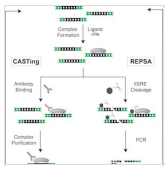

Jul 31, 2020

Restriction Endonuclease Protection Assays using Infrared-Fluorescent Probes

Michael Van Dyke[1], Isabelle Gracien[1]

[1]Department of Chemistry and Biochemistry, Kennesaw State University

1 *Works for me* dx.doi.org/10.17504/protocols.io.bi5ikg4e

Michael Van Dyke
Department of Chemistry and Biochemistry, Kennesaw State Uni...

ABSTRACT

Many proteins sequence-specifically bind duplex DNA, e.g., transcriptional regulatory proteins. Analysis of their interactions can be performed by a variety of methods, including electrophoretic mobility shift assays (EMSA) and quantitative DNase I footprinting. Here we describe an additional electrophoretic method, restriction endonuclease protection assays (REPA), to qualitatively and quantitatively study the interactions of thermophilic transcription regulatory proteins to PCR-generated, infrared-fluorescent DNA probes. REPA utilizes type IIS restriction endonucleases (IISRE), which cleave double-stranded DNA without specificity at a fixed distance from their recognition sequence. Thus, IISREs can be used to probe the occupancy of a suitably situated DNA-binding site for a variety of ligands. REPA has certain advantages as it does not require the maintenance of ligand-DNA complex stability during gel electrophoresis, as is the case with EMSA and is technically far less challenging than quantitative DNase I footprinting.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Van Dyke M, Gracien I. Restriction endonuclease protection assays using infrared-fluorescent probes. Protocols.io, 7/2020. doi: 10.17504/protocols.io.bi5ikg4e

DOI

dx.doi.org/10.17504/protocols.io.bi5ikg4e

PROTOCOL CITATION

Michael Van Dyke, Isabelle Gracien 2020. Restriction Endonuclease Protection Assays using Infrared-Fluorescent Probes. **protocols.io** dx.doi.org/10.17504/protocols.io.bi5ikg4e

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Van Dyke M, Gracien I. Restriction endonuclease protection assays using infrared-fluorescent probes. Protocols.io, 7/2020. doi: 10.17504/protocols.io.bi5ikg4e

KEYWORDS

IR fluorescence imaging, PCR, protein-DNA interactions, REPA

CREATED

Jul 29, 2020

LAST MODIFIED

Jul 31, 2020

PROTOCOL INTEGER ID

39818

GUIDELINES

Protocol development supported by a grant from the NSF (CHE-1709263).

MATERIALS

NAME	CATALOG #	VENDOR
Gel Loading Dye Orange (6X) - 4.0 ml	B7022S	New England Biolabs
FokI - 1,000 units	R0109S	New England Biolabs

SAFETY WARNINGS

Protocol deemed safe for use in an undergraduate research environment.

BEFORE STARTING

1. REPA requires the use of suitably designed test and control DNA probes, the former containing a ligand-binding site overlapping with a IISRE cleavage site and the latter having a IISRE cleavage site but lacking a ligand-binding site. Criteria for the design of IR fluorophore-labeled DNA probes suitable for REPA may be found at https://dx.doi.org/10.17504/protocols.io.wfjfbkn. Sequences of a test DNA probe for the *Thermus thermophilus* HB8 transcriptional regulatory protein TTHA0167 (SbtR) and a control probe are shown in Figure 1. Note that for proper REPA analysis, the test and control probes should be designed so that they have the same IISRE recognition sequences but different lengths, both for the intact DNAs and their labeled cleavage products.
2. IR fluorophore-labeled DNA probes for REPA are routinely synthesized by PCR. Detailed instructions may be found at https://dx.doi.org/10.17504/protocols.io.mbdc2i6. Note that we find the PCR synthesis of probes containing IRDye® 800-labeled primers typically has smaller yields than probes synthesized with IRDye® 700-labeled primers. Thus, one should plan accordingly.
3. REPA, as a cleavage protection assay, requires constancy in reaction conditions, especially the time of IISRE cleavage. Such constancy benefits from having an organized workspace. An example of an organized workspace, where all needed items are within easy reach, is shown in Figure 2.
4. Restriction endonuclease cleavage optimization. Before performing REPA, it is best to determine the amount of IISRE needed to give optimal cleavage of both test and control DNA probes. Optimal cleavage can range from 80 – 99%, depending on whether the REPA is for quantitative binding affinity determination (requiring cleavage protection in the linear range) or as a prelude for REPSA selections (which benefits from near-complete DNA cleavage). Optimization can be performed initially using ten-fold serial dilutions of IISRE, followed by two-fold serial dilutions in the range of interest once it has been roughly determined. Operationally, the same steps used in REPA are used, minus the ligand and its binding reaction. These are described in detail in the following protocol.

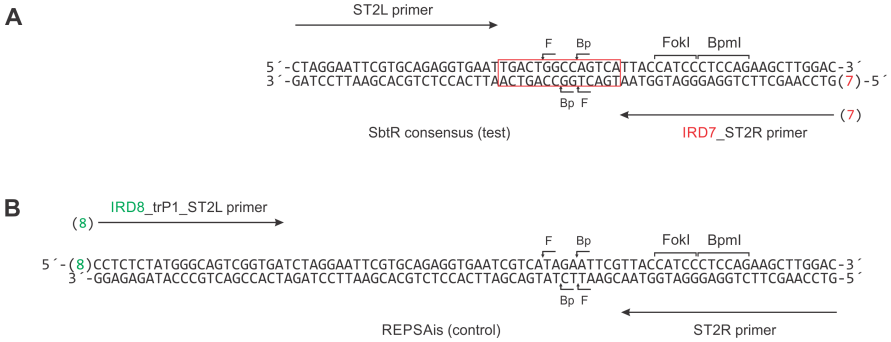

Figure 1. Sequences of the IR fluorophore-modified REPA probes (**A**) SbtR consensus test probe and (**B**) REPSAis control probe. Features: (red box) SbtR consensus binding sequence, (brackets) type IIS restriction endonuclease FokI or BpmI recognition sequence, (angled arrows) cleavage sites for (F) FokI or (Bp) BpmI. Horizontal arrows above and below sequences correspond to the primers used for PCR amplification. (7, 8) indicate 5'-end modification with IRDye® 700 or 800, respectively. The 63-bp SbtR consensus test probe is IRDye® 700-labeled on its bottom strand and yields a 34/30-bp labeled fragment upon FokI cleavage. The 86-bp REPSAis control probe is IRDye® 800-labeled on its top strand and yields a 52/56-bp labeled fragment upon FokI cleavage.

Citation: Michael Van Dyke, Isabelle Gracien (07/31/2020). Restriction Endonuclease Protection Assays using Infrared-Fluorescent Probes. https://dx.doi.org/10.17504/protocols.io.bi5ikg4e

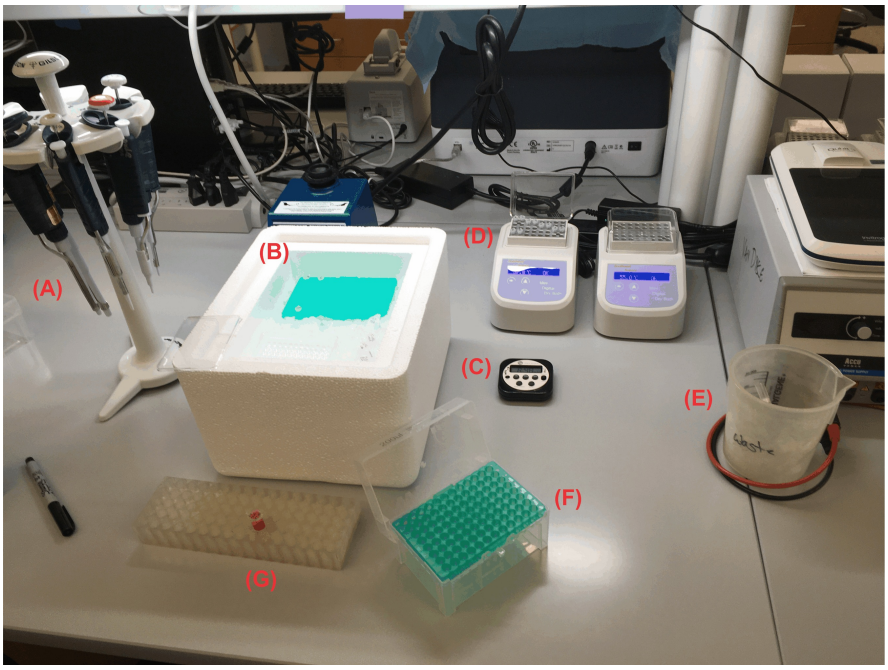

Figure 2. Workspace for REPA. (**A**) Micropipettes. P2 and P10 are primarily used for REPA. (**B**) Crushed ice container for the storage of reagents, sample dilutions, and assembled reactions. Insert from a 200 µL racked tip box can be used to assemble reactions; microwell Terasaki plates can be used for serial dilutions. (**C**) Timer. (**D**) Mini dry bath hot block heaters for protein-DNA binding reactions and IISRE cleavage reactions. We use Southwest Science SH100 digital dry baths with SWMINI-02 block inserts, suitable for PCR tubes. (**E**) Waste beaker. (**F**) Rack for stopped reactions. An empty 200 µL racked tip box works well for this purpose. (**G**) FokI Loading Dye. 6x Orange Gel Loading Dye + 1% SDS. Keep at room temperature to prevent SDS precipitation.

REPA set up

1 **Plan REPA reactions.** For the example shown here, we investigated the concentration dependence of the *Thermus thermophilus* HB8 transcriptional regulator SbtR to inhibit IISRE FokI cleavage of an IRD7-labeled (red) test DNA containing the SbtR consensus binding sequence as compared to an IRD8-labeled (green) control DNA. Reactions (10 µL)contained 1 nM each of the test and control DNA and a suitable IISRE cleavage buffer (1x NEB CutSmart®), with the variable being SbtR concentration in the range 1-200 nM. Uncut DNA and FokI cleavage control reactions were also included. Table 1 shows the assembly plan used for this REPA experiment.

Table 1. REPA Reaction Plan

Sample	Experiment	Master Mix	SbtR?	FokI?
Sample	Uncut DNA control	8 µL	2 µL 1x PDB	(none)
Sample	FokI cleavage control	8 µL	1 µL 1x PDB	1 µL 0.4 U FokI
Sample	200 nM SbtR	8 µL	1 µL 20 µM SbtR	1 µL 0.4 U FokI
Sample	100 nM SbtR	8 µL	1 µL 1/2 SbtR	1 µL 0.4 U FokI
Sample	50 nM SbtR	8 µL	1 µL 1/4 SbtR	1 µL 0.4 U FokI
Sample	25 nM SbtR	8 µL	1 µL 1/8 SbtR	1 µL 0.4 U FokI
Sample	13 nM SbtR	8 µL	1 µL 1/16 SbtR	1 µL 0.4 U FokI
Sample	6.3 nM SbtR	8 µL	1 µL 1/32 SbtR	1 µL 0.4 U FokI

Citation: Michael Van Dyke, Isabelle Gracien (07/31/2020). Restriction Endonuclease Protection Assays using Infrared-Fluorescent Probes. https://dx.doi.org/10.17504/protocols.io.bi5ikg4e

Sample	3.1 nM SbtR	8 µL	1 µL 1/64 SbtR	1 µL 0.4 U FokI
Sample	1.6 nM SbtR	8 µL	1 µL 1/128 SbtR	1 µL 0.4 U FokI

2 **Assemble REPA reactions.** To ensure consistency among reactions, we routinely make a Master Mix containing the constant elements (DNA, buffer, water). Here, our Master Mix contained 52.5 µL water, 10.5 µL 10x NEB CutSmart® buffer, and 10.5 µL each 10 nM IRD7_ST2_SbtR_wt (test) and 10 nM IRD8_trP1_REPSAis (control) DNAs. Note the slight excess of reagents in the prepared Master Mix, to offset any potential pipetting errors. Mixed by brief vortexing and consolidated by brief minifuge centrifugation, 8 µL aliquots of Master Mix were then placed in a series of labeled PCR tubes (1-10) pre-chilled on crushed ice (Figure 3).

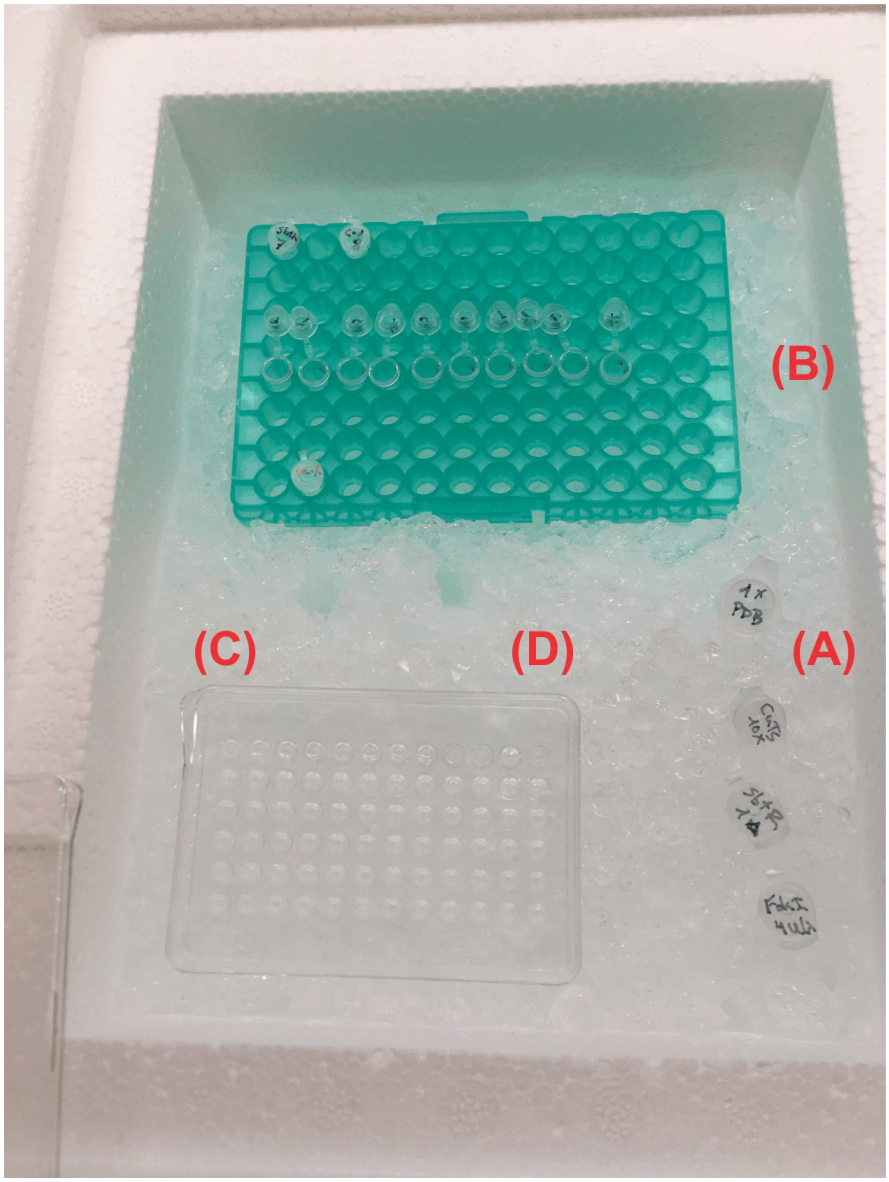

Figure 3. REPA reaction preparation. All samples should be kept on ice. (**A**) Reagents needed for assembling Master Mix. (**B**) 200 µL racked tip box containing PCR tubes labeled 1 – 10, for assembling REPA reactions. (**C**) Terasaki plates with 5 µL 1x PDB in microwells 1 – 7, for two-fold SbtR serial dilutions. (**D**) Terasaki plate microwell with 9 µL 1x PDB, for FokI dilution.

3 **Make serial dilutions of the ligand under investigation.** Two-fold serial dilutions of a freshly prepared 20 µM SbtR solution were made in 1x Protein dilution buffer (PDB, 100 mM NaCl, 20 mM Tris-Cl, 0.05% Tween 20, pH 7.8 @ 25

Citation: Michael Van Dyke, Isabelle Gracien (07/31/2020). Restriction Endonuclease Protection Assays using Infrared-Fluorescent Probes. https://dx.doi.org/10.17504/protocols.io.bi5ikg4e

°C), giving the range 16 – 1000 nM SbtR. This was achieved by aliquoting 5 µL 1x PDB in each of seven microwells on a Terasaki plate on ice, adding 5 µL 20 µM SbtR to the rightmost well, and mixing by repeated pipetting (8x). Such was repeated sequentially rightward using just diluted SbtR until the series 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 was made. Afterward, 1 µL of each SbtR dilution was added to the appropriate reaction tube #3 – #10, and the samples mixed by repeated flicking followed by a sharp wrist shake to consolidate the sample in the bottom of the tube. 1x PBD was added to the control reactions #1 and #2, volumes indicated above, and mixed likewise.

4 **Prepare FokI dilution.** Commercial FokI (NEB, typically 4 – 5 U/ µL) was diluted to its optimal concentration for REPA as determined earlier through restriction endonuclease cleavage optimization experiments (see Before Starting 4., above). We used a 0.4 U/ µL FokI dilution in the present example, which typically provides near-complete (> 90%) DNA cleavage under our standard reaction conditions. Dilutions (1:9) were made with 1x PDB in a Terasaki plate microwell on ice and remained stable for at least 1 h before use.

REPA execution

5 **REPA timecourse.** REPA experiments consist of the following steps: (A) assemble reactions on ice, (B) protein-DNA binding reaction, (C) sample pre-equilibration for IISRE [if necessary], (D) IISRE cleavage reaction, (E) cleavage reaction stop, (F) native PAGE electrophoresis, (G) imaging by IR fluorescence, and (H) quantitative densitometry. Of these, Steps (B) – (E) are time-dependent. A timeline outlining these steps for the SbtR REPA experiment is shown in Figure 4.

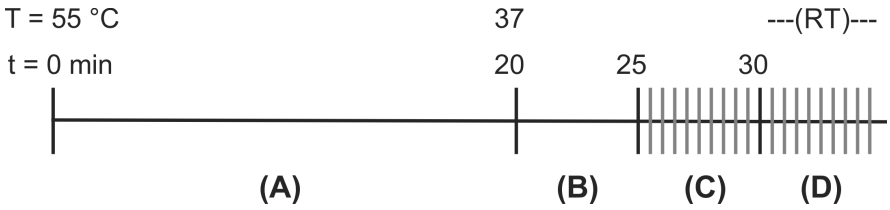

Figure 4. Timeline for REPA with SbtR. (**A**) Incubate samples at 55 °C for 20 min. to facilitate specific SbtR binding. (**B**) Pre-equilibrate samples at 37 °C for at least 5 min before FokI cleavage. (**C**) To one sample every 30 sec., add 1 µL diluted FokI to initiate cleavage reaction. Continue incubation at 37 °C for precisely 5 min. (**D**) Stop cleavage reaction by the addition of 4 µL FokI Loading Buffer and store sample at room temperature.

6 **Protein binding reaction.** Before challenge by the IISRE, the protein under investigation needs to be incubated with the test and control DNAs to allow equilibration. As SbtR is a thermophilic protein, incubations were performed at an elevated temperature (55 °C) for 20 min using a digital mini dry bath with a block that accommodates 200 µL PCR tubes. 20m

7 **Sample pre-equilibration for IISRE cleavage.** Given that FokI is a mesothermic IISRE, samples need to be pre-equilibrated to its optimum reaction temperature prior to initiating cleavage. We found that a 5 min pre-equilibration at 37 °C is usually sufficient. To affect such, samples were transferred together from a 55 °C dry bath to a 37 °C dry bath. Note that pre-equilibration can occur for more extended periods (> 60 min) with no adverse consequences for SbtR binding. However, we routinely limit pre-equilibration to < 10 min. 5m

8 **IISRE cleavage. After pre-equilibration.** IISRE cleavage was initiated by the addition of 1 µL 0.4 U/µL FokI to Tube #2 using a P2 micropipette. The sample was mixed thoroughly by finger flicking twice and snap shaking tube to consolidate liquid in tube bottom. The tube was returned to 37 °C dry bath block, and incubation allowed to resume for precisely 5 min. At that point, the cleavage reaction was stopped by the addition of 4 µL FokI loading buffer (FLB, 6x Orange Loading Dye with the addition of 1% SDS), mixed thoroughly as above, and storage at room temperature. With practice, it is possible to initiate IISRE cleavage for a new sample every 30 sec. Thus, with appropriate staging, one can have ten samples subjected to IISRE cleavage simultaneously. Table 2 illustrates the time points used in our SbtR REPA experiment.

Table 2. SbtR REPA Timepoints

Sample	Experiment	Time (FokI)	Time (FLB)
1	Uncut DNA control	(n/a)	t = 4:30
2	FokI cleavage control	t = 0	5:00
3	200 nM SbtR	0:30	5:30

Citation: Michael Van Dyke, Isabelle Gracien (07/31/2020). Restriction Endonuclease Protection Assays using Infrared-Fluorescent Probes. https://dx.doi.org/10.17504/protocols.io.bi5ikg4e

4	100 nM SbtR	1:00	6:00
5	50 nM SbtR	1:30	6:30
6	25 nM SbtR	2:00	7:00
7	13 nM SbtR	2:30	7:30
8	6.3 nM SbtR	3:00	8:00
9	3.1 nM SbtR	3:30	8:30
10	1.6 nM SbtR	4:00	9:00

Note that as Sample #1 is the uncut DNA control and does not require any FokI addition, FLB may be added at any time after the pre-equilibration step. We routinely choose the t = 4:30 timepoint as this provides a comparable time of exposure at 37 °C.

REPA analysis

9 **Gel electrophoresis.** DNA products in these samples were resolved by native PAGE using hand cast 9.5%:0.5% acrylamide:bisacrylamide, ½x TBE, 10 cm vertical minigels. Electrophoresis was performed initially at 50 V for 5 min, followed by 100 V for 1 h, at which point the Orange G dye had approached the bottom of the gel. A detailed description of our gel electrophoretic technique may be found at https://dx.doi.org/10.17504/protocols.io.mcyc2xw.

10 **IR fluorescence imaging.** Resolved DNAs were visualized by IR fluorescence using a LI-COR Odyssey Imager and LI-COR Image Studio™ 5.2 software. A general overview may be found at https://dx.doi.org/10.17504/protocols.io.mcyc2xw. Data from our SbtR REPA experiment are shown in Figure 5. Note that as the glass plates used in casting and running the polyacrylamide gel are transparent at near-IR wavelengths. Thus, it is not necessary to remove the gel from these but instead image the entire assembly directly.

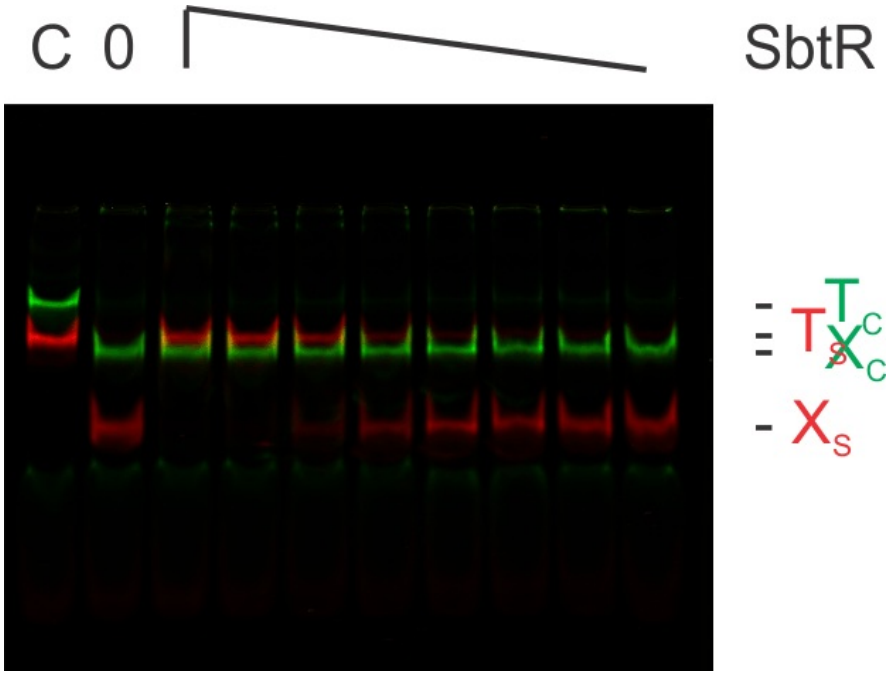

Figure 5. SbtR-binding to its consensus sequence as determined by REPA. Shown are LI-COR images of IRD7-labeled SbtR-consensus DNA (red) and IRD8-labeled REPSAis control DNA (green) subjected to FokI cleavage following binding reactions in the presence of (left to right) 0, 200, 100, 50, 25, 13, 6.3, 3.1, and 1.6 nM SbtR. (C) Uncleaved DNA control lane. (T) Intact, uncleaved DNA, (X) cleaved DNA.

11 **Densitometry.** Labeled DNA products were quantitated using the DNA analysis module in LI-COR Image Studio™ 5.2 software. A detailed procedure may be found at https://dx.doi.org/10.17504/protocols.io.mcyc2xw. For two-label experiments such as REPA, we find that analyzing each channel independently is preferred. The densitometry of test and control DNAs from our SbtR REPA experiment is shown in Figure 6 with the data analysis in Table 4.

Citation: Michael Van Dyke, Isabelle Gracien (07/31/2020). Restriction Endonuclease Protection Assays using Infrared-Fluorescent Probes. https://dx.doi.org/10.17504/protocols.io.bi5ikg4e

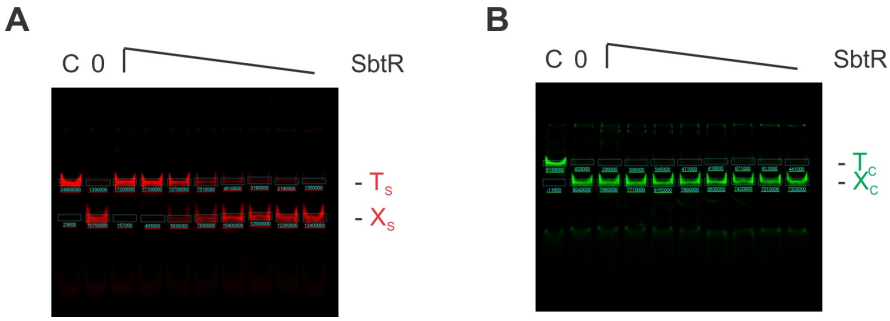

Figure 6. Quantitation of SbtR-binding to its consensus sequence as determined by REPA. Shown are the analyses of the image shown in Figure 5, specifically (**A**) the 700 nm channel, which corresponds to the IRD7-labeled SbtR-consensus DNA (Test, red), and (**B**) the 800 nm channel, which corresponds to the IRD8-labeled REPSAis control DNA (Control, green). In each lane, uncut DNA is on top and cleaved DNA below. Quantitation values for each are shown below each corresponding band and are tabulated in Table 4.

12 **Cleavage protection determination.** Cleavage protection was determined using the following equation:

$$\% \text{ cleavage} = X/(X + T)$$

where X is the cleaved DNA for one probe (test or control), and T is its corresponding intact DNA. For our SbtR REPA experiment, the calculated % cleavage values as a function of SbtR concentration are shown in Table 4 and graphically in Figure 7 for both the test probe, which contains a consensus SbtR binding site and a control DNA. From this study, we find that the IC_{50} for FokI cleavage inhibition was 25 nM SbtR under these reaction conditions. This value compares favorably with those determined previously (https://dx.doi.org/10.1371/journal.pone.0159408).

Table 4. REPA Results

Sample	Experiment	Test (T)*	Test (X)	Test (%X)	Control (T)	Control (X)	Control (%X)
1	Uncut DNA control	2490	2.36	0	9130	0	0
2	FokI cleavage control	133	1070	89	43.3	8040	99
3	200 nM SbtR	1720	15.7	1	29.9	7960	100
4	100 nM SbtR	1710	49.3	3	35.9	7710	100
5	50 nM SbtR	1370	393	22	54.5	8150	99
6	25 nM SbtR	751	793	51	47.1	7900	99
7	13 nM SbtR	461	1540	77	41.6	8630	100
8	6.3 nM SbtR	319	1250	80	67.1	7420	99
9	3.1 nM SbtR	219	1230	85	61.1	7010	99
10	1.6 nM SbtR	135	1240	90	44.1	7320	99

*Pixel values are in thousands (*i.e.*, 2490 shown = 2490000 measured).

Citation: Michael Van Dyke, Isabelle Gracien (07/31/2020). Restriction Endonuclease Protection Assays using Infrared-Fluorescent Probes. https://dx.doi.org/10.17504/protocols.io.bi5ikg4e

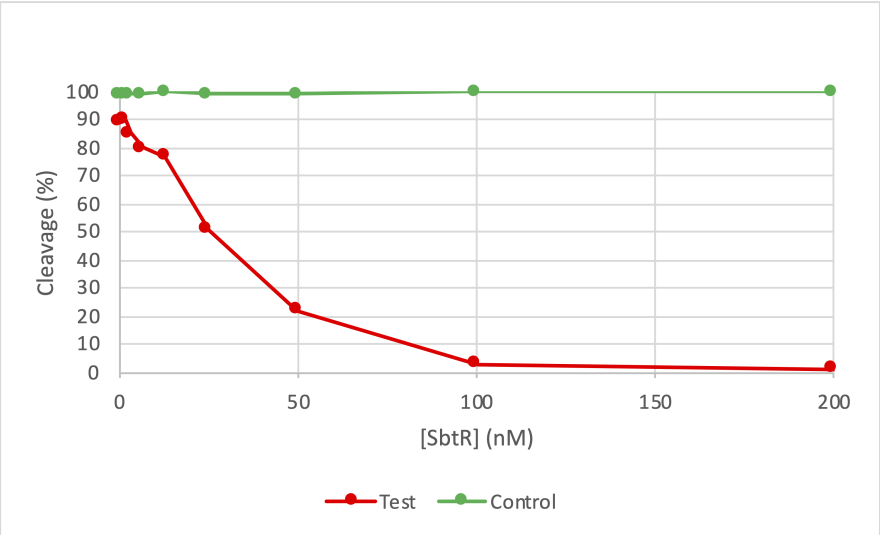

Figure 7. Graphical representation of FokI cleavage inhibition to IRD7-labeled SbtR-consensus DNA (Test, red) or IRD8-labeled REPSAis control DNA (Control, green) as a function of SbtR concentration. Data are from Table 4.

Citation: Michael Van Dyke, Isabelle Gracien (07/31/2020). Restriction Endonuclease Protection Assays using Infrared-Fluorescent Probes. https://dx.doi.org/10.17504/protocols.io.bi5ikg4e