Optimizing Pyrogen Testing of Intravenous Immunoglobulins (IVIG) with the Monocyte Activation Test

With the animal-based Rabbit Pyrogen Test set to be discontinued in Europe in January 2026, companies will need to consider the Monocyte Activation Test (MAT) to test products at risk of non-endotoxin pyrogen (NEP) contamination. When implementing the MAT, though, quality control (QC) laboratories must demonstrate that potential product interferences can be mitigated. Here, we describe a strategy for optimizing the MAT preparation to overcome interference in intravenous immunoglobulin preparations, based on feasibility studies from Sanquin Diagnostic Services.

For many decades, pyrogen testing of injectables and parenteral pharmaceuticals has been a critical part of ensuring patient safety. As pyrogen testing evolves, regulatory agencies are increasingly embracing animal-free testing methods in line with initiatives to reduce, refine, and replace analytical tests that consume experimental animals (otherwise known as 'the 3Rs').

For example, the animal-based Rabbit Pyrogen Test (RPT) will finally be discontinued in Europe as of January 2026, as part of the pyrogenicity strategy of the European Pharmacopoeia (Ph. Eur.) Commission. 57 Ph. Eur. texts have been revised to omit the RPT, and companies are being asked to establish a suitable *in vitro* pyrogen test for new pharmaceuticals.[1] This change is guided by a new general chapter on Pyrogenicity (5.1.13), which was published in January 2025 and will be implemented in July 2025 (Ph. Eur. 11.6-11.8, 07/2025). The chapter requires that companies

conduct a risk assessment for NEP contamination before defining a testing strategy with the Monocyte Activation Test (MAT), bacterial endotoxin tests (the LAL test or its recombinant version, recombinant Factor C), or both.

The MAT works by measuring the innate human immune system's response to pyrogens, where human monocytes respond to endotoxins or NEPs by secreting pro-inflammatory cytokines, such as interleukin-6 (IL-6). These cytokines are then measured to provide a readout of pyrogenicity of the tested substance. Conducting the MAT is simple, comprising only three steps (Figure 1).

Crucially, product matrices can interfere with *in vitro* test systems for endotoxin and pyrogen testing, including the MAT. As such, during MAT preparatory testing, labs must determine if (and how) their specific product interferes with the assay reagents, and how to overcome this interference.

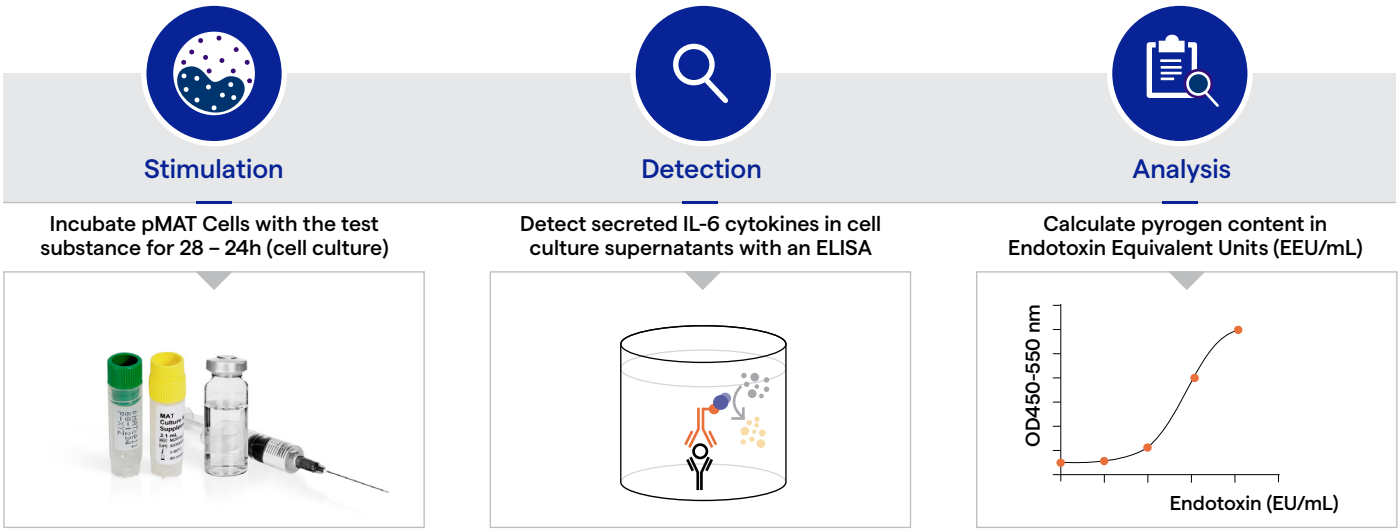

Figure 1.
The three steps of the MAT assay: Stimulation, Detection, Analysis.

Options for overcoming interferences include:

- Diluting the product up to the maximum valid dilution (MVD)
- Adjusting product pH
- Selecting a different serum source for the MAT (human serum vs. fetal bovine serum)
- Increasing buffering capacity
- Adding divalent cations
- Adding dispersants
- Heat inactivation of proteins

Here, we show a strategy for optimizing the MAT to overcome pH-related interference in intravenous immunoglobulin (IVIG) preparations, based on feasibility testing studies from Sanquin Diagnostic Services, a part of Sanquin Blood Supply Foundation based in Amsterdam, The Netherlands. Sanquin was instrumental in developing the MAT and has decades of MAT experience.

Overcoming pH-related interferences when testing IVIG preparations with the MAT

IVIGs are one of the major therapeutic products of plasma fractionation and are used to address conditions such as immunodeficiency disorders and autoimmune diseases. IVIGs are available in freeze-dried and liquid formats, with liquid preparations being more common. This is because liquid formats lead to less product aggregation compared to freeze-dried formats, mitigating activation of the complement cascade. However, while better for patients, liquid IVIG preparations have a lower pH (4.5 – 5.5), which can interfere with pyrogen testing.

Since certain plasma derived medicinal products (PDMPs) are at risk of NEP contamination (a fact that has been well documented for human albumin preparations in particular) testing IVIGs with the MAT is recommended.[2,3,4]

Implementation of the MAT in a laboratory is described in detail in Ph. Eur. general chapter 2.6.30 Monocyte activation test.[5] as well as in our whitepaper *Adopting the Monocyte Activation Test for More Sustainable Pyrogen Testing*.[6] Broadly, preparatory testing involves the following: Creating a valid endotoxin standard curve; preparing multiple dilutions of your product (from undiluted up to the MVD); testing each dilution with and without a positive product control (PPC) spike, determining the sample dilution with optimal spike recovery; detecting at least two non-endotoxin ligands for pattern recognition receptors (e.g., peptidoglycans, lipoteichoic acids, synthetic bacterial lipoproteins, flagellins, crude bacterial whole cell extracts, etc.), and testing for interference of the product with the ELISA readout.

To validate your product with the MAT test system, you must demonstrate valid recovery of spiked endotoxin (lipopolysaccharide, LPS) and at least one NEP spike. LPS recovery must be within the acceptance criteria of 50 – 200%, although achieving between 70% and 150% recovery during preparatory testing is recommended to allow for small variations that might occur between different product batches. For NEPs, recovery should be ≥50%.[5]

Evaluating LPS recovery in freeze-dried and liquid IVIGs

Sanquin Diagnostic Services evaluated LPS recovery with the MAT for both freeze-dried and liquid IVIG formulations using a MAT kit with pooled cryopreserved peripheral blood mononuclear cells (PBMCs) and human serum as a supplement during cell stimulation.

Reagents used

Cat. no.	Description
00296408	PyroCell® Human Serum Rapid Kit
12-722F	Iscove's Modified Dulbecco's Medium, IMDM
E0150000	Reference Standard Endotoxin (EDQM)
M090001/03 NL	Phosphate Buffered Saline (Fresenius Kabi)

The contaminant limit concentration (CLC) for 5% IVIG was 0.5 endotoxin equivalent units per mL (EE/mL). The limit of detection (LOD) per well for the test was 0.03 EE/mL, based on historical data. The maximum valid dilution (MVD) was 16x.

Three product dilutions (4x, 8x, and 16x) of freeze-dried and liquid IVIGs were prepared in complete medium containing human serum. For information purposes, two higher product dilutions (32x and 64x) of the liquid IVIGs were added to the study. The same dilutions were spiked with reference standard endotoxin (RSE) at the estimated middle of the standard curve. The ideal spike recovery was set at 70 – 150%.

MAT results from the freeze-dried IVIG preparations showed acceptable LPS recovery in all three dilutions (Table 1). Results from the liquid IVIG preparations, however, showed inadequate LPS recovery at below or close to 50% in all tested dilutions (Table 2). The team concluded that the poor recovery was likely due to the lower pH of the liquid preparation (pH = 4.5 – 5.5) relative to the freeze-dried preparation (neutral pH), where the lower pH could have impacted the ability of the monocytes to detect and respond to LPS.

Dilution	Recovery (%)	EE/mL unspiked	EE/mL* dilution
4	112	<0.007	0.044
8	82	0.011	0.069
16	79	0.009	0.155

Table 1.
LPS recovery in preparations of freeze-dried IVIGs tested with the MAT.

Dilution	Recovery (%)	EE/mL unspiked	EE/mL* dilution
4	52	0.056	0.224
8	49	0.042	0.336
16	56	0.042	0.672
32*	80	0.017	0.544
64*	112	0.02	1.28

Table 2.
LPS recovery in liquid IVIGs tested with the MAT. The 32x and 64X dilutions were above the MVD and were for informational purposes only.

Exploring methods to overcome pH-related iInterference

The team then explored two ways to neutralize the pH of the liquid IVIG preparation to overcome the interference with the MAT.

Exploration 1:
Increasing product pH by titration with NaOH

As a first attempt, the team increased the pH of the product to pH 6.5 by titrating it with NaOH before dilutions were subjected to the MAT. The pH modified dilutions were then compared to the same dilutions without pH adjustment. All other conditions in the protocol remained the same (e.g., MAT reagents, dilution factors, diluents).

The pH-modified dilutions showed a slightly improved LPS recovery compared to the dilutions that were not modified (Table 3). However, only the highest (16x) dilution showed acceptable recovery suggesting that the inhibition observed in the liquid IVIG preparation is not solely pH mediated.

	Dilution	Recovery (%)	EE/mL unspiked	EE/mL* dilution
Batch A	4	37	0.051	0.21
	8	54	0.010	0.008
	16	58	0.015	0.24
Batch A (pH modified)	4	49	0.041	0.16
	8	65	<0.009	0.07
	16	73	<0.009	0.15

Table 3.
LPS recovery in two batches of liquid IVIGs tested with the MAT: Batch A was without pH modification, and batch B was modified via addition of NaOH.

Exploration 2:
Increasing the buffering capacity with phosphate buffered saline (PBS) solution

In the next exploration, the team increased the buffering capacity of the product matrix, which, in this case, also led to a more neutral pH.

The test protocol was identical to the initial test except that the first product dilution (2x) was prepared with phosphate buffered saline (PBS). The subsequent 1:1 dilution with complete medium led to a 4x final dilution of the product in the test well. The 8x and 16x test well dilutions were also prepared in complete medium, increasing the relative amount of complete medium and decreasing the relative amount of PBS. This approach allowed the team to compare readouts to an endotoxin standard curve prepared with complete medium. Preparing all dilutions in PBS lowered the quantification values (data not shown).

With this approach LPS recoveries in all dilutions were valid (Table 4).

Dilution	Recovery (%)	EE/mL per well	EE/mL Product
4	**94**	0.082	0.328
8	**70**	0.022	0.176
16	**73**	0.015	0.24

Table 4.
Recovery of LPS from sequential dilutions of liquid IVIGs. The first dilution was prepared in PBS. Complete Medium was used for further dilutions from this dilution.

Key takeaways

- Liquid IVIG preparations can lead to non-valid LPS spike recoveries with the MAT (low product pH causes interference)
- Out of the two methods trialled to overcome the pH-related interference, increasing buffering capacity was a suitable treatment option, leading to valid spike recoveries in all tested dilutions of liquid IVIGs
- At least a 1:2 dilution of the test matrix with PBS is necessary for valid LPS spike recovery of liquid IVIGs. Subsequent dilutions should be in complete medium to use the endotoxin standard curve prepared with complete medium.

Evaluating NEP recovery in liquid IVIG preparations

Next, the team investigated whether the liquid IVIG product matrix interferes with MAT detection of NEPs. As per the revised Ph. Eur. general chapter 2.6.30. Monocyte activation test,[6] companies must demonstrate 50 – 200% PPC recovery of at least one relevant NEPs (although two is recommended). Given the potential for synergy between the product and the spiked NEP, recoveries of ≥50% are sufficient.

A search of the scientific literature indicated that FLA-ST and PGN were real-world NEP contaminants of pharmaceuticals, and so these were used for spiking.

PGN-Sandi Ultrapure	tlrl-sipgn
FLA-ST Ultrapure	tlrl-epstfla-5
FLA-BS Ultrapure	tlrl-pbsfla
PAM3CSK4	tlrl-pms
HKSA	tlrl-hksa
R848	tlrl-r848-1

Non-Endotoxin Pyrogens were purchased from Invivogen (formulation: "ultra-pure", endotoxin tested)

Dilutions were prepared as per Exploration 2 for LPS recovery. However, FLA-ST and PGN were spiked separately into the product, instead of LPS.

While there was poor recovery of FLA-ST in all dilutions, acceptable recovery of PGN was shown in the 8x and 16x dilutions (Table 5). Since the spiked FLA-ST was derived from salmonella, the team concluded that the poor recovery may be explained by the presence of neutralizing antibodies in the IVIG sample.

Dilution	FLA-ST recovery (%)	PGN recovery (%)
4	1	39
8	6	59
16	20	82

Table 5.
Recovery of two NEPs in liquid IVIG preparations tested with the MAT.

Exploration 3: Spiking a larger panel of NEPs

Given the low recovery of FLA-ST, the team spiked a larger panel of NEPs using the same protocol. The spiked NEPs were FLA-BS (since humans do not typically have antibodies against Bacillus subtilis, from which this NEP is derived), HKSA, R848, and PAM3CSK4.

FLA-BS recovery was poor in all dilutions (Table 6). However, acceptable recoveries were demonstrated in at least one dilution of each of the remaining three NEPs.

Dilution	FLA-BS recovery	HKSA recovery	R848 recovery	PAM3CSK4 recovery
4	10	116	23	43
8	18	128	166	54
16	33	116	892	88

Table 6.
Recovery of four NEPs in liquid IVIG preparations tested with the MAT.

Key takeaways

- Companies evaluating the compatibility of the MAT with IVIGs need to be careful when selecting NEP spike preparations
- Since several PDMPs contain neutralizing antibodies, labs may find that several NEPs do not recover well
- In particular, NEPs from microorganisms such as Salmonella may not be good candidates for spiking IVIG samples

Effectively optimizing and deploying the MAT

Regulatory agencies are increasingly embracing animal-free pyrogen testing methods. In Europe, changing regulations will eliminate the RPT as a compendial testing method, requiring companies to consider the MAT for testing products with a risk of NEP contamination. To deploy the MAT, however, companies need to ensure it is suitable for their specific product through a preparatory test and identify effective ways to overcome any potential product interference.

Here we demonstrated, through studies conducted by Sanquin Diagnostic Services, that increasing buffering capacity using PBS solution may help overcome interference and achieve valid recovery of LPS in liquid IVIG preparations. We also showed that laboratories should choose NEP spike preparations carefully when it comes to NEP recovery testing with IVIG products. A discussion of these experiments can also be found in the webinar "Insights from Testing Various IVIG Formulations in the Monocyte Activation Tests".

Implementing and optimizing the MAT for your specific product does not need to be difficult, costly, or time consuming. With decades of experience in endotoxin and pyrogen testing, Lonza has the expertise and technical know-how to help you through the process. Contact us for more information about the MAT, and for dedicated support with implementing the MAT in your laboratory.

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