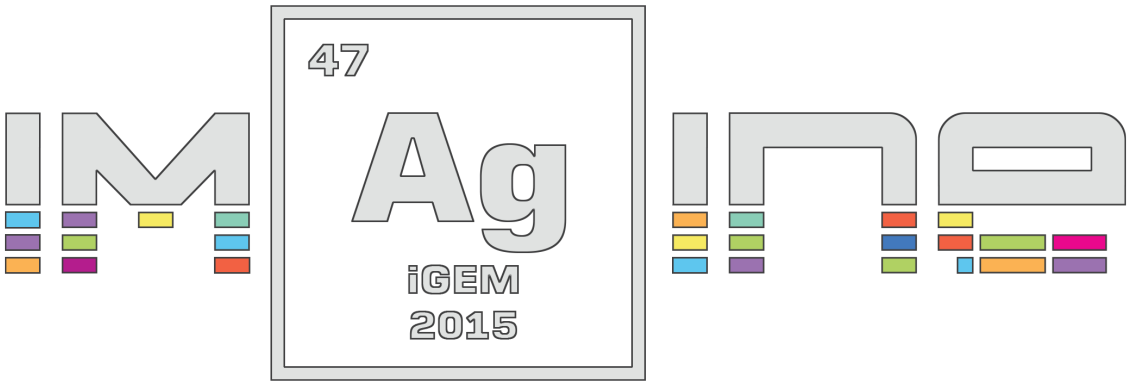
iGem 2015



Protocols and Procedures

NAIT iGem 2015

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PCR

Make PCR Master Mix

Before beginning, ensure all reaction components and properly thawed and mixed.

I. Calculate the required volumes of each component based on the following table:

Component	50µL Reaction	Final Concentration
PCR Grade Water	Up to 50 µL	N/A
2X KAPA HiFi HS	25.0 µL	1X
10 µM Forward Primer	1.5 µL	0.3 µL
10 µM Reverse Primer	1.5 µL	0.3µL
Template DNA	As required	As Required

- Reaction Volumes may be adjusted between 10 - 50 µL
- < 1 ng less complex DNA (0.1 to 1.0 ng) per 50 ≠ L

Set Up Individual Reactions

I. Transfer the appropriate volumes of PCR master mix, template, and primer to individual PCR tubes
II. Cap or seal individual reactions.
III. Mix and centrifuge briefly.

Run PCR

NOTE: A PCR gradient was conducted to determine the optimal annealing temperature needed for best results. We observed that the best annealing temperature was 61°C.

I. Perform PCR with the following Cycle Protocol:

Step	Temperature	Duration	Cycles
Initial Denaturation	95°C	3 min	1
Denaturing	98°C	20 sec	25
Annealing	61°C	30 sec	25
Extension	72°C	15 sec	25
Final Extension	72°C	1 min	1

NOTE: PCR products can be left overnight at 4°C

Run Agarose gel electrophoresis to confirm that PCR was successful

Agarose gel Protocol

I. Mix 99mL of TA buffer with 1g of Agarose and mix on a hotplate until boil
II. Add 10µL of ethidium bromide after boil and pour into Agarose casting tray
III. Insert comb at desired depth
IV. Let stand until gel is formed
V. Add samples into well of desire on Agarose gel
VI. Run gel at 70V for desired time

QIAquick PCR Purification

I. Add 5X Buffer PB to 1X of the PCR reaction and mix into a separate 1.5 mL micro centrifuge tube. If the colour of the mixture is orange or violet, add 10 µK 3M sodium acetate, pH 5.0 and mix. The colour of the mixture will turn yellow
II. Place a QIAquick column into a provided 2 mL collection tube and then place into centrifuge and transfer mix
III. To bind DNA, apply the samples to the QIAquick column and centrifuge at 13 000 rpm from 60s. Discard the fluid that is in the collection tube (aka the flow-through). Place column back into the same tube.
IV. Centrifuge the column once more in the provided 2 mL collection tube for 1 minute to remove residual wash buffer
V. Dab the bottom of the column on Kim Wipe tissue.
VI. Place each column in a clean 1.5 mL micro centrifuge tube
VII. Add 50 µL of EB to the column (Aim for the center of the membrane inside the column)
VIII. Centrifuge at 13 000 RPM for 60 s
IX. DO NOT THROW OUT FLOW THROUGH. Using a micropipette, set at 65 µL, take flow through and add it once more to the column
X. Centrifuge again (13 000 RPM, 60s)
XI. Dispose of column (Purified DNA should now be in the 1.5 mL microfuge tube)

Digestion and Ligation

Digestion of vectors and inserts

Two enzymes are needed in order to cut the DNA stands at specific restriction sites

I. Find the protocol of restriction enzymes being used
II. Mix the following components:

Enzyme 1	1µL
Enzyme 2	1µL
DNA	1µg
Buffer	5µL
Nuclease Free H_2O	Up to 50µL

*Ratio of enzymes to DNA is 1:1 and water is added to maintain the concentration based on each enzyme

III. Let reaction occur for 1 hour

rSAP Protocol

Removal of phosphorylated ends
Preformed after digestion of psB1C3

Protocol

I. Prepare sample using the following

rSAP	1 Unit
DNA	1pmol of DNA ends
Nuclease Free H_2O	Up to 20 µL
CutSmart Buffer (10X)*	2µL

*Provided with rSAP
[total volume should equal 20µL]

II. Incubate at 37°C for 30 minutes
III. Stop the reaction by heat inactivation ay 65°C for 5 minutes

Ligation of Vectors and Inserts

I. Set up the following reaction mixture on ice:

Component	20 µL Reaction
10X T4 Ligase Buffer	2µL
Vector DNA (4kb)	50 ng (0.020 pmol)

Insert DNA (1kb)	37.5 ng (0.60 pmol)
Nuclease Free H_2O	Up to 20 µL
T4 DNA Ligase	1 µL

II. Gently mix the reaction by pipetting up and down and microfuge briefly
III. For cohesive ends, incubate at 16°C overnight or at room temperature for 10 minutes
IV. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours
V. Heat inactivate at 65°C for 10 minutes
VI. Chill on ice and transform 1-5 µL if the reaction into 50 µL competent Cells

Transforming Bacteria

Transformation

I. Thaw tube of BL21 (DE3) competent E. coli cells on ice for 10 min
II. Add 1-5 µL containing 1pg-100ng of plasmid DNA to the cell mixture. Carefully flick the tube 4-5 times to mix cells and DNA. (DO NOT VORTEX)
III. Place the mixture on ice for 30 minutes (DO NOT MIX)
IV. Heat shock at exactly 42°C for exactly 10 seconds. (DO NOT MIX)
V. Place on ice for 5 Minutes. (DO NOT MIX)
VI. Pipette 950µL of room temperature SOC into the mixture.
VII. Place at 37°C for 60 minutes. Shake vigorously (250RPM) or rotate
VIII. Warm selection plates to 37°C
IX. Mix the cells thoroughly by flicking the tube and inverting, then perform several 10-fold serial dilutions in SOC
X. Spread 50-100µL of each dilution onto a selection plate and incubate overnight at 37°C. alternatively, incubate at 30°C for 24-36 hours or 25°C for 48 hours

Validating the Transformation

Colony PCR (KAPA HiFi Hotstart)

I. Prepare the PCR master mix using the following

Component	50µL Reaction*	Final Concentration
PCR Grade Water	Up to 50 µL	N/A
2X KAPA HiFi HS	25.0 µL	1X
10 µM Forward Primer **	1.5 µL	0.3 µL
10 µM Reverse Primer**	1.5 µL	0.3µL
Colony DNA	As required	As Required

* Reaction Volumes may be adjusted between 10 - 50 µL

** Provided by iGem

II. Transfer the appropriate volumes of PCR master mix and colony DNA to individual PCR tubes

III. Cap or seal individual reactions.

IV. Mix and centrifuge briefly.

V. Preform PCR using the following cycle protocol

Step	Temperature	Duration	Cycles
Initial Denaturation	95°C	3 min	1
Denaturing	98°C	20 sec	25
Annealing	61°C	30 sec	25
Extension	72°C	15 sec	25
Final Extension	72°C	1 min	1

Agarose Gel Protocol (1 Gel)

I. Mix 99mL of TA buffer with 1g of Agarose and mix on a hotplate until boil

II. Add 10µL of ethidium bromide after boil and pour into Agarose casting tray

III. Insert comb at desired depth

IV. Let stand until gel is formed

V. Add samples into well of desire on Agarose gel

VI. Run gel at 70V for desired time

Sample Prep

I. Add 10µL sample with 2µL sample buffer for each well. ~1:5 ratio

NOTE: allow for excess reagents if needed

Review Results

I. Any samples under 339 base pairs in length were determined to be unsuccessful as they had no insert

Protein Expression, Isolation and Purification

Protein expression using BL21(DE3)

I. Transform expression plasmid into BL21 (DE3). Plate on antibiotic selection plates and incubate overnight at 37°C
II. Re-suspend a single successful colony (determined from validating the transformation in 10mL liquid culture with antibiotic
III. Incubate at 37°C until optical density reaches an absorbance of 0.4-0.8
IV. Induce with 4 or 40µL of 100mM stock of IPTG (final concentration of 40 or 400µM) and induce for 3 to 5 hours at 37°C
V. For large scale, inoculate 1L of liquid medium (with anitboitic) with freshly grown colony or 10 mL of freshly grown culture. Incubate at 37°C until optical density reaches an absorbance of 0.4-0.8. Add 40 or 400µM IPTG and express protein using optimal time and temperature determined in small scale trial

Protein Purification under Denaturing

Ni-NTA Spin Kit

NOTE: due to dissociation of urea used pH values of buffers will need to be check and if necessary adjusted

NOTE: this protocol is suitable for use with frozen cell pellets. Cell pellets frozen for at least 30 minutes at -20°C can be lysed by re-suspending in lysis buffer and adding Benzonase Nuclease (3units/mL culture volume). Fresh pellets require sonication or homogenization in addition. To the addition of 3 units/mL culture volume Benzonase Nuclease and 1mg/mL culture volume lysozyme.

I. Thaw cells for 15 minutes and re-suspend in 700µL buffer B-7M urea and add 3 units/mL culture volume Benzonase Nuclease
   NOTE: Cells from 5 mL cultures are usually used, but culture volume used depends on protein expression level. Re-suspending pellet in 700µL buffer will allow recovery volume of cleared lysate of approx. 600µL
II. Incubate cells with agitation for 15 minutes at room temperature. Solution should become translucent when lysis is complete.
   NOTE: buffer B is the preferred lysis buffer, as the cell lysate can be analyzed directly by SDS-PAGE. If the cells or the protein do not solubilize buffer A must be used.
III. Centrifuge lysate at 12,000xg for 15-30 minutes at room temperature to pellet the cellular debris. Collect supernatant.
   NOTE: save 20µL of the cleared lysate for SDS-PAGE analysis
IV. Equilibrate a Ni-NTA spin column with 600µL buffer B-7M urea. Centrifuge for 2 minutes at 890xg (2900RPM).
   NOTE: the spin columns should be centrifuged with an open lid to ensure that the centrifugation step is completed after 2 minutes.
V. Load up to 600µL of the cleared lysate supernatant containing the GxHis-tagged protein onto a pre-equilibrated Ni-NTA spin column. Centrifuge for 5 minutes at 270xg (1600RPM), and collect the flow-through
   NOTE: to ensure sufficient binding, it is important not to exceed 270xg (1600RPM) when centrifuging Ni-NTA spin columns
VI. Wash Ni-NTA spin column with 600µL buffer C. Centrifuge for 2 minutes at 890xg (2900RPM)
VII. Repeat step VI
VIII. Elute the protein with 200µL buffer E. Centrifuge for 2 minutes at 890xg (2900RPM) and collect the elute
IX. Repeat step VIII

SDS PAGE and Silver Staining

Make PA Gel

Separating Gel

I. Level glass plates in the casting frame and place the frame within the casting stand. Ensure the plates are locked into place.
II. Place the following solutions in a 50 mL falcon tube:
   Mix:

- 4.2 mL dH2O
- 2.5 mL TRIS 4X Buffer
- 3.3 mL Polyacrylamide Solution.

III. Mix and invert six times.
IV. Add 33.3µL of APS (Ammonium Persulfate) into the falcon tube.
V. Mix and invert six times.
VI. Add 6.7µL of TEMED to the falcon tube.
NOTE: Must be done in a fume hood. TEMED is toxic.
VII. Mix and invert six times. Solution will harden in roughly 15 minutes.
VIII. Add solution in between glass plates using a pipette aid roughly 3/4 the way up the front plate.
IX. Fill remaining space with ethanol to ensure an even, level gel. Ethanol also gets rid of SDS bubbles.
X. Once gel is solidified, remove the ethanol using a Kim Wipe ensuring the area in between the plates is now dry.

Stacking Gel:

I. Place the following solutions in a 50ml falcon tube:
Mix:
- 3.05 mL dH2O
- 1.25 mL Stacking Buffer Solution
- 650 µL Polyacrylamide Solution

II. Mix and invert six times.
III. Add 25µL of APS (Ammonium Persulfate) into the falcon tube.
IV. Mix and invert six times.
V. Add 5µL of TEMED to the falcon tube.
NOTE: Must be done in a fume hood. TEMED is toxic.
VI. Mix and invert six times.
VII. Fill in the remaining area in between the glass plates with the stacking solution and place comb inside.
VIII. After gel is solidified, remove from casting frame and place in plastic wrap. Store in the fridge.
IX. Gel is ready for use

Run Electrophoresis

I. Prepare samples that will be run in the gel electrophoresis.
II. Mix 15µl of each sample with 5µl of sample buffer solution. A 3 to 1 ratio is used. A maximum of 30µl can be inserted into each well.
III. Place glass plates in the electrode assembly and into the tank cell.
IV. Fill tank with 1X electro buffer solution. Using a loading pipette and tips, load desired samples into wells along with a molecular weight ladder sample.
V. Connect the lid to the tank and place the correct cables onto the tank lid.

Run gel at 120 volts for 60-90 minutes or until desired bands are about the length of the gel.

Silver Stain Plus

I. Once SDS-PAGE is complete, remove gels from the glass plates and remove the stacking gel layer using a paper towel.
II. Place gel in a glass container, wash with 200ml distilled water for 5 minutes. Repeat the wash step 4X. (Shake gel back and forth in shaker) Decant water into waste container.
III. Mix a solution with 90ml of 10% acetic acid and 90ml of 10% methanol in a clean beaker. Add 10ml of fixative enhancer solution into the beaker and pour over the gel. Let sit for 20 minutes on shaker, or up to a maximum of overnight, depending on the desired sensitivity of the gel.
IV. Decant solution into a proper waste container.
V. Wash gel once again with 200ml of distilled water for 5 minutes on shaker. Repeat 4X. Decant water into waste container.
VI. Mix both parts of stain solution into two separate falcon tubes:

Mix 1	**Mix 2**
2.5 mL of Silver Staining Complex Solution	1.25g of Development Accelerator
2.5 mL of Reduction Moderator Solution	25mL of DH20
2.5 mL of Image Development Reagent	
17.5 mL of DH20	

VII. Mix both solutions together until each are dissolved, pour over gel. Let sit for up to 20 minutes or until desired bands show.
VIII. Decant solution into proper waste container.
IX. Prepare a stop solution containing 90ml of 10% acetic acid and 90ml of 10% methanol. Pour over gel and shake for 15- 20 minutes.
X. Decant solution into proper waste container.
XI. Wash gel with 200ml of DH20 for 5 minutes on shaker. Repeat 2X.
XII. Review results