Cloning – Gateway BP-Reaction II

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Version: 1.0 - Print Version (.PDF)

ATTENTION: This is expensive. One reaction as described here is ca. 5 Euro!

1. Design PCR-Primers with attB1.1 and attB2.1 sites
2. Gel-purify you PCR product
3. Make sure you have a PCR product with attB1.1 and attB2.1 and one DONR™clone
4. Measure the DNA concentration of both constructs
5. Calculate the amount in ng needed of PCR product (25 fmol each):
   ng needed = (length of the PCR product in **bp**) **x 0.0165**
6. Calculate the volume in µl needed of DONR™ plasmid (75 ng):
   µl needed = **75 ng** needed / (concentration in **ng/µl**)
7. Prepare in a new eppi the Gateway® reaction:

PCR-product	(? ng)
pDONR™-vector	(75 ng)

   add water to a total volume of **4µl**

8. Remove the 5 x **BP-Clonase™ II** enzyme mix from -20°C
9. Pipett **1 µl** of this **BP-Clonase™II** enzyme mix to the Gateway® reaction
10. Store the enzyme mix and buffer immediately at -20°C !!!
11. Incubate at room temperature for **1 hour** or over night
12. Add **0,5 µl** of **Proteinase K** solution and incubate for **10 min** at **37°C (IMPORTANT)**
13. Transform DH5α bacteria
14. Plate bacteria with proper antibiotic selection

Materials needed:

BP-Clonase™ II enzyme mix (# 11789-020) by Invitrogen

Commented Protocol:

1. Design PCR-Primers with attB1.1 and attB2.1 sites

Check out the protocol on primer design. In short - you should take care of some things. The Gateway® clones have a reading frame which should be kept. Design primers that the PCR product starts with a ATG and ends with a STOP-codon or the last aminoacid (if you want to make a fusion protein). Primer3plus is a powerful tool helping you to pick primers with the right annealing temperature which should be 60°C. Try to avoid self similarity and other things as usual, but because you are very limited in the position of the primers (its start and stop), I only care about annealing temperature and give it a try. Then just add to the primer which binds the start codon the attB1.1-sequence at his 5' End . To the primer which binds the stop codon or the last aminoacid add the attB2.1-sequence at his 5' End . The open reading frame is indicated and you should change the last two NN to code for an aminoacid of your choice. Good luck for the PCR! Because of the long 5' overhang and the restrictions on picking the primers, getting the PCR to work can be tricky.

Improved and more efficient att sites used to recombine into pDONR 221:
attB1.1 GGG-GCA-ACT-TTg-tac-aaa-aaa-gtt-gNN
attB2.1 GG-GGC-AAC-TTT-GTA-CAA-Caa-agt-tgN

The original att sites used to recombine into pDONR 221:
attB1 GGGG-ACA-AGT-TTg-tac-aaa-aaa-gca-ggc-tNN
attB2 GGG-GAC-CAC-TTT-GTA-CAA-Gaa-agc-tgg-gtN

The att sites used to recombine into pDONR P4-P1R:
attB4 GGGG-ACA-ACT-TTg-tat-aga-aaa-gtt-gNN
attB1 GGG-GAC-TGC-TTT-TTT-GTA-Caa-act-tgN

The att sites used to recombine into pDONR P2R-P3:
attB2 GGGG-ACA-GCT-TTc-ttg-tac-aaa-gtg-gNN
attB3 GGG-GAC-AAC-TTT-GTA-TAA-Taa-agt-tgN

2. Gel-purify you PCR product

Purification of the PCR-product is needed to get rid of smaller side-products, which remove primer-dimers which can result in false positive colonies. Remember that you want to clone DNA, so the cutting should be made on the weakest UV-light available and as fast as possible. And of course you **NEVER** make a picture of the gel before. Use the kid for gel-purification available in your lab.

3. Make sure you have a PCR product with attB1.1 and attB2.1 and one DONR™clone

You need a PCR product with the attB1.1 and attB2.2 and the DONR™ vector MUST have attP1 and attP2 sites, or it will not work.

The amount of plasmids is not soo important as in a multiple Gateway® reaction, because it is more efficient. If you want to optimize you can calculate equimolar amounts of both plasmids as described in the How to measure DNA. Here we use double the amount of DEST-vector, because most of the ones we use are round and about double the size of the ENTR™ clones.

4. Measure the DNA concentration of both constructs

The amount of ENTR™ is not so important as in a multiple Gateway® reaction, because it is more efficient. If you want to optimize you can calculate equimolar amounts of both plasmids as described in the multiple Gateway® protocol. Here we use double the amount of DEST-vector, because most of the ones we use are round and about double the size of the ENTR™ clones.

5. Calculate the amount in ng needed of PCR product (25 fmol each):

ng needed = (length of the PCR product in **bp**) **x 0.0165**

6. Calculate the volume in µl needed of DONR™ plasmid (75 ng):

µl needed = **75 ng** needed / (concentration in **ng/µl**)

The DONR-vector should be tested for low background colonies (due to a mutated ccdB-gene) when transferred in DH5alpha-bacteria.

7. Prepare in a new eppi the Gateway® reaction:

PCR-product	(? ng)
pDONR™-vector	(75 ng)

add water to a total volume of **4µl**

8. Remove the 5 x BP-Clonase™ II enzyme mix from -20°C

It is most efficiently mixed by pipetting up and down, do not vortex.

9. Pipett 1 µl of this BP-Clonase™II enzyme mix to the Gateway® reaction

This is expensive stuff don't leave it to rot in the ice-bucket!

10. Store the enzyme mix and buffer immediately at -20°C !!!

The enzymes looses 50% activity after 15 freeze-thaw cycles. The advantage of BP-Clonase™II is that it can be stored at -20 °C because it contains already the buffer. This is expensive stuff don't leave it to rot in the ice-bucket!

11. Incubate at room temperature for 1 hour or over night

Incubation over-night will enhance the reaction ca. 5-10 fold. This is especially important for PCR products over 5.000 bp.

12. Add 0,5 µl of ProteinaseK solution and incubate for 10 min at 37°C (IMPORTANT)

This step will enhance the reaction ca. **100 fold!!!!**. This is different to the LR-reactions which are only enhanced 2 fold by adding the proteinase K!!!!

13. Transform DH5α bacteria

For electro competent cells use 1-2 µl, for chemical competent all.

14. Plate bacteria with proper antibiotic selection

The resulting ENTR™-vectors are kanamycin resistant.

Known Issues:

- The reaction is very efficient. You can obtain about 200 colonies of which about 95 % are correct.
- BP reactions work better with linear templates like PCR-products. If you want to use plasmids, linearize them first with a suitable restriction enzyme.
- The obtained plasmids are big. To check for correct clones digest with Sty I and in parallel with Eco RI and Hind III. Compare the pattern of bands with the predicted band size to find the correct clones.

References and Comments:

The protocol is a evolution of the supplied informations, mainly because they were not clear enough and complex. It uses only half of the recommended amounts to be more cost effective. I did it as described before several times sucessfully.

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