for checking RNA. A denaturing gel is not necessary for doing a quick check. Here is an example of how the RNA gel should look with RNA from mouse liver using our PowerLyzer UltraClean Tissue and Cells RNA Kit.

In this example, 5 ul of RNA was run because liver has very high yields of RNA. If you overload the gel, you will not get good clear separation of the bands.

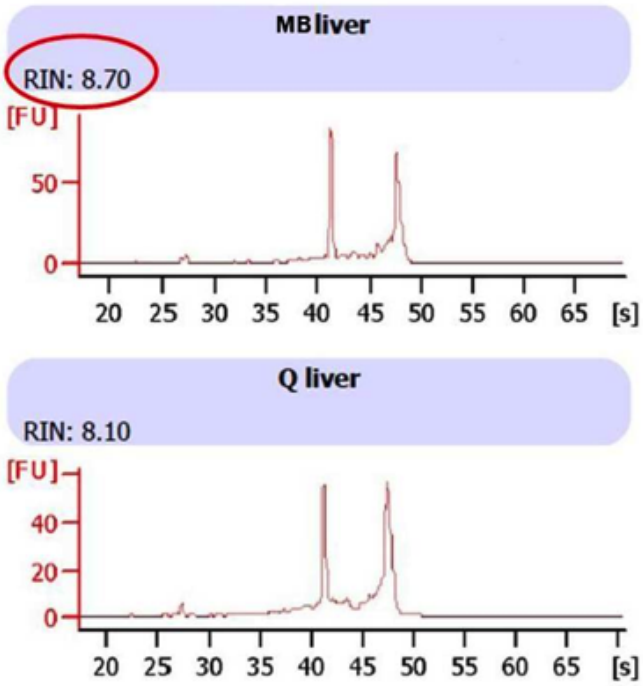
With high quality RNA, the upper band (28S) should look double the intensity of the lower band (18S) and appear crisp and sharp. If the homogenization was performed well, DNA in the upper



part of the gel should be absent. If the upper band 28S appears to be equal intensity to the lower band, it indicates some level of degradation has occurred. Smearing between the 28S and 18S is normal as this is where most of the mRNA will migrate. Smearing far below the 28S band is never a good sign.

There is another way to analyze RNA integrity and that is using the Agilent Bioanalyzer or similar type instrument. This instrument is nice because it uses only 1-2 ul of RNA and it measures the sizes of the rRNA bands to deliver what is called a RIN number. The RNA Integrity Number (RIN) is a way to standardize the quality of the RNA between preps.

Here is an example of an Agilent Bioanalyzer result. These are liver RNA samples analyzed with the Eukaryotic Total RNA Nano Series II kit. The RIN numbers are shown for two samples processed using bead beating and then purified using theMO BIO UltraClean Tissue and Cells RNA Kit or a Competitor's kit that



also uses a spin filter.

The sizes coming from the Bioanalyzer are smallest to largest, left to right. The peak between 45-50 seconds is the 28S rRNA and the second peak around 41-42 seconds is the 18S. If the RNA were degraded, we would see some blips or peaks on the scan around 35-40 seconds and earlier. The larger the RNA, the later it appears. If genomic DNA were contaminating the RNA, it would appear as a peak on the right.

Greater detail on both the Nanodrop and Agilent Bioanalyzer can be found in this helpful document put together by Biomedical Genomics.

Not everyone has the ability to run the Agilent Bioanalzyer in their lab. The disadvantages are that it is a very expensive instrument and the kits for analyzing RNA are also very expensive. If you

have a core facility on campus, they may allow you to run samples on it, but they'll usually charge a price to offset the cost of the kits. But that's why you have options. Using an agarose gel to check integrity and presence or absence of DNA and then the nanodrop for checking purity and yields, you'll be able to discern whether you have high quality and yields of RNA.

And one last note about DNA contamination….

DNA contamination in the RNA can be a hassle, especially for those of you working in microbial genetics, where primers cannot be designed to cross intron-exon boundaries. On-Spin Column DNase removal systems are one effective way to remove the DNA before elution of RNA. By letting the DNase soak into the membrane, it works to digest and remove the DNA before the RNA is eluted. But often times it is not enough. If the sample or cells were stored in RNALater or RNAProtect Bacteria Reagent, the DNA will become much more resistant to DNase treatment. In these cases, DNase treatment in solution is the better way to go, where the DNase has full access to the DNA instead of trying to work around the confines of a silica membrane. The disadvantage of using DNase in solution is that it must be inactivated before PCR using either EDTA and heat. To overcome this problem, we developed the RTS DNase Kit, a room temperature stable high velocity DNase enzyme that is easily removed using a DNase binding resin. It is extremely gentle on your RNA and highly effective at removing the DNase.

Recent publication: UltraClean Tissue & Cells RNA Isolation Kit

1. Splice-Mediated Motif Switching Regulates Disabled-1 Phosphorylation and SH2 Domain Interactions

Zhihua Gao, Ho Yin Poon, Lei Li, Xiaodong Li, Elena Palmesino, Darryl D. Glubrecht, Karen Colwill, Indrani Dutta, Artur Kania, Tony Pawson, and Roseline Godbout Mol. Cell. Biol., Jul 2012; 32: 2794 – 2808.

Getting the RNA You Want

We know many of our customers like to be selective about their RNA. That's because, most of our RNA technical questions involve a desire to retain or exclude certain varieties of RNA. It's not always possible to get what you want; but sometimes by making slight adjustments to the extraction protocol, it is possible to get what you need. In fact, in a previous MO BIO blog article [microRNA from Fresh Tissue and FFPE Samples using MO BIO Kits with Modified Protocols] we discussed how to bring in very small sized RNA when using our tissue extraction kits.

Since many of our customers are now turning their efforts towards extracting RNA from more difficult samples (AKA dirtier), we figured it would be a good time to talk about some of our newer RNA kits: PowerMicrobiome RNA, PowerPlant RNA, PowerWater RNA and PowerBiofilm RNA. All four of these RNA Isolation kits use the same combination of two solutions to bind the RNA in your cell lysate to a silica spin column. It's the ratio of these two binding solutions that determines the size of the RNA that will bind and subsequently the size of the RNA you extract. Everything smaller than the cutoff point will wash off the column. Everything above the cutoff will stay on the column and will come out in your elution. You can alter the binding solutions to change the cutoff point but you'll always lose everything smaller than the cutoff.

So how can this be used to your advantage? First, let's talk about the sizes and types of RNA that are in cells. The ribosomal RNA (rRNA) will be by far the most abundant type of RNA you isolate. That's because rRNA makes up about 80% of the total RNA in each cell. In prokaryotic cells you'll find three major varieties of rRNA: 23S (2906 nt), 16S (1542 nt) & 5S (120 nt). In eukaryotic cells you'll find 28S (5070 nt), 18S (1869 nt), 5.8S (156nt) and 5S (121 nt). If you run your sample on a standard 1% agarose gel, you can see the two largest rRNA bands running side-by-side. The second most abundant (~15%) RNA in cells

is transfer RNA (tRNA). The tRNA is used to translate codons to amino acids in the ribosome. It's pretty small, ranging from between 70 and 90 nucleotides.

The third most common RNA, and probably the target for most of our customers, is messenger RNA (mRNA). The mRNA is used to convey the genetic code for proteins from the genome to the ribosomes. Therefore its size is as varied as the proteins it encodes for. Although most of it will be in the 2000 base range, you can find mRNA as small as 500 and as large as 14,000 bases. Unfortunately, this variation makes it difficult to select for it by size. On a gel, it looks like a faint smear mixed in with the rRNA. Virtually all of the rest of the RNA in the cell consists of microRNA (miRNA, ~22 nucleotides), used for gene regulation in eukaryotic cells or small interfering RNA (siRNA, 20-25 bp), which impedes the expression of some genes.

Okay, so here's the scoop. If you follow the standard kit protocol, you'll isolate all RNA greater than around 60 nucleotides. This means you will get rRNA, mRNA and tRNA but lose the miRNA and siRNA. To pull in smaller RNA you'll need to lower the size cut-off by altering the binding mixture. The standard binding mixture is 1 volume of binding solution (PM3, PR3, PWR3, or BFR4, depending on the kit), 1 vol of 100% ethanol and 1 volume of lysate, a 1:1:1 mixture. To lower the size cutoff, you'll need to add an additional volume of 100% ethanol, so a 1:2:1 mixture. This will allow you to isolate pretty much all of the RNA that's there. If you want to raise the size cutoff, you'll need to replace the solution containing 100% ethanol (PM4, PR4, PWR4, or BFR5, depending on the kit) with 70% ethanol. This will give you just mRNA and the two largest rRNAs. If you are studying mRNA, this helps reduce the background RNA a little bit.

We thought this might be a litte confusing. So we've created a handy chart to help. You can use the alternate bind with any of the kits in the chart. Each of these kits also includes our strongest inhibitor removal technology (IRT). Note that you cannot use this technique with our RNA PowerSoil kit or the UltraClean Microbial RNA kits. If you have any questions regarding the technique please contact us at technical support: technical@mobio.com

References

1. Molecular Cell Biology. 4th edition. Lodish H, Berk A, Zipursky SL, et al. New York: W. H. Freeman; 2000.

	RNA Isolated		
	mRNA,16S/23S rRNA	tRNA,mRNA, 5S/16S/23S rRNA	miRNA,siRNA, tRNA,mRNA, 5S/16S/23S rRNA
PowerMicrobiome Binding Mixture	1 vol PM3 1 vol 70% EtOH 1 vol lysate	1 vol PM3 1 vol PM4 1 vol lysate	1 vol PM3 2 vol 100% EtOH 1 vol lysate
PowerPlant Binding Mixture	1 vol PR3 1 vol 70% EtOH 1 vol lysate	1 vol PR3 1 vol PR4 1 vol lysate	1 vol PR3 2 vol 100% EtOH 1 vol lysate
PowerWater Binding Mixture	1 vol PWR3 1 vol 70% EtOH 1 vol lysate	1 vol PWR3 1 vol PWR4 1 vol lysate	1 vol PWR3 2 vol 100% EtOH 1 vol lysate
PowerBiofilm Binding Mixture	1 vol BFR4 1 vol 70% EtOH 1 vol lysate	1 vol BFR4 1 vol BFR5 1 vol lysate	1 vol BFR4 2 vol 100% EtOH 1 vol lysate

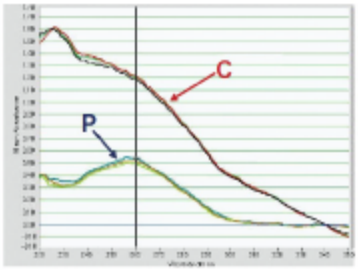
How Dirty is DNA Without IRT?

Scientists often come to us with their dirty little DNA problems. Samples like soil, feces, and blood (oh my!) can make extracting DNA challenging because they are high in compounds like humic acids, polysaccharides, heme, or dyes. These bind to the DNA and inhibit enzymes used in downstream applications like PCR and sequencing. MO BIO uses patented Inhibitor Removal Technology® (IRT), a method to remove these substances in many of our kits. It is very effective at removing the inhibitory compounds without significantly decreasing DNA yield. But how well does it really work? Good scientists want to see the data. Recently we received the following request…

Q: Do you have comparative data showing extracted DNA from identical samples with and without Inhibitor Removal Technology® (IRT)?

The answer is yes. We have done a number of quantitative assessments demonstrating the importance of using IRT for the treatment of environmental samples during nucleic acid extraction and purification. A summary of this data was presented in a poster at the ASM General Meeting in 2010. One experiment involved isolating DNA from 0.1 g of compost using either the IRT containing MO BIO PowerSoil® DNA Isolation Kit or a competitor's kit that does not contain IRT. All samples were quantified on the Nanodrop and run on a gel, then subjected to end-point PCR using universal Streptomyces 16S rRNA primers with the Kapa2G Fast HotStart Readymix (MO BIO Laboratories). Figure 3A (below) shows the purified DNA curves and gel results. Figure 3B shows the absorbance values of the purified DNA. Of note is that the competitor's kit (C) appeared to have more than double the yield of the PowerSoil® kit (P) based on just the A260 reading. But the gel shows that the apparent increased yield was due largely to cross-absorbance from the high level of humic acid contamination (based on the low 230/260 ratio combined with

high 340 readings, Fig. 3B) plus degraded RNA that co-purified with the DNA (Fig. 3A). Most importantly, however, inhibitor carry-over was confirmed with PCR. (Fig. 3C).



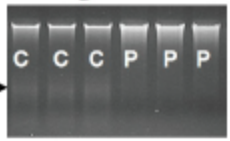
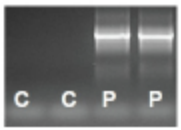


Figure 3A – Compost DNA curves and gel results for PowerSoil® (P) and a competitor's (C) kit. Arrow denotes degradation.

Figure 3B – Absorbance values of purified DNA from compost using PowerSoil® (P) and a competitor's (C) kit.

	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
C	59.38	1.188	0.807	1.47	0.78	50.00	230	1.527	0.636
C	60.71	1.214	0.818	1.48	0.78	50.00	230	1.565	0.652
C	60.30	1.206	0.829	1.46	0.79	50.00	230	1.539	0.768
P	25.58	0.512	0.268	1.91	1.63	50.00	230	0.314	0.056
P	24.60	0.492	0.262	1.88	1.60	50.00	230	0.308	0.043
P	26.97	0.539	0.287	1.88	1.55	50.00	230	0.349	0.052

Figure 3C – PCR results from compost using PowerSoil® (P) and a competitor's (C) kit.



Another experiment involved comparing two of our own water kits; one with IRT (PowerWater®) and one without IRT (RapidWater®). Other than the IRT they are identical. Comparable amounts of DNA were extracted as shown on the gel in Figure 4A, but the Real-time PCR results show lower and more consistant Cq values when IRT was used.

DNA doesn't have to look dirty to be dirty! With this water DNA sample, even the purity measurements by Nanodrop looked good… but the qPCR results show it is not the case.
Most samples are not as tricky as this water sample from Elliott Bay, WA. However, no matter what your sample is, MO BIO has a way to extract it. And with IRT, you can be sure it will amplify too.

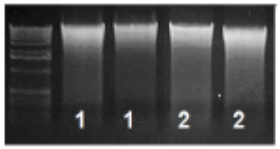


Figure 4A - Genomic DNA from Elliot Bay samples. PowerWater® DNA Isolation Kit (1); RapidWater® DNA Isolation Kit (2)

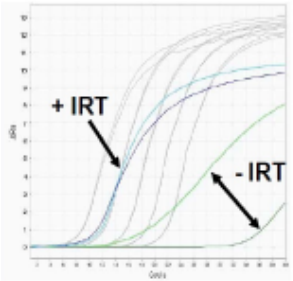


Figure 4B - Real-time PCR results from Elliot Bay. IRT containing PowerWater® DNA Isolation Kit (Blue); RapidWater® DNA Isolation Kit (Green); standard curve (Gray).

MO BIO DNA and RNA isolation products utilizing the IRT include: PowerBiofilm™, PowerSoil®, PowerMax®, PowerPlant®, PowerClean®, PowerWater®, PowerFood® and BiOstic®Bacteremia DNA Isolation Kit.

Misconceptions about DNA Quantification

Problems achieving high yield and purity are exaggerated in environmental samples because of the added complexity of microorganism lysis and inhibitor removal. Quantifying the nucleic acids in these samples is the easy part. But if you don't know what to look for, you can easily make mistakes in interpreting the results, which can lead to a lot of repeat work or missed critical information in your experiments.

Let's discuss some of the common misconceptions surrounding DNA isolation and quantification and what problems to look out for before going to the next step with your sample.

1. True or False: A higher UV A260 reading means more DNA.

False. A high A260 reading does not always mean high genomic DNA yields. One of the main reasons for a high A260 reading that does not correlate to genomic DNA is the absorbance of UV due to highly degraded DNA or RNA. Degraded RNA absorbs a high level of UV and results in a boost to the A260 reading. The method used to purify the DNA after lysis will determine what is present in the final sample. Many purification methods do not separate the small DNA and RNA from the high molecular weight gDNA. The PowerSoil DNA Isolation Kit does.

The only way to know what you really have in your sample is to run 5-10 µl on an agarose gel. This will give you a clear indication as to whether you have predominantly genomic DNA or a mix of nucleic acids sheared to varying lengths.

2. True or False: Bead beating always gets higher yields of DNA

False. Not all samples need to be homogenized with high velocity bead beaters. Typically for RNA extraction of tissues and plants, definitely use the strongest method available to you. You

want to break the genomic DNA down in size. For genomic DNA from a variety of environmental samples, gentle methods such as vortexing with beads will isolate high molecular weight DNA. When measuring the yields on a spec, as mentioned above, the more sheared the DNA, the higher the absorbance reading. This does not mean more DNA was isolated. When qPCR is performed, less DNA will be added to the reaction due to the false high reading, leading to higher Cq values and inaccurate quantification in the sample.

For microbial DNA from soil, the bead beater can sometimes do more damage than good. For DNA from fungus and spores, a thorough discussion on ways to optimize the lysis using different beads and heat has been described in detail. We also give some recommendations for using the bead beater and soil based on published references.

The best approach to ensure the integrity of the DNA is to run a gel in addition to the Nanodrop or UV scan so you can make a better assessment of what you really have. If you see a smear on your gel, then the bead beating was too hard.

3. True or False: If the sample looks clear, it is free of humic and fulvic acids

False. Humic acids give the sample the characteristic brown color so if your DNA elutes with color, you know you've got a lot of contamination. Even if it looks clear, there can still be low levels of humic and fulvic acid or even polysaccharide contamination in the sample. Using a kit with Inhibitor Removal Technology such as PowerSoil, PowerWater, and coming soon, PowerBiofilm, will ensure that a clear eluate is actually clean.

In addition to the A260 reading for yields, a low 260/230 ratio can be indicative that the sample still has some organic contaminants. A ratio above 1.5 is ideal. A ratio below 1.0 has significant contaminants present that may interfere with enzymatic applications.

4. True or False: If my soil has low amounts of DNA, I need to try stronger methods for lysis

Depends on the soil. If your soil has low amounts of DNA, you may need to start with more. Some soils, such as clay soils and sediments, may benefit from harder bead beating and the use of glass beads for homogenization, such as the bead tubes that are included in the PowerLyzer PowerSoil DNA Isolation Kit.

What exactly should the yield of DNA be in soil?

The yield in soil varies greatly but for a rich organic soil with a high microbial load, yields of DNA will range between 20-30 µg per gram of soil (5-7 µg/ PowerSoil prep). According toWhitman et. al (1998), rich top soil contains 1-2×109cells/gram of soil (1). Using an E.coligenome as an example, this equates to 5-10 µg of DNA in a gram of soil or around 1-3 µg of DNA per PowerSoil DNA Kit prep. Eluted in 50 ul, the concentration to expect for microbial rich soil is around 20-60 ng/µl. If the genome of the organisms in soil are double the size of E.coli, then yields of 40-120 ng/µl are in the correct range for microbe dense soil.

Most of the soil we use in our lab are not at the high end of microbial load, so yields of 10-20 ng/ul are not uncommon for average soil preps.

Given this information, if another isolation method gives you yields far above this range, it is not all DNA. The DNA is either contaminated with UV absorbing PCR inhibitors or mostly degraded RNA. Either way, the information obtained from genotyping will not be as complete or accurate as clean pure microbial DNA obtained from using PowerSoil and UltraClean Soil Kits.

Make sure to always check the yields on a gel and for even greater accuracy, use qPCR.

5. True or False: PCR is the best way to check for inhibition

True. The only way to know if the sample is inhibitor free is to use it in an enzymatic reaction. Even better is to use qPCR and perform serial 10 fold dilutions and check the efficiency of amplification using a primer pair for 16S rDNA. It is likely that there will be some background amplification in the water control because most PCR mixes have background bacterial DNA, but the difference in Cq value between the samples and the control will be far away enough to not matter (usually 6-10 cycles).

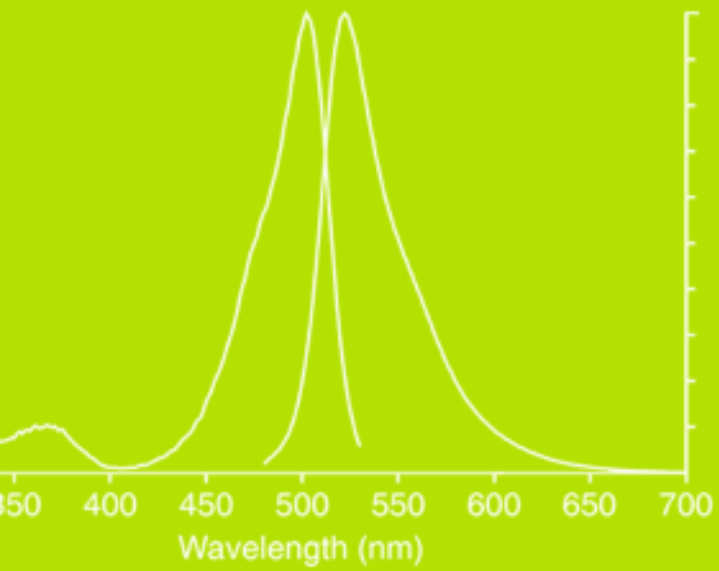
With qPCR, the desired result is a change of ~3.3 cycles between each 10-fold dilution. This indicates perfect doubling each cycle and is a sign that the sample is inhibitor free. What you may see is the first sample (undiluted) is shifted to the right and then the rest of the samples fall into place. This indicates that there is some inhibiting substances in the DNA. Remember not to add too much DNA. 1-2 µl per 50 ul PCR is adequate or around 10-100ng for the first sample and then dilute from there. If the first sample amplifies to soon (and falls in the baseline for the instrument, where fluorescence is subtracted out as background), it will cause some problems with the standard curve so you may want to start with 10 ng and dilute from there.

Another good approach is to set up a PCR reaction that always works (such as for a plasmid) and then spike in 1 µl of the DNA from the environmental sample. If it causes the PCR to fail, or reduces the amount of product, it indicates inhibition.

Summary

These technical tips do apply to more than just soil and water samples. Even DNA from blood or tissues can be affected by inhibitors causing problems with absorbance readings and inaccuracies in quantification. The best approach is to always run a quick agarose gel to go with your Nanodrop results so you can see the integrity and composition of the sample along with the yield. Additionally, PCR or qPCR can help get a more exact quantification of the amount of gDNA or number of microbes in the starting sample.

The Difference Between the Nanodrop and Fluorescent Dye for Quantifying DNA



Plasmid DNA isolation is so routine today in labs that you pretty much expect to get DNA back, even when you make a mistake. But are you getting back only DNA? It turns out that plasmid preps are the perfect application to demonstrate a basic difference in two methods for DNA quantification: spectrophotometry (Nanodrop) vs. fluorescent dye (Picogreen).

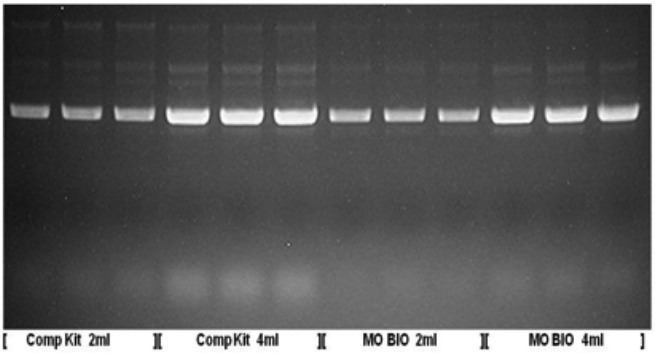
Like most labs, we use the Nanodrop to quantify nucleic acids. It is easy, sensitive, and no standard curve is needed. However, when we compared the quantification results from plasmid preps using the Nanodrop and Picogreen, between our plasmid kit and a competitor, the results were very surprising. But before we go any further…

What is Picogreen?

Picogreen is a dye that, when bound to double-stranded sequences, and excited by light at 485nm, emits fluorescence at 530nm. Using a fluorescent plate reader or a Qubit, it allows you to determine the amount of DNA in a sample in the presence of contaminants that influence the UV absorbance at wavelength 260, such as RNA or salt and guanidine contamination. It is a useful tool for getting a more exact concentration of DNA from a prep that contains RNA.

How does this relate to plasmid preps?

Well, remember that RNase you added into your Tris resuspension solution? This is supposed to digest the RNA which is abundant in the healthy logarithmically growing E.coli culture you prepared for your plasmid prep. Then, ideally, the digested RNA is washed out of the silica spin column so that you obtain only high purity DNA. What surprised us was just how much RNA was still contaminating plasmid DNA. See the example below. Using agarose gel electrophoresis, the fuzzy banding from RNA contamination is clearly visible low in the gel.



*RNase A was added fresh to the resuspenson buffer prior to performing the preps with the competitor kit but not with the MO BIO Kit.

However, check out what that RNA contamination does to the spectrophotometer readings as compared to picogreen:

If all you did was measure your DNA on a spectrophotometer,

	Nanodrop ng/µl	Picogreen ng/µl
Comp Kit 2 ml	59	16
Comp Kit 2 ml	39	16
Comp Kit 2 ml	41	17
Comp Kit 4 ml	81	33
Comp Kit 4 ml	97	35
Comp Kit 4 ml	80	32
MO BIO 2 ml	22	16
MO BIO 2 ml	23	16
MO BIO 2 ml	20	16
MO BIO 4 ml	46	30
MO BIO 4 ml	43	29
MO BIO 4 ml	44	32

and compared kits, you would certainly think that the first kit is better. The gel would show you they are equal, but the spec reading would make you think you had double the DNA.

However, the picogreen results tell a different story. When RNA is present, the artificial boost in yield is anywhere from double to triple the actual amount. Using the MO BIO Kit, the yield you get on the spec is much closer to the actual yield.

What does this mean to you?

It means that when setting up restriction digests for cloning experiments, or sending plasmid off for sequencing, that you will have accurate starting amounts of DNA moving forward. This is especially important when you are trying to troubleshoot cloning experiments and set up accurate ratios of insert to plasmid.

I know not everyone has the ability to measure their DNA with picogreen. And it may not always be important to know exactly how much you have. We don't use it for everything either. Just keep in mind that when you see the fuzzy banding on the bottom of your gel in your plasmid DNA, your plasmid yields may actually be half what you think, so plan accordingly. Or, try the MO BIO plasmid DNA kit and you won't have to worry about it.

We see this issue come up for genomic DNA preps as well. Our advice? Always use at least two methods for checking your DNA: an agarose gel and a spectrophotometer or picogreen measurement. The fact is that using only one method will not give you enough information about your sample to ensure success in the steps that follow.

Where Did My DNA Go? Tips for DNA Clean-Up

Genomic DNA clean-up is a technique that is very common but still causes many people to suffer from separation anxiety. Here's a look at some tips and tricks to improve your DNA clean-up and avoid the loss of precious samples.

Why is my DNA "dirty"?

Here's the scene: You've collected a set of samples, they could be plants, seeds, stool, gut material, soil, water, FFPE or anything that is known to contain PCR inhibitors. You isolate DNA using your typical method and the DNA fails to amplify in PCR. You need every last molecule for whole genome shotgun sequencing…. what do you do?

Secondary clean-up of DNA

The PowerCleanβ DNA Clean-up Kit provides a quick, easy and reliable secondary clean-up method to purify previously isolated genomic DNA from any source. The kit contains MO BIO's patented Inhibitor Removal Technologyβ (IRT), a proven method of removing PCR inhibitors including humic acids, polysaccharides, polyphenolics, lipids, heme and more.

Going back to the sad story of your precious samples that failed to amplify… After learning about PowerCleanβ, You run the samples through the kit to isolate clean DNA. But wait… the spec readings have changed! Before clean-up it said you have 300 ng/µl of DNA and now it says you have 30 ng/µl. It's your worst nightmare. Your DNA has been lost!

Don't worry, we're here to tell you that your DNA is safe and sound - and clean. There's some confusion about how to tell what you had before and after clean up using standard quantification lab techniques.

PCR inhibitors influence DNA quantification

Previously, we explained how PCR inhibitors wreak havoc on UV260 wavelength readings and inflate DNA yields. This is important because many of the organic inhibitors found in plants,

seeds, stool, gut material, soil, water and FFPE tissues co-absorb at the 260 wavelength and interfere with accuracy in DNA yield readings. Degraded RNA, which will be co-isolated with CTAB or phenol:chloroform methods, also interferes with absorbance readings. Silica spin filter kits that do not have a method for inhibitor removal and use very strong binding salts that do not discriminate between DNA and RNA binding are also problematic. All of these factors are going to cause problems when interpreting your DNA yield.

The good news is, once these interferences are removed, the new absorbance readings represent the true DNA yields. Let's look at a real life example of how this will look using standard laboratory techniques; the NanoDrop and agarose gel electrophoresis. Beginning with the NanoDrop (Figure 1), we see the results of the same soil extracted with and without Inhibitor Removal

Sample ID	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 rev
No IRT	59.38	1.188	0.807	1.47	0.78	50.00	230	1.527	0.696
No IRT	60.71	1.214	0.818	1.48	0.76	50.00	230	1.565	0.652
No IRT	60.30	1.205	0.828	1.46	0.78	50.00	230	1.539	0.768
IRT	25.58	0.512	0.268	1.91	1.63	50.00	230	0.314	0.040
IRT	24.60	0.492	0.262	1.88	1.60	50.00	230	0.308	0.043
IRT	26.97	0.539	0.287	1.88	1.55	50.00	230	0.349	0.052

Figure 1. NanoDrop readings for samples with (No IRT) and without (IRT) PCR inhibitors.

The 260/230 ratios in Figure 1 tell us that there is a problem with this DNA. In the samples with inhibitors (No IRT), the readings are below 1.0, indicating a high level of impurities. We can get another key piece of information from this data; the 340 reading. The samples isolated with no IRT have an elevated 340 value compared with the pure samples isolated with IRT. They are almost 10X higher. Humic acids will optimally absorb at wavelength 320 so the high absorbance at 340 reflects the carry-over of humic acids in the final DNA. The 260/230 ratio, combined with the high 230 absorbance, tells us this DNA is not clean due to the presence of organic compounds.

NanoDrop also provides graphical representation of the absorbance data across all of the wavelengths (Figure 2). We clearly see a high level of interference across all of the wavelengths being measured. It starts high and stays amplified. The 260 reading is caught in middle of this inhibitor-fest and consequently is way above where it should be.

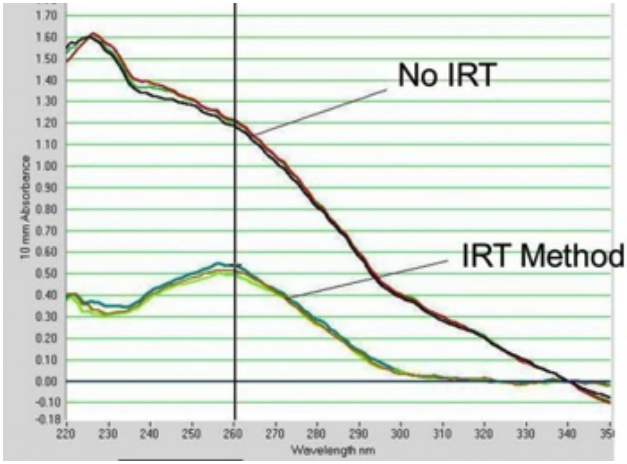


Figure 2. NanoDrop absorbance data for samples isolated with and without IRT.

However, we know that it is still difficult for some people to believe that this is not all DNA. In this case, best way to confirm your results is with an agarose gel picture. The gel picture has information that is not visible on the NanoDrop; integrity of the DNA and a visual representation of the yields (Figure 3). Although the NanoDrop indicates that the No IRT samples are more than double the yields of the IRT samples, the gel picture shows us that the yields of the No IRT samples are actually lower. The contaminants make analysis confusing. This is why we always recommend checking your DNA with two methods – NanoDrop along with a gel picture or PicoGreen reading.

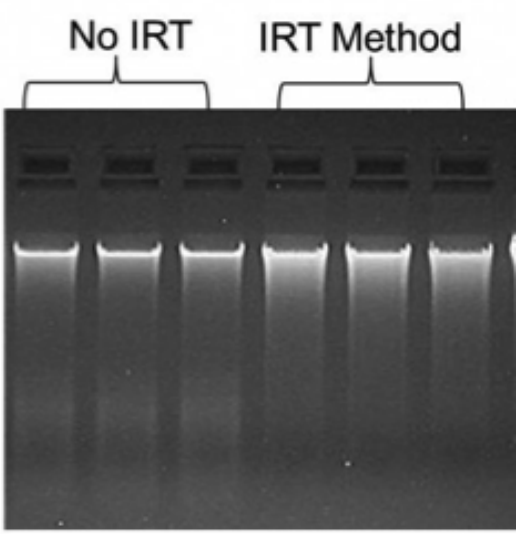


Figure 3. Gel analysis of samples isolated with and without IRT.

So what happens when you clean dirty DNA samples up using the PowerCleanβ Kit? You remove these interfering compounds from the DNA and, as a result, you see a drop in the yield reading. In this case it would be more than 50%. However, this decrease in yield is not due to the loss of high molecular weight DNA. It is loss of the compounds you don't want in your prep. It is now pure DNA.

RNA contamination can inflate DNA yields

Depending on the method used, RNA will co-extract with the DNA. It may not be intact, but if it is there it will absorb UV. Any method using phenol or chloroform to extract nucleic acids will co-isolate the RNA, and methods using strong binding salts and ethanol in equal volume will also result in RNA co-isolation. Because the NanoDrop cannot differentiate between DNA and RNA, this is where a second method such as PicoGreen comes in handy, because it measures dsDNA only. An agarose gel picture is also helpful because the RNA smear can be visualized.

In this article, The Difference between the NanoDrop and fluorescent dye for quantification of DNA, we show how much RNA can impact your yield readings.

We can demonstrate this by examining plasmid DNA purified from 4 ml of overnight LB E.coli culture using a popular plasmid prep kit on an agarose gel (Figure 4). Here, we can very easily see the degraded RNA smear at the bottom of the agarose gel. The average NanoDrop reading for these 3 samples was 81 ng/µl, while the corresponding PicoGreen reading averaged just 16 ng/µl. RNA absorbance can impact readings by as much 70% for some samples. This amount of inaccuracy in your DNA sample can lead to pretty big errors later on.

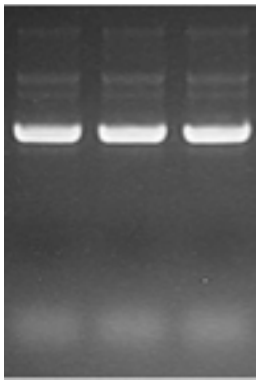


Figure 4. Agarose gel analysis of plasmid DNA isolated from 4 ml of LB E.coli culture

Accurate yields and clean DNA

So the take home message is this: examine DNA samples before clean up and after clean up on an agarose gel. Take a look at the intensity (yield) and integrity (size range of the DNA) and compare with your second method (NanoDrop or Picogreen) to check for the presence of RNA or inhibitors.

You can save yourself anxiety and time if you do a simple check to see what you are starting with. Remember, the PowerCleanβ Kit can only give back what you put in. You want clean DNA. But knowing how much DNA you had to begin with will calm your nerves and give you the assurance you need about the outcome.

A Quick Guide for Troubleshooting Problems with PCR Amplification

PCR is one of the most common techniques performed in virtually all molecular labs today. It is so routine, that when something goes wrong, it can be exceptionally frustrating. No one wants to spend time troubleshooting a problem that is as simple as mixing a few solutions together in a tube and putting it into a machine. We need fast answers so we can go on with our research.

Recently in our labs, we encountered unexpected problems while doing qPCR and PCR experiments. As a result, we were reminded of some valuable lessons. I would like to impart them to you here today along with additional advice for troubleshooting PCR problems that usually crop up when you least expect it.

End-Point PCR

Let's begin with mystery of the failing 16S end-point PCR.

With end-point PCR and a 2X Ready-Mix, there's not much that can go wrong. As long as the primers are added (they were) and they are fresh (they were brand new) and others were using them with success (they were), then I can rule out the primers.

And fortunately, I had some controls in my experiment. I was evaluating a panel of different soils and was working with a difficult agricultural sample type that appears to have a lot of fertilizers or chemicals present, thus always gives weak amplification in PCR but should amplify without dilution. Included in this run was positive control DNA from a soil that always amplifies and it worked as it should. So I was able to rule out the enzyme kit as the problem.

So what's left? Well, while standing by the thermal cycler, watching it begin the hot start, I noticed it was going into a 10 minute hotstart. I thought it was strange since the kit is "fast" and it needs only a 2 minute hotstart. And then after the reaction was finished, I noticed other changes to the protocol. The extension cycle should be 10 seconds but it was reduced to only 1 second.

The answer to this mystery?

Someone had changed the saved program for our Kapa Fast PCR

run and forgot to change it back. I changed the program back and all the samples worked as they should.

You would think that because the PCR worked for some soils but not others, that the program wouldn't matter. But it does. Apparently, when the sample is difficult to begin with, having the cycling conditions just slightly not optimal can cause negative results. With control DNA it worked fine. The morale of this first story is: when your PCR stops working, check your machine and make sure someone didn't modify your program.

I should note that our typical advice is to dilute the samples 1:10 when they do not work undiluted and this always amplifies. But I was using a soil I know works undiluted so I couldn't rest until I had it right.

qPCR Troubleshooting

Around the same time, we were faced with our first ever qPCR assay that did not work. We tried everything from re-calibrating the instrument, re-ordering primers, to running an assay that works in the machine with the chemistry to show that the enzyme and machine were not to blame. Then we also tried adjusting the annealing temperature, time, and extension time. Nothing worked. The melt curve data was most informative. We could see that the amplification was not specific. There were multiple curves in all the reactions. When this happens, it is usually a poor design of the assay.

So we went back to the original paper that we took the assay from and sure enough, when we looked at their data a little more closely, their efficiency data wasn't very good either. They didn't show melt curve data but we suspected the assay worked the same for them as it did for us: poorly. But it was published anyway. The lesson here is: when you take an assay from a paper, check that they reported all the necessary information according to the MIQE guidelines. Researchers need to give full details about their qPCR assays including the PCR efficiency and sensitivity.

We found another assay for the organism that is giving us the 95% PCR efficiency we are used to and the melt curves show only one peak.

Sometimes it's not always a bad thing when a PCR fails. If we never had to troubleshoot, we would never learn anything. Here is more advice for troubleshooting PCR problems. If you have a specific problem and aren't sure what to do, leave us a comment or email me at technical@mobio.com.

10 Tips for PCR Troubleshooting

1. When working with an existing assay, always have a positive control (and a negative control) so that you can rule out a problem with the primers, enzyme or a machine setting. Check your program and make sure it's correct.

2. When designing a brand new assay and testing it for the first time, include a positive control reaction so that if the new assay fails, you know you should focus on the primer design and not the chemistry.

3. If the PCR products appear as a smear, you may need to increase the annealing temperature or decrease the magnesium (if you added Mg yourself and it wasn't already in the mix.) You may also be adding too much template.

4. If your amplification is weak or non-existent, many things could be happening. Dilution of the template 1:10 will let you know if the issue is a PCR inhibitor. Try diluting the DNA first if this is an environmental sample. If not, try bringing the annealing temperature down a couple degrees or adding additional

magnesium. Conversely, the template may be GC rich and you may need a longer hotstart or an additive to help melt the template.

5. Make sure you are following the protocol for your kit, including the amount of time it needs for enzyme activation and the cycling times. Each manfacturers kit is different and optimized for their chemistry.

6. Check your DNA template on a gel AND a spectrophotometer or with picogreen. Don't trust the reading alone. Make sure you have DNA (and not RNA) and that the yield looks accurate to the Nanodrop or picogreen reading.

7. With qPCR, set up 10 fold dilutions of template for the standard curve and use at least 5 dilutions to have the best sensitivity and linearity. Your assay is only accurate down to the lowest Cq that you can detect that is linear in the assay. Once the assay loses linearity, those sample past that point cannot be accurately quantified. A perfect assay will have a slope of -3.3, meaning that every 10 fold dilution is 3.3 cycles higher. This is 100% doubling in each cycle.

8. Some SYBR Green kits use three step cycling (denaturing, annealing, extension) and some use two step cycling (denaturing and annealing/extension combined at 60C). Follow the directions for your kit.

9. When you open a new enzyme kit of a different lot or get new primers, repeat the standard curve again. Make sure you get the same Cq values for the same dilutions. If the primer synthesis was poor, you'll be able to catch it right away. If the curve is different, you will be able to calculate the data correctly and avoid misinterpretation of the results.

10. Signs that the primer design is a problem are mutiple melt curve peaks, non-specific amplification, and poor PCR efficiency. On an agarose gel, the assay should give you one single band. It may be the oligo synthesis but usually it is the design. There are a number of PCR additives you can try that may help if you have no choice but to design an assay in a troublesome area for polymerase.

NTC contamination issues

No template control problems affect everyone at some point in their PCR career. I wanted to devote extra attention to addressing this annoying, but common problem. Here are some possible reasons for PCR contamination and solutions for solving the issue.

1. If you are amplifying with 16S primers, the contamination is probably coming from the enzyme. This is very common and difficult to avoid. If this is a gene specific primer and you have contamination, then it may be true contamination of a reagent. Here is what we recommend for eliminating the chance of false positives:

2. Designate a separate area of the lab as a PCR station and do not use it for anything else. Ideally, this is in a different room than where the DNA is prepped and PCR products are handled and analyzed.

3. Purchase a set of PCR only pipettors and do not use them for anything else.

4. Wipe down your PCR area and pipettors with a Lab Cleaner that removes nucleic acids and follow that with wiping the surfaces

down with 70% ethanol to remove the cleaner

.

5. Always use aerosol resistant tips.

6. Keep clean water in the PCR only area for use with PCR only

.

7. Add the positive control DNA at your bench, after the NTC reaction has been closed. Do not bring your test samples to the PCR area.

8. Aliquot your primers and your enzyme mix if you purchase large volumes. If something does come up positive, you can always throw away the small aliquot and grab a fresh tube. This way you don't need to throw out an entire kit or batch of primers.

Good Enough for Next Generation Sequencing?

Since Next Generation Sequencing (NGS) was introduced onto the market less than a decade ago, the technology has undergone rapid growth and improvement. Run speeds have increased, costs have gone down, and the sheer number of bases sequenced per run has improved so significantly that NGS is now within reach for most researchers. Consequently, scores of scientists are trying NGS for the first time. And they have a lot of questions, some of which have been thrown our way here at technical support. The most common question we get is whether or not DNA isolated using a particular MO BIO DNA Isolation kit is suitable for Next Generation Sequencing.

Why the concern? For a NGS run to be successful, it is critical that the DNA be free of contaminants like salts, protein, EDTA, and any other gunk that might be carried over from the original sample. This can be a real problem if you are starting with "difficult" material, as a lot of our customers do. Some recent examples include bird feces, insect guts, cactus pulp, pipe slime, peat moss, pig slop, sewer water and even crude oil-contaminated soil. (Sounds like episodes from "Dirty Jobs") It can be a real challenge to get clean DNA from such sources. Is it possible? Heck yes. Many of our customers are already doing next generation sequencing with DNA isolated using MO BIO kits. Others are using the MO BIO PowerClean DNA Clean-up kit to make their contaminated DNA suitable for NGS. IRT, our patented inhibitor removal technology is very effective at removing most of the substances that can interfere with NGS.

Since we've had customers ask us for references, below we've listed a brief selection. We've tried to choose publications covering a variety of sample types, NG sequencing platforms, and MO BIO isolation kits. Hope this helps your research and your next generation endeavors. Let us know if you have another reference you think should be included.

- **Virus, UltraClean Microbial DNA Isolation Kit**
  Roche 454 Pyrosequencing
  Metagenomic Analysis of the Viral Communities in Fermented Foods
  Eun-Jin Park, Kyoung-Ho Kim, Guy C. J. Abell, Min-Soo Kim, Seong Woon Roh, and Jin-Woo Bae. Appl. Envir. Microbiol., Feb 2011; *77*: 1284 – 1291.

- **Bacteria, UltraClean Microbial DNA Isolation Kit, Pyrosequencing**
  Genome Sequence of a Novel Species, Propionibacterium humerusii
  Susan M. Butler-Wu, Dhruba J. Sengupta, Weerayuth Kittichotirat, Frederick A. Matsen, III, and Roger E. Bumgarner J. Bacteriol., Jul 2011; 193: 3678

- **DNA, PowerClean DNA Clean-Up Kit, Pyrosequencing**
  Lachnospiraceae and Bacteroidales Alternative Fecal Indicators Reveal Chronic Human Sewage Contamination in an Urban Harbor
  Ryan J. Newton, Jessica L. VandeWalle, Mark A. Borchardt, Marc H. Gorelick, and Sandra L. McLellan. Appl. Envir. Microbiol., Oct 2011; *77*: 6972 – 6981.

- **Deep Sea Sediments, PowerMax DNA Isolation Kit, Genome Analyzer GAII instrument (Illumina)**
  Ultra-deep sequencing of foraminiferal microbarcodes unveils hidden richness of early monothalamous lineages in deep-sea sediments
  Béatrice Lecroq, Franck Lejzerowicz, Dipankar Bachar, Richard Christen, Philippe Esling, Loïc Baerlocher, Magne Østerås, Laurent Farinelli, and Jan Pawlowski. PNAS, Aug 2011; 108: 13177 – 13182.

- **Symbiotic-fungus, PowerSoil DNA Isolation Kit, Pyrosequencing**
  Distinct Ectomycorrhizospheres Share Similar Bacterial Communities as Revealed by Pyrosequencing-Based Analysis of 16S rRNA Genes
  S. Uroz, P. Oger, E. Morin, and P. Frey-Klett. Appl. Envir. Microbiol., Apr 2012; 78: 3020 – 3024.

- **Metal-Contaminated Stream Sediment, PowerSoil DNA Isolation Kit, GS 454 FLX Pyrosequencing**
  Mercury and Other Heavy Metals Influence Bacterial Community Structure in Contaminated Tennessee Streams
  Tatiana A. Vishnivetskaya, Jennifer J. Mosher, Anthony V. Palumbo, Zamin K. Yang, Mircea Podar, Steven D. Brown, Scott C. Brooks, Baohua Gu, George R. Southworth, Meghan M. Drake, Craig C. Brandt, and Dwayne A. Elias, Appl. Envir. Microbiol., Jan 2011; *77*: 302 – 311.

- **Skin Swabs, PowerSoil DNA Isolation Kit,**

Pyrosequencing
Forensic identification using skin bacterial communities
Noah Fierer, Christian L. Lauber, Nick Zhou, Daniel McDonald, Elizabeth K. Costello, and Rob Knight
PNAS, Apr 2010; 107: 6477 – 6481.

- **Oral swabs, PowerSoil DNA Isolation Kit, 454 Life Sciences FLX Sequencer**
The bacterial microbiota in the oral mucosa of rural Amerindians Monica Contreras, Elizabeth K. Costello, Glida Hidalgo, Magda Magris, Rob Knight, and Maria G. Dominguez-Bello, Microbiology, Nov 2010; 156: 3282 – 3287.

- **Breast Tissue, FFPE DNA Isolation Kit, SOLiD Sequencing**
Identification of high-confidence somatic mutations in whole genome sequence of formalin-fixed breast cancer specimens Yost SE, Smith EN, Schwab RB, Bao L, Jung H, Wang X, Voest E, Pierce JP, Messer K, Parker BA, Harismendy O, Frazer KA Nucleic Acids Res. 2012 Apr 6, 1-12