5' S1 Nuclease protection protocol

Protocol Used in: Lyons SM, Cunningham CH, Welch JD, Groh B, Guo AY, Wei B, Whitfield ML, Xiong Y, Marzluff WF (2016). Nucleic Acids Research. 44(19): 9190-9205

Making your radioactive probe

Considerations for making a probe before you start:

a. You will need the gene you are interested in cloned into some sort of vector
b. You will need a restriction site within the cDNA sequence (R1) and one upstream of the transcription start site (R2) or within the vector
c. Make sure that when cutting R1 and R2 you will be able to gel purify a distinct band (no double bands)
d. Since you will be phosphorylating with T4 PNK, there are not as many restrictions on R1 as there are with a 3' S1. However, restriction enzymes leaving a 5' overhang are preferred as they are more readily phosphorylated then 3' overhangs or blunt ends. Phosphorylation of a 5' recessed end (3' overhang) may require addition of PEG for efficient phosphorylation. See Sambrook and Russell
e. Typically, you want the distance from transcription start site to R1 to be >100nts

1. Cut ~5 μg of plasmid containing you cloned gene at R1
2. Prior to dephosphorylation, either heat inactivate restriction enzyme if CIP can be used in current buffer, or Clean up with PCR clean up kit. Elute in 30 μl of H_2O
3. Set up dephosphorylate reaction of cut DNA*:
    a. 30 μL cut DNA
    b. 3.5 μL 10X CutSmart Buffer (NEB)
    c. 1.5 μL QuickCIP (NEB M0525)

    *If you only heat inactivated the restriction enzyme, Add CIP to heat inactivated reaction
4. Incubate at 37^{o}C for 30 minutes. It is important to dephosphorylate with CIP and then phosphorylate with T4 PNK by the forward reaction rather than using T4 PNK alone using the exchange reaction. The forward reaction leads to a higher specific activity probe.
5. Clean up through PCR column eluting in 30 μL
6. Set up phosphorylation reaction:
    a. 30 μL eluted DNA
    b. 5 μL of 10X T4 PNK buffer (for Forward reaction)
    c. 12.5 μL of [γ-^{32}P]-ATP
    d. 2.5 μL of T4 PNK (NEB M0201S)
7. Incubate at 37^{o}C for 30 minutes
8. Stop reaction by adding 2 μL of 0.5 M EDTA and heating to 65^{o}C for 15 minutes
9. Run through illustra MicroSpin G-50 columns (GE LifeSciences 27533001) to remove unincorporated nucleotides
10. Ethanol precipitate with 3M Sodium Acetate
11. Resuspend in 20 μL dH_2O
12. Cut DNA with R2 following NEB protocols.
13. Run on an agarose gel and gel purify the band corresponding to your probe using Qiagen Kit eluting in 30 μL dH_2O
14. Take counts of eluted probe. Anywhere from 1000 cpm – 50000 cpm is within the acceptable range for an S1 probe.

Hybridize probe to RNA

Considerations before hybridizing probe:

a. Each probe is going to have its own temperature for optimal hybridization. Since your probe is dsDNA, too low of a temperature will lead to reannealing of dsDNA and too high of temperature will prevent hybridization with target RNA. Typically, hybridization at 52$_o$C is sufficient, but occasionally a temperature gradient is necessary to determine the proper temperature. A PCR machine is ideal for such a gradient.
b. Amount of cellular RNA needed is dependent upon abundance of target message. In general, S1 assays are more sensitive than northern blots, so, the amount of RNA used for a northern is typically sufficient for S1 assays. However, if you're doing an S1 on a histone RNA, 3 – 5 μg of cellular RNA is recommended since you will be detecting only histone message (e.g. Hist2H3C), but, if you are doing a northern for a H3 message, you are detecting multiple messages.
c. If you poly(A) select your RNA, do not do so with glycogen as it has an inhibitory effect on S1 nuclease. Instead, use carrier RNA for precipitation.
d. Two controls are needed at this point, (1) Probe + S1 and (2) 10% Probe. For these add the same amount of yeast or *E. coli* tRNA as cellular RNA you added to your experimental samples. These 2 controls will give you an idea of the efficiency of the S1 digestion

1. In a microcentrifuge tube mix a volume of probe equivalent to 1000 cpm with an amount of RNA needed to detect your target RNA. Probe may need to be diluted.
2. Ensure that all of your microcentrifuge tubes contain the same amount of liquid. This is important because you want all of you samples to dry at the same time.
3. In a speed-vac, dry RNA and Probe
4. Resuspend in 10 μL of S1 Hybridization buffer
5. Heat at ~100$_o$C for 10 minutes
6. Hybridize overnight at proper hybridization temperature. This must be empirically determined.

Digestion of unhybridized probe

1. Set up nuclease reaction*:
    a. 10 μL hybridized RNA/Probe
    b. 10 μL 10X S1 nuclease buffer
    c. 1 μL S1 nuclease (Promega M5761)
    d. q.s. to 100 μL with dH_2O

    *DO NOT add enzyme to your 10% probe control otherwise its not a control
2. Incubate at room temperature for 1.5 hours
3. Add 1 μL of glycogen and ethanol precipitate (phenol/chloroform extraction is not necessary)
4. Resuspend in 20 μL RNA loading dye containing both bromophenol blue and xylene cyanol and run on a urea/acrylamide gel. The percentage of gel must be determined based on the size of your probe and your expected protected fragment.
5. Dry gel and expose to phosphor screen or film.

S1 hybridization Buffer

80% Formamide, 40mM PIPES (pH 6.4), 500 mM NaCl, 1 mM EDTA