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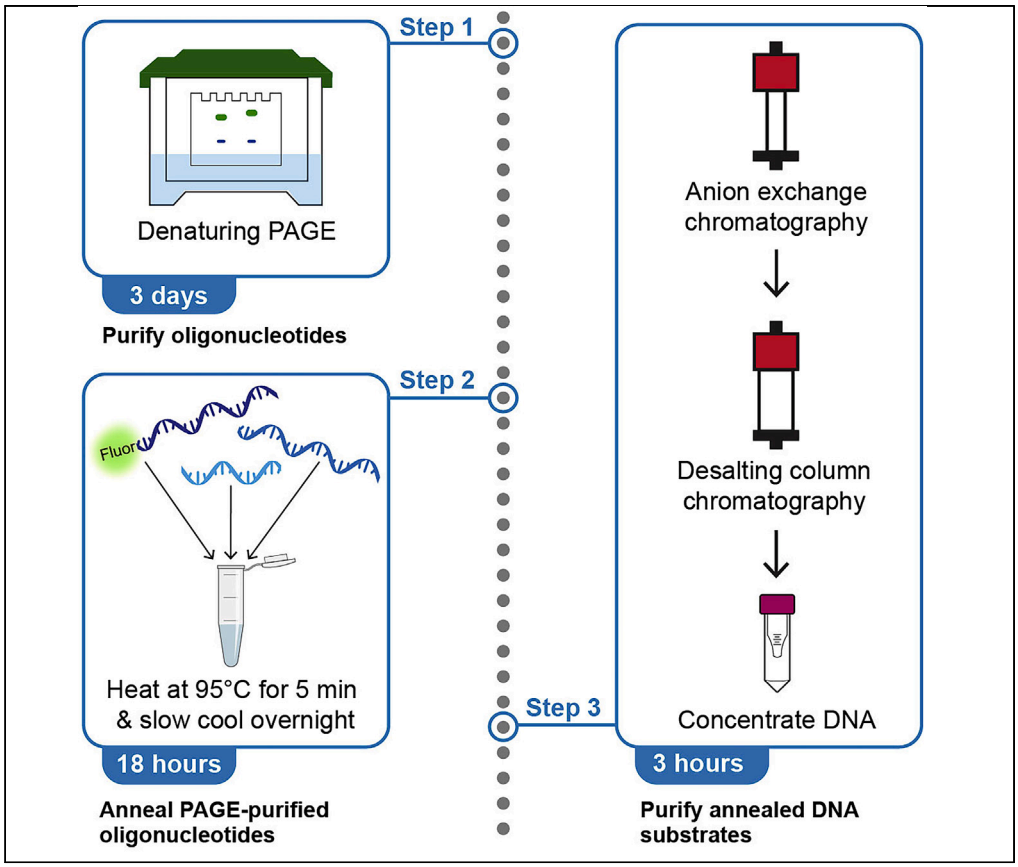
Protocol

Annealing and purification of fluorescently labeled DNA substrates for *in vitro* assays



We present a protocol to generate high-quality fluorescently labeled DNA substrates that can be used for biochemical assays, including DNA-binding and nuclease activity assays. We describe polyacrylamide-gel-electrophoresis-based purification of DNA oligonucleotides, followed by annealing the oligonucleotides and purifying the annealed substrates using anion-exchange chromatography. This protocol circumvents the use of radioisotopes, which require training and dedicated equipment for safe handling and necessitate specialized waste disposal. This protocol is amenable to varying lengths of oligonucleotides and DNA substrates.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Reproducible and cost-effective approach to purify oligonucleotides using PAGE

Robust protocol to produce high-quality fluorescent DNA substrates for biochemical assays

Steps to purify the annealed substrates using anion-exchange chromatography

Adaptable to varying lengths of oligonucleotides and DNA substrates

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Annealing and purification of fluorescently labeled DNA substrates for *in vitro* assays

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SUMMARY

We present a protocol to generate high-quality fluorescently labeled DNA substrates that can be used for biochemical assays, including DNA-binding and nuclease activity assays. We describe polyacrylamide-gel-electrophoresis-based purification of DNA oligonucleotides, followed by annealing the oligonucleotides and purifying the annealed substrates using anion-exchange chromatography. This protocol circumvents the use of radioisotopes, which require training and dedicated equipment for safe handling and necessitate specialized waste disposal. This protocol is amenable to varying lengths of oligonucleotides and DNA substrates.

For complete details on the use and execution of this protocol, please refer to Payliss and Tse et al. (2022).[1]

BEFORE YOU BEGIN

All forms of life use DNA-binding proteins to execute fundamental biological processes that are essential for survival, such as replication, transcription, and DNA repair. Notable examples of these proteins include DNA polymerases, helicases, topoisomerases, transcription factors, and nucleases. The identification and functional characterization of these proteins have benefitted greatly from biochemical studies using synthetic DNA substrates produced by annealing oligonucleotides. Here we present a reliable protocol to purify and anneal oligonucleotides to produce high-quality DNA substrates for *in vitro* assays.

In theory, DNA substrates can be prepared from any complementary or partially complementary DNA sequences. However, it is important to use oligonucleotides that do not self-anneal. Other parameters, such as thermostability, should be considered by increasing oligonucleotide length or GC content if reactions are performed at elevated temperatures. Specific recognition sequences can also be included in the substrate design (e.g., transcription factor binding motifs or restriction enzyme target sequences). Depending on the research aims, the DNA substrates can be incubated with the protein of interest in its purified[1–5] or partially purified form.[6,7] Additionally, users have the option to assay DNA binding or cleavage.

Traditionally, DNA substrates are prepared by annealing complementary oligonucleotides, one of which is 5′-end-labeled with ^{32}P.[1,3,8–19] However, there are caveats to using radioisotopes, most of which arise from the potential health hazards of radioactivity. For example, users require radiation training and a dedicated workspace and equipment for the safe handling, storage, and disposal of radioactive materials. One experimental limitation of using radiolabeled

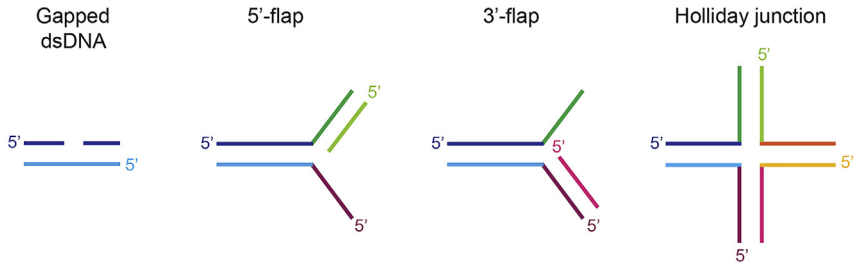

Figure 1. Schematic illustration of branched DNA substrates formed by annealing partially complementary oligonucleotides

The branched DNA structures shown are thought to mimic DNA intermediates that form during cellular DNA replication and repair.[23,24] Oligonucleotides with complementary sequences will anneal and are depicted using light and dark shades of a given color (e.g., the dark blue oligonucleotide is perfectly complementary to the light blue oligonucleotide).

DNA substrates stems from the lack of distinct radioisotopes, which precludes the design of substrates with differentially labeled oligonucleotides (i.e., the multiplex design). This is a drawback for nuclease activity assays that aim to identify the incision sites because multiple sets of DNA substrates must be prepared, each of which contains the radiolabel on a different strand.[3,20]

Fluorophores offer a safe alternative to conventional radioisotopes and procedures can be performed in the standard laboratory workspace. The vast selection of fluorophores allows for the flexible design of DNA substrates with unique fluorophores on each oligonucleotide.[4,5] The stability of fluorophores compared to radioisotopes also means that users can generate and store large quantities of fluorescently labeled DNA substrates. The disadvantages of using fluorophores are discussed in the limitations of this protocol.

We present a detailed procedure to generate fluorescently labeled DNA substrates from PAGE-purified oligonucleotides. Purified oligonucleotides are essential to remove the shorter DNA species that contaminate commercially available synthetic oligonucleotides and can complicate the interpretation of experimental results.[20,21] The annealed structures are subsequently purified using anion exchange chromatography to remove excess unlabeled oligonucleotides and incomplete DNA structures formed during annealing.[22] Using appropriate oligonucleotides, this protocol can be applied to construct different types of branched DNA substrates (Figure 1). In addition, the protocol is amenable to producing substrates with different fluorophores, including 6-carboxyfluorescein (FAM), Cyanine 3 (Cy3), or Cyanine 5 (Cy5).

Design DNA oligonucleotides and substrates

Timing: 1–5 h

1. Careful consideration should be given to the design of branched DNA substrates and oligonucleotide sequences.
   a. Specific recognition sequences supported by experimental data may be included in the oligonucleotide design.
   b. It is advisable to avoid sequences that self-anneal. Additionally, the reaction conditions, including temperature and the concentrations of monovalent and divalent cations, should be borne in mind to ensure that the melting temperature of the DNA substrate is significantly higher than the reaction temperature.

Note: There are several freely available software applications that can be used to determine the properties of a given oligonucleotide sequence. We recommend OligoAnalyzer™ (Integrated DNA Technologies) and OligoCalc (Northwestern University).[25]

Alternatives: Several research groups have published extensively with branched DNA substrates constructed from different oligonucleotide sequences, each of which can be considered to represent experimentally validated sequences and substrates. As such, users are encouraged to perform a comprehensive literature search prior to designing DNA substrates for their system. The oligonucleotide sequences used in this protocol originate from the laboratory of Dr. Stephen West.

2. Determine which oligonucleotide(s) should be labeled with a fluorophore and the position of the modification (e.g., 5′-FAM). See troubleshooting 1.

⚠ CRITICAL: If two or more oligonucleotides are to be fluorescently labeled, it is important to choose fluorophores with well-separated excitation and emission spectra (e.g., FAM and Cy5). To that end, SpectraViewer (ThermoFisher Scientific) offers an easy and convenient online tool to compare the compatibility and excitation/emission wavelength of many different fluorophores.

Purchase DNA oligonucleotides

Timing: 1–5 days

3. Purchase oligonucleotides in lyophilized format from a commercial supplier (e.g., Integrated DNA Technologies, MilliporeSigma).

Note: Since this protocol describes how to PAGE-purify oligonucleotides, users can select the most cost-effective purification option, such as standard desalting. However, since PAGE-purification results in a relatively low yield (20%–70%, depending on length), users are advised to choose the synthesis scale that yields at least 10 nmol of starting product.

Alternatives: In our experience, in-house PAGE purification is more cost-effective than purchasing PAGE-purified oligonucleotides. However, we encourage users to perform their own cost-benefit analysis since prices may vary depending on supplier and geographical location.

4. Store lyophilized oligonucleotides at −20°C until ready for PAGE-purification.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
40% Acrylamide/bis-acrylamide solution (19:1)	BioShop	Cat #: ACR003.500
30% Acrylamide/Bis-acrylamide solution (37.5:1)	BioShop	Cat #: ACR010.500
100% Ethyl alcohol (absolute ethanol)	MedStore	Cat #: 22734-P006-EAAN
Ammonium persulfate (APS)	BioShop	Cat #: AMP001.100
Boric acid	BioShop	Cat #: BOR001.1
Bromophenol blue	BioShop	Cat #: BRO777.5
Ethylenediaminetetraacetic acid (EDTA)	BioShop	Cat #: EDT001.500

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Formamide	BioShop	Cat #: FOR001.500
Glacial acetic acid	BioShop	Cat #: ACE333.4
Hydrochloric acid (12.1 N) (HCl)	BioShop	Cat #: HCL333.500
Isopropanol	Caledon	Cat #: 8600-1-40
Magnesium chloride ($MgCl_2$)	BioShop	Cat #: MAG520.1
Orange G, disodium salt	BioShop	Cat #: ORA787.25
Stains-All	MilliporeSigma	Cat #: E9379-1G
Sodium acetate	BioShop	Cat #: SAA310.500
Sodium chloride	BioShop	Cat #: SOD001.5
Tetramethylethylenediamine (TEMED)	BioShop	Cat #: TEM001.50
Tris base	BioShop	Cat #: TRS001.5
Urea	BioShop	Cat #: URE001.1
Xylene cyanol FF	BioShop	Cat #: XYC001.5
Critical commercial assays		
HiTRAP Desalting Column, 5 mL	Cytiva	29-0486-84
HiTRAP Q HP Column, 1 mL	Cytiva	17-1153-01
Ultra-4 Centrifugal Unit, 3 kDa MWCO	Amicon	UFC800324
Oligonucleotides		
Oligo 1	Payliss and Tse et al.[1]	FAM-ACGCTGCCGAATTCTACCAGTGCC TTGCTAGGACATCTTTGCCCACCTGCAG GTTCACCC
Oligo 2	Payliss and Tse et al.[1]	GGGTGAACCTGCAGGTGGGCAAAGAT GTCCATCTGTTGTAATCGTCAAGCTTT ATGCCGT
Oligo 3	Payliss and Tse et al.[1]	ACGGCATAAAGCTTGACGATTACAAC AGATCATGGAGCTGTCTAGAGGATC CGACTATCG
Oligo 4	Payliss and Tse et al.[1]	CGATAGTCGGATCCTCTAGACAGCTC CATGTAGCAAGGCACTGGTAGAATTC GGCAGCGT
Oligo 2.5	Payliss and Tse et al.[1]	GGGTGAACCTGCAGGTGGGCAAAGATGTCC
Oligo 3.5[a]	Payliss and Tse et al.[1]	CATGGAGCTGTCTAGAGGATCCGACTATCG
Oligo 5	This paper	FAM-ACGCTGCCGAATTCTACCAGTGCCTTGCTA
Oligo 6	This paper	TAGCAAGGCACTG
Oligo 7 [a]	This paper	ATTCGGCAGCGT
Software and algorithms		
Fluorescence SpectraViewer	Thermo Fisher Scientific	https://www.thermofisher.com/order/fluorescence-spectraviewer#!/
OligoAnalyzer™ Tool	Integrated DNA Technologies	https://www.idtdna.com/pages/tools/oligoanalyzer
OligoCalc	Kibbe[25]	http://biotools.nubic.northwestern.edu/OligoCalc.html
Other		
PROTEAN II xi large format vertical electrophoresis cell and gel casting system (16 × 20 cm gel size; 1.5 mm thick) (or similar)	Bio-Rad	Cat #: 1651812
PROTEAN II xi comb, 5-well, 1.5 mm	Bio-Rad	Cat #: 1651883
PowerPac High-Voltage Power Supply (or similar)	Bio-Rad	Cat #: 1645056
ÄKTA Pure FPLC (or similar)	Cytiva	N/A
500 µL sample loop	Cytiva	Cat #: 18111399
1 mL sample loop	Cytiva	Cat #: 18111401
Typhoon FLA 9500, including BPB1 (530DF20) filter	Cytiva	Cat #: 29-0040-80

[a] Oligonucleotides contain a 5′-phosphate.

MATERIALS AND EQUIPMENT

10× Tris-Borate-EDTA Buffer (10× TBE)		
Reagent	Final concentration	Amount
Tris base	890 mM	216 g
Boric acid	890 mM	110 g
EDTA disodium salt, dihydrate	20 mM	14.8 g
ddH_2O	N/A	x L to a total volume of 2 L
Total	**10×**	**2 L**
Store the solution at room temperature (25°C). Use within 6 months.		

10% Ammonium Persulfate (10% APS)		
Reagent	Final concentration	Amount
Ammonium persulfate	10% (w/v)	1 g
ddH_2O	N/A	x mL to a total volume of 10 mL
Total	**N/A**	**10 mL**
Store the solution at 4°C. Use within 1 month.		

⚠ CRITICAL: Ammonium persulfate is a strong oxidizing agent and irritant. Users should exercise caution when handling and storing this reagent.

2× Denaturing Loading Dye		
Reagent	Final concentration	Amount
Formamide	90% (v/v)	9 mL
10× TBE	1×	1 mL
Bromophenol blue	0.25% (w/v)	0.025 g
Xylene cyanol FF	0.25% (w/v)	0.025 g
Total	**2×**	**10 mL**
Store the solution at room temperature (25°C). Use within 6 months.		

⚠ CRITICAL: Formamide is a reproductive toxin and irritant. Users should exercise caution when handling this reagent.

15% Denaturing Polyacrylamide Gel		
Reagent	Final concentration	Amount
40% Acrylamide/Bis-acrylamide solution (19:1)	15%	37.5 mL
10× TBE	1×	10 mL
Urea	7 M	42 g
10% APS	0.09% (v/v)	900 µL
TEMED	0.075% (v/v)	75 µL
ddH_2O	N/A	x mL to a total volume of 100 mL
Total	**N/A**	**100 mL**

⚠ CRITICAL: Acrylamide/Bis-acrylamide solution, urea, APS and TEMED are toxic and users should handle them with care. The gel solution should be prepared in a fume hood.

Note: For a denaturing acrylamide gel of 16 cm × 20 cm (1.5 mm thick), 100 mL of gel solution is sufficient. PAGE gels are best prepared on the day of the experiment and allowed to polymerize for at least 1 h at room temperature.

Note: Urea takes time to dissolve. If necessary, the 10× TBE, 40% Acrylamide/Bis-acrylamide, urea, and water can be added to a glass beaker and slowly heated with gentle stirring on a hot plate in the fume hood. The mixture should be allowed to cool to room temperature before adding the 10% APS and TEMED. The gel will begin to polymerize quickly after the addition of the 10% APS and TEMED, so make sure that you have assembled the glass plates and casting tray.

10% Native Polyacrylamide Gel		
Reagent	Final concentration	Amount
30% Acrylamide/Bis-acrylamide solution (37.5:1)	10%	13.3 mL
10× TBE buffer	1×	4 mL
10% APS	1% (v/v)	400 μL
TEMED	0.1% (v/v)	40 μL
ddH_2O	N/A	x mL to a total volume of 40 mL
Total	**N/A**	**40 mL**

⚠ CRITICAL: Acrylamide/Bis-acrylamide solution, APS and TEMED are toxic and users should handle them with care.

Note: For a native acrylamide gel of 17 cm × 11 cm (1.5 mm thick), 40 mL of gel solution is sufficient. PAGE gels are best prepared on the day of the experiment and allowed to polymerize for at least 1 h at room temperature.

Tris-EDTA (TE) Buffer		
Reagent	Final concentration	Amount
1 M Tris-Cl, pH 8.0	100 mM	10 mL
0.5 M EDTA, pH 8.0	10 mM EDTA	2 mL
ddH_2O	N/A	x mL to a total volume of 100 mL
Total	**N/A**	**100 mL**
Store the solution at room temperature (25°C). Use within 6 months.		

Note: Adjust the pH of the final solution to 8.0 (25°C) using strong acid or base. Filter sterilize using a 0.2 μm PES membrane.

Stains-All Staining Solution		
Reagent	Final concentration	Amount
Stains-All	0.1% (w/v)	0.1 g
Formamide	10% (v/v)	10 mL
Isopropanol	20% (v/v)	20 mL
ddH_2O	N/A	70 mL
Total	**N/A**	**100 mL**

⚠ CRITICAL: Formamide is a reproductive toxin and irritant. Users should exercise caution when handling this reagent.

Note: Stains-All solution should be made fresh before staining the gel.

3 M Sodium Acetate (CH_3COONa), pH 5.2		
Reagent	Final concentration	Amount
CH_3COONa	3 M	61.5 g
ddH_2O	N/A	x mL to a total volume of 250 mL
Total	**3 M**	**250 mL**
Store the solution at room temperature (25°C). Use within 6 months.		

Note: Adjust the pH of the solution to 5.2 using glacial acetic acid. Leave the solution at room temperature (25°C) overnight (12–16 h) and check the pH the next day. If necessary, re-adjust to pH 5.2 using glacial acetic acid.

⚠ CRITICAL: Acetic acid is corrosive, flammable, and an irritant. Users should exercise caution when handling this reagent. Solutions should be pH-adjusted in a fume hood.

1 M Tris-Cl, pH 7.5		
Reagent	Final concentration	Amount
Tris base	1 M	121.1 g
12.1 N HCl	N/A	x mL to pH 7.5 (25°C)
ddH_2O	N/A	x mL to a total volume of 1 L
Total	**1 M**	**1 L**
Store the solution at room temperature (25°C). Use within 6 months.		

Note: The pH of Tris solutions is temperature-dependent and it is important to adjust the pH of the solution after it has equilibrated to the temperature that will be used in the experiment.

⚠ CRITICAL: HCl is toxic and causes severe burns and eye damage. Users should exercise caution when handling this reagent and solutions should be pH-adjusted in a fume hood.

5 M Sodium Chloride (NaCl)		
Reagent	Final concentration	Amount
NaCl	5 M	146.1 g
ddH_2O	N/A	x mL to a total volume of 500 mL
Total	**5 M**	**500 mL**
Store the solution at room temperature (25°C). Use within 6 months.		

1 M Magnesium Chloride ($MgCl_2$)		
Reagent	Final concentration	Amount
$MgCl_2$	1 M	50.8 g
ddH_2O	N/A	x mL to a total volume of 250 mL
Total	**1 M**	**250 mL**
Store the solution at room temperature (25°C). Use within 6 months.		

10× DNA Hybridization Buffer		
Reagent	Final concentration	Amount
1 M Tris-Cl, pH 7.5	500 mM	25 mL
5 M NaCl	1 M	10 mL
1 M $MgCl_2$	100 mM	5 mL
Nuclease-free ddH_2O	N/A	10 mL
Total	**10×**	**50 mL**
Store the solution at room temperature (25°C). Use within 6 months.		

Note: Adjust the pH of the final solution to 7.5 (25°C) using concentrated HCl or NaOH. The solution should be filtered through a 0.2 μm PES filter before use.

DNA Binding Buffer		
Reagent	Final concentration	Amount
1 M Tris-Cl, pH 7.5	10 mM	5 mL
5 M NaCl	50 mM	5 mL
Nuclease-free ddH_2O	N/A	490 mL
Total	**N/A**	**500 mL**
Store the solution at 4°C. Use within 6 months.		

Note: Adjust the pH of the final solution to 7.5 (4°C) using concentrated HCl or NaOH. The solution should be filtered through a 0.2 μm PES filter before use.

DNA Elution Buffer		
Reagent	Final concentration	Amount
1 M Tris-Cl, pH 7.5	10 mM	5 mL
5 M NaCl	1 M	100 mL
Nuclease-free ddH_2O	N/A	395 mL
Total	**N/A**	**500 mL**
Store the solution at 4°C. Use within 6 months.		

Note: Adjust the solution to pH = 7.5 (4°C) using concentrated HCl or NaOH. The solution should be filtered through a 0.2 μm PES filter before use.

DNA Storage Buffer		
Reagent	Final concentration	Amount
1 M Tris-Cl, pH 7.5	50 mM	25 mL
1 M $MgCl_2$	5 mM	2.5 mL
Nuclease-free ddH_2O	N/A	472.5 mL
Total	**N/A**	**500 mL**
Store the solution at room temperature (25°C). Use within 6 months.		

Note: Adjust the pH of the final solution to 7.5 (25°C) using concentrated HCl or NaOH. The solution should be filtered through a 0.2 μm PES filter before use.

STEP-BY-STEP METHOD DETAILS

Purification of DNA oligonucleotides using denaturing PAGE and ethanol precipitation

Timing: 3 days

Oligonucleotides are heat denatured in the presence of formamide and then resolved through a preparative denaturing polyacrylamide gel (steps 1–3). After electrophoresis, the full-length product is identified by staining (step 4), eluted from the gel by diffusion (steps 4–5), and concentrated by ethanol precipitation (steps 6–8). The purpose of this step is to obtain highly pure full-length oligonucleotides that are free of contaminating shorter oligonucleotide species. This is critical to obtain high-quality and reproducible results.

Denaturing polyacrylamide gels can resolve oligonucleotides from 2 to 300 bases, depending on the percentage of polyacrylamide used (see Table 1). In this section, we describe how to purify synthetic oligonucleotides using 15% denaturing PAGE and ethanol precipitation.

Table 1. Concentrations of acrylamide for optimal separation of DNA using denaturing PAGE[a]

Acrylamide (%)	Effective range of separation (bp)	Migration of xylene cyanol FF[b]	Migration of bromophenol blue[b]
5.0	80–500	260	60
8.0	60–400	160	45
12.0	40–200	70	20
15.0	25–60	60	15
20.0	< 30	45	12

[a]Adapted from Green and Sambrook.[26]
[b]The numbers provided are the approximate sizes (in base pairs) of double-stranded DNA with which the dye co-migrates.

1. Prepare the 15% denaturing polyacrylamide gel.
   a. Assemble the equipment that you will need for casting the gel, including the glass plates (16 × 20 cm gel size), 1.5 mm spacers, and a 5-well comb, according to the manufacturer's instructions.

   ⚠ CRITICAL: This protocol involves loading large amounts of DNA into each well of the polyacrylamide gel. To prevent cross-sample contamination, it is important to leave 1 empty well between consecutive samples. We recommend using a 5-well comb and loading 50 nmol to 2 μmol of DNA per lane. The total sample volume should be ≤ 350 μL of sample per lane (maximum volume per lane ≈ 400 μL).

   b. Prepare the 15% denaturing polyacrylamide gel solution, as described above.

   Note: Once the polyacrylamide gel solution has cooled to room temperature, it can be removed from the fume hood and the gel can be cast at room temperature.

   c. Immediately after adding the 10% APS and TEMED to the gel solution, swirl gently to mix and then use a 25 mL serological pipette to transfer the 15% acrylamide/bis-acrylamide gel solution into the glass plates. See troubleshooting 2.
   d. Allow the gel to polymerize at room temperature for at least 1 h.
   e. Once the gel has polymerized, assemble the electrophoresis unit according to the manufacturer's instructions and fill with 1× TBE buffer.
   f. Carefully remove the comb. Flush the wells with 1× TBE buffer using a 30 mL plastic syringe and 21-gauge needle to remove unpolymerized gel solution and settled urea.
   g. Pre-run the gel at 15 W for 90 min at room temperature in 1× TBE buffer.
2. Prepare oligonucleotides for denaturing PAGE.
   a. If working with lyophilized oligonucleotides, resuspend in 2× or 1× denaturing loading dye to a final concentration of 50 mM. Vortex to mix.
   b. If the oligonucleotides are already in solution, mix the volume needed for 1–2 μmol DNA with an equal volume of 2× denaturing loading dye. Vortex to mix.

   Note: We recommend loading a maximum of 2 μmol DNA per lane when using the 5-well comb (see step 1a above).

   c. Heat the samples for 5 min at 95°C.

   ⚠ CRITICAL: The purpose of heating the oligonucleotides in denaturing loading dye is to remove DNA secondary structures. As such, samples should be loaded quickly after heating to prevent the reformation of DNA secondary structures.

3. Perform electrophoresis.
   a. Before loading the samples, flush the gel wells with 1× TBE buffer using a 30 mL plastic syringe and 21-gauge needle to remove the settled urea.

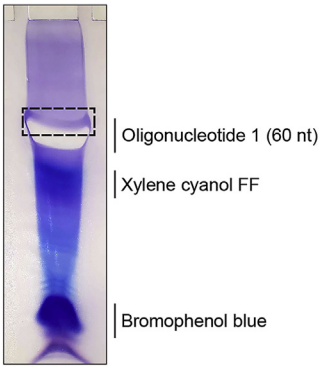

Figure 2. Purification of synthetic DNA oligonucleotides by preparative denaturing PAGE

Representative results showing the separation of 100 nmol oligonucleotide 1 (60 nt) by 15% denaturing PAGE. Electrophoresis was performed for 2.5 h at 15 W, after which time DNA was detected by immersion in Stains-All staining solution. The predominant band represents the full-length oligonucleotide, of which the upper half was excised (indicated by the dashed lines) and eluted from the gel by diffusion. See step-by-step method details: purification of DNA oligonucleotides using denaturing PAGE and ethanol precipitation (steps 1–4) for further details.

b. Carefully load the samples.
c. When purifying fluorescently labeled oligonucleotides, cover the electrophoresis tank with aluminum foil to prevent quenching (photobleaching) the fluorophore.
d. Electrophoresis is performed at room temperature for approximately 2.5 h at 15 W, or until desired separation is achieved (see Table 1). See troubleshooting 3.

Note: In a 15% denaturing polyacrylamide gel, bromophenol blue migrates around 15 nt and xylene cyanol FF migrates around 60 nt (Table 1). If the oligonucleotide migrates at the same position as the dyes, the loading buffer can be prepared with a different tracking dye or without the tracking dyes. See troubleshooting 3.

4. DNA staining and elution by diffusion.
   a. After electrophoresis, carefully transfer the gel to a glass tray. Submerge the gel in Stains-All Solution and mix with gentle rocking or agitation for 10 min at room temperature.
   b. Destain the gel by submerging in nuclease-free ddH_2O and mixing with gentle rocking or agitation for 15 min at room temperature (Figure 2).

Note: You should observe one predominant product, representing the full-length oligonucleotide, and a series of shorter oligonucleotides that co-migrate as a smear of lower molecular weight products (Figure 2).

   c. Using a clean scalpel for each product, excise the upper half of the most intense band to avoid shortened DNA products. Transfer the gel slice to a 15 mL conical tube.
   d. Elute each oligonucleotide in at least 500 µL TE buffer, depending on the size of the gel piece. There should be enough TE buffer to completely submerge the gel, with a visible layer of solution (≥ 200 µL) on top. See troubleshooting 4.
      i. To facilitate diffusion, crush the gel into small pieces using the end of a plastic inoculating loop or spreader. To do so, press the gel fragments against the sides of the tube several times.
   e. Incubate the conical tubes at room temperature (25°C) overnight (12–16 h), either on a spinning wheel or with end-over-end rotation.

⚠ CRITICAL: It is important to minimize the exposure of fluorescently labeled DNA to light. Users should cover the glass tray and all conical or microfuge tubes in aluminum foil.

Alternatives: After the oligonucleotide of interest is excised from the gel, the DNA can also be eluted by electroelution, as described by Lopez-Gomollon and Nicolas.[21]

5. Separate the eluted DNA from the polyacrylamide gel fragments by centrifugation.
   a. On the following day, centrifuge the conical tubes containing the gel/DNA solution at 3,220 × *g* for 2 min (4°C).
      i. Carefully pipette the supernatant, such that no gel pieces are transferred, into another 15 mL conical tube that can withstand high centrifugal force (≥ 18,000 × *g*). See troubleshooting 5.
   b. Wash the crushed gel with 0.5–1 mL of TE buffer and mix by gentle inversion.
   c. Centrifuge the conical tubes containing the crushed gel solution at 3,220 × *g* for 2 min (4°C).
      i. Carefully transfer the supernatant into the 15 mL conical tube containing the eluted DNA solution (step 5.a.i). Mix by gentle inversion.
   d. Discard the conical tube containing the crushed gel as hazardous chemical waste.
   e. Proceed to the next step with the eluted DNA.
6. Perform ethanol precipitation.
   a. To the eluted DNA solution, add 3 M sodium acetate, pH 5.2 to a final concentration of 0.3 M (i.e., 1/10th of the total supernatant volume). Mix by gentle inversion.
   b. Add 3 volumes of ice-cold absolute ethanol and mix by gentle inversion.
   c. Incubate the tubes upright at −20°C overnight (12–16 h), protected from light.

   ⏸ **Pause point:** The DNA/ethanol solution can be stored at −20°C for several days.

   d. The next day, retrieve the conical tubes from the −20°C freezer and spin down at 18,000 × *g* for 1 h (4°C).

   Note: Take note of the orientation of the tubes in the rotor and the approximate location of the DNA pellet after centrifugation. Users may find it useful to draw a circle around the DNA pellet.

   e. Carefully pour off the supernatant, taking care not to dislodge the pellet.
   f. Gently wash the pellet with 3–5 mL of ice-cold 70% ethanol (made from absolute ethanol) taking care to not dislodge the pellet.
   g. Centrifuge at 18,000 × *g* for 30 min (4°C).
   h. Carefully pour off the supernatant, taking care not to dislodge the pellet.
   i. Invert the tube on a Kimwipe for 5–10 min to drain off the ethanol and then turn the tube upright to air dry the pellet for 10 min (room temperature).
   j. Use a wide-bore pipette tip to resuspend the pellet in 200–500 μL of TE buffer. Do not pipette up and down more than 2 times. Instead, resuspend by tapping or gentle agitation. See troubleshooting 6.

   Alternatives: If wide-bore pipette tips are not available, users can cut the tip off of a 1 mL pipette tip.

   k. Transfer the purified oligonucleotide solution into an appropriate microfuge tube.

   Note: Unlabeled oligonucleotides can be stored in regular 1.5 mL microfuge tubes, whereas fluorescently labeled oligonucleotides should be stored in amber or black 1.5 mL microfuge tubes.

7. Determine oligonucleotide concentration using spectrophotometry (A_{260}).

Note: The final yield will vary depending on the length of the oligonucleotide, but users can generally expect to recover 20%–70% of the starting material.

8. Store purified oligonucleotides at −20°C.

Annealing and purification of fluorescently labeled DNA substrates

Timing: 2 days

In this step, we describe how to anneal PAGE-purified oligonucleotides to form the DNA substrate. The unlabeled oligonucleotides are added in excess of the fluorescently labeled oligonucleotide. As such, the fluorescently labeled DNA substrate must be purified from the excess unlabeled oligonucleotides. This is accomplished using anion exchange chromatography. The final yields range from 30% to 50%, depending on the DNA substrate.

9. Anneal PAGE-purified oligonucleotides.
   a. Pipette 2.5 nmol of fluorescently labeled oligonucleotide and 3.75 nmol of each unlabeled oligonucleotide into a black 1.5 mL microfuge tube (i.e., 1:1.5 mol ratio of fluorescently labeled to unlabeled oligonucleotides).
   b. Add 50 μL of 10× DNA hybridization buffer and bring the volume up to 500 μL using DNase-free H_2O. Mix well.
   c. Fill a glass beaker with H_2O and place on a heat plate set to 100°C. Bring to 95°C (with gentle stirring).
   d. Place the annealing reactions in a Styrofoam microfuge tube holder and immerse in boiling water for 2 min.
   e. After 2 min, turn off the heat source.
   f. Leave the microfuge tubes in the beaker and allow to slowly cool to room temperature overnight (12–16 h). See troubleshooting 7.

⚠ CRITICAL: Steps 10 and 11 are performed at 4°C. All solutions must be filtered through a 0.2 μm PES membrane prior to use to prevent particulates from entering the FPLC.

10. Purify the annealed DNA substrate using anion exchange chromatography.
    a. Connect a 1 mL HiTRAP Q HP column to an FPLC system, such as the ÄKTA™ Pure, according to the manufacturer's instructions.
       i. Equilibrate the column with 5 column volumes (CV) of ddH_2O, followed by 5 CV of DNA elution buffer (i.e., buffer B), and 5 CV of DNA binding buffer (i.e., buffer A) at a flow rate of 1 mL/min.
    b. Attach a 500 μL sample loop to the injection valve.
       i. Wash the sample loop with 2.5 mL (5 loop volumes) of ddH_2O, followed by 2.5 mL of DNA storage buffer, flow rate = 1 mL/min.

    Note: Our protocol describes a 500 μL annealing reaction, which is loaded into a 500 μL sample loop (i.e., filled-loop mode). Users can adjust the volume of the sample loop according to the volume of the annealing reaction.

    c. Program the sample run using the following parameters:
       i. Empty the sample loop with 1 mL of DNA binding buffer, flow rate = 1 mL/min. Collect the flow-through from an FPLC outlet line.
       ii. Wash the column with 10 CV of DNA binding buffer, flow rate = 1 mL/min.
       iii. Elute the DNA in 500 μL fractions with a 20 CV linear gradient from 100% DNA binding buffer (i.e., buffer A) to 100% DNA elution buffer (i.e., buffer B), flow rate = 1 mL/min.

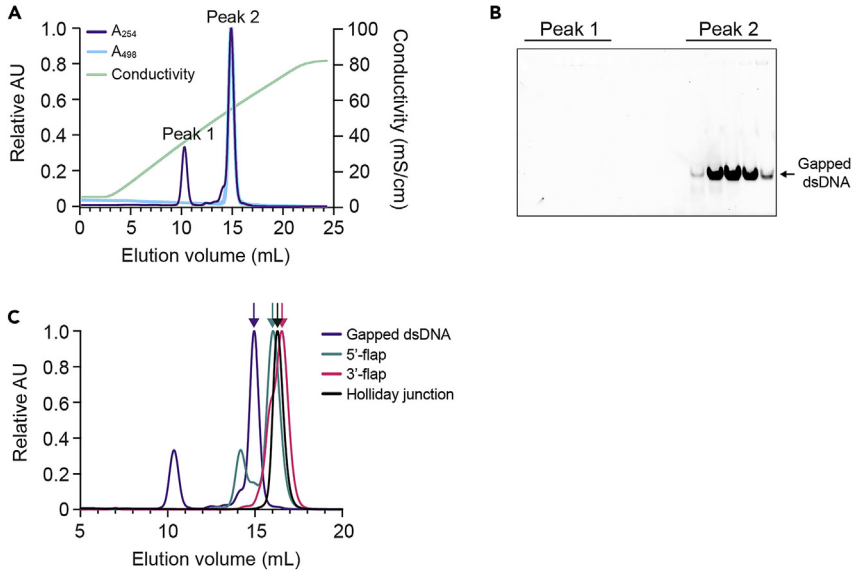

Figure 3. Purification of fluorescently labeled DNA substrates by anion exchange chromatography

Combinations of PAGE-purified oligonucleotides were annealed to produce the DNA substrates, as indicated in Table 2. The correctly annealed fluorescently labeled DNA substrates are purified using anion exchange chromatography.

(A) Elution profile for FAM-labeled double-stranded DNA (dsDNA) containing a 5 nt gap, showing absorbance at 254 nm (DNA, purple trace) and 498 nm (FAM, blue trace) in relative absorbance units (AU), and conductivity (mS/cm, green trace). Absorbance is scaled relative to the most intense peak in the elution profile. Excess unlabeled oligonucleotides elute in peak 1 and the correctly annealed fluorescently labeled DNA substrate elutes in peak 2.

(B) The elutions from anion exchange chromatography in (A) were separated by 10% native PAGE and imaged on a Typhoon FLA 9500 laser-scanning platform using the BPB1 (530DF20) filter (Cytiva).

(C) Elution profiles for FAM-labeled gapped dsDNA, 5′-flap, 3′-flap, and Holliday junction DNA substrates, showing absorbance at 254 nm in relative absorbance units (AU). For each substrate, absorbance is scaled relative to the most intense peak in the elution profile. Arrows indicate the peak fractions containing the correctly annealed fluorescently labeled DNA substrates. See step-by-step method details: annealing and purification of fluorescently labeled DNA substrates (step 10) for further details.

iv. Extend the elution with a 5 CV step at 100% DNA elution buffer (i.e., buffer B), flow rate = 1 mL/min. Continue to collect 500 µL fractions during this step.

v. If the FPLC is equipped with a variable wavelength UV detector, set the detector to monitor at 260 nm and the excitation wavelength of your fluorophore (e.g., 498 nm for FAM).

d. Using a plastic syringe, slowly inject the annealing reaction into the sample loop, ensuring that no air is injected into the FPLC. Do not remove the syringe from the FPLC.

e. Start the sample run.

f. Identify the peak fractions by absorbance at 260 nm and the excitation wavelength of your fluorophore or native PAGE analysis (Figures 3A–3C). See troubleshooting 8.

g. Pool the peak fractions containing the fluorescently labeled DNA substrate into a new tube. The total volume should be approx. 1.5 mL.

11. Buffer exchange into DNA storage buffer.

a. Attach a 5 mL HiTRAP Desalting column to an FPLC system, such as the ÄKTA™ Pure, according to the manufacturer's instructions.

i. Equilibrate the column with 5 CV of ddH_2O, followed by 5 CV of DNA storage buffer, flow rate = 5 mL/min.

b. Attach a sample loop to the injection valve. The loop volume should be selected according to the sample volume.

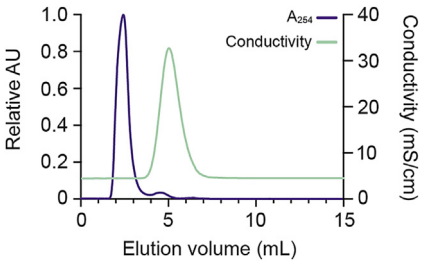

Figure 4. Buffer exchange of fluorescently labeled DNA substrates by desalting column chromatography

Representative elution profile for FAM-labeled gapped dsDNA fractionated by desalting column chromatography showing absorbance at 254 nm (DNA, purple trace) in relative absorbance units (AU) and conductivity (mS/cm, green trace). Absorbance is scaled relative to the most intense peak in the elution profile. See step-by-step method details: annealing and purification of fluorescently labeled DNA substrates (step 11) for further details.

i. Wash the sample loop with 5 loop volumes of ddH_2O, followed by 5 loop volumes of DNA storage buffer, flow rate = 5 mL/min.

Note: The HiTRAP Desalting column is packed with Sephadex G-25 Superfine resin (fractionation range approx. 800 to 5,000 Da), which provides for fast separation of high- and low-molecular weight species. The recommended sample volume for the 5 mL HiTRAP Desalting column is 0.1–1.5 mL per run. We recommend using a 1 mL sample loop and injecting the DNA sample (step 10.g) in 1 mL increments (i.e., filled-loop mode). However, users can adjust the size of sample loop according to the sample volume.

c. Program the sample run using the following parameters:
   i. After sample injection, empty the sample loop with 1 mL of DNA storage buffer, flow rate = 5 mL/min. Collect the flow through in 500 μL fractions.
   ii. Elute the DNA in 500 μL fractions with 1 CV of DNA storage buffer, flow rate = 5 mL/min.
   iii. Re-equilibrate the column with 2 CV of DNA storage buffer, flow rate = 5 mL/min. This step is needed to reduce the salt concentration before the next sample injection.
   iv. If the FPLC is equipped with a variable wavelength UV detector, set the detector to monitor at 260 nm and the excitation wavelength of your fluorophore.
d. Using a plastic syringe, slowly inject the DNA solution into the sample loop, ensuring that no air is injected into the FPLC. Do not remove the syringe from the FPLC.
e. Start the sample run.
f. Repeat steps 11.d to 11.e until all of the DNA sample has been purified over the desalting column.
g. Identify the peak fractions by absorbance at 260 nm and the excitation wavelength of your fluorophore (optional).
   i. The conductivity trace should be used to guide the selection of peak fractions; users should only pool fractions that are well-resolved from the salt peak (Figure 4).
h. Pool the peak fractions together in a new tube.

12. Sample concentration and storage.
   a. Concentrate the DNA in a 15 mL 4 kDa MWCO centrifugal device (4°C), as per the manufacturer's instructions.

Note: The final concentration of the DNA sample depends on the downstream application. For nuclease activity assays, we concentrate the solution to approximately 1 μM.

b. Transfer the concentrated DNA substrate into an appropriate microfuge tube.

Note: Unlabeled DNA can be stored in regular 1.5 mL microfuge tubes, whereas fluorescently labeled DNA should be stored in amber or black 1.5 mL microfuge tubes.

c. Determine the DNA concentration by spectrophotometry (A_{260}).
d. Store the purified DNA substrates at −20°C. Users may choose to aliquot the substrate to avoid multiple freeze-thaw cycles, depending on the final volume and concentration.

Table 2. Combinations of PAGE-purified oligonucleotides annealed to produce branched DNA substrates

DNA substrate	Fluorescently labeled oligonucleotide	Unlabeled oligonucleotides
Holliday junction	Oligo 1 (5′-FAM)	Oligos 2, 3, and 4
5′-flap	Oligo 1 (5′-FAM)	Oligos 4 and 2.5
3′-flap	Oligo 1 (5′-FAM)	Oligos 4 and 3.5
Gapped dsDNA	Oligo 5 (5′-FAM)	Oligos 6 and 7

Oligonucleotide sequences are provided in the key resources table.

Note: The extinction coefficient of the DNA substrate is the sum of the extinction coefficients of the component oligonucleotides. Extinction coefficients can be determined using one of several freely available online tools, including OligoAnalyzer™ (Integrated DNA Technologies) or OligoCalc (Northwestern University).[25]

EXPECTED OUTCOMES

Denaturing PAGE analysis and staining of synthetic oligonucleotides is expected to reveal one predominant product, representing the full-length oligonucleotide, and a series of shorter oligonucleotides that represent the -1, -2, etc. products. This gives the appearance of one intense 'blob' and a smear of lower molecular weight products (Figure 2).

The anion exchange chromatogram should contain one or two distinct peaks at A_{260} (Figure 3A). The correctly annealed DNA substrates elute from the anion exchange column at a relatively high concentration of NaCl (i.e., near the end of the elution) whereas the excess unlabeled oligonucleotides elute earlier (Figure 3A). If the FPLC is equipped with a variable UV-VIS detector, users can also monitor absorbance at the wavelength associated with the fluorophore (e.g., 498 nm for FAM). In this case, the chromatogram is expected to contain one distinct peak, which represents the fluorescently labeled DNA substrate (Figure 3A).

As an additional check, the fractions from anion exchange chromatography can be analyzed by native PAGE and in-gel fluorescence, where gels are imaged using an appropriate scanner (e.g., Typhoon FLA 9500, Cytiva). As shown in Figure 3B, the excess unlabeled oligonucleotides elute in the first peak and fluorescently labeled DNA substrates elute in the second peak. Importantly, we observe similar elution patterns for multiple types of branched DNA substrates using the anion exchange chromatography method described here (Figure 3C and Table 2).

Solutions containing a high concentration of fluorescently labeled oligonucleotides or substrates will also have a faint color that corresponds to the fluorophore modification. For example, after anion exchange chromatography, the peak fractions containing FAM-labeled DNA substrates will have a pale yellow-green hue (data not shown).

After anion exchange chromatography, the fluorescently labeled DNA substrate is in a relatively high ionic strength buffer, which could interfere with downstream DNA-binding or enzyme activity assays. Application of the fluorescently labeled DNA substrate over the HiTRAP Desalting column is needed for sample desalting and buffer exchange. Visual inspection of the A_{260} and conductivity traces reveal that the peak fractions containing the DNA substrate elute earlier than the salt peak (Figure 4). The conductivity trace should be used to guide the selection of peak fractions for subsequent DNA concentration: users should only pool peak fractions that are well-resolved from the salt peak.

This protocol will yield approximately 2 μg of high-quality fluorescently labeled DNA substrate.

LIMITATIONS

Our protocol is optimized for the purification and annealing of DNA oligonucleotides ranging in length from 10 to 60 nt. This has been applied successfully to unlabeled oligonucleotides and oligonucleotides modified with different fluorescent dyes, including FAM, Cy3, and Cy5, as well as biotin. Nevertheless, the current protocol should be tested and specific steps optimized when working with oligonucleotides that are < 10 nt or > 60 nt.

While there are many advantages to working with fluorescence compared to radioisotopes, there are also some limitations that should be considered. One is the relatively high cost of the modified oligonucleotides, due to the fact that fluorescent labels cannot be added enzymatically and need to be incorporated during oligonucleotide synthesis. Specialized equipment is also needed to detect fluorophores, including optical filters for the selective excitation and detection of fluorescence at different wavelengths. These limitations are arguably offset by the safety, speed, and ease-of-handling afforded by fluorescent dyes. Another limitation is that some fluorescent labels may not be as sensitive as radioisotopes in some applications. As such, users should consider performing a side-by-side comparison of both detection methods.

It is also important to appreciate that fluorophores are relatively large chemical moieties that can alter the binding properties of the interaction partners. The fluorescent properties of dyes can also vary considerably with the local chemical environment. For example, fluorescein and its phosphoramidite derivative called 6-carboxyfluorescein (FAM) are commonly used to label oligonucleotides. These fluorophores are sensitive to pH variations and are highly susceptible to nucleobase-quenching, most notably by guanine proximity.[27–30] The extent of sequence-dependent fluorescence quenching can also be influenced by dye placement; that is, whether the dye is attached to the 5′- or the 3′-end of the oligonucleotide.[27–30] Nucleobase identity can also influence Cy3/Cy5 fluorescence intensity.[31–33] These factors should be considered when designing fluorescently labeled DNA oligonucleotides and substrates (see troubleshooting 1).

TROUBLESHOOTING

Problem 1

Which fluorophore should I select to label the oligonucleotide? Should the fluorophore be attached to the 5′-end or the 3′-end? See before you begin: design DNA oligonucleotides and substrates (step 2).

Potential solution

As discussed in the limitations, users should consider the availability of equipment to detect fluorescence before selecting a specific dye. The oligonucleotide sequence should also be considered to avoid fluorescence-quenching. As such, no general guidelines can be given on fluorophore selection or placement. Instead, users should consider multiple experimental parameters when selecting a fluorescent dye (e.g., equipment availability, cost, yield, and assay sensitivity), while also consulting the literature on sequence-dependent fluorescence quenching.

Problem 2

There are air bubbles trapped in the preparative denaturing polyacrylamide gel after pouring. See step-by-step method details: purification of DNA oligonucleotides using denaturing PAGE and ethanol precipitation (step 1.c).

Potential solution

The glass plates must be scrupulously clean and free of particulates and debris to avoid trapping air bubbles while pouring the gel. Before and after each use, we recommend cleaning the glass plates with warm water containing a small amount of dish detergent to remove residual polyacrylamide gel bits, followed by several rinses in ddH_2O and a final rinse with 95% ethanol.

If air bubbles are trapped during pouring, users can gently tap the glass plate to dislodge the air bubbles. If the air bubble is trapped near the top of the gel, use a P200 pipette loaded with a flat gel-loading tip to remove the air bubble. Press the pipette push button to the first stop and carefully insert the pipette tip into the air bubble. Release the push button slowly and smoothly (to the top position) to aspirate the air in the bubble.

Problem 3

The oligonucleotide migrates at the same position as the bromophenol blue or xylene cyanol FF tracking dye. See step-by-step method details: purification of DNA oligonucleotides using denaturing PAGE and ethanol precipitation (step 3.d).

Potential solution

It will be difficult to discern the full-length oligonucleotide product from the tracking dye if the two species co-migrate during denaturing PAGE (refer to Table 1 for the approximate migrations of bromophenol blue and xylene cyanol FF). There are two solutions to this problem. First, the oligonucleotides can be prepared in 2× denaturing loading buffer *without* the tracking dyes. Alternatively, the bromophenol blue and xylene cyanol FF can be substituted with 0.2% (w/v) Orange G, which migrates with the buffer front in a 15% polyacrylamide gel. In each case, the formamide tracking dye solution containing bromophenol blue and xylene cyanol FF can be loaded into an unused well to monitor the extent of migration during electrophoresis.

Problem 4

How can I maximize the recovery of oligonucleotides during elution from a crushed polyacrylamide gel? See step-by-step method details: purification of DNA oligonucleotides using denaturing PAGE and ethanol precipitation (step 4.d).

Potential solution

The elution by diffusion method, or "crush and soak" technique, is a standard and simple method to recover fragments of DNA from polyacrylamide gels.[21,34] The drawback is that the process is relatively inefficient, with recovery yields ranging from 20% to 70%, depending on the size of the oligonucleotide. To maximize recovery, we recommend crushing the polyacrylamide gel into 1 mm pieces, using approximately 2 volumes of elution buffer for every 1 volume of polyacrylamide gel, and ensuring adequate mixing on the spinning wheel or during end-over-end rotation. The recovery of long oligonucleotides ($\geq$ 60 nt) may be increased further by incubation at 37°C.

Problem 5

I contaminated my oligonucleotide sample with crushed polyacrylamide gel fragments. How can I remove these before ethanol precipitation? See step-by-step method details: purification of DNA oligonucleotides using denaturing PAGE and ethanol precipitation (step 5.a).

Potential solution

Care must be taken to prevent contaminating the solution containing eluted DNA with crushed polyacrylamide gel fragments. In the event that this occurs, pellet the gel fragments by centrifugation at 3,220 × *g* for 2–5 min and then transfer the clarified supernatant to a fresh tube. Users may also consider using a gel-loading tip to pipette the supernatant containing the eluted DNA.

Problem 6

I am experiencing difficulties in resuspending the pellet recovered after ethanol precipitation. See step-by-step method details: purification of DNA oligonucleotides using denaturing PAGE and ethanol precipitation (step 6.j).

Potential solution

For difficult-to-resuspend pellets, try adding TE buffer in 100 μL increments and incubating at 37°C with gentle agitation for 20–30 min. Alternatively, incubate at 4°C with gentle agitation overnight (12–16 h). If this does not solve the problem, then the DNA sample is likely contaminated with small gel fragments (see troubleshooting 5).

Problem 7

Why do I need to slowly cool the annealing reaction overnight (12–16 h)? See step-by-step method details: annealing and purification of fluorescently labeled DNA substrates (step 9.f).

Potential solution

Slow cooling facilitates hydrogen-bonding (hybridization) between perfectly complementary oligonucleotide sequences, as opposed to hybridization between partially complementary sequences.

Problem 8

During anion exchange chromatography, I monitored absorbance at 260 nm and the excitation wavelength of my fluorophore (e.g., 498 nm for FAM). A comparison of the raw traces shows that the intensity of the peak containing the correctly annealed DNA substrate is significantly greater fat 260 nm than at 498 nm. Why? See step-by-step method details: annealing and purification of fluorescently labeled DNA substrates (step 10.f).

Potential solution

DNA absorbs light most strongly at 260 nm and the fluorophore, in this case FAM, absorbs light most strongly at 498 nm. The A_{260} of the peak containing the correctly annealed DNA substrate reflects the contribution of each oligonucleotide in the final DNA substrate whereas A_{498} provides a read-out of the FAM-labeled oligonucleotide only. Additional factors may also be contributing to fluorescence quenching (see Limitations).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Haley D.M. Wyatt (haley.wyatt@utoronto.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze any datasets/code.

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AUTHOR CONTRIBUTIONS

Y.W.E.T., H.Y.Y., and H.D.M.W. collected the data. Y.W.E.T. and H.D.M.W. wrote and edited the protocol and corresponding figures. The work was supervised by H.D.M.W. Funding was secured by H.D.M.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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