

Overview:

The purpose of CEPAD-PCR is to allow for enhanced screening of point mutations created using CRISPR-editing. This is done by introducing silent mutations in addition to the desired point mutation to reduce sequence similarity between wildtype and the mutant. Internal primers are then designed in opposite directions at the site of the nucleotide changes. In combination with two flanking primers at different distances from the internal primers, this allows the wildtype and mutant products to be different sizes and separate on an agarose gel.

This protocol uses the Co-CRISPR method published by Paix et al. (Paix et al 2015) as a basis for its CRISPR edit design and screening and only lists the deviations from that protocol.

CRISPR edit design

1. Determine the site of the desired mutation(s).
2. Design the desired changes such that the maximum number of nucleotides are changed.
3. Depending on the nucleotides changed above, choose one of the following options:
 - a. If you can change all three nucleotides in the codon, no further mutations are necessary. Three contiguous nucleotides should be sufficient.

Example (Red are changed nucleotides):

```
1.  S  ->  A
    AGT -> GCC
```

- b. If you were unable to change all three nucleotides, but nucleotide 1 or 2 has been changed, introduce a silent mutation in the preceding amino acid to change its codon. If you are unable to change the codon of the preceding amino acid, go to option c. You should (at worst) be left with two or more contiguous nucleotide changes and another nucleotide change which is not contiguous.

Examples (Red are changed nucleotides):

```
1.  A | F  ->  A | L
    GCC|TTT -> GCG|CTG
```

```
2.  A | S  ->  A | K
    GCC|AGT -> GCG|AAA
```

Note: if you are unable to change the codon of the preceding amino acid, go to the next option

- c. Look for a serine, leucine, or arginine residue nearby. With one of these amino acids, you should be able to change both the 1st and 3rd nucleotide in the codon (with serine you can change the second one as well). Changing these two nucleotides should be sufficient to make detection allele-specific.

Examples (Red are changed nucleotides):

1. S -> S
TCA -> **AGT**

2. L -> L
CTG -> **TTA**

3. R -> R
AGG -> **CGT**

Primer design

4. Design two internal primers in opposite directions which overlap by 3 to 4 nucleotides on their 3' ends. The 3' nucleotide of each primer should be a changed nucleotide from above. Primer length should be optimized to maintain a constant T_m among both internal primers.

Requirements:

- One of these primers should be a forward primer and the other a reverse primer
- One of these should be the wildtype-specific primer and contain no nucleotide changes
- The other should be the mutant-specific primer and contain all nucleotide changes

Note: To check the T_m of a primer, you can use a primer T_m calculator such as the one from Thermofisher ([Calculator](#))

5. Design two flanking (external) primers at different distances from the internal primers from step 4. These primers should also have a similar T_m compared to the internal primers. This allows each internal primer to amplify a product with a different size, allowing for the detection of both homozygotes and heterozygotes.

Primer optimization

6. Optimization is performed on N2 animals (or the injected strain). Lysis and PCR reactions are performed using standard single worm PCR conditions. Since the CRISPR edit has not yet been created, primers should be optimized with the three gradient PCR reactions below. Choose an annealing temperature which fulfills all three of the following requirements:
 - a. Outer primers only:
 - Only the full-length band appears
 - b. Wildtype primers only:
 - Only the wildtype band appears
 - c. All primers:
 - No mutant band appears

Screening

7. Screening is performed using standard single worm PCR conditions with the addition of all four PCR primers.