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Supporting information for article:

Synchrotron-based macromolecular crystallography module for an undergraduate biochemistry lab course

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Supplementary Methods

Stiers, et al.

F. tularensis Histidine Acid Phosphatase (FtHAP) MX Module:

This protocol covers the steps for production of FtHAP, as well as the four units of the MX module. Steps 1-3 below occur prior to the MX module, which begins with crystallization (step 4). Instructions relevant to instructor/TA are shown in italics. The protocol is designed to provide sufficient protein for 8 crystallization screens (16 students); it should be scaled accordingly for different team/class sizes. Each step is designed to occur within the time constraints of one 4-hour laboratory class (some require outside time as noted), with a separation of a day or more between steps. The expression vector is available upon request from the authors.

1) Transformation of expression vector
2) Growing bacterial cultures
3) Purification of FtHAP

→Start MX module

4) Set up crystallization screens
5) Harvest and ship crystals
6) Diffraction data collection, processing & transfer
7) Refinement & model building

Additional Materials and Information

- Learning questions for students
- Table S1. List of needed supplies and vendors
- Table S2 . Stock solutions needed for crystallization screens
- Appendix: Eight crystallization screens for FtHAP

>>All plates and cell cultures in the following protocol are in Luria-Bertani (LB) media with 100 μg/L ampicillin

1. Transformation of expression construct

Required time: ~20 minutes working time; 12 hr. growth time

The gene for FtHAP is available in a pET20b expression vector (Novagen) with an N-terminal His(6)affinity tag. Transform 50 ng of the vector into *E. coli* BL21DE3 (or other competent line) cells for expression using a standard heat shock protocol.

Plate transformation mixture and incubate at 37° C overnight. Store plate at 4° C until used in Step 2.

If schedules permit, students can do the transformation in a laboratory prior to starting cultures. Otherwise this step can be done by the TA/instructor outside of class.

After transformation, it may be useful to conduct a small-scale protein expression test prior to growing the larger cultures in step 2 (below). Pick 2-3 colonies and grow mini-cultures (5 mL) overnight in LB/amp at 37° C. Centrifuge cultures, disrupt cells with B-PER® reagent (Thermo Scientific) or similar detergent formulation, and separate the soluble and insoluble fractions of the cells with centrifugation. Protein expression can be assessed by SDS-PAGE. Look for a strong band of MW ~37 kDa in the soluble fraction. For future use, make glycerol stocks from multiple colonies with good expression of soluble FtHAP and store at -80° C.

2. Growing bacterial cultures for protein expression (large and small scale)

A. Large-scale culture (1 liter total; provides ~10 mg protein for MX unit)

Required time: 45 minutes working time; 20 hrs. growth time

A large culture may be grown and protein purified by TA/instructor to provide backup protein for MX, in case of failure by one or more student teams during purification.

Bacterial cultures for FtHAP expression can be grown as in *Felts et al., Acta Cryst. (2006). F62, 32-25*. Alternatively, a simpler approach can be taken as follows. Select one colony from the transformation plate (Step 1) and grow overnight in 20 mL of LB media at 37° C. Divide culture in half and inoculate 2 x 500 mL flasks of LB media with 100 μg/mL ampicillin. Grow for ~4 hrs (OD 600 of ~0.4-0.6); chill on ice or at 4° C for 10 minutes. Induce with IPTG (final concentration 0.4 mM) and grow for another 4 hours at 37° C. Collect cells by centrifugation at 8000 rpm for 15 minutes at 4° C. Pellets should be flash frozen in liquid N_2 and stored at -80° C.

Alternatively, if cultures will be used within a day or two, they can be kept at 4° C and centrifuged immediately prior to cell lysis.

B. Small-scale cultures (done by each student team; provides ~1 mg/ml per team)

Required time: 30 minutes working time; 20 hrs. growth time

Grow one (or two, if backup desired) 50 mL cultures per student team (eight) for purification, following the protocol above, without the expansion into a larger flask.

Small-scale cultures reduce the needed supplies/media, shaker space, and growth time. If these are not limiting factors, larger-scale cultures can be easily done by students.

Cultures can be grown by the students, by the TA/instructor outside of class, or by a combination of both, depending on schedule and available equipment. For instance, cultures can be inoculated and left to grow during class, then induced by the students before they leave, grown for another ~3-4 hrs., then removed from the shaker by the TA and kept at 4° C until students are available to spin them down.

3. Purification of FtHAP (large and small scale)

A. Large-scale purification of one-liter cell pellet using 30 ml column

Required time: ~6.5 hours working time; +32 hours for dialysis

Resuspend frozen cell pellets in the equilibration buffer (EB: 20 mM $NaPO_4$ pH 7.4, 10 mM Imidazole, and 1 M NaCl) containing 0.5 mM TLCK, 0.5 mM PMSF, 2 mM $CaCl_2$, 2 mM $MgSO_4$, and 10 μg/mL DNase. Lyse cells with a French Press at 16,000 psi. Centrifuge lysate for 30 minutes at 15,000 rpm, leaving soluble FtHAP in the supernatant. Pour supernatant into a column loaded with Ni^{2+} affinity resin previously equilibrated in EB. Seal column, mix by repeated inversion of the column manually into a slurry for batch-binding, and put on an orbital shaker for 30 minutes at 4° C. Allow resin to settle and collect flow-through. Pass ten column volumes of EB over the column, followed by five column volumes of the wash buffer (20 mM $NaPO_4$, pH 7.4, 25 mM Imidazole, 1 M NaCl). Elute protein with ~two column volumes of 20 mM $NaPO_4$, pH 7.4, 400 mM Imidazole, 1 M NaCl and collect fractions of ~10 mL.

Assess aliquots from each fraction by SDS-PAGE. Fractions containing protein should be combined and dialyzed step-wise as follows: EB - overnight; 20 mM $NaPO_4$ pH 7.4, 0.75 M NaCl

- 8hrs; 20 mM $NaPO_4$ pH 7.4, 0.5 M NaCl – overnight. Concentrate dialyzed sample to 10 mg/mL, add 0.1% sodium azide, flash freeze and store at -80° C.

For crystallization screens, a total of ~10 mg (1 mL of 10 mg/mL) FtHAP should be prepared by the TA/instructor. Aliquot into separate tubes for the students (~50 μL needed per crystal screen).

B. Small-scale purification using 10 mL column

Required time: 6 hours working time; 52 hrs. total (with dialysis)

Grow a 50 mL culture overnight as in 3.A. Centrifuge and resuspend in 5 mL of 20 mM TRIS-HCl, pH 7.8, 1.0 M NaCl , 2 mM β-mercaptoethanol (BME) and 1 mM PMSF for lysis. Put samples on ice and lyse with sonication (15 minutes with two second pulses at 40% output and 10 second intervals between pulses). Centrifuge lysed cells for 5 minutes using a table-top centrifuge at highest speed. Transfer supernatant to a clean tube and keep on ice. Set up samples for overnight dialysis into 5 mM Tris Maleate, pH 7.4, 750 mM NaCl, and 2 mM BME.

Samples need to moved into the following buffers for dialysis outside of class time: 5 mM Tris Maleate, pH 7.4, 500 mM NaCl, 2 mM BME for ~6 hours, then overnight in 5 mM Tris Maleate, pH 7.4, 150 mM NaCl, 2 mM BME.

Students should be provided with a disposable plastic column containing 1 mL of Ni-affinity resin. Pass ten column volumes of equilibration buffer (EB: 5 mM Tris Maleate, pH 7.4, 150 mM NaCl) supplemented with 20 mM Imidazole through the column. Batch-binding can be done as described in 3.A. Collect flow through. Wash column with ten column volumes of Wash Buffer (EB with 40mM Imidazole). Elute protein with Elution Buffer (EB plus 300 mM Imidazole) in 1 mL fractions. Collect samples throughout the purification and analyze by SDS-PAGE. *Purified protein should be supplemented with glycerol to 50% and stored at -20° C until needed for kinetics.*

Protein purified from a 50 mL culture should provide a sufficient protein at an appropriate concentration for kinetics assays (~1 mg of protein at 1 mg/ml). If students wish to prepare enough for crystallization, a larger culture size should be used and arrangements for protein concentration will need to be made.

4. Crystallization screens

Required time: ~ 1.5 hrs. working time; ~2 days for crystal growth

Initial crystallization conditions were taken from: Felts et al., Acta Cryst. (2006). F62, 32-25. The optimized condition for the 'guaranteed' crystal growth (discussed below) is: 10% Tacsimate pH 7.0, 0.1M HEPES pH 7.0, 19% w/v PEG 3350. Eight different crystallization screens were designed, to give each student team a unique experience (see Appendix end of this protocol). The screens produce varied crystal morphologies, precipitation, microcrystals, etc. Variables include buffer (pH), precipitant concentration, etc. Many other combinations are possible. Each screen contains 4 wells with "guaranteed" conditions for growing crystals (see red boxes on crystallization screens). Either hanging or sitting drop vapor diffusion formats can be utilized for the crystallization screens. The latter avoids the need for coverslips and greased trays; the former tends to produce somewhat larger crystals in our experience.

Stock solutions required for preparing the well conditions in the various screens are found in ***Table S2****. See screens 1-8 attached at end of this protocol.*

- Using the provided screens and stock solutions, students should first calculate the amount of each stock solution and H_2O needed for the wells of their crystallization plate, such that the final volume of each well is 0.5 mL.
- Pipet required amount of necessary solutions into each well of a 24-well plate, being careful to change pipet tips to prevent cross contamination.
- After the well solutions are completed, prepare the crystallization drops by pipetting 2 μl protein (10 mg/mL) then adding 2 μl well buffer to the protein drop. If a sitting drop plate is used, seal with tape after drops are completed. If a hanging drop tray is used, seal each well by inverting coverslip after each drop is prepared.

5. Harvest and ship crystals

Required time: ~ 3.5 hrs. working time (30 minutes per student team); 1-2 days for shipping crystals to beam linea

The cryoprotectant solution for FtHAP crystals grown using the "guaranteed condition" (see Appendix) is: 10% v/v tacsimate, pH 7.0, 0.1 M HEPES, pH 7.0, 24% (w/v) PEG 3350, and 25% (v/v) PEG 200. Avoid letting the crystals sit in the cryoprotectant for more than 10 minutes, as this will diminish crystal quality and they will eventually dissolve.

Crystal harvesting and cryoprotection will likely be challenging for the students and requires time for demonstrating the process to each student team. The protocol below describes use of Hampton Research supplies ***(Table S1****); alternatives are available at other vendors.*

Supplies needed:

- 0.2 mL of cryoprotectant solution per well per condition (i.e., if each team harvests 2 crystals from the guaranteed condition, need 3.2 mL minimum for the class).
- 10 liters liquid N_2 in pourable storage dewar
- 2 dissecting microscopes – large platforms for plates highly preferable
- 2 shallow form dewars, filled with liquid N_2
- 2 tall dewars for temporary storage of canes, filled with liquid N_2
- >4 Vial Clamps™ for handling CrystalCap vials under liquid N_2 or when transferring crystals to canes
- >2 CrystalWand Magnetic™ for crystal handling
- >4 pairs of cryo-gloves
- >10 cryocanes
- >20 CrystalCaps (magnetic) equipped with Microtubes (pins) holding loops of varying sizes, with matching CrystalCap vials

Crystal harvesting follows the steps below. Wear cryo-gloves when appropriate, such as when pouring liquid N_2, transporting dewar filled with liquid N_2, and touching tools that have been chilled in liquid N_2. It is assumed that the students will wear safety glasses/goggles throughout the entire laboratory experience. Cryocanes and cryovials should be labeled before being immersed in liquid N_2.

1. Fill shallow dewar with liquid N_2
2. Use the microscope to identify crystal to be harvested
3. Cut square in tape around well (sitting-drop plate) or invert coverslip (hanging drop plate)
4. Add 20 μL of cryoprotectant to drop, mix **gently** by pipetting
5. Remove 20 μL solution from drop
6. Repeat steps 5-6 twice
7. Choose CrystalCap/pin with loop of appropriate size for selected crystal (approximately same dimensions); attach to magnetic wand
8. Place tongs and CrystalCap vial in shallow dewar to cool
9. Extract crystal from drop by picking up in loop.
10. **Quickly** immerse crystal in liquid N_2, maneuver inside CrystalCap vial, and press plunger on CrystalWand to release CrystalCap/pin into vial.
11. Place labeled cryocane into tall dewar filled with liquid N_2.
12. Transfer crystal in CrystalCap/vial assembly from the shallow dewar into the lowest open position on the cryocane
13. Repeat 1-12 for 2-3 crystals per student team

The following videos may be useful references:

(https://www.youtube.com/watch?v=i2G1fYtjXt8 and
https://www.youtube.com/watch?v=FENUWRYXMOM and
https://www.youtube.com/watch?v=QcsaWowulDM)

Crystals stored in canes are ready for transfer to the cryoshipper, which should be pre-cooled with liquid N_2. Cooling the cryoshipper requires about 10 L of liquid N_2. It may be desirable to ship a batch of crystals to the beam line prior to the shipment of student crystals, in case of damage to the cryoshipper or delays in shipping. These can also be used for test data collection to check crystal quality, cryoprotection, and remote access to the beam line prior to the scheduled student data collection.

6. Data collection, processing & transfer

Required time: ~3.5 hours

*The instructions below are customized for remote data collection at ALS beamline 4.2.2**.*** *The setup allows all students to view diffraction images on a large screen and control data collection in real time. Data collected at other beamlines/synchrotrons will require modifications according to local requirements.*

Setup for Data Collection and Processing

- Control unit (laptop) connected to the classroom projector with audio
  - Connected to Ethernet line if possible – Wifi may be unreliable and slow
- NoMachine remote desktop software installed on control unit
  - This enables a remote connection to the beam line computer and control of the robot without time lag
- Arrange with beamline staff scientist to be on standby during the student data collection time to assist with any problems at the beamline
- All laboratory laptops need to have Phenix, Coot, and PyMOL installed prior to class
  The instructor should launch the data collection software (e.g. BLU-ICE) and establish control of the beamline before the students attempt to collect data. For each group, the following generic steps are suggested. The details of each step will vary depending on the beamline.
  6.1. Direct the robot to transfer the desired crystal to the goniometer.
  6.2. Center the crystal.

6.3. Collect test diffraction images at two angles (e.g., 0, 90°) using a short exposure (0.5-1 s) and oscillation angle of about 0.5°. Set the detector distance so that the edge of the detector corresponds to ~2.5 Å resolution.
6.4. If diffraction to at least ~2.5 Å is observed, proceed to **6.5**; otherwise go to **6.1.**
6.5. Repeat **6.3** at 2-3 different detector distances to identify a distance that optimally captures the full extent of the diffraction resolution.
6.6. If time permits, repeat **6.3** with the exposure time increased to 5 s. Repeat **6.3** with the oscillation angle increased to 5° and 0.1°.
6.7. Collect a complete data set of 180° (3 minutes at ALS 4.2.2).
6.8. Go to **6.1** to analyze another crystal, if time permits.

Data Reduction

Processing of the files can begin immediately after a reasonable dataset is collected for each team. At ALS 4.2.2, data can be processed automatically via the 'doit' script on the beamline computer. We recommend that this be done by the TA/instructor or beamline personnel. A generic protocol is below:

doit /*complete_path*/root_0001.img
aimit_final *res*
cp mydata_uniqueifyF.mtz root.mtz

Note:
complete_path = the complete path of the directory containing the images.
root = the root name of the diffraction images.
res = the high resolution cutoff. Change this value to obtain desired I/sigma threshold in the highest resolution bin (>=1.5 generally).

7. Refinement and model building

Required time: ~4 hrs.

If local experts are available to assist, students can begin refinement/model building in the same class period as they collect their data, taking full advantage of rapid synchrotron data collection.

Preparation of input models

Input models for refinement/model building may vary according to goals of the class. This can be as simple as chain A of PDB ID 3IT1. If desired, each student team can be given a

different model, so they have unique problems to solve. Many simple variations of the input model are possible; see text.

Refinement & model building

These come naturally to some students and with great difficulty to others. Prior instruction on Coot, including a demonstration of modeling tools, would be useful if possible. Use of Density Fit Analysis and the other validation tools is recommended. This section of the MX module demands a full laboratory period of 1-on-1 instruction with student teams.

7.1. Start Phenix on the laptop
7.2. Create a new project
7.3. Choose phenix.refine from the Refinement pane
7.4. Use the "Add file" button to input a starting coordinate file (.PDB) and the reflection file (.mtz).
7.5. Select the space group as $P4_12_12$.
7.6. Press the Run button. Coot should open, and maps should soon appear.

Making figures with PyMOL

It is a good learning experience for students to use their refined structures to make a publication quality figure of FtHAP with PYMOL. This allows them to connect the MX module with illustrations of proteins that they have seen in textbooks. Previous instruction with PyMOL (earlier in the class) will facilitate this. Images may vary depending on the goals of the instructor, and can be included in lab notebooks or formal student reports.

A tutorial for PyMOL is available at:
https://pymolwiki.org/index.php/Practical_Pymol_for_Beginners

Learning questions for students

Below is a selection of questions that can be given to the students for consideration during the MX module, or used as a basis for informal discussions during the laboratory periods.

Crystallization:

- How pure does a protein need to be to crystallize?
- How does one assess protein purity?
- Based on the results of the crystallization screens, what factors affect crystallization of FtHAP and how?

Crystal harvesting:

- Why is X-ray data typically collected at cryo-temperatures?
- Why do crystals need to be cryo-protected?
- Which buffer component provides cryoprotection?
- What is the physical mechanism of ice suppression?

Data collection:

- Assess the effects on diffraction of the following parameters:
    - Length of exposure
    - Crystal to detector distance
    - Phi angle
- How is resolution observed on a diffraction image?
- What is the shadow in the middle of the image?
- Identify a strong reflection that has resolution of about 10 A and another with resolution of about 3 A.
    - Which reflection has a higher scattering angle?
- Identify the highest resolution reflection on the image.
- Do you see any evidence of ice from the diffraction image?
- What features are observed in high quality diffraction images?

Structure refinement and model building:

- How does the electron density tell you the direction of the polypeptide chain?
    - Hint: Look for bumps in the electron density that indicate the carbonyl bonds of the backbone.
- Verify that peptide bonds are planar.
- Calculate the Ramachandran map and keep the plot open.
    - Use the space bar to jump along the polypeptide chain and watch how the phi,psi value changes.
    - Correlate phi,psi with the secondary structure that you see in the model.
- Find an ion pair.
    - Write the residue names and the interaction distance.

- Compare the density for a Ser to that of a Cys
    - Comment on the density that represents the side chain -OH and -SH groups.
    - Why are they different?
    - Hint: X-rays interact with electrons.
- Compare the density for a Val to that of a Thr
    - Comment on the density that represents the side chain -OH and -CH3 groups.
- Which element is conspicuously absent from the model and why?
    - Hint: X-rays interact with electrons.
- View the electron density from other groups to see how the resolution affects the quality of the electron density map.
- Sequencing by density exercise.
    - Close your eyes and ask your partner to navigate to a region of the structure and turn off the model but keep the electron density visible.
    - Open your eyes and determine the sequence for 5 contiguous residues using only the density as your guide.
    - Check your answer by turning on the model.
- Active site exercise
    - Find the catalytic His residue; which atom is the nucleophile?
    - Is there density near the catalytic His that is not accounted for by the model? What does this density represent?
        - Hint: think about all the components of the reservoir.
- There are many strong spherical electron density features that are not accounted for by the protein model. What do these represent?
    - Hint: what is the major component of the reservoir?
- Enable Environment Distances and then find an alpha helix.
    - Obtain the distances for several n to n+4 hydrogen bonds.
    - What is the variation in the hydrogen bonding distance?
- Find an Asn or Gln residue.
    - Notice that the density features representing the side chain carbonyl and amino groups are not sufficiently different to distinguish between these two groups. This leads to an ambiguity in the model that must be resolved.
    - Why isn't the density sufficient to resolve the ambiguity? Hint: X-rays interact with electrons.
    - How can we decide where the carbonyl and amino groups should be modeled?
    - Convince yourself that a similar ambiguity exists for His.

Table S1. List of reagents and vendors

Item	Hampton Research Item No.	Item No. of our components
VDX™ Plate with sealant (24)	HR3-172	-
22 mm x 0.22 mm Siliconized square cover slides (~1000)	HR3-217	-
CrystalCap ALS Copper HT, 0.2-0.3 mm CryoLoop - 30 pack	HR8-190	-
CrystalCap Vial	HR4-904	-
Ammonium Sulfate	HR2-541	Fisher A702-500
BIS-TRIS	HR2-783	Fisher BP301-500
Citric Acid	HR2-757	Fisher BP339-500
HEPES	HR2-733	Fisher BP152-500
TRIS-HCl	HR2-725	Fisher BP152-500
PEG 3350	HR2-527	Integra 25322-68-3
Tacsimate pH 7.0 (100%) 200 mL	HR2-755	-
Sodium Malonate	HR2-707	-
Potassium Sodium Tartrate Tetrahydrate	HR2-539	-
Sodium Chloride	HR2-637	Fisher S271-3
Lithium Sulfate Monohydrate	HR2-545	Acros Organics 10377-48-7
Magnesium Chloride Hexahydrate	HR2-803	Fisher M33-500
Sodium Formate	HR2-765	Fisher S648-500
DL-Malic Acid	HR2-761	Acros Organics 617-48-1
PEG Monomethyl ether 5,000	HR2-615	-
CrystalWand™ Magnetic	HR4-729	-
Vial Clamp - Curved 45°/135	HR4-671	-
CryoCane 5 Vial Holder	HR4-709	-
CryoSleeve	HR4-708	-
Cryogenic Foam Dewar (2 liter) - Tall	HR4-675	-
Cryogenic Foam Dewar (500 ml) - Small	HR4-662	-
Cryo Express Dry Shipper (CX100)	NA	MiTeGen TW-CX100
Shipping case for CX100	NA	MiTeGen TW-CX10-8C00
Ninja ice gloves	NA	Ninja Ice HPT NI00

*This column specifies source of supplies used for the MX unit; Hampton Research numbers are provided for convenience. Dash (-) indicates Hampton Research items were used. No problems with reproducibility of crystallization have been observed when changing sources or batches of chemical.

Table S2. Stock solutions needed for crystallization screens

No.	Precipitant(s), salts	Buffer
1	3.0 M Ammonium Sulfate	0.5 M BIS-TRIS-varying pH 0.5 M Citric Acid-varying pH 0.5 M HEPES-varying pH 0.5 M Tris-varying pH
2	50% w/v PEG 3350, 3.0 M Ammonium Sulfate, 3.4 M Sodium Malonate, 2.0 M Potassium Sodium Tartrate Tetrahydrate, 1.0 M Sodium Chloride	1.0 M Tris, pH 8.5,
3	50% w/v PEG 3350, 1.0 M Sodium Chloride, 1.0 M Lithium Sulfate Monohydrate, 1.0 M Magnesium Chloride Hexahydrate, 3.4 M Sodium Malonate	1.0 M Tris, pH 8.5
4	50% w/v PEG 3350, 1.0 M Magnesium Chloride Hexahydrate, 3.4 M Sodium Malonate, 1.0 M Sodium Formate, 1.0 M DL-Malic Acid	
5	3.0 M Ammonium Sulfate	0.5 M BIS-TRIS, pH 5.5 1.0 M Citric Acid, pH 3.5 1.0 M HEPES, pH 7.5 1.0 M Tris, pH 8.5
6	100% v/v Tacsimate pH 7.0, 50% PEG Monomethyl ether 5,000	1.0 M HEPES-pH 7
7	3.0 M Ammonium Sulfate	0.5 M BIS-TRIS pH 5.5 0.5 M BIS-TRIS, pH 6.25, 1.0 M HEPES, pH 7.5, 1.0 M HEPES, pH 8.5
8	50% w/v PEG 3350, 100% v/v Tacsimate, pH 7.0	1.0 M HEPES-pH 7.0,
All	100% v/v Tacsimate, pH 7.0, 50% w/v PEG 3350	1.0 M HEPES-pH 7.0

Appendix

Eight crystallization screens used for undergraduate MX module. Conditions for each well of a 24-well plate are given. Conditions in red boxes are highly likely to produce crystals suitable for data collection. Other wells are used to demonstrate the effects of different variables on crystallization.