Troubleshooting Forum

Molecular Biology Techniques Q&A

Site-directed mutagenesis: colony growth

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 don't I get many colonies following transformation of the *DpnI*-digested PCR product? (Thread 21770)

Q I have been working on site-directed mutagenesis (SDM) for over a month, but still haven't been able to get the mutation I need. I used Fermentas *Pfu* polymerase to amplify my vector with insert and saw two bands following the PCR. I then set up a *DpnI* digestion of the PCR products using 3 μ L PCR product, 1 μ L *DpnI*, 1 μ L buffer, and 5 μ L water to make a total volume of 10 μ L. I digested the product for 90 min at 37°C. When I ran a gel following digestion, I didn't see any bands at all, but I transformed the JM109 cells anyway using 1.5 μ L of the digested product. After overnight incubation at 37°C, I had only a single colony. The colony did not grow in Luria Bertani (LB) broth with ampicillin or on an LB plate. I think this colony must contain my mutant, but what went wrong? Why won't it grow?

A When I do SDM, I add the *DpnI* and its buffer directly into the PCR reaction. I don't know if that will make much difference for your results, but I thought I would let you know since that is easier. I also don't worry much about checking each step on gels since I am most concerned about the end results. You mentioned that you got only a single colony after incubating at 37°C overnight. Were those cells incubated with LB only or with LB plus antibiotic? If you added the antibiotic, did you incubate at 37°C for 1 h without antibiotic first? I suggest you check your competent cells. Did you include any controls?

A I recommend that you use the Stratagene kit; it makes the procedure quite simple. Adding 1 μ L *DpnI* to 3 μ L PCR product seems like a lot to me. I usually add 1 μ L *DpnI* straight into my PCR reaction as described in the previous post, but I don't add the *DpnI* buffer. I incubate this for 2 h at 37°C. The single colony you found sounds like an artifact. You should check to see if the bacteria are viable by growing some of the transformed cells on an LB plate without Amp/Kan. I would also add a plate with cells transformed with the original plasmid. Unfortunately, since you are not using the kit, there are no easy controls to see if the mutagenesis worked.

A If you did not see any bands in the agarose gel after *DpnI* digestion, that probably means you had very little PCR amplification. You should try to improve the PCR by using additives such as DMSO or betaine. Then after the amplification finishes, add 1 μ L *DpnI* directly to the 50- μ L PCR reaction tube and incubate at 37°C for 1 h. Use the *DpnI*-digested DNA for transformation. After *DpnI* digestion, you should be able to see at least a faint band on an agarose gel.

A This might be a simple and obvious question, but it has raised problems for me in the past: are you sure your vector confers ampicillin resistance? You mentioned that you saw a band on your gel following PCR, but not after *DpnI* digestion. To me, this indicates that you overdigested your PCR product. As others have stated, your ratio of *DpnI* to product is atypical and may be too high. This will depend on your concentration of *DpnI* of course, if you are using a stock of a different concentration than we expect. If you have too much *DpnI*, you could get non-specific cutting. Your *DpnI* or water may be contaminated by other nucleases that are not specific to methylated DNA or you may be denaturing your DNA during the *DpnI* incubation somehow. If the hot top is on, your tube might be heating high enough to break down DNA. That is a long shot, but it's something you

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could check. As long as you use more of your PCR product in the reaction, you shouldn't need to add the *DpnI* buffer on top of the PCR buffer.

A I also add 10–20 U *DpnI* directly into my 50- μ L PCR reaction, using only the PCR buffer. But this is with the Stratagene *Pfu* Turbo, Ultra, or UltraII. Before assuming this will work for you, you will need to check the Fermentas buffer. I think the Stratagene buffer is proprietary, but *DpnI* might tolerate a range of buffers. You just need to be sure that *DpnI* will be active and specific in your PCR buffer. How much plasmid template did you start with? Stratagene recommends 10 ng, but says that you can vary this amount if you have problems. You might be able to try a range of 1–50 ng.

You may also want to consider your PCR conditions. You shouldn't exceed 18 cycles. Extending around a large plasmid can require generous extension times. Stratagene recommends 68°C extension temperatures, as 72°C can cause strand displacement and ruin the PCR product. Some primers, especially the longer ones used for SDM, can form secondary structures and not work well. You could try shifting the location of your primers a bit. High competence bacteria can also help. The Stratagene protocol does note that results can be acceptable even if you see no product on an agarose gel.

Q I tried using the Stratagene kit originally, but didn't get any results so I decided to stop using it to cut costs. I am certain that my vector is ampicillin-resistant; I'm using a pgl3 vector. I'm not sure about the competency of the bacteria. How will growing the bacteria for 1 h without the ampicillin test for competency? I use these same cells for cloning and they work well. I will try testing them anyway since they may have lost competency over time. How much of the *DpnI*-digested product do you use for transformation?

A If you transform 2 μ L intact plasmid at $\sim$ 100 ng/ μ L, you should get a lot of colonies. In fact, they should be uncountable if the cells have good competency. If you see $\leq$ 100 colonies on your plate, you have very poor competent cells that are not suitable for transforming a mutagenesis or ligation product. I frequently use 2.5 μ L (but you can use $\leq$ 4 μ L) of the *DpnI*-digested product in a 50- μ L aliquot of XL1-blue or DH5 α cells. If $>$ 10% of your total volume is *DpnI* product, you risk inhibiting the transformation with the PCR or *DpnI* buffer.

A I use the QuikChange method without the kit as well. Originally, I also expected to see thousands of colonies, but in practice, I usually only get $\sim$ 100 after plating 20–25 μ L competent cells transformed with 5% of the PCR product. I plate by spinning down the transformation in a microfuge tube, pouring off most of the supernatant, resuspending the remaining supernatant, and streaking that on the plate. I always use highly competent cells ($>10^9$ / μ g), so I'd expect proportionately fewer colonies with less-competent cells. The number of colonies can also be template-dependent; recently a certain template gave me $<$ 20 cells per transformation. I would not have found any with less-competent cells, so it is very important to test the competence of your cells. When testing for competence, I usually get far fewer colonies than expected from intact plasmid. Perhaps this is because the DNA is not supercoiled or modified (e.g., methylated). Has anyone else noticed this? Also, in certain cloning experiments I've found the mutagenesis primer partially duplicated in the final clone's sequence. Does anyone know how to prevent this?

A I have experienced that too; it can happen when you use reverse complementary primers. Any time I see this, I just sequence another clone from the same plate and I always get a valid result.

A I have also seen strange results like that. In my case, I think it happened because my template was GC-rich even in the areas I wanted to mutate. I usually just ignore these things when they come up and sequence another clone.

Selected and edited by Kristie Nybo, Ph.D.

BioTechniques 50:87–89 (February 2011)
doi 10.2144/000113609

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