Making Calcium Competent Cells protocol

Day 1

1. Streak out frozen glycerol stock of bacterial cells (Top10, DH5α, etc.) onto an LB plate (no antibiotics since these cells do not have a plasmid in them). Work sterile. Grow plate overnight at 37°C.

Day 2

1. Autoclave:

   1 L LB (or your preferred media)

   1 L of 100 mM CaCl2

   1 L of 100 mM MgCl2

   100 mL of 85 mM CaCl2, 15% glycerol v/v

   4 centrifuge bottles and caps Lots of microfuge tubes

2. Chill overnight at 4°C:

   100 mM CaCl2

   100 mM MgCl2 85 mM CaCl2, 15% glycerol v/v

   Centrifuge rotor

3. Prepare starter culture of cells Select a single colony of E. coli from fresh LB plate and inoculate a 10 mL starter culture of LB (or your preferred media – no antibiotics). Grow culture at 37°C in shaker overnight.

4. Notes:

   • You will have extra CaCl2 and MgCl2. These solutions can be saved and reautoclaved for the next time you make competent cells.

   • You can also substitute other media like SOB, 2xYT, etc. for the LB if you prefer.

• All glassware should be detergent free. Presence of detergent reduces competency of cells.

Day 3

1. Inoculate 1 L of LB media with 10 mL starter culture and grow in 37°C shaker. Measure the OD600 every hour, then every 15-20 minutes when the OD gets above 0.2.

2. When the OD600 reaches 0.35-0.4, immediately put the cells on ice. Chill the culture for 20-30 minutes, swirling occasionally to ensure even cooling. Place centrifuge bottles on ice at this time.

IMPORTANT NOTES:

• It is important not to let the OD get any higher than 0.4. The OD should be carefully monitored and checked often, especially when it gets above 0.2, as the cells grow exponentially. It usually takes about 3 hours to reach an OD of 0.35 when using a 10 mL starter culture.

• It is also very important to keep the cells at 4°C for the remainder of the procedure. The cells, and any bottles or solutions that they come in contact with, must be pre-chilled to 4°C.

3. (Spin #1) Split the 1 L culture into four parts by pouring about 250 mL into ice cold centrifuge bottles. Harvest the cells by centrifugation at 3000g (~4000 rpm in the Beckman JA-10 rotor) for 15 minutes at 4°C. 4. Decant the supernatant and gently resuspend each pellet in about 100 mL of ice cold $MgCl_2$. Combine all suspensions into one centrifuge bottle. Make sure to prepare a blank bottle as a balance. 5. (Spin #2) Harvest the cells by centrifugation at 2000g (~3000 rpm in the Beckman JA-10 rotor) for 15 minutes at 4°C. 6. Decant the supernatant and resuspend the pellet in about 200 mL of ice cold $CaCl_2$. Keep this suspension

on ice for at least 20 minutes. Start putting 1.5 mL microfuge tubes on ice if not already chilled. 7. (Spin #3) Harvest the cells by centrifugation at 2000g (~3000 rpm in the Beckman JA-10 rotor) for 15 minutes at 4°C. At this step, rinse a 50 mL conical tube with ddH2O and chill on ice. 8. Decant the supernatant and resuspend the pellet in ~50 mL of ice cold 85 mM CaCl2, 15% glycerol. Transfer the suspension to the 50 mL conical tube. 9. (Spin #4) Harvest the cells by centrifugation at 1000g (~2100 rpm in the Beckman GH-3.8 rotor) for 15 minutes at 4°C. 10. Decant the supernatant and resuspend the pellet in 2 mL of ice cold 85 mM CaCl2, 15% glycerol. The final OD600 of the suspended cells should be ~ 200-250. 11. Aliquot 50 µL into sterile 1.5 mL microfuge tubes and snap freeze with liquid nitrogen. Store frozen cells in the -80°C freezer.

Fast digestion protocol

1. Prepare the reaction mixture at room temperature in the order indicated.
2. Mix gently and spin down.
3. Incubate at 37^{o}C in a heat block or water thermostat for 5 min.
4. Inactivate the enzyme(optional).

Ligation protocol

1. Set up the following reaction in a microcentrifuge tube on ice.
2. Gently mix the reaction by pipetting up and down and microcentrifuge briefly.
3. For cohesive(sticky) ends incubate at 16^{o}C overnight or room temperature for 10 minutes.
4. For blunt ends or single base overhangs, incubate at 16^{o}C overnight or room temperature for 2h (alternatively, high concentration T4 DNA ligase can be used in a 10-minute ligation).

5. Heat inactivate at 65 ^{o}C for 10 minutes.
6. Chill on ice and transform 1-5ul of the reaction into 50ul of competent cells.

Transformation with protocol

1. Resuspend DNA in selected wells in the Distribution Kit with 10ul dH2O. Pipet up and down several times, let sit for a few minutes. Resuspension will be red from cresol red dye.
2. Label 1.5ml tubes with part name or well location. Fill lab ice bucket with ice, and pre-chill 1.5 tubes in a floating foam tube rack.
3. Thaw competent cells on ice: This may take 10-15 min for 260ul stock. Dispose of unused competent cells. Do not refreeze unused cells, as it will drastically reduce transformation efficiency.
4. Pipette 50 ul of competent cells into 1.5 ml tube: 50ul in a 1.5ml tube per transformation. Tube should be labeled, pre-chilled, and in floating tube rack for support. Keep all tubes on ice. Don't forget a 1.5ml tube for your control.
5. Pipette 1ul of resuspended DNA into 1.5ml tube: Pipette from well into appropriately labeled tube. Gently pipette up and down a few times. Keep all tubes on ice.
6. Pipette 1ul of control DNA into 1,5ml tube: Pipette 1ul of 10pg/ul control into your control transformation. Gently pipette up and down a few times. Keep all tubes on ice.
7. Close 1.5ml tubes, incubate on ice for 30 min: Tubes may be gently agitated/flicked to mix solution, but return to ice immediately.
8. Heat shock tubes at 42^{o}C for 45 seconds.
9. Incubate on ice for 5 min.

10. Pipette 950 LB media to each transformation.
11. Incubate at 37 °C for 1h, shaking at 200-300rmp.
12. Pipette 100ul of each transformation onto petri plates.
13. Spin down cells at 3000rmp for 5 min and discard 800 of the supernatant. Resuspend the cells in the remaining 100ul, and pipette each transformation onto petri plates.
14. Incubate transformations overnight(14-18 hr) at 37 °C.
15. Pick single colonies: grow up cells and miniprep, also make glycerol stocks.
16. Count colonies for control transformation.

Test silicate production protocol

Start new culture with 3 tubes for each colony.

Wait for the beginning of log phase.

Induce with 1% of Arabinose 1X

Wait 4hours;

Add silicate standard to a concentration of 60µM;

Wait overnight.

Silicatein Staining protocol

Add 1/1000 of Rhodamine 123

Wait 10 min

Wash the cells :

Centrifuge the cells 2 min at 14000rpm

| Remove the supernatant

5X | Add PBS

| Centrifuge the cells 2 min at 14000rpm

Make microscope slide with the cells :

Put a drop of water on the slide

Scratch the pellet with a tip and stir it in the droplet.

Put the cover slip

Pass quickly in the burner to seal it.

Protein Extraction protocol

Materials:

- Liquid culture of cells expressing protein of interest
- BugBuster reagent (5 ml per gram of wet cell paste)

Protocol:

1. Harvest cells from liquid culture by centrifugation at 10,000 × g for 10 min using a weighed centrifuge tube. For small scale extractions (1.5 ml or less), centrifugation can be performed in a 1.5ml tube at 14,000–16,000 × g. Decant and allow the pellet to drain, removing as much liquid as possible. Determine the wet weight of the pellet.

2. Resuspend the cell pellet in room temperature BugBuster reagent by pipetting or gentle vortexing, using 5 ml reagent per gram of wet cell paste. This typically corresponds to about 2.5 ml per 50ml culture. For small cultures, use up to 1/5 culture volume for resuspension (e.g., use 300 µl BugBuster for 1.5ml cultures). There are no adverse effects to using larger volumes of BugBuster, if needed.

3. Incubate the cell suspension on a shaking platform or rotating mixer at a slow setting for 10–20 min at room temperature.

4. Remove insoluble cell debris by centrifugation at 16,000 × g for 20 min at 4°C.

5. Transfer the supernatant to a fresh tube. For SDS-PAGE analysis, remove a small sample (25–50 µl) and combine with equal volume of 4X SDS Sample Buffer (Cat. No. 706073). Immediately heat for 3 min at 85°C to denature proteins and then store at –20°C until SDS-PAGE analysis.

SDS-PAGE gel Protocol

1. Run SDS-PAGE.

2. Wet membrane in H2O. Soak membrane in transfer buffer for 10 min.

3. Set up transfer from the gel to a nylon membrane in transfer buffer.

4. Place “transfer sandwich” in semi-dry transfer chamber. Run at 23 V for 30 min for 0.75 and 1.0 mm gels or 40 min for 1.5 mm gel.

5. Block blot by soaking in blotting buffer for 1 hr with shaking. Alternatively, blocking can be done with as much as 10% milk and 0.5% Tween 20 to reduce background.

6. To 10 ml blocking solution, add primary antibody at proper dilution. Incubate the membrane for 1 hr with shaking. Alternatively, incubation with 1º Ab can be done ON @ 4 ºC,

7. Wash 2x briefly with PBS-T, then wash 3x with PBS-T for 5 min.

8. To 10 ml PBS-T, add secondary antibody at proper dilution. Incubate the membrane for 1 hr with shaking.

9. Wash 2x briefly with PBS-T, then wash 3x with PBS-T for 5 min.

10. Detection by ECL. Expose blot to film for 15 sec – 5 min.

Alizarin Red S staining Protocol

The organic dye Alizarin red S (ARS) will produce a pink to red stain on any carbonate that will react with dilute acid. The reaction between carbonates and acid is usually controlled (1 to 2 minutes at 25oC for thin sections) so that the more reactive minerals, such as calcite and aragonite, stain red but the less reactive ones, such as dolomite and siderite, remain unstained.

Preparation of Alizarin red S and potassium ferricyanide stain

1) To 300ml of 0.5% HCl add 0.6g Alizarin red S and filter

2) To 200ml of 0.5% HCl add 4g potassium ferricyanide

3) mix the two solutions.

Staining Procedure

1) Sections can be etched before staining in 1% HCl for 5 seconds

2) Stain sections for 30 to 60 seconds in combined stain until ferroan and non ferroan varieties are differentiated. CO2 bubbles evolve from the reacting surface. Agitate the section and withdraw from staining solution briefly to break any CO2 bubbles that stick to the surface and prevent the stain from reaching with that part of the section. If the bubbles are not released the section it will have many small unstained circles.

3) Rinse the section **gently** with deionised water. The stain is delicate and is easily removed - don't jet water at the surface or touch it. Once dry the stain is more durable but if you wish to archive the stained section it is best to cover slip.

4) Allow the stained section to dry by stacking vertically so the water runs off. The stain is water soluble so dry as quickly as possible without touching the stained surface.

Miniprep protocol

1. Pellet 1–5 ml bacterial overnight culture by centrifugation at >8000 rpm (6800 x g) for 3 min at room temperature (15–25°C).

2. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.

3. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 min. If using LyseBlue reagent, the solution will turn blue.

4. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. If using LyseBlue reagent, the solution will turn colorless.

5. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. October 2010 Quick-StartProtocol Sample & Assay Technologies

6. Apply the supernatant from step 5 to the QIAprep spin column by decanting or pipetting. S Centrifuge for 30–60 s and discard the flow-through, or z apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source.

7. Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB. S Centrifuge for 30–60 s and discard the flow-through, or z apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source.

Note: This step is only required when using endA+ strains or other bacteria strains with high nuclease activity or carbohydrate content.

8. Wash the QIAprep spin column by adding 0.75 ml Buffer PE. S Centrifuge for 30–60 s and discard the flow-through, or z apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source.

Transfer the QIAprep spin column to the collection tube. 9. Centrifuge for 1 min to remove residual wash buffer.

10. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

QIAquick PCR Purification Kit Protocol using a microcentrifuge

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene. For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil). 2. If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. Place a QIAquick spin column in a provided 2 ml collection tube.

4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.

5. Discard flow-through. Place the QIAquick column back into the same tube. Collection tubes are re-used to reduce plastic waste.

6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.

7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min. IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation. PCR Purification Spin Protocol

8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge. IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer. 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. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
10. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel. Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.