Dear Customer,

The QIAGEN® Bench Guide is designed to help you with your laboratory work. Background information, protocols, hints, and tips are provided for purification and analysis of plasmid DNA, genomic DNA, RNA, and proteins, as well as recipes for buffers and solutions. Organized so that you can find the information you need quickly and easily, the Bench Guide will be a useful resource for everyone — from newcomers to the lab to experienced molecular biologists.

In addition to the information provided here, QIAGEN offers a wide range of literature for specific applications. To find out more about these specialized resources, please turn to page 101. Technical information is also provided on the QIAGEN web site at www.qiagen.com. For an overview of the QIAGEN web site, please turn to page 102.

If you have questions about any of the information in the Bench Guide, or about molecular biology in general, please call your local QIAGEN Technical Service Department or your local distributor (see back cover for contact information).

Wishing you continued success with your research projects,

Your QIAGEN Team

Plasmid DNA

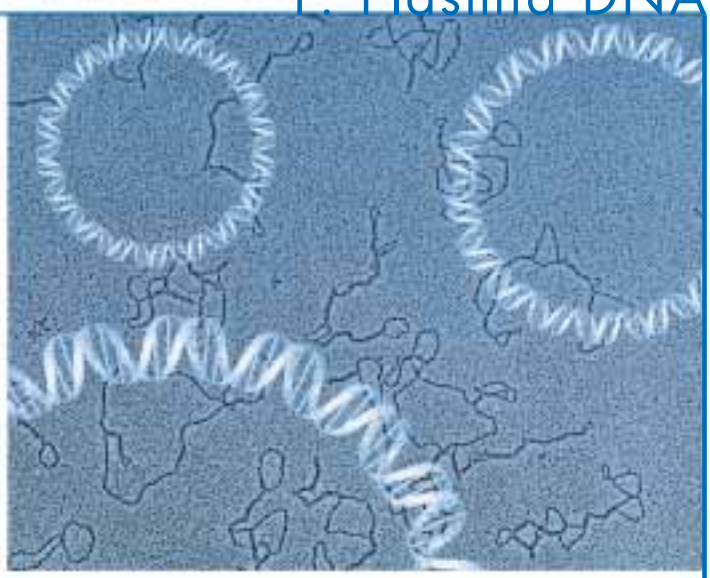
Genomic DNA

RNA

Protein

Appendix

1. Plasmid DNA



Contents

What is Plasmid DNA?

Bacterial plasmids are closed circular molecules of double-stranded DNA that range in size from 1 to >200 kb. They are found in a variety of bacterial species, where they behave as additional genetic units inherited and replicated independently of the bacterial chromosome. However, they rely upon enzymes and proteins provided by the host for their successful transcription and replication.

Plasmids often contain genes that code for enzymes that can be advantageous to the host cell in some circumstances. The encoded enzymes may be involved in resistance to, or production of, antibiotics, resistance to toxins found in the environment (e.g., complex organic compounds), or the production of toxins by the bacteria itself.

Once purified, plasmid DNA can be used in a wide variety of downstream applications such as sequencing, PCR, expression of proteins, transfection, and gene therapy.

This chapter describes common plasmid DNA procedures, including how to make and transform competent cells, how to culture and handle plasmid-containing cells, and how to purify and quantify plasmid DNA.

Competent Cells and Transformation

Protocol 1. Preparation of competent *E. coli*

Protocol 1

Cells that have the ability to take up DNA (from a variety of sources) are termed "competent". Several techniques exist to prepare competent cells and one such technique for preparing competent *E. coli* is given below.

Note: Cells prepared using this protocol are not suitable for electroporation.

Materials

- *E. coli* cells in glycerol stock vial
- LB medium
- LB-agar plates
- Appropriate selective antibiotics
- TFB1 buffer
- TFB2 buffer

For buffer and media compositions, see "Bacterial Culture Media and Buffers", page 92.

1. Remove a trace of *E. coli* cells from the glycerol stock vial with a sterile toothpick or inoculating loop, and streak it out on LB-agar plates containing an appropriate concentration of the relevant selective antibiotic(s) (see "Antibiotics", page 5). If the host strain has already been cultured and stored at 2–8°C (cultures can be stored at 2–8°C for up to 3 months without any significant loss of viability), streak out bacteria from those stocks.
2. Incubate at 37°C overnight.
3. Pick a single colony and inoculate 10 ml LB medium containing relevant antibiotic(s). Grow overnight at 37°C.

protocol continues overleaf

Protocol 1. Continued

4. Add 1 ml overnight culture to 100 ml prewarmed LB medium containing the relevant antibiotic(s) in a 500 ml flask, and shake at 37°C until an OD_{600} of 0.5 is reached (approximately 90–120 min).
5. Cool the culture on ice for 5 min, and transfer the culture to a sterile, round-bottom centrifuge tube.
6. Collect the cells by centrifugation at low speed (5 min, 4000 x *g*, 4°C).
7. Discard the supernatant carefully. Always keep the cells on ice.
8. Resuspend the cells gently in cold (4°C) TFB1 buffer (30 ml for a 100 ml culture) and keep the suspension on ice for an additional 90 min.
9. Collect the cells by centrifugation (5 min, 4000 x *g*, 4°C).
10. Discard the supernatant carefully. Always keep the cells on ice.
11. Resuspend the cells carefully in 4 ml ice-cold TFB2 buffer.
12. Prepare aliquots of 100–200 µl in sterile microcentrifuge tubes and freeze in liquid nitrogen or a dry-ice–ethanol mix. Store the competent cells at –70°C.

Tip Several commercial kits (e.g., the QIAGEN® PCR Cloning^{plus} Kit for cloning of PCR products) offer ready-to-use competent cells for optimized transformation.

Protocol 2. Transformation of competent *E. coli* cells

Protocol 2

Transformation is the process in which plasmid DNA is introduced into a bacterial host cell. Several methods exist for transformation of bacterial cells, one of which is given below.

Materials

- Competent *E. coli* cells (see Protocol 1, above)
- SOC medium
- LB-agar plates

For buffer and media compositions, see "Bacterial Culture Media and Buffers", page 92.

1. Transfer an aliquot of the DNA to be transformed (10 µl or less) into a cold sterile 1.5 ml microcentrifuge tube, and keep it on ice.
2. Thaw an aliquot of frozen competent *E. coli* cells on ice.
3. Gently resuspend the cells and transfer 100 µl of the cell suspension into the microcentrifuge tube with the plasmid DNA, mix carefully, and keep on ice for 20 min.
4. Transfer the tube to a 42°C water bath or heating block for 90 s.
5. Add 500 µl SOC medium to the cells and incubate for 60–90 min at 37°C.

Tip Shaking increases transformation efficiency.

6. Plate out 50, 100, and 200 µl aliquots on LB-agar plates containing the relevant antibiotic(s). Incubate the plates at 37°C overnight until colonies develop.

Positive control to check transformation efficiency

Transform competent cells with 1 ng of a control plasmid containing an antibiotic resistance gene. Plate onto LB-agar plates containing the relevant antibiotic(s). Compare the number of colonies obtained with the control plasmid to the number obtained with the plasmid of interest to compare transformation efficiency.

Negative control to check antibiotic activity

Transform cells with 20 µl of TE. Plate at least 200 µl of the transformation mix on a single LB-agar plate containing the relevant antibiotic(s). An absence of colonies on the plates indicates that the antibiotic is active.

Growth and Culture of Bacteria*

Bacterial culture media and antibiotics

Liquid media

Liquid cultures of *E. coli* can generally be grown in LB (Luria-Bertani) medium. Please note that a number of different LB broths are commonly used. We recommend using the LB composition given in "Bacterial Culture Media and Buffers" (page 92) to obtain the highest yields of plasmid DNA.

Sterilizing media

Sterilize liquid or solid media by autoclaving, using a pressure and time period suitable for the type of medium, bottle size, and autoclave type.

Tip It is advisable to autoclave liquid medium in several small bottles rather than in one large vessel, to avoid possible contamination of an entire batch. Fill bottles only 3/4 full with medium and loosen the caps before autoclaving to avoid hot medium boiling over. Tighten caps once the media is cool (<40°C) to maintain sterility.

Tip Antibiotics and medium supplements such as amino acids are degraded by autoclaving. Antibiotics should be added to liquid medium immediately prior to use from stock solutions that have been filter-sterilized, distributed into aliquots, and stored in the dark at –20°C (see "Antibiotics", page 5).

** More extensive coverage of microbiological technique can be found in current manuals: see references 1 and 2, page 21.*

Solid media

E. coli strains can generally be streaked and stored, for a short period of time, on LB plates containing 1.5% agar and the appropriate antibiotic(s).

Preparation of LB-agar plates

Prepare LB medium according to the composition given in "Bacterial Culture Media and Buffers", page 92. Just before autoclaving, add 15 grams agar per liter and mix. After autoclaving, swirl the medium gently to distribute the melted agar evenly throughout the solution.

Tip Cool autoclaved agar medium to below 50°C (when you can hold it comfortably) before adding heat-sensitive antibiotics and nutrients. Mix thoroughly before pouring.

Tip Pour plates in a laminar-flow hood or on a cleaned bench surface next to a Bunsen burner. Use 30–35 ml medium per standard 90 mm petri dish.

Tip After pouring plates, any air bubbles may be removed by passing the flame of a Bunsen burner **briefly** over the surface.

Dry plates by removing the lids and standing the plates in a laminar-flow hood for 1 hour; with the covers slightly open in a 37°C incubator for 30 minutes; or left upside down with lids on at room temperature overnight.

Tip Store plates inverted at 4°C in a dark room or wrapped in aluminum foil to preserve light-sensitive antibiotics. Do not store for longer than 1 month as antibiotics may degrade. Label plates with the date and the antibiotic used.

Antibiotics

Bacterial strains carrying plasmids or genes with antibiotic selection markers should always be cultured in liquid or on solid medium containing the appropriate selective agent. Lack of antibiotic selection can lead to loss of the plasmid carrying the genetic marker and potentially to selection of faster-growing mutants!

Tip Prepare stock solutions of antibiotics separately from batches of liquid or solid media, sterilize by filtration, aliquot, and store in the dark at –20°C. Recommended stock and working concentrations for commonly used antibiotics are shown in **Table 1**.

Tip Before adding antibiotics to freshly autoclaved medium, ensure that the medium has cooled to below 50°C.

Table 1. Concentrations of commonly used antibiotics

Antibiotic	Stock solutions		Working concentration (dilution)
	Concentration	Storage	
Ampicillin (sodium salt)	50 mg/ml in water	–20°C	100 µg/ml (1/500)
Chloramphenicol	34 mg/ml in ethanol	–20°C	170 µg/ml (1/200)
Kanamycin	10 mg/ml in water	–20°C	50 µg/ml (1/200)
Streptomycin	10 mg/ml in water	–20°C	50 µg/ml (1/200)
Tetracycline HCl	5 mg/ml in ethanol	–20°C	50 µg/ml (1/100)
Carbenicillin	50 mg/ml in water	–20°C	50 µg/ml (1/1000)

Storage of *E. coli* strains

There are different methods for storing *E. coli* strains depending on the desired storage time. Glycerol stocks and stab cultures enable long-term storage of bacteria, while agar plates can be used for short-term storage. When recovering a stored strain, it is advisable to check that the antibiotic markers have not been lost by streaking the strain onto an LB-agar plate containing the appropriate antibiotic(s).

Protocol 3. Preparation of glycerol stocks

Protocol 3

E. coli strains can be stored for many years at –70°C in medium containing 15% glycerol.

Prepare glycerol stocks of bacteria as follows:

1. Add 0.15 ml glycerol (100%) to a 2 ml screw-cap vial and sterilize by autoclaving.

Tip Vials of sterilized glycerol can be prepared in batches and stored at room temperature until required.

2. Add 0.85 ml of a logarithmic-phase *E. coli* culture (see "Growth of *E. coli* cultures", page 7) to the vial of pre-sterilized glycerol.
3. Vortex the vial vigorously to ensure even mixing of the bacterial culture and the glycerol.
4. Freeze in an dry ice–ethanol bath or liquid nitrogen and store at –70°C.

Tip Avoid repeated thawing and re-freezing of glycerol stocks as this can reduce the viability of the bacteria.

Tip For precious strains, storage of two stock vials is recommended.

Protocol 4. Preparation of stab cultures

Protocol 4

E. coli strains can also be stored for up to 1 year as stabs in soft agar. Stab cultures can be used to transport or send bacterial strains to other labs.

Prepare stab cultures as follows:

1. Prepare and autoclave 0.7% LB agar (standard LB medium containing 7 g/liter agar) as described in "Bacterial Culture Media and Buffers", page 92.
2. Cool the LB agar to below 50°C (when you can hold it comfortably) and add the appropriate antibiotic(s). While still liquid, add 1 ml agar to a 2 ml screw-cap vial under sterile conditions, then leave to solidify.

Tip Vials of agar can be prepared in batches and stored at room temperature until required.

3. Using a sterile straight wire, pick a single colony from a freshly grown plate and stab it deep down into the soft agar several times.
4. Incubate the vial at 37°C for 8–12 h leaving the cap slightly loose.
5. Seal the vial tightly and store in the dark, preferably at 4°C.

Growth of *E. coli* cultures

Figure 1 shows the sequence of steps necessary to go from a stored stock of bacteria to a liquid culture for plasmid isolation. Bacterial stocks should always be streaked onto selective plates prior to use, to check that they give rise to healthy colonies carrying the appropriate antibiotic resistance. Stocks can potentially contain mutants arising from the cultures used to prepare them, or can deteriorate during storage.

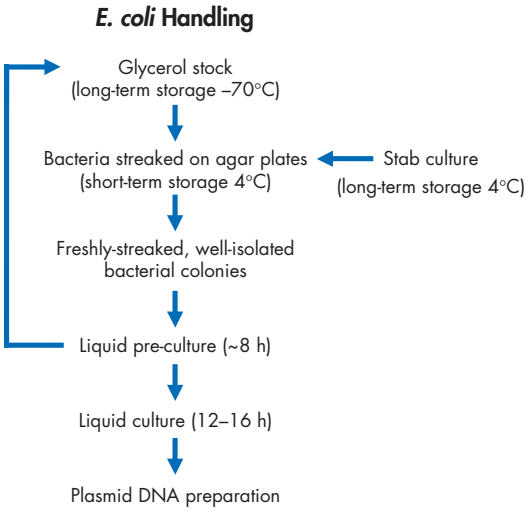


Figure 1. *Essential steps for storage and handling of* E. coli.

Protocol 5. Recovery of single colonies from stored cultures

Protocol 5

Plates of streaked bacteria can be sealed with Parafilm® and stored upside-down at 4°C for several weeks.

Bacteria should always be streaked onto plates containing the appropriate antibiotic to ensure that selective markers are not lost.

To obtain isolated colonies, streak an agar plate as follows:

1. Flame a wire loop, and cool on a spare sterile agar plate.
2. Using the wire loop, streak an inoculum of bacteria (from a glycerol stock, stab culture, or single colony on another plate) across one corner of a fresh agar plate, as shown in **Figure 2**.
3. Flame and cool the wire loop again. Pass it through the first streak and then streak again across a fresh corner of the plate.
4. Repeat again to form a pattern as in **Figure 2**.
5. Incubate the plate upside down at 37°C for 12–24 h until colonies develop.
6. Inoculate liquid cultures from a healthy, well-isolated colony, picked from a freshly streaked selective plate. This will ensure that cells growing in the culture are all descended from a single founder cell, and have the same genetic makeup.

Tip Culture volumes >10 ml should not be inoculated directly from a plate, but with a pre-culture of 2–5 ml diluted 1/500 to 1/1000.

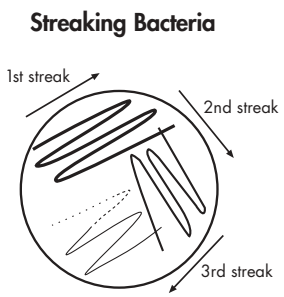


Figure 2. *Streaking bacteria on agar plates.*

E. coli growth curve

The growth curve of an *E. coli* culture can be divided into distinct phases (Figure 3). **Lag phase** occurs after dilution of the starter culture into fresh medium. Cell division is slow as the bacteria adapt to the fresh medium. After 4–5 hours the culture enters **logarithmic (log) phase,** where bacteria grow exponentially. Cells enter **stationary phase** (~16 hours) when the available nutrients are used up. The cell density remains constant in this phase. Eventually the culture enters the **phase of decline,** where cells start to lyse, the number of viable bacteria falls, and DNA becomes partly degraded.

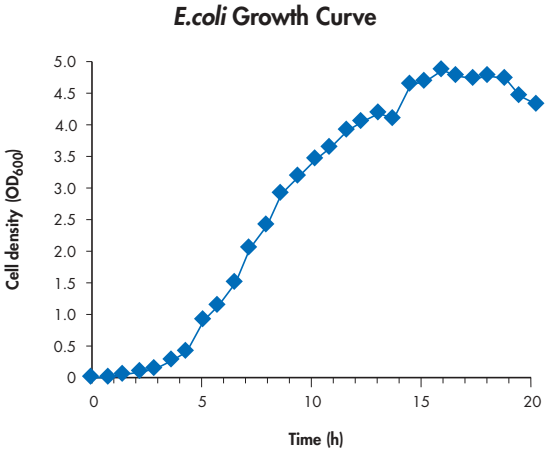


Figure 3. Growth curve of E. coli *in LB medium. Host strain: DH5α™; plasmid: pUC21. High OD_{600} readings were calculated by diluting the sample to enable photometric measurement in the linear range of 0.1–0.5 OD_{600}*

Measuring cell density

The growth curve of a bacterial culture can be monitored photometrically by reading the optical density at 600 nm (Figure 3). Note however that photometric measurements of cell density can vary between different spectrophotometers.

Tip Calibrate your spectrophotometer by determining the number of cells per milliliter giving a particular OD_{600} reading. Plate serial dilutions of a culture on LB agar plates and calculate the number of cells per milliliter in the original culture. This is then set in relation to the measured OD_{600} value.

Tip High OD_{600} readings should be calculated by diluting the sample in culture medium to enable measurement in the linear range of 0.1–0.5 OD_{600}.

Tip Another way of estimating the amount of cell harvest is to assess the pellet wet weight. Typically a 1 liter, overnight culture of *E. coli* within a cell density of 3–4 x 10^9 cells per milliliter corresponds to a pellet wet weight of approximately 3 grams.

Protocol 6. Preparation of bacteria for plasmid preps

To prepare the bacterial culture for your plasmid prep, follow the steps below.

1. Prepare a starter culture by inoculating a single colony from a freshly grown selective plate into 2–10 ml LB medium containing the appropriate antibiotic. Grow at 37°C for ~8 h with vigorous shaking (~300 rpm).

Tip It is often convenient to grow the starter culture during the day so that the larger culture can be grown overnight for harvesting the following morning.

2. Dilute the starter culture 1/500 to 1/1000 into a larger volume of selective LB medium.

Tip Use a flask of at least 5 times the volume of culture to ensure sufficient aeration.

Tip Do not use a larger culture volume than recommended in the protocol, as use of too many cells will result in inefficient lysis and reduce the quality of the preparation.

3. Grow the culture at 37°C with vigorous shaking (~300 rpm) for 12–16 h.

Tip Growth for 12–16 h corresponds to the transition from logarithmic into stationary growth phase (see Figure 3), when cell density is high (3–4 x 10^9 cells per ml) and RNA content of cells is low.

Tip Growth of cultures is dependent on factors such as host strain, plasmid insert and copy number, and culture medium. To determine the optimal harvesting time for a particular system, monitor the cell density and the growth of the culture by measuring the OD_{600} (see previous section).

4. Harvest the bacterial culture by centrifugation at 6000 x *g* for 15 min at 4°C. Remove all traces of the supernatant. The cells are now ready for the lysis procedure.

Tip The procedure may be stopped at this point and continued later by freezing the cell pellets obtained by centrifugation. The frozen cell pellets can be stored at –20°C for several weeks.

Lysis of Bacterial Cells for Plasmid Purification

Effective lysis of bacterial cells is a key step in plasmid isolation as DNA yield and quality depend on the quality of cell lysate used for the purification.

Alkaline lysis

Alkaline lysis is one of the most commonly used methods for lysing bacterial cells prior to plasmid purification (3, 4). Production of alkaline lysates involves four basic steps (**Figure 4**):

1. Resuspension

Harvested bacterial cells are resuspended in Tris·Cl–EDTA buffer containing RNase A.

Tip Ensure that bacteria are resuspended completely leaving no cell clumps in order to maximize the number of cells exposed to the lysis reagents.

Tip Buffer volumes for alkaline lysis in QIAGEN plasmid purification procedures are optimized for particular culture volumes in LB medium. Do not use a culture volume larger than recommended in the protocol as this will lead to inefficient lysis and reduce the quality of the plasmid preparation.

Tip For large scale purification of low-copy plasmids, for which larger cultures volumes are used, it may be beneficial to increase the lysis buffer volumes in order to increase the efficiency of alkaline lysis and thereby the DNA yield.

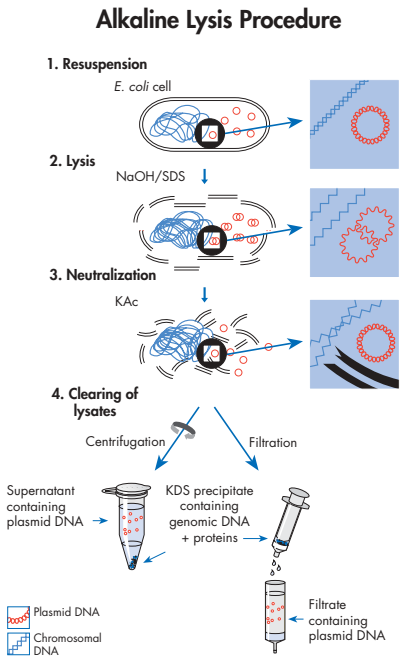


Figure 4. *Principle of alkaline lysis.*

2. Lysis

Cells are lysed with NaOH/SDS. Sodium dodecyl sulfate (SDS) solubilizes the phospholipid and protein components of the cell membrane, leading to lysis and release of the cell contents. NaOH denatures the chromosomal and plasmid DNA, as well as proteins. The presence of RNase A ensures that liberated cellular RNA is digested during lysis.

Tip If after addition of lysis buffer (NaOH/SDS) the solution appears very viscous and is difficult to mix, this indicates excess biomass in the lysate step. This results in insufficient cell lysis and it is recommended to double the amount of lysis and neutralization buffers used.

Tip Avoid vigorous stirring or vortexing of the lysate as this can shear the bacterial chromosome, which will then copurify with the plasmid DNA. The solution should be mixed gently but thoroughly by inverting the lysis vessel 4–6 times.

Tip Do not allow the lysis to proceed for longer than 5 minutes. This is optimal for release of the plasmid DNA, while avoiding irreversible plasmid denaturation.

3. Neutralization

The lysate is neutralized by the addition of acidic potassium acetate. The high salt concentration causes potassium dodecyl sulfate (KDS) to precipitate, and denatured proteins, chromosomal DNA, and cellular debris are coprecipitated in insoluble salt-detergent complexes. Plasmid DNA, being circular and covalently closed, renatures correctly and remains in solution.

Tip Precipitation can be enhanced by using chilled neutralization buffer and incubating on ice.

4. Clearing of lysates

Precipitated debris is removed by either centrifugation or filtration, producing cleared lysates.

Tip QIAGEN lysate filtration technologies ensure removal of all precipitated material without centrifugation, considerably reducing plasmid purification time.

Purification of plasmid DNA from cleared bacterial lysates was traditionally performed using cesium chloride (CsCl) ultracentrifugation. Today, a variety of commercially available plasmid purification kits offer easy procedures for different throughput requirements and applications.

Isopropanol Precipitation of DNA

Alcohol precipitation is commonly used for concentrating, desalting, and recovering nucleic acids. Precipitation is mediated by high concentrations of salt and the addition of either isopropanol or ethanol. Since less alcohol is required for isopropanol precipitation, this is the preferred method for precipitating DNA from large volumes. In addition, isopropanol precipitation can be performed at room temperature, which minimizes co-precipitation of salt that may interfere with downstream applications.

This section provides hints on how to perform an effective isopropanol precipitation and to help ensure maximum recovery of DNA. The range of values given reflects protocol variation depending on the scale and type of preparation.

Protocol 7. Isopropanol precipitation procedure

Protocol 7

1. Adjust the salt concentration if necessary, e.g., with sodium acetate (0.3 M, pH 5.2, final concentration) or ammonium acetate (2.0–2.5 M, final concentration).
2. Add 0.6–0.7 volumes of room-temperature isopropanol to the DNA solution and mix well.

Tip Use all solutions at room temperature to minimize co-precipitation of salt.

3. Centrifuge the sample immediately at 10,000–15,000 x *g* for 15–30 min at 4°C.

Tip Centrifugation should be carried out at 4°C to prevent overheating of the sample. (When precipitating from small volumes, centrifugation may be carried out at room temperature.)

4. Carefully decant the supernatant without disturbing the pellet.

Tip Marking the outside of the tube or uniformly orienting microcentrifuge tubes before centrifugation allows the pellet to be more easily located. Pellets from isopropanol precipitation have a glassy appearance and may be more difficult to see than the fluffy salt-containing pellets that result from ethanol precipitation.

Tip Care should be taken when removing the supernatant as pellets from isopropanol precipitation are more loosely attached to the side of the tube.

Tip Carefully tip the tube with the pellet on the upper side to avoid dislodging the pellet.

Tip For valuable samples, the supernatant should be retained until recovery of the precipitated DNA has been verified.

5. Wash the DNA pellet by adding room-temperature 70% ethanol. This removes co-precipitated salt and replaces the isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

protocol continues overleaf

Protocol 7. Continued

6. Centrifuge at 10,000–15,000 x *g* for 5–15 min at 4°C.

Tip Centrifuge the tube in the same orientation as previously to recover the DNA in a compact pellet.

7. Carefully decant the supernatant without disturbing the pellet.

Tip The QIAprecipitator™ (QIAGEN) ensures recovery of precipitated DNA without centrifugation, considerably reducing plasmid purification time and eliminating the risk of DNA pellet loss.

8. Air-dry the pellet for 5–20 min (depending on the size of the pellet).

Tip Do not overdry the pellet (e.g., by using a vacuum evaporator) as this will make DNA, especially high-molecular-weight DNA, difficult to redissolve.

9. Redissolve the DNA in a suitable buffer.

Tip Choose an appropriate volume of buffer according to the expected DNA yield and the desired final DNA concentration.

Tip Use a buffer with a pH ≥8.0 for redissolving, as DNA does not dissolve easily in acidic buffers. (If using water, check pH.)

Tip Redissolve by rinsing the walls to recover all the DNA, especially if glass tubes have been used. To avoid shearing DNA do not pipet or vortex.

Tip High-molecular-weight DNA should be redissolved very gently to avoid shearing, e.g., at room temperature overnight or at 55°C for 1–2 h with gentle agitation.

A Guide to Analytical Gels

This section is aimed at providing useful hints for effective gel analysis of nucleic acids. Firstly, the basic steps involved in pouring an agarose gel for DNA analysis are outlined. Subsequent sections look at loading and running the gel and visualization of the DNA.

Principle of gel analysis

Gels allow separation and identification of nucleic acids based on charge migration. Migration of nucleic acid molecules in an electric field is determined by size and conformation, allowing nucleic acids of different sizes to be separated. However, the relationship between the fragment size and rate of migration is non-linear, since larger fragments have greater frictional drag and are less efficient at migrating through the polymer.

Agarose gel analysis is the most commonly used method for analyzing DNA fragments between 0.1 and 25 kb. Other specialized analytical gel methods exist for analyzing extremely large or small DNA molecules. Detailed information on all types of analytical gels can be found in current molecular biology manuals (1, 2).

Pouring an agarose gel

Agarose concentration

The concentration of agarose used for the gel depends primarily on the size of the DNA fragments to be analyzed. Low agarose concentrations are used to separate large DNA fragments, while high agarose concentrations allow resolution of small DNA fragments (**Table 2**).

Table 2. Concentration of agarose used for separating DNA of different sizes

Agarose concentration (% w/v)	DNA fragment range (kb)
0.3*	5–60
0.5	1–30
0.7	0.8–12
1.0	0.5–10
1.2	0.4–7
1.5	0.2–3
2.0*	0.05–2

Adapted from references 1 and 2.

** Most gels are run using standard agarose, although some special types of agarose are available for particular applications and for very high or low agarose concentrations. For example, low-melt agarose allows in situ enzymatic reactions and can be used for preparative gels.*

Electrophoresis buffers

The most commonly used buffers for agarose gel electrophoresis are TBE (Tris-borate–EDTA) and TAE (Tris-acetate–EDTA) (see "Agarose Gel Electrophoresis Buffers for Analysis of DNA", page 93). Although more frequently used, TAE has a lower buffering capacity than TBE and is more easily exhausted during extended electrophoresis. TBE gives better resolution and sharper bands, and is particularly recommended for analyzing fragments <1 kb.

The drawback of TBE is that the borate ions in the buffer form complexes with the *cis*-diol groups of sugar monomers and polymers, making it difficult to extract DNA fragments from TBE gels using traditional methods.

Tip QIAGEN Systems for gel extraction utilize optimized buffers for efficient extraction of DNA fragments from both TBE and TAE gels.

Protocol 8. Pouring the gel

1. Prepare enough 1x running buffer both to pour the gel and fill the electrophoresis tank.
2. Add an appropriate amount of agarose (depending on the concentration required) to an appropriate volume of running buffer (depending on the volume of the gel tray being used) in a flask or bottle.

Tip The vessel should not be more than half full. Loosely cover the vessel to minimize evaporation. **Note:** The cover should not be airtight.

Tip Always use the same batch of buffer to prepare the agarose as to run the gel, since small differences in ionic strength can affect migration of DNA.

3. Heat the slurry in a microwave or boiling water bath, swirling the vessel occasionally, until the agarose is dissolved.

Tip Ensure that the lid of the flask is loose to avoid buildup of pressure. Be careful not to let the agarose solution boil over as it becomes superheated.

Tip If the volume of liquid reduces considerably during heating due to evaporation, make up to the original volume with distilled water.

4. Cool the agarose to 55–60°C. Add ethidium bromide if desired (see page 14).
5. Pour the agarose solution onto the gel tray to a thickness of 3–5 mm. Insert the comb either before or immediately after pouring. Leave the gel to set (30–40 min).

Tip Ensure that there is enough space between the bottom of the comb and the glass plate (0.5–1.0 mm) to allow proper formation of the wells and avoid sample leakage.

6. Carefully remove the comb and adhesive tape, if used, from the gel. Fill the tank containing the gel with electrophoresis buffer.

Tip Add enough buffer to cover the gel with a depth of approximately 1 mm liquid above the surface of the gel. If too much buffer is used the electric current will flow through the buffer instead of the gel.

Running an agarose gel

Preparation of samples

Agarose gel analysis with ethidium bromide staining allows detection of DNA amounts from as little as 20 ng up to 500 ng in a band (5 mm wide x 2 mm deep). Loading of larger amounts of DNA will result in smearing of the DNA bands on the gel.

Tip Samples must always be mixed with gel loading buffer prior to loading (see below).

Tip Be sure that all samples have the same buffer composition. High salt concentrations will retard the migration of the DNA fragments.

Tip Ensure that no ethanol is present in the samples, as this will cause samples to float out of the wells.

Gel loading buffers and markers

Gel loading buffer (see "Agarose Gel Electrophoresis Buffers for Analysis of DNA", page 93) must be added to the samples before loading and serves three main purposes:

1. To increase the density of the samples to ensure that they sink into the wells.
2. To add color to the samples through use of dyes such as bromophenol blue, Orange G, or xylene cyanol, facilitating loading.
3. To allow tracking of the electrophoresis due to co-migration of the dyes with DNA fragments of a specific size.

Molecular-weight markers should always be included on a gel to enable analysis of DNA fragment sizes in the samples. See "Commonly Used DNA Markers in Agarose Gel Electrophoresis", page 93 for commonly used markers.

Protocol 9. Electrophoresis

1. Apply samples in gel loading buffer to the wells of the gel.

Tip Prior to sample loading, rinse wells with electrophoresis buffer. Make sure that the entire gel is submerged in the running buffer.

Tip Once samples are loaded, do not move the gel tray/tank as this may cause samples to float out of the wells.

2. Connect the electrodes so that the DNA will migrate towards the anode (positive electrode).

Tip Electrophoresis apparatus should always be covered to protect against electric shocks.

3. Turn on the power supply and run the gel at 1–10 V/cm until the dyes have migrated an appropriate distance. This will depend on the size of DNA being analyzed, the concentration of agarose in the gel, and the separation required.

Tip Avoid use of very high voltages which can cause trailing and smearing of DNA bands in the gel, particularly with high-molecular-weight DNA.

Tip Monitor the temperature of the buffer periodically during the run. If the buffer becomes heated, reduce the voltage.

Tip Melting of an agarose gel during electrophoresis is a sign that the voltage is too high, that the buffer may have been incorrectly prepared or has become exhausted during the run.

Visual analysis of the gel

Staining

To allow visualization of the DNA samples, agarose gels are stained with an appropriate dye. The most commonly used dye is the intercalating fluorescent dye ethidium bromide, which can be added either before or after electrophoresis (see **Table 3**). Alternatives include commercial dyes such as SYBR® Green.

Tip Stock solutions of ethidium bromide (generally 10 mg/ml) should be stored at 4°C in a dark bottle or bottle wrapped in aluminum foil.

Addition of ethidium bromide prior to electrophoresis — add ethidium bromide to a final concentration of 0.5 µg/ml to the melted and subsequently cooled agarose, i.e., just before pouring the gel.

Tip Mix the agarose–ethidium bromide solution well to avoid localized staining.

Addition of ethidium bromide after electrophoresis — soak the gel in a 0.5 µg/ml solution of ethidium bromide (in water or electrophoresis buffer) for 30–40 minutes.

Tip Rinse the gel with buffer or water before examining it to remove excess ethidium bromide.

Tip Staining buffer can be saved and re-used.

Note: Ethidium bromide is a powerful mutagen and is very toxic. Wear gloves and take appropriate safety precautions when handling. Use of nitrile gloves is recommended as latex gloves may not provide full protection. After use, ethidium bromide solutions should be decontaminated as described in commonly used manuals (1, 2).

Table 3. Comparison of ethidium bromide staining methods

Addition of ethidium bromide prior to electrophoresis	Addition of ethidium bromide after electrophoresis
Faster and more convenient procedure	Slower procedure requiring additional step
Allows monitoring of migration throughout the procedure	Does not allow monitoring of migration during electrophoresis
Requires decontamination of gel tanks and comb	No decontamination of gel tanks and comb necessary
More ethidium bromide is required	Usually less ethidium bromide is required
Electrophoretic mobility of linear DNA fragments is reduced by ~15%	No interference with electrophoretic mobility

Visualization

Ethidium bromide–DNA complexes display increased fluorescence compared to the dye in solution. This means that illumination of a stained gel under UV light (254–366 nm) allows bands of DNA to be visualized against a background of unbound dye. The gel image can be recorded by taking a Polaroid™ photograph or using a gel documentation system.

Tip UV light can damage the eyes and skin. Always wear suitable eye and face protection when working with a UV light source.

Tip UV light damages DNA. If DNA fragments are to be extracted from the gel, use a lower intensity UV source if possible and minimize exposure of the DNA to the UV light.

Agarose Gel Analysis of Plasmid DNA

The main uses of agarose gels for plasmid DNA analysis are:

- Analysis of the size and conformation of nucleic acids in a sample
- Quantification of DNA (see page 16)
- Separation and extraction of DNA fragments

Analysis of a purification procedure

Figure 5 shows how agarose gel electrophoresis can be used to analyze the nucleic acid content of samples taken during a plasmid purification procedure. The gel demonstrates successful plasmid purification using anion-exchange columns as well as some atypical results.

M: Lambda DNA digested with *Hin*dIII.

1: Cleared lysate containing supercoiled (lower band) and open circular plasmid DNA (upper band) and degraded RNA (smear at the bottom of the gel).

2: Flow-through fraction containing only degraded RNA (the plasmid DNA is bound to the anion-exchange resin in the column).

3: Wash fraction to ensure that the resin in the column is cleared of RNA and other contaminants (plasmid DNA remains bound to the column).

4: Eluate containing pure plasmid DNA in supercoiled and open circular forms.

Analysis of the Plasmid Purification Procedure

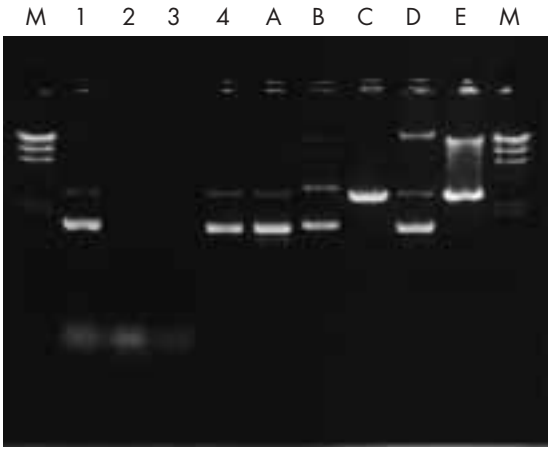


Figure 5. Agarose gel analysis of a plasmid purification procedure using QIAGEN anion-exchange tips. Samples were taken at different stages of the procedure. 2 µl of each sample was run on a 1% agarose gel. ***M:*** *lambda-HindIII markers.*

Lanes A–E illustrate some atypical results that may be observed in some preparations, depending on plasmid type and host strain.

A: Supercoiled (lower band) and open circular form (upper band) of the high-copy plasmid, pUC18, with an additional band of denatured supercoiled DNA migrating just beyond the supercoiled form.

B: Multimeric forms of supercoiled plasmid DNA (pTZ19) that may be observed with some host strains and should not be mistaken for genomic DNA. Multimeric plasmid DNA is easily distinguished from genomic DNA by restriction digestion.

C: Linearized form of plasmid pTZ19 after restriction digestion with *Eco*RI.

D: Sample contaminated with bacterial chromosomal DNA (uppermost band).

E: *Eco*RI digestion of a sample contaminated with bacterial genomic DNA, which gives a smear above the plasmid DNA.

Tip With large-constructs such as BAC, PAC, and P1 DNA, the supercoiled form migrates at a slower rate than the linear form. Furthermore, large-construct DNA >50 kb is often difficult to distinguish from genomic DNA by agarose gel analysis.

Gel extraction

Agarose gels can be used for separation and extraction of DNA fragments, for example, a specific DNA fragment from a PCR or restriction digestion reaction.

Tip Ensure that the percentage of agarose used for the gel allows good separation of DNA fragments for easy excision.

Tip Run agarose gels for DNA extraction at a low voltage. This will enable efficient separation of DNA bands without smearing, facilitating excision of the gel slice.

Tip Excise the fragment quickly under low-strength UV light to limit DNA damage.

DNA fragments can be extracted quickly and efficiently from agarose gels using silica-gel–based purification. Silica-gel–based methods typically result in higher and more reproducible recoveries than other gel extraction methods, such as electroelution, and require no phenol extraction or ethanol precipitation. In a typical silica-gel–based purification procedure, the agarose gel slice is first solubilized. DNA is then bound to the silica-gel material in the presence of high concentrations of chaotropic salts. A wash step removes impurities, and DNA is then eluted in low-salt buffer.

Tip QIAGEN offers three kits for silica-gel–based purification of differently sized DNA fragments from agarose gels, which differ in methodology and elution volumes:

- Purification of fragments between 70 bp and 10 kb in a spin-column format which can be used in a microcentrifuge or on a vacuum manifold.
- Purification of fragments between 70 bp and 4 kb in a spin-column format, in elution volumes of only 10 µl.
- Purification of fragments between 40 bp and 50 kb using silica-gel particles.

Polyacrylamide gel electrophoresis (PAGE)

As an alternative to agarose gel electrophoresis, polyacrylamide gels can be used for the analytical or preparative separation of small, double-stranded DNA fragments. This method is applicable to DNA fragments from 10 to 1000 bp. The resolution and capacity of polyacrylamide gels is higher than that of agarose gels. However, agarose gels are much easier to pour and run, and in the vast majority of cases deliver acceptable resolution. Protocols for PAGE of DNA can be found in standard molecular biology texts (1, 2).

Quantification of DNA

Reliable measurement of DNA concentration is important for many applications in molecular biology. Plasmid DNA quantification is generally performed by spectrophotometric measurement of the absorption at 260 nm, or by agarose gel analysis. In this section, we examine some critical factors for quantification, such as the effect of solvents, phenol, and RNA contamination on absorption.

DNA quantification by spectophotometry

Plasmid DNA concentration can be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer using a quartz cuvette. For reliable DNA quantification, A_{260} readings should lie between 0.1 and 1.0. An absorbance of 1 unit at 260 nm corresponds to 50 µg plasmid DNA per ml (A_{260} = 1 ⇒ 50 µg/ml).* This relationship is only valid for measurements made at neutral pH, therefore, samples should be diluted in a low-salt buffer with neutral pH (e.g., Tris·Cl, pH 7.0). An example of the calculation involved in nucleic acid quantification when using a spectrophotometer is provided in "Spectrophotometric Measurement of Nucleic Acid Concentration", page 91.

When working with small amounts of DNA, such as purified PCR products or DNA fragments extracted from agarose gels, quantification via agarose gel analysis may be more effective (see "DNA quantification by agarose gel analysis", page 17).

Tip If you will use more than one quartz cuvette to measure multiple samples, the cuvettes must be matched.

* *Based on a standard 1 cm path length.*

Effects of solvents on spectrophotometric readings

Absorption of nucleic acids depends on the solvent used to dissolve the nucleic acid (6). A_{260} values are reproducible when using low-salt buffer, but not when using water. This is most likely due to differences in the pH of the water caused by the solvation of CO_2 from air. A_{260}/A_{280} ratios measured in water also give rise to a high variability between readings (**Figure 6**) and the ratios obtained are typically <1.8, resulting in reduced sensitivity to protein contamination (6). In contrast, A_{260}/A_{280} ratios measured in a low-salt buffer with slightly alkaline pH are generally reproducible.

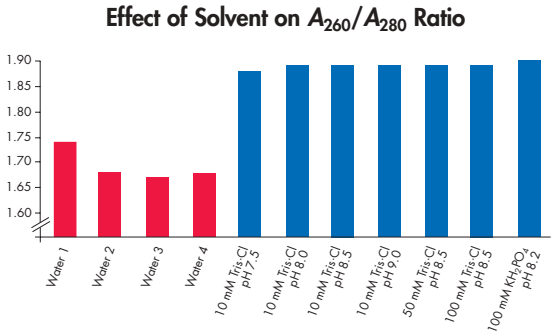


Figure 6. A_{260}/A_{280} *ratios were measured in different solvents. Red bars represent readings in ultrapure water, taken at different times after dissolution of DNA. Blue bars represent readings in various low-salt buffers as indicated.*

Effect of RNA contamination on spectrophotometric readings

Plasmid DNA preparations can contain RNA contamination, for example, when the RNase A treatment during alkaline lysis does not degrade all RNA species. Since spectrophotometric measurement does not differentiate between DNA and RNA, RNA contamination can lead to overestimation of DNA concentration. RNA contamination can sometimes be detected by agarose gel analysis with routine ethidium bromide staining, although not quantified effectively. RNA bands appear faint and smeary and are only detected in amounts ≥25–30 ng (0.5:1 RNA:DNA ratio).

RNA contamination of plasmid DNA can be a concern depending on the method used for plasmid preparation. Methods using alkaline lysis with phenol extraction cannot separate RNA from plasmid DNA, leading to high levels of RNA contamination. In contrast, QIAGEN offers advanced silica-gel–membrane and anion-exchange resin technologies that ensure plasmid DNA is virtually free of RNA (7–9).

Effect of phenol on spectrophotometric readings

Phenol has an absorption maximum of 270–275 nm, which is close to that of DNA. Phenol contamination mimics both higher yields and higher purity, because of an upward shift in the A_{260} value leading to over-quantification of DNA (**Figure 7**).

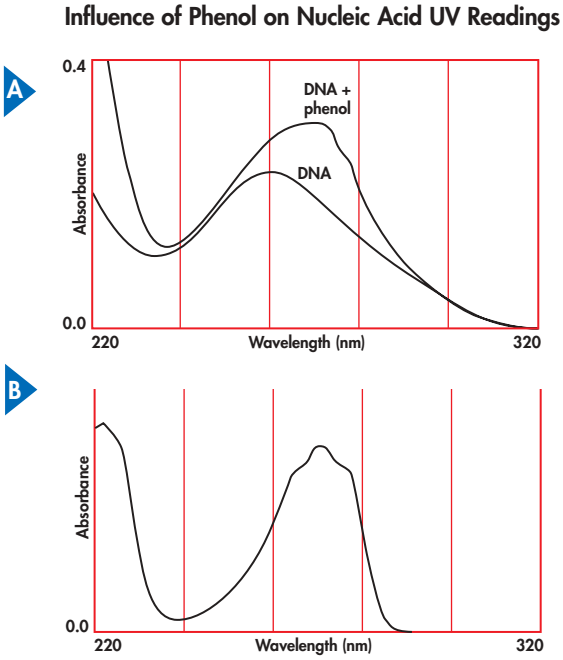


Figure 7. Effect of phenol on quantification of DNA. ***A:*** *DNA was purified on a silica membrane and split into two 100 µl aliquots. 1 µl of a 1:1000 dilution of phenol in water was added to one sample (final phenol dilution 1:100,000) and the UV absorbance spectrum of both samples was measured.* ***B:*** *UV scan of phenol, diluted 1:5000 in water.*

DNA quantification by agarose gel analysis

Agarose gel analysis enables quick and easy quantification of DNA (5), especially for small DNA fragments (such as PCR products). As little as 20 ng DNA can be detected by agarose gel electrophoresis with ethidium bromide staining. The DNA sample is run on an agarose gel alongside known amounts of DNA of the same or a similar size. The amount of sample DNA loaded can be estimated by comparison of the band intensity with the standards either visually (**Figure 8**) or using a scanner or imaging system.

Tip Be sure to use standards of roughly the same size as the fragment of interest to ensure reliable estimation of the DNA quantity, since large fragments interchelate more dye than small fragments and give a greater band intensity.

More precise agarose gel quantification can be achieved by densitometric measurement of band intensity and comparison with a standard curve generated using DNA of a known concentration. In most experiments the effective range for comparative densitometric quantification is between 20 and 100 ng.

Tip The amount of DNA used for densitometric quantification should fall within the linear range of the standard curve.

See "A Guide to Analytical Gels", page 11, for further information on agarose gel electrophoresis.

Agarose Gel Analysis of Plasmid DNA

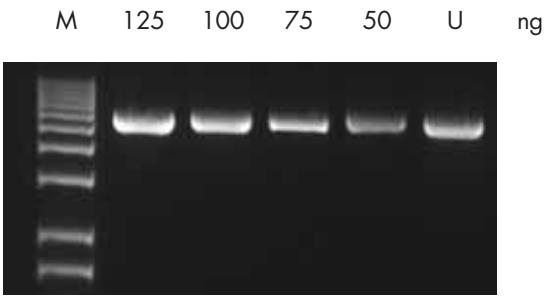


Figure 8. *An unknown amount of a 5.5 kb DNA fragment* ***(U)*** *was run alongside known quantities (as indicated in ng) of the same DNA fragment on a 1% TAE agarose gel. The unknown sample contained ~85 ng DNA, as estimated by visual comparison with the standards.* ***M:*** *1 kb DNA ladder.*

Restriction Endonuclease Digestion of DNA

Principle of restriction digestion

DNA for downstream applications is usually digested with restriction endonucleases. This yields DNA fragments of a convenient size for downstream manipulations.

Restriction endonucleases are bacterial enzymes that bind and cleave DNA at specific target sequences. Type II restriction enzymes are the most widely used in molecular biology applications. They bind DNA at a specific recognition site, consisting of a short palindromic sequence, and cleave within this site, e.g., AGCT (for *Alu*I), GAATTC (for *Eco*RI), and so on. Isoschizomers are different enzymes that share the same specificity, and in some cases, the same cleavage pattern.

Tip Isoschizomers may have slightly different properties that can be very useful. For example, the enzymes *Mbo*I and *Sau*3A have the same sequence specificities, but *Mbo*I does not cleave methylated DNA, while *Sau*3A does. *Sau*3A can therefore be used instead of *Mbo*I where necessary.

Selecting suitable restriction endonucleases

The following factors need to be considered when choosing suitable restriction enzymes:

- Fragment size
- Methylation sensitivity
- Blunt-ended/sticky-ended fragments
- Compatibility of reaction conditions (where more than one enzyme is used)

Fragment size

Restriction enzymes with shorter recognition sequences cut more frequently than those with longer recognition sequences. For example, a 4 base pair (bp) cutter will cleave, on average, every 4^4 (256) bases, while a 6 bp cutter cleaves every 4^6 (4096) bases.

Tip Use 6 bp cutters for mapping genomic DNA or YACs, BACs, or P1s, as these give fragments in a suitable size range for cloning.

Blunt-ended/sticky-ended fragments

Some restriction enzymes cut in the middle of their recognition site, creating blunt-ended DNA fragments. However, the majority of enzymes make cuts staggered on each strand, resulting in a few base pairs of single-stranded DNA at each end of the fragment, known as "sticky" ends. Some enzymes create 5' overhangs and others create 3' overhangs. The type of digestion affects the ease of downstream cloning:

- Sticky-ended fragments can be easily ligated to other sticky-ended fragments with compatible single-stranded overhangs, resulting in efficient cloning.
- Blunt-ended fragments usually ligate much less efficiently, making cloning more difficult. However, any blunt-ended fragment can be ligated to any other, so blunt-cutting enzymes are used when compatible sticky-ended fragments cannot be generated – for example, if the polylinker site of a vector does not contain an enzyme site compatible with the fragment being cloned.

Methylation

Many organisms have enzymes called methylases that methylate DNA at specific sequences. Not all restriction enzymes can cleave their recognition site when it is methylated. Therefore the choice of restriction enzyme is affected by its sensitivity to methylation. In addition, methylation patterns differ in different species, also affecting the choice of restriction enzyme.

Tip Methylation patterns differ between bacteria and eukaryotes, so restriction patterns of cloned and uncloned DNA may differ.

Tip Methylation patterns also differ between different eukaryotes (see page 33), affecting the choice of restriction enzyme for construction genomic DNA libraries.

Compatibility of reaction conditions

If a DNA fragment is to be cut with more than one enzyme, both enzymes can be added to the reaction simultaneously provided that they are both active in the same buffer and at the same temperature. If the enzymes do not have compatible reaction conditions, it is necessary to carry out one digestion, purify the reaction products (for example using the MinElute™ Reaction Cleanup Kit), and then perform the second digestion.

Components of a restriction digest

- Water
- Buffer
- DNA
- Enzyme

DNA

The amount of DNA digested depends on the downstream application. For mapping of cloned DNA, 0.2–1 µg DNA per reaction is adequate.

Tip DNA should be free of contaminants such as phenol, chloroform, ethanol, detergents, or salt, as these may interfere with restriction endonuclease activity.

Enzyme

One unit of restriction endonuclease completely digests 1 µg of substrate DNA in 1 hour. However, supercoiled plasmid DNA generally requires more than 1 unit/µg to be digested completely. Most researchers add a ten-fold excess of enzyme to their reactions in order to ensure complete cleavage.

Tip Ensure that the restriction enzyme does not exceed more than 10% of the total reaction volume, otherwise the glycerol in which the enzyme is supplied may inhibit digestion.

Reaction volume

Most digests are carried out in a volume between 10 and 50 µl. (Reaction volumes smaller than 10 µl are susceptible to pipetting errors, and are not recommended.)

Protocol 10. Setting up a restriction digest

Protocol 10

1. Pipet reaction components into a tube and mix well by pipetting.

Tip Thorough mixing is extremely important.

Tip The enzyme should be kept on ice and added last.

Tip When setting up large numbers of digests, make a reaction master mix consisting of water, buffer, and enzyme, and aliquot this into tubes containing the DNA to be digested.

2. Centrifuge the tube briefly to collect the liquid at the bottom.
3. Incubate the digest in a water bath or heating block, usually for 1–4 h at 37°C. However, some restriction enzymes require higher (e.g., 50–65°C) while others require lower (e.g., 25°C) incubation temperatures.
4. For some downstream applications it is necessary to heat-inactivate the enzyme after digestion. Heating the reaction to 65°C for 20 min after digestion inactivates the majority of enzymes that have optimal incubation temperature of 37°C.

Tip Some restriction enzymes are not fully inactivated by heat treatment. The MinElute Reaction Cleanup Kit provides complete removal of restriction enzymes and salts following digestion.

Ligation of DNA

In order to construct new DNA molecules, DNA must first be digested using restriction endonucleases (see "Restriction Endonuclease Digestion of DNA", page 18). The individual components of the desired DNA molecule are purified and then combined and treated with DNA ligase. The products of the ligation mixture are introduced into competent *E. coli* cells and transformants are identified by appropriate genetic selection. Appropriate control ligations should also be performed (See Protocols 1 and 2, pages 2 and 3).

Removal of 5' phosphates from linearized vector DNA can help prevent vector self-ligation and improve ligation efficiency. To remove 5' phosphates from DNA, add calf intestinal phosphate (CIP) buffer and 1 U CIP and incubate for 30–60 minutes at 37°C. Once the reaction is complete, inactivate CIP by heating to 75°C for 15 minutes.

Protocol 11. Ligation of DNA and subsequent transformation

1. A typical ligation reaction is set up as follows:
   - Component DNAs (0.1–5 µg)
   - Ligase buffer
   - 1 µl 10 mM ATP
   - 20–500 U T4 DNA ligase
2. Incubate for 1–24 h at 15°C.

Tip Simple ligations with two fragments having 4 bp 3' or 5' overhanging ends require much less ligase than more complex ligations or blunt-end ligations. The quality of the DNA will also affect the amount of ligase needed.

Tip Ligation of sticky-ends is usually carried out at 12–15°C to maintain a balance between annealing of the ends and the activity of the enzyme. Higher temperatures make annealing of the ends difficult, while lower temperatures diminish ligase activity.

Tip Blunt-end ligations are usually performed at room temperature since annealing is not a factor, though the enzyme is unstable above 30°C. Blunt-end ligations require about 10–100 times more enzyme than sticky-end ligations in order to achieve an equal efficiency.

3. Introduce 1–10 µl of the ligated products into competent *E. coli* cells and select for transformants using the genetic marker present on the vector (for further information, see Protocols 1 and 2, pages 2 and 3).
4. From individual *E. coli* transformants, purify plasmid or phage DNAs by miniprep procedure and determine their structures by restriction mapping.

Tip It is highly recommended to include two controls in every transformation experiment:
- A "mock" transformation without DNA.
- A transformation reaction with a known amount of closed circular plasmid DNA.

Controls are essential if things go wrong. For example, colonies on plates that receive mock-transformed bacteria may indicate that the medium lacks the correct antibiotic. An absence of colonies on plates receiving bacteria transformed with plasmids under construction can only be interpreted if a positive control using a standard DNA has been included. See page 4 for further information on transformation controls.

QIAGEN offers a wide range of products for the preparation and isolation of plasmid DNA, DNA cleanup, and PCR fragment cloning (including competent cells), for all throughput and purity requirements. For further information about QIAGEN products and literature please refer to the *QIAGEN Product Guide*, visit us online at **www.qiagen.com**, or contact QIAGEN Technical Services or your local distributor.

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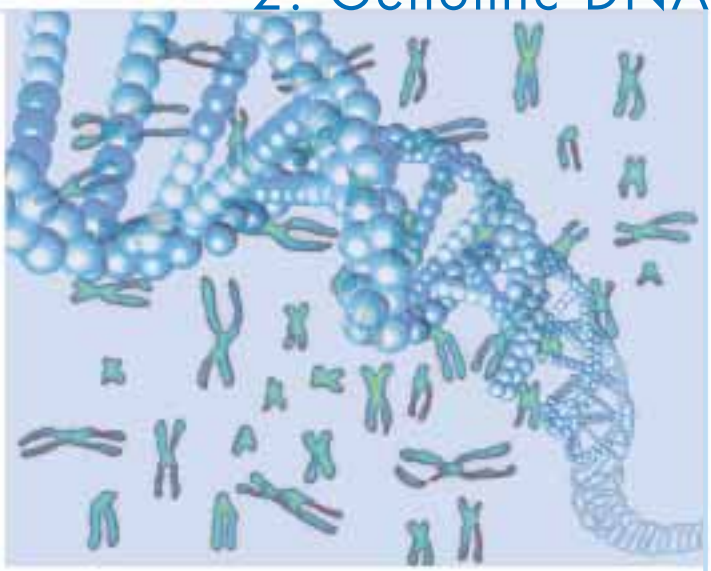
2. Genomic DNA



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What is Genomic DNA?

Genomic DNA constitutes the total genetic information of an organism. The genomes of almost all organisms are DNA, the only exceptions being some viruses that have RNA genomes. Genomic DNA molecules are generally large, and in most organisms are organized into DNA–protein complexes called chromosomes. The size, number of chromosomes, and nature of genomic DNA varies between different organisms (Table 1). Viral DNA genomes are relatively small and can be single- or double-stranded, linear or circular. All other organisms have double-stranded DNA genomes. Bacteria have a single, circular chromosome. In eukaryotes, most genomic DNA is located within the nucleus (nuclear DNA) as multiple linear chromosomes of different sizes. Eukaryotic cells additionally contain genomic DNA in the mitochondria and, in plants and lower eukaryotes, the chloroplasts. This DNA is usually a circular molecule and is present as multiple copies within these organelles.

Genomic DNA contains genes, discrete regions that encode a protein or RNA. A gene comprises the coding DNA sequence as well as the associated regulatory elements that control gene expression. Nuclear eukaryotic genes also contain noncoding regions called introns. The number of genes varies widely between different organisms. Coding DNA represents only a small fraction of eukaryotic genomic DNA: the bulk of the DNA is noncoding, much of which is made up of repetitive sequences. Some noncoding DNA has structural and regulatory functions, however, the function of most of this DNA is largely unknown.

The number of copies of each genetic locus present in a cell, or ploidy, also varies between organisms. The somatic (body) cells of organisms that reproduce sexually are usually diploid, having two sets of homologous chromosomes and hence two copies of each genetic locus, while the germ (reproductive) cells are haploid and have only one copy of each chromosome. Prokaryotic cells are haploid. Some plants are polyploid, for example, modern wheat, which is hexaploid (six copies of each chromosome).

This chapter describes considerations for isolation of genomic DNA from different sample sources, as well as commonly used techniques for analysis of genomic DNA.

Table 1. Sizes and molecular weights of various genomic DNAs

Organism	Base pairs per haploid genome	Molecular weight of genome (daltons)	Number of chromosomes
SV40	5243	3.4×10^6	
ΦX174	5386	3.5×10^6	
Adenovirus 2	35,937	2.3×10^7	
Lambda	48,502	3.2×10^7	
Escherichia coli	4.7×10^6	3.1×10^9	x = 1
Saccharomyces cerevisiae	1.5×10^7	9.8×10^9	2x = 32
Dictyostelium discoideum	5.4×10^7	3.5×10^{10}	x = 6
Arabidopsis thaliana	7.0×10^7	4.6×10^{10}	2x = 10
Caenorhabditis elegans	8.0×10^7	5.2×10^{10}	2x = 12
Drosophila melanogaster	1.4×10^8	9.1×10^{10}	2x = 8
Gallus domesticus (chicken)	1.2×10^9	7.8×10^{11}	2x = 78
Mus musculus (mouse)	2.7×10^9	1.8×10^{12}	2x = 40
Rattus norvegicus (rat)	3.0×10^9	2.0×10^{12}	2x = 42
Xenopus laevis	3.1×10^9	2.0×10^{12}	2x = 36
Homo sapiens	3.3×10^9	2.1×10^{12}	2x = 46
Zea mays	3.9×10^9	2.5×10^{12}	2x = 20
Nicotiana tabacum	4.8×10^9	3.1×10^{12}	2x = 48

Adapted from references 1 and 2.

General Remarks on Handling Genomic DNA

DNA is a relatively stable molecule. However, introduction of nucleases to DNA solutions should be avoided as these enzymes will degrade DNA. Genomic DNA consists of very large DNA molecules, which are fragile and can break easily. To ensure the integrity of genomic DNA, excessive and rough pipetting and vortexing should be avoided. DNA is subject to acid hydrolysis when stored in water, and should therefore be stored in TE buffer (see "Commonly Used Buffers and Solutions", page 90) or Buffer AE from QIAGEN.

Sample Storage Prior to Isolation of Genomic DNA

The quality of the starting material affects the quality and yield of the isolated DNA. The highest DNA yield and quality is achieved by purifying genomic DNA from freshly harvested tissues and cells. If samples cannot be processed immediately after harvesting, they should be stored under conditions that preserve DNA integrity. In general, genomic DNA yields will decrease if samples, particularly animal samples, are stored at either 2–8°C or –20°C without previous treatment. In addition, repeated freezing and thawing of frozen samples should be avoided as this will lead to genomic DNA of reduced size, and in clinical samples, to reduced yields of pathogen DNA (e.g., viral DNA). Recommendations for storage of different starting materials are discussed below.

Blood

An anticoagulant should be added to blood samples that will be stored. For example, blood samples treated with heparin or EDTA can be stored at 2–8°C for a few days, or at –20°C or –80°C for a few weeks. Alternatively, blood samples can be treated with ACD Solution B (0.48% citric acid, 1.32% sodium citrate, 1.47% glucose; use 1 ml per 6 ml blood) and stored for at least 5 days at 2–8°C or 1 month at –20°C. For long-term storage, blood nuclei can be prepared and stored at –20°C.*

Other clinical samples

Most biological fluids (e.g., plasma, serum, and urine) and stool samples can be stored at 2–8°C for several hours. Freezing at –20°C or –80°C is recommended for long-term storage. Swabs can be stored dry at room temperature.

Animal tissue

Freshly harvested tissue can be immediately frozen and stored at –20°C, –80°C, or in liquid nitrogen. Lysed tissue samples can be stored in a suitable lysis buffer for several months at ambient temperature.†

Animal and human tissues can also be fixed for storage. We recommend using fixatives such as alcohol and formalin; however, long-term storage of tissues in formalin will result in chemical modification of the DNA. Fixatives that cause cross-linking, such as osmic acid, are not recommended if DNA will be isolated from the tissue. It is also possible to isolate DNA from paraffin-embedded tissue.

* *Blood & Cell Culture Kits from QIAGEN provide buffers and a protocol for preparation of cell nuclei.*

† *e.g., Buffer ATL from DNeasy® Tissue Kits.*

Animal, yeast, and bacterial cell cultures

Centrifuge harvested cell cultures, remove the supernatant, and then store the cells at –20°C or –80°C. Alternatively, animal cell nuclei can be prepared and stored at –20°C.*

Plant tissue

Fresh leaves and needles from most plant species can be stored for up to 24 hours at 4°C without affecting DNA quality or yield. In general, samples that will be stored for longer than 24 hours should be stored at –80°C. However, some samples (e.g., tree buds) can be stored for several days at 4°C. Tissues stored at 4°C should be kept in a closed container to prevent dehydration. Large samples (e.g., branches) can be stored in a plastic bag containing a wet paper towel.

If it is not practical to store frozen samples, a number of methods are available for drying plant tissue, for example, silica gel, food dehydrators, or lyophilizers (3). To prevent DNA degradation, material should be completely desiccated in less than 24 hours. Dried samples should be kept in the dark at room temperature under desiccating or hermetic conditions for long-term storage. Depending on how the sample was handled, the DNA in herbarium and forensic samples may be degraded. Disrupted plant material can be stored in a suitable lysis buffer for several months at ambient temperature.†

Fungal material

Mycelium should be harvested directly from a culture dish or liquid culture. For liquid cultures, the cells should be pelleted by centrifugation and the supernatant removed before DNA isolation or storage. Harvested samples can be either directly frozen or freeze dried, and stored at –80°C.

* *Blood & Cell Culture Kits from QIAGEN provide buffers and a protocol for preparation of cell nuclei.*

† *e.g., Buffer AP1 from DNeasy Plant Kits.*

Sample Disruption for Isolation of Genomic DNA

Complete disruption and lysis of cells walls and plasma membranes of cells and organelles is an absolute requirement for all genomic DNA isolation procedures. Incomplete disruption results in significantly reduced yields.

Disruption methods

Lysis buffer

Disruption generally involves use of a lysis buffer that contains a detergent (for breaking down cellular membranes) and a protease (for digestion of protein cellular components). The choice of protease depends on the lysis buffer used. Both QIAGEN® Proteinase K and QIAGEN Protease have high activity in commonly used buffers for DNA isolation, however QIAGEN Proteinase K is recommended for buffers containing SDS and >8 mM EDTA.

Some sample types require additional treatment for efficient lysis; this is described in more detail below. Specific recommendations for different sample sources are described in “Special considerations for isolating genomic DNA from different sample sources”, page 26.

Disruption using rotor–stator homogenizers

Rotor–stator homogenizers thoroughly disrupt animal and plant tissues in 5–90 seconds depending on the toughness of the sample. The rotor turns at very high speed causing the sample to be disrupted by a combination of turbulence and mechanical shearing. Foaming of the sample should be kept to a minimum by using properly sized vessels, by keeping the tip of the homogenizer submerged, and by holding the immersed tip to one side of the tube. Rotor–stator homogenizers are available in different sizes and operate with probes of different sizes. Probes with diameters of 5 mm and 7 mm are suitable for volumes up to 300 µl and can be used for homogenization in microfuge tubes. Probes with a diameter of 10 mm or above require larger tubes.

Disruption using the Mixer Mill MM 300 and other bead mills

In disruption using a bead mill, the sample is agitated at high speed in the presence of beads. Disruption occurs by the shearing and crushing action of the beads as they collide with the cells. Disruption efficiency is influenced by:

- size and composition of beads
- ratio of buffer to beads
- amount of starting material
- speed and configuration of agitator
- disintegration time

The optimal beads to use are 0.1 mm (mean diameter) glass beads for bacteria, 0.5 mm glass beads for yeast and unicellular animal cells, 3–7 mm stainless steel beads for animal tissues, and 3–7 mm stainless steel or tungsten carbide beads for plant and fungal tissues. It is essential that glass beads are pretreated by washing in concentrated nitric acid. Alternatively, use commercially available acid-washed glass beads (e.g., SIGMA, cat. no. G1145, G1277, or G8772). All other disruption parameters must be determined empirically for each application.

Tip The *DNeasy 96 Plant Handbook* from QIAGEN contains guidelines for disruption of plant material using the Mixer Mill MM 300.

Disruption using a mortar and pestle

For disruption using a mortar and pestle, freeze the sample immediately in liquid nitrogen and grind to a fine powder under liquid nitrogen. Transfer the suspension (tissue powder and liquid nitrogen) into a liquid-nitrogen–cooled, appropriately sized tube and allow the liquid nitrogen to evaporate without allowing the sample to thaw. Add lysis buffer and continue as quickly as possible with the isolation procedure.

Special considerations for isolating genomic DNA from different sample sources

Some sample sources contain substances that can cause problems in DNA isolation and analysis. Special considerations are required when working with these sample sources. In this section, considerations for working with a number of different sources are discussed.

Blood

Blood samples are routinely collected for clinical analysis. Blood contains a number of enzyme inhibitors that can interfere with downstream DNA analysis. In addition, common anticoagulants such as heparin and EDTA can interfere with downstream assays. DNA isolation from blood requires a method to provide high-quality DNA without contaminants or enzyme inhibitors.

Erythrocytes (red blood cells) from birds, fish, and frogs contain nuclei and hence genomic DNA, while those from mammals do not. Since healthy mammalian blood contains approximately 1000 times more erythrocytes than nuclei-containing leukocytes (white blood cells, comprising lymphocytes, monocytes, and granulocytes) removing the erythrocytes prior to DNA isolation can give higher DNA yields. This can be accomplished by several methods. One is selective lysis of erythrocytes, which are more susceptible than leukocytes to hypotonic shock and burst rapidly in the presence of a hypotonic buffer. Alternatively, Ficoll® density-gradient centrifugation can be performed to recover mononuclear cells (lymphocytes and monocytes) and remove erythrocytes. This technique also removes granulocytes. A third method is to prepare a leukocyte-enriched fraction of whole blood, called buffy coat, by centrifuging whole blood at 3300 x *g* for 10 minutes at room temperature. After centrifugation, three different fractions are distinguishable: the upper clear layer is plasma; the intermediate layer is buffy coat; and the bottom layer contains concentrated erythrocytes.

Blood samples, including those treated to remove erythrocytes, can be efficiently lysed using lysis buffer and protease or proteinase K. Along with the animal's genomic DNA, viral and bacterial DNA can also be isolated from blood samples (see page 27).

Other clinical samples

Most biological fluids can be treated in the same way as blood samples for isolation of DNA. Isolation of DNA from stool samples is more difficult, as stool typically contains many compounds that can degrade DNA and inhibit downstream enzymatic reactions.

Tip The QIAamp® DNA Stool Kit from QIAGEN provides InhibitEX™ and Buffer ASL, unique reagents that efficiently absorb inhibitory compounds in stool samples for efficient removal by centrifugation.

Animal tissues and cell culture

Animal cell cultures and most animal tissues can be efficiently lysed using lysis buffer and protease or proteinase K. Fresh or frozen tissue samples should be cut into small pieces to aid lysis. Mechanical disruption using a homogenizer, mixer mill, or mortar and pestle prior to lysis can reduce lysis time. Skeletal muscle, heart, and skin tissue have an abundance of contractile proteins, connective tissue, and collagen, and care should be taken to ensure complete digestion with protease or proteinase K.

For fixed tissues, the fixative should be removed prior to lysis. Formalin can be removed by washing the tissue in PBS. Paraffin should similarly be removed from paraffin-embedded tissues by extraction with xylene followed by washing with ethanol.

Tip The *DNeasy Tissue Kit Handbook* and *QIAamp DNA Mini Kit Handbook* from QIAGEN provide protocols for isolation of DNA from fixed and paraffin-embedded tissues.

Yeast cell cultures

Yeast cell cultures must first be treated with lyticase or zymolase to digest the cell wall. The resulting spheroplasts are collected by centrifugation and then lysed using lysis buffer and proteinase K or protease.

Tip The *DNeasy Tissue Kit Handbook* from QIAGEN provides a protocol for isolation of DNA from yeast.

Bacteria

Many bacterial cell cultures can be efficiently lysed using lysis buffer and protease or proteinase K. Some bacteria, particularly Gram-positive bacteria, require pre-incubation with specific enzymes (e.g., lysozyme or lysostaphin) to lyse the rigid, multilayered cell wall.

Bacterial DNA can also be isolated from a wide variety of clinical samples. Bacterial cells should be pelleted from biological fluids, and the DNA isolated as for bacterial cell cultures. Swab samples should be pretreated with fungicide before centrifugation of bacterial cells.

Tip The *QIAamp DNA Mini Kit Handbook* from QIAGEN provides protocols for isolation of bacterial DNA from various clinical samples, including biological fluids and swaps (e.g., eye, nasal, and pharyngeal).

DNA viruses

In clinical applications viral DNA is typically isolated from cell-free body fluids, where their titer can be very low. Virus particles may need to be concentrated before DNA isolation by ultracentrifugation, ultrafiltration, or precipitation. Addition of carrier DNA may also be necessary during DNA isolation when the expected yield of DNA is low. Integrated viral DNA is prepared using the same procedure as for isolation of genomic DNA from the relevant sample. Bacteriophage, such as M13 and lambda, are isolated from infected bacterial cultures. The bacterial cells must be removed from the culture by centrifugation prior to isolation of viral DNA.

Tip The *QIAamp DNA Blood Mini Kit*, *QIAamp DNA Stool Mini Kit*, and *QIAamp UltraSens Virus Kit Handbooks* from QIAGEN provide protocols for isolation of viral DNA from various clinical samples, including blood, stool, plasma, serum, urine, and cerebrospinal fluid. QIAGEN also offers the QIAGEN Lambda and the QIAprep® M13 systems for isolation of lambda and M13 DNA, respectively.

Plants

Isolation of DNA from plant material presents special challenges, and commonly used techniques often require adaptation before they can be used with plant samples. Several plant metabolites have chemical properties similar to those of nucleic acids, and are difficult to remove from DNA preparations. Co-purified metabolites and contaminants introduced by the purification procedure, such as salts or phenol, can inhibit enzymatic reactions or cause variations in UV spectrophotometric measurements and gel migration.

DNA isolation is often improved by using plants grown under conditions that do not induce high levels of plant metabolites. Because of the great variation among plants, it is difficult to make general statements about growth conditions to use. However, as a general guideline, it is recommended to use healthy, young tissues when possible. DNA yields from young tissues are often higher than from old tissue because young tissue generally contains more cells than the same amount of older tissue. Young tissue of the same weight also contains fewer metabolites. In addition, many protocols for "home-made" DNA isolation methods recommend growing plants in darkness for 1–2 days before harvesting to prevent high-level accumulation of plant metabolites.

Plant tissue and filamentous fungi should be mechanically disrupted using a mixer mill or mortar and pestle prior to lysis.

Tip DNeasy Plant Kits from QIAGEN efficiently remove plant metabolites during isolation of DNA from a wide variety of plant species, including very demanding sources.

Storage, Quantification, and Determination of Quality and Yield of Genomic DNA

Storage of DNA

For long-term storage, DNA should be dissolved in TE buffer (see "Commonly Used Buffers and Solutions", page 90) or Buffer AE from QIAGEN, and stored at –20°C. DNA stored in water is subject to acid hydrolysis. Any contaminants in the DNA solution may lead to DNA degradation. Avoid repeated freeze-thawing as this will lead to precipitates. We recommend storing genomic DNA samples in aliquots.

Quantification of DNA

Spectrophotometry and fluorometry are commonly used to measure DNA concentration. Spectrophotometry can be used to measure microgram quantities of pure DNA samples (i.e., DNA that is not contaminated by proteins, phenol, agarose, or RNA). Fluorometry is more sensitive, allowing measurement of nanogram quantities of DNA, and furthermore the use of Hoechst 33258 dye allows specific analysis of DNA.

Spectrophotometry

DNA concentration can be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer using a quartz cuvette. For greatest accuracy, readings should be between 0.1 and 1.0. An absorbance of 1 unit at 260 nm corresponds to 50 µg genomic DNA per ml (A_{260} =1 ⇒ 50 µg/ml)*. This relation is valid only for measurements made at neutral pH, therefore, samples should be diluted in a low-salt buffer with neutral pH (e.g., Tris·Cl, pH 7.0). An example of the calculation involved in nucleic acid quantification when using a spectrophotometer is provided in "Spectrophotometric Measurement of Nucleic Acid Concentration", page 91).

Tip If you will use more than one cuvette to measure multiple samples, the cuvettes must be matched.

Tip Spectrophotometric measurements do not differentiate between DNA and RNA, so RNA contamination can lead to overestimation of DNA concentration.

Tip Phenol has an absorbance maximum of 270–275 nm, which is close to that of DNA. Phenol contamination mimics both higher yields and higher purity, because of an upward shift in the A_{260} value.

Fluorometry

Fluorometry allows specific and sensitive measurement of DNA concentration by use of the fluorochrome Hoechst 33258, which shows increased emission at 458 nm when bound to DNA. This dye has little affinity for RNA, allowing accurate quantification of DNA samples that are contaminated with RNA.

DNA standards and samples are mixed with Hoechst 33258 and measured in glass or acrylic cuvettes using a scanning fluorescence spectrophotometer or a dedicated filter fluorometer set at an excitation wavelength of 365 nm and an emission wavelength of 460 nm. The sample measurements are then compared to the standards to determine DNA concentration.

Tip As Hoechst 33258 preferentially binds AT-rich DNA, use standards with a similar base composition to the sample DNA.

* *Based on a standard 1 cm path length.*

Purity of DNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/ A_{280}) provides an estimate of DNA purity with respect to contaminants that absorb UV light, such as protein. The A_{260}/ A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/ A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/ A_{280} ratio and reduced sensitivity to protein contamination (4). For accurate A_{260}/ A_{280} values, we recommend measuring absorbance in a slightly alkaline buffer (e.g., 10 mM Tris·Cl, pH 7.5). Make sure to zero the spectrophotometer with the appropriate buffer.

Pure DNA has an A_{260}/ A_{280} ratio of 1.7–1.9. Scanning the absorbance from 220–320 nm will show whether there are contaminants affecting absorbance at 260 nm. Absorbance scans should show a peak at 260 nm and an overall smooth shape.

Tip Phenol has an absorbance maximum of 270–275 nm, which is close to that of DNA. Phenol contamination mimics both higher purity and higher yields, because of an upward shift in the A_{260} value.

RNA contamination

Depending on the DNA isolation method used, RNA will be co-purified with genomic DNA. RNA may inhibit some downstream applications, but it will not inhibit PCR. Spectrophotometric measurements do not differentiate between DNA and RNA, so RNA contamination can lead to overestimation of DNA concentration.

Treatment with RNase A will remove contaminating RNA; this can either be incorporated into the purification procedure or performed after the DNA has been purified. Prior to use, ensure that the RNase A solution has been heat-treated to destroy any contaminating DNase activity. Alternatively, use DNase-free RNase purchased from a reliable supplier.

Tip Advanced anion-exchange technology from QIAGEN allows isolation of high-molecular-weight genomic DNA that is free of RNA.

Integrity and size of genomic DNA

The integrity and size of genomic DNA can be checked by pulse-field gel electrophoresis (PFGE) using an agarose gel (see "A Guide to Analytical Gels", page 34). Genomic DNA isolated using anion-exchange chromatography (e.g., using QIAGEN Genomic-tips) should be sized up to 150 kb with an average length of 50–100 kb (**Figure 1**). DNA of this size is suitable for Southern analysis, library construction, genome mapping, and other demanding applications. Genomic DNA purified using silica-gel–membrane technology (e.g., using the DNeasy and QIAamp Systems) should be sized up to 50 kb with fragments of 20–30 kb predominating. DNA of this size is ideal for analysis by PCR.

DNA yield

Genomic DNA yields depend on the size, type, age, and quality of the starting material. The DNA content of the cell type used also affects yields: a tissue composed of small cells will have a higher cell density, and therefore is likely to contain more DNA than a sample of the same size comprised of larger cells. DNA content also depends on the genome size and ploidy.

Agarose Gel Analysis of Genomic DNA

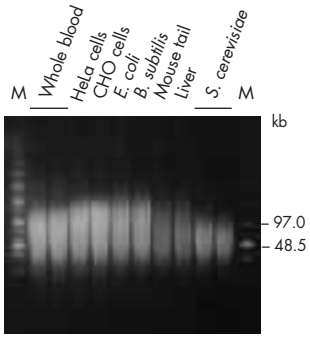

Figure 1. *Pulse-field gel electrophoresis of DNA (2 µg) purified with QIAGEN Genomic-tips.* ***M:*** *markers.*

Isopropanol Precipitation of Genomic DNA

Alcohol precipitation is commonly used for concentrating, desalting, and recovering nucleic acids. Precipitation is mediated by high concentrations of salt and the addition of either isopropanol or ethanol. Since less alcohol is required for isopropanol precipitation, this is the preferred method for precipitating DNA from large volumes. In addition, isopropanol precipitation can be performed at room temperature, which minimizes co-precipitation of salt that interferes with downstream applications.

Protocol 1. Isopropanol precipitation procedure

Protocol 1

1. Adjust the salt concentration if necessary, for example, with sodium acetate (0.3 M, pH 5.2, final concentration) or ammonium acetate (2.0–2.5 M, final concentration).
2. Add 0.6–0.7 volumes of room-temperature isopropanol to the DNA solution and mix well.

Tip Use all solutions at room temperature to minimize co-precipitation of salt.

Tip Do not use polycarbonate tubes for precipitation as polycarbonate is not resistant to isopropanol.

3. Centrifuge the sample immediately at 10,000–15,000 x *g* for 15–30 min at 4°C.

Tip Centrifugation should be carried out at 4°C to prevent overheating of the sample. (When precipitating from small volumes, centrifugation may be carried out at room temperature.)

Tip Genomic DNA can alternatively be precipitated by spooling the DNA using a glass rod following addition of isopropanol. The spooled DNA should be transferred immediately to a microfuge tube containing an appropriate buffer and redissolved (see step 9, page 31).

protocol continues overleaf

Protocol 1. Continued

4. Carefully decant the supernatant without disturbing the pellet.

Tip Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Pellets from isopropanol precipitation have a glassy appearance and may be more difficult to see than the fluffy salt-containing pellets resulting from ethanol precipitation.

Tip Care should be taken when removing the supernatant as pellets from isopropanol precipitation are more loosely attached to the side of the tube.

Tip Carefully tip the tube with the pellet on the upper side to avoid dislodging the pellet.

Tip For valuable samples, the supernatant can be retained until recovery of the precipitated DNA has been verified.

5. Wash the DNA pellet by adding 1–10 ml (depending on the size of the preparation) of room-temperature 70% ethanol. This removes co-precipitated salt and replaces the isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

6. Centrifuge at 10,000–15,000 x *g* for 5–15 min at 4°C.

Tip Centrifuge the tube in the same orientation as previously to recover the DNA into a compact pellet.

7. Carefully decant the supernatant without disturbing the pellet.

8. Air-dry the pellet for 5–20 min (depending on the size of the pellet).

Tip Do not overdry the pellet (e.g., by using a vacuum evaporator) as this will make DNA, especially high-molecular-weight DNA, difficult to redissolve.

9. Redissolve the DNA in a suitable buffer.

Tip Choose an appropriate volume of buffer according to the expected DNA yield and the desired final DNA concentration.

Tip Use a buffer with a pH of 7.5–8.0, as DNA does not dissolve easily in acidic buffers. (If using water, check pH.)

Tip Redissolve by rinsing the walls to recover all the DNA, especially if glass tubes have been used. To avoid shearing the DNA, do not pipet or vortex.

Tip High-molecular-weight DNA, such as genomic DNA, should be redissolved very gently to avoid shearing, e.g., at room temperature overnight or at 55°C for 1–2 h with gentle agitation.

Restriction Endonuclease Digestion of Genomic DNA

Many applications require conversion of genomic DNA into conveniently sized fragments by restriction endonuclease digestion. Restriction endonucleases are bacterial enzymes that bind and cleave DNA at specific target sequences. Type II restriction enzymes are the most widely used in molecular biology applications. These enzymes bind DNA at a specific recognition site, consisting of a short palindromic sequence, and cleave within this site, for example, AGCT (for *Alu*I), GAATTC (for *Eco*RI), and so on. Isoschizomers are different enzymes that share the same specificity and in some cases, the same cleavage pattern.

Tip Isoschizomers may have slightly different properties that can be very useful. For example, the enzymes *Mbo*I and *Sau*3A have the same sequence specificities, but *Mbo*I does not cleave methylated DNA, while *Sau*3A does. *Sau*3A can therefore be used instead of *Mbo*I where necessary.

Selecting suitable enzymes

The following factors need to be considered when choosing suitable restriction enzymes:

- Fragment size
- Blunt-ended/sticky-ended fragments
- Methylation sensitivity
- Compatiblity of reaction conditions (when carrying out digests with more than one enzyme)

Fragment size

Restriction enzymes with shorter recognition sequences cut more frequently than those with longer recognition sequences. For example, a 4 base pair (bp) cutter will cleave, on average, every 4^4 (256) bases, while a 6 bp cutter cleaves every 4^6 (4096) bases.

Tip Use 6 bp cutters for mapping genomic DNA, as these give fragments in a suitable size range for cloning.

Blunt-ended/sticky-ended fragments

Some restriction enzymes cut both DNA strands at the same position, creating blunt-ended DNA fragments. However, the majority of enzymes make cuts staggered on each strand, resulting in a few base pairs of single-stranded DNA at each end of the fragment, known as "sticky" ends. Some enzymes create 5' overhangs and others create 3' overhangs. The type of digestion affects the ease of downstream cloning:

- Sticky-ended fragments can be easily ligated to other sticky-ended fragments with compatible single-stranded overhangs, resulting in efficient cloning.
- Blunt-ended fragments usually ligate much less efficiently, making cloning more difficult. However, any blunt-ended fragment can be ligated to any other, so blunt-cutting enzymes are used when compatible sticky-ended fragments cannot be generated — for example, if the polylinker site of a vector does not contain an enzyme site compatible with the fragment being cloned.

Methylation

The genomic DNA of some organisms is methylated at specific sequences (e.g., the dinucleotide sequence CpG). Methylation patterns differ in different species, affecting the choice of restriction enzyme:

- The CpG dinucleotide occurs about five times less frequently in mammalian DNA than would be expected by chance, and most restriction enzymes with a CpG dinucleotide in their recognition site do not cleave if the cytosine is methylated. Therefore many enzymes with CpG in their recognition site, such as *Eag*I, *Not*I, and *Sal*I, cleave mammalian DNA only rarely.
- Drosophila, Caenorhabditis, and some other species do not possess methylated DNA, and have a higher proportion of CpG dinucleotides than mammalian species. Rare-cutter enzymes therefore cleave more frequently in these species.
- Plant DNA is highly methylated, so for successful mapping in plants, choose enzymes that either do not contain a CpG dinucleotide in their recognition site (e.g., *Dra*I or *Ssp*I) or that can cleave methylated CpG dinucleotides (e.g., *Bam*HI, *Kpn*I, or *Taq*I).

Compatibility of reaction conditions

If DNA is to be cut with more than one enzyme, both enzymes can be added to the reaction simultaneously provided that they are both active in the same reaction buffer and at the same reaction temperature. If the enzymes do not have compatible reaction conditions, perform one digestion, purify the reaction products, for example by isopropanol precipitation, and then perform the second digestion.

Components of a restriction digest

- Water
- DNA
- Buffer
- Enzyme

DNA

The amount of DNA digested depends on the downstream application and the genome size of the organism being analyzed. We recommend using a minimum of 10 µg DNA per reaction for Southern blotting of mammalian and plant genomic DNA.

Tip DNA should be free of contaminants, such as phenol, chloroform, ethanol, detergents, or high salt concentrations, as these may interfere with restriction endonuclease activity.

Enzyme

One unit of restriction endonuclease completely digests 1 µg of substrate DNA in 1 hour. However, genomic DNA generally requires more than 1 unit/µg to be digested completely. Most researchers add a ten-fold excess of enzyme to their reactions in order to ensure complete digestion.

Tip Ensure that the restriction enzyme does not exceed more than 10% of the total reaction volume, as otherwise the glycerol in which the enzyme is supplied may inhibit digestion.

Reaction volume

Most digests are carried out in a volume between 10 and 50 µl. (Reaction volumes smaller than 10 µl are susceptible to pipetting errors, and are not recommended.)

Protocol 2. Setting up a restriction digest

1. Pipet reaction components into a tube and mix well by pipetting.

Tip Thorough mixing is extremely important.

Tip The enzyme should be added last and kept on ice when not in the freezer.

Tip When setting up large numbers of digests, make a reaction master mix consisting of water, buffer, and enzyme, and pipet aliquots into tubes containing the DNA to be digested.

2. Centrifuge the tube briefly to collect the liquid at the bottom.
3. Incubate the reaction in a water bath or oven, usually for 1–4 h at 37°C. Restriction enzymes isolated from thermophilic bacteria require higher incubation temperatures, e.g., 50–65°C.
4. For some downstream applications it is necessary to heat-inactivate the enzyme after digestion. The majority of enzymes that have an optimal incubation temperature of 37°C are inactivated by heating the reaction to 65°C for 20 min after digestion.

Tip Some restriction enzymes are not fully inactivated by heat treatment. Restriction enzymes can be removed from reactions by purification using DNeasy Kits.

A Guide to Analytical Gels

Gels allow separation and identification of nucleic acids based on charge migration. Migration of nucleic acid molecules in an electric field is determined by size and conformation, allowing nucleic fragments of different sizes to be separated. However, the relationship between the fragment size and rate of migration is non-linear, since larger fragments have greater frictional drag and are less efficient at migrating through the polymer.

Agarose gel analysis is the most commonly used method for analyzing DNA fragments between 0.1 and 25 kb, while pulse-field gel electrophoresis enables analysis of DNA fragments up to 10,000 kb. This section provides useful hints for effective gel analysis of genomic DNA.

Pouring an agarose gel

Agarose concentration

The concentration of agarose used for the gel depends primarily on the size of the DNA fragments to be analyzed. Low agarose concentrations are used to separate large DNA fragments, while high agarose concentrations allow resolution of small DNA fragments (**Table 2**). Most gels are run using standard agarose, although some special types of agarose are available for particular applications. For example, low-melt agarose allows in situ enzymatic reactions and can therefore be used for preparative gels. Genomic DNA can be isolated directly from cells immobilized in low-melt agarose gels.*

Tip Use ultrapure-quality agarose since impurities such as polysaccharides, salts, and proteins can affect the migration of DNA. Agarose quality is particularly important when running high-percentage agarose gels.

* *See reference 5 for further information.*

Table 2. Concentration of agarose used for separating DNA fragments of different sizes

Agarose concentration (% w/v)	DNA fragment range (kb)
0.3*	5–60
0.5	1–30
0.7	0.8–12
1.0	0.5–10
1.2	0.4–7
1.5	0.2–3
2.0*	0.1–2

Adapted from references 1 and 5.

** Most gels are run using standard agarose, although some special types of agarose are available for particular applications, and for very high or low agarose concentrations. For example, low-melt agarose allows in situ enzymatic reactions and can be used for preparative gels.*

Electrophoresis buffers

The most commonly used buffers for agarose gel electrophoresis are TBE (Tris-borate–EDTA) and TAE (Tris-acetate–EDTA) (see "Agarose Gel Electrophoresis Buffers for Analysis of DNA", page 93). Although more frequently used, TAE has a lower buffering capacity than TBE and is more easily exhausted during extended electrophoresis. TBE should be used for pulse-field gel electrophoresis due to the high voltages used in this procedure.

Protocol 3. Pouring the gel

1. Prepare enough 1x electrophoresis buffer both to pour the gel and fill the electrophoresis tank.
2. Add an appropriate amount of agarose (depending on the concentration required) to an appropriate volume of electrophoresis buffer (depending on the type of electrophoresis apparatus being used) in a flask or bottle.

Tip The vessel should not be more than half full. Cover the vessel to minimize evaporation.

Tip Always use the same batch of buffer to prepare the agarose as to run the gel since small differences in ionic strength can affect migration of DNA.

3. Heat the slurry in a microwave or boiling water bath, swirling the vessel occasionally, until the agarose is dissolved.

Tip Ensure that the lid of the flask is loose to avoid buildup of pressure. Be careful not to let the agarose solution boil over as it becomes super-heated.

Tip If the volume of liquid reduces considerably during heating due to evaporation, make up to the original volume with distilled water. This will ensure that the agarose concentration is correct and that the gel and the electrophoresis buffer have the same buffer composition.

4. Cool the agarose to 55–60°C.
5. Pour the agarose solution onto the gel tray to a thickness of 3–5 mm. Insert the comb either before or immediately after pouring the gel. Leave the gel to set (30–40 min).

Tip Ensure that there is enough space between the bottom of the comb and the glass plate (0.5–1.0 mm) to allow proper formation of the wells and avoid sample leakage.

Tip Make sure that there are no air bubbles in the gel or trapped between the wells.

6. Carefully remove the comb and adhesive tape, if used, from the gel. Fill the tank containing the gel with electrophoresis buffer.

Tip Add enough buffer to cover the gel with a depth of approximately 1 mm liquid above the surface of the gel. If too much buffer is used the electric current will flow through the buffer instead of the gel.

Running an agarose gel

Agarose gel electrophoresis allows analysis of DNA fragments between 0.1 and 25 kb (e.g., genomic DNA digested with a frequently cutting restriction endonuclease), while pulse-field gel electrophoresis enables analysis of DNA fragments up to 10,000 kb (e.g., undigested genomic DNA or genomic DNA digested with rare cutting restriction endonucleases). The amount of genomic DNA loaded onto a gel depends on the application, but in general, loading of too much DNA should be avoided as this will result in smearing of the DNA bands on the gel.

Gel loading buffer (see "Agarose Gel Electrophoresis Buffers for Analysis of DNA", page 93) must be added to the samples before loading and serves three main purposes:

1. To increase the density of the samples to ensure that they sink into the wells on loading.
2. To add color to the samples through use of dyes such as bromophenol blue or xylene cyanol, facilitating loading.
3. To allow tracking of the electrophoresis due to co-migration of the dyes with DNA fragments of a specificsize.

Molecular-weight markers should always be included on a gel to enable analysis of DNA fragment sizes in the samples. See "Commonly Used DNA Markers in Agarose Gel Electrophoresis", page 93) for commonly used markers.

Protocol 4. Preparation of samples

Protocol 4

1. Add 1 volume of gel loading buffer to 6 volumes DNA sample and mix.

Tip Samples should always be mixed with gel loading buffer prior to loading on a gel.

Tip Do not use sample volumes close to the capacity of the wells, as samples may spill over into adjacent wells during loading.

Tip Be sure that all samples have the same buffer composition. High salt concentrations, for example in some restriction buffers, will retard the migration of the DNA fragments.

Tip Ensure that no ethanol is present in the samples, as this will cause samples to float out of the wells on loading.

Protocol 5. Agarose gel electrophoresis

Protocol 5

1. Apply samples in gel loading buffer to the wells of the gel.

Tip Prior to sample loading, remove air bubbles from the wells by rinsing them with electrophoresis buffer. Make sure that the entire gel is submerged in the electrophoresis buffer.

Tip To load samples, insert the pipet tip deep into the well and expel the liquid slowly. Take care not to break the agarose with the pipet tip.

Tip Once samples are loaded, do not move the gel tray/tank as this may cause samples to float out of the wells.

Tip Be sure to always include at least one lane of appropriate molecular-weight markers.

2. Connect the electrodes so that the DNA will migrate towards the anode (positive electrode).

Tip Electrophoresis apparatus should always be covered to protect against electric shocks.

protocol continues overleaf

Protocol 5. Continued

3. Turn on the power supply and run the gel at 1–10 V/cm until the dyes have migrated an appropriate distance. This will depend on the size of DNA being analyzed, the concentration of agarose in the gel, and the separation required.

Tip Avoid use of very high voltages which can cause trailing and smearing of DNA bands in the gel, particularly with high-molecular-weight DNA.

Tip Monitor the temperature of the buffer periodically during the run. If the buffer becomes overheated, reduce the voltage.

Tip Melting of an agarose gel during the electrophoresis is a sign that the buffer may have been incorrectly prepared or has become exhausted during the run.

Tip For very long runs, e.g., overnight runs, use a pump to recycle the buffer.

Protocol 6. Pulse-field gel electrophoresis

Protocol 6

1. Apply samples in gel loading buffer to the wells of the gel.

Tip Pulse-field gel electrophoresis uses high voltages, so TBE buffer, which has greater buffering capacity than TAE buffer, should be used.

Tip Prior to sample loading, remove air bubbles from the wells by rinsing them with electrophoresis buffer. Make sure that the entire gel is submerged in the running buffer.

Tip To load samples, insert the pipet tip deep into the well and expel the liquid slowly. Take care not to break the agarose with the pipet tip.

Tip Once samples are loaded, do not move the gel tray/tank as this may cause samples to float out of the wells.

Tip Be sure to always include at least one lane of appropriate molecular-weight markers.

2. Connect the electrodes so that the DNA will migrate towards the anode (positive electrode).

Tip Electrophoresis apparatus should always be covered to protect against electric shocks.

3. Turn on the power supply and run the gel at 170 V with a switch interval of 5–40 s until the dyes have migrated an appropriate distance. This will depend on the size of DNA being analyzed, the concentration of agarose in the gel, and the separation required.

Tip Monitor the temperature of the buffer periodically during the run. If the buffer becomes heated, reduce the voltage.

Tip Melting of an agarose gel during the electrophoresis is a sign that the buffer may have been incorrectly prepared or has become exhausted during the run.

Tip For very long runs, e.g., overnight runs, use a pump to recycle the buffer.

Visual analysis of the gel

Staining

To allow visualization of the DNA samples, agarose gels are stained with an appropriate dye. The most commonly used dye is the intercalating fluorescent dye ethidium bromide, which can be added either before or after the electrophoresis (see **Table 3**). Alternatives include recently introduced commercial dyes such as SYBR® Green.

Tip Stock solutions of ethidium bromide (generally 10 mg/ml) should be stored at 4°C in a dark bottle or bottle wrapped in aluminum foil.

Addition of ethidium bromide prior to electrophoresis — add ethidium bromide at a concentration of 0.5 µg/ml to the melted and subsequently cooled agarose, that is, just before pouring the gel.

Tip Mix the agarose–ethidium bromide solution well to avoid localized staining.

Addition of ethidium bromide after electrophoresis — soak the gel in a 0.5 µg/ml solution of ethidium bromide (in water or electrophoresis buffer) for 30–40 minutes.

Tip Rinse the gel with buffer or water before examining it to remove excess ethidium bromide.

Tip Staining buffer can be saved and re-used.

Note: Ethidium bromide is a powerful mutagen and is very toxic. Wear gloves and take appropriate safety precautions when handling. Use of nitrile gloves is recommended as latex gloves may not provide full protection. After use, ethidium bromide solutions should be decontaminated as described in commonly used manuals (1, 5).

Table 3. Comparison of ethidium bromide staining methods

Addition of ethidium bromide prior to electrophoresis	Addition of ethidium bromide after electrophoresis
Faster and more convenient procedure	Slower procedure requiring additional step
Allows monitoring of migration during electrophoresis	Does not allow monitoring of migration during electrophoresis
Requires decontamination of gel tanks and comb	No decontamination of gel tanks and comb necessary
More ethidium bromide is required	Usually less ethidium bromide is required
Electrophoretic mobility of linear DNA fragments is reduced by ~15%	No interference with electrophoretic mobility

Visualization

Ethidium bromide–DNA complexes display increased fluorescence compared to the dye in solution. This means that illumination of a stained gel under UV light (254–366 nm) allows bands of DNA to be visualized against a background of unbound dye. The gel image can be recorded by taking a Polaroid™ photograph or using a gel documentation system.

Tip UV light can damage the eyes and skin. Always wear suitable eye and face protection when working with a UV light source.

Tip UV light damages DNA. If DNA fragments are to be extracted from the gel, use a lower intensity UV source if possible and minimize exposure of the DNA to the UV light.

DNA Analysis by Southern Blotting

Southern blotting is a widely used technique that allows analysis of specific DNA sequences. DNA is usually first converted into conveniently sized fragments by restriction digestion. The DNA is next run through an agarose gel (5). Southern blotting (named after its inventor, E.M. Southern) refers to the transfer of the DNA to a nylon or nitrocellulose membrane by capillary transfer. The DNA of interest can be identified by hybridization to radioactive or chemiluminescent probes and visualized by autoradiography or staining.

Many variations on the Southern blotting procedure exist. A standard protocol is described here. See "Southern Transfer Buffers and Solutions" (page 94) for recipes for buffers and solutions.

Equipment required

- Whatman® 3MM filter paper
- Blotting membrane
- Paper towels, a stack of approximately 15–20 cm
- Plastic wrap
- Two glass or Plexiglas® plates
- Buffer tray (e.g., glass casserole dish) capable of holding 1–2 liters of buffer
- Support (to be placed in the buffer tray)
- Flat weight, approximately 1 kg
- Oven, at 80°C, or UV transilluminator
- Orbital shaker

Preparation of gels for Southern blotting

Fragmentation of large DNA molecules (optional)

DNA fragments longer than 10 kb do not transfer to blotting membranes efficiently. In order to facilitate their transfer, these fragments are reduced in size, either by acid depurination or by UV irradiation.

Acid depurination — immediately after gel electrophoresis, place the gel in a solution of 0.2 M HCl, so that it is completely covered. Agitate gently for 10 minutes. During this period the color of the bromophenol blue in the samples will change from blue to yellow, indicating that the gel has been completely saturated with the acid. Rinse the gel briefly in distilled water.

Tip The depurination step should not last too long, since very short fragments attach less firmly to the membrane.

Tip Depurinated gels may yield "fuzzy" bands on the final autoradiograph, presumably because of increased diffusion of the DNA during transfer. Depurination is therefore recommended only when fragments larger than 10 kb are to be transferred.

UV irradiation — expose the gel to UV light at a wavelength of 254 nm from a source operating at 30 W, for 30–60 seconds.

Denaturation

Double-stranded DNA must be denatured in order to create suitable hybridization targets. Completely cover the gel with denaturation buffer (see "Southern Transfer Buffers and Solutions", page 94) and incubate for 30 minutes with gentle shaking. If acid depurination was used to denature the DNA, the bromophenol blue will return to its original color during this incubation.

Neutralization

Remove the denaturation buffer and completely cover the gel in neutralization buffer (see "Southern Transfer Buffers and Solutions", page 94). Incubate for 30 minutes with gentle shaking.

Southern blotting

Protocol 7. Assembling the blotting apparatus

1. Place a support larger than the gel in a tray containing 10x SSC (see "Southern Transfer Buffers and Solutions", page 94), and cover the support with a glass or Plexiglas plate (see **Figure 2**).
2. Cut two lengths of Whatman 3MM paper wider than the gel, long enough to fit under the gel and reach to the bottom of the dish on either side (see **Figure 2**). Wet the sheets briefly in 10x SSC, and place them on the glass plate. Remove any air bubbles between the paper and the support by rolling a pipet several times back and forth over the surface.
3. Cut one sheet of blotting membrane and two sheets of Whatman 3MM paper about 1 mm larger than the gel on each side.

Tip Always wear gloves when working with blotting membranes. Handle membranes carefully by the edges or using clean blunt-ended forceps.

4. Place the prepared gel upside-down on the platform. Remove any air bubbles trapped between the gel and the platform by rolling a pipet several times back and forth over the gel.
5. Surround the gel with plastic wrap. This ensures that the 10x SSC moves only through the gel and not around it.
6. Place the precut blotting membrane on top of the gel so that it covers the entire surface. Do not move the blotting membrane once it has been placed on the gel. Remove any air bubbles between the paper and the support as described in step 4.
7. Briefly wet the two precut sheets of Whatman 3MM paper in 10x SSC, and place them on top of the nylon membrane (**Figure 2**). Again, remove any trapped air bubbles as described in step 4.
8. Place a 15–20 cm stack of dry paper towels on top of the filter paper.

Tip Make sure that the plastic wrap surrounding the gel prevents contact of the paper towels with the 10x SSC and the wet filter paper under the gel. Ensure that the towels do not droop over since they can cause liquid to flow around the gel instead of through it.

9. Place a second glass or Plexiglas plate on top of the paper towels. Place the weight on top of the plate (**Figure 2**).
10. Let the transfer proceed for 12–18 h.

Tip Transfer efficiency is improved by removing the wet paper towels and replacing them with dry ones at least once during the transfer.

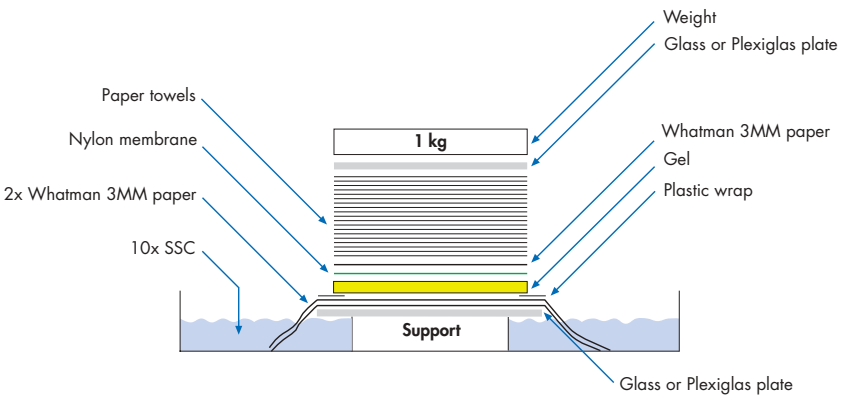

Figure 2. *Southern blot setup.*

Protocol 8. Fixing the DNA to the blot

1. After the transfer is complete, remove the weight, paper towels, and the two sheets of filter paper. Turn over the gel and the blotting membrane together, and lay them, gel-side up, on a sheet of dry filter paper. Mark the positions of the gel lanes on the membrane using a ballpoint pen or a soft-lead pencil. Peel the gel from the membrane. If desired, keep the gel to assess the effiency of DNA transfer, otherwise discard.

Tip Before removing the gel from the blotting membrane, ensure that the gel lanes are marked so that they can be later identified.

Tip In order to assess the efficiency of DNA transfer, stain the gel with ethidium bromide after blotting to see how much DNA remains.

2. Fix the DNA to the blot, either by baking (step 2A) or by UV-crosslinking (step 2B).

Tip UV-crosslinking generally gives better results and enhanced sensitivity compared with baking. However, effective crosslinking requires optimization of the system.

2A. To fix the DNA to the membrane by baking, first let the blot air-dry on a sheet of filter paper, then place between two sheets of filter paper, and bake at 80°C for 2 h.

2B. To fix the DNA by UV-crosslinking, first protect the surface of the membrane by covering the UV source (e.g., a transilluminator) with plastic wrap. Then take the damp blot and expose the side with DNA to the UV source for a predetermined length of time.

Tip It is important to optimize the system for UV-crosslinking. To do this, prepare a blot with several control DNA samples. Cut the blot into separate strips for each lane, and irradiate each blot for different times, varying from 0.5 to 5 min. Hybridize all the blots together and determine which time gives the optimal signal intensity. It is important to use the same conditions (UV wavelength, distance from UV source) for each experiment. It is also important to calibrate the system routinely, as the energy emitted from a UV bulb is reduced with use.

Tip UV light can damage the eyes and skin. Always wear suitable eye and face protection.

3. If the blot will not be used immediately, store it at room temperature covered in plastic wrap.

Guidelines for PCR

PCR is a powerful tool that allows amplification of specific DNA sequences. Prerequisites for successful PCR include the design of optimal primer pairs, the use of appropriate primer concentrations, and optimization of the PCR conditions.

Tip Genomic DNA sized up to 50 kb, such as that isolated using DNeasy and QIAamp Kits, shows the highest amplification efficiency under normal PCR cycling conditions.

Primer design

The following points should be considered when designing primers for PCR.

Length: 18–30 nucleotides

GC content: 40–60%

T_m:

- Simplified formula for estimating melting temperature (T_m): T_m = 2°C x (A+T) + 4°C x (G+C)
- Whenever possible, design primer pairs with similar T_m values.
- Optimal annealing temperatures may be above or below the estimated T_m. As a starting point, use an annealing temperature 5°C below T_m.

Sequence:

- Avoid complementarity of two or three bases at the 3' ends of primer pairs to reduce primer–dimer formation.
- Avoid mismatches between the 3' end of the primer and the target-template sequence.
- Avoid runs of 3 or more G or C at the 3' end.
- Avoid a 3'-end T. Primers with a T at the 3' end have a greater tolerance of mismatch.
- Commercially available computer software (e.g., Primer Designer 1.0, Scientific Software, 1990; Oligo, Rychlik and Rhoads, 1989) can be used for primer design.

Concentration:

- Spectrophotometric conversion for primers: 1 A_{260} unit = 20–30 µg/ml.
See "Spectrophotometric Measurement of Nucleic Acid Concentration", page 91 for an example of the calculation for determining nucleic acid concentration when using a spectrophotometer.
- Molar conversions:

Primer length	pmol/µg	20 pmol
18mer	168	119 ng
20mer	152	132 ng
25mer	121	165 ng
30mer	101	198 ng

- Use a concentration of 0.1–0.5 µM of each primer. For most applications, a primer concentration of 0.2 µM will be sufficient.

Storage: Lyophilized primers should be dissolved in a small volume of distilled water or TE to make a concentrated stock solution. Prepare small aliquots of working solutions containing 10 pmol/µl to avoid repeated thawing and freezing. Store all primer solutions at –20°C. Primer quality can be checked on a denaturing polyacrylamide gel; a single band should be seen.

PCR conditions

The primer and Mg^{2+} concentration in the PCR buffer and annealing temperature of the reaction may need to be optimized for each primer pair for efficient PCR. In addition, PCR efficiency can be improved by additives that promote DNA polymerase stability and processivity or increase hybridization stringency, and by using strategies that reduce nonspecific primer–template interactions (1). Use of high-purity reagents is also essential for successful PCR, especially for amplification of rare templates, for example, single copy genes in genomic DNA or pathogenic viral DNA sequences in genomic DNA isolated from an infected organism.

Tip Q-Solution and QIAGEN PCR buffer, together with QIAGEN *Taq* DNA Polymerase or HotStarTaq™ DNA Polymerase, provide efficient template amplification without the need for optimization, even for templates that have extensive secondary structure or that are GC- rich (6).

Inclusion of control reactions is essential for monitoring the success of PCR reactions. Wherever possible, a positive control should be included to check that the PCR conditions used can successfully amplify the target sequence. As PCR is extremely sensitive, requiring only a few copies of target template, a negative control containing no template DNA should always be included to ensure that the solutions used for PCR have not become contaminated with the template DNA.

Tip PCR setup should be performed in a separate area from PCR analysis to ensure that reagents used for PCR do not become contaminated with PCR products. Similarly, pipets used for analysis of PCR products should never be used for setting up PCR.

QIAGEN offers a wide range of products for isolation of genomic DNA from various sources and for all throughput requirements, as well as products for PCR analysis. For further information about QIAGEN products and literature please refer to the *QIAGEN Product Guide*, visit us online at **www.qiagen.com**, or contact QIAGEN Technical Services or your local distributor.

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6. Critical Factors for Successful PCR, *QIAGEN*

3. RNA

Contents

What is RNA?

RNA is a biological macromolecule that serves a number of different functions. Messenger RNA (mRNA), transcribed from DNA, serves as a template for synthesis of proteins. Protein synthesis is carried out by ribosomes, which consist of ribosomal RNA (rRNA) and proteins. Amino acids for protein synthesis are delivered to the ribosome on transfer RNA (tRNA) molecules. RNAs are also part of riboproteins involved in RNA processing.

In addition, many viruses contain RNA as their genome instead of DNA, and RNA species called ribozymes catalyze biochemical reactions, similar to enzymes. Since RNA is able to perform functions usually associated with DNA and proteins, it has been suggested that RNA was the original biological molecule, with subsequent evolution of DNA and proteins.

A typical mammalian cell contains 10–30 pg total RNA. The majority of RNA molecules are tRNAs and rRNAs. mRNA accounts for only 1–5% of the total cellular RNA although the actual amount depends on the cell type and physiological state. Approximately 360,000 mRNA molecules are present in a single mammalian cell, made up of approximately 12,000 different transcripts with a typical length of approximately 2 kb (**Tables 1** and **2**, page 46). Some mRNAs comprise as much as 3% of the mRNA pool whereas others account for less than 0.01%. These "rare" or "low abundance" messages may have a copy number of only 5–15 molecules per cell. However, these rare species may account for as much as 11,000 different mRNA species, comprising 45% of the mRNA population (**Table 3**, page 46.)*

** For more information, see reference 1.*

While the genes of an organism are relatively fixed, the mRNA population represents how genes are expressed under any given set of conditions. Analysis of RNA by hybridization technologies, including northern blotting and microarray analysis, or by RT-PCR can provide a good reflection of an organism's gene-expression profile.

Compared to DNA, however, RNA is relatively unstable. This is largely due to the presence of ribonucleases (RNases), which break down RNA molecules.

RNases are very stable, do not require cofactors, are effective in very small quantities, and are difficult to inactivate. RNase contamination can come from human skin and dust particles, which can carry bacteria and molds. Isolation and analysis of RNA therefore requires specialized techniques.

This chapter describes procedures for successful stabilization, purification, and analysis of RNA.

Table 1. **RNA content of a typical human cell**

Total RNA per cell	~10–30 pg
Proportion of total RNA in nucleus	~14%
DNA:RNA in nucleus	~2:1
mRNA molecules	$2 \times 10^5 - 1 \times 10^6$
Typical mRNA size	1900 nt

Table 2. **RNA distribution in a typical mammalian cell**

RNA species	Relative amount
rRNA (28S, 18S, 5S)	80–85%
tRNAs, snRNAs, low MW species	15–20%
mRNAs	1–5%

Table 3. **mRNA classification based on abundance**

Abundance class	Copies/cell	Number of different messages/cell	Abundance of each message
Low	5–15	11,000	<0.004%
Intermediate	200–400	500	<0.1%
High	12,000	<10	3%

Table 4. **RNA content in various cells and tissues**

Source		Total RNA (µg)	mRNA (µg)
Cell cultures*	–	30–500	0.3–25
	NIH/3T3	120	3
	HeLa	150	3
	COS-7	350	5
Mouse — developmental stages†	Unfertilized egg	0.43 ng	nd
	Oocyte	0.35 ng	nd
	2-cell	0.24 ng	nd
	8–16-cell	0.69 ng	nd
	32-cell	1.47 ng	nd
	13-day-old embryo	450	13
Mouse tissue‡	Brain	120	5
	Heart	120	6
	Intestine	150	2
	Kidney	350	9
	Liver	400	14
	Lung	130	6
	Spleen	350	7

** 10^7 cells † Per organism ‡ 100 mg nd = not determined*

General Remarks on Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, all plasticware or glassware should be treated to eliminate possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the isolation procedure. In order to create and maintain an RNase-free environment, the following precautions should be followed while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep isolated RNA on ice when aliquots are pipetted for downstream applications.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended when working with RNA. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Non-disposable plasticware

Non-disposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH, 1 mM EDTA followed by RNase-free water (see "Solutions", page 48). Alternatively, chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240°C for at least 4 hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate). Fill glassware with 0.1% DEPC (0.1% in water), incubate overnight (12 hours) at 37°C, and then autoclave or heat to 100°C for 15 minutes to eliminate residual DEPC.

** DEPC is a suspected carcinogen and should be handled with great care. Wear gloves and use a fume hood when using this chemical.*

Electrophoresis tanks

Electrophoresis tanks should be cleaned with detergent solution (e.g., 0.5% SDS), thoroughly rinsed with RNase-free water, and then rinsed with ethanol* and allowed to dry.

Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC.† DEPC is a strong, but not absolute, inhibitor of RNases that works by covalently modifying RNases.

** Plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier's instructions.*

† DEPC is a suspected carcinogen and should be handled with great care. Wear gloves and use a fume hood when using this chemical.

Protocol 1. Preparation of RNase-free solutions

1. Add 0.1 ml DEPC to 100 ml of the solution to be treated. Shake vigorously to bring the DEPC into solution.
2. Incubate for 12 h at 37°C.
3. Autoclave for 15 min to remove any trace of DEPC.

Tip DEPC will react with primary amines and cannot be used directly to treat Tris buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer.

Tip Trace amounts of DEPC will modify purine residues in RNA by carboxymethylation. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 min. Carboxymethylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified.

Tip RNeasy® buffers from QIAGEN are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

Stabilization of RNA in Biological Samples

In order to ensure accurate gene-expression analyses, it is important that the RNA analyzed truly represents the in vivo gene expression of the sample. This is complicated by the fact that changes can occur during handling of the sample and isolation of the RNA.

Once a biological sample is harvested, its RNA becomes extremely unstable. There are two major types of artifacts that can occur. Downregulation of genes and enzymatic degradation of RNA result in an artificial reduction of both nonspecific and specific mRNA species. At the same time, certain genes can be induced during handling and processing of the sample. The combination of these two effects can result in a transcription profile that differs from the true in vivo gene-expression pattern.

Immediate stabilization of the RNA expression pattern is a prerequisite for accurate gene-expression analysis. Traditionally, samples harvested for RNA analysis are immediately frozen in liquid nitrogen and stored at –80°C until processed. Stabilization reagents such as RNA*later*™ RNA Stabilization Reagent for tissues, RNAprotect™ Bacteria Reagent for bacteria, and the PAXgene™ Blood RNA System for blood can alternatively be used to stabilize RNA in biological samples.

Disruption and Homogenization of Starting Materials for Isolation of RNA

Efficient disruption and homogenization of the starting material is an absolute requirement for all total RNA isolation procedures. Disruption and homogenization are two distinct steps.

Disruption: Complete disruption of cells walls and plasma membranes of cells and organelles is absolutely required to release all the RNA contained in the sample. Different samples require different methods to achieve complete disruption. Incomplete disruption results in significantly reduced yields.

Homogenization: Homogenization is necessary to reduce the viscosity of the cell lysates produced by disruption. Homogenization shears the high-molecular-weight genomic DNA and other high-molecular-weight cellular components to create a homogeneous lysate. Incomplete homogenization results in inefficient binding of RNA and therefore significantly reduced yields.

Some disruption methods simultaneously homogenize the sample while others require an additional homogenization step. **Table 5** (page 51) gives an overview of different disruption and homogenization methods suitable for various starting materials. It can be used as a guide to choose the appropriate method for the starting material with which you are working. The disruption and homogenization methods listed in **Table 5** are described in more detail below.

Note: After storage in RNA*later* RNA Stabilization Reagent, tissues become slightly harder than fresh or thawed tissues. Disruption and homogenization of this tissue, however, is usually not a problem.

Disruption and homogenization using rotor–stator homogenizers

Rotor–stator homogenizers thoroughly disrupt and simultaneously homogenize, in the presence of lysis buffer, animal tissues in 5–90 seconds depending on the toughness of the sample. Rotor–stator homogenizers can also be used to homogenize cell lysates. The rotor turns at a very high speed causing the sample to be disrupted and homogenized by a combination of turbulence and mechanical shearing. Foaming of the sample should be kept to a minimum by using properly sized vessels, by keeping the tip of the homogenizer submerged, and by holding the immersed tip to one side of the tube. Rotor–stator homogenizers are available in different sizes and operate with differently sized probes. Probes with diameters of 5 mm and 7 mm are suitable for volumes up to 300 µl and can be used for homogenization in microfuge tubes. Probes with a diameter of 10 mm or above require larger tubes.

Disruption and homogenization using the Mixer Mill MM 300 and other bead mills

In disruption using a bead mill, the sample is agitated at high speed in the presence of beads. Disruption and simultaneous homogenization occur by the shearing and crushing action of the beads as they collide with the cells. Disruption efficiency is influenced by:

- Size and composition of beads
- Ratio of buffer to beads
- Amount of starting material
- Speed and configuration of agitator
- Disintegration time

The optimal beads to use are 0.1 mm (mean diameter) glass beads for bacteria, 0.5 mm glass beads for yeast and unicellular animal cells, and 3–7 mm stainless steel beads for animal and plant tissues. Plant material as well as the beads and disruption vessels must be precooled in liquid nitrogen, and disruption should be performed without lysis buffer. It is essential that glass beads are pretreated by washing in concentrated nitric acid Alternatively, use commercially available acid-washed glass beads (e.g., SIGMA, cat. no. G1145, G1277, or G8772). All other disruption parameters must be determined empirically for each application.

Tip A protocol for mechanical disruption of yeast cells with glass beads is included in the *RNeasy Mini Handbook* and the *RNeasy Midi/Maxi Handbook* since this is the most widespread application for bead-milling. In addition, the *RNeasy Mini Handbook* contains guidelines for disruption and homogenization of RNA*later* stabilized tissues using the Mixer Mill MM 300 and stainless steel beads.

Disruption using a mortar and pestle

For disruption using a mortar and pestle, freeze the sample immediately in liquid nitrogen and grind to a fine powder under liquid nitrogen. Transfer the suspension (tissue powder and liquid nitrogen) into a liquid-nitrogen-cooled, appropriately sized tube and allow the liquid nitrogen to evaporate without allowing the sample to thaw. Add lysis buffer and continue as quickly as possible with the procedure.

Note: Grinding the sample using a mortar and pestle will disrupt the sample, but it will not homogenize it. Homogenization must be performed separately before proceeding.

Table 5. Guide to disruption and homogenization methods for different samples

Starting material	Disruption method	Homogenization method	Comments
Cultured animal cells	Addition of lysis buffer	Rotor–stator homogenizer or QIAshredder™ homogenizer* or syringe and needle needed for cytoplasmic RNA	If ≤1 x 10^5 cells are processed, lysate can be homogenized by vortexing. No homogenization protocol.
Animal tissue	Rotor–stator homogenizer	Rotor–stator homogenizer	Simultaneously disrupts and homogenizes.
	Mortar and pestle	QIAshredder homogenizer* or syringe and needle	Rotor–stator homogenizer usually gives higher yields than mortar and pestle.
	Mixer Mill MM 300 (recommended for RNA*later* stabilized tissues)	Mixer Mill MM 300	The Mixer Mill MM 300 gives results comparable to using a rotor–stator homogenizer.
Bacteria	Enzymatic (lysozyme) digestion followed by addition of lysis buffer	Vortex	If more than 5 x 10^8 cells are being processed further homogenization using QIAshredder homogenizer* or a syringe and needle may increase yield.
	Mixer Mill MM 300	Mixer Mill MM 300	Bead-milling simultaneously disrupts and homogenizes; bead-milling cannot be replaced by vortexing.
Yeast	Enzymatic (lyticase/zymolase) digestion of cell wall followed by lysis of spheroplasts by addition of lysis buffer	Vortex	
	Mixer Mill MM 300	Mixer Mill MM 300	Bead-milling simultaneously disrupts and homogenizes; bead-milling cannot be replaced by vortexing.
Plants and filamentous fungi	Mortar and pestle	QIAshredder homogenizer*	Mortar and pestle cannot be replaced by rotor–stator homogenizer.

Homogenization using QIAshredder homogenizers

Use of QIAshredder modules* is a fast and efficient way to homogenize cell and tissue lysates without cross contamination of the samples. The lysate (maximum volume 700 µl) is loaded onto the QIAshredder spin column sitting in a 2 ml collection tube, spun for 2 minutes at maximum speed in a microfuge and the homogenized lysate collected.

Homogenization using a syringe and needle

Cell and tissue lysates can be homogenized using a syringe and needle. High-molecular-weight DNA can be sheared by passing the lysate through a 20-gauge (0.9 mm) needle, attached to a sterile plastic syringe, at least 5–10 times or until a homogeneous lysate is achieved. Increasing the volume of lysis buffer may be required to facilitate handling and minimize sample loss.

** QIAshredder homogenizers are supplied with the RNeasy Plant Mini Kit and can be purchased separately for use with the RNeasy Mini Kit.*

Special Considerations for Isolation of RNA from Different Sample Sources

Some sample sources have differences in their RNA or contain substances that can cause problems in RNA isolation and analysis. Special considerations are required when working with these sample sources. In this section, considerations for working with a number of different sources are discussed.

Plants

Isolation of RNA from plant material presents special challenges, and commonly used techniques often require adaptation before they can be used with plant samples. Several plant metabolites have chemical properties that are similar to nucleic acids, making them difficult to remove from RNA preparations. Co-purified metabolites (such as polysaccharides, polyphenolics, and flavones) and contaminants introduced by the purification procedure (such as salts or phenol) can inhibit enzymatic reactions or cause variations in UV spectrophotometric measurements and gel migration. Additional problems with RNA isolation from plant material include pipetting errors due to increased viscosity and RNA degradation during storage.

RNA isolation is often improved by using plants grown under conditions that do not induce high levels of plant metabolites. Because of the great variation among plants, it is difficult to make general statements about growth conditions to use. However, as a general guideline, it is recommended to use healthy, young tissues when possible. RNA yields from young tissues are often higher than from old tissue because young tissue generally contains more cells than the same amount of older tissue. Young tissue of the same weight also contains fewer metabolites. In addition, many protocols for "home-made" RNA isolation methods recommend growing plants in darkness for 1 to 2 days before harvesting to prevent high-level accumulation of plant metabolites.

Heart, muscle, and skin tissue

RNA isolation from skeletal muscle, heart, and skin tissue can be difficult due to the abundance of contractile proteins, connective tissue, and collagen. In order to remove these proteins, which can interfere with RNA isolation, the sample needs to be treated with a protease. However, the protease digest needs to be carried out under conditions that do not allow RNA degradation.*

Bacteria

Bacterial mRNAs differ from eukaryotic mRNAs in a number of essential features. Prokaryotic mRNAs have no 5' cap and only rarely have poly-A tails. The absence of a poly-A tail means that mRNA isolation by hybrid capture is not possible. In addition, oligo-dT primers cannot be used to prime first-strand cDNA synthesis so random primers need to be used instead.

In addition, bacterial mRNAs are highly unstable, with an average half-life of about 3 minutes for fast growing bacteria. Sometimes the bacterial mRNA begins to degrade while it is still being translated. This can be a big problem for researchers trying to isolate mRNA from bacteria. Since mRNAs are very rapidly turned over in bacteria, gene-expression studies are even more difficult in prokaryotes than in eukaryotes. To accurately preserve gene-expression patterns and to maximize the amount of fully intact mRNA isolated, samples need to be stabilized prior to sample harvesting and processing.

Tip RNAprotect Bacteria Reagent allows stabilization of RNA in bacterial cells.

** A specialized protocol that includes a proteinase K digestion is included in the RNeasy Mini Handbook (3rd edition, June 2001) and the RNeasy Midi/Maxi Handbook (2nd edition, June 2001).*

Blood

Blood samples are routinely collected for clinical analysis. Blood contains a number of enzyme inhibitors that can interfere with downstream RNA analysis. In addition, common anticoagulants such as heparin and EDTA can interfere with downstream assays. RNA isolation from blood requires a method to provide high-quality RNA without contaminants or enzyme inhibitors.

Erythrocytes (red blood cells) of human blood do not contain nuclei and are therefore not important for RNA isolation since they neither synthesize nor contain RNA. The target of isolation from whole blood is leukocytes (white blood cells), which are nucleated and contain RNA. Leukocytes consist of 3 main cell types: lymphocytes, monocytes, and granulocytes.

Since healthy blood contains approximately 1000 times more erythrocytes than leukocytes, removing the erythrocytes simplifies RNA isolation. This can be accomplished by selective lysis of erythrocytes, which are more susceptible than leukocytes to hypotonic shock and burst rapidly in the presence of a hypotonic buffer.*

A common alternative to erythrocyte lysis is Ficoll® density-gradient centrifugation. In contrast to erythrocyte-lysis procedures, Ficoll density-gradient centrifugation only recovers mononuclear cells (lymphocytes and monocytes) and removes granulocytes. Mononuclear cells isolated by Ficoll density-gradient centrifugation can then be processed for RNA isolation as with other animal cells.

** The QIAamp® procedure uses optimized conditions for selective lysis to allow fast removal of erythrocytes without affecting the stability of leukocytes.*

RNA viruses

Some viruses have a single- or double-stranded RNA genome (see **Table 6**, page 54). Viral RNA is typically isolated from cell-free body fluids, where their titer can be very low. Virus particles may need to be concentrated by ultracentrifugation, ultrafiltration, or precipitation before RNA isolation. Addition of carrier RNA may also be necessary during RNA isolation when the expected yield of RNA is low.

A major problem with viral RNA is that it typically has a high degree of secondary structure. This can make downstream analysis especially difficult. Many reverse transcriptases have difficulty transcribing through complex RNA secondary structure.† In addition, RNA viruses have a high mutation rate due to inaccurate copying when they replicate. Therefore it is often difficult to obtain a homogeneous population for analysis.

Free circulating RNA in sera

RNA associated with proteolipids has been detected in the serum of some cancer patients. The concentration is approximately tenfold higher than free circulating DNA in human plasma, and the RNA is relatively stable, with a half-life of about 2 days in human whole blood. Nonetheless, the RNA can be degraded by repeated freeze–thaw cycles. As with viral RNA in cell-free body fluids, addition of carrier RNA may be necessary during RNA isolation of this RNA.

† Omniscript™ and Sensiscript™ Reverse Transcriptases have a high affinity for RNA that allows them to read through complex RNA secondary structures through which other RTs cannot.

Table 6. Selected viruses

Family	Selected viruses	Genome
Adenoviridae	Adenovirus	ds DNA
Arenaviridae	Lassa virus	ss RNA
Bornaviridae	Borna disease virus	ss RNA
Bunyaviridae	Hantaan virus	ss RNA
Caliciviridae	Hepatitis E virus, Norwalk virus	ss RNA
Filoviridae	Ebola virus	ss RNA
Flaviviridae	Hepatitis C and G viruses, Dengue virus	ss RNA
Hepadnaviridae	Hepatitis B virus	ss/ds DNA
Herpesviridae	Herpesviruses (HSV; CMV; EBV; HHV6, 7, 8)	ds DNA
Papovaviridae	Human papillomavirus, JC virus	ds DNA
Paramyxoviridae	Parainfluenza virus, respiratory syncytial virus, rubulavirus	ss RNA
Parvoviridae	Parvovirus B19 (erythrovirus)	ss DNA
Picornaviridae	Coxsackie virus, foot-and-mouth disease virus, hepatitis A virus, poliovirus, rhinovirus	ss RNA
Reoviridae	Rotavirus	ds RNA
Retroviridae	Human foamy virus, human immunodeficiency virus, human T-cell leukemia virus	ss RNA
Rhabdoviridae	Rabies virus	ss RNA

Storage, Quantification, and Determination of Quality of Total RNA

Storage of RNA

Purified RNA can be stored at –20°C or –70°C in water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer using quartz cuvettes. To ensure significance, readings should be between 0.15 and 1.0. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (A_{260} =1 ⇒ 40 µg/ml)*. This relation is valid only for measurements made at neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a low-salt buffer with neutral pH (e.g., 10 mM Tris·Cl, pH 7.0). An example of the calculation involved in nucleic acid quantification when using a spectrophotometer is provided in "Spectrophotometric Measurement of Nucleic Acid Concentration", page 91). As discussed below (see "Purity of RNA", page 56), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1M NaOH, 1 mM EDTA followed by washing with RNase-free water (see "Solutions", page 48). Use the buffer in which the RNA is diluted to zero the spectrophotometer.

Tip If you will use more than one cuvette to measure multiple samples, the cuvettes must be matched.

Quantification of small amounts of RNA and of mRNA can be performed using ethidium bromide staining, as described in Protocol 2 (page 55).

* *Based on standard 1 cm path length.*

Protocol 2. Quantification of mRNA by ethidium bromide staining

The following protocol is routinely used at QIAGEN for quantifying mRNA isolated by hybrid capture using Oligotex® mRNA Kits.

1. Make a series of RNA reference solutions by diluting a stock solution containing a known amount of RNA (e.g., total RNA). We recommend using a series of dilutions in RNase-free water with concentrations of 0, 2, 4, 6, 10, and 12 ng/µl (1 ng/µl = 1 µg/ml).
2. Prepare a solution of 1.5 µg/ml ethidium bromide* in 0.1 M ammonium acetate.
3. Mix 2 µl of each RNA reference with 8 µl of the ethidium bromide solution.
4. Prepare three different dilutions of the mRNA solution to be measured. These dilutions should be chosen so that at least one lies within the range of the series of RNA references.
5. Mix 2 µl of each mRNA dilution with 8 µl of the ethidium bromide solution.

** Ethidium bromide is mutagenic and moderately toxic. Wear gloves and take appropriate cautions when using.*

6. Pipet all mixtures (10 µl each) onto a UV transilluminator.† Keep the UV lamp turned off while pipetting. To avoid contaminating the UV transilluminator surface with ethidium bromide, cover it first with disposable, transparent, plastic wrap.
7. Turn on the UV lamp,† and photograph the samples, taking care to adjust the timing and aperture settings so that the differences in signal intensity of the different RNA standards are clearly distinguishable.
8. Compare the signal intensities of the mRNA dilutions with the series of RNA reference solutions. Determine which RNA reference(s) is nearest in signal intensity to the mRNA dilution(s).
9. The original concentration of this RNA reference then corresponds to the mRNA concentration in the mRNA dilution. Multiply by the dilution factor to find the mRNA concentration in the original, undiluted sample.

   For example, if a 10-fold dilution of the mRNA gives the same signal intensity as the 6 ng/µl RNA reference, then the original, undiluted mRNA sample has a concentration of 10 x 6 ng/µl = 60 ng/µl. In this case, a 5-fold dilution of the mRNA should give the same signal intensity as the 12 ng/µl RNA reference (5 x 12 ng/µl = 60 ng/µl).

† UV radiation is dangerous, especially to the eyes. Make sure that the UV source is appropriately shielded. Wear a face shield that blocks UV radiation while the transilluminator is switched on.

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination (2).

For accurate ratios, we recommend measuring absorbance in a low-salt buffer with slightly alkaline pH (e.g., 10 mM Tris·Cl, pH 7.5). Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1* in 10 mM Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution.

For determination of RNA concentration, however, we still recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 40 µg/ml RNA) is based on an extinction coefficient calculated at neutral pH (see "Quantification of RNA", page 54).

** Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.*

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. To prevent any interference by DNA in RT-PCR applications, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified (see "General guidelines for designing standard RT-PCR primers", page 67). Alternatively, DNA contamination can be detected on agarose gels following RT-PCR by performing control experiments in which no reverse transcriptase is added prior to the PCR step or by using intron-spanning primers. For sensitive applications (such as differential display) or if it is not practical to use splice-junction primers, DNase digestion of the purified RNA with RNase-free DNase is recommended.

Tip Using RNeasy Kits, DNA can be removed with an optional on-column DNase digestion using the RNase-Free DNase Set. The DNase is efficiently washed away in the subsequent wash steps. Alternatively, after purification, the RNA can be treated with DNase (see "DNase I Digest of RNA Prior to RT-PCR", page 65). The RNA can then be repurified, or after heat inactivation of the DNase, the RNA can be used directly in downstream applications.

Isolation of cytoplasmic RNA is particularly advantageous in applications where the absence of DNA contamination is critical, since the intact nuclei are removed.[†]

[†] *Using RNeasy cytoplasmic protocols, DNase digestion is generally not required: most of the DNA is removed with the nuclei, and the RNeasy silica-gel–membrane technology efficiently removes nearly all of the remaining small amounts of DNA without DNase treatment. However, even further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan® RT-PCR analysis with a low-abundant target). Using the cytoplasmic protocol with the optional DNase digestion results in undetectable levels of DNA, even by sensitive quantitative RT-PCR analyses.*

Integrity of RNA

The integrity and size distribution of total RNA can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining (see "A Guide to Analytical Gels" below). The respective ribosomal bands (**Table 7**) should appear as sharp bands on the stained gel. 28S ribosomal RNA bands should be present with an intensity approximately twice that of the 18S rRNA band (**Figure 1**). If the ribosomal bands in a given lane are not sharp, but appear as a smear of smaller sized RNAs, it is likely that the RNA sample suffered major degradation during preparation.

Table 7. Size of ribosomal RNAs from various sources

Source	rRNA	Size (kb)
E. coli	16S	1.5
	23S	2.9
S.cerevisiae	18S	2.0
	26S	3.8
Mouse	18S	1.9
	28S	4.7
Human	18S	1.9
	28S	5.0

Analysis of Total RNA

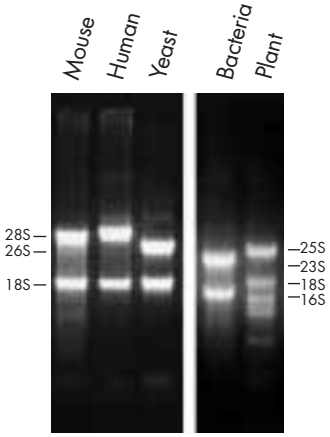

Figure 1. *Formaldehyde agarose gel of total RNA isolated from the indicated sources using RNeasy kits. 10 µg RNA was loaded per lane.*

A Guide to Analytical Gels

Principle of denaturing gel analysis

Formaldehyde agarose gels allow separation and identification of RNA based on charge migration. Unlike DNA, RNA has a high degree of secondary structure, making it necessary to use a denaturing gel. Formaldehyde in the gel disrupts secondary RNA structure so that RNA molecules can be separated by their charge migration.

In an electric field, nucleic acid molecules migrate towards the anode due to negatively charged phosphates along the backbone. The migration of denatured RNA molecules is determined by their size; however, the relationship between the fragment size and rate of migration is nonlinear, since larger fragments have a greater frictional drag and are less efficient at migrating through the gel.

Agarose gel analysis is the most commonly used method for analyzing RNA species, which generally correspond in size to the resolution range of an agarose gel. Small RNA fragments, such as tRNAs or 5S rRNAs, can be analyzed by polyacrylamide gel electrophoresis. Detailed information on all types of analytical gels can be found in current molecular biology manuals (3, 4). This section describes formaldehyde agarose gel electrophoresis.

Preparing formaldehyde agarose gels for RNA analysis

The following protocol for formaldehyde agarose (FA) gel electrophoresis is routinely used at QIAGEN and gives enhanced sensitivity for gel and subsequent analysis (e.g., northern blotting). A key feature of this protocol is the concentrated RNA loading buffer that allows a larger volume of RNA sample to be loaded onto the gel than provided for in conventional protocols

Agarose

The concentration of agarose used for the gel determines the size range of RNA fragments that can be resolved. For most RNA species of interest, a concentration of 1.0–1.2% (w/v) agarose will give best results. For resolution of large mRNA species, it may be helpful to reduce the agarose concentration. For analysis of smaller mRNAs, the agarose concentration can be raised to 2%. With small RNA species, such as tRNAs or 5S rRNAs, polyacrylamide gel electrophoresis is recommended.

Tip Use ultrapure-quality agarose since impurities such as polysaccarides, salts, and proteins can affect the migration of RNA

** Plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier's instructions.*

Protocol 3. Pouring the gel

1. Prepare enough 10x FA gel buffer to pour the gel and to make enough FA gel running buffer (see "Formaldehyde Agarose Gel Electrophoresis Buffers for Analysis of RNA", page 94) to fill the electrophoresis tank.
2. Mix an appropriate amount of agarose, 10x FA gel buffer, and RNase-free water in a flask or bottle. To prepare FA gel of size 10 x 14 x 0.7 cm, mix:

   - 1.0–1.2 g agarose
   - 10 ml 10x FA gel buffer
   - Add RNase-free water to 100 ml

Tip If smaller or larger gels are needed, adjust the quantities of components proportionately. The vessel should be no more than half full. Cover the vessel to minimize evaporation.

3. Heat the mixture in a microwave or boiling water bath, swirling the vessel occasionally until the agarose is dissolved.

Tip Ensure that the lid of the flask is loose to avoid buildup of pressure. Be careful not to let the agarose solution boil over as it becomes super-heated.

Tip If the volume of liquid reduces considerably during heating due to evaporation, make up to the original volume with RNase-free distilled water. This will ensure that the agarose concentration is correct.

4. Cool the agarose to 65–70°C in a water bath. Stir or swirl occasionally to prevent uneven cooling.
5. After cooling, add 1.8 ml of 37% (12.3 M) formaldehyde and 1 µl of a 10 mg/ml ethidium bromide stock solution.

Tip Formaldehyde is toxic. Use a fume hood to avoid inhalation. Wear gloves and take appropriate safety precautions when handling.

Tip Make sure that the solution has cooled sufficiently before adding formaldehyde and ethidium bromide. Formaldehyde is volatile and may evaporate if added to a solution that is too hot.

Tip Ethidium bromide in the gel allows visualization of the RNA with UV light. Ethidium bromide is toxic and a powerful mutagen. Wear gloves and take appropriate safety precautions when handling. Use of nitrile gloves is recommended as latex gloves may not provide full protection. After use, ethidium bromide solutions should be decontaminated as described in commonly used manuals.

Tip Stock solutions of ethidium bromide (generally 10 mg/ml in water) should be stored at 2–8°C in a dark bottle or a bottle wrapped in aluminum foil.

protocol continues overleaf

Protocol 3. Continued

6. Pour the agarose solution onto the gel tray in a fume hood to a thickness of 3–5 mm. Insert the comb either immediately before or immediately after pouring. Let the gel set for at least 30 min.

Tip Ensure that there is enough space between the bottom of the comb and the gel tray (0.5–1.0 mm) to allow proper well formation and avoid sample leakage.

Tip Make sure that there are no air bubbles in the gel or trapped between the wells. Air bubbles can be carefully removed with a Pasteur pipet before the gel sets.

Tip Thicker gels can be used to increase the amount of sample volume that can be loaded. Thinner gels generally transfer better in northern blotting, but smaller sample volumes can be used.

Tip The thickness of the comb affects the sharpness of bands in the gel. A thinner comb gives sharper bands, but less sample can be loaded per well.

7. Leaving the comb in the gel, place the gel in the electrophoresis tank. Fill the tank with 1x FA gel running buffer.

Tip Add enough buffer to cover the gel with approximately 1 mm of liquid above the surface of the gel. If too much buffer is used, the electric current will flow through the buffer instead of the gel.

8. Carefully remove the comb from the gel. Prior to running, let the gel equilibrate in 1x FA gel running buffer for at least 30 min.

Running and analyzing formaldehyde agarose gels for RNA analysis

RNA loading buffer

RNA loading buffer (see "Formaldehyde Agarose Gel Electrophoresis Buffers for Analysis of RNA", page 94) must be added to samples before loading them on a gel. The loading buffer serves three main purposes:

1. To denature the RNA sample prior to loading.
2. To increase the density of the samples to ensure that they sink into the wells on loading.
3. To add color to the samples through the use of dyes, facilitating loading and visualization on the gel while running.

A key feature of the concentrated RNA loading buffer described in "Formaldehyde Agarose Gel Electrophoresis Buffers for Analysis of RNA" (page 94) is that it allows a larger volume of RNA sample to be loaded onto the gel than conventional protocols allow.

Electrophoresis buffers

RNA gels are run at a lower pH than DNA gels since RNA has a lower pK_a than DNA. Furthermore, unlike DNA, RNA is susceptible to alkali cleavage at high pH. RNA gels should therefore be run at neutral pH. MOPS (3-[N-morpholino]propanesulfonic acid) is the most commonly used buffer for RNA gels due to its high buffering capacity at pH 7.0. Formaldehyde is included in the running buffer to keep the RNA denatured. Formaldehyde is also added to the agarose gel.

Tip Electrophoresis tanks should be cleaned with detergent solution (e.g., 0.5% SDS), thoroughly rinsed with RNase-free water, and then rinsed with ethanol* and allowed to dry.

* *Plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier's instructions.*

Protocol 4. Sample preparation for electrophoresis

Protocol 4

1. Add 1 volume of 5x RNA loading buffer to 4 volumes of RNA sample (for example, 5 µl of loading buffer and 20 µl of RNA) and mix.

Tip Samples should always be mixed with RNA loading buffer prior to loading on a gel.

Tip Do not use sample volumes close to the capacity of the wells as samples may spill over into adjacent wells during loading.

Tip Be sure that all samples have the same buffer composition. High salt concentrations will retard the migration of RNA molecules.

Tip Ensure that no ethanol is present in the samples, for example, carried over from purification procedures. Ethanol may cause samples to float out of the wells on loading.

2. To denature RNA, incubate for 3–5 min at 65°C. Chill on ice.

Protocol 5. Electrophoresis

Protocol 5

1. Apply denatured samples to the wells of the gel. The gel should be submerged in electrophoresis buffer in the electrophoresis tank prior to loading.

Tip Prior to sample loading, remove air bubbles from the wells by rinsing them with electrophoresis buffer.

Tip Make sure that the entire gel is submerged in the FA gel running buffer.

Tip To load samples, insert the pipet tip deep into the well and expel the liquid slowly. Take care not to break the agarose with the pipet tip.

Tip Once samples are loaded, do not move the gel tray/tank as this may cause samples to float out of the wells.

Tip Be sure to include at least one lane of appropriate molecular-weight markers.

2. Connect the electrodes of the electrophoresis apparatus so that the RNA will migrate towards the anode or positive lead (usually red).

Tip The electrophoresis apparatus should always be covered to protect against electric shock.

Tip Run the gel in a fume hood to avoid exposure to formaldehyde fumes from the gel and running buffer.

3. Turn on the power supply, and run the gel at 5–7 V/cm until the bromophenol blue dye has migrated approximately 2/3 of the way through the gel.

Tip Avoid use of high voltages, which can cause trailing and smearing of RNA bands.

Tip Monitor the temperature of the buffer periodically during the run. High temperature can cause partial melting of the gel and distortion of the bands. If the buffer becomes significantly heated, reduce the voltage.

Tip For very long runs (e.g., overnight runs), use a pump to recycle the buffer.

Visual analysis of the gel

Ethidium bromide in the gel allows visualization of the RNA with UV light. Ethidium bromide is toxic and a powerful mutagen. Wear gloves and take appropriate safety precautions when handling. Use of nitrile gloves is recommended, as latex gloves may not provide full protection. After use, ethidium bromide solutions should be decontaminated as described in commonly used manuals (3, 4).

Tip Stock solutions of ethidium bromide (generally 10 mg/ml in water) should be stored at 2–8°C in a dark bottle or a bottle wrapped in aluminum foil.

Visualization

Ethidium bromide–RNA complexes display increased fluorescence compared to the uncomplexed dye in solution. This means that illumination of a stained gel under UV light (254–366 nm) allows bands of RNA to be visualized against a background of unbound dye. The gel image can be recorded by taking a Polaroid™ photograph or using a gel documentation system.

Tip UV light can damage the eyes and skin. Always wear suitable eye and face protection when working with a UV light source.

Tip UV light damages RNA. If RNA fragments are to be extracted from the gel, use a lower intensity UV source if possible, and minimize exposure of RNA to the UV light.

Analysis of total RNA

The integrity and size distribution of total RNA can be checked by observing the stained RNA. The respective ribosomal bands should appear as sharp bands on the stained gel (see **Table 7** and **Figure 1**, page 57). If the ribosomal bands in a given lane are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the RNA sample suffered major degradation during preparation. The 28S ribosomal RNA band should be present at approximately twice the intensity of the 18S rRNA band. Since the 28S rRNA is more labile than the 18S rRNA, equal intensities of the two bands generally indicates that some degradation has occurred.

RNA analysis by northern blotting

Principle of RNA blotting

After separating RNA molecules based on charge migration in a denaturing gel, RNA molecules in the gel are transferred to a nylon or nitrocellulose membrane by capillary transfer. The RNA of interest can then be identified by hybridization to radioactive or chemoluminescent probes and visualized by autoradiography or photography.

Since DNA blotting is commonly referred to as "Southern blotting", after its inventor E.M. Southern, the term "northern blotting" was coined for this analogous RNA-blotting process.

The northern blotting procedure

The following protocol for northern blotting is routinely used at QIAGEN. This procedure is intended for use with a standard formaldehyde agarose gel, prepared and run as previously described (pages 57–61).

Blotting membrane

Northern blotting is generally carried out by immobilization of the RNA on nylon or nitrocellulose membranes. Positively charged nylon membranes are generally recommended over nitrocellulose because of their greater strength and ease of handling.

Tip Always wear gloves while working with blotting membranes. Handle membranes carefully by the edges or using clean blunt-ended forceps.

Transfer buffer

Formaldehyde agarose RNA gels are generally blotted using a high-salt buffer such as 20x SSC (see "Northern Transfer Solution", page 95). Prepare 1.2 liters of 20x SSC. For larger RNA gels (>100 ml volume), use 2.4 liters of 20x SSC.

Tip Save 200 ml of the 20x SSC solution, and dilute it twofold to make 10x SSC for soaking the gel and washing the blot after transfer.

Equipment required

- Whatman® 3MM filter paper
- Paper towels, a stack of approximately 15–20 cm
- Plastic wrap
- Two glass or Plexiglas® plates
- Buffer tray (e.g., glass casserole dish) capable of holding 1–2 liters of buffer
- Flat weight, approximately 1 kg
- RNase-free water (200 ml)
- 0.05 M NaOH (200 ml)

Protocol 6. Presoaking filter paper and blotting membrane

1. Cut one sheet of nylon membrane and two sheets of Whatman 3MM paper about 1 mm larger than the gel on each edge.
2. Cut two lengths of Whatman paper wider than the gel, long enough to fit under the gel and reach to the bottom of the dish on either side (see **Figure 2**).
3. Wet the nylon membrane in water. Then soak the Whatman paper and nylon membrane in 20x SSC for 1–2 min.

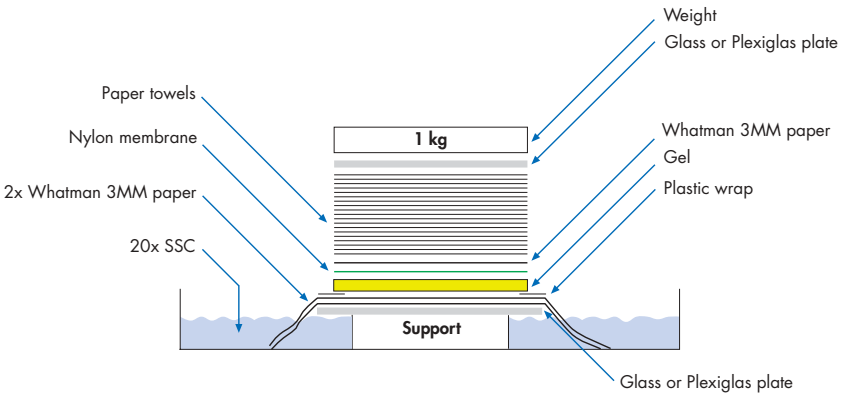

Figure 2. *Northern blot setup.*

Protocol 7. Capillary transfer

1. Fill the buffer tray with 1 liter 20x SSC. Place a glass or Plexiglas plate across the tray or on top of a support (**Figure 2**).
2. Place the two lengths of presoaked filter paper over the glass or Plexiglas plate so that the ends contact the bottom of the tray (**Figure 2**). Remove any air bubbles between the sheets of filter paper and the plate by rolling a pipet several times back and forth over the surface.
3. Immediately after gel electrophoresis, soak the gel for 10 min, with gentle shaking, in 200 ml RNase-free water and then for 15 min in 200 ml 0.05 M NaOH. Finally, soak the gel for 10 min in 200 ml 10x SSC to neutralize the NaOH.

Tip Dilute 20x SSC twofold to make 10x SSC.

Tip The gel contains formaldehyde to denature the RNA. Formaldehyde is toxic. Use a fume hood to avoid inhalation. Wear gloves and take appropriate safety precautions.

4. Position the gel upside-down on the filter paper covering the plate.
5. Place a sheet of plastic wrap over the gel. Use a sheet large enough to cover the surface of the filter paper on the glass or Plexiglas plate. Using a clean scalpel or razor blade, carefully cut the plastic wrap around the gel. Remove the piece over the gel so that the remaining plastic wrap surrounds the gel. This ensures that the transfer buffer moves only through the gel and not around it.
6. Place the presoaked nylon membrane on top of the gel so that it covers the entire surface (**Figure 2**). Do not move the nylon membrane once it has been placed on the gel. Remove any air bubbles between the membrane and the gel by gently rolling a pipet several times back and forth over the surface.
7. Place the two presoaked sheets of Whatman 3MM paper on top of the nylon membrane (**Figure 2**). Again, remove any air bubbles by gently rolling a pipet several times back and forth over the surface.
8. Place a 15–20 cm stack of dry paper towels on top of the filter paper (**Figure 2**).

protocol continues overleaf

Protocol 7. Continued

Tip Make sure that the plastic wrap around the gel prevents contact of the paper towels with the transfer buffer and the wet filter paper under the gel. Ensure that the towels do not droop over since they can cause liquid to flow around the gel instead of through it.

9. Place a second glass or Plexiglas plate on top of the paper towels. Place the 1 kg weight on top of the plate (**Figure 2**).

10. Let the transfer proceed for 12–18 h.

Tip Remove the wet paper towels and replace them with dry ones at least once during the transfer. If necessary, add more transfer buffer to the buffer tray.

Tip The gel contains formaldehyde which will diffuse out of the gel during transfer. Formaldehyde is toxic. Perform the transfer in a fume hood to avoid inhalation. Wear gloves and take appropriate safety precautions when handling.

Protocol 8. Fixing the RNA to the blot

1. After the transfer is complete, remove the weight, paper towels, and the two sheets of filter paper. Turn over the gel and the nylon membrane together, and lay them, gel-side up, on a dry sheet of filter paper. Mark the positions of the gel lanes on the membrane using a ball-point pen or a soft-lead pencil. Peel the gel from the membrane and discard it.

Tip Make sure to mark the gel lanes before removing the gel from the nylon membrane! Without this marking, you won't be able to tell which lane is which.

Tip Most of the formaldehyde in the gel transfers into the paper towels and the upper sheets of filter paper. Dispose of them according to your institution's waste-disposal guidelines.

2. Wash the nylon membrane for 1 min in 100–200 ml 10x SSC.

Tip Dilute 20x SSC twofold to make 10x SSC. This wash step is critical to remove any agarose that adheres to the blot.

3. Fix the RNA to the blot by baking (step 3A) or UV-crosslinking (step 3B).

Tip UV-crosslinking generally gives better results and enhanced sensitivity compared to baking. However, proper crosslinking requires prior optimization of the system.

3A. To fix the RNA by baking, first let the blot air-dry on a dry sheet of filter paper, then place between two sheets of filter paper. Bake for 30 min to 2 h at 80°C in a vacuum oven.

3B. To fix the RNA by UV-crosslinking, take the damp blot and expose the side with the RNA to UV irradiation (e.g., with a UV transilluminator) for a determined length of time.

Tip To determine the proper conditions for UV irradiation, the system must first be empirically tested and optimized. To do this, take an RNA blot with several lanes containing identical RNA samples. Cut the blot into separate strips for each lane, and irradiate each for different times, varying from 0.5 to 5 min. After hybridization, determine which time gives the optimal signal intensity. Be sure to use the same conditions (UV wavelength, distance from UV source) for each experiment. In addition, the system should be routinely calibrated to determine that the intensity of the UV irradiation remains unchanged.

Tip UV light can damage the eyes and skin. Always wear suitable eye and face protection.

4. If the blot is not to be used immediately, store it at 4°C, wrapped in plastic wrap.

DNase I Digest of RNA Prior to RT-PCR

Protocol 9. DNase I digestion of RNA

This protocol can be used with any RNA isolation method. RNase-Free DNase I is available from a number of commercial suppliers.

Tip When using RNeasy Kits or the QIAamp RNA Blood Mini Kit, DNase digestion is generally not required since the silica-membrane, spin-column technology used in these kits efficiently removes the majority of the DNA without DNase treatment. Further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., quantitative RT-PCR with a low-abundance target). Residual DNA can be removed by DNase I digestion following RNA isolation, as described below, or using the QIAGEN RNase-Free DNase Set for on-column DNase digestion.

1. Mix the following in a microcentrifuge tube:
   - 1–2 µg RNA (contaminated with genomic DNA)
   - 2 µl 10x DNase buffer (500 mM Tris·Cl, pH 8.0; 50 mM $MgCl_2$; 10 mM DTT)
   - 10 units RNase inhibitor
   - 0.5 Kunitz units* DNase I, RNase-free
   - Make the volume up to 20 µl with water

Tip Use of RNase-free reagents and plasticware is recommended.

** Kunitz units are the commonly used units for measuring DNase I, defined as the amount of DNase I that causes an increase in A_{260} of 0.001 per minute per milliliter at 25°C, pH 5.0, with highly polymerized DNA as the substrate (5). Use the appropriate conversion factor for DNase I preparations that are not quantified in Kunitz units.*

2. Incubate for 30 min at 37°C.
3. Add 2 µl of mM EDTA.

Tip Larger amounts of EDTA can inhibit RT-PCR.

4. Incubate for 5 min at 65°C to inactivate the DNase.

Tip Do not exceed the recommended inactivation time or temperature. Longer times or higher temperatures can cause degradation of the RNA.

5. Use up to 10 µl of the DNase-treated RNA solution in the RT reaction.

Guidelines for RT-PCR

To perform PCR using RNA as a starting template, the RNA must first be reverse transcribed into cDNA in a reverse transcription (RT) reaction. RT and PCR can be carried out either sequentially in the same tube (1-step RT-PCR) or separately (2-step RT-PCR). One-step RT-PCR requires gene-specific primers.

Tip QIAGEN offers Omniscript and Sensiscript RT Kits for reverse transcription. Omniscript RT is specially designed for reverse transcription using 50 ng to 2 µg per reaction. Sensiscript RT is optimized for use with very small amounts of RNA (1 pg – 50 ng). QIAGEN also offers the QIAGEN OneStep RT-PCR Kit for easy and sensitive one-step RT-PCR.

Protocol 10. 2-step RT-PCR

Protocol 10

1. Mix the following reagents in a microcentrifuge tube:
   - 2.0 µl 10x Buffer RT
   - 2.0 µl dNTP Mix (5 mM each dNTP)
   - 2.0 µl oligo-dT primer (10 µM)
   - 1.0 µl RNase inhibitor (10 units/µl)
   - 1.0 µl RT*
   - Template RNA (generally 50 ng – 2 µg)†
   - Add RNase-free water to a final volume of 20 µl.
2. Incubate at 37°C for 60 min.*
3. Add an aliquot of the finished reverse-transcription reaction to the PCR mix.

Tip No more than 1/5 of the final PCR volume should derive from the finished reverse-transcription reaction.

4. Carry out PCR with *Taq* DNA polymerase.

Tip QIAGEN offers *Taq* DNA Polymerase and HotStarTaq™ DNA Polymerase for efficient amplification without the need for optimization.

General guidelines for PCR can be found in "Guidelines for PCR", pages 42–43.

* *When using Omniscript or Sensiscript RT from QIAGEN. For other enzymes, refer to supplier's instructions.*

† *Sensiscript RT can be used with 1 pg – 50 ng RNA.*

General guidelines for designing standard RT-PCR primers

To avoid amplification of contaminating genomic DNA, primers for RT-PCR should be designed so that one half of the primer hybridizes to the 3' end of one exon and the other half to the 5' end of the adjacent exon (see **Figure 3A**). Such primers will anneal to cDNA synthesized from spliced mRNAs, but not to genomic DNA.

To detect amplification of contaminating DNA, RT-PCR primers should be designed to flank a region that contains at least one intron (see **Figure 3B**). Products amplified from cDNA (no introns) will be smaller than those amplified from genomic DNA (containing introns). Size difference in products is used to detect the presence of contaminating DNA.

If only the mRNA sequence is known, choose primer annealing sites that are at least 300–400 bp apart. It is likely that fragments of this size from eukaryotic DNA contain splice junctions. As explained in the previous point and in **Figure 3**, such primers may be used to detect DNA contamination.

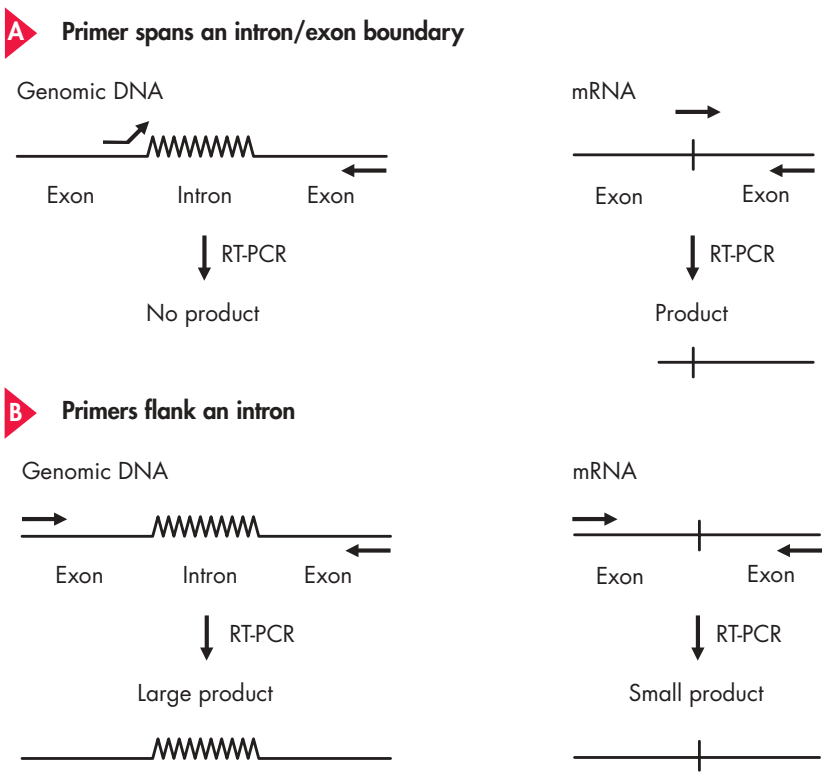

Figure 3. *Primer design to* ***A*** *eliminate or* ***B*** *detect amplification from contaminating genomic DNA.*

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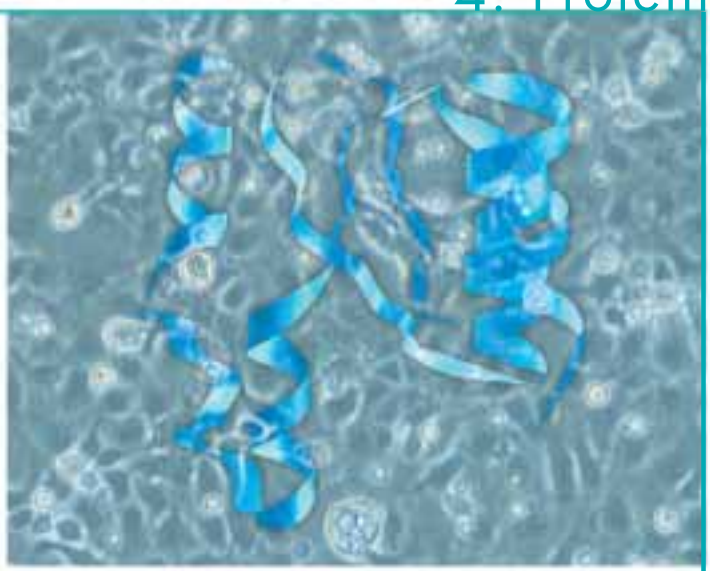
4. Protein



Contents

What is a Protein?

The word protein is derived from the Greek *proteios*, meaning "of the first rank". The term was coined in 1838 by the Swedish scientist Jöns Berzelius, to reflect the importance of this group of molecules.

A stretch of DNA called a gene carries the information required to build a protein. It is believed that there are around 30,000 genes in the human genome but more than 300,000 proteins in the human proteome, making proteins the most abundant class of all biological molecules. The difference between the number of genes and proteins is due the fact that one gene is able to give rise to more than one protein, and that once produced, proteins can be chemically modified (usually by other proteins) to change their properties and activities.

The building blocks of proteins are amino acids. There are twenty naturally occurring amino acids (**Table 1**) from which all natural proteins are constructed. All twenty are based on a common structure and differ in the chemical properties of their so-called side-chains. Some (e.g., tryptophan and phenylalanine) are strongly hydrophobic, while others (e.g., lysine and aspartic acid) carry an ionic charge at physiological pH, making them hydrophilic. Amino acids are linked together by peptide bonds to form protein chains. The sequence of amino acids in a protein and the way the protein chain is folded determine its properties.

The advances made in molecular biology over the past few decades have greatly improved the study of proteins. Previously, the only way to obtain a specific protein was to purify it from the natural source, a procedure that was often extremely inefficient and time-consuming. With the advent of recombinant molecular biological techniques it is possible to clone the DNA that encodes the protein of interest into an expression vector and express the protein in bacteria, usually *E. coli*. The universality of the genetic code that translates a DNA sequence into a protein allows proteins from any organism to be expressed quickly and in large amounts.

This chapter describes procedures for expression, analysis, detection, and assay of proteins.

Table 1. The naturally occurring amino acids.

Amino acid	3-letter code	1-letter code	Amino acid	3-letter code	1-letter code
Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamic acid	Glu	E	Serine	Ser	S
Glutamine	Gln	Q	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Iso	I	Valine	Val	V

Expression of Proteins in *E. coli*

Expression of a recombinant protein can be approached in general by constructing a plasmid that encodes the desired protein, introducing the plasmid into the required host cell, growing the host cells and inducing protein expression, and then lysing the cells, purifying the protein, and performing SDS-PAGE analysis to verify the presence of the protein (**Figure 1**).

The protocols and recommendations given in the Plasmid DNA chapter for the handling and transformation of *E. coli* are also valid for the production of recombinant proteins. With careful choice of host strains, vectors, and growth conditions, most recombinant proteins can be cloned and expressed at high levels in *E. coli*.

Optimal growth and expression conditions for the protein of interest should be established with small-scale cultures before large-scale protein purification is attempted.

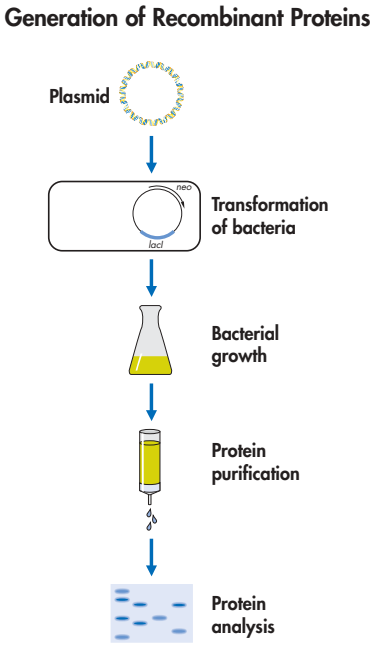


Figure 1. *Overview of the steps involved in expression and analysis of recombinant proteins.*

Basic principles

This section discusses critical factors to be considered when expressing foreign proteins in *E. coli*.

Culture media

The media of choice for the growth of *E. coli* cells containing an expression plasmid are LB medium and its modifications, 2x YT, or Super Broth, each containing the relevant selective antibiotic(s). Initially it is advisable to try expression in all three media in parallel, and to do a time course analysis to monitor growth and expression after induction. Striking differences between the level of expression in different media and at different times are often observed.

Maintenance of the expression plasmid

Poor plasmid maintenance in the cells can lead to low expression levels. Ampicillin is an unstable antibiotic and is rapidly depleted in growing cultures due in part to the β-lactamase secreted by resistant bacterial cells. It is important to check plasmid levels by plating cells from the expression culture on plates with and without ampicillin. If the stability of the expression construct is a problem, the cultures should be grown in the presence of 200 µg/ml ampicillin, and the level should be maintained by supplementing ampicillin during long growth periods. Alternatively, the cultures may be grown in the presence of carbenicillin, a more stable β-lactam, at 50 µg/ml (see "Antibiotics", page 5).

Small-scale expression cultures

Small-scale expression and purification experiments are highly recommended and should be performed before proceeding with a large-scale preparation. In many cases aliquots of the cells can be lysed in a small volume of sample buffer and analyzed directly by SDS-PAGE. The use of small expression cultures provides a rapid way to judge the effects of varied growth conditions on expression levels and solubility of recombinant proteins. Expression levels vary between different colonies of freshly transformed cells, and small-scale preparations permit the selection of clones displaying optimal expression rates.

Induction of protein expression

The method used for induction of protein expression is dependent on the plasmid vector and *E. coli* strain used. Protein expression can be induced by a raising of the incubation temperature or by the addition of an inducing chemical such as isopropyl-β-D-thiogalactoside (IPTG) to the culture medium. Details of induction methods and the plasmids they relate to can be found in standard molecular biology texts (1,2).

Time-course analysis of protein expression

To optimize the expression of a given protein construct, a time-course analysis by SDS-PAGE (Protocol 5, page 75) of the level of protein expression is recommended. Intracellular protein content is often a balance between the amount of soluble protein in the cells, the formation of inclusion bodies, and protein degradation. By checking the protein present at various times after induction, the optimal induction period can be established (**Figure 2**).

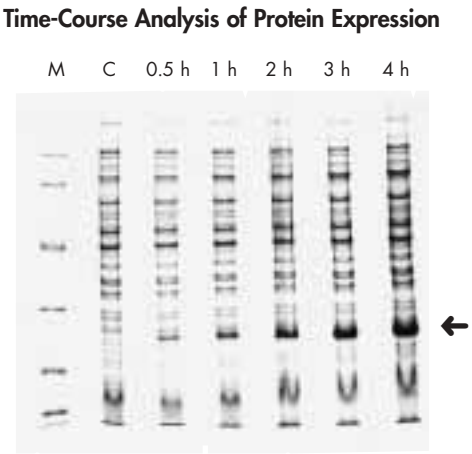


Figure 2. *Time course analysis of the expression of dihydrofolate reductase (arrowed). Aliquots were removed at the times indicated and analysed by SDS-PAGE.* ***C****: uninduced control.* ***M****: markers.*

Colony blots

We recommend the colony-blot procedure (**Figure 3**; Protocol 1, page 72) to identify clones expressing a protein and to distinguish semi-quantitatively between expression rates. This can be an advantage for selecting clones after transformation, since freshly transformed colonies may differ significantly in their expression rates. Using this method, colonies subsequently found to be expressing proteins at rates as low as 0.1 to 0.5 mg/liter are easily distinguished from colonies that do not express protein.

Note: If using the QIA*express*® Expression System, the small size of the His tag means that small peptides (<30 amino acids) expressed from QIA*express* vectors without an insert are degraded within the cells, and will not yield a false positive signal in the detection procedure. Other commonly used vectors that encode larger affinity tags may lead to expression of a small, but stable and detectable translation product even without an insert. This will lead to false positive signals from colonies that harbor the expression vector without insert, which may be indistinguishable from the signals from colonies expressing the desired protein.

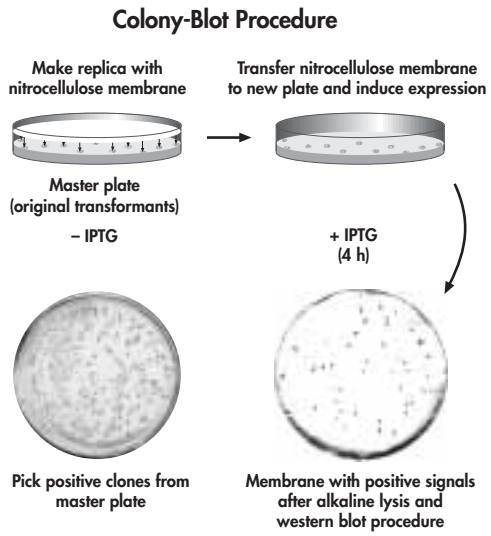


Figure 3. *Detection of positive expression clones by colony blotting.*

Protocol 1. Preparation of colony blots

Chemiluminescent substrates are not recommended for use with colony blots.

Materials

- LB-agar plates with appropriate antibiotics
- Nitrocellulose membrane discs (e.g., Millipore®, cat. no. HATF 085 25)
- Blunt-ended forceps
- Syringe needle
- Polystyrene dishes
- Inducer of protein expression (e.g., IPTG)
- 10% SDS solution
- Denaturing solution
- Neutralization solution
- 20x SSC
- Whatman® 3MM filter paper, 5 sheets

For buffer and reagent compositions, see "Colony-Blot Solutions", page 95. To make LB-agar plates, see "Preparation of LB-agar plates", page 5.

1. Plate freshly transformed cells on LB-agar plates containing the appropriate antibiotics, and incubate overnight.

Tip After spreading the transformation mix, dry the plates inverted with the lids slightly open until small wrinkles develop on the surface of the agar. To prevent smearing, incubation should not be started until all of the liquid has been absorbed into the agar.

Tip To avoid expression of toxic proteins in the absence of an inducer (a result of "leaky" promoters) and to maintain plasmid stability, incubation can be carried out at 30°C.

Tip If the expressed protein is not toxic and the plasmids are stable, incubation can be carried out at 37°C, but care should be taken that the colonies do not become too large.

2. Remove the plates from the incubator, open lids slightly, and allow any condensation to dry for 10 min.
3. Place a dry, numbered nitrocellulose filter on the agar surface in contact with the colonies, taking care not to introduce air bubbles.

Tip Hold the filter on opposite sides with blunt-ended forceps, and lower gently onto the agar surface, making contact first along the middle and then lowering (but not dropping) the sides.

Tip Number filters with a water-resistant marking pen or pencil.

4. Using a syringe needle, pierce the filter and agar at asymmetric positions to facilitate proper alignment following detection. Grip filter on the sides with blunt-ended forceps, and peel it off in one movement.
5. Transfer filter (colony side up) to a fresh LB-agar plate and induce expression, e.g., by using a plate containing antibiotics and 250 µM IPTG (see "Induction of protein expression", page 71). Avoid introducing air bubbles.

Tip Hold the filter on opposite sides with blunt-ended forceps, and lower gently onto the agar surface, making contact first along the middle and then lowering (but not dropping) the sides.

6. Incubate plates for 4 h at 37°C. Place master plates in a 30°C incubator for 4 h to allow colonies to regrow.
7. Prepare a set of polystyrene dishes for colony lysis and binding of protein to the filters. Each dish should contain a sheet of Whatman 3MM paper soaked with the following solutions:

   Dish 1) 10% SDS solution
   Dish 2) Denaturing solution
   Dish 3) Neutralization solution
   Dish 4) Neutralization solution
   Dish 5) 2x SSC

Note: Discard excess fluid so that paper is moist but not wet. Excess liquid promotes colony swelling and diffusion and will result in blurred signals.

protocol continues overleaf

Protocol 1. Continued

8. Place the nitrocellulose filters (colony side up) on top of the paper in each of these dishes, taking care to exclude air bubbles (colonies above air bubbles will not lyse properly and will generate a higher background in the final staining step).

   Incubate sequentially in the dishes (prepared in step 7), at room temperature as follows:

   Dish 1) 10% SDS solution 10 min
   Dish 2) Denaturing solution 5 min
   Dish 3) Neutralization solution 5 min
   Dish 4) Neutralization solution 5 min
   Dish 5) 2x SSC 15 min

9. Continue with the protocol for immunodetection using a chromogenic substrate (Protocol 11, page 82).

Tip Due to the problem of high background, protocols using chemiluminescent substrates are not recommended for detection after colony blotting.

Note: At times there is only a slight difference between colonies which express protein and those that do not. Shorter staining times are required with this procedure. A 2–3 min staining time is usually sufficient, but it is very important to monitor color development at this stage.

Tip If it is still difficult to differentiate between positive clones and background, the cause of the high background should be determined. The following controls should be included:

1. A plate of host bacteria without the expression plasmid
2. A plate of host bacteria harboring the expression plasmid without the insert
3. A colony-blot treated only with secondary antibody prior to detection
4. A positive control expressing the protein of interest, if possible

Protocol 2. Growth of standard expression cultures (100 ml)

1. Inoculate 10 ml culture medium containing relevant antibiotics in a 50 ml flask. Grow the cultures overnight at 37°C.
2. Inoculate 100 ml prewarmed medium (with antibiotics) with 5 ml of the overnight culture and grow at 37°C with vigorous shaking until an OD_{600} of 0.6 is reached (30–60 min).

Tip Take a 1 ml sample immediately before induction. This sample is the noninduced control. Pellet cells and resuspend them in 50 µl 5x SDS-PAGE sample buffer (see "SDS-PAGE Buffers and Solutions for Analysis of Proteins", page 96). Store at –20°C until SDS-PAGE analysis.

3. Induce expression (e.g., by adding IPTG to a final concentration of 1 mM).
4. Incubate the cultures for an additional 4–5 h.

Tip Collect a second 1 ml sample. This sample is the induced control. Pellet cells and resuspend them in 100 µl 5x SDS-PAGE sample buffer. Freeze and store the sample at –20°C until SDS-PAGE analysis.

Tip If expressing a protein for the first time, take a 1 ml sample every hour and treat as above to produce a time-course of expression.

5. Harvest the cells by centrifugation at 4000 x *g* for 20 min.
6. Freeze the cells in dry ice–ethanol or liquid nitrogen, or store cell pellet overnight at –20°C.

Protocol 3. Culture growth for preparative purification (1 liter)

1. Inoculate 20 ml culture medium containing the relevant antibiotics. Grow overnight at 37°C with vigorous shaking.
2. Inoculate a 1 liter culture 1:50 with the noninduced overnight culture. Grow at 37°C with vigorous shaking until an OD_{600} of 0.6 is reached.

Tip Take a 1 ml sample immediately before induction. This sample is the noninduced control. Pellet cells and resuspend in 50 µl 5x SDS-PAGE sample buffer (see "SDS-PAGE Buffers and Solutions for Analysis of Proteins", page 96). Store at –20°C until SDS-PAGE analysis.

3. Induce expression (e.g., by adding IPTG to a final concentration of 1 mM).
4. Incubate the culture for an additional 4–5 h.

Tip Collect a second 1 ml sample. This is the induced control. Pellet cells in a microcentrifuge tube and resuspend in 100 µl 5x SDS-PAGE sample buffer. Store at –20°C until SDS-PAGE analysis.

5. Harvest the cells by centrifugation at 4000 x *g* for 20 min. Freeze the cells in dry ice–ethanol or liquid nitrogen, or store cell pellet overnight at –20°C.

Protein Purification

The expression and purification of recombinant proteins facilitates production and detailed characterization of virtually any protein. Although a wide variety of heterologous expression systems have been developed and are currently used to produce recombinant proteins, the purification of the proteins obtained can still be problematic. Classical purification procedures can be employed, but in most cases recombinant DNA techniques permit the construction of fusion proteins in which specific affinity tags are added to the protein sequence of interest; the use of these affinity tags simplifies the purification of the recombinant fusion proteins by employing affinity chromatography methods. Ideally a tag should be small, and have a minimal effect on the structure, activity, and properties of the recombinant protein.

Different affinity tags have different sizes and properties. The 6xHis tag used in the QIA*express* system has a size of just 0.84 kDa, compared to 26 kDa for the glutathione S-transferase tag, 30 kDa for protein A, and 40 kDa for maltose-binding protein. The FLAG® tag consists of just 8 amino acids, but is highly immunogenic, which means that the FLAG tag must be removed before a recombinant protein can be used to produce antibodies. In contrast, the His tag has extremely low immunogenicity and rarely interferes with protein structure or function, making His-tagged proteins suitable for all kinds of downstream applications without cleaving the tag.

To learn more about the QIA*express* System for cloning, expression, purification, and assay of His-tagged proteins, visit us at **www.qiagen.com** or contact QIAGEN Technical Services or your local distributor.

Protein Analysis

SDS polyacrylamide gel electrophoresis

Principle of SDS-PAGE analysis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) involves the separation of proteins based on their size. By heating the sample under denaturing and reducing conditions, proteins become unfolded and coated with SDS detergent molecules, acquiring a high net negative charge that is proportional to the length of the polypeptide chain. When loaded onto a gel matrix and placed in an electric field, the negatively charged protein molecules migrate towards the positively charged electrode and are separated by a molecular sieving effect. After visualization by a protein-specific staining technique, the size of a protein can be estimated by comparison of its migration distance with that of a standard of known molecular weight. It is also possible to blot the separated proteins onto a positively charged membrane and to probe with protein-specific antibodies in a procedure termed western blotting (see Protocol 7, page 78).

Acrylamide concentration

The concentration of acrylamide used for the gel depends on the size of the proteins to be analyzed. Low acrylamide concentrations are used to separate high molecular weight proteins, while high acrylamide concentrations are used to separate proteins of low molecular weight (Table 2, page 76). Improved resolution of protein bands is achieved by the use of a discontinuous gel system having stacking and separating gel layers.

Preparation of dilute or salt-containing samples for SDS-PAGE

Acid precipitation of proteins (see Protocol 4 below) can be carried out prior to SDS-PAGE analysis in order to concentrate dilute samples or to remove high concentrations of salts that may interfere with the SDS-PAGE procedure.

Protocol 4. TCA precipitation of proteins

Protocol 4

1. Dilute samples to 100 µl; add 100 µl 10% trichloroacetic acid (TCA).
2. Leave on ice for 20 min; centrifuge for 15 min in a microcentrifuge.
3. Wash pellet with 100 µl of ice-cold ethanol, dry, and resuspend in 5x SDS-PAGE sample buffer (see "SDS-PAGE Buffers and Solutions for Analysis of Proteins", page 96). Boil for 7 min at 95°C, and then load samples immediately onto a gel for SDS-PAGE.

Protocol 5. Separation of proteins by SDS-PAGE

Protocol 5

Materials

- Gel apparatus and electrophoresis equipment
- 30% acrylamide/0.8% bis-acrylamide stock solution*
- 2.5x separating gel buffer
- 5x stacking gel buffer
- TEMED (N,N,N',N'-tetramethylethylenediamine)
- 10% ammonium persulfate
- Butanol
- 5x electrophoresis buffer
- 5x SDS-PAGE sample buffer
- Protein samples

Tip Use only high-quality reagents and water for SDS-PAGE. Gel buffers and self-prepared acrylamide/bis-acrylamide stock solutions should be filtered, degassed, and stored at 4°C.

For buffer and reagent compositions, see "SDS-PAGE Buffers and Solutions for Analysis of Proteins", page 96.

** Acrylamide is a potent neurotoxin and is absorbed through the skin. Take appropriate safety measures particularly when weighing solid acrylamide/bisacrylamide, and also when working with the solutions and gels.*

protocol continues overleaf

Protocol 5. Continued

Table 2. Compositions and separation properties of SDS-PAGE gels*

Gel acrylamide concentration (%)	Linear range of separation (kDa)	30 % acrylamide/ 0.8% bis-acrylamide stock solution (ml)	2.5x separating gel buffer (ml)	Distilled water (ml)
15.0	12–43	2.75	2.2	0.55
10.0	16–68	1.83	2.2	1.47
7.5	36–94	1.38	2.2	1.92
5.0	57–212	0.92	2.2	2.38

Adapted from reference 2.

** Volumes given are used for an 8 x 8 or 8 x 10 cm, 1 mm thick minigel, final volume 5.5 ml.*

1. Assemble gel plates with spacers according to the manufacturer's instructions.

Tip The plates should be thoroughly cleaned and dried before use.

2. Mark the level to which the separating gel should be poured — a few millimeters below the level where the wells will be formed by the comb.

3. Mix the following in a beaker or similar vessel (for a 12% acrylamide 8 x 8 or 8 x 10 cm, 1 mm thick, minigel).
   - 2.2 ml 30% acrylamide/0.8% bis-acrylamide stock solution
   - 2.2 ml 2.5x separating gel buffer
   - 1.1 ml distilled water
   - 5 µl TEMED

   The volumes of acrylamide/bis-acrylamide solution and water should be adjusted according to the percentage acrylamide required (dependent on the size of protein to be separated; see **Table 2** above).

Tip The size of the gel apparatus used will determine the volumes of gel solutions necessary.

4. Just before pouring, add 50 µl 10% ammonium persulfate, and mix well. Pour the gel between the assembled gel plates to the level marked in step 2. Overlay with butanol.

Tip Water can be used instead of butanol when using apparatus that may be damaged by the use of butanol — see the manufacturer's instructions.

Tip As soon as ammonium persulfate is added, the gel should be poured quickly before the acrylamide polymerizes.

Tip Prepare ammonium persulfate solution freshly each time it is required.

5. After polymerization is complete (around 20 min), pour off butanol, rinse with water and dry.

Tip Water remaining on the plates can be removed using pieces of filter paper.

6. For the stacking gel, mix the following:
   - 0.28 ml 30% acrylamide/0.8% bis-acrylamide stock solution
   - 0.33 ml 5x stacking gel buffer
   - 1 ml distilled water
   - 2 µl TEMED

7. Just before pouring, add 15 µl 10% ammonium persulfate, and mix well. Pour on top of the separating gel. Insert comb, avoiding introduction of air bubbles.

Tip As soon as ammonium persulfate is added the stacking gel should be poured quickly, before the acrylamide polymerizes.

protocol continues overleaf

Protocol 5. Continued

Tip With a marker pen, mark and/or number the positions of the wells before removing the comb. This aids loading of samples.

8. After the stacking gel polymerizes (around 10 min), the gel can be placed in the electrophoresis chamber. Fill the chamber with electrophoresis buffer and remove the comb.
9. Before loading, add 1 volume 5x SDS-PAGE sample buffer to 4 volumes of protein sample (i.e., add 2 µl sample buffer to 8 µl sample giving a final volume of 10 µl). Vortex briefly and heat at 95°C for 5 min.

Tip During heating at 95°C, release pressure build up in tubes by briefly opening lids, or piercing a small hole in the lid with a needle. After heating, samples should be briefly centrifuged and vortexed.

10. Load samples and run gel. For electrophoresis conditions refer to the recommendations provided by the manufacturer of the apparatus.

Tip Before loading the samples, rinse out wells with 1x electrophoresis buffer using a suitable syringe and needle.

Tip Load empty wells with 1x SDS-PAGE sample buffer to ensure that sample lanes do not spread out.

Tip Ensure that the electrodes are correctly connected. The proteins will migrate towards the positive (labeled +, usually red) electrode.

Tip Running the gel until the bromophenol blue dye reaches the bottom edge usually gives a satisfactory spread of protein bands.

Visualization of proteins in SDS-PAGE gels

Visualization of protein bands is carried out by incubating the gel with a staining solution. The two most commonly used methods are Coomassie® and silver staining. Silver staining is a more sensitive staining method than Coomassie staining, and is able to detect 2–5 ng protein per band on a gel. Many protocols are available but in order to increase reproducibility, use of a commercially available kit, such as the Bio-Rad® Plus Silver Staining Kit (cat. no.161-0449EDU), is recommended. Silver staining of proteins depends on the reaction of silver with sulfhydryl or carboxyl moieties in proteins and is therefore not quantitative, with some proteins being poorly stained by silver. In addition, after silver staining the protein becomes oxidized and cannot be used for further applications, such as sequencing. Coomassie staining, though less sensitive, is quantitative and Coomasie-stained proteins can be used for downstream applications.

Protocol 6. Coomassie staining

Protocol 6

Materials

- Coomassie staining solution
- Destaining solution
- SDS polyacrylamide gel containing separated proteins (see Protocol 5, page 75)

For buffer and reagent compositions, see "Coomassie Staining Solutions", page 97.

1. Incubate the gel in Coomassie staining solution for between 30 min and 2 h with gentle shaking. Coomassie Brilliant Blue R reacts nonspecifically with proteins.
2. Gently agitate the stained gel in destaining solution until the background becomes clear (1–2 h).

Tip A folded paper towel placed in the destaining bath will soak up excess stain and allow the re-use of destaining solution.

After destaining the proteins appear as blue bands against a clear gel background. Typically, bands containing 50 ng protein can be visualized.

Western blotting

Following electrophoresis, proteins in a polyacrylamide gel can be transferred to a positively charged membrane (e.g., Schleicher and Schuell BA85) in a buffer-tank–blotting apparatus or by semi-dry electroblotting as described below. With the semi-dry electroblotting method, the gel and membrane are sandwiched between two stacks of filter paper that have been pre-wet with transfer buffer. The membrane is placed near the anode (positively charged), and the gel is placed near the cathode (negatively charged). SDS-coated, negatively charged proteins are transferred to the membrane when an electric current is applied. With the tank-blotting method, a blotting cassette is submerged in a tank for blotting (Figure 4). Tank blotting can be performed over extended periods since the buffer capacity is far greater than that with semi-dry transfer systems. Results obtained with the tank-blotting method are typically better, with more efficient transfer, particularly of large proteins. Transfer efficiency can be checked by staining proteins on the membrane using Ponceau S (Protocol 8, page 79). Once transferred to the membrane, the proteins can be probed with epitope-specific antibodies or conjugates.

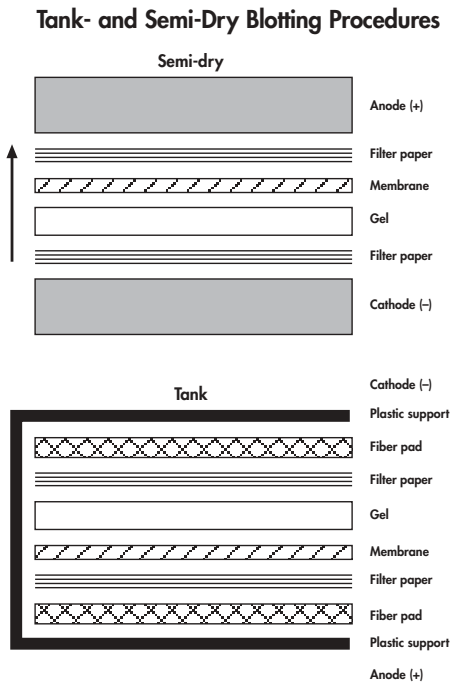


Figure 4. Schematic of tank- and semi-dry blotting methods. Arrows show direction of protein transfer.

Protocol 7. Western transfer

Protocol 7

Materials

- Transfer apparatus
- Filter paper (e.g., Whatman 3MM)
- Positively-charged membrane (e.g., Schleicher and Schuell BA85)
- SDS polyacrylamide gel containing separated proteins (see Protocol 5, page 75)
- Transfer buffer (semi-dry or tank-blotting)

For buffer and reagent compositions, see "Western Transfer Buffers and Solutions", page 97.

1. Cut 8 pieces of filter paper and a piece of membrane to the same size as the gel.

Tip To avoid contamination, always handle the filter paper, membrane, and gel with gloves.

2. Incubate membrane for 10 min in semi-dry or tank-blotting transfer buffer.
3. Soak filter paper in semi-dry or tank-blotting transfer buffer.

4A. **Semi-dry transfer:**
Avoiding air bubbles, place 4 sheets of filter paper on the cathode (negative, usually black), followed by the gel, the membrane, 4 sheets of filter paper, and finally the anode (positive, usually red). See Figure 4.

4B. **Tank-blotting:**
Avoiding air bubbles, place 4 sheets of filter paper on the fiber pad, followed by the gel, the membrane, 4 sheets of filter paper, and finally the second fiber pad (see Figure 4).

Tip Air bubbles may cause localized nontransfer of proteins. They can be removed by gently rolling a Pasteur pipet over each layer in the sandwich.

5. Carry out the transfer procedure. For current, voltage, and transfer times specific to your apparatus, consult the manufacturer's instructions.

protocol continues overleaf

Protocol 7. Continued

Tip Time of transfer is dependent on the size of the proteins, percentage acrylamide, and gel thickness. Transfer efficiency should be monitored by staining (see below). The field strength required is determined by the surface area and thickness of the gel: 0.8 mA/cm^2 is a useful guide (1 h transfer).

6. After transfer, mark the orientation of the gel on the membrane.

Protocol 8. Ponceau S staining

Protocol 8

1. Incubate membrane in Ponceau S staining solution (see "Western Transfer Buffers and Solutions", page 97) with gentle agitation for 2 min.
2. Destain in distilled water until bands are visible.

Tip Check that proteins of different sizes have been transferred uniformly to the membrane. Hydrophobic proteins may be more efficiently transferred by increasing the percentage of methanol in the transfer buffer.

3. Mark membrane using a suitable pen (i.e., one not containing water-soluble ink) or pencil, or cut as desired.

Dot blots

Dot blotting is a simple, convenient method for detection of proteins in crude lysates or solutions without the need for separation by SDS-PAGE. This method is especially useful as a simple control because it avoids problems that may be due to the western transfer process. Any components that interfere with binding or bind nonspecifically, however, will not be spatially separated from the protein and will interfere with the intensity of signals. Suitable controls should always be employed to compensate for this.

Protocol 9. Preparation of dot blots

Protocol 9

Materials

- Nitrocellulose membrane (e.g., Schleicher and Schuell BA85)
- Protein samples
- Dilution buffer for native or denaturing conditions

For buffer and reagent compositions, see "Protein Dot-Blot Preparation Buffers", page 98.

1. Dilute protein samples in buffer to final protein concentrations of 1–100 ng/µl.

Tip The protein of interest is diluted in dilution buffer for denaturing conditions, dilution buffer for native conditions, or another preferred buffer.

2. Apply 1 µl samples of diluted protein directly onto membrane. It is also possible to use crude cell lysate and apply 1 µl samples with an estimated concentration of 1–100 ng/µl protein.

Note: Under native conditions especially, the antibody epitope must be at least partially exposed to allow antibody binding. In most cases diluting the protein with buffer containing denaturing reagents will increase epitope exposure and give better results.

Tip To differentiate between nonspecific and positive signals, an extra sample containing 1 µl of a cell extract of the host strain without plasmid (or other suitable control) should also be applied to the membrane and treated together with the protein of interest.

3. After applying the samples, the membrane should be dried for a short time at room temperature before proceeding with the detection process.

Tip For larger sample volumes, suitable equipment is available from several suppliers.

4. Proceed with immunodetection (Protocol 10 or 11, page 81 or 82, respectively).

Protein Detection

Specific antibody-mediated detection of proteins on a membrane

Working with antibodies

Antibodies are proteins synthesized by an animal in response to the presence of a foreign substance (antigen). By injecting an antigen into an animal, after a certain time, antibodies of a class termed IgG (immunoglobulin G) that react specifically with the introduced protein can be harvested from the animal's serum. Each antibody has a specific affinity for a particular region of the antigen. This region is termed an epitope. The antibody –epitope interaction can be utilized for highly specific and sensitive detection of a protein that has been immobilized on a membrane, in a process termed immunodetection. The antibody that binds specifically to the protein of interest is termed the primary antibody and is often obtained from rabbits or mice. The primary antibody is applied to the membrane and allowed to bind to the target protein. In order to locate the primary antibody (and therefore the protein of interest), a secondary antibody is required. The secondary antibody recognizes and binds to all IgG antibodies from another animal species. It is important that the secondary antibody used in an experiment is directed against IgGs from the species of origin of the primary antibody. For example, if the primary antibody was generated in a mouse, then a goat anti-mouse secondary antibody can be used for detection. The secondary antibody is usually chemically coupled to a reporter, which allows detection and visualization of the antibody. Fluorescing molecules, or enzymes that produce colored or luminescent reaction products, are typically used as reporter groups. A primary antibody chemically coupled to a reporter enzyme is termed a conjugate, and can be used for direct detection without the use of a secondary antibody.

Detection of a protein on a membrane

After protein transfer from an SDS-PAGE gel to a membrane (see Protocol 7), the remaining protein-free sites on the membrane must be blocked. This prevents the primary or secondary antibody from binding directly to the membrane and giving rise to a high background signal. Several blocking reagents are in common use, including nonfat dried milk, BSA, and casein. After blocking, the primary antibody is added and allowed to bind to the protein (**Figure 5**, step 1). After washing (which removes nonspecifically bound antibody), the secondary antibody is added, to detect where the primary antibody has bound (**Figure 5**, step 2). After another wash step, the location of the secondary antibody, (and therefore the primary antibody and the protein of interest) is determined by adding a substrate for the enzyme conjugated to the secondary antibody (**Figure 5**, step 3). Substrates are available that give rise to a colored compound (chromogenic detection), or to the emission of light (chemiluminescent detection), at the reaction site. The use of an antibody that reacts specifically with an epitope commonly introduced into a recombinant protein (such as QIAGEN Anti·His Antibodies which detect 6xHis tags) eliminates the need for a protein-specific antibody, and allows the use of one antibody for the detection of all proteins containing this feature. Coupling a reporter enzyme directly to such antibodies eliminates the need for a secondary antibody, and delivers significant time savings. Detailed information on immunodetection procedures can be found in current molecular biology manuals (1, 2).

Immunodetection Procedure

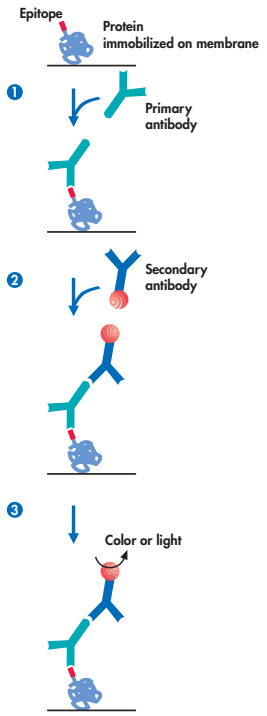


Figure 5. Immunodetection of a protein immobilized on a membrane.

Protocol 10. Immunodetection (chemiluminescent detection method)

Materials

- Western blot (see Protocol 7, page 78)
- TBS buffer
- TBS-Tween®/Triton® buffer
- Blocking buffer*
- Primary (protein-specific) antibody stock solution
- Secondary antibody-enzyme conjugate stock solution
- Secondary antibody dilution buffer
- Chemiluminescent substrate†

For buffer and reagent compositions, see "Immunodetection Buffers", page 98.

** For chemiluminescent detection, BSA does not sufficiently block nonspecific binding of the secondary antibody to the membrane, so milk powder should be used to dilute the secondary antibody. However, in some cases, dilution of antibody in a buffer containing milk powder can lead to reduced sensitivity. If this is the case, the primary antibody should be diluted in BSA solution, and the secondary antibody in milk powder solution. Alternatively, if alkali-soluble casein (Merck, cat. no. 1.02241) is available in your country, it can be used as a blocking reagent throughout the chemiluminescent detection protocol.*

† CDP-Star® from Tropix, Inc. can be used with alkaline phosphatase-(AP)conjugated secondary antibodies, and the ECL™ system from Amersham Pharmacia Biotech can be used in combination with horseradish peroxidase-(HRP) conjugated secondary antibodies.

Perform all incubation and wash steps on a rocking platform or orbital shaker.

1. Wash membrane twice for 10 min each time with TBS buffer at room temperature.
2. Incubate membrane for 1 h in blocking buffer at room temperature.

Tip Seal the vessel used for incubation with plastic film to prevent the membrane from drying out.

3. Wash membrane twice for 10 min each time in TBS-Tween/Triton buffer at room temperature.
4. Wash membrane for 10 min with TBS buffer at room temperature.
5. Incubate membrane with primary antibody solution (1/1000–1/2000 dilution of primary antibody stock solution in blocking buffer) at room temperature for 1 h.

Tip Make sure that the membrane is fully coated by the antibody solution. Do not allow the membrane to dry out.

Tip To reduce the volume of antibody required, the membrane can be sealed in a plastic bag.

6. Wash membrane twice for 10 min each time in TBS-Tween/Triton buffer at room temperature.
7. Wash membrane for 10 min in TBS buffer at room temperature.
8. Incubate the membrane with a dilution of secondary antibody in 10% nonfat dried milk in TBS for 1 h at room temperature. Dilute the secondary antibody according to the manufacturer's recommendations.

Tip Ensure that your secondary antibody is directed against the species of origin of your primary antibody!

Tip Milk powder is needed to reduce background because BSA does not block sufficiently for the very sensitive chemiluminescent detection method.

Tip Use the lowest recommended concentration to avoid false signals.

9. Wash 4 times for 10 min each time in TBS-Tween/Triton buffer at room temperature.

protocol continues overleaf

Protocol 10. Continued

10. Perform chemiluminescent detection reaction, cover the membrane with thin plastic wrap, and expose to X-ray film according to the manufacturer's recommendations.

Tip Ensure that you use the correct chemiluminescent detection substrate, i.e., an AP substrate for AP conjugates, or an HRP substrate for HRP conjugates!

Tip Blots can be wrapped in plastic wrap and stored at 4°C. Protocols exist for stripping the blot, which can subsequently be reprobed with a different antibody (3).

Protocol 11. Immunodetection (chromogenic detection method)

Protocol 11

Materials

- Western blot (see Protocol 7, page 78)
- TBS buffer
- TBS-Tween/Triton buffer
- Blocking buffer*
- Primary (protein-specific) antibody stock solution
- Secondary antibody-enzyme conjugate stock solution
- Chromogenic substrate

For buffer and reagent compositions, see "Immunodetection Buffers", page 98. A list of chromogenic substrates is provided in "Chromogenic Substrates for Immunoblotting Procedures", page 99.

** 3% BSA (w/v) can be used both as the blocking buffer and secondary antibody dilution buffer for chromogenic detection.*

Perform all incubation and wash steps on a rocking platform or orbital shaker.

1. Follow steps 1–7 of Protocol 10, page 81.
2. Incubate the membrane with secondary antibody solution diluted in 3% BSA (w/v) in TBS for 1 h at room temperature. Dilute according to the manufacturer's recommendations.

Tip Ensure that your secondary antibody is directed against the species of origin of your primary antibody!

Tip Use the lowest recommended concentration to avoid false signals.

3. Wash 4 times for 10 min each time in TBS-Tween/Triton buffer at room temperature.
4. Stain with AP or HRP staining solution until the signal is clearly visible (approximately 5–15 min). Do not shake blots during color development.

Tip Ensure that you use the correct chromogenic detection substrate, i.e., an AP substrate for AP conjugates, or an HRP substrate for HRP conjugates!

5. Stop the chromogenic reaction by rinsing the membrane twice with water.
6. Dry the membrane and photograph as soon as possible as the colors will fade with time. The product formed when using HRP is particularly unstable.

Protein Assay

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a method that is analogous to immunodetection of proteins on a membrane, and is used for the quantitative assay of proteins in solution. In an ELISA, proteins are immobilized on a solid support (e.g., the wells of a 96-well plate) and used as capture molecules to bind the protein that is being assayed. After a wash step to remove nonspecifically bound material, a secondary antibody — specific for the protein being assayed — is added. This secondary antibody is usually conjugated to an enzyme that allows its detection by chromogenic or chemiluminescent methods.

In one type of ELISA assay, an antibody that binds an epitope on a target protein is immobilized, and a test solution added. The immobilized antibody will capture any target protein present in the sample. A wash step removes nonspecifically bound material, and subsequently a second antibody is added that reacts with a second epitope on the protein (see **Figure 6A**).

Alternatively, a protein can be immobilized on a solid support, and antibodies reacting with the protein can be detected and quantitated in a test solution by the addition of a secondary antibody that reacts with the primary antibody (see **Figure 6B**). This example is presented in Protocols 12 and 13.

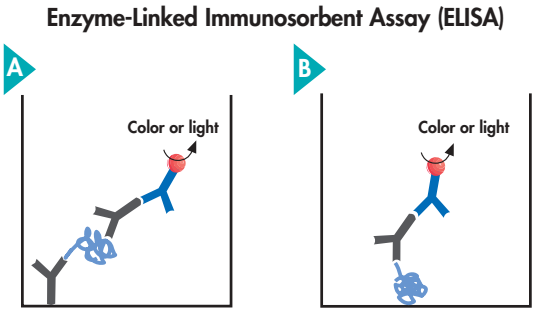


Figure 6. *Two common types of ELISA.* ***A****: An antibody is immobilized on a solid support and captures proteins in solution.* ***B****: A protein is immobilized and captures antibodies in solution.*

Protocol 12. Coating 96-well microplates with protein for ELISA

Protocol 12

This procedure is used to immobilize proteins onto the inner surfaces of 96-well microplates. The proteins can then be assayed using a primary and secondary antibody in a process analogous to that used for detection of proteins on western blots.

Important notes before starting

- The ease with which proteins bind to polystyrene plates is very much dependent on the particular protein. Optimization of binding conditions is necessary. Refer to the manufacturer's instructions.
- As a starting point, three buffers at different pH values should be compared.
- Binding may be carried out at 4–37°C. Successful binding may depend on the stability of the protein.

Materials

- Suitable 96-well microplates

Coating buffers:

- PBS, pH 7.2
- 50 mM sodium carbonate, pH 9.6
- 50 mM sodium carbonate, pH 10.6
- Microplate blocking buffer

For buffer and reagent compositions, see "Coating and Assay Buffers and Solutions for ELISA Procedures", page 99.

protocol continues overleaf

Protocol 12. Continued

1. Serially dilute the protein to be immobilized in coating buffer(s).
2. Add 200 µl of the protein solution to each well, and incubate overnight at 4°C.
3. Wash wells 4 times with PBS. Soak wells for 10–60 s per wash, and dry the wells by tapping the plate on paper towels.
4. Block wells with 250 µl of microplate blocking buffer for 2 h at room temperature (20–25°C) on a shaker platform.

Tip After blocking, plates can be dried overnight at 20–25°C, but sensitivity of the assay will be reduced.

Tip After drying, it may be possible to store the plates at 4°C for a period of time before use, but this will depend on the specific protein to be assayed.

5. Wash wells 4 times with PBS. Soak wells for 10–60 s per wash, and dry the wells by tapping the plate on paper towels.
6. Proceed with the protocol for assay of proteins with a protein specific antibody (Protocol 13, below).

Protocol 13. Assay of immobilized proteins with a protein specific antibody

Protocol 13

Antibodies can be used to assay proteins that are immobilized directly to the well surfaces of a 96-well polystyrene microplate (Protocol 12, above). The sensitivity of assays performed in this way depends largely on the particular protein to be assayed.

Important notes before starting

- Binding of detection antibodies should be carried out for at least 1 h at room temperature. If the concentration of the protein to be detected is very low or if the epitope is partly hidden, incubation times of 2–4 h or overnight may increase sensitivity.
- Best results will be obtained if all steps are carried out on a shaker. If there is no shaker available, incubation times should be increased (up to 2–3 h at room temperature or overnight at 4°C) or the incubation temperature should be raised to allow sufficient diffusion of molecules.
- Antibody dilution depends on the individual antibody used. Please refer to manufacturer's recommendations or begin at concentrations useful for western-blot or dot-blot analyses and try further dilutions. Usually primary monoclonal antibody at 0.1 µg/ml to 1 µg/ml will yield satisfactory results. Each antibody should be titrated over this range of concentrations to determine the optimal dilution.
- Suitable negative controls are essential. Assays should always be performed in parallel with samples without any proteins (lysis/dilution buffer alone, reagent blank) and with samples similar to those assayed but lacking the target protein (e.g., lysate from *E. coli* transformed with vector lacking the protein-encoding insert). These controls should be incubated with antibodies and the remaining assay components.

  Note: This protocol is intended to be used as an example. Optimal conditions for each individual protein and antibody should be determined.
- If establishing a new assay system, the binding of the protein or antibody to the solid support should be optimized first (incubation time and amounts of protein). Primary antibody or other secondary components of the assay should be optimized afterwards.

protocol continues overleaf

Protocol 13. Continued

Materials

- 96-well microplates coated with protein (from Protocol 12, page 83)
- PBS/BSA
- PBS
- Anti-target–protein antibody
- Secondary-antibody conjugate
- Substrate for alkaline phosphatase or horseradish peroxidase or one of the alternative substrates for horseradish peroxidase as described in "Substrates and Solutions for Protein Assay Procedures", page 100. Buffers and substrates indicated for alternative use will yield higher sensitivity, but dependent on the antibody system used, they may also lead to increased background signals.

For buffer and reagent compositions, see "Substrates and Solutions for Protein Assay Procedures", page 100.

1. Add 200 µl of Anti-target–protein antibody diluted 1/2000 in PBS/BSA. Cover plate, and incubate for 1–2 h at RT.

Tip For higher sensitivity, antibody binding can be performed overnight at 4°C.

2. Wash wells 4 times with PBS-Tween. Soak wells for 10–60 s per wash, and dry the wells by gently tapping the plate on paper towels after the wash.
3. Dilute secondary antibody in PBS/BSA according to the manufacturer's recommendations. Add 200 µl of the diluted antibody to each well, and incubate at room temperature for 45 min.
4. Wash wells 4 times with PBS-Tween. Soak wells for 10–60 s per wash, and dry the wells by gently tapping the plate on paper towels.
5. Add 200 µl of substrate solution, and monitor color development in a microplate reader.

Tip Substrate solution should always be prepared immediately before use.

Tip Monitor color development over a period of 45 min, or add 50 µl stopping reagent after a specific time and measure product. When testing a new assay system, a time-course of color development should be carried out to determine optimal development time and temperature.

Tip If the reaction is stopped the signal will increase slightly, depending on the substrate used, and the color will be stable for a certain period of time.

Quantifying proteins using the Bradford method

The Bradford method is a quantitative protein assay method, based on the binding of a dye, Coomassie Brilliant Blue, to a protein sample, and comparing this binding to a standard curve generated by the reaction of known amounts of a standard protein, usually BSA.

For this assay, protein samples should be diluted in an appropriate buffer (generally the same buffer in which they are dissolved). The BSA standard curve should be prepared using the same buffer.

Protocol 14. Bradford assay procedure

Protocol 14

Materials

- BSA standard solution (1 mg/ml*)
- Bradford assay dye reagent (available commercially, e.g., Bio-Rad Protein Assay Dye Reagent Concentrate, cat. no. 500-0006)
- Protein dilution buffer

Proteins should be diluted in the buffer in which they are dissolved. Use the same buffer to prepare the standard curve.

** Concentration of the BSA standard solution should be measured photometrically. A 1 mg/ml solution of BSA should have an A_{280} of 0.66.*

1. Prepare a standard curve by pipetting together carefully the solution volumes listed in **Table 3** overleaf corresponding to 0, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml BSA. The end volume of all samples should be 200 µl.
2. Dilute an aliquot of the dye reagent concentrate 1:5 with distilled water†. Pipet 20 µl of each BSA dilution into a plastic cuvette, add 1 ml diluted dye reagent, mix well, and incubate for 5 min at room temperature.
3. Measure the OD at 595 nm for each sample, and plot the standard curve.
4. Prepare a second standard curve by pipetting together carefully the solution volumes listed in **Table 3** corresponding to 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml BSA. The end volume of all samples should be 200 µl.
5. Dilute an aliquot of the dye reagent concentrate 1:5 with distilled water.† Pipet 20 µl of each BSA dilution into a plastic cuvette, add 1 ml diluted dye reagent, mix well, and incubate for 5 min at room temperature.
6. Measure the OD at 595 nm for each sample, and plot the standard curve.

protocol continues overleaf

† Stored in the dark, diluted dye reagent is stable for 2 weeks. Write the date of preparation on the bottle and cover with aluminum foil.

Protocol 14. Continued

7. Prepare dilutions of the protein sample of interest and test 20 µl aliquots in duplicate as above. It is important that the protein samples to be tested are handled in exactly the same manner as the samples used in generating the standard curves. Calculate the protein concentration of the test samples by comparing the OD_{595} with the standard curve(s), taking into account dilution factors (Figure 7).

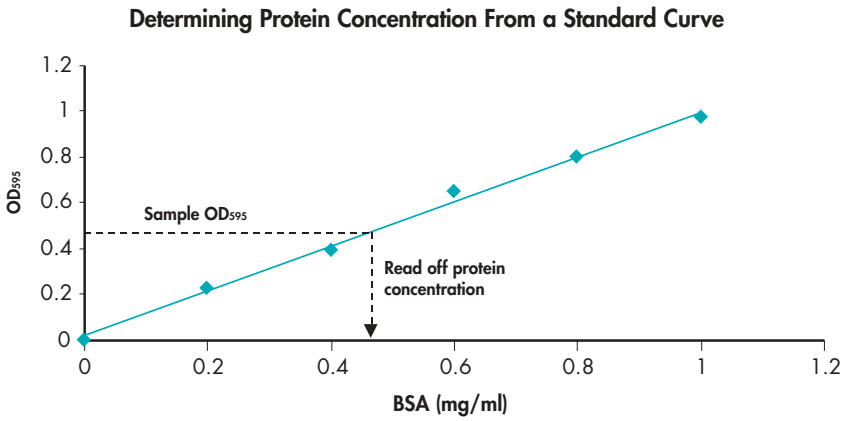


Figure 7. Standard curve generated using the Bradford method.

Table 3. Standard curve samples for Bradford protein assay

BSA conc. (mg/ml)	Volume of BSA standard solution (µl)	Volume of protein dilution buffer*(µl)
0	–	200
0.05	10	190
0.1	20	180
0.2	40	160
0.3	60	140
0.4	80	120
0.5	100	100
0.6	120	80
0.8	160	40
1.0	200	–

* *Use the same buffer as that used to dilute the protein of interest.*

QIAGEN offers a wide range of products and resources for the expression, purification, detection, and assay of proteins. *The QIAexpressionist* is a comprehensive manual that provides background information and protocols for the cloning, expression, and purification of 6xHis-tagged proteins expressed using QIAGEN pQE vectors. The *QIAexpress Detection and Assay Handbook* provides detailed information and protocols for the detection and assay of 6xHis-tagged proteins. Many of the protocols in these manuals can be adapted to general use. For further information about QIAGEN products and literature please refer to the *QIAGEN Product Guide*, visit us online at **www.qiagen.com**, or contact QIAGEN Technical Services or your local distributor.

References

1. Ausubel, F.M., et al. (1999) Current Protocols in Molecular Biology. *New York: John Wiley and Sons.*

2. Sambrook, J. and Russell, D. (2001) Molecular Cloning: A Laboratory Manual. *3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.*

3. Kaufmann S.H., Ewing, C.M., and Shaper, J.H. (1987) The erasable Western blot. Anal. Biochem. ***161****, 89.*

5. Appendix

Contents

continues overleaf

5. Appendix

Contents

Continued

This Appendix provides recipes for the media, buffers, and solutions that are used with the protocols described in the previous chapters. Please be aware that different versions of the same medium, buffer, or solution may exist, so the recipes described here may differ slightly from those in other molecular biology manuals. The recipes described here are those used by the scientists at QIAGEN, and provide optimal results. This Appendix also contains additional useful information for analysis of nucleic acids and proteins, as well as information about different technical resources offered by QIAGEN.

General Guidelines for Preparation and Storage of Buffers and Solutions

Preparing buffers and solutions

The following guidelines should be followed when preparing buffers and solutions for use in the procedures described in this Bench Guide.

1. It is generally easier to start by weighing dry components. Carefully weigh out the desired amount of each component.

Tip Large amounts of components can be weighed directly into the vessel in which they will be dissolved, e.g., measuring cylinder or beaker.

Tip For smaller amounts, use weighing boats and add each component sequentially once the correct amount has been weighed out. Rinse weighing boats with distilled water over the buffer vessel to ensure that all the substance weighed out has been added.

2. Measure liquid components using a measuring cylinder and add to the dry components. Rinse the measuring cylinder with a small amount of distilled water and empty into the vessel.
3. Add distilled water (or the appropriate solvent) to approximately 90% of the desired volume.

continues overleaf

4. Stir the solution using a stir bar and a magnetic stirrer, and adjust the pH with the appropriate acid or base to the desired value using a calibrated pH meter.

Tip Add acid or base dropwise and allow the pH to stabilize before adding further acid or base.

Tip Buffer pH changes with temperature. If you will be working with buffers at a specific temperature, e.g., 4°C, prepare buffers using water at 4°C. Ensure that your pH meter is adjusted to compensate for the change in temperature.

5. Add distilled water to the desired volume.
6. Filter-sterilize buffer through a 0.45 µm filter, or autoclave.

Tip Do not autoclave solutions that will be used in experiments that are sensitive to bacterial endotoxins. Filter-sterilize such solutions instead.

Storing buffers and solutions

In general, buffers and solutions should be stored at 2–8°C. Solutions containing unstable compounds (e.g., antibiotics) should be stored in aliquots at –20°C. Bottles should be clearly labeled with the name of the buffer/solution, the pH, the components and their concentration, and the date of preparation. When preparing buffers from stock solutions, check and, if necessary, adjust the pH before adjusting the buffer to the final volume.

Commonly Used Buffers and Solutions

Buffer	Components per liter	
20x SSC	NaCl Sodium citrate·$2H_2O$ *Adjust pH to 7.0 with NaOH*	175.3 g 88.2 g
0.5 M EDTA, pH 8.0	Ethylenediaminetetraacetic acid (EDTA)·$2H_2O$ NaOH *Adjust pH to 8.0 with NaOH* *EDTA will not go into solution until the pH is about 8.0*	186.1 g ~20 g
1 M Tris·Cl	Tris base *Adjust to desired pH with HCl*	121.1 g
TE, pH 7.4	1 M Tris·Cl, pH 7.4 0.5 M EDTA, pH 8.0	10 ml 2 ml
10% SDS	Sodium dodecyl sulfate (SDS) *Adjust pH to 7.2 with HCl* *There is no need to sterilize 10% SDS*	100 g

Spectrophotometric Measurement of Nucleic Acid Concentration

Spectrophotometric conversions for calculating the concentration of nucleic acids from their absorbance at 260 nm (A_{260}) are given in **Table 1**.

Table 1. Spectrophotometric conversions

1 A_{260} unit	Concentration (µg/ml)*
dsDNA	50
ssDNA	33
RNA	40
Oligonucleotides	20–30

Adapted from reference 1.

** This relationship is only valid for measurements made at neutral pH, and is based on a standard 1 cm path length.*

An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 µl

Dilution = 10 µl of RNA sample + 490 µl distilled water (1/50 dilution).

Measure absorbance of diluted sample in a quartz cuvette.

A_{260} = 0.23

Concentration of RNA sample = Spectrophotometric conversion x A_{260} x dilution factor
= 40 x 0.23 x 50
= 460 µg/ml

Total yield = concentration x volume of sample in milliliters
= 460 µg/ml x 0.1 ml
= 46 µg

Tip Spectrophotometric measurements must be taken using a quartz cuvette. If you use more than one cuvette to measure multiple samples, they must be matched.

Tip To ensure readings fall within the linear range, values should lie between 0.1 and 1.0.

Tip Dilute samples in a low-salt buffer with neutral pH (e.g., 10 mM Tris·Cl, pH 7.0) when making spectrophotometric measurements for determining nucleic acid concentration.

Tip Spectrophotometric quantification of DNA is accurate only when the sample is not contaminated with RNA, and vice versa.

Tip Phenol has an absorbance maximum of 270–275 nm, which is close to that of DNA and RNA. Phenol contamination mimics both higher yields and higher purity because of an upward shift in the A_{260} value.

Bacterial Culture Media and Buffers

Media

Medium	Components per liter	
LB	Tryptone	10 g
	Yeast extract	5 g
	NaCl	10 g
	Autoclave	
LB agar*	Tryptone	10 g
	Yeast extract	5 g
	NaCl	10 g
	Agar: for 1.5% LB agar	15 g
	for 0.7% LB agar	7 g
	Autoclave	

Medium	Components per liter	
SOC	Tryptone	20 g
	Yeast extract	5 g
	NaCl	0.5 g
	Dissolve, then add:	
	250 mM KCl	10 ml
	2 M $MgCl_2$	5 ml
	Autoclave, cool, then add:	
	1 M sterile glucose†	20 ml

** For details on preparing LB-agar plates, see "Preparation of LB-agar plates", page 5.*

† Do not sterilize by autoclaving! Filter the solution through a 0.2 µm filter instead.

Buffers for preparing competent *E. coli*

Buffer	Composition of working solutions	Components per liter	
TFB1	100 mM RbCl	RbCl	12.1 g
	50 mM $MnCl_2$	$MnCl_2 \cdot 4H_2O$	9.9 g
	30 mM potassium acetate	Potassium acetate	2.9 g
	10 mM $CaCl_2$	$CaCl_2$	1.1 g
	15% glycerol	Glycerol	15 ml
	pH 5.8	*Adjust pH to 5.8*	
		Sterilize by filtration	
TFB2	10 mM MOPS	MOPS	2.1 g
	10 mM RbCl	RbCl	1.2 g
	75 mM $CaCl_2$	$CaCl_2$	8.3 g
	15% glycerol	Glycerol	15 ml
	pH 6.8	*Adjust pH to 6.8 with KOH*	
		Sterilize by filtration	

Agarose Gel Electrophoresis Buffers for Analysis of DNA

Buffer	Composition of working solution		Components		
			Stock solution components per liter		
TAE	**1x**	40 mM Tris·acetate 1 mM EDTA	**50x**	Tris base Glacial acetic acid 0.5 M EDTA, pH 8.0	242 g 57.1 ml 100 ml
TBE	**0.5x**	45 mM Tris·borate 1 mM EDTA	**5x**	Tris base Boric acid 0.5 M EDTA, pH 8.0	54 g 27.5 g 20 ml
			Components per 10 ml		
Gel loading buffer	**6x**	0.25% bromophenol blue 0.25% xylene cyanol FF 40% (w/v) sucrose*		Bromophenol blue Xylene cyanol FF Sucrose*	25 mg 25 mg 4 g

** 15% Ficoll (Type 400) or 30% glycerol can be used instead of sucrose.*

Commonly Used DNA Markers in Agarose Gel Electrophoresis

λ *Hin*dIII	λ *Hin*dIII–*Eco*RI	λ *Eco*RI	Φ *X*174 *Hae*III	100 bp ladder†	1 kb ladder‡
23,130	21,226	21,226	1,353	2,072	12,216
9,416	5,148	7,421	1,078	1,500	11,198
6,557	4,973	5,804	872	1,400	10,180
4,361	4,268	5,643	603	1,300	9,162
2,322	3,530	4,878	310	1,200	8,144
2,027	2,027	3,530	281	1,100	7,126
564	1,904		271	1,000	6,108
125	1,584		234	900	5,090
	1,375		194	800	4,072
	947		118	700	3,054
	831		72	600	2,036
	564			500	1,636
	125			400	1,018
				300	517
				200	506
				100	396
					344
					298
					220
					201
					154
					134
					75

Sizes in base pairs

† *Invitrogen, cat. no. 15628-019.*

‡ *Invitrogen, cat. no. 15615-0116.*

Southern Transfer Buffers and Solutions

Buffer	Composition of working solution	Components per liter	
Denaturation buffer	1.5 M NaCl 0.5 M NaOH	NaCl NaOH	87.7 g 20 g
Neutralization buffer	1 M Tris·Cl 1.5 M NaCl pH 7.4	Tris base NaCl *Adjust pH to 7.4 with HCl*	121.1 g 87.7 g
20x SSC	3 M NaCl 0.3 M sodium citrate	NaCl Sodium citrate·$2H_2O$ *Adjust pH to 7.0 with NaOH*	175.3 g 88.2 g

Formaldehyde Agarose Gel Electrophoresis Buffers for Analysis of RNA

Buffer	Composition of working solution		Components		
			Stock solution components per liter		
FA gel buffer*	**1x**	20 mM MOPS 5 mM sodium acetate 1 mM EDTA pH 7.0	**10x**	MOPS, free acid Sodium acetate·H20 0.5 M EDTA, pH 8.0 *Adjust pH to 7.0 with NaOH*	41.9 g 6.8 g† 20 ml
			Components per liter		
FA gel running buffer	**1x**	1x FA gel buffer 2.5 M formaldehyde‡		10x FA gel buffer 37% (12.3 M) formaldehyde RNase-free water§	100 ml 20 ml 880 ml
			Components per 10 ml		
RNA loading buffer	**5x**	0.25% bromophenol blue 4 mM EDTA 0.9 M formaldehyde‡ 20% glycerol 30.1% formamide 4x FA gel buffer		Bromophenol blue 0.5 M EDTA, pH 8.0 37% (12.3 M) formaldehyde Glycerol Formamide 10x FA gel buffer *Stable for ≈ 3 months at 2–8°C*	25 mg¶ 80 µl 750 µl 2 ml 3.084 ml 4 ml

* *FA gel buffer turns yellow during autoclaving. This has no effect on gel electrophoresis.* † *Alternatively, 4.1 g anhydrous sodium acetate*

‡ *Toxic and/or mutagenic. Take appropriate safety measures.* § *See page 48.*

¶ *Alternatively, use 16 µl of a saturated aqueous bromophenol blue solution instead of 25 mg powder. To make this solution, add solid bromophenol blue to distilled water, mix, and continue to add more bromophenol blue until no more will dissolve. Centrifuge to pellet the undissolved powder, and carefully pipet the saturated supernatant.*

Northern Transfer Solution

Buffer	Composition of working solution	Components per liter	
20x SSC	3 M NaCl 0.3 M sodium citrate	NaCl Sodium citrate·$2H_2O$ *Adjust pH to 7.0 with NaOH*	175.3 g 88.2 g

Colony-Blot Solutions

Solution	Composition of working solution	Components per liter	
10% SDS	10% (w/v) SDS	SDS *Adjust pH to 7.2 with HCl*	100 g
Denaturing solution	0.5 M NaOH 1.5 M NaCl	NaOH NaCl	20 g 87.7 g
Neutralization solution	1.5 M NaCl 0.5 M Tris·Cl pH 7.4	NaCl Tris base *Adjust pH to 7.4 with HCl*	87.7 g 60.6 g
20x SSC	3 M NaCl 0.3 M sodium citrate	NaCl Sodium citrate·$2H_2O$ *Adjust pH to 7.0 with HCl*	175.3 g 88.2 g

SDS-PAGE Buffers and Solutions for Analysis of Proteins

Solution	Composition of working solution	Components	
		Components per liter	
30% acrylamide/ 0.8% bis-acrylamide stock solution*	30% acrylamide 0.8% bis-acrylamide (*N,N'*-methylene-bis-acrylamide)	Acrylamide Bis-acrylamide	300 g 8 g
2.5x separating gel buffer	1.875 M Tris·Cl 0.25% SDS pH 8.9	Tris base SDS *Adjust pH to 8.9 with HCl*	227.1 g 2.5 g
5x stacking gel buffer	0.3 M Tris·phosphate 0.5% SDS pH 6.7	Tris base SDS *Adjust pH to 6.7 with phosphoric acid*	36.3 g 5 g
5x electrophoresis buffer	0.5 M Tris base 1.92 M glycine 0.5% SDS	Tris base Glycine SDS *pH should be 8.8 without adjustment*	60.6 g 144.1 g 5 g
		Components per 10 ml	
5x SDS-PAGE sample buffer	0.225 M Tris·Cl, pH 6.8 50% glycerol 5% SDS 0.05% bromophenol blue 0.25 M dithiothreitol (DTT)†	1 M Tris·Cl, pH 6.8 Glycerol SDS Bromophenol blue 1 M DTT	2.25 ml 5 ml 0.5 g 5 mg 2.5 ml

* *Can be purchased as a ready-to-use solution from several companies, e.g., Rotiphorese® Gel 30 (Roth, cat. no. 3029.1) or Bio-Rad® 30% Acrylamide/Bis Solution (Bio-Rad, cat. no. 161-0158). Acrylamide is a potent neurotoxin and is absorbed through the skin. Take appropriate safety measures particularly when weighing solid acrylamide/ bisacrylamide, and also when working with solutions and gels.*

† *Do not autoclave solutions containing DTT.*

Coomassie® Staining Solutions

Solution	Composition of working solution	Components per 100 ml	
Coomassie staining solution	0.05% (w/v) Coomassie Brilliant Blue R-250 40% (v/v) ethanol 10% (v/v) glacial acetic acid 50% (v/v) water	Coomassie Brilliant Blue R-250* Ethanol *Dissolve, then add:* Glacial acetic acid Water *Filter before use*	50 mg 40 ml 10 ml 50 ml
Destaining solution	40% (v/v) ethanol 10% (v/v) glacial acetic acid 50% (v/v) water	Ethanol Glacial acetic acid Water	40 ml 10 ml 50 ml

* *e.g., SIGMA, cat. no. B 0149.*

Western Transfer Buffers and Solutions

Solution	Composition of working solution	Components	
		Components per liter	
Semi-dry transfer buffer	25 mM Tris base 150 mM glycine 10% (v/v) methanol	Tris base Glycine Methanol *pH should be 8.3 without adjustment*	3.0 g 11.3 g 100 ml
Tank-blotting transfer buffer	25 mM Tris base 150 mM glycine 20% (v/v) methanol	Tris base Glycine Methanol *pH should be 8.3 without adjustment*	3.0 g 11.3 g 200 ml
		Components per 100 ml	
Ponceau S staining solution	0.5% (w/v) Ponceau S 1% (v/v) glacial acetic acid	Ponceau S Glacial acetic acid	0.5 g 1 ml

Protein Dot-Blot Preparation Buffers

Solution	Composition of working solution	Components per liter	
Dilution buffer for denaturing conditions	8 M urea 100 mM NaH_2PO_4 10 mM Tris·Cl pH 8.0	Urea $NaH_2PO_4·H_2O$ Tris base *Adjust pH to 8.0 with HCl*	480.5 g 13.8 g 1.2 g
Dilution buffer for native conditions	50 mM NaH_2PO_4 300 mM NaCl pH 8.0	$NaH_2PO_4·H_2O$ NaCl *Adjust pH to 8.0 with NaOH*	17.5 g 6.9 g

Immunodetection Buffers

Buffer*	Composition of working solution	Components per liter	
TBS buffer	10 mM Tris·Cl 150 mM NaCl pH 7.5	Tris base NaCl *Adjust pH to 7.5 with HCl*	8.8 g 1.2 g
TBS-Tween®/Triton® buffer	20 mM Tris·Cl 500 mM NaCl 0.05% (v/v) Tween 20 0.2% (v/v) Triton X-100[†] pH 7.5	Tris base NaCl Tween 20 Triton X-100 *Adjust pH to 7.5 with HCl*	2.4 g 29.2 g 500 µl 2 ml
Blocking buffer	3% (w/v) BSA in TBS buffer *Alternative:* 1% (w/v) alkali-soluble casein in TBS buffer	BSA[‡] *Dissolve in TBS buffer* Alkali-soluble casein[§] *Dissolve in TBS buffer*	30 g 10 g
Secondary antibody dilution buffer	10% (w/v) nonfat dried milk powder in TBS buffer *Alternative:* 1% (w/v) alkali-soluble casein in TBS buffer	Nonfat dried milk powder *Dissolve in TBS buffer* Alkali-soluble casein[§] *Dissolve in TBS buffer*	100 g 10 g

* *Buffers containing BSA or milk powder should be prepared freshly each time they are required. Store other buffers at 2–8°C to avoid microbial spoilage. Do not use azide as a bactericide as this will inhibit the peroxidase detection reaction.*

[†] *SIGMA, cat. no. x-100* [‡] *SIGMA, cat. no. A7906.* [§] *Merck, cat. no. 1.02241.*

Chromogenic Substrates for Immunoblotting Procedures

Chromogenic substrate*	Abbreviation	Reaction product
Peroxidase substrates		
3,3'-Diaminobenzidine	DAB	Brown, insoluble
3-Amino-9-ethylcarbazole	AEC	Red, insoluble
4-Chloro-1-naphthol	4C1N	Blue, insoluble
Alkaline phosphatase substrate		
5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium	BCIP/NBT	Blue, insoluble

* *Prepare solutions for alkaline phosphatase or horseradish-peroxidase reaction immediately before use.*

Coating and Assay Buffers and Solutions for ELISA Procedures

Solution	Composition of working solution	Components per liter	
PBS	50 mM potassium phosphate 150 mM NaCl pH 7.2	0.5 M K_2HPO_4 0.5 M KH_2PO_4 NaCl *pH should be 7.2 without adjustment*	71.7 ml 28.3 ml 8.8 g
50 mM sodium carbonate, pH 9.6	50 mM Na_2CO_3 pH 9.6	$Na_2CO_3 \cdot H_2O$ *Adjust pH to 9.6 with NaOH*	6.2 g
50 mM sodium carbonate, pH 10.6	50 mM Na_2CO_3 pH 10.6	$Na_2CO_3 \cdot H_2O$ *Adjust pH to 10.6 with NaOH*	6.2 g
PBS/BSA†	0.2% BSA in PBS	BSA *Dissolve in PBS buffer*	2 g
Microplate blocking buffer†	2.0% sucrose 0.1% BSA 0.9% NaCl	Sucrose BSA NaCl	20 g 1 g 9 g

† *Buffers containing BSA or milk powder should be prepared freshly each time they are required.*

Substrates and Solutions for Protein Assay Procedures

Substrate*/Solution	Components	
Phosphate–citrate buffer, pH 5.0	0·2 M Na_2HPO_4	51.5 ml
	0·1 M citric acid	48.5 ml
Substrate for alkaline phosphatase		
p-Nitrophenyl Phosphate (pNPP)	pNPP	50 mg
	1 M diethanol-amine; 0.01% $MgCl_2 \cdot 6\ H_2O$, pH 9.8	10 ml
Substrate for horseradish peroxidase		
2,2'-Azino-bis[3-Ethylbenz-thiazoline-6-Sulfonic Acid] (ABTS®)	ABTS	10 mg
	Phosphate–citrate buffer	10 ml
	Immediately before use add	
	30% H_2O_2	2 µl
Alternative substrates for horseradish peroxidase†		
o-Phenylenediamine (OPD)	OPD	10 mg
	Phosphate–citrate buffer	10 ml
	Immediately before use add	
	30% H_2O_2	2 µl
3,3',5,5'-Tetramethylbenzidine (TMB)	TMB	1 mg
	DMSO	1 ml
	Dissolve then add	
	Phosphate–citrate buffer	9 ml

* *Prepare substrates immediately before use.*

† *These substrates will yield higher sensitivity, but depending on the antibody systems used, they can also lead to increased background signals.*

Details of substrates for protein assay procedures

Substrate	Wavelength for monitoring color development	Stopping reagent‡	Wavelength for determining stopped product
pNPP	405 nm	3 M NaOH	405 nm
ABTS	415 nm	1% SDS	415 nm
OPD	450 nm	3 M HCl or 3 M H_2SO_4	492 nm
TMB	370 nm or 650 nm	2 M H_2SO_4	450 nm

‡ *If the reaction is stopped, the signal will increase slightly, depending on the substrate used, and the color will be stable for a period of time.*

Further information about buffers and solutions for molecular biology can be found in commonly used manuals (e.g., 1,2).

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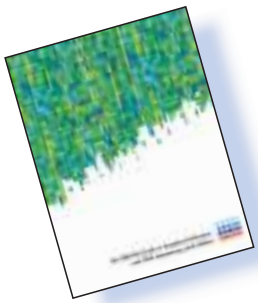

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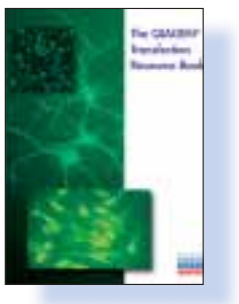

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References

1. Ausubel, F.M. et al., eds. (1999) Current Protocols in Molecular Biology, *New York: John Wiley and Sons.*

2. Sambrook, J. and Russell, D. (2001) Molecular Cloning: A Laboratory Manual. *3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.*

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