The Slacker Way (kit-free) for Making Fusion Proteins for Antibody Generation/Affinity Purification (K. Oegema)

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Step 1

PCR Design

Design oligos for making 150-250 aa pieces of protein of interest. Design oligos for 2-3 such pieces per protein; one N-Ter, one C-ter and (if protein is large) one middle piece. To design the primers, I like to use MIT's Primer3 program (http://www.genome.wi.mit.edu//cgi-bin/primer/primer3_www.cgi). Use sites on either end - preferably Bam HI, Eco RI, Xho I or Sal I with 3 or 7 bp "clamps" on either end to ensure that the restriction enzyme can cut at the end of the PCR fragments. For Eco RI, Sma I and BamHI I have used 3 bp clamps something like CGC. For Sal I and Xho I, I usually use a 7 bp clamp something like "CTCTTCC". The sequences of the primers can be varied to avoid internal hybridization of the primers or pairing of the primers with each other. We have also succesfully used the SmaI site (remember digests for this enzyme are at 25C). Bgl II also produces ends that can be cloned into the BamHI site if you get desperate for sites. Design sites to clone inframe with GST into Pharmacia pGEX6P-1,2,3 vector series. There are stop codons in all three frames after the polylinker so it should not be necessary to insert a stop codon in the C-terminal primer.

Generate protein sequence files of the precise fusion proteins and calculate the MW and predicted pI of of the fusion proteins. The pI is useful when figuring out how to couple the protein to affigel.

Obtain source material for PCR

- If you have a clone of the gene of interest you are set. Alternatively:
- For yeast, genomic DNA is good for most genes (the SGD database has a list of all genes with introns)
- For worms use cDNA; if not available use first strand cDNA generated using RT-PCR of mRNA
- For frogs - use cDNA library (use purified library DNA and not phage). As for worms, RT-PCR is also an option
- For mammals - as per the slacker way, you can buy 1st strand cDNA from Clontech for most tissues and cell lines you can think of. We have had good luck using this as a source for PCR

Step 2

PCR

PCR pieces of interest using a 25-30 cycle standard PCR protocol (100 µl reaction vol). I often use Taq because it is usually the most robust particulary when PCRing from libraries (this is in contrast to Pretty Fucking Useless). Screening eliminates all but missense mutations. For antibody production, missense mutations are not a big deal and so I don't worry about it. Check PCR reactions by gel to make sure you have product of correct size.

Add 1/9 vol 3M NaAc, pH 5.2 to the PCR reactions and then Phenol/Chloroform the reaction (DO NOT SKIP THIS!) - just one Phenol/Chloroform extraction is sufficient (i.e. no need to do chloroform alone, etc. but if you're anal you can go ahead and do what you like). Precipitate DNA with 2 vol EtOH, pellet, wash and resuspend in 50 µl TE

Step 3

PCR Reaction Processing

Digest PCR reaction in a final volume of 50-100 µl with appropriate enzyme. Subsequent cloning is best if you digest with each enzyme individually, cleaning up the reaction by some method in between but, since this is the slacker way, you can go ahead and digest together. I usually digest overnight (Karen) but Arshad does his digests for the standard amount of time.

Phenol/Chloroform and EtOH ppt the digested PCR reaction as per step 2. Resuspend in 20 µl of TE. If you want, you can check the DNA by gel (you should have seen a reasonable pellet).

Processing the vector

Digest the vector as requred in parallel with the PCR reaction. Karen always CIPs the vectors adding 1 ul of CIP to the digestion and incubating at 37C for 1 hour. Arshad never CIPs the reactions and has not had much problem with bkgd (He always does sequential digests for vectors). Gel purify the vector. Once again, one phenol/chloroform extraction/EtOH ppt after vector digestion but before gel purification greatly eases cloning.

Ligations

Ligate the PCR fragment into the gel purified and appropriately digested pGEX6P vector. For ligations, I use ~0.3 µl insert from a 100 µl 30 cycle Taq PCR reaction processed as above.

Ligate at RT for 2-10 hrs (depending on your schedule). Transform ligation reaction directly into competent BL21 cells (use 40 µl of competent cells and 1-2 µl ligation) and leave plates at 37°C until colonies are "picking" size (do not leave too long!)

Step 4

Expression Screening

Setup 7-8 2 ml cultures at 28°C, inoculated with one colony from each successful ligations (ligation success is scored here just by # of colonies). Also setup 2 cultures from colonies on the vector only plate. Shake ON for 16 hours at 28°C. Growing the cultures at 28C prevents them from overgrowing.

The next morning, inoculate 0.5 ml LB+Amp with 50 µl of overnight culture and put at 37°C for one hour. Put the rest of the 2 ml ON culture in a 2 ml eppie, spin at 6K for 1', remove supe and freeze pellet at -20°C (this will be used for a mini prep later).

To the 0.5 ml cultures at 37°C, add IPTG to 1.0 mM (5 µl of 0.1M IPTG) and shake cultures an additional hour at 37°C

Pellet induced cultures at 6K for 1' in µfuge, add 100 µl 2X Laemmli SB and put at 95°C for 7'-10' vortexing vigorously (2-3X each tube).

Load 10 µl of sample on 12% SDS-PAGE gels. Always load a vector only lane for comparison. Coomassie stain and destain the gels.

Compare the induced cultures for the various constructs to the vector only lane. You want to see good expression of a fusion protein of the correct size. Success will vary significantly but it is best to look for good expressors and avoid the troublesome ones (which will only cause more trouble later). See later for an example.

Select 2-4 good expressors of the correct size for each construct and miniprep DNA for these from the 2 ml ON cultures frozen earlier. Throw out all the other frozen cell pellets.

Check the miniprepped DNA for presence of insert by digest - run the digested PCR reaction (ligation insert) alongside for comparison. All expressor colonies should have inserts of the right size. This miniprep DNA will be the master plasmid stock so store it well labeled, etc. Each miniprep is good for over 300 transformations so there is no need to do a maxi prep. You will only use this DNA for transformation 3-4 times and never again! (remember this is the slacker way...)

Often there are FPs where none of the colonies look good - in this case just throw out all the frozen pellets. This is why it is important to design 2-3 FPs per protein. The aim is to get at least one good one per protein. In our experience it is often best (and fastest) to redesign the FPs than to try and get troublesome ones to work. The goal here is not functional analysis (where you have no choice) but simply antibody production. Often making a FP shorter will make expression better but may reduce antigenicity. Unless we have no choice, we set our lower size limit to ~150 aa.

Step 5

Large Scale Expression

Transform 1 µl of 1/5 dilution of the miniprep DNA into BL21 cells. 37°C for ~9 hours. Pick one colony into 100 ml LB+Amp and leave ON at 28°C for ~9-10 hrs (OD should be ~0.5-1). Inoculate 1L of LB+Amp with 20 ml of ON and incubate at 25-28°C till OD is 0.5-1. Add IPTG to 1.0 mM and incubate at same temp for 3-4 hours. Save 0.5 ml culture before IPTG addition and before pelleting induced cultures. Run on gel to check that induction is OK.

Pellet cells in 1L bottles (3200 rpm 10', brake=3), resuspend in ice-cold PBS, pellet 50 ml conicals or in GSA bottles (8K 10') and freeze in tubes/bottles or scrape out cells and freeze directly in liquid N2 for grinding.