A Method for Assessing the Quality of Synthetic Oligonucleotides Using Pre-cast Polyacrylamide Minigels

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Introduction

Methods for assessing the quality of synthetic oligonucleotides produced in a core laboratory setting must be easy, rapid, and provide adequate identification of failed syntheses using a minimal amount of sample. While capillary gel electrophoresis (CGE) offers excellent resolution of synthesized products (1), this method can be costly (requiring expensive instrumentation and supplies), time-consuming (average run times of 30 minutes per oligonucleotide using a Beckman P/ACE system), space-consuming (most systems require significant laboratory space), and can be extremely sensitive to overloading (2) thus producing artifacts such as peak-splitting (unpublished observations). After CGE analysis of over 3000 oligonucleotides (synthesized on Perkin Elmer/Applied Biosystems [Foster City, CA] Model 394 DNA synthesizers), we sought a simpler, less expensive, and faster method for routine quality analysis.

Methods based on polyacrylamide gel electrophoresis (PAGE) are widely used for analyses of DNA including synthetic oligonucleotides. Since the mobility of oligonucleotides through the gel is influenced primarily by length and, to a lesser degree, by the sequence or base composition, sample oligonucleotides cannot always be directly compared to markers of the same length (3). PAGE can, however, detect the presence of truncated sequence products (failure sequences), and can thus provide a useful assessment of oligonucleotide quality. Most methods for analyzing oligonucleotides using PAGE are labor-intensive and require gels at least 15 to 20 cm in length. Denaturing minigels (8 cm in length) pre-cast in disposable cassettes are commercially available in appropriate acrylamide concentrations and thicknesses to allow for resolution of small amounts of most oligonucleotides synthesized in the average core facility. We examined the resolution capabilities of some of these commercially available minigels and established an appropriate standard operating procedure for routine screening of general quality of oligonucleotides of 15 or more bases in length.

Methods

Design of Test Oligonucleotides. To test gel resolution, a series of oligonucleotides was designed to represent a sampling that spans the median size range of oligonucleotides synthesized in our resource laboratory. These oligonucleotides begin with 16 bases of equal, random A, G, C, and T composition. The 3' base was omitted from the design of the 16mer to simulate an n-l synthesis failure. A 28mer was designed by adding 12 randomized, equal A, G, C, and T composition bases to the 3' end of the 16mer sequence. The 3' base of the 28mer was omitted to create the 27mer. This pattern was used to design oligonucleotides of 43, 44, 75, and 76 bases in length.

Gel Electrophoresis. Pre-cast minigels (8 cm x 8 cm gel size, 1 mm thick, Novex, San Diego, CA) containing Tris base, boric acid, EDTA, and 7 M urea (TBE-urea) in acrylamide concentrations of 15% with 15 wells (catalog number EC68855), 15% with 10 wells (catalog number EC6885), and 10% with 10 wells (catalog number EC6875) were tested. TBE-urea minigels containing 20% acrylamide from Oxford Glycosystems, (Rosedale, NY), hand-cast TBE-urea minigels containing 20% and 15% acrylamide, and TBE-urea gradient minigels containing 4 to 15% acrylamide (test gels supplied by Novex), were also tested, but did not give adequate resolution or were not reproducible in quality. Running buffer consisting of 89 mM Tris base, 89 mM boric acid, and 1.6 mM EDTA was obtained by diluting 5X Novex solution (catalog number LC6675) with distilled water. Samples were resuspended in sample buffer composed of 45 mM Tris base, 45 mM boric acid, 1 mM EDTA, 6% ficoll, 0.005% bromophenol blue, 0.025% xylene cyanol and 3.5 M urea obtained by diluting 2X Novex solution (catalog number LC6876) with distilled water. Electrophoresis was performed at constant voltage (180 volts) using a Schleicher & Schuell Profile system (equivalent to a Novex XCell II Mini-Cell) and a Model 250 power supply (Life Technologies, Gaithersburg, MD). To stain gels, ethidium bromide (0.0005%) in distilled water was tried, but some oligonucleotides did not stain or staining intensity varied. Methylene blue (0.02%) in distilled water was found to stain all the test oligonucleotides with equal intensity and was used to stain all gels shown below. Distilled water was used for destaining. Gel images were digitized and printed using an AlphaImager 2000 (Alpha Innotech Corp., San Leandro, CA) digital imaging system.

To determine the appropriate amount of oligonucleotide needed to produce a distinct, visible, sharp band and to allow for adequate visualization of an n-1 band, approxi-

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mately 100-200 ng (0.003-0.006 ODU at 260 nm) of selected oligonucleotides were electrophoresed on 15% gels. It was determined that loading approximately 100 ng (0.003 ODU) produced optimal results for visualization of bands (data not shown). To determine the appropriate electrophoresis conditions to allow detection of n-1 sequences and to provide visualization of possible shorter failure sequences, each test oligonucleotide alone and mixes of oligonucleotide pairs representing n and n-1 sequences were placed on different gel types and electrophoresed for various times as listed in Table 1. After electrophoresis, gels were stained, destained, photographed, and evaluated.

Mass Spectrometry. Because we have a MALDI-TOF mass spectrometer (Perseptive Biosystems Voyager-DE RP Biospectrometry Workstation) in our laboratory, we routinely analyze oligonucleotides that are not visible by PAGE (oligonucleotides less than 15 bases in length) and those suspected of failure as judged by PAGE. If such instrumentation is available, the following method may be used to analyze oligonucleotides. Dry 200 pmoles of the oligonucleotide in a 0.5-mL microcentrifuge tube using a vacuum centrifuge. Dissolve the oligonucleotide in 4 µL of distilled water. Add 1 µL of 0.1 M ammonium citrate and 3 µL of matrix solution (20 mg/mL 3-hydroxypicolinic acid in 50% acetonitrile, 0.1% trifluoroacetic acid) to the solution. Add a few cation-exchange beads, prepared as previously described (4), to the mixture and deionize the sample at 50°C for 30 minutes. Apply the sample to the sample plate, trying to avoid the ion-exchange bead, and allow to dry. Collect spectra as positive ions at 25000V in the linear mode.

Results

Conditions were determined that provided adequate resolution of full-length oligonucleotides from smaller sequences and that provided visualization of possible shorter failure sequences (see Table 2).

Table 2. Gel concentrations and electrophoresis times for PAGE assessment of synthetic oligonucleotides.

Oligo Length	*Gel %*	*Electrophoresis Time (min.)**
<15	submit for mass spectrometry analysis	
15-35	15	45-55
36-50	15	55-65
51-80	10	50-55
80-100	10	60
>100	10	70

*Gels are run at a constant voltage of 180 volts.

The best resolution for oligonucleotides 15 and 16 bases in length (Figure 1), 27 and 28 bases in length (Figure 2) and 43 and 44 bases in length (Figure 3) were obtained using minigels containing 15% acrylamide with 10 wells (note the differing electrophoresis time in each case). We also compared the 10-well gels to those containing 15 wells and found that gels containing 15 wells gave poorer resolution of n and n-1 bands for test oligonucleotides from 15 to 44 bases in length than gels containing 10 wells (Figure 4). Minigels containing an acrylamide concentration of 10% were also tested. These gels provided poorer resolution between n and n-1 bands (Figure 5, panel B) than minigels containing 15% acrylamide (Figure 5, panel A) for oligonucleotides 15 to 44 bases in length. Oligonucleotides of 76

Table 1. Gel types, electrophoresis times, and oligonucleotides tested using TBE-urea minigels containing 15% or 10% acrylamide electrophoresed at 180 volts.

Gel Type		*Electrophoresis Time*	
(%)	*(well)*	*(minutes)*	*Lengths of Oligonucleotides Tested*
15	15	45	15, 16, 15+16, 27, 28, 27+28, 43, 44, 43+44, 75, 76, 75+76
15	15	55	15, 16, 15+16, 27, 28, 27+28, 43, 44, 43+44, 75, 76, 75+76
15	15	65	15, 16, 15+16, 27, 28, 27+28, 43, 44, 43+44, 75, 76, 75+76
15	15	90	15, 16, 15+16, 27, 28, 27+28, 43, 44, 43+44, 75, 76, 75+76
15	10	45	15, 16, 15+16, 27, 28, 27+28, 43, 44, 43+44
15	10	55	15, 16, 15+16, 27, 28, 27+28, 43, 44, 43+44
15	10	65	27, 28, 27+28, 43, 44, 43+44, 75, 76, 75+76
15	10	90	27, 28, 27+28, 43, 44, 43+44, 75, 76, 75+76
10	10	40	15, 16, 15+16, 27, 28, 27+28, 43, 44, 43+44
10	10	50	27, 28, 27+28, 43, 44, 43+44, 75, 76, 75+76
10	10	60	27, 28, 27+28, 43, 44, 43+44, 75, 76, 75+76
10	10	70	27, 28, 27+28, 43, 44, 43+44, 75, 76, 75+76
10	10	80	27, 28, 27+28, 43, 44, 43+44, 75, 76, 75+76

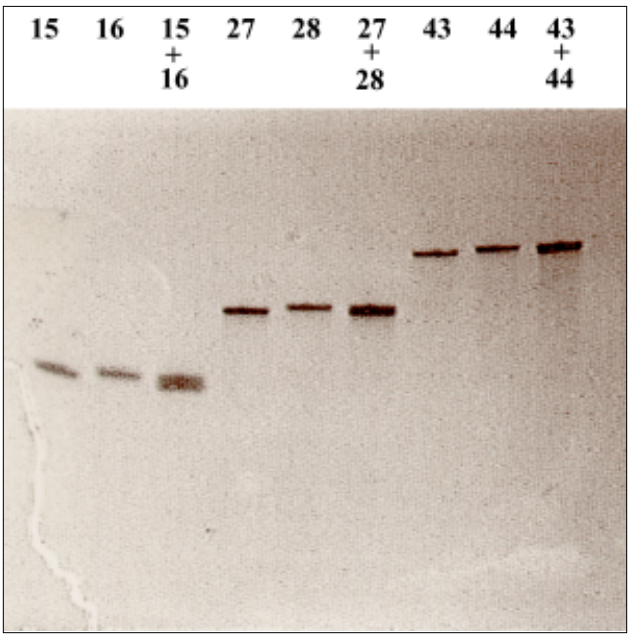

Figure 1. Resolution of oligonucleotides 15 and 16 bases in length electrophoresed for 45 minutes on a minigel containing 15% acrylamide with 10 wells. Oligonucleotide lengths are listed above each sample.

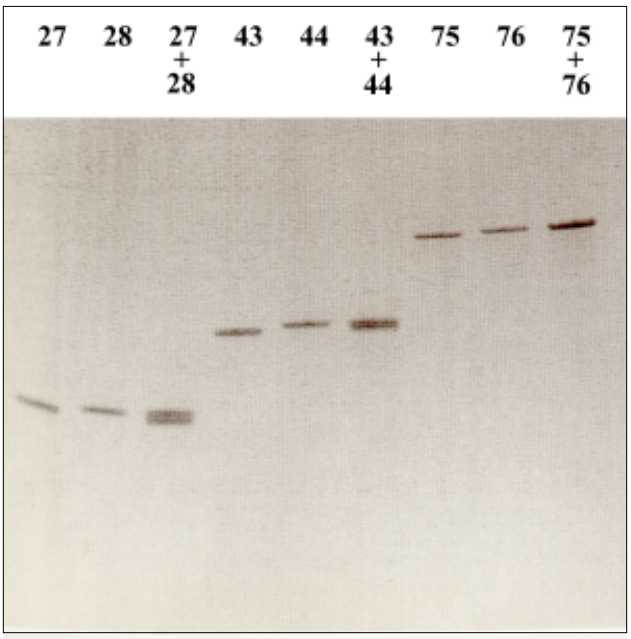

Figure 3. Resolution of oligonucleotides 43 and 44 bases in length electrophoresed for 65 minutes on a minigel containing 15% acrylamide with 10 wells. Oligonucleotide lengths are listed above each sample.

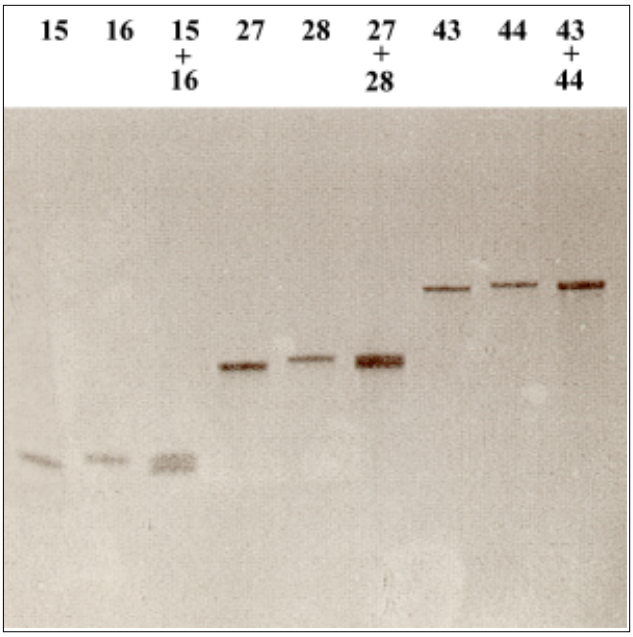

Figure 2. Resolution of oligonucleotides 27 and 28 bases in length electrophoresed for 55 minutes on a minigel containing 15% acrylamide with 10 wells. Oligonucleotide lengths are listed above each sample.

and 75 bases could not be resolved on either minigels containing 15% or 10% acrylamide with electrophoresis times up to 90 minutes (data not shown). However, electrophoresis of oligonucleotides up to approximately 120 bases in length on 10% acrylamide gels can be used to assess general oligonucleotide quality and to detect failure sequences smaller than n-1 in length. Oligonucleotides less than 15 bases in length are often not visible on minigels containing either 15% or 10% acrylamide.

Oligonucleotides less than 15 bases, and those that do not meet the criteria for passing PAGE analysis, can be subjected to MALDI-TOF mass spectrometry analysis as described in Methods. A single peak corresponding to the theoretical mass denotes a successful synthesis. Often the observed mass is 18 Da lower than the theoretical mass, presumably due to a dehydration, the source of which is not known. This small mass difference is easily distinguished from the much larger mass differences resulting from a failure in synthesis of the oligonucleotide. A mass peak corresponding to an adduct of the oligonucleotide with 3-hydroxypicolinic acid is typically observed at a mass 139 Da higher than the major peak (data not shown).

Based on these results, the following standard operating procedure was established to provide a rapid and easy quality control method for synthetic oligonucleotides from 15 to >100 bases in length.

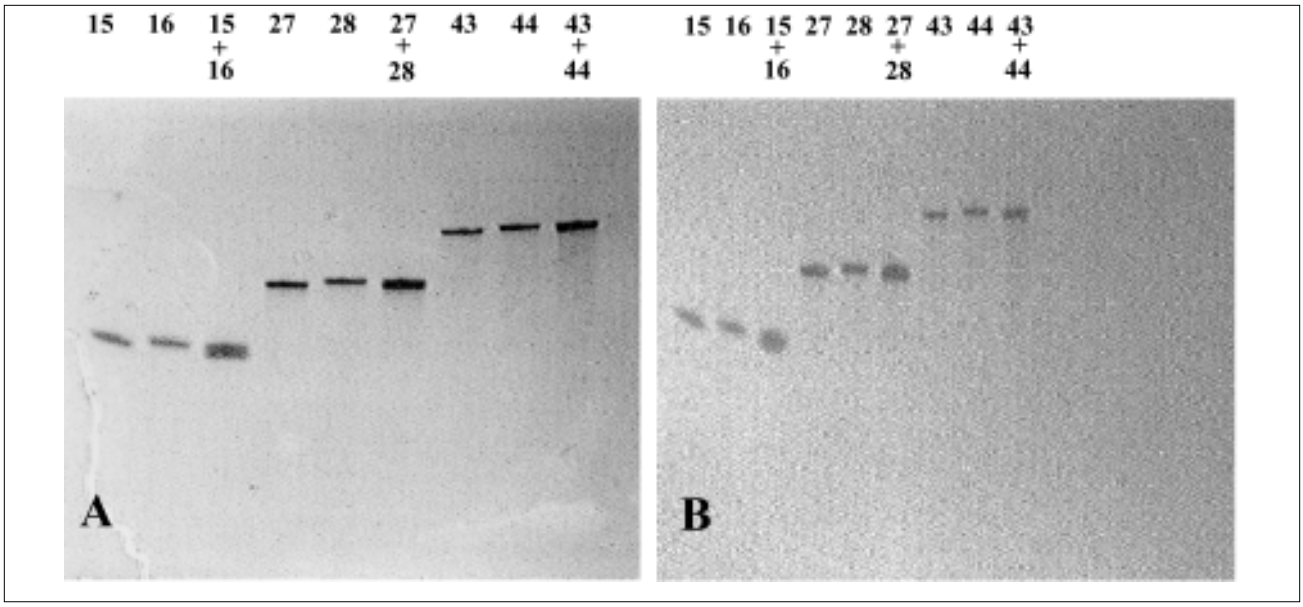

Figure 4. Resolution of oligonucleotides 15 to 44 bases in length electrophoresed for 45 minutes on a minigel containing 15% acrylamide with 10 wells (Panel A) or 15 wells (Panel B). Oligonucleotide lengths are listed above each sample.

Standard Operating Procedure

1. Group oligonucleotides in ascending order by length, along with appropriate markers. Markers are oligonucleotides of varying lengths and base compositions synthesized by our resource laboratory. Oligonucleotides less than 15 bases in length will not resolve on these gels and should be submitted for mass spectrometry analysis, if available. Select the appropriate 10-well pre-cast gel using Table 2.

2. Aliquot 100 ng (0.003 ODU) of oligonucleotide in a 0.5-mL tube and dry in a vacuum centrifuge without heat for at least 10 minutes.

3. Resuspend the sample in 6 µL of sample buffer and vortex.

4. Heat the sample at 100°C for 2 minutes. Chill the sample on ice.

5. Place the appropriate pre-cast minigel in the Schleicher & Schuell Profile system and fill the inner chamber completely. Fill the outside chamber approximately 1/3 full with running buffer.

6. Flush gel sample wells with running buffer and immediately load 6 µL of sample per well.

7. Electrophorese at constant voltage (180 volts) for the appropriate time as listed in Table 2.

8. Disassemble the gel apparatus and remove the gel from the disposable cassette. Stain the gel in methylene blue staining solution for 15 minutes. Destain the gel in distilled water until the background is low (at least 30 minutes). The stain may be used for several gels. If bands appear light after 15 minutes of staining, discard the stain and replace with fresh stain, stain for 15 minutes, then destain.

9. Photograph or digitize and print the gel image.

10. Analyze the gel for oligonucleotide purity and approximate length. Samples are judged as passing if the stained gel band meets all of the following criteria: a) a single, sharp band, b) band mobility within 2 mm of a marker of the same length, and c) the band is stained with the approximate intensity of the marker of the same length.

An example of samples that contained multiple, diffuse bands and were thus judged as failed is shown in Figure 6. If gel analysis reveals multiple, fuzzy, or smeared bands, the sample has failed quality control and should be re-synthesized. To determine the nature of the synthesis problem, submit the sample for more extensive analysis using mass spectrometry. If bands run distinctly faster or slower than markers, submit the sample for more extensive analysis using mass spectrometry to determine if the mobility shift is due to base composition or to a failed synthesis.

Conclusions

Pre-cast, commercially available minigels can provide a fast and convenient method for screening the general, overall quality of synthetic oligonucleotides. Ten-well gels produce the best resolution of n and n-1 failure sequences for oligonucleotides 15 to 44 bases in length. Oligonucleotides ranging from 15 to 76 bases in length can be resolved from failure sequences in run times of 70 minutes or less. Two gels can be run at the same time in the same gel apparatus

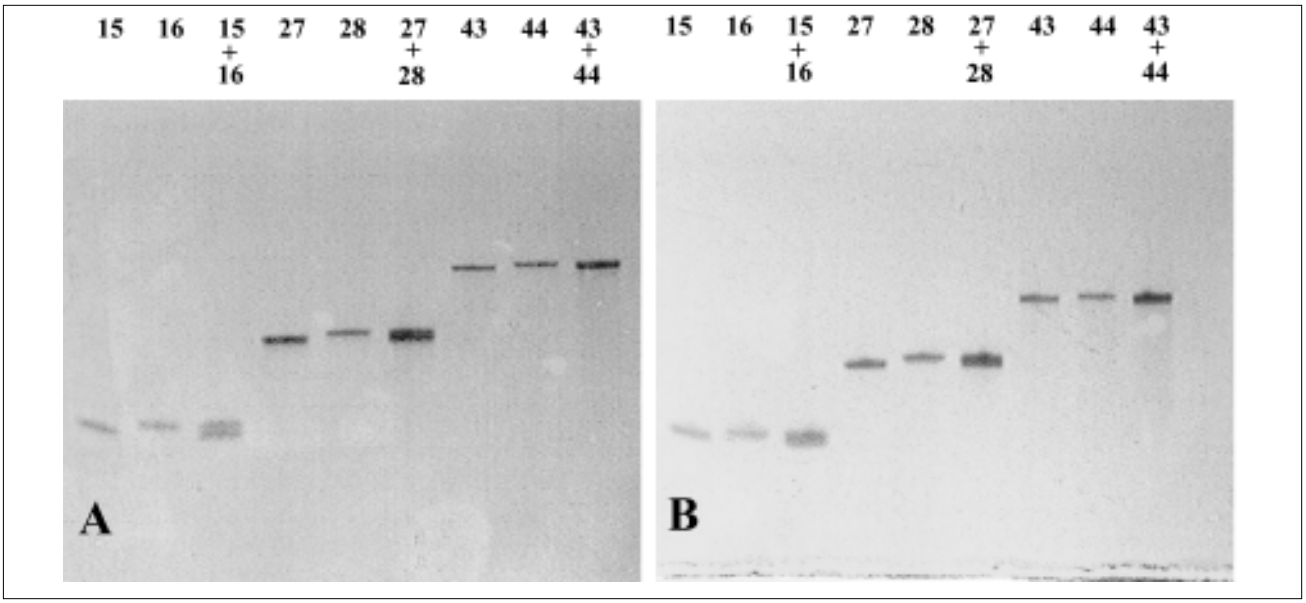

Figure 5. Resolution of oligonucleotides from 15 to 44 bases in length electrophoresed on a minigel containing 15% acrylamide for 55 minutes (panel A) or 10% acrylamide for 40 minutes (panel B). Oligonucleotide lengths are listed above each sample.

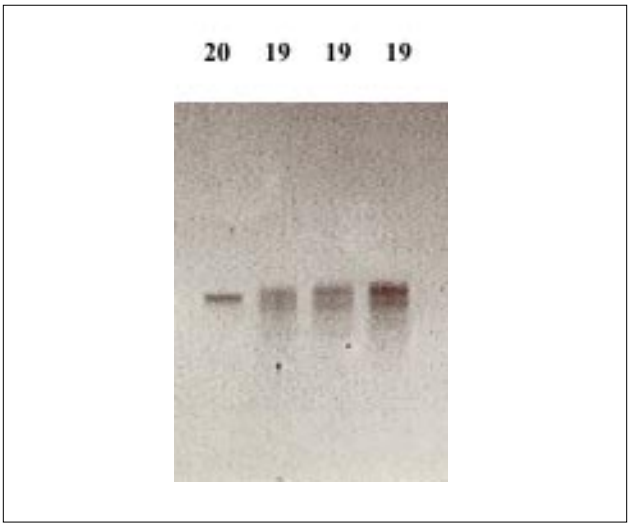

Figure 6. Example of oligonucleotides that failed PAGE quality analysis. Oligonucleotides were electrophoresed on a minigel containing 15% acrylamide for 50 minutes. Oligonucleotides tested included a 20-mer marker and three 19-mer failed oligonucleotides. Oligonucleotide lengths are listed above each sample.

thus increasing the number of oligonucleotides screened in a single run. This gel analysis system requires very little bench space and, by using commercially made buffers and gels pre-cast in disposable cassettes, labor costs are minimal.

References

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