Around-the-horn PCR and cloning

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Abstract

This protocol is designed for 'around-the-horn' or 'divergent' PCR, where primers go around most or all of a plasmid but are pointed away from each other so they generate a linear product. Note that this protocol is written for Q5 polymerase, but works fine with other polymerases. To switch polymerases, just change the PCR reaction setup.

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Before start

Program the thermocycler with the PCR program in the main protocol

Materials

- Q5 Hot Start High-Fidelity DNA Polymerase - 500 units M0493L by New England Biolabs
- Phusion Hot Start Flex DNA Polymerase - 100 units M0535S by New England Biolabs
- HotStart ReadyMix (KAPA HiFi PCR kit) KK2601 by Kapa Biosystems
- dNTP 639125 by Takara
- forward primer (25 uM) by Contributed by users
- reverse primer (25 uM) by Contributed by users
- template (5ng/ul) by Contributed by users

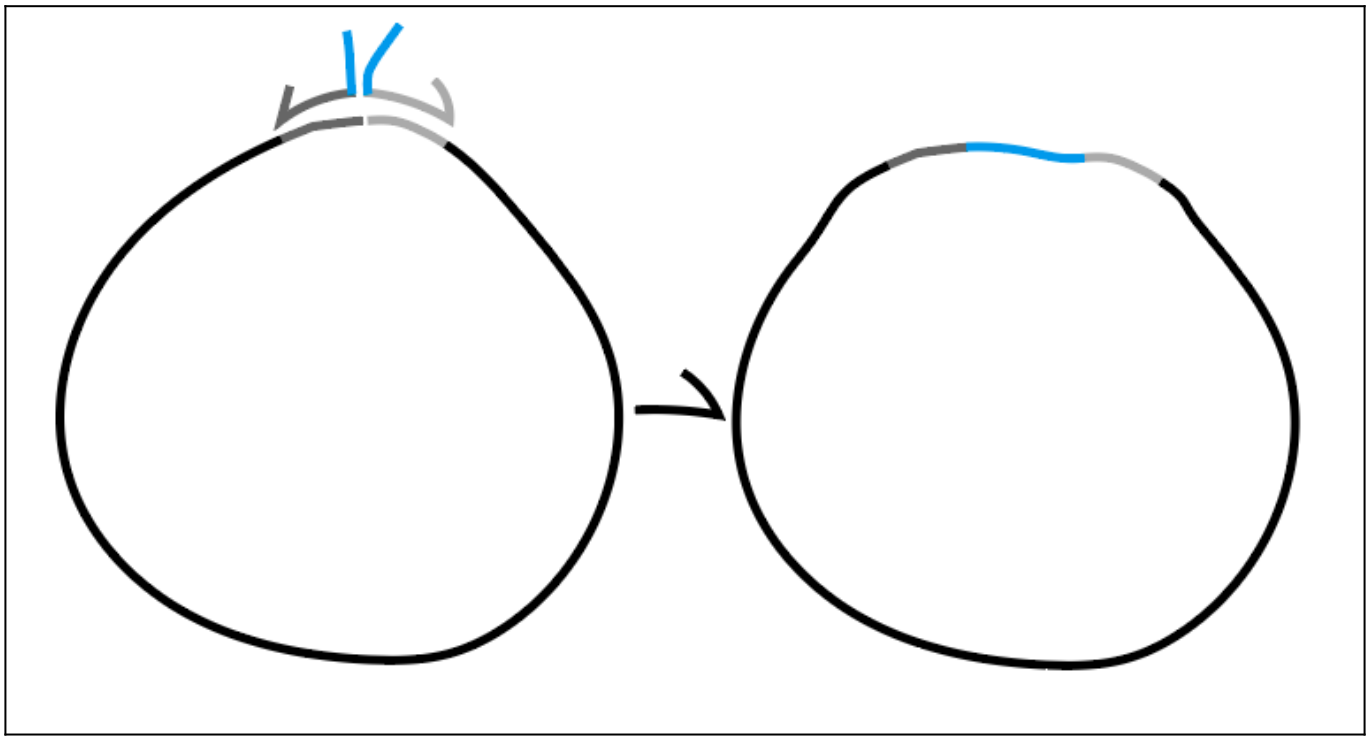
Protocol

Design primers

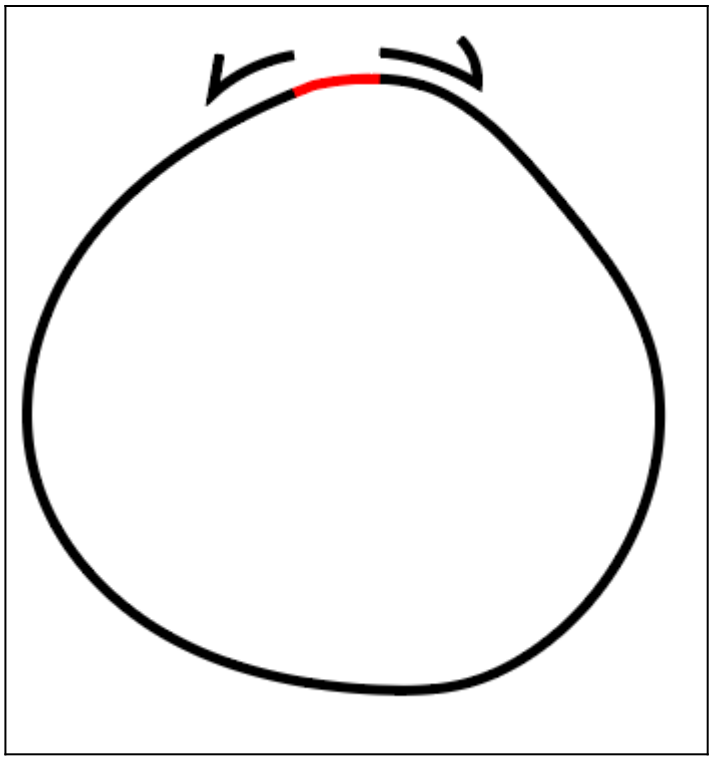
Step 1.

The basic idea of this protocol is that primers head 'away' from each other on a plasmid backbone, which gives you a lot of flexibility in what you can do. Downsides are that it generates a linear product instead of a circular one so you have to phosphorylate and ligate the PCR products and that the PCR takes a while.

Insertion (region in blue are inserted):



Deletion (region in red is deleted):



Note that deletions and insertions can be combined. This strategy can be helpful when designing products for Gibson cloning.

Set up the PCR

Step 2.

Mix the following on ice.

Reagent	Volume for 1 reaction
Q5 buffer	10 ul
dNTPs (10 mM)	1 ul
forward primer (25 uM)	1 ul
reverse primer (25 uM)	1 ul
template (5 ng/ul)	5 ul
Q5 polymerase	0.5 ul
ddH2O	31.5 ul

Run the PCR

Step 3.

Run this PCR:

95 degrees for 2 minutes

95 degrees for 15 seconds
65 degrees for 15 seconds
72 degrees for 10 minutes

repeat the above 30 times

72 degrees for 15 minutes

note that the extension times at 72 degrees can be adjusted to the plasmid. In general I allow 1 minute for each kb of plasmid.

Remove the template DNA

Step 4.
This strategy will have high background unless you remove the template DNA. DNA from most E. coli strains is methylated and can be degraded by the relatively nonspecific restriction enzyme Dpn1.

Add 1 ul Dpn1 to each PCR tube and incubate for 30 minutes to overnight at 37 degrees.

Purify the PCR product

Step 5.
In general I recommend gel purifying PCR products from these reactions both to further get rid of template DNA and to avoid cloning any truncated PCR products.

Purify the PCR product

Step 6.
Pour a 1% agarose gel in 0.5X TBE.

75 ml 0.5X TBE
750 mg agarose

mix and microwave until boiling and clear - about 90 seconds

Mix and check that the agarose is dissolved.

Add 7.5 ul SYBR safe

Purify the PCR product

Step 7.
Casting gel

Assemble the gel casette with combs. Use combs that are big enough to accomodate the entire PCR. Typically these have four or five lanes per gel. Can use two combs per gel.

Pour hot agarose into casette and let cool to RT.

Purify the PCR product

Step 8.
Running gel

Put RT gel into a tank with 0.5X TBE

Ensure gel is submerged in TBE.

Load ladder into one well. Typically 10 ul ladder is sufficient, even in large lanes.

Load samples into remaining lanes.

Run gel at 120V for 30 minutes

Purify the PCR product

Step 9.
Cut PCR product bands

Image gel on the blue light imager

Prepare one 1.5 ml tube for each successful band

Cut band out with a clean razor blade and transfer to tube.

Purify the PCR product

Step 10.
Gel purify the PCR product according to a mini-spin protocol, eluting in 15 ul. Quantify the product using a nanodrop. Good yields are 50 ng/ul, often yields are 10 ng/ul (which can still work).

Phosphorylate, ligate, and transform

Step 11.
Ligation of unphosphorylated DNA can be accomplished by simutaneous phosphorylation using T4 PNK and ligation with T4 DNA ligase. The reaction setup is simple, but ensure you use T4 DNA ligase buffer and **not** T4 PNK buffer, since PNK buffer has no ATP.

Phosphorylate, ligate, and transform

Step 12.

Phosphorylation

Mix the following in a tube:

- 1 ul 10X T4 ligase buffer
- 1 ul PNK
- 50 ng of gel purified PCR product
- water to 9 ul

Incubate for 30 minutes at 37 degrees

Phosphorylate, ligate, and transform

Step 13.

Ligation

Move to RT, add
1 ul T4 DNA ligase
Incubate for 1 or more hours at RT (1 hour typically sufficient)

Phosphorylate, ligate, and transform

Step 14.

Transformation:

Thaw competent cells from -80 on ice

Add 4 ul reaction to 33 ul competent cells in a microfuge tube

Incubate on ice for 25 minutes

Heat shock at 42 degrees for 1 minute

Incubate on ice for 2 minutes

Add 180 ul LB or SOC media

Shake at 37 degrees for 1 hour

Plate 75 ul on a plate with proper antibiotic