

Making 15% gel for SDS

The following instructions make 6 SDS gels.

- Prepare the molds.
- In a 50mL falcon tube, pour 45mL of 15% Acrylamide solution (7mL per gel, and a little extra) made up of:
 - 50mL 1.5M Tris at pH 8.8
 - 75mL 40% Acrylamide
 - 2mL 10% SDS
- Add 0.45mL of 10% APS solution to the falcon tube, mix by inversion
 - We want the final concentration of APS in the gel to be 0.1%, so for 45mL of solution we need 0.45mL of 10% APS solution
- Add 45 μ L of 100% TEMED to the falcon tube, mix by inversion
 - We want the TEMED to be 0.1% of the solution
- Take 7mL of solution from the falcon tube with a pipette and let it flow into the mold. Fill a little over the fill line, as the gel shrinks when it polymerizes
- Pour 1mL of 100% isopropanol on top of the gel slowly to cover it. This prevents the gel from drying up.
- 20 minutes later, check if the gel has polymerized by inverting and flicking the leftover solution in the falcon tube
- When the gel is solid, pour out the isopropanol and place the gel molds upside down to let them drip dry for about one minute
- In a 15mL falcon tube, add 12mL of 4% stacking gel (you need 2mL per gel)
- Add 120 μ L of 10% APS solution to the 15mL falcon tube, mix by inversion
- Add 12 μ L of 100% TEMED to the 15mL falcon tube, mix by inversion
- Take 1mL of solution from the falcon tube in a pipette, place the pipette at a 45° angle on the side of the gel mold and push forcefully to fill any air pockets formed by the shrinking of the gel
- Add an extra mL of the solution from the falcon tube to the top of the gel, then place the comb in the gel to form the wells.
- The gels can be kept in the refrigerator, enveloped in tissue soaked in water and kept in a closed plastic bag

Running 15% SDS-PAGE Gels

- Place the gel in 1X Lamelli buffer (Glycine, Tris buffer and SDS).
- Flush the wells using a 200 μ L pipette.
- Load 2 μ L of Page Ruler Plus Prestained Ladder in the first well.
- Add the samples of interest in the remaining wells.
- Run the gel at 200V for 1 hour.
- When the gel has run, rinse the gel with water to remove any leftover buffer.
- Separate the gel from the glass plates and drop it into a square Tupperware filled with water.
- Pour out the water and cover the gel with Coomassie blue stain.
- Microwave the gel until the liquid boils. Then, let the gel stain for 20 minutes on the rotating plate. The Coomassie blue stain is made up of:

- 50% methanol
 - 10% acetic acid
 - 1g/L chromatic blue R350
- Pour out the stain and cover the gel with destain. The destain is made up of:
 - 50% methanol
 - 15% acetic acid
- Microwave the gel until the destaining liquid boils.
- Place folded pieces of paper on each side of the Tupperware to absorb the blue dye. Leave it to destain for at least 30 minutes on the shaking plate.
- Place the gel on the light revealer to visualize the results.

Protein Expression

- Inoculate E. coli BL21 transformed with the construct of interest in a LB liquid culture containing ampicillin.
- Let the culture grow at 37°C until it reaches an OD₆₀₀ ~0.6.
- Cool the liquid culture down by keeping it at 15°C for 30min.
- Add IPTG 1mM to the liquid culture and induce expression for 24 hours at 15°C.

Protein Purification

After inducing expression in a 1-liter culture for 24 hours at 15°C, proceed with the following steps:

The 1-liter culture is divided in 3 containers: one is used as a test purification, one for the bacterial lysate SDS-PAGE gel, and the last one is used for the actual purification.

- Remove 10mL from the liter culture and put it in a 15mL falcon tube.
- Remove 0.5mL from the liter culture and put it in an Eppendorf tube.
- Pour the remainder of the liter culture in the container used for the big centrifuge.

Treat the 3 volumes simultaneously.

Buffers

Cold Lysis Buffer

- 50mM sodium phosphate pH 8.5
- 300mM NaCl
- 5mM imidazole
- 0.5mg ml⁻¹ lysozyme
- 1mM phenylmethanesulphonyl fluoride

Elution Buffer

- 50mM sodium phosphate pH 8.5
- 300mM NaCl
- 300mM imidazole

Bacterial Lysate SDS-PAGE Gel

- Put 0.5mL of the liquid culture in an Eppendorf tube. Centrifuge it for 1 minute at 14000 rpm. Remove the supernatant.
- Add 100μL of 1X SDS loading dye to the pellet and resuspend it.

- Boil it for 10 minutes at 95°C.
- Centrifuge for 5 minutes at 14000 rpm. All the bacterial proteins will remain in the supernatant.
- At this step, you can optionally freeze the Eppendorf at -20°C.
 - When removing from the freezer, boil the Eppendorf at 95°C for 5 minutes
 - Then centrifuge the Eppendorf for 2 minutes at maximum speed
- Load 10µL of the supernatant onto the gel. You want to see a more distinct line at your desired protein's size, showing that the overexpression was successful.

Protein Test Purification

- Remove 10mL from the liter culture and put it in a falcon tube.
- Centrifuge it at 5'000 rpm for 10 minutes
- Pour out supernatant.
- At this point, you could freeze the pellet at -20°C if you chose to do so.
- Add 1mL of lysis buffer to the cell pellet and resuspend the pellet.
- Sonicate the solution. After doing so, always keep it on ice. Set the pulse at 001.0S, 001.0S. Set the amplitude at 40%.
 - Press start, then wait for 15 pulses. Press stop, then remove the tube and place on ice for a minute. Repeat this step once more.
- Transfer the solution in a 1.5mL Eppendorf tube.
- Centrifuge it for 10 minutes at full speed and 4°C.
- Prepare the magnetic beads for the his-tag affinity column purification:
 - In a 1.5mL Eppendorf tube, add 70µL of well suspended His Mag Sepharose Ni bead solution.
 - Put this Eppendorf in the 12-tube magnetic separation rack. Remove the liquid (it contains ethanol).
 - Take the tube out of the rack, add 200µL of water and resuspend the beads. Place the tube back on the rack and remove the water once more. This step is used to wash the remaining ethanol away.
 - Add 200µL of Cold Lysis Buffer and resuspend the beads.
- Add the supernatant to the tube containing the magnetic beads.
- Put this tube in the rotating machine in the cold room for 45 minutes.
- Place the tube in the magnetic separation rack. Remove the supernatant.
- Then do 3 washes. For each wash, add 1mL of Cold Lysis Buffer then mix with beads. Suction out the supernatant after placing the tube in the separation rack.
- After removing the supernatant from the last wash, add 30µL of elution buffer. Let it sit for 2 minutes.
- In a 1.5mL Eppendorf tube, add:
 - 20µL of the solution containing the eluted recombinant proteins.
 - 20µL of 2x SDS loading dye
- Boil it at 95°C for 5 minutes and use 8µL to run a 15% SDS-PAGE gel.

Protein Large-Scale Purification

- After pouring the liquid culture in the centrifuge container, centrifuge it with a counterweight at 5000rpm for 15 minutes.
- Pour out the supernatant.
- Resuspend the cell pellet in 30mL Cold Lysis Buffer in a 50mL falcon tube.
- At this step, you could optionally freeze your cells at -80°C.

- To thaw the big cell pellet and Cold Lysis Buffer solution kept at -80°C, place the falcon tube in warm water. When the contents are almost completely thawed out, place the tube on ice.
- Fill up the falcon tube with Cold Lysis Buffer so that it contains 50mL of solution.
- Pour the contents of the falcon tube in a beaker containing a magnetic rod. Place on magnetic stirrer at 800RPM until the cell chunks are dissolved.
- To make the Cold Lysis Buffer more efficient, we want to add PMSF and Lysozyme to the solution to get the following concentrations:
 - 0.5mg/mL of lysozyme (L6876 Lysozyme from Sigma Aldrich)
 - 1mM of PMSF

For our 50mL solution, we therefore add 500μL of 100mM PMSF, drop by drop using a pipette. We then add 25mg of lysozyme powder, measure on a precise scale. Pour the powder all at once in the beaker and leave it on the magnetic stirrer until fully dissolved.

- Place the beaker in the refrigerator for 30 minutes to incubate at 4°C.
- After 30 minutes, put the beaker back on the magnetic stirrer for a few seconds to mix up the cells that have partially settled. Then, place the beaker on ice.
- Sonicate the cells. Set the sonication for 10 minutes at 1 second on, 1 second off.
- Pour equal volumes of the sonicated cell solutions in two centrifugeable SS34 tubes. Keep these tubes on ice and make sure they are the exact same weight.
- Centrifuge the two SS34 tubes at 18'000 rpm for 40 minutes at least. Pour the supernatant in a 50mL bottle.
- Prepare the His-tag affinity column by cleaning out the pump and removing the air inside. Equilibrate the column with the cold lysis buffer.
- Run the supernatant through the column (2mL/minute).
- Elute the protein from the his-tag affinity column using the elution buffer. Collect the flowthrough in a 96 well fraction collector plate.
- The chromatogram shows us which fractions from the collector plate should contain our protein. By analyzing the peaks in the chromatogram, we select the fractions to be tested with the SDS gel.
- Label Eppendorf tubes for the selected fractions and add:
 - 10μL of 2x SDS loading dye
 - 10μL of the corresponding fraction
- Heat the Eppendorf tubes for 5 minutes at 95°C and load 8μL to the gel.
- When the fractions containing our protein of interest have been identified, remove them from the collection plate and put them in a falcon tube. Keep this tube on ice.

- Measure the concentration of the fractions collected in the falcon tube using the nanodrop.
- Concentrate the sample using a vivaspin turbo tube 10'000 MWCO.
- Do a gel filtration and collect the flowthrough into collecting fractions.
- Run a gel to determine which fractions contain the protein.
- Pull the right fractions into a 2mL Eppendorf tube.
- Measure the concentration using ProtParam from Expasy.
- Concentrate the protein to the desired concentration.
- Make aliquots, snap freeze them and store the protein at -80°C.

Measure the concentration using the Bradford Method

This can be used when the amino acid sequence of the protein doesn't contain any Tryptophan. In that case, the nanodrop doesn't work and alternative methods have to be used.

- Start with 25uL of BSA at 2mg/mL and make 2-fold dilutions (6 tubes).
- Start with 5uL of our protein and make 2-fold dilutions as well.
- Add 800uL of H₂O and 1uL of protein from each dilution into spectrophotometer cuvettes.
- Add 200uL Bradford Reagent to each cuvette.
- Make a negative control containing 800uL water and 200uL reagent (no protein).
- Measure the absorbance of the BSA dilutions.
- Compare the absorbance of our protein to the absorbance of BSA to determine the concentration. Make sure the curve is linear.

If the protein weighs 10kDa, then we have 1mM for 12.5mg/mL. This allows us to determine the concentration in μ M of our protein.