Centurion University
Shaping Lives... Empowering Communities...

WORKSHOP ON
PROTEIN PURIFICATION & SDS-PAGE

INSTITUTION'S INNOVATION COUNCIL (Ministry of HRD Initiative)

4 QUALITY EDUCATION

- Bacterial protein expression
- Protein purification strategies
- SDS-PAGE
- Result analysis

RESOURCE PERSONS

Dr. Shaikh Nausad Hossain
CSIR-Pool Scientist
DBT-Institute of Life Sciences
Bhubaneswar, Odisha, India

Dr. Gagan Kumar Panigrahi
Assistant Professor
Department of Zoology
Center for Genetics and Genomics
CUTM, Bhubaneswar, Odisha, India

Date: February 01, 2023
Time: 9:30 AM - 4:30 PM
Address: Centurion University of Technology and Management, Bhubaneswar Campus

RS. 1000/- ONLY
(KIT, CERTIFICATE & LUNCH)

REGISTRATION LINK

https://forms.gle/g26gf3Mc61x2YhBr8

Web: https://workshop.cutm.ac.in
Email: zoologyevents@cutm.ac.in

DEPARTMENT OF ZOOLOGY & CENTER FOR GENETICS AND GENOMICS

FACULTY COORDINATOR:

Dr. Pradip Kumar Prusty
Assistant Professor
Department of Zoology
Center for Genetics and Genomics
Mob: 8249485242
Email: pradipkumar.prusty@cutm.ac.in

FOR QUERIES CONTACT:

Dr. Yashaswi Nayak
Dean, School of Applied Sciences
Associate Professor & Head,
Department of Zoology
Mob: 9861522222
Email: yashaswi.nayak@cutm.ac.in

Dr. Rukmini Mishra
Associate Professor & Head,
Department of Botany, Center for Genetics and Genomics
Mob: 7077320293
Email: rukmini.mishra@cutm.ac.in

Centurion University
Shaping Lives... Empowering Communities...

WORKSHOP ON PROTEIN PURIFICATION AND SDS-PAGE

Organized by

Department of Zoology and Center for Genetics and Genomics

S.I. No.	Name of the participant	Affiliation
1	Bishwajeta Jena	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
2	Ipsita Patro	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
3	Bilash Ranjan Nayak	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
4	Samapika Chaudhury	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
5	Monalisha Rautaray	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
6	Romyaprakash prusty	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
7	Ipsita Nayak	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
8	Adyasha Singh	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
9	Ruchismita Mohanty	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
10	Binapani Sahu	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
11	Mahima Manojyoti Aran	C.V. Raman Global University, Bhubaneswar, Odisha, India
12	Manusruti Sahu	C.V. Raman Global University, Bhubaneswar, Odisha, India
13	Tan Thakur	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
14	Gargi Patel	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
15	Abhipsa Samal	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
16	Payal Patel	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
17	Bharati Gupta	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
18	Sthitaprajnya Mishra	Centurion University of Technology and Management, Bhubaneswar, Odisha, India
19	Anjali Lata sahoo	Centurion University of Technology and Management, Bhubaneswar, Odisha, India

Centurion University
Shaping Lives... Empowering Communities...

WORKSHOP ON PROTEIN PURIFICATION & SDS-PAGE

PROGRAM SCHEDULE ON 01-02-2023

10.00 AM - 10.30 AM	**Registration & Inaugural address**
	Inaugural address by Dr. Yashaswi Nayak, Dean SoAS
	Venue: CR-18 (Aryabhatta Building)
10.30 AM - 10.45 AM	**Tea & Snacks**
	Venue: In front of CR-18 (Aryabhatta Building)
10.45 AM - 11.10 AM	**Lecture: Basics of Protein Purification & SDS-PAGE**
	By: Dr. Shaikh Nausad Hossian (Resource Person)
	Venue: CR-18 (Aryabhatta Building)
11.15 AM - 01.00 PM	**Demonstration and hands-on practice of protein purification, SDS-PAGE and Western (in two batches)**
	By: Dr. Gagan Kumar Panigrahi & Dr. Shaikh Nausad Hossian (Resource Persons)
	Venue: Environmental Sciences Lab (1st Floor, Aryabhatta Building)
1.00 PM - 01.20PM	**Visit to Fish Carnival**
	Venue: Madhusudan Building (5th Floor, Faculty Pantry)
1.20 PM - 01.50PM	**Lunch**
	Venue: Pantry, 1st Floor, Aryabhatta Building
2.00 PM - 03.30 PM	**Demonstration and hands-on practice of protein purification, SDS-PAGE and Western (in two batches)**
	By: Dr. Gagan Kumar Panigrahi & Dr. Shaikh Nausad Hossian (Resource Persons)
	Venue: Environmental Sciences Lab (1st Floor, Aryabhatta Building)
3.30 PM - 03.45 PM	**Result analysis**
	By: Dr. Gagan Kumar Panigrahi & Dr. Shaikh Nausad Hossian (Resource Persons)
	Venue: CR-18 (Aryabhatta Building)
3.45 PM - 04.15 PM	**Discussion and feedback**
	By: Dr. Gagan Kumar Panigrahi & Dr. Shaikh Nausad Hossian (Resource Persons)
	Venue: CR-18 (Aryabhatta Building)
4.15 PM - 04.45 PM	**Valedictory**
	Coordinators: Dr. Pradip Kumar Prusty
	Venue: CR-18 (Aryabhatta Building)
4.45 APM - 5.10 PM	**Tea, Snacks & group photo session**
	Venue: In front of CR-18 (Aryabhatta Building)

Definition of Protein Purification

The expression of recombinant proteins, especially using bacterial vectors and hosts, is a mature technology. The problem is how to isolate it in an active form. Purification of recombinant protein is an important technology in biological research. To study the particular function and structure of a protein, researchers must isolate and purify the recombinant protein from the organism. The protein purification method mainly uses the similarity and difference between different recombinant proteins. Non-proteinaceous materials can be removed based on the similarity between proteins, and the target recombinant protein then can be isolated and purified based on the differences between proteins.

Protein tags are a useful and convenient tool for improving solubility of recombinant proteins, streamlining protein purification, and allowing an easy way to track proteins during protein expression and purification. In addition, protein tags are a useful tool for tracking proteins and processes directly in live cells using microscopy or indirectly using Western blot, immunoprecipitation, or immunostaining.

Protein Purification Practice

The final purification process ideally consists of sample preparation, including extraction and clarification when required followed by above described three phases of purification. The number of steps will always depend on purity required and intended use of protein.
An analytical purification generally utilizes three properties to separate proteins. First, proteins- may be purified according to their isolectric points by running them through a pH graded gel or an ion exchange column. Second, proteins can be separated according to their size or molecular weight via size exclusion chromatography or by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) analysis. Proteins are often purified by using 2D-PAGE and are then analyzed by peptide mass fingerprinting to establish the protein identity. This is very useful for scientific purposes and the detection limits for protein are nowadays very low and Nano gram amounts of protein are sufficient for their analysis.

How to Apply Purification Principles:

Selection and Combination of Purification Techniques: The aim of this combination is to evolve a fastest route to a product of required purity. For any chromatographic separation each different technique will offer a different performance with respect to recovery, resolution, speed and capacity. A technique can be optimized to focus on one of these parameters; for example, resolution to achieve the best between two parameters such as speed and capacity.

Purification of a Tagged Protein:

Adding a tag to the protein gives the protein a binding affinity it would not otherwise have. Usually the recombinant protein is the only protein in the mixture with this affinity, which aids in separation. The most common tag is the Histidine-tag (His-tag) that has affinity towards nickel or cobalt ions. Thus by immobilizing nickel or cobalt ions on a resin, an affinity support that specifically binds to histidine tagged proteins can be created.

Affinity Tag Definition

Protein tags are convenient for improving solubility of recombinant proteins, streamlining protein purification, and allowing an easy way to track proteins during protein expression and purification. Perhaps the most common application of protein tags involves the addition of a purification 'tag', also called affinity tag, which provides a standardized method to purify the fused recombinant protein.

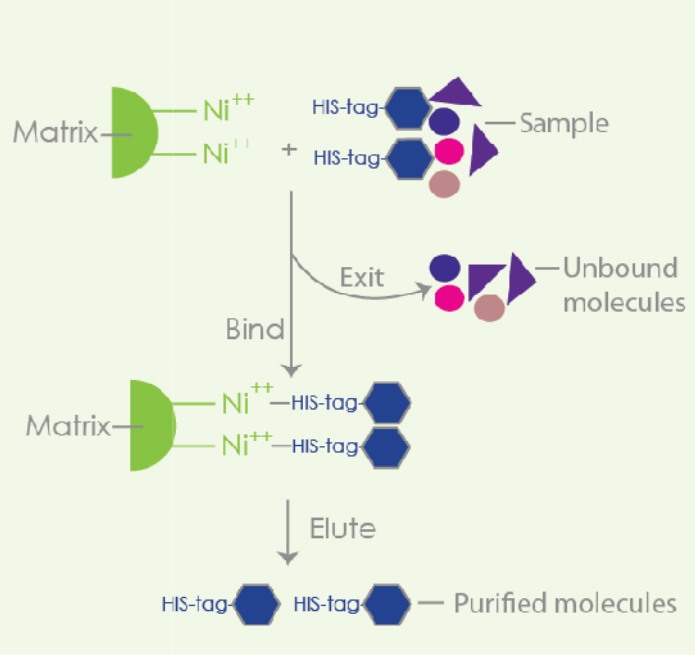
Affinity tag:

Affinity tag refers to a short peptide added to either the N- or C-end of a recombinant protein to facilitate purification of the expressed protein and the affinity tag sequence usually contains from several to hundreds of amino acids. Many tags can also provide additional functions unrelated to purification, such as facilitating detection of the target protein or improving the solubility of the target recombinant protein.

Usage of Affinity Tag in Purification of Recombinant Protein

Affinity fusion tags are widely used in purification of recombinant protein.Different affinity tags have their specific application in recombinant protein purification depend on the properties of the target protein, the elution, the expression systems and the specific conditions of affinity column.

After the expression of the tagged fusion protein, the sample is added to the purification column where the affinity tag can be adopted together with the target protein. A selective interaction with the specific affinity tag and elution under special conditions result in a highly efficient one-stage purification of the desired product.In some cases, it is necessary to restore the biological activity and structural conformation of the recombinant protein, some fusion tags that allowing the refolding of the desired protein are required. Moreover, application of affinity tags can also lower material and time expenditures for obtaining the desired recombinant protein.



Evaluating Purification Yield:

The most general method to monitor the purification process is by running a SDS PAGE, of the different steps. This method only gives a rough measure of the amounts of different proteins in the mixture, and it is not able to distinguish between proteins with similar molecular weight. In order to evaluate the process of multistep purification, the amounts of the specific protein have to be compared to the amount of total protein.

Work flow

Purification of Campylobacter Invasive Antigen B (CiaB), expressed in E. coli expression system, using affinity purification.

Protein Expression:

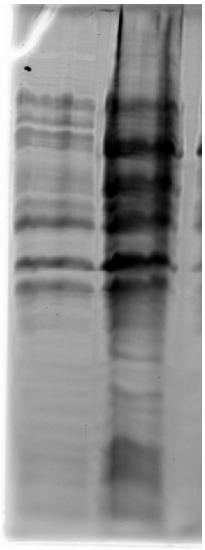
Small scale expression test was performed by growing the transformed colonies from LB plated in 10 ml of LB broth containing ampicillin. Various expression parameters such as growth temperature, IPTG concentration, and duration of post induction incubation were optimized.The best condition for the optimum expression of the protein was found to be induction at 18^0 C in presence of 500µm concentration of IPTG for the duration of 16hrs post induction.

Small Scale protein expression

SDS-PAGE Analysis of Un-induced & Induced CiaB protein expression:

Lanes 1 2

1. Un-induced Expressed CiaB
2. Induced Expressed CiaB



SDS-PAGE Gel Picture of expressed protein

Un-induced and Induced CiaB protein band corresponding to the size of 73 kDa was found in SDS-PAGE. Induced protein shows thick band while UN-induced shows faint or degraded band.

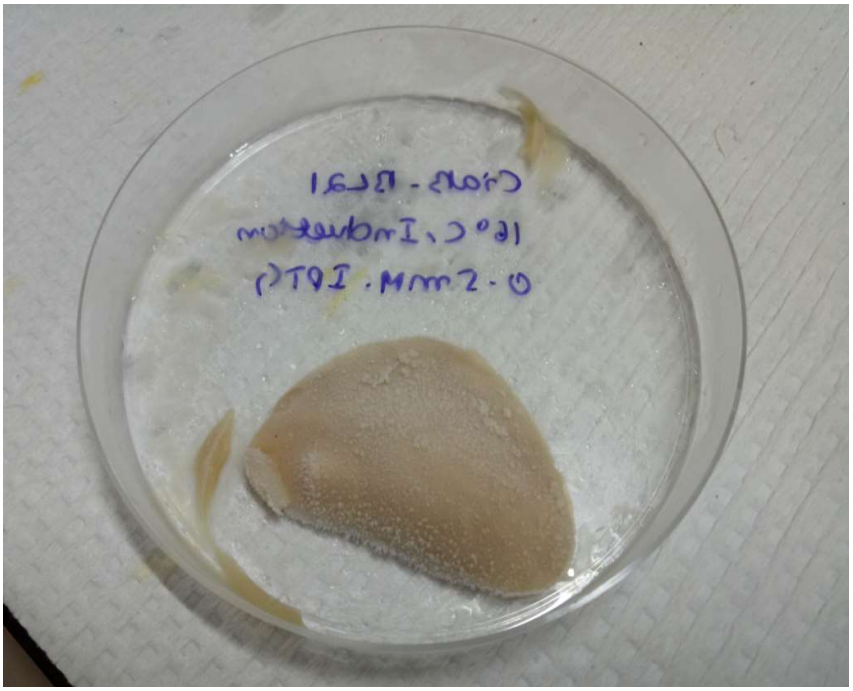
Mass expression of CiaB protein:

Based on the optimized expression protocol transformed cells grown in 2L LB broth and the cell were harvested after the post induction. 10g of wet pellets were harvested from 2L culture. The cell pellet was used for protein purification.

Large scale protein expression

***E.coli* cells harvesting by centrifugation:**

Cell pellets harvested by centrifugation and stored at -20^0 C



Cell pellets after centrifugation of large scale expressed protein

Resuspension and sonication of cells:

Complete resuspension was confirmed by homogenous solution in lysis buffer which appeared milky white without any cell clumps as shown in the picture.

Resuspension

Resuspended Cells

Cell lysis & Soluble protein separation:

After complete round of sonication the resuspended milky white cell suspension turned translucent, which indicated the complete lysis of *E.coli* cells. The long centrifiguation step separated the pellet which contains the cell debris & the supernatant with soluble proteins among which our CiaB protein was present.

Sonicated Cells

Before centrifugation sonicated cell

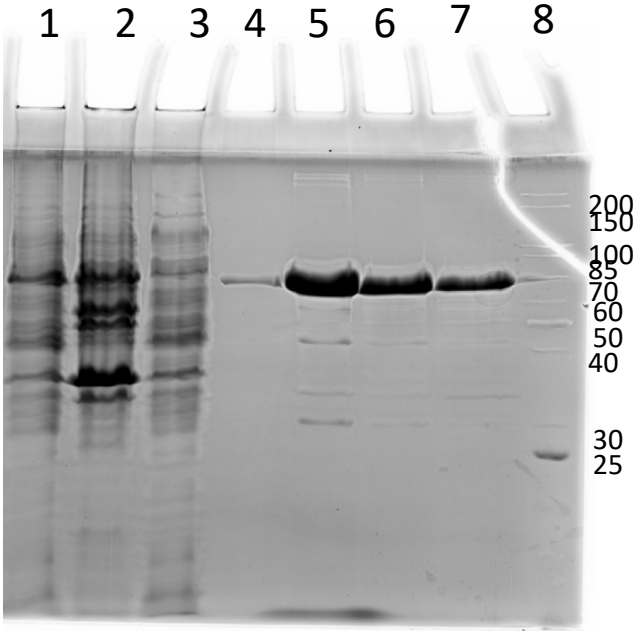
After Centrifugation

Affinity purification using Ni-NTA bead:

Affinity purification includes steps such as binding of target protein, washing off non-specifically bound proteins, elution with varying concentration of imidazole. Samples were stored from each step of purification for running SDS-PAGE. During washing steps, in presence of low concentration of Immidazole (50mM), non-specifically bound proteins were eluted. Which were having lower affinity than the targeted CiaB protein.When the concentration of Immidazole was increased in Buffers step-wise, CiaB protein elutes. The presence of CiaB in the eluted sample was confirmed by the protein band in the SDS-PAGE corresponding to the size of 73 kDa.

Affinity purification using Ni-NTA bead

SDS-PAGE ANALYSIS OF CiaB Protein Purification



SDS-PAGE Gel Picture of purified proteins

L1: superernatant; L2: pellet, L3: Flow through; L4:Wash; L5:Elution1; L6: Elution 2; L7: Elution 8; L8: Molecular weight ladder.

Elute 1: 50mM Immidazole; Elute 2: 300mM Immidazole; Elute 3: 1M Immidazole.

SDS- PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis)

Electrophoresis is the migration of charged molecules in solution in response to an electric field. The rate of migration of protein molecules in electrophoresis largely depends on the charge, size and shape of the molecules. When proteins are treated with sodium dodecyl sulphate (SDS), an anionic detergent; SDS denatures and binds to proteins fairly specifically in a mass ratio of 1.4:1. Thus, it confers a negative charge to the protein in proportion to its length. Normally a reducing agent such as 2-mercaptoethanol or dithiothreitol is induced in order to separate the polypeptide subunits to ensure a separation based exclusively on molecular weight. In denaturing SDS-PAGE separations therefore, migration is determined solely by the molecular size of the protein molecules. As a tool, SDS-PAGE is used analytically to separate the proteins in a complex mixture and to determine the molecular weight of individual proteins.

Reagents

i) Acrylamide stock (30%)

Acrylamide	29.2 g
N, N'- bis-acrylamide	0.8 g
Distilled water	Up to 100 ml

The ingredients will be mixed thoroughly with the help of a magnetic stirrer and filtered through a Whatman No 1 filter paper and stored in an amber coloured bottle at 4º C.

ii) 1.5M Tris HCl, pH 8.8

Tris	18.15 g
Distilled water	80 ml

The pH is adjusted to 8.8 with conc. HCl and the volume is made up to 100 ml with distilled water.

iii) 0.5M Tris HCl, pH 6.8

Tris	3.03 g
Distilled water	40 ml

The pH is adjusted to 6.8 with conc. HCl and the volume is made up to 50 ml with distilled water.

iv) 10% SDS

SDS	1 g
Distilled water	10 ml

v) 10% APS (Ammonium persulphate)

APS	0.01 g
Distilled water	0.1 ml

vi) TEMED (N, N, N', N' – Tetramethylethylenediamine)

vii) 4X Sample buffer

30% Glycerol	7.5 ml
1M Tris HCl, pH 6.8	5.0 ml
12% SDS	3g
6% β- Mercaptoethanol	1.5 ml
0.05% ServaBlue	0.0125g
Distilled water	Make upto 25ml

viii) Electrode buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3)

Tris	0.9 g
Glycine	4.32 g
SDS	0.3 g
Distilled water	300 ml

ix) CBB Staining solution

Coomassie brilliant blue (R-250)	0.5 g
Methanol	200 ml
Acetic acid	40 ml
Distilled water	160 ml

x) Destaining solution

Methanol	150 ml
Acetic acid	35 ml
Distilled water	315 ml

Gel polymerization mixture (for 2 gels)

Reagents	Separating gel	Stacking gel
	10% acrylamide	5% acrylamide
Acrylamide stock (30%)	3.34 ml	0.67 ml
Tris-HCl, pH 8.8	2.5 ml	-
Tris-HCl, pH 6.8	-	0.50 ml
SDS (10%)	100 µl	40 µl
APS	5.0 µl	6.0 µl
TEMED	5.0 µl	6.0 µl
Distilled water	4.0 ml	2.79 ml

Western blotting

Western blotting is a technique to identify a specific protein of interest in an electrophoresed gel. Protein samples separated by SDS-PAGE are transferred electrophoretically to PVDF membrane. Proteins immobilized on PVDF are incubated with an antibody (first antibody) against a specific protein. A second antibody-enzyme conjugate (antibody against the first antibody conjugated to an enzyme) is then allowed to react with the first antibody. Adding an appropriate substrate for the enzyme in the conjugate, a product is developed at the antigen-antibody complex site to identify the specific protein band.

Reagents

i) Transfer buffer (25mM Tris base, 192mM Glycine and 20% methanol)

Tris	3.03 g
Glycine	14.4 g
Distilled water	800 ml

Methanol	200 ml

ii) TBS (Tris buffer saline, 20 mM Tris,150 mM Sodium Chloride, pH 7.4)

Tris	2.42 g
Sodium chloride	8.76 g
Distilled water	900 ml

The pH of the solution was adjusted to 7.4 with conc. HCl and the volume was adjusted to 1000ml with distilled water.

iii) TBST

TBS	200 ml
Tween20	0.1 ml

iv) Blocking agent

Skim milk powder	2.5 g
TBS	50 ml

Method

Following SDS-PAGE the gel is equilibrated in transfer buffer for 30 minutes at room temperature. A PVDF membrane is cut slightly larger than the size of the gel and the membrane is made wet in transfer buffer (To avoid membrane contamination, gloves should be used while handling the membrane). Two fiber pads and two pre-cut filter papers are soaked in transfer buffer. Trapping of air bubbles are avoided in the fiber pads and filter papers during the wetting process. The complete transfer unit is closed; power supply is connected and is made to run at 100 volts for 2 hour. After the run is over, the cassette is disassembled and the PVDF membrane is removed carefully. The membrane is incubated in blocking agent for 3 hours at room temperature. The membrane is washed thrice with TBST at 15 minute time interval. The membrane is incubated in an antibody solution (against the protein of interest) for one hour and washed thrice with TBST. Antibody-enzyme conjugate is added and incubated for one hour. Again the membrane is washed with TBST for three times and then the specific substrate is added for the enzyme used in conjugate. Luminol solution is applied prior to auto-radiography.

Centurion University

WORKSHOP ON PROTEIN PURIFICATION AND SDS-PAGE

Organized by

Department of Zoology and Center for Genetics and Genomics

01-02-2023

S.I. No.	Name of the participant	Signature
1	Bishwajeta Jena	Bishwajeta Jena
2	Ipsita Patro	Ipsita Patro
3	Bilash Ranjan Nayak	Bilash Ranjan Nayak
4	Samapika Chaudhury	Samapika Chaudhury
5	Monalisha Rautaray	Monalisha Rautaray
6	Romyaprakash Prusty	Romyaprakash Prusty
7	Ipsita Nayak	Ipsita Nayak
8	Adyasha Singh	Adyasha Singh
9	Ruchismita Mohanty	Ruchismita Mohanty
10	Binapani Sahu	Binapani Sahu
11	Mahima Manojyoti Aran	Mahima Manojyoti Aran
12	Manusruti Sahu	Manusruti Sahu.
13	Tan Thakur	Tan Thakur
14	Gargi Patel	Gargi Patel.
15	Abhipsa Samal	Abhipsa Samal
16	Payal Patel	Payal Patel
17	Bharati Gupta	Bharati Gupta
18	Sthitaprajnya Mishra	Sthitaprajnya Mishra
19	Anjali Lata Sahoo	Anjali Lata Sahoo.
20.	Sonit Bhusan Patanaik	Sonit Bhusan Patanaik