Troubleshooting Forum

Molecular Biology Techniques Q&A

Cloning Splice Variants

This month's question from the Molecular Biology Forums (online at molecularbiology.forums.biotechniques.com) comes from the "DNA and General PCR 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 clone splice variants from a single cDNA carrying all possible exons? (Thread 31603)

Q I have a cDNA clone containing 6 exons. From this cDNA carrying exons A-B-C-D-E-F, I would like to create cDNAs with exons A-C-D-F and A-B-E-F. However, no suitable restriction sites to do this are available. Since synthetic DNA is so expensive, can anyone suggest another way I can clone these isoforms from the cDNA that I have?

A The only way I can think of is site-directed mutagenesis to remove certain exons. There are PCR-based commercial kits to do this.

Q How many base pairs can I remove using site-directed mutagenesis?

A If you make primers with built-in restriction sites to flank the exon you want to delete, you can digest and religate after PCR. There should be no limit to the number of base pairs you can delete. But also keep in mind that the product will not be exactly the isoform you want; there will be two to three extra codons in the place of the deleted exon because of the restriction site.

A If the alternative splicing isoforms are specific to certain tissues, you can use cDNAs from those tissues and primers for exons A and F to get your desired clones. If you don't have access to the different tissues or cell lines you need, you might try PCR mutagenesis. This can be done with a kit or simply by standard PCR and cloning. You can remove as many bases as you like with PCR. If you use a proofreading polymerase, you can create blunt ends that can be phosphorylated and ligated together.

A It is preferable to use a restriction enzyme that generates an overhang if such cut sites can be created using silent mutations near the junction. An alternative to PCR of each end and blunt end cloning is to make complementary primers that cross the deletion location, such as in the Quikchange method. I often perform that procedure without the kit by using my own proofreading PCR enzyme. The chances of success go down with larger deletions, but I have successfully deleted up to 2kb this way.

A I prefer not to amplify the cDNA by PCR and religate it through blunt-end ligation. If one base is removed from the primers by a nonspecific nuclease, you will introduce a frame-shift mutation. It is better to do it through sticky end ligation even though this will give you extra codons.

Commercial mutagenesis kits such as Quikchange destroy the template with *DpnI* endonuclease to remove any remaining wild-type clones.

A Are you recommending digesting the template over a blunt ligation? Or are you not sure this can be done properly without the kit? I do this procedure without the kit using *DpnI* from NEB by following the same protocol included in the kit. In place of mystery solution Q, I use 3% DMSO. I recommend using the kit for the first time, or in general if money is not an issue, but I found it to be unnecessary once I was familiar with the method.

A In my earlier post, I was trying to make two points. First, sticky-end ligation is less risky than blunt-end ligation of the PCR fragment. Second, the template should be destroyed with *DpnI* to avoid parental background clones.

As far as using a kit, I think that is just a matter of preference; experienced personnel should be able to follow the online protocol without using the kit.

A Another option is recombination PCR using 4 primers. Many kits call for PCR to replicate the entire plasmid, but you can also use similar mutagenesis primers in combination with other primers that recognize the ends of the sequence. First, generate 2 PCR products: from primer A to Primer B/E and from primer B/E to primer F. Purify the PCR products, combine them in an equimolar ratio, and run 2 cycles of PCR without any primers. This allows the complementary region in the B/E overlap to anneal and generates a full length template. Then perform another PCR with primers A and F and you can amplify the mutated construct. I have had good success using this method, but I find the kits to be a bit faster and less labor intensive.

A You might use Webcutter to identify silent restriction sites that can be used to design restriction enzyme primers without adding any codons.

Q Recombination PCR sounds interesting, but I'm not sure I understand the method. Could you please elaborate on the technique or post the reference? Also, I always treat my PCR products with *DpnI* to remove template unless I plan to gel purify.

A The idea is that you create two PCR products overlapping at the mutation point. The length of the overlap is sufficient to act as a primer itself, so the two PCR products can be mixed together, allowing those overlap regions to prime the

other strand to create a full length PCR product. Usually this is done by using the complement of the first reverse primer as the forward primer for the next piece, although isn't required, so if it improves your primer design, you can shift one a few nucleotides up or downstream. Since you want to cause a deletion instead of a point mutation, I think you will need to make a long primer. Try one normal length primer for each side of the deletion, and a complementary one for the other PCR product.

After amplifying the two primary PCR products, separate these from the template by gel purification or *DpnI* treatment and mix them together. I'm not sure about the purpose of the two cycles with no primer. Add only the outer primers and amplify again. Use fewer cycles than usual at this step since you are only trying to link the pieces together; additional cycles will increase your mutation risk. The two PCR products should knit together to create the full length gene with the deletion in the center. Finally, digest with restriction enzyme for enzyme sites on the outer primers (A and F) and ligate into the prepared vector. If you have convenient unique restriction sites close to the deletion, you won't need to amplify the entire gene, just replace part of the gene and reduce the mutation risk and sequencing efforts required. The other mutagenesis method is easier because it doesn't require ligation, vector preparation, or two stage PCR, but it does introduce some mutation risk across the entire vector.

A I think the two cycles without primers are optional, but they seem to improve the technique in my hands. Essentially, primers A and F have no template since you have only put in two overlapping fragments. Running a few cycles of PCR without primers allows the fragments to anneal in their overlap regions and use the overlap as a primer to generate the full length template. Then you can add the primers at the ends and amplify the full length product. I prefer to put my construct into a cloning plasmid, perform the mutagenesis, and sequence the insert fully. I then subclone just the insert into an expression plasmid. As mentioned already, there is a risk of introducing point mutations throughout the plasmid when using the kits, so I wouldn't recommend doing site directed mutagenesis on an expression plasmid unless you plan to sequence the whole thing.

A I agree that it's safer to reclone the insert, although this can be done in whatever vector you happen to be using, including the expression vector. I have never gone to that effort; instead, I take advantage of the speed of the mutagenesis method to directly change my vector. Most mutations that matter will be selected against because the plasmid will not be selected or reproduce if the origin, selection marker, or other important areas are mutated. There is a risk that the promoter or other expression sites may be mutated, so I always pick a few spare clones as backups in case one doesn't express. Starting with nanogram quantities of DNA and using only 17 PCR cycles does not leave much chance for mutation when using a high fidelity proof reading enzyme. If sequencing the insert is not sufficient assurance, then it is a good idea to cut out the insert and religate it into a final vector or use the other overlap method discussed.

A Another more straight forward option is PCR SOEING where you sew together the different exons. The original method

was described in *BioTechniques*: Gene splicing by overlap extension. *BioTechniques*. 1990 May;8(5):528-35. With this method, you can create many different combinations.

Q I will try these methods, one for the first isoform and another one for second isoform since it looks like it will be more convenient to clone one isoform using the overlap method and the other one using mutagenesis.

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