Cloning ML04.001.001

Author:	ERG	**Valid from:**	2011-06-10	**Expired:**	
Responsible:	CSJ				
link:	\\Geusnt1\geokem1\Microlab_protocols\Protocols_active\04 Cloning & sequencing\ML_04_001_001_cloning.doc				

Kit for cloning: TOPO TA Cloning invitrogen

Preparation of agar plates for culturing the clones:

- Prepare 20 g LB-Agar in 500 mL MilliQ for autoclaving (yields about 25 plates)
- For each cloning reaction prepare 6 plates
- Cool down the medium to roughly 50 ^{0}C
- Add 500 µL Kanamycin (Standard with 50 mg/mL in sterile MilliQ) (→ plates have about 50µg/ml) (is placed in -20º freezer) to the liquid LB-agar and stir gently before pouring the plates (~20 mL per plate)
- Leave the plates to dry 20 min in the laminar flow with open lids. Store dark and dry place until the day before cloning (+4°C)
- Day before cloning (can also be done at day of cloning):
    - Incubate the plates at at 37 ^{0}C upside down 30 min before addition of X-gal
    - Dilute the X-gal in dimethylformamide (Cupboard for hazardous compounds in the weighing room) to a concentration of 40mg/mL
      It is possible to prepare this standard in a centrifuge cap, then wrap in aluminium foil (as X-Gal is sensitive to light!) and store at -20°C
    - Add 40µL X-gal per plate in the laminar flow. Leave to dry for 1 hour, and store at 37 ^{0}C until use the following day
- At the day of cloning, it is important to aereate the plates for 15 min in the laminar flow (lights off!). Before use for selective spreading of the cloning colonies, make sure to reheat the plates to 37 ºC (for at least 30min!)

Transformation

Preparation of the cloning vector:

Kit: TOPO TA Cloning invitrogen – *stored in -20º freezer*

Always use filter-tips during cloning.
Keep the cloning vector on ice when nothing else is mentioned.
Remember to pre-heat a water bed to 42ºC.
Remember to defreeze the SOC medium at room temperature – *stored in 80^{0}C freezer*

For each cloning reaction mix the following ingredients **(on ice)**:

1. 2 µl PCR product
2. 1 µl Salt solution
3. 2 µl H_2O
4. 1 µl TOPO Vector (= plasmid)

Stir gently with pipette tip

- Incubate at room temperature for 10 min (*integration of PCR-product in vector*)
- Transfer the reactions back to the ice tray

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Insertion of the cloning vector into the competent cells (E. Coli):

The Competent cells are stored at -80ºC – The component cells should be completely thawed (defrost).
!!! Only thaw the competent cells on ice, don´t let them thaw at room temperature or with hands!!!

1. Add 2 µl Vector reaction-mix (as prepared) to each tube of competent cells – stir gently with pipette tip
2. Incubate on ice for 10 min
3. Heat-shock treatment for 30 sec. at 42ºC (**no shaking**)
   (*increase of permeability of cell membrane, transfer vector in E.coli*)
4. **Immediately** transfer back to the ice tray
5. Add 250 µl SOC medium (Room temperature) to each reaction
6. Shake **horizontally** at 200 rpm at 37ºC for 1 hour
7. Plating:
   a. Spread 50 µL undiluted reaction mix on 3 plates.
   b. From each reaction mix make a 5-fold dilution of 30 µl + 120 µl SOC medium
   c. From the 5-fold diluted reaction mix spread 50 µL on 3 plates
8. Store the left transformation mix at +4°C, if further plating of cells is needed on next day
9. Incubate the plates at 37 ^{0}C **for 24 hours**
10. Transfer the desired no. of white colonies (successful clones) to 96-microtiter trays or culture vials with **TB dry** (medium especially for E.coli) **or LB** liquid + kanamycin (50 µg/mL) with sterile toothpicks. For culture vials the medium volume should be 5 mL
11. Close the microtiter trays or culture vials with breathable sealing and incubate at 37 ^{0}C **for roughly 24 hours**.
12. Proceed with plasmid-extraction procedure (E.g. Mobio Ultraclean 6 minute Mini Plasmid Prep Kit Cat No# 12300-100).

NB. If you wish to store additional clones for future analysis, it is wise to store them in 96-wells microtiter plates (as step 11). Add 100 µL medium + kanamycin (50 µg/µL) to each well. After 24 hours of growth at 37ºC, add 100 µL (80% in water) glycerol to each well and store the plate at -80ºC. (This long term storage is only for the ones who want to revive the cells in the future)