Cloning - TOPO-Reaction

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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 ca. 14 Euro and should only be spend if you are convinced it will work!

1. Make a good PCR reaction in 50 µl volume with special **TOPO-Primers**
2. Load the PCR-product on an agarose gel, run it and cut out band
3. Purify the DNA and elute in **50 µl**
4. Load 5µl of the purified PCR-product with marker
5. **Estimate the amount of DNA** in ng per µl relative to the marker
6. Calculate the amount of DNA needed
   ng needed = (length of the PCR product in bp) x 0.007
7. Pipett the exact amount needed and add water up to **4 µl**
8. Add **1 µl diluted salt solution** and mix well
9. Pipett **2.5 µl of this solution** in a new eppi
10. Prepare everything for transformation of the bacteria
11. Add **0.5 µl of the TOPO-vector** and mix well
12. Incubate at room temperature for **15 min**
13. **Transform bacteria immediately**
14. Plate on KANA-plates

How it works:

In the kit the vector comes with a covalently bound topoisomerase I (that is what makes the kit so expensive). The topoisomerase will join the PCR-product into the vector and cut itself out. (Check the manual for more details).

Materials needed:

pENTR™ Directional TOPO® Cloning Kit (# K2400-20) by Invitrogen

Commented Protocol:

1. Make a good PCR reaction in 50 µl volume with special TOPO-Primers

Check the homepage for protocols on this issue. In short add CACC to the forward primer and take care that the reverse primer does not have a CACC or similar sequence. Also include a Kozak sequence, meaning A/G at position -3 and a G at position 4 relative to ATG. A good 5' end for a primer would be C-ACC-**ATG**-GXX-XXX with the ATG of your protein bold. Use a proofreading polymerase for blund end PCR products. DO NOT use Taq-polymerase.

2. Load the PCR-product on an agarose gel, run it and cut out band

Purification of the PCR-product is needed to get rid of smaller side-products, which are more efficiently cloned in the vector. 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.

3. Purify the DNA and elute in 50 µl

Use the kid for gel-purification available in your lab.

4. Load 5µl of the purified PCR-product with marker

5. Estimate the amount of DNA in ng per µl relative to the marker

Should be easy. Compare the density of the band with a band of same density of the marker. Then check how much ng the band of the marker has and divide it by 5. That's the amount of purified PCR-Product per µl you have got.

6. Calculate the amount of DNA needed

ng needed = (length of the PCR product in bp) x 0.007

This is absolutely essential. At this step you try to calculate how much PCR-product you need to get a 1:1 ratio (on a molar ratio) of vector to PCR-product (10 fmol). If you use too much PCR-product each side of the vector will react with an other fragment and make a linear product, which bacteria cannot amplify! So calculate it and trust it. Then you can get 40-80% correct clones.

7. Pipett the exact amount needed and add water up to 4 µl

8. Add 1 µl diluted salt solution and mix well

For electro-competent cells the salt solution has to be diluted 1:4, otherwise the transformation will not be efficient any more.

9. Pipett 2.5 µl of this solution in a new eppi

I prefer to make a mix and use half of it because pipetting is more accurate and easier.

10. Prepare everything for transformation of the bacteria

Important, because the next steps are time-critical.

11. Add 0.5 µl of the TOPO-vector and mix well

0.5 µl is difficult to pipet. LOOK at your tip and CHECK that you loaded 0.5 µl in it!!! Sometimes the surface tension holds the vector back and nothing is in the tip!!!

12. Incubate at room temperature for 15 min

Not longer! The protocol states 5 min. I always try to incubate between 10-15 min but never longer. Although it should be more efficient with longer times I never had good results with longer incubations.

13. Transform bacteria immediately

That's why everything had to be prepared!

14. Plate on KANA-plates

Containing 50 µg / ml Kanamycin

Known Issues:

- The TOPO® reaction is very efficient for small fragments and gets less and less efficient the bigger the fragments are (problems start at 2500 bp). Anyway I cloned lots of constructs of 4500 bp and even 6000 bp are possible, but you have to check then up to 100 colonies to find a correct one.
- Inversions of the whole fragment, insertions of some bp in front of the CACC and deletions of some bp are quite common (although I never had any of them with this protocol, but that does not mean anything). Also primer can have errors during synthesis. Check carefully the sequences from the attL-sites over the primer sequences into the amplified fragment. I have NEVER seen a mutation in a PCR fragment amplified with a proofreading polymerase (according to my protocols).
- Sometimes, especially with small fragments sequencing does not work. Then the attL-sites bind to each other and make problems. If you digest before sequencing with Asc I for the forward primer and Not I for the reverse primer, sequencing works fine. Another option is to use the attL-Blocker (a primer with modified 3'-End), check the special protocol.

References and Comments:

I developed this protocol myself, because the supplied instructions were not clear enough and the students in our lab failed. I did it as described before many times and never had any problems (also following the students).

How to cite this page in publications:

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<http://www.untergasser.de/lab/protocols/topo_reaction_v1_0.htm>.

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