

6-1707

DNase I protocol for footprinting ToxR membranes

1. Synthesis of klenow filled in probes

Qiagen prep 200-300 mls of strain carrying plasmid of interest (tip 100). Resuspend plasmid in 50-100 uls TE pH 7.4. You want a concentrated DNA sample, so take this into consideration when you grow up the strain and when you resuspend the DNA pellet.

After checking the plasmid on an agarose gel, set up a restriction enzyme digest of it. Your strategy is to cut with one enzyme, fill in the 5' overhang with ^{32}P , and then drop out the labelled insert with a second restriction enzyme. Which enzymes do you use? Well, you want the DNA binding site to be approximately in the middle of the probe. I have generated good footprints with 88 bps and 107 bps on both sides of the footprinted region with the pTLI2 probe, also with 77 bps and 76 bps on both sides of the footprinted region with the pLS716 probe. Also, I have never used a probe for footprinting that is larger than 240 bps in length. A probe that is too long cannot be used. (I would say >300 bps is too long, but I am not sure).

cut 20 uls of Q-prep DNA with restriction enzyme 1 in a 30 ul volume. (normal incubation period)

klenow fill in reaction.
to 30 ul restriction digest, add:
2.5 ul 2mM mix of dGTP, dTTP, dATP
3.0 ul alpha ^{32}P dCTP
1.0 ul klenow

RT 10 min.
70° C 15 min. to inactivate klenow

add second restriction enzyme (normal incubation period)

2. isolation and purification of klenow filled in probes

Run reactions on a 6% polyacrylamide-1x TBE non-denaturing gel. Use 1x TBE as running buffer. Use thin spacers because a thinner gel allows for better elution. You can run the gel at RT at 80V. Running the gel takes a relatively long time, usually 6-7 hours but it can go longer than this. I would say 10 hour max.

Note on loading this gel: because the gel is so thin, you cannot use normal yellow tips for loading. I use tips that have a long, thin end on them. I get these from Mike Imperiale's lab. The thin end can reach the well bottom, whereas yellow tips are too thick to reach the well bottom.

To isolate the probe fragments, break apart the glass plates. Lay saran wrap over the gel. Take film, tape, sharpie, timer, scissors, gel, and film cassette to the darkroom. Using the safety light, cut 3 triangle notches out of a piece of film. Put the film on the gel that is covered with saran wrap and tape the film to the gel. With the sharpie, outline the film on the gel, make sure to outline the triangle notches. Expose this for 10 minutes. Develop.

Intense probe bands should be visible. Using the film as a template, cut out the probe fragments from the gel (you have to lay the gel on top of the film). Add one gel slice to an eppendorf tube containing 300 ul elution buffer.

Elution buffer: 0.5 M NH₄OAc pH 7.5
0.1% SDS
1 mM EDTA

elute O/N at 37°C

The next day, transfer supe to clean tube and add fresh 300ul elution buffer to the gel slice and continue eluting for most of the day.

EtOH precipitate probes.
to 300 ul elution buffer, add: 30 ul 3M NaOAc pH 5.2
825 ul 100% cold EtOH

normal EtOH ppt. (you can go -20°C O/N or -70°C for half an hour)
Spin tubes 30 min max speed at 4°C.
do one 70% wash. Spin for 5 min.
resuspend in 100 ul TE pH 7.4

count 1 ul in scintillation counter (a good probe will be 50,000-400,000 cpm per ul)
dilute probe to 20,000 cpm per ul in TE pH 7.4

3. gel shifts

the goal of the gel shift is to determine the amount of ToxR membrane that will shift 100% of the probe.

use 1 ul of probe (20,000 cpm) in each reaction

typical reaction:

4 ul 5x binding buffer 10 mM tris-HCl pH 7.4
1mM EDTA (use pH 8 to make buffer)
50 ug per ml BSA
5mM NaCl
50mM KCl

2 ul 0.1 mg per ml salmon sperm DNA
1 ul probe (20,000cpm)
_ ul membrane
_ ul MQH₂O

20ul final volume

bind at 30°C 30 min.

add: 5.5 ul MQH₂O
3.0 ul 50mM MgCl₂

70% uncut?
90% uncut
37°C
↓
5%

37°C — 50% uncut probe is 10%

not
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JM3 K(300)
1108-

of...
mem... what...

1.5 ul 20 mM CaCl₂
3 ul 5x binding buffer containing 10% glycerol (for density)

incubate RT 2 minutes

the reason for adding Mg and Ca is that DNase I needs these ions for activity. However, these ions can disrupt protein-DNA interactions. So in the gel shift, you're testing to see if Mg and Ca disrupt DNA binding by your protein of interest. You may want to do a side-by-side comparison of binding in the presence and absence of Ca and Mg. Ca and Mg do not affect ToxR binding to the *ctx* or *toxT* promoters.

run on a 6% polyacrylamide -1x TBE gel. Use 1x TBE as running buffer. Use thick spacers for the gel shift. Before pre-running gel, add 20ul of 5% thioglycolate to each well. pre-run gel at 120V for 30 min. at 4°C. Load gel, run at 120V for approximately 6 hours.

dry gel 80°C 1 hour
expose at -70°C with screens

note on gel shifts: it typically requires 1-2 mg per ml protein in the binding rxn to shift 100% of the *toxT* and *ctx* probes.

note on 5x binding buffer: I don't use glycerol in the binding rxn because that's what I used for hydroxyl radical footprinting and also I figured the footprinting rxn's would be "greasy" enough with the addition of whole membrane fractions. I believe the glycerol is used to give the rxn enough density to float to bottom of the well. Glycerol is not necessary for efficient binding.

you can load 10x DNA dyes to use as a marker. The slow dye(xylene cyanol) runs at 260 bp on a 5% non-denaturing polyacrylamide gel and at 160 bp on an 8% non-denaturing polyacrylamide gel.

4. Determine membrane: DNase I ratio for efficient cleavage.

use DNase I dilutions of 1:250, 1:500, 1:1000, 1:2000

set up the the following rxn in however many tubes you will need.
the volumes are increased 3.5x compared to gel shift reactions.
(also set up a rxn that will get no DNase I)

14 ul 5x binding buffer
7 ul 0.1 mg per ml salmon sperm DNA
3.5 ul probe (70,000 cpm)
45.5 ul MQH₂O

incubate 30°C 30 min to mimick a true binding rxn

make up DNaseI dilutions in MQH₂O (keep on ice)

to the rxns add:
17 ul MQH₂O

10 ul 50 mM MgCl₂
2 ul 50 mM CaCl₂
1 ul of diluted DNaseI

RT 2 min.

add 100 ul STOP (200 mM NaCl, 2 mM EDTA, 1% SDS)

note: in the actual footprinting reactions, you will do phenol:chloroform extractions at this point. However, when you are only cleaving the naked probe, it is not necessary.

EtOH ppt.

add: 20 ul 7.5 M NH₄OAc pH 7.5
550 ul 100% EtOH
1 ul glycogen

std. incubation period (I usually go -20°C O/N, but -70°C for 0.5 hours works just fine)

spin down in 4°C 30 min max speed

2 70% EtOH washes (spin 5 min in between each one)

resuspend in 5 ul TE + 5 ul formamide stop dyes

(volumes vary depending on if you will load entire rxn in one lane, or split the rxn between two lanes. For one lane, halve the TE and stop dyes and load the 5 ul in one lane).

run on denaturing polyacrylamide-urea-TBE gel

I typically use a 6% polyacrylamide, 6.88 M urea, 1x TBE gel
Use 1x TBE as running buffer

I pre-run the gel 10 min 1500V

heat samples 90°C 5 min before loading

load 5 ul per lane

dry gel for one hour at 80°C

expose at -70°C with screens

5. footprinting reactions

based on the DNase I cleavage reactions and the gel shift, you will know how much DNase I and membrane to use in the actual footprinting reaction.

set up binding reactions: (volume is increased 3.5x relative to gel shift reaction volume)

14 ul 5x binding buffer

7 ul 0.1 mg per ml salmon sperm DNA

3.5 ul probe

_ membrane

_ MQH₂O

final volume 70 ul

incubate 30°C 30 minutes

make up DNase I dilution in MQ H₂O keep on ice

add: 17 ul MQH₂O
10 ul 50 mM MgCl₂
2 ul 50 mM CaCl₂
1 ul DNase

RT 2 min

add 100 ul STOP (same stuff used in step 4 above)

phenol extract the reactions 2-3 times
chloroform:isoamyl (24:1) extract reactions 2-3 times

this step is important because you need to degrade as much of the membrane as possible and get it removed from the aqueous. The cleaner the aqueous is, the sharper the bands are on the gel.

EtOH ppt.
add 20 ul 7.5 M NH₄OAc pH 7.5
550 ul 100% EtOH
1 ul glycogen

standard incubation (I usually go -20°C O/N, but -70°C for 0.5 hours works just fine)

spin down reaction 4°C max speed for half an hour
do two 70% EtOH washes (spin 5 min in between each one)

resuspend in 5 ul TE + 5 ul formamide stop dyes

(again, this can be halved if you want to load entire reaction in one lane)

from this point, everything is the same as in part 4.

final notes: you can skip part 4, but be prepared to have used the wrong DNase I dilution and you will wind up having to repeat the footprinting reactions again. This wastes membrane, which you go through fairly quickly because you have to use so much in the binding reactions.

✓ to generate a sequencing ladder to map the binding site, you can order a primer with its 5' end corresponding to the 5' end of your probe. Simply sequence the plasmid that contains the insert you're using as a probe.

You should see a pretty good footprint signal come up O/N with a -70°C exposure utilizing screens.