

## TRIPLE TEMPLATE PCR FOR INSERTION OF YFP.

Dave Jackson/ Chuck Kopec, 2/20/2002.

This is the sequence of the insert in the plasmid I made for the original cloning procedure; the following 2 primers were used to amplify EYFP, and the product cloned into a TOPO vector:

3'sfi1ala-YFP            TTGGCCGCTGCGGCCGCAGCAGCAGCAGCTGGATC  
5'fse1gly-YFP            AAGGCCGGCCTGGAGGTGGAGGTGGAGCTGTGAGCA

### Plasmid name YFP #3

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TAA GGC CGG CCT GGA GGT GGA GGT GGA GCT GTG AGC  
AAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACC  
TACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCCGTGCCCTGGCCCCACCCTCGTGACCACCTTCGGCTACGGCCTGCAGTGCTTCGCCCCGCTAC  
CCCGACCACATGAAGCAGCAGCACTTCTTCAAGTCCGCCATGCCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCC  
GAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAAC  
AGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACCTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTAC  
CAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCCACAACCACTACCTGAGCTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATG  
GTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAG GAT CCA GCT GCT GCT GCT GCT GCG GCC GCA GCG GCC AAA  
AGG

We next amplified from the above plasmid using the YFP left and YFP right primers, below, to produce the “TTYFP” fragment. Note that the linker sequences have changed to be more of a gly/ ala/ hybrid, this was necessary to avoid Primer3 having a heart attack. Also, the sfi and fse sites are still present.

YFP LEFT	GGC	CGG	CCT	GGA	GGT	GGA	GGT	GGA	GCT	GTG	AGC	A
	G	R	P	G	G	G	G	G	A	V	S	
YFP RIGHT	GGC	CCC	AGC	GGC	CGC	AGC	AGC	ACC	AGC	AGG	ATC	
	D	P	A	G	A	A	A	A	A	G	A	

So, to do TTPCR, you need to add the following linker sequences to P2 and P3. Note that these are in the +1 reading frame. So think about how these run in to the gene specific parts of P2 and P3, basically they just run into in frame codons. (Examples of the primers we have used successfully are on the next page).

**Linker at 5' end of P2** 5' TCC ACC TCC ACC TCC AGG CCG GCC 3' (gene specific part starts here, top strand, in +1 reading frame).

**Linker at 5' end of P3** 5' T GGT GCT GCT GCG GCC GCT GGG GCC 3' (gene specific part starts here, bottom strand, in minus1 reading frame).

### PRIMER 3 scores:

OLIGO	<u>start</u>	<u>len</u>	<u>tm</u>	<u>gc%</u>	<u>any</u>	<u>3' seq</u>
YFP LEFT	4	34	87.66	70.59	8.00	2.00 GGCCGGCCTGGAGGTGGAGGTGGAGCTGTGAGCA
YFP RIGHT	1	33	90.43	75.76	8.00	4.00 GGCCCCAGCGGCCGCGAGCAGCACCAGCAGGATC
P2 LINKER	840	24	79.87	75.00	8.00	8.00 TCCACCTCCACCTCCAGGCCGGCC
P3 LINKER	1	25	86.77	80.00	8.00	7.00 TGGTGCTGCTGCGGCCGCTGGGGCC

### Successful TTPCR primer examples:

3707 P1 CACCGGATGCTCGATGGTTTCTCC  
 3707 P2 TCCACCTCCACCTCCAGGCCGGCC GAGAGCAAGGGGACGGTTGT  
 3707 P3 TGGTGCTGCTGCGGCCGCTGGGGCC CTCTCCGCCGCTCCTCA  
 3707 P4 TCGAAGGTCGAATGAAGATGG

4557 P1 CACCTGCCAAAGGAGTATTTGGTTCA  
 4557 P2 TCCACCTCCACCTCCAGGCCGGCC GTGTACAACACGGGAGGAAACTT  
 4557 P3 TGGTGCTGCTGCGGCCGCTGGGGCC GCTATTAAAGAAGCTTTCGGTGTTCT  
 4557 P4 CGTCGAAATCTATGGCTCCAC

VIP1 P1 CACCTCCCCCTGTATGGGTGTCTG  
 VIP1 P2 TCCACCTCCACCTCCAGGCCGGCC CGATGGCTGCCCCGTTTGT  
 VIP1 P3 TGGTGCTGCTGCGGCCGCTGGGGCC CCAAGCTACATGGATTTCACCA  
 VIP1 P4 CGACGGTTAAAGGAGCTGTTG

**NOTE THAT IF YOU USE EXTAQ, THE ENDS OF THE PCR PRODUCTS SHOULD BE “POLISHED” USING PFU TAQ PRIOR TO THE TRIPLE PCR, OTHERWISE THE “A” OVERHANG ON THE 3’ END COULD LEAD TO ERRORS.**