Chapter 4

DNA Aptamer Generation by Genetic Alphabet Expansion SELEX (ExSELEX) Using an Unnatural Base Pair System

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Abstract

Genetic alphabet expansion of DNA using unnatural base pair systems is expected to provide a wide variety of novel tools and methods. Recent rapid progress in this area has enabled the creation of several types of unnatural base pairs that function as a third base pair in polymerase reactions. Presently, a major topic is whether the genetic alphabet expansion system actually increases nucleic acid functionalities. We recently applied our unnatural base pair system to in vitro selection (SELEX), using a DNA library containing four natural bases and an unnatural base, and succeeded in the generation of high-affinity DNA aptamers that specifically bind to target proteins. Only a few hydrophobic unnatural bases greatly augmented the affinity of the aptamers. Here, we describe a new approach (genetic alphabet *Ex*pansion *SELEX*, ExSELEX), using our hydrophobic unnatural base pair system for high affinity DNA aptamer generation.

Key words Genetic alphabet expansion, Unnatural base pair, Aptamer, SELEX, In vitro selection, PCR

1 Introduction

Replication and transcription of nucleic acids rely on two sets of base pairs, A–T and G–C, and thus the functions of nucleic acid molecules, such as aptamers that specifically bind to target molecules, generated by in vitro selection (SELEX) involving PCR amplification [1, 2], are restricted within this limited number of only four natural base components. To further increase the affinity and stability of nucleic acid aptamers and the success rates of aptamer generation, several modified components based on the natural bases have been applied to SELEX [3–5]. However, these natural-base modification methods have not yet substantially improved the affinities of aptamers with high specificity to target proteins.

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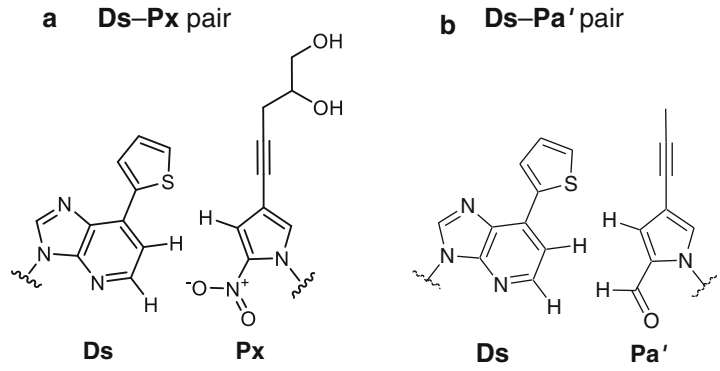

Fig. 1 Chemical structures of the Ds–Px and Ds–Pa′ pairs. (**a**) The Ds–Px pair functions as a third base pair in PCR with high efficiency and fidelity, by using both the Ds and Px substrates. (**b**) The Ds–Pa′ pair is employed for replacement PCR, by using just the Pa′ substrate, without dDsTP

Another approach toward the substantial improvement of SELEX might be the expansion of the genetic alphabet of nucleic acids. As compared to antibodies composed of 20 different amino acids, standard nucleic acids consist of only four nucleotide bases with similar chemical and physical properties. Thus, increasing the number of nucleotides by creating artificial extra bases (unnatural bases) with different properties had been expected to augment nucleic acid functionality, such as aptamer affinities. As a requirement for use in the genetic alphabet expansion in SELEX, an unnatural base should function as a third base pair with its counterpart in PCR amplification.

So far, three types of unnatural base pairs have been created for conventional PCR amplification [6–10]. Among them, the hydrophobic base pair between 7-(2-thienyl)imidazo[4,5-*b*]pyridine (Ds) and 2-nitro-4-propynylpyrrole (Px) that we developed exhibits high fidelity and efficiency in PCR, by either DeepVent or AccuPrime*Pfx* DNA polymerase (Fig. 1a) [7, 8].

Recently, we developed a new SELEX method using the Ds–Px pair (genetic alphabet *Ex*pansion *SELEX*, ExSELEX), for generating DNA aptamers from a library containing hydrophobic Ds bases (Fig. 2) [11]. We added only the Ds base as the fifth base to the library for two reasons: (1) the hydrophobic Ds base strengthens the interactions with hydrophobic portions in target proteins (increasing the chemical diversity), and (2) in the absence of the pairing partner Px bases in the library, the Ds bases cannot pair in the tertiary structures of each DNA fragment (increasing the structural diversity).

A major problem in developing ExSELEX is the difficulty in determining the sequence of each aptamer in the isolated DNA library, after finishing several rounds of selection and PCR amplifi-

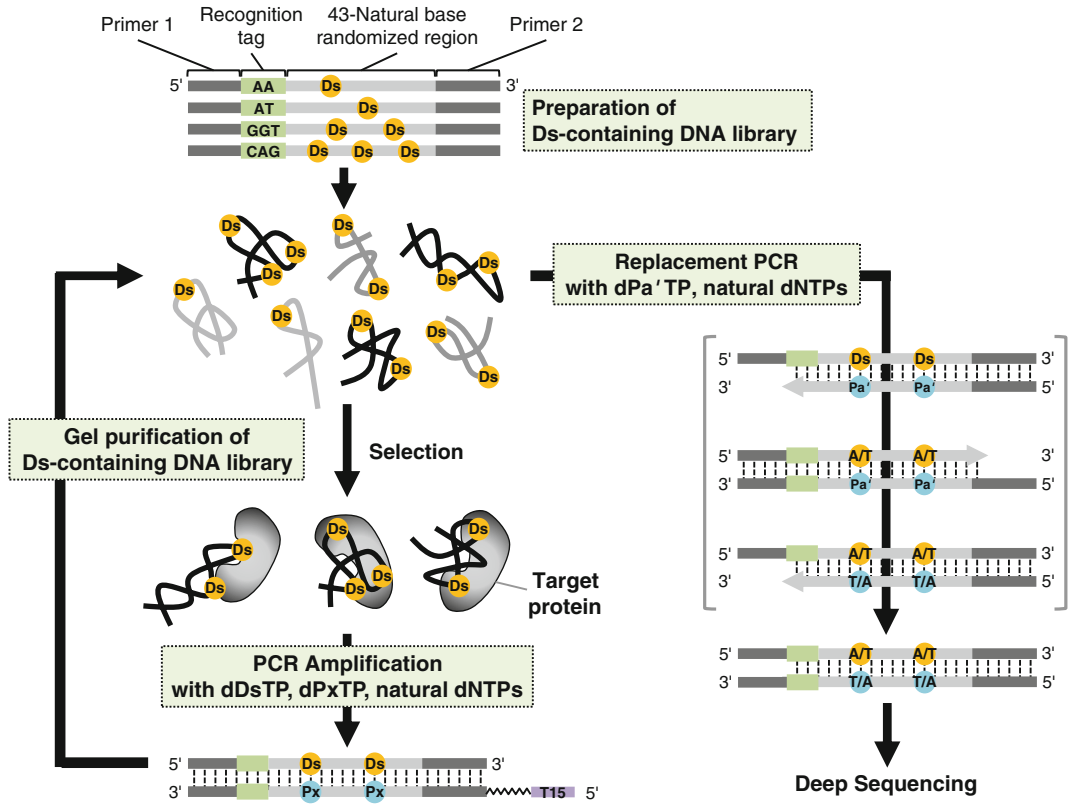

Fig. 2 Scheme of genetic alphabet expansion SELEX (ExSELEX) for generating DNA aptamers from a library containing hydrophobic Ds bases. The steps described in this chapter are highlighted in *boxes*

cation. In conventional SELEX, each aptamer sequence can be determined by cloning and sequencing, or deep sequencing using a next-generation sequencer. However, at present, we have no means of utilizing these methods and sequencers involving unnatural base pairs. To address this difficulty, we developed a new method for ExSELEX, using a DNA library containing more than 20 different sublibraries (Fig. 2) [11].

Each sublibrary consists of a 43-natural-base randomized region, in which one to three Ds bases are embedded at different predetermined positions in each sublibrary, a recognition tag sequence with two or three natural bases, which is specific to each sublibrary, and PCR primer sequences at both termini. Each sublibrary is chemically synthesized and mixed to prepare the ExSELEX DNA library.

In ExSELEX, conventional methods are employed for the selection step of isolating DNA fragments that bind to target proteins from the library. After selection, the isolated DNA fragments are amplified by PCR using AccuPrime*Pfx* DNA polymerase with the four natural base substrates and two unnatural base substrates, Ds and Px. For PCR in ExSELEX, AccuPrime *Pfx* DNA poly-

merase is better than DeepVent DNA polymerase. Although the fidelity of the Ds–Px pair using DeepVent DNA polymerase is slightly higher than that using AccuPrime *Pfx* DNA polymerase, the PCR amplification efficiency and sequence independence using AccuPrime *Pfx* DNA polymerase are superior to those using DeepVent DNA polymerase [8]. To separate the single-stranded Ds-containing DNA fragments from the amplified duplexes, PCR is performed using a primer linked with an extra oligo-T (T15) sequence, via a C12 alkyl spacer.

After several rounds of selection and PCR amplification, the Ds bases in the selected DNA library are replaced with natural bases (mainly adenine bases) by PCR amplification in the absence of dDsTP and dPxTP (replacement PCR). However, the mutation of the unnatural to natural bases by replacement PCR is inefficient, due to the high fidelity of the Ds–Px pair in PCR. Thus, another unnatural base substrate, a nucleoside triphosphate of 4-propynylpyrrole-2-carbaldehyde (Pa′) [6, 12], is added as a mediator from the unnatural to natural base replacement in PCR. The fidelity of Pa′ as a pairing partner of Ds is lower than that of the Px base, and thus the misincorporation rate of natural base substrates opposite Pa′ is increased after the Pa′-incorporation into DNA opposite Ds (Fig. 1b). For replacement PCR, we use TITANIUM Taq or AccuPrime*Pfx* DNA polymerase. After replacement PCR, each sequence consisting of natural bases is determined by deep sequencing, and the original Ds positions in each sequence are identified from the recognition tag sequence. From the determined sequences, Ds-containing DNA aptamers are chemically synthesized, and the affinity of each DNA aptamer is evaluated by binding assays with the target proteins [11].

Here, we describe the several steps focused on the preparation of the Ds-containing DNA library, the PCR amplification, and the replacement PCR in ExSELEX, since any conventional processes can be chosen, depending on the target proteins, for the other steps (selection method and sequencing determination) [13–18]. However, the unnatural base pair technology is quite new, and the present ExSELEX method may be modified according to future improvements in the unnatural base pair systems.

2 Materials

The common materials used in the experiments are listed below (*see* **Note 1**).

1. Apparatus for gel electrophoresis (*see* **Note 2**).
2. Nuclease-free, deionized, sterile water.
3. 40 % acrylamide–bis (19:1) solution (*see* **Note 3**).

4. Nuclease-free 10× TBE: 0.89 M Tris base, 0.89 M boric acid, and 0.02 M EDTA (*see* **Note 2**).
5. Urea (ultrapure grade).
6. Acrylamide–bis solution with urea in 1× TBE (*see* **Note 4**).
7. APS: 10 % ammonium persulfate (w/v). This can be stored at 4 °C for 1–2 months (*see* **Note 5**).
8. ≥99 % TEMED: *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (*see* **Note 5**).
9. Polyacrylamide gel containing 7 M urea (*see* **Note 5**).
10. TLC plate containing a UV fluorescent indicator (*see* **Note 6**).
11. Handheld UV lamp (254 nm).
12. Saran Wrap.
13. Razor blade.
14. Filter units: Ultrafree-CL and Steriflip (pore size 0.22 μm, Millipore) (*see* **Note 7**).
15. 3 M sodium acetate, pH 5.2 (*see* **Note 8**).
16. 99.5 % ethanol (*see* **Note 8**).
17. Denaturing gel-loading buffer: 10 M urea solution in 1× TBE (*see* **Note 9**).
18. 2 mM each dNTP mix: Mix equal amounts of standard deoxyribonucleoside triphosphates of the natural bases (dATP, dTTP, dCTP, and dGTP), and dilute into a 2 mM working solution. Aliquots of the solution should be stored at −10 to −30 °C.
19. DNA ladder marker (e.g., 10, 25, or 100 bp ladder).
20. 10 mg/ml ethidium bromide (*see* **Notes 2** and **10**).
21. 4 % agarose gel: To prepare a 4 % agarose gel, add 4 g of NuSieve 3:1 Agarose (Lonza) to 100 ml of 1× TBE, and heat until the agarose is completely dissolved. Add 10 mg/ml ethidium bromide at the final proportion of 0.1 μl/ml, pour the gel in a gel-casting chamber, insert the comb and let cool at room temperature for at least 30 min.
22. Agarose-loading buffer: 30 % glycerol, 0.025 % (w/v) BPB.

2.1 Preparation of Ds-Containing DNA Library

1. Chemically synthesized Ds-containing DNA sublibraries (*see* **Note 11**): The DNA library with the sequence, 5′-CTGT CAATCGATCGTATCAGTCCAC-$Y2_{-3}$-N_{43}-GCATGACTCG AACGGATTAGTGACTAC-3′, is designated as N43Ds and used in our aptamer selection (*see* **Note 12**) [11]. Y_{2-3} is a recognition tag sequence with two or three natural bases. N_{43} is a randomized sequence with 43 natural bases, in which one to three Ds bases are embedded at different predetermined positions in each sublibrary. Underlined sequences are constant

regions for annealing sites with forward and reverse primers in PCR amplification.

2. 8 % polyacrylamide gel, 7 M urea (20 cm × 40 cm, 2 mm thickness) (*see* **Notes 2**, **4**, and **5**).

2.2 Preparation of Ds-Containing DNA Library by PCR Amplification

1. Ds-containing DNA library.
2. Primers: For the PCR amplification of the N43Ds library, the following two primers can be used: Fwd27 primer, 5′-TTCTGTCAATCGATCGTATCAGTCCAC-3′, and T15-L-Rev29 primer, 5′-TTTTTTTTTTTTTTT-C12spacer-AAGTAG TCACTAATCCGTTCGAGTCATGC-3′ (*see* **Note 13**) [11].
3. 2.5 U/μl AccuPrime *Pfx* DNA polymerase (Life Technologies): The components are AccuPrime *Pfx* DNA polymerase (2.5 U/μl), 50 mM $MgSO_4$, and 10× AccuPrime *Pfx* Reaction Mix, which includes final concentrations of 1 mM $MgSO_4$ and 0.3 mM each natural dNTP. In PCR amplification involving the Ds–Px pair, we additionally supplement the reaction mixture with 0.1 mM each natural dNTP, 0.5 mM $MgSO_4$, and 0.05 mM each of dDsTP and Diol1-dPxTP (*see* **Note 14**).
4. 0.5 mM dDsTP (TagCyx Biotechnologies) (*see* **Note 15**) [6].
5. 0.5 mM Diol1-dPxTP (TagCyx Biotechnologies) (*see* **Note 15**) [8].
6. 10 % polyacrylamide gel, 7 M urea (16 cm × 16 cm, 2 mm thickness) (*see* **Notes 2**, **4**, and **5**).

2.3 Replacement PCR

1. Ds-containing DNA library.
2. Primers: Fwd27 primer, 5′-TTCTGTCAATCGATCGTATCA GTCCAC-3′, and Rev29 primer, 5′-AAGTAGTCACTAAT CCGTTCGAGTCATGC-3′ [11].
3. 10× TITANIUM Taq PCR Buffer (Clontech).
4. 50× TITANIUM Taq DNA polymerase (Clontech).
5. 0.25 mM dPa′TP (TagCyx Biotechnologies) (*see* **Note 15**) [6, 12].
6. PCR product purification kit: e.g., Wizard® SV Gel and PCR Clean-Up System, MinElute PCR Purification Kit (QIAGEN).

3 Methods

3.1 Preparation of Ds-Containing DNA Library

1. Set up gel electrophoresis to purify each sublibrary (*see* **Note 2**): Attach the gel plate to the electrophoresis apparatus, fill the upper and lower reservoirs with 1× TBE as a running buffer, and connect to an electric power supply. Prior to loading the sample, run for 30–60 min until the temperature of the gel reaches around 50 °C.

2. Prepare the loading sample: Dissolve DNA fragments in 200 μl of water and mix with 200 μl of denaturing gel-loading buffer. Heat the solution at 75 °C for 3 min.
3. Turn off the power supply, flush out the urea leached from the wells with 1× TBE, and then load the sample in each well.
4. Electrophorese until the marker dyes reach the predetermined positions. Generally, stop the electrophoresis when the xylene cyanol dye reaches about one-fourth of the distance from the bottom of the gel.
5. After electrophoresis, remove the gel plate from the apparatus. Detach one glass plate, and cover the gel with Saran Wrap. Turn the glass plate over, and remove the other glass plate from the gel. Place the gel on a fluorescent TLC plate. Check the shadow band corresponding to the DNA fragments on the gel by illumination with a handheld UV lamp at 254 nm (*see* **Note 6**).
6. Excise the bands with a clean razor blade, transfer the gel slices to a 50-ml conical tube, and crush the slices (*see* **Note 16**). Add 5.4 ml of sterile water, and incubate the tube for about 10 h at 37 °C with gentle agitation.
7. Pass the eluted solution through a 0.22 μm Steriflip filter (*see* **Note 7**).
8. Place 1.2 ml aliquots of the filtrate (about 4.8 ml) into four 5 ml tubes. Add 120 μl of 3 M sodium acetate, and 2.5 ml of ethanol per tube. After mixing, store them at −20 °C for 1 h. Recover the precipitated DNA fragments by centrifugation (*see* **Note 8**).
9. Add 0.1 ml water to each tube and incubate for 3 min at 75 °C, to dissolve the purified DNA fragments.
10. Combine the solutions in a single 1.5 ml tube, and determine the oligonucleotide concentration from its UV absorbance in an appropriate buffer, such as TE buffer.
11. Make the Ds-containing DNA library: Mix equal amounts of each sublibrary, and dilute to a 10 μM working solution. Store the solution in aliquots at −10 to −30 °C.

3.2 Preparation of Ds-Containing DNA Library by PCR Amplification

1. Prepare a master mix in a 1.5 ml tube, for 9-tube PCR reactions on a 0.1 ml scale (*see* **Note 17**). *See* Table 1.
2. Transfer a 60 μl volume from the master mix to a thin-wall PCR tube, add 40 μl of water and mix the solution (designated as Non-Template-Control, "NTC").
3. To the remaining master mix (480 μl), add 320 μl of Ds-containing DNA library and mix the solution, and then aliquot 100 μl volumes from the solution into eight thin-wall PCR tubes (designated as "DNA plus").

Table 1
Protocol for setting up the PCR reaction using the modified nucleotide dDsTP

Component	Volume (μl)	Final concentration in PCR
10× AccuPrime*Pfx* Reaction Mix	90	1×
20 μM Fwd27 primer	45	1 μM
20 μM T15-L-Rev29 primer	45	1 μM
2 mM each dNTP mix	45	0.4 mM in total
10 mM $MgSO_4$	45	1.5 mM in total
0.5 mM dDsTP	90	0.05 mM
0.5 mM Diol1-dPxTP	90	0.05 mM
Water	72	
2.5 U/μl AccuPrime*Pfx* DNA polymerase	18	0.5 U/μl

4. Place the "DNA plus" and "NTC" reaction tubes in a thermal cycler, and perform PCR with a program consisting of *N* cycles at 94 °C for 30 s and 65 °C for 2 min 30 s (*see* **Note 17**).
5. Combine the "DNA plus" PCR reactions into a single 1.5-ml tube.
6. Mix 5 μl each of "DNA plus" and "NTC" reaction with 2 μl agarose-loading buffer and load the samples onto a 4 % agarose gel. Load a DNA ladder marker in one lane.
7. Run the gel at 150 V with 1× TBE as the running buffer for 15 min, and detect the dsDNA bands with a UV-lamp.
8. Set up the gel electrophoresis to purify the PCR-amplified, Ds-containing DNA library (*see* **Note 2**): Attach the gel plate to the electrophoresis apparatus, fill the upper and lower reservoirs with 1× TBE as a running buffer, and connect to an electric power supply. Prior to loading the sample, run the gel for about 30 min until the temperature of the gel reaches around 50 °C.
9. Prepare the loading sample: Add 400 μl of denaturing sample solution to the "DNA plus" PCR samples (total about 800 μl, prepared in **step 4**). Heat the solution at 75 °C for 3 min.
10. Turn off the power supply, flush out the urea leached from the wells with 1× TBE, and then load the sample inside three large wells (400 μl × 3) (*see* **Note 18**).

11. Run the gel until the marker dyes reach the predetermined positions. Generally, stop the electrophoresis run when the bromophenol blue dye has almost reached the bottom of the gel.
12. After gel electrophoresis, remove the gel plate from the apparatus. Detach one glass plate, and cover the gel with Saran Wrap. Turn the glass plate over, and remove the other glass plate from the gel. Place the gel on a fluorescent TLC plate. Check the shadow bands corresponding to the DNA fragments on the gel (*see* **Note 19**), by illumination with a hand-held UV lamp at 254 nm (*see* **Note 6**).
13. Excise the band corresponding to the Ds-containing DNA library (*see* **Note 19**), with a clean, disposable razor blade, transfer each gel slice to three 2-ml tubes, and crush the slices against the wall of the tube (*see* **Note 16**). Add 1 ml of water to each tube, and incubate the tube for 10 h at 37 °C with gentle agitation.
14. Pass the eluted solution through a 0.22-μm Ultrafree-CL filter unit (*see* **Note 7**).
15. Mix the filtrate with 1/10 volume of 3 M sodium acetate, and 2 volume of ethanol. After mixing, store at −20 °C for 1 h. Recover the precipitated DNA fragments by centrifugation (*see* **Note 8**).
16. Dissolve the purified DNA library in 50–200 μl of water. Determine the DNA concentration from its UV absorbance.

3.3 Replacement PCR

1. Prepare the PCR reaction mix in a thin-wall tube. *See* Table 2 for a 100-μl PCR reaction.
2. Perform the PCR reactions in a thermal cycler with the following PCR conditions: 10–12 cycles at 94 °C for 30 s and 65 °C for 2 min 30 s (*see* **Note 20**).
3. Mix 5 μl of the PCR sample with 2 μl agarose-loading buffer, and load the sample on a 4 % agarose gel. Load a DNA ladder maker in one lane.
4. Run the gel at 150 V with 1× TBE as a running buffer for 15 min, and detect the dsDNA bands with a UV-lamp.
5. Purify the PCR products by using PCR product purification kits, to remove unconsumed primers, dNTPs, and other materials.
6. Determine the DNA concentrations from the UV absorbance and subject the samples to sequencing procedures, such as cloning and preparation of DNA templates for deep sequencing.

Table 2
Protocol for setting up the PCR reaction using the modified nucleotide dPa′TP

Component	Volume (μl)	Final concentration
10× TITANIUM Taq PCR Buffer	10	1×
20 μM Fwd27 primer	5	1 μM
20 μM Rev29 primer	5	1 μM
2 mM each dNTP mix	15	0.3 mM each
0.25 mM dPa′TP	20	0.05 mM
Water	3	
50× TITANIUM Taq PCR Buffer	2	1×
5 nM Ds-containing DNA library	40	2 nM (*see* **Note 20**)

4 Notes

1. It is important to take care to avoid nucleases and DNA contamination from conceivable sources. Use gloves during experiments, and DNA AWAY (Molecular BioProducts, Inc.) is effective to remove DNA contamination on laboratory instruments and glass/plastic-ware.
2. To analyze small PCR products (less than 100 bp), we use a commercially supplied agarose gel electrophoresis system or a vertical slab electrophoresis system with a denaturing polyacrylamide gel (glass plate size: 16 cm × 16 cm, thickness: 1 mm). For rapid confirmation of PCR products with no need for purification and quantification, we analyze the products on a 4 % agarose gel with ethidium bromide, using 1× TBE as the running buffer. For purification and quantification of the products, we generally use a denaturing polyacrylamide gel. For the purification of chemically synthesized DNA fragments (0.2-μmol scale synthesis), we use a denaturing gel electrophoresis system with a longer plate size (20 cm × 40 cm, 2 mm thickness), because higher resolution is needed to separate a one-base difference in oligonucleotides. A power supply with a temperature probe is useful for automatic and precise temperature control during the denaturing gel electrophoresis. We usually set the temperature at 45–50 °C and use 1× TBE as the running buffer.
3. Monomeric acrylamide is neurotoxic, and care should be taken to avoid exposure. Store the solution at 4 °C with protection from light.

4. To make denaturing polyacrylamide gels, it is useful to have a stock solution, which is kept at 4 °C. For example, to make a 10 % acrylamide–bis solution with 7 M urea (400 ml), mix 100 ml of 40 % acrylamide–bis (19:1) solution, 40 ml of 10× TBE, and 168 g of urea, and bring the volume to 400 ml with water. To remove impurities, filtration is recommended, but not required. The optimal acrylamide–bis percentage depends on the nucleotide length to be separated. The percentage of polyacrylamide in the gel should be lower with longer oligonucleotides. In general, for >90-mer oligonucleotides, an 8 % denaturing polyacrylamide is used, with 10 % for 45–120-mer, 15 % for 25–50-mer, and 20 % for <30-mer.
5. For example, to prepare a 20 cm × 40 cm (2 mm) gel, mix 130 ml acrylamide–bis solution with 650 μl of APS. Just before pouring, add 130 μl of TEMED, and mix the solution by gentle swirling. Immediately pour the gel mix between the gel plates and insert the gel comb. For oligonucleotide purification, making the gel on the previous day is recommended to ensure complete polymerization, although the gel usually polymerizes in about 30 min. After removing the comb, promptly wash out the wells thoroughly. To prepare a 16 cm × 16 cm (2 mm) gel, 40 ml acrylamide–bis solution, 200 μl of APS, and 40 μl of TEMED are used.
6. Highly concentrated oligonucleotides (>0.1 OD units) within a gel can be visualized by ultraviolet (UV) shadowing, without staining. Place the gel, covered with Saran Wrap, onto the TLC plate containing a fluorescent indicator, and shine UV light from a handheld UV lamp. The regions with highly concentrated nucleotides can be detected as “shadows” on the plate, due to their absorbance of the illuminated UV.
7. Before ethanol precipitation of the purified oligonucleotides eluted from the polyacrylamide gel slices, the solution should be filtered through a filter unit, to remove the small gel slices. Otherwise, these impurities will also be precipitated, and not only inhibit the concentration determination by UV absorbance, but also impede further applications.
8. For ethanol precipitation, mix the solution containing DNA with 1/10 vol. of 3 M sodium acetate and 2–3 vol. of ethanol, and store the solution at −10 to −30 °C (for at least 30 min). After centrifugation (10,000–12,000 × *g* for 40 min) at 4 °C, wash the precipitate with 500 μl of pre-chilled 70 % ethanol per tube. The ethanol residue is evaporated with a centrifugal evaporator. In the ethanol precipitation of DNA libraries used for binding with the target protein, co-precipitating agents, such as glycogen, should not be used, since such agents might affect the interactions of the DNA library with the target.

9. The solution should be filtered and stored in aliquots at −10 to −30 °C. Before use, incubate the aliquot at 75 °C to completely dissolve the urea. Alternatively, a denaturing solution with a dye, such as 0.05 % (w/v) xylene cyanol or 0.05 % (w/v) bromophenol blue as a marker dye, can also be used. In that case, keep in mind that the migration of the dye should not correspond with that of the desired fragments on the gel.
10. Ethidium bromide intercalates in DNA and is highly toxic, and care should be taken to avoid exposure.
11. Ds-containing DNA fragments can be purchased from custom suppliers, or chemically synthesized with an automated DNA synthesizer using dDs-amidite (dDs-CE Phosphoramidite, Cat. Nos: 10-1521-90 for 100 μmol, and 10-1521-02 for 0.25 g, Glen Research). In general, we perform the chemical synthesis on the 0.2 μmol scale by the conventional phosphoramidite method. The protecting group removal in the synthetic reactions (i.e., deprotection) is usually performed by heating the preparation at 55 °C for 6 h, after the fragment release from the CPG column by an incubation at room temperature for an hour in a concentrated NH_4OH solution. The NH_4OH is evaporated to dryness with a centrifugal evaporator. Afterwards, the fragments are subjected to purification by denaturing gel electrophoresis.
12. Keep in mind that the location of the Ds bases might affect the PCR amplification efficiency. In general, we separate two Ds bases by at least six natural bases when designing Ds-containing sublibraries [7, 11]. For more efficient PCR amplification with reduced sequence bias, the separation of two Ds bases by at least eight natural bases is recommended.
13. The T15-L-Rev29 primer is the Rev29 primer linked with an extra oligo-T (T15) sequence via the C12 alkyl spacer. In PCR products, the Ds-containing strand sequence is 5′-TTCTGTCAATCGATCGTATCAGTCCAC-Y2$_{-3}$-N43-GCATGACTCGAACGGATTAGTGACTACTT-3′ and the Px-containing strand sequence is composed of 5′-TTTTT TTTTTTTTTT-C12-spacer and the sequence complementary to the Ds-containing strand.
14. The amplification efficiency and fidelity of the Ds–Px pairing during PCR depend on the DNA polymerases used in the reactions [8]. Among the commercially available DNA polymerases, Deep Vent DNA polymerase with 3′→5′ exonuclease activity (exo+) and AccuPrime*Pfx* DNA polymerase, which is used in this protocol, are suitable for PCR amplification involving Ds and Px. The 3′→5′ exonuclease activity removes the unnatural substrates misincorporated opposite the natural

bases during PCR, which is important for the high Ds–Px pairing selectivity [8]. In the one-way incorporation of modified dPxTP opposite Ds in templates, TITANIUM Taq DNA polymerase can also be used [19].

15. Store the unnatural base substrate solution (dDsTP [6], dDiol1-PxTP [8], and dPa′TP [6, 12]) at −10 to −30 °C, and minimize repeated freeze–thaw cycles. Without repeated freeze–thaw cycles, these substrates are stable for at least a year. We generally dilute the original stock solution to a 0.5 or 0.25 mM working solution, and store aliquots with appropriate volumes.
16. To easily crush gel slices, use a disposable BioMasher stir bar (BioMasher II for a 1.5–2-ml tube and BioMasher V for a 50-ml conical tube), instead of a disposable pipette tip or a spatula.
17. The total PCR reaction volume depends on the amount of PCR products that will be sufficient for further use. In general, eight PCR (0.1-ml scale) reactions with a sufficient PCR cycle number will yield about 100–300 pmol of Ds-containing DNA library after purification on a denaturing gel. The amplification yield depends on both the number of PCR cycles and the amount of input DNA. Before large scale PCR amplification, assessing the appropriate number of PCR cycles is highly recommended by real-time PCR with a 25-μl reaction volume, in the presence of 30,000–75,000-fold diluted SYBR Green I (Lonza), to determine the number of PCR cycles sufficient for amplification. Too many PCR cycles might increase the unnatural to natural base mutations, as well as the amounts of undesired longer and/or shorter by-products.
18. We generally make a 6-well slot gel with a 2 mm thickness, and about 400 μl of the sample solution can be loaded per well. We load as much of the sample solution per well as possible into the middle slots, and load a marker dye on both sides.
19. Two bands will be detected at the upper position for xylene cyanol. The upper band corresponds to the Px-strand with an extra oligo-T (T15) sequence via the C12 alkyl spacer, and the lower band corresponds to the Ds-strand, i.e., the Ds-containing DNA library of interest. Depending on the number of PCR cycles employed, unconsumed primers might be detected at the positions between the xylene cyanol and the bromophenol blue.
20. Lower amounts of the Ds-containing DNA library can be used, with an appropriate increase in the PCR cycle number.

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