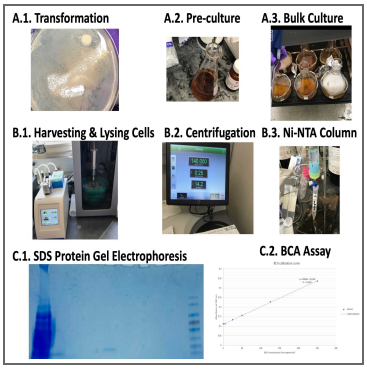

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Expression and Purification of SARS-CoV-2 Main Protease (Nonstructural Protein 5, NSP5)

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ABSTRACT

SARS-CoV-2 is the virus that caused the global COVID-19 pandemic in 2020-2023. This protocol describes how to express and purify the main protease of SARS-CoV-2 (nonstructural protein 5, NSP5) with a C-terminal hexahistidine tag. SARS-CoV-2 main protease is the enzyme (protein) consisting of 306 amino acid residues, responsible for the release of other important proteins in the virus' life cycle (i.e. NSPs 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16), including RNA polymerase (NSP12), which copies the virus' genome in the host. The inhibition of SARS-CoV-2 main protease activity is a strategy to kill the virus.

- The purified protein in this protocol was used to biochemically characterize SARS-CoV-2 main protease and identify a small molecule inhibitor (i.e. arteannuin B) in a previously published manuscript from our research lab: Varela, K.; Arman, H.D.; Berger, M.S.; Sponsel, V.M.; Lin, C.-H.A.; Yoshimoto, F.K. Inhibition of Cysteine Proteases via Thiol Michael Addition Explains the Anti-SARS-CoV-2 and Bioactive Properties of Arteannuin B. *J. Nat. Prod.* 2023, 86, 1654-1666.
- The purified protein was determined to be active through an assay using a model peptide substrate (4-fluoro-FLQS sequence). The peptide product (4-fluoro-FL) was detected using liquid chromatography mass spectrometry.
- The protease activity was inhibited with arteannuin B, a natural product isolated from the plant, *Artemisia annua*. Arteannuin B blocked the activity of the enzyme by forming a covalent bond with the active site cysteine residue of SARS-CoV-2 main protease (cysteine-145). Covalent bond formation was determined by mass spectrometry.

MATERIALS

A.1. Transformation of Plasmid into Bacteria (Day 1)

Materials:

Protocol status: Working
We use this protocol and it's working

Created: Sep 18, 2023

Last Modified: Oct 16, 2023

PROTOCOL integer ID: 87963

Keywords: SARS-CoV-2, Protein expression and purification, Cysteine protease, Enzyme, Virus, COVID-19, Biochemistry, Transformation, Induction, Nickel NTA Column Chromatography, Centrifugation, Ultracentrifugation, SDS Protein Gel Electrophoresis, Coomassie Brilliant Blue, BCA Assay, Ultrasonic Dismembrator, Cell Lysis, Chaperones, Plasmid DNA, Incubator shaker, Micropipette, UV-Vis Spectrophotometer, BL-21 Competent cells

Funders Acknowledgement:
Max and Minnie Tomerlin Voelcker Fund
Grant ID: The Max and Minnie Tomerlin Voelcker Fund Young Investigator Award

a. -78 ºC freezer
b. ice in bucket
c. Nitrile gloves
d. Lab coat
e. Safety glasses
f. BL21 cells (Thermo Scientific Catalog number: EC0114)
g. 2 mL microcentrifuge tube
h. SOC (Super Optimal broth with Catabolite repression) Media (Thermo Scientific Catalog number: 15544034)
i. plasmid DNA (encoding cDNA of desired protein as described in reference [1])
j. plasmid of chaperone protein DNA (to help with protein folding)
k. floating foam tube rack (Fisherbrand, Catalog number: 36-099-2328)
l. incubator shaker (Eppendorf New Brunswick Innova 44)
m. incubator set to 37 ºC
n. water bath set at 42 ºC
o. LB agar plate with ampicillin and kanamycin (as described in reference [1])
p. micropipettes (1-10 µl, 20-100 µl, 100 µl-1000 µl)
q. micropipette tips
r. spreader to spread transformed cells onto LB agar plate

A.2. Pre-Culture (Before Bulk Culture) (Day 2)

Materials:
a. Nitrile gloves
b. Lab coat
c. Safety glasses
d. autoclave
e. autoclave gloves
f. autoclave trays
g. autoclave tape
h. aluminum foil
i. cart (to hold autoclave bin and supplies)
j. Analytical balance
k. 250 ml Erlenmeyer flask
l. 5 g of TB media
m. 125 ml of deionized water
n. ampicillin solution (25 mg of ampicillin in 250 µl of deionized water)
o. kanamycin solution (12.5 mg of kanamycin in 250 µl of deionized water)
p. Incubator shaker

A.3. Bulk Culture (Induction of Protein Expression) (Day 3 then Days 4-6)

Materials:

a. Nitrile gloves
b. Lab coat
c. Safety glasses
d. 6 1-L Fernbach flasks
e. Incubator shaker (Innova)
f. Analytical balance
g. 47.5 g of TB media **for each 1-L of bulk culture (6 L total)**
h. 5 ml of glycerol **for each 1-L of bulk culture (6 L total)**
i. Autoclave
j. thiamine solution (2.38 g thiamine in 14 ml of water)
k. Double beam UV/Vis spectrophotometer (VWR, UV-6300PC)
l. 1 ml disposable cuvette (to check the optical density 600 nm after inducing protein expression)
m. IPTG solution (1.67 g of IPTG in 14 ml of water for the induction of cysteine protease)
n. arabinose solution (6.3 g of arabinose in 14 ml of water for the induction of chaperone proteins)
o. Graduated cylinder (100 mL)
p. Overnight pre-culture solution from **Section A.2.**
q. Refrigerator
r. Ti 45 rotor (for the ultracentrifuge in the next section)

B.1. Harvesting and Lysing the Cells (Day 7)

Materials:

a. Nitrile gloves
b. Lab coat
c. Safety glasses
d. Centrifuge (Beckman Coulter, Avanti JXN-26)
e. Ohaus scale (2kg-5lb Capacity, Harvard Trip I400/I500 Series)
f. 6x 1 L centrifuge bottles
g. 500 mL beaker
h. Stir bar
i. Stir plate
j. Cold box (refrigerator)
k. Analytical balance
l. Buffer A:
   (i) Tris HCl (1 M, 50 mL),
   (ii) water (450 mL),
   (iii) sucrose (85.6 g),
   (iv) EDTA disodium salt (93 mg))

m. Spatula
n. Rubber policeman
o. Sonic dismembrator (Fisherbrand, Model CL-334)
p. Bucket of ice

B.2. Centrifuging the Lysed Cells (Day 7)

Materials:
a. Nitrile gloves
b. Lab coat
c. Safety glasses
d. Centrifuge (Beckman Coulter, Avanti JXN-26)
e. Ohaus scale (2kg-5lb Capacity, Harvard Trip I400/I500 Series)
f. Lysed Cells from Step 15 in the previous section (Purification of the Proteins I)
g. Ultracentrifuge (Beckman Coulter, Optima XPN-80)
h. 6x ultracentrifuge tubes (for Ti-45 rotor)
i. Cooled down Ti-45 Rotor from Section **A.3.** (for ultracentrifuge)
j. Bucket of ice
k. Beckman Coulter tube removal tool (Beckman Coulter 301875) (can also use skinny spatula)

B.3. Purification of the Supernatant from the Ultracentrifuge Step (Day 7)

Materials:
a. Nitrile gloves
b. Lab coat
c. Safety glasses
d. Ni nitriloacetic acid (NTA) resin (500 ml commercial bottle, Thermo Scientific Product #88223)
- Up to three chromatography columns were run (50 mL volume of Ni NTA resin for each run) depending on how pure the protein looks by SDS protein gel electrophoresis (usually two columns may be enough)

e. Column
f. Adaptors for column
g. Cold box (refrigerator)
h. Stir plate
i. Stir bar
j. Analytical balance
k. Spatula
l. 50 mL Plastic disposable syringe

m. Clamp to hold the column
n. Stand to hold the clamp for the column
o. 50 mL Falcon tubes to collect the protein fractions (up to 20)
p. Tube rack to hold the 50 mL Falcon tubes
q. Sharpee to label Falcon tubes

r. Buffers for eluting the protein out of the nickel column:

-Buffer B (500 mL volume of: 100 mM pH 7.4 KPhos, 100 µM DTT, 20% glycerol (v/v)):
(i) K_2HPO_4 (potassium phosphate dibasic, 6.059 g)
(ii) KH_2PO_4 (potassium phosphate monobasic, 2.07 g)
(iii) Glycerol (100 mL)
(iv) Water (400 mL)

-Buffer C (300 mM imidazole of "Buffer B"):
(i) Buffer B (200 mL)
(ii) Imidazole (4.08 g)

-Buffer D (40 mM imidazole of "Buffer B"):
(i) Buffer B (87.7 mL)
(ii) Buffer C (13.3 mL)

-**Alternative Method for Buffer D** (40 mM imidazole of "Buffer B"):
(i) Buffer B (500 mL)
(ii) 1.35 g of imidazole

-Buffer E (70 mM imidazole of "Buffer B"):
(i) Buffer B (87.7 mL)
(ii) Buffer C (13.3 mL)

-**Alternative Method for Buffer E** (70 mM imidazole of "Buffer B"):
(i) Buffer B (200 mL)
(ii) 0.91 g of imidazole

s. Amicon filter (30 kDa, Millipore Sigma, catalog number: UFC903024)
t. Centrifuge (Beckman Coulter, Avanti JXN-26)

C.1. Analysis of the Purified Proteins I: SDS Protein Gel Electrophoresis (Days 7-8)

Materials:

a. Nitrile gloves
b. Lab coat
c. Safety glasses
d. Power Supply for Electrophoresis (Thermo Scientific, EPS 300X)
e. Invitrogen Gels (4-20% Tris-Glycine gel, 1.0 mm x 15 well, Catalog #: XP04205BOX)
f. 1-10 µL micropipette
g. Gel loading tips for 10 µL micropipette (longer tips)
h. Protein Ladder (10-250 kDa protein ladder, Thermo Scientific, Catalog #: 26619)
i. pH meter for making solutions (SI Analytics, Lab 850)
j. Analytical balance (to weigh miligram to g quantities)
k. pipettes (100-1000 µL volume)
l. 1000 µL pipet tips
m. 50 mL Falcon tube
n. Denaturing solution for proteins (**10 mL of 4x stock solution**):
(i) 1 M Tris HCl buffer, pH 6.8 **[Amount: 2.5 mL]** (121.1 g of tris base in 800 mL of water and the pH
is adjusted using a pH meter to 6.8 using concentrated HCl)
(ii) Water **[Amount: 0.5 mL]**
(iii) Sodium dodecyl sulfate (SDS) **[Amount: 1.0 g]**
(iv) Bromophenol blue **[Amount: 0.8 mL of 0.1% w/v solution]** (Dissolve 10 mg of bromophenol
blue in 1 mL of water to make a 0.1% w/v solution)
(v) Glycerol **[Amount: 4.0 mL]**
(iv) Beta-mercaptoethanol **[Amount: 2.0 mL]**
o. Eluted protein samples from Ni-column (**Section B.3.**, **Step 15**)
p. Styrofoam floaty (to heat 2-mL microcentrifuge tubes)
q. 2 mL microcentrifuge tubes (at least 10, for each protein sample)
r. Water bath at 90 ºC
s. Running Buffer (10X, pH 8.3):
(i) Tris base (30 g)
(ii) Glycine (144 g)
(iii) Sodium dodecyl sulfate (10 g)
(iv) Water (1000 mL)
t. Running Buffer (1X - **make fresh or gel will not stain properly - it will smear**):
(i) 10X Running Buffer (100 mL)
(ii) Water (900 mL)
u. Pyrex Crystallizing Dish (for staining solution, 740 mL capacity)
v. Pyrex Crystallizing Dish (for destaining solution, 740 mL capacity)
w. Aluminum foil
x. Saran wrap
y. Staining solution for protein gel (500 mL)
(i) Coomassie brilliant blue G-250 (1 g)
(ii) Methanol (500 mL)

(iii) Water (400 mL)
(ii) Acetic acid (100 mL)

z. Destaining solution for protein gel (500 mL)
(i) Acetic acid (100 mL)
(ii) Methanol (100 mL)
(iii) Water (800 mL)

C.2. Analysis of the Purified Proteins II: BCA Assay to Quantify Purified Protein (Day 8)

Materials:

a. Nitrile gloves
b. Lab coat
c. Safety glasses
d. Double beam UV/Vis spectrophotometer (VWR, UV-6300PC)
e. BCA assay kit (Thermo Scientific, Pierce^{TM} Bicinchoninic Acid) Protein Assay Kit, Product #:
23225):
(i) BCA Protein Assay Reagent A (Product # 23228)
(ii) BCA Protein Assay Reagent B (Product # 1859078)
(iii) Bovine Serum Albumin (BSA) Standard (2 mg/mL concentration) (Product # 23209)
f. 50 mL Falcon tube to make the working reagent (WR)
g. Styrofoam floaty
h. 20x 2 mL microcentrifuge tubes
i. 37 ºC shaking water bath
j. 1 mL plastic cuvettes
k. Microsoft Excel on computer to make the calibration curve of Solutions A-F (Abs at 562 nm vs. concentration, y vs. x)

SAFETY WARNINGS

Proper personal protective equipment was worn at all times:
-Safety glasses
-Nitrile gloves
-Lab coat

Chemical Fumehood was used when the protein denaturing solution was made and employed (beta-mercaptoethanol has a strong odor)

Table of Contents: Expression and Purification of SARS-CoV-2...

1 **Table of Contents for Expression and Purification of SARS-CoV-2 Main Protease**

A.1. Expression of Proteins in Bacteria I - Transformation (Day...

2 **A.1. Transformation of Plasmid into Bacteria (Day 1)**

Materials:

a. -78 ºC freezer
b. ice in bucket
c. Nitrile gloves
d. Lab coat
e. Safety glasses
f. BL21 cells (Thermo Scientific Catalog number: EC0114)
g. 2 mL microcentrifuge tube
h. SOC (Super Optimal broth with Catabolite repression) Media (Thermo Scientific Catalog number: 15544034)
i. plasmid DNA (encoding cDNA of desired protein as described in reference [1])
j. plasmid of chaperone protein DNA (to help with protein folding)
k. floating foam tube rack (Fisherbrand, Catalog number: 36-099-2328)
l. incubator shaker (Eppendorf New Brunswick Innova 44)
m. incubator set to 37 ºC
n. water bath set at 42 ºC
o. LB agar plate with ampicillin and kanamycin (as described in reference [1])
p. micropipettes (1-10 µl, 20-100 µl, 100 µl-1000 µl)
q. micropipette tips
r. spreader to spread transformed cells onto LB agar plate

Procedure:

1. Thaw BL21 cells in ice bucket
2. Add 1 µl plasmid DNA for protease expression and 1 µl of plasmid DNA for chaperone proteins to BL21 cells with the 1 µl pipette
3. Leave cells with plasmid on ice for 30 minutes
4. Heat shock the cells for 30 seconds at 42 ºC in the water bath
5. Cool cells back on ice for 20 minutes
6. Add SOC media (200 µl)
7. Shake the cells at 225 rpm at 37 ºC in the incubator shaker
8. After 1.5 hr, spread the SOC media that was shaken in step 7 on the LB agar plate
9. Leave LB agar plate in 37 ºC incubator overnight
10. If there is extra time, do **Steps 1-6** in the next section (**Section A.2.**, Day 2) if there is time

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A.2. Expression of Proteins in Bacteria II - Pre-Culture (Day 2)

3 **A.2. Pre-Culture (Before Bulk Culture) (Day 2)**

Materials:

a. Nitrile gloves
b. Lab coat
c. Safety glasses
d. autoclave
e. autoclave gloves
f. autoclave trays
g. autoclave tape
h. aluminum foil
i. cart (to hold autoclave bin and supplies)
j. Analytical balance
k. 250 ml Erlenmeyer flask
l. 5 g of TB media
m. 125 ml of deionized water
n. ampicillin solution (25 mg of ampicillin in 250 µl of deionized water)
o. kanamycin solution (12.5 mg of kanamycin in 250 µl of deionized water)
p. Incubator shaker

Procedure:

1. Add 5 g of TB media to 125 ml of deionized water
2. **for tomorrow's experiment with the **bulk culture*** **(Section A.3.)**: add 47.5 g of TB media to 1 L

of deionized water in a Fernbach flask. Do this step for 6 different Fernbach flasks
3. Cap the TB media solutions with aluminum foil and add autoclave tape on the top
4. Autoclave the solutions in steps 1 and 2 (~1-2 hours)
5. Once the autoclaved solutions are cooled to room temperature, add 100 µl of ampicillin solution and 100 µl of kanamycin solution to the preculture media.
6. Also add 1 ml of ampicillin solution and 1 ml of kanamycin solution to each 1 L of the bulk culture media after the autoclaved media are cooled to room temperature.
7. Pick a colony of transformed bacteria using a 100 µl pipette with a pipet tip
8. Inoculate a colony of bacteria into the preculture media (125 ml of TB media) and shake overnight at 225 rpm, 37 ºC

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h

A.3. Expression of Proteins in Bacteria III - Bulk Culture (Day ...

4 **A.3. Bulk Culture (Induction of Protein Expression) (Day 3 then Days 4-6)**

Materials:

a. Nitrile gloves
b. Lab coat
c. Safety glasses
d. 6 1-L Fernbach flasks
e. Incubator shaker (Innova)
f. Analytical balance
g. 47.5 g of TB media **for each 1-L of bulk culture (6 L total)**
h. 5 ml of glycerol **for each 1-L of bulk culture (6 L total)**
i. Autoclave
j. thiamine solution (2.38 g thiamine in 14 ml of water)
k. Double beam UV/Vis spectrophotometer (VWR, UV-6300PC)
l. 1 ml disposable cuvette (to check the optical density 600 nm after inducing protein expression)
m. IPTG solution (1.67 g of IPTG in 14 ml of water for the induction of cysteine protease)
n. arabinose solution (6.3 g of arabinose in 14 ml of water for the induction of chaperone proteins)
o. Graduated cylinder (100 mL)
p. Overnight pre-culture solution from **Section A.2.**
q. Refrigerator

r. Ti 45 rotor (for the ultracentrifuge in the next section)

Procedure:

1. Turn on the UV/Vis spectrophotometer to warm up the lamp (takes about 15 minutes)
2. Add 18 ml of preculture to 1 L of the bulk culture solution (Day 3)
3. Add 2 ml of thiamine solution to each 1 L of the bulk culture
4. Shake the 6 1-L Fernbach flasks at 190 rpm, 37 ºC
5. After 2.5 hr, the optical density at 600 nm (OD_{600}) was checked on a UV/Vis spectrophotometer, which confirmed absorbance at 0.7
6. After confirming OD_{600} was 0.7, each 1 L of bulk culture was induced with 2 ml of IPTG solution and 2 ml of arabinose
7. The temperature of the shaker was lowered to 28 ºC and the shaking was reduced to 150 rpm
8. The 6 L of bulk culture continued to shake at 150 rpm at 28 ºC for 72 hours (Days 4-6)
9. Place the Ti 45 rotor in the refrigerator to cool down the rotor the day before using the ultracentrifuge (for the ultracentrifuge in Section **B.2.**)

h

B.1. Purification of the Proteins I: Harvesting and Lysing the …

5 **B.1. Harvesting and Lysing the Cells (Day 7)**

Materials:

a. Nitrile gloves
b. Lab coat
c. Safety glasses
d. Centrifuge (Beckman Coulter, Avanti JXN-26)
e. Ohaus scale (2kg-5lb Capacity, Harvard Trip I400/I500 Series)
f. 6x 1 L centrifuge bottles
g. 500 mL beaker
h. Stir bar
i. Stir plate
j. Cold box (refrigerator)
k. Analytical balance
l. Buffer A:
   (i) Tris HCl (1 M, 50 mL),
   (ii) water (450 mL),
   (iii) sucrose (85.6 g),
   (iv) EDTA disodium salt (93 mg))
m. Spatula

n. Rubber policeman
o. Sonic dismembrator (Fisherbrand, Model CL-334)
p. Bucket of ice

Procedure:

1. Pellet the 6 L of bulk culture from the expression using the 6x 1 L centrifuge bottles
2. When measuring out the bulk culture into the bottles, use the Ohaus balance to ensure that the bottles weigh the same before adding them to the rotor
3. Also do not fill up the bottles passed 3/4 of volume capacity to avoid the liquid from splashing out during centrifugation
4. The centrifuge was set to 4,000 rpm (5,000 x g) at 0 ºC for 30 minutes
5. After the pellets were formed, the supernatant was transferred out of the bottles into a 4 L Erlenmeyer flask and set aside
6. The rest of the 6 L bulk culture was added to the bottles and centrifuged to pellet the cells once again
7. A 500 mL beaker was tared
8. Buffer A was made: (i) Tris HCl (1 M, 50 mL), (ii) water (450 mL), (iii) sucrose (85.6 g), (iv) EDTA disodium salt (93 mg)
9. Once the cells were completely pelleted and the pellets were transferred to the tared beaker. The weight of the pellets were taken (~100 g).
10. 100 mL of Buffer A was added to the pellet
11. All subsequent steps were performed at 0 ºC
12. The pellet suspension in Buffer A was sonicated for 1 minute under ice
13. The sonicated suspension was placed under ice for 1 minute
14. Steps 12 and 13 were repeated two more times
15. The solution after Step 14 was added to centrifuge bottles

h

B.2. Purification of the Proteins II: Centrifuging the Lysed Cel...

6 B.2. Centrifuging the Lysed Cells (Day 7)

Materials:

a. Nitrile gloves
b. Lab coat
c. Safety glasses
d. Centrifuge (Beckman Coulter, Avanti JXN-26)
e. Ohaus scale (2kg-5lb Capacity, Harvard Trip I400/I500 Series)
f. Lysed Cells from Step 15 in the previous section (Purification of the Proteins I)

g. Ultracentrifuge (Beckman Coulter, Optima XPN-80)
h. 6x ultracentrifuge tubes (for Ti-45 rotor)
i. Cooled down Ti-45 Rotor from Section **A.3.** (for ultracentrifuge)
j. Bucket of ice
k. Beckman Coulter tube removal tool (Beckman Coulter 301875) (can also use skinny spatula)

Procedure:

1. Turn on the ultracentrifuge and start the vacuum (set to 0 µm)
2. Using the Centrifuge (JXN-26), the cells were centrifuged at 14,000 x g (0 ºC) for 1 hour
3. The resulting supernatant was transferred to 6 ultracentrifuge bottles (the liquid was only added 3/4 to the capacity of the bottles)
4. The cooled down Ti-45 rotor is removed from the refrigerator
5. The ultracentrifuge bottles are properly balanced and placed in the Ti-45 rotor using the Ohaus balance
6. Centrifuge the supernatant from Step 3 at 142,000 x g (0 ºC) for 2 hours
7. Repeat Steps 3 and 4 if any supernatant was left over after completing Step 3 the first time
8. The more that **Step 5** is performed, there will be more pellet collected, which will help with the flow of the column with the eluent in **Section B.3.**

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B.3. Purification of the Proteins III: Protein Purification by Ni-...

7 **B.3. Purification of the Supernatant from the Ultracentrifuge Step (Day 7)**

Materials:

a. Nitrile gloves
b. Lab coat
c. Safety glasses
d. Ni nitriloacetic acid (NTA) resin (500 ml commercial bottle, Thermo Scientific Product #88223)
- Up to three chromatography columns were run (50 mL volume of Ni NTA resin for each run) depending on how pure the protein looks by SDS protein gel electrophoresis (usually two columns may be enough)

e. Column
f. Adaptors for column
g. Cold box (refrigerator)
h. Stir plate
i. Stir bar
j. Analytical balance

k. Spatula
l. 50 mL Plastic disposable syringe
m. Clamp to hold the column
n. Stand to hold the clamp for the column
o. 50 mL Falcon tubes to collect the protein fractions (up to 20)
p. Tube rack to hold the 50 mL Falcon tubes
q. Sharpee to label Falcon tubes

r. Buffers for eluting the protein out of the nickel column:

-Buffer B (500 mL volume of: 100 mM pH 7.4 KPhos, 100 µM DTT, 20% glycerol (v/v)):
(i) K_2HPO_4 (potassium phosphate dibasic, 6.059 g)
(ii) KH_2PO_4 (potassium phosphate monobasic, 2.07 g)
(iii) Glycerol (100 mL)
(iv) Water (400 mL)

-Buffer C (300 mM imidazole of "Buffer B"):
(i) Buffer B (200 mL)
(ii) Imidazole (4.08 g)

-Buffer D (40 mM imidazole of "Buffer B"):
(i) Buffer B (87.7 mL)
(ii) Buffer C (13.3 mL)

-**Alternative Method for Buffer D** (40 mM imidazole of "Buffer B"):
(i) Buffer B (500 mL)
(ii) 1.35 g of imidazole

-Buffer E (70 mM imidazole of "Buffer B"):
(i) Buffer B (87.7 mL)
(ii) Buffer C (13.3 mL)

-**Alternative Method for Buffer E** (70 mM imidazole of "Buffer B"):
(i) Buffer B (200 mL)
(ii) 0.91 g of imidazole

s. Amicon filter (30 kDa, Millipore Sigma, catalog number: UFC903024)
t. Centrifuge (Beckman Coulter, Avanti JXN-26)

Procedure:
1. Prepare the nickel column by shaking the resin bottle so the solid is evenly dispersed in the solution
2. Pour the nickel resin into the column (approximately 35-50 mL volume)
3. Let the liquid filter out of the column so the nickel resin is packed - use either gravity or push

the liquid out using the 50 mL disposable syringe attached to the cap connecting the column

4. Once the resin has settled in the column and there is a bed of liquid (1-2 cm) above the resin, load the column with the supernatant from **Section B.2. (Step 6)**.
5. Use the syringe to help with the flow of the solution through the column
6. Once the liquid reaches 1-2 cm above the resin, run 200 mL of Buffer B through the column
7. Collect the eluent with 50 mL Falcon tubes
8. Once Buffer B is completely added (leave 1-2 cm of the liquid above the resin bed), then add Buffer D (40 mM imidazole, 100 mL) and collect the eluent with 50 mL Falcon tubes
9. Once Buffer D is completely added (leave 1-2 cm of the liquid above the resin bed), then add Buffer E (70 mM imidazole, 100 mL) and collect the eluent with 50 mL Falcon tubes
10. Once the Buffer E is completely added (leave 1-2 cm of the liquid above the resin bed), then add Buffer C (300 mM imidazole, 100 mL) and collect the eluent with 50 mL Falcon tubes
11. After all of the Buffer C flows through the column, separately concentrate the different collected fractions in **Steps 7-10 (Section B.3.)** using four different Amicon filters (use a different Amicon filter for the different Buffers used to elute off of the column B, C, D, and E) - label the Amicon filters with the buffer being used.
12. Specifically, add 3-5 mL of the eluted buffer from the column (e.g. Buffer B) into the top of the Amicon filter. In a second Amicon filter tube, add a different eluted buffer (e.g. Buffer C
13. Centrifuge the Amicon filters using the Avanti JXN-26 with the proper adaptor to fit into the centriduge at 4000 x g at 0 C for 1 hour
14. After 1 hour, remove the liquid that filters through in the tube
15. Repeat **Steps 12-14** until the protein samples are concentrated to a desired volume

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C.1. Analysis of the Purified Proteins I: SDS Protein Gel Electr...

8 **C.1. Analysis of the Purified Proteins I: SDS Protein Gel Electrophoresis (Days 7-8)**

Materials:

a. Nitrile gloves
b. Lab coat
c. Safety glasses
d. Power Supply for Electrophoresis (Thermo Scientific, EPS 300X)
e. Invitrogen Gels (4-20% Tris-Glycine gel, 1.0 mm x 15 well, Catalog #: XP04205BOX)
f. 1-10 µL micropipette
g. Gel loading tips for 10 µL micropipette (longer tips)
h. Protein Ladder (10-250 kDa protein ladder, Thermo Scientific, Catalog #: 26619)
i. pH meter for making solutions (SI Analytics, Lab 850)
j. Analytical balance (to weigh miligram to g quantities)
k. micropipettes (100-1000 µL volume)

l. 1000 µL pipet tips
m. 50 mL Falcon tube
n. Denaturing solution for proteins (**10 mL of 4x stock solution**):
(i) 1 M Tris HCl buffer, pH 6.8 **[Amount: 2.5 mL]** (121.1 g of tris base in 800 mL of water and the pH
is adjusted using a pH meter to 6.8 using concentrated HCl)
(ii) Water **[Amount: 0.5 mL]**
(iii) Sodium dodecyl sulfate (SDS) **[Amount: 1.0 g]**
(iv) Bromophenol blue **[Amount: 0.8 mL of 0.1% w/v solution]** (Dissolve 10 mg of bromophenol blue in 1 mL of water to make a 0.1% w/v solution)
(v) Glycerol **[Amount: 4.0 mL]**
(iv) Beta-mercaptoethanol **[Amount: 2.0 mL]**
o. Eluted protein samples from Ni-column (**Section B.3.**, **Step 15**)
p. Styrofoam floaty (to heat 2-mL microcentrifuge tubes)
q. 2 mL microcentrifuge tubes (at least 10, for each protein sample)
r. Water bath at 90 ºC
s. Running Buffer (10X, pH 8.3):
(i) Tris base (30 g)
(ii) Glycine (144 g)
(iii) Sodium dodecyl sulfate (10 g)
(iv) Water (1000 mL)
t. Running Buffer (1X - **make fresh or gel will not stain properly - it will smear**):
(i) 10X Running Buffer (100 mL)
(ii) Water (900 mL)
u. Pyrex Crystallizing Dish (for staining solution, 740 mL capacity)
v. Pyrex Crystallizing Dish (for destaining solution, 740 mL capacity)
w. Aluminum foil
x. Saran wrap
y. Staining solution for protein gel (500 mL)
(i) Coomassie brilliant blue G-250 (1 g)
(ii) Methanol (500 mL)
(iii) Water (400 mL)
(ii) Acetic acid (100 mL)
z. Destaining solution for protein gel (500 mL)
(i) Acetic acid (100 mL)
(ii) Methanol (100 mL)
(iii) Water (800 mL)

Procedure:
1. Start a water bath at 90 ºC in a chemical fume hood
2. Add 10 µL of protein sample to a 2 mL microcentrifuge tube that is labeled
3. Add 20 µL of denaturing solution to each of the tubes in **Step 2** (above)
4. Place tubes in styrofoam floaty
5. Heat the tubes at 90 ºC for 5 minutes in a fume hood

6. Remove the comb on the protein gel (the comb can be used to open the casing of the gel in **Step 21**, see below)
7. Remove the tape on the base of the gel to expose the gap in the plastic casing to the running buffer
8. Fit the gel into the cassette for the gel apparatus - make sure the wells face inward
9. Fill the chamber with 1X running buffer both outside and inside the chamber (~500 mL)
10. Load 10 microliter of protein into the well of each gel
11. For the loading of the protein ladder use 5 microliter from the commercially available tube
12. Fit the casing of the gel apparatus on the top of the chamber and screw on the cables
13. Turn on the power supply at 200 V
14. Run the gel for 30 minutes
15. While the gel is running, bubbles can be seen as the voltage is turned on
16. Once the proteins have traveled far enough on the gel (look at the protein ladder lane to see if the band has traveled near the end of the gel), stop the power supply
17. After turning off the power supply, remove the cables on the top of the cassette
18. Pour the staining solution in one of the Pyrex crystallizing dishes
19. Pour the destaining solution in the second Pyrex crystallizing dish
20. Unscrew the gel out of the cassette
21. Using the comb from **Step 6** (see above) or a spatula, separate the two pieces of the plastic casing of the gel
22. Place the gel in the staining solution in the Pyrex crystallizing dish and cover with aluminum foil
23. Let the gel sit in the staining solution for 30 minutes
24. Transfer the gel carefully to the destaining solution and cover with aluminum foil
25. Leave the gel in the destaining solution overnight (Day 7 to Day 8)
26. The next morning, gently remove the gel with nitrile gloves on
27. Place gel on saran wrap

h

h

C.2. Analysis of the Purified Proteins II: BCA Assay to Quantif...

9 **C.2. Analysis of the Purified Proteins II: BCA Assay to Quantify Purified Protein (Day 8)**

Materials:

a. Nitrile gloves
b. Lab coat
c. Safety glasses
d. Double beam UV/Vis spectrophotometer (VWR, UV-6300PC)
e. BCA assay kit (Thermo Scientific, Pierce™ Bicinchoninic Acid) Protein Assay Kit, Product #: 23225):
   (i) BCA Protein Assay Reagent A (Product # 23228)
   (ii) BCA Protein Assay Reagent B (Product # 1859078)
   (iii) Bovine Serum Albumin (BSA) Standard (2 mg/mL concentration) (Product # 23209)
f. 50 mL Falcon tube to make the working reagent (WR)
g. Styrofoam floaty
h. 20x 2 mL microcentrifuge tubes
i. 37 ºC shaking water bath
j. 1 mL plastic cuvettes
k. Microsoft Excel on computer to make the calibration curve of Solutions A-F (Abs at 562 nm vs. concentration, y vs. x)

Procedure:

1. Turn on the UV-Vis spectrophotometer (to warm up the lamp - about 15 minutes)
2. Make working reagent (WR): Add 25 mL BCA reagent A and 0.5 mL reagent B in a 50 mL Falcon tube
3. Make solutions A-F (for the calibration curve): (**Solution A:** 700 µL of water and 100 µL of bovine serum albumin (BSA) stock), (**Solution B:** 400 µL of water and 400 µL of solution A), (**Solution C**: 400 µL of water and 300 µL of solution B), (**Solution D**: 400 µL of water and 300 µL of solution C), (**Solution E**: 400 µL of water and 300 µL of solution D), (**Solution F**: 400 µL of water)
4. Take 6 microcentrifuge tubes and label them A, B, C, D, E, and F
5. Take 100 µL of each sample and add 0.93 mL of WR
6. For example, to the tube labeled "A" add 100 µL of solution A then add 0.93 mL of WR, etc.
7. Label a 2 mL microcentrifuge with the protein sample that will be added to the tube (e.g. "Buffer B" for protein sample eluted with buffer B from the column in **Section B.3.**)
8. Repeat **Step 7** (above) for all protein samples to be quantified
9. Add 0.93 mL of WR and then add 100 µL of the protein sample
10. Shake the samples at 37 ºC at 100 rpm for 15 minutes
11. After 15 minutes, measure the absorbance of each sample at 562 nm with the spectrophotometer
12. Record the absorbance for each sample and save the values
13. Input the values for the calibration curve into an excel spreadsheet (y-values)
14. The x-values correspond to the known concentrations of solutions A-F
15. Add a trendline to the plot generated for the calibration curve (y=mx+b)
16. Using the curve that is generated in **Step 15**, determine the concentration of the protein samples

10 **D. Photos of Select Materials.**

A.1. -78 °C Freezer.

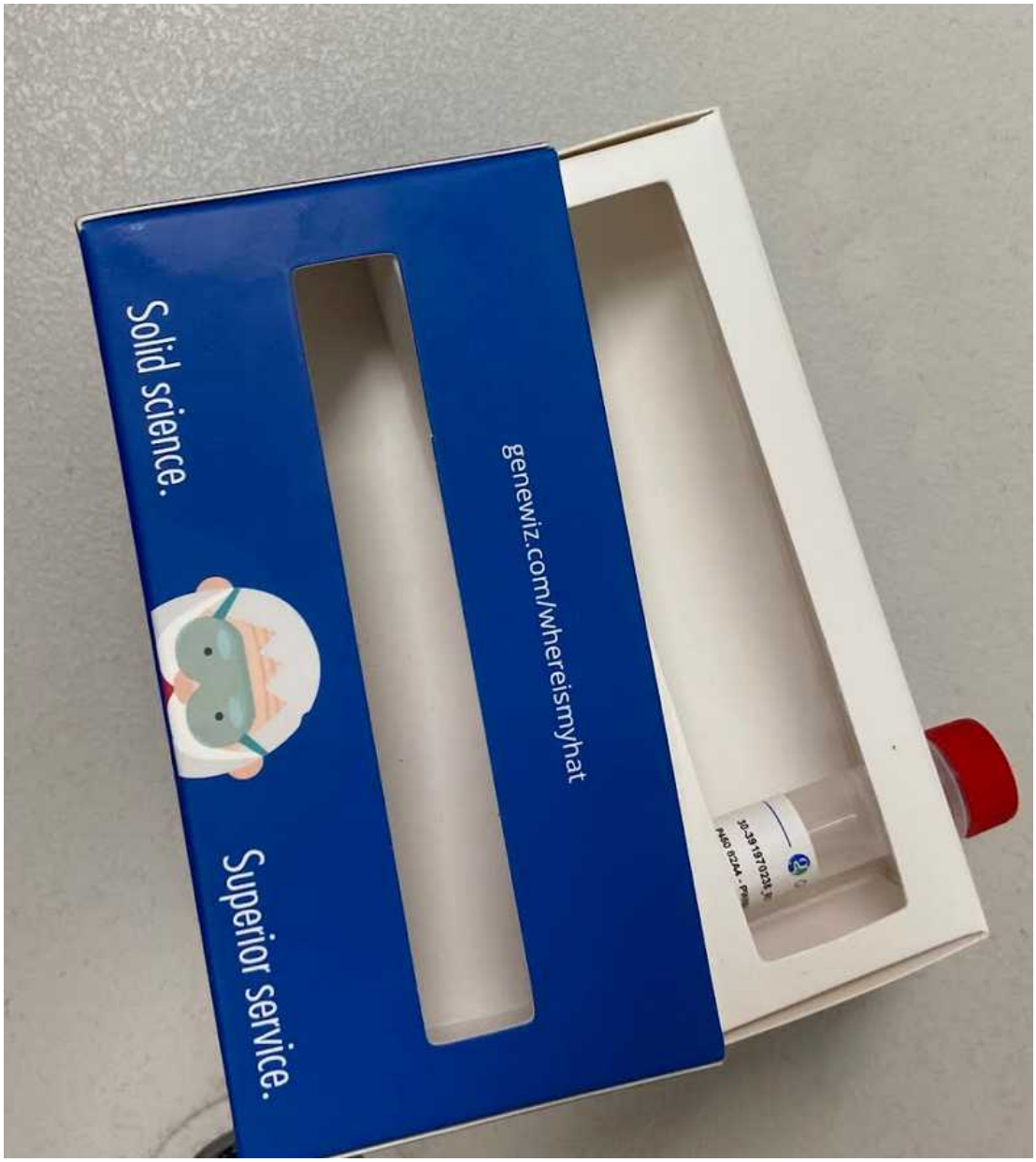
A.1. incubator shaker (Eppendorf New Brunswick Innova 44).

A.1. Shaking water bath 42 °C.

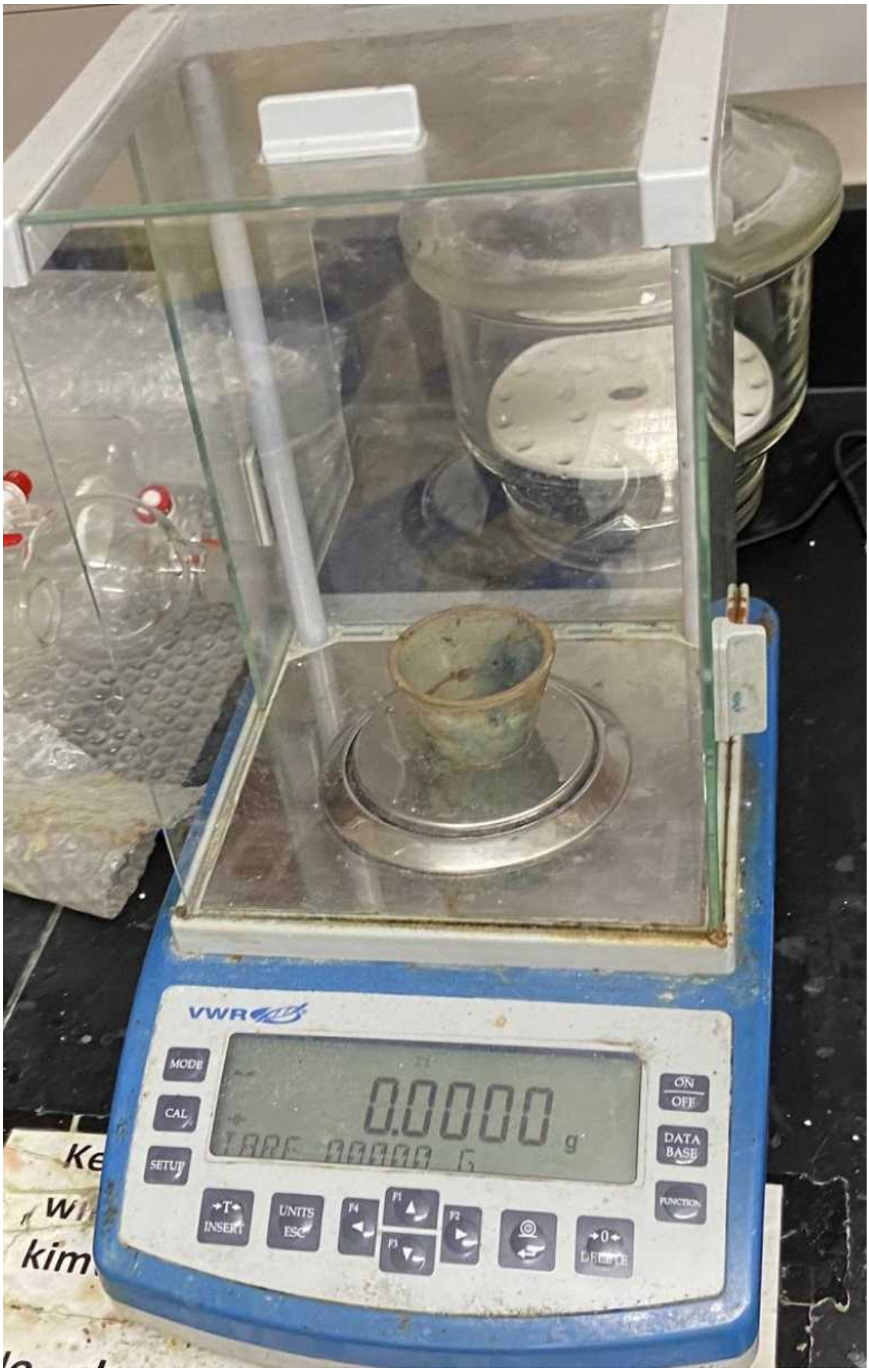
A.1. Incubator set to 37 °C.

A.1. Micropipettes (1-10 µl, 20-100 µl, 100 µl-1000 µl).

A.1. Micropipette tips. (Bottom image: gel loading tip)

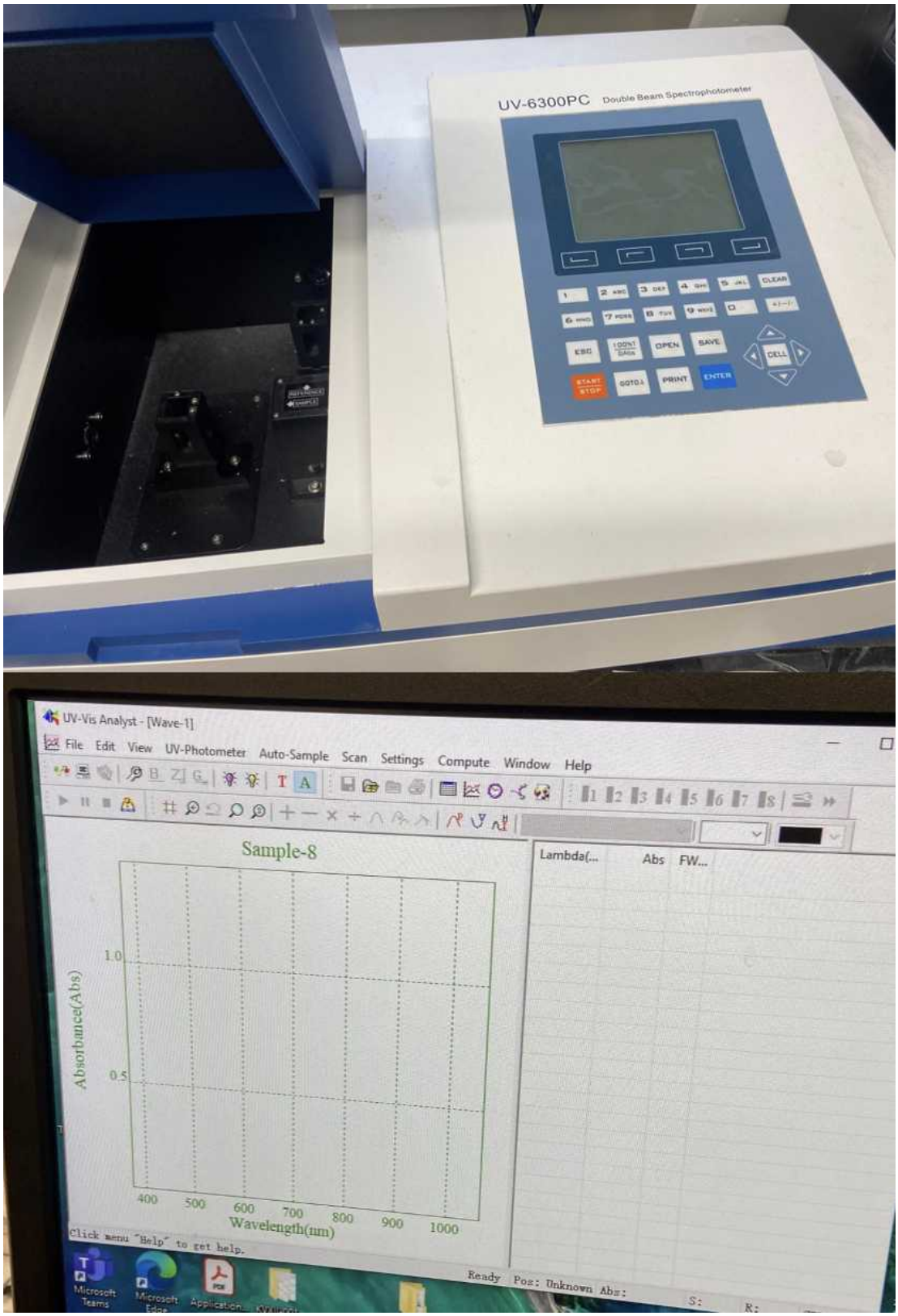


A.1. Plasmid DNA (encoding cDNA of desired protein).



A.2. Analytical balance.

A.3. 6 1-L Fernbach flasks.



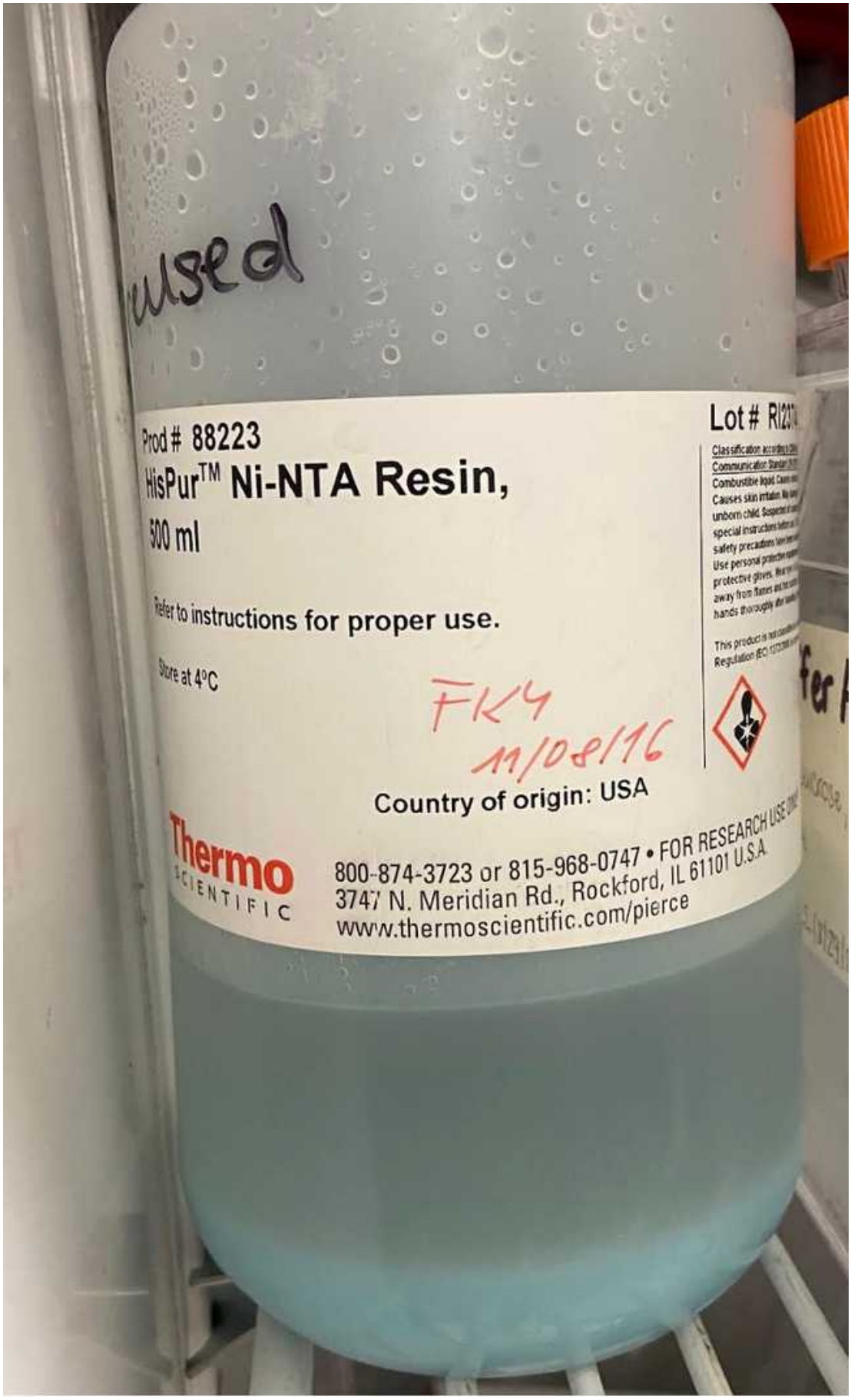
A.3. Double beam UV/Vis spectrophotometer (VWR, UV-6300PC).

B.1. Centrifuge.

B.1. Sonic dismembrator (Fisherbrand, Model CL-334).

B.2. Ti-45 rotor for the ultracentrifuge tubes.

B.2. Ultracentrifuge.

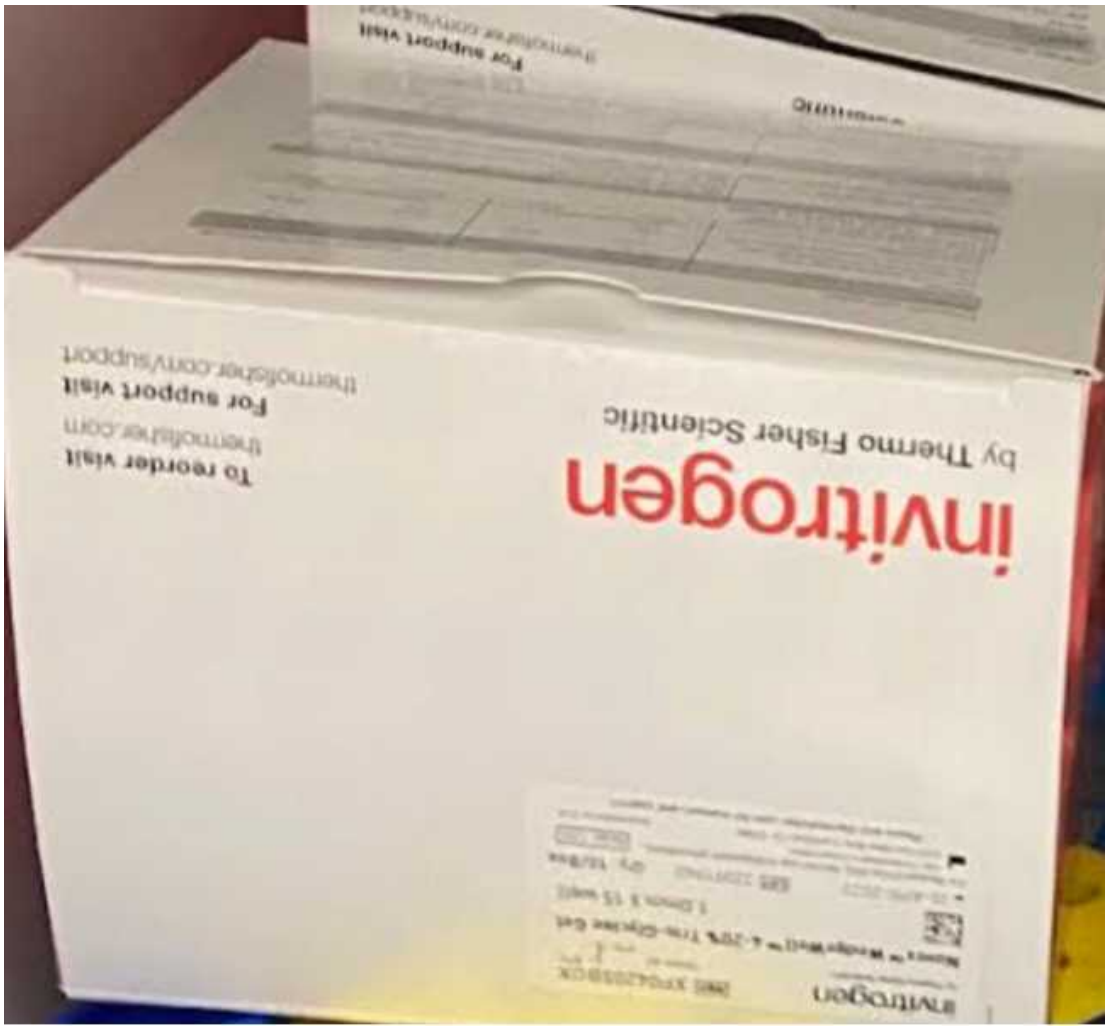


B.3. Nickel NTA resin.

B.3. Column setup to purify protein.

B.3. Tube rack to hold the 50 mL Falcon tubes.

C.1. Protein ladder.

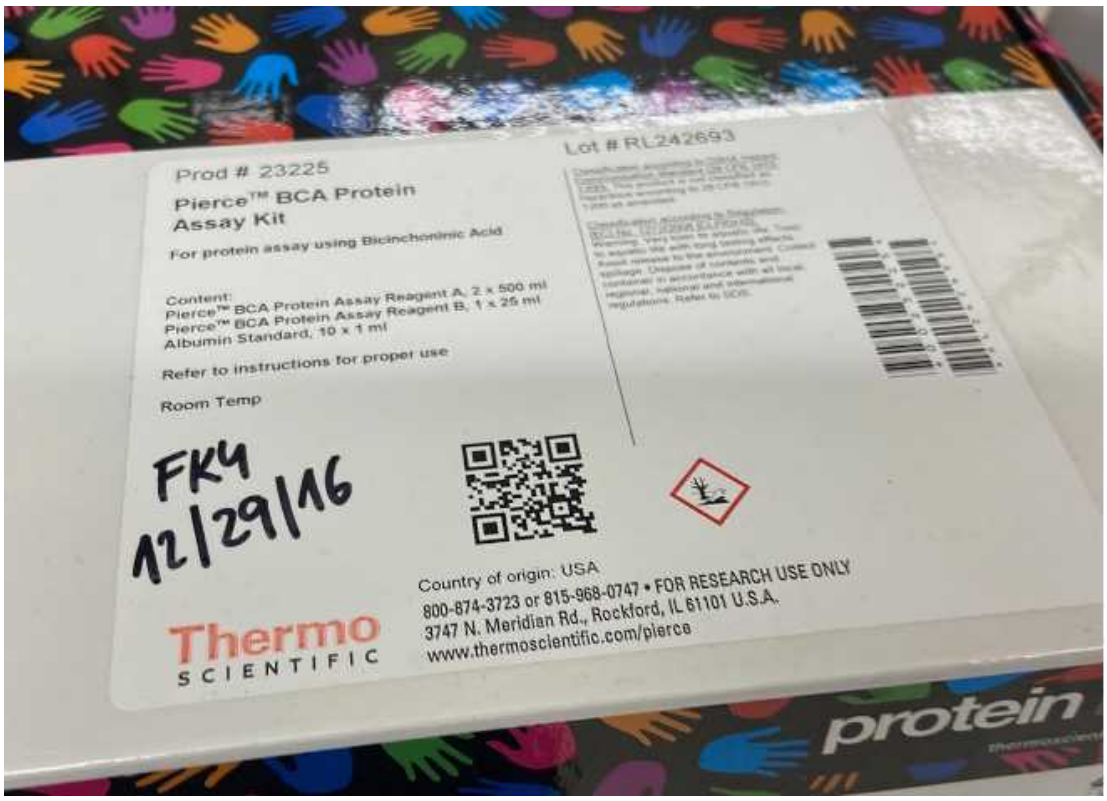


C.1. Protein gels.

C.1. SDS Protein gel electrophoresis apparatus.


MeOH
AcOH
No. 1395
100

C.1. Coomassie blue stain solution.



C.2. BCA protein assay kit.