



Live Webinar Q&A Sheet:

Therapeutic Proteins in Biological Fluids: Size Characterization Using AF4 with Fluorescence and SPR

The recorded webinar may be viewed from the [FFF-MALS](#) webinars page. These questions were submitted by live viewers. Additional information on Eclipse™, VISION™, DAWN® and FFF-MALS may be found in the Wyatt website Resources section under [Webinars](#), [Application Notes](#), and [Bibliography](#), as well as on the corresponding [Product page](#) and [Solutions](#) page of our web site.

Please contact info@wyatt.com with any additional questions.

Q: What is the maximum sample load for separation with FFF?

A: The exact amount depends on the sample type, the requirements of the resolution and the channel footprint. The analytical channels usually take a few µg up to tens of µg, while with the semi-preparative channel, milligram quantities can be injected.

Q: In complex biological samples, can we be sure that the separation by FFF is strictly by size and can we expect elution of homogeneous fractions from the FFF channel?

A: We can expect fractions eluting from the FFF separation to have the same hydrodynamic radius. This is true in the absence of non-ideal interactions with the membrane. But that does not necessarily mean the fractions are homogeneous in composition. In a complex biological matrix, a mixture of different species will elute which have the same size, but can be different proteins, lipoproteins and complexes of species which happen to have the same size. In this case additional specific analytical methods are needed to identify the component of interest. This can be SPR or mass spectrometry or a biological assay.

Q: How complicated is the method development in FFF for a complex sample?

A: If one has an estimate of the minimum and maximum sizes that needs to be separated, it is straightforward to develop a flow program that will provide good separation using Wyatt's FFF method development software, [VISION DESIGN](#). A batch DLS measurement of the sample, e.g. using the [DynaPro® NanoStar® DLS instrument](#), is usually sufficient to get a good approximation of the minimum and maximum size. But there are other parameters which need to be optimized, such as membrane type, the composition of the carrier solution and the injected amount. In most cases, good starting parameters can be found in the published data on similar samples.



- Q: What new sensors were added to the AF4 to measure all relevant system parameters?*
- A: Eclipse NEON includes multiple internal sensors to measure pressure, flow rates, solvent density and also valve positions. Taken together, the readings reflect the system status. Any deviation from the expected values can be detected and a conclusion on necessary corrective measures is drawn in real time. An example is a mismatch between the flow rate reported from the pump and the inlet flow rate measured by the Eclipse. If the inlet flow rate is lower than the pump flow rate, the cause of the mismatch might be leakage; otherwise it points to a malfunction of the pump.
- Q: With regards to detection as mentioned, will MALDI-TOF perform the same as MALS?*
- A: MALDI-TOF determines molar mass, similar to MALS, and in that respect they can be seen as similar. But MALDI-TOF cannot be done online. The sample is spotted on a disc and then scanned off-line by the mass spectrometer. There are also many limitations in ionizing an inhomogeneous sample and MALDI-TOF only works for lower molar mass macromolecules. It will not work for particles and large components for which FFF is mostly applied. Therefore, MALS and DLS are the primary detection methods coupled to FFF to determine size.
- Q: When you did the case 1 experiment, what kinds of cross gradient was used? Linear or non-linear?*
- A: A constant cross flow was used to separate the protein fraction of the serum, because this results in optimal resolution of albumin, antibodies and their oligomers. In order to elute the large sizes, the cross flow is reduced in a fast linear decay (an exponential decay would also work).
- Q: Do you have any special cleaning procedure for your detectors?*
- A: Usually flushing the system with a 20/80 % mixture of ethanol and water is sufficient. The detector chain itself can be cleaned with harsher solutions like acids or strong basic detergents, or with protease solutions to remove protein deposits.
- Q: What is the peak before F1 for stressed Ab in serum?*
- A: These are the albumin peaks which elute early compared to the antibodies.
- Q: What is the volume and protein concentration requirement for the AF4 method?*
- A: The volume is variable, because during focusing the solvent in which the sample was dissolved permeates through the membrane. Injection volumes depend on the capability of the injector, but it's the quantity of sample that is relevant for detectability. For proteins, typical minimum amounts are a few micrograms.

- Q: Did you perform specific studies to assess whether dilution induces alterations on proteins?*
- A: Generally, dilution can cause alterations, like dissociation of dynamic complexes or even aggregates. We can determine if this occurs by injecting different sample amounts and observing a change in the results, but this was not observed in the present study.
- Q: Is it possible to apply this method to characterize a conjugate which has MW of 10^6 Da with monomer 2×10^4 Da?*
- A: Yes, there is no reason, why that should not be possible. The molar mass range is well within the capability of FFF.
- Q: What is the best choice for membrane protein separation, SEC or FFF?*
- A: For membrane proteins a detergent must be added to the carrier solution in order to solubilize the protein, and the detergent concentration must be maintained. However, in FFF the permeability of the membrane may be higher for the detergent than for the protein, leading to loss of detergent and the proteins may aggregate or adhere to surfaces. This can complicate FFF method development, so if the separation works on SEC, that is usually the preferred separation method.
- Q: Which protein or polymer is used for the calibration when a 100 kDa membrane used?*
- A: Calibration is not necessary for molar mass determination in the SPR assay, which is aimed at quantitation. For purposes of estimating size from retention time, the effective height of the channel can be calibrated using a large protein like ferritin or thyroglobulin which does not permeate a 100 kDa membrane.
- Q: What can you tell us about the time required for aggregate analysis? Is it time consuming?*
- A: The time-consuming step is setup of the system and calibration of the SPR instrument for the protein of interest. The actual analysis time is between 20 and 30 minutes, depending on the run time of the method.
- Q: Have you studied proteins that have a concentration-dependent reversible self-association property with this technique?*
- A: Not up to this point. Generally, the change of concentration during focusing and elution will shift the equilibrium of the complex. Retention depends on the average state of the complex during migration in the channel. The eluting fraction is diluted, and this will shift the equilibrium towards the components forming the complex. The analysis of the results can be complicated. A better choice for studying reversible self-association is [CG-MALS](#) which uses the [DAWN[®] MALS instrument](#) with a [Calypso[®] composition-gradient system](#).



Q: What's the molar-mass resolution we can expect from AF4-MALS?

A: AF4 can separate two monodisperse populations which differ in molar mass by 40% or in radius by 15%. The resolution in terms of detecting a change in size as fractions elute is very high – on the order of 2-3%.

Q: What is the dilution factor in the AF4 channel?

A: The injected sample is first concentrated at the membrane to higher values compared to the original solution. This can be a factor of 10x or even 100x at high cross-flow rates. Then the sample zone is diluted at the outlet of the channel. This dilution can be calculated: it depends on the size of the sample and increases with the square of the cross-flow rate, and is generally on the order of 100x – 1000x. Using the Dilution Control Module™ option for Eclipse, the dilution can be reduced by a factor of up to 10, which means that the sample can be retrieved at roughly the original injected concentration.

Q: Do you know if this kind of analysis is used for quality control of the current COVID19 vaccines?

A: AF4-MALS is used extensively in the development of COVID-19 vaccines by most of the leading vaccine producers. It is generally not used for the final release assay or batch control. This may change in the future because AF4-MALS can determine multiple critical quality attributes in a single run.