

Theoretical and practical course in protein biochemistry, biophysics and structural biology

Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos

Faculdade de Medicina de Ribeirão Preto

20 to 31 March 2023



**UNIVERSITY OF
CAMBRIDGE**

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THE
ROYAL
SOCIETY

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Version: 30/3/23

Timetable (Overview)

Day 1 (Monday 20 March) – arrive by 9:30 am

Welcome

Seminar: Protein expression systems

Lab: Large-scale protein expression and Biochemical calculations

Day 2 (Tuesday 21 March) – arrive by 9:30 am

Seminar: Bioinformatics for protein expression

Lab: Harvest cell cultures, prepare buffers and protein bioinformatics

Day 3 (Wednesday 22 March) – arrive by 9:00 am

Seminar: Protein purification part 1, lysis and affinity chromatography

Lab: Protein affinity purification and dialysis

Day 4 (Thursday 23 March) – arrive by 9:30 am

Seminar: Protein purification part 2, size-exclusion and other chromatography techniques

Lab: Size exclusion chromatography and pouring SDS-PAGE gels

Day 5 (Friday 24 March) – arrive by 9:30 am

Seminar: Protein purification part 3, quality control and protein storage

Lab: SDS-PAGE, storing protein samples and revision

Day 6 (Monday 27 March) – arrive by 9:00 am

Seminar: Biochemistry of proteins interactions

Lab: GST pull-down

Day 7 (Tuesday 28 March) – arrive by 9:00 am

Seminar: Biophysics of protein interactions: measuring affinities

Lab: Surface plasmon resonance (Biacore)

Day 8 (Wednesday 29 March) – arrive by 10:00 am

Workshop: Surface plasmon resonance data interpretation

Seminar: Looking at protein structures

Computer lab: Visualising proteins using ChimeraX

Day 9 (Thursday 30 March) – arrive by 10:00 am

Seminar: Protein structure determination

Computer lab: Visualising proteins using Coot

Day 10 (Friday 31 March) – arrive by 10:00 am

Seminar: Protein structure prediction and AlphaFold

Computer lab: Structure prediction

Wrap-up

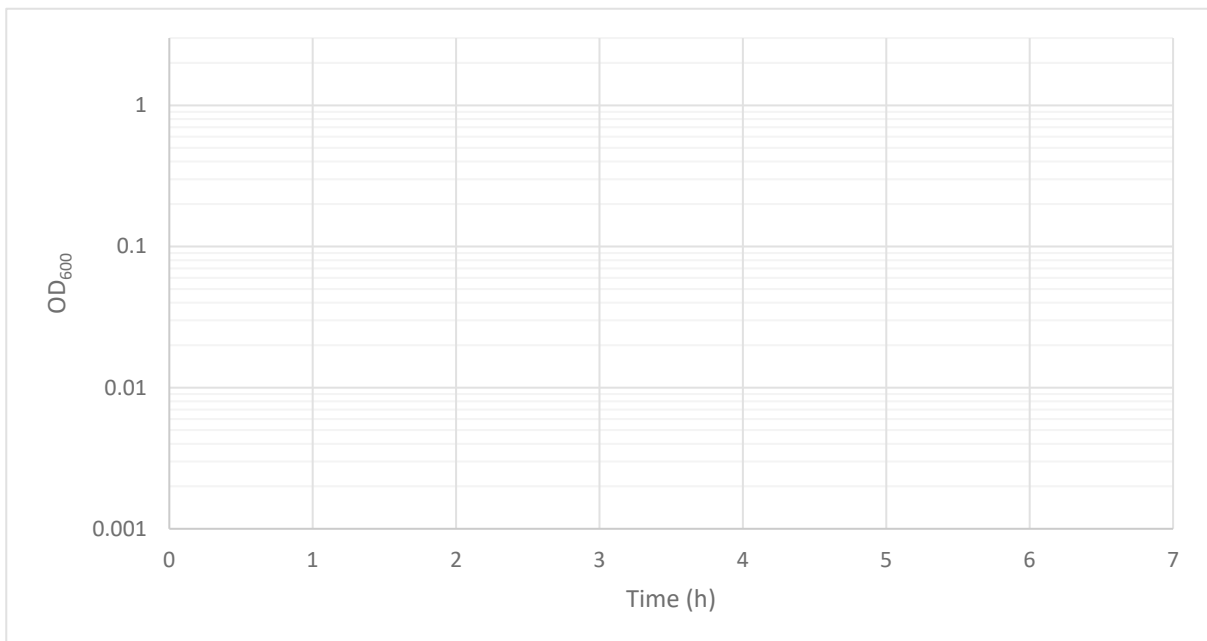
Day 1 – Large-scale protein expression and biochemical calculations

Large-scale protein expression

Today we are performing the grow-up of the bacteria, transformed with the plasmid encoding your protein of interest (a fluorescent protein or the anti-GFP nanobody).

Last week the demonstrators performed the bacterial transformation ([Protocol 1](#)) and they did an overnight starter culture ([Protocol 2, Day 1](#)). Today you need to monitor the growth of the bacteria by measuring the optical density at 600 nm (OD_{600}) and inducing protein expression when the cells are in mid-log phase (OD_{600} is between 0.8 to 1.2; [Protocol 2, Day 2](#)).

Time (h)	OD_{600}



Biochemical calculations

Below are some 'standard' biochemical calculations that you need to be able to perform when working in a protein chemistry lab. You will need some of these numbers for the experiments later in the week, so **check your answers with a demonstrator** before finishing for the day.

Useful equations:

Henderson-Hasselbalch equation: $\text{pH} = \text{pKa} + \log_{10}([\text{base}]/[\text{acid}])$

Relative centrifugal force (RCF, $\times g$): $\text{RCF} = \text{RPM}^2 \times r \times 1.118 \times 10^{-5}$

Where RPM = revolutions per minute *and* r = radius of centrifuge (in cm)

Beer-Lambert law: $A = E_{0.1\%} \times c \times l$

Where A = absorbance, $E_{0.1\%}$ = extinction coefficient (in $(\text{mg}/\text{mL})^{-1}\text{cm}^{-1}$) *and* l = path length (in cm)

Question 1:

You are growing a 100 mL culture of bacteria and you will induce expression with IPTG. Your IPTG stock is at 2000 \times concentration. How much volume of IPTG must you add to induce the culture with 1 \times IPTG?

Question 2:

How much of the following stock solutions will you need to make 1 L (1000 mL) of dialysis buffer (20 mM HEPES pH 7.5, 150 mM NaCl)?

- 1 M HEPES pH 7.5
- 5 M NaCl
- MilliQ water

Question 3:

How much of the following solutions will you need to make 200 mL of NiNTA wash buffer (20 mM HEPES pH 7.5, 20 mM imidazole pH 7.5, 500 mM NaCl)?

- 1 M HEPES pH 7.5
- 2 M imidazole pH 7.5
- 5 M NaCl
- MilliQ water

Question 4:

You want to make a 5 mL solution of 50 mM reduced L-glutathione (GSH). The molecular weight of GSH is 307.32 g/mol. How much GSH powder will you need to weigh out?

Question 5:

You're making a cell lysis buffer by adding 200 μL 10% TWEEN-20 and 40 μL 0.5 M MgCl_2 to 40 mL of NiNTA wash buffer. What is the final concentration of TWEEN-20 and MgCl_2 in the lysis buffer?

Question 6:

You're pouring a NiNTA column. The resin is supplied as a 50% slurry in 20% ethanol. How much resin do you need to pour out to make a 1 mL NiNTA column?

Question 7:

You need to centrifuge your protein at 14,000 $\times g$ for 10 min. The radius of your centrifuge rotor is 8.2 cm, what speed do you need to set your centrifuge to (in RPM).

Question 8:

The molecular weight of water is 18.02 g/mol. What is the molarity of pure water?

Question 9:

The density of 2-mercaptoethanol is 1.11 g/mL and its molecular weight is 78.13 g/mol. What is the molarity of pure (100% v/v) 2-mercaptoethanol?

Question 10:

You measure the absorbance at 280 nm of your protein solution to be 1.6. The protein has an extinction coefficient ($E_{0.1\%}$) of 1.2 and the path length of your spectrophotometer is 1 cm. What is the concentration of your protein in mg/mL?

The protein has a molecular weight of 26 kDa. What is the molarity of the protein sample (in μM)?

Question 11:

You have a stock of concentrated hydrochloric acid. The bottle says that it is a 36% w/v HCl solution and that the density of the solution is 1.179 kg/L. The molecular weight of HCl is 36.46 g/mol. What is the molarity of your hydrochloric acid?

Question 12:

You want to make 1 L of a 1 M stock of Tris pH 7.5 (at 20°C). The pKa of Tris is 8.07 and you have both Tris.HCl (Tris acid; $M_w = 157.60$ g/mol) and Tris base ($M_w = 121.14$ g/mol). How much Tris acid and Tris base should you weight out to make a pH 7.5 stock buffer?

Day 2 – Harvesting cells, preparing buffers and protein bioinformatics

Protein expression

You will need to harvest your cell pellet ([Protocol 2, Day 3](#)) and store it at -80°C for processing later in the week. Make sure you label your tube well (or perhaps you won't need to?!).

You will also need to decontaminate your flasks – follow the instructions from the demonstrators.

Preparing buffers

Everyone will need to make the following buffers:

- 200 mL of **wash buffer** ([Protocol 3](#) or [Protocol 4](#), as appropriate for your protein)
- 1 L of **dialysis buffer** ([Protocol 5](#))

And if you're purifying a His-tagged protein you'll also need to prepare:

- 50 mL **elution buffer** ([Protocol 3](#))

All buffers will need to be made using MilliQ water and 0.2 µm filtered. **Check your calculations with a demonstrator** before starting to make the buffers.

Protein bioinformatics

The vector maps for the protein expression plasmids can be downloaded from the course website (<http://www.atomicvirology.path.cam.ac.uk/brazil>). Using the bioinformatics tools that you learnt about in the seminar (e.g. ApE and ProtParam), note down the following details about your protein of interest:

Number of amino acids:

Molecular weight:

Theoretical isoelectric point (pI):

E_{0.1%} (assuming all cysteines are reduced):

Day 3 – Protein affinity purification and dialysis

Protein affinity purification

This is the big day – time to purify your protein. Notes are as follows:

- Follow [Protocol 3](#) if you're purifying a His-tagged protein or [Protocol 4](#) if your protein is GST tagged
- You will perform the cell lysis in the Falcon tube containing your pellet – you can make up the lysis buffer in a measuring cylinder and then just pour it into the Falcon tube
- Tell a demonstrator when your cell pellet is resuspended in lysis buffer and they will help you use the sonicator
- Use some MilliQ water to check that your Econo-Column flows well before pouring your resin into it – if it doesn't pour well contact a demonstrator
- Make sure that you keep your cell lysate and wash buffer cold (on ice) until you've performed the affinity purification and washed the resin (i.e. until you're ready to elute the protein off the resin)
- Once you have performed the elution, discuss with a demonstrator which fractions to pool and use for dialysis
- Store all your SDS-PAGE samples in a small, labelled bag at -20°C ready for SDS-PAGE

Dialysis

- Pool your eluted fractions ready for dialysis ([Protocol 5](#))
- Ask a demonstrator for dialysis tubing and make sure you are careful when pouring your protein into the tubing (do this above a beaker to contain and recover any spills)
- We will do all the dialysis together in on big beaker, so once you have your sample ready hand it to the demonstrator along with your dialysis buffer
 - o The demonstrators will swap the dialysis over to the second beaker of buffer tomorrow morning

Cleaning and storing affinity resin

- Follow the relevant protocol for your resin, [Protocol 3](#) if you're using NiNTA or [Protocol 4](#) if you're using GSH resin
- These resins are *expensive* and we want to get multiple uses out of them, so it's important to clean them immediately after your dialysis is set up
- 6 M guanidine hydrochloride is a potent denaturant so be sure to wear eye protection when working with it
- When you have finished the cleaning, contact a demonstrator who will collect the cleaned affinity resin

Day 4 - Size exclusion chromatography and pouring SDS-PAGE gels

Size-exclusion chromatography (SEC)

The demonstrators will have equilibrated the SEC column ready for you to inject your protein ([Protocol 6](#)). We can only inject one protein at a time, so different groups will run their proteins throughout the day. You will need to recover your protein from the dialysis tubing into 2 x 2 mL microcentrifuge tubes and centrifuge it for 10 min at $\geq 14,000\times g$ before you inject the protein onto the AKTA. For the sake of speed we won't be concentrating the dialysed proteins before injecting onto the AKTA – this might lower our yield a bit but don't worry, we will have plenty of protein for the experiments next week.

Once your SEC run has finished, discuss with the demonstrator which fractions are likely to contain your protein based on the elution profile. Remove and store at 4°C the fraction tubes that are likely to contain your protein.

Pouring SDS-PAGE gels

The recipe provided in [Protocol 7](#) is for two SDS-PAGE gels. We will use one tomorrow and the other next week, so we will want to store them at 4°C in cling film. Mark which gel is yours with a marker pen. We will use a 15-well comb when pouring the gels. Remember that unpolymerized acrylamide is highly toxic so treat it with care.

Day 5 – SDS-PAGE, storing protein samples and revision

Preparing SDS-PAGE samples

You have 15 lanes on your gel and will want to use one of these for molecular weight standards. That means you can choose up to 9 fractions from your SEC elution to run on the gel.

Make up your SDS-PAGE samples in PCR tubes as follows:

Sample name	Sample (μL)	MilliQ water (μL)	5 \times SDS-PAGE loading buffer (μL)
Pre-induction bacteria	5	15	5
Post-induction bacteria	5	15	5
Cleared lysate	1	19	5
Affinity resin flow-through	1	19	5
Pooled affinity resin eluate	1	19	5
SEC fractions	5	15	5

Load 2.5 μL of the molecular weight standards and 9 μL per well of your boiled gel samples then run the gel as detailed in [Protocol 7](#).

Storing samples

Once your gel has sufficiently destained, discuss with a demonstrator which samples to pool. Pre-rinse an Amicon concentrator as detailed in [Protocol 6 \(Prepare sample\)](#) and pool the desired fractions into the concentrator. Concentrate to ~ 1 mL, move it from the concentrator into a microcentrifuge tube and measure the A_{280} of your protein sample. Calculate the concentration of your protein using the Beer-Lambert law, write this on the microcentrifuge tube and give this number to the demonstrators. Store your purified protein at 4°C.

Revision and homework

This has been a busy week, so once your protein sample is stored at 4°C you're welcome to stay and ask the demonstrators questions about the experiments done this week, or to leave so you can do your own self-directed revision.

However, you have **one essential piece of homework**. You need to work out how much of your purified protein you would need to dilute in buffer to get 1 mL at 10 μM protein.

Day 6 – GST pull-down

Equilibrate resin and start bait incubation: before lunch

Each group will equilibrate enough resin for four pull-down experiments. You will perform pull-downs as follows:

1. 2.5 μM GST-tagged anti-GFP nanobody (bait) + 10 μM EGFP (prey)
2. 2.5 μM GST-tagged anti-GFP nanobody (bait) + 10 μM other fluorescent protein* (prey)
3. 2.5 μM GST-tagged control protein (bait) + 10 μM EGFP (prey)
4. 2.5 μM GST-tagged control protein (bait) + 10 μM other fluorescent protein* (prey)

* Either mTurquoise2, Venus or mCherry - you choose!

The demonstrators will distribute 1 mL of each GST-tagged bait protein to each group. Prepare the resin and start the incubation as described in [Protocol 8](#) and leave your bait incubating with the prey over lunch. Make sure you save some of your bait proteins for making the SDS-PAGE samples.

Complete GST pull-down and boil SDS-PAGE samples: after lunch

Before removing your bait and washing your resin, make up 1 mL of each prey protein at 10 μM concentration (use the buffer in [Protocol 8](#) and the calculation you did as homework over the weekend). Make sure you save some of your prey proteins for making the SDS-PAGE samples.

While the beads are incubating with the prey make up your GSH elution buffer and prepare the tubes with 5 \times SDS-PAGE loading buffer, ready to add the eluted protein.

Once you have eluted your protein, make all your SDS-PAGE samples and freeze them at -20°C (no need to boil today, we'll do that tomorrow when we run the gel).

Day 7 – SDS-PAGE of GST pull-down and surface plasmon resonance

GST pull-down SDS-PAGE: before lunch

Boil your SDS-PAGE samples from yesterday and load them onto an SDS-PAGE gel. Don't forget to also load some molecular weight standards. Set the gel to run over lunch – we can run it at a lower voltage to make sure it doesn't run too far!

GST pull-down SDS-PAGE: after lunch

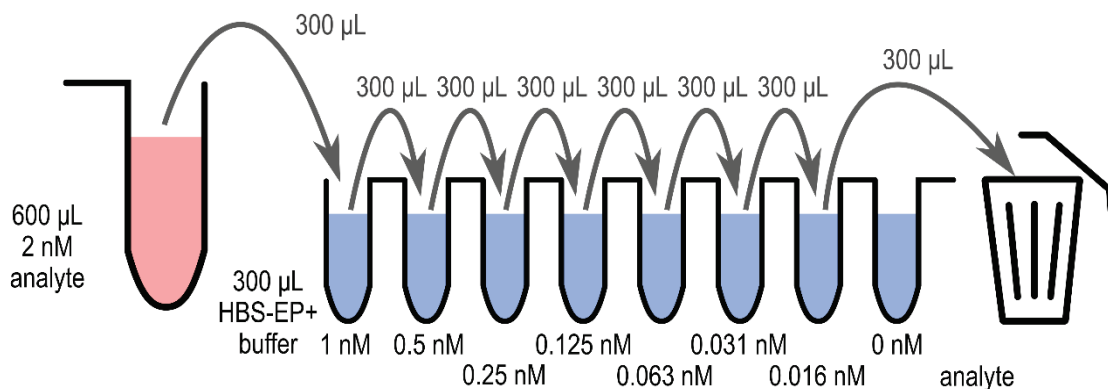
Stain and destain your SDS-PAGE samples. Compare your results with the other groups. Which fluorescent proteins bind to the anti-GFP nanobody?

Surface plasmon resonance (Biacore)

The demonstrators have already prepared a CM5 surface with the GST-tagged anti-GFP nanobody as the ligand. We need you to prepare the analyte.

We will do a two-fold serial dilution of analyte (fluorescent protein) from 2 nM downwards (that's right – 2 nM! You don't need much protein for Biacore!). Make 600 μL of your fluorescent protein in the HBS-EP+ buffer that will be supplied. You won't be able to make this big a dilution all in one go, so use multiple dilutions of no more than 1 in 100 (10 μL per 1 mL) to make the relevant intermediate stocks. Once the analyte solution is prepared, give it a 'hard spin' ($\geq 14,000\times g$ for 10 min) and transfer the protein to a fresh tube to ensure that we don't have any particulate matter in the sample.

Use your 2 nM analyte solution to perform the serial dilution as follows:



You will need to prepare 7 microcentrifuge tubes with 300 μL HBS-EP+ each before starting the serial dilution. Make sure that you mix the sample well at each step of the serial dilution (I generally pipette up and down 10 times slowly).

Once you have prepared your analyte you will need to transfer 280 μL of each dilution to a 7 mm tube ready for use on the Biacore. Label the sides of the tubes before you add the analyte dilutions and make sure that you don't have any bubbles at the bottom of the tube! Cap each tube with an orange lid. The demonstrators will take everyone over to the Biacore in small groups so that you can see how the instrument works and how to set up an experiment.

Days 8, 9 and 10 – Visualising proteins and structure prediction

These practical sessions will be held in the computer lab. You will need to make sure you bring along a laptop and an external mouse for these days. All the details of the activities can be found on the course website (<http://www.atomicvirology.path.cam.ac.uk/brazil>).

Protocol 1: Bacterial transformation

Reagents:

- Competent *E.coli* cells
- Growth medium (2×TY, SOC or SOB) with no antibiotic
- Agar plates (2×TY or LB) with antibiotic
 - o Most plasmids will confer Ampicillin resistance (100 µg/mL) or kanamycin resistance (50 µg/mL). If you are transforming into bacterial with a pLysS or pLysE plasmid you will also require chloramphenicol (25 µg/mL)¹

Protocol

1. Thaw 50 µL of competent cells on ice and immediately add 1 µL (≥ 1 ng) of purified plasmid DNA (purified using miniprep kit or similar)²
2. Incubate on ice for 30 min
 - a. During this incubation you can start pre-warming your agar plates @ 37°C
3. Heat shock in a 42°C water bath for 30s
4. Recover on ice for 2 min
5. Add 250 µl growth medium **with no antibiotics**
6. Resuscitate bacteria by growing at 37°C in a shaking incubator for 1 hour
7. Plate 50 µl cells onto agar plate containing the appropriate antibiotic(s)³
8. Incubate the plate at 37°C overnight in a warm room or over the weekend at room temperature

Notes:

- For transformations with ampicillin resistance only you can skip the heat-shock and recovery step and just plate the cells onto pre-warmed agar plates after the incubation on ice
 - o And if you're in a hurry you can cut the incubation down to 5 min, but transformation efficiency may drop

¹ Make antibiotic stocks at 1000× (e.g. 100 mg/mL ampicillin). Note that chloramphenicol stocks should be prepared up in ethanol as it is not soluble in water.

² For newly ligated plasmid constructs, following cloning and ligation, add 3 µl plasmid DNA to the cells

³ For newly ligated plasmid constructs plate all cells

Protocol 2: Large-scale bacterial protein expression

Reagents:

- Agar plates containing bacterial cells (BL21 pLysS) transformed with your expression plasmid
- Growth medium (2×TY)
- Selection antibiotics
 - o Generally ampicillin (100 mg/mL for 1000× stock) or kanamycin (50 mg/mL for 1000× stock) plus chloramphenicol (25 mg/mL in ethanol for 1000× stock)
- IPTG (0.8 M for 2000× stock)
- Cuvettes for measuring OD₆₀₀

Protocol

Day 1:

1. Prepare two 5 mL 2×TY cultures with appropriate antibiotics at 1× concentration (e.g. 100 µg/mL ampicillin and 25 µg/mL chloramphenicol) and inoculate each with a single bacterial colony from your plates
2. Place starter cultures on a shaking platform at 220 rpm, 37°C and grow overnight

Day 2:

3. Prepare medium for the large-scale cultures by adding antibiotic at 0.5× concentration (e.g. 50 µg/mL ampicillin and 12.5 µg/mL chloramphenicol)
4. Before inoculating large-scale cultures, take a sample of medium and place in a sterile tube to use as your blank for OD₆₀₀ measurements.
5. Pool the overnight starter cultures¹ and use to inoculate the large-scale cultures with 1/1000 volume of starter culture (e.g. 100 µL per 100 mL of culture)
6. Place large-scale cultures on a shaking platform at 220 rpm, 37°C and incubate
7. From 2 hours post-inoculation, take OD₆₀₀ measurements every hour to track bacterial growth
8. When OD₆₀₀ reaches 0.8–1.2, drop growth temperature to 22°C and incubate for 30 min
9. *Take a small sample of culture (100 µL) for SDS-PAGE (pre-induction)*
10. Induce cells by adding 1× concentration of IPTG (e.g. 0.4 mM final [IPTG])
11. Continue incubating cells on shaking platform at 220 rpm, 22°C and grow overnight

Day 3:

12. *Take a small sample of culture (100 µL) for SDS-PAGE (post-induction)*
13. Harvest cells by centrifugation at 5000×g for 15 min
14. Discard supernatant², transfer pellet to 50 mL Falcon tube and store at -70°C

Notes:

- You can do the grow-up in one day (works best for T7 express pLysY cells from NEB) by following the above protocol with the following modifications:
 - o Step 2: Inoculate your starter cultures first thing in the morning and grow at 37°C
 - o Step 5: When cultures are turbid (~2–4 hrs), pool and inoculate large-scale cultures with 1/200 volume (e.g. 500 µL per 100 mL of culture)
- For some proteins you obtain a higher yield by inducing for 2–4 hours at 37°C, rather than dropping the temperature to 22°C and inducing overnight

¹ Assuming both overnight cultures are turbid, if not just use whichever one worked

² Follow local safety guidelines for decontamination of bacterial supernatant

Protocol 3: NiNTA affinity chromatography (His-tagged proteins)

Reagents:

- MilliQ water¹
- 200 mL **wash buffer** (20 mM HEPES pH 7.5, 20 mM imidazole pH 7.5, 500 mM NaCl)
- 50 mL **elution buffer** (20 mM HEPES pH 7.5, 250 mM imidazole pH 7.5, 500 mM NaCl)
- 10% v/v TWEEN-20
- 2-mercaptoethanol
- 0.5 M MgCl₂
- DNase (e.g. Bovine pancreas DNaseI, Merck #D4527)
- EDTA-free protease inhibitor cocktail (Merck P8849)
- Hen egg-white lysozyme (Merck #L4919 or similar)
- NiNTA agarose resin (Qiagen #30230)²
- BioRad Econo-Columns (10 x 1 cm; #7371012) or similar
- Bradford reagent (Bio-Rad Protein Assay Dye #5000006)
- Clear-bottom microtitre plate (Greiner 655101 or similar)

Protocol

1. Prepare 40 mL lysis buffer by adding 200 µL 10% TWEEN-20, 4 µL 2-mercaptoethanol, 40 µL 0.5 M MgCl₂, 40 U DNase, 40 µL protease inhibitor cocktail and ~5 mg hen egg white lysozyme to 40 mL **wash buffer**³
2. Resuspend cell pellet in lysis buffer at 4°C by gently vortexing and/or pipetting up and down⁴
3. Lyse cells on ice by disrupting with a sonicator at 50% amplitude for 5×30s bursts, allowing cells to recover for 30s between bursts
4. Transfer lysate to a centrifuge tube and clarify by centrifuging at ≥20,000×g for 30 min at 4°C
5. While lysate is clearing, pour 2 mL of NiNTA (50% slurry) into Econo-Column for a 1 mL column volume (c.v.)
 - a. Wash NiNTA resin with 10 c.v. MilliQ water
 - b. Wash NiNTA resin with 20 c.v **wash buffer**
 - c. Stop column and leave resin sitting in ~1 c.v. **wash buffer**
6. Transfer cleared lysate to 50 mL Falcon tubes
7. *Take a small sample (10 µL) of the cleared lysate for SDS-PAGE*
8. Add washed NiNTA resin to cleared lysate and incubate at 4°C on a rolling bed / rotating wheel for 60 min⁵
9. Apply cleared lysate plus NiNTA resin to Econo-Column, collecting flow-through
10. *Take a small sample (10 µL) of the NiNTA flow through for SDS-PAGE*
11. Wash resin with 20 c.v. of **wash buffer** at 4°C
12. Add 200 µL / well of 1× Bradford reagent to 10 wells of the clear microtitre plate
13. Prepare 1.5 mL microcentrifuge tubes (10) to collect elution fractions
14. Elute the protein using **elution buffer** as 900 µL fractions
 - a. For each, qualitatively measure the amount of eluted protein by taking 5 µL, adding

¹ All buffers and other reagents for protein chemistry must be prepared in MilliQ water

² Other Ni agarose resins work well too, follow their guidance for [imidazole] in wash and elution buffers

³ Scale up for larger cultures – I use 150 mL of lysis buffer for a 1–2 L culture

⁴ For larger cultures, transfer lysate to a beaker and use a magnetic stirrer to resuspend

⁵ Do not use a magnetic stirrer to incubate resin with cleared lysate, the stirrer will crush the beads and reduce the column performance

to the Bradford reagent in the microtitre plate and mixing vigorously

15. Pool fractions with substantial protein (fractions that turn the Bradford reagent blue)

16. **Either** proceed directly to size exclusion chromatography **or** dialyse to remove imidazole¹

Cleaning and regenerating NiNTA resin

Reagents:

- MilliQ water
- NiNTA cleaning solution (6 M guanidine hydrochloride, 0.2 M acetic acid)
- 20% (v/v) ethanol

Protocol:

1. Wash resin with 2 c.v. cleaning solution
2. Wash resin with 20 c.v. MilliQ water
3. Wash resin with 20 c.v. 20% ethanol
4. Resuspend resin in 20% ethanol and transfer to Falcon tube
5. Store at 4°C for re-use (resin can be used at least 5 times)
 - a. For more stringent cleaning, including stripping and re-loading with Ni²⁺, consult the product handbook

¹ Imidazole is a chaotropic agent that makes proteins very unhappy, so you want to remove it as fast as possible

Protocol 4: GSH affinity chromatography (GST-tagged proteins)

Reagents:

- MilliQ water¹
- 200 mL **wash buffer** (20 mM HEPES pH 7.5, 300 mM NaCl, 1 mM dithiothreitol)
- Reduced L-glutathione (Merck #G4251 or similar)
- 5N sodium hydroxide
- 10% v/v TWEEN-20
- 2-mercaptoethanol
- 0.5 M MgCl₂
- DNase (e.g. Bovine pancreas DNaseI, Merck #D4527)
- EDTA-free protease inhibitor cocktail (Merck P8849)
- Hen egg-white lysozyme (Merck #L4919 or similar)
- Glutathione Sepharose 4B (GSH) resin (Cytiva #17-0756-05)
- BioRad Econo-Columns (10 x 1 cm; #7371012) or similar
- Bradford reagent (Bio-Rad Protein Assay Dye #5000006)
- Clear-bottom microtitre plate (Greiner 655101 or similar)

Protocol

1. Prepare 40 mL lysis buffer by adding 200 µL 10% TWEEN-20, 4 µL 2-mercaptoethanol, 40 µL 0.5 M MgCl₂, 40 U DNase, 40 µL protease inhibitor cocktail and ~5 mg hen egg white lysozyme to 40 mL **wash buffer**²
2. Resuspend cell pellet in lysis buffer at 4°C by vortexing and/or pipetting up and down³
3. Lyse cells on ice by disrupting with a sonicator at 50% amplitude for 5×30s bursts, allowing cells to recover for 30s between bursts
4. Transfer lysate to a centrifuge tube and clarify by centrifuging at ≥20,000×g for 30 min at 4°C
5. While lysate is clearing, pour 2 mL of GSH resin (50% slurry) into Econo-Column for a 1 mL column volume (c.v.)
 - a. Wash GSH resin with 10 c.v. MilliQ water
 - b. Wash GSH resin with 20 c.v **wash buffer**
 - c. Stop column and leave resin sitting in ~1 c.v. **wash buffer**
6. Transfer cleared lysate to 50 mL Falcon tubes
7. *Take a small sample (10 µL) of the cleared lysate for SDS-PAGE*
8. Add washed GSH resin to cleared lysate and incubate at 4°C on a rolling bed / rotating wheel for 60 min⁴
9. Apply cleared lysate plus GSH resin to Econo-Column, collecting flow-through
10. *Take a small sample (10 µL) of the GSH flow through for SDS-PAGE*
11. Wash resin with 20 c.v. of **wash buffer** at 4°C
12. While resin is washing, prepare the **elution buffer**:
 - a. Resuspend 153.66 mg reduced L-glutathione in 20 mL wash buffer for 25 mM glutathione
 - b. **Important:** Add ~110 µL 5N sodium hydroxide to the elution buffer to neutralise,

¹ All buffers and other reagents for protein chemistry must be prepared in MilliQ water and 0.2 µm filtered

² Scale up for larger cultures – I use 150 mL of lysis buffer for a 1–2 L culture

³ For larger cultures, transfer lysate to a beaker and use a magnetic stirrer to resuspend

⁴ Do not use a magnetic stirrer to incubate resin with cleared lysate, the stirrer will crush the beads and reduce the column performance

because reduced glutathione is highly acidic

- c. Measure pH of the elution buffer with a pH strip or pH meter to ensure pH ~7.5
13. Add 200 μ L / well of 1 \times Bradford reagent to 10 wells of the clear microtitre plate
14. Prepare 1.5 mL microcentrifuge tubes (10) to collect elution fractions
15. Elute the protein using **elution buffer** as 900 μ L fractions
 - a. The GST:GSH interaction has a very slow off rate, so incubate each fraction with the resin for 2 min before allowing it to flow through
 - b. For each, qualitatively measure the amount of eluted protein by taking 5 μ L, adding to the Bradford reagent in the microtitre plate and mixing vigorously
16. Pool fractions with substantial protein (fractions that turn the Bradford reagent blue)
17. **Either** proceed directly to size exclusion chromatography **or** dialyse to remove reduced glutathione

Cleaning and regenerating GSH resin

Reagents:

- MilliQ water
- GSH cleaning solution (6 M guanidine hydrochloride)
- 20% (v/v) ethanol

Protocol:

1. Wash resin with 2 c.v. cleaning solution
2. Wash resin with 20 c.v. MilliQ water
3. Wash resin with 20 c.v. 20% ethanol
4. Resuspend resin in 20% ethanol and transfer to Falcon tube
5. Store at 4°C for re-use (resin can be used up to 5 times¹)

¹ *E. coli* encode a protease that will slowly cleave the reduced glutathione from the GSH resin over time, so yields will fall if the resin is re-used too many times

Protocol 5: Protein dialysis

Reagents:

- Dialysis buffer (20 mM HEPES pH 7.5, 150 mM NaCl)
- 3.5 kDa MWCO Snakeskin dialysis tubing (ThermoFisher #68035)
- Dialysis clips, large beaker and magnetic stirrer

Protocol:

1. Cut an appropriate length of dialysis tubing
 - a. Tubing can accommodate ~3.5 mL per cm, but add significant extra length for closing the tubing at each end
2. Close one end of the dialysis tubing (either with clips or tie a knot)
3. Pour protein solution into the dialysis tubing
 - a. Pour over a beaker or similar so you can rescue any spills
4. Close other end of the dialysis tubing, leaving a small air gap in the tubing so it will float
5. Place dialysis tubing into a beaker with 100-fold excess of dialysis buffer (e.g. dialyse 10 mL of protein solution in 1L of buffer)
6. Incubate dialysis buffer either at room temperature or 4°C, stirring the dialysis solution with a magnetic stirrer, for at least 4 hours
7. Transfer dialysis tubing into a fresh beaker of dialysis buffer either at room temperature or 4°C overnight, stirring the dialysis solution with a magnetic stirrer, for at least 4 hours

Protocol 6: Size-exclusion chromatography (SEC) using an AKTA Start

Reagents:

It is **essential** that all buffers have been 0.2 µm filtered and are free of particulates

- MilliQ water
- **SEC buffer** (20 mM HEPES pH 7.5, 150 mM NaCl)
- SEC column (HiLoad Superdex 75 16/600 pg¹)
- 5 mL polypropylene fraction collection tubes (Greiner #115201 or similar)
- 15 mL 10 kDa MWCO Amicon concentrator (Millipore UFC901024)
- **SEC cleaning buffer** (0.2 M sodium hydroxide)
- **SEC storage buffer** (20% (v/v) ethanol, 0.2 M sodium acetate)²

Protocol:

Equilibrate column:

1. Place the pump tubing into the peristaltic pump and clip closed
2. Place inlet line A into MilliQ and perform a pump wash:
 - a. Method Run > Pre-Defined > System Preparation > Pump Wash
 - i. PumpWashInlet = A
 - ii. PumpWashVolume = 10 mL
 - iii. No result
3. Start a Manual Run at 0.5 mL/min and rinse the system with MilliQ water
 - a. Ensure you click the “Wash valve” to direct flow through the UV and conductivity, and wait for these to stabilise
4. Connect the column using ‘drop-to-drop’ method, dry connectors and then stop flow
5. Place inlet A into SEC buffer, inlet B into MilliQ water, and run equilibration program (~4 hrs)
 - a. Method Run > User Defined > Shared Methods > Sxx 16_600 > Eq from EtOH
6. While equilibration is running, fill Frac30 fraction collector carousel with 5 mL tubes

Prepare sample:

1. Pre-rinse concentrator by adding 5 mL SEC buffer and centrifuging at 16°C³, 4000×g for 1 min, then discard buffer and flow-through
2. Add pooled/dialysed protein sample to concentrator and centrifuge at 16°C, 4000×g for 5 min
3. Mix the sample in the concentrator using a pipette to prevent over-concentration at filtration membrane
 - a. If concentrating > 15 mL then top up concentrator and save flow-through in a Falcon tube
4. Repeat steps 2 and 3 until sample volume is ~3.5 mL
5. Transfer sample to two 2 mL microcentrifuge tubes and centrifuge at 16°C, 14,000×g for 10 min in a benchtop centrifuge before proceeding immediately to injecting sample and starting the SEC
6. *Take a small sample (10 µL) of your concentrated affinity column eluate for SDS-PAGE*

¹ For proteins with $M_w \leq \sim 60$ kDa, for larger proteins use a Superdex 200 or Superose 6 column

² For S200 and Superose 6 columns, storage buffer is 20% ethanol

³ Solutions concentrate much more slowly at 4°C, so if a protein needs to be kept cold set the centrifuge to 8°C and if it's stable at room temperature (most proteins are) then set the centrifuge to 16°C, to keep the sample from overheating

Inject sample and perform SEC:

1. Make sure:
 - a. AKTA start is not running
 - b. Fractionation dispenser is above the inner ring and Frac30 is filled with tubes
 - c. 5 mL injection loop is connected
 - d. Injection valve is on 'Load Sample'
2. Rinse loop with >2 loop volumes (e.g. 10 mL) of **SEC buffer**
3. Rinse loop with another 2 loop volumes of **SEC buffer – do not remove syringe**
4. Switch injection valve to 'Inject to column'
5. Remove buffer syringe, aspirate sample into a fresh 5 mL syringe and then place syringe with sample into the injection port – **do not inject sample**
6. Switch injection valve is on 'Load Sample'
7. Inject sample – **do not remove syringe**
8. Start run
 - a. Method Run > User Defined > Shared Methods > Sxx 16_600 > Run Sxx 16_600
 - b. Remember to save the results somewhere sensible
9. When prompted, switch injection valve to 'Inject to column' and click 'Confirm and Continue'
10. **Set a timer for 10 min and wait**
 - a. Use this time to make sure you don't have any leaks in the system
11. When prompted, switch injection valve to 'Load Sample' and click 'Confirm and Continue'
 - a. It is **vitaly important** you do this without delay, as otherwise you could lose your sample
12. Set a timer for 75 min to swap fraction dispenser from inner to outer ring
13. When prompted, swap fraction dispenser from inner to outer ring and click 'Confirm and Continue'
14. Wait until SEC finishes and then store samples and/or analyse by SDS-PAGE

Cleaning SEC column:

1. Wash the sample loop with >2 loop volumes of MilliQ water
 - a. And >2 loop volumes of 20% ethanol if not using again for a while
2. Place the sample tube in 0.2 M **SEC cleaning buffer** (>50 mL)
3. Method Run > Pre Defined > System Preparation > Pump Wash
 - a. PumpWashInlet = Sample
 - b. PumpWashVolume = 4 mL
 - c. No Result
4. Place inlet line A into MilliQ water (>400 mL) and line B into **SEC storage buffer** (>200 mL)
5. Method Run > User Defined > Shared Methods > Sxx 16_600 > CIP and store in EtOH (~6 hrs)
6. Remove the column from the AKTA Start, sealing ends appropriately
7. Prime sample inlet in 20% ethanol as in Step 3
8. Unclip the pump tubing from the sample pump

Protocol 7: SDS-PAGE

Reagents:

- MilliQ water
- 5× SDS-PAGE loading buffer (250 mM Tris pH 6.8, 10% w/v SDS, 25% v/v glycerol, 0.02% w/v bromophenol blue, 5% v/v 2-mercaptoethanol)
- Gel pouring ingredients:
 - 30% w/v 37.5:1 acrylamide:bis-acrylamide
 - 10% w/v ammonium persulfate (APS)
 - *N,N,N',N'*-Tetramethylethane-1,2-diamine (TEMED)
 - 10% sodium dodecyl sulfate (SDS)
 - 1.5 M Tris pH 8.8
 - 0.5 M Tris pH 6.5
- SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% w/v SDS, pH 8.3)
- Coomassie stain (0.25% w/v Coomassie brilliant blue R250, 50% v/v ethanol, 10% v/v glacial acetic acid)
- Destain (10% v/v ethanol, 10% v/v glacial acetic acid)
- SDS-PAGE casting stand and running assembly (e.g. BioRad Mini-PROTEAN)
- SDS-PAGE standards (e.g. BioRad Precision Plus Dual Color Standards, #1610374)

Protocol:

Pouring gel:

1. Assemble gel casting stand with clean gel plates
2. Prepare resolving gel in Falcon tube using the recipe below, adding the APS to initiate the polymerisation last
3. Swiftly pour the gel between the gel plates
4. Cover gel with a small amount of 100% isopropanol
5. Keep left-over resolving gel in the Falcon tube so you can see when the gel is set

10 mL Resolving gel (2 gels)	9%	10%	12%	15%	18%
MQW (mL)	4.3	4.0	3.3	2.3	1.3
1.5 M Tris.HCl pH 8.8 (mL)	2.5	2.5	2.5	2.5	2.5
30% Acrylamide (mL)	3.0	3.3	4.0	5.0	6.0
10% SDS (μL)	100	100	100	100	100
10% APS (μL)	100	100	100	100	100
TEMED (μL)	10	10	10	10	10

6. Once resolving gel is set, decant off isopropanol, rinse top of gel with deionised water and dry
7. Insert combs half-way (not fully) between top of the gel plates
8. Prepare stacking gel from 4% gel stock, adding APS last to initiate the polymerisation
9. Pipette stacking gel between gel plates and then fully insert comb
10. Keep left-over stacking gel in microcentrifuge tube so you can see when gel is set

2 mL Stacking gel (2 gels)	
4% Stacking gel stock (μL)	2000
10% APS (μL)	10
TEMED (μL)	10

4% Stacking gel stock – can store at 4°C for at least 3 months	
MQW (mL)	60.7
0.5 M Tris.HCl pH 6.8 (mL)	25.0
10% SDS (mL)	1.0
30% Acrylamide (mL)	13.3

Preparing samples, running gel and staining:

1. Add appropriate volume of 5× SDS-PAGE loading buffer to sample for a 1× concentration
 - a. E.g. Add 10 µL 5× SDS-PAGE loading buffer to 40 µL of sample
2. Boil sample at 95°C for 5 min¹
3. While sample is boiling, prepare SDS-PAGE apparatus, fill tank with SDS-PAGE running buffer and then remove comb
 - a. If gel is slightly old, it's sometimes helpful to use a P200 and pipette SDS-PAGE running buffer into each well to ensure that they are free of material
4. Allow sample to cool to room temperature and centrifuge quickly to collect sample in bottom of tube
5. Load standards and samples into gel
6. Assemble gel apparatus, connect to powerpack and perform electrophoresis at 200 V for ~40 min until dye front is at the bottom of the gel
7. Disassemble gel apparatus and plates, and carefully transfer gel into a weigh boat / petri dish with deionised water
8. Decant deionised water and add Coomassie stain to cover gel
 - a. Optional: Heat gel in stain for 1 min in a microwave to speed up the staining process, but make sure stain does not boil
9. Incubate gel for ≥ 15 min on a rocking platform
10. Decant stain and re-use or dispose of according to local safety rules
11. Add destain to cover gel
 - a. Optional: Heat gel in destain for 1 min in a microwave to speed up the staining process, but make sure stain does not boil
12. Incubate gel for ≥ 60 min on a rocking platform
 - a. Optional: Add a tissue (Kimwipe) to destain to absorb stain and accelerate destaining
13. If necessary, repeat destaining
14. Once sufficiently destained, transfer gel to deionised water and image using phone camera or gel dock system

Notes:

- Un-polymerised acrylamide is **highly toxic**, so caution is needed when handling it
- Poured gels can be stored at 4°C for at least one week. To store, wrap in cling film with a moist piece of paper towel
- SDS-PAGE samples can be stored at -20°C but it's generally best to re-boil the sample after thawing

¹ It's generally most convenient to prepare SDS-PAGE samples in thin-walled PCR tubes and boil them in a thermal cycler. I set the programme to actively cool the sample to room temperature after the boil.

Protocol 8: GST pull-down

Reagents:

- **Wash buffer** (20 mM HEPES pH 7.5, 200 mM NaCl, 0.1% v/v TWEEN 20¹, 1 mM DTT, 1 mM EDTA)
- Reduced L-glutathione (Merck #G4251 or similar)
- Elution buffer (wash buffer supplemented with 50 mM reduced L-glutathione – see [Notes](#))
- Glutathione Sepharose 4B (GSH) resin (Cytiva #17-0756-05)
- 5× SDS-PAGE loading buffer (250 mM Tris pH 6.8, 10% w/v SDS, 25% v/v glycerol, 0.02% w/v bromophenol blue, 5% v/v 2-mercaptoethanol)
- Purified **bait** and **prey** proteins

Protocol:

For each sample:

1. Dispense 40 µL of Glutathione Sepharone 4B (ensure fully resuspended) into a 1.5 mL microcentrifuge tube (eppi)
2. Perform 2 washes as follows:
 - a. Add 400 µL of wash buffer to each well
 - b. Mix by gentle inversion for 1–2 min
 - c. Pellet beads by centrifuging at 500×g for 2 min
3. Carefully remove and discard supernatant, being careful not to aspirate beads²
4. Add 2.5 µM **bait protein** (400 µL final volume, 1 nmol/tube) to eppi with GSH beads
 - a. *Keep a sample of the bait protein to run on a gel as input (20 µL sample + 5 µL 5× loading buffer, load 10 µL/well)*
5. Mix by gentle inversion on a rotating wheel for 30-120 min
6. Pellet beads by centrifuging at 500×g for 2 min
7. Carefully remove and discard supernatant, being careful not to aspirate beads
8. Perform 3 washes as follows:
 - a. Add 400 µL of **wash buffer** to each well
 - b. Mix by gentle inversion for 1–2 min
 - c. Pellet beads by centrifuging at 500×g for 2 min
 - d. Carefully remove and discard supernatant, being careful not to aspirate beads
9. Add 10 µM **prey protein** (400 µL final volume, 4 nmol/tube) to eppi with GSH beads
 - a. *Keep a sample of prey protein to run on gel as an input (20 µL sample + 5 µL 5× loading buffer, load 10 µL/well)*
10. Mix by gentle inversion on a rotating wheel for 30-120 min
11. Pellet beads by centrifuging at 500×g for 2 min
12. Carefully remove and discard supernatant, being careful not to aspirate beads
13. Perform 3 washes as follows:
 - a. Add 400 µL of **wash buffer** to each well
 - b. Mix by gentle inversion for 1–2 min
 - c. Pellet beads by centrifuging at 500×g for 2 min
 - d. Carefully remove and discard supernatant, being careful not to aspirate beads
14. Add 40 µL of **elution buffer** to each eppi
15. Mix by gentle inversion for 1–2 min

¹ NP-40 (IPEGAL CA-630) or TWEEN 20 in the buffers help stop the resin clumping.

² Leave a tiny bit of supernatant on the beads. You can also put a P10 tip that has been crushed with a pair of tweezers on the end of a P1000 tip to minimise aspiration of beads throughout this protocol.

16. Pellet beads by centrifuging at $\geq 16,000\times g$ for 5 min
17. While beads are being centrifuged, add 10 μL of 5 \times SDS-PAGE loading buffer to a fresh tube¹
18. Remove eluted protein supernatant and transfer to tube with 5 \times SDS-PAGE
19. Boil sample at 95°C for 5 min
 - a. *Use samples for SDS-PAGE, loading 10 μL per well*

Notes:

- This experiment can be performed at room temperature or 4°C, depending on the optimal stability of your protein (room temperature should generally be fine, and may indeed be better if a conformational change of the bait or prey is required for binding)

To make elution buffer:

- Add 7.7 mg GSH and 5.3 μL 5N sodium hydroxide to 500 μL **wash buffer**
- Check pH using a pH strip and adjust if necessary

¹ It's generally most convenient to prepare SDS-PAGE samples in thin-walled PCR tubes and boil them in a thermal cycler. I set the programme to actively cool the sample to room temperature after the boil.

Appendix: Useful Recipes

NB: MQW = MilliQ water, RT = room temperature

Protein expression and purification:

1000× Ampicillin (96 × 500 µL, 100 mg/mL)

- 5 g Ampicillin
- To 50 mL with MQW
- Aliquot and store @ -20°C

1000× Chloramphenicol (40 mL, 25 mg/mL)

- 1 g Chloramphenicol
- To 40 mL with 100% ethanol
- Store @ -20°C

2000× IPTG (96 × 500 µL, 0.8 M)

- 9.532 g IPTG
- To 50 mL with MQW
- Aliquot and store @ -20°C

400 U / aliquot DNase (96 × 200 µL)

- 40,000 U DNase (Sigma D4527-40KU)
- To 20 mL with MQW
- Aliquot and store @ -20°C

1000× DTT (96 × 500 µL, 1 M)

- 7.7125 g DTT
- To 50 mL with MQW
- Aliquot and store @ -20°C

25 mM GSH (20 mL)

- 153.66 mg reduced L-glutathione
- 105 µL 5N sodium hydroxide
- To 20 mL with buffer

2M HEPES pH 7.5 (100 mL)

- 47.66 g HEPES free acid in MQW
- Adjust pH to 7.5 by dropwise addition of 5 N sodium hydroxide
- MQW to 100 mL
- 0.2 µm filter and store @ 4°C

SDS-PAGE:

5× SDS-PAGE Running Buffer (5L)

- 75 g Tris base
- 360 g Glycine
- 25 g SDS powder (or 250 mL 10% SDS)
- Distilled water to 5L

10% APS

- 1 g APS
- To 10 mL with MQW
- Store @ -20°C in 1 mL aliquots

1.5 M Tris pH 8.8 (500 mL)

- 18.45 g Tris HCl
- 76.95 Tris base
- To 500 mL with MQW
- 0.2 µm filter and store @ RT

0.5 M Tris pH 6.8 (100 mL)

- 7.47 g Tris HCl
- 0.32 g Tris base
- To 100 mL with MQW, 0.2 µm filter

5× SDS PAGE loading buffer (10 mL)

- 5 mL 0.5 M Tris pH 6.8
- 1 g SDS
- 2.5 mL 100% glycerol
- 5 mg Bromophenol Blue
- 2 mL MQW
- Store @ RT
- Add 50 µL 2-mercaptoethanol per 1 mL, just before using

Coomassie stain (500 mL)

- 1.25 g Coomassie Brilliant Blue R250
- 250 mL Ethanol
- 50 mL Glacial acetic acid
- 200 mL RO water

Destain (5 L)

- 500 mL Ethanol
- 500 mL Glacial acetic acid
- 4L RO water

GST pull-downs:

Wash buffer recipe for 12 samples (50 mL)

- 1 mL 1 M Tris pH 7.5
- 2 mL 5M NaCl
- 500 µL 10% v/v TWEEN 20
- 100 µL 0.5 M EDTA pH 8.0
- 50 µL 1 M DTT
- MilliQ water to 50 mL