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Polymerase Chain Reaction (PCR) V.2

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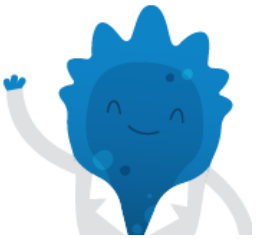

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External link: https://www.addgene.org/protocols/pcr/

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Protocol status: Working
We use this protocol and it's working

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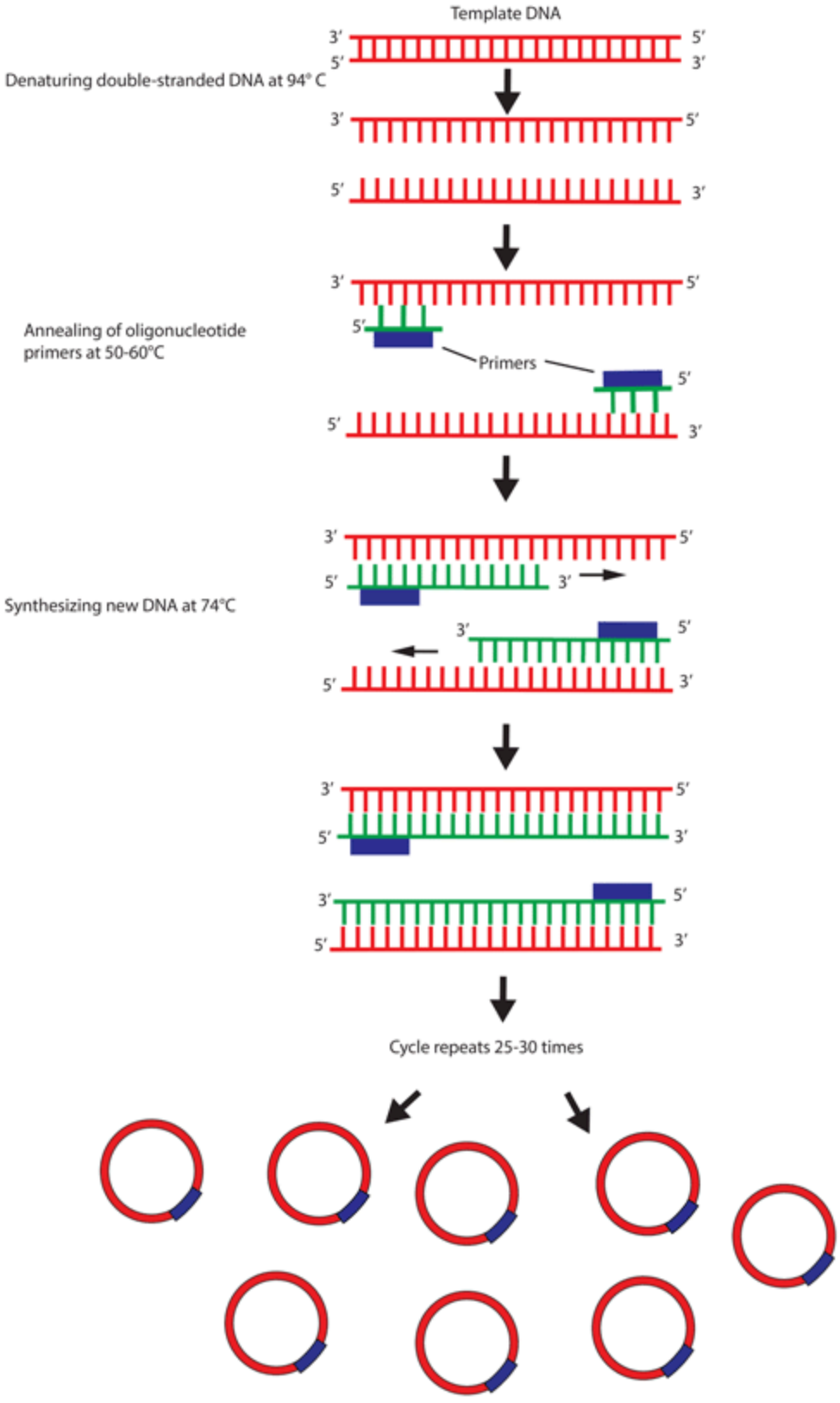
Abstract

This protocol is for performing Polymerase Chain Reaction (PCR). To see the full abstract and additional resources, visit **https://www.addgene.org/protocols/pcr/**.

Basic PCR Program

1. **Initial Denaturation for** 00:02:00 **at** 94 °C **:** This initiation step heats the double stranded DNA template strand to the point where the strands start denaturing and the hydrogen bonds are broken between the nucleotide base pairs.
2. **Denature** 00:00:30 **at** 94 °C **:** Continued denaturation of double stranded DNA.
3. **Anneal primers for** 00:00:30 **at** 55 °C **:** The forward and reverse primers are stable within this temperature range to anneal to each of the single stranded DNA template strands. The DNA polymerase is also stable enough to now bind to the primer DNA sequence.
4. **Extend DNA for** 00:01:00 **at** 74 °C **:** The Taq polymerase has an optimal temperature around 70 °C - 75 °C so this step enables the DNA polymerase to synthesize and elongate the new target DNA strand accurately and rapidly.
5. **Repeat steps 2-5, 25-30 times.**
6. **Final Extension for** 00:05:00 **at** 74 °C **:** A final extension to fill-in any protruding ends of the newly synthesized strands.


Template DNA
Denaturing double-stranded DNA at 94° C
Annealing of oligonucleotide primers at 50-60°C
Primers
Synthesizing new DNA at 74°C
Cycle repeats 25-30 times

Guidelines

Tips and FAQ

If your target DNA sequence is GC-rich, increase the time of the denaturing.

Your 5′ and 3′ primers should be designed to have similar melting temperatures (Tm). Set the annealing temperature to 5 °C lower than the Tm of your primers. If you are getting non-specific PCR products, increase the annealing temperature step-wise by 1 °C - 2 °C .

The rate of DNA synthesis is ~1-2 kb/min. The extension time can be adjusted according to the length of the target sequence.

How do I design primers? See our PCR cloning protocol for more detailed instructions on primer design.

What do I do if my PCR reaction isn't working? Try adding 1 µL of 25mM $MgCl_2$and/or 1 µL DMSO to each reaction. The ideal setup for this troubleshooting step is to do one reaction with each, and one reaction with both. $MgCl_2$ acts to supplement the amount supplied by the buffer stock, where it is known to form a gradient when frozen. DMSO helps with denaturation of the DNA, particularly for GC-rich templates.

What does each ingredient specifically do?

- **Template DNA** contains the portion of DNA that we are interested in amplifying for analysis and manipulation. It is essential that the template DNA and primer are complementary for a PCR reaction to work efficiently.
- **Taq Buffer** with $MgCl_2$ provides an optimal and stable chemical environment for the DNA polymerase to work adequately. Divalent cations such as Mg^{2+} and Mn^{2+} stabilize the buffer solution. These cations can also be used for PCR-mediated DNA mutagenesis. A higher cation concentration increases the error rate of the DNA polymerase.
- **Deoxynucleoside Triphosphates (dNTPs)** are the building blocks added one at a time to the new DNA strand by the DNA polymerase.
- **Forward and Reverse Primers** hybridize and are complementary to the 3′ ends of the sense and anti-sense strands of the template DNA. They are strands of nucleic acid that are starting points for DNA elongation and synthesis.
- **Taq DNA polymerase** is a special DNA polymerase that can withstand radical temperature changes during a typical PCR reaction. The DNA polymerase has an optimum temperature around 70 °C and is the molecule responsible for driving the DNA synthesis.

- **Sterile dH_2O** is used to fill the remaining 50 µL PCR reaction. Its solvent and buffer capabilities provide a suitable environment for the DNA amplification reaction.

Materials

Equipment

- Thin-walled PCR tubes
- Ice Bucket
- PCR Machine
- Agarose Gel

Reagents for each 50 µL PCR reaction:

- 2 µL Template DNA (10 ng - 500 ng)
- 5 µL 10X Taq buffer with $MgCl_2$
- 1 µL dNTP mix (10 mM each nt)
- 2.5 µL Forward Primer (10 µM stock)
- 2.5 µL Reverse Primer (10 µM stock)
- 36.8 µL Sterile dH_2O
- 0.2 µL Taq DNA Polymerase (5 units/µL)

Troubleshooting

1 Design Primers. See our protocol on **how to design a primer**.

Note

Primer3 is an excellent resource for choosing primers.

2 Place thin-walled PCR tubes On ice .

3 Set up a 50 µL PCR reaction (Keep all your reagents on ice):

- 2 µL Template DNA (10 ng - 500 ng)
- 5 µL 10X Taq buffer with $MgCl_2$
- 1 µL dNTP mix (10 mM each nt)
- 2.5 µL Forward Primer (10 µM stock)
- 2.5 µL Reverse Primer (10 µM stock)
- 0.2 µL Taq DNA Polymerase (5 units/µL)
- 36.8 µL Sterile dH_2O (variable)

Note

If you are doing multiple PCR reactions, save time by creating a “master mix,” which minimizes the number of small volumes you need to pipet. If you are using the same template DNA for all your reactions, the Template DNA can be added to the master mix. Forward and Reverse Primers **DO NOT** get added to a master mix.

4 Place reaction tubes in PCR machine.

5 Set annealing temperature 5 °C below the primer melting temperature (Tm).

6 Set extension step at 00:01:00 - 00:02:00 per kilobase of product depending on whether you are using a polymerase with proofreading capabilities.

Note

note, see manufacturer's instructions for specific instructions about extension time and temperatures.

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7 Initial Denaturation for 00:02:00 at 94 °C .

8 Denature for 00:00:30 at 94 °C .

9 Anneal primers for 00:00:30 at 55 °C (or 5°C below Tm).

10 Extend DNA for 00:02:00 at 74 °C .

11 Repeat steps 2-4 for 25-30 cycles.

12 Final Extension for 00:05:00 at 74 °C .

13 Run 2 µL on a gel to check size and concentration of PCR product.

Master Mix Protocol

14 Multiply the volume of each reagent by the number of individual PCR reactions you wish to perform and add 10% extra to account for pipetting error.

In this example, we have 7 different PCR reactions (7 unique primer pairs), so we multiply each volume by 7.

15 In a single 1.5mL tube combine the following:

- **10X Taq buffer with $MgCl_2$**: 5 µL x 7 reactions = 35 µL total + 10% (3.5 µL) = 38.5 µL
- **dNTP mix (10 mM each nt)**: 1 µL x 7 reactions = 7 µL total + 10% (0.7 µL) = 7.7 µL
- **Template DNA**: 2 µL x 7 reactions = 14 µL total + 10% (1.4 µL) = 15.4 µL
- **Sterile dH_2O**: 36.8 µL x 7 reactions = 257.6 µL + 10% (25.7 µL) = 283.3 µL
- **Taq DNA polymerase**: 0.2 µL x 7 reactions = 1.4 µL + 10% (0.14 µL) = 1.54 µL

16 Mix the contents by gently pipetting up and down several times. Keep tube On ice .

17 Add the forward and reverse primers to the thin-walled PCR tubes.

Note

Do this before adding the master mix so you know that the primers have been added, pipet the forward primer onto one side of the tube wall and the reverse primer onto the other.

18 Add the master mix to the thin-walled PCR tubes. Put 50 µL – 2.5 µL (fwd primer) – 2.5 µL (rev primer) = 45 µL volume of master mix to add to each PCR tube.

19 Secure the tops to the PCR tubes, gently tap each tube to bring all the liquid to the bottom before placing it in the PCR machine.

Diluting Primers

20 Most people order primers from a company, which synthesizes and ships them as a lyophilized powder. The researcher then needs to reconstitute their primers in liquid, normally sterile dH_2O. To make a 100uM stock of any primer, add a number of µl of dH_2O

equal to the number of nanomoles of DNA times 10. For example, if your lyophilized DNA is 38.5nm, add 385 µL of water.

21 After making your 100uM stock, immediately make a working concentration of each primer (10uM) by making a 1:10 dilution of the stock. For example, add 100 µL of primer stock to 900 µL of dH_2O.