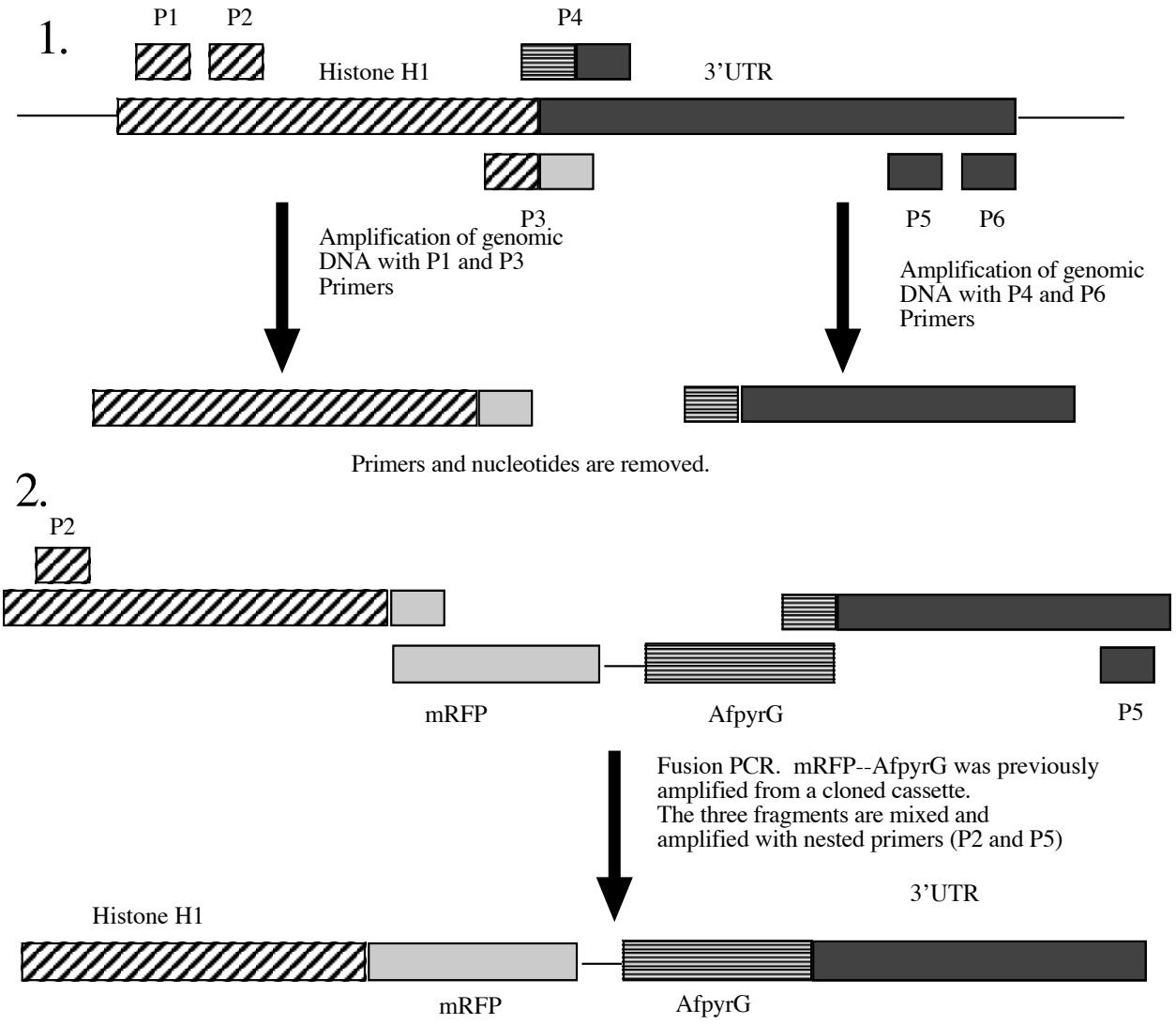


Linear fragment suitable for transformation after removal of primers and nucleotides.

The figure above is a schematic diagram for generation of a linear fragment to tag the C-terminus of Histone H1 with monomeric red fluorescent protein (mRFP). The same approach can be used to tag the C-termini of genes with GFP and other fluorescent moeties or epitope tags. It can also be used to replace genes or to replace the promoters of genes with regulatable promoters such as alcA. The following procedure is very robust and reliable in our hands. It consistently produces fusion PCR products that are adequately pure to be used without band purification to transform *nkuA* deletion strains. A benefit over a previously published procedure (Yang *et al*., 2004, Eukaryot. Cell 3:1359-1362) is that the overhangs that allow fusion PCR are on the primers that are used to amplify the genomic fragments (primers P3 and P4 specifically). This saves two

primers from the previous procedure and allows the cassette (mRFP--AfpyrG in this case) to be amplified once, frozen and aliquots used in many fusion PCR reactions.

First one needs to order six primers (P1-P6) as shown in step one. We normally use homologous flanking regions about 1000 bp in length so P2 and P3 would normally be about 1000 bp apart as would P4 and P5. P1 is just "outside" P2 and P6 is just "outside" P5. P1, P2, P5, and P6 are normally about 18-21 bp in length and have a melting temperature of about 60ºC using the following formula for melting temperature. T_m= [(C + G) X 4] + [(A + T) X 2] where C = the number of C's in the primer, G = the number of G's in the primer, etc. T_m is of course in ºC. Longer primers do not work better and often work worse than primers of this length.

Primers P3 and P4 are longer.

P3 Using P3 as an example:

 This segment is identical to the antisense of the 3' end of the Histone H1 coding region. It is 18-20 bp in length and has a melting temperature of approximately 60ºC.

 This segment is identical to the 5' end of the cassette.

We normally include a "GA linker" at the 5' ends of fluorescent proteins and other C-terminal tags. This is a nucleotide sequence that encodes five glycines and five alanines (GAGAGAGAGA). This sequence of amino acids is quite flexible and makes it less likely that the fluorescent protein will interfere with the structure of the C-terminal region of the protein to be tagged. To minimize nucleotide repetitiveness in the sequence we use the following sequence (designed by the Osmani lab):

5' ggagctggtgcaggcgctggagccggtgcc 3'

We have incorporated this into all the fluorescent protein cassettes we use for C-terminal tagging (GFP, mCherry, tdTomato and mRFP) and so this portion of P3  is the same from one experiment to the next (21 bp). The entire primer is about 40 bp in length.

The design parameters for P4 are essentially the same as for P3. One portion  corresponds to the 3' UTR of the selectable marker (in this case the *pyrG* gene of *Aspergillus fumigatus*) and the other portion  corresponds to a region in the 3' UTR of the targeted gene (in this case Histone H1).

First round amplification is carried out with 100 ng of (CsCl purified) genomic DNA for each of the two reactions. P1 and P3 are used to amplify one fragment and P4 and P6 the other.

Our PCR conditions are:

Enzyme used - Accuprime Pfx (INVITROGEN, CAT# 12344-024 in the US) (for amounts, consult manufacturer's protocol). Other enzymes may be used but this enzyme works well in our hands and has strong proofreading activity to minimize sequence changes.

Primer concentration: 300 nM final concentration for each primer

94°C, 2 min (Required to activate the Accuprime Pfx)

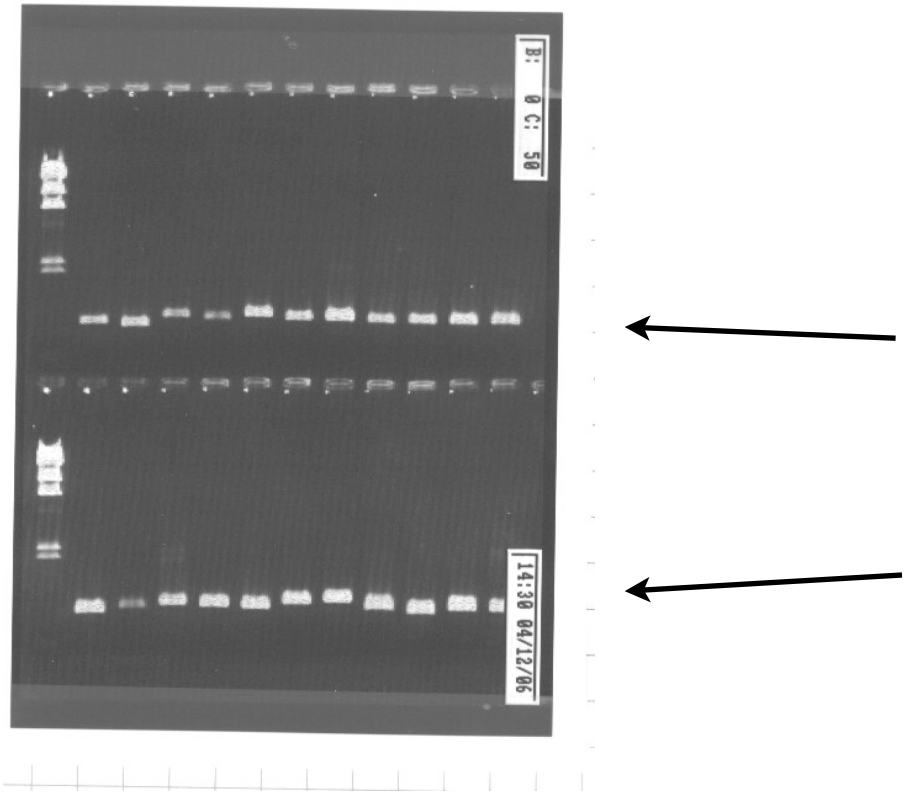
30 cycles:
94°C, 20 sec (denaturation)
Ramp (slope) down to 70°C at maximum rate (most primers will not anneal in this step)
70°C, 1 sec
Ramp down to annealing temperature at 0.1°C/sec

Note: Ramp rates (change in temperature per second also known as slopes) are important. Primers will anneal and extend while the machine is ramping down to and up from the annealing temperature. Since different PCR machines have different rates of temperature increase and decrease, a program developed for one machine will not necessarily give the same results on another machine. (The maximum ramp rate for older machines tends to be much less than for new machines.) We use a relatively slow ramp rate that works well and gives essentially identical results on different machines.

55°C, 30 sec (annealing step--annealing temperature is usually 5 ºC below the primer Tm)
Ramp up to extension temperature at 0.2°C/sec
68°C, 1 min (extension, change according to size; extension time is 1 min/kb of the fragment being amplified)
Ramp up at maximum rate to 94°C

Primers and nucleotides are removed before proceeding to the fusion PCR step. We normally use Qiagen's QIAquick PCR Purification Kit (catalog # 28194 in the US). We normally check the purified products by agarose gel electrophoresis, but the amplification almost never fails unless there was an error in the design of the primers.

Below are two gels from the first round PCR of our most recent tagging experiment. In each case the two flanking sequences for 11 different genes were correctly amplified. Amplified bands are shown with arrows.



Fusion PCR

The two amplified flanking sequences and the cassette are mixed and amplified using nested primers (P2 and P5). **In our hands, using nested primers, as opposed to using the original primers (P1 and P6), greatly increases the specificity of the amplification .**

Our PCR conditions are:

Enzyme used - Accuprime Taq hi fi (INVITROGEN, CAT# 12346-086) (for amounts, consult manufacturer's protocol) Note: This is not the same enzyme that is used for the first round PCR. Accuprime Taq hi fi works better for long fragments than Accuprime

pfx. Accuprime Taq hi fi does contain a proofreading enzyme but the fidelity of DNA replication is not as high as that of Accuprime pfx.

Template used – 2 flanking DNA + cassette DNA (use approximately equal amounts; 0.5 μl of each cleaned PCR product. The PCR amplified DNA is not quantified with any precision. The cassette is normally amplified once, cleaned, frozen at -80ºC and thawed and an aliquot used for each fusion PCR. The cassette does not need to be amplified each time.)
Primer concentration: 400 nM final concentration for each primer

94°C, 2 min (to activate Accuprime Taq hi fi)

10 cycles:
94°C, 20 sec (denaturation)
Ramp down to 70°C at maximum rate (most primers should not anneal in this step)
70°C, 1 sec
Ramp down to annealing temperature at 0.1°C/sec
55°C, 30 sec (annealing step, the annealing temp is normally 5ºC below the primer $T_{m.}$)
Ramp up to extension temperature at 0.2°C/sec
68°C, 5 min (extension, the extension time is normally 1 min/kb of the expected fusion PCR fragment)
Ramp up at maximum rate to 94°C

5 cycles:
94°C, 20 sec (denaturation)
Ramp down to 70°C at maximum rate
70°C, 1 sec
Ramp down to annealing temperature at 0.1°C/sec
55°C, 30 sec (annealing)
Ramp up to extension temperature at 0.2°C/sec
68°C, 5 min extension time for the first cycle. **Increase extension time for each subsequent cycle by 5 sec as enzymes are losing potency. The last cycle will be 5 min 20 sec**
Ramp up at maximum rate to 94°C

10 cycles:
94°C, 20 sec (denaturation)
Ramp down to 70C at maximum rate (most primers will not anneal at this temp range)
70°C, 1 sec
Ramp down to annealing temperature at 0.1°C/sec
55°C, 30 sec (annealing, change according to primer Tm)
Ramp up to extension temperature at 0.2°C/sec
68°C, 5 min 20 sec extension time for first cycle. **Increase extension time by 20 sec for every subsequent cycle as enzymes will be losing potency. The last cycle will be 9 min 20 sec.**

Ramp up at maximum rate to 94°C

The final fused product should be purified with the QIAquick kit (elution from the column is in 50 µl of TE buffer) before transforming into *A. nidulans*. We use approximately 10 µl of the fusion PCR product for transforming protoplasts in a volume of 100 µl.

Below is an agarose gel showing fusion PCR from our most recent tagging experiment (GFP tagging the C-terminus of 11 proteins). In each lane the predominant product has the expected size for the fusion PCR product (arrow). Each has some high molecular weight DNA (probably dimers and trimers of the correct fragment). The QIAquick-purified PCR products were transformed (without gel purification) into an *nkuA* deletion strain. Protoplasts were made from 1 X 10^8 germinating conidia and divided into 14 aliquots of 100 µl each (11 fusion PCR products plus a previously constructed fragment and positive and negative controls). Approximately 200 transformants were obtained with each fusion PCR product. The vast majority of the transformants we have tested so far from this transformation have been correct. They give the anticipated diagnostic PCR pattern and the expected localization pattern. We haven't carried out Southern hybridizations on these as yet, but in previous experiments, heterologous integrations (in addition to the correct integrations) have been very rare.