Aalto University
School of Chemical Engineering

Laboratory course in Biosystems and Biomaterials

Part III – Recombinant antibody production from yeast and analytics

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Outline of experiment

Yeast has been widely used as an expression system for the production of heterologous proteins. A lot of factors involved in the expression and secretion process have been addressed in research, like expression levels, protein folding, vesicular transport, secretion efficiency, and degradation. In eukaryotes secreted proteins are targeted initially to the endoplasmic reticulum (ER). In contrast to prokaryotes, the eukaryotic cell has separate and specialized compartments of which the ER is designed for protein folding and sorting. Protein synthesis occurs in the cytosol but for many proteins, relocation into ER starts already during translation. After passing the ER membrane through the protein translocation complex, the nascent polypeptide encounters multiple aspects that facilitate the protein to achieve its native conformation. The ER harbors an oxidative environment and a diverse set of folding factors, which together enhance the rate in which the protein acquires its structural characteristics dictated by the amino acids sequence. Only correctly folded proteins will exit the ER and be secreted outside the cell.

There are three major categories of folding factors: molecular chaperones, protein disulfide isomerases (PDIs), and peptidyl-prolyl *cis-trans* isomerases (PPIases). The ER has its own set of molecular chaperones that are distinct from cytosolic chaperones albeit their mechanism of action is the same. Chaperones bind to the hydrophobic patches of the polypeptide preventing the protein from aggregation. In the course of their action, chaperones bind and hydrolyze ATP. The hydrolysis and exchange of nucleotide (ADP to ATP) can be facilitated by additional proteins called co-chaperones, which speed up the binding cycle of the corresponding chaperone. PDIs on the other hand catalyze the formation and mixing of disulfide bonds, which form between two cysteine residues. Disulfide bonds are common in secreted proteins as these covalent bonds stabilize the native conformation in the varying extracellular environment. In the case of disulfide bonds, the oxidative environment of ER becomes important as it favors the formation and stabilization of the bonds. PPIases target certain prolines in the polypeptide: the backbone atoms of this amino acid can occur also in *cis*-configuration (naturally around 10 %) instead of the common *trans*-bond. This property is important in some proteins and switching from the *trans* to *cis* is catalyzed by PPIases. After the peptide backbone has achieved *cis*-conformation, the folding protein is freed from the angle strain caused by unfitting backbone conformations.

Although yeast is successfully used to produce and secrete insulin (by Novo Nordisk), other proteins are much more recalcitrant for expression in yeast. One of the most difficult proteins to express in yeast is the full-length antibody.

IgG-antibodies are tetrameric, 150-kDa proteins that are secreted by the plasma cells of the mammalian immune system to fight against disease-causing agents. The subunits of IgG enter the ER separately, in which the complete protein is assembled. The folding of IgG employs all types of folding factors: chaperones, PPIases, and PDIs, which catalyze both the formation of inter- and intrachain disulfide bonds. The light chains and heavy chains do not fold separately but require interaction with each other to complete the process. Assembly of the IgG-tetramer is catalyzed sequentially by the different folding factors as shown in Figure 1.

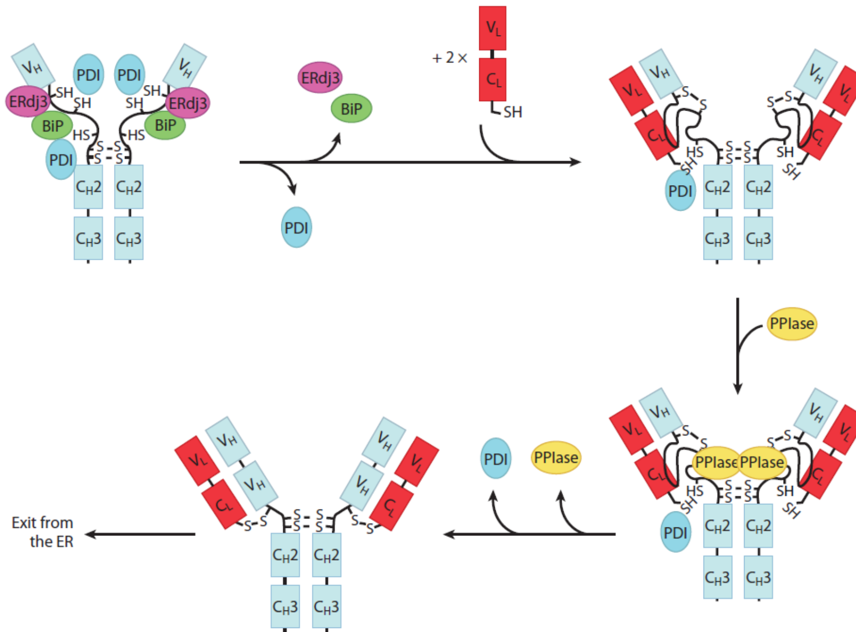


Figure 1: The folding process of IgG. (Braakman and Bulleid 2011).

Expression of selected folding factors as discussed above might be able to improve folding and finally lead to an improved production.

Many of the genes, which are involved in protein folding in the ER are under control of the unfolded protein response (UPR). UPR increases expression of chaperones and other folding factors when it senses accumulation of folding intermediates, misfolded proteins, and protein aggregates. Protein misfolding can be triggered by elevated temperatures, defects in the redox balance, defects in glycosylation and also by overexpression of recombinant proteins which is generally denoted as ER stress. If the sensing mechanism is impaired ER stress can dramatically lower the cellular fitness. Up to 300 genes are under UPR control and their induction aims to lower the folding stress in the ER by increasing the folding efficiency, degrading misfolded proteins and by increasing the cellular capacity to secrete proteins.

In the Part III experiments, the *S. cerevisiae* strain YLK20, derived from the parental strain W303α is used. YLK20 contains two deletions Δ*vps*30 and Δ*alg*3.

Strain name	Genotype
Δ*vps*30 Δ*alg*3 (YLK20)	*leu2*-3,112 *trp1*-1 *can1*-100 *ura3*-1 *ade2*-1 *his3*-11,15 Δ*vps*30::KanMX Δ*alg*3::NatMX

These yeast strains are transformed with plasmids for the overexpression of the antibody (named pAX544) and plasmids for expression of selected genes involved in the protein folding. Plasmid pAX544 carries a *URA3*, the other plasmids a *LEU2* selection marker.

Plasmids containing the ORF for genes used in this experiment	
Plasmid	***Protein expressed***
pJR034	***CRP5*** encodes a peptidyl-prolyl *cis-trans* isomerase (PPIases) and catalyzes the *cis-trans* isomerization of peptide bonds N-terminal to proline residues

pJR001	***PDI1*** encodes a protein disulfide isomerase (PDI) important for disulfide-bond formation in the ER
pIRE1	***IRE1*** encodes the protein which acts as an ER stress sensor which upon activation triggers the unfolded protein response (UPR).
pKK1	***HAC1*** encodes the transcriptional activator responsible for the induction of UPR
pEK7	**none,** *empty control plasmid*

For more information about yeast genes and other information regarding *Saccharomyces cerevisiae* see also *http://www.yeastgenome.org/*

References

Feige *et al*. How antibodies fold. Trends Biochem Sci 35:189-198.
Braakman and Bulleid, 2011. Protein folding and modification in the mammalian endoplasmic reticulum. Annu Rev Biochem. 80:71-99.

Schedule – Overview of the procedures

Days 1 and 2 are used to cultivate the cells; days 3 to 5 are used to prepare the samples for the analytics and perform the analytical tests.

For each procedure, a detailed protocol is appended at the end of the course manual.

Cultivation of yeast strains (in pairs)

Day 1. Expression tests – Preculture inoculation

All cultivations are done using Synthetic dropout media, which is a chemically defined media. To maintain plasmids, the strains are grown in SD media lacking leucine and uracil. Each group will grow the strains assigned to them.

1) Pick a single colony from each strain and transfer it to a cultivation tube containing 3 mL SD -Leu -Ura (with glucose as a carbon source)
    i) incubate overnight at 28 ˚C, 230 rpm.

Day 2. Expression tests, continued

Cultivation of the strains

1. Prepare the SD –Leu –Ura media from the stock solutions provided in a 50 ml Falcon tube. Label the Falcon carefully with your name.

Concentration of stock solution	Final concentration	Volumes for 50 ml media
2x SD -Leu -Ura	1x	
40% glucose	2%	
500 mM sodium phosphate buffer, pH 6.5	50 mM	
10 mg/ml BSA	100 µg/ml	
H_20 to 50 ml		

2. Transfer 14 ml of the media into 100 ml shake flasks, prepare three flasks (control and 2 plasmids)

3. Measure the OD_{600} of all the precultures. Add 0.1 ml culture to 0.9 ml dH_2O in the cuvette. Mix the suspension carefully just before measuring!

4. Transfer x ml of preculture equivalent to 7.5 OD units of cells to an Eppendorf tube and collect cells by centrifugation (3500 rpm, 5 min) (Hint: for calculating the volume needed: divide the total amount of cells needed (7.5 OD) by the measured OD_{600})

5. Remove supernatant and resuspend cell pellet in 1 ml sterile dH_2O.

6. Add the 1 ml cell suspension to the shake flasks containing the media. This will yield a starting OD of 0.5.

7. Start growing at 28˚ C, 180 rpm for 16 to 20 hours.

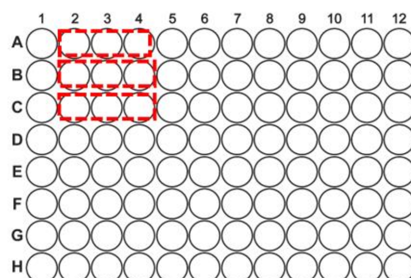
Sample preparation for analytics

The purpose of the analytics is to determine the quantities (ELISA) of the produced antibodies and to check the integrity of the antibody (Immunoblotting). For test the samples need to be prepared in a specific manner.

Day 3. Sample collection and starting analytics.

You will extract the total proteins from **cell pellets** and verify the presence and integrity of the antibody with immunoblotting. In addition, you will test the amount of secreted antibody from the **culture supernatant**.

1. Measure the OD_{600} of all the cultures after overnight cultivation (Add 0.1 ml culture to 0.9 ml dH_2O in the cuvette). Mix the suspension carefully just before measuring!
2. Transfer cultures into 15 mL Falcon tubes and put the tubes on ice.
3. Centrifuge the tubes containing cultures for 5 min at 3900 rpm.
    a. Transfer 1,9 ml of the supernatant to a fresh tube 2 mL tube. Discard the remaining solution. The collected supernatant will be used for ELISA.
    b. Store the remaining cell pellets on ice.
4. Continue with the culture supernatants.
    a. Add 100 µL of 20x PBT (PBS with 0.05 % Tween-20) to the supernatants in 2 ml tubes and mix.
    b. Transfer 3 x 300 µL of each supernatant to a 96-well plate, do this for all strains. (Replica plate A) NB. Mark the position of your samples!
    c. Repeat this (identical replica plate B)



    d. The 96-well plates (two replica plates) are stored in the freezer until ELISA measurements.

5. Extract the total protein from cell pellets for immunoblotting (Protocol 3.1). Start at step 4, steps 1 to 3 are already done. We prepare samples in the presence of a reducing agent (DTT). DTT will break the disulfide bonds in the proteins.
6. Prepare SDS-PAGE-gels according to the protocol 3.2. We need 12.5 % SDS-PAGE gels.

Gels can be prepared several days ahead if they do not dry out. Thus, after casting the gels the ready gels are wrapped in wet tissue paper, placed in plastic bags, and stored at 4 °C.

Day 4, Analytics, continued

1. Run the cell pellet samples on the SDS-PAGE gels (Protocol 3.3)
2. After the SDS-PAGE gel run is completed, start immunoblotting protocol (Protocol 3.4).
    a. Transfer proteins to membranes (steps 1 - 5)
    b. block membranes o/n (step 6)
3. **While the gels are running**, prepare 96-well plates for the ELISA (Protocol 3.5), coating of plates.

Day 5. Analytics, continued

1. Continue with immunoblotting protocol (Protocol 3.4).
    i. We use an antibody for heavy chain detection, the full name of the antibody is “Goat anti-human IgG peroxidase conjugate”.
2. Continue with ELISA (Protocol 3.5)

Protocols

3.1. Preparation of cell extracts

The content of Western blot samples is basically the same as prepared for normal SDS-PAGE. However, the specificity and sensitivity of Western blotting enables that higher amounts of protein can be loaded to the gel to detect and quantify proteins that are present in low amounts. In the first protocol, the cells are destroyed to extract the intracellular proteins for analysis. Protease inhibitor should be used as intracellular proteases can degrade the target molecule.

NOTE: To avoid sample degradation it is important to keep the samples on ice during this protocol!

1. At the designated time point the culture is transferred to a falcon tube and put on ice
2. After OD_{600} measurement the cells are spun down for 5 minutes, 3900 rpm, + 4 ˚C.
3. The supernatant is collected to a separate tube.
4. Resuspend the cell pellet at a concentration of 0.05 OD units/µl in ddH_2O.
   - calculate the total amount of cells (OD units) = OD_{600} reading x culture volume
   - Volume ddH_2O needed = OD units / 0.05 OD units/µl
5. 200 µl of suspension is transferred to an Eppendorf tube and spun down for 5 minutes, 5000 rcf + 4 ˚C.
6. Remove the supernatant by pipetting.
7. The pellet is resuspended in 200 µL sample buffer containing protease inhibitor cocktail and DTT.
8. 100 µL of acid washed glass beads are added.
9. Samples are vortexed for 10 minutes at full speed in the cold room.
10. To remove foam, the samples are spun down at full speed for 5 minutes + 4 ˚C.
11. Transfer the supernatants to new Eppendorf tubes.
12. The samples are boiled for 5 minutes at 80 ˚C.

3.2. SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins according to their molecular weight. Note that unpolymerized acrylamide and TEMED are poisonous, prepare in a fume hood.

Materials:

- 10 % APS
- TEMED
- 40 % acrylamide

- 4x Separating buffer (1.5 M Tris-HCl pH 8.8, 0.4 % SDS)
- 2x Stacking gel (0.25 M Tris-HCl pH 6.8, 0.2 % SDS)
- Sterile H_2O

Casting the gel

1. Check that the glasses are clean. Wash and rinse with water and ethanol if necessary.
2. Set up the casting gear. Press glasses against each other so that the lower end is even (check by placing on the table for example). You may check with water that the set-up doesn't leak. Remove the water by pouring and Whatman filter paper.
3. Mark the border of separating and stacking gel by measuring 0.5 cm below the comb.
4. Mix the separating gel according to the table below. The mixture is enough for two gels. APS and TEMED are responsible for the polymerization so add these last.

acrylamide end concentration %	4 x separating buffer (ml)	H_2O (ml)	40 % Acrylamide (ml)	10 % APS (µl)	TEMED (µl)
12.5	2.5	4.375	3.125	60	12.5

5. Pipette the mixture between the glasses till the marking.
6. Add 500 µl saturated butanol or isopropanol evenly on top of the gel.
7. Let the separating gel solidify (takes around 45 minutes).
8. Mix the stacking gel according to the scheme.
    a. The amounts below are for two gels.

acrylamide end concentration %	2 x stacking buffer (ml)	H_2O (ml)	40 % Acrylamide (ml)	10 % APS (µl)	TEMED (µl)
3	3.8	3.2	0.6	60	12.5

9. Pour butanol/isopropanol out and rinse with ethanol/water. Dry with Whatman-pieces.
10. Add the stacking gel –mixture on top of the gels. Push the combs inside and let the stacking gel solidify (for about 20 minutes).

11. You can use the gel for running right away or store it at +4 ˚C wrapped in moist paper and plastic foil.

3.3. Running SDS-PAGE gels

Protein samples are separated on SDS-PAGE according to their size. Depending on the size of the protein of interest, acrylamide concentration is adjusted which increases / decreases pore size in the gel matrix. After running of gels, proteins can be visualized with stains, or the gels are used for immunoblotting.

Materials:

- SDS-PAGE running buffer.
- Protein size marker
- Positive control

1. Set up the running device with two gels on each side. Try to run gels of the same percentage in the same container.
2. Load 5 µl of positive control in the first well on each gel.
3. Load 5 µl of protein standard in the second well on each gel.
4. Load 10 µl of each cell extract sample
5. Run gel first at 80 V until the front reaches the separating gel border, and the molecular weight standard has started to separate.
6. Then run at 120 V until the front comes out in the bottom of the gel. Running the gel takes from 60 to 90 minutes.

3.4. Immunoblotting

From the gel the proteins are transferred, "blotted", to a nitrocellulose membrane, to which the proteins bind by hydrophobic interactions. On the membrane the proteins are easily accessible for probes and detection.

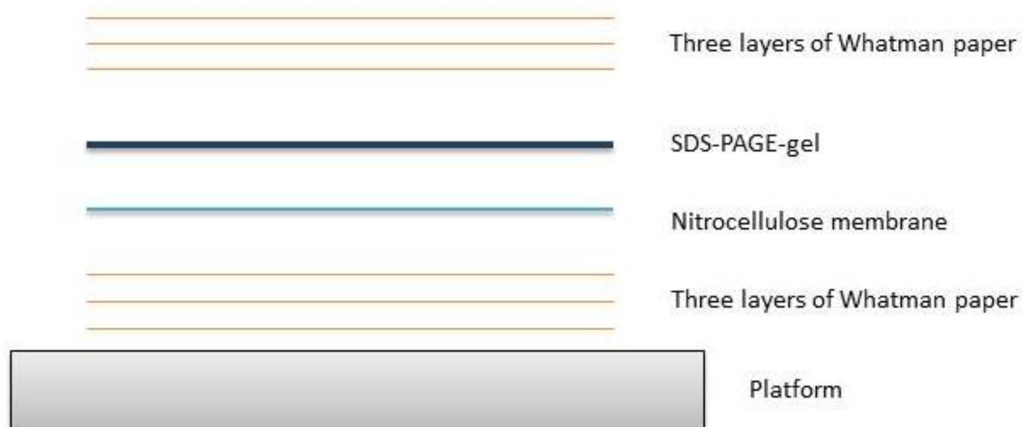
Materials:

- Nitrocellulose membrane
- Whatman paper
- Transferring buffer (contains methanol)
- TBS-T (TBS with 0.1 % Tween-20)
- 5 % milk (w/v) in TBS-T
- 1 % milk (w/v) in TBS-T
- ECL detection kit

- Detection antibodies

Transfer

1. Cut nitrocellulose membrane to meet the size and shape of the separation gel. Do not touch the actual membrane with your hands, use tweezers.
2. Soak the pieces of Whatman paper and the nitrocellulose membrane in transferring buffer.
3. After the run, remove the concentration gel and soak the separation gel in transferring buffer for a few.
4. Set up the blotting layers on the platform in the order shown in the picture below. Two gels fit on one platform. Roll out possible air bubbles with a tube.
5. Blot for 30 min at 2.5 A, 25 V.



Blocking and detection

6. Continue with the nitrocellulose membrane only. Discard the other components. By using tweezers, move the membrane to a container with 12 ml of 5 % milk in TBS-T. Block the membrane at +4 ˚C on a shaker platform overnight.
7. Add 3 µl of detection antibody to 12 ml of 1 % milk in TBS-T. Incubate membrane in antibody solution at RT for 60 min on a shaker platform.
8. Rinse membrane twice with TBS-T.
9. Wash membrane with TBS-T by incubating it in 15-20 ml TBS-T on a shaker platform for 5 minutes. Repeat for three times in total.
10. Cut a suitable surface from see-through plastic for the membrane. Remove TBS-T and place the membrane on the plastic. Dry excess TBS-T gently with a piece of Whatman paper.
11. Mix Solutions 1 and 2 from the detection kit in 1:1 ratio. You need 2 ml per membrane.
12. Pipette the mixed detection solution evenly on the membrane. Cover with see-through plastic and press gently to get the liquid even. Incubate in detection solution for 5 minutes.

13. Pour the extra liquid out and dry with Whatman paper. The chemiluminescence reaction will continue for around one hour, take a picture from the membrane inside that time window. Start with 1 min exposure time.

3.5. ELISA: Enzyme-linked immunosorbent assay

Materials:

- PBS
- PBT (PBS with 0.05 % Tween-20)
- Coating: Goat anti-human IgG (SIGMA, #2316) Fc specific 2.2 mg/mL. Working solution is made by diluting 19,1 µL in 10 mL PBS, mix well before use.
- Standard: You will receive human IgG standard antibody (Myeloma) diluted in PBT with a concentration of 1 µg/mL. Make serial 1:2 dilutions in 1,5 mL volumes to the following concentrations: 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625 and 0.0078125 µg/mL.
- Detection antibody: Goat anti-human IgG peroxidase conjugated (SIGMA, #A0170) Fc Specific 7,1 mg/ml. Diluted 1:4000 in PBT (3 µl per 12 ml), mix well before use.
- *Substrate: Making a 10 mg/mL ortho-phenylenediamine (OPD, a peroxidase substrate) solution by weighing at least 50 mg of OPD. 200 µL of the 10 mg/mL* OPD is diluted in 10 mL of 0.05 M citrate-phosphate buffer pH 5, Shortly before use add 3 µL of 30% H_2O_2. Target concentration of OPD is 0.2 mg/ml. Mix well before use.
  - **Note: be careful as OPD is toxic and collect all OPD-containing waste in the designated container!**
- 1 M H_2SO_4 (for stopping the reaction)

Protocol with 96-well plates:

All incubation steps are carried out on a rocker platform!

1. Wells are coated with 100 µl goat anti-human IgG (4.2 µg/ml in PBS) per well by incubation overnight at 4˚C on a shaking platform.
2. The next day, the plate is washed with 200 µl PBT per well and then removing the liquid, repeat 5 times.
3. For blocking, the plate is incubated with 200 µl PBT per well for 45 minutes at RT on a shaking platform, the plate is covered with a lid or foil.
4. The plate is washed with 200 µl PBT per well and then removing the liquid, repeat 5 times.
5. Standards and samples are added 200 µl per well according to pipetting schema and incubated for 1.5 hour at RT.
6. The plate is washed with 200 µl PBT per well and then removing the liquid, repeat 5 times.
7. Detection antibody is added 100 µl per well and incubated for 1 hour at RT on a shaking platform; the plate is covered with a lid or foil.

8. The plate is washed with 200 µl PBT per well and then removing the liquid, repeat 5 times.
9. Substrate is added 100 µl per well and incubated for 8 minutes. Each row is pipetted with 10 seconds intervals to ensure same incubation time for each well.
10. Reaction is stopped by addition of 100 µl 1 M H_2SO_4 per well, with the same 10 seconds intervals.
11. The signal is measured on plate reader at 490 nm.

Media recipes

Minimal Medium (for selection of prototrophs)

Yeast Nitrogen Base w/o amino acids	0.67 %	6.7 g
Glucose	2 %	20.0 g
Agar (for plates)	2 %	20.0 g

Adjust volume to 1000 ml with ddH2O and autoclave.

SD Medium (Synthetic Complete Dextrose)

Mix C- and N-source from Minimal Medium (Nitrogen Base and Glucose) and add amino acids from the drop-out mix mentioned in list below.

Drop-Out Mix

Amino acids provided in SYNTHETIC COMPLETE SUPPLEMENT MIXTURE (SCSM). For selection of auxotrophic markers, the respective amino acid(s) are left out.

Compound	mg/l medium
Adenine	21
Alanine	85
Arginine	85,6
Asparagine	85,6
Aspartic acid	85,6
Cysteine	85,6
Glutamine	85,6
Glutamic acid	85,6
Glycine	85,6
Histidine	85,6
Inositol	85,6
Isoleucine	85,6
Leucine	173,4
Lysine	85,6
Methionine	85,6
PABA	8,6
Phenylalanine	85,6
Proline	85,6
Serine	85,6
Threonine	85,6
Tryptophane	85,6
Tyrosine	85,6
Uracil	85,6
Valine	85,6

Buffers and solutions

	Final concentration	For 1 liter
10x SDS Running Buffer (pH ~ 8.3)		
Tris base	0.25 M	30.3 g
Glycine	1.92 M	144.0 g
SDS	1%	10.0 g

Adjust volume to 1000 ml with ddH2O
It is not recommended to adjust pH! If you add HCl to the buffer, the additional Cl^- ions may interfere with the electrophoresis resulting in very long run-times.

4x Separating Gel Buffer (pH 8.8)

Tris base	1.5 M	182.0 g
SDS (20 % stock solution)	0.4 %	20.0 ml

Adjust pH to 8.8 with HCl
Adjust volume to 1000 ml with ddH2O

2x Stacking Gel Buffer (pH 6.8)

Tris base	0.25 M	30.0 g
SDS (20 % stock solution)	0.2%	10.0 ml

Adjust pH to 6.8 with HCl
Adjust volume to 1000 ml with ddH2O

4x Sample Buffer (Lämmli) *for 40 ml*

1M Tris-HCl (pH 6.8)	0.25 M	10.0 ml
SDS	8 %	3.2 g
Glycerol (80%)	40 %	20.0 ml
β-Mercaptoethanol	20 %	8.0ml
Bromophenolblue	0.2 %	8.0 mg

Adjust volume to 40 ml with ddH2O, mix well and aliquot, store at -20°C

Western Blot Transfer Buffer (semi-dry blotting) For 1 liter

10x SDS Running Buffer	100 ml
Methanol	200 ml
ddH2O	700 ml

PBS 20x (phosphate buffered saline)

NaCl	2.7 M	160.0 g

KCl	0.05 M	4.0g
*Na2HPO4	0.2 M	28.8 g
KH2PO4	0.35 M	4.8g

Adjust volume to 1000 ml with ddH2O, autoclave, store at RT
Usually, pH is around 8.3 when buffer is concentrated 20x. If you need a certain pH, first dilute 20x stock in 800 ml, then adjust pH to e.g. 7.4 with HCl or 10 % KOH and adjust volume to 1000 ml with ddH2O.

TBS (tris buffered saline)	Final concentration	For 1 liter
Tris-HCl (Tris base, adjust pH to 7.5 with HCl)	50 mM	6.1 g
NaCl	150 mM	8.8 g

PBS-T, TBS-T:
PBS containing 0.05 % Tween 20
TBS containing 0.1 % Tween 20