Analysis and Purification of Oligonucleotides by Polyacrylamide Gel Electrophoresis (PAGE)

Introduction

Polyacrylamide gel electrophoresis (PAGE) is widely used for oligonucleotide analysis and purification. The first section of this chapter discusses the basic methods (apparatus, solutions and electrophoresis conditions) required for the successful analysis and purification of oligonucleotides. Because the protocols for autoradiography, analytical ultraviolet (UV) shadowing and gel purification vary, these topics are discussed subsequently. A polymerized, cross-linked acrylamide gel matrix between two glass plates is common to all three methods.

The ends of the gel matrix are immersed in buffer chambers containing an electrolytic salt solution through which an electric field of controlled voltage or current is applied. Charged molecules migrate through the gel matrix, separating on the basis of charge and mass. The equation for electrophoretic velocity is complex, but the predominant factors effecting separation are charge and mass. Molecular shape, net hydrophobicity, size, interactions with the gel matrix and other parameters also influence the rate at which a molecule travels toward the opposite electrode.

Basic Methods

Apparatus

The most convenient and familiar format for PAGE is the slab gel apparatus. Many dimensions, shapes and configurations are available. The lengths and widths of the gel are not critical as long as the minimum length is 15–20 cm. Spacers between 0.4 and 1.6 mm are typically used. The thinnest are used for analytical gels of ^{32}P-labeled oligonucleotides, while the thickest are used for preparative gels. The most critical choice to be made is the width of the sample wells. Maximum resolution of preparative samples is obtained by having the sample band as narrow as possible. Combs that form wells approximately 1 cm wide are useful for sample loads of about 1 optical density unit (A_{260nm}). Power supplies capable of providing 3000 V and 300 mA with a constant power option are best.

WARNING ***Before conducting experiments with radioisotopes such as ^{32}P, consult your institution's Radiation Safety Officer for proper procedures in ordering, handling and disposing of radioactive materials.***

Solutions

Acrylamide Stock Solution: Prepare a stock solution of 38% acrylamide and 2% bisacrylamide. High-quality acrylamide and bisacrylamide are available from several commercial sources and can be used without further purification. To prepare this solution, weigh 380 g of acrylamide and 20 g of bisacrylamide. Dissolve with deionized water to a final volume of 1 L. Filter the solution and store it at 4 ^{o}C.

WARNING ***Acrylamide in monomeric form is a neurotoxin and a suspected carcinogen. Wear gloves and a dust mask when weighing.***

10X TBE: This solution is used in the gel itself and with dilution (1X TBE) as the electrophoresis buffer. 10X TBE is 0.89 M Tris base, 0.89 M borate and 25 mM EDTA. To prepare this buffer, weigh 108 g of tris(hydroxymethyl)aminomethane, 55 g of boric acid and 9.3 g of disodium EDTA dihydrate**.** Dissolve with deionized water to a final volume of 1 L. Store the solution at 4 °C. Filtration will prevent precipitation of TBE, adding to its shelf life. The stock is stable for many months but is susceptible to bacterial growth and should be inspected before use.

1.6% Ammonium Persulfate (APS): This is prepared as a 1.6% (w/v) solution in distilled water. Because it is the initiator of polymerization, a fresh stock should be made every week. Store it at 4 °C. Alternatively, solid ammonium persulfate may be used.

Table 3-1. Recipes for Polyacryamide Gels

Gel thickness (mm)	Total vol. (mL)	% gel (%)	40% acryl. (mL)	Urea (g)	10X TBE (mL)	APS (mL)	TEMED (µL)
0.4	100	8	20	42	10	4.4	60
				42	10	4.4	60
				42	10	4.4	60
				42	10	4.4	60
0.8	150	8	30	63	15	6.6	100
		12	45	63	15	6.6	100
		20	75	63	15	6.6	100
1.6	250	8	50	105	25	11	150
				105	25	11	150
				105	25	11	150

Recipes are for 30 x 40-cm gels.

Pouring the Gel

The glass plates should be thoroughly washed, rinsed with acetone or ethanol, dried and assembled prior to preparing the polyacrylamide solution.

1. Measure out the 40% acrylamide and 10X TBE. Bring to final volume with deionized water.

2. Add this solution and the appropriate amount of urea to a flask (with a side arm) and mix until the urea has completely dissolved. If the gel is to be used for autoradiography, filtering the solution may help prevent extraneous exposure spots on the film. For UV shadow gels, filtering is not necessary.
3. Add the APS, then mix and degas the solution under vacuum for several minutes.
4. Add the TEMED (tetramethylethylene diamine (Aldrich T2, 250-0)), mix for approximately 30 s, pour the gel (use a pipet to minimize spillage) and insert the comb. Be sure to dislodge any trapped air bubbles, especially in the wells. Wait 1-2 h before using the gel.

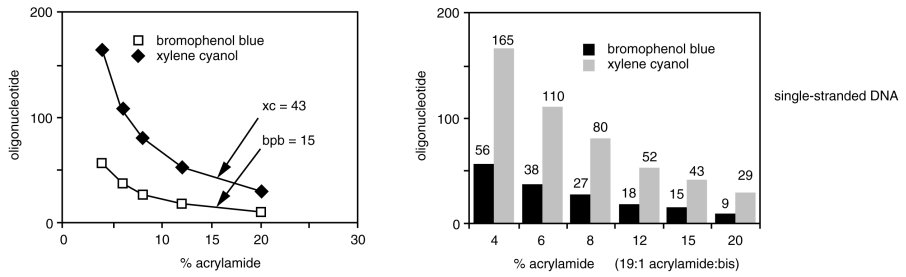
Electrophoresis Conditions

Denaturing and Tracking: Denaturing conditions should be maintained with single-stranded DNA oligonucleotides to minimize the formation of secondary structures caused by hydrogen bonding. Both the high concentration of urea (7 M) in the gel and sample loading in formamide serve as denaturants. However, secondary structure effects can be present in the gel, even in 7 M urea. Colored tracking dyes, such as bromophenol blue and xylene cyanol, can be loaded on the gel in adjoining lanes. This helps to determine the migration points of the oligonucleotides not visible during electrophoresis.

Gel Percentage and Migration Rates: PAGE is useful for the analysis and purification of oligonucleotides of any length. The gel (polyacrylamide) concentration should be varied, depending on the oligonucleotide length. Choice of gel percentage is a compromise between speed and resolution. Higher concentrations of polyacrylamide decrease the average pore size of the gel, slowing migration and increasing separation of sample molecules. According to oligonucleotide size, the recommended polyacryl-amide concentrations are:

Table 3-2. Migration Rate Table

Oligonucleotide size	Polyacrylamide concentrations (%)
<25	20
25-50	15
50-90	12
>90	8



Both the length and, to a lesser extent, the sequence of an oligonucleotide affect its mobility through the gel. The order of nucleotide migration is C>A>T>G, with C the most rapid. Oligonucleotides of the same length but with different sequences will migrate at slightly different rates. Therefore, comparison of an **oligonucleotide size marker** with the sample oligonucleotide to verify the correct length is only marginally accurate. Figure 3-1 shows the migration of different sequences of the same length in a single gel. Figure 3-2 shows a UV shadow gel of 32-mers that differ only in four bases at their 3' ends. The results clearly show the effect of base composition.

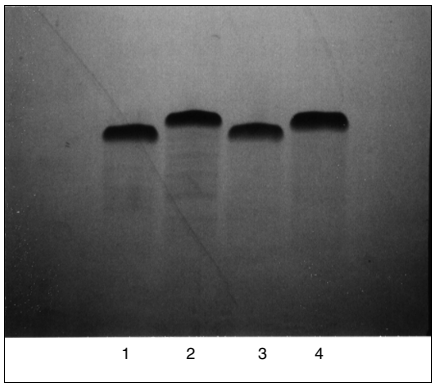


Figure 3-1. *UV shadow gels showing the migration of 18-mers of different sequence. Lane 1: $(AT)_{9^\circ}$ Lane 2: $(GT)_{9^\circ}$ Lane 3: $(CT)_{9^\circ}$ Lane 4: $(T)_{18^\circ}$*

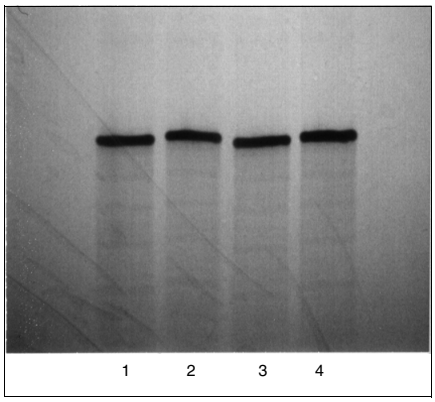


Figure 3-2. *32-mers that differ only in their 3' ends. The 3' ends are: Lane 1: AAAA. Lane 2: GGGG. Lane 3: CCCC. Lane 4: TTTT.*

Loading and Running: After the gel has been allowed to polymerize, usually for 1–2 h or longer, it is placed on the electrophoresis apparatus, and the upper and lower buffers (lX TBE) are added. Before the sample is loaded, the wells must be flushed with approximately 1 mL of 1X buffer in a syringe to remove debris and urea that can affect resolution. The gel is then prerun with a lane of bromophenol blue and xylene cyanol in formamide. Prerunning elevates the temperature of the gel, which helps to melt secondary structures and serves as visual confirmation that the gel is functioning properly.

If an oligonucleotide is suspected of forming secondary structures, heating it to 90 °C for several minutes in loading buffer and immediately placing it on ice just prior to loading

may help. Slow cooling stabilizes secondary structures. The warming of the gel and the sample serves to aid in the denaturation process. The running wattage of the gel should generate sufficient heat to warm the gel plates but allow handling for 1 min without burning the skin. Attaching a metal plate to the gel apparatus results in even distribution of the heat. This ensures against cracking the gel plates and melting the polyacrylamide. It also minimizes "smiling" or "frowning" of the gel samples.

After prerunning and just prior to loading, the sample wells are flushed a second time to remove urea. The samples are carefully loaded onto the lower surface of the sample well. The sample is dissolved in a 9:1 mixture (v/v) of formamide to 1X TBE. To avoid shearing or uneven loading, sample volume should not exceed 10 µL per 1.5-cm well for analytical size (0.4 mm) gels. A Hamilton-type syringe or a flat-tipped pipetter should be used to load the sample. Once the samples are loaded, electrophoresis should be initiated immediately to avoid diffusion.

UV Shadow Analysis

UV shadowing, the visualization technique most commonly used for purification, can also be used for analysis of crude mixtures. UV shadowing is less labor intensive than radiolabeling, and it often provides sufficient resolution for routine analysis. After electrophoresis, the gel is transferred to a fluorescent media, commonly a 20 x 20-cm plastic-wrapped preparative TLC plate and visualized under short-wavelength UV light. This method is direct, not dependent on further treatment or reactions, such as staining or labeling.

Sample Preparation

UV shadow analysis requires a higher amount of oligonucleotide than autoradiography; however, the amounts required (0.5–2 optical density units (ODU) are still only a small fraction of a typical synthesis. Desalting is not usually necessary for UV shadow analysis of oligonucleotides shorter than 50 bases. The sample may be dried down and loaded directly from the ammonia deprotect solution. Desalting sometimes reduces smearing on gels and gives a better picture. After quantitating the oligonucleotide, the following amounts should be dried down for analysis on an 0.8-mm-thick gel:

Number of Bases	ODU to be Loaded
< 25	0.5–1.7
25–50	0.8–1.4
> 50	1.5–2.0

A larger quantity of oligonucleotide may be necessary to analyze syntheses with low coupling yields.

Electrophoresis Conditions

Using the highest gel concentration for a given oligonucleotide length will maximize resolution. The width of the comb teeth should be about 1 cm. A gel length of 15–20 cm with

0.8-mm spacers is useful for the analysis of oligonucleotides up to approximately 70 bases. For oligonucleotides longer than approximately 70 bases, 5' ^{32}P end labeling and autoradiography gives better resolution.

1. When polymerization is complete (~ 1–2 h), fill the upper and lower buffer chambers with 1X TBE.
2. Prerun the gel for 30–60 min at a constant power of 15–25 W. The temperature of the gel plates should be less than 55 ×C.
3. Turn off the power.
4. Use a syringe to flush each well with approximately 1 mL of 1X TBE. Be careful to flush out all urea and gel debris.
5. Load each sample in 5–10 mL of 9:1 (v/v) of formamide to 1X TBE.
6. Load 5–10 mL of dye (bromophenol blue and xylene cyanol) in formamide into an empty outer well to aid in tracking the migration of the oligonucleotide.
7. Electrophorese at 15–25 W constant power until the desired species has migrated one-half to two-thirds the length of the gel.

The above conditions were optimized on Hoefer gel electrophoresis systems (models SE 400 and SE 600).

Visualization and Interpretation

Gently pry apart the glass plates with a nonmetallic wedge and place the gel on a fluorescent, preparative TLC plate (20 x 20 cm) that has been covered with plastic wrap. The oligonucleotides may be visualized under a short-wave UV lamp (240–300 nm). The UV-absorbing oligonucleotide appears as a shadow; it absorbs UV light and masks the emission of fluorescence in the plate. The gel may be photographed using an instant camera, black and white film, and a green filter.

Interpretation of analytical UV shadowing is subjective. Comparison with oligonucleotides in adjacent lanes of known quality and length may prove helpful.

Autoradiography

As oligonucleotide length increases, the preferred method of analysis and purification by PAGE changes. For short oligonucleotides, UV shadowing offers quick and easy high-resolution analysis. For oligonucleotides longer than approximately 70 bases, 5' ^{32}P labeling and autoradiography give better resolution of the crude mixtures.

Sample Preparation

Before the oligonucleotide undergoes electrophoresis, it must be:

1. quantitated by absorbance;
2. desalted (usually necessary only for autoradiographic analysis; see Chapter 5 and Appendix C for desalting methods);
3. evaporated to dryness.

The surface area of the sample well on a thin gel is smaller than that on a thicker gel. Consequently, normal loading of an oligonucleotide sample on a thin gel may clog it, causing much of the sample to be retained in the well. By decreasing the amount of sample loaded, the oligonucleotide mixture will enter the gel easily and give excellent resolution. Typically, 1–20 pmol of crude oligonucleotide are radiolabeled for autoradiography and loaded onto a 0.4-mm x 40-cm gel for proper resolution.

The most common method of radiolabeling is 5' end labeling. T4 polynucleotide kinase is used to catalyze the phosphorylation of the 5' hydroxyl. As shown in Figure 3-3, [γ ^{32}P] ATP serves as the phosphate donor. This method can be used at different scales, depending on the quantity and length of the oligonucleotide to be labeled. Variations are also used when phosphorylating oligonucleotides for ligations as well as for radiolabeled probes.

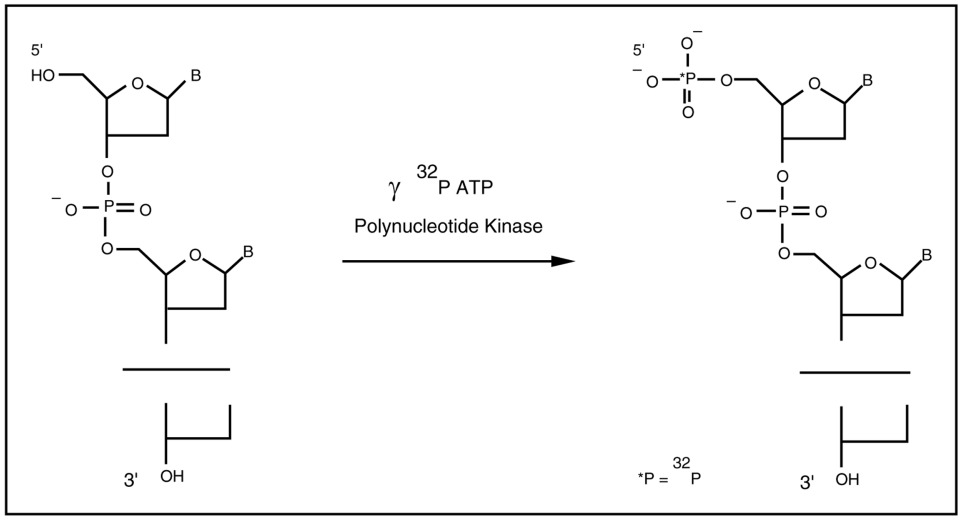


Figure 3-3. *5' ^{32}P labeling reaction*

When analyzing crude compounds, an excess of both hot and cold ATP must be present in the reaction for competitive labeling of each oligonucleotide species in a sample. At limiting ATP concentrations (an excess of oligonucleotide relative to ATP), the kinase enzyme will phosphorylate oligonucleotides of approximately 10–20 bases before phosphorylating longer sequences. The small quantity of radiolabeled ATP will not produce the excess needed for efficient phosphorylation. A proper molar excess is achieved with the addition of unlabeled ATP.

5' Radiolabeling Protocol

The oligonucleotide should be dried in an Eppendorf tube. It is usually not necessary to purify or desalt sample oligonucleotides for kinase analysis. They may be used after loading directly from the ammonia deprotection solution. Extremely long or impure syntheses may benefit from a simple ethanol precipitation or desalting operation. The amount of each sample to be labeled can vary, depending upon the activity of the ^{32}P ATP and the desired autoradiography film exposure time. The following protocol is proven and provides for analyzing a sample twice with an approximate film exposure time of 1–2 h, assuming that

the ^{32}P ATP is fresh. If the radiolabeled ATP is older than 2 weeks, twice the amount may need to be added to achieve a exposure time of 1–2 h. The half-life of ^{32}P ATP is 14.3 days.

To measure the samples, convert absorbance (ODU) to picomole amounts, using the following approximation: 1 mmol of oligonucleotide = 10 ODU/base (e.g., 10 pmol of a 50-mer = 0.005 ODU).

Number of Bases	pmol to be Labeled
< 40	5
40–70	10
> 70	20–50

It is necessary to make a serial dilution of the sample in water to avoid aliquoting sub-microliter volumes. This molar conversion from absorbance is a useful guide, although it assumes that the entire sample is full-length product oligonucleotide, which is usually not the case.

For each sample, prepare a master mix of the following:

- 4 µL of 100 µM **cold** ATP in water (400 pmol)
- 1 µL of kinase buffer (0.25 M Tris-HCl, pH-7.6; 0.1 M $MgCl_2$; 0.1 M DTT (dithiothreitol)). Kinase buffer should be stored frozen and discarded after 6 months.
- 0.2 µL of 10 mM spermidine
- 1 µL (10 units) of T4 polynucleotide kinase (New England Biolabs, 201S)
- 1.5 µL of γ ^{32}P ATP (3000 Ci (111TBq)/mmol) (NEN DuPont, NEG-002H; or Amersham, PB-168)

1. Vortex the master mix and spin it for a few minutes in a microcentrifuge, observing the appropriate radiation safety procedures.
2. Dispense 6 µL of the master mix into each sample tube.
3. Vortex, spin and heat the sample tubes at 37 °C for 45–60 min.
4. Cool, spin and add 14 µL of formamide/dye mix (0.1% bromphenol blue, 0.1% xylene cyanol) to each sample tube.
5. Vortex and spin the sample tubes. The dye-containing samples are stable. They may be stored in a refrigerator or freezer and are ready for loading on the gel.

Recommended Gel Configurations		
Plates	20–40 cm	(length)
Spacers	0.4 mm	(depth)
Wells	8–16 mm	(width)

Electrophoresis Conditions

1. Attach the gel plate assembly to the gel apparatus and clamp an aluminum plate to the surface.
2. Fill the upper and lower buffer chambers with 1X TBE.
3. Apply a constant power of ~60 W and prerun the gel for approximately 1 h. The aluminum plate should be warm but not hot.
4. Turn off the power.
5. Rinse out any urea that has diffused into the wells, using a syringe filled with 1X TBE.
6. Carefully load 10 µL of each sample and electrophorese at about 60 W constant power. When the aluminum plate reaches a stable temperature, it should be warm but not too hot to touch (approximately 55 °C). Higher temperatures, caused by too much power, may crack the glass plates, resulting in leaking and shorting. The exact run time depends on the size of the oligonucleotide, but it should be sufficient to allow the desired species to migrate approximately two-thirds of the gel length.

The above conditions were optimized on a Model SG-600-33 Adjustable Electrophoresis System (C.B.S. Scientific Co.).

Visualization and Interpretation

1. When the electrophoresis is complete, turn off the power and remove the gel plate assembly. Be careful to properly dispose of the radioactive lower buffer solution, according to Federal and local regulations.
2. Separate the glass plates by gentle prying with a nonmetallic wedge. The gel should remain attached to one of the plates, preferably the bottom one.
3. Wrap the gel with plastic wrap.

   Alternatively, lay a discarded, clean X-ray film over the gel and turn the gel over so that the film is on the bottom. Carefully remove the plate (try to prevent tears and air bubbles in the gel), leaving the gel adhering to the film. Then wrap the film in plastic wrap and smooth out most of the wrinkles.
4. Place the gel inside a light-tight film cassette containing an intensifying screen.
5. In a darkroom, lay a sheet of unexposed X-ray film on the gel.
6. Close the cassette and store it at -70 °C during exposure.

The radioactively labeled oligonucleotide molecules emit beta particles that expose the X-ray film. When the film is placed between the gel and an intensifying screen, exposure will be accelerated. The quality of results obtained from analyzing crude products by autoradiography is depicted in Figure 3-4.

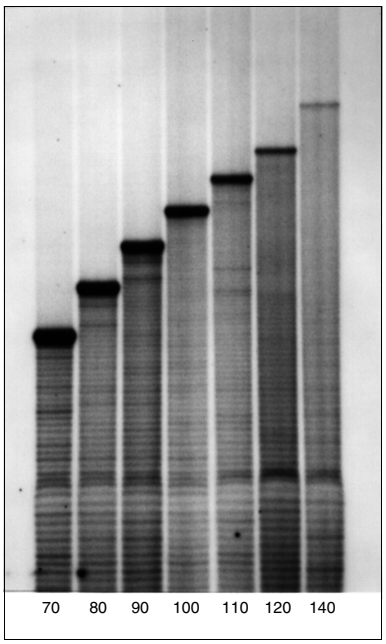


Figure 3-4. *Autoradiograph of crude oligonucleotides.*

Exposure time is critical. Remember that the total incorporated radioactivity is distributed among several (sometimes many) species, depending on the length of the oligonucleotide. An exposure time that is too long will overrepresent failure sequences because total exposure of silver grains at the product band will no longer respond to beta emissions. In other words, when a band achieves a certain blackness, it will not blacken further. The lighter bands, however, will continue to darken. This effect may mislead the viewer as to the relative amounts of product and failure sequences. An exposure that is too short will not sufficiently reveal failure sequences. Figure 3-5 shows the effect of exposure on the interpretation of results.

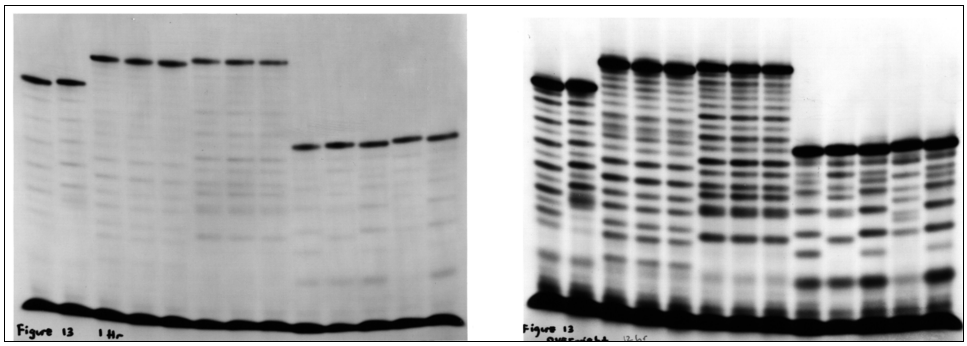


Figure 3-5. *Light (1 h) and dark (12 h) exposure of oligonucleotides of varying length and sequence.*

Interpretation of an autoradiogram is usually subjective. Visual inspection of the intensity of the major product band and comparison with the failure sequences is often sufficient. Comparison with oligonucleotides of known size and quality may also be useful.

Densitometry

Densitometry can be used to quantify bands and spots on autoradiograms and stained gels. Densitometers measure the portion of incident light that is either reflected or transmitted by a sample. Quantitation by measuring reflection is affected by small changes in surface texture, and it measures only the portion of the sample that is on the surface. For this reason, densitometers that measure reflection are usually reserved for opaque samples, such as TLC (thin-layer chromatography) plates. Other densitometers quantify light absorbance by measuring transmittance and optical density.

The densitometry signal is converted to peak format when a lane from a gel is scanned. Lane 1 of the autoradiogram in Figure 3-6 gives little information until scanned by densitometry.

The most accurate systems scan the entire gel, allowing a 3-D integration of each band. Other systems scan individual or multiple tracks on each band or lane. Because gels rarely have band widths less than 1–2 mm, a resolution of 100 µm (10 points/ mm) is usually sufficient. The system should be capable of measuring both incident and transmitted light. This prevents changes in the intensity of the incident light from distorting the data.

Problems often arise when very dark bands are seen on the film. Dark spots absorb most of the light incident upon them, which reduces the number of photons that reach the detector. This results in a low signal-to-noise ratio. A higher signal-to-noise ratio may be maintained by using a strong source of incident light, such as a laser. Other factors to consider are the optical resolution of the system and the method of analog-to-digital signal conversion. The percentage of error is lower if the transmittance is converted to optical density units before digitization.

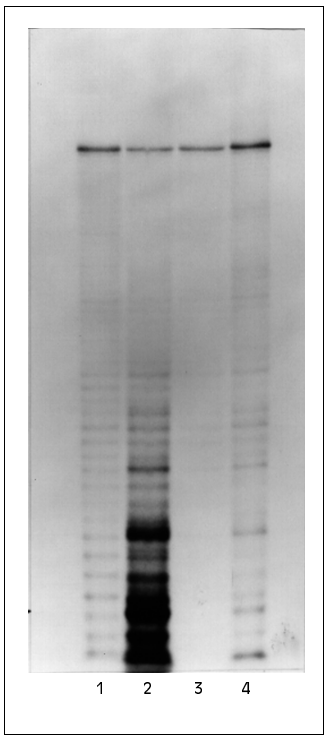


Figure 3-6. *Densitometry trace showing the quantitation of the main product and failure sequences of the 60-mer in Lane 1 of the Autoradiograph.*

Staining

Gel staining with a DNA-specific stain, such as methylene blue, may be used to visualize oligonucleotides. Staining can be particularly useful if the gel is to be preserved, but it is not recommended if the analyzed sample is to be used in subsequent applications. As an analytical tool, staining has limited sensitivity; sequences present in low concentrations may not be visible. Nevertheless, staining is more sensitive than UV shadowing and is considered easier than radiolabeling (see the 5' Radiolabeling Protocol, page 3-7).

Methylene blue and Stains-All are useful for staining oligonucleotides. For example, the gel, after electrophoresis and removal from the plates, may be soaked in a shallow pan with 200 mg of methylene blue per liter of water for approximately 15–30 min. Drain the pan and rinse the gel of all excess, unbound dye for several minutes. Stained oligonucleotides are visualized as blue bands and may be photographed under ambient light.

Ethidium bromide staining is a well-established technique for visualizing double-stranded DNA fragments. Unfortunately, it is not efficacious for visualizing short, single-stranded DNA fragments such as synthetic oligonucleotides. The UV irradiation required to visualize ethidium bromide damages DNA and compromises its usefulness. Also, the intercalation of ethidium bromide into short, single-stranded DNA is inefficient, highly sequence-dependent and sometimes undetectable in sequences shorter than 25 bases. We do not recommend ethidium bromide staining for analysis of oligonucleotides.

Purification

The goal of electrophoretic gel purification is separation of the desired product from other, contaminating oligonucleotides in the crude mixture. The key steps in this process are gel separation, UV-shadowing, product excision from the gel and recovery of the product from the gel matrix. PAGE purification is excellent for separation by length. Figure 3-7 depicts a gel of crude mixtures and their subsequently purified products.

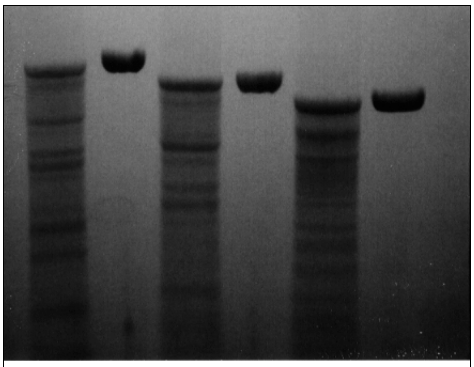


Figure 3-7. *Comparison of crude and gel-purified 85-, 75- and 65-mers. 15% polyacrylamide. Loading: 1.5 ODU of crude or 0.45 ODU of gel-purified oligonucleotide.*

The methods used in gel purification are like those used in analysis, with two major differences:

1. Preparative gels are usually thicker gels, used to accommodate the loading of a larger quantity of oligonucleotide.
2. The oligonucleotide also has to be loaded in a sufficient concentration to allow recovery of ample amounts of the desired species from the gel matrix.

Sample Preparation

DNA samples for purification are quantitated and prepared in much the same way as for analysis. In some cases, especially for long oligonucleotides, desalting is recommended before electrophoresis. Dissolve the dried oligonucleotide in loading media (9:1 (v/v) formamide/1X TBE) to a concentration of 1–2 ODU per µL. Samples that do not readily dissolve should be heated to approximately 60 °C and vortexed. To avoid possible contamination and masking of the product, do not load marker dyes with the sample. Marker dyes such as bromophenol blue and xylene cyanol should be loaded in the outer wells to aid in tracking the migration of the oligonucleotide (refer to Table 3-2).

Electrophoresis Conditions

Electrophoresis conditions for purification are similar to those for analysis. The primary differences are the gel thickness and the sample concentration. The height of the gel will depend on the length of the oligonucleotide; longer oligonucleotides require longer gels to

obtain sufficient resolution. A gel length of 15–20 cm will adequately resolve oligonucleotides up to 50 bases; for longer oligonucleotides, a 40-cm gel is recommended. The thickness of the gel and the width of the comb teeth will determine the well surface area and ultimately the amount of crude mixture that can be loaded. For example, a gel 1.5 mm thick and comb teeth 2–3 cm wide allow for the loading of 10–12 ODU (A_{260nm}) of oligonucleotide.

The increased thickness of preparative gels requires higher power settings (30–50 W constant power) and/or longer electrophoresis times to obtain maximum resolution. To achieve optimal product separation, let the sample migrate as far down the gel as possible. Because the goal is separation of the oligonucleotide from the crude mixture, it is acceptable to run the truncated sequences off the bottom of the gel. Ideally, the product band should be allowed to migrate at least two-thirds the length of the gel.

Visualization and Product Excision

Gently pry apart the glass plates with a nonmetallic wedge and place the gel on a fluorescent TLC plate covered with plastic wrap. The oligonucleotides are visualized using a short-wavelength UV lamp (approximately 240 nm). It is important that a short-wave UV lamp be used. DNA does not absorb much above 280–290 nm. Take care to minimize the exposure of the oligonucleotide to UV light, which can cause thymidine dimerization.

Figure 3-8 and Figure 3-9 are photographs of a preparative gel (before and after product band excision) illuminated by a hand-held UV light source. When excising the oligonucleotide, remember that the UV lamp must be held **directly** overhead to avoid errors. This prevents movement of the shadow from its correct position on the product band to another position that could include contaminants.

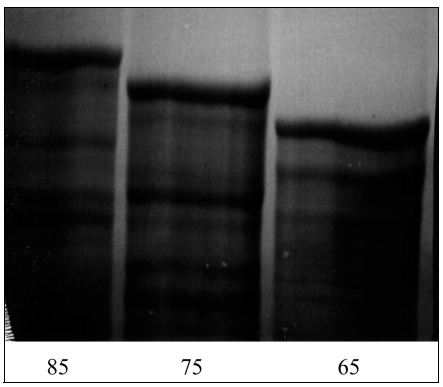


Figure 3-8. *Preparative gel. 15% polyacrylamide, 1.5-mm thick, loading 10 ODU of each crude oligonucleotide mixture.*

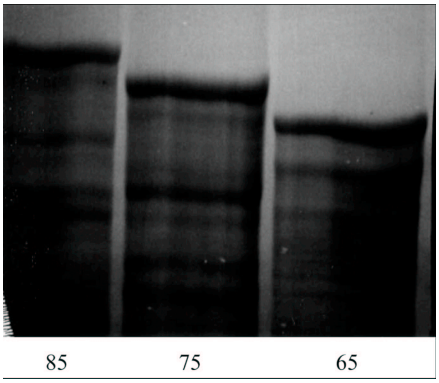


Figure 3-9. *Excision of 85-, 75- and 65-mer product bands from a preparative gel.*

The product is excised with a clean razor blade. If the oligonucleotide is not degenerate, the cuts should be slightly to the interior of the product band to eliminate contamination from failure sequences lacking only a single nucleotide. Conversely, to avoid missing some of the possible sequences in a degenerate product mixture, the cuts should be at or slightly outsidethe diffuse product band. The excised band is then placed in a tube or a fritted column.If more than one sequence is run on the same gel, care should be taken in handling to prevent cross-contamination.
Once the product band has been excised from the gel, it is necessary to recover the oligonucleotide from the acrylamide gel material. The two most common ways are either **soaking** or **electroelution.** Both methods are effective, but soaking is often the method of choice because it is inexpensive, easy and can be accomplished without monitoring. Product recovery yields are 10–80% of the initial oligonucleotide loading. The quantity of product recovered depends on the concentration of failure sequences in the sample. The gel slice should be soaked in at least 1 mL of any of the following extraction solutions:
• 0.5 M NaCl, 2 M triethylammonium acetate
• 50 mM triethylammonium acetate
• 0.5 M NaCl, 0.1 M Tris-HCl (pH 7.0) containing 1 mM EDTA
• Deionized water

1. Incubate the gel slice at room temperature for at least 12 h.
2. Decant and save the solution.
3. Remove dissolved urea, salts and gel debris with the Oligonucleotide Purification Cartridge (OPC) desalting protocol

Note that the OPC desalting procedure, unlike the purification procedure, is for trityl-off oligonucleotides. As with any purification method, oligonucleotide recovery should be verified by UV absorbance. Unlike the OPC purification protocol, the desalting protocol can yield up to 50 ODU of desalted oligonucleotide because no ammonia is used.

Triethylammonium is the counter ion, bound to DNA that has been desalted or purified by the OPC. If it is necessary to remove the triethylammonium counter ion, ethanol precipitation with 1.0 M sodium chloride is advised.

OPC Desalting Protocol

1. Pass 5 mL of acetonitrile through the OPC (ABI Part # 400771) to waste followed by 5 mL of 2 M TEAA (ABI part# 400613).
2. Dissolve the oligonucleotide in 1–3 mL of aqueous solution (e.g., 0.1 M TEAA). The loading solution should not contain organic solvents or ammonium hydroxide.
3. Pass the diluted solution through the OPC at a rate of about 1 drop/s.
4. Collect the eluate and pass it through a second time.
5. Pass 15 mL of 0.1 M TEAA through the OPC to waste.
6. Elute dropwise with 1 mL of 50% acetonitrile in water (v/v) and collect the desalted oligonucleotide in a suitable (e.g., a 1.5-mL Eppendorf) vial.

Differences in the Purification of Long and Short Oligonucleotides

Crude samples of long oligonucleotides contain substantially more truncated sequences than do crude samples of shorter oligonucleotides because of the higher number of coupling cycles. Hence, the product is in a lower concentration relative to the crude sample of a

shorter oligonucleotide. For this reason, it is necessary to load more long oligonucleotide sample on a preparative gel to visualize the product. Because the amount of oligonucleotide that can be loaded on a gel is limited, prepurification techniques such as OPC are recommended.

Diagnosing Synthesis Problems

Oligonucleotide analysis by autoradiography or UV shadowing may be used to diagnose synthesis problems. Figure 3-10 and Figure 3-11 depict examples of "problem" syntheses. Coupling failures, such as those seen in Figure 3-10, may be caused by old tetrazole, old or wet phosphoramidites or improper flow rates.

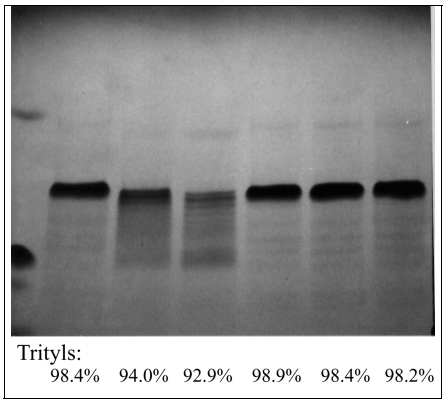


Figure 3-10. *A comparison of successful and failed 18-mer syntheses, showing the correlation between trityl and gel analysis.*

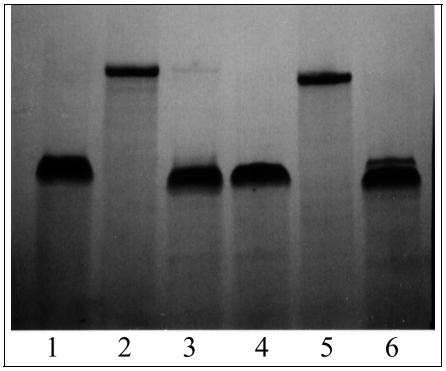


Figure 3-11. *Gel analysis of oligonucleotides from "problem" syntheses. Lane 1: 18-mer. Lane 2: 36-mer. Lane 3: 36-mer with no iodine delivery (no oxidation) after base 18 (note faint 36-mer band). Lane 4: 36-mer with a coupling failure at base 18 (tetrazole depletion) and efficient capping. Lane 5 36-mer with a coupling failure at base 19 (phosphoramidite depletion) and failure to cap. Lane 6: 18-mer with incomplete detritylation after base 6.*

Problem Areas

Sequence and base composition can have a marked effect on the electrophoretic behavior of an oligonucleotide. The most common problem associated with the sequence is secondary structure formation caused by hydrogen bonding. Secondary structures are most often, but not exclusively, associated with palindromic sequences and sequences rich in guanosine. Because secondary structures are in equilibrium with denatured molecules or other secondary structures, banding on the gel can be complex, making interpretation of the gel difficult. Figure 3-12 shows a UV shadow gel of a 20-mer that has secondary structure problems. In this figure, the 20-mer has a major band migrating at approximately the rate of a 10-mer.

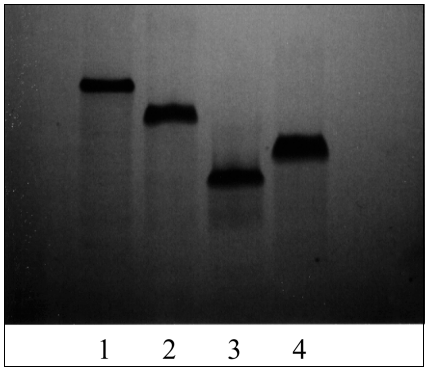


Figure 3-12. *An example of the effects of secondary structure on oligonucleotide migration. Lane 1: 25-mer. Lane 2: 20-mer 5' $(AGTC)_5$ 3'. Lane 3: 20-mer with secondary structure 5' GGT GGC CAC CAT GGT GGC CC 3'. Lane 4: 15-mer.*

The mixed base sites of oligonucleotides are often referred to as degenerate or redundant. In the synthesis of mixed probes of high degeneracy, the inherent complexity of the crude product will reduce resolution. Because of base composition differences between the oligonucleotides in the mixture, the product band will appear broader than normal. Figure 3-13 shows a highly degenerate 20-mer in the crude and purified state. Lanes 3 and 4 show the slight PAGE analytical difference between an incompletely deprotected (lane 3) and fully deprotected oligonucleotide (lane 4).

Sample preparation can also affect gel migration. Samples with the DMT group on the 5' end will not end label with T4 polynucleotide kinase. Hence, product quality will appear minimal at best by kinase analysis. DMT-bearing oligonucleotides analyzed on UV shadow gels will show a slightly retarded product band. Some detritylation may occur during sample preparation and electrophoresis. Compared to deprotected samples, samples with base-protecting groups remaining will appear as slightly higher molecular weight products because the base-protecting groups hinder migration. Additionally, the base-protecting groups hamper the ability of T4 polynucleotide kinase to end label the product molecules.

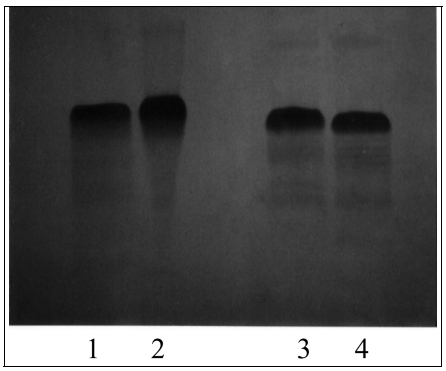


Figure 3-13. *Examples of the effects of degeneracies and of incomplete base deprotection on UV shadow gel analysis. Lane 1: crude 20-mer with 16 fourfold degenerate positions. Lane 2: Same 20-mer after OPC purification. Lane 3: 18-mer with incomplete deprotection (stored at -20 °C immediately after cleavage). Lane 4: Same 18-mer after 8 h of deprotections at 55 °C.*

To achieve complete quantitative base deprotection, samples should be incubated for a minimum of 8 h at 55 °C in concentrated, fresh ammonia. Oligonucleotides synthesized with Fast Oligonucleotide Deprotection (FOD) reagents require 1 h at 55 °C in concentrated ammonia for complete deprotection. Excessive salt in the oligonucleotide sample may affect migration and may interfere with enzymatic labeling reactions. Fresh kinase reaction buffer should be used because it contains DTT (see the 5' Radiolabeling Protocol, page 3-7). The loss of DTT through degradation over time can strongly affect 5'-labeling efficiency.

Other sample-related problems are caused by:

- inaccurate quantitation of the sample;
- inactive enzyme;
- inaccurate ATP concentrations.

There is a marked effect on the results obtained when enzymatically phosphorylating with kinase under limiting ATP concentrations. If radioactive ATP is the sole source of ATP, there will not be sufficient reagent to use as the phosphorylating agent. Under these conditions, the enzyme will preferentially label shorter oligonucleotides. By adding **cold** (unlabeled) ATP to the reaction mixture, efficient phosphorylation can be accomplished independent of the length of the molecules.

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