Multiplexed Immunoassay analyses at the Australian Proteome Analysis Facility (APAF)

Multiplexed Immunoassays (MIAs) are advanced techniques used in biomedical research and clinical studies for the academic and pharmaceutical sectors. They are used to simultaneously determine the abundance of multiple analytes from a single sample. Typically quantifying up to 80 proteins, biomarkers or hormones per well, the strength of this platform is the low sample requirement and the ability to collect hundreds, or thousands of data points in a single day.

Our Multiplexed Immunoassay services utilise the gold-standard Luminex 200 system to provide precise and comprehensive identification and quantification of key analytes from various sample biofluids, including plasma, serum, cell lysate, tears, saliva, and urine. MIAs are targeted tools that provide valuable insights into the molecular composition of biospecimens the biological changes that underpin them.

Most importantly, APAF supports our MIA clients through a highly collaborative approach, which extends from support in choosing the right panel, through to interpretation of the results. With a decade of experience in the technique, APAF extends this knowledge and guidance to all our clients to get the best quality data out of the analysis.

Applications of Multiplexed Immunoassay Analysis

- **Biomarker Discovery**: High-throughput identification of novel disease biomarkers in cell lysates, serum, or tissue samples by screening multiple targets at once.
- **Immune Response Profiling**: Assesses cytokine and chemokine levels to understand immune system dynamics during infection, inflammation, or therapy.
- **Drug and Vaccine Development**: Monitors inflammatory responses and safety profiles by measuring stress or damage markers pre- and post-treatment.
- **Clinical Diagnostics**: Facilitates disease diagnosis or progression monitoring using pre-configured vendor panels for established protein biomarkers.
- **Pathway Analysis**: Supports exploration of complex signalling pathways as orthogonal support to proteomic investigations.

Benefits of Multiplexed Immunoassays

- **Simultaneous detection** and quantitation of up to 80x analytes in the same well.
- **Low volume required** (10-25uL) from precious samples, with improved per-sample cost.
- **Comprehensive data** from a single experiment, detecting biomarkers as low as a few pg/mL.
- **Increased throughput and efficiency**, detecting tens of thousands of read-outs per hour.

Challenges and considerations with Multiplexed Immunoassays

- **Kit cost** can be a significant barrier and *is not a guarantee of success*.
- **Low sensitivity from matrix effects**, particularly when detecting low-abundance biomarkers in a complex sample matrix (i.e., lysate or tissue homogenates).
- **Dynamic range can be a challenge** when target analytes can span a wide range of concentrations in an assay, necessitating different dilution requirements. As a result, highly variable analytes can fall above or below the range of the standards.
- **Cross reactivity** of capture and detection antibodies to non-target antigens, leading to false-positive results. This can be a significant challenge, and minimisation is essential for ensuring specificity and accuracy. The use of commercially, or vendor-validated kits is advised.
- **Variability** can undermine the reliability and reproducibility of the assay, stemming from biological, technical and batch effects.
- **Data analysis** can be a challenge, but APAF's Bioinformatics Team are positioned to assist with visualisation and interpretation.

A key consideration is the balance of sample number against the number of technical replicates. For a 96-well assay, 16-wells are consumed by the standards and blanks if run in technical duplicate. This leaves 80 wells available for unknown, control or reference samples. This means that the following number of samples can be assayed upon the one plate:

Number of technical replicates per sample	Number of unknowns, controls or reference samples per plate
1	80
2	40
3	26
4	20

For each of the above reasons, APAF highly recommends discussing your needs, goals and design with our experienced staff *prior* to the commencement of the project.

Method Summary

Multiplexed Immunoassay analysis services are ISO 17025-accredited at APAF, and involve the following general steps (Figure 1):

1. Sample filtration (if sufficient sample volume is available)
2. Bead Preparation – Beads that are pre-coated with capture antibodies for the target analytes are dispensed and washed.
3. Sample Incubation – Target analytes bind to the beads.
4. Detection Antibody Addition – Biotinylated detection antibodies bind to captured analytes.
5. Reporter Addition – Streptavidin-PE binds to biotin for signal generation.
6. Data Acquisition – Beads are analysed by a flow-based Luminex 200 reader.
7. Data Analysis – Fluorescence quantification and concentration calculation.

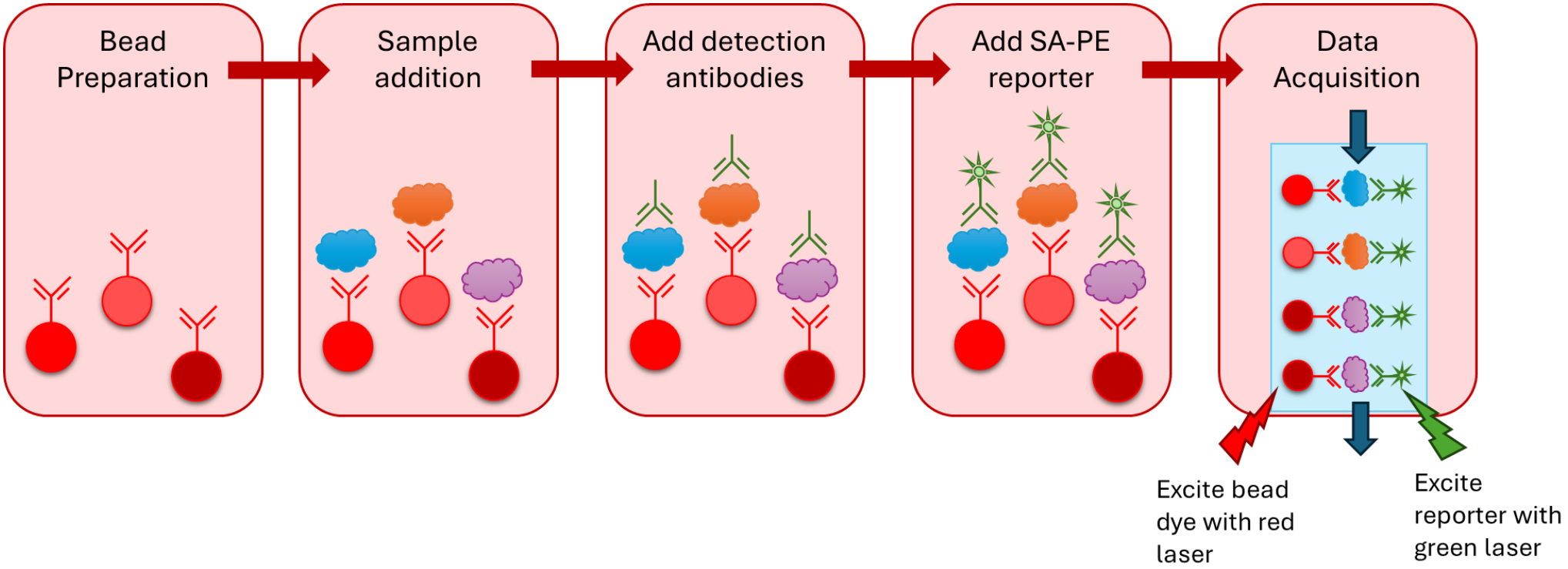


Figure 1. Schematic graphic of multiplexed immunoassay workflow.

Notes:

- Stages listed here are general in nature, and manufacturer's instructions are strictly adhered to. Different kits/vendors may employ individual incubation times and conditions.
- Filtration of samples is recommended to ensure that the microfluidics of the Bioplex 200 system are not blocked/restricted during data acquisition, which impairs bead classification and fluorescence signal accuracy.

Data reporting

APAF's standard procedure for multiplexed immunoassay analysis is to analyse a client sample in technical duplicate, with the data provided as a multi-tabbed excel attachment with a PDF report containing pertinent project information.

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

Data Formatting

APAF offers two standard output formats for multiplexed immunoassay analysis results, at the discretion of APAF staff:

1. A PDF report coversheet, with a Microsoft Excel attachment listing respective values for each given analyte (i.e., single-analyte view).
2. A PDF report coversheet, with a Microsoft Excel attachment listing respective values for each given parameter (i.e., multi-analyte view).

Interpreting your results

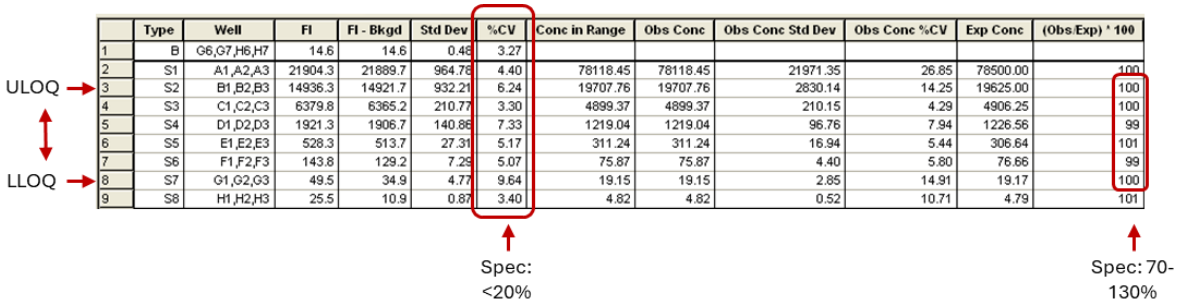
APAF is committed to supporting our clients in terms in interpreting the data that is generated from this technique and often incorporate a scheduled debrief in the project to address client queries and/or concerns. When assessing the assay performance, it is important to consider factors such as:

Specificity

Investigate potential for antibody-related cross-reactivity stemming from the presence of interfering compounds from the sample matrix. This is an important consideration for and is assessed by the kit vendor through antigen detection/exclusion experiments.

Assay working range

The working range of concentrations within the assay varies between analytes and kits due to the multiplexed nature of these products. Precise and accurate quantitations employ analyte-specific boundaries that are defined by the lower limit of quantitation (LLOQ) and the upper limit of quantitation (ULOQ). The two main parameters dictating these boundaries are intra-assay precision (%CV) and standard curve recovery (Figure 2).



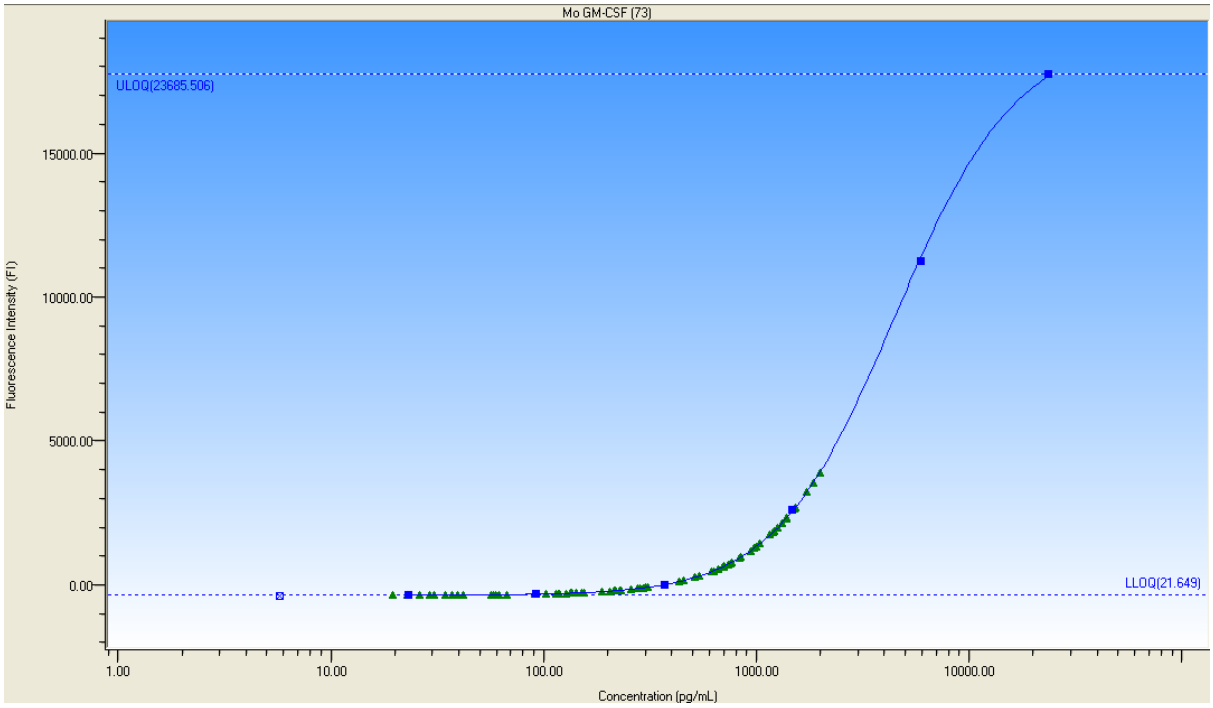
	Type	Well	FI	FI - Bkgd	Std Dev	%CV	Conc in Range	Obs Conc	Obs Conc Std Dev	Obs Conc %CV	Exp Conc	(Obs/Exp) * 100
1	B	G6,G7,H6,H7	14.6	14.6	0.48	3.27						
2	S1	A1,A2,A3	21904.3	21889.7	964.78	4.40	78118.45	78118.45	21971.35	28.85	78500.00	100
3	S2	B1,B2,B3	14936.3	14921.7	932.21	6.24	19707.76	19707.76	2830.14	14.25	19625.00	100
4	S3	C1,C2,C3	6379.8	6365.2	210.77	3.30	4899.37	4899.37	210.15	4.29	4906.25	100
5	S4	D1,D2,D3	1921.3	1906.7	140.86	7.33	1219.04	1219.04	96.76	7.94	1226.56	99
6	S5	E1,E2,E3	528.3	513.7	27.31	5.17	311.24	311.24	16.94	5.44	306.64	101
7	S6	F1,F2,F3	143.8	129.2	7.29	5.07	75.87	75.87	4.40	5.80	76.66	99
8	S7	G1,G2,G3	49.5	34.9	4.77	9.64	19.15	19.15	2.85	14.91	19.17	100
9	S8	H1,H2,H3	25.5	10.9	0.87	3.40	4.82	4.82	0.52	10.71	4.79	101

Figure 2. Intra-assay precision (%CV) and standard curve recovery.

Sensitivity (Limit of Quantification; LOQ)

The lowest detectable amount of an analyte (i.e., LOD), is often as the concentration of the analyte corresponding to the median fluorescent intensity (MFI) of the background, plus two or three standard deviations (SD) of the mean background MFI. This value corresponds to the lowest analyte concentration that can be distinguished from zero (i.e., background) but does not guarantee precision/accuracy at that level.

As MIA seeks to provide robust quantitative data, LOQ values represent the lowest or (highest) concentration of a cytokine that can be accurately and reliably quantified with a defined level of precision and accuracy. In effect, these values define the dynamic range of the assay, delineating the limits of reliable quantification. From these upper (ULOQ) and lower (LLOQ) boundaries, the corresponding concentration values can be assessed against the logistic-5PL regression (Figure 3):



$$y = d + \frac{(a-d)}{(1+(\frac{x}{c})^{b})^{g}}$$

d = response at zero concentration
a = response at infinite concentration
c = midpoint concentration
b = slope factor
g = asymmetry factor
x = concentration
y = response

Figure 3. Standard curve with 5-parameter logistic (5PL) regression. Standards are shown as blue squares, and unknown samples as green triangles. ULOQ and LLOQ values are shown as dashed lines. The above figure demonstrates that most observed data points fell within the assay working range, with some at (or below) the lower bounds, thus suggesting conservative interpretation of those values.

Accuracy

The closeness of agreement between the value and the accepted reference value is used to assess the fit of a curve to the standard points by calculating the ratio of the observed concentration to the expected concentration of each standard point (see Figure 4).

Obs Conc	Exp Conc	(Obs/Exp) * 100
78118.45	78500.00	100
19707.76	19625.00	100
4899.37	4906.25	100
1219.04	1226.56	99
311.24	306.64	101
75.87	76.66	99
19.15	19.17	100
4.82	4.79	101

$$Recovery\ percentage\ = \frac{Observed\ Concentration}{Expected\ Concentration}\ x\ 100\%$$

Figure 4. Calculation and assessment of standard curve recovery (Obs/Exp)*100%).

Precision

The closeness of agreement amongst a series of measurements obtained from multiple sampling of the same homogenous sample is highly important and is further defined by intra- and inter-assay precision. Intra-assay precision is an expression of within-run repeatability. Inter-assay precision is an expression of plate-to-plate consistency (Figures 2 and 5, shown as %CV).

IFN-g (34)	Intra-assay CV for Standards (mean FI of Run 1)		
Exp Conc (pg/mL)	Mean FI	Std Dev	%CV
71885	6837.5	77.1	1.1
17971.3	5409.5	0.7	0.0
4492.8	2677.5	194.5	7.3
1123.2	728.0	25.5	3.5
280.8	177.0	9.9	5.6
70.2	52.3	2.5	4.7
17.6	28.0	0.0	0.0
4.4	24.0	0.0	0.0

Figure 5a. Intra-assay variability and precision of Run 1 (IFN-γ).

IFN-g (34)	Inter-assay CV - Standard mean FI						
Exp Conc (pg/mL)	Run 1	Run 2	Run 3	Run 4	Mean	Std Dev	%CV
71885	6837.5	6787.5	6466	6806.5	6724.4	173.5	2.6
17971.3	5409.5	5384.5	5266.5	5354	5353.6	62.4	1.2
4492.8	2677.5	2936	2514.8	2769.5	2724.5	176.0	6.5
1123.2	728	821.8	722.5	730.5	750.7	47.5	6.3
280.8	177	194	180	181.3	183.1	7.5	4.1
70.2	52.3	64.8	57.5	59.8	58.6	5.2	8.9
17.6	28	36.5	30.5	34.5	32.4	3.8	11.9
4.4	24	28.8	27	30	27.5	2.6	9.5

Figure 5b. Inter-assay variability and precision of Runs 1-4 (IFN-γ).

Sample Submission and Shipment to APAF

For biofluids such as plasma, serum, tear or urine samples, we recommend sending 25-40µL of material for each technical replicate required in a sealed container under appropriate temperature conditions. We appreciate that this is not often available, and we scale as best we can to the available material, and provide the above suggestion only as an ideal amount that allows for filtration and representative sampling. Where the available sample amount is lower than this, we recommend discussing this with an APAF team member to determine whether a dilution is recommended/appropriate.

Please note that low-abundance analytes can be diluted below the working range of the standard curve, and should multiple assays be requested, APAF would require a commensurate increase in the amount of sample volume to be provided.

For most complex sample types, we highly recommend filtering the biofluid through a 0.22µm centrifugal filter at the time of collection, and before initial storage/freezing. This allows for representative sampling, and reduces the risk of particulates, debris, or other constituents from impeding the microfluidics, and thereby impacting the quality of the data, or potentially terminating the run. Should samples have been previously prepared (i.e., unfiltered), we advise against unnecessary freeze-thaws, and can perform the filtration for you as a sample preparation service.

For cell or tissue lysates, we recommend including a buffer blank to assess for any potential interferences (see Specificity).

When ready to despatch, please direct the parcel, with an appropriate amount of dry ice, 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,

The Protein Analysis Team