# Sedimentation Equilibrium Analytical Ultracentrifugation

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## Calculating $\varepsilon_{230}$ (molar extinction coefficient at 230 nm)

1. Collect UV spectra of your peptide in triplicate
2. According to Beer's Law, $\varepsilon_{230} = \varepsilon_{280}(A_{230}/A_{280})$
    - Where $\varepsilon_{280}$ is the molar extinction coefficient of your peptide according to the number of tryptophan and tyrosine residues it contains
    - If there are no Trp or Tyr residues, use $\varepsilon_{257}$ according to the number of its phenylalanine residues
3. For the molar extinction coefficient, use the average value calculated from your three trials

## Sample preparation

1. For each peptide, prepare samples at three concentrations:
    a. ~0.8 AU (in this case, absorbance units) @ 280 nm
    b. ~0.3 AU @ 280 nm
    c. ~0.3 AU @ 230 nm
        i. Where c = Abs/(ε•l), where l is the path length of the cell (1.2 cm)
        ii. I usually round these concentrations to the nearest multiple of 10 or 25 so I don't have to later explain why I collected data at 36 μM
        iii. From the highest concentration to the lowest should be about a full order of magnitude
2. Prepare 150 μL of peptide in buffer at each concentration

## Cleaning the AU cell

1. Untorque to loosen (using clamp)
2. Unscrew (using black tool)
    a. Take extra care if recollecting sample
    b. Tap gently if necessary (can also freeze for a couple minutes, but make sure the center doesn't fall out)
3. Remove sample (if applicable) and disassemble
4. Rinse windows with DI water
5. Pour alconox in 50 mL Falcon tube (enough to submerge window) and insert one window
    a. Repeat for the other window in a separate tube

6. Sonicate the windows in alconox (~5 min timer)
7. Pour out alconox, then secure window between index finger and thumb and wash with DI water and EtOH
8. Dry with $N_2$(g) – make sure residue is completely blown off, then place the dry windows on a Kim wipe on a paper towel
9. Rinse the 6-well centerpiece thoroughly (~2 min) with DI water and EtOH
10. Dry with $N_2$(g)
11. Place dry centerpiece on clean paper towel
12. For all other components:
    a. Wash with DI water and EtOH, dry with $N_2$(g), place on paper towel
    b. White rings are disposable and may be discarded
13. Relabel metal cell housing with position # (1, 2, or 3)

## Assembling the AU cell and loading sample

1. Place white window gasket in black window holder
2. Place brown liner within the holder, with the opening opposite the notch
3. Dust off the window with $N_2$(g), then wipe with lens paper
    a. Can wipe hard to remove residue only after all the dust has been removed, otherwise window can get scratched
4. Place window in holder
    a. Arrow on the edge of the window lines up with the notch
5. Repeat process for the other window
    a. Place window assemblies on lens paper
6. Dust off centerpiece and cell housing with $N_2$(g)
7. Insert centerpiece into cell housing
    a. Insert into the larger half so that the ridge and the ledge fit together
8. Place window assembly in bottom, so that the model number can be read right side up
    a. Line up notch and ridge
9. Insert screw gasket (brown ring), then screw ring
    a. Worn out side reading “Out” facing out
    b. Use black screw tool until resistance
10. Use torque clamp to tighten screw
    a. First time, torque to 60
        i. Set black needle to 0, then point red needle to the desired level and tighten
11. Load samples, taking care not to drip
    a. Model # facing upwards (opening up)
    b. Holes on left
    c. Sample in top row: high to low from left to right
    d. Buffer in bottom row

    e. 120 µL sample, 125 µL buffer
12. Place window assembly on top
13. Insert screw gasket then screw ring (“Out” facing out)
14. Screw til resistance with black screw tool
15. Use torque clamp to tighten
    a. Torque to 60
    b. Alternate top and bottom sides up to 90, then 125

## Loading the rotor

1. Tear off a small piece of Kim wipe (~0.5” x 3”) to make for a snug fit
2. Insert cell assembly opposite the counterbalance
    a. Part # facing in
3. Cut off excess Kim wipe
4. Line up diameter of centerpiece with the etched lines on the bottom of the rotor
    a. Use a straight edge for reference

## SE-AU experiment setup

1. Load rotor into instrument and attach detector
    a. Close door and press “Vacuum”
2. On computer, open Proteome Lab
3. File – new
    a. NoName99.SCN
4. Cell 2 – Wavelength
    a. 200-300 nm
    b. Enter initials and sample name under “Comment”
5. Detail – Browse – select data folder
    a. OK
6. Options – Radial calibration before first scan
    a. OK
7. XL Settings:
    a. Speed: 3000, Time: Hold, Temp: 25
8. When pressure on the instrument is below 600 microns, click Start Single Scan
    a. It will take ~10 min to calibrate and acquire a wavelength scan
9. Cell 2 – Equilibrium
    a. W1 = 280
10. Detail
    a. Second wavelength = 230
    b. Radial Step size = 0.001
    c. Replicates = 10
    d. Centerpiece = 6

e. Browse – select data folder

11. Options – uncheck radial calibration
12. Method – see below
13. When pressure is below 100 microns, click Start Method Scan

## Sedimentation equilibrium method

1. "Scan" tab
2. NoName99.SCN window – "Method" button

| Step | Speed | Accel/Decel | Delay | Temp | # |
|---|---|---|---|---|---|
| 1 | 36,000 | 400 | 0:00 | 25 | 1 |
| 2 | 36,000 | 400 | 1:00 | 25 | 1 |
| 3 | 36,000 | 400 | 1:00 | 25 | 1 |
| … | 36,000 | 400 | 1:00 | 25 | 1 |
| ~60 | 36,000 | 400 | 1:00 | 25 | 1 |

3. Click OK, Click Yes
4. When pressure is below 100 microns, click Start Method Scan
5. At ~ 24 hours, use "matching" to confirm the sample is at equilibrium (see below)
6. Once equilibrium data has been collected at 36,000 rpm:
    a. Scan – Stop Scan
7. NoName99.SCN window – "Method" button

| Step | Speed | Accel/Decel | Delay | Temp | # |
|---|---|---|---|---|---|
| 1 | 36,000 | 400 | 0:00 | 25 | 1 |
| 2 | 42,000 | 400 | 0:00 | 25 | 1 |
| 3 | 42,000 | 400 | 1:00 | 25 | 1 |
| … | 42,000 | 400 | 1:00 | 25 | 1 |
| ~60 | 42,000 | 400 | 1:00 | 25 | 1 |

8. Collect data at four speeds:
    a. 36k, 42k, 50k, and 60k rpm
9. After 60,000 rpm run is complete:
    a. NoName99.SCN window – "Method" button

| Step | Speed | Accel/Decel | Delay | Temp | # |
|---|---|---|---|---|---|
| 1 | 60,000 | 50 | 0:10 | 25 | 1 |
| 2 | 50,000 | 50 | 0:10 | 25 | 1 |
| 3 | 40,000 | 50 | 0:10 | 25 | 1 |
| 4 | 30,000 | 50 | 0:10 | 25 | 1 |
| 5 | 20,000 | 50 | 0:10 | 25 | 1 |

    b. Options – check "Stop XL after last scan"

c. OK
d. Click “Start Method Scan”

## Matching

1. Use the Match feature within Heteroanalysis to ensure that your peptide sample has reached equilibrium at each speed
2. Open Heteroanalysis
3. Click “Match” from the top toolbar
    a. Cell Number 2
    b. Loading Options:
        i. Load Every Nth File
        ii. N = 2
4. “Load Files”
    a. First perform matching on the odd numbered samples (280 nm), then repeat for the evens (230 nm)
5. “Set Range”
    a. Set the blue bars around each sample well individually
        i. For 280 nm, look only at the first two wells (higher concentrations)
        ii. For 230 nm, look only at the third well (lowest concentration)
    b. Click “Match Files”
6. “Results”
    a. When RMSD holds constant for 3 successive time points, this indicates that your sample has reached equilibrium
    b. Ensure that equilibrium has been satisfactorily achieved in each well
7. When you are convinced that your sample is at equilibrium at the current speed, return to the instrument and set it for the next fastest speed

## Calculating vbar

1. Refer to Durchschlag, H. & Zipper, P. “Calculation of the partial volume of organic compounds and polymers”. *Progr. Colloid Polym. Sci.* **1994**, 94, 20-39.
    a. http://www.springerlink.com/content/r5xp4k0765271k72/fulltext.pdf
2. Based on the chemical structure of your peptide, calculate the partial volume according to Durchschlag and Zipper
3. vbar = (partial volume)/MW

## Data processing

1. Open Heteroanalysis
2. Options – Set vbar / vbar (A)
3. Set density (for aqueous phosphate buffers, I typically use 1.00674)

4. Source Files tab:
    a. Change Folder – wherever the data is
    b. Select File – most recent scan at each speed (ie at equilibrium)
    c. Centerpiece – 6 channels
    d. File types – IP, RA
    e. Click “Load”
    f. Repeat for each speed
5. Loaded Data tab:
    a. Click “Edit” for each loaded file
    b. Left click drag box to include only those data points along curve that fulfill $0.1 \leq \text{data} \leq 1.0$
        i. Click “Delete outside”
    c. Repeat for each file
    d. Model – Ideal (to start with)
    e. Click “Setup Fit”
6. Fit Control tab:
    a. MW – enter correct value
    b. Ext. Coeff. – enter calculated value x 1.2 (to account for path length)
        i. Hold Ctrl while clicking this box to fill down
    c. Float MW, Baseline, and Ref. Conc.
7. Return to Loaded Data tab and switch model to Monomer-N-mer
    a. Lock MW
    b. Float Ln Ka, Baseline, and Ref. Conc.
    c. Begin by floating n, then lock n to each oligomeric state between 2 and 10 (or as high as necessary) to find best fit
        i. When fitting data, you may have to adjust values and floats until you achieve good fits

## Exporting Data

1. Graphs – Export
2. Select desired Graph
    a. This is annoying and tedious because the graphs are just numbered and you have to count which one you want based on the logfile
3. Double click within the graph window
    a. “Editing” window pops up
    b. Select “Export” tab, then “Data” tab (text file will already be selected)
    c. Click Save
        i. Choose desired destination and give the file a descriptive name