

## Zonal Sedimentation Analysis on Sucrose Gradients

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**[Abstract]** Zonal sedimentation analysis on sucrose gradients allows estimation of the molecular size of an individual protein or a protein complex by centrifugation at a constant speed under non-denaturing conditions. This method is particularly suitable for globular proteins like the influenza A virus (IAV) protein hemagglutinin (HA). Here, I describe step by step a protocol used to evaluate the oligomeric state of recombinant HA trimers (Magadan *et al.*, 2013).

### **Materials and Reagents**

1. Trimerized recombinant HA (reCHA<sub>3</sub>) derived from influenza A/Puerto Rico/8/34 (PR8) virus (Magadan *et al.*, 2013)
2. Gel filtration protein standards [carbonic anhydrase (29 kDa), ovalbumin (43 kDa), conalbumin (75 kDa), aldolase (158 kDa), and ferritin (440 kDa)] (GE, catalog numbers: 28-4038-41 and 28-4038-42)
3. 4x NuPAGE LDS sample buffer (Life Technologies, catalog number: NP0007)
4. NuPAGE Novex 4-12% Bis-Tris protein gels (Life Technologies, catalog number: NP0321PK2)
5. NuPAGE MES SDS running buffer (Life Technologies, catalog number: NP000202)
6. Ponceau S solution (Sigma-Aldrich, catalog number: P7170)
7. 5% acetic acid
8. Blotting grade blocker nonfat dry milk (Bio-Rad Laboratories, catalog number: 170-6404XTU)
9. 1x PBS (Life Technologies, catalog number: AM9624)
10. Tween-20 (Sigma-Aldrich, catalog number: P1379)
11. A home-made, conformation-independent mouse monoclonal antibody to denatured HA1 (clone CM-1)
12. A rabbit polyclonal anti-mouse HRP-conjugated antibody (Dako, catalog number: P0260)
13. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, catalog number: 34077)
14. High purity sucrose (Thermo Fisher Scientific, catalog number: NC0110097)

15. UltraPure 5 M NaCl (Life Technologies, catalog number: 24740-011)
16. UltraPure 0.5 M EDTA (pH 8.0) (Life Technologies, catalog number: 15575-020)
17. Sucrose gradient (see Recipes)

### **Equipment**

1. Refractometer (Bausch & Lomb Incorporated)
2. 14 x 89 mm Ultra Clear tubes (Beckman Coulter, catalog number: 344059)
3. Pipettor
4. 1.5 ml micro-centrifuge tubes
5. An ultracentrifuge equipped with a SW41 rotor (Beckman Coulter)
6. Nitrocellulose blotting membranes (0.45  $\mu$ m pore size) (Life Technologies, catalog number: LC2000)
7. A chamber to run mini-gels [I routinely use the XCell SureLock Mini Cell electrophoresis system (Life Technologies, catalog number: EI0001).]
8. A Mini Trans-Blot Cell (Bio-Rad Laboratories, catalog number: 170-3930)
9. Carestream Kodak BioMax XAR films (Sigma-Aldrich, catalog number: F5388)
10. Kodak X-OMAT 2000A processor or equivalent
11. A cassette for autoradiography

### **Software**

1. ImageJ software (<http://rsbweb.nih.gov/ij>)

### **Procedure**

- A. Zonal sedimentation on a sucrose gradient
  1. Simultaneously load 6  $\mu$ g of recHA<sub>3</sub> and 60  $\mu$ g of each protein standard by pipetting them (use a 10  $\mu$ l tip connected to a pipettor) just below the top of a 5-25% sucrose gradient containing a 60% sucrose cushion.
  2. Ultracentrifuge the sucrose gradient for 16 h at 35,000 rpm, 4 °C.
  3. Place the tube containing the sucrose gradient in a tightly fitted rack to avoid any undesired movement.
  4. Carefully place a 1 ml tip connected to a pipettor just below the top of the sucrose gradient.
  5. Manually collect fractions of 250  $\mu$ l by slow pipetting.
  6. Transfer fractions to new 1.5 ml micro-centrifuge tubes.

7. Repeat steps A4 and A6 until the sucrose gradient is completely fractionated.
8. Combine 15  $\mu$ l of each fraction with 5  $\mu$ l of 4x LDS sample buffer.
9. Boil samples for 5 min.

#### B. SDS-PAGE and Western blotting

1. Load 15  $\mu$ l of every sample onto protein mini-gels.
2. Run for ~1 h at constant 50 mA/gel.
3. Transfer proteins to nitrocellulose membranes for ~1 h at constant 300 mA.
4. Stain membranes with 10 ml 0.1% Ponceau S solution in 5% acetic acid for 5 min. Rinse membranes with water. At this point, it is possible to see the protein standards on the stained membranes.
5. Scan or take a picture of the stained membranes.
6. Block membranes with 10 ml 5% nonfat milk/1x PBS for 30 min at room temperature.
7. Wash membranes with 10 ml 0.5% Tween-20/1x PBS for 10 min at room temperature with vigorous shaking.
8. Incubate membranes with 10 ml neat mouse hybridoma supernatant containing the anti-HA antibody for at least 2 h at room temperature with slow rocking.
9. Wash membranes 3 times with 10 ml 0.5% Tween-20/1x PBS for 10 min at room temperature with vigorous shaking.
10. Incubate membranes with 10 ml anti-mouse HRP-conjugated antibody diluted 1:3,000 in 5% nonfat milk/1x PBS for 1 h at room temperature with slow rocking.
11. Wash membranes 3 times with 10 ml 0.5% Tween-20/1x PBS for 10 min at room temperature with vigorous shaking.
12. Incubate membranes with 5 ml SuperSignal West Pico chemiluminescent substrate for 5 min at room temperature with slow rocking.
13. Expose films to membranes at room temperature.
14. Develop films using a Kodak X-OMAT 2000A processor or equivalent.
15. Please refer to Figure 5 on our prior publication (Magadan *et al.*, 2013) for representative results and conclusions.

#### C. Calculation of the recHA3 molecular size

1. Measure the intensity of each protein standard band on stained membranes with Ponceau S using the Image J software. Then, plot the distribution of the protein standards on the different sucrose gradient fractions.
2. Repeat step C1 measuring the distribution of recHA<sub>3</sub> obtained by Western blotting.
3. By comparing both plots, it is evident that recHA<sub>3</sub> (~200 kDa) sediments as discrete peaks immediately following the fractions containing aldolase (158 kDa).

## **Recipes**

### 1. Sucrose gradient

Prepare a 60% sucrose stock by dissolving high purity sucrose in 50 mM Tris-HCl (pH 7.5) and 100 mM NaCl

Measure the refractive index (RI) of the 60% sucrose solution using a refractometer (RI=1.4418 at room temperature)

Make 25%, 22.5%, 20%, 17.5%, 15%, 12.5%, 10%, 7.5%, and 5% sucrose solutions using the 60% sucrose stock solution described above in this recipe

Pour a 2 ml 60% sucrose cushion in a 14 x 89 mm tube and then 1 ml of the following sucrose solutions by careful pipetting: 25%, 22.5%, 20%, 17.5%, 15%, 12.5%, 10%, 7.5%, and 5%

Equilibrate the sucrose gradient for at least 1 h at room temperature

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## **References**

1. Magadan, J. G., Khurana, S., Das, S. R., Frank, G. M., Stevens, J., Golding, H., Bennink, J. R. and Yewdell, J. W. (2013). [Influenza A virus hemagglutinin trimerization completes monomer folding and antigenicity](#). *J Virol* 87(17): 9742-9753.