Cloning – Multiple Gateway Reaction II without a Mastermix

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ATTENTION: This is not something what is done with low quality material or just to try. One reaction as described here is 20 Euro and should only be spend if you are convinced it will work!

1. Make sure you have **three different (!) ENTR and one DEST clones** for multiple Gateway
2. **Measure the DNA concentration** of all constructs
3. **Calculate the amount** in ng needed of each ENTR clone
   Without Master mix you need 10fmol of each ENTR:
   ng needed = (length of the plasmid in **bp**) **x 0.0066**
4. Calculate the amount in µl needed of each plasmid
   µl needed **= ng** needed / (concentration in **ng/µl**)
5. Obtain a tested DEST-vector (20 fmol needed)
   ng needed = (length of the plasmid in **bp**) **x 0.0132**
6. Prepare in a new Eppi the multiple Gateway reaction
   10 fmol of each of the 3 ENTR-clones
   20 fmol DEST-vector
   add water to a total volume of **8 µl**
7. Remove the 5 x LR-Clonase **Plus** Enzyme mix from -20°C and vortex 2 x 2 sec
   This is expensive stuff don't leave it to rot in the ice-bucket!
8. Add **2 µl** of the Enzyme mix to the multiple Gateway reaction and mix well
9. Store the enzyme mix and buffer **immediately at -20°C !!!**
10. Incubate at room temperature for 16 hours or **over night**
11. Add **1 µl of Proteinase K** solution and incubate for 10 min at 37°C
12. **Transform DH5α bacteria**
    For electro competent cells use 2 µl, for chemical competent the complete mix.
13. Plate bacteria with proper antibiotic selection

Useful Formula:

Desired amount in **ng** =
= amount in **fmol x** length of plasmid in **bp** (660 fg / 1 fmol) x (1 ng / 1000000 fg)

Which is equal to:
Desired amount in **ng** = amount in **fmol x** length of plasmid in **bp x 0.00066** x (ng / fmol)

Materials needed:

Gateway® Enzyme: LR-Clonase ™ II Plus Kit (# 12538-120) by Invitrogen

Commented Protocol:

1. Make sure you have three different (!) ENTR and one DEST clones for multiple Gateway

You need one ENTR™ clone with attL4 and attR1, one with the "classical" combination attL1 and attL2 and one with attR2 and attL3. The DEST ™ vector MUST have attR4 and attR3 sites, or it will not work. Most Errors are made at that step when people by accident use two ENTR™ clones with identical att-sites and lack one pair due to that.

2. Measure the DNA concentration of all constructs

The amount of ENTR is very important for this 4-plasmid-combination-reaction. Measure the amount of each plasmid again to avoid variations between different measurements. If you want to be really sure, than use 1 µg of each plasmid, linearize them by restriction digest and compare the amount on gel (all bands should be of same idensity).

3. Calculate the amount in ng needed of each ENTR clone

Without Master mix you need 10fmol of each ENTR:
ng needed = (length of the plasmid in **bp**) **x 0.0066**

I would prefer the MasterMix, because it's a little more handy, but I guess its personal choice.

4. Calculate the amount in µl needed of each plasmid

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

This should be easy.

5. Obtain a tested DEST-vector (20 fmol needed)

ng needed = (length of the plasmid in **bp**) **x 0.0132**

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

6. Prepare in a new Eppi the multiple Gateway reaction

10 fmol of each of the 3 ENTR-clones
20 fmol DEST-vector
add water to a total volume of **8 µl**

You can also make this reaction in a total volume of 5 µl, but I prefer 10 µl because I am afraid that 5 µl dry out during the incubation over night.

7. Remove the 5 x LR-Clonase Plus Enzyme mix from -20°C and vortex 2 x 2 sec

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

8. Add 2 µl of the Enzyme mix to the multiple Gateway reaction and mix well

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

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

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

10. Incubate at room temperature for 16 hours or over night

I would not shorten this incubation, it is aready not givig so many clones.

11. Add 1 µl of Proteinase K solution and incubate for 10 min at 37°C

This step will enhance the reaction ca. 2fold.

12. Transform DH5α bacteria

For electro competent cells use 2 µl, for chemical competent the complete mix.

Electroposation requires low concentrations of salts, that's why we can not use too much.

13. Plate bacteria with proper antibiotic selection

The pDEST R4-R3 is ampicilin resistant, all our Binary-vectors are spectinomycin resistant. If you are not sure, check first.

Known Issues:

- The reaction is not very efficient. You can obtain 20 - 200 colonies of which 70 - 80 % are correct.
- 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:

I developed this protocol myself, because the supplied instructions were not clear enough and complex. I did it as described before many times and never had any problems (also the students). If it didn't work most of the cases two ENTR™ clones with identical Att-sites were used.

Please visit Invitrogen for further information and for the acquisition of the needed materials.

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