3' S1 Nuclease Protection Assay

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 probe before you start:

a. You will need your gene of interest cloned into some sort of vector
b. You will need a restriction site within your cDNA sequence (R1) and one downstream of the 3' end processing site or if you're interested in misprocessing, within the vector (R2).
c. If run-on transcription is a possibility of misprocessing, R2 absolutely must be within the vector (i.e. not in genomic DNA downstream of the proper 3'end processing site). Otherwise, you will never be able to distinguish undigested probe from run-on transcripts.
d. Make sure that when cutting R1 and R2 you will be able to gel purify a distinct band (no double bands)
e. R1 must leave a 5' overhang so that it can be filled in by Klenow. Also, one of the nucleotides to be filled in must be cytosine if your using [α-^{32}P] dCTP as your radionuclide
f. Typically, you want the distance from R1 to the 3' processing site to be >100nts

1. Cut ~5 μg of plasmid containing your gene of interest with at R1
2. Heat inactivate enzyme if possible and clean up with PCR clean up column or Phenol/chloroform extract. Elute or resuspend in 30 μL.
3. Fill in with Klenow fragment by setting up following reaction:
    a. 30 μL of cut DNA
    b. 5 μL of [α-^{32}P] dCTP
    c. 5 μL of 10 mM dATP, dGTP, dTTP mix (3.3 mM each)
    d. 5 μL of 10X NEB Buffer
    e. 3 μL of Klenow fragment (NEB M0210)
    f. 2 μL of water
4. Incubate at 37^{o}C for 30 minutes
5. Remove unincorporated nucleotides by running through G50 column
6. Ethanol precipitate DNA. G50 column will add EDTA to the eluate which will chelate out Mg^{2+} ions necessary for restriction enzymes.
7. Resuspend in 20 μL of dH_2O
8. Cut labeled DNA at R2
9. Run on agarose gel and gel purify proper band using Qiagen columns. Elute in 30 μL.
10. Determine counts of eluted probe. Anywhere from 1000 cpm – 50000 cpm is within the acceptable range. However, if more than one cytosine is present in the fill in reaction, cpm/μL can be much higher.

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 an 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 tube 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

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 it is 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 pre-ran urea/acrylamide gel
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