

Bioc471a/571a Homework 5 - Due at the start of class September 25

1. (2 pts) Why is it not a good idea to have RNase H activity present in the first strand cDNA reaction, but it is required for the second strand reaction in the replacement strategy? Does the replacement strategy result in synthesis of full-length cDNA products that include the first nucleotide of the mRNA transcript? Explain.

RNaseH cleaves DNA-RNA hybrids and during first strand synthesis it will cleave the RNA template strand in molecules containing partially extended primers. In the strand replacement strategy, RNA primers are generated by RNaseH cleavage of the RNA-DNA hybrid generated by first strand synthesis. The difference between the RNaseH-associated activity of reverse transcriptase, and addition of RNaseH to the second strand reaction, is the ability to control when RNA cleavage will take place, i.e., after the RNA template has been fully extended.

The RNA replacement strategy does not include cDNA products with the first nucleotide because the RNA primer used to generate the 5' end of the cDNA is degraded.

2. (2 pts.) Based on the flow scheme for oligonucleotide adaptors in figure 5.4, and use of the lZapII cDNA cloning vector, would blue-white screening of plaques distinguish between recombinants that contain EcoRI adapted cDNA inserts, and those that only have EcoRI adaptor dimers (false-positive)? Explain. If after isolating a recombinant cDNA clone from this library you discover that the insert contains an internal Eco RI site, what two explanations could account for this and how would you distinguish between these possibilities?

Adaptor primers would ligate into the lZap vector the same way an insert would, and since the adaptor dimer is 26 bp long, it would disrupt the LacZ alpha fragment coding sequence and score as a recombinant (white colony).

If an EcoRI-adapted cDNA insert contained an internal EcoRI site, it could either be a naturally occurring EcoRI site, or evidence that two cDNA fragments ligated together prior to being inserted into the vector; this arrangement would also create an "internal" EcoRI site. These two possibilities could be distinguished by obtaining the DNA sequence around the internal EcoRI site. If the internal EcoRI sequence was flanked by NotI recognition sequences, then two cDNA fragments must have ligated together. However, if the only sequences that match the adaptor were found to be that of EcoRI (GAATTC), then it must be coming from a natural EcoRI site that could subsequently be confirmed by genomic sequencing.

3. (2 pts.) Based on the flow scheme for oligonucleotide screening of a cDNA library in figure 5.6, how many different oligonucleotide sequences are represented in the NT probe pool? What is the rationale for using the two probes in successive screenings, i.e., screen first with the NT probe and then the CT probe? Will any bona fide cDNAs be missed using this strategy? Explain.

Based on the degeneracy at each position, the total number of oligonucleotides in the pool would have to be 64 (1 x 2 x 2 x 4 x 2 x 2).

By using the two probes in successive order, you have a better chance of identify bona fide cDNAs because false-positive hybridizations in the first screening are eliminated if they don't hybridize to the CT probe. The one problem with this strategy is if a bona fide cDNA were to have a 3' termini within the "RAV" gap region (or only contain a small portion of the CT probe), then a potentially important 5' clone might not be pursued due to lack of hybridization to the CT probe.

4. (2 pts.) What three criteria must be met for a functional cDNA screening strategy to work using the plasmid sib selection approach? Describe the functional screening assay initially used by Caterina et al. (Nature 389:816-824, 1997) to identify the cDNA insert encoding VR1. Describe the assay they used to quantitate the binding specificity of VR1 for capsaicin relative to compounds in the poblano verde chili.

The three criteria for a functional cDNA screen to work are 1) the assay must have a very high signal to noise ratio, i.e., a positive signal from a single plasmid in a large pool must be able to score positive in the assay, 2) the library must contain a large number of full-length clones, and 3) the activity must be encoded by a single polypeptide.

The initial assay used by Caterina et al. to identify VR1 was based on transfecting the plasmid pools into human cells and then comparing Ca⁺⁺ binding activity (using the fluorescent calcium-binding dye FURA) in cells treated +/- capsaicin.

Once VRI was identified and characterized, a more quantitative assay was performed using patch clamping in regions of a Xenopus oocyte membrane following injection of in vitro synthesized VR1 mRNA.

5. (2 pts.) What are three key functional components required for functional cDNA screening using the yeast two-hybrid strategy? What function in the bait protein would give a high background signal, i.e., show a positive signal even in the absence of a target protein? What is the advantage of having both the Gal4-dependent His3 and lacZ reporter genes in the host yeast strain?

The three functional components of yeast two-hybrid screening are 1) the Bait vectore expressing a fusion gene containing the Gal4 DBD fused to the coding sequence of the Bait protein, 2) the target cDNA library containing random cDNAs fused to the Gal4 activation domain, and 3) a yeast strain containing Gal4 reprotor genes (LacZ and/or His 3) integrated into the yeast genome.

A bait protein containing an edogenous transcriptional activation domain would not work because the DBD-Bait fusion protein would activate the reporter genes in the absence of a Bait-Target protein interaction.

The advantage of two reporter genes is that it decreases the chance of a false postive because both genes need to be responsive. Moreover, the His3 reporter gene is a selection and the LacZ gene is a screen.