NPB General Lab Protocols

Time frame

	Monday	**Tuesday**	**Wednesday**	**Thursday**	**Friday**
1st week	•Testing cell competency (1) •Make buffers	•make competent cells (2)	•transformation of plasmids (3) ◦pGEX-PyVP1 ◦pET41a(+)	•pick colonies (4a)	•Mini-Prep (4b) •DNA restriction digest (5)
2nd week	•Maxiprep (14) •PCR (6) •purity check (7) •PCR purification (8) •purity check (7) • PCR restriction (9) •setup sequencing (13)	•DNA purification (10) •Ligate VP1-PCR with pET-41a(+)	•transformation of ligation (3)	•pick colonies (4a)	•Mini-Prep (4b) •DNA restriction digest (5)
3rd week	•Maxiprep (14) •purity check (7) •setup sequencing (13)	•setup culture for expression (15)	•collect protein •sample preparation (16) •run PAGE (16)	•Western Blot (17)	

Protocol 0. Calibration of pipettes.

As per laboratory training procedure.

Protocol 1. Testing Cell competency and validation of phenotype.

1. Both competent and non-competent cells are stored in a quarantine container upon arrival (-80ºC).
2. An aliquot of the cells is thawed on ice (4ºC).
3. Following aseptic techniques and microbial streaking protocols given during lab training; the stock is streaked onto LB-agar plates (10g tryptone/peptone digest of casein, 5g yeast extract, 5g NaCl, 15g agar – the pH is adjusted using indicator strips to ~pH 7 using either 1ml 1N NaOH or 0.2ml of 5N NaOH and ddH_2O added to make the media up to 1L; this is autoclaved at 121ºC for 20min and placed in the slow-cool autoclave cycle that sustains the temperature at 60ºC until pouring). Each batch of plates receives either Ampicillin (100µg/ml), Kanamycin (50µg/ml), Chloramphenicol (170µg/ml), a combination of these or no antibiotic, [antibiotics are made up as per the table 1]).
4. Plates are incubated at 37°C for 16h.

Table 1: Common antibiotics and their concentrations:

Antibiotic	Concentration of stock solution	Final concentration
Ampicillin	100mg/ml in H_2O	100µg/ml
Kanamycin	34mg/ml in EtOH	170µg/ml
Chloramphenicol	10mg/ml in H_2O	50µg/ml

Protocol 2. Making Ca-competent E. coli JM109 and BL21(DE3) pLysS cells (Sambrook and Russell, 3rd Edition, pg 1.109-1.118).

1. From the appropriate plate, transfer a selected single colony to 100ml of LB media (in at least a 500ml baffled flask; 10g tryptone, 5g yeast extract, 5g NaCl, pH is adjusted to 7 with 1 ml 1N NaOH if necassary, ddH_2O up to 1 L, autoclave). Incubate the culture for 3h at 37°C with 160-200rpm agitation, monitoring OD_{600} every 30min (as per UV-VIS in lab training).
2. Harvest the culture when the OD_{600} reaches to 0.35, {modification: 1 OD is ~10^9 cells/ml and is cell-type specific, this information is recorded on the MSDS supplied with the product}.
3. Transfer the bacterial cells to 2 sterile, ice-cold tubes.
4. Balance the tubes and centrifuge at a low speed (2,700 x g for 10min at 4°C).
5. Pour off the media [into an autoclave waste container] from the cell pellets and completely drain residual media by standing the tubes on paper towel in an inverted position for 10min.
6. Resuspend each pellet in 30ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80mM $MgCl_2$, 20mM $CaCl_2$).
7. Centrifuge at 2,700 x g for 10min at 4ºC.
8. Remove the supernatant and completely drain residual media as per step 5.
9. Resuspend the pellet in 2ml of ice-cold 0.1M $CaCl_2$ for each 50ml of original culture.
10. Add 70µl of DMSO to each tube of 2ml cell suspension. Mix gently by swirling, and store the suspension on ice for 15min.
11. Add an additional 70µl of DMSO to each suspension. Mix gently by swirling, and then return the suspension to ice while aliquoting out.
12. Working quickly, dispense 50-100µl aliquots of the suspensions into chilled, sterile microfuge tubes. The expected number of cells in each vial is ~1 - 2.42 x 10^8 cells/ml and is cell type-and condition specific (see MSDS).
13. {Modification: Snap freeze the competent cells by rotating the tightly closed tubes into a beaker of dry ice while squirting MeOH (100%) onto the tube}.
14. Store the frozen competent cells immediately at -80°C until needed.

Protocol 3. Transformation of plasmid DNA into Ca-competent cells (Sambrook and Russell, 3rd Edition, pg 1.118).

1. Pre-incubate LB-agar plates at 37°C for 2h before transformation.
2. Thaw Ca-competent cells on ice (4ºC).
3. Add plasmid DNA (no more than 25ng in a volume of 5µl or less) to each tube of 50-100µl of prepared competent cells. Mix the contents of the tubes by gently swirling and immediately store the tubes on ice for 30mins.
4. Transfer the tubes to a rack placed in a preheated water bath (42ºC) and incubate for 90secs. Warning! Do not shake the tubes.
5. Transfer the tubes to ice (4ºC). Incubate on ice for 2 min.
6. Add 800µl SOC medium (4g Tryptone, 1g Yeast extract, 500mg NaCl, 2ml 250mM KCl, deionised water up to 190ml, adjust pH 7.0, autoclave, then add 1ml of 0.45µm filtered 2M $MgCl_2$, 4ml 1M Glucose) to each tube. Incubate the tubes at 37ºC with gentle shaking (no more that 50rpm) for 45min.
7. {Modification: Transfer 50µl of the transformed cells onto agar plates each containing appropriate selective antibiotics (ampicillin, kanamycin or chloramphenicol) and spread the cell suspension thoroughly on the surface using a sterile rod. Ensure there will be less than 500 colonies per plate for suitable growth and selection ($<10^3$ cells)}.
8. Transfer plates to the incubator. Incubate (agar side up) at 37ºC for ~16h.

Protocol 4. Mini-scale preparation (mini-prep) of plasmid DNA (Sambrook and Russell, 3rd Edition, pg 1.32-1.34; A8.9- A8.16).

1. Inoculate 2ml of LB media (10g tryptone, 5g yeast extract, 5g NaCl, pH is adjusted to 7 with 1 ml 1N NaOH if necassary, ddH_2O up to 1L, autoclave) containing the appropriate antibiotic (table 1) with a single colony of transformed bacteria. Incubate the culture overnight at 37ºC with vigorous shaking (200-300 rpm).
2. Pour 1.5 ml of the culture into a microfuge tube. Centrifuge at maximum speed (21,885 x *g*) for 30 seconds at 4ºC. Important! Keep the unused portion of the original culture at 4ºC for other applications, e.g. preparing glycerol stocks and maxi-scale DNA preparation.
3. {Modification: Remove the media, and dry the bacterial pellet in an inverted position for 10min}.
4. Resuspend the pellet in 100µl of ice cold Alkaline lysis solution I (50mM Glucose, 25mM Tris-HCl, 10mM EDTA, pH 8.0, autoclave for 20min at 121ºC and store at 4ºC) by vortexing.
5. Add 200µl of freshly prepared Alkaline lysis solution II (0.2N NaOH, 1%(w/v) SDS) to each bacterial suspension. Mix the contents gently by inverting the tubes. Important! Do not vortex or vigorously mix the solutions as this increases genomic DNA, RNA and protein contamination. The solution will become very viscous.
6. Incubate at room temperature for exactly 5min.
7. Immediately add 150µl of Alkaline lysis solution III (60ml 5M K.acetate, 11.5ml glacial acetic acid, 28.5ml deionised water pH 6.5) and mix the contents gently to avoid spillage of cellular debris as per step 5. Viscosity is reduced and a white precipitate forms (proteins, cellular debris and salt). Store the tube for 5min on ice.
8. Centrifuge 5min at maximum speed (21,885 x *g*) at 4°C.
9. Transfer the supernatant to a fresh tube and add 2 volumes of 100 % EtOH at RT. Mix the solution by gentle vortexing and incubate on ice for 5min.
10. Centrifuge at a maximum speed for 5min at 4 ºC.
11. Remove the supernatant and add up to 1ml of cold 70% EtOH to the pellet at the opposite side to the pellet. Warning! Do not break the pellet.
12. Recover DNA by centrifugation at maximum speed for 2min at 4 ºC (21,885 x *g*).
13. {Modification: Dry the pellet inverted at RT for 10min}.
14. Dissolve the nucleic acids in 50µl of ddH_2O or TE (pH 8.0) [buffer list] containing 20µg/ml RNase A. Incubate at 37ºC for 1-2h.
15. Store the DNA solution at -20ºC.

Protocol 5. The validation of plasmid DNA by restriction digestion.

1. Single and double restriction enzyme digests are conducted as per the following example:

Component	**pGexPyVP1**	**pGexPyVP1**	**pGexPyVP1**
DNA	1 µg	1 µg	1 µg
Buffer BamHI	2µl	-	-
Buffer R+	-	2µl	-
Buffer Y-Tango	-	-	4 µl
BamHI	1 unit	-	1 unit
XhoI	-	1 unit	1 unit
Deionised H_2O	Up to total volume of 20 µl	Up to total volume of 20 µl	Up to total volume of 20 µl

2. Incubate at 37ºC, 1h.
3. Prepare 1% (w/v) agarose gel (high melting point) and run gel electrophoresis [(*Protocol 6*)]. Add 3µl 6x Gel loading dye into each, and load 20µl to each well.

Protocol 6. Amplifying VP1 gene from pGexPyVP1 (Promega, PCR core systems, Technical Bulletin, pg 4-5).

1. Set-up 3 PCR reactions in PCR tubes as per the following table, For –ve control, add H_2O instead of the template.

Volume	**Component of Master mix**
5µl	$MgCl_2$-free 10x Reaction buffer
3µl	$MgCl_2$, 25mM solution. Completely thaw on ice and mix.
10-50ng	Template (pGexPyVP1)
1µl	dNTP mix
1µl	Forward primer (For VP1 EcoRI,
1µl	Reverse primer (VP1revSalI,
0.25µl	Taq polymerase
Add to 50µl.	H_2O

Primers have been designed as following. Also see Primers for detail.

EcoRI

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5′-ACCggAATTCATggCCCCCAAAAgAAAAAgC-3′
PyVP1       5′-ATggCCCCCAAAAgAAAAAgCggCgTCTCT...-3′
1-30        5′-TACCgggggTTTTCTTTTTCgCCgCAgAgA...-3′

PyVP1       5′-...AAAACAAAgACTgTATTTCCTggAAATTAA-3′
1125-1155   3′-...TTTTgTTTCTgACATAAAggACCTTTAATT-3′
                    3′-CTgACATAAAggACCTTTAATTCAgCTgtggtgg-5′
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SalI

2. Place tubes in a thermocycler that has been pre-heated to 95 °C. Start the thermal cycling program as given below.

Initial denaturation	95°C	2min	1 cycle
Denaturation	95°C	0.5min	
Annealing	55°C	0.5min	35 cycles
Extension	68°C	1.0min	
Final extension	68°C	10min	1 cycle
Storing	4°C	∞	

Protocol 7. Checking purity by running gel electrophoresis.

The following protocol uses gel electrophoresis system from Biorad.

1. Prepare 1% (w/v) agarose gel with SybrSafe stain by combining following;

    - 0.3 g DNA grade agarose
    - Add 1x TAE buffer (40mM Tris-acetate, 1mM EDTA, pH 8.3) up to 27ml.
    - Melt the mixture in microwave for 2min or until completely dissolved at medium temperature.
    - 3ml SybrSafe (manufacturer)

2. Seal both ends of gel tray using masking tape.
3. Pour the melted agarose into the tray. Warning! – hot agarose can damage the tray. Place the comb. Cool down at RT. Gently remove the comb when the gel is solidified. Remove tapes. Place the tray in gel tank. Fill the gel tank with fresh 1x TAE buffer up to the marked level.
4. Prepare samples for loading as below;
   5µl PCR product
   2µl 6x DNA loading dye
   5µl ddH_2O

5. Load samples (12µl each) onto the gel. Load 3 µl DNA ladder.
6. Run the gel at 120V for approx. 30min (visual control). In a 1% (w/v) agarose gel, the front of bromophenol blue runs at ~500bp.
7. View the gel under UV Gel Logic system with filter 535 as per laboratory training guidelines.
8. Check size and purity of PCR products.
9. PCR product can be stored at -20 °C until needed.

Protocol 8. PCR purification with Qiagen QIAQuick PCR clean up kit. Given here are copies of the manufacturer's instructions.

In case the visual control (*Protocol 7*) of the PCR purity revealed no contaminating bands, use the QIAQuick PCR cleanup procedure:

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 8). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the new MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

Notes: • Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

• All centrifuge steps are at 13,000 rpm (~17,900 x *g*) in a conventional tabletop microcentrifuge.

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.

For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).

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

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

4. Discard flow-through. Place the QIAquick column back into the same tube.

Collection tubes are re-used to reduce plastic waste.

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

6. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.

IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

7. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

8. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or H2O to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

In case the visual control (*Protocol 7*) of the PCR purity revealed even miniscule contaminating bands, use the QIAQuick gel extraction procedure:

This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. This kit can also be used for DNA cleanup from enzymatic reactions (see page 8). For DNA cleanup from enzymatic reactions using this protocol, add 3 volumes of Buffer QG and 1 volume of isopropanol to the reaction, mix, and proceed with step 6 of the protocol. Alternatively, use the new MinElute Reaction Cleanup Kit.

Notes: • The yellow color of Buffer QG indicates a pH ≤7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- Isopropanol (100%) and a heating block or water bath at 50°C are required.
- All centrifugation steps are carried out at 13,000 rpm (~17,900 x *g*) in a conventional table-top microcentrifuge.
- 3 M sodium acetate, pH 5.0, may be necessary.

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

Minimize the size of the gel slice by removing extra agarose.

2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).

For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.

IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).

If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow. The adsorption of DNA to the QIAquick membrane is efficient only at pH 7.5.

Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. Add 1 gel volume of isopropanol to the sample and mix.

For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.

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

7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.

The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.

8. Discard flow-through and place QIAquick column back in the same collection tube.

Collection tubes are re-used to reduce plastic waste.

9. (Optional): Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.

This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription or microinjection.

10. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.

Note: If the DNA will be used for salt sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.

11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min

at 13,000 rpm (~17,900 x *g*).

IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

13. To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or H2O to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.

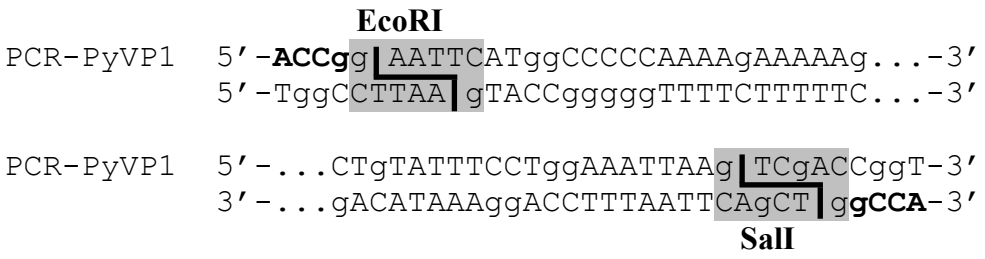
IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

Protocol 9. Restriction digestion of PCR product and vector (protocol from Roche).

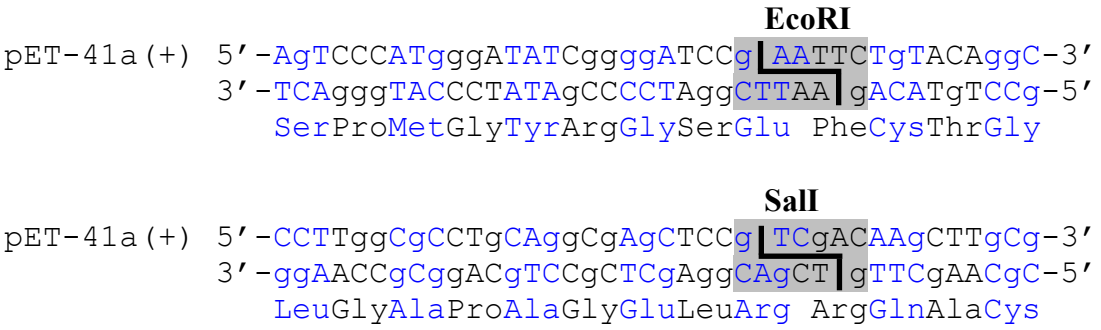
1. Set-up restriction digestion for the purified PCR product and vector as below in 1.5 ml microcentrifuge tubes.

Component	PCR product	pET-41(a+)
DNA	45 µl solution	5 µg
10 x SURE/Cut Buffer H	6 µl	12.5 µl
Deionized H_2O	Up to total volume of 60µl	Up to total volume of 125µl
EcoRI	1 unit/µg DNA	5 units
SalI	1 unit/µg DNA	5 units

PCR-products are cleaved as shown below;



Vector is cleaved as shown below;



2. Incubate on heating block at 37°C. Monitor digestion of the vector by running gel electrophoresis following *Protocol 7*. Include uncut vector for comparison.
3. When the digestion is complete, run electrophoresis using LMT gel following *Protocol 9*.

Protocol 10. Purifying PCR product and vector after restriction digestion through low-melting temperature (LMT) agarose (Sambrook and Russell, 3rd Edition, pg 5.30-5.31).

1. Prepare 1% (w/v) LMT agarose gel (Progen) with SybrSafe stain by combining following;
   0.3g DNA grade low melting point agarose
   Add 1x TAE buffer (40mM Tris-acetate, 1mM EDTA, pH 8.3) up to 27ml.
   Melt the mixture in microwave for 2min at low-medium temperature.
   3ml SybrSafe.
2. Prepare samples and run gel electrophoresis following *Protocol 7*.
3. When the run is complete, view the gel under UV. Cut the bands off the gel using a clean razor blade and transfer them to 1.5 ml microcentrifuge tubes. View the remaining gel under UV to ensure complete removal. Use a new razor for every band.
4. Weigh the gel slice as per in lab training using an analytical balance and add 5 volumes of LMT elution buffer (20mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0)
5. Close tubes and incubate at 65°C until gel fully completely dissolves.
6. Cool the solution down to RT, and then add an equal volume of equilibrated phenol (pH 8.0). Vortex the mixture for 20sec. Recover the aqueous phase (upper phase) by centrifugation at 4,000 x *g* for 10min at 20°C.
7. Extract the aqueous phase once with phenol:chloroform (1:1, v/v) and once with chloroform.
8. Transfer the aqueous phase to a fresh centrifuge tube. Add 0.2 volume of 10M ammonium acetate and 2 volumes of absolute EtOH. Store the mixture for 10 min at RT, and then recover the DNA by centrifugation at maximum speed for 20mins at 4°C.
9. Wash the DNA pellet with up to 1ml of 70% EtOH. Caution! Do not break the pellet.
10. Dissolve in an appropriate volume (10-20µl) of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0).
11. Run another gel electrophoresis (normal melting point) to check the amount and quality of recovered of DNA.

Protocol 11. Quantifying amount of DNA using UV spectroscopy (Sambrook and Russell, 3rd Edition, pg A8.20-21).

The following protocol uses Shimadzu UV spectrophotometer and software and complies with in-house lab training.

1. Turn on the UV-VIS.
2. Fill a quartz cuvette with the same buffer used to dilute the DNA and place it in the reference slot.
3. Fill a quartz cuvette with the same buffer used to dilute the DNA and place it in the sample slot.
4. Run 'Baseline' starting from 400nm to 190nm.
5. Using a fine pipette tip, completely remove the buffer in the sample slot without displacing the cuvette.
6. Dilute the selected volume of DNA solution with the same buffer as 2 and 3 above to a total volume of 80µl (e.g. 5µl in 80µl, 1:16 dilution). Put the diluted DNA solution in the measuring cell.
7. Set the measuring wavelength at 400nm. Press 'Autozero'.
8. Press 'Start'. The spectrum should read zero at 400nm. Use the method button to set scan range from 400 – 220nm. Scan from 400 – 220nm, visually confirming the baseline is smooth from 400 – 330nm.
9. Press 'Go To λ' and type in 260. Record the absorbance. Press 'Go To λ' and type in 280. Record the absorbance again.
10. Calculate the concentration of the undiluted DNA solution by assuming that 1 OD_{260} implies a 50µg/ml DNA concentration. Determine purity of the DNA solution by calculating the OD_{260}:OD_{280} ratio. Optimal purity is implied by a ratio of 1.8-2.0.
11. Zoom in on the 400 – 250nm region to fill the view with the nucleic acid and protein profiles. Print the spectrum for profile recording in lab book.

Protocol 12. Ligation of PCR insert into vector.

Following protocol uses T4 DNA ligase (Fermentas).

1. Calculate the amount of insert in the ligation mixture using the following. Typically add 200ng of vector DNA with the molar ratio of insert:vector being 5:1. For this example, size of vector is 5870bp and size of insert is 1155bp.

   $$\frac{\text{(ng of vector)(kb size of insert)}}{\text{kb size of vector}} \times \text{(molar ratio of insert/vector)} = \text{ng of insert}$$

2. Set up ligation reactions as following

Components	pET-41(a+) with VP1	pET-41(a+) self-ligation	No ligase control
Vector DNA	200ng	200ng	200ng
PCR product (VP1 insert)	as determined	-	as determined
10x ligation buffer	2µl	2µl	2µl
T4 ligase	1 unit	1 unit	-
Deionized water	to 20µl	to 20µl	to 20µl

3. Incubate the mixture O/N at 16˚C. The ligation should result in following product.

```
     pET-41a(+)              PyVP1          pET-41a(+)
5′-ggATCCgAATTCATggCC...AATTAAgTCgACAAgCTTgCg-3′
3′-CCTAggCTTAAgTACCgg...TTAATTCAgCTgTTCgAACgC-5′
   GlySerGluPheMetAla    AsnEndValArgGlnAlaCys
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   Both the self-ligation control as well as the no-ligase control should yield no or very few colonies, while the ligation reaction results in 100-200 colonies.
4. Inactivate T4 ligase by heating the reaction mixture at 65°C for 10min.
5. Use up to 3µl for transformation into Ca-competent JM109 cells following *Protocol 2*.
6. Plate on LB agar plates containing the appropriate antibiotic (see table 1).
7. After incubation at 37°C for ~16h, pET-41(a+) with VP1 ligation mixture should show many colonies (at least >100) where as the other mixtures show few.
8. Pick 24 colonies and miniprep DNA (Protocol 3). Clearly mark and number the selected colonies.
9. Set up a restriction digestion with appropriate enzymes. For pGEX-VP1 you can use the restriction digest given in *Protocol 5*.
10. Run gel electrophoresis to check digestion pattern (*Protocol 4*). It is recommended to set up single digests first and run the gel before proceeding to double digests.
11. Select a successful clone and set up sequencing reactions.

Protocol 13. Setting up sequencing reactions.

1. Determine the concentration using UV spectrophotometer (*Protocol 10*).
2. Set up following reactions in clean 1.5ml microfuge tubes.

Component	**Reaction 1**	**Reaction 2**	**Reaction 3**
pET41(a+)-VP1	1µg	1µg	1µg
Primer (amount)	VP1-GST (6.4pmol)	VP1K199E-S (6.4pmol)	VP1K312G-AS (6.4pmol)
Deionized water	to 8µl	to 8µl	to 8µl

It is important to choose the primers to match the cycling conditions.

i. 94°C for 5min
ii. 96°C for 10sec
iii. 50°C for 5sec
iv. 60°C for 4min

3. Clearly label the tube. Fill in the submission form online. Print out the form and bring it with samples to AGRF.
4. Analyze the sequences by doing pair-wise alignment with the wild-type murine polyoma VP1 sequence (M34958). *AGRF Access Code*

Protocol 14. Maxiprep of pET41a(+)-VP1 (Qiagen) and preparing glycerol stock (Sambrook and Russell, 3rd Edition, pg A8.5).

1. Inoculate 250ml LB media containing kanamycin with 10µl of a positively tested Miniprep sample (*Protocol 11/12*). Incubate at 37°C O/N.
2. Prepare glycerol stock by mixing 750µl of the culture with 250µl of sterile 60% glycerol. Mix thoroughly and freeze immediately at -80˚C.
3. To recover the bacteria, scrape the frozen surface of the culture with a sterile inoculating loop, and then immediately streak the bacteria that adhere to the needle onto the surface of an LB agar plate containing kanamycin. Return the frozen culture to storage at -80°C. Incubate the plate overnight at 37°C.
4. Use this culture to prepare DNA. Purify plasmid using Qiagen Plasmid Maxi kit following the manufacturer's protocol as follows:

1. Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for ~8 h at 37°C with vigorous shaking (~300 rpm).
Use a tube or flask with a volume of at least 4 times the volume of the culture.
2. Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids inoculate 25 ml or 100 ml medium. For low-copy plasmids, inoculate 100 ml or 500 ml medium. Grow at 37°C for 12–16 h with vigorous shaking (~300 rpm).
Use a flask or vessel with a volume of at least 4 times the volume of the culture. The culture should reach a cell density of approximately 3–4 x 109 cells per ml, which typically corresponds to a pellet wet weight of approximately 3 g/liter medium (see page 68).
3. Harvest the bacterial cells by centrifugation at 6000 x *g* for 15 min at 4°C.
6000 x *g* corresponds to 6000 rpm in Sorvall® GSA or GS3 or Beckman™ JA-10 rotors. Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained. If you wish to stop the protocol and continue later, freeze the cell pellets at –20°C.
4. Resuspend the bacterial pellet in 4 ml or 10 ml Buffer P1.
For efficient lysis it is important to use a vessel that is large enough to allow complete mixing of the lysis buffers. Ensure that RNase A has been added to Buffer P1. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.
5. Add 4 ml or 10 ml Buffer P2, mix gently but thoroughly by inverting 4–6 times, and incubate at room temperature for 5 min.
Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification from CO2 in the air.
6. Add 4 ml or 10 ml of chilled Buffer P3, mix immediately but gently by inverting 4–6 times, and incubate on ice for 15 min or 20 min.
Precipitation is enhanced by using chilled Buffer P3 and incubating on ice. After addition of Buffer P3, a fluffy white material forms and the lysate becomes less viscous. The precipitated material contains genomic DNA, proteins, cell debris, and SDS. The lysate

should be mixed thoroughly to ensure even potassium dodecyl sulfate precipitation. If the mixture still appears viscous and brownish, more mixing is required to completely neutralize the solution.

7. Centrifuge at ≥20,000 x *g* for 30 min at 4°C. Remove supernatant containing plasmid DNA promptly.

Before loading the centrifuge, the sample should be mixed again. Centrifugation should be performed in non-glass tubes (e.g., polypropylene). A centrifugal force of 20,000 x *g* corresponds to 12,000 rpm in a Beckman JA-17 rotor or 13,000 rpm in a Sorvall SS-34 rotor. After centrifugation the supernatant should be clear.

Note: Instead of centrifugation steps 7 and 8, the lysate can be efficiently cleared by filtration using a QIAfilter Midi or Maxi Cartridge (see page 70 in QIAGEN manual).

8. Centrifuge the supernatant again at ≥20,000 x *g* for 15 min at 4°C. Remove supernatant containing plasmid DNA promptly.

This second centrifugation step should be carried out to avoid applying suspended or particulate material to the QIAGEN-tip. Suspended material (causing the sample to appear turbid) can clog the QIAGEN-tip and reduce or eliminate gravity flow. Remove a 240 µl or 120 µl sample from the cleared lysate supernatant and save for an analytical gel (sample 1) in order to determine whether growth and lysis conditions were optimal.

9. Equilibrate a QIAGEN-tip 100 or QIAGEN-tip 500 by applying 4 ml or 10 ml Buffer QBT, and allow the column to empty by gravity flow.

Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the QIAGEN-tip to drain completely. QIAGEN-tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

10. Apply the supernatant from step 8 to the QIAGEN-tip and allow it to enter the resin by gravity flow.

The supernatant should be loaded onto the QIAGEN-tip promptly. If it is left too long and becomes cloudy due to further precipitation of protein, it must be centrifuged again or filtered before loading to prevent clogging of the QIAGEN-tip. Remove a 240 µl or 120 µl sample from the flow-through and save for an analytical gel (sample 2) in order to determine the efficiency of DNA binding to the QIAGEN Resin.

11. Wash the QIAGEN-tip with 2 x 10 ml or 2 x 30 ml Buffer QC.

Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first wash is sufficient to remove all contaminants in the majority of plasmid DNA preparations. The second wash is especially necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used. Remove a 400 µl or 240 µl sample from the combined wash fractions and save for an analytical gel (sample 3).

12. Elute DNA with 5 ml or 15 ml Buffer QF.

Collect the eluate in a 10 ml or 30 ml tube. Use of polycarbonate centrifuge tubes is not recommended as polycarbonate is not resistant to the alcohol used in subsequent steps. Remove a 100 µl or 60 µl sample of the eluate and save for an analytical gel (sample 4).

. If you wish to stop the protocol and continue later, store the eluate at 4°C. Storage periods longer than overnight are not recommended.

13. Precipitate DNA by adding 3.5 ml or 10.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥15,000 x *g* for 30 min at 4°C. Carefully decant the supernatant.

All solutions should be at room temperature in order to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. A centrifugal force of 15,000 x *g* corresponds to 9500 rpm in a Beckman JS-13 rotor and 11,000 rpm in a Sorvall SS-34 rotor. Alternatively, disposable conicalbottom centrifuge tubes can be used for centrifugation at 5000 x *g* for 60 min at 4°C. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

14. Wash DNA pellet with 2 ml or 5 ml of room-temperature 70% ethanol, and centrifuge at ≥15,000 x *g* for 10 min. Carefully decant the supernatant without disturbing the pellet.

Alternatively, disposable conical-bottom centrifuge tubes can be used for centrifugation at 5000 x *g* for 60 min at 4°C. The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

15. Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume of buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5).

Redissolve the DNA pellet by rinsing the walls to recover all the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. Overdrying the pellet will make the DNA difficult to redissolve. DNA dissolves best under slightly alkaline conditions; it does not easily dissolve in acidic buffers.

Protocol 15. Expression of recombinant proteins in an expression host. Based on 'QIAexpressionist (version 5)'(QIAGEN), 'The Recombinant Protein Handbook – protein amplification and simple purification Ed AB', 'GST Gene Fusion System Ed AA' (GE Healthcare) and B-Per product guide (Pierce).

> ***Note:*** *In order to derive the most suitable expression conditions for a protein it is most appropriate obtain an uninduced growth curve and screen growth temperature, concentration of the inducer (e.g. IPTG), media type, OD_{600} at induction and harvest, media:flask volume ratios and rate of aeration. Other factors will also influence the global bioprocess (e.g. the contamination spectrum). The following are standard practices or initial conditions and are typically employed to screen a large amount of transformants or produce protein for laboratory-scale experiments. Warning! Growth of cultures >400ml now requires an IBC clearance and suitable risk assessment. Cultures greater than this volume require the operator be licensed to do so*.

1. Transform the plasmid into Ca competent expression cells (BL21(DE3)pLysS in this example) as per Protocol 2 and 3.
2. Plate onto LB agar plates containing suitable antibiotics (Table 1).
3. Select single colonies and inoculate into 10 - 100ml of the desired Expression Media (See Media section, e.g. M9*, 2YT, TB, LB), but containing a suitable antibiotic in a 50 – 500ml baffled flask.
4. Incubate at 30ºC – 37ºC overnight with vigorous shaking (180-220rpm) Note: growth is typically 37ºC unless toxic protein or excess proteolytic digestion is noted. In general the temperature is dependent on expression conditions and ranges typically between 20 – 42ºC.
5. The volume:flask ratio is most ideal at 1:5 or less for baffled flasks during growth and expression.
6. {Modification: Warning! Measure the OD_{600} with appropriate dilutions so the test sample is below OD_{600} = 0.4. Warning! OD_{600} values above 0.4 are not proportional to the cell density}.
7. Equilibrate the Expression media to the appropriate temperature in the incubator for at least 1h.
8. {Modification: Add a suitable amount of the culture so the starting OD_{600} of the Expression culture is at least 1:20 the expected final OD_{600}. A starting OD_{600} ~0.05 or less is suitable for all media types to ensure the culture history is removed during expression}.
9. Grow the cultures at the selected temperature with vigorous aeration (180 – 220rpm) to an OD_{600} that is mid-log (typically 0.6 – 0.8 for LB media) and induce with a moderate amount of the inducer (for IPTG this is typically 1mM).
10. Maintain the culture for up to 6h (typically 2 – 5h for most laboratory expressions).

When analyzing the expression on SDS-PAGE collect and prepare the samples in the following manner:

a. Monitor OD_{600} (test solutions to be below 0.4) and collect samples at appropriate time intervals (e.g. every hour) include a time 0 at the time of induction. The samples should be collected in duplicate and to a standardized OD_{600} by

adjusting the volume appropriately. I.e., the OD_{600} should be collected to allow dilution for downstream processing to a final "in gel" OD_{600} equivalence of material that is between 0.075 – 0.15/well loaded by dilution for SDS-PAGE applications. {Modification: Total OD_{600} dilutions ≤ 0.15/well are good for Western blotting, but can exhibit poor resolution of low molecular weight bands by SDS-PAGE, therefore 0.15/well is often more appropriate for SDS-PAGE. As an example, of how to calculate loadings, an OD_{600} of 0.5 is chosen as the starting point for induction, and 1ml of this material is collected, at T= 1h, the OD_{600} is 1, and so only 500µl is collected to standardize the concentration to an OD_{600} of 0.5. These pellets are resuspended in 1x gel loading buffer in a volume of 100µl (OD_{600} eqv of 0.5/100 = 0.005OD/µl), then a total of 15µl of the sample needs to be loaded to give a final OD_{600} eqv/well of 0.075. Note: it is not necessary to collect 1ml of sample, however, the amount of sample and the resuspension is critical as too large a pellet and too small a volume cause excessive viscosity}.

b. Collection of samples is achieved by centrifugation at maximum speed (21,885 x *g*) for 5 (4˚C). Pour off the supernatant and store the pellets at -80ºC until needed.

Protocol 16. Sample preparation for SDS-PAGE and Western Blotting. Based on Invitrogen's NuPAGE Technical Guide (Version E).

1. {Modification: Thaw the protein/cell pellet samples on ice and place an aliquot of the selected marker (Magicmarker or Mark12) on ice}.
2. Add an appropriate amount of NuPage 4x sample buffer to create a 1x working solution (106 mM Tris-HCl, 141 mM Tris, 2 % SDS, 0.51 mM EDTA, 10 % Glycerol, 0.22 mM G250, pH 8.5) for a final loading volumes and protein concentration or OD_{600} values so that no less than 20ng of protein are loaded per well for SDS-PAGE (detection limit 10ng). For Western blotting, the amount should be determined experimentally and is protocol and detection system-dependent, but lower ends of SDS-PAGE concentrations are most suitable. The 10-well gels take up to 20µl of sample and 17-well gels take up to 15µl of sample. Note: The amount of salt, strength of buffer and pH of the sample can affect the migration and should be minimized through appropriate dilution. An example is provided in the table below showing other sample components:

Sample	Xµl to no less than 20ng/well e.g. 10µl
NuPage sample buffer (4x)	5µl
10x Nu-PAGE reducing agent (500mM DTT)	2µl
Deionized H2O	Up to desired volume, 3µl in this example
Total Volume	**20µl**

Note: The Mark12 standard can be loaded (5µl) as is or prepared in sample buffer as for a sample. {Modification: The Magic Marker (3µl) is in a Lammaeli buffer and must be treated as a sample to ensure correct migration in the Bis-Tris/MOPS system}.

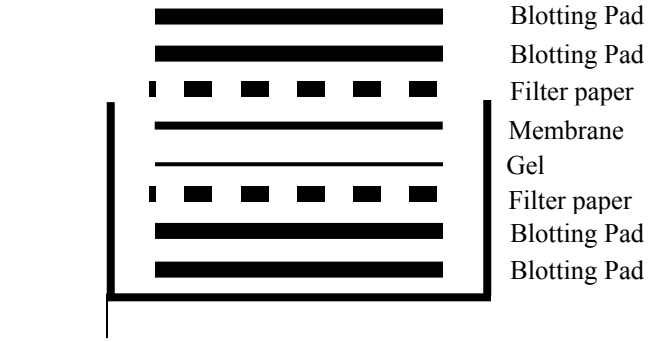
3. {Modification: Disperse the sample preparation solution to homogeneity by pipetting or vortexing as appropriate. Ensure the sample is not overly turbid or viscous. If so, adjust the volumes and concentrations with more sample buffer components}.
4. {Modification: Briefly centrifuge the sample to pull all liquids off the sides of the tubes (maximum speed, 20sec, 4ºC)}.
5. Heat the sample at 70°C for 10min.
6. While sample is heating, prepare the gel(s) and chamber as follows:
    i. Lock the gel into the chamber with either a second gel or blank chamber dam on the electrical frame and using the lever-lock. Ensure you have peeled off the white tape on the base of the gel(s). Ensure the gel(s) and dam extend to the bottom of the chamber and the electrical frame contact (cathode) is slotted into the tank cathode position.
    ii. Add 500µl NuPage antioxidant into the inner chamber and fill the inner chamber with 200ml, and outer chamber with 600ml of 1x running buffer (50mM MOPS, 50mM Tris, 0.1 % SDS, 1mM EDTA, pH 7.7).
    iii. {Modification: Remove the comb(s) and rinse the wells by pipetting ~ 1.5x the well volume with 1x MOPS running buffer to remove excess salt ions from storage}.

7. {Modification: Centrifuge the samples at maximum speed (21,885 x *g*, 5min, 4ºC) and immediately transfer a suitable volume to chilled tubes resting on ice. The standards do not need to be transferred but should be cooled before loading. Note: If gels are being repeated or samples used in other applications, it is often necessary to reheat and recentrifuge the samples. If the samples are noted to be very viscous, increase the centrifugation time as appropriate}.
8. Load the gels asymetrically.
9. Run the gel(s) at starting values of no more than 100-115mA/gel (typically 200V) to final current values of 60-70mA/gel. Voltages should not exceed 200V, nor should they be excessively low (<150V). Very high currents indicate high salt conditions and will lead to anomalous migration. A typical run is 180 – 200V for 45 - 50min.
10. {Modification: Once completed (dye almost leaving gel), turn off the power and empty the running buffer from the tank into a recycling Schott bottle (1x running buffer can be used twice before being discarded, but should not be used if more than two days old). Wash the tank with tap water (with the gels and frame *in situ*) to remove excess SDS, then remove and wash the gel cassette(s)}.
11. Carefully open the gel cassette(s) using the gel knife and remove the bottom of the gel and lanes if desired. For Coomassie staining continue, for Western blotting go to *Protocol 16*.
12. Transfer by inversion to a small container or tray and wash the gel with tap water.
13. Stain the gel(s) using coomassie stain (0.1% (v/v) coomassie G250 Brilliant blue, 40% (v/v) Ethanol, 10% (v/v) Acetic acid) so the stain covers the gel. Staining is improved by agitation on a rotating platform and by avoiding methanol and acetate evaporation by using a sealed container or placing foil over the tray. Incubate at RT for 45min - 3h with gentle shaking {Modification: overnight is ok as required}.
14. {Modification: Recycle the staining solution to the “Used Stain” Schott bottle}.
15. {Modification: Wash the stained gel in tap water until the stain leaches less heavily from the gel and container walls and then} cover in a suitable volume of destain (10% (v/v) Ethanol, 7.5% (v/v) Acetic acid) and incubate until the gel background is suitably decoloured (3h – O/N) by agitation on a shaker. {Modification: destaining can be accelerated by changing the destain solution at hourly intervals and soaking the gel in tap water until the gel swells again}.
16. Collect the image using the Gel Logic as per in lab training.

Protocol 17: Western Blotting. Based on Invitrogen's NuPAGE Technical Guide (Version E), GE Healthcare's 'GST Western BlottingDetection Kit For the detection of GST fusion proteins on Western Blots using enhanced Chemiluminescence' (Rev-C), Sigma-Aldrich's 'ANTI- GLUTAHIONE-S-TRANSFERASE (GST) ALKALINE PHOSPHATASE CONJUGATE' product data sheet (SN/AC 4/01) and 'GST-Tag Monoclonal Antibody product sheet' (TB325 Rev.C 0503) (Novagen).

Note: All protocols except Novagen use either PBS or TBS. The Novagen protocol is the only one that recognizes the problems of the blocking agents to alter the pH and thereby affect the binding of the Ab's to the antigens and is the most suitable to achieve very good results, whereas other methods are similar, the quality of the transfer is decreased.

1. Pre-soak the blotting pads, filter paper and membrane for at least 5min in 1x transfer buffer (1x NuPage transfer buffer, comprising 25mM Bicine, 25mM Bis-Tris.base, 1mM EDTA, pH 7.2 plus 10%v/v Methanol for transferring one gel and 20% (v/v) Methanol for transferring two gels).
2. Wash the gel once disassembled (as per *Protocol 15* above) and assemble the transfer as shown below:



3. If preparing a second transfer, place an extra blotting pad on the anode side of the first filter paper, add an extra filter paper, then the second membrane and filter paper as per normal. The transfer closest to the cathode will always transfer with the highest efficiency. Note: When assembling the transfer remove air bubbles by rolling a test tube gently over the surfaces while applying extra buffer. Easier transfer is obtained using 0.45µm PVDF or nitrocellulose membranes, and greater retention over longer transfer periods is obtained with 0.22µm membranes.
4. Place the anode (+) core on top of the pads and loosely transfer this to the X-Cell II Blot Module to fit horizontally across the bottom of the unit and lock the module together while pouring the transfer buffer into the inner chamber of the blot module. Do not fill all the way to the top as this generates extra conductivity and heat, reducing the efficiency of the transfer.
5. Fill the outer chamber with deionised water.
6. Transfer at 30V for 1-2h (longer times depending on membrane size and number of gels). Expected currents are; start: 170mA, end: 110mA.
7. {Modification: Disassemble the module and wash the nitrocellulose membrane(s) in ddH_2O for 2min. All washing steps are enhanced by agitation on an automated

shaking table – do not pour solutions directly onto the membrane and treat them gently}.

8. Wash the membrane twice (5min each) using 20ml of 1x TBS (150mM NaCl, 10mM Tris-HCl pH 7.5).
9. Incubate for 30min in 20ml blocking solution with gentle agitation [Blocking solution must be made fresh and in the following manner: A 1-5% (w/v) Casein (milk powder) is prepared in a solution of 5x TBS.
    (a) The dry powder is added to 70% of the final volume of ddH_2O. Allow the Casein to hydrate for at least 10min.
    (b) Add up to 350µl of 10M NaOH per 100ml of blocking solution in small increments to solubilise the suspension and achieve the correct pH (measured by pH tape) of 7.5. This needs to be done slowly and carefully.
    (c) Add appropriate amounts of stock 1M Tris.HCl and 5M NaCl to make the 5x TBS blocking solution final concentrations of 50mM Tris, 750mM NaCl. The pH should be close to 7.5 and is adjusted as necessary.
    (d) Store at 4ºC.

Note: PVDF requires 5% (w/v) Casein blocking solution working concentration. Blocking of PVDF is best with longer times of 30min – 1h.

10. Wash twice for 5min each with 20ml 1x TBST (TBST is 500mM NaCl, 20mM Tris-HCl, 0.05% (v/v) Tween-20, 0.2% (v/v) Triton X-100, pH 7.5). Warning! Do not aggressively agitate or over wash or the blocking solution will shed from the membrane. {Modification: Alternative is to use 50% dilute blocking solution with 0.1% (v/v) Tween-20 for this washing step and proceed to step 12}.
11. Wash once for 5min with 20ml of 1x TBS.
12. Prepare 1° Ab solution (in this case rabbit:anti-VP1 or rabbit:anti-GST antibodies) in a 50 ml Falcon tube to a suitable dilution (1:10,000 in this case) diluted with 20ml of 0.5x blocking solution (i.e. 2µl Ab is placed in the falcon tube and 10ml of blocking solution is added and diluted with 10ml ddH_2O). Incubate the membrane with the Ab solution for 1h.
13. Wash twice for 5min each using 1xTBST.
14. Wash once for 5min in TBS. {modification: 0.5x blocking solution}.
15. Prepare 2° anti-rabbit IgG antibody as per Step 12 and incubate each membrane in 20ml of this solution at RT for 1h.
16. Wash membranes five times with 1x TBST. Important! Membranes must be washed properly for best signal:noise ratios.
17. Wash for 5min with 1x TBS.
18. Prepare substrate solution by dissolving 1 tablet of BCIP/NBT in 10ml deionized water (5ml is sufficient for a single blot). Incubate membranes in this solution for 5 – 15min. Important! Incubation must be in the dark (cover) and with absolute minimal agitation or there will be fattening of the bands and “fuzzy ends” as an artifact of the process. Wash the blot thoroughly in ddH_2O to stop the reaction and store in the light.
19. Collect and image using the Gel Logic as per lab training protocols.

Materials description

Item	Supplier	Catalogue #	Batch #
SURE/Cut Buffer H (10x)	Roche	1417991	10377720
Gel-loading buffer (6x)	Fermentas	R0611	
Agar	Spectrum	AG110	RC1619
Agarose (high-melting-point)	Progen	200-0011	274030011
Agarose (low-melting-point)	Progen	200-0030	06020030A
Ampicillin sodium salt	Sigma	A9518-5G	113K0522
Anti-GST antibody	Sigma	A-7340	073K4808
BamHI	Fermentas	ER0051	5821
BCIP/NBT Western Blot substrate	Sigma	B-5655	024K8200
Boric acid	Merck Pty Ltd	10058.3R	34652
Bromophenol Blue	Sigma-Aldrich	113491-5G	09315MA
$CaCl_2$	Univar	A127-500G	AF309129
Chloramphenicol	Sigma	C0378-5G	013K0152
Chloroform	Biolab	BSPCL728.2.5	AH309112
DMSO	Ajax	A2225-500 ML	AH403195
DNA size standard	Fermentas	SM0331	2501
EcoRI	Roche	703737	92879322
EDTA	Univar	663-500G	F2C036
Ethanol	UQ Chemical Store		
BamHI buffer (10x)	Fermentas	B57	2510
Filter paper	Whatman	1001 150	D1365282
Formic Acid	Ajax Finechem	A2471-500ML	AA401005
G250 stain	Sigma	B0770-25G	093K0580
Glacial acetic acid	LabScan	A8401	03020168
Glucose	Chem Supply	GA018	222957
Glycerol	Univar	A242-2.5L	AH309109
HCl	Univar	A256-2.5L	AA403013
Isopropanol	Biolab	BSPPL865.2.5	AH309103
K_2HPO_4	Univar	A2221-500G	AF309205
Kanamycin Sulfate	Gibco	11815-032	1206950
KH_2PO_4	Chem Supply	PA009	221807
$MgCl_2$	Sigma	M3634-100G	043K0146
$MgSO_4$	Univar	A302-500G	AF402202
Na_2HPO_4	Chem Supply	SA026	222643
NaCl	Sigma-Alidrich	S-9888	063K0016
NaOH	Univar	A482-500G	AF407330
NdeI	Fermentas	ER0581	2814
NH_4Cl	Schlarlav	AM0273	55391
Nitrocellulose membrane	Pall life sciences	83783H	40380
pET-41(a+)	Novagen	70556-3	N51459
Pfu Ultra Polymerase	Stratagene	200524-51	0130485
Phenol – Buffer Saturated pH 7.5	Invitrogen	15513047	1199161
Potassium Acetate	Merck	10350.4X	33820
Potassium Chloride	Biolab	BSPPL402.550	32904
Protein Marker (SDS-PAGE)	Invitrogen	LC5677	MRK40628
RNAse A	Fermentas	EN0531	1311
SalI	Roche	348783	10064922
SDS	Amresco	0227-1KG	1813B54
SDS-PAGE gel	Invitrogen	NP0329 BOX	4090373
SYBR Safe DNA Gel Stain	Molecular Probes	533101	S2E13-1

TBE Buffer 5× stock solution	Sigma-Aldrich	T4415	22K8412
Tris	Invitrogen	15504020	1175829
Tris-HCl	Sigma-Alidrich	T32531KG	054K5413
Peptone (Amyl media)	Amyl Media	RM271	7983
XhoI	Fermentas	ER0691	1911
Yeast Extract	Becton, Dickinson & Company	211929	4075311
T4 DNA ligase	Fermentas	EL0014	1712
T4 DNA ligase buffer	Fermentas		3604
NuPage transfer buffer (20x)	Invitrogen	NP0006	
NuPage antioxidant	Invitrogen	NP0005	
Tween-20			
NaOH			

Instruments

Item	**Supplier**	**Catalogue #**	**Serial #**
Western Blot Module	Invitrogen	E19051	1199620-045
Protein electrophoresis cell	Invitrogen	XCell SureLock™	
DNA electrophoresis system	Biorad		
UV spectrophotometer	Shimadzu	UV2450	A10834137007 CS
Thermocycler	Integrated Sciences	HBPX2220	PX220191

Primers

Primer	*Sequence*	*Company*	*Cat. No.*	*Conc* µg/µl	*Conc* µM	*Tm* °C
VP1 For	5'-ATggCCCCCAAAAgAAAAAgC-3'	Proligo	166467	0.73	81	58
VP1 Rev(24)	5'-TTAATTTCCAggAAATACAgTCTT-3'	Proligo	171439	0.73	100	48
VP1 GST For	5'-gAAgCTATCCCACAAATTg-3'	Micromon	26498	0.32	55.37	46.6
VP1 K199 For	5'-ATCAAAACAATCACAAAggAggACATggTCAACAA-3'	Micromon	25982	0.398	36	60.8
VP1 K312 Rev	5'-ATTTTTgACCCATCTTCCTCTCAgggTgATTTTgAA-3'	Micromon	26181	0.156	14.28	62.1
For VP1 EcoRI	5′-ACCggAATTCATggCCCCCAAAAgAAAAAgC-3′	Proligo	168063	0.95	100	72
VP1 Rev SalI	5′-ggTggTgTCgACTTAATTTCCAggAAATACAgTC-3′	Proligo	166478	0.71	68	66

Recipes

Alkaline lysis solution I
50mM Glucose
25mM Tris-HCl (pH 8.0)
10mM EDTA (pH 8.0)
Autoclave for 20 minutes at 121°C and store at 4°C.

Alkaline lysis solution II (prepare fresh)
0.2N NaOH
1%(w/v) SDS

Alkaline lysis solution III (pH 6.5)
60ml 5M K acetate
11.5ml glacial acetic acid
28.5ml deionized water
Store at 4°C and transfer to an ice-bucket before use.

Antibiotics

Antibiotic	*Stock Solution*	*Working Concentration*
Ampicillin	100mg/ml in H_2O	100µg/ml
Chloramphenicol	34mg/ml in ethanol	170µg/ml
Kanamycin	10mg/ml in H_2O	50µg/ml

Stored at -20 °C.

Ammonium Acetate (10 M)
77g ammonium acetate
Make up to 100ml with de-ioinized water
Sterilize by filtration with 0.45µm filter.
Store solution in tightly sealed bottles at 4°C

Dithiothreitol (DTT, 1M)
154.25mg/ml DTT
1ml ddH_2O
Store at -80°C

IPTG (1M stock)
238mg IPTG
1ml H_2O
Filter sterilize through 0.45µl filter.
Store at -20°C.

LB medium (1L)
10 g tryptone
5 g yeast extract
10 g NaCl
H_2O up to 1 L

2YT medium (1L)
16 g tryptone
10 g yeast extract
5 g NaCl
H_2O up to 1 L

$MgCl_2$-$CaCl_2$ solution
80 mM $MgCl_2$
20 mM $CaCl_2$
Autoclaved. Stored at 4 ºC.

PBS (1x)
137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
2 mM KH_2PO_4

Phenol : chloroform
50% Phenol (Buffer Saturated, pH 7.5)
50% Chloroform

T.E. Buffer
10mM Tris-HCl, pH 8.0
1mM EDTA, pH 8.0
Autoclave at 121ºC for 20 minutes.

1× T.A.E Buffer, pH 8.3
40mM Tris-acetate
1mM EDTA

1M Tris-HCl, pH 8.0
1M Tris-HCl
Adjust pH to 8.0 by adding NaOH

LMT Elution Buffer
20mM Tris-HCl, pH 8.0
1mM EDTA, pH 8.0

SOC Medium
4g Tryptone

1g Yeast extract
500 mg NaCl
2ml 250mM KCl
Add deionized water up to 190ml
Adjust pH to 7.0 with 1N NaOH
Autoclave for 20 minutes at 121°C.
Add 1ml 2M $MgCl_2$ – prepare separately and autoclave for 20 minutes at 121°C.
Add 4ml 1M Glucose – sterilize by filtration through 0.22µm-filter.

LB agar plates
10 g tryptone
5 g yeast extract
10 g NaCl
15 g Agar
H_2O up to 1 L
Autoclave.
Cool down to 55 °C. Add appropriate antibiotic to the final concentrations as given below.

Antibiotic	*Stock Solution*	*Working Concentration*
Ampicillin	50mg/ml in H_2O	100µg/ml
Chloramphenicol	34mg/ml in ethanol	170µg/ml
Kanamycin	10mg/ml in H_2O	50µg/ml

Pour into Petri-dishes 90 mm diameter in a safety cabinet. Store at 4 °C.

NuPage SDS sample buffer (4x)

106 mM Tris-HCl
141 mM Tris
2 % SDS
0.51 mM EDTA
10 % Glycerol
0.22 mM G250

For 100 ml,
6.66 g Tris-HCl
6.82 g Tris
8.0 g SDS
0.06 g EDTA
10 ml Glycerol
7.5 ml G250 1% solution
Add H_2O up to 100 ml
Adjust pH ~ 8.5
Store at 4 °C.

MOPS running buffer (5x)
50 mM MOPS
50 mM Tris
0.1 % SDS
1 mM EDTA

For 500 ml,
26.15 g MOPS
15.15 g Tris
2.5 g SDS
0.75 g EDTA
Add H_2O up to 500 ml
pH ~ 7.7 exact. Do not use NaOH or HCl to adjust pH.
Store at 4 °C. Stable for 6 months.

NuPage Transfer buffer (20x)
25 mM Bicine
25 mM Bis-Tris (free base)
1 mM EDTA
pH 7.2

SDS-PAGE stain (100 ml)
100 mg G250 Brilliant blue
40 ml Ethanol
10 ml Acetic acid
50 ml H_2O

SDS-PAGE destain (200 ml)
20 ml Ethanol
15 ml Acetic acid
H_2O to 100 ml