Purification of Oligonucleotides Using Denaturing Polyacrylamide Gel Electrophoresis

BASIC PROTOCOL

Several methods exist for the purification of oligonucleotides following chemical synthesis (*UNIT 2.11*). The advantages of purification on denaturing polyacrylamide gels are speed, simplicity, and high resolution. These gels can resolve oligonucleotides from 2 to 300 bases long, depending on the percentage of polyacrylamide used (see Table 2.12.1). This method is thus useful for isolating not only chemically synthesized deoxyribonucleotides (*UNIT 2.11*) but also small RNAs or other single-stranded oligonucleotides. After gel setup, samples are loaded onto a urea-based denaturing gel, separated by electrophoresis, and finally recovered from the crushed gel slice by freeze/thaw and elution.

Materials

Nucleic acid samples
Urea
10× and 1× TBE buffer, pH 8 (*APPENDIX 2*)
40% acrylamide/2% bisacrylamide (*UNIT 7.6*)
TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine)
10% ammonium persulfate (APS) in water (store ≤1 month at 4°C)
Urea loading buffer (see recipe)
3 M sodium acetate, pH 5.2 (*APPENDIX 2*)
TE buffer, pH 7.5 (*APPENDIX 2*)

Acrylamide gel electrophoresis apparatus
Glass plates, spacers, and combs for pouring gels
50°, 60° and 90°C water baths
DC power supply
Thin-layer chromatography (TLC) plate with fluorescent indicator (e.g., Silica Gel F-254 or IB-F, Merck)
Hand-held short-wave (254-nm) UV lamp
Small-bore (5-ml) syringe (e.g., Becton Dickinson)
15-ml centrifuge tube capable of withstanding high temperatures
Rotary shaker
0.2-µm filter (Gelman Sciences)

Additional reagents and equipment for oligonucleotide deprotection (*UNIT 2.11*), gel electrophoresis (*UNIT 2.7*), phenol extraction, and ethanol and butanol precipitation (*UNIT 2.1*)

Prepare the sample

1. Follow the appropriate deprotection protocol (*UNIT 2.11*) to prepare the nucleic acid sample for electrophoresis.

   Be sure to lyophilize the sample to dryness. The samples will generally appear as an off-white powder following deprotection and lyophilization. If a yellowish liquid or crusty pellet remains, rather than an off-white powder, resuspend the pellet in 0.5 ml distilled water and add $^1/_{10}$ vol of 3.0 M sodium acetate, pH 5.2. Add 3 vol of 100% ethanol and chill ~20 min at –80°C to precipitate. Centrifuge 10 min at 16,000 × g , 4°C. Decant and save the supernatant. Wash the pellet in 70% ethanol and lyophilize to dryness.

Contributed by Andrew Ellington and Jack D. Pollard, Jr.
Current Protocols in Molecular Biology (1998) 2.12.1-2.12.7

Table 2.12.1 Concentrations of Acrylamide Giving Optimum Resolution of DNA Fragments Using Denaturing PAGE[a]

Acrylamide (%)	Fragment sizes separated (bases)	Migration of bromphenol blue (bases)	Migration of xylene cyanol (bases)
30	2-8	6	20
20	8-25	8	28
10	25-35	12	55
8	35-45	19	75
6	45-70	26	105
5	70-300	35	130
4	100-500	~50	~230

[a]Data, from Maniatis et al. (1975), are for single-stranded DNA; RNA will migrate slightly more slowly than DNA of the same sequence and length.

Prepare the gel

2. Assemble the gel-casting apparatus.

   Gel spacer and casting systems have been developed to avoid leakage. Those that avoid the need to seal the gel with tape are best, and recently gel casting boots that lack bottom spacers have become available (GIBCO/BRL). Greasing the side/bottom spacers or pouring an agarose plug for the gel is not necessary if some care is taken to ensure that the bottom of the plate assembly is completely sealed. The gel plates should be thoroughly cleaned by washing them with warm soapy water and then rinsing them in 70% ethanol/water. However, if the plates are particularly dirty or if the complete removal of any residual nucleic acids is required, the plates may be soaked in 0.1 M NaOH for 30 min prior to washing. If the gel is particularly thin (<1 mm), silanizing one or both plates (APPENDIX 3B) facilitates post-electrophoretic separation of the gel from the plate.

3. Prepare the gel solution (see Table 2.12.1 for appropriate acrylamide concentrations for resolving single-stranded DNAs). For a denaturing acrylamide gel of 20 cm × 16 cm × 1.6 mm, 60 ml of gel solution is sufficient, made by mixing the following:

   25.2 g urea (7 M final)
   6 ml 10× TBE buffer
   Desired amount of 40% acrylamide/2% bisacrylamide needed for resolution
   H_2O to 60 ml final.

   CAUTION: *Acrylamide is a neurotoxin; always wear gloves, safety glasses, and a surgical mask when working with acrylamide powder.*

   Commercially prepared polyacrylamide solutions (National Diagnostics) are available and highly recommended since they have long shelf lives and avoid manipulation of the neurotoxic acrylamide powder.

   Pick a concentration of acrylamide that will allow the single-stranded nucleic acid to migrate approximately one-half to three-fourths of the way through the gel when the loading dye has reached the bottom of the gel. This allows for good separation of non-full-length and full-length products.

   Use a flask that has a wide mouth and a spout for pouring.

4. Heat the mixture to speed its dissolution by immersing the flask in a 60°C water bath or under hot running tap water. Once most of the urea and acrylamide have dissolved, vigorously agitate the solution for ~20 min with magnetic stirring to ensure complete mixing.

5. Add 40 µl TEMED and swirl the flask to ensure thorough mixing. Immediately add 300 µl of 10% APS and mix thoroughly. Pour the acrylamide between the gel plates and insert the comb. Clamp the comb in place at the top of the gel to avoid separation of the gel from the plates as the acrylamide polymerizes. Allow the gel to polymerize for ~30 min.

   IMPORTANT NOTE: *Polymerization begins as soon as the TEMED and APS are mixed, so all succeeding steps must be performed promptly.*

   CAUTION: *Be sure to wear safety glasses while pouring the gel, since it is easy to splash the neurotoxic unpolymerized acrylamide.*

   For thick gels, the acrylamide can be poured directly from the mixing flask, but for thinner ones, a large syringe fitted with a needle is useful. By pouring the gel slowly with a tilt of 45° relative to the bench top and starting from one corner, bubbles may largely be avoided. Also, letting the gel polymerize while it is lying flat helps minimize undesirable hydrostatic pressure on the gel bottom.

   TEMED may be stored indefinitely at 4°C, but the ability of APS to efficiently initiate the free radical–induced acrylamide polymerization diminishes greatly over time. Make a new APS stock every month and store at 4°C.

Run the gel

6. After polymerization is complete, remove the comb and any bottom spacers from the gel. Wash the gel plates free of spilled acrylamide, and be sure that the spacers are properly seated and clean.

7. Fill the lower reservoir of the electrophoresis tank with 1× TBE. Initially, place the gel into the lower tank at an angle to avoid formation of air bubbles between the plates and the gel bottom. Clamp the gel plates to the top of the electrophoresis tank and fill the upper reservoir with 1× TBE so that the wells are covered.

   A syringe with a bent needle may be used to remove air bubbles trapped under the gel, which will disrupt the current flow.

8. Using a DC power supply, prerun and warm the gel for at least 30 min at 20 to 40 V/cm (constant voltage).

9. Add 1× urea loading buffer to the oligonucleotide pellet from step 1 and resuspend it by heating it 5 min at 90°C.

   The amount of sample that can be loaded depends on the efficiency of the synthesis reaction. At least 10 µg of material in a single band 2 cm wide is required to cast a clear UV shadow. The longer the oligonucleotide, the less full-length product will be obtained.

   Use an amount of loading buffer that is consistent with loading ~25% of a 0.2-µmol synthesis of a 20-mer oligonucleotide per 2 cm × 2 cm × 1.6 mm well. This will give sharp bands with good resolution. Up to 4-fold more may be added, but the resolution will suffer.

10. Rinse the wells thoroughly with 1× TBE solution immediately prior to gel loading.

    The 7 M urea dissolved in the gel will start to diffuse from the wells, creating a dense layer at the bottom of the wells that prevents sample loading and decreases resolution. Rinsing eliminates this problem.

11. Load the samples.

    Tracking dyes such as bromphenol blue and xylene cyanol may be added to the samples or in empty lanes to monitor migration (see Table 2.12.1 for migration data).

12. Electrophorese the gel at 20 to 40 V/cm (constant voltage) until the positions of the tracking dye(s) from the loading buffer indicate that the oligonucleotide has migrated one-half to three-fourths of the way through the gel.

    The speed of electrophoresis is directly proportional to the voltage gradient across the gel. The current in the circuit and the heat generated for higher-percentage gels (>15% acrylamide) are corresponding smaller, since the increased acrylamide concentration leads to greater resistance. While some heating of the gel during electrophoresis is desirable since it helps to denature the sample, temperatures >65°C should be avoided. All gels should be monitored to make sure that they do not generate so much heat that the plates crack. For example, while a 20% gel can be electrophoresed at 800 V with few problems, an 8% gel run under the same conditions would likely generate too much heat for the apparatus to dissipate.

13. When the oligonucleotide is sufficiently resolved, turn off the power supply and detach the plates from the electrophoresis tank. Pry off the top plate. Cover the gel with plastic wrap (taking care to avoid bubbles and folds) and invert the plate onto a TLC plate with a fluorescent indicator. Using a spatula, peel a corner of the gel away from the plate and onto the plastic wrap. Pry off the remaining plate and place another sheet of plastic wrap on top of the gel.

Recover the oligonucleotide

14. Visualize the bands on the gel by briefly exposing them to short-wave (254 nm) radiation from a handheld lamp. The bands will appear as black shadows on a green background. Outline the bands using a marking pen.

    Avoid unnecessarily long UV exposure, which will damage the nucleic acids.

    The desired band is generally the darkest one on the gel (excluding material that runs at the dye front); it should also be the slowest-migrating band unless deprotection was incomplete. Lighter bands containing partially protected oligonucleotides, if present, will migrate considerably above the major fully deprotected band. If the stepwise efficiency of the synthesis is low, a smear may be seen instead of a clear band. The top of the smear should be cut out of the gel.

    Unpolymerized acrylamide absorbs strongly at 211 nm and may also cause shadowing that is confined to the edges and wells of the gel.

15. Cut out the bands directly with a clean scalpel or razor blade.

16. Chop the gel slabs into fine particles by forcing the gel through a small-bore syringe to aid the diffusion of the oligonucleotide from the matrix. Place the crushed gel slab in a 15-ml centrifuge tube capable of withstanding high temperatures.

17. Add 3 ml TE buffer for every 0.5 ml of gel slab. Freeze the sample for 30 min at −80°C or until frozen solid. Quickly thaw it in a hot water bath (~50°C) and let soak 5 min at 90°C. Elute on a rotary shaker overnight at room temperature.

    This freeze/rapid thaw approach (Chen and Ruffner, 1996) greatly decreases elution time and increases yield by allowing ice crystals to break apart the acrylamide matrix. Recovery of a 20-mer oligonucleotide is typically 80% after 3 hr of rotary shaking, making this technique comparable in yield to electroelution (UNIT 2.7).

    Since elution is a diffusion-controlled process, using more buffer will aid in elution efficiency. Also, note that longer oligonucleotides will take longer to diffuse from the gel. If speed is essential and high yields are dispensable, enough sample can be obtained for most experiments in only a few hours of extraction. Increasing the temperature to 37°C will also speed the process. Yield may be increased by repeated elutions.

18. Centrifuge the tube 2 min at 1000 × *g*, room temperature, to pellet the gel fragments. Use a syringe to remove the supernatant, then filter off any remaining acrylamide

fragments by passing the suspension through an 0.2-μm filter and into a fresh 15-ml centrifuge tube.

19. Concentrate the sample by extracting against 1 vol *n*-butanol. Remove the upper butanol layer and repeat until the volume of the lower, aqueous layer is convenient for precipitation.

    About ⅕ vol of the aqueous layer is extracted into the organic butanol layer for every volume of butanol used. If too much butanol is added and the water is completely extracted into the butanol, simply add more water and concentrate again.

20. Add 3.0 M sodium acetate, pH 5.2 (to a final concentration of 0.3 M). Add 2 vol of 100% ethanol to precipitate DNA or 3 vol for RNA. Chill 20 min at −20°C. Pellet the oligonucleotide by centrifuging 10 min at 12,000 × *g* , 4°C.

    Do not attempt to precipitate small oligonucleotides (20 bases) in the presence of ammonium ions. If the samples prove refractory to precipitation, use a 1:1 (v/v) mix of ethanol/acetone or 6 vol of acetone for precipitation. A rinse with 95% ethanol will remove undesired salts.

21. Redissolve the oligonucleotide in TE buffer if appropriate.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Urea loading buffer

8 M urea
20 mM EDTA
5 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
0.5% (w/v) xylene cyanol, bromphenol blue, or both

Add 1 vol loading buffer to sample if a solution, or enough to dissolve a dry sample.

COMMENTARY

Background Information

The traditional alternative to gel purification of oligonucleotides has been high-performance liquid chromatography (HPLC). Although alkali perchlorate salts HPLC systems can achieve very high resolution of small and medium-sized oligonucleotides (<60 bases), electrophoresis provides superior capacity and resolution over a greater range of sizes and is simpler to set up and operate. Separation times using HPLC may be faster (<30 min) than for gels, but the time required for initialization of the system and product workup tend to negate this advantage.

Purification of oligonucleotides on low-pressure reverse-phase cartridges is technically simpler than gel electrophoresis, and faster (<2 hr). However, these cartridges offer no separation of desired product from failed sequences and, if not used properly, allow contamination of the final product with low-molecular-weight compounds that often inhibit subsequent enzymatic manipulation of oligonucleotides. For short oligonucleotides synthesized in high yield, very simple purification methods (e.g., gel filtration or ethanol precipitation) are adequate for some applications, such as sequencing or PCR primers, that do not require absolutely homogeneous material.

The high resolution and capacity of polyacrylamide gels makes them the method of choice for the purification of oligonucleotides. Urea disrupts hydrogen bonding between bases and thus allows oligonucleotides to be resolved almost exclusively on the basis of molecular weight as opposed to secondary structure. However, it should be noted that oligonucleotides of equivalent length but different sequence will still migrate slightly differently. Thus, mixed sequences will appear as broader bands than homogeneous sequences (Applied Biosystems, 1984). Also, RNA eletrophoreses through the gel more slowly than does DNA of comparable size. Finally, when modified nu-

cleotides have been incorporated into the nucleic acids, the compatibility of their chemistries with that of the acrylamide matrix should be checked before PAGE purification (in particular, oligonucleotides bearing thio groups seem to undergo Michael addition to the acrylamide, which renders them irreversibly capped).

Critical Parameters

For most applications, the separation of oligonucleotides from mononucleotides and protecting groups provides adequate purification. In those cases where separation of oligonucleotides from nearby failure sequences is essential, however, the most critical parameters to be considered are the percentage of acrylamide and the amount of sample loaded. If maximum resolution is desired, then only 50 to 100 µg of material should be loaded per 2 cm × 2 cm × 1.6 mm well. The percentages of acrylamide that will give optimal resolution for different-sized oligonucleotides are given in Table 2.12.1, and can also be determined empirically by running a small portion of the starting material on trial gels and staining with ethidium bromide. By running long (20- to 30-cm) gels, oligonucleotides of lengths (n) up to 100 bases can be cleanly separated from the $n - 1$ and $n + 1$ products. If an oligonucleotide contains extensive self-complementary sequences or polyguanosine tracts, it may not be completely denatured in 7 M urea, and thus it may not be possible to separate it cleanly from failed synthesis products. To overcome this difficulty, samples can be electrophoresed on gels containing 20 M formamide instead of urea (Frank et al., 1981).

Troubleshooting

All of the problems that apply to nondenaturing PAGE are relevant here (see *UNIT 2.7*, Commentary). However, most failures in purification will occur because the initial synthesis reaction has been inefficient. In almost all cases, it is better to resynthesize a poor-yielding oligonucleotide than to attempt to isolate a small amount of full-length product from a starting material seriously contaminated with failure sequences. If the oligonucleotide cannot be resynthesized, relatively small amounts of product can be visualized by autoradiography providing the starting material is end-labeled with polynucleotide kinase and [γ-^{32}P]ATP (the starting material should not contain residual ammonium, which inhibits the enzyme; see *UNIT 3.10*). It is convenient to end label a small amount of starting material with radioactive ATP; the remainder should be phosphorylated using nonradioactive ATP so that it will not migrate differently from the labeled tracer. Smaller amounts of starting material should be loaded on thinner (~0.75- to 1.0-mm) gels in narrower lanes (~1.0 cm).

Anticipated Results

In general, the yield of purified oligonucleotides from denaturing PAGE decreases as the percentage of acrylamide increases. With crushed gel slices, an average yield of 50% may be expected.

Greater recoveries can be obtained by increasing the volume of elution solution added to the gel slice or by performing serial elutions from the same gel slice. Methods employing more active transfers (e.g., electroelution; Smith, 1980; Vorndam and Kerschner, 1986) may give more efficient recoveries. Also, samples (especially large synthetic RNAs) that prove particularly refractory to elution with aqueous buffers may be eluted easily with 6 vol formamide (for >5 hr at room temperature) followed by a brief (~1 hr) elution with an aqueous buffer. Isoamyl alcohol may be used to concentrate the formamide/aqueous buffer extracts to a convenient precipitation volume (J. Urbach, pers. comm.).

Time Considerations

It is usually most convenient to set up and run the gel on one day, elute the oligonucleotide overnight, then phenol extract and ethanol precipitate the sample the following day. However, a deprotected oligonucleotide can be ready for molecular biology applications in as little as 6 hr: setup and polymerization of gel, 1 hr; running of gel, 2 hr; fragment elution, 2 hr; product recovery, 1 hr.

Literature Cited

Applied Biosystems. 1984. Evaluation and Purification of Synthetic Oligonucleotides. User Bulletin no. 13. Applied Biosystems, Foster City, Calif.

Chen, Z. and Ruffner, D.E. 1996. Modified crush-and-soak method for recovering oligodeoxynucleotides from polyacrylamide gel. *Biotechniques* 21:820-822.

Frank, R., Muller, D., and Wolff, C. 1981. Identification and suppression of secondary structures formed from deoxyoligonucleotides during electrophoresis in denaturing polyacrylamide gels. *Nucl. Acids Res.* 9:4967-4979.

Maniatis, T., Jeffrey, A., and deSande, H.V. 1975. Chain length determination of small double- and single-stranded DNA molecules by polyacrylamide gel electrophoresis. *Biochemistry* 14:3787-3794.

Smith, H.O. 1980. Recovery of DNA from gels. *Methods Enzymol.* 65:371-379.

Vorndam, A.V. and Kerschner, J. 1986. Purification of small oligonucleotides by polyacrylamide gel electrophoresis and transfer to diethylaminoethyl paper. *Anal. Biochem.* 152:221-225.

Contributed by Andrew Ellington
University of Texas
Austin, Texas

Jack D. Pollard, Jr.
Harvard Medical School and
Massachusetts General Hospital
Boston, Massachusetts