TARDIGARD

protocol

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PCR amplification

PCR (polymerase chain reaction) is a common and relatively easy way to amplify target sequence. With the help of high-fidelity thermostable dna polymerase, thermocycler, suitable template and our designed primer. The target sequence could be amplified within 1-2 hours depending on the length of the sequence. In addition, it can amplify DNA with possibility of expanding the DNA sequence at the start or/and end of the sequence with carefully designed primers.

Purpose: Amplify our target protein sequence

Add restriction enzyme recognition site at the start & end of our target sequence

Notice: it is essential to work with gloves and clean bench in order to protect the DNA from DNase activity

Estimated time: 1.5~2h

a. Primer design

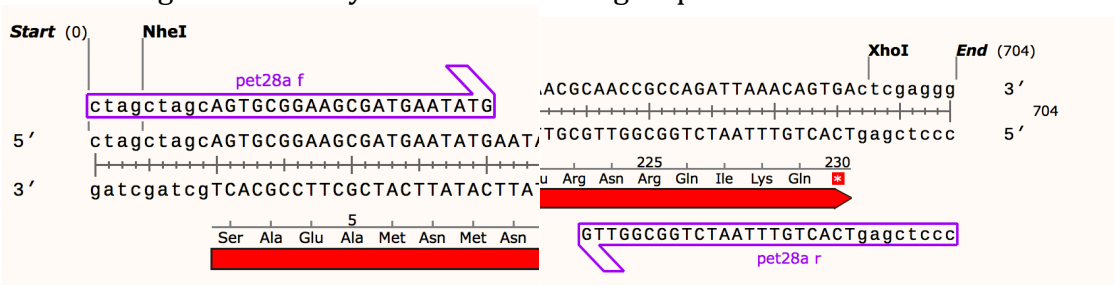
Common Tips

1. Usually we design two primers, one forward primer and one reverse primer. This enable us to isolate and obtain the exact sequence that we want by flanking and defining the target sequence.
2. In ideal situation, we want the annealing between primer and template as strong as possible, especially at the end. CG have 3 hydrogen bond, AT have two hydrogen bond. So GC combination is stronger than AT combination. Stronger annealing and higher annealing temperature can be achieved by increasing GC content.
3. The length of primer can be adjust according to needs, but the complementary part should be at least 18bp and the total length is usually 18 ~ 30 bp, better not exceed 60 bp . (primers under 60 base pairs are within certain price category, the synthesis above 60 are less reliable and the company would do further purification which increase the price)
4. If the annealing temperature is too low, when temperature increased in PCR, this primer will not have enough energy to bind to template and PCR will not work. The acceptable range are 55°C ~ 75°C
5. The forward and backward Tm value should be approximately the same with difference within 2 °C

6. Usually the CG content should be approximately 40%~60% .
7. Classically we write DNA sequence in 5' to 3' direction. So when sending the "primer list" that need to be synthesis to companies, be sure to write the reverse complementary sequence for the reverse primer.
8. All primer should be active and complementary with template at 3' end because this is where the DNA polymerase can bind to.

Design Primer with Sticky Ends

1. Primer with sticky ends are often use to add restriction enzyme cutting site; short sequence like RBS or homologous sequence for more advanced assembly method (e.g. Gibson assembly, One step cloning) or homologues recombination in order to integrate the target sequence into bacterial chromosome.
2. In this example, we add the site NheI and XhoI which enable restriction digestion and ligation of sticky ends in the coming steps.



Checking your Primer

1. Check the secondary structure of your primer, make sure the free energy have a low absolute value and the 3' end is free to anneal to template.
2. Check complementary between two primers
3. Check complementary between primers and DNA template, make sure there is single annealing site to ensure specific sequence is obtained.

a. PCR mix preparation

Reagent	Volume	Final Concentration
PrimeSTAR Max Premix(2×)	25 μl	1×
Primer 1	10 ~ 15 pmol	0.2~0.3 μM
Primer 2	10 ~ 15 pmol	0.2~0.3 μM
Template	<200 ng*	
Add ddH2O	Up to 50 μl	

a. PCR programme setting

1	-	98°C	30min
2	X30	98°C	10sec

3	X30	55°C(your Tm value)	5sec/15sec
4	X30	72 °C	5sec/kb
5	-	72°C	10min
6	-	12°C	hold

*Notice

- in step 3 annealing, Tm value can be calculated with our online-calculator

-annealing time (s3) should be decreased to 5 if 15sec result in smear and low resolution/sharpness

-PCR product purification is needed before carry out your next step

-if multiple band is obtained, gel purification should be considered