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DNA Purification

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WHAT CRITERIA COULD YOU CONSIDER WHEN SELECTING A PURIFICATION STRATEGY?

How Much Purity Does Your Application Require?

What contaminants will affect your immediate and downstream application(s)? As discussed below and in Chapter 1, "Planning for Success in the Laboratory," time and money can be saved by determining which contaminants need not be removed. For example, some PCR applications might not require extensively purified DNA. Cells can be lysed, diluted, and amplified without any further steps. Another reason to accurately determine purity requirements is that yields tend to decrease as purity requirements increase.

How Much Nucleic Acid Can Be Produced from a Given Amount of Starting Material?

While it is feasible to mathematically calculate the total amount of nucleic acid in a given sample, and values are provided in the research literature (Sambrook et al., 1989; Studier and Moffat, 1986; Bolivar et al., 1977; Kahn et al., 1979; Stoker et al., 1982), the yields from commercial purification products and noncommercial purification strategies are usually significantly less than these maxima, sometimes less than 50%. Since recoveries will vary with sample origin, consider making your plans based on yields published for samples similar if not identical to your own.

Do You Require High Molecular Weight Material?

The average size of genomic DNA prepared will vary between commercial products and between published procedures.

How Important Is Speed to Your Situation?

Some purification protocols are very fast and allow isolation of nucleic acids within 30 minutes, but speed usually comes at the price of reduced yield and/or purity, especially when working with complex samples.

How Important Is Cost?

Reagents obviously figure into the cost of a procedure, but the labor required to produce and apply the reagents of purification should also be considered.

How Important Is Reproducibility (Robustness) of the Procedure?

Some methods will not give consistent quality and quantity. When planning long-term or high-throughput extractions, validate your methods for consistency and robustness.

What Interferes with Nucleic Acid Purification?

Nuclease

One of the major concerns of nucleic acid purification is the ubiquity of nucleases. The minute a cell dies, the isolation of DNA turns into a race against internal degradation. Samples must be lysed fast and completely and lysis buffers must inactivate nucleases to prevent nuclease degradation.

Most lysis buffers contain protein-denaturing and enzyme-inhibiting components. DNases are much easier to inactivate than RNases, but care should be taken not to reintroduce them during or after purification. All materials should be autoclaved or baked four hours at 300°F to inactivate DNases and RNases, or you should use disposable materials. Use only enzymes and materials guaranteed to be free of contaminating nucleases. Where appropriate, work on ice or in the cold to slow down potential nuclease activity.

Smears and lack of signal, or smeared signal alone, and failure to amplify by PCR are indicative of nuclease contamination. The presence of nuclease can be verified by incubating a small aliquot of your sample at 37°C for a few hours or overnight, followed by evaluation by electrophoresis or hybridization. If nuclease contamination is minor, consider repurifying the sample with a procedure that removes protein.

Shearing

Large DNA molecules (genomic DNA, bacterial artificial chromomoses, yeast artificial chromosomes) can be easily sheared during purification. Avoid vortexing, repeated pipetting (especially through low-volume pipette tips), and any other form of mechanical stress when the isolate is destined for applications that require high molecuar weight DNA.

Chemical Contaminants

Materials that interfere with nucleic acid isolation or downstream applications involving the purified DNA can originate from the sample. Plants, molds, and fungi can present a challenge because of their rigid cell wall and the presence of polyphenolic components, which can react irreversibly with nucleic acids to create an unusable final product.

The reagents of a DNA purification method can also contribute contaminants to the isolated DNA. Reagents that lyse and solubilize samples, such as guanidinium isothiocyanate, can inhibit some enzymes when present in trace amounts. Ethanol precipitation of the DNA and subsequent ethanol washes eliminate such a contaminant. Phenol can also be problematic. If you experience problems with DNA purified by a phenol-based strategy, apply chloroform to extract away the phenol. Phenol oxidation products may also damage nucleic acids; hence re-distilled phenol is recommended for purification procedures.

A mixture of chloroform and phenol is often employed to maximize the yield of isolated DNA; the chloroform reduces the amount of the DNA-containing aqueous layer at the phenol interphase. Similar to phenol, residual chloroform can be problematic, and should be removed by thorough drying. Drying is also employed to remove residual ethanol. Overdried DNA can be difficult to dissolve, so drying should be stopped shortly after the liquid can no longer be observed. Detailed procedures for the above extraction, precipitation and washing steps can be found in Sambrook, Fritsch, and Maniatis (1989) and Ausubel et al. (1998).

Ammonium ions inhibit T4 polynucleotide kinase, and chloride can poison translation reactions (Ausubel et al., 1998). The common electrophoresis buffer, TBE (Tris, borate, EDTA) can inhibit enzymes (Ausubel et al., 1998) and interfere with transformation due to the increased salt concentration (Woods, 1994). Phosphate buffers may also inhibit some enzymes, namely T4 Polynucleotide kinase (Sambrook et al., 1989), alkaline phosphatase (Fernley, 1971), Taq DNA polymerase (Johnson et al., 1995), and Poly A polymerase from *E. coli* (Sippel, 1973). Agarose can also be a problem but some enzyme activity can be recovered by adding BSA to 500 μg/ml final concentration (Ausubel et al., 1998). EDTA can protect against nuclease and heavy metal damage, but could interfere with a downstream application.

The anticoagulant heparin can contaminate nucleic acids isolated from blood, and should be avoided if possible (Grimberg et al., 1989). Taq DNA polymerase is inhibited by heparin, which

can be resolved by the addition of heparinase (Farnert et al., 1999). Heparin also interacts with chromatin leading to release of denatured/nicked DNA molecules (Strzelecka, Spitkovsky, and Paponov, 1983). Narayanan (1996) reviews the effects of anticoagulants.

What Practices Will Maximize the Quality of DNA Purification?

The success of DNA purification is dependent on the initial quality of the sample and its preparation. It would be nice to have a simple, straightforward formula that applies to all samples, but some specimens have inherent limitations. The list below will help guide your selection and provide remedies to nonideal situations:

1. Ideally start with fresh sample. Old and necrotic samples complicate purification. In the case of plasmid preparations, cell death sets in after active growth has ceased, which can produce an increase in unwanted by-products such as endotoxins that interfere with purification or downstream application.

   The best growth phase of bacterial cultures for plasmid preparations may be strain dependent. During the log phase of bacterial culture, actively replicating plasmids are present that are "nicked" during replication rather than being supercoiled. Still some researchers prefer mid to late log phase due to the high ratio of DNA to protein and low numbers of dead cells. Others only work with plasmids that have grown just out of log phase to avoid co-purification of nicked plasmid.

   If old samples can't be avoided, scaling up the purification can compensate for losses due to degradation. PCR or dot blotting is strongly recommended to document the integrity of the DNA.

2. Process your sample as quickly as possible. There are few exceptions to this rule, one being virus purification. When samples can't be immediately purified, snap freeze the intact sample in liquid nitrogen or hexane on dry ice (Franken and Luyten, 1976; Narang and Seawright, 1990) or store the lysed extract at –80°C. Commercial products, such as those from Ambion, Inc., can also protect samples from degradation prior to nucleic acid purification. Samples can also be freeze-dried, as discussed below in the question, *How Can You Maximize the Storage Life of Purified DNA?*.

3. Thorough, rapid homogenization is crucial. Review the literature to determine if your sample requires any special physical or mechanical means to generate the lysate.

4. Load the appropriate amount of sample. Nothing will impair the quality and yield of a purification strategy more than overloading the system. Too much sample can cause an increase in the viscosity of the DNA preparation and lead to shearing of genomic DNA. If you do not know the exact amount of starting material, use 60 to 70% of your estimate.

How Can You Maximize the Storage Life of Purified DNA?

The integrity of purified DNA in solution could be compromised by nuclease, pH below 6.0 and above 9.0, heavy metals, UV light, and oxidation by free radicals. EDTA is often added to chelate divalent cations required for nuclease activity and to prevent heavy metal oxidative damage. Tris-based buffers will provide a safe pH of 7 to 8 and will not generate free radicals, as can occur with PBS (Miller, Thomas, and Frazier, 1991; Muller and Janz, 1993). Free-radical oxidation seems to be a key player in breakdown and ethanol is the best means to control this process (Evans et al., 2000).

Low temperatures are also important for long-term stability. Storage at 4°C is only recommended for short periods (days) (Krajden et al., 1999). Even though some studies have shown that storage under ethanol is safe even at elevated temperatures (Sharova, 1977), better stability is obtained at –80°C. Storage at –20°C can lead to degradation, but this breakdown is prevented by the addition of carrier DNA. RNA stored in serum has also been shown to degrade at –20°C (Halfon et al., 1996).

Another approach for intermediate storage is freeze drying DNA-containing samples intact (Takahashi et al., 1995). The DNA within freeze-dried tissue was stable for 6 months, but RNA began degrading after 10 weeks of storage. The control of moisture and temperature had a significant effect on shelf life of samples. The long term stability of DNA-containing samples is still being investigated (Visvikis, Schlenck, and Maurice, 1998), but some companies offer specialized solutions (e.g., RNA Later™ from Ambion, Inc.) allowing storage at room temperature.

ISOLATING DNA FROM CELLS AND TISSUES

What Are the Fundamental Steps of DNA Purification?

The fundamental processes of DNA purification from cells and tissues are sample lysis and the segregation of the nucleic acid away from contaminants. While DNA is more or less universal to all species, the contaminants and their relative amounts will differ

considerably. The composition of fat cells differs significantly from muscle cells. Plants have to sustain high pressure, contain chloroplasts packed with chromophores, and often have a very rigid outer cell wall. Bacteria contain lipopolysaccharides that can interfere with purification and cause toxicity problems when present in downstream applications. Fibrous tissues such as heart and skeletal muscle are tough to homogenize. These variations have to be taken into consideration when developing or selecting a lysis method.

Lysis

Detergents are used to solubilize the cell membranes. Popular choices are SDS, Triton X-100, and CTAB(hexadecyltrimethyl ammonium bromide). CTAB can precipitate genomic DNA, and it is also popular because of its ability to remove polysaccharides from bacterial and plant preparations (Ausubel et al., 1998).

Enzymes attacking cell surface components and/or components of the cytosol are often added to detergent-based lysis buffers. Lysozyme digests cell wall components of gram-positive bacteria. Zymolase, and murienase aid in protoplast production from yeast cells. Proteinase K cleaves glycoproteins and inactivates (to some extent) RNase/DNase in 0.5 to 1% SDS solutions. Heat is also applied to enhance lysis. Denaturants such as urea, guanidinium salts, and other chaotropes are applied to lyse cells and inactivate enzymes, but extended use beyond what is recommended in a procedure can lead to a reduction in quality and yield.

Sonication, grinding in liquid nitrogen, shredding devices such as rigid spheres or beads, and mechanical stress such as filtration have been used to lyse difficult samples prior to or in conjunction with lysis solutions. Disruption methods are discussed at *http://www.thescientist.com/yr1998/nov/profile2_981109.html*.

Segregation of DNA from Contaminants

The separation of nucleic acid from contaminants are discussed below within the question, *What Are The Strengths and Limitations of Contemporary Purification Methods?*

DNA Precipitation

To concentrate nucleic acids for resuspension in a more suitable buffer, solvents such as ethanol (75–80%) or isopropanol (final concentration of 40–50%) are commonly used in the presence of salt to precipitate nucleic acids (Sambrook, Fritsch, and Maniatis,

1989; Ausubel et al., 1998). If volume is not an issue, ethanol is preferred because less salt will coprecipitate and the pellet is more easily dried. Polyethylene glycol (PEG) selectively precipitates high molecular weight DNA, but it is also more difficult to dry and can interfere with downstream applications (Hillen, Klein, and Wells, 1981). Trichloroacetic acid (TCA) precipitates even low MW polymers down to (5 kDa) (*http://biotech-server.biotech.ubc.ca/biotech/bisc437/lecture/e-na-isoln/na-isoln3.html*), but nucleic acids cannot be recovered in a functional form after precipitation.

Salt is essential for DNA precipitation because its cations counteract the repulsion caused by the negative charges of the phosphate backbone. Ammonium acetate is useful because it is volatile and easily removed, and at high concentration it selectively precipitates high molecular weight molecules. Lithium chloride is often used for RNA because Li^+ does not precipitate double-stranded DNA, proteins, or carbohydrates, although the single-stranded nucleic acids must be above 300 nucleotides. To efficiently precipitate nucleic acids, incubation at low temperatures (preferably ≤–20°C) for at least 10 minutes is required, followed by centrifugation at 12,000 × *g* for at least five minutes. Temperature and time are crucial for nucleic acids at low concentrations, but above 0.25 mg/ml, precipitation may be carried out at room temperature. Additional washing steps with 70% ethanol will remove residual salt from pelleted DNA. Pellets are dried in a speed vac or on the bench and are resuspended in water or TE (10 mM Tris, 1 mM EDTA). Do not attempt to precipitate nucleic acids below a concentration of 20 ng/ml unless carrier such as RNA, DNA, or a high molecular weight co-precipitant like glycogen is added. In the range from 20 ng/ml to 10 μg/ml, either add carrier or extend precipitation time, and add more ethanol. Polyethylene glycol (PEG) precipitation is even more concentration dependent and will only work at DNA concentrations above 10 μg/ml (Lis and Schleif, 1975). Pellets will dissolve better in low-salt buffers (water or TE) and at concentrations below 1 mg/ml. Gentle heating can also help to redissolve nucleic acids

What Are the Strengths and Limitations of Contemporary Purification Methods?

Salting out and DNA Precipitation

Mechanism

Some of the first DNA isolation methods were based on the use of chaotropes and cosmotropes to separate cellular components

based on solubility differences (Harrison, 1971; Lang, 1969). A chaotrope increases the solubility of molecules ("salting-in") by changing the structure of water, and as the name suggests, the driving force is an increase in entropy. A cosmotrope is a structure-maker; it will decrease the solubility of a molecule ("salting-out"). Guanidium salts are common chaotropes applied in DNA purification. Guanidinium isothiocyanate is the most potent because both cation and anion components are chaotropic. Typical lyotropes used for salting out proteins are ammonium and potassium sulfate or acetate. An all solution based nucleic acid purification can be performed by differentially precipitating contaminants and nucleic acids.

Cells are lysed with a gentle enzyme- or detergent-based buffer (often SDS/proteinase K). A cosmotrope such as potassium acetate is added to salt out protein, SDS, and lipids but not the bulk of nucleic acids. The white precipitate is then removed by centrifugation. The remaining nucleic acid solution is too dilute and in a buffer incompatible with most downstream applications, so the DNA is next precipitated as described above.

Features

Protocols and commercial products differ mainly in lysis buffer composition. Yields are generally good, provided that sample lysis was complete and DNA precipitation was thorough. These procedures apply little mechanical stress, so shearing is generally not a problem.

Limitations

If phenolic contaminants (i.e., from plants) are a problem, adding 1% polyvinylpyrrolidine to your extraction buffer can absorb them (John, 1992; Pich and Schubert, 1993; Kim et al., 1997). Alternatively, add a CTAB precipitation step to remove polysaccharides (Ausubel et al., 1998).

Extraction with Organic Solvents, Chaotropes, and DNA Precipitation

Mechanism

Chaotropic guanidinium salts lyse cells and denature proteins, and reducing agents (β-mercaptoethanol, dithiothreitol) prevent oxidative damage of nucleic acids. Phenol, which solubilizes and extracts proteins and lipids to the organic phase, sequestering them away from nucleic acids, can be added directly to the lysis buffer, or a phenol step could be included after lysis with either

GTC- or SDS-based buffers as above. GTC/phenol buffers often require vortexing or vigorous mixing.

The affinity of nucleic acids for this two-phase extraction system is pH dependent. Acidic phenol is applied in RNA extractions because DNA is more soluble in acidic phenol; smaller DNA molecules (<50 kb) will be found in the organic phase and larger DNA molecules (>50 kb) in the interphase. When purifying RNA via this procedure, it is essential to shear the DNA to ensure a light interphase.

Phenol titrated to a pH of 8 is used to separate DNA from proteins and lipids, since DNA is insoluble in basic phenol. Whether protocols call for a GTC/phenol, a GTC, or an SDS based step followed by phenol, it is best to follow a phenol extraction with chloroform in order to extract residual phenol from the aqueous phase. Phenol is highly soluble in chloroform, and chloroform is not water soluble. Remaining lipids may also be removed by this step. Phenol extractions are followed by nucleic acid precipitation steps as described above.

Features

Though caustic and toxic, this strategy still has wide use because yield, purity, and speed are good, and convenient for working with small numbers of samples.

Limitations

If lysis is incomplete, the interphase between organic and aqueous layers becomes very heavy and difficult to manipulate, and may trap DNA. Phenol is not completely insoluble in water, so if chloroform steps are skipped, residual phenol can remain and interfere with downstream applications. High salt concentrations can also lead to phase inversion, where the aqueous phase is no longer on top (problematic if colorless phenol is used). Diluting the aqueous phase and increasing the amount of phenol will correct this inversion. When working with GTC/phenol-based extraction buffers, cross-contamination of RNA with DNA, and vice versa, is frequent.

Glass Milk/Silica Resin-Based Strategies

Mechanism

Nucleic acids bind to glass milk and silica resin under denaturing conditions in the presence of salts (Vogelstein and Gillespie, 1979). Recent findings indicate that binding of some nucleic

acids might even be feasible under nondenaturing conditions (Neudecker and Grimm, 2000). The strong, hydrophobic interaction created in the presence of chaotropic substances can be easily disrupted by removal of salt. The adsorption is followed by wash steps, usually with salt/ethanol which will not interfere with the strong binding of nucleic acids but will wash away remaining impurities and excess chaotrope. Depending on the protocol, this can be followed by a low salt/ethanol wash step that can lead to a reduction in yield. Finally nucleic acids are eluted from the glass in a salt or TE buffer. Nucleic acids are then ready for use.

Most methods create a denaturing adsorption environment by using guanidium salts for one-step lysis and binding. The strength of the binding depends on the cation used to shield the negative charges of the phosphate backbone and the pH (Romanowski et al., 1991). Slightly acidic pH and divalent cations, preferably magnesium, seem to work best.

Differences between glass milk, silica resin, and powdered glass consist mainly in capacity and adsorption strength, a function of impurities present in the binding resins. Diatomaceous earths seem to have an especially high binding capacity (*http://www.nwfsc.noaa.gov/protocols/dna-prep.html*). Pure silica oxide has the lowest affinity to nucleic acids (Boom et al., 1990), but this can improve recovery even though initial binding capacity is lower.

Glass milk is silica presuspended in chaotropic buffer, whereas the silica resin is a solid, predispensed matrix usually found in spin or vacuum flow-through format. Glass milk gives more flexibility for scale of prep, predispensed resin is more convenient for high-throughput applications. Glass milk or silica-based kits are available from numerous vendors, and even though the basic principle is the same, there can be significant differences in efficiency, purity, and yield.

Features

DNA purification based on hydrophobic adsorption to glass or silica is fast, simple, straightforward, and scalable. No additional time-consuming and yield-reducing precipitation steps are required. Depending on binding and wash buffer composition, very good yield and purity values are obtained. This purification approach can also allow restriction digestion/ligation reactions directly on the glass surface, improving transformation efficiency of complex ligation mixtures (Maitra and Thakur, 1994).

Limitations

One of the dangers of silica-based strategies is underloading the sample. Even though yields are good, there is always sample loss due to some remaining material on the resin or filter. The smaller the DNA fragment, the tighter is the interaction. Oligonucleotide primers are actually removed because binding to the glass becomes virtually irreversible. Underloading can become a critical issue when working with small samples and large volumes of glass milk or silica filter.

Some of the older methods utilized unstable buffer components, such as NaI, that tended to oxidize over time, leading to very poor recoveries. Some procedures required the addition of reagents to produce functional wash or elution buffers. If the concentrations were incorrect, or if volatile reagents (i.e., ethanol) were added and the buffers stored long term, these buffers lost their effectiveness. Incomplete sample lysis can be problematic because intact cells may also bind to silica and lyse under low- or no-salt elution conditions, leading to degradation of nucleic acids. Incomplete ethanol removal after wash steps will cause the problems described earlier for ethanol precipitation (discussed below under the question *What Are The Fundamental Steps Of DNA Purification?*). Ethanol must be completely removed from the samples after wash steps to avoid problems such as diffusion out of agarose gel wells ("unloadable" DNA/RNA) or undigestable DNA. Overdrying will lead to irreversible binding of nucleic acids to the resin severely impairing yields.

Anion Exchange (AIX) Based Strategies

Mechanism

Nucleic acids are very large anions with a charge of –1/base and –2/bp; hence they will bind to positively charged purification resins (commonly referred to as anion exchangers). After washes in low-salt buffers, the DNA is eluted in a high-salt buffer. AIX strategies are applied to purify genomic and plasmid DNA.

Logic might suggest that the greater the strength of the anion exchanger, the more DNA it would bind (and more tightly), which would make for superior DNA purification. In practice, however, if an anion exchanger is too strong, most DNA is never recovered. This is especially problematic when working with small samples and with spun column formats. Forcing liquid through porous chromatography resins via centrifugation does not allow for even flow rates, hence resolution is poor. For this reason some spun column plasmid purification procedures advise the recovery of

only a portion of the potential total material to avoid contamination by genomic DNA. Procedures where the buffer flows through columns packed with AIX resins under the force of gravity (as in standard column chromatography) can overcome this problem, but are slower. Gravity flow-based columns can clog if lysis is incomplete or if removal of protein or lipid is incomplete. Resolution is very much flow rate dependent, and tight control of linear flow rates on HPLC or FPLC™ systems are superior to gravity flow and/or spun column formats when it comes to resolution and scale-up.

Features

These methods can produce very pure DNA, but the yields in small-scale applications tend to be low, especially in spun column formats.

Limitations

Not the most robust method, and recoveries tend to be lower, and the final elution step of AIX protocols involves high-salt buffers. The 0.7 to 2 M sodium chloride eluate needs to be desalted, usually by a precipitation step, which decreases recovery and increases the overall procedure time. The binding capacities tend to be low (0.25–2 mg/ml of resin), increase with pH, and decrease with increasing size of the DNA. The amount of RNA present in the sample will also affect binding capacity because RNA will compete with DNA for binding.

Hydroxyapatite (HA) Based Strategies

Mechanism

Nucleic acids bind to crystalline calcium phosphate through the interaction of calcium ions on the hydroxyapatite and the phosphate groups of the nucleic acids. An increase in competing free phosphate ions from 0.12 to 0.4 M will elute nucleic acids, with single-stranded nucleic acids eluting before double-stranded DNA. The entire experiment needs to be run at 60°C for thermal elution (Martinson and Wagenaar, 1974) or in the presence of formamide at room temperature (Goodman et al., 1973).

Sodium phosphate buffers are most commonly used; the phosphate salt affects the selectivity of the resin (Martinson and Wagenaar, 1974). Nucleic acids may also be eluted by increasing the temperature until nucleic acid strands melt and elute from the column.

Features

Excellent separation of single-stranded from double-stranded DNA molecules.

Limitations

The quality and performance of hydroxyapatite can vary from batch to batch and between manufacturers. Thermal elution procedures require reliable temperature control, but fluctuations occur because of lack of heat-regulated chromatography equipment. These elevated temperatures can also produce bubbles in the buffer that can interfere with the separation. Hydroxyapatite has poor mechanical stability. Hydroxyapatite procedures often employ high-salt buffers and lead to sample dilution, requiring an additional precipitation step.

For these reasons hydroxyapatite is not extensively referenced. It is mostly limited to subtractive cDNA cloning (Ausubel et al., 1998), removal of single-stranded molecules, and DNA reassociation analysis (Britten, Graham, and Newfeld, 1974).

What Are the Steps of Plasmid Purification?

Alkaline Lysis and Boiling Strategies

Mechanism (Small Scale)

Plasmid purification holds a special challenge because the target DNA must be purified from DNA contaminants. Isolation strategies take advantage of the physical differences between linear, closed, and supercoiled DNA. Alkaline lysis (Birnboim and Doly, 1979), boiling, and all other denaturing methods exploit the fact that closed DNA will renature quickly upon cooling or neutralizing, while the long genomic DNA molecules will not renature and remain "tangled" with proteins, SDS, and lipids, which are salted out. Whether boiling or alkaline pH is the denaturing step, the renaturing step is usually performed in the cold to enhance precipitation or salting-out of protein and contaminant nucleic acids.

Buffer 1 of an alkaline lysis procedure contains glucose to buffer the effects of sodium hydroxide added in step 2, and lysozyme, to aid cellular breakdown which prevents plasmid from becoming trapped in cellular debris. Buffer 2 contains SDS and NaOH. SDS denatures proteins and NaOH denatures DNA, both plasmid and genomic, and proteins, and partially breaks down RNA. Buffer 3 contains an acidic potassium acetate solution that will salt out proteins by complexing SDS with potassium and precipitating out a mix of SDS, K^+, proteins, and denatured genomic

DNA. Supercoiled plasmids and RNA molecules will remain in solution.

Another method lyses cells by a combination of enzymatic breakdown, detergent solubilization, and heat (Holmes and Quigley, 1981). The lysis buffer usually contains lysozyme, STE, and Triton X-100 or CTAB. Bacterial chromosomal DNA remains attached to the membrane and precipitates out. Again, the aqueous supernatant generated by this method contains plasmid and RNA.

Polyethylene glycol (PEG) has been used to separate DNA molecules by size, based on it's size-specific binding to DNA fragments (Humphreys, Willshaw, and Anderson, 1975; Hillen, Klein, and Wells, 1981). A 6.5% PEG solution can be used to precipitate genomic DNA selectively from cleared bacterial lysates. Trace amounts of PEG may be removed by a chloroform extraction.

Isolation of plasmid DNA by cesium chloride centrifugation in the presence of ethidium bromide (EtBr) is especially useful for large-scale DNA preparations. The interaction of EtBr with DNA decreases the density of the nucleic acid; because of its supercoiled conformation and smaller size, plasmid incorporates less EtBr than genomic DNA, enhancing separation on a density gradient.

Chromatographic methods such as anion exchange and gel filtration may also be used to purify plasmids after lysis. For chromatography, RNA removal prior to separation is essential because the RNA will interfere with and contaminate the separation process. RNase A treatments (Feliciello and Chinali, 1993), RNA-specific precipitation (Mukhopadhyay and Mandal, 1983; Kondo et al., 1991), tangential flow filtration (Kahn et al., 2000), and nitrocellulose filter binding (Levy et al., 2000a, 2000b) have been employed to desalt, concentrate, and generally prepare samples for column purification.

Limitations

The efficiency of plasmid purification will vary with the host cell strain due to differences in polysaccharide content and endonuclease—End A+ strains such as HB101 (Ausubel et al., 1998). Recombination impaired hosts are often selected when producing plasmids prone to deletion and rearrangement of cloned inserts (Summers and Sherratt, 1984; Biek and Cohen, 1986). The University of Birmingham's Web site gives useful links to research strain genotypes and characteristics at *http://web.bham.ac.uk/bcm4ght6/res.html*, as does the *E. coli* Genetic Stock Center at Yale Univeristy (*http://cgsc.biology.yale.edu*).

Whenever nucleic acids are denatured, there is a risk of irreversible denaturation. Never increase the denaturation time beyond what is recommended, and ensure that pH values are accurate for neutralization. Prolonged high pH or heat exposure may lead to more contamination with genomic DNA (Liou et al., 1999) and nicked, open, and irreversibly denatured plasmid. The pH of solution 3 of an alkaline lysis procedure needs to be pH 5.5 to precipitate out SDS/protein/genomic DNA. Effects of changing criticial parameters have been studied in detail (Kieser, 1984). These protocols have been modified to purify cosmids, but larger DNA molecules will not renature as well as small plasmids. Most methods work well for plasmids up to 10 kb; above 10 kb, denaturation has to be milder (Hogrefe and Friedrich, 1984; Azad, Coote, and Parton, 1992; Sinnett, Richer, and Baccichet, 1998).

The yield of low copy number plasmids can be improved dramatically by adding chloramphenicol (Norgard, Emigholz, and Monahan, 1979) or spectinomycin (300 μg/ml; Amersham Pharmacia Biotech, unpublished observations), which prevent replication of chromosomal but not plasmid DNA. However, extended exposure to such agents have also been shown to damage DNA in vitro (Skolimowski, Knight, and Edwards, 1983).

Resources

Plasmid purification methodology could fill an entire book of its own. Traditional chromatography has been applied to isolate large- and small-scale preparations of plasmid from a variety of hosts. Techniques include gel filtration, anion exchange, hydrophobic interaction chromatography, single-strand affinity matrix (Pham, Chillapagari, and Suarez, 1996; Yashima et al., 1993a, b), triple helix resin, silica resin, and hydroxyapatite in a column as well as microtiter plate format. Plasmid purification procedures are reviewed in O'Kennedy et al. (2000), Neudecker and Grimm (2000), Monteiro et al. (1999), Ferreira et al., 2000. Ferreira et al. (1999), Ferreira et al. (1998), Huber (1998), Lyddiatt and O'Sullivan (1998), and Levy et al. (2000a).

CsCl Purification

Mechanism

The separation of DNA from contaminants based on density differences (isopycnic centrifugation) in CsCl gradients remains an effective if slow method. High *g* forces cause the migration of dense Cs^+ ions to the bottom of the tube until centripetal force and force of diffusion have reached an equilibrium.

Within a CsCl gradient, polysaccharides will assume a random coil secondary structure, DNA a double-stranded intermediate density conformation, and RNA, because of its extensive secondary structure, will have the highest density. Dyes that bind to nucleic acids and alter their density have been applied to enhance their separation from contaminants. The binding of EtBr decreases the apparent density of DNA. Supercoiled DNA binds less EtBr than linear DNA, enhancing their separation based on density differences. CsCl centrifugation is most commonly applied to purify plasmids and cosmids in combination with EtBr. Ausubel et al. (1998) also provides protocols for the isolation of genomic DNA from plants and bacteria.

Features

Cesium gradient formation requires long periods (at least overnight) of ultracentrifugation and are caustic, yet remain popular because they produce high yield and purity and are more easily scaled up.

Limitations

GC content of DNA correlates directly to its density. Equilibrium density of DNA can be calculated as $1.66 + 0.098 \times \%GC$ (Sambrook, Fritsch, and Maniatis, 1989). The density of very GC-rich DNA can be sufficiently high as to cause it to migrate immediately adjacent to RNA in a CsCl gradient. If too much sample is loaded onto a gradient, or if mistakes were made during preparation of the gradient, separation will be incomplete or ineffective.

Affinity Techniques

Triple helix resins have been used to purify plasmids and cosmids (Wils et al., 1997). This approach takes advantage of the adoption of a triple rather than a double helix conformation under the proper pH, salt, and temperature conditions. Triple helix affinity resins are generated by insertion of a suitable homopurine sequence into the plasmid DNA and crosslinking the complement to a chromatographic resin of choice. The triple helix interaction is only stable at mild acidic pH; it dissociates under alkaline conditions. The interaction at mildly acid pH is very strong (Radhakrishnan and Patel, 1993). This strong affinity allows for extensive washing that can improve the removal of genomic DNA, RNA, and endotoxin during large-scale DNA preparations.

A radically different approach applies covalent affinity chromatography to trap contaminants. Some of the examples include

a chemically modified silica resin that irreversibly binds protein via an imide bond (Ernst-Cabrera and Wilchek, 1986), and a modified silica resin that covalently binds to polysaccharides via a cyclic boric acid ester, trapping proteins in the process. This latter reaction was initially applied to purify tRNA (McCutchan, Gilham, and Soll, 1975); it is described in greater detail by O'Neill et al. (1996). Some commercial products use salts to generate an irreversible protein precipitate that forms a physical barrier between the aqueous nucleic acid and the solid protein phase. Affinity-based technologies are also described at *http://www.polyprobe.com/about.htm* and at *http://www.edgebio.com*.

Features

Affinity techniques can produce excellent yields. Impressive purity is achieved if the system is not overloaded; if need be, the affinity steps can be repeated to further enhance purity. These methods are especially recommended when sample is precious and limited or purity requirements are very high.

Limitations

Cost, which may be minimized by reuse of resin. However cleaning of resin and its validation may be problematic.

WHAT ARE THE OPTIONS FOR PURIFICATION AFTER IN VITRO REACTIONS?

Spun Column Chromatography through Gel Filtration Resins

Mechanism

As in standard, column-based gel filtration (size exclusion) chromatography, a liquid phase containing sample and contaminant passes through a resin. The smaller molecules (contaminant) enter into the resin's pores, while the larger molecules (desired product) will pass through without being retained. Properly applied, this procedure can accomplish quick buffer exchange, desalting, removal of unincorporated nucleotides, and the elimination of primers from PCR reactions (gel filtration spin columns will *not* remove enzyme from a reaction; this requires organic extraction) to name a few applications.

Features and Limitations

These procedures are fast, efficient, and reproducible when the correct resins and centrifugation conditions are applied to the

appropriate samples. Viscous solutions are not compatible with this technique.

One should not approach spun column, size exclusion chromatography with a care-free attitude. The exclusion limits based on standard chromatography should not be automatically applied to spun columns. Spinning makes such standard chromatography data obsolete. Before you apply a resin or a commercial spun column in an application, verify that the product has been successfully used in your particular application. Just because a resin has a pore size that can exclude a 30 nucleotide long oligo isn't a guarantee that a column with this resin will remove all or even most of the primer from a PCR reaction.

Manufacturers will optimize the columns and/or the procedures to accomplish a stated task. The presence of salt (100–150 mM NaCl) improves the yield of radiolabeled probes from one type of spun column, but the presence of Tris can interfere with the preparation of templates for automated sequencing (Amersham Pharmacia Biotech, unpublished observations, and *Nucleic Acid Purification Guide*, 1996). Too much *g* force, and the contaminants can elute with the desired product; too little *g* force, and the desired product is not eluted. If the volume you're eluting off the spun column is much greater or less than the volume you've loaded, the applied *g* force is no longer correct.

If you plan to create a spun column from scratch, consider the following:

- Sample volumes should be kept low with respect to the volume of resin, usually below a tenth to a twentieth of the column volume to allow for good resolution.
- Gel filtration resin will not resolve components efficiently (purity >90%) unless the largest contaminant is at least 20 times smaller than the smallest molecule to be purified.
- Desalting, where the size difference between ions and biomolecule is >>1:20, works well even at high flow rates.

Filter Cartridges

Mechanism

Filtration under the influence of vacuum suction or centrifugation operates under principles similar to gel filtration. Semipermeable membranes allow passage of small molecules such as salts, sugars, and so forth, but larger molecules such as DNA are retained. Since the retentate rather than an eluate is collected, samples will be concentrated. Ultrafiltration and microfiltration are reviewed by Munir (1998) and Schratter et al., (1993).

Features and Limitations

Filtration procedures are fast and reproducible provided that the proper *g* force or vacuum are applied. Membranes can clog from debris when large molecules accumulate at the membrane surface (but don't pass through), forming a molecule-solute gel layer that prevents efficient removal of remaining contaminants. As with gel filtration spun columns, filtration will not remove enzymes from reaction mixes unless the enzyme is small enough to pass through the membrane, which rarely is the case.

Silica Resin-Based Strategies

Mechanism

The approach is essentially identical to that described for silica resins used to purify DNA from cells and tissue, as described above.

Features and Limitations

Advantages and pitfalls are basically the same. Recoveries from solutions are between 50 to 95% and from agarose gels, 40 to 80%. Fragments smaller than 100 bp or larger than 10 kb (gel), or 50 kb (solution), are problematic. Small fragments may not elute unless a special formulation of glass milk is used (e.g., Glass Fog™ by 5′-3′ Eppendorf), and large fragments often shear and give poor yield. Depending on the capture buffer formulation, RNA and single-stranded molecules may or may not bind.

When using silica resins to bind nonradioactively labeled probes, investigate the stability of the label in the presence of chaotrope used for the capture and washing steps. Chaotropes create an environment harsh enough to attack contaminants such as proteins and polysaccharides, so it would be prudent to assume that any protein submitted to such an environment will lose its function. Nucleic acids covalently tagged with horseradish peroxidase or alkaline phosphatase are less likely to remain active after exposure to harsh denaturants. The stability of the linker connecting the reporter molecule to the DNA should also be considered prior to use.

Also consider the effect of reporter molecules/labels on the ability of DNA to bind to the resin. Nucleic acids that elute well in the unlabeled state may become so tightly bound to the resin by virtue of their label that they become virtually "sorbed out" and hence are unrecoverable. This is a notable concern when the reporter molecule is hydrophobic.

Isolation from Electrophoresis Gels

This subject is also addressed in Table 8.4 of Chapter 8, "Electrophoresis." Purification through an electrophoresis gel (refered to hereon as *gel purification*) is the only choice if the objective is to simultaneously determine the fragment size and remove contaminants. It could be argued that gel purification is really a two-step process. The first step is filtration through the gel and separation according to size. The second step is required to remove impurities introduced by the electrophoresis step (i.e., agarose, acrylamide, and salts). There are several strategies to isolate DNA away from these impurities, as summarized in Table 7.1 and discussed in detail below.

All these procedures are sensitive to the size and mass of the amount of gel segment being treated. The DNA should appear on the gel as tight bands, so in the case of agarose gels, combs must be inserted straight into the gel. When isolating fragments for cloning or sequencing, minimize exposure to UV light; visualize the bands at 340 nm. Any materials coming in contact with the gel slice should be nuclease free. Crush or dice up the gel to speed up your extraction method.

Polyacrylamide Gels

Crush and Soak

With time, nucleic acids diffuse out of PAGE gels, but recovery is poor. The larger the fragment size, the longer is the elution time required for 50% recovery. Elevated temperatures (37°C) accelerate the process. A variation of the crush and soak procedure is available at *http://www.ambion.com/techlib/tb/tb_171.html*. A procedure for RNA elution is provided at *http://grimwade.biochem.unimelb.edu.au/bfjones/gen7/m7a4.htm*.

Electroelution

Depending on the instrumentation, electroelution can elute DNA into a buffer-filled well, into a dialysis bag, or onto a DEAE cellulose paper strip inserted into the gel above and below the band of interest. Inconsistent performance and occasionally difficult manipulations make this approach less popular.

Specialized Acrylamide Crosslinkers

These are discussed in Chapter 8, "Electrophoresis."

Table 7.1 Comparison of Nucleic Acid Punfication Methods from Gel and/or Solution

Method	Used for	Yield	Speed	Benefits	Limitations
l.m.p. agarose with or without agarase, or phenol[a] (Ausubel et al., 1998, Hengen, 1994)	DNA fragments and/or plasmids	Up to 70%; typically 50%	From 0.5 to 2 h depending on downstream purification method chosen	Agarase especially useful for large fragments or cosmids, since treatment is very gentle; some applications may allow treatment directly in melted gel slice (e.g., ligation, labeling with Klenow)	Requires an additional purification step; carrier often required for precipitation because solutions are dilute; extraction with phenol is caustic, especially hot phenol
"Freeze and squeeze" (Benson and Spencer, 1984, Ausubel et al., 1998)	DNA, RNA fragments	40–60% (for fragments up to 5 kb, above that, lower)	Slow; freeze for at least 15′ or up to 2 h, then follow with precipitation	Very gentle; good for larger molecules; very inexpensive	Low yield
"Crush and soak" (for acrylamide gels) Sambrook, Fritsch, and Maniatis, 1989	Mostly RNA, but works for any nucleic acid	40–70% depending on elution time, concentration, etc.	2–4 h depending on fragment size	Allows high sample loads; best for reactions generating larger quantities of probe (in vitro transcription) to compensate for low recoveries	Significant chance of contamination when working with radioactivity; poor recovery
Gel filtration, desalting Ausubel et al., 1998; Sambrook, Fritsch, and Maniatis, 1989	DNA and RNA; fragment size must be well above exclusion limit of resin	>90% for fragments above exclusion limit	3–15 min, depending on column format (gravity flow vs. spun column); primer removal protocols might require 30 min	Fast, with high purity and yield	Often leads to dilution of sample; only removes small contaminants, difficult to monitor separation of noncontaminants without radioactivity
Glass milk/Na I (Ausubel et al., 1998; Hengen, 1994)	Usually DNA fragments and plasmids from agarose gel or solution	50–75% from solution, 40–70% from gel	0.25–1.5 h	Fast, versatile; removes most major contaminants (proteins, primers, salts); efficient one-step purification	Yield; Na I stability; shearing

Silica/guanidinium salts (Ausubel et al., 1998; Gribanov et al., 1996; Boom et al., 1990; Vogelstein and Gillespie, 1979)	Usually DNA fragments and plasmids from agarose gel or solution	80–90% from solution, up to 80% from gel	5 min from solution; up to 1 h from gel	Fast, versatile; removes most major contaminants (proteins, primers, salts); efficient one-step purification	Shearing; yields very much resin/protocol-dependent
Filter cartridges in combination with freezing or without freezing (Leonard et al., 1998; Blattner et al. 1994; Li and Ownby, 1993; Schwarz and Whitton, 1992)	Concentration and desalting of DNA/RNA samples; desalting of freeze-squeeze eluted agarose gel slices	Up to 95% depending on fractionation range of membrane and nonspecific interaction with membrane	Often 2–5 min; depends on required concentration and salt tolerance of downstream applications	Fast; simultaneous desalting and concentration possible	Molecular size cutoff is not always well defined; not recommended for primer removal unless size cutoff well above primer size; will not remove large contaminants like proteins
Ethanol or isopropanol precipitation (Sambrook, Fritsch, and Maniatis, 1989, Ausubel et al., 1998)	Any nucleic acid as long as concentration is >10 μg/ml and at least 0.1 M monovalent cations are present	Up to 95% depending on protocol	20 min-overnight depending on sample concentration	Easy to monitor (visible pellet); noncaustic, robust, high yields, versatile in combination with different precipitation salts	More time-consuming; difficult for multiple samples, pellet may be lost; may not remove protein contaminants
Electroelution (Ausubel et al., 1998; Bostian, Lee, and Halvorson, 1979, Dretzen et al., 1981, Girvitz et al., 1980; Henrich, Lubitz, and Fuchs, 1982; Smith, 1980; Strongin et al., 1977; Tabak and Flavell, 1978)	Mostly DNA fragments from gel; elution onto DEAE membrane does not work well for fragments >2 kb	Up to 90% for fragments <1 kb, very small fragments between 50–60%, large fragments as low as 20%	2–4 h; or 1–3 h for DEAE elution	Few reagents required; not caustic/toxic; yields for fragments up to 1 kb are quite high	More difficult to monitor; only for fragments from 0.05–20 kb; need to be combined with a second method

Source: Data in table aside from references also based on average values found in catalogs and online of the following manufacturers: Ambion, Amersham Pharmacia Biotech, Amresco, Bio101, BioRad, Biotronics, BioTecx, Bioventures, Boehringer Mannheim/Roche, Clontech, CPG, Dynal, Edge Biosystems, Epicentre, FMC, Genhunter, Genosys, Gentra Systems, GIBCO Life Technologies, Invitrogen,Ligochem, 5′ 3′ (Eppendorf), Macromolecular Resource Center, Maxim Biotech, MBI Fermentas PerSeptive (now Life Technologies), Nucleon, Promega, Qiagen, Schleicher & Schuell, Sigma, Stratagene, USB, Worthington. For additional data, see DeFrancesco (1999), who provides a fragment purification products table and a comparison of size, agarose limitiations, buffer compatibility, time requirements, yield, capcity, and volume for isolation of DNA from agarose gels.

[a] If hot phenol is used, avoid phenol chloroform which can severly impair yields (Ausubel et al., 1998).

Agarose

Detailed procedures regarding the methodology discussed below are available at *http://www.bioproducts.com/technical/dnarecovery.shtml#elution*.

Freeze and Squeeze

Comparable to crush and soak procedures for polyacrylamide gels, this method is easy and straightforward, but it suffers from poor yields.

Silica-Based Methods

Silica or glass milk strategies are fast and efficient because the same buffer can be used for dissolving the gel and capturing the nucleic acid. Problems may arise when agarose concentrations are very high (larger volumes of buffers are required, reducing DNA concentration), nucleic acid concentration is very low (recovery is poor), fragment size is too small or large (irreversible binding and shearing, respectively), or if agarose dissolution is incomplete. Finally, some silica resins will not bind nucleic acids in the presence of TBE. When in doubt use TAE buffers (Ausubel et al., 1998).

Low Melting Point Agarose (LMP Agarose)

LMP agarose melts between 50 and 65°C. Some applications tolerate the presence of LMP agarose (Feinberg and Vogelstein, 1984), but for those that don't, DNA can be precipitated directly or isolated by phenol treatment (*http://mycoplasmas.vm.iastate.edu/lab_site/methods/DNA/elutionagarose.html*). Another option is to digest the agarose with agarase. This DNA can either be used directly for some applications or be precipitated to remove small polysaccharides and concentrate the sample. Glass beads are another way to follow up on melting your agarose slice as mentioned above. The negative aspect of LMP agarose is that sample load and resolution power are lower than in standard agarose procedures.

What Are Your Options for Monitoring the Quality of Your DNA Preparation?

The limitations of assessing purity by $A_{260}:A_{280}$ ratio are described in Chapter 4 (spectrophotometer section). Nevertheless, $A_{260}:A_{280}$ ratios are useful as a first estimation of quality. For northerns and southerns, try a dot blot. Success of PCR reactions can be scouted out by amplification of housekeeping genes. If

restriction fragments do not clone well, try purifying a control piece of DNA with the same method and religate.

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8

RNA Purification

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SELECTING A PURIFICATION STRATEGY

Do Your Experiments Require Total RNA or mRNA?

One of the first decisions that the researcher has to make when detecting or quantitating RNA is whether to isolate total RNA or poly(A)-selected RNA (also commonly referred to as mRNA). This choice is further complicated by the bewildering array of RNA isolation kits available in the marketplace. In addition the downstream application influences this choice. The following section is a short primer in helping make that decision.

From a purely application point of view, total RNA might suffice for most applications, and it is frequently the starting material for applications ranging from the detection of an mRNA species by Northern hybridization to quantitation of a message by

RT-PCR. The preference for total RNA reflects the challenge of purifying enough poly(A) RNA for the application (mRNA comprises <5% of cellular RNA), the potential loss of a particular message species during poly(A) purification, and the difficulty in quantitating small amounts of purified poly(A) RNA. If the data generated with total RNA do not meet your expectations, using poly(A) RNA instead might provide the sensitivity and specificity that your application requires. The pros and cons with either choice are discussed below. Your experimental data will provide the best guidance in deciding whether to use total or poly(A) RNA. Be flexible and open minded; there are many variables to consider when making this decision.

Two situations where using poly(A) RNA is essential are cDNA library construction, and preparation of labeled cDNA for hybridization to gene arrays. To avoid generating cDNA libraries with large numbers of ribosomal clones, and nonspecific labeled cDNA it is crucial to start with poly(A) RNA for these procedures.

The next section gives a brief description of the merits and demerits of using total RNA or poly(A) RNA in some of the most common RNA analysis techniques. Chapter 14, "Nucleic Acid Hybridization," discusses the nuances and quirks of these procedures in greater depth. For detailed RNA purification protocols, see Krieg (1996) Rapley and Manning (1998), and Farrel (1998).

Northern Hybridizations

Northern analysis is the only technique available that can determine the molecular weight of an mRNA species. It is also the least sensitive. Total RNA is most commonly used in this assay, but if you don't detect the desired signal, or if false positive signals from ribosomal RNA are a problem, switching to poly(A) RNA might be a good idea. Since only very small amounts of poly(A) RNA are present, make sure that it is feasible and practical to obtain enough starting cells or tissue. Theoretically you could use as much as 30 μg of poly(A) RNA in a Northern, which is the amount found in approximately 1 mg of total RNA. Will it be practical and feasible for you to sacrifice the cells or tissue required to get this much RNA? If not, use as much poly(A) RNA as is practical.

One drawback to using poly(A) RNA in Northern hybridizations is the absence of the ribosomal RNA bands, which are ordinarily used to gauge the quality and relative quantity of the RNA samples, as discussed later in this chapter. Fortunately there are other strategies besides switching to poly(A) RNA that can be used to increase the sensitivity of Northern hybridizations. You could alter the hybridization conditions of the DNA probe

(Anderson, 1999), or you could switch to using RNA probes in the hybridization, which are 3- to 5-fold more sensitive than DNA probes in typical hybridization buffers (Ambion Technical Bulletin 168, and references therein). Dramatic differences in the sensitivity of Northern blots can also be seen from using different hybridization buffers.

If you remain dissatisfied with the Northern data, and you are not interested in determining the size of the target, switching to a more sensitive technique such as nuclease protection or RT-PCR might help. Nuclease protection assays, which are 5- to 10-fold more sensitive than traditional membrane hybridizations, can accommodate 80 to 100 μg of nucleic acid in a single experiment. RT-PCR can detect extremely rare messages, for example, 400 copies of a message in a 1 μg sample as described by Sun et al. (1998). RT-PCR is currently the most sensitive of the RNA analysis techniques, enabling detection and quantitation of the rarest of targets. Quantitative approaches have become increasingly reliable with introduction of internal standards such as in competitive PCR strategies (Totzke et al., 1996; Riedy et al., 1995).

Dot/Slot Blots

In this procedure, RNA samples are directly applied to a membrane, either manually or under vacuum through a filtration manifold. Hybridization of probe to serial dilutions of sample can quickly generate quantitative data about the expression level of a target. Total RNA or poly(A) RNA can be used in this assay. Since the RNA is not size-fractionated on an agarose gel, a potential drawback to using total RNA in dot/slot blots is that signal of interest cannot be distinguished from cross-hybridization to rRNA. Switching to poly(A) RNA as the target source might alleviate this problem. However, it is crucial that relevant positive and negative controls are run with every dot/slot blot, whether the source of target nucleic acid is total RNA or poly(A) RNA.

Hybridization to Gene Arrays and Reverse Dot Blots

Gene arrays consist of cDNA clones (sometimes in the form of PCR products, sometimes as oligonucleotides) or the corresponding oligos spotted at high density on a nylon membrane, glass slide, or other solid support. By hybridizing labeled cDNA probes reverse transcribed from mRNA, the expression of potentially hundreds of genes can be simultaneously analyzed. This procedure requires that the labeled cDNA be present in excess of the target spotted on the array. This is difficult to achieve unless poly(A) RNA is used as template in the labeling reaction.

Ribonuclease Protection Assays

Either total RNA or poly(A) RNA can be used as starting material in nuclease protection assays. However, total RNA usually affords enough sensitivity to detect even rare messages, when the maximum amount (as much as 80 to 100 μg) is used in the assay. If the gene is expressed at extremely low levels, requiring week-long exposure times for detection, a switch to poly(A) RNA might prove beneficial and may justify the added cost. Although very sensitive, nuclease protection assays do require laborious gel purification of the full-length probe to avoid getting confusing results.

RT-PCR

RT-PCR is the most sensitive method for detecting and quantitating mRNA. Theoretically, even very low-abundance messages can be detected with this technique. Total RNA is routinely used as the template for RT-PCR, (Frohman, 1990) but some cloning situations and rare messages require the use of poly(A) RNA (Amersham Pharmacia Biotech, 1995).

Note that one school of thought concerning RT-PCR considers it advisable to treat the sample RNA with DNase I, since no purification method produces RNA completely free of contaminating genomic DNA. RT-PCR is sensitive enough that even very small amounts of genomic DNA contamination can cause false positives. A second school of thought preaches avoidance of DNase I, as discussed in Chapter 11, "PCR."

cDNA Library Synthesis

As mentioned earlier, high-quality mRNA that is essentially free of ribosomal RNA is required for constructing cDNA libraries. Unacceptably high backgrounds of ribosomal RNA clones would be produced if total RNA were reverse transcribed to prepare cDNA.

Is It Possible to Predict the Total RNA Yield from a Certain Mass of Tissue or Number of Cells?

The data provided in this section are based on experimentation at Ambion, Inc. using a variety of samples and different purification products. The reader is cautioned that these are theoretical estimates, and yields can vary widely based on the type of tissue or cells used for the isolation, especially when dealing with difficult samples, as discussed later. The importance of rapid and complete tissue disruption, and homogenizing at subfreezing tem-

peratures cannot be overemphasized. In addition, yields from very small amounts of starting material are subject to the law of diminishing returns. Thus, if the option is available, always choose more starting material rather than less. Samples can be pooled together, if possible, to maximize yields.

For example, 5 mg of tissue or 2.5×10^6 cells yields about 10 μg of total RNA, comprised of 8 μg rRNA, 0.3 μg mRNA, 1.7 μg tRNA, and other RNA. In comparison, 1 g of tissue or 5×10^8 cells yields about 2 mg of total RNA, comprised of 1.6 mg rRNA + 60 μg mRNA + 333 μg tRNA and other RNA.

Is There Protein in Your RNA Preparation, and If So, Should You Be Concerned?

Pure RNA has an $A_{260}:A_{280}$ absorbance ratio of 2.0. However, for most applications, a low $A_{260}:A_{280}$ ratio probably won't affect the results. Researchers at Ambion, Inc. have used total RNA with $A_{260:280}$ ratios ranging from 1.4 to 1.8 with good results in RNase protection assays, Northern analysis, in vitro translation experiments, and RT-PCR assays. If protein contamination is suspected to be causing problems, additional organic extractions with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 mixture) may remove the contaminant. Residual phenol can also lower the $A_{260}:A_{280}$ ratio, and inhibit downstream enzymatic reactions. Chloroform/isoamyl alcohol (24:1) extraction will remove residual phenol. Chapter 4, "How to Properly Use And Maintain Laboratory Equipment," discusses other artifacts that raise and lower the $A_{260:280}$ ratio. Some tissues will consistently produce RNA with a lower $A_{260:280}$ ratio than others; the $A_{260:280}$ ratio for RNA isolated from liver and kidney tissue, for example, is rarely above 1.7.

Is Your RNA Physically Intact? Does It Matter?

The integrity of your RNA is best determined by electrophoresis on a formaldehyde agarose gel under denaturing conditions. The samples can be visualized by adding 10 μg/ml of Ethidium Bromide (EtBr) (final concentration) to the sample before loading on the gel. Compare your prep's 28S rRNA band (located at approximately 5 Kb in most mammalian cells) to the 18S rRNA band (located at approximately 2.0 Kb in most mammalian cells). In high-quality RNA the 28S band should be approximately twice the intensity of the 18S band (Figure 8.1).

The most sensitive test of RNA integrity is Northern analysis using a high molecular weight probe expressed at low levels in the tissues being analyzed. However, this method of quality control is very time-consuming and is not necessary in most cases.

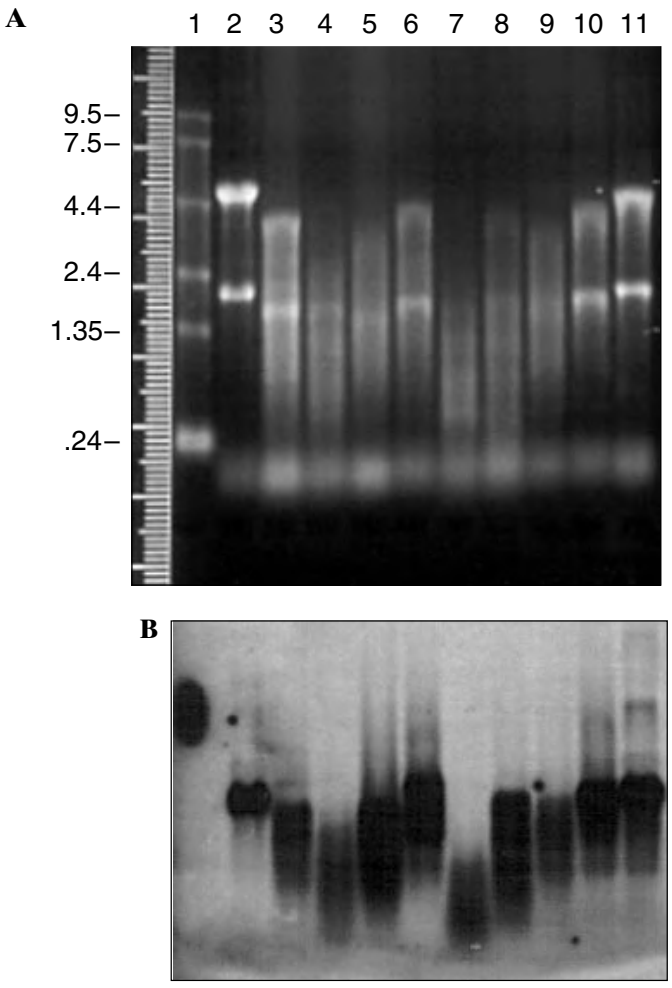

Figure 8.1 Assessing quality of RNA preparation via agarose gel electrophoresis (*A*) This gel shows total RNA samples (5µg/lane) ranging from high-quality, intact RNA (lane 2) to almost totally degraded RNA (lane 7). Note that as the RNA is degraded, the 28S and 18S ribosomal bands become less distinct, the intensity of the ribosomal bands relative to the background staining in the lane is reduced, and there is a significant shift in their apparent size as compared to the size standards. (*B*) This is an autorad of the same gel after hybridization with a biotinylated GAPDH RNA probe followed by nonisotopic detection. The exposure is 10 minutes the day after the chemiluminescent substrate was applied. Note that the signal in lane 2, from intact RNA, is well localized with minimal smearing, whereas the signals from degraded RNA samples show progressively more smearing below the bands, or when the RNA is extremely degraded, no bands at all (lane 7). Reprinted by permission of Ambion, Inc.

Northern analysis is not tolerant of partially degraded RNA. If samples are even slightly degraded, the quality of the data is severely compromised. For example, even a single cleavage in 20% of the target molecules will decrease the signal on a Northern blot by 20%. Nuclease protection assays and RT-PCR analyses will tolerate partially degraded RNA without compromising the quantitative nature of the results.

Which Total RNA Isolation Technique Is Most Appropriate for Your Research?

There are three basic methods of isolating total RNA from cells and tissue samples. Most rely on a chaotropic agent such as guanidium or a detergent to break open the cells and simultaneously

inactivate RNases. The lysate is then processed in one of several ways to purify the RNA away from protein, genomic DNA, and other cellular components. A brief description of each method along with the time and effort involved, the quality of RNA obtained, and the scalability of the procedures follow.

Guanidium-Cesium Chloride Method

Slow, laborious procedure, but RNA is squeaky clean; unsuitable for large sample numbers; little if any genomic DNA remains.

This method employs guanidium isothiocyanate to lyse cells and simultaneously inactivate ribonucleases rapidly. The cellular RNA is purified from the lysate via ultracentrifugation through a cesium chloride or cesium trifluoroacetate cushion. Since RNA is more dense than DNA and most proteins, it pellets at the bottom of the tube after 12 to 24 hours of centrifugation at ≥32,000 rpm.

This classic method yields the highest-quality RNA of any available technique. Small RNAs (e.g., 5S RNA and tRNAs) cannot be prepared by this method as they will not be recovered (Mehra, 1996). The original procedures were time-consuming, laborious, and required overnight centrifugation. The number and size of samples that could be processed simultaneously were limited by the number of spaces in the rotor. Commercial products have been developed to replace this lengthy centrifugation (Paladichuk, 1999) with easier, less time-consuming methods. However, if the goal were to isolate very high-quality RNA from a limited number of samples, this would be the method of choice (Glisin, Crkuenjakov and Byus, 1974).

Single- and Multiple Step Guanidium Acid-Phenol Method

Faster, fewer steps, prone to genomic DNA contamination, somewhat cumbersome if large sample numbers are to be processed.

The guanidium-acid phenol procedure has largely replaced the cesium cushion method because RNA can be isolated from a large number of samples in two to four hours (although somewhat cumbersome) without resorting to ultracentrifugation. RNA molecules of all sizes are purified, and the technique can be easily scaled up or down to process different sample sizes. The single-step method (Chomczynski and Sacchi, 1987) is based on the propensity of RNA molecules to remain dissolved in the aqueous phase in a solution containing 4 M guanidium thiocyanate, pH 4.0, in the presence of a phenol/chloroform organic phase. At this low pH, DNA molecules remain in the organic phase, whereas proteins and other cellular macromolecules are retained at the interphase.

It is not difficult to find researchers who swear by GITC—phenol procedures because good-quality RNA, free from genomic DNA contamination is quickly produced. However, a second camp of researchers avoid these same procedures because they often contain contaminating genomic DNA (Lewis, 1997; S. Herzer, personal communication). There is no single explanation for these polarized opinions, but the following should be considered.

Problems can occur in the procedure during the phenol/chloroform extraction step. The mixture must be spun with sufficient force to ensure adequate separation of the organic and aqueous layers; this will depend on the rotor as can be seen in Table 8.1. For best results the centrifuge brake should not be applied, nor should it be applied to gentler settings.

The interface between the aqueous and organic layers is another potential source of genomic contamination. To get high-purity RNA, avoid the white interface (can also appear cream colored or brownish) between the two layers; leave some of the aqueous layer with the organic layer. If RNA yield is crucial, you'll probably want as much of the aqueous layer as possible, again leaving the white interface. In either case you can repeat the organic extraction until no white interface is seen.

Residual salt from the precipitation step, appearing as a huge white pellet, can interfere with subsequent reactions. Excessive salt should be suspected when a very large white pellet is obtained from an RNA precipitation. Excess salt can be removed by washing the RNA pellet with 70% EtOH (ACS grade). To the RNA pellet, add about 0.3 ml of room temperature (or –20°C) 70% ethanol per 1.5 ml tube or approximately 2 to 3 ml per 15 to 40 ml tube. Vortex the tube for 30 seconds to several minutes to dislodge the pellet and wash it thoroughly. Recover the RNA with a low speed spin, (approximately 3000 × *g*; approximately 7500 rpm in a microcentrifuge, or approximately 5500 rpm in a SS34 rotor), for 5 to 10 minutes at room temperature or at 4°C.

Table 8.1 Spin Requirements for Phenol Chloroform Extractions

Volume Tube	Speed	Spin Time
1.5 ml	10,000 × *g*	5 minutes
2.0 ml	12,000 × *g*	5 minutes
15 ml	12,000× *g*	15 minutes
50 ml	12,000× *g*	15 minutes

Remove the ethanol carefully, as the pellets may not adhere tightly to the tubes. The tubes should then be respun briefly and the residual ethanol removed by aspiration with a drawn out Pasteur pipet. Repeat this wash if the pellet seems unusually large.

Non-Phenol-Based Methods

Very fast, clean RNA, can process large sample numbers, possible genomic contamination.

One major drawback to using the guanidium acid-phenol method is the handling and disposal of phenol, a very hazardous chemical. As a result phenol-free methods, based on the ability of glass fiber filters to bind nucleic acids in the presence of chaotropic salts like guanidium, have gained favor. As with the other methods, the cells are first lysed in a guanidium-based buffer. The lysate is then diluted with an organic solvent such as ethanol or isopropanol and applied to a glass fiber filter or resin. DNA and proteins are washed off, and the RNA is eluted at the end in an aqueous buffer.

This technique yields total RNA of the same quality as the phenol-based methods. DNA contamination can be higher with this method than with phenol-based methods (Ambion, Inc., unpublished observations). Since these are column-based protocols requiring no organic extractions, processing large sample numbers is fast and easy. This is also among the quickest methods for RNA isolation, usually completed in less than one hour.

The primary problem associated with this procedure is clogging of the glass fiber filter by thick lysates. This can be prevented by using a larger volume of lysis buffer initially. A second approach is to minimize the viscosity of the lysate by sonication (on ice, avoid power settings that generate frothing) or by drawing the lysate through an 18 gauge needle approximately 5 to 10 times. This step is more likely to be required for cells grown in culture than for lysates made from solid tissue. If you are working with a tissue that is known to be problematic (i.e., high in saccharides or fatty acids), an initial clarifying spin or extraction with an equal volume of chloroform can prevent filter-clogging problems. A reasonable starting condition for the clarifying spin is 8 minutes at $7650 \times g$. If a large centrifuge is not available, the lysate can be divided into microcentrifuge tubes and centrifuged at maximum speed for 5 to 10 minutes. Avoid initial clarifying spins on tissues rich in glycogen such as liver, or plants containing high molecular-weight carbohydrates. If you generate a clogged filter, remove the remainder of the lysate using a pipettor, place it on top of a fresh filter, and continue with the isolation protocol using both filters.

What Protocol Modifications Should Be Used for RNA Isolation from Difficult Tissues?

RNA isolation from some tissues requires protocol modifications to eliminate specific contaminants, or tissue treatments prior to the RNA isolation protocol. Fibrous tissues and tissue rich in protein, DNA and RNases, present unique challenges for total RNA isolation. In this section we address problems presented by difficult tissues and offer troubleshooting techniques to help overcome these problems. A separate section will discuss the homogenization needs of various sample types in greater detail.

Web sites that discuss similar issues are *http://www.nwfsc.noaa.gov/protocols/methods/RNAMethodsMenu.html* and *http://grimwade.biochem.unimelb.edu.au/sigtrans.html.*

Fibrous Tissue

Good yields and quality of total RNA from fibrous tissue such as heart and muscle are dependent on the complete disruption of the starting material when preparing homogenates. Due to low cell density and the polynucleate nature of muscle tissue, yields are typically low; hence it is critical to make the most of the tissue at hand. Pulverizing the frozen tissue into a powder while keeping the tissue completely frozen (use a chilled mortar and pestle) is the key to isolating intact total RNA. It is critical that there be no discernible lumps of tissue remaining after homogenization.

Lipid and Polysaccharide–Rich Tissue

Plant and brain tissues are typically rich in lipids, which makes it difficult to get clean separation of the RNA and the rest of the cellular debris. When using phenol-based methods to isolate total RNA, white flocculent material present throughout the aqueous phase is a classic indicator of this problem. This flocculate will not accumulate at the interface even after extended centrifugation. Chloroform : isoamyl alcohol (24 : 1) extraction of the lysate is probably the best way to partition the lipids away from the RNA. To minimize loss, back-extract the organic phase, and then clean up the recovered aqueous RNA by extraction with phenol : chloroform : isoamyl alcohol (25 : 24 : 1).

When isolating total RNA from plant tissue using a non-phenol-based method, polyvinylpyrrolidone-40 (PVP-40) can be added to the lysate to absorb polysaccharide and polyphenolic contaminants. When the lysate is centrifuged to remove cell debris, these contaminants will be pelleted with the PVP (Fang, Hammar, and Grumet, 1992; see also the chapter by Wilkins and Smart, "Isolation of RNA from Plant Tissue," in Krieg, 1996, for a list of refer-

ences and protocols for removing these contaminants from plant RNA preps). Centrifugation on cesium trifluoroacetate has also been shown to separate carbohydrate complexes from RNA (Zarlenga and Gamble, 1987).

Nucleic Acid and Nuclease-Rich Tissue

Spleen and thymus are high in both nucleic acids and ribonuclease. Good homogenization is the key to isolating high-quality RNA from these tissues. Tissue samples should be completely pulverized on dry ice, under liquid nitrogen, to facilitate rapid homogenization in the lysis solution, which inhibits nucleases. Cancerous cells and cell lines also contain high amounts of DNA and RNA, which makes them unusually viscous, causing poor separation of the organic and aqueous phases and potentially clogging RNA-binding filters. Increasing the ratio of sample mass : volume of lysis buffer can help alleviate this problem in filter-based isolations. Multiple acid–phenol extractions can be done to ensure that most of the DNA is partitioned into the organic phase during acid-phenol-based isolation procedures. Two to three extractions are usually sufficient; one can easily tell if a lysate is viscous by attempting to pipet the solution and observing whether it sticks to the pipette tip. The DNA in the lysate can alternatively be sheared, either by vigorous and repeated aspiration through a small gauge needle (18 gauge) or by sonication (10 second sonication at 1/3 maximum power on ice, or until the viscosity is reduced).

Hard Tissue

Hard tissue, such as bone and tree bark, cannot be effectively disrupted using a Polytron™ or any other commonly available homogenizer. In this case heavy-duty tissue grinders that pulverize the material using mechanical force are needed. SPEX Certiprep, Metuchen, NJ, makes tissue-grinding mills that chill samples to liquid nitrogen temperatures and pulverize them by shuttling a steel piston back and forth inside a stationary grinding vial.

Bacteria and Yeast

Bacterial and yeast cells can prove quite refractory to isolating good-quality RNA due to the difficulty of lysing them. Another problem with bacteria is the short half-life of most bacterial messages. Lysis can be facilitated by resuspending cell pellets in TE and treating with lysozyme, subsequent to which the actual

extraction steps are performed. A potenial drawback of using lytic enzymes is that they can introduce RNases. Use the highest-quality enzymes to reduce the likelihood of introducing contaminants. Yield and quality from phenol-based extraction protocols can also be improved by conducting the organic extractions at high temperatures (Lin et al., 1996).

Lysis of yeast cells is accomplished by vigorous vortexing in the presence of 0.4 to 0.5 mm glass beads. If using a non-phenol-based procedure for RNA isolation, the lysis can be monitored by looking for an increase in A_{260} readings. Yeast cells can also be treated with enzymes such as zymolase, lyticase, and glucolase to facilitate lysis (Ausubel et al., 1995).

Is a One-Step or Two-Step mRNA–(poly(A) RNA)– Purification Strategy Most Appropriate for Your Situation?

One-step procedures purify poly(A) RNA directly from the starting material. A two-step strategy first isolates total RNA, and then purifies poly(A) RNA from that.

Sample Number

One-step strategies involve fewer manipulations to recover poly(A) RNA. When comparing different one-step strategies, consider that two additional washing steps multiplied by 20 samples can consume significant time and materials, and arguably, faster purification strategies decrease the chance of degradation. Centifugation, magnetics, and other technologies sound appealing and fast, but the true speed of a technique is determined by the total manipulations in a procedure. High-throughput applications such as hybridization of gene arrays are usually best supported by one-step purification procedures.

Sample Mass

The percentage of poly(A) RNA recovery is similar between one- and two-step strategies. So, when experimental sample is limited, a one-step procedure is usually the more practical procedure.

Yield

Commercial one-step products are usually geared to purify small (1–5 μg) or large (25 μg) quantities of poly(A) RNA, and manufacturers can usually provide data generated from a variety of sample types. If you require more poly(A) RNA, a two-step procedure is usually more cost effective.

How Many Rounds of Oligo(dT)–Cellulose Purification Are Required?

One round of poly(A) RNA selection via oligo(dT)–cellulose typically removes 50 to 70% of the ribosomal RNA. One round of selection is adequate for most applications (i.e., Northern analysis and ribonuclease protection assays). A cDNA library generated from poly(A) RNA that is 50% pure is usually sufficient to identify most genes, but to generate cDNA libraries with minimal rRNA clones, two rounds of oligo(dT) selection will remove approximately 95% of the ribosomal RNA. Remember that 20 to 50% of the poly(A) RNA can be lost during each round of oligo(dT) selection, so multiple rounds of selection will decrease your mRNA yield. The use of labeled cDNA to screen gene arrays is severely compromised by the presence of rRNA-specific probes, so two rounds of poly(A) selection might be justified.

Which Oligo(dT)–Cellulose Format Is Most Appropriate?

Resins

Commercial resins are derivatized with oligo(dT) of various lengths at various loading capacities—mass of oligo(dT) per mass of cellulose. The linkage between the oligo(dT) and celluose is strong but not covalent; some nucleic acid will leave the resin during use. Oligo(dT) chains 20 to 50 nucleotides long, bound to cellulose at loading capacities of approximately 50 mg/ml, are commonly used in column and batch procedures. Some suppliers refer to this as Type 7 oligo(dT)-cellulose. The word "Type" refers to the nature of the cellulose. Type 77F cellulose is comprised of shorter strands than Type 7, and it does not provide good flow in a chromatography column. Type 77F does work very well in a batch mode, binding more mRNA than Type 7.

Column Chromatography

Oligo(dT)-cellulose can be scaled up or down using a variety of column sizes. Column dimension isn't crucial, but the frit or membrane that supports the oligo(dT)-cellulose is. The microscopic cellulose fibers can clog the frits and filter discs in a gravity chromatography column. Test the ability of several ml of buffer or water to flow through your column before adding your RNA sample. If your column becomes clogged during use, resuspend the packed resin with gentle mixing, and prepare a new column using a different frit, or do a batch purification on the rescued resin as described below. Some commercial products pack oligo(dT)-cellulose in a syringelike system so that the plunger can forcefully

push through the matrix. The frits in these push-systems accommodate flow under pressure. Applying pressure to a clogged, standard oligo(dT)-cellulose chromatography column usually worsens matters. Occasionally air bubbles become trapped within the spaces of the frit. Gentle pressure or a very gentle vacuum applied to the exit port of the column can release these trapped bubbles and improve flow.

Batch Binding or Spin Columns

Batch binding consists of directly mixing the total RNA with oligo(dT)-cellulose in a centrifuge tube, and using a centrifuge to separate the celluose from the supernate in the wash and elution steps. Batch binding and washing of the matrix and spun columns circumvent the problems of slow flow rates, and clogged columns often experienced with gravity-driven chromatography. Scaling reactions up and down is convenient and economical, using the guidelines of 100 A_{260} units of total RNA per 0.5 g of oligo(dT)-cellulose. Increasing the incubation times for the poly(A) RNA hybridization to the oligo(dT)-cellulose can sometimes increase yields by 5 to 10%.

Tissues that lyse only with difficulty, and viscous lysates, can interfere with oligo(dT) binding by impeding the movement of oligo(dT)–coated particles. Additional lysis buffer, or repeated passage through a fine-gauge (21 gauge) needle with a syringe to shear the DNA and proteins, can reduce this viscosity. Lysates with excessive amounts of particulates should be cleared by centrifugation before attempting to select poly(A) RNA.

Can Oligo(dT)-Cellulose Be Regenerated and Reused?

Oligo(dT)-cellulose can theoretically be regenerated and reused, but the reader is strongly recommended not to do so. The hydroxide wash that regenerates the resin should destroy any lingering mRNA, but it is difficult to prove 100% destruction. Also the more a reagent is manipulated, the more likely it is to become contaminated with trace amounts of RNase. However some researchers still reuse oligo(dT)-cellulose until poor flow or reduced binding leads them to prepare fresh oligo(dT)-cellulose. Be especially wary of regenerated oligo(dT)-cellulose that appears pink or slimy.

If you must reuse oligo(dT)-cellulose, first wash it with 10 bed volumes of elution buffer followed by 10 bed volumes of 0.1 N NaOH. (One bed volume equals the volume of cellulose settled in the column.) The NaOH degrades any RNA remaining after elution. After the 0.1 N NaOH treatment, wash the oligo(dT)-

cellulose with 10 bed volumes of water followed by 10 bed volumes of absolute alcohol. If the regenerated oligo(dT)-cellulose is to be stored for longer than a couple of weeks, dry it under a vacuum and store it with desiccant at –20°C. For short-term storage, refrigerate at 4°C after the NaOH and water washes; desiccation isn't required.

If the oligo(dT)-cellulose is to be reused immediately after removing residual RNA with the NaOH wash, equilibrate the column in 10 bed volumes of elution buffer followed by 10 bed volumes of binding buffer. The column is now ready for sample application.

To use resin that has previously been regenerated and stored, resuspend the oligo(dT)-cellulose in elution buffer, pour into the column, and wash with 10 bed volumes of binding buffer.

Can a Kit Designed to Isolate mRNA Directly from the Biological Sample Purify mRNA from Total RNA?

One-step procedures that obtain mRNA from intact cells or tissue typically employ a denaturing solution to generate a lysate, which is directly added to the oligo(dT)-cellulose. Washing with specific concentrations of salt buffers ultimately separates poly(A) RNA from DNA and other RNA species.

Typically total RNA can be substituted into one-step procedures by skipping the homogenization steps, adjusting the salt concentration of the total RNA to 500 mM and adding this material to the oligo(dT)-cellulose. Consult the manufacturer of your product for their opinion on this approach, and verify the binding capacity of the oligo(dT)-cellulose for total RNA.

MAXIMIZING THE YIELD AND QUALITY OF AN RNA PREPARATION

What Constitutes "RNase-Free Technique"?

Fundamentals

RNase contamination is so prevalent, special attention must be given to the preparation of solutions. Solutions should be prepared in disposable, RNase-free plasticware or in RNase-free glassware prepared in the lab. Glassware can be made RNase-free by baking at 180°C for 8 hours to overnight, or by treating with a commercial RNase decontaminating solution. Alternatively, RNase can be removed by filling containers with 0.1% DEPC, incubating at 37°C for 2 hours, rinsing with sterile water and

then either heating to 100°C for 15 minutes, or autoclaving for 15 minutes to eliminate RNase.

Electrophoresis apparatus used for RNA analysis can be made RNase-free by filling with a 3% hydrogen peroxide solution, incubating for 10 minutes at room temperature and rinsing with DEPC-treated water.

When preparing RNase-free solutions, wear gloves and change them often. Regardless of the method used to prepare RNase-free solutions, keep in mind that they can easily become contaminated after preparation. This occurs when solutions are open and used regularly, or when they are shared with others. It is wise to prepare small volumes of solutions and aliquot larger volumes into RNase-free containers. Solutions should be clearly labeled "RNase-free" to avoid contamination and should only be used with RNase-free pipettes and pipette tips. Also adhere to the maxim "when in doubt, throw it out."

How Does DEPC Inhibit RNase?

The most common method of preparing RNase-free solutions is diethylpyrocarbonate (DEPC) treatment. DEPC inactivates RNases by carboxyethylation of specific amino acid side chains in the protein (Brown, 1991). DEPC is a suspected carcinogen, and it should always be used with the proper precautions.

How Are DEPC-Treated Solutions Prepared? Is More DEPC Better?

Most protocols specify adding DEPC to solutions at a concentration 0.1%, followed by mixing and room temperature incubation for several hours to overnight. The container lid should be loosened for the extended incubation because a considerable amount of pressure can form during the reaction. Finally, the solution is autoclaved; this inactivates the residual DEPC by hydrolysis, and releases CO_2 and EtOH as by-products.

The EtOH by-product can combine with trace carboxylic acid contaminates in the vessel to form volatile esters, which impart a slightly fruity smell to the solution. This does not mean that trace DEPC remains in solution. DEPC has a half-life of 30 minutes in water, and at a DEPC concentration of 0.1%, solutions autoclaved for 15 minutes/liter can be assumed to be DEPC-free. Be aware that increasing the concentration of DEPC to 1% can inhibit more RNase but can also inhibit certain enzymatic reactions, so more is usually not better.

Should You Prepare Reagents with DEPC-Treated Water, or Should You Treat Your Pre-made Reagents with DEPC?

Some researchers prefer to DEPC-treat preprepared solutions, while others opt for preparing DEPC-treated water first and combining it with ultrapure RNase-free powdered reagents. It should be noted that many reagents commonly used in RNA studies contain primary amines, such as Tris, MOPS, HEPES, and PBS, and cannot be DEPC-treated because the amino group "sops up" the DEPC, making it unavailable to inactivate RNase. These solutions should be prepared with ultrapure reagents and DEPC-treated water. When preparing solutions in this manner, use RNase-free spatulas and magnetic stirrers, wear gloves and change them often. Spatulas and magnetic stirrers can be made RNase-free by soaking in 0.1% DEPC followed by autoclaving (as described above for containers) or by using a commercial RNase decontamination solution according to the manufacturer's directions. Either method of solution preparation is acceptable. Other options are commercially prepared RNase-free solutions available from several vendors, or recently-introduced alternatives to DEPC treatment.

How Do You Minimize RNA Degradation during Sample Collection and Storage?

RNase is present in all cells and tissues; hence they must be immediately inactivated when the source organism dies. Samples should be immediately frozen in liquid nitrogen, or immediately disrupted in a chaotropic solution (i.e., GITC). In some cases RNase activity can eventually be restored even in the presence of a chaotrope if the extract is not frozen (Amersham Pharmacia Biotech, unpublished observations). In other experiments homogenized tissue has been stored for at least one week at room temperature, or two months at 4°C without any loss of RNA in a lysis buffer (Ambion, Inc., unpublished observations). A commercial RNase inhibitor also exists that can prevent RNA degradation within mammalian tissue, cells, and some plant tissues stored above freezing temperature for long periods. However periodically sampling the integrity of RNA purified from frozen stock materials is recommended in light of reports of RNA degradation in samples frozen under protective conditions.

Mammalian Tissues and Cells

Tissues can be harvested and immediately immersed in liquid nitrogen. However, large pieces of tissue do not freeze instantaneously, allowing RNase to degrade RNA found in the interior of

the sample. The smaller the tissue pieces, the faster it freezes. Once frozen, tissue should be immediately moved to a –70°C freezer, or stored on dry ice until it can be transferred to a freezer for long-term storage. In frozen tissue, RNA may be stable indefinitely, but periodic sampling for RNase degradation is recommended to avoid unpleasant surprises.

If the sample tissue is relatively soft (see the discussion of disruption methods below), and samples are few, they can be harvested directly into the lysis solution, immediately homogenized, and stored up to 12 months at –70°C without affecting RNA quality. Such lysates can be thawed on ice, an aliquot removed for processing, and refrozen. Firm or hard tissue requires more physical disruption as described below.

Mammalian cells are typically easy to homogenize. After a quick wash in culture media to remove debris, pipetting or vortexing in the presence of lysis solution will usually suffice. Cell lysates should be stored at –70°C. Alternatively, washed cell pellets can be quick-frozen by immersing the tube containing the pellet into liquid nitrogen. The tube can then be transferred to –80°C for long-term storage. The disadvantage to freezing cell pellets is that except for very small ones, they will have to be pulverized in liquid nitrogen for RNA isolation.

Bacteria and Yeast

Most gram-negative bacteria can be pelleted and frozen. Small samples (milliliters) of *E. coli* can be lysed and frozen as described above for mammalian cells; larger volumes (liters) will require enzymatic digestion or isolation procedures that incorporate lysis (e.g., an SDS lysis/isolation procedure). Some gram-positive bacteria and most yeast cells resist disruption and require more aggressive methods as described below.

How Do You Minimize RNA Degradation during Sample Disruption?

Fast and complete lysis of any sample is arguably the most critical element of RNA purification. When purifying RNA from a sample type for the first time, test your homogenization procedure for speed, efficiency, and ease of use in a small-scale experiment. A purification procedure involving 20 precious samples is the wrong time to discover the practical limits of an extraction procedure.

RNase inhibition provided by chaotropes and other reagents can be overwhelmed by adding too much starting material. Follow your procedure's recommendation. Scale up if necessary.

Monitor Disruption

Disruption can usually be monitored by close inspection of the lysate. Visible particulates should not be observed, except when disrupting materials containing hard, noncellular components, such as connective tissue or bone. Disruption of microorganisms, such as bacteria and yeast, can be monitored by spectrophotometry. The A_{260} reading should increase sharply as lysis begins and then level off when lysis is complete. Lysis can also be observed as clarification in the suspension or by an increase in viscosity.

Mammalian Tissues and Cells

Most animal tissues can be processed fresh (unfrozen). It is important to keep fresh tissue cold and to process it quickly (within 30 minutes) after dissection. If tissues are necrotic, the RNA can begin degrading in vivo. Ideally pre-dispense the lysis solution into the homogenizer, and then add the tissue and begin homogenizing. Samples should never be left sitting in lysis solution undisrupted.

Electronic rotor-stator homogenizers (e.g., Polytron) can effectively disrupt all but very hard or fibrous tissues. In addition, they do the job rapidly. If you have access to an electronic homogenizer, for most tissues, you should use it. If you can only use manual homogenizers, soft tissues can be thoroughly disrupted in a Dounce homogenizer, but firm tissues, however, especially connective tissues, will be homogenized more thoroughly in a ground glass homogenizer or TenBroeck homogenizer (available from Bellco, Vineland, NJ). Very hard tissues such as bone, teeth, and some hard tumors may require a milling device as described for yeast. A comparison of tissue disrupters is described in Johnson (1998). Enzymatic methods may also be used for specific eukaryotic tissues, such as collagenase to break down collagen prior to cell lysis.

Animal tissues and any type of relatively large cell pellets that have been frozen after collection must be disrupted by grinding in liquid nitrogen with a mortar and pestle. During this process it is important that the equipment and tissue remain at temperatures well below 0°C. The tissue should be dry and powdery after grinding. After grinding, thoroughly homogenize the sample in lysis solution using a manual or electronic homogenizer. Processing frozen tissue this way is cumbersome and time-consuming, but very effective.

Mammalian cells are normally easy to disrupt. Cells grown in suspension are collected by centrifugation, washed in cold 1× PBS,

and resuspended in a lysis solution. Lysis is completed by immediate vortexing or vigorous pipetting of the solution. Rinse adherent cells in cold 1× PBS to remove culture medium. Then add lysis solution directly to the plate or flask, and scrape the cells into the solution. Finally, transfer the cells to a tube and vortex or pipette to completely homogenize the sample. Placing the flask or plate on ice while washing and lysing the cells will further protect the RNA from endogenous RNases released during the disruption process.

Plant Tissues

Soft, fresh plant tissues can often be disrupted by homogenization in lysis solution alone. Other plant tissues, like pine needles, can be frozen with liquid nitrogen, then ground dry. Some hard woody plant materials may require freezing and grinding in liquid nitrogen or milling. The diversity of plants and plant tissue make it impossible to give a single recommendation for techniques specific to your tissue. (See Croy, 1993, and Krieg, 1996, for guidance in preparing RNA from plant sources.)

Yeast and Fungi

Lysozyme and zymolase are frequently used with bacteria and yeast to dissolve cell walls, envelopes, coats, capsules, capsids, and other structures not easily sheared by mechanical methods (Ausubel et al., 1995). Sonication, homogenization, or vigorous vortexing in a lysis solution usually follows enzymatic treatment. Yeast can be extremely difficult to disrupt because their cell walls may form capsules or nearly indestructible spores. Bead mills that vigorously agitate a tube containing the sample, lysis buffer, and small beads will completely disrupt even these tough cells within a few minutes. Bead mills are available from Biospec Products, Inc., Bartlesville, OK, and Bio 101, Vista, CA. Alternatively, yeast cell walls can be lysed with hot phenol (Krieg, 1996) or digested with zymolase, glucalase, and/or lyticase to produce spheroplasts, which are readily lysed by vortexing in a lysis solution. Check that the enzyme you select is RNase-free.

To disrupt filamentous fungi, scrape the mycelial mat into a cold mortar, add liquid nitrogen, and grind to a fine powder with a pestle. The powder can then be thoroughly homogenized or sonicated in lysis solution to completely solubilize (Puyesky et al., 1997).

Bacteria

Bacteria, like plants, are extremely diverse; therefore it is difficult to make one recommendation for all bacteria. Bead milling

will lyse most gram-positive and gram-negative bacteria, including mycobacteria (Cheung et al., 1994; Mangan et al., 1997; Kormanec and Farkasovshy, 1994). Briefly, glass beads and lysis solution are added to a bacterial cell pellet, and the mixture is milled for a few minutes. Some gram-negative bacteria can be lysed by sonication in lysis solution, but this approach is sufficient only for small cultures (milliliters), not large ones (liters).

Bacterial cell walls can be digested with lysozyme to form spheroplasts, which are then efficiently lysed with vigorous vortexing or sonication in sucrose/detergent lysis solution (Reddy and Gilman, 1998). Gram-positive bacteria usually require more rigorous digestion (increased incubation time and temperature, etc.) than gram-negative organisms (Krieg, 1996; Bashyam and Tyage, 1994).

Is There a Safe Place to Pause during an RNA Purification Procedure?

Ideally RNA should be purified without interruption, no matter which procedure is used. If a pause is unavoidable, stop when the RNA is precipitated or is in the presence of a chaotrope. For example, when using an organic isolation procedure, the RNA isolation can be stopped when the samples have been homogenized in a chaotrophic lysis solution. They can be stored for a few days at –20°C or –80°C without degradation.

What Are the Options to Quantitate Dilute RNA Solutions?

Spectrophotometry

The most common quantitative approach is to dilute a small volume of the RNA prep to meet the sample volume requirement of the cuvette. If the concentration of your RNA stock is low, the absorbance of the diluted RNA may fall outside the linear range of the spectrophotometer (see Chapter 4, "How to Properly Use and Maintain Laboratory Equipment").

Cuvettes are commercially available to accommodate sample volumes below 10 μl; some instruments can accept capillaries that hold less than 1 μl. If your spectrophotometer can tolerate these cuvette's minute sample windows, sample dilution might be unnecessary.

Dilute solutions can be concentrated by precipitation and microfiltration. Centrifugation-based RNase-free concentrators are available from Millipore corporation. (Bedford, MA), and glycogen enhances the precipitation of RNA from dilute solutions (Amersham Pharmacia Biotech, *MRNA Purification Kit Instruction Manual*, 1996). Adjust the NaCl concentration of 1.0 ml of an

aqueous solution of RNA to 300 mM using a 3 M NaCl stock prepared in 10 mM Tris, 1 mM EDTA, pH 7.4. Add 10 μl of a 10 mg/ml glycogen solution (prepared in RNase-free water). Next, add 2.5 ml of ice-cold ethanol. Mix. Chill at –20°C for at least 2 hours, then centrifuge at 4°C for 10 minutes at 12,000 × g to recover the precipitated RNA. Be aware that since it is from a biological source, glycogen can contain protein (e.g., RNase) and nucleic acid (e.g., DNA) contaminants.

The riskiest option is to place your undiluted RNA prep into a cuvette. If this is your only option, carefully rinse the quartz cuvette with concentrated acid (check with your cuvette supplier to determine acid stability) followed by extensive rinsing in RNase-free water. Avoid hydrofluoric acid, which etches quartz and UV grade silica. Concentrated hydrochloric and nitric acid are tolerated by cuvettes of solid quartz or silica, but can damage cuvettes comprised of glued segments. A better option is to treat the cuvette with a commercial RNase decontamination solution.

Fluorometry

An alternative quantitation strategy is staining RNA with dyes such as Ribogreen®, SYBR®Green, and SYBR Gold (all available from Molecular Probes, Eugene, OR). Ribogreen is the most sensitive of these dyes for RNA; it is designed to be detected with a fluorometer for RNA quantitation in solution. With Ribogreen and a fluorometer, 1 to 10 ng/ml RNA can be detected. In contrast, both SYBR Green and SYBR Gold are designed to quantify RNA in a gel-based format, and they require the use of a densitometer or other gel documentation system that allows pixel values to be converted into numerical data. This method provides only rough approximations of the RNA loaded on a gel; it is valid for concentrations of 1 to 5 μg/lane. These dyes do not bind irreversibly to the RNA and do not have negative effects on downstream applications.

WHAT ARE THE OPTIONS FOR STORAGE OF PURIFIED RNA?

RNase activity and pH >8 will destroy RNA. For short-term storage of a few weeks or less, store your RNA in RNase-free Tris-EDTA or 1 mM EDTA at –20°C in aliquots. For long-term storage, RNA should be stored in aliquots at –80°C in TE, 1 mM EDTA, formamide, or as an ethanol/salt precipitation mixture.

TROUBLESHOOTING

A Pellet of Precipitated RNA Is Not Seen at the End of the RNA Purification.

The RNA Pellet Is There, but You Can't See It

- Pellets containing 0.5 to 2.0 μg of RNA should be visible but might not be as obvious as DNA pellets of the same mass. RNA pellets can range from clear to milky white in appearance. Pellets typically form near the bottom of the tube, but can also smear along the side depending on the rotor angle. Colored coprecipitants can help to visualize RNA pellets, but use them only if they are RNase-free. Marking the centrifuge tube to indicate the anticipated location of the pellet can help locate barely visible pellets.
- Remove the solution used to precipitate the RNA. This sometimes makes the pellet easier to see.
- Proceed as if a pellet is present, and quantitate the solution via a spectrophotomoter, fluorometer, or electrophoresis.

The RNA concentration was too low for precipitation by standard techniques

- The efficiency of RNA precipitation can be increased by adding 50 to 150 μg/ml glycogen or 10 to 20 μg/ml linear acrylamide to typical salt/ethanol precipitations. Glycogen does not appear to inhibit cDNA synthesis, Northern, or PCR reactions, but it may contain DNA, which could result in confusing RT-PCR results. Linear acrylamide is free of contaminating nucleic acids, but it is neurotoxic. Exercise great caution when handling RNA precipitated with acrylamide. Refer to manufacturers' Material Safety Data Sheets for more information on toxicity of linear acrylamide solutions.

The RNA pellet is truly absent

Sample Source Issues
Was the sample obtained from an unhealthy source? Did the tissue appear to be necrotic?
Was the sample quantity insufficient for the purification procedure?
Storage Issues
When originally isolated, was the sample allowed to linger at room temperature, or was it flash frozen immediately?

Was it stored in a frost-free freezer, hence subjected to thawing?
Was the pH of the stored preparation below 8.5?

Homogenization Issues

Was the sample immediately homogenized, or was it left intact for any period of time?
Was the extraction fast, thorough, and complete? Was the RNA too dilute to be effectively precipitated?

Was the Pellet Accidentally Discarded While Removing a Supernatant?

Nonsiliconized tubes decrease the likelihood of this happening.

A Pellet Was Generated, but the Spectrophotometer Reported a Lower Reading Than Expected, or Zero Absorbance

Refer to the troubleshooting example in Chapter 2, "Getting What You Need from a Supplier."

Did the RNA completely dissolve? Are visible pellet remnants (usually small white flecks) visible?

- Heat the RNA to 42°C, and vortex vigorously. Remove remaining debris by centrifugation. Overdried RNA pellets can be extremely difficult to resuspend; avoid drying with devices like a Speed Vac.

RNA Was Prepared in Large Quantity, but it Failed in a Downstream Reaction: RT PCR is an Example

Is the RNA at fault?

- Did the first strand cDNA synthesis reaction succeed, and the PCR reaction fail?
- Was the quality of the RNA evaluated?
- Was total RNA or poly(A)RNA used in the reaction? Using poly(A)RNA might work where total RNA failed.
- Was the poly(A)RNA purified once or twice on oligo(dT)cellulose. A second round will increase purity but will decrease yield up to 50%.

Is the RT-PCR reaction at fault?

- Did you test the positive control RNA and PCR primers?
- Did you test your gene specific PCR primers?

My Total RNA Appeared as a Smear in an Ethidum Bromide-stained Denaturing Agarose Gel; 18S and 28S RNA Bands Were not Observed

The RNA was degraded

Is it an electrophoresis artifact?

Did the RNA markers produce the correct banding pattern? If not, the buffers and loading dye could be the problem.

Could the gel be overloaded? 10 to 30 μg/lane of RNA is the maximum amount that should be loaded.

Only a Fraction of the Original RNA Stored at –70°C Remained after Storage for Six Months

The RNA is degraded.

Was the RNA stored as a wet ethanol precipitate or in formamide?

Was the RNA stored as aliquots?

Was the pH of the stored preparation <8.5?

Was the RNA frozen immediately after it was isolated?

Did you verify the calculations used to quantitate the RNA?

The RNA adsorbed to the walls of the storage container.

Is the RNA concentration <0.5 μg/ml, which increases the impact of loss due to adsorbtion?

Is the storage vessel siliconized, which decreases the risk of adsorbtion?

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