

7.13 Experimental Microbial Genetics

Fall 2008

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Bootcamp Day 5 - Gel Extraction of correctly sized PA14 genomic DNA fragments and BamHI linearized, dephosphorylated pMQ64, Ligation and Transformation

A. GEL EXTRACTION QIAQUICK GEL EXTRACTION KIT PROTOCOL

This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. Read through the QIAquick® Spin Handbook posted at the 7.17 Stellar website for in depth information about this protocol.

Notes:

- The yellow color of Buffer QG indicates a pH ≤ 7.5 .
- Add ethanol (96–100%, check cap to see if it has already been added) to Buffer PE before use (see bottle label for volume).
- Isopropanol (100%) and a heating block or water bath at 50°C are required.
- All centrifugation steps are carried out at 13,000 rpm (~17,900 x g) in a conventional table-top microcentrifuge.
- 3 M sodium acetate, pH 5.0, may be necessary.
- Prewarming the eluent buffer to 50°C will increase DNA yields.

1. Excise the DNA fragment from the agarose gel with a clean razor.

Minimize the size of the gel slice by removing extra agarose.

2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 μ l).

For example, add 300 μ l of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by inverting the tube every 2–3 min during the incubation.

IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).

If the color of the mixture is orange or violet, add 10 μ l of 3 M sodium acetate, pH 5.0, and mix.

The color of the mixture will turn to yellow. The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤ 7.5 . Buffer QG contains a pH indicator, which is yellow at pH ≤ 7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

Note: if your DNA is running with the bromophenol blue or xylene cyanol FF in the loading dye the QG buffer may turn blue, but probably no need to adjust the pH in this case.

5. Add 1 gel volume of isopropanol to the sample and mix.

For example, if the agarose gel slice is 100 mg, add 100 μ l isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb,

addition of isopropanol has no effect on yield.

6. Place a QIAquick spin column in a provided 2 ml collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.
The maximum volume of the column reservoir is 800 μ l. For sample volumes of more than 800 μ l, simply load and spin again.
8. Discard flow-through and place QIAquick column back in the same collection tube.
Collection tubes are re-used to reduce plastic waste.
9. Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.
This step will remove all traces of agarose.
10. To wash, add 0.75 ml of Buffer PE to QIAquick column, leave for 2-5mins and centrifuge for 1 min.
11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 13,000 rpm (~17,900 $\times g$).
IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
13. To elute DNA, add 30-50 μ l EB or H₂O (depending on desired concentration of sample, generally inserts for ligation should be eluted in 30 μ l so they are more concentrated) to the center of the QIAquick membrane, wait 1 min, and then centrifuge the column for 1 min.
IMPORTANT: Ensure that the water is dispensed directly onto the QIAquick membrane for complete elution of bound DNA (but don't touch the membrane with your tip). The average eluate volume is 48 μ l from 50 μ l elution buffer volume, and 28 μ l from 30 μ l.
Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at -20°C as DNA may degrade in the absence of a buffering agent.

B. LIGATION USING NEB QUICK LIGASE

1. Combine 50 ng of vector with insert at molar ratios of 1:3, 1:1, and 3:1. Adjust volume to 10 μ l with dH₂O. In addition, you will need to do several controls. Set up one reaction with no insert, and two reactions with no vector (one for each insert). Lastly, set up one reaction with no insert AND no vector. Think: why are these controls important? What do you expect to see? What does it mean if you see something else?

To calculate molar ratios of Vector:Insert use the equation:

$$[(\text{ng of vector} \times \text{size of insert (in kb)}) \div \text{size of vector (in kb)}] \times \text{molar amount of (insert} \div \text{vector)} = \text{ng of insert}$$

Example:

How much 500 bp insert DNA needs to be added to 100 ng of 3.0 kb vector in a ligation reaction for a desired vector:insert ratio of 1:3?

$$[(100 \text{ ng vector} \times 0.5 \text{ kb insert}) \div 3.0 \text{ kb vector}] \times (3 \div 1) = 50 \text{ ng insert}$$

Today the exact volumes of everything to add to the ligation will be given to you, but in the future you will calculate this for yourself using the above equation.

2. Add 10 μl of 2X Quick Ligation Buffer and mix.
3. Add 1 μl of Quick T4 DNA Ligase and mix thoroughly.
4. Centrifuge briefly and incubate at room temperature (25°C) for 5-10 minutes.
5. Chill on ice, then transform or store at -20°C .

6. Do not heat inactivate.

Heat inactivation dramatically reduces transformation efficiency.

C. TRANSFORMATION OF DH5 α -T1^R CHEMICALLY COMPETENT CELLS

Genotype DH5 α -T1^R: F- ϕ 80lacZΔM15 Δ (lacZYA-argF)U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 tonA (confers resistance to phage T1)

General Handling:

Be extremely gentle when working with competent cells. Competent cells are highly sensitive to changes in temperature or mechanical lysis caused by pipetting. Transformation should be started immediately following the thawing of the cells on ice. **Mix by swirling or tapping the tube gently, not by pipetting or vortexing.**

You will need the following items for transformation:

- 37°C shaking and non-shaking incubator
- 10 cm diameter LB agar plates with appropriate antibiotic & X-gal
- Ice bucket with ice
- 42°C water bath

Before Starting

- Pour LB agar plates with appropriate antibiotic (this should be done WELL in advance of starting this protocol; see note below)
- Equilibrate a water bath to 42°C
- Warm the vial of SOC medium to 37°C
- Spread 40 μl of 40 mg/ml X-Gal onto LB agar plates with antibiotic (see section on blue white screening below)
- Warm the plates in a 37°C incubator for 30 minutes
- Obtain a floating tube holder that will hold all transformation tubes so that they can all be put into a 42°C water bath at once.

Blue-White Color Screening

Blue-white color screening for recombinant plasmids is available when transforming this host strain with a plasmid that provides α -complementation. When *lacZ* is expressed by this strain in the presence of the chromogenic substrate X-gal, colonies containing plasmids with inserts will be white, while colonies containing plasmids without inserts will be blue. DH5 α -T1^R *E. coli* **does not require IPTG** to induce expression from the *lac* promoter.

Plates for Blue-White Color Screening

Spread 40 μ l of 40 mg/ml X-gal (prepared in dimethylformamide (DMF)) on solidified LB agar plates + appropriate antibiotic at least 30 minutes prior to plating the transformations. For consistent color development across the plate, pipette the X-gal into a 50-100 μ l pool (depending on how dry the plates are) of SOC or LB medium and then spread the mixture across the plate.

Transformation Procedure

The instructions provided below are for general use. Plasmid DNA should be free of phenol, ethanol, protein, and detergents for maximum transformation efficiency. **NOTE:**

Although the protocol calls for SOC media to recover these cells, LB is fine for routine transformations with plasmid DNA. For ligations, SOC is preferable. The SOC medium recipe, should you need to make it, is at the end of this protocol.

1. Briefly centrifuge the ligation reaction and place on ice.
2. Thaw, on ice, one 50 μ l vial of cells for each ligation/transformation.
3. Pipette 1 to 5 μ l of each ligation reaction directly into the competent cells and mix by tapping gently.

Note: NEB suggests transforming your cells with 5 ng of ligation mixture. Adding more than 5 μ L of the ligation mixture may disrupt salt concentrations and make your transformation less efficient.

Do not mix cells by pipetting up and down. Store the remaining ligation reaction at -20°C.

4. Incubate the vial on ice for 30 minutes.
5. Incubate for exactly 30 seconds in the 42°C water bath. Do not mix or shake.
6. Remove vial from the 42°C bath and place immediately on ice.
7. Add 250 μ l of pre-warmed SOC medium to each vial. (SOC is a rich medium; sterile technique must be practiced to avoid contamination.)
8. Place the vial in a microcentrifuge rack on its side and secure with tape to avoid loss of the vial. Shake the vial at 37°C for exactly 1 hour at 225 rpm in a shaking incubator. Alternatively, tape your tubes to the roller drums.
9. Don't forget to spread Xgal on your plates if you plan to use blue/white screening!
10. Spread 20 μ l to 200 μ l from each transformation vial on separate prewarmed and labeled LB agar plates. In general, it is recommended that you plate two different volumes to ensure that you will get individual colonies on at least one plate. For bootcamp, plate equal portions of the cells to two plates (~150 μ l/plate).
11. If you have remaining transformation reaction, store it at +4°C and plate out the next day, if desired.
12. Wrap the plates in parafilm, invert them and incubate at 37°C overnight.
13. In general, once you have colonies, select a few for analysis via plasmid isolation, restriction digest analysis, PCR, or sequencing. For bootcamp, see Day 6 protocol.

Preparation of Agar Plates:

LB Agar Plates (2%) – We can get agar from the kitchen. Pour plates about 2 days before required (they need time to dry). This means you need to be organized because you will need to order it in advance on the order sheet!!! Autoclave to melt agar. Let cool (high temperatures will inactivate the antibiotics) until hand warm/hot, about 55°C (agar should still be molten!). Add the appropriate concentration of your desired antibiotic. Pour into petri dishes (~25 ml/100-mm plate). Leave to dry 1-2 days. Store upside down in bags the plates came in at 4°C. Use within a month.

SOC Recipe (per one liter)

2% tryptone
0.5% yeast extract
10 mM sodium chloride
2.5 mM potassium chloride
10 mM magnesium chloride
10 mM magnesium sulfate
20 mM glucose