
However the figures are illegible, so I have my own version attached here. We use standard ‘adaptamer’ sequences so that we can use the same *K. lactis* primers again and again – they are labeled A and B – you can substitute A and B with any ~20bp sequence of homology. In your case you want to make A the first 20bp of your ACT1 PCR product and B the last 20bp – unless your mutations are near (<100bp) the beginning or end of the gene.

This figure is just FYI since you already have your ACT1 mutations. You should however design up- and down- primers that are simply ~20bp near the 5’ and 3’ ends of the ACT1 gene, respectively.

I have illustrated amplifying an entire ORF, in reality this can be any >500bp sequence, with the mutation >100bp from either end. Potentially multiple mutations can be combined.

Your A adaptamer is 20bp or so from the 5’ end of the ACT1 PCR product (to complement the up-primer) your B adaptamer is 20bp or so from the 3’ end of ACT1 PCR product (to complement the down-primer). The *K. lactis* sequences are below.
Here are the \textit{K. lactis} sequences for your primers:

To amplify the 'K.l. UP' use primers E-KL-5' and KLi-3' (in scheme above)
\textit{Use pWJ1164 as a template to generate an 836bp product}

\textbf{E-KL-5'}

\begin{verbatim}
5' to 3' GTGATTCTGGGTAGAAGATCG
\end{verbatim}

***Note this primer should have ~20bp at the 5' end identical to the 3' end of ACT1 PCR product (FRAGMENT X) to enable you to make a fusion (this is your B adaptamer in the scheme above).

\textbf{KLi-3'}

\begin{verbatim}
5' to 3' GAGCAATGAACCCAATAACGAAATC
\end{verbatim}

To amplify 'K.l. DOWN' use primers KLi-5' and D-KL-3' (in scheme above)
\textit{Use pWJ1165 as a template to generate an 893bp product}

\textbf{KLi-5'}

\begin{verbatim}
5' to 3' CTTGACGTTCGTTCGACTGATGAGC
\end{verbatim}

\textbf{D-KL-3'}

\begin{verbatim}
5' to 3' CGATGATGTAGTTTCTGGTT
\end{verbatim}

***Note this primer should have ~20bp at the 5' end identical to the 5' end of the ACT1 PCR product (FRAGMENT X) to enable you to make a fusion (this is your A adaptamer in the scheme above).

And now for the transformation.
Recombination tends to occur near the ends of the PCR products, but it is possible that recombination will occur internally giving URA3+ cells that lack the desired mutation in one or both new copies of the knock in – particularly true if your mutation confers slow growth or death! Therefore isolate several transformants and pop-out each one separately and check by sequencing.
gene to be modified in the genome

Transform with Fusion L & Fusion R

Fusion L

Fusion R

knock-in

select on -Ura medium

K.L.URA3

select on 5FOA

pop-out

confirm with sequencing using primers outside amplified region

good idea to include RFLP in your mutation to make genotyping easier