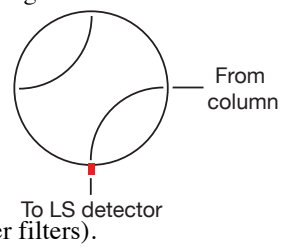
Multi-Angle light scattering (Agard Lab)
Columns: Shodex KW-802.5 or KW-804: http://www.shodex.net/index.php?seitenid=1&prodcat=72&expd_prod=110

Overall Rules:

- Shodex KW-804 and KW-802.5 columns are available.
    - Outside users: It's fine to use these for trial experiments, but if you're going to use SEC-MALS regularly, please buy your own columns. They are about $1200.
- Andrew must approve your samples before you inject them.
    - Be prepared to demonstrate that you won't be injecting aggregated protein into the system.
    - This means you cannot run a void peak from an S200 (or similar) run.
- Samples must be 0.1 µ filtered immediately prior to your run. If you cannot filter your sample, you cannot run it.
- The most important rule: **No rapid changes in pressure**. Once the column is flowing, do not stop it or pause it without slowly ramping it down. Rapid changes in pressure cause shedding of the column matrix. This is the #1 source of noise in light scattering measurements.
- Always prepare enough sample so that you load >50 µL, otherwise you will get air into the sample loop.

Preparing the system for an experiment:

- Select appropriate column
    - Put on new column: arrow on column indicates flow direction. Screw the bottom of the column on first, then set flow to ~0.3 mL/min and fill up the port on the top of the column. Turn off flow, then screw in top connection making sure not to introduce air.
- Change post-column valve to direct flow to light scattering as shown:



- Filter buffer with 0.1 µ filter (smaller than normal buffer filters).
    - Outside users: Ask if you need a filter. We're happy to provide filters in a pinch but regular users should buy their own.
- Put line A1 into your buffer, then run pump wash in UNICORN: Manual > Pump > Pump Wash > Execute
- If you wish to collect fractions be sure the delay volume is set correctly in UNICORN:
    - This must be set before you start the method in UNICORN.
    - System > Settings > Specials > FracParameters > DelayVol set to 0.929 mL.
    - If UNICORN won't let you change the DelayVol, close the Settings window, click End in the menu bar at the top, then try to set the DelayVol again.
- Equilibrate column by slowly increasing flow rate:
    - UNICORN method: Dan/LSkw8seriesRun or Dan/LSkw8seriesGlycerol
    - Takes about 2 hrs to reach full flow rate (0.35 mL/min, or 0.25 mL/min with buffer with 10% glycerol) and for buffer to fully enter column.
    - Pressure at maximum flow rate is typically ~1.7-1.8 MPa.
    - Equilibration can be done overnight to reach lowest baseline noise level. If you do this, wait until max flow rate is reached, then click Hold in the menu bar in UNICORN. Make sure you have enough buffer to last overnight.
    - The UNICORN method will begin ramping down the flow rate after 600 min (not including time in 'Hold' mode). If you need more time, be sure to click Hold before 600 min.
- Purge the flow cell in the differential refractive index detector (dRI; Optilab T-rEX) by selecting [Purge On]. The button will turn yellow while the flow cell is purging.
    - When the line on the detector levels out turn it back to [Purge Off]. The button turns green.
    - If you are using a buffer containing glycerol, purge the flow cell for as long as possible before doing an experiment, preferably overnight. Otherwise you will have a really difficult time getting a flat refractive index baseline.

Running a sample:

- Ideally, your first sample should be ~250 µg BSA (50 µL of 5 mg/mL) dissolved in your mobile phase.
    - This allows you to make sure the system is working correctly, and also allows for normalization of the light scattering detectors and for band broadening correction to be performed.
    - You can skip the normalization if you know the last normalization was performed recently in a similar buffer to the one you are using, but if it's significantly different (i.e. glycerol, different [salt]) or its been a while since a normalization it's a good idea to do it again.
    - Likewise, the default band broadening correction parameters may be okay, but will likely need to be changed for different columns, buffers, flow rates, etc.
- Somewhere between 10 and 50 µg of protein is a good place to start, though how much is necessary varies greatly across different samples.
    - It will probably require some optimization to get good signal – see "How much protein should I use?", available on the Agard Lab SEC-MALS Google Group.
    - The dRI detector is extremely sensitive. Even very small differences between your sample buffer and the mobile phase can cause dramatic spikes in the dRI signal at the end of the column run. This can make data analysis difficult, especially with late-eluting proteins. It is a good idea to dialyze or desalt your protein into the column buffer, especially if the buffers are very different.

- **Filter your sample (0.1 µ centrifugal filters) immediately before loading.**
- Wash the syringe with your mobile phase.
- Make sure the injection valve is set to Load in UNICORN, then wash the loop with your mobile phase (2-3 times)
- Inject ~55ul of sample into the loop (a bit more than 50 uL so you are sure the loop is full)
    - Do not remove the syringe from the injection port or else air will enter the sample loop and be injected onto the column.
- Set up the MALS run in Astra:
    - File > New > Experiment from Method…
    - Choose either LSkw8series or LSkw8seriesglycerol, then click Create.
    - Click the green triangle 'Run' button.
    - The "Basic Collection" window will appear. Do not click OK yet!
- Inject sample manually in UNICORN:
    - Manual > Flow path > Injection valve > Inject > Execute
    - Immediately click OK in Astra.
    - Wait until ~100 µL has been injected, then set injection valve back to Load in UNICORN.
- Takes 36 min per sample run (~50 for mobile phases with glycerol)
- If you want to collect fractions, go to Manual > Frac > Fractionation, set the volume (0.15 – 0.2 mL typically), check that it will be dispensed into an empty well of the plate (change TubeNumber if necessary), then click execute.
    - It will take a couple minutes for your sample to travel through the tube to the fraction collector, so don't be alarmed when the fraction collector doesn't immediately do anything.
    - Remember to go to Manual > Frac > FractionationStop when the run is done or it will drip buffer all over the fraction collector.

Analysis:
- Your results show up in the list on the left-hand side of the Astra window.
- Click the '+' next to 'Procedures' to expand the list. Double click to open a procedure.
- Once you're done with a procedure, click 'Apply' (keeps Procedure window open) or 'OK' (applies changes then closes Procedure window).
- Basic Collection:
    - Once your run is done, check the quality of the data from each LS detector.
        - Click the checkboxes in the list on the right side of the window to show the signal from each detector.
        - LS detectors 1 & 2 will have no useful signal due to refraction by the glass flow cell (see page 3-17 in the DAWN HELEOS manual). Any signal you see here is from stray light in the system. Data from these detectors is excluded from analysis by default.
        - You will probably see that the high and low angle detectors LS 3-4 and 17-18 have either no useful signal or extremely noisy signal. Make a note of the detectors with bad signal – you can exclude these detectors' data from the analysis later on.
    - Click 'OK'.
- Despiking:
    - Default settings are almost always okay.
    - If your light scattering data is particularly noisy, it may help to increase the despiking level from 'Normal' (default) to 'Heavy'.
    - Select a despiking level using the drop-down menu in the upper left corner. Look at the graph to see if the noise level looks better.
    - Click 'Apply' or 'OK'
- Baselines:
    - Click 'Autofind' in the upper left and check the results for all detectors using the list on the right side of the window. The baseline can be adjusted manually by clicking and dragging the handles at the ends of the baseline indicator.
    - Alternatively, set baseline manually by clicking and dragging. Start with LS 11 (the 90° detector) then click 'Set All' to apply baseline setting to all detectors. Check all the detectors using the list on the right hand side of the window.
    - Check the dRI baseline carefully – it usually requires different settings than the LS baselines.
    - If some of the light scattering signals look really bad (usually the low/high angle detectors 1-4 and 17-18), it's okay to ignore them. You can exclude the data from these detectors later.
    - Click 'Apply' or 'OK'
- Band Broadening
    - Background info:
        - Peaks will broaden out as they travel from the LS detector to the dRI detector due to diffusion. Additionally, the two instruments have different sensitivities. Together, these effects make a peak have a different shape in the LS channel than in the dRI channel. This results in a characteristic "frowning" molecular weight curve across the peak, with the molecular weight systematically overestimated at the apex of the peak and underestimated at the tails. This can be corrected with the "Band Broadening" procedure.
    - Make sure the BSA monomer peak is selected in the "Peaks" procedure described below. This lets you check the quality of the band broadening correction.
    - In the "Band Broadening" procedure, check the "Instrument" drop-down menu in the upper left: "Optilab rEX" should be selected.
    - In the graph, select the entire BSA monomer peak, making sure not to include the left-hand tail if it looks like the dimer peak is overlapping with the monomer.
    - Below the graph, make sure that both "Generic UV Device" and "DAWN HELEOS" are enabled.
    - Click "Reset" for both "Generic UV Device" and "DAWN HELEOS" to reset the correction parameters to the

defaults.
    - At the top of the window, click “Perform Fit”. The graph will update with the correction applied to the data. The signals from the UV, LS, and dRI detectors should overlay one another much better now.
    - Click the “Broadened” button to turn on/off the broadening correction in the graph so you can compare the corrected and un-corrected data.
    - Go to the “EASI Graph” (see below) and check the quality of the correction. Ideally, the molecular weight should be flat across the entire BSA monomer peak. If you select the entire range of BSA peaks, you should see that the molecular weight steps up gradually from monomer to dimer without any fluctuations between the peaks.
    - In the “Band Broadening” procedure, click “Apply” or “Okay”.
- Alignment:
    - **Do not adjust this if you are doing Band Broadening correction.** The band broadening correction also takes care of alignment.
    - Select a well-defined peak then click ‘Align’. This will adjust the inter-detector delay volume so that the light scattering and refractive index signals overlay.
    - If a peak contains a monodisperse species, the light scattering and refractive index signals should overlay more or less exactly. If multiple species in your sample elute at the same time or your peaks are very close together, it may be impossible for the signals to overlay exactly. If this is the case for your sample, you may have to play around with the delay volume and check the end results in the EASI graph (see below). You can manually adjust the delay volume in the table below the graph.
    - Alternatively, if you’ve run a BSA sample, you can determine the delay volume using the BSA monomer peak, then apply that delay volume to your other experiments.
    - Click ‘Apply’ or ‘OK’.
- Peaks:
    - Select the peaks for which you want to determine molecular weight.
    - Repeat as needed for additional peaks.
    - To delete a peak, click in the table below the graph then hit the delete key.
    - Click ‘Apply’ or ‘OK’.
- Normalization:
    - Can be performed with any small (<10 nm), monodisperse, highly concentrated particle, usually BSA.
    - In the ‘Peaks’ procedure, select the entire BSA monomer peak.
    - In the ‘Normalization’ procedure, set the radius to 3.48 nm, then click normalize. The normalization coefficients will update.
    - In the “Details” section at the bottom of the Normalization window, make sure the “Normalization Type” is set to “Area”
    - Click ‘Apply’ or ‘OK’.
- Molar Mass & Radius from LS:
    - Use this procedure to check that your normalization coefficients are good and to exclude data from detectors with bad signal.
    - The ‘results graph’ on the left shows (roughly speaking) light scattering as a function of scattering angle. If the normalization is good, all the points should lie on the red fit line. The fit is calculated for each ‘slice’ through the peak. You can view each slice by clicking on the ‘control graph’ on the right side, then using the left and right arrow keys to view the data for each slice.
    - Common problems with normalization:
        - Points ‘zig-zag’ on either side of the fit line.
            - Try adjusting the peak selection and re-normalizing.
        - One point is very off relative to the others.
            - Try adjusting the peak selection and re-normalizing. If this doesn’t help, look at the raw data from that detector to make sure it’s not especially noisy or spiky. If it looks like the data quality is bad, exclude it from analysis as described below.
        - Points at very high and low angles are very off.
            - These are the noisiest positions from which to measure light scattering and these data are down-weighted in the analysis. It’s okay to exclude them from analysis as described below.
    - Excluding detectors with bad data: Click the ‘+’ next to ‘Enable detectors’ in the table below the graphs, then uncheck the bad detectors. Detectors 1 & 2 should always be disabled when collecting data in aqueous buffers.
- EASI Graph:
    - Graph shows Rayleigh ratio and differential refractive index, and the calculated molecular weight for the selected peaks.
    - You can choose which data to show by right clicking the graph, choosing Edit, then (un)checking the boxes next to Rayleigh ratio, etc.
    - You may want to change your peak selection a bit to cut out noisy portions on the edges of the peaks.
    - Disabling detectors with noisy signals can also improve the results.
    - If the line showing molecular weight is slanted, re-running the band broadening correction (or alternatively adjusting the inter-detector delay volume with the ‘Alignment’ procedure) can help to straighten it out. Remember that the line will only be exactly horizontal for monodisperse species if band broadening correction has been done. If you have co-eluting species (e.g. a dimer dissociating to a monomer), the line is expected to slant.
    - If you want to export the data for plotting in different software, right click the graph, choose Edit, then click Export…
- Reports:
    - Reports let you see the numerical value of the calculated molecular weight.
    - Click ‘Report (Summary)’, then scroll down to Results
        - M_w is the weight-averaged molar mass that is directly determined from the fit to light scattering and refractive index data.

        - The percentage error shown is derived from uncertainties that are experimentally characterized in each run. Sources of uncertainty include noise in the light scattering and refractive index signals (measured at the beginning and end of the run) and uncertainty associated with the fitting procedures. These errors are propagated through all of the molecular weight calculation procedures to yield the value shown. Sources of uncertainty that are <u>not</u> included in this percentage error include errors in instrument calibration, errors in determining the normalization coefficients, and errors in the *dn*/*dc* value used to calculate protein concentration.
        - For a more detailed explanation, see page E-21 in the Astra manual (pdf on desktop).
    - Check the calculated mass to make sure all of your protein is eluting and not sticking to the column.
        - Expand the peak in the 'Peaks' procedure so that all dRI signal above the baseline is included.
        - Compare the calculated mass to the mass you injected. Hopefully the values are fairly close. If not, that means your sample is getting stuck somewhere in the system, <u>which is bad</u>. If your sample recovery is poor, <u>please don't keep injecting it</u> to prevent the column and/or system from becoming clogged. Try changing your buffer conditions to see if recovery improves.

When you are done:

- If you clicked 'Hold' in UNICORN, make sure to click continue.
- <u>Without pausing or stopping the flow,</u> quickly switch the buffer line into filtered 0.02% NaN_3 or MilliQ H_2O.
- Open the purge valve on the dRI detector.
- The UNICORN methods are set to slowly ramp the pressure down after 600 minutes (not including 'Hold' time).
- When the method finishes, remove the column from the system, screw in the plugs (<u>make sure they're actually plugs and not connectors!</u>) and place in the drawer in the cold room.