Supplementary Protocol for DNA Nanoswitches: A quantitative platform for gel-based biomolecular interaction analysis, MA Koussa, K Halvorsen, A Ward, WP Wong, Nature Methods (2015).

Preface:

This document serves as a detailed protocol to provide you with all the information needed to implement the nanoswitch platform in your lab. The protocol will walk you through all the steps required to form nanoswitches, and use them to make on-rate and off-rate measurements. The protocol goes through the specific steps required to make measurements on the ubiquitous biotin-streptavidin system, with notes about where changes can be made to adapt the platform to your system of interest. While supplies last, we are happy to provide a starter kit, which contains all of the oligonucleotides needed to perform these measurements, to any interested labs. The detailed contents of the kit are described in section 2 of this protocol. Anyone interested in obtaining a starter-kit for their lab should visit http://wonglab.tch.harvard.edu/nanoswitch/ .

Protocol:

1. Linearizing the M13 Scaffold

To begin one must first prepare the linear single-stranded M13 scaffold. We do this by using a synthetic oligonucleotide complementary to the site where we want to cut that creates a double stranded region allowing the restriction enzyme BtsCI to cleave the M13 scaffold. The following will walk you through the procedure needed to make the linear scaffold.

Note: We recommend using Eppendorf LoBind tubes and Corning DeckWorks Lowbind Tips (Corning Product #s: 4121 and 4147) for all liquid handling.

1.1 Reagents needed:

- 1.1.1 250µg/ml (~100nM) Circular single-stranded M13 DNA (New England Biolabs Catalog#: N4040S)
- 1.1.2 100µM Synthetic oligonucleotide complementary to the BtsCI restriction site (sequence 5'->3': CTACTAATAGTAGTAGCATTAACATCCAATAAATCATACA).
- 1.1.3 BtsCI (20,000 units/ml) Restriction Enzyme (New England Biolabs Catalog#: R0647S or R0647L)
- 1.1.4 10x NEBuffer 2 (New England Biolabs Catolog #: B7002S)

1.1.5 Nuclease Free Water (Invitrogen Ultrapure Distilled Water DNAse and RNAse free Catalog # 10977-015)

1.2 Mix the following in a PCR tube (Axygen PCR-02D-C)

1.2.1 ____ 5µl of 100nM circular single-stranded M13 DNA

1.2.2 ____ 2.5µl of 10x NEBuffer 2

1.2.3 ____ 0.5µl of 100µM BtsCI restriction-site complimentary-oligonucleotide

1.2.4 ____ 16µl of Nuclease Free Water

1.2.5 ____ Pipette to mix thoroughly being sure to avoid air bubbles

1.3 ____ Place the tube in a thermocycler and subject to the following linearization protocol:1

1.3.1 ____ Bring to 95° C and hold for 30 seconds

1.3.2 ____ Cool to 50° C

1.3.3 ____ Add 1µl of the BtsCI enzyme

- Mix thoroughly with a pipette to ensure enzyme is well mixed. The enzyme is in glycerol and will sink to the bottom if not mixed well. It is best to use a pipette set to ~10µl to do this as the 1µl pipette may not provide adequate mixing. Failure to do this may greatly lower linearization efficiency.

1.3.4 ____ Allow the mixture to sit at 50° C for 1 hour

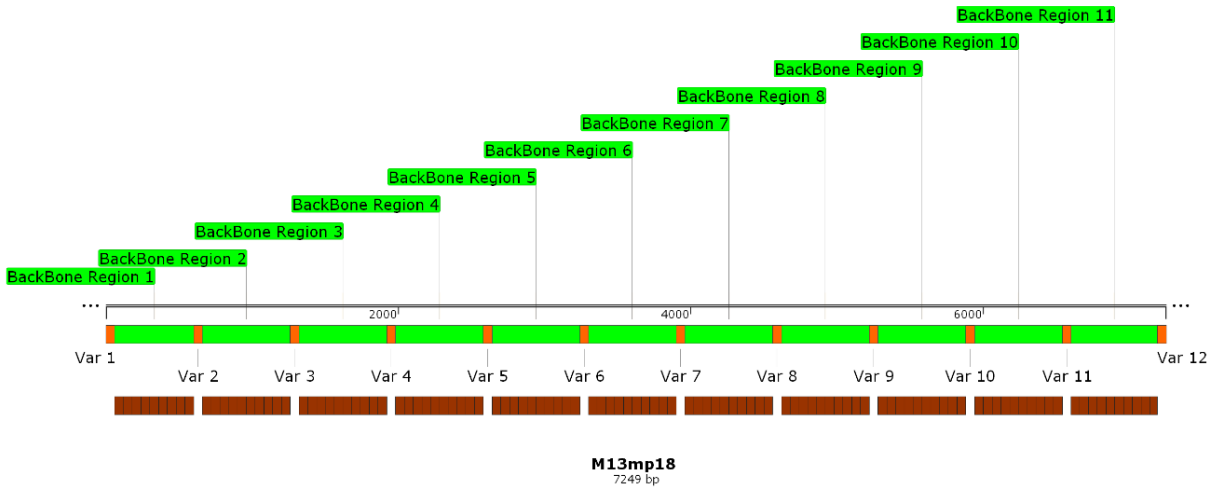
1.3.5 ____ Bring the mixture up to 95° C for 1 minute to heat deactivate the enzyme

- For convenience you may want to set the thermocycler to hold at 4°C after completion

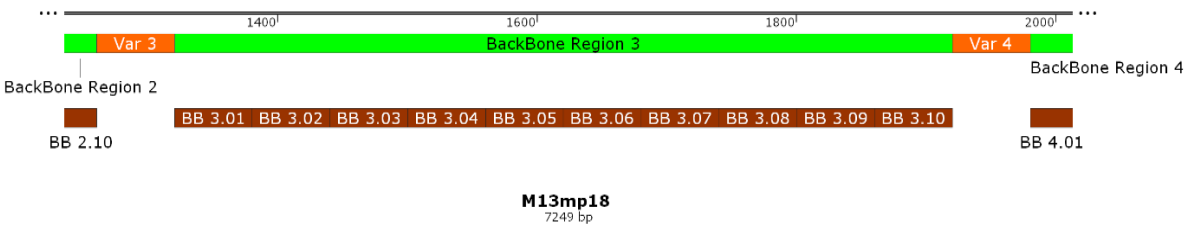
1.3.6 This will result in 25µl of ~20nM linearized circular M13 in NEBuffer 2

- We have scaled this up as much as 4 times resulting in 100µl of final product with no obvious reduction in linearization efficiency
- Additionally we have found the linearization to be equally efficient in NEBuffers 2, 2.1,4, and cutsmart
- To aid in quantification the linearized DNA can be spiked with 1 µl BstNI Digest of pBR322 DNA (New England Biolabs). This provides low molecular weight bands which can be used as intensity references to combat errors in pipetting at later stages

Now that linear single-stranded scaffold is made you will need the set of oligonucleotides that are complementary to the scaffold. We have chosen to use 60 nt oligonucleotides. This yields 120 60 nt oligonucleotides and one 49 nt oligonucleotide. We have divided these oligonucleotides into two groups to avoid having to mix 121 oligonucleotides for each construct. A mixture of 12 oligonucleotides evenly spaced along the backbone are each stored in their own tubes and referred to as variable regions or Vars. The remaining 109 oligonucleotides are all pre mixed together and we refer to these as backbone oligonucleotides. We have created a SnapGene DNA file which has these regions highlighted and labeled (in the SnapGene file, the bottom strand is the M13 sequence while the top strand represents the oligonucleotide sequences). SnapGene Viewer is freely available software that can be downloaded at: http://www.snapgene.com/products/snapgene_viewer/ . Images exported from SnapGene can be found below. The SnapGene file can be found at: https://drive.google.com/file/d/0B_Sa-wyS5qEUaldiUFlGbHkwczQ/view?usp=sharing , an excel file with the 109 backbone oligonucleotides can be found at: https://drive.google.com/file/d/0B_Sa-wyS5qEUcEF3OF9ZQkRTeWM/view?usp=sharing , and an excel file with the 12 variable oligonucleotides can be found at: https://drive.google.com/file/d/0B_Sa-wyS5qEUbTVHdUp6V0NUUjQ/view?usp=sharing.c Additionally, these files are available in the online supplementary materials on nature method's website.



Linearized M13 showing the 12 evenly spaced Var 1-Var 12(orange) oligonucleotides interrupted by 11 back bone regions (green), each of which is filled with 10 backbone oligonucleotides (maroon) (with the exception of backbone region 1 which only has 9 backbone oligonucleotides). In the following image we have zoomed in on the region between Var 3 and Var 4.



2. Materials available upon request:

To facilitate trying the nanoswitch platform in your lab, we have ordered some materials for starter kits that we will provide while supplies last. We will provide a mixture of the 109 backbones, a mixture of the 10 Vars (Vars 1, 2, 3, 5, 6, 7, 9, 10, 11, and 12), and a mixture of Var 4-3'biotin and 5'biotin-Var 8. The 109 mix can be used in conjunction with the provided Var oligos to create scaffolds capable of forming loops between Vars 4 and 8 upon the addition of streptavidin. Additionally, one could use the 109 mix in conjunction with 12 separately purchased Var oligonucleotides (or the 10 var mix and separately purchased Var 4 and Var 8 oligonucleotides) which can be functionalized to your specific needs. Protocols for functionalizing oligonucleotides for incorporation into this scaffold can be found in Halvorsen et. al 2011 and Koussa et. al 2014.

Below we provide a protocol for using the included 109 mix, the 10 Var Mix, and the mix of the two biotinylated oligonucleotides, to make biotin-SA on-rate and off-rate measurements.

3. Building the construct:

3.1 Hybridize the 121 oligonucleotides to the scaffold

3.1.1 ____ Hydrate provided oligonucleotides

3.1.2 ____ Hydrate the 109 backbone oligonucleotide mixture with nuclease free water to achieve a total oligonucleotide concentration of 100μM (10μl per nanomole).

3.1.3 ____ Hydrate the 10 var mixture with nuclease free water to achieve a total oligo concentration of 100μM (10μl per nanomoles).

3.1.4 ____ Hydrate the mixture of the two biotinylated vars with nuclease free water to achieve a total oligonucleotide concentration of 100μM (10μl per nanomoles)

3.1.5 ____ Create a mixture of 119 unfunctionalized oligonucleotides

- To help ensure that each scaffold has all of its oligonucleotides hybridized we recommend putting the oligonucleotides in at a 10 fold excess to the scaffold. As having many excess biotinylated oligonucleotides would be costly and problematic for on-rate experiments, the biotinylated oligonucleotides will only be put in at a 4x excess to the scaffold. As all of the oligonucleotides are mixed in an equimolar fashion, adding 1.09 μl of the 109 mixture is simply like adding 0.01 μl of each of the 109 oligonucleotides at 100μM.

3.1.6 ____ Mix the following in an Eppendorf DNA LoBind tube (0030 108.035)

a. ____ 10.9μl of the 100μM 109 backbone mix

b. ____ 1.0 μl of the 10 Var mixture

3.2 Mix the single stranded scaffold, unfunctionalized oligonucleotides, and functionalized oligonucleotides

3.2.1 Mix the following in an Axygen PCR tube

a. ____ 5μl of 20nM linear single-stranded M13 DNA (see Section 1)

b. ____ 1.19μl the mixture of 119 unfunctionalized oligonucleotides

- This is essentially like adding 0.01 μl of each oligonucleotide at 100μM which gives a 10 fold excess to the 5μl of 20nM scaffold

c. ____ 0.8 µl of 100x dilute biotinylated Var mixture (1µl of the mixture of functionalized Var 4 and Var 8 + 99µl of nuclease free water)
    - This is essentially like adding 0.004 µl of each of the biotinylated Vars at 100µM thus giving a 4 fold excess compared to the scaffold
d. ____ 0.22 µl of 10x NEBuffer 2
e. ____ Pipette to mix thoroughly

3.2.2 Place the mixture in a thermocycler and subject it to the following protocol

a. ____ Heat the sample to 90° C
b. ____ Cool the sample 1°C per minute until it reaches 20°
    - Note: if non-thermostable custom functionalized-oligonucleotides are used these oligonucleotides should not be added in until after the mixture has reached a temperature compatible with the functionalized oligonucleotide
    - For convenience you may want to set the thermocycler to hold at 4°C after completion

3.3 Optional PEG precipitation step to remove excess oligonucleotides.

Large pieces of DNA such as the scaffold will precipitate out, while smaller pieces of DNA such as the oligonucleotides will remain in the supernatant. (This is not necessary for the biotin-streptavidin experiments but may be useful for custom applications).

Note: that the precipitation efficiency is very sensitive to the concentration of the PEG so it helps to make a large stock of PEG that you can calibrate so that you do not have to calibrate your concentration every time you make a new PEG stock

3.3.1 Make a 30% (by weight) stock of 8k PEG (Polyethylene Glycol 8000 Amresco 0159-500G)

a. ____ Add 15g of 8k PEG to a 50 ml falcon tube
b. ____ Bring this up to 50 ml with nuclease free water

3.3.2 Make 2 dilutions of your PEG stocks in protein LoBind tubes as follows (As mentioned above, the efficiency is very sensitive to PEG concentration thus the following percentages may not work for you and you may have to tweak the percentages for your 30% stock

a. 4.75% PEG in 30mM $MgCl_2$ (This is higher so that when mixed with the 40µl of DNA the final PEG concentration will be ~3.5%)
    i. ____ 38 µl of 30% PEG stock
    ii. ____ 24 µl of 300mM $MgCl_2$
    iii. ____ 178 µl of nuclease-free water
b. 3.5% PEG in 30mM $MgCl_2$
    i. ____ 28 µl of 30% PEG stock
    ii. ____ 24 µl of 300mM $MgCl_2$
    iii. ____ 188 µl of nuclease-free water

3.3.3 Dilute your hybridized construct to 40 µl in 30mM $MgCl_2$ in a DNA LoBind tube

- For example, if you have 8 µl of the hybridized DNA/oligonucleotide mix then to this mix you should add 4 µl of 300mM $MgCl_2$ and 28 µl of nuclease-free water

3.3.4 Perform the PEG precipitation

a. ____ Add 115 µl of 4.75% PEG and mix thoroughly
b. ____ Centrifuge at 16,000 rpm for 30 minutes at room temperature
c. ____ Carefully remove the top 145 µl
    - This should be done very carefully as it is easy to disturb the pellet it should take roughly 30 seconds to slowly pipette out the supernatant in one smooth draw. It helps to constantly be pipetting near the fluid air boundary as to be as far away from the pellet as possible
d. ____ Add 115 µl of 3.5% PEG and mix thoroughly
e. ____ Centrifuge at 16,000 rpm for 30 minutes at room temperature

f. Carefully remove the top 115 µl
    - This should be done very carefully as it is easy to disturb the pellet it should take roughly 30 seconds to slowly pipette out the supernatant in one smooth draw. It helps to constantly be pipetting near the fluid air boundary as to be as far away from the pellet as possible
g. ____ Add 115 µl of 3.5% PEG and mix thoroughly
h. ____ Centrifuge at 16,000 rpm for 30 minutes at room temperature
i. ____ Carefully remove the top 115 µl
    - This should be done very carefully as it is easy to disturb the pellet it should take roughly 30 seconds to slowly pipette out the supernatant in one smooth draw. It helps to constantly be pipetting near the fluid air boundary as to be as far away from the pellet as possible
j. The remaining 10 µl should have the hybridized DNA scaffold free of any detectable amount of free floating oligonucleotides

This construct is now ready for use in on-rate experiments.

4. On-rate Experiments:

To avoid the formation of aggregates (streptavidin molecules linking multiple scaffolds) we work at low construct concentrations (i.e. the concentration of the construct should be lower than the effective concentration of the two biotins on the scaffold, which in the case of Vars 4 and 8, is ~30nM. The construct prepared in the above protocol (3.2) is at a concentration of ~16nM. To ensure that aggregation is not an issue we will dilute this 100 fold.

4.1 Dilute the construct 100 fold to achieve a concentration of 160 pM. (note if you used oligonucleotides at lower concentration your construct may already be less concentrated than 16nM if you used different volumes in creating your construct you must calculate the final construct concentration)

4.1.1 ____ In an Eppendorf protein LoBind tube(0030 108.094), mix 1µl of your construct with 99µl of the buffer in which you wish to do the experiment

One buffer condition that we recommend for biotin streptavidin experiments is 1x NEBuffer 2 supplemented with an additional 150mM NaCl (total 200mM NaCl)

4.1.2 To make 2 ml of this mix the following

a. ____ 200 µl of 10x NEBuffer 2
b. ____ 100 µl of 3M NaCl in nuclease-free water
c. ____ 1,700 µl of nuclease-free water

To avoid capping (i.e. the binding of two streptavidin molecules to one construct thus rendering that construct unloopable) the streptavidin concentration should also be well below the effective concentration of the ligands on the scaffold which for Vars 4 and 8 is ~30nM. To achieve this we will dilute the streptavidin to ~6nM so that when mixed in equal volumes with the scaffold the final concentration of streptavidin is one-tenth of the effective concentration of the two biotins on the loop.

1mg/ml streptavidin (Rockland catalog number: S000-01) has a concentration of ~15µM diluting this 2,500 fold will bring the streptavidin to a nominal concentration of 6nM. We measured the concentration of our streptavidin, and its activity (using a HABA assay) and found that our

results closely match the data sheet provided by Rockland yielding a final streptavidin concentration of 6.88 nM which when mixed 1:1 with the construct yielded a streptavidin concentration of 3.44nM

4.2 Dilute the streptavidin 2,500 fold

4.2.1 ____ In an Eppendorf protein LoBind tube, mix 4µl of 1mg/ml streptavidin with 196µl of the same buffer used to dilute the construct. This yields a ~300nM stock of streptavidin

4.2.2 ____ In another Eppendorf protein LoBind tube, mix 4µl of the ~300nM streptavidin with 196µl of the same buffer used to dilute the construct. This yields a ~6nM stock of streptavidin (This low concentration solution should be made fresh before each experiment)

4.3 Preparation for on-rate experiments

To do the on-rate measurement equal volumes of 160pM construct and 6nM streptavidin will be combined. Loops will immediately start forming. To halt loop formation the construct can be quenched with free biotin to occupy all free sites on streptavidin thus preventing further loop closure events. If fractions of the construct are quenched with biotin at different times this yields an on-rate time series.

The rate of loop closure with 3 nM streptavidin and 80 pM scaffold saturates after roughly 2 minutes. A good series of time points to cover this range well is 5 seconds, 10 seconds, 15 seconds, 20 seconds, 30 seconds, 40 seconds, 60 seconds, 80 seconds, 120 seconds, 360 seconds.

As you will only have 5 seconds between the first couple of time points you will want to prepare all of your tubes and quencher ahead of time. Below we provide a very detailed approach to doing these experiments which we have found greatly facilitates performing on-rate measurements.

To prepare the biotin quencher you will want to make a saturated solution of biotin (Sigma-Aldrich B4501-100MG) (0.22mg/ml ~1mM) in nuclease free water. To ensure the solution is

saturated we recommend having excess powder in the tube. The tube can be vortexed then centrifuged before each use.

4.4 Performing an on-rate experiment with the above mentioned time series:

4.4.1 ____ 10 Eppendorf protein LoBind tubes should be labeled and arranged in an easily accessible manner in a tube rack.

4.4.2 ____ 2 µl of the saturated biotin solution should be pipetted into the very bottom of each tube

- Tube caps can be closed now, but during the on-rate experiments it will help save time to leave all your tubes open
- We recommend having an ample supply of tips 1-200µl LowBind tips and three pipettors at hand (1 that can pipette 60 µl and two that can pipette 10 µl)

4.4.3 ____ Add 60 µl of the 160 pM construct to a new protein LoBind tube

4.4.4 ____ Load tips onto all three pipettors and have them at close reach

4.4.5 ____ Set a timer, that counts up after the alarm rings, for 30 seconds

4.4.6 ____ Aspirate 60 µl of the ~6 nM streptavidin mixture into the pipette

4.4.7 ____ When the alarm sounds mix 60 µl of streptavidin with the 60 µl of DNA

- Do not handle the tube near the bottom as this will heat the sample to your body temperature yielding an artificially higher room-temperature on rate.

4.4.8 ____ Grab the first 10 µl pipettor and aspirate 10 µl of the mixture

4.4.9 ____ When the count-up timer hits 5 seconds pipette the mixture into the bottom of the 5 second tube esnsuring good mixing with the 2 µl of saturated biotin

4.4.10 ____ Grab the second pipette and repeat this procedure for the 10 second tube

4.4.11 ____ Now continue with the remaining time points changing tips between samples

Notes:

This procedure can take a couple of tries to nail the early time points. We recommend practicing with empty tubes to get the timing and positioning down. Additionally having more time between samples i.e. 10 seconds rather than 5 may help for the first couple of experiments.

We have also found that doing the experiment on parafilm can help with speed, but we tend to prefer using the low bind tubes

There should be roughly 20 μl of the mixture left after completion. This can be left unquenched, or can be quenched at a later time point to provide a true plateau value

5. Pouring, running, and staining the gel:

We recommend running the loops on a 0.7% agarose gel in 0.5x TBE at 4V/cm (100V in a 25cm Owl gel box) for 100 minutes. It is important to use TBE instead of TAE, since the separation of looped from unlooped is significantly better in TBE under these conditions. Other running buffers have not been extensively tested. Other agarose percentages can be used, but we recommend staying between 0.6% and 1%. Adjusting times and voltages to suit your needs is possible, but we have not extensively tested this, so it is recommended to follow the protocol for a baseline before tweaking parameters.

5.1 Preparing and running the gel:

5.1.1 ____ Add 0.7 g of ultrapure agarose (Invitrogen catalog # 16500-500) to a 500 ml Erlenmeyer flask

5.1.2 ____ Make 1 L of 0.5x TBE by diluting 50 ml of 10x TBE (BioRad 161-0770EDU) with 950 ml of Reverse Osmosis, MilliQ, or double-distilled water

5.1.3 ____ Add 100 ml of 0.5x TBE and a magnetic stir bar to the Erlenmeyer flask

- It is important to use the same buffer for making the gel and for running buffer. Even slight mismatches in buffer can cause gel artifacts that make quantification difficult. Make sure you have enough 0.5x TBE to make the gel and to fill the gel box with running buffer.

5.1.4 ____ Cover the top of the flask with aluminum foil to prevent vapor from escaping

5.1.5 ____ Place the Erlenmeyer flask on a hot plate with stirring capability

5.1.6 ____ Bring to a rolling boil while stirring at ~400 rpm, and allow to boil for several seconds

5.1.7 ____ Cool the flask until it is cool enough that it can be held comfortably in a gloved hand for 60 seconds

- If the gel cools too much it will not pour evenly. It is better to error on the side of too hot than too cold

- We find that cooling by running cool water over the flask for a minute or submerging in ice water for 20 seconds tends to provide quick and adequate cooling
- Ensure that you swirl the contents while doing this so that liquid doesn't gel at the walls of the flask

5.1.8 ____ Pour the gel into a molding tray with a gel comb containing at least 13 wells and allow to solidify

- We find that pouring the gel in the cold room (4°C) helps expedite the gelling process (10 minutes in the cold room should be ample time, at 25°C the gel should be allowed at least 45 minutes to solidify)

5.1.9 ____ To each of the 12 µl samples (2 µl biotin + 10 µl construct/streptavidin) add 2 µl of 6x-ficoll loading-dye (Promega catalog # G1881)

5.1.10 ____ We recommend running a ladder alongside the on rate samples to aid in verification of the looped and unlooped bands

5.1.11 ____ To prepare the ladder mix the following

i. ____ 0.5 µl of 1kb extension ladder (Invitrogen catalog # 10511-012)

ii. ____ 4 µl of 6x ficoll loading dye

iii. ____ 19.5 µl of 0.5x TBE

5.1.12 ____ Fill the gel box with the 0.5x TBE running buffer (to the fill line)

- It is important to use the same buffer for making the gel and for running buffer. Even slight mismatches in buffer can cause gel artifacts that make quantification difficult. Make sure you have enough 0.5x TBE to make the gel and to fill the gel box with running buffer.

5.1.13 ____ Load 4 µl of the ladder mix into the first lane

5.1.14 ____ Load the full 14 µl of each of the time points into the next 10 lanes

- Additionally 10 µl of the unquenched sample can be mixed with 2 µl of 6x ficoll loading dye and the 12 µl can be loaded

5.1.15 ____ In the final lane load 4 μl of the ladder mix

- Having a ladder on both sides helps if there is skew at the running or imaging stages

5.1.16 ____ Run the gel by applying 4V/cm (distance measured electrode to electrode) for 100 minutes

- Note that if the interaction of interest has a lifetime shorter than the gel run time, the loops will fall apart during the gel running process and no looped band will be seen
- To circumvent this we have shown separation of the looped and unlooped, for a Var 4 – Var 8 construct, in as little as 9 minutes at 300 V (supplementary Figure s3)
- Additionally for short lived interactions, crosslinkers, such as glutaraldehyde, could be used to stabilize the loops to prevent loop opening during the gel running process

5.2 Staining the gel:

We recommend using SYBR-gold (Life technologies catalog # S-11494) for maximal sensitivity

5.2.1 ____ In a container slightly larger than the gel dilute the SYBR-gold 1 in 10,000 in running buffer (i.e. 6 μl of stock SYBR-gold in 60 ml of 0.5x TBE)

5.2.2 ____ Allow the gel to sit in stain on a rocker for at least 30 minutes

- one hour usually leads to more even staining

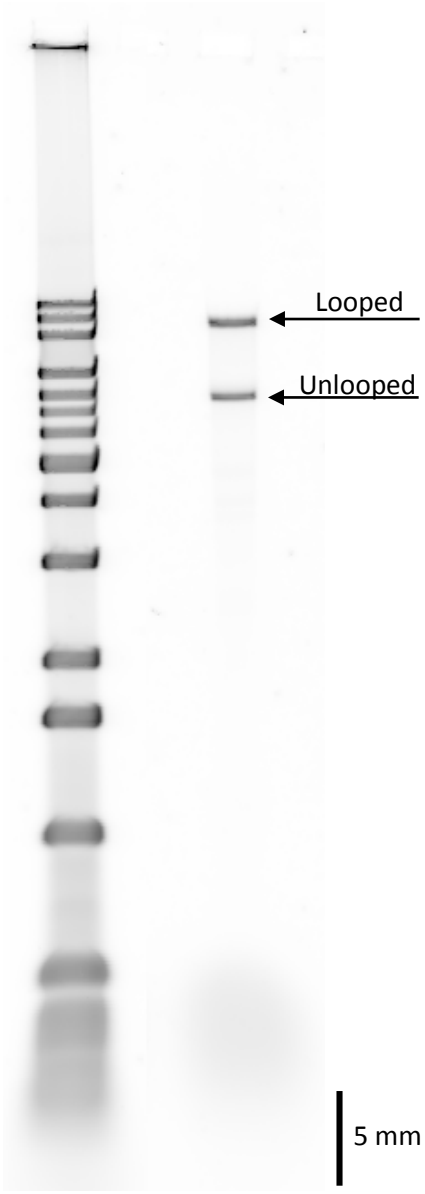
5.3 Imaging the gel:

We have found similar results using several imagers (including a point and shoot camera) but for the highest sensitivity and repeatability we recommend using a laser scanning gel imager such as the Typhoon FLA 9000. Note that some gel imaging formats save with non-linear intensity profiles. This will affect your analysis as intensity will no longer be linearly proportional to the amount of material. Either make sure that you save the images with a linear intensity profile or that you linearize the images with freely available tools in imageJ (see online methods).

SYBR-gold can be excited at 488. On the Typhoon FLA 9000 we usually image using the standard SYBR-gold settings with a PMT value of 500 and a resolution of 100μm or 10μm if we want to resolve two bands that are very close to one another.

See online methods and supplemental for details about image analysis, quantification, and kinetic modeling.

Below is an image of a gel prepared, run, and stained under these conditions showing where the looped and unlooped bands appear relative to the 1kb extension ladder (Invitrogen catalog # 10511-012).



6. Performing Off-rate experiments:

To perform off-rate experiments you must first form loops and allow them to equilibrate. This can be done by following the steps in the on-rate experiment with the exception of the quenching steps (4.1-4.3, 4.4.3, and 4.4.6). Simply mix equal volumes of 160 pM construct and 6nM streptavidin, and allow this to form loops for 10 minutes.

To perform the off-rate experiments samples of loops will be allowed to sit in quenching conditions for different amounts of time with the idea being that if a loop opens it will do so irreversibly as the now available binding site will quickly be quenched by the large excess of free floating biotin in solution. To avoid having to run many gels it is easiest to do the longest time point first so that all the samples can be run on the same gel. For example, if you wanted your longest time point to be 72 hours you would mix 10 μl of loops with 2 μl of saturated biotin 72 hours before you planned on running the gel.

An example time series for the biotin streptavidin system would be to take time points over ~5 days at room temperature in the presence of 200 mM salt. Alternatively, for a quicker experiment time points can be taken over 3 hours at 50°C and 200 mM NaCl.

To form the loops follow the protocols above to generate linear DNA(1), hybridize on the functionalized and unfunctionalized oligonucleotides(3), dilute the construct (4.1), and dilute the streptavidin(4.2).

Once you have the required materials (See 1-4.3) You may proceed to do an off-rate experiment:

6.1 Forming loops

- 6.1.1 ____ Add 70 μl of the 160 pM construct to a new protein LoBind tube
- 6.1.2 ____ Add 70 μl of the 6nM streptavidin to the 40 μl of DNA
- 6.1.3 ____ Allow this to sit at 25° C for 10 minutes to allow loop formation to go to completion

The following protocol illustrates an off-rate with the following time points 0, 1 min, 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 90 min, 120 min, and 180 min.(These times do not need to be adhered to strictly, but this range of times is good to show a significant off rate).

6.2 Performing an off-rate experiment with the above mentioned time points:

- 6.2.1 ____ Select a date and time when you wish to run the gel
- 6.2.2 ____ 180 min before running the gel, mix 10 µl of the looped mixture with 2 µl of saturated biotin in an Eppendorf protein LoBind tube
- 6.2.3 ____ Allow this to sit at 50°C (in a thermocycler or water bath)
- 6.2.4 ____ 120 min before running the gel, mix 10 µl of the looped mixture with 2 µl of saturated biotin in another Eppendorf protein LoBind tube
- 6.2.5 ____ Allow this to sit at 50°C (in a thermocycler or water bath
- 6.2.6 ____ 90 min before running the gel, mix 10 µl of the looped mixture with 2 µl of saturated biotin in another Eppendorf protein LoBind tube
- 6.2.7 ____ Allow this to sit at 50°C (in a thermocycler or water bath)
- 6.2.8 ____ Cast a gel and get everything ready to run the gel (see section 5)
- 6.2.9 ____ 60 min before running the gel, mix 10 µl of the looped mixture with 2 µl of saturated biotin in another Eppendorf protein LoBind tube
- 6.2.10 ____ Allow this to sit at 50°C (in a thermocycler or water bath)
- 6.2.11 ____ 45 min before running the gel, mix 10 µl of the looped mixture with 2 µl of saturated biotin in another Eppendorf protein LoBind tube
- 6.2.12 ____ Allow this to sit at 50°C (in a thermocycler or water bath)
- 6.2.13 ____ 30 min before running the gel, mix 10 µl of the looped mixture with 2 µl of saturated biotin in another Eppendorf protein LoBind tube
- 6.2.14 ____ Allow this to sit at 50°C (in a thermocycler or water bath)
- 6.2.15 ____ 15 min before running the gel, mix 10 µl of the looped mixture with 2 µl of saturated biotin in another Eppendorf protein LoBind tube
- 6.2.16 ____ Allow this to sit at 50°C (in a thermocycler or water bath)
- 6.2.17 ____ 10 min before running the gel, mix 10 µl of the looped mixture with 2 µl of saturated biotin in another Eppendorf protein LoBind tube

6.2.18 ____ Allow this to sit at 50°C (in a thermocycler or water bath)

6.2.19 ____ 5 min before running the gel, mix 10 μl of the looped mixture with 2 μl of saturated biotin in another Eppendorf protein LoBind tube

6.2.20 ____ Allow this to sit at 50°C (in a thermocycler or water bath)

6.2.21 ____ 1 min before running the gel, mix 10 μl of the looped mixture with 2 μl of saturated biotin in another Eppendorf protein LoBind tube

6.2.22 ____ Allow this to sit at 50°C (in a thermocycler or water bath)

6.2.23 ____ 10 μl of an unquenched sample held at the same temperature can be used as your 0 point

6.2.24 ____ Add 2 μl of 6x ficoll loading dye to each of the samples right before loading and running the gel

- Some of the liquid may have condensed on the cap. Mild centrifugation can help ensure that all of your volumes are the same

6.2.25 ____ Load, run, stain, and image the gel as described in section 5

Note: if you wish to do the off-rate in different solution conditions, all you need to do is alter the buffer in which you dilute your streptavidin and your construct. If you wish to do the off rate at a different temperature you simply need to let the samples sit in a water bath or thermocycler set to the desired temperature rather than at room temperature.

See online methods, Halvorsen et. al 2011 and Koussa et. al 2014 for information on how to implement the nanoswitch platform for non-biotin-streptavidin systems.