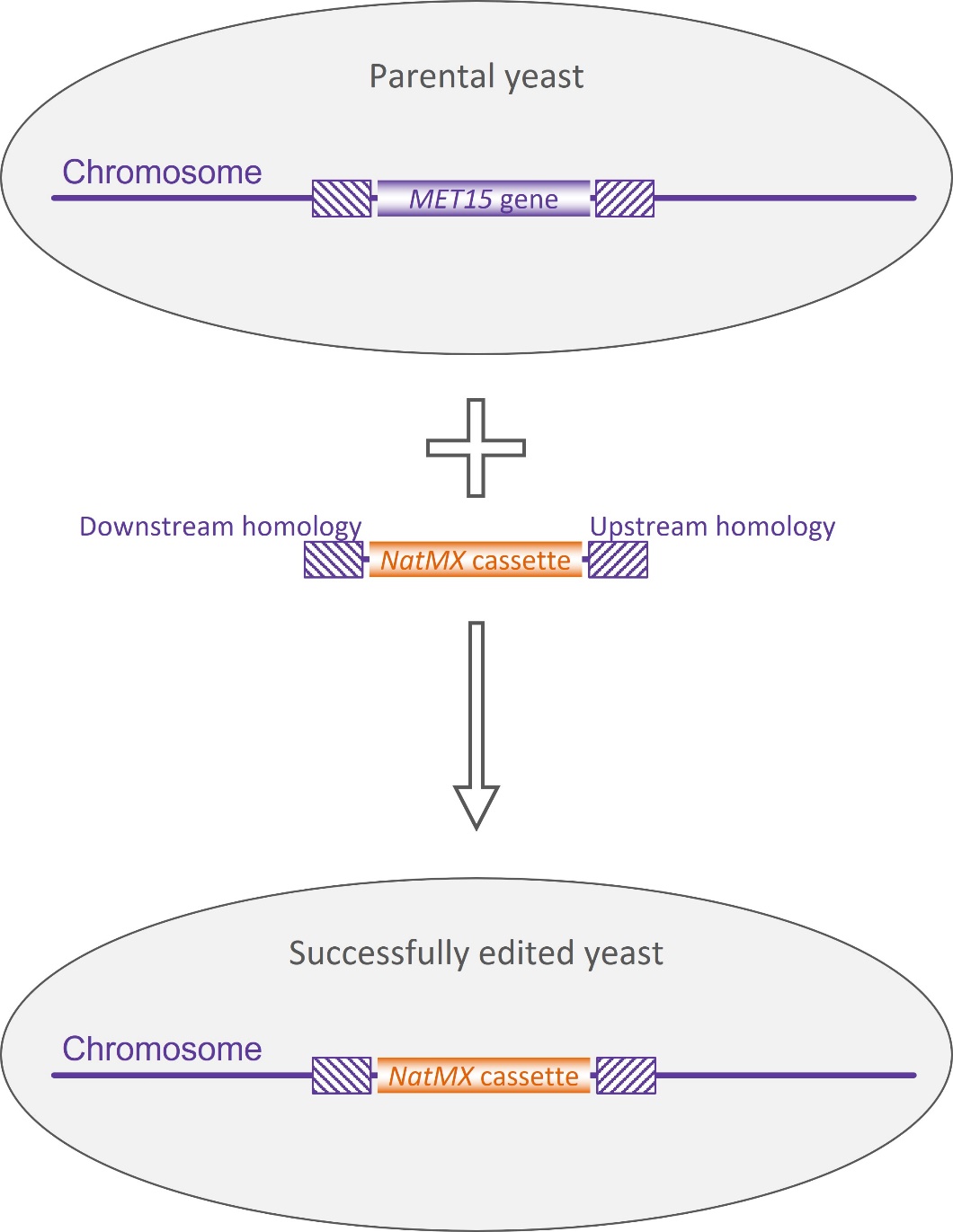
CRISPRmas Practicals – PCR Primer Design

## Overview of the Gene Editing Experiment

Figure 1 is a reminder of what we hope will happen during the gene editing process. As you can see, a parental yeast cell and a successfully edited yeast cell will have different genotypes and, therefore, different genomic DNA sequences. If we could isolate genomic DNA from our yeast colonies (we can!), we can use PCR to distinguish between the different DNA sequences and confirm the success (or failure!) of our gene editing.

**Figure 1: Gene editing of *MET15*.** The parentalyeast has an intact *MET15* gene (purple). During gene editing, yeast cells are supplied with a repair template that consists of the *NatMX* cassette (orange) cloned between two regions of homology to the *MET15* gene (boxes with purple diagonal fill). If gene editing is successful, the *MET15* gene will be replaced by the *NatMX* cassette. The surrounding chromosome sequences (including the upstream and downstream homology regions) are unchanged.



## What Primers Do You Need?

Now I would like you to design some primers that will allow you to confirm the successful integration of the *NatMX* cassette at the *MET15* locus. Here is an outline of the primers that I think that you will need to get you started:

1. **A positive control.** Your PCR reaction will use yeast genomic DNA as a template. A negative result in the PCR reaction could be because your yeast DNA isolation protocol did not work, and you have no template DNA! You need to be able to prove that you have successfully purified genomic DNA and that a negative result is a real result, not just a technical issue.
2. **Presence/absence of the *Nat*MX cassette.** You will need a pair of primers that confirm whether the *NatMX* cassette is present in your yeast cells.
3. **Presence/absence of the *MET15* gene.** This is not strictly necessary, but it might be useful to confirm whether the *MET15* gene is intact (small insertions/deletions are unlikely to be distinguished by PCR, however).
4. **Correct integration of the *NatMX* cassette.** Although it is relatively unlikely, it is possible for the *NatMX* cassette to integrate into a random position in the yeast genome. You will need a pair of primers that will confirm that the *NatMX* cassette is integrated into the *MET15* locus, rather than elsewhere in the genome.

You should also plan some negative controls to prove that your primers are specific. You need to be able to show that a positive result is a real result, rather than a non-specific PCR product. What could be a sensible negative control for these primer pairs? There are a few options here.

You are obviously going to need more than one pair of primers! But it is fine to use the same primer in more than one PCR reaction.

## Plan the Primer Position and Orientation

The first step is to decide where your primers will bind and what direction they will amplify DNA (we will worry about primer sequences in the next step!) Remember that, in order to get a PCR product, you need a **pair of primers** that will amplify the DNA between them. One primer will bind to the start of the DNA sequence to be amplified (this is often referred to as the **forward** primer). The other (**reverse**) primer will bind to the end of the DNA sequence. As a pair, they will exponentially amplify the DNA sequence between them, producing a PCR product that can be analysed by agarose gel electrophoresis.

At the end (in the “Useful Information” section is a printout of the possible genomic conformations above. Use these to plan where your primers will bind and in which direction they will amplify. Drawing arrows on the figures would be a good way to do this (perhaps in pencil, just in case!) And label the primers so that you can distinguish between them. Numbers are fine, but it can be useful to give primers informative names (for example, MET15\_forward or MET15\_F for short).

Make sure that your primers allow you to address the points raised above (in the “What Primers do you Need?” section). If you are unsure, get a demonstrator to look at them before you move on to the next step. You should also outline the expected results for the different possibilities (for example, size of PCR product / no PCR product).

## Design the Primer Sequence

Once you have decided on the location and orientation of your primers, have a go at designing the sequence of some of them. You can do as many of these as you like, but you should have a go at designing at least one pair of primers (forward and reverse).

There are some important things to understand about primers for PCR (see figure 2):

1. Primers bind template DNA in an antiparallel manner, in the same way that the two strands of a DNA molecule are antiparallel. This means that the 5’-3’ orientation of the template and of the primer are opposite to each other.
2. Each primer in a pair will bind to opposite strands of the template DNA.
3. Primer extension is always in the 5’-3’ direction.

**Figure 2: Primer hybridisation in PCR.** Primers are the reverse complement of the sequence to which they hybridise. The forward primer binds to the bottom strand of DNA and synthesises DNA in a 5’-3’ direction. The reverse primer binds to the top strand of DNA and synthesises DNA in the opposite direction (this is still the 5’-3’ direction).



There are also some rules and guidelines when designing primers:

### Rules – these are things you must do!

* Primers are always written in the **5’ to 3’** direction.
* Primers must be the **reverse complement** of the sequence to which they need to hybridise.
* You need to design a forward **and** a reverse primer.
* Primers must be a minimum of 17 bp in size.

### Guidelines – less strict but should be considered

* Aim for primers that are 18-28 nucleotides in length.
* When designing a pair of primers, they should be a few hundred base pairs away from each other. You are aiming to produce a PCR product of approximately 400-600 bp in size (the specific size is not critical; designing good primers is more important).
* The GC composition of a primer should ideally be in the region of 50-60%.
* Primers should have a melting temperature (Tm) of 60-65°C – see below for how to calculate this. Primer pairs should have similar Tm values to each other.
* Avoid long sequences of repeated nucleotides.
* Avoid sequences within the same primer (or between pairs of primers) that might be able to anneal to each other. This is difficult when designing primers by hand, so don’t worry about it too much today (in real life, software will check this for you).
* It is a good idea to check that your primer doesn’t have similarity to other sequences. We would normally do this using an online similarity checking tool (BLAST) but don’t worry about this today.

### Calculating Melting Temperature (Tm)

A quick and easy way of estimating primer melting temperature (which you can use today) is the Wallace Rule. The Wallace Rule states that, for every G or C nucleotide, add 4°C to your Tm; for every A or T nucleotide, add 2°C to your Tm:

This is clearly just an estimate (although it works surprisingly well!) and there are more complex calculation methods that are much more accurate. Nowadays, we often use primer design software which carry out these more accurate calculations automatically and check for other issues, such as self-annealing of primers.

### DNA Sequences

The DNA sequences that you need are at the end (in that “Useful Information” section again) and you should make a note of your primer sequences in your lab book. The relevant information that you should include is:

* The DNA sequence (obviously!) written 5’-3’
* Length of primer (in base pairs, bp)
* Melting temperature (Tm)
* GC content as a percentage
* Which region of the template DNA your primer binds to (i.e., which nucleotides)
* What size PCR product you would get with the primer pair you designed

## Useful Information

### Gene Editing – Possible Outcomes

Diagram, text

Description automatically generated

### *MET15* DNA Sequence

This is the DNA sequence (coding strand only!) of the *MET15* gene and its surrounding chromosomal region. The protein-coding Open Reading Frame (ORF) is shown in bold, with the start codon highlighted in green and the stop codon highlighted in blue. The upstream and downstream homology sequences are underlined.

GTTGATTTTTATTCCAACACTAAGAAATAATTTCGCCATTTCTTGAATGTATTTAAAGATATTTAATGCTATAATAGACATTTAAATCCAATTCTTCCAACATACAATGGGAGTTTGGCCGAGTGGTTTAAGGCGTCAGATTTAGGTGGATTTAACCTCTAAAATCTCTGATATCTTCGGATGCAAGGGTTCGAATCCCTTAGCTCTCATTATTTTTTGCTTTTTCTCTTGAGGTCACATGATCGCAAAATGGCAAATGGCACGTGAAGCTGTCGATATTGGGGAACTGTGGTGGTTGGCAAATGACTAATTAAGTTAGTCAAGGCGCCATCCTCATGAAAACTGTGTAACATAATAACCGAAGTGTCGAAAAGGTGGCACCTTGTCCAATTGAACACGCTCGATGAAAAAAATAAGATATATATAAGGTTAAGTAAAGCGTCTGTTAGAAAGGAAGTTTTTCCTTTTTCTTGCTCTCTTGTCTTTTCATCTACTATTTCCTTCGTGTAATACAGGGTCGTCAGATACATAGATACAATTCTATTACCCCCATCCATACA**ATGCCATCTCATTTCGATACTGTTCAACTACACGCCGGCCAAGAGAACCCTGGTGACAATGCTCACAGATCCAGAGCTGTACCAATTTACGCCACCACTTCTTATGTTTTCGAAAACTCTAAGCATGGTTCGCAATTGTTTGGTCTAGAAGTTCCAGGTTACGTCTATTCCCGTTTCCAAAACCCAACCAGTAATGTTTTGGAAGAAAGAATTGCTGCTTTAGAAGGTGGTGCTGCTGCTTTGGCTGTTTCCTCCGGTCAAGCCGCTCAAACCCTTGCCATCCAAGGTTTGGCACACACTGGTGACAACATCGTTTCCACTTCTTACTTATACGGTGGTACTTATAACCAGTTCAAAATCTCGTTCAAAAGATTTGGTATCGAGGCTAGATTTGTTGAAGGTGACAATCCAGAAGAATTCGAAAAGGTCTTTGATGAAAGAACCAAGGCTGTTTATTTGGAAACCATTGGTAATCCAAAGTACAATGTTCCGGATTTTGAAAAAATTGTTGCAATTGCTCACAAACACGGTATTCCAGTTGTCGTTGACAACACATTTGGTGCCGGTGGTTACTTCTGTCAGCCAATTAAATACGGTGCTGATATTGTAACACATTCTGCTACCAAATGGATTGGTGGTCATGGTACTACTATCGGTGGTATTATTGTTGACTCTGGTAAGTTCCCATGGAAGGACTACCCAGAAAAGTTCCCTCAATTCTCTCAACCTGCCGAAGGATATCACGGTACTATCTACAATGAAGCCTACGGTAACTTGGCATACATCGTTCATGTTAGAACTGAACTATTAAGAGATTTGGGTCCATTGATGAACCCATTTGCCTCTTTCTTGCTACTACAAGGTGTTGAAACATTATCTTTGAGAGCTGAAAGACACGGTGAAAATGCATTGAAGTTAGCCAAATGGTTAGAACAATCCCCATACGTATCTTGGGTTTCATACCCTGGTTTAGCATCTCATTCTCATCATGAAAATGCTAAGAAGTATCTATCTAACGGTTTCGGTGGTGTCTTATCTTTCGGTGTAAAAGACTTACCAAATGCCGACAAGGAAACTGACCCATTCAAACTTTCTGGTGCTCAAGTTGTTGACAATTTAAAGCTTGCCTCTAACTTGGCCAATGTTGGTGATGCCAAGACCTTAGTCATTGCTCCATACTTCACTACCCACAAACAATTAAATGACAAAGAAAAGTTGGCATCTGGTGTTACCAAGGACTTAATTCGTGTCTCTGTTGGTATCGAATTTATTGATGACATTATTGCAGACTTCCAGCAATCTTTTGAAACTGTTTTCGCTGGCCAAAAACCATGA**GTGTGCGTAATGAGTTGTAAAATTATGTATAAACCTACTTTCTCTCACAAGTACTATACTTTTATAAAACGAACTTTATTGAAATGAATATCCTTTTTTTCCCTTGTTACATGTCGTGACTCGTACTTTGAACCTAAATTGTTCTAACATCAAAGAACAGTGTTAATTCGCAGTCGAGAAGAAAAATATGGTGAACAAGACTCATCTACTTCATGAGACTACTTTACGCCTCCTATAAAGCTGTCACACTGGATAAATTTATTGTAGGACCAAGTTACAAAAGAGGATGATGGAGGTTTCTTTACAATAAAGAAGCACATGTGTGTTAACGTTTTTAGTATTTGCTTGTTATGTAAATCAGGAAAACTTCGCGGGATTTGGTTGGATGCTACTTTCCATACAATAAATATTATAGATCTAAAAAGCCAAATTACAAGTAAAGATTAGTAAAGCTGTTGGAATTCCATCGTTGATAAAAATGTTAGTTATTAAATATAAAAGTCAGAATAGGTGAACTTGGATTTAATTGTTGGCATTTCGTTGCTGCTAGAGGCCATA

### DNA Sequence of the *NatMX* Cassette

This is the DNA sequence (coding strand only!) of the *NatMX* cassette. The protein-coding Open Reading Frame (ORF) of the resistance gene is shown in bold, with the start codon highlighted in green and the stop codon highlighted in blue. The remaining sequences include promoter and terminator sequences.

GACATGGAGGCCCAGAATACCCTCCTTGACAGTCTTGACGTGCGCAGCTCAGGGGCATGATGTGACTGTCGCCCGTACATTTAGCCCATACATCCCCATGTATAATCATTTGCATCCATACATTTTGATGGCCGCACGGCGCGAAGCAAAAATTACGGCTCCTCGCTGCAGACCTGCGAGCAGGGAAACGCTCCCCTCACAGACGCGTTGAATTGTCCCCACGCCGCGCCCCTGTAGAGAAATATAAAAGGTTAGGATTTGCCACTGAGGTTCTTCTTTCATATACTTCCTTTTAAAATCTTGCTAGGATACAGTTCTCACATCACATCCGAACATAAACAACC**ATGGGTACCACTCTTGACGACACGGCTTACCGGTACCGCACCAGTGTCCCGGGGGACGCCGAGGCCATCGAGGCACTGGATGGGTCCTTCACCACCGACACCGTCTTCCGCGTCACCGCCACCGGGGACGGCTTCACCCTGCGGGAGGTGCCGGTGGACCCGCCCCTGACCAAGGTGTTCCCCGACGACGAATCGGACGACGAATCGGACGACGGGGAGGACGGCGACCCGGACTCCCGGACGTTCGTCGCGTACGGGGACGACGGCGACCTGGCGGGCTTCGTGGTCATCTCGTACTCGGCGTGGAACCGCCGGCTGACCGTCGAGGACATCGAGGTCGCCCCGGAGCACCGGGGGCACGGGGTCGGGCGCGCGTTGATGGGGCTCGCGACGGAGTTCGCCGGCGAGCGGGGCGCCGGGCACCTCTGGCTGGAGGTCACCAACGTCAACGCACCGGCGATCCACGCGTACCGGCGGATGGGGTTCACCCTCTGCGGCCTGGACACCGCCCTGTACGACGGCACCGCCTCGGACGGCGAGCGGCAGGCGCTCTACATGAGCATGCCCTGCCCCTAA**TCAGTACTGACAATAAAAAGATTCTTGTTTTCAAGAACTTGTCATTTGTATAGTTTTTTTATATTGTAGTTGTTCTATTTTAATCAAATGTTAGCGTGATTTATATTTTTTTTCGCCTCGACATCATCTGCCCAGATGCGAAGTTAAGTGCGCAGAAAGTAATATCATGCGTCAATCGTATGTGAATGCTGGTCGCTATACTG