Primary *in vivo* C-terminal HA_3 tagging

NOTE: Protocol uses long PCR primers with approximately 50bp of homology to the 3' end of the gene of interest. These are used to amplify the HA_3 epitope tag and the KL-TRP gene from plasmid SB1006 (pKL-Trp.HA_3). This product is then transformed into the *S.cerevisiae* strain of choice and integrates by homologous recombination. The *K.lactis* Trp complements *trp*$^-$ in yeast, but has little primary sequence homology so should not integrate at the *TRP* locus.

A few general things:

A. Primers are ≈ 70bp long – they must be PAGE purified (not cheap)
B. Remove the stop codon when designing the primers
C. You're restricted to the 3' end of the gene (obviously) so they might be theoretically terrible primers (with lots of internal homology, etc) – go for it.
D. Ensure you check with BLAST whether the primers will anneal anywhere else other than the gene of interest.

1. The PCR step and purification of the PCR products

I generally use Denville TaqPro (low fidelity; according to Tim the error rate is $1.5x10^{-5}$ after 70 cycles, or ≈ $1x10^{-6}$/cycle). The higher-fidelity Denville SurePol is always great if you're worried, although the yield tends to be lower (are you really that worried?). A hot start is essential. Gel purification of the products is not a bad idea but not usually done.

(i) Make primer pair sets at 2.5μM (10x). It is best to employ a hot start which helps to overcome any internal homology and the resulting formation of primer dimers etc. Each reaction is 50μl final in thin-walled PCR tubes. All mixtures are made on ice.
(ii) Per tube add 10μl ddH_2O, 5μl 2.5μM primer mix, 1μl SB1006 (pKL-Trp.HA_3 plasmid template). Place at 94°C for 8 minutes. While incubating make a stock on ice containing appropriate Taq buffer, $MgCl_2$ (if required), NTPs (10x, 2.5mM), ddH_2O and TaqPro (0.5μl per 50μl reaction). Pause the PCR machine, place the PCR tube on ice for a minute or so and add the rest of the reaction mix. Flash spin and place back in the PCR machine.
(iii) Standard cycle is at 94°C for 45secs, 50°C for 45secs, 72°C for 2m for 35x cycles. If you want you can add a final step of 94°C for 10min, 16°C final (this is good if you're running the reaction overnight). **NB.** If using the small Techne machine be careful about the lid. The Techne machine ramps very quickly so the reaction should take about 2.5 – 3 hours.
(iv) If you want to gel purify the products (usually don't bother): 1% Agarose TGE gel for 90 minutes. Product size is approximately 1.1kB. Cut out the gel slice and geneclean (or otherwise) purify the DNA. Resuspend in a final volume of 30μl.

2. Transforming the yeast

Any yeast strain should work as long as it has a Trp auxotrophy. It is always possible that the tag, if it incorporates correctly, will interfere with the function of the protein: keep this in mind. If the intent is simply to tag the protein with the intention of immunoprecipitating it or using for ChIPs then I use strain YSB726 (haploid) or its diploid parent YSB455.

(v) Innoculate the yeast strain into 10mls of the appropriate medium overnight at the appropriate temperature (30°C for standard strains).

(vi) Next morning allow the tubes to sit on the bench for about 20 minutes before sub-innoculating 1:10. (This is a handy method of observing possible bacterial contamination. If the prep is yeast only they will settle quickly such that the top few mm of the culture tube will clarify).

(vii) Sub the culture 1:10 and place back in the incubator with rotation for approximately 4 hours – the intention is to use the yeast for transformation when they are in exponential growth phase.

(viii) Spin down the yeast pellet (should get ≈250µl packed cell volume, pcv), wash with 1ml ddH_2O (transfer to and flash spin in an ependorff) and once with 1ml LiTE. Resuspend in 500µl LiTE. You require 50µl per transformation, so transfer as desired to fresh ependorff tubes. Add 5µl of sheared single-stranded salmon sperm DNA (10mg/ml, previously boiled (500µl aliquots at –20°C, boil one and store at 4°C for long term use) per 50µl of above and mix by a quick vortex.

(ix) Add 2 - 5µl of transforming DNA (in this case the PCR product from **step iv**) per tube. Add 250µl PLATE per tube. Mix by a quick vortex. Incubate 30m at 30°C with agitation (give it a flick occasionally; this timing is not crucial - if you forget about it an hour or even overnight won't kill them). Incubate 1 – 2h at 42°C; longer incubations at this temperature may result in higher transformation efficiencies (**NB.** If you're working with a temperature sensitive (*ts*) strain

(x) Flash spin in microfuge (because of the high viscosity of the PEG the quality of the pellet in this case will be very different: a longer flash spin is used, and a significant number of cells will be along the side of the tube – careful not to lose them. Remove the supernatant, resuspend cells in 300µl ddH_2O and spread on TRP^- plates. Incubate 3 – 4 days at 30°C; colonies should start to appear after 2 – 3 days but usually take longer. Streak any positives on TRP^- and number them: it is important to do this so you don't mix up your clones. You should get >90% positive clones (appropriate integration, etc) but rarely a sequence will be more difficult to target (eg. when a family of genes have significant homology). Again consider that if the protein you're working on has an important domain at the carboxy-terminus, the tag could interfere with function: keep this in mind (eg. if your gene is essential, the slow-growing colonies may be the right ones).

LiTE
0.1M Lithium Acetate(50% Stock)
1x TE (10mM Tris.Cl pH7.9, 1mM EDTA)

PLATE
40% PEG 3500 or less (50% stock)
0.1M Lithium Acetate (1M Stock)
1x TE (10mM Tris.Cl pH7.9, 1mM EDTA)

3. Yeast protein extraction

While you can check if the PCR product is integrated at the right place (by genomic DNA extraction and Southern or PCR analysis) this isn't much use : you have to show that the protein is appropriately tagged and this will require westerns with the **12CA5** Ab. There are protocols for all of these options. Note that *S.cerevisiae* are extremely difficult to break open compared to other eukaryotes – this means specialized protocols are required for getting at internal stuff.