Troubleshooting Forum

Molecular Biology Techniques Q&A

Knockout mice

This month's question from the Molecular Biology Forums (online at molecularbiology.forums.biotechniques.com) comes from the "General Methods" section. Entries have been edited for concision and clarity. Mentions of specific products and manufacturers have been retained from the original posts, but do not represent endorsements by, or the opinions of, *BioTechniques*.

Why do I still detect the full length protein in mouse heart tissue where it has been knocked out? (Thread 18428)

Q I am working with some knock-out mice generated by Cre-LoxP. These have been used in many labs where they were considered true knockouts. The researcher who created the mice reported that exon 3 was deleted by flanking it with LoxP sites, followed by subsequent removal through Cre expression. Removal of exon 3 linked exon 2 to exon 4, causing a frame shift and introducing a stop codon. To verify that this resulted in complete knockout of gene expression, the original researchers performed RT-PCR and Western blot on RNA and proteins extracted from the uterus of mice showing that RNA and protein were not produced there anymore.

I am interested in studying the heart of these mice. I began my studies by checking the tissue at the genomic, RNA, and protein levels. DNA sequence analysis confirmed the exon is actually deleted in all tissues I have checked, but Taqman analysis showed that the sequence after exon 3 is present in all tissues. The researchers who created the mice said it is quite possible that full length RNA is produced without exon 3, but the resulting RNA is unstable and would rapidly degrade. If that is the case, why am I able to isolate and sequence the RNA?

To further investigate, I checked for protein expression, which should not be present in any tissues since the deletion of exon 3 introduces a stop codon in exon 4. My Western blots showed protein expression in the heart tissue of these mice but confirmed that there was no expression in the uterus. Cre expression resulted in a germ line deletion of exon 3, so no cells should contain this protein. What is going on?

A It's possible that the transcript is spliced differently in the heart than in the uterus. A change in the splice regulatory system might activate a cryptic splice site in heart tissue that brings the downstream sequence back into frame. In wild type mice, splicing might occur at a site that is deleted in the knockout mice, leading to the use of the poor-consensus cryptic site in the heart tissue of the knockout mice. Obviously I don't have any data to support this idea, but it seems like you need to explore any possibilities.

A Does your antibody detect the predicted truncated protein or do you see a full length protein on your Western

blots? Can you detect any alternatively spliced isoforms? Is it possible that your gene has a copy somewhere else in the genome that is used in tissues other than the uterus?

Q I thought about alternative splicing. This receptor has some known isoforms, some of which play an important role in the development of breast cancer. So if I pursue this question, how can I collect evidence for an isoform in the heart created through a cryptic splice site? I did RNA-seq to try to see what was going on, but in those experiments, I could only see the expected frame shift that generates the stop codon.

A Your RNA-seq results don't necessarily show the absence of alternative splice forms. Sequencing always depends on your choice of primers. If the expressed, alternatively spliced isoform lacks one of the priming sites, it won't be amplified. So your sequencing results will only show the transcript with the frame shift. You might try 5'-RACE.

Where does the antibody bind? That region must be conserved between the isoforms since you detect the longer protein in your Western blots. If you are using a commercial antibody and don't have this information, just try calling the company. They probably will be willing to share this information or at least narrow the binding site to a particular region on the protein.

Q With Western blot, I detect the full length protein along with the other known splice variants in the hearts of my wild type and knockout mice. They show the same protein pattern, including four bands. In the ovaries of the knockout mice, I detect nothing; in their uteri I detect a band possibly arising from a splice variant since the size is smaller than expected for the full length protein.

I do not know if my gene of interest has a copy somewhere else in the genome. It has not been described anywhere, so I think it is unlikely. Previous researchers performed Southern blots, so they should have been able to detect any other existing copies of the gene in the genome.

A Try RT-PCR to pull out the cryptically spliced mRNA and map it to the genomic sequence. Then you will be able to design a steric-blocking oligo to inhibit splicing at that cryptic site. It is difficult to deliver such oligos to intact adult organisms, but you could isolate primary cardiomyo-

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cytes and demonstrate the lack of the cryptically spliced form in parallel cultures with and without splice-blocking oligo treatment.

This paper describes how to deliver the oligo into cardiomyocytes: Masaki M et al. Smad1 protects cardiomyocytes from ischemia-reperfusion injury. *Circulation*. 2005 May 31;111(21):2752-9. Epub 2005 May 23.

Q When sequencing, I chose primer pairs in different exons, beginning with the first exon and ending in the second, third, fourth, and even fifth exons. Subsequently the amplicons were sequenced and all showed the expected sequencing results.

My antibody binding site is conserved. The antibody binds to a known sequence at the C-terminal end of the protein in exon 8. This area should never be detected in the knockout mice since the protein should not be transcribed beyond the newly generated stop codon in exon 4.

A The paper I previously cited is just a proof of principle for introducing a splice-blocking oligo to cultured primary myocytes. If you are considering splice-blocking as an experimental technique, this paper will give you the necessary details: Morcos PA. Achieving targeted and quantifiable alteration of mRNA splicing with Morpholino oligos. *Biochem Biophys Res Commun*. 2007 Jun 29;358(2):521-7. Epub 2007 May 7.

A From the position of antibody binding, it seems clear that the only plausible answer is that you are detecting the full length protein. You will have to trust the data. There may be an alternate first exon, or different exons. Or maybe exons 1-3 are simply skipped.

If you are certain that the band you see on your Western blot represents the full-length protein (and not a protein of approximately the same length but carrying a different N-terminus), then you should explore the idea that another copy of the gene, or perhaps a closely related gene, is expressed in the heart.

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