Protein Purification [P]

Notebook for protein expression in WK6 strain, following [C01] notebook where nanobodies were inserted into vectors with his- and avi- tag.

We used a nickel-resin based purification protocol with our his-tagged nanobodies. Specifically, we will use FPLC to wash and elute our nanobody once it is loaded into a ni-resin column. After isolating our nanobody we will perform a desalting procedure to replace the imidazole buffer the protein currently resides in. By running it through a desalting column, we can isolate our protein in PBS 5% glycerol buffer without imidazole. In this buffer, we biotinylated the nanobodies using BirA, which is possible because our nanobodies have an Avi-Tag. Finally, we will desalt again to isolate proteins from the substrates in the biotination reaction. In the end they will be in 1X PBS with 5% glycerol and ready for subsequent assays and procedures.

Lysis Protocol Overview

1. Inoculate single colony of WK6 transformant with nanobody-avi plasmid into 5mL LB with amp, 2% glucose, 1mM MgCl2 and grow overnight at 37C 170rpm
2. Inoculate 10mL culture TB (and phosphates added = TBII) with amp, 2% glucose, 1mM MgCl2. Grow at 37C 170RPM.
    a. Once OD reaches 0.7, spike with 1mM IPTG
    b. Incubate overnight at **28C 170RPM**
3. Lysis and Protein Purification
    a. Transfer to high speed centrifuge tubes and pellet at 9000g for 15 min, removing supernatant with pipette
    b. For 10mL of lysate, resuspend with 150uL of TES buffer and place in ice for 1 hour on orbital shaker in cold room
    c. Add 300uL of ¼ TES buffer, and place in ice for 45 minutes on orbital shaker in cold room
    d. Centrifuge at 10,000g at 4C for 30 minutes, and collect supernatant into eppendorf tube
    e. Run a SDS-PAGE gel to verify whether procedure was successful

Protein Purification Protocol Overview

We performed nickel-resin based purification protocol with our His-tagged nanobodies. Specifically, we used FPLC to wash and elute our protein from a lysate loaded into a Ni-resin column.

1. Running Lysate into column:
    a. Automated pump to load onto column in 556 far right corner. Pump lysate through into column, will take about 45mins. Once finished can remove column and bring to FPLC machine
2. FPLC Elution:

a. Wash the pumps for a few minutes to replace buffer in wiring, then add nickel column.
b. First wash with wash buffer (sans imidazole) for about 8 minutes. This contains low imidazole concentration, 20mM. Flow rate can be 1mL/min. UV spectra over time will first have a spike, then should be constant.
c. Add gradient to go to 100% buffer B over 8 minutes. The UV will increase at a constant rate due to imidazole. The imidazole concentration in the elution buffer is 250mM.
    i. Once the slope increases, this indicates the protein is being eluted. This will probably occur somewhere between 30-70% elution buffer, it has varied between experiments.
    ii. Collect by executing “Fraction900” 5mL
        1. To stop collection early, change flow rate to 0mL
    iii. Make sure the falcon tube is oriented correctly to collect the elution.
d. Cleaning column:
    i. Let flow at 100% imidazole for a few minutes, then decrease to 0% imidazole. Let wash for 5-10 minutes to remove all imidazole.

Desalting Protocol Overview

Desalting was performed using an FPLC machine to quickly replace the elution buffer with 1X PBS and 5% glycerol.

1. Preparation:
    a. Rinse off deslating injection column with milliQ water
    b. Adjust capacity to volume of elution of protein (ex: 5mL) and pour in.
    c. Screw in cap securely
    d. Add milliQ water to other side and screw in caps
2. Secure injection column and desalting column with correct wiring orientation. An example image is shown below:

3. Initiation
    a. Change flow-pump to on
        i. Adjust flow-pump to 2 mL/min, pressure should be no more than ~0.5 mAU
        ii. This pumps water above the injection column and pushes the sample out.
4. Once the sample is completely dispensed, change flow-pump back to “load”
    a. Important to do! Otherwise pressure in injection tube might break apparatus
5. Watch UV for spike. First spike will be the protein, so collect that.
    a. Same collection method: “Frac900” execution, approximately same volume as elution volume
6. Clean up:
    a. Let buffer continue washnig to let imidazole flow out column. You can stop or do next protein once UV peak shows imidazole went through.
7. Closing program:
    a. Flow-rate to 0mL

b. Hit “End”
c. Can turn off machine

Biotinylation Protocol Overview

We used BirA enzyme to biotinylate our nanobodies which contain an Avi-tag. This is essential future experiments using these proteins because it allows us to bind them to streptavidin-coated sensors for kinetic testing, and agarose beads for when we biopan for our second nanobody dimer binder.

This is similar to a DNA restriction digest because an enzyme catalyzes the reaction. You mix the following in some manner:

1. Desalted nanobody
2. BirA MixA, and BirA MixB - the reaction buffers that also contain biotin
3. BirA - The enzyme

Here is a typical reaction recipe:

1. 4mL of desalted nanobody
2. 0.5 mL of BirA mixA
3. 0.5 mL of BirA mixB
4. 3uL BirA enzyme

Incubation: Let incubate for one hour away from light at room temperature.

Desalting of Biotinylated Protein Protocol Overview

Perform desalting of biotinylated protein identical to the previous method. The purpose of this is to isolate the protein in a buffer without the reagents from the biotinylation reaction (ATP, biotin, and other buffer components).

Resources

Protein expression and induction protocol used for WK6 strain found in this paper, starting at step 70.
https://www.nature.com/articles/nprot.2014.039.pdf

Summary of Progress (8/22)

Cloning was partially successful last week. [C01] project is continuing, but we are also proceeding with 4 successful clones based on sequencing verification. Recently we also identified 9 other successful clones (1 is redundant - [To do - insert explanation of sequencing]).

We've transformed the 4 successful plasmids from earlier this week into WK6 competent cells provided by the Gu lab using our traditional protocol for DH5-alpha chemically competent cells (which is identical to the protocol for NEB High Efficiency cells).

WK6 Strain

[To do - insert why Wk6 strain is used] WK6 strain of E. Coli serves the same purpose as BL21 but sometimes works when BL21 doesn't. A tradeoff with this strain is that it grows very slowly.

Inoculated 10mL TB with phosphates, glucose, MgCl2 at 12:35pm. Incubated at 37C 170RPM
Reached OD 0.07 at 8:40pm, and OD 0.189 at 9:40pm.

[P.1] Copied from [C01]: [C01.23] Transformations

- 1 M glucose stock solution created, put in autoclave at setting 4 along with CaCl2 liquids
    - Added 45 g glucose to 250 mL of
    - https://www.elabprotocols.com/protocols/#!protocol=248

Transformations into WK6 strain E. Coli:

New label	Corresponds to:	Concentration Plasmid	Volume plasmid added:
C01.23-2	13-2 / 19-1	120.6	0.7uL
C01.23-5	13-5 / 19-2	33.8	2uL
C01.23-11	13-11 / 19-3	129.9	0.7uL
C01.23-1	16-1.2	268.4	0.33uL

Note on nomenclature:

The **-1, -2, -5, -11** is important to maintain throughout procedures. These note the nanobody insert. Please do not reset the index on sample labeling (ex: marking them as -1,-2,-3,-4) because this makes it necessary to cross reference.

-1 through -6 denotes artemisinin binders, -7 through -12 denotes vitamin biD binders.

[P.2] Lysis and PAGE Gel

Goal

Lyse WK6 strains with nanobodies expressed to see if they are expressed well.

Making Starter Culture

8/21/18
Karl
Inoculated single colony of WK6 transformant with nanobody-avi plasmid into 5mL LB with amp, 2% glucose, 1mM MgCl2 and grew overnight at 37C 170rpm

Inoculating into TB Culture

8/22/18
Karl
Inoculated 10mL culture TB (and phosphates added = TBII) with amp, 2% glucose, 1mM MgCl2. Grew at 37C 170RPM.

Notes:

- OD growth slow
- (12:35) Inoculated culture with 200uL starter culture
- (8:40) All ODs are around 0.07
- (9:40) OD = 0.185
- (10:30) OD = 0.36
- (11:30) OD = 0.681, 0.655, 0.666, 0.650 for -1, -2, -5, -11 respectively

Spiked cultures with IPTG to 1mM by adding 10uL to each.

- To spike to 1mM IPTG final concentration, add 1uL of 1M IPTG for every mL of culture. In other words, 1M IPTG is 1000X concentration just like an antibiotic stock solution.
- IPTG was given by Gu lab. It's stored in -20C fridge ([P]/[C01] box)
- Incubated overnight at **28C 170RPM**

Lysis

8/23/18
Angie, Chemay

Carried out lysis according to Nature protocol (Lysis Protocol Overview).

Notes:
Shoukai noted that our final product was cloudier than expected and centrifuged it at 10,000g for additional 30 min.

Ran a SDS-PAGE gel; Gu lab loaded the sample for us (loaded in ascending order: -1, -2, -5, -11).

Final products were stored in left -20C freezer ([P]/[C01] box)

- Labeled as C01.24-1, C01.24-2, C01.24-5, C01.24-11

[P.3] Lysis & Protein Purification

8/28/18
Jay, Kara, Krithi

Carried out lysis according to Nature protocol (step 3 in Lysis Protocol Overview).

Samples given to Shoukai for SDS-PAGE gel.

[P.4] TB Culture Inoculation

8/30/18
Angie, Aerilynn, Kara

Using TBI media made by Aimee and Angie on 8/29, and overnight C01.23-2 starter culture prepared by Jay on 8/29, and TBII

Notes:

- Inoculated into 1L TB media as opposed to 10mL
- (12:30) Put into shaker; started later than expected since we needed to autoclave the 2L flask
- (1:47) Added glucose and $MgCl_2$ not already in starter culture late
- (~1:50) Flask broke as we were putting into the shaker, step 2 will need to be pushed back a day
- Shaker also stopped working, likely a result of the spill

Prepared a new starter culture of C01.23-2 for tomorrow

- Used bacterial glycerol stock **93** (C01.23-2 prepared by Chemay on 8/22) from -80C fridge
- 10mL LB/amp + 100uL 1M $MgCl_2$ + 400uL 50% Glucose + 5 uL glycerol stock

Starter cultures stored in Gu's shakers next to high speed centrifuge at 5:20PM.

[P.5] Redo of TB Culture Inoculation

8/31/18
Aerilynn, Karl

Notes:

- (9:20) -2 nanobody culture ready for incubation
- (9:40) -2 nanobody culture placed into shaker
- (10:15) Other cultures placed into shaker

Buffers

Also prepared 500mL TES buffer. From nature protocol for 1L:
TES buffer Dissolve 24.22 g of Tris, 0.19 g of EDTA and 171.15 g of sucrose in 1 liter of ddH2O. Adjust the pH to 8.0 with HCl. TES buffer can be stored for months at 4 °C. TES/4 buffer Dilute 250 ml of TES buffer in 750 ml of ddH2O. This buffer can be stored for months at 4 °C.

500mL volume, also adjusted for EDTA dihydrate disodium salt:
TES buffer: Dissolve 12.11 g of Tris, 0.12 g of EDTA dihydrate and 85.575 g of sucrose in 0.5 liter of ddH2O. Adjust the pH to 8.0 with HCl. TES buffer can be stored for months at 4 °C.
TES/4 buffer: Dilute 250 ml of TES buffer in 750 ml of ddH2O. This buffer can be stored for months at 4 °C.

Harvest Cells

9/1/18
Karl

1. Harvested 1L culture by dividing into 2 500mL nalgene centrifuge tubes provided by Shoukai the day prior. Centrifuge 9000x g 15mins 4C. Notes on use:
    a. These tubes do not need to be autoclaved prior to use, just completely clean inside. We are only harveesting Nbs, and they won't cloned into anything else in the future
    b. Centrifuge use:
        i. This rotor is large and very heavy. Place in carefully
        ii. Had to add WD-40 to get inner screw to turn.
        iii. Tighten outer screw first (secure lid to rotor), then inner screw(secure to spindle that rotor is mounted on). You shouldn't be able to lift the rotor out once it is secured.

iv. Make sure 2 bottles are balanced within 3g. To balance, use larger scale that is really old. It can handle larger weights (up to 820g).

2. Placed nalgene tubes in -80C freezer
3. Harvested 10 mL cultures via tabletop centrifuge. Pelleted at 14000 rpm for 30s several times to collect all culture in 1 single tube.

[P.6] Starter Culture Inoculation

9/5/18
Karl, Aerilynn, Kara, Grace, Angie

Made a starter culture of **-4, -6,** and **-8** for TB innoculation tomorrow

- 10mL of LB/Amp/MgCl2 solution (prepared by GK 8/21)
- 400uL of 50% glucose (prepared by KA 8/27)
- Took colonies from plates C01.28-4, C01.28-6, and C01.28-8
    - C01.28-4 only had one colony to share, so it's possible there may be lower growth in this tube compared to others
- Placed in shaker at 3pm

9/6/18
Grace
Made glycerol stocks of **-4, -6,** and **-8** and stored in Bacteria Glycerol Stocks in -80C

[P.7] Octet and PAGE Gel

9/6/18
Karl, Angie

Finished protein purification on and desalted **-2;** also ran diluted samples through Octet system using SSA biosensors

- Nanodrop for **-2 desalt** OD280=0.230
- Shoukai aided us with each procedure; the Octet is in molES and needs to be reserved

Ran a PAGE gel on samples **-1, -2, -4, -5, -6, -8, -11** and **-2 desalt**

- Stained and let sit overnight

[P.8] Starter Culture Inoculation

9/7/18
Aerilynn

Inoculated nanobody **-8** from glycerol stock **107**

- 10mL of LB/amp/MgCl2 + 400uL of 50% glucose
- Used sterile wooden stick to scrape ice from glycerol stock then swirled stick around in solution
- Culture placed in shaker at 170RPM and 37C at 1:20PM

[P.X] Notes on Procedures for Protein Purification

What we have already completed for -8 Nb

1. Inoculate starter culture
2. Inoculate 1L culture and induce with IPTG at OD 0.7 for protein expression
3. Pellet, resuspend, and lyse cells. Collect clarified lysate with protein.

Overview of Protein Purification Procedures

1. Inoculate starter Culture
    a. Need to prepare substantive biomass before inoculating 1L culture.
2. Inoculate 1L culture with starter culture, induce protein expression with IPTG at correct OD (0.5-0.7 usually)
3. Run lysate through Ni-NTA column to bind His-proteins to resin.
    a. Our proteins will bind to the Ni resin in the lysate buffer conditions
4. Wash and elute column to collect purified Nanobody with FPLC machine
    a. Get rid of unwanted proteins and other lysate debris
5. Run purified protein through desalting column to remove imidazole
    a. Imidazole will inhibit subsequent steps
6. Biotinylate Nanobody in 1hr reaction (very similar to setting up digest reaction)
    a. So the nanobodies can bind to streptavidin, much like biotinylated small molecules!
7. Desalt again
    a. Isolate proteins from excess biotin and enzymes from reaction

8. (Post purification) Test kinetics using Octet
    a. Verify if it actually binds well to molecule at low concentrations
    b. Need info to set up round 2 of biopanning
    c. If poor, need to test other proteins

[P.9] Starter Culture Inoculation -4

9/11/18
Kara

Notes:
Gu Lab borrowed 250g of potassium phosphate dibasic anhydrous, then they gave us another partially used bottle in return.

Made and autoclaved 1L of TB using the following recipe:

- 24g yeast extract
- 20g tryptone (granulated)
- 8mL 50% glycerol
- ~892mL of milliQ water

Added 100 mL of TBI and 0.1 g amp salt (for 100 ug/mL final concentration) when inoculating with -4 starter culture (10mL). Placed in Gu Lab shaker by the high speed centrifuge at 3:45pm.

TB culture was later induced by Karl around 9:30pm.

[P.10] -6 Purification

Purification

9/12/18
Angel, Jay, Karl, Kara

- Took 1 L culture of Colony 28-4 from yesterday
- Lysis, purification, and desalting of Nbs -4 and -8
    - Initial Results indicate potentially poor yields, though this is only qualitative. Tomorrow we can measure the concentration at absorbance 280nm using a a nanodrop.
- Nb -6 10mL LB + Amp starter culture put in shaker
    - Timestamp: 2:27pm

Induction

9/13/18
Karl

Inoculated with Nb-6 starter culture, which was highly saturated, at 12:40pm. The TB did not contain phosphate buffer, and instead extra TBI and 0.1% glucose + 100 ug/mL Amp. We saved a blank of this mixture before inoculating with the culture.

Growth was slow; OD at 4:04pm was 0.098

[P.11] Purification, Biotinylation and Desalting Nbs -4, -8

Lysis, purification, and desalting of Nbs -4 and -8

9/12/18
Karl

Initial results indicate potentially poor yields, though this is only qualitative. Tomorrow we can measure the concentration at absorbance 280nm using a a nanodrop.

Biotinylation, Desalting

9/13/18
Karl

Biotinylation occurred using exact same recipe as before:

- 4mL desalted Nb - added 400uL H2O to one of them to fill to 4mL
- 0.5mL of Bio MixA
- 0.5mL Bio MixB
- 3uL BirA

We have < 500uL of each MixA/B, and a bit more of BirA enzyme. Stored back in -20C [P] notebook. Covered in foil because reaction is light sensitive.

[P.12] [C01.12] Transformations into WK6

9/13/18

Karl

Transformed **-4, -6, -8** nanobodies into WK6 to carry out protein purification later on in the week.

Final Concentrations

- -4: 0.049 mg/mL
- -6: 0.298 mg/mL
- -8: 0.064 mg/mL

[P.13] -2 Purification

9/28/18
Karl

1. Inoculated 5mL LB + Amp + Glucose _ MgCl2, using media made on 9/14 from 4C fridge
    a. Used glycerol stock **93**
2. Grown at 37C 170rpm overnight (Shaker by J505)

Plans for Saturday

1. Make at least 1L TBI
2. Inoculate larger culture with supplemented LB for tomorrow (using starter culture from Friday)
    a. Try to inoculate first thing Sat morning to let it grow at least 20 hours

Plans for Sunday

1. Inoculation and Induction of 1L TB with -2 strain
    a. Will probably take 10 hours
    b. We can improve this by
        i. Inoculating with denser culture
        ii. More culture

First Attempt TB Inoculation

9/30/18
Jay, Aimee

Inoculated 1L TB media, which did not grow correctly. A starter culture of nanobody **-2** was remade in TB with Amp, glucose, and MgCl2.

The TB culture was still left in the shaker at 32C and 170RPM overnight so we could evaluate whether any growth would occur the next day.

Second Attempt TB Inoculation

10/1/18
Angie

Inoculated 1L of TB with **-2** starter culture from yesterday.

Notes:

- (12:40) OD 0.284
- (1:30) OD 0.466
- (2:00) OD 0.564
- (2:15) Induced culture with 1mL of 1M IPTG and placed in shaker by Gu lab at 28C, 170RPM

Merged aliquots of 1M IPTG together and stored in left 20C fridge in [P]/[C01] box (there is about 0.5mL left).

Lysis

10/2/18
Angie, Karl

Pelleted culture from yesterday, and stored in -80C freezer