7.02 Fall 2001 Recitation #5

Outline:
- Northern blot washes
- Probe detection
- Interpretation of results
- Zebrafish observation

I. Northern blot washes:

Stringency: relative ability of the probe to bind to an imperfect target.

- As in the hybridization, during the washes we again modulate the stringency to get rid of the probe that may have hybridized to unintended targets, while allowing hybridization to intended targets
  - We [again] use:
    - **Salt concentration**
      - salt (Na+) present in the SSC buffer neutralizes the negative charges on the RNA/DNA backbones, thus allowing probe:target binding to occur
        - 2X SSC: high salt wash, allows some non-specific binding to occur
        - 0.5X SSC: low salt wash, increases stringency by allowing some RNA/DNA repulsion to occur
    - **Temperature**
      - The higher the temperature, the more disruption in base pairing H-bonding
        - Room temp: low stringency wash
        - 68°C: high stringency wash, allows only specific hybridization between target and probe to persist
- By hybridizing at a lower stringency, we allow more time for the probe to anneal to the intended target. Then, we slowly raise the stringency in order to get rid of the probe annealed to unintended, non-specific RNA targets.

Step	Stringency	Solution	Purpose
Prehyb	Low	Caesin/Formamide/SDS@50°C	Caesin/SDS blocks non specificbinding sites on filter. Formamide allows binding to occur at lower temperatures by disrupting DNA-RNA bp interactions (H-bonds).
Hyb	Low	Prehyb+probe@50°C	Hyb solution promotes probe/target binding in a low stringency situation.
High Salt Wash	Med/Low	2xSSC+0.1%sds@RT	Med/Low stringency washes away most of probe but still some non-specific binding exists.
Low Salt Wash	High	0.5xSSC@68°C	Last high stringency wash gets rid ofmost/all non-specific probe:non-target interactions while allowing onlyexact probe:target sequence matches to stay on the filter.

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***two 15-min low and high stringency washes were done for you by the TAs**

II. Probe detection:

- the probe contains modified bases with digoxygenin covalently attached to uracils. We can utilize this modified base to visualize the where the probe is bound on the blot relative to all the other nucleic acids on the blot.
    a) digoxygenin is recognized by an anti-DIG antibody
    b) the antibody is conjugated to the enzyme alkaline phosphatase
    c) alkaline phosphatasewill cleave the substrate CSPD
    d) cleaved CSPD give off a luminescent product
    e) the luminescent product can be detected using film!

- Your blots were rinsed in washing buffer and incubated in blocking buffer by the TAs, so **today**, start with the *detection* step in the protocol
    - Things to remember:
        - After incubation in CSPD solution drain most of it off
        - to wrap the blot in saran wrap:
            - spread a piece of saran wrap on your bench large enough to wrap the membrane **once**
            - lay down the membrane onto the Saran wrap with the RNA side down (the markings for the **ladder** are on the **RNA** side)
            - wrap the blot in the saran wrap completely so no membrane is exposed, but there's only one layer of saran wrap on the RNA side.
            - label your blot with bench number and time when you began your 37°C incubation
- your blots will be put on film for 10-15 minutes
- you will get your developed film back in about 1 hour

III. Interpretation of results:

- From your film you can determine:
    1. stages at which *zcyt-1* is expressed
    2. relative levels of *zcyt-1* expression
    3. size of *zcyt-1* transcript
- we need our controls to interpret our results
    - positive control?
    - negative control?

Positive ctrl	Negative control	Interpretation
+	-	There was little RNA or it was degraded so that the probe wasnot able to detect the target RNA.
+	+	Difficult to interpret; stringency isn't high enough.
-	-	One of the steps of the procedure (probe labeling/ hybridizationor detection) has failed.

IV. Zebrafish

- Retinoic Acid Experiment:
  - Tally of results seen by class:

- Conclusions from last semester:
  - Embryos most susceptible to RA when treated at the very beginning of gastrulation
  - results seen even with the lowest concentration of RA, but the effects were mild and treated embryos survived
    - Examples of defects from 0.1 uM RA treatment:
      - delayed development (consistent)
      - bent tail (consistent)
      - loss of pigmentation in the body
      - uneven placement of eyes (one more anterior than other)
      - weaker circulation
  - Increasing the RA concentration made the effects more severe; circulation never developed and embryos died after about 60 hrs
    - Examples of defects from 0.5uM and 1um RA treatments:
      - very delayed development
      - blob-like body
      - no distinct segmentation
      - lack of clear backbone ending
      - less defined eyes
      - lack of head features
      - spasms