



Cloning of PCR amplicons in competent cells of *E. coli* using Invitrogen TOPO® TA Cloning kit

1. Subject

Using the TOPO® TA cloning kit it is possible to transfer PCR amplicons into *E. coli* cells containing the plasmid vector pUC18. Once the *E. coli* cells are transformed DNA can be extracted easily. Part of the plasmid containing the insert (amplicon) is submitted to PCR using M13 forward and reverse primers.

2. Principle

E. coli cells can be made competent chemically or by electroporation. The kit uses chemically competent cells. These cells are able to take up foreign DNA, e.g. amplicons. The material is added to competent cells on ice. During a heat shock at 42°C the cells are transformed. It is said that only one type of DNA is taken up per cell. The plasmid vector pCR®2.1-TOPO® has a single 3'-thymidine (T) overhang and topoisomerase I is covalently bound to this vector (the vector is "activated").

Taq-polymerase has a non-template dependent terminal transferase activity that adds a single deoxy-adenosine (A) to the 3' ends of PCR products, but only when the primers used during PCR do NOT have a 5' phosphate added. Otherwise the PCR product will not ligate into pCR®2.1-TOPO®. Once the product is inside the cell, they are incubated, harvested and DNA is extracted.

Selection of the right colonies ("recombinants") is done on Luria-Bertani (LB) medium containing ampicilline, because the competent *E. coli* carry the ampR-gene (ampicilline resistance). Furthermore, only those *E. coli* cells with the insert will not express the lacZ gene, showing white colonies on LB medium containing X-gal, whereas cells without insert will turn light to dark blue.

3. Material and reagents

3.1 TOPO TA cloning kit containing pCR®2.1-TOPO® (Invitrogen, K4500-01)

- plasmid DNA (10 ng/μl) in buffer with phenol red (= vector)
- PCR buffer 10x
- Salt solution
- dNTP mix
- M13 forward (5'-GTA AAA CGA CGG CCA G-3')
- M13 reverse (5'-CAG GAA ACA GCT ATG AC-3')
- control template
- control PCR primers
- sterile water
- SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose)
- 20 vials of chemically competent cells (TOP 10 cells; purple cap)
- pUC18 control DNA (dilute 1:500 in TE buffer)
- pCR®2.1-TOPO® vector (yellow cap)

3.2 sterile microcentrifuge tubes 500 μl, 1.5 ml

3.3 Drygalsky spatel

3.4 micropipets 0.5-50, 50-200, 200-1000 μl

3.5 sterile pipettips (white, yellow, blue)

3.6 incubator 37°C, shaking and non-shaking

3.7 X-gal stock solution (Promega, V3941)

3.8 ampicilline (Fluka, 10044)

3.9 tryptone

3.10 yeast extract

3.11 NaCl

3.12 petri dishes with notches

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- 3.13 NaOH
- 3.14 agar
- 3.15 pH-meter
- 3.16 styropor trays
- 3.17 (crushed) ice
- 3.18 water bath 42°C
- 3.19 single use filter unit 0.2 µm (Schueller?)
- 3.20 heating block
- 3.21 microcentrifuge (Eppendorf 5410)

4. Solutions

4.1 LB (Luria-Bertani) medium

Dissolve 10 g tryptone [3.9], 5 g yeast extract [3.10], 10 g NaCl [3.11] in 950 ml ultrapure water. Adjust pH to 7.0 with NaOH [4.3] and bring total volume to 1000 ml. Add 15 g agar [3.15]. Autoclave 20 min. at 15 psi. Cool to 55°C and add 2 ml ampicilline stock solution [4.4], 800 µl X-gal stock solution [3.7] and pour into petri dishes [3.12]. Let harden, invert and store at 4°C, in the dark.

4.2 NaOH 0.5 M solution

Dissolve 5 g NaOH in 250 ml ultrapure water.

4.3 ampicilline stock solution (50 mg/ml)

Dissolve 500 mg ampicilline [3.8] in 10 ml sterile water. Filter sterilize with 0.2 µm single use filter unit [3.19]. Store at -20°C, but not longer than 6 months.

5. Protocol

Transformation preparation for each sample

- 5.1 Equilibrate a water bath [3.18] to 42°C.
- 5.2 Warm the vial of SOC medium to room temperature (if stored at 4°C).
- 5.3 Warm 3 selective plates [4.1] to room temperature.
- 5.4 Thaw on ice water 1 vial of One Shot (TOP 10) cells for each transformation and one for the control reaction.

Start cloning reaction

- 5.5 Mix 1 µl salt solution, X µl PCR template [6.6], 4-X µl H₂O and 1 µl TOPO® vector (red). Mix gently. For the negative control: 1 µl salt solution, 2 µl pUC18 control DNA, 2 µl H₂O and 1 µl TOPO® vector.
- 5.6 Incubate 5 min. [6.1] at room temperature (22-23°C).
- 5.7 Cool reaction on ice.

Actual transformation

- 5.8 Add 2 µl of mix [5.7] to a vial of One Shot chemically competent E. coli (TOP 10; purple cap) cells and mix gently. Add also 2 µl of the negative control to one tube of One Shot cells.
- 5.9 Incubate 15 min. on ice.
- 5.10 Heat-shock cells for 30 sec. at 42°C [5.1] without shaking.
- 5.11 Transfer the tubes with cells to ice immediately.
- 5.12 Add 250 µl SOC medium [5.2].
- 5.13 Close tubes tightly and shake horizontally (65 rpm) at 37°C for 1 hour [6.2].
- 5.14 Spread 50, 75 and 100 µl of each transformation on prewarmed selective plates [5.3] and incubate overnight at 37°C.
- 5.15 Prewarm a selective plate [4.1] for every sample.
- 5.16 Pick ~50 white colonies [6.3], one blue colony and transfer to a selective plate [5.15].
- 5.17 Incubate overnight at 37°C.
- 5.18 Put the plates at 4°C for at least 1 hour to get a better colour distinction.
- 5.19 Number positive clones [6.4] sequentially [8.1].

DNA extraction

- 5.20 Isolate all cells of every numbered colony and suspend in 100 µl sterile ultrapure water by vortexing. At least one blue colony has to be extracted as a negative control.
- 5.21 Incubate at 98°C for 10 min. in a heating block [3.20].
- 5.22 Transfer tubes on ice and cool.

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- 5.23 Spin down cell debris in a microcentrifuge [3.21] at 14.000 rpm for 2 min.
- 5.24 Pipette 80 µl supernatant to sterile microcentrifuge tubes (1.5 ml) and store at 4°C until use.

Preparing PCR reaction

- 5.25 Prepare PCR [6.5] reaction mix for all samples with M13 forward and reverse primers (25 µg/per reaction).
- 5.26 Check the results of the PCR on 1% agarose gel.
- 5.27 Make a photograph [6.7] of the gel using the ImageMaster VDS system
- 5.28 Purify positive PCR amplicons with GFX-columns.
- 5.29 Sequence using M13 forward and reverse primers.

6. Remarks

- 6.1 Incubation time may be increased up to 30 min. to improve the yield of transformed cells.
- 6.2 During incubation the cells start replicating and the ampR-gene is expressed.
- 6.3 Check the colonies very well. Sometimes colonies seem to be white, but at closer look they contain some blue pigment. Doñt use these colonies, but only the complete white colonies. The blue colony checks the quality of the selective plate and can be used as negative control during PCR [5.20].
- 6.4 Even after transferring the complete white colonies it can happen, that again some of them turn light blue or show blue edges. These colonies should be skipped too and should not be harvested for DNA extraction.
- 6.5 The amount of template is 5 µl per reaction. This means, that the amount of H₂O should be adjusted accordingly and 45 µl of mix is added to obtain a 50 µl reaction. The primers used at DSMZ are different from the ones published in the instruction manual (DSMZ: M13 forward [5'-GTA AAA CGA CGG CCA GT-3], M13 reverse [5'-GGA AAC AGC TAT GAC CAT G-3]). The PCR conditions are as follows: initial denaturation at 94°C, 3 min.; 28 cycles (93°C, 1 min.; 52°C, 1 min.; 72°C, 2 min.); final elongation at 72°C, 3 min. Cool to 8°C. The right samples are those samples which have the expected size. The negative control (blue colony) shows a band at 204 bp, the "recombinants" have amplicons with length original amplicon + ~204 bp.
- 6.6 The amount of template can be 0.5 µl and up to 4 µl (maximum). This depends on the original amplicon concentration. A normal PCR reaction gives a good yield and 2 µl is sufficient in most cases.
- 6.7 The negative control (blue colony) shows a band at 204 bp, the "recombinants" have amplicons with length original amplicon + ~204 bp.

7. Literature

- 7.1 Instruction Manual of the Invitrogen TOPO TA Cloning kit (Invitrogen, K4500-01) .
- 7.2 (Book about cloning)

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