IspG Procedures

- Make sure the anaerobic tent will be available on the days you plan to use it.
- Make sure the autoclave/incubator will be available.

- For the transformation of the host cells (E. coli XL-Blue cells) follow the procedures that comes with the cells.

Growing *E. coli* XL-Blue Cells on Agar Plates

Preparing Plates

- Measure out 20 g of Difco LB Agar for every half-liter (500 mL) of dd water. Suspend the agar in the solution (use a stir bar) until thoroughly mixed; retrieve the stir bar before pouring the agar solution into an autoclavable bottle.
- Autoclave (setting 9, see above). While the solution is autoclaving, turn on one of the water baths located in the main room (357) next to the flow box. Adjust settings and monitor the water bath until the thermometer reads 55°C.
- When the autoclave is done, remove the bottle and place it in the water bath until it reaches approximately the same temperature as the water. When this is the case, remove the bottle from the water bath and place it in the flow box.
- Put on gloves and spray them with EtOH. Add 5 mL of 10 mg/mL filter-sterilized ampicillin and swirl the bottle to mix. Pour the agar solution into a clean plate until it just covers the bottom, then put the lid on the plate and carefully move it. Swirl the bottle every so often to keep the agar from hardening/settling.
- Once the bottle is empty/nearly so, immediately rinse it with water.
- Allow the plates to harden in the flow box for at least 2-3 hours. Protect from UV radiation with aluminum foil, if necessary.
- Once the plates have solidified, invert them, wrap in aluminum foil in stacks of 5-6, and store in the refrigerator until you need them.

Inoculating plates

- Make sure the flow box in room 357 is sterile and not currently in use.
- Retrieve the appropriate glycerol stock solution from the -80 °C freezer, as well as an unused plate from the fridge, the spreader and its beaker containing alcohol (if there is no alcohol in the beaker, add enough to cover the bottom of the spreader), gloves, SOC medium, the sterile yellow pipettor tips, and the 2-20 µl and 10-100 µl pipettors.
- Put everything you will need into the flow box, including the small propane tank located beneath the table in front of the box, then don the gloves and spray them with the 70% alcohol solution located in a spray bottle in front of the box. Let your hands dry inside the box, and be careful what you touch from now on.
- Open the gas on the propane tank and light the flame using the striker. Carefully retrieve the spreader from the alcohol solution, making sure the triangular portion has been immersed. Wave this portion through the flame, making sure that the ethanol ignites. Carefully balance the spreader on top of the beaker so that the triangular portion does not touch anything. Turn off the gas and let the spreader cool for 10-15 minutes. To check the temperature, gently touch the triangular portion to a corner of the plate agar. If the agar appears to melt or deform, then the spreader is probably too hot. Use this time to label the plate with the name of the organism/protein and the date of inoculation.
- Pipette 40-50 µl of SOC onto the agar surface, then use another tip to obtain 2-5 µl of culture from the stock solution. Use the spreader to gently distribute the solution across the surface of the plate. After a few minutes, invert the plate and put it in a 37 °C incubator for 24 hours.
- Heat-sterilize the spreader for each plate you make.

Growing *E. coli* XL-Blue Cells in Culture

- For the initial cultures (5 and 100 mL), you will use stock SOC medium. This is generally stored in the refrigerator after its preparation, so check there first. If there is none, if it has become turbid due to growth of microorganisms or if you are preparing a 1 L culture, you will need to make the SOB/SOC solutions as described below.

Preparing sterile stock solutions

i. Make sure there is SOC medium in the refrigerator. If not, prepare an additional liter below.
ii. For a 1 L culture (SOB medium):
1.20.0 g of tryptone
2.5.0 g of yeast extract
3.0.5 g of NaCl
4.1 L distilled, deionized H_2O (from the container above the sink marked EZPure)
iii. Prepare the solution in the appropriate flask (for cell growth, in a 2800 mL flask covered at the top with aluminum foil; for stock SOC solution, in an autoclavable (plastic-encased) bottle with the lid on loosely). Add a strip of autoclave tape (white with white stripes) to the aluminum foil/cap.

iv. Put the flasks in one of the three autoclavable containers (white, labeled Nalgene along the sides) and add a little water in the bottom of the container.
v. Take the container down the hall (room 357) to the autoclave. Open the autoclave and put the container inside, making sure that nothing touches the side of the autoclave.
vi. Close the door of the autoclave securely (door closed light on bottom right of control panel will light up)
vii. Press the third button on the top row of the autoclave instrument panel. It looks like a flask. On the display, the number 9 should appear, as part of a string of digits that looks like this: 09-250 20 00 00P. When this appears, press Start. Sign in on the log sheet and come back in about an hour.

viii. While the solution is autoclaving, prepare 1 M $MgCl_2$ and 1 M $MgSO_4$ solutions, in volumes of 10 mL for each liter of SOB solution that you have prepared. Also prepare 20 mL of 20% w/v glucose (dextrose).

ix. When the autoclave has completed its cycle, the display will read CYC FINISH. You may then remove your containers and solution, provided you are wearing thick heatproof gloves.
x. When the bottle is cool enough for you to touch it without feeling burnt, put it in the air-flow box located next to the cold room. Bring your solutions of $MgCl_2$ and $MgSO_4$ with you, as well as a 10 mL syringe and a filter (0.22 μm).
xi. Put gloves on and spray them with the nearby 70% EtOH solution to sterilize them. When the alcohol has evaporated, use the syringe to draw up 10 mL of the $MgCl_2$. Carefully open the filter container and thread the filter onto the syringe. Try to avoid touching the filter, especially the tip. The green rim is the best place to grab it. Slowly inject the $MgCl_2$ into your autoclaved solution. Remove the filter, placing it back into the container

in the same orientation that it came out. Fill the syringe with 10 mL of the $MgSO_4$ solution, add the filter as before, and inject into the autoclaved solution. Repeat with the glucose, adding 20 mL of glucose for each liter of medium. The SOB solution has now become SOC solution!

xii. If the solution is for stock purposes (later use), put it in the refrigerator. Otherwise, add any other ingredients (antibiotics, culture) and incubate.

Inoculation of a 5 mL culture (and 100 mL):

i. Take these items to the flow box:
   1. the agar plate of your culture
   2. the 1 mL and 2-20 µl, pipettors and tips (autoclaved)
   3. an autoclaved 25-50 mL Erlenmeyer flask
   4. the bottle of SOC medium from the fridge
   5. a centrifuge tube of ampicillin from the -30 °C freezer
   6. gloves

ii. Perform the necessary sterilization procedures and put these items in the box.

iii. Pipette 5 mL of the SOC medium into the Erlenmeyer flask.

iv. Add ampicillin, 12.5 µl (10 mg/ml stock).

v. Using a sterile pipette tip from the 2-20 µL pipettor, carefully remove an isolated culture from the plate without damaging the agar too much. Drop the entire tip into the 5 mL solution.

vi. Put the culture into the incubator.

vii. The procedure for preparing the 100 mL culture is similar, except this time you add 100 mL of SOC medium (use an autoclaved graduated cylinder) to a much larger flask (250-500 mL). Use a sterile 1 mL pipettor tip to transfer all of the 5 mL culture to the 100 mL flask and incubate.

viii. Add ampicillin, 250 µl (10 mg/ml stock) and $FeCl_3$, 30 µl (1 M stock)

ix. Put the flask in the shaker/incubator. The time scale to follow is on the supporting information.

Inoculation of a 1 L culture:

i. Take the 100 mL culture out of the incubator.

ii. Measure 25-50 (depends on how many liters of culture you are inoculating) mL of culture into an autoclaved graduated cylinder and add to 1 L SOC medium.

iii. Add ampicillin, 2.5 ml (10 mg/ml stock).

iv. Incubate in large shaker for 2-3 hours, and then extract 1 mL of culture. Check OD at 600 nm. If it is between 0.4-0.6, add 1 mL of 0.1 g/L IPTG

v. Wait 7-8 hours after initial incubation, then check the culture's OD again. This time, dilute the culture 10x (100 µl culture in 900 µl dd water). If the OD is 3 (corrected for dilution), the cells are ready to harvest.

Material:

- $MgCl_2$, 1 M
- $MgSO_4$, 1 M
- ampicillin, 10 mg/ml
- $FeCl_3$, 1M
- IPTG 0.4 M

Growing *E. coli* XL-Blue Cells in Culture

- Label the lids of 3 large centrifuge bottles (for every L of cell culture) (white caps) with tape and a sharpie. Weigh each bottle and its lid together and record the weight on a piece of paper. Be sure to keep the bottle with its lid.
- Fetch the cell cultures and carefully pour them into the bottles. Make sure each bottle is at least 2/3 full, and match their weights to within 0.1 g. Keep the bottles of similar weights together. Put the lids on tightly.
- Carry the bottles down the hall to room 357. Obtain the rotor matching the centrifuge you wish to use from the cold room. Put in the centrifuge and the centrifuge cups, making sure the ones of equal weight are on opposite sides of the centrifuge to balance each other out. Set the centrifuge for 5 x 1000 rpm for 20 minutes.
- Once the centrifuge is done, retrieve the cups, wipe out the rotor if necessary, turn the centrifuge off and return the rotor to the cold room.
- Take the cups back to the lab. Pour out the liquid; there should be a pellet of cells in the bottom. Weigh the container and pellet and record the weight with the weights of the cups and lids from earlier. Subtracting the cup and pellet weight from the weight of the cup alone will give you the weight of the cell pellet.
- Put the cups containing the cell pellets in the -30 °C freezer if you will use them soon and in the -80 °C freezer if you will use them later. Add Lysol to the supernatant and let it sit for at least 24 hours before throwing it out.

Protein purification

Cell extract:

- Get the cell pellets out of the freezer and let them thaw a few minutes.
- Make sure there is everything in the tent that you need, including: several beakers of different sizes, bottles, the sonicator cup, clean test tubes, etc.
- Remove the lids of the centrifuge cups and bring them and a red insulated bowl of ice inside the tent.
- If you only had 1 L of cell culture, resuspend the cell pellets in a total of 50 mL of *buffer A*. If you had more than 1 L, use 100 mL to resuspend the cell pellets. To do this, add a small amount of buffer to each cup and swirl gently. Use a pipettor tip (the 1000 µl one) to break up clumps of cells into a homogenous solution. Clamp the sonicator cup firmly in the ice bucket and pour the cell solution into it. Try to keep ice out of the sonicator cup. Repeat until all of the cell culture is in the sonicator cup. You can wash with more of the buffer to get as many of the cells as possible out of the centrifuge cup.
- Retrieve the sonicator and position it so that the tip is a half inch into the cell solution, but not touching the sides or bottom of the sonicator cup. Clamp it firmly into place.
- Turn on the sonicator (button beneath bottom right side). Set the timer to 3 minutes, 0.5 s pulse on, 0.5 s pulse off, amplitude (using dial on side) 75 %. Press the start button and leave the sonicator alone for about 6 minutes. Sonicate 2 more times on the same settings, giving the culture a couple of minutes to cool down between periods of sonication.
- Turn off the sonicator and remove it from the solution, carefully wiping the tip clean. Pour the solution into two 45-Ti centrifuge cups (should have already been in the tent to become anaerobic). Weigh them (with their lids!) to make sure they are within 0.1 g of each other. Screw the caps on tightly, and remove them from the tent.
- Bring them into the lab and centrifuge them on our machine. Use the 45-Ti rotor, located in the small brown fridge in the lab. Set the centrifuge to 16000 rpm for 20 minutes (approximately) and put the cups in the rotor. Make sure that the washers in the rotor are aligned and secure so that a vacuum may be formed. Shut the door, turn on the vacuum (vacuum, Enter), set the rotor (4, Enter), and then press start. The centrifuge will beep when it's done. Turn off the vacuum and wait a minute before opening the door. Remove the centrifuge cups and put them back in the tent.

Heat treatment for *Thermus thermophilus* protein:

- Pour the supernatant (try to avoid pouring out the precipitate) into a small bottle. Stopper it and put a lid on it, then remove it from the tent and heat it in the water bath set at 65 C for 30 minutes. After this, put it back in the tent and pour it into new centrifuge cups and centrifuge the solution at 25000 rpm for 25 minutes. Pour out the supernatant into a beaker for the next step.

- Use one of the small Millipore filters (0.2 um) already open and in the tent to filter the protein, in a similar fashion to filter-treating the solutions and antibiotics above. Put the filtered protein into a different beaker. You will notice as you filter more protein that it becomes harder and harder to depress the syringe, and you may even have to get another filter (not more than two – very expensive!!!).

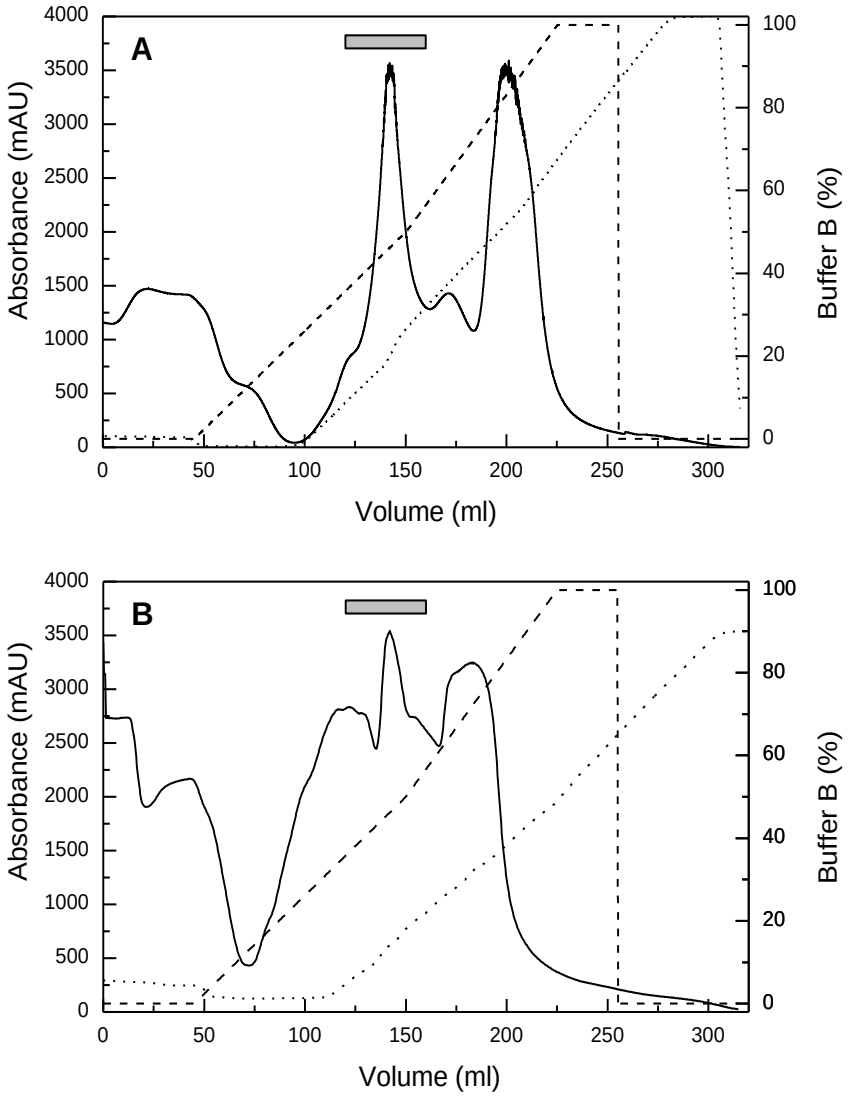
DEAE Sepharose column:

- For this step the 1.6 × 40 cm diethylaminoethyl (DEAE) sepharose column is used. This is a weak anion exchanger.
- Begin by washing the column using the wash program. Make sure the column is connected to the P-1 pump and that the intakes for pumps A and B are in the appropriate (respective) buffers, in this case *buffer A and B* (Buffer B is normally the high salt buffer).
- Now you have to get the protein on the column. Before you connect the pump first Hook up the pump (small gray box) to the column. Put the pump intake in a small beaker of buffer and turn it on a low flow rate (1-2). Connect the dripping connector to the top of the column, unscrewing the top cap and placing it aside before screwing together the column and connector. Unscrew the bottom cap of the column and connect the base of the column to another tube leading to an empty bottle. Turn off the pump and replace the buffer solution with your protein solution. Turn the pump back on and set it for 3-4. Leave the pump alone, but check it periodically to make sure the intake still has access to protein solution. As the amount of solution decreases, you can tilt the beaker, and when it is almost empty, add a few mL of buffer to the beaker to wash excess protein from the tubes and pump through the column.
- When the loading is completed disconnect the P-1 pump and reconnect the column to the FPLC system. Now the purification program can be started. The protein will be eluted using a linear gradient from 0 to 1 M NaCl in buffer A.
- Fractions containing dark brown color were pooled. Generally, faction would have to be checked for activity and/or any other properties that are unique for the enzyme of interest. In this case the GcpE enzyme is very abundant and is the most intensely colored enzyme. Therefore pooling the dark colored fraction will work in this case. (See also the SDS-PAGE results below)

- The next column is a Mono Q column, which is a strong anion exchanger. For to enzyme to be able to bind to this column the salt concentration in the sample has to be lowered significantly. For this purpose the sample is diluted 10x with buffer A and concentrated to a volume of **10 ml** in an Amicon filtration unit (10 kDa cut-off filter).

Material:

- Buffer A: 20 mM Tris-HCl pH 8.0
- Buffer B: 20 mM Tris-HCl pH 8.0
  1 M NaCl



DEAE column profile of the purification of the GcpE protein. Two profiles are shows with either low amount of protein loaded – 1-2 L cell culture (**A**) or high amount of protein loaded – 4 L cell culture (**B**). The solid line is the absorbance at 280 nm. The dashed line represents the % concentration of buffer B used. The dotted lines represent the % conductivity. Note that the % Buffer is what is programmed and is added to the top of the column, while the conductivity is measured at the column exit and coincides with the time of the absorption measurement. The protein eluted between 18% and 32% buffer B, indicated by the gray bar.

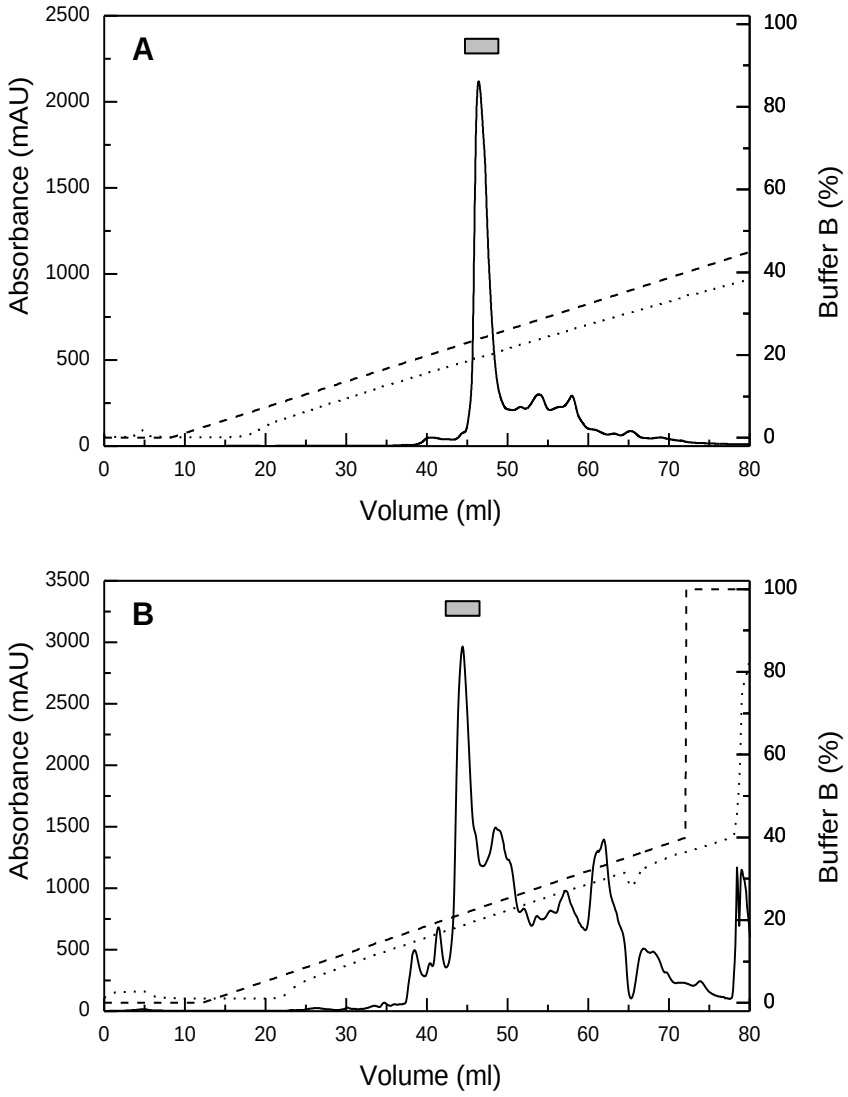
Mono Q column:

- For this step the 1 × 10 cm Mono Q column is used. This is a strong anion exchanger.
- Begin by washing the column using the wash program. Make sure the column is connected to the FPLC and that the intakes for pumps A and B are in the appropriate (respective) buffers, in this case *buffer A and B* (Buffer B is normally the high salt buffer).

- This column is packed with a very fine material and runs at a much higher pressure than the DEAE sepharose column. In this case the P-1 pump cannot be used to load the protein sample, but the enzyme has to be loaded into the sample loop (10 ml) first.
- The default setting for the port is 'LOAD', which means that the sample can be injected into the loop without the need to change the setting of the port. Make sure you wash the loop with regular buffer first.
- When the sample loop is filled, the sample has to be loaded onto the column. For this change the port setting to 'INJECT'. Use pump A to load the sample onto the column.
- ***Dependent of the amount of cells you started with, you might not be able to lead all the protein onto the column at once.*** You might have to do 2, 3 or four runs. In that case you will only load 5, 3.3, or 2.5 ml protein sample at-a-time.
- After the sample is loaded make sure you reset the port to 'LOAD'.
- Now the purification program can be started. The protein will be eluted using a linear gradient from 0 to 1 M NaCl in buffer A.
- Fractions containing dark brown color are pooled (normally two fration). Again, the GcpE enzyme is very abundant and is the most intensely colored enzyme. Therefore onlu pooling the dark colored fractions. (See also the SDS-PAGE results below)

Material:

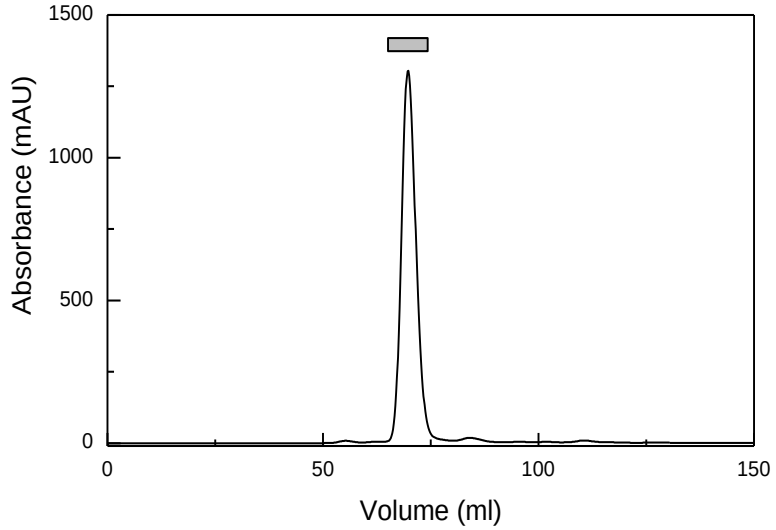
- Buffer A: 20 mM Tris-HCl pH 8.0
- Buffer B: 20 mM Tris-HCl pH 8.0
  1 M NaCl



DEAE column profile of the purification of the GcpE protein. Dependent on how much protein has been loaded on the previous column (DEAE sepharose) the protein profile might show a well isolated protein peak indication high protein purity (**A**) or a peak that still shows significant overlap with other protein peaks (**B**). The solid line is the absorbance at 280 nm. The dashed line represents the % concentration of buffer B used. The dotted lines represent the % conductivity. The protein eluted between 18% and 32% buffer B, indicated by the gray bar.

Superdex 200 column:

- Generally the protein is pure enough for EPR experiments after the first two columns. If higher purity is needed, however, the protein can be further purified by gel filtration using an XK 16/60 superdex 200 column.
- In this case only ‘buffer A’ containing 100 mM NaCl is needed.
- The column has to be loaded via the sample loop. Note that for a good separation of peaks the sample loop with a volumn of 100 µl has to be used. This means that the protein has to be concentrated to this volume before the column run.



Superdex 200 column profile of the purification of the GcpE protein. The position of the GcpE peak is indicated by the gray bar.