AALTO-HELSINKI 2021 iGEM PROTOCOLS

REAGENT PREPARATION

LB AGAR PLATE PREPARATION

INTRODUCTION

Preparation of 500mL LB agar solution, makes ~20-30 plates

MATERIALS

› Autoclave
› Water bath
› Pipettes
› 1L bottle
› LB agar powder (OR use a premix LB agar powder according to instructions on container):
    › Agar
    › Tryptone
    › NaCl
    › Yeast extract
› dH_2O
› NaOH for pH adjustment

PROCEDURE

LB Agar recipe

1. Weigh out 7.5 g agar, 5 g tryptone, 5 g sodium chloride (NaCl) and 2.5 g yeast extract (OR use a premix LB agar powder according to instructions on container) and add to a 1 L bottle.

2. Measure out approximately 400 mL of distilled water and add to the bottle.

3. Shake the bottle to dissolve the reagents.

4. Once the reagents have fully dissolved, adjust the pH to 7.0 by using sodium hydroxide (NaOH) solution.

5. Once the pH is adjusted, top up the solution to 500 mL by using distilled water.

6. To sterilize, autoclave the solution on a liquid cycle (20 min at 15 psi).

7. Leave the solution to cool to approximately 55°C, or warm enough to be held in your hand.

8. When cooled, add the appropriate antibiotic into the solution and swirl to mix.

Preparing plates

9. Carefully pour a thin layer of solution into the Petri dishes to cover the bottom of the plate (approximately 10 - 20 mL per plate). *Try to avoid transferring or creating any bubbles.*

10. Leave the plates to set (30 min) before storing them in the fridge.

SOC MEDIA PREPARATION

INTRODUCTION

Preparation of 1L SOC media

https://www.sigmaaldrich.com/catalog/product/sigma/h8032?lang=fi&region=FI

SOC Medium, shorthand for Super Optimal broth with Catabolite repression, is a nutritionally rich bacterial culture medium. SOC medium is identical to SOB medium, except that it contains 20 mM glucose.

MATERIALS

› Hanahan's Broth (SOB Medium) powder
› Distilled water
› 1 M glucose solution (glucose + deionized H_2O, requires filter sterilization)
› Flask
› Autoclave
› 0.22-µm filter

PROCEDURE

1. Weigh 28 g of Hanahan's powder.

2. Suspend the powder in 980 ml distilled water. Stir to dissolve (heat if necessary)

3. Autoclave for 15 minutes at 121°C to sterilize (liquid cycle).

4. After the SOB medium has been autoclaved, allow it to cool to 60°C or less.

5. Add 20 mL of a sterile 1 M solution of glucose. (This solution is made by dissolving 18 g of glucose in 90 mL of deionized H_2O. After the sugar has dissolved, adjust the volume of the solution to 100 mL with deionized H_2O and sterilize by passing it through a 0.22-µm filter.)

PREPARATION OF YPD MEDIUM

INTRODUCTION

Preparation of 1L of YPD medium

Makes enough competent cells for 5-10 transformations

REFERENCE

https://barricklab.org/twiki/bin/view/Lab/ProtocolsLithiumAcetateTransformation

MATERIALS

› Bacto agar
› Bacto peptone
› Glucose (40% w/v)
› Yeast extract
› Stir bar/plate

PROCEDURE

1. To an autoclavable flask, add: (total volume is 1L)

Reagent	Liquid	Agar Plates
Bacto agar	--	24 g
Bacto peptone	20 g	20 g
Yeast extract	10 g	10 g
Water	950 mL	950 mL

2. Autoclave the mixture

Liquid media

3. Add 50 mL of sterile 40% (w/v) glucose. Mix. Allow to cool before use.

Agar plates

4. While stirring the autoclaved mixture on a magnetic stir plate, add 50 mL of sterile 40% glucose per liter of media.

5. While still warm, pour media into 9-cm diameter plastic Petri dishes. Allow agar mixture to cool and solidify.

E.COLI GLYCEROL STOCK PREPARATION

INTRODUCTION

Preparation of glycerol E. coli seed stock for -80°C freezer

REFERENCE

Labcourse manual, Laboratory Course in Biosystems and Biomaterials Engineering and https://www.addgene.org/protocols/create-glycerol-stock/

MATERIALS

› E. coli seed stock
› Sterile loops
› Glycerol
    › 50%
› Pre-chilled cryotubes
    › Normal eppendorfs work as well for shorter storage time
› Box for tubes
› Ice

PROCEDURE

Liquid culture (day 1)

1. Take glycerol seed stock from the freezer

2. Scrape some of the frozen bacteria off of the top with a sterile loop into 4 ml of LB medium

and shake overnight (12-18 hrs) at 37 °C

3. Check that the solution has a cloudy haze

Seed stock preparation

4. Add glycerol to 25%. Mix gently

5. Aliquot 1 ml samples to tubes (on ice)

6. Place in -80°C freezer indefinitely

S.CEREVISIAE GLYCEROL STOCK PREPARATION

INTRODUCTION

Preparation of glycerol S.cerevisiae seed stock for -80°C freezer

REFERENCE

Taken from Cell Biology Protocols
https://www.sciencegateway.org/protocols/cellbio/yeast/ygs.htm

MATERIALS

› S.cerevisiae seed stock
› Sterile loops
› Glycerol
    › 50%
› Pre-chilled cryotubes
    › Normal eppendorfs work as well for shorter storage time
› Box for tubes
› Ice

PROCEDURE

1. Grow 10mL liquid culture to stationary.

2. Autoclave 30% (v/v) glycerol.

3. Add 0.5 ml yeast culture to 0.5 ml 30% (v/v) glycerol in 1.5 ml cryovials or sterile eppendorf tubes.

4. Invert tubes to mix cells and glycerol, freeze with liquid nitrogen and store at -80 °C.

Sterile DMSO can be used instead of glycerol.

To revive stored cells, scraping off splinters of solid ice with a sterile toothpick or inoculation loop and streaking onto a YPD plate. Avoid complete thawing of the stock.

CLONING PREPARATION

PREPARATION OF COMPETENT E.COLI CELLS

INTRODUCTION

Preparation of E. coli cells that are ready for transformation.

REFERENCE

From Sesilja

MATERIALS

› E. coli culture
› LB media
› LB plates with proper antibiotic
› 0.1 M $CaCl_2$
› ice-cold
› 0.1 M $CaCl_2$ containing 15% glycerol
› ice-cold
› Pre-chilled tubes
› 1.5 ml

PROCEDURE

The day before

1. Place the 0.1 M $CaCl_2$ solution and 0.1 M $CaCl_2$ solution containing 15% glycerol at 4 °C overnight.

2. Label 1.5mL Eppendorf tubes and put into an empty box. Store box in -20°C freezer overnight. Put 1mL tips in -20°C freezer. Prepare 30 tubes/strain.

Day 1

3. Inoculate several colonies in 30 mL LB medium within a 100 mL flask.

4. Subculture at 30°C with shaking till OD_{600} reaches ~ 0.25-0.3 (about 3-4 hours subculture time). (You can grow the cells at 37°C for the first 1-2 hours, until you can see some growth, and then transfer to 30°C.)

5. Pour the culture in 50mL Falcon tubes and chill on ice for 15 minutes.

6. Cool down centrifuge.

7. Centrifuge at 4°C at 1000 x g for 10 minutes.

8. Discard the supernatant and resuspend the pellet with 12 mL ice-cold 0.1 M $CaCl_2$ solution.

9. Keep cells on ice again for 30 minutes.

10. Centrifuge cells at 1000 x g at 4°C for 10 minutes.

11. Discard the supernatant and resuspend pellet with 1.5 mL ice-cold 0.1 M $CaCl_2$ solution containing 15% glycerol.

12. Pipette 100 µL of cell suspension into -80°C frozen eppendorfs, freeze in liquid nitrogen and transfer them to -80°C freezer.

PREPARATION OF COMPETENT S.CEREVISIAE CELLS

INTRODUCTION

Preparation of *S.cerevisiae* cells that are ready for transformation.

Makes enough competent cells for 10-20 transformations

REFERENCE

Adapted from: Gietz, R. & Schiestl, Robert. (2007). Frozen competent yeast cells that can be transformed with high efficiency using the LiAc/SS carrier DNA/PEG method. Nature protocols. 2. 1-4. 10.1038/nprot.2007.17.
https://www.researchgate.net/publication/51383033_Frozen_competent_yeast_cells_that_can_be_transformed_with_high_efficiency_using_the_LiAcSS_carrier_DNAPEG_method

MATERIALS

› FCC (frozen competent cell) solution
  › Glycerol 5% (v/v)
  › DMSO 10% (v/v)
  › Water
› 2xYPD medium (double amount of components in the same amount of water)
› *S.cerevisiae* culture
› Sterile water
› 50mL and 200mL flask tubes, 50mL Falcon tubes, Eppendorfs

PROCEDURE

1. O.N. culture at 30ºC, 200rpm. The culture should be grown in flasks instead of tubes so the cells are properly aerated.

   A. Yeast without a plasmid: growth in 25mL of liquid 2xYPD medium.
   B. Yeast with a plasmid: growth in 200mL of the appropriate selective medium

2. Measure OD_{600}. To do so, dilute 100uL of culture in 900uL of water.

   Remember: 10^6cells = 0.1(OD_{600})

   $0{,}1mL \cdot x = 1mL \cdot OD_{600} \cdot (10^6 cells/mL) / 0.1(OD_{600})$

   $x = OD_{600} \cdot 10^8 cells/mL$

3. Add $5 \cdot 10^8$ cells to 100mL of 2xYPD medium in a flask.

V (volume to add) = $5 \cdot 10^8$ cells / x

4. Incubate flask at 30ºC, 200rpm, until the cell titer is at least $2 \cdot 10^7$ cells / mL (about 4h).

5. Transfer the culture to 50mL Falcon tubes (25mL per tube) and harvest the cells by centrifugation at 3000g for 5min, RT.

6. Wash each Falcon tube in 12,5mL of sterile water, and then resuspend in 250uL of sterile water. Transfer this volume to an Eppendorfs, (join the volumes from 2 Falcon tubes so that at the end you have 2 Eppendorfs containing 500uL each). Centrifuge at 3000g for 5min, RT.

7. Resuspend the cell pellet of each Eppendorf in 500uL of FCC solution.

8. Dispend 50uL samples into an appropriate number of 1.5mL Eppendorf tubes.

9. Place tubes into a rack and freeze at -80ºC.

> Note! It is better if samples freeze slowly, so Styrofoam racks are recommended, as well as using some additional insulation such as Styrofoam chips or newspaper to reduce the air space around the box.

RESUSPENSION OF IDT FRAGMENTS

INTRODUCTION

Resuspension of gene fragments from IDT for use

REFERENCE

From IDT spec sheet

MATERIALS

› IDT fragments
› TE

PROCEDURE

1. Before opening, centrifuge tubes at a minimum of 3000 x g

Makes sure fragments are at the bottom of the tube

2. Add appropriate amount of TE for target concentration of 10 ng/µL

Fragment size	Volume of TE to add
250 ng	25 µL
500 ng	50 µL
1000 ng	100 µL

3. Vortex

4. Incubate at 50C for 20 minutes

5.Vortex and centrifuge

iGEM DNA KIT PLATE PREPARATION

INTRODUCTION

Instruction on how to use iGEM distribution DNA kit plates

Note: There is an estimated 2-3ng of DNA in each well. When following this protocol, assume that you are transforming with 200-300pg/µL

Note: There is not enough DNA in each well to perform anything but transformations.

MATERIALS

› dH_2O
› competent *E.coli* cells
› LB plates with antibiotics
› LB media with antibiotics

PROCEDURE

Resuspending dried DNA

1. With a pipette tip, punch a hole through the foil cover into the corresponding well of the part that you want. Make sure you have properly oriented the plate. Do not remove the foil cover, as it could lead to cross contamination between the wells.

2. Pipette 10µL of dH2O into the well. Pipette up and down a few times and let sit for 5 minutes to make sure the dried DNA is fully resuspended. The resuspension will be red, as the dried DNA has cresol red dye. We recommend that you do not use TE to resuspend the dried DNA.

3. Transform 1µL of the resuspended DNA into your desired competent cells, plate your transformation with the appropriate antibiotic and grow overnight.

4. Pick a single colony and inoculate broth (again, with the correct antibiotic) and grow for 16 hours.

5. Use the resulting culture to miniprep the DNA AND make your own glycerol stock (for further instruction on making a glycerol see this page). We recommend using the miniprepped DNA to run QC tests, such as restriction digests and sequencing.

CLONING

CloneJET BLUNT END CLONING

INTRODUCTION

For cloning of IDT fragments into backbones for level 0 plasmids

REFERENCE

From Thermo Scientific

MATERIALS

› IDT fragments
› ice
› CloneJET PCR cloning kit
› 2X reaction buffer
› pJET1.2/blunt cloning vector
› nuclease-free water
› T4 DNA ligase

PROCEDURE

1. Combine the following to create a final volume of 20µL:

***Final volumes increased to 30µL*

Component	Volume
2X Reaction buffer	10 µL
DNA fragment	0.15 pmol ends*
pJET1.2/blunt cloning vector	1 µL (0.05 pmol ends)
Nuclease-free water	Up to 19 µL
T4 DNA ligase	1 µL

Do all of this on ice!!!

DNA Fragment	Volume to add*
AC T7 promoter + RBS	1.7 µL
FC T7 promoter + RBS	1.7 µL
GC T7 promoter + RBS	1.7 µL
CD AHRtr CDS	11.2 µL**

CD ARNTtr CDS	13.5 µL**
CD AIP CDS	9.5 µL**
DF GST + T7 Terminator	6.9 µL
DF Linker + T7 Terminator	1.8 µL
DG GST + T7 Terminator	6.9 µL
DG Linker + T7 Terminator	1.8 µL
DH Linker + T7 Terminator	1.8 µL
DH GST + T7 Terminator	6.9 µL
AF sfGFP level 1 plug	8.2 µL
FG sfGFP level 1 plug	8.2 µL
GH sfGFP level 1 plug	8.2 µL
AH sfGFP level 2 plug	8.2 µL

2. Vortex briefly and centrifuge for 3-5 seconds

3. Incubate mixture at room temperature for 5 minutes

4. Ligation mixture ready for transformation

TRANSFORMATION OF E.COLI

INTRODUCTION

Transformation of *E.coli* cells with plasmid of interest

Remember to include a negative control without plasmid DNA. Calculate the required amount of plasmid (25 ng) for step 2 based on NanoDrop measurement results

REFERENCE

Biolab III laboratory course manual

MATERIALS

› Competent cells
› Isolated plasmids
› LB medium
› LB agar plate
› Ice
› Heat block 42°C
› Shaker 37°C
› Incubator 37°C

PROCEDURE

Transformation process

1. Make a mini water bath by pipetting water into dry heat block wells (optional) and warm the block to 42°C

2. Pipette 1-5 µL of plasmid (25 ng) into the Eppendorf tube that contains the competent cells, mix gently.

3. Incubate tubes on ice for 30 minutes.

4. Incubate tubes in 42°C in a water bath for 45 sec = heat shock

5. Incubate tubes on ice for 5 minutes.

6. Add 500 µL of LB medium (without antibiotic).

7. Incubate cells at 37°C and 200 rpm for 1 hour.

8. Collect cells by centrifugation (3 mins at 3300 g / ~5900 rpm) and remove about 450-500 µL supernatant

9. Resuspend cells in the remaining supernatant (about 50 µL) and plate onto a LB-agar plate with AMP. Let the plate gel surface dry (the liquid absorbs into the gel) before proceeding to incubation.

10. Incubate overnight at 37°C or over weekend in room temperature, gel-side up.

TRANSFORMATION OF S.CEREVISIAE (Lithium Acetate)

INTRODUCTION

Transformation of yeast cells with plasmid of interest

REFERENCE

https://barricklab.org/twiki/bin/view/Lab/ProtocolsLithiumAcetateTransformation

MATERIALS

› 1 M LiAc

1. Dissolve 10.2 g of LiOAc in 100 mL of water
2. Autoclave
3. Store at room temperature

› TE buffer

› 1mM Na_2EDTA
› 10mM Tris-HCl
› pH 8.0
› Sterile ddH2O for a final volume of 25mL

› Single-stranded carrier DNA

1. Dissolve 50 mg of salmon sperm DNA in 25mL of sterile TE buffer in a conical flask, using a magnetic stir plate at 4ºC. Should take up to few hours, can be speeded up by drawing DNA up and down a pipette until no visible DNA is seen.
2. Dispense 10 samples of 1.0 mL into 1.5 mL Eppendorfs and the remainder in 15 mL Falcon tube (as a stock). Store them at -20ºC.
3. Before use, denature the carrier DNA in a boiling water bath for 5 min and chill immediately in ice. Denature carrier DNA can be boiled three or four times without significant loss of activity.

› PEG solution

1. Warm about 30 mL ddH2O in microwave
2. Add 50 g PEG 3350 and stir the solution until it gets dissolved.
3. Make the volume up to 100mL in a 100mL measuring cylinder and mix thoroughly.
4. Autoclave.
5. Store at RT tightly capped.

PROCEDURE

1. Prepare frozen competent cell transformation mix for the planned number of transformations plus one extra. You can also include an extra tube for a negative control tube for no plasmid DNA.

Transformation mix components per reaction	Volume (uL)
PEG (50%(w/v))	260
LiAc 1.0 M	36
Single-stranded carrier DNA (2.0 mg / mL)	50
Plasmid DNA plus sterile water	14
Total volume	360

The master mix should contain PEG, the LiAc, the ssDNA (and maybe the water), for the planned number of transformations plus the extra one (and maybe the negative control one as well). Divide it then in aliquots and add the plasmid DNA (and maybe the water, if not added before).

2. Thaw cell samples in a 37ºC water bath for 15-30s.

3. Centrifuge at 13000g, 2min, RT, and remove the supernatant.

4. Add transformation mix to the pellets and vortex vigorously to resuspend the pellet.

5. Incubate in a 42ºC water bath for 20-60min depending on the strain.

6. Centrifuge the tubes at 13000g, 30s, RT. Remove the supernatant with a micropipette.

7. Pipette 1.0mL of:

A. Prototrophic gene selection: use sterile water. Vortex to mix thoroughly and resuspend the pellet.

B. Eukaryotic antibiotic gene selection: use YPD liquid medium. Vortex to mix thoroughly and resuspend the pellet, and then incubate for 2-3h at 30ºC to ensure good expression from the input plasmid DNA.

8. Plate the cells onto plates containing the appropriate selection medium. You can do a diluted plate, containing 100uL of the cell solution, and a concentrated plate, by centrifuging the rest of the cell solution (13000g, 5min, RT), resuspending it in 100uL of sterile water and plating it then.

iGEM TRANSFORMATION

INTRODUCTION

Transformation using distribution kit parts, recommended method by iGEM

MATERIALS

› Floating foam tube rack
› Ice & ice bucket
› Lab timer
› 42°C water bath
› 37°C incubator
› Sterile spreader or glass beads
› Pipettes and tips (10µl, 20µl, 200µl recommended)
› Microcentrifuge

PROCEDURE

Transformation

1. Resuspend DNA in selected wells in the Distribution Kit with 10µl dH20. Pipette up and down several times, let sit for a few minutes. Resuspension will be red from cresol red dye.

2. Label 1.5ml tubes with part name or well location. Fill lab ice bucket with ice, and pre-chill 1.5ml tubes (one tube for each transformation, including your control) in a floating foam tube rack.

3. Thaw competent cells on ice: This may take 10-15min for a 260µl stock. Dispose of unused competent cells. Do not refreeze unused thawed cells, as it will drastically reduce transformation efficiency.

4. Pipette 50µl of competent cells into 1.5ml tube: 50µl in a 1.5ml tube per transformation. Tubes should be labeled, pre-chilled, and in a floating tube rack for support. Keep all tubes on ice. Don't forget a 1.5ml tube for your control.

5. Pipette 1µl of resuspended DNA into 1.5ml tube: Pipette from well into appropriately labeled tube. Gently pipette up and down a few times. Keep all tubes on ice.

6. Pipette 1µl of control DNA into 2ml tube: Pipette 1µl of 10pg/µl control into your control transformation. Gently pipette up and down a few times. Keep all tubes on ice.

7. Close 1.5ml tubes, incubate on ice for 30min: Tubes may be gently agitated/flicked to mix solution, but return to ice immediately.

8. Heat shock tubes at 42°C for 45 sec: 1.5ml tubes should be in a floating foam tube rack. Place in water bath to ensure the bottoms of the tubes are submerged. Timing is critical.

9. Incubate on ice for 5min: Return transformation tubes to ice bucket.

10. Pipette 950µl SOC media to each transformation: SOC should be stored at 4°C, but can be warmed to room temperature before use. Check for contamination.

11. Incubate at 37°C for 1 hours, shaking at 200-300rpm

12. Pipette 100µL of each transformation onto petri plates Spread with sterilized spreader or glass beads immediately. This helps ensure that you will be able to pick out a single colony.

13. Spin down cells at 6800g for 3 mins and discard 800µL of the supernatant. Resuspend the cells in the remaining 100µL, and pipette each transformation onto petri plates Spread with sterilized spreader or glass beads immediately. This increases the chance of getting colonies from lower concentration DNA samples.

14. Incubate transformations overnight (14-18hr) at 37°C: Incubate the plates upside down (agar side up). If incubated for too long, colonies may overgrow and the antibiotics may start to break down; untransformed cells will begin to grow.

15. Pick single colonies: Pick single colonies from transformations: do a colony PCR to verify part size, make glycerol stocks, grow up cell cultures and miniprep.

16. Count colonies for control transformation: Count colonies on the 100µl control plate and calculate your competent cell efficiency. Competent cells should have an efficiency of 1.5x10^8 to 6x10^8 cfu/µg DNA.

PLASMID DNA EXTRACTION & PURIFICATION

INTRODUCTION

Extraction of plasmids from transformed *E. coli* cells to later observe successful uptake of gene insert

MATERIALS

› 96-100% ethanol
› heat block
› transformed culture
› Buffer A1
› Buffer A2
› Buffer A3
› Buffer A4
› Buffer AE (elution buffer)
› Spin columns
reagents found in Miniprep kit

PROCEDURE

Cultivate and harvest cells

1. Obtain 1-5 mL of culture and centrifuge for 30 seconds at 11 000 x g

Cell lysis

2. Add 250µL of Buffer A1 and resuspend pellet. Transfer from conical tube to spin column.

Make sure no cell clumps remain before adding Buffer A2

3. Add 250µL of Buffer A2 and mix gently by inverting tube 6-8 times. Do not vortex.

Check Buffer A2 for precipitated SDS prior to use. If a white precipitate is visible, warm the buffer for several minutes at 30–40 °C until precipitate is dissolved completely. Mix thoroughly and cool buffer down to room temperature (18–25 °C).

4. Incubate at room temperature for up to 5 minutes or until lysate is clear.

5. Add 300µL of Buffer A3 and mix by inverting the tube 6-8 times until blue samples turn colourless. Do not vortex.

Clarification of lysate

6. Centrifuge for 5 minutes at 11 000 x g at room temperature. Repeat in case supernatant isn't clear.

Bind DNA

7. Place spin column in a collection tube (2mL) and decant supernatant (or pipette a maximum of 750µL) into column.

8. Centrifuge for 1 minute at 11 000 x g

9. Discard flowthrough and place spin column back into collection tube

Repeat to load remaining lysate until none is left.

Wash silica membrane

10. Add 600µL Buffer A4 and centrifuge for 1 min at 11 000 x g

11. Discard flowthrough and place spin column back into the empty collection tube.

Can place tubes in a 50°C heat block opened to allow ethanol to evaporate

Dry silica membrane

12. Centrifuge for 2 minutes at 11 000 x g and discard collection tube

Elute DNA

13. Place spin column in a 1.5 mL microcentrifuge tube

14. Add 50µL of Buffer AE and incubate at room temperature for 1 minute

15. Centrifuge for 1 min at 11 000 x g

RESTRICTION-DIGESTION

INTRODUCTION

Digestion of DNA using restriction FastDigest enzymes

MATERIALS

› Nuclease-free water
› DNA
› 10X FastDigest Buffer or 10X FastDigest green buffer
› FastDIgest enzymes

PROCEDURE

DIgestion

NOTE! if using more than one restriction enzyme and if they require different temperatures, complete first lower temperature digestion then add second restriction enzyme and raise temperature to second enzyme requirements.

1. Prepare reaction mixture as below at room temperature:

Component	Volume		
	Plasmid DNA	Unpurified PCR product	Genomic DNA
Nuclease-free water	15µl	17µl	30µl
10X FastDigest Buffer	2µl	2µl	5µl
DNA	2µl (up to 1µl)	10µl (~0.2µg)	10µl (5µg)
FastDigest enzyme	1µl	1µl	5µl
Total Volume	20µl	30µl	50µl

NOTE! Add FastDIgest enzyme last

2. Incubate at 37°C for 15 minutes. Time sensitive!

3. Transfer immediately to ice.

ASSAYS

PCR

INTRODUCTION

PCR master mix preparation and basic template to set up the reaction in the PCR machine.

REFERENCE

From: MMB-117 lab course, generalization of the protocol that was given.

MATERIALS

› PCR machine
› DNA template
› dNTPs
› Forward and reverse primers
› DNA polymerase
› Water

PROCEDURE

1. Prepare master mix:

	Volume for 1 sample (final volume = 25 uL)	Volume for n tubes (master mix)
10X buffer	2.5 uL	2.5 x (n+1)
dNTP (10mM)	0.5 uL	0.5 x (n+1)
Primer forward (10uM)	0.5 uL	0.5 x (n+1)
Primer reverse (10uM)	0.5 uL	0.5 x (n+1)
DNA polymerase	0.5uL	0.5 x (n+1)
DNA template	Equivalent to approx 10ng of DNA template as final concentration	Not added to the master mix
Water	Up to 25 uL	Up to 25 x (n+1)

Note: n+1 master mix is prepared because there are always pipetting errors. It is better to prepare volume for more reactions than the needed ones because you might run short of volume if you go with the exact number.

Note: polymerase should be added last.
Note: it is also necessary to add a positive (control DNA template) and a negative (no DNA template) control. Therefore, you must prepare 3 more reactions (pos+neg+excess) than the samples that you have. For example, 10 samples would mean 10+pos+neg+1 = 13-tube master mix

2. Divide the content of the master mix in small PCR tubes according to the number of samples.

3. Add each sample to its correspondent tube.

4. Put samples in the PCR machine and configure the programme. Programme temperatures, times and cycles depend on the polymerase that is being used, the primers annealing temperature and the length of the DNA template. Reference below:

T (ºC)	Time	Cycles
95 (depends on the length of the DNA template)	1min	-
95 55 (depends on primers annealing temperature) 72 (depends on optimal DNA polymerase temperature)	30s 30s 1min	30 to 40 (depends on the length and the concentration of DNA template)
72	10min	-
4 (storage purposes)	∞	

PCR CLEAN UP

INTRODUCTION

Does PCR cleanup, DNA concentration and removal of salts, enzymes, etc. from enzymatic reactions (SDS < 0.1%).

REFERENCE

https://www.takarabio.com/assets/documents/User%20Manual/NucleoSpin%20Gel%20and%20PCR%20Clean up%20User%20Manual_Rev_04.pdf

MATERIALS

› Nucleospin Gel and PCR Clean-up kit

  › *NOTE! Wash buffer NT3 must be prepared before use (add 96-100% pure ethanol; 4:1 parts ethanol to wash buffer NT3 → ex. 24mL ethanol to 6mL NT3)*

› dH_2O

PROCEDURE

PCR clean-up

1. Mix 1 volume sample with 2 volumes buffer NT1 (100 µL sample and 200 µL NT1)

NOTE! for <30 µL sample volume, adjust reaction volume to 50-100 µL with dH_2O

(NOTE! for removal of small fragments, dilutions of NT1 can be used. More info in documentation)

2. Place a NucleoSpin Gel and PCR Clean-up Column into a collection tube (2 mL)

3. Load up to 700 µL sample in tube

Bind DNA

4. Centrifuge: 11000g (~10800 rpm), 30 s

5. Discard flow-through

6. If needed repeat step 3-5 to load remaining sample

Wash silica membrane

7. Add 700 µL Buffer NT3

8. Centrifuge: 11000g (~10800 rpm), 30 s

9. Discard flow-through

10. Recommended: Repeat steps 7-9

Minimizes chaotropic salt carry-over and improve A260/A230 values

Dry silica membrane

11. Centrifuge: 11000g (~10800 rpm) 1 min

NOTE! make sure the column does not come in contact with flow-through when removing it

12. (Extra step: incubate columns for 2-5 min at 70 °C) Total removal of ethanol, ethanol may inhibit enzymatic reactions

13. Place NucleoSpin Gel and PCR Clean-up column into a 1.5 mL tube

Elute

14. Add 15-30 µL Buffer NE

15. Incubate: 1 min (18-25 °C)

16. Centrifuge: 1 min 11000g (~10800 rpm)

NOTE! DNA recovery of larger fragments (>1 kb) can be increased by multiple elution steps with fresh buffer, heating to 70 °C and incubation for 5 min. see documentation for more information.

17. Storage in - 20°C freezer

AGAROSE GEL ELECTROPHORESIS

INTRODUCTION

Determine the expected size of the DNA vector. The vector size determines the percentage of the gel you will be making.

DNA size resolution (kb)	Percent agarose gel (w/v)
1kb-30kb	0.5%
800bp-12kb	0.7%
500bp-10kb	1.0%
400bp-7kb	1.2%
200bp-3kb	1.5%
50bp-2kb	2.0%

Note: Remember to check tray size before starting this protocol. You can try the volume of the tray with water. Here we use 150 ml volume. Note: Remember to check the percentage of agarose gel required, usually gels are made between 0.7% and 2.0%. This protocol is for 1% agarose gel.

REFERENCE

From: Biolab III manual, basic steps for preparing, loading and running agarose gel electrophoresis.

MATERIALS

Preparation of gel

› Agarose powder
› 1x TAE (tris-acetate-EDTA) buffer, maybe needs to be made from 50x stock (To make 1x TAE from 50X TAE stock, dilute 20ml of stock into 980 ml of deionized water)
› 500 ml microwavable flask
› 10,000X SYBR Safe DNA Gel Stain concentrate
› Agarose gel comb
› Agarose gel tray
› Microwave

Loading and running the gel

› Thermo Scientific 6X DNA Loading Dye
› DNA samples
› Control sample

› Ladder: GeneRuler Mix, Thermo Scientific SM0333

PROCEDURE

Preparing agarose gel

1. Measure 1.5 g agarose powder and add it to a 500 ml flask.

2. Add 150 ml 1xTAE buffer to the microwavable flask

3. Melt the agarose in a microwave until the solution becomes clear. Heat the solution in the microwave for several short intervals - do not let the solution boil for long periods as it may boil out of the flask. For example, microwave for 30-45 sec, stop and swirl.

4. Let the solution cool to about 50-55°C, swirling the flask occasionally to cool evenly.
50°C = about when you can comfortably keep your hand on the flask

5. Add correct amount of SYBR Safe (1:10 000) to the solution using a pipette and mix it properly by gently swirling the beaker.

Dilute the concentrated stain 1:10 000 in agarose gel buffer
1:10 000 --> =1/10 000 * volume of solution
1/10 000*150 ml = 0.0150 ml = **15.0 µl of SYBR Safe**
Work cautiously, SybrSafe is a carcinogenic material!

6. Seal the ends of the casting tray with two layers of tape or use the gel caster with rubber stoppers.

7. Place the combs in the gel casting tray.

8. Pour the melted agarose solution into the casting tray slowly to avoid bubbles and let cool until it is solid (should appear milky white).

Pour only the appropriate amount of solution, the combs should not be totally submerged in the gel! At room temperature, it takes 20-30 min to get solid.

9. When solid, pull out the combs and remove tape or stoppers.

10. Place the gel in the electrophoresis chamber.

11. Add enough 1x TAE buffer so that there is about 2-3 mm of buffer over the gel. Now the gel is ready for loading.

Loading the gel

12. Add loading buffer to DNA samples. Add 1 volume of loading dye to 5 volumes of sample. Pipette up and down to mix the loading dye. (Example: Add 5 µl of 6X Sample Loading Buffer to each 25 µl PCR reaction)

13. Record the order of each sample, control and ladder.

14. Pipette 5 µl of the DNA size standard/molecular weight ladder into at least the first well on the gel.

15. Carefully pipette desired amount (5-10 µl for PCR check-up, 20 µl for fragment cutting (as much as fits in the well)) of each sample/loading buffer mixture into separate wells in the gel.

Running the gel

16. Place the lid on the gel box, connecting the electrodes.

17. Connect the electrode wires to the power supply, making sure the positive (red) and negative (black) are correctly connected.

*Remember - **Run to Red.** The DNA is negatively charged and will run towards the positive electrode.*

18. Turn on the power supply to about 80-100 volts.

*Maximum allowed voltage will vary depending on the size of the electrophoresis chamber – **it should not exceed 5 volts/cm** between electrodes!*

19. Check to make sure the current is running through the buffer by looking for bubbles forming on each electrode.

20. Check to make sure that the current is running in the correct direction by observing the movement of the blue loading dye – this will take a couple of minutes.

21. Let the power run until the blue dye approaches the end of the gel (when the dye line is about 80% of the way down the gel).

22. Turn off power.

23. Disconnect the wires from the power supply.

24. Remove the lid of the electrophoresis chamber.

25. Using gloves, carefully remove the tray and gel.

SAMPLE PREPARATION FOR WESTERN BLOT

INTRODUCTION

Preparing sample for protein extraction

REFERENCE

From https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6376.pdf

MATERIALS

› TBS (ice-cold)
› Lysis buffer (RIPA - ice cold)
› 50 mM Tris-HCl, pH 8.0
› 150 mM NaCl
› 1% Nonidet P-40 (NP-40) or 0.1% Triton X-100 0.5% sodium deoxycholate
› 0.1% sodium dodecyl sulphate (SDS)
› 1 mM sodium orthovanadate
› 1 mM NaF
› Protease inhibitors tablet (Roche)
› ice
› cell scraper (cold)
› pre-cooled centrifuge
› Loading buffer (2x Laemmli buffer)
› 4% SDS
› 10% 2-mercaptoethanol
› 20% glycerol
› 0.004% bromophenol blue
› 0.125 M Tris-HCl
Check the pH and adjust to pH 6.8 if necessary.

PROCEDURE

Preparation of Samples

1. Place the cell culture dish in ice and wash the cells with ice-cold Tris-buffered saline (TBS).

2. Aspirate the TBS, then add ice-cold lysis RIPA buffer (1 ml per 100 mm dish).

3. Scrape adherent cells off the dish using a cold plastic cell scraper and gently transfer the cell suspension into a precooled microcentrifuge tube.

4. Maintain constant agitation for 30 min at 4°C.

5. If necessary, sonicate 3 times for 10–15 sec to complete cell lysis and shear DNA to reduce sample viscosity.

6. Spin at 16,000 x g for 20 min in a 4°C precooled centrifuge.

7. Gently remove the centrifuge tube and place it on ice. Transfer the supernatant to a fresh tube, also kept on ice, and discard the pellet.

8. Remove a small volume (10–20 µl) of lysate to perform a protein assay. Determine the protein concentration for reach cell lysate.

9. If necessary, aliquot the protein samples for long-term storage at –20°C. Repeated freeze and thaw cycles cause protein degradation and should be avoided.

10. Take 20 µg of each sample and add an equal volume of loading 2x Laemmli sample buffer.

11. Boil each cell lysate in sample buffer at 95°C for 5 min.

12. Centrifuge at 16,000 x g in a microcentrifuge for 1 min.

SDS-PAGE

INTRODUCTION

Technique used to separate proteins by size.

The linearized protein is made to migrate electrophoretically using a running buffer through a polyacrylamide gel that contains pores wherein they get separated based on their size.

REFERENCE

From Rupesh and http://www.ispybio.com/search/protocols/sds%20protocol1.pdf

MATERIALS

› dH_2O
› 30% acrylamide
› Tris-HCl
  › 1.5 M (pH 8.8) for separation/resolving gel
  › 1.0 M (pH 6.8) for stacking gel
› 10% SDS
› 10% APS
› TEMED
› Running buffer (TRIS/glycine/SDS)
› 25mM TRIS
› 190 mM glycine
› 0.1% SDS
› Loading dye
› Protein ladder

PROCEDURE

Preparation of Separation Gel (10%)

1. Set casting frames (two glass plates in casting frames) on casting stands

2. Mix reagents in the following order, swirl gently but thoroughly:

Component	Volume required for 20 mL
Double distilled water	7.9 mL
30 % acrylamide	6.7 mL

1.5 M Tris (pH 8.8)	5.0 mL
10% SDS	0.2 mL
10% APS	0.2 mL
TEMED	0.008 mL

After adding TEMED and APS to the SDS-PAGE separation gel solution, the gel will polymerize quickly, so add these two reagents when ready to pour.

3. Depending on desired thickness of casting gel, pipette appropriate amount of gel solution into the gap between the glass plates. Leave ~2 cm below the bottom of the comb for the stacking gel. Make sure to remove bubbles.

Thickness of the gel	Volume of stacking gel	Volume of separating gel
0.75 mm	2 ml	4 ml
1.0 mm	3 ml	6 ml
1.5 mm	4 ml	8 ml

4. Layer the top of the gel with isopropanol. This will help to remove bubbles at the top of the gel and will also keep the polymerized gel from drying out.

In ~30 min, the gel should be completely polymerized.

5. Remove the isopropanol and wash out the remaining traces of isopropanol with distilled water.

Preparation of Stacking Gel (5%)

6. Mix reagents in the following order:

Component	Volume required for 9 mL
Double distilled water	4.1 mL
30 % acrylamide	1.0 mL
1.0 M Tris (pH 6.8)	0.75 mL
10% SDS	0.06 mL
10% APS	0.06 mL
TEMED	0.006 mL

7. Pour stacking gel on top of the separation gel (until overflow).

8. Add combs to make wells without trapping air under the teeth. In ~30 min, the stacking gel should become completely polymerized.

Running the gel

9. Clamp gel into apparatus, and fill both buffer chambers with gel running buffer according to the instructions for the specific apparatus.

Running buffer will need to be diluted to desired concentrations

10. Load 20 µg of sample (from preparation protocol), loading dye and molecular mass protein markers into wells for separation by electrophoresis.

11. Run at 100V for 1.5 hours

WESTERN BLOT

INTRODUCTION

Transfer of proteins, separated by size through SDS – PAGE, from polyacrylamide gel to PVDF membrane

Western blot works on the principle of electrophoresis where the proteins, negatively charged due to SDS, migrate from polyacrylamide gel in the negative electrode to PVDF membrane in the positive electrode.

REFERENCE

From https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6376.pdf

MATERIALS

› Transfer buffer
› 25mM TRIS
› 190 mM glycine
› 20% methanol

For proteins larger than 80kD, SDS should be included at a final concentration of 0.1%

› Ponceau S staining buffer
› 0.2% (w/v) Ponceau S
› 5% glacial acetic acid
› TRIS-buffered saline with Tween 20 (TBST) buffer
› 20mM TRIS, pH 7.5
› 150 mM NaCl
› 0.1% Tween 20
› Blocking buffer
› 3% bovine serum albumin (BSA) in TBST
› Stripping buffer
› 20 ml 10% SDS
› 12.5 ml 0.5M TRIS-HCl pH 6.8
› 67.5 ml ultrapure water
› 0.8 ml 2-mercaptoethanol

PROCEDURE

Transferring protein from gel to membrane

1. Place the gel in 1x transfer buffer for 10–15 min.

2. Assemble the transfer sandwich and make sure no air bubbles are trapped in the sandwich.

The blot should be on the cathode and the gel on the anode.

3. Place the cassette in the transfer tank and place an ice block in the tank.

4. Transfer overnight in a cold room at a constant current of 10 mA.

Antibody Incubation

1. Briefly rinse the blot in water and stain it with Ponceau S solution to check the transfer quality.

2. Rinse off the Ponceau S stain with three washes with TBST.

3. Block in 3% BSA in TBST (blocking buffer) at room temperature for 1 hr.

4. Incubate overnight in the primary antibody solution against the target protein at 4°C.

Note: The antibody should be diluted in the blocking buffer according to the manufacturer's recommended ratio. Primary antibody may be applied to the blot for 1–3 hr at room temperature depending on antibody quality and performance.

5. Rinse the blot 3–5 times for 5 min with TBST.

6. Incubate in the HRP-conjugated secondary antibody solution for 1 hr at room temperature.

Note: The antibody can be diluted using 5% skim milk in TBST.

7. Rinse the blot 3–5 times for 5 min with TBST.

Imaging and data analysis

1. Apply the chemiluminescent substrate to the blot according to the manufacturer's recommendation.

2. Capture the chemiluminescent signals using a CCD camera-based imager.

Note: The use of film is not recommended in this step because of its limited dynamic range.

3. Use image analysis software to read the band intensity of the target proteins.

Stripping and reprobing

1. Warm the buffer to 50°C.

2. Add the buffer to the membrane in a container designated for stripping. Incubate at 50°C for up to 45 min with some agitation.

3. Rinse the blot under running water for 1 hr.

4. Transfer the membrane to a clean container, wash 5 times for 5 min with TBST.

5. Block in 3% BSA in TBST at room temperature for 1 hr.

6. Incubate overnight in the primary antibody solution (against the loading control protein) at 4°C.

Note: The antibody should be diluted in the blocking buffer at the manufacturer's recommended ratio.

7. Rinse the blot 3–5 times for 5 min with TBST.

8. Incubate in the HRP-conjugated secondary antibody solution for 1 hr at room temperature.

Note: The antibody can be diluted using 5% skim milk in TBST. 9. Rinse the blot 3–5 times for 5 min with TBST.

Imaging and data analysis

1. Apply the chemiluminescent substrate to the blot following the manufacturer's suggestions.

2. Capture the chemiluminescent signals using a CCD camera-based imager.

Note: The use of film is not recommended in this step because of its limited dynamic range.

3. Use image analysis software to read the band intensity of the loading control proteins.

4. Use the loading control protein levels to normalize the target protein levels.