Troubleshooting Forum

Molecular Biology Techniques Q&A

5' RACE

This month's question from the Molecular Biology Forums (online at molecularbiology.forums.biotechniques.com) comes from the "RNA 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*.

Can I avoid cloning when performing 5' RACE? (Thread 28053)

Q I am studying alternative splicing and the 5' UTR of a particular gene in dogs. To do this, I plan to do gene-specific 5' RACE, followed by sequencing. I want to omit the cloning steps; I don't see any need for them since I will be using specific primers.

I extracted RNA from skin punches, but had slight problems with the quality after Tri-reagent isolation. The 260/230 ratios are low. I plan to do additional ethanol washes to see if that will solve the problem.

In the meantime, I started working out the RT-PCR using an RNA isolation that was not too poor in quality. I am using a very long 62 bp reverse primer that binds to the 3' end of the mRNA. I get a clear band at about ~500-600 bp, which is the expected size. I get a few extra bands as well, but they are very weak and may be the result of un-spliced or degraded RNA. It's also possible that they come from gDNA contamination since this RNA definitely has gDNA in it. The gDNA product should be 3 kb instead of 500bp and since I use elongation times of only 1 min, I don't expect to see much gDNA amplification.

I did reverse transcription first to create the single stranded cDNA, followed by PCR to generate double-stranded cDNA, and then PCR for my specific amplified sequence. I sequenced both the double stranded cDNA and the amplification product. For both samples, a signal was produced, but the background was too high to allow the sequence to be readable.

I don't know how to increase the specificity or which product I should sequence to get the most valuable information. Would increasing the temperature of the reverse transcription reaction increase specificity? I wonder if my reverse primer is simply too long or nonspecific. Should I create a single stranded cDNA using oligo (dT) primers, make double stranded cDNA using oligo (dT), and then use gene specific primers to amplify the cDNA?

Can anyone advise me regarding RT-PCR using sequence-specific primers combined with oligo (dT) primers and direct sequencing without a cloning step?

What quality of RNA is required for 5' RACE? The kit I'm using selects for mRNA only by degrading all RNA without a cap.

A If you get a clean single band when performing ordinary RT-PCR, sequencing the PCR product can save time over cloning. However, you do not have a clean single band. You are doing RACE with specificity only at one end, which reduces the likelihood of getting a specific product.

With RACE, you might also be amplifying transcripts that start from alternate sites, have alternate splicing, or are short if the cap degradation doesn't work perfectly. You cannot expect to always get a clean single band. So since that is true, why don't you want to clone? If it worked well with your first attempt, you could sequence the PCR product and avoid cloning. But since it did not, cloning may actually save time in the end.

RNA purity is not always essential if you can get the PCR to work. It sounds like this is true in your case since you see the desired size of PCR product.

Priming the reverse transcription step does not improve specificity even with a gene-specific primer. If the gene specific primer is 60bp, unless it's mostly A/T, it will be below the needed melting temperature and you will not be able to expect specificity. If it contains mostly A/T and is not random, that could also cause poor specificity. From your question, I wasn't sure if you were using that long primer in the later PCR steps, but I would advise against it because the low specificity of the RT would then be carried into the PCR, which is where the process becomes specific. Oligo-dT or random priming is sufficient for the RT, and gene specific priming is okay as long as you use different primers for the PCR.

To determine which product to sequence, the first strand RT reaction product will not give you a clean sequence. But if you are referring to the product of the first PCR amplification with a second nested PCR reaction following it, you may sequence a clean band that you get at any stage. You are more likely to find the correct gene sequence after the second nested PCR.

If you saw a predominant band of the predicted size, you may not need to worry much about increasing specificity; you could clone it. Or you might cut the product out of a gel, amplify a few more cycles if needed and then see if it's clean enough to sequence from the 3' end. I have not done specific reverse transcription reactions myself and would not expect that to work, although better reagents may be available now. The PCR should be done with an annealing temperature that is less than 5°C below the predicted melting temperature. The melting temperature is only

an estimate, so you can change this if you don't see a product. You may raise your annealing temperature if the reaction is not specific.

A second nested PCR reaction is a powerful way to increase specificity and can be essential if nonspecific primers are used on one end as done in RACE. A third nesting is not out of the question. Even if the priming is specific, there are other reasons one may not get a single band. (I mentioned some above: alternate splices, alternate start sites, short RT extensions, secondary structure in the RNA that can cause RT stops, incomplete splicing, degraded RNA, etc.). So, the answer is to increase the PCR annealing temperature to close to the primer T_m . You may even need to redesign the primers. But you don't need to worry much about the specificity of the RT.

Since you are doing RACE, I assume the 5' sequence is unknown. Because there are reasons for varying 5' lengths, sequencing is more likely to be clean from the 3' end.

Q I think you are exactly correct. I was using a non-specific reverse primer for cDNA synthesis and PCR, resulting in non-specific PCR products and bad sequence reads. I will design nested reverse primers for PCR to get a more specific product.

The reverse transcriptase I am using, MuLV RNase H-, doesn't have RNase H activity. Do I need to do an RNase H digest after first strand synthesis?

A RNase H digestion of first strand cDNA is not necessary. I usually do not do it.

A I remember the Superscript RT protocol first offered RNaseH treatment as an option, but now it is included in the main protocol. The information accompanying the kit says RNaseH is reported to help in some cases. So in general, it looks like this step is not required, but it may help in certain sequence-specific situations.

A RNase H destroys the RNA, but the initial denaturation step of the PCR will be enough to denature any RNA-DNA duplex. I found that RNase H-treated cDNA became unstable upon storage at -20°C , so I no longer treat the first strand cDNA with RNase H.

A But without RNase treatment after the reverse transcription first strand cDNA synthesis, isn't it possible that the subsequent PCR may amplify the remaining RNA? If so, this would influence the quantity and quality of synthesized cDNA and alter gene expression data. Are the 95°C steps for denaturation during cycling sufficient to destroy all the existing RNAs, including the RNA-DNA duplex?

A The RNA cannot serve as template for DNA polymerase in PCR. The initial denaturation step should separate the RNA from the cDNA, but not destroy the RNA. RNase H is needed only if you are amplifying very long cDNA fragments.

A I have never had problems with cDNA storage after RNase H treatment, but I store the cDNA at -80°C . This leaves much less of a chance of thawing than in -20°C freezers. I remember reading from Invitrogen that in many cases RNase H is unnecessary, but in certain cases they had received reports of problems when it wasn't used. They didn't specify what problems might arise without RNaseH treatment, but now they include this step in the main protocol.

Selected by Kristie Nybo, Ph. D.

BioTechniques 52:135-137 (March 2012) doi 10.2144/000113817

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