

NIAID Tetramer Core Facility Protocols



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Note: We have listed the specific equipment that is used at the Tetramer Core Facility. This does not exclude the use of comparable equipment.

Preparation of Inclusion Bodies

Materials:

Disposable cuvettes
1 L Beckman polypropylene screw top centrifuge bottles with bottle sleeves
50 ml polypropylene screw top centrifuge tubes
15 ml polypropylene screw top centrifuge tubes
100 ml polypropylene beaker
500 ml disposable polypropylene beaker
25 X 89 mm Beckman Polyallomer Tubes
Plastic stir rod
Fisher Scientific stirring plate
Beckman CS-15R centrifuge with FO 630 rotor
Beckman JA-6 centrifuge with JS 4.2 rotor
Fisher Scientific 550 Sonic dismembrator with CL4 Ultrasonic converter and cabinet
Fisher Scientific Lab Jack

Reagents:

LB media
1.0 M IPTG
50 mg/ml lysozyme
1.0 M MgCl₂
2 mg/ml Dnase I in 50% glycerol, 75 mM NaCl
Triton-X 100
1M DTT

Resuspension Buffer (pH 8.0):

50 mM Tris-HCL (pH 8.0)
25% (W/V) sucrose
1 mM EDTA
0.1% (w/v) NaAzide
10 mM DTT (add fresh)

Wash Buffer (pH 8.0):

50 mM Tris-HCl (pH 8.0)
0.5% Triton-X100
100 mM NaCl
1 mM EDTA
0.1% Azide
1mM DTT (add fresh)

Wash Buffer without Triton X-100 (pH 8.0):

50 mM Tris-HCl (pH 8.0)
100 mM NaCl
1 mM EDTA
0.1% Azide
1mM DTT (add fresh)

Urea Solution (pH 6.0):

25 mM MES (pH 6.0)
8M urea
10 mM EDTA
0.1mM DTT (add fresh)

Method:

1. Inoculate a 100 ml LB culture with the desired clone.
2. Incubate overnight at 37°C on a horizontal shaker. Prewarm LB media for step #3 in warm room or incubator.
3. Inoculate each of 6 X 1L LB cultures containing antibiotics with 12 ml of the above culture.
4. Incubate the cultures on horizontal shaker until the OD at 600 nm reaches 0.6 OD. At this point, take a 1 ml pre-induction sample and freeze the cell pellet at -20°C. Induce the remaining 6L of culture by the addition of 1.0 M IPTG to final concentration of 1 mM.
5. Incubate the culture on a horizontal shaker for an additional 4 hours at 37°C. Take a 0.5 ml post-induction sample (freeze pellet at -20°C).
6. Transfer cultures to 1 L Beckman polypropylene screw top centrifuge bottles with bottle sleeves. Centrifuge the cultures in the Beckman Avanti J-20 centrifuge with JLA 8.1000 rotor at 4,000 RPM for 15 minutes at 4°C.
7. Pool pellets and resuspend in with resuspension buffer for a total volume of 60 ml. Store the resuspended cultures at -80°C in two 50 ml polypropylene screw top centrifuge tubes. These may be stored for a few weeks before proceeding.
8. Thaw bacteria resuspensions and pool in a 100 ml polypropylene beaker containing a 1/2 inch stir bar.
9. Stir mixture on a stir plate at half speed.
10. To stirring mixture add drop wise: 1.2 ml 50 mg/ml lysozyme (final = 1 mg/ml), 300 ul 1.0 M MgCl₂ (final=5mM), 1.5 ml of 2 mg/ml Dnase I in 50% glycerol containing 75 mM NaCl, 600 ul Triton-X 100 (final= 1%), 600 ul 1M DTT (final=10 mM).
11. Place the 100 ml beaker containing the bacterial lysate into a disposable 500 ml polypropylene beaker containing ice such that the smaller beaker is held firmly in place.

12. Place the above on the lab jack inside the sonicator cabinet and insert the CL4 ultrasonic converter into the 100 ml beaker containing the bacterial lysate. Adjust the lab jack such that the ultrasonic converter is at the maximum depth without touching the beaker.
13. Sonicate the solution for 1.5 min at 0.5 sec alternations on power 4.
14. Transfer the lysates to three 25 X 89 mm Beckman polyallomer tubes and centrifuge in the Beckman CS-15R centrifuge with FO 630 rotor at Max RPM for 10 minutes at 4°C.
15. Decant the supernatant and add 1-2 ml wash buffer to the pellet.
16. Dissociate the pellet using a plastic stir rod, add wash buffer to 15 ml, and continue to stir until the pellet is dispersed.
17. On ice, sonicate solution for 1.5 min at 0.5 sec alternations on power 4.
18. Centrifuge the samples in the Beckman CS-15R centrifuge with FO 630 rotor at Max RPM for 10 minutes at 4°C.
19. Decant supernatant.
20. Repeat this wash step 3X, or until pellet appears clean (white) and supernatant is clear.
21. Resuspend pellets in wash buffer without Triton X-100 as above.
22. Centrifuge in a Beckman CS-15R centrifuge with FO 630 rotor at Max RPM for 10 minutes at 4°C.
23. Decant the supernatant and resuspend the pellet in 200-1000 ul of ddH₂O. Once a white paste has formed resuspend the pellets in a total 10-30 ml urea solution. (ie-3 pellets in 10-30 ml 8 M Urea)
24. Centrifuge the 10 ml of inclusion bodies in a Beckman CS-15R centrifuge with FO 630 rotor at Max RPM for 10 minutes at 4°C.
25. Transfer the supernatant to a 15 ml polypropelene screw top centrifuge tube.
26. Dilute 2 ul of the above inclusion bodies in 98 ul of urea solution and take a UV scan from 240 nm to 320 nm.
27. Calculate the protein concentration. Aliquot 250, 500 or 1000 nmol fractions into screwcap tubes.
28. Quick freeze the aliquots in liquid nitrogen and store at -80°C.
29. Run pre-induction, post-induction, and a final inclusion body sample on a 10-12% SDS PAGE gel.

Folding MHC-1 Monomers

Materials:

Stir bar

Stir plate
10° C water incubator or refrigerated incubator
1L beaker
5 ml syringe
26 gauge needle
P1000 pipetter
1000 ul pipette tips

Reagents:

Folding Buffer (pH 8.0):

400 mM L-Arginine
100 mM Tris
2 mM EDTA

Injection Buffer (pH 4.2):

3 M Guanidine HCl
10 mM NaAcetate
10 mM EDTA

Heavy chain of choice
Light chain of choice
Peptide of choice
Reduced form Glutathione
Oxidized form Glutathione
100 mM PMSF

**This protocol describes a 1L folding reaction. Larger or smaller folding reactions can be scaled up or down as needed. The Garboczi protocol is included as a reference.*

Method:

1. Prechill 1 L of folding buffer in a 1L beaker containing a stir bar to 10° C using a water bath or refrigerated incubator.
2. To cold folding buffer add reduced glutathione to 5mM, oxidized glutathione to 0.5 mM, and PMSF to 0.2 mM.
3. Calculate the necessary quantities of heavy chain (1 uM final), light chain (2 uM final), and peptide (30 mg/L final).
4. Add heavy chain and light chain each to 5.0 ml of injection buffer and load each into a separate 5 ml syringe at room temperature.
5. Dilute the peptide in 500 ul ddH₂O or DMSO depending the amino acid sequence of the peptide.
6. Place the 1 L beaker containing the folding buffer on a stir plate and stir on high speed. (Avoid “foaming” of reaction)
7. Add peptide dropwise to the stirring reaction using a pipetter.
8. Forcefully inject heavy chain and light chain to the stirring reaction through a 26 gauge needle as near to the stir bar as possible. The folding reaction should become slightly opaque after adding light chain.
9. Replace the folding reaction at 10° C and incubate overnight.
10. In the morning, add heavy chain ONLY (now 2uM) as above and replace at 10° C.
11. In the evening, add heavy chain ONLY (final 3uM) as above and replace at 10° C.
12. Incubate overnight at 10° C.

Concentrating Folding Reactions

Materials:

Amicon 500 ml stir cell
N₂ tank with regulator
Biomax 30 membrane

Reagents:

20 mM Tris (pH 8.0)

Method:

1. Remove the base, membrane holder, and O-ring from the concentrator.
2. Place a Biomax 30 membrane, shiny side up, on the membrane holder.
3. Place the O-ring on top of the filter such that it fits within the inner circumference of the membrane holder.
4. Insert the membrane holder, membrane, and O-ring assembly into the bottom of the concentrator body.
5. Screw in the base into the body such that the membrane holder is held firmly in place.
6. Attach the elastomeric tubing to the elutant drain of the membrane holder.
7. Add the pre-filtered or pre centrifuged folding reaction to be concentrated to the stir cell.
8. Insert the stirrer assembly into the stir cell.
9. Place the cap assembly on the stir cell such that the tube fitting assembly will reside opposite the elastomeric tubing.
10. Place the stir cell within the stand assembly. The base of the stir cell should fit into a circular groove in the stand assembly. Flip the black pressure relief valve on the cap assembly into the up position.
11. Place the end of the elastomeric tubing into a container to collect the elutant.
12. Attach the tube fitting assembly to the cap assembly.
13. Place the concentrator/dialysis selector to gas and the appropriate color coded button on the manifold to the on position.
14. Open the main valve on the tank, the gauge on the right hand side of the regulator should indicate that there is gas in the tank.
15. The small black knob on the left underside of the regulator should be in the open position. The valve is in the open position unless it is completely tightened clockwise.
16. Turn the large black knob on the front of the regulator clockwise until the pressure on the left most gauge reads 60 psi.
17. Reverse steps to refill the concentrator.
18. Concentrate the folding reaction to approximately 7 ml

Buffer Exchange

We have two protocols to exchange buffer

Dialysis

De-salting Column

Buffer Exchange by Dialysis

Materials:

Spectrapor 7 Dialysis Tubing and Clips

1L Beaker

1XPBS

100 mM PMSF

7 ml Concentrated Folding Reaction

Reagents:

Biotinylation buffer

100 mM Tris (pH 7.5)

200 mM NaCl

5 mM MgCl₂

Method:

1. Prepare in 1L beaker with stir bar 1L of Biotinylation Buffer. Chill to 4°C
2. Prepare dialysis tubing (accommodates 1.8 ml per cm of tubing). For 10 ml of concentrate, use 11 cm of tubing (double the capacity to allow for expansion during dialysis).
3. Clip tubing on one end with clip of appropriate size. Carefully pipet concentrated reaction into tubing, and clip the other end, leaving room for expansion.
4. Place tubing in beaker and set beaker on stir plate in cold room (4°C)
5. Spin at medium/low speed for 2-3 hours, or until solution precipitates in tubing.
6. Carefully pipet reaction out of tubing and divide into eppendorf tubes (~1.5ml per tube) to be microcentrifuged.
7. Centrifuge the reaction in a cold micro centrifuge at max speed to remove precipitate.

Buffer Exchange by Desalting Column

Materials:

PD-10 Desalting Columns, Amersham Pharmacia

Reagents:

Biotinylation buffer

100 mM Tris (pH 7.5)

200 mM NaCl

5 mM MgCl₂

The PD- 10 columns are designed for buffer exchange of proteins. The beads in the columns have pores (6000 MW) that are smaller than most proteins, but are bigger than small molecule buffer components. This means that the proteins have a "shorter" path through the column than the buffer that the protein was in. The proteins come out in the buffer that the column was equilibrated in, rather than in their original buffer.

The columns are uniformly packed so that you can apply your protein in exactly 2.5 ml, and it will then come off in 3.5 ml. These are the recommended volumes when using the columns. They can be used to desalt smaller volumes (between 1.0 and 2.5 ml), but use of protein volumes less than 2.5 ml will require careful monitoring of protein fractions coming off the column.

Method:

1. Slice bottom plug off new columns with a razor blade. If you are reusing the columns, simply remove the bottom cap.
2. Remove cap and decant buffer off the top.
3. Equilibrate the column with 25 ml of the biotinylation buffer. The volume of the space above the top frit is approximately 5 ml. You can fill it with the buffer and let it drain 5 times, for a total of 25 ml.
4. Add exactly 2.5 ml of your protein. You will not collect the flow through as this 2.5 ml drains -- it will go to your waste container.
5. When the 2.5 ml of protein has completely entered the column, switch to 15 ml collection tubes at the bottom of the column.
6. Add exactly 3.5 ml of buffer, and let it drain all the way into the column, collecting the sample. You will have 3.5 ml of sample from each column. All of your protein will have eluted from the column.
7. For reuse of the columns, first wash with 10 ml of d.i. H₂O, then 25 ml of either 20% ethanol of PBS + 0.1% sodium azide.

*Note: To avoid cross contamination, it is not recommended to reuse columns for **different** reactions.*

Biotinylation of MHC-1 Monomers

Reagents:

100mM Biotin in Tris-base
100 mM ATP *** pH 7.0 ***
100 mM PMSF in isopropanol
1 mg/ml Leupeptin in H₂O
1 mM Pepstatin in methanol

Method:

Add the following to Buffer Exchanged Reaction:

Biotin to a final concentration of 400 uM
ATP to a final concentration of 5 mM
PMSF to a final concentration of 200 uM
Leupeptin to a final concentration of 1 ug/ml
Pepstatin to a final concentration of 1 uM

7.5 ul BIR A enzyme (2mg/ml)

Incubate overnight at room temperature.

Centrifuge the biotinylation reaction in a cold micro centrifuge at max speed to remove precipitate before applying to s300 column.

S300 Column Chromatography

Materials:

HiLoad Pump P-50 (Pharmacia)
Monitor UV-1 (Pharmacia)
RediFrac Fraction Collector (Pharmacia)
Chart Recorder (Pharmacia)
HiPrep 26/60 Sephacryl S-300 Size exclusion column (Pharmacia)

Reagents:

Buffer

20mM Tris pH 8.0; 150mM NaCl at 4°C.

Method:

Although injection protocols will differ due to the specifics of your set-up, it is important to not introduce air on to the column.

1. Make sure there is sufficient buffer to complete run.
2. Set manual valve to connect injection port to waste port. Remove any air bubbles in the system by pushing buffer through the system. Move the valve to a position half way between the injection port and the column port.
3. Attach a syringe containing the biotinylated sample to the injection port.
4. Move the valve to connect the injection port and the column port.
5. Slowly inject the sample onto the column. **** Do not introduce air into the system****
6. Move valve to connect column port to pump port.
7. Start pump at a flow rate of 2 ml per minute. Start chart recorder. Collect 4 minute fractions (8ml).

Mono Q Column Chromatography

Materials:

FPLC
MonoQ HR5/5 column
ultrafree-15 centrifugal filtering device
ultrafree-4 centrifugal filtering device
50 ml Polypropylene centrifuge tube
IEC GP8R centrifuge

Reagents:

20mM Tris pH 8.0
20mM Tris pH 8.0 ; 500mM NaCl
1xPBS with 1 ug/ml Leupeptin, 1 uM Pepstatin and 2mM EDTA.

Method:

Buffer Exchange Centrifugal Filtration

1. Pour relevant S300 fractions corresponding to folded MHC-1 monomers into a single ultrafree-15 centrifugal filtering device and close cap.
2. Place the ultrafree-15 centrifugal filtering device in a 50 ml centrifuge tube.
3. Centrifuge the ultrafree-15 centrifugal filtering device at 2000 g at 4 °C in the IEC GP8R centrifuge until the volume has reached below 1 ml (15-30 minutes depending on concentration of sample. Determine empirically).
4. Add additional fractions to be concentrated to the ultrafree-15 centrifugal filtering device and repeat centrifugation.
5. To exchange the buffer fill the ultrafree-15 centrifugal filtering device with 20 mM Tris (pH 8.0), mix by inversion, and repeat centrifugation. Concentrate to volume \leq 1ml.
6. Bring volume up to 10ml with 20mM Tris pH 8.0

Ion-Exchange Chromatography

1. Before beginning Mono Q column chromatography first check the levels of the A and B buffers.
2. On our system valve one position one is inject (add to loop) valve one position two is load (sample to column). **Note: the needle can only be removed from the injection port when valve 1 is in position 2 or else an air bubble will form in the line and be injected onto the column).**
3. Attach the clean and filled (buffer A) 10 ml super loop to valve 1.
4. Fill the small fraction collector rack with 56 disposable 1.5 ml polypropylene tubes.
5. Place the collector rack onto the fraction collector and place the fraction collector arm against the first tube.
6. Load the buffer A filled superloop:

- A) Press [MANUAL] button on FPLC to turn on the keypad.
 - B) Press [STEP FORWARD] button 4 times until screen reads "VALVE 1.1.1.1.1.0 0" to open the valve mode.
 - C) Set valve 1 to position 2 by entering [1] [.] [2] [DO STORE].
 - D) Set a flow by pressing [4] [.] [0] [DO STORE].
 - E) After the super loop has emptied, remove the syringe from the injection port.
 - F) Refill the syringe with the 10ml S300 reaction sample.
 - G) Insert the syringe into the injection port and inject sample.
 - H) Press [END] button on keypad
 - H) Start Method from keypad or by remote control through FPLC Director program
7. Pool fractions that correspond to biotinylated MHC-1 monomers and concentrate in an ultrafree-4 centrifugal filtering device as previously described. Concentrate to volume of $\leq 300\text{ul}$.
 8. Exchange the buffer with PBS containing 1X protease inhibitors and 2 mM EDTA. Concentrate to volume of $\leq 300\text{ul}$.
 9. Repeat buffer exchange, and concentrate to volume of $\leq 300\text{ul}$
 10. Measure OD, determine protein concentrations, and aliquot in 200 ug fractions.
 11. For storage, snap freeze in liquid nitrogen and store at -80°C . This protects the samples from any contaminating protease.

Biotinylation Assay

Materials:

Streptavidin 0.8 mg/ml
ddH₂O
2x SDS loading dye

Method:

Add 2ug of each MHC-1 monomer to one of two eppendorf tubes either label "+" or "-".

QS the "-" tube to 10 ul with ddH₂O

To the "+" tube add 5 ul of Streptavidin and qs to 10 ul with ddH₂O.

Incubate both tubes at room temperature for 1 hour.

Prepare samples for nondenaturing electrophoresis by adding 10 ul of 2X loading dye to each sample tube only. Do not boil samples. Do not add DTT.

Load samples onto a 12% PAGE gel.

Tetramer Preparation

Materials:

Your Favorite Fluorescent Label attached to Streptavidin
Flow Cytometer

Method:

MHC tetramers are typically prepared with most of the commonly used fluorescent labels used for flow cytometry. One major advantage of the technique is that the label is already attached to the streptavidin molecule, eliminating the need for further chemical modification of the MHC/peptide complex. The choice of label is based upon the signal-to-noise ratio of the label, the capabilities of the flow cytometer that will be used for the assay, and the availability of other fluorescent antibodies used in conjunction with the tetramer. Tetramers labeled with phycobiliproteins such as phycoerythrin (PE) or allophycocyanin (APC) usually give the brightest signals and have been used in most experiments. In our lab, we prefer to use the APC label for the tetramers, reserving the FITC or PE channels for other commercially available phenotyping reagents. However, many commonly used flow cytometers do not have the capacity to measure APC (e.g. the FACScan, the Coulter EPICS XL); when using these instruments, PE is the recommended choice for the tetramer label.

Calculate the total moles of biotinylated MHC in the purified stock. As noted above, we recommend that biotinylated MHC be stored in 100 μ l aliquots at a concentration of 2 mg/ml.

Calculate the concentration of biotin binding sites in the streptavidin stock.

To perform this calculation, it is necessary to know the concentration of the streptavidin conjugate, whether the concentration is expressed in terms of streptavidin content alone or in terms of the total mass of streptavidin conjugate, and the ratio of label to streptavidin. As an example, Molecular Probes provides streptavidin-PE conjugates at a concentration of 1 mg/ml. The concentration refers to the mass of the total conjugate, not just the streptavidin moiety. In addition, the material from Molecular Probes is size selected to have a 1:1 ratio of phycoerythrin:streptavidin. The average molecular weight of a 1:1 streptavidin-PE conjugate is 300,000 Da. Therefore, the concentration of biotin binding sites is:

$$(1 \text{ mg/ml}) * (1 \text{ g/1000mg}) * (1000 \text{ ml/l}) * (1 \text{ mol/300,000g}) * 4 \text{ Biotin BS/mol} = 1.33 \times 10^{-5} \text{ mol/l}$$

This is equivalent to 1.33×10^{-11} moles per μ l.

A table of the average number of biotin binding sites for additional streptavidin conjugates is included below.

Label	Molecular Weight	mg/ml	Moles BiotinBS / ul
Phycoerythrin	300,000	1	1.33x 10 ⁻¹¹
Allophycocyanin	164,000	1	2.44 x 10 ⁻¹¹
FITC	60,000	1	6.67 x10 ⁻¹¹

Calculate the volume of streptavidin conjugate that must be added to give a 1:1 ratio of biotinylated MHC to biotin binding sites.

For example, when adding streptavidin-APC to 200 ug biotinylated MHC, the proper total volume of streptavidin-APC is 174 ul.

Add the streptavidin slowly—in 1/10 volume aliquots—waiting 10-15 minutes between each addition. This process performed at room temperature, and the tube containing the MHC and streptavidin is stored in the dark (e.g., in a drawer).

If the volume of streptavidin required has been overestimated, this procedure will produce the maximum concentration of tetramers because all biotin binding sites will be saturated after the early additions.

Store the tetramer at 4°C in an amber polypropylene tube in a covered box. Tetramer stocks prepared in this way have been used for 3-6 months.

As with most phycobiliprotein conjugates, freezing of the tetramers is not generally recommended, although reagents that have been frozen may retain some activity. Although we have not investigated this, storage of tetramers in the presence of excess peptide and/or beta-2m may increase the stability of the tetramer.

Tetramer Staining Protocol

Basic Principles

To conserve reagents, we try to keep the staining reactions at as low a volume as possible. In practice, we usually add 20ul of a 2x stock of stain to 20 ul of a 2x stock of cells for a total volume of 40 ul. This is just a guideline, and you could go higher or lower in volume.

All stainings are carried out at 4°C. Sometimes, we obtain higher intensity tetramer stains if we incubate at room temperature, but some surface markers—particularly CD62L—are sensitive to the higher temperature.

Titer your reagents before performing a big experiment. The tetramer stocks are typically used at a final dilution of 1:100, but your mileage may vary.

With some tetramers (but not most) we have observed CD8 mediated binding. In other words, the tetramer binds to all CD8+ cells. The CD8-mediated binding (I hesitate to call it non-specific) can usually be blocked by inclusion of some CD8 antibodies that block the interaction. In other experiments, it seems as if there are steric effects such that as long as the CD8 mAb is labeled with PE (240 kDa), the non-antigen specific effects are abrogated. We're still investigating this.

For fresh lymphoid cells (PBMC, lymph nodes, splenocytes), we typically stain 1-2x10⁶ cells. For clones and CTL lines, you can probably get away with as few as 2x10⁵ cells. When staining clones, it might be worthwhile to add cells from a clone with a different specificity as an internal negative control. We usually do this at a ratio of 10:90 specific clones:non-specific clone.

Method:

1. Prepare PBMC, splenocyte, or lymph node cells at a concentration of 5 x 10⁷ cells per ml in your favorite FACS buffer (FB, usually PBS + 2% calf serum + 0.1% sodium azide).
2. Add 20 ul to each well of a microtiter plate.
3. Add 20 ul of each 2x staining stock containing all of your labeled reagents. Mix by pipetting up and down. Avoid bubbles as much as possible, at this and the washing steps.
4. Incubate on ice in the dark for 60 minutes.
5. Add 150 ul FB. Spin 5 minutes at 1200 RPM. Decant supe by "flicking" into sink or bucket of bleach. If you feel more comfortable aspirating, that's fine. We stopped aspirating for all staining because of our experience with intracellular cytokine staining protocols; in the presence of saponin, you lose more cells. However, with infectious/hazardous samples, aspirating may be better.
6. Repeat previous wash step 2 more times.
7. Resuspend cells in 200 ul 1% paraformaldehyde (PFA) in PBS. PFA appears to develop some "green" fluorescence detectable in the FITC channel over time. Ideally, it should be prepared weekly, but we don't always do this.