# Size-exclusion chromatography (SEC) analyses at the Australian Proteome Analysis Facility (APAF)

**Size exclusion chromatography (SEC)**, also known as **gel filtration chromatography**, is a technique used in biochemistry and molecular biology to separate molecules based on their size.

At APAF, SEC is employed to profile the composition and molecular weight distribution of proteins or peptides in a sample.

Our SEC services provide comprehensive characterisation/s of a variety of sample types, including purified proteins, peptide digests, food products, and biological samples. SEC is a versatile tool in many scientific fields, providing valuable insights into the molecular composition of samples and for understanding protein structure, function, and assessment of degradation and/or aggregation.

Standard SEC services are ISO 17025-accredited.

## Applications of Size-exclusion chromatography

Size exclusion chromatography (SEC) is a gentle technique that relies upon a porous matrix within the chromatography column to separate smaller molecules that take longer to travel through the column than the larger molecules which elute sooner.

SEC affords high resolution and reproducibility and is a non-destructive technique that preserves the biological activity of sensitive biomolecules. It has multiple applications, including:

- **Molecular Weight Determination**: SEC characterises the molecular weight distribution of proteins, peptides, and other macromolecules. This is crucial for understanding the properties and behaviour of these substances or indicating potential aggregation/degradation.
- **Biopharmaceuticals**: SEC is utilised to ensure the purity and quality of biopharmaceutical products, such as monoclonal antibodies or vaccines.
- **Quality Control**: SEC is a standard technique in quality control processes for verifying the consistency and stability of biopolymer products.
- **Protein Purification**: SEC may be employed to purify proteins of particular sizes, enabling researchers to remove aggregates or contaminants from protein samples.
- **Polysaccharide Analysis**: SEC is employed to analyse the size distribution of polysaccharides, which is important in food science and biotechnology.

## Challenges and Considerations

- **Defined Molecular Weight ranges**: SEC has a limited separation range and can be less effective for very small or very large molecules.
- **Sample Preparation**: Proper preparation is crucial to avoid contamination and ensure accurate results. As buffer constituents can interfere with separation, we are happy to assist you with advice and assistance to ensure a robust result.
- **Sensitivity and Specificity**: Choosing the right buffer/matrix is pivotal to balance protein solubility with structure and stability.

## Method Summary

The process generally involves:

- **Instrumental setup and the running of standards**. Freshly prepared mobile phase and standards are essential to reliable separation. To accurately determine molecular sizes, the column is calibrated using standards of known molecular weight/s. This calibration curve enables the correlation of the elution volume with the molecular size.

- **Sample preparation**. Dry samples are resuspended in an appropriate buffer, whilst liquid samples are diluted in the mobile phase. Sample suspensions are then passed through a 0.22µm filter to remove microparticles or debris.

- **Samples injected onto the column**. The SEC column is packed with a porous matrix, with pores of specific sizes. The sample mixture is injected into the column, and as the sample travels through the column, molecules interact with the porous beads. Larger molecules cannot enter the pores and thus travel quickly through the column. Smaller molecules enter the pores and take a longer, more convoluted path, slowing their progress
    - Note: The separation is based purely on size of the molecules. Molecular weights can easily shift following aggregation, degradation or post-translational modification.

- **Detection**: As the molecules exit the column, they pass through a UV-Vis spectrophotometer. The detector records the elution profile, producing a chromatogram that displays the separation of the molecules.
    - Note: refractive index detectors, or light scattering detectors available at other MQ facilities.

- **Data Analysis:** The chromatogram is analysed to profile the size distribution of the molecules. Peaks on the chromatogram correspond to different molecular sizes, allowing for the identification and quantification of the components in the sample.

**Key Points:**

- **Resolution:** The resolution of SEC depends upon the pore size, the presence of challenging components such as lipids or detergents within the matrix, and the magnitude of the range of the molecules being separated.

- **Buffer:** The choice of buffer can affect the separation, as it needs to maintain the stability and solubility of the molecules. Our methods favour native (i.e., physiological conditions) conditions, but other approaches can be utilised on prior agreement.

- **Reduction**: Some complex sample types may benefit from being reduced prior to separation using dithiothreitol, or similar compounds.

- **Flow Rate:** The flow rate of the mobile phase can influence the separation efficiency. Our methods employ slower flow rates to improve resolution.

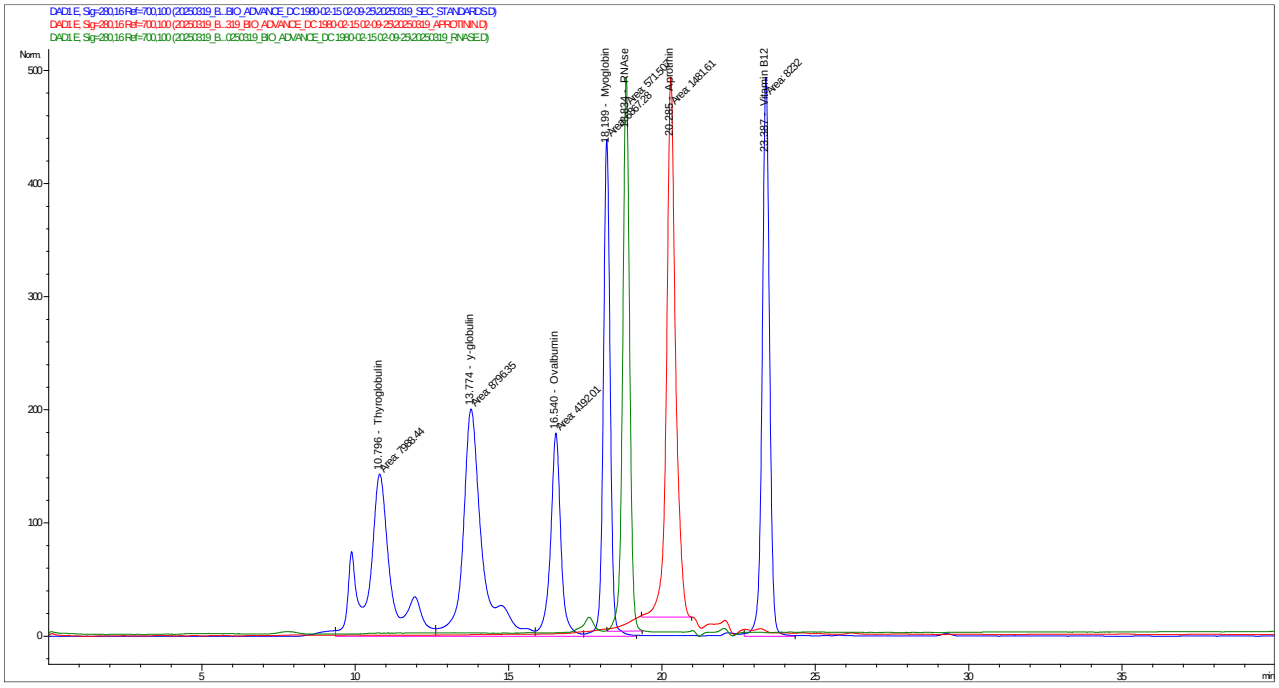
**Molecular weight profiles available**

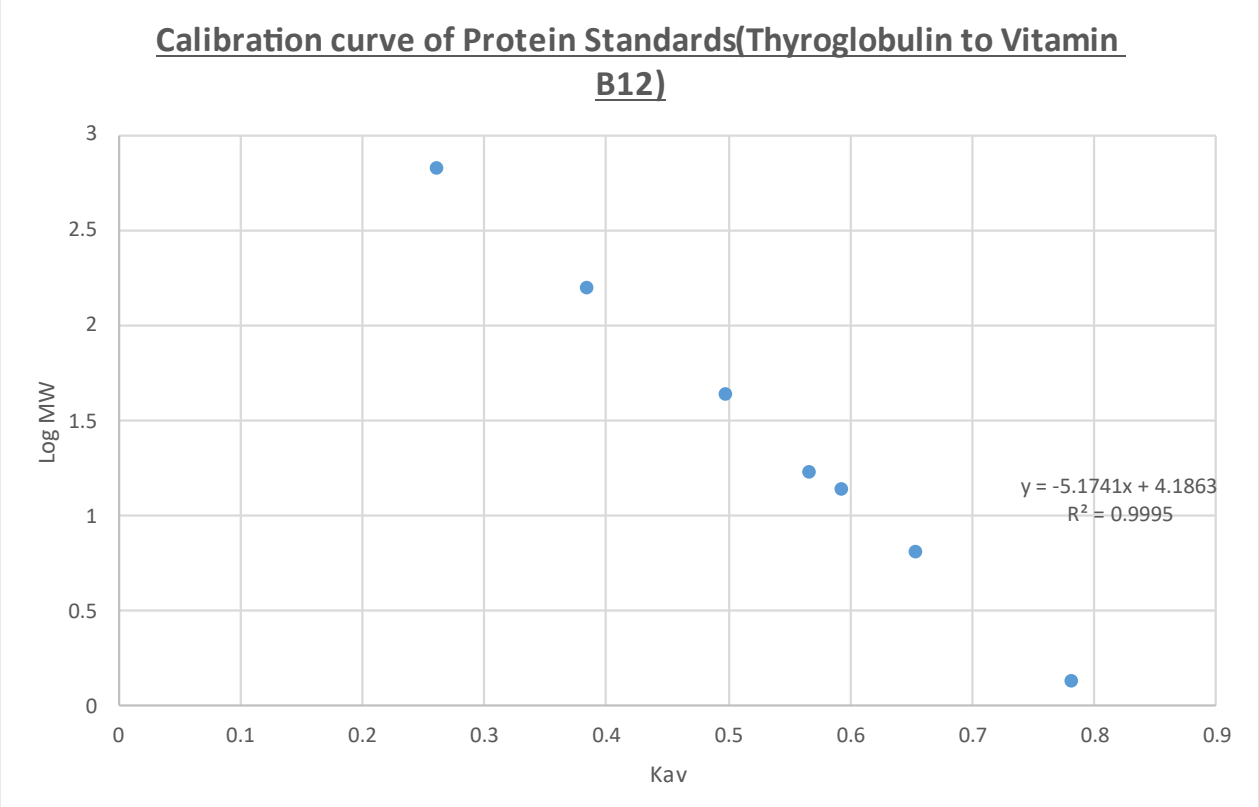
The table below outlines the molecular weight ranges that are quantified by stock columns: Custom columns can be provided by the client, or ordered with sufficient lead-in time (chargeable).

| Column type | Ideal Molecular weight range | General applications | Notes |
|---|---|---|---|
| Agilent BIO-SEC-3 | 500kDa – 5kDa | Antibody characterisation | For use with purified proteins or antibodies, only |
| Agilent AdvanceBio | ~750 kDa – 5 kDa | General molecular weight profiling of biological samples or complexes | |
| Superdex 200 Increase | ~600 kDa – 10 kDa | Molecular weight profiling of complex samples and challenging matrices | Small bead size and narrow particle size distribution provide high resolution. Longer run times but more amenable to harsher sample types. |
| Superdex 75 | ~70 kDa – 3 kDa | General molecular weight profiling of protein digests | |
| Superdex Peptide HR | 15,000 Da – 250 Da | Peptide molecular weight profiling | |
| Agilent GF-250 | 400 kDa -4,000 Da | General molecular weight profiling of biological samples or complexes | |
| Jupiter C4 | >10 kDa | Profiling protein interactions, aggregation | For use under non-native conditions. May require method development for challenging analytes. |
| Additional columns can be sourced with sufficient lead-in time | | | |

**Note**: mass ranges are idealised for globular proteins and may not be appropriate for all analytes of interest or sample types.

## Example Chromatogram – Molecular weight standards and system suitability



## Data reporting

APAF's standard procedure for Size-exclusion chromatography is to analyse a client sample and then to report the standard molecular weight window results upon a NATA-endorsed report. APAF offers additional reporting options, however these must be discussed prior to the commencement of the project and may be subject to respective pricing.

Should you require custom molecular weights to be reported, please notify the Technology Manager – Protein Analysis, Dr. David Cantor (david.cantor@mq.edu.au) at the time of quoting or *before* the report has been released. Once the report has been issued, any requests to report individual replicate data will incur an additional reporting charge.

Any modifications to the standard workflow should be identified and discussed with the Technology Manager prior to commencing any project.

## Data Formatting

APAF offers a standard output formats for SEC analysis results:

- A PDF report, containing project details, standards, relevant blanks, molecular weight calculations, sample chromatograms and Molecular weight profiles.

Should you require small sample number projects to be reported in Microsoft Excel format, please notify APAF staff by listing the requirement on the APAF Service Request Form.

## Interpreting your results

Interpreting the results of SEC experiments can be nuanced and multifactorial. APAF is happy to assist you in interpreting the results and in drawing valid conclusions from the data.

## Complimentary MW analysis by Mass Photometry

Size-exclusion chromatography results can be complimented with Mass Photometry at APAF. Mass photometry is a label-free technique that measures the molecular weight of individual molecules in solution by detecting their light scattering when they transiently interact with a surface.

Mass photometry is a cutting-edge method for analysing biomolecules from 30 kDa to 5 MDa in size whilst still in their native state, providing insights into protein complexes and assemblies, protein-protein interactions, affinity and dynamics, and structural heterogeneity.

## Sample Submission and Shipment to APAF

For most sample types, we recommend sending 1-10g of material in a sealed container under appropriate temperature conditions. This allows for representative sampling, and repeat analysis, should it be required. Where the available sample amount is lower, we recommend discussing this with an APAF team member to determine whether a scaled-down assay is possible/appropriate.

When ready to despatch, please direct the parcel to the following address, with attention to the Protein Analysis team:

- Science Stores, Faculty of Science and Engineering, 14 Eastern Road room 186, Macquarie University, NSW, 2109, Australia

**Further Enquiries**

Please see our website for more information on the Australian Proteome Analysis Facility:

https://www.mq.edu.au/research/research-centres-groups-and-facilities/facilities/macquarie-analytical-and-fabrication-facility/australian-proteome-analysis-facility

Please direct enquiries or requests for further information to info.apaf@mq.edu.au, or to Dr. David Cantor (david.cantor@mq.edu.au).

Thank you,

Protein Analysis Team