An In Vitro Selection Protocol for Threose Nucleic Acid (TNA) Using DNA Display

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ABSTRACT

Threose nucleic acid (TNA) is an unnatural genetic polymer composed of repeating threofuranosyl sugars linked by 2′ and 3′ phosphodiester bonds. TNA is capable of forming antiparallel Watson-Crick duplex structures in a self-pairing mode, and can also cross-pair opposite complementary strands of DNA and RNA. The solution NMR structure of a self-complementary TNA duplex reveals that TNA adopts an A-form helical structure, which explains its ability to exchange genetic information with natural genetic polymers. In a recent advance, a TNA aptamer was evolved from a pool of random sequences using an engineered polymerase that can copy DNA sequences into TNA. This unit details the steps required to evolve functional TNA molecules in the laboratory using a method called DNA display. Using this approach, TNA molecules are physically linked to their encoding double-stranded DNA template. By linking TNA phenotype with DNA genotype, one can select for TNA molecules with a desired function and recover their encoding genetic information by PCR amplification. Each round of selection requires ~3 days to complete and multiple rounds of selection and amplification are required to generate functional TNA molecules. *Curr. Protoc. Nucleic Acid Chem.* 57:9.8.1-9.8.19.

Keywords: threose nucleic acid (TNA) • oligonucleotide • in vitro selection • DNA display • aptamer

INTRODUCTION

This unit describes the methodology for selecting protein-binding TNA aptamers using DNA display. This approach utilizes a self-priming, hairpin library to covalently link each TNA molecule to its encoding DNA template strand. The technique itself is general and could be applied to evolve other xenonucleic acid molecules (XNA) as long as the requisite polymerase is available to copy the DNA library into the desired XNA polymer. This approach is particularly useful when an XNA-dependent DNA polymerase is not available to copy the XNA molecules back into DNA for amplification by PCR. The protocols discuss generation of the self-priming hairpin library (see Basic Protocols 1 and 2), transcription of the library into a pool of DNA-displayed TNA molecules (see Basic Protocol 3), separation of protein-binding TNA molecules using capillary electrophoresis (CE)-based selection (see Basic Protocol 4), and amplification of the enriched pool of molecules for a subsequent round of selection (see Basic Protocols 5 and 6). Functional molecules are then identified by DNA sequencing. CE-based selections usually require three to four rounds for functional molecules to dominate the pool with each round requiring 2 to 3 days to complete.

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BASIC PROTOCOL 1

GENERATION OF THE DNA HAIRPIN LIBRARY

The DNA hairpin library is generated by ligating a linear single-stranded DNA library to a synthetic DNA strand that forms a self-complementary hairpin structure (Fig. 9.8.1). The synthetic DNA library is chemically synthesized with fixed-sequence, primer-binding sites flanking each side of the random region. The DNA library can be purchased from a commercial vendor or synthesized in-house using a solid-phase DNA synthesizer (Bradley et al., 2000). The DNA hairpin strand used for primer extension and strand displacement is ligated to the 3′ end of the DNA library. This step does not require a DNA splint, since the stem-loop structure is partially complementary to a region of the DNA library.

Materials

DNA hairpin:
5′/Phos/actacgtacccacaacctcggccgtaccacggtacgtagtgacactcgtatgcagtagcc 3′
10× T4 DNA ligase buffer
Library: 5′ TGTCTACACGCAAGCTTACA-N_{50}-GGCTACTGCATACGAGTGTC 3′
100,000 U/μL T4 DNA ligase (NEB)

1.5-mL microcentrifuge tube (Eppendorf)
16° and 90°C heating blocks

Ligate hairpin to DNA library

1. To a 1.5-mL microcentrifuge tube, add:

   40 μL of 100 μM DNA hairpin
   100 μL of 10× T4 DNA ligase buffer
   740 μL of ddH_2O.

   The DNA hairpin requires a 5′ phosphate for ligation to the DNA library. The oligonucleotide can be chemically phosphorylated during solid-phase oligonucleotide synthesis or enzymatically modified after the synthesis is complete. Enzymatic phosphorylation requires incubating the synthetic DNA oligonucleotide with T4 polynucleotide kinase and ATP. It is important to heat denature and anneal the hairpin prior to incubation with the DNA library.

2. Denature and anneal the hairpin by heating tube 5 min on a 90°C heating block and cooling on ice.

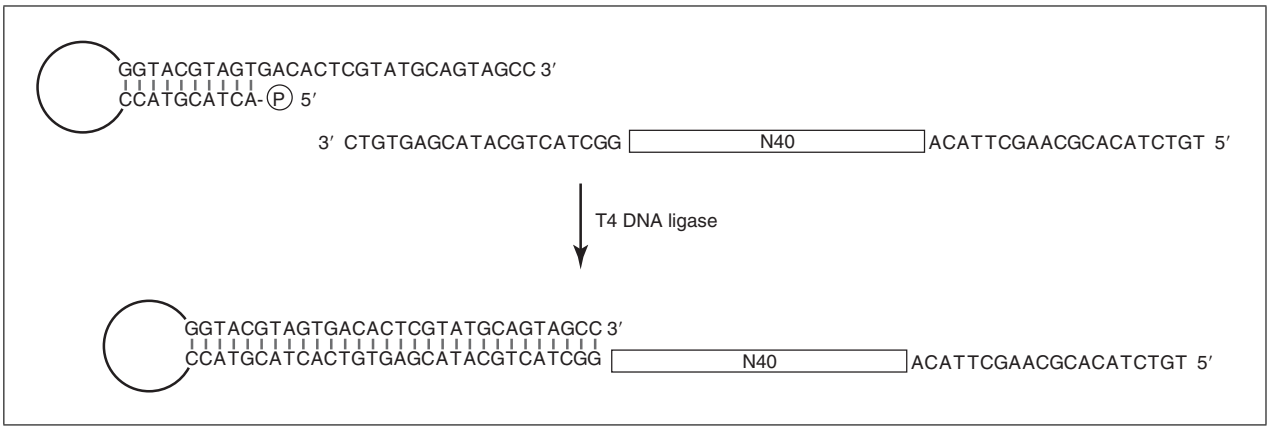

Figure 9.8.1 Generation of the DNA hairpin library. A 5′-phosphorylated hairpin is ligated to the complementary region of the DNA library using T4 DNA ligase. The product of this reaction is the self-priming DNA library used to synthesize the TNA library.

3. Add the following reagents to the ligation reaction:

   20 μL of 100 μM DNA Library (~1×10^{15} unique DNA molecules)
   100 μL of T4 DNA ligase.

4. Incubate overnight at 16°C.

PURIFICATION OF THE DNA HAIRPIN LIBRARY

BASIC PROTOCOL 2

The DNA hairpin-library is purified by denaturing polyacrylamide gel electrophoresis (PAGE), isolated from the gel, and recovered by electroelution and ethanol precipitation. A standard protocol is given here.

Materials

Acrylamide concentrate (see recipe, also available from National Diagnostics)
Acrylamide diluent (see recipe, also available from National Diagnostics)
10× TBE buffer (see recipe)
10% (w/v) ammonium persulfate (APS, EMD Biosciences)
N′,N′,N′,N′,tetraethylmethylenediamine (TEMED, Pierce)
Hairpin template reaction product (see Basic Protocol 1)
Urea (Sigma)
PAGE running dye (see recipe)
3 M sodium acetate, pH 5.2 (NaOAc, Sigma)
Absolute ethanol (Sigma)
70% ethanol, −20°C

Gel plates (19.7 × 16– and 19.7 × 18.5–cm)
Spacers (1.5-mm thick)
Comb (2-well with two marker lanes)
100-mL beaker
Magnetic stir bar and stir plate
PAGE electrophoresis apparatus
50-mL plastic syringe
Power supply
90°C heating block
Plastic transfer pipets (pulled capillary)
Spatula
Plastic wrap
UV-active thin-layer chromatography (TLC) plate
Handheld UV lamp (254-nm)
Black permanent marker
Razor blade or scalpel
Electroelution apparatus and cassettes
Electroelution membranes
1.5-mL microcentrifuge tubes (Eppendorf)
Vortex
Refrigerated microcentrifuge
Spectrophotometer

Prepare urea-PAGE gel

1. Prepare the gel plates, spacers, and comb using the manufacturer's recommended protocol.

   Purification gels are 1.5-mm thick. Using a comb with one or two wells is recommended.

2. In a 100-mL beaker, combine the following to prepare a 10% urea-PAGE gel:

20 mL acrylamide concentrate
25 mL acrylamide diluent
5 mL of 10× TBE.

3. Stir the mixture using a magnetic stir bar and magnetic stir plate until the solution is homogeneous.
4. Add 400 μL of 10% APS and 20 μL TEMED to the solution and stir for 30 sec.
5. Carefully pour the acrylamide solution into the prepared gel plates, ensuring that there are no leaks or air bubbles present between the plates.
6. Insert the comb at the top of the gel and dislodge any air bubbles by tapping on the glass.
7. Allow the solution to polymerize between the glass plates (typically 30 min).

Pre-run urea-PAGE gel

8. Carefully remove the comb and bottom spacer from the gel plates making sure not to damage the gel.
9. Rinse the gel with tap water to remove excess acrylamide or urea that may form on the outside of the glass plates.
10. Place the gel plates into the gel electrophoresis apparatus and secure the plates as suggested by the manufacturer.
11. Fill the bottom reservoir of the gel apparatus with 1× TBE buffer. Displace air bubbles that form in the bottom spacer region using a syringe containing 1× TBE buffer.
12. Fill the top reservoir of the electrophoresis apparatus with 1× TBE buffer until the solution is ~1 cm above the gel.
13. Rinse the top well with 1× TBE buffer to remove any residual polyacrylamide.
14. Connect the apparatus to the power supply and pre-run the gel for 30 min at a constant power of 20 W.

    The ideal wattage for the gel will heat the glass plates so they are warm to the touch. Temperatures greater than ~70°C can cause the glass to break.

15. While the gel is pre-running, prepare the DNA sample by adding 50% (w/v) urea and denature the sample by heating for 5 min at 90°C.

Load and run the urea-PAGE gel

16. Once the gel has finished pre-running, disconnect the gel apparatus from the power supply and rinse the wells with 1× TBE to remove any residual urea.

    Rinsing the wells prior to sample loading helps to visualize the sample as it is being loaded into the wells. The sample can also be mixed with dye to aid in visualization.

17. Load the sample into the well using a pulled plastic transfer pipet or micropipet equipped with gel loading tips.
18. Load 5 μL PAGE running dye into a small well on the side of the gel.
19. Reconnect the gel apparatus to the power supply and run the gel for 1.5 hr at a constant power of 20 W.

    The approximate location of the DNA in the gel can be inferred in relation to the running dye (see Table 9.8.1).

Table 9.8.1 Migration of Running Dye in Relation to DNA Oligonucleotide Length in Nucleotides (nt) in Different Percentages of Polyacrylamide Gels

Acrylamide (%)	Bromophenol blue (nt)	Xylene cyanol (nt)
6	25	103
8	18	75
10	14	58
12	11	48
14	9	40
16	8	35
18	7	31
20	6	28

Extract oligonucleotide from gel

20. Carefully separate the glass plates and remove the gel using a spatula to peel the gel away from the glass.

21. Wrap the gel in a single layer of plastic wrap (front and back).

22. Place the gel on a UV-active TLC plate and identify the DNA band using a handheld UV lamp set at a wavelength of 254 nm.

    The TLC plate will glow green in the presence of 254-nm UV light. Oligonucleotides present in the gel will absorb the UV light and produce a shadow on the TLC plate. Hold the UV lamp perpendicular to the gel to ensure that the shadow overlays with the DNA in the gel.

23. Using a black permanent marker, trace the DNA band on the surface of the plastic wrap.

    This step should be done quickly to avoid damage to the DNA by UV irradiation. An example of the gel electrophoretic mobility of the hairpin, library, and ligation product is shown in Figure 9.8.2.

    Wear protective glasses to protect your eyes from UV light.

24. Turn the UV lamp off and cut the DNA band out of the gel using a clean razor blade or scalpel.

Prepare electroelution apparatus

25. Assemble the electroelution apparatus according to the manufacturer's instructions.

    Crush and soak (gel diffusion; Sambrook and Russell, 2001) can be used as an alternative method to recover DNA from the gel pieces when an electroelution apparatus is not available.

26. Place the gel slices between the permeable and impermeable membranes.

27. Electroelute the DNA from the gel slices according to the manufacturer's instructions.

28. Run the current in the reverse direction for 30 sec at 200 V to dislodge any DNA that may have concentrated on the surface of the membrane.

29. Transfer the solution between the permeable and impermeable membranes to 1.5-mL microcentrifuge tubes with a maximum volume of 400 μL per tube.

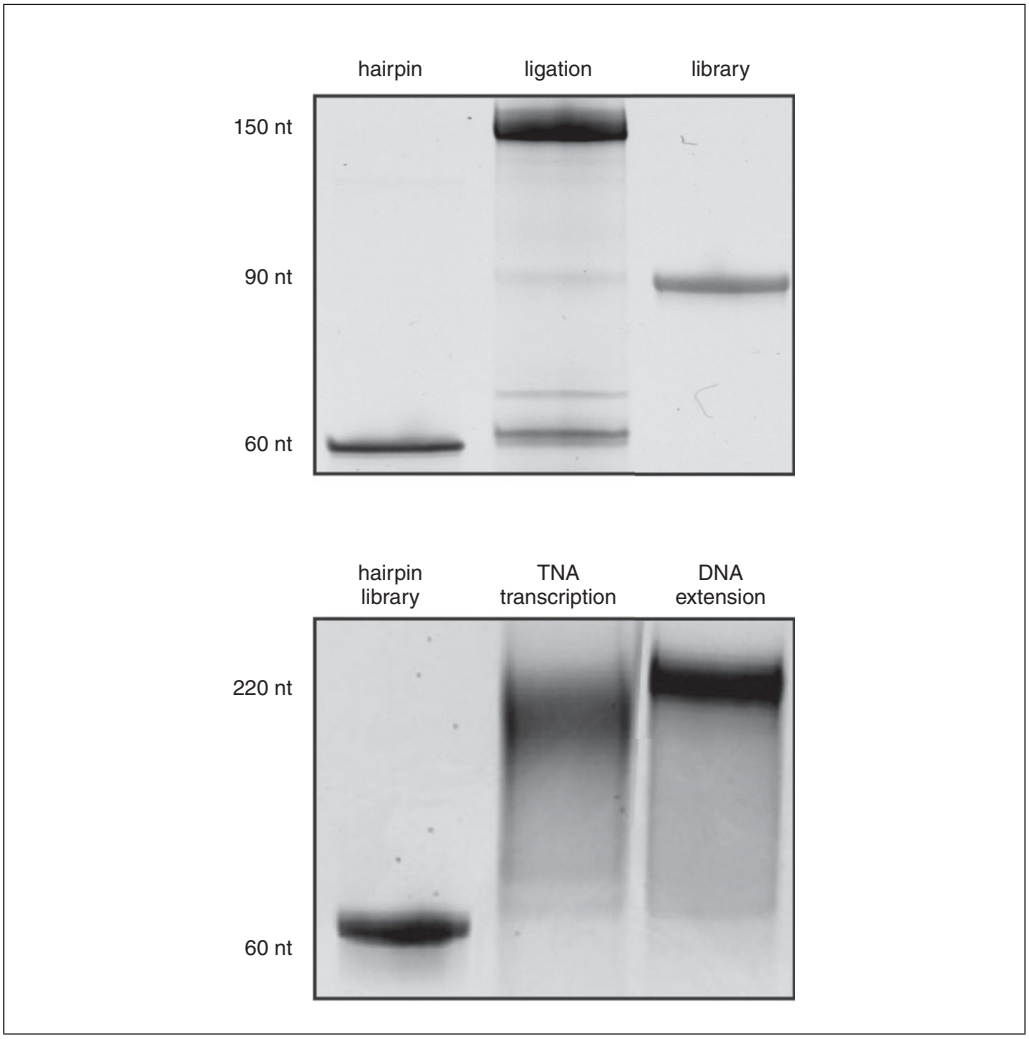

Figure 9.8.2 Electrophoretic mobility of libraries and TNA products. The 60-nt hairpin and 90-nt library combine to form a 150-nt hairpin library (top). The ligation efficiency ranges from 90% to 100%. TNA transcription of the hairpin library proceeds with high efficiency as compared to DNA extension of the same library. TNA migrates slightly faster than DNA in PAGE analysis.

Precipitate oligonucleotide with ethanol

30. Add a volume of 3 M NaOAc (pH 5.2) that is 1/10 the volume of the solution in the 1.5-mL microcentrifuge tube.
31. Add a volume of absolute ethanol that is 2.5 times the volume of solution in the 1.5-mL microcentrifuge tube. Mix the samples by inverting the tube several times.
32. Incubate each tube for 1 hr at 4°C.
33. Briefly vortex the sample to dislodge any material from the tube walls.
34. Remove tubes and microcentrifuge the mixture for at least 30 min at 18,000 × *g*, 4°C.

    The centrifugation step is a crucial part of the precipitation. The sample should be centrifuged for no less than 30 min.

35. Locate a white/translucent pellet near the bottom of the tube.
36. Remove and discard the supernatant.

37. Gently add 1 mL of cold (–20°C) EtOH to the tube, carefully invert the sample several times, and discard the supernatant. If the pellet becomes dislodged during the washing step, repeat the centrifugation step before moving forward.

38. With the pellet adhered to the surface, gently tap the tube against a paper towel to remove excess EtOH.

39. Resuspend DNA in 25 μL of ddH_2O.

40. Determine concentration of DNA by measuring UV absorbance of the oligonucleotide at 260 nm. Calculate concentration using Beer's Law: $A = E \times b \times c$, where A is the UV absorbance at 260 nm, E is the extinction coefficient of the oligonucleotide, b is the concentration in molarity, and c is the path-length of the UV cuvette.

    The average extinction coefficient for the hairpin template can be found using DNA calculators freely available on the Internet, e.g., IDT's OligoAnalyzer (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/).

GENERATION OF THE TNA LIBRARY USING DNA DISPLAY

BASIC PROTOCOL 3

The TNA library is constructed by extending the DNA hairpin library with TNA. This produces a library of DNA-TNA hairpins that are connected by an internal stem-loop structure. To display each TNA molecule on its encoding double-stranded DNA molecule, the TNA strand is displaced from the DNA strand by extending a DNA primer annealed to the stem loop region with DNA (Fig. 9.8.3).

Materials

10× Thermopol buffer (NEB)
DNA hairpin library
10 mM $MnCl_2$ (Sigma)
2 U/μL Therminator DNA polymerase (NEB)
200 μM (each) tNTPs
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol saturated with 10 mM Tris·Cl, pH 8.0 and 1 mM EDTA
100 μM strand displacement DNA primer: 5′FAM-AAG GCT ACT GCA TAC GAG TGT CAC TAC GTA CCG TGG TAC GGC CGA GGT TG 3′
5 mM (each) dNTPs

1.5-mL microcentrifuge tubes (Eppendorf)
Thermal cycler
PCR tubes
Vortexer
Microcentrifuge
NAP-5 columns (GE Healthcare)
Lyophilizer
Spectrophotometer

Perform TNA transcription of hairpin library

1. To a 1.5-mL microcentrifuge tube, add the following reagents:

    20 μL 10× Thermopol buffer
    20 μL 10 μM self-priming DNA library
    44 μL ddH_2O.

2. Denature the sample for 5 min at 95°C and cool for 10 min on ice to generate the hairpin structure at the 3′ end of the DNA library.

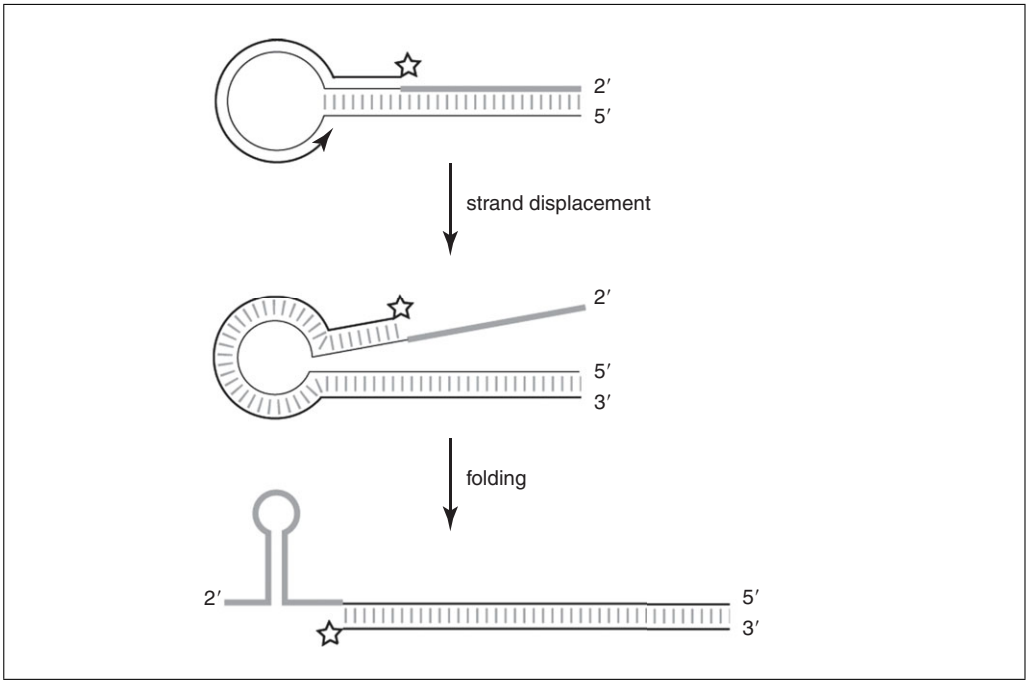

Figure 9.8.3 Displacement of the TNA molecule from its template. To liberate the TNA molecule from the DNA template, a DNA primer is annealed to the hairpin and extended by Therminator DNA polymerase. The primer is FAM-labeled for detection during capillary electrophoresis.

3. To a PCR tube on ice, add the following reagents:

   6 μL $MnCl_2$
   10 μL Therminator DNA polymerase.

   It is important to premix the $MnCl_2$ and Therminator DNA polymerase to ensure the enzyme is saturated with manganese ions.

4. Pre-heat DNA library to 55°C on a heating block.
5. Add enzyme mixture from step 3.
6. Start the reaction by adding 100 μL of tNTPs.
7. Incubate for 6 to 10 hr at 55°C.

   At this step, the transcription yield may be monitored by polyacrylamide gel electrophoresis. An example of the expected mobility for an unextended and extended library is shown in Figure 9.8.2.

Perform phenol-chloroform extraction to remove Theminator DNA polymerase

8. Add an equal volume of phenol/chloroform/isoamyl alcohol to the TNA extension product.
9. Vortex or mix vigorously.
10. Microcentrifuge 2 min at 18,000 × *g* (13,000 rpm), room temperature to separate the organic and aqueous phases and allow the sample to stand for a few minutes (until both layers are completely clear) at room temperature until both layers are clear.
11. Transfer the aqueous top layer containing the TNA extension product to a new 1.5-mL microcentrifuge tube.

    The extraction can be repeated to improve purity. However, once is generally sufficient to remove the polymerase.

Perform NAP-5 purification of TNA extension products

12. Remove cap and lid from the NAP-5 column and allow the storage buffer to drain.
13. Rinse the column three times with 3.5 mL ddH_2O, each time.
14. Add the TNA extension product to the column and allow the liquid to completely enter the column bed.
15. Add 1 mL ddH_2O and collect the elution into a 1.5-mL microcentrifuge tube.
16. Lyophilize the TNA extension product to dryness.
17. Resuspend the TNA extension product in 20 μL ddH_2O.
18. Quantify the concentration by UV absorbance as described in Basic Protocol 2.

The extinction coefficient of TNA is estimated using the extinction values for DNA.

Perform strand invasion to liberate TNA molecule

19. Combine the following reagents in a PCR tube:

    20 μL TNA-DNA library
    1 μL strand displacement DNA primer
    10 μL 10× Thermopol buffer
    14 μL ddH_2O.

20. Denature oligonucleotide 5 min at 90°C and cool for 10 min on ice to anneal primer.
21. Bring reaction to 55°C (1 min) and add 50 μL dNTPs.
22. Incubate reaction 2 min at 55°C.
23. Add 5 μL Therminator DNA polymerase.
24. Incubate 30 min at 55°C.
25. Incubate 90 min at 65°C.

Perform NAP-5 purification of TNA-DNA fusion molecules

26. Remove cap and lid from the NAP-5 column and allow storage buffer to drain.
27. Rinse the column three times with 3.5 mL ddH_2O, each time.
28. Add the TNA-DNA fusion product to the column and allow the liquid to completely enter the column bed.
29. Add 1 mL ddH_2O and collect the elution into a 1.5-mL microcentrifuge tube.
30. Lyophilize TNA extension product to dryness.
31. Resuspend the TNA extension product in 20 μL ddH_2O.
32. Quantify the concentration by UV absorbance as described in Basic Protocol 2.

SELECTION OF AN ANTI-THROMBIN TNA APTAMER BY CAPILLARY ELECTROPHORESIS

BASIC PROTOCOL 4

Capillary electrophoresis is an analytical technique to separate molecules based on differences in their mass-to-charge ratio (Mendonsa and Bowser, 2004). TNA molecules that are bound to the protein target will have an increase in their mass-to-charge ratio, which shifts their mobility in the capillary relative to the library of unbound molecules. The population of bound molecules can be isolated by directing the capillary to a collection vial at the appropriate time. As an example, this protocol describes selection of aptamers

against human α-thrombin. However, this is a general approach that could be applied to other peptide and protein targets.

Materials

TNA-DNA fusion molecules (see Basic Protocol 3)
10 μM human α-thrombin (Hematologic Technologies Inc.)
10× thrombin selection buffer (see recipe)
1% polyvinylpyrrolidine (PVP, Sigma)

200-μL PCR tubes (Eppendorf)
2-mL Glass vials (Beckman Coulter)
Glass capillary (0.1-mm i.d.; 60-cm total length)
Heating loop
Beckman ProteomeLab PA 800 protein characterization system (or another suitable system with appropriate fluorescence equipment; excitation, 488 nm; emission, 520 nm)

Incubate TNA-DNA fusion molecules with thrombin

1. Combine the following reagents in a 200-μL PCR tube:

   2.5 μL ddH_2O
   10 μL 10 μM TNA-DNA fusion molecules
   1 μL 10 μM thrombin
   1.5 μL 10× thrombin selection buffer.

   To maintain selective pressure on the population of evolving molecules, decreasing the ratio of human thrombin to TNA over the course of the selection is recommended. In the past, ratios of 10:1, 1:1, and 1:10 for rounds 1 through 3 of a selection have been used.

2. Incubate 1 hr at room temperature.

Prepare capillary electrophoresis machine

3. Prepare the following 2-mL glass vials and label them as follows:

   Water rinse: 1 mL ddH_2O
   Buffer rinse: 1 mL thrombin selection buffer
   Water dip: 1.5 mL ddH_2O
   PVP rinse: 500 μL PVP solution
   Sample: incubated DNA-TNA fusion molecules
   Anode buffer: 1 mL thrombin selection buffer
   Cathode buffer: 1 mL thrombin selection buffer.

   The sample vial contains a spring to hold the small PCR tube in place.

4. Cut a 0.1-mm i.d. glass capillary to a final length of 60 cm.

5. Generate a window according to manufacturer's recommended length by inserting the capillary through a heating loop. Wipe the capillary with a Kimwipe to remove any remaining debris.

6. Carefully slide capillary into the cassette holder and attach it to the protein characterization system.

7. Turn on the laser and detector.

8. Run the following protocol with their corresponding vials to prepare capillary for separation:

Water rinse	1 min	20 psi
Buffer rinse	1 min	20 psi
Water dip	10 sec (removes excess buffer from capillary exterior)	
PVP rinse	1 min	20 psi (coats capillary with PVP)
Buffer rinse	2 min	20 psi (removes excess PVP and prepares for separation)

Separate bound from unbound molecules by capillary electrophoresis

9. Inject 70 nL of sample by placing the capillary in the sample tube and running 5 sec at 0.5 psi.
10. Dip capillary in the water dip vial to remove excess sample.
11. Move capillary ends to the anode and cathode buffer vials. Apply 10 kV constant for 30 min and monitor the fluorescence emission over time.
12. Repeat steps 9 through 11 four times for a total of five separations.

GENERATION OF ENRICHED DNA POOL

BASIC PROTOCOL 5

Molecules that survive the selection are amplified by PCR and the resulting double-stranded DNA product is separated into single-stranded material to regenerate the library for the next round of selection. The first step is to determine the appropriate number of cycles for PCR amplification, paying special attention not to over amplify the DNA pool. This step is often called test PCR or cycle optimization. Since amplifying a pool is costly in terms of time and money, any optimization of the PCR should take place on a small scale. The more involved large-scale amplification can then be carried-out using the optimized PCR conditions.

Materials

10× Thermopol buffer
5 mM (each) dNTPs
Biotinylated regeneration primer: 5′/5Biosg/GGCTACTGCATACGAGTGTCA CTACGTACCGTGGTACGGCCGAGGTTGTG 3′
PBS1: 5′ TGTCTACACGCAAGCTTACA 3′
Recovered selection output
2 U/μL *Taq* DNA polymerase
6× agarose loading dye
Ultrapure agarose powder
0.5× TBE buffer (see recipe)
10 mg/mL ethidium bromide
100-bp DNA ladder
PCR purification kit (Qiagen)

200-μL PCR tubes
Thermal cycler
500-mL flask
Gel casting tray and comb
Agarose gel electrophoresis apparatus
Power supply
UV imaging source
Spectrophotometer

Perform cycle optimization of selection output

1. Combine the following reagents into a 200-μL PCR tube:

73 μL ddH_2O
10 μL 10× Themopol buffer
8 μL dNTPs
1 μL Biotinylated regeneration primer
1 μL PBS1
5 μL selection output (5% total volume)
2 μL 2 U/μL *Taq* DNA polymerase.

Run a no-template control to ensure that the reagents are not contaminated prior to amplification. Contamination would introduce non-functional molecules into the enriched pool that would diminish the efficiency of the selection. However, it is important to note that the polymerase tends to amplify material regardless of template during the later cycles (from 28 to 35).

2. Place tube in the thermal cycler and run the following program:

1 cycle:	2 min	95°C
30 cycles:	15 sec	95°C
	15 sec	60°C
	45 sec	72°C

3. At the end of every third extension steps (72°C step), transfer 5 μL of PCR reaction to a tube containing 1 μL of 6× agarose loading dye for a total of 30 cycles, resulting in ten tubes.

The addition of a pause step between every three cycles in the PCR program ensures that an aliquot is not missed. It is best to remove the aliquot as soon as the designated extension step ends.

Prepare agarose gel

The agarose gel may be prepared while the cycle optimization is running so that it is ready for use when finished.

4. In a 500-mL flask, combine 3 g of ultrapure agarose powder with 120 mL of 0.5× TBE buffer.
5. Gently mix the solution.
6. Heat the solution in a microwave for 1 min at maximum power.
7. Mix the solution vigorously to ensure even heat distribution.
8. Microwave the solution for an additional 1 min.
9. Remove the solution from the microwave and add 2 μL ethidium bromide.

CAUTION: *The solution and flask are very hot. Use the appropriate safety precautions. Additionally, ethidium bromide is a toxin. Be very careful. If the laboratory is not equipped to handle ethidium bromide, Sybr Safe (Invitrogen) may be used instead.*

10. Mix the solution gently ensuring an even distribution of ethidium bromide without creating too many air bubbles.

Remove any superficial bubbles with a pipet tip.

11. Carefully pour the heated solution into an agarose casting tray with the appropriate comb.
12. Allow gel to solidify for ~30 min at room temperature.
13. Carefully remove the comb ensuring that the wells stay intact.

Load sample and run agarose gel

14. In an agarose gel electrophoresis apparatus, place the gel into its appropriate location.
15. Fill the apparatus with enough 0.5× TBE to cover the gel by ~0.5 cm.
16. Load a 100-bp DNA ladder into the first well according to the manufacturer's recommendations.
17. Load the complete 6 μL from each sample into subsequent wells.
18. Attach the lid of the electrophoresis unit and plug into a power supply.
19. Run the agarose gel 40 min at a constant 90 V.
20. Image by the gel UV.
21. Identify the cycle number just before the amplification plateaus.

    Over amplification of a DNA library can produce multiple PCR products that are observed on an agarose gel. To avoid this problem, the optimal number of PCR cycles should be determined for each round of selection by performing one or more test PCR reactions on a small amount of the library (see Bradley et al., 2000 for examples).

Large-scale PCR of selection output

22. Set up 19 additional PCR reactions, using the same conditions determined by cycle optimization.
23. Perform the thermal cycle program for the total cycles identified in step 21.
24. Clean up the reactions using Qiagen's PCR purification kit following the manufacturer's recommendations.

    Each of the 19 reactions should be passed through the same Qiagen column to maximize the final concentration. Elute the column in ddH_2O rather than elution buffer.

25. Measure concentration by UV absorbance using Beer's Law (see Basic Protocol 2).
26. Save resulting PCR product at −20°C. For storage >1 year, store at −80°C.

GENERATION OF SINGLE-STRANDED HAIRPIN TEMPLATES FOR A NEW SELECTION ROUND

BASIC PROTOCOL 6

After the large-scale PCR amplification is complete, a portion of the amplified DNA will be used to generate single-stranded templates for the next round of selection. In this step, the non-template strand is immobilized on a streptavidin-agarose resin using a biotinylated tether. Base-mediated melting of the DNA duplex will denature and remove the non-biotinylated top strand.

Materials

Biotinylated DNA (see Basic Protocol 5)
Streptavidin-agarose resin (Pierce)
Streptavidin binding buffer (see recipe)
100 mM NaOH
100 mM HCl
3 M sodium acetate, pH 5.2 (NaOAc, Sigma)
5 mg/mL glycogen (Pierce)
Absolute ethanol (Sigma)
70% ethanol, −20°C

Small chromatography column (BioRad)
Rotator
1.5-mL microcentrifuge tubes (Eppendorf)
Vortexer
Refrigerated microcentrifuge
Spectrophotometer

Bind and elute biotinylated DNA on streptavidin agarose

1. Combine 25 μL biotinylated DNA and 175 μL streptavidin binding buffer.

   CAUTION: *Do not use >50% of the PCR product. If the selection output runs out, then the selection round must be repeated. If more material is needed, amplify more DNA by PCR before moving on.*

2. Add 100 μL streptavidin-agarose resin to a small chromatography column.
3. Wash column five times with 1 mL streptavidin binding buffer, each time, to equilibrate the resin.
4. Cap the bottom of the column and add the biotinylated DNA. Mix by gently tapping the side of the column. Cap the top of the column.
5. Incubate 30 min at room temperature with rotation or by gently flicking the column every few minutes.
6. Uncap the top and bottom of the column and collect the flow through in a 1.5-mL microcentrifuge tube.
7. Wash column five times with 1 mL streptavidin binding buffer, each time, to remove any unbound molecules.
8. Cap the bottom of the column and add 100 μL of 100 mM NaOH and incubate 10 min at room temperature. Uncap the bottom of the column. Collect the elution.
9. Repeat five more times for a total of six separate NaOH elutions.
10. Neutralize each of the elutions by adding 100 μL of 100 mM HCl to each tube.

Precipitate oligonucleotide with ethanol

11. Add a volume of 3 M NaOAc (pH 5.2) that is 1/10 the volume of solution in the 1.5-mL microcentrifuge tube.
12. Add 4 μL glycogen to each tube.

    Glycogen acts as a carrier molecule for precipitating small amounts of material.

13. Add a volume of absolute ethanol that is 2.5 times the volume of solution in the 1.5-mL microcentrifuge tube. Mix the samples by inverting the tube several times.
14. Incubate each tube 1 hr at 4°C.
15. Briefly vortex the sample to dislodge any material from the tube walls.
16. Remove tubes and centrifuge mixture for at least 30 min at 18,000 × *g* (13,000 rpm), 4°C.

    The centrifugation step is a crucial part of the precipitation. The sample should be centrifuged for no less than 30 min.

17. Locate a white/translucent pellet near bottom of tube.
18. Remove and discard supernatant.

19. Gently add 1 mL of cold (–20°C) EtOH to the tube and carefully invert the sample several times and discard the supernatant. If the pellet becomes dislodged during the washing step, repeat the centrifugation step before moving forward.

20. With the pellet adhered to the surface, gently tap the tube against a paper towel to remove excess EtOH.

21. Resuspend DNA pellet in 10 μL ddH_2O.

22. Determine concentration of the DNA by measuring the UV absorbance of the oligonucleotide at 260 nm. Calculate concentration using Beer's Law $A = E \times b \times c$, where A is the UV absorbance at 260 nm, E is the extinction coefficient of the oligonucleotide, b is the concentration in molarity, and c is the path-length of the UV cuvette.

    The average extinction coefficient for the hairpin template can be found using DNA calculators freely available on the Internet, e.g., IDT's OligoAnalyzer (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/).

REAGENTS AND SOLUTIONS

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

Acrylamide concentrate (29:1)

241.6 g acrylamide
8.3 g methylene bisacrylamide
7.5 M urea
ddH_2O to 1 L
Store in amber bottle up to 1 year at room temperature

CAUTION: *Acrylamide and bisacrylamide are hazardous. Use appropriate safety precautions and laboratory apparel.*

Acrylamide diluent

7.5 M urea (Sigma)
Store in amber bottle up to 1 year at room temperature

Agarose loading dye, 6×

10 mM Tris·Cl (*APPENDIX 2A*)
60 mM EDTA
50% glycerol
0.05% Orange-G dye
Store up to 1 year at room temperature

PAGE running dye

0.05% (w/v) bromophenol blue
0.05% (w/v) xylene cyanol
1× TBE (see recipe)
Store up to 1 year at room temperature

Streptavidin binding buffer

10 mM Tris·Cl (*APPENDIX 2A*)
50 mM NaCl
1 mM EDTA
Store up to 6 months at room temperature

TBE buffer, 10×

1 M Tris·Cl (*APPENDIX 2A*)
1 M boric acid
10 mM EDTA, pH 8
Store up to 6 months at room temperature

Thrombin selection buffer, 10×

50 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
100 mM NaCl
1 mM $MgCl_2$
Store up to 6 months at room temperature

COMMENTARY

Background Information

Threose nucleic acid

α-L-Threofuranosyl-(3′-2′) nucleic acid (TNA) is an unnatural genetic polymer composed of repeating threofuranosyl nucleotides linked by vicinally connected 2′,3′-phosphodiester bonds (Schöning et al., 2000, 2002). TNA has attracted significant attention as an RNA analog for biomedicine because TNA polymers are resistant to nuclease degradation and maintain stable Watson-Crick duplex structures opposite complementary strands of DNA and RNA (Yu et al., 2013). This latter observation is remarkable considering that TNA polymers have a backbone repeat unit that is one atom shorter than the backbone repeat unit found in DNA and RNA. The solution NMR structure of a self-complementary TNA octamer reveals that TNA-TNA duplexes adopt an A-form helical structure that closely approximates the helical geometry of A-form RNA (Pallan et al., 2003; Ebert et al., 2008). The structural parameters of chimeric TNA-DNA and TNA-RNA duplexes are not yet known.

In addition to its possible applications in biomedicine, TNA has also been the focus of numerous studies aimed at evaluating TNA as an ancestral genetic material that may have contributed to the origin and early evolution of life on Earth (Engelhart and Hud, 2010; Joyce, 2012). According to the pre-RNA world hypothesis, life arose through a series of discrete chemical steps in which prebiotic chemistry gave rise to different types of information-carrying biopolymers. Then, at some later point in history, these pre-RNA world polymers were superseded by RNA-based life forms that gave rise to modern life with DNA genomes and protein enzymes. In this regard, TNA is an attractive RNA progenitor candidate, because threose is a chemically simpler sugar to synthesize under prebiotic conditions than ribose, and TNA has the ability to exchange genetic information with RNA. This later point is important because it provides a realistic model for the transfer of genetic information between successive genetic systems.

Motivated by the desire to create biologically stable molecules for molecular medicine and biotechnology and interest in life's origins, the authors have developed an in vitro selection strategy to evaluate the functional properties of TNA. Key to the development of this technology was the discovery that a genetically engineered form of the native DNA polymerase from the *Thermococcus* species 9°N functions as an efficient DNA-dependent TNA polymerase (Ichida et al., 2005b). This enzyme, known commercially as Therminator DNA polymerase, can copy three-nucleotide DNA libraries into TNA with high efficiency and fidelity; however, the polymerase stalls when it encounters G residues in the DNA template. Therminator DNA polymerase carries the mutation A485L in addition to the exonuclease silencing mutations D141A and D143A. Because a TNA-dependent DNA polymerase was not available to reverse transcribe TNA polymers back into DNA for amplification using the polymerase chain reaction (PCR), the authors devised a one-enzyme selection strategy that establishes a genotype-phenotype link by displaying individual TNA molecules on their encoding double-stranded DNA sequence (Ichida et al., 2005a). With this strategy, it is possible to select for a particular TNA function (phenotype) and recover the encoding genetic information (genotype) by amplifying the attached DNA sequence by PCR. In a recent demonstration, the authors have successfully used this approach to isolate a TNA aptamer that binds to human α-thrombin with high affinity and high specificity (Yu et al., 2012).

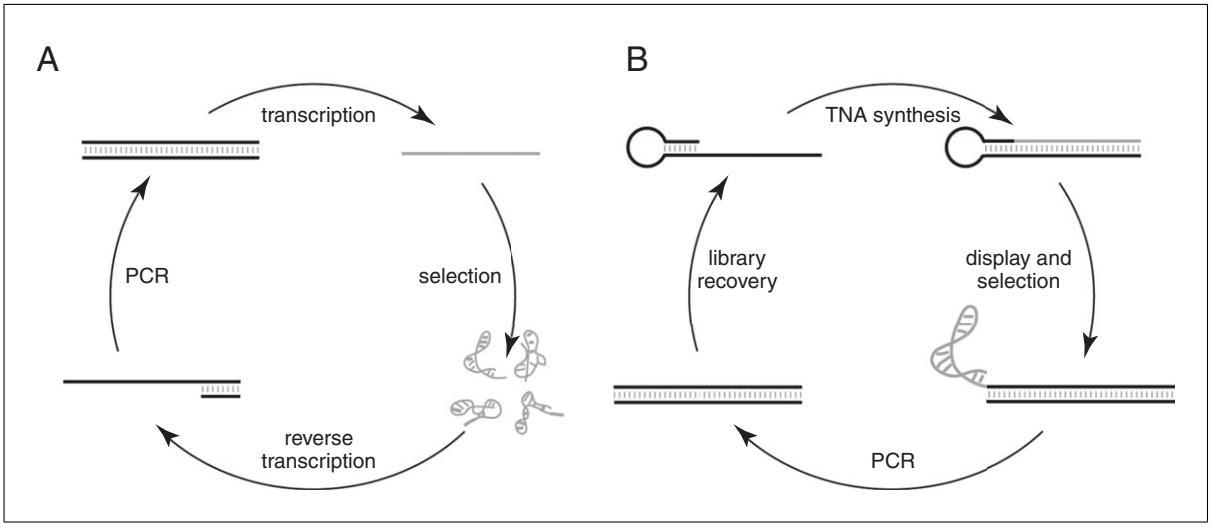

Figure 9.8.4 Traditional in vitro selection versus DNA display. (**A**) Traditional in vitro selection (SELEX) of RNA molecules involves transcribing a DNA library into RNA, selecting for binding, and amplifying the selected molecules by reverse-transcription PCR. (**B**) For DNA display, the selection process involves extending a self-priming DNA library with TNA to create a pool of TNA-DNA hairpin sequences. The TNA strand is then displaced in a second primer extension step in which a DNA primer annealed to the stem loop region is extended across the DNA region of the DNA-TNA hairpin. TNA molecules displayed on their double-stranded DNA templates are incubated with a target molecule and functional molecules are recovered and amplified by PCR.

This unit describes the steps required to synthesize and evolve functional TNA molecules using DNA display. A general overview of the methodology is first described followed by a detailed step-by-step protocol for each step in the synthesis and selection process.

Overview of in vitro selection

In vitro selection, also known as SELEX (systematic evolution of ligands by exponential enrichment; *UNIT 9.1*) is a powerful molecular biology technique commonly used to evolve nucleic acid molecules that can bind to a desired target or catalyze a specific chemical reaction. Starting from a large library of nucleic acid molecules (typically 10^{15} different sequences), functional molecules are isolated through iterative rounds of in vitro selection and amplification (Figure 9.8.4A; Joyce, 1994; Gold et al., 1995). In a manner analogous to natural selection, molecules that survive the selection increase in abundance, while molecules that lack the desired function go extinct. Amplification is typically performed using PCR, but it is also possible to amplify molecules under isothermal conditions using RNA transcription. By increasing the selective pressure, e.g., by reducing the incubation time or substrate concentration, it is possible to identify individual molecules with superior functional properties. In vitro selection has been used to study basic questions in molecular evolution as well as develop molecules with practical applications in molecular medicine. In addition to Macugen, which is an RNA aptamer approved by the FDA for the treatment of macular degeneration, many aptamers are now in clinical trials in the U.S. and Europe (Ni et al., 2012).

Library modification for selecting functional TNA molecules

Each library in an in vitro selection experiment has two fundamental components, the variable region and two constant primer-binding sites that are used for amplification. The composition of the random region can vary depending on the needs of the individual researcher, but in many cases random regions of 40 to 50 unbiased nucleotides (equal distribution of A, C, G, and T) are sufficient to identify functional molecules with ligand binding affinity. The fixed-sequence regions are designed as orthogonal primer binding sites that can be used to selectively amplify only those molecules that are present in the library. It is advisable to perform a negative control, no-template PCR reaction before and during the selection to ensure that PCR contaminants are not being introduced into the pool during library amplification.

For more than two decades, in vitro selection experiments have been performed on DNA and RNA. Moving beyond natural genetic polymers (or close structural analogues thereof) requires polymerases that can transcribe, reverse transcribe, and amplify the

nucleic acid sequences encoded in unnatural genetic polymers (Fig. 9.8.4B). Recent advances in nucleic acid chemistry and protein evolution strategies have made it possible to copy artificial genetic polymers using genetically engineered DNA polymerases (Pinheiro et al., 2012). In the case of TNA, for example, Therminator DNA polymerase has been identified as an engineered enzyme that can copy DNA templates into TNA (Ichida et al., 2005b; Yu et al., 2013). Since a reverse transcriptase that converts TNA sequences into DNA sequences was not available at that time, the authors devised a one-enzyme in vitro selection strategy that involves displaying TNA molecules on their encoding double-stranded DNA sequence (Ichida et al., 2005a). This was achieved by extending a library of self-priming DNA templates with TNA to produce a library of DNA-TNA hairpin structures. Next, the TNA strand is displaced using a strand invasion strategy that involves extending a DNA primer annealed to the loop region with DNA. The product of the strand displacement step is a TNA molecule that is covalently linked to its own double-stranded DNA message. With this configuration, it is possible to select TNA molecules with a desired function and recover their encoding genetic information by amplifying the DNA by PCR. This protocol describes the library modifications required to display TNA molecules on their encoding DNA templates.

Critical Parameters and Troubleshooting

There are a few critical parameters that need to be addressed when transcribing TNA polymers using Therminator DNA polymerase. First, the $MnCl_2$ solution must be freshly prepared. $MnCl_2$ oxidizes in aqueous solutions, which changes its activity in the polymerase extension assay. It is possible to store the solution for up to 1 week at −20°C; however, this can lead to differences in TNA synthesis efficiency. Also, $MnCl_2$ and Therminator DNA polymerase should be pre-mixed prior to adding the primer template complex. Adding all of the reagents at the same time to the reaction tube produces significantly less TNA product.

Each step of the reaction can be monitored by polyacrylamide gel electrophoresis. However, it is important to note that TNA does not stain with ethidium bromide or SYBR green. The DNA portion of the DNA display molecule will stain with these reagents.

During the capillary electrophoresis separation step, researchers should collect all of the fractions to determine which vial contains the protein-bound TNA molecules. The bound complex can be identified using a PCR assay to see which tube contains the TNA-DNA fusion molecules. Once these vials are identified, they can be combined and amplified on a larger scale to regenerate the library for another round of in vitro selection and amplification.

Anticipated Results

Generating the hairpin library is a relatively efficient procedure. Ligation efficiency will range from 90% to 100%. However, PAGE purification of the reacted molecules is oftentimes inefficient. About 30% to 50% recovery of the input should be expected after purification. It is advised that the researcher take these losses into account when preparing the starting libraries.

Both TNA transcription of three nucleotide libraries and strand displacement reactions are relatively efficient with >90% conversion to full-length product. Electrophoretic mobility assays can be used to monitor extension efficiencies by running a small portion of the sample before and after each step. It is advised to use ~1 pmol of material for radioactive and 10 pmol of material for fluorescent analysis.

Capillary electrophoresis is an efficient means for separating bound versus unbound molecules. Selections using this methodology tend to be finished in three or fewer rounds of selection.

It is notable that cycle optimizations tend to require fewer cycles as the selection progressed as the number of bound molecules increases.

Time Considerations

Each round of DNA display selection will take 3 days to complete. However, the entire selection can be completed in ~2 weeks, since capillary electrophoresis selections generally require only three or four rounds of selection. This is a significantly shorter time frame than traditional selections, which often require ten or more rounds of selection to complete.

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