Design and PCR Synthesis of Infrared Fluorophore-Labeled Modular DNA Probes

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ABSTRACT

IR fluorophore-modified DNAs and IR fluorescence imaging provide a safe and effective alternative for investigating ligand-DNA interactions than those involving radioactivity. These DNAs can be synthesized by conventional phosphoramidite chemistries and are commercially available. However, they are relatively expensive, which can be prohibitive if many different DNA probes are required. Described here is the design of modular DNA probes that can be synthesized by PCR using conventional templates and a defined set of IR fluorophore-modified primers. These have been found effective for investigating protein-DNA interactions in a variety of assays, including Electrophoretic Mobility Shift Assays (EMSA), Restriction Endonuclease Protection Assays (REPA), and the iterative selection method Restriction Endonuclease Protection, Selection, and Amplification (REPSA).

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

Protocol development supported by grants from the NIH (1R15GM104833-01) and NSF (MCB-1714778).

MATERIALS

NAME	CATALOG #	VENDOR
Taq DNA Polymerase with Standard Taq Buffer - 400 units	M0273S	New England Biolabs

MATERIALS TEXT

DNA oligonucleotides, including unlabeled and IR fluorophore-labeled primers and modular templates, purified

Modular DNA probe design

1 A modular DNA probe is designed to be capable of amplification by PCR. Thus, it needs to contain two flanking regions of defined sequence and a central region containing those sequences under investigation. Length and sequence of the flanking regions are dictated by their role in the annealing of PCR primers but may also contain defined sequences for additional roles (e.g., recognition sequences for type IIS restriction endonucleases, for use in REPA). Typical primers range from 20 – 40 nucleotides in length and have a GC base content of 40 – 60%. Programs such as Primer3web and IDT OligoAnalyzer can be used to design effective primers. The central region's length and sequence will be dependent upon the ligand-DNA interactions being investigated. Figure 1 shows an example of a modular DNA probe from our laboratory that has been used to study a series of mutations in the consensus binding site of a *Thermus thermophilus* HB8 transcriptional regulatory protein SbtR by both EMSA and REPA. Important elements within the modular DNA probe are indicated.

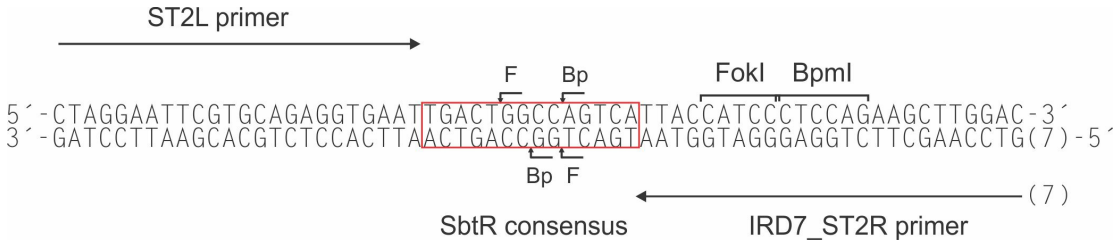

Figure 1. Sequence of the modular IR fluorophore-modified DNA probe *SbtR consensus DNA*. Elements: the SbtR consensus binding sequence is indicated by a red box, and the recognition sequences and cleavage sites for the type IIS restriction endonucleases FokI and BpmI are indicated by brackets and arrows, respectively. The sequences corresponding to the two primers are indicated by horizontal arrows. These also define the two flanking regions in the modular DNA probe. (7) 5' IRDye® 700 modification.

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2 DNA primers and templates used in the PCR synthesis of modular IR fluorophore-modified DNA probes may be obtained commercially. Our laboratory routinely uses materials from Integrated DNA Technologies (IDT), although other manufacturers that can synthesize IR fluorophore-modified oligonucleotides will suffice. One primer needs to be modified with a 5′ IR fluorophore while the other does not. As our laboratory uses a LI-COR Odyssey IR fluorescence imager, we use oligonucleotides modified with either IRDye® 700 or IRDye® 800, which are optimized for this instrument. Note that DNA probes modified with IRDye® 700 exhibit superior chemical and physical properties (e.g., recovery following multiple freeze-thaw cycles) as compare to those modified with IRDye® 800. Thus, IRDye® 700-modified probes are our preferred standards. Table 1 shows a series of oligonucleotides used in the synthesis of DNA probes for the study of SbtR-DNA binding. Note that while the resulting double-stranded DNA probes are 63 bp, the template oligonucleotides are only 59 nucleotides. This was done to avoid mandatory oligonucleotide purifications costs and had no appreciable effect on the quality of the resulting IRDye® 700-modified DNA probes.

Table 1. Oligonucleotides

Name	Sequence	Length	Purif.	Use
ST2L	CTAGGAATTCGTGCAGAGGTGAAT	24	Desalt	PCR primer
IR7_ST2R	/5IRD700/GTCCAAGCTTCTGGAGGGATGGTAA	25	HPLC	5′ IRDye® 700-modified PCR primer
ST2_SbtR_R7_wt	AGGAATTCGTGCAGAGGTGAATTGACTGGCCAGTCA TTACCATCCCTCCAGAAGCTTGG	59	Desalt	SbtR consensus DNA probe precursor
ST2_SbtR_R7_m1	AGGAATTCGTGCAGAGGTGAATAGACTGGCCAGTCA TTACCATCCCTCCAGAAGCTTGG	59	Desalt	SbtR mutant 1 DNA probe precursor
ST2_SbtR_R7_m2	AGGAATTCGTGCAGAGGTGAATTCACTGGCCAGTCA TTACCATCCCTCCAGAAGCTTGG	59	Desalt	SbtR mutant 2 DNA probe precursor
ST2_SbtR_R7_m3	AGGAATTCGTGCAGAGGTGAATTGTCTGGCCAGTCA TTACCATCCCTCCAGAAGCTTGG	59	Desalt	SbtR mutant 3 DNA probe precursor
ST2_SbtR_R7_m4	AGGAATTCGTGCAGAGGTGAATTGAGTGGCCAGTCA TTACCATCCCTCCAGAAGCTTGG	59	Desalt	SbtR mutant 4 DNA probe precursor
ST2_SbtR_R7_m5	AGGAATTCGTGCAGAGGTGAATTGACAGGCCAGTCA TTACCATCCCTCCAGAAGCTTGG	59	Desalt	SbtR mutant 5 DNA probe precursor
ST2_SbtR_R7_m6	AGGAATTCGTGCAGAGGTGAATTGACTCGCCAGTCA TTACCATCCCTCCAGAAGCTTGG	59	Desalt	SbtR mutant 6 DNA probe precursor
ST2_SbtR_R7_m7	AGGAATTCGTGCAGAGGTGAATTGACTGCCCAGTCA TTACCATCCCTCCAGAAGCTTGG	59	Desalt	SbtR mutant 7 DNA probe precursor

For mutant probe precursors, mutated base indicated in red font.

Modular IR-fluorophore DNA probe synthesis by PCR

3 Modular DNA probes are synthesized by PCR in our laboratory using Taq DNA polymerase and a standard Taq buffer from New England Biolabs (NEB). Please refer to their protocols.io procedure (https://doi.org/10.17504/protocols.io.ch7t9m) for experimental details. Our set up and execution follows theirs exactly, with the following exceptions:

1. We use a 1.1-fold excess of the unlabeled primer (0.22 µM final concentration). In the example provided that would be ST2L. Use of an excess unlabeled primer is to ensure that nearly all of the IR fluorophore-modified primer (IRD7_ST2R) will be incorporated into the desired double-stranded DNA probe and that minimal quantities of IR fluorophore-modified single-stranded DNA result.
2. We routinely use 2 ng template DNA in our PCR reactions. Given their relatively low molecular weight (1.8 kg/mole), this ensures that we seed our reactions with a fairly large amount of template (0.1 pmoles). This allows us to reduce the number of PCR cycles necessary to exhaust most all PCR primers.
3. Thermocycling conditions for PCR are dependent on the primers chosen, their final concentrations, and the thermophilic DNA polymerase and buffers used. For reagents provided by NEB, their online tool Tm Calculator is quite useful for determining these values. For our example, annealing would be performed at 54 °C. Thus, our standard thermocycling conditions for PCR are: (1) initial denaturation [95 °C, 30 s], (2) 20 cycles of denaturation [95 °C, 30 s], annealing [54 °C, 30 s], and elongation [68 °C, 60 s], (3) final extension [68 °C, 300 s], and (4) hold [4 °C, indefinitely].

4 After PCR synthesis is completed, samples are stored at –20 °C and thawed on ice when needed. Purification is not indicated as the highly hydrophobic IR fluorophores tend to adhere to most commercial matrices used for oligonucleotide purification (Qiagen MinElute PCR Purification Kit, Zymo Research Oligo Clean & Concentrator™), thereby significantly reducing overall yields.

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5 PCR probes are quantitatively and qualitatively assayed by Qubit and native PAGE, respectively. Protocols for each may be found at https://doi.org/10.17504/protocols.io.k5pcy5n and https://doi.org/10.17504/protocols.io.mcyc2xw. Expected results are 2+ ng/µL double-stranded DNA with 99+% present in a single electrophoretic species.

6 It is our experience that these impure DNA probes do not adversely impact their use in routine protein-DNA analyses (EMSA, REPA), especially when they constitute 1-10% of the final reaction volume. Examples of the use of IR fluorophore-labeled DNA probes in EMSA and REPA are shown in Figures 2 and 3, respectively.

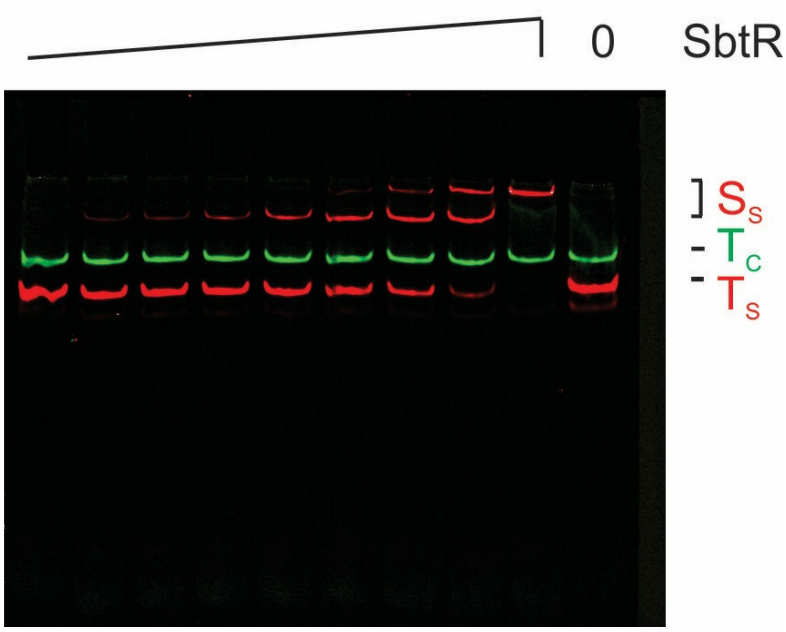

Figure 2. SbtR binding to its consensus sequence as analyzed by EMSA and IR fluorescence imaging. Shown are native PAGE-resolved DNA species formed following incubation with increasing SbtR concentrations. (T_S) Unbound IRDye® 700-labeled SbtR consensus DNA probe. (T_C) Unbound IRDye® 800-labeled control DNA probe. (S_S) SbtR-IRDye® 700-labeled SbtR consensus DNA complexes. Figure reprinted from https://doi.org/10.1371/journal.pone.0159408.

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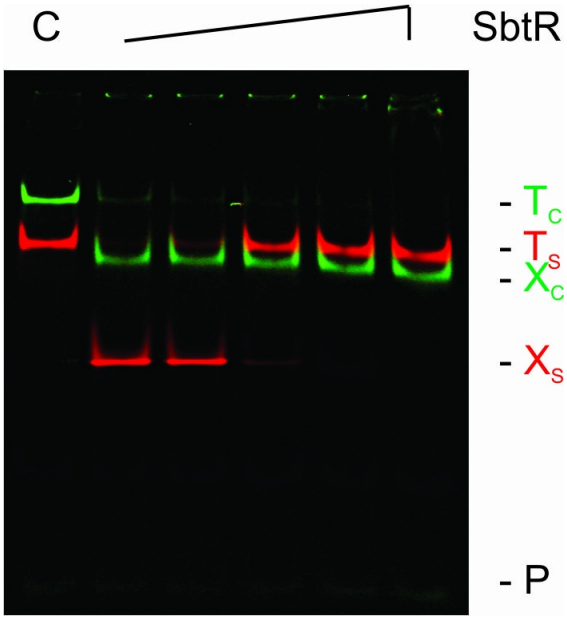

Figure 3. SbtR binding to its consensus sequence as analyzed by REPA and IR fluorescence imaging. Shown are native PAGE-resolved DNA species formed following incubation with increasing SbtR concentrations and subsequent cleavage by the type IIS restriction endonuclease FokI. (T_C) Uncleaved IRDye® 800-labeled control DNA probe. (T_S) Uncleaved IRDye® 700-labeled SbtR consensus DNA probe. (X_C) Cleaved IRDye® 800-labeled control DNA probe. (X_S) Cleaved IRDye® 700-labeled SbtR consensus DNA probe. (C) Uncleaved DNA control. Figure reprinted from https://doi.org/10.1371/journal.pone.0159408.

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