NPB General Lab Protocols

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 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 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.

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			

Recipes

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