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# Fit for purpose testing and independent GMP validation of the monocyte activation test

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ABSTRACT

The present study describes the "fit for purpose" testing and the independent product-specific GMP validation of the monocyte activation test (MAT) to detect pyrogenic and pro-inflammatory contaminants, MAT Method A, Quantitative Test (European Pharmacopoeia, Ph. Eur. chapter 2.6.30, 2017). A fit for purpose study was carried out to ensure that the chosen MAT set-up (cryopreserved PBMC, IL-6 detection) can reliably discriminate between batches of product containing pyrogenic contaminants below the contaminants limit concentration, CLC, from batches containing pyrogenic contaminants above the CLC. Such testing is carried out once, before the chosen MAT set-up is used for subsequent product testing to show that the incidence of false positives (pyrogen-negative (<CLC) batches testing as pyrogen-positive (>CLC) batches) and – especially – false negatives (pyrogen-positive (>CLC) testing as pyrogen-negative (<CLC)) is low. This study also afforded the opportunity to collect an independent body of validation data for comparison with that obtained previously (Daniels et al., 2022) to evaluate the robustness of MAT Method A and its fitness to replace the rabbit pyrogen test (RPT) where this has not already happened.

## Introduction

The monocyte activation test, MAT, for pyrogenic and pro-inflammatory contaminants was introduced into the European Pharmacopoeia (*Ph. Eur.*) as a 'non-animal' replacement for the rabbit pyrogen test, RPT, in 2010 and revised in 2017 (European Pharmacopoeia chapter 2.6.30, 2017). However, the MAT is not a compendial method in various other pharmacopoeias, notably the United States Pharmacopoeia (USP), with the MAT defined in the USP as "an alternative method" to the RPT (US Pharmacopoeia USP <151>, 2017). In practice this means that the US FDA requires data from the RPT (US Pharmacopoeia USP <151>, 2017) whereas the EMA and other regulatory agencies require data from the MAT, requiring pharmaceutical manufacturers to carry out both the MAT and the RPT on at least 3 process performance qualification, PPQ, production batches of new parenteral products (as well as the bacterial endotoxins test, BET (European Pharmacopoeia chapter 2.6.14, 2012)).

Recently, a GMP validation of *Ph. Eur.* MAT Method A, Quantitative Test was published (Daniels et al., 2022), though this publication did not include what we refer to here as "fit for purpose" testing since such testing is not a pharmacopeial requirement. The fit for purpose testing is carried out once before the chosen MAT set-up is used for product testing to show that the incidence of false positives (pyrogen-negative (<CLC) batches testing as pyrogen-positive (>CLC) and – especially – false negatives (pyrogen-positive (>CLC) testing as pyrogen-negative (<CLC) batches) is low and to collect an independent body of validation data for comparison with that obtained previously (Daniels et al., 2022) to evaluate the robustness of MAT Method A and its fitness to replace the RPT, where this has not already happened. So, the study described below was carried out with two objectives:

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(i) to determine whether or not the chosen MAT set-up (cryopreserved PBMC, IL-6 detection) can reliably discriminate "pyrogen-negative (<CLC) batches" from "pyrogen-positive (>CLC)" batches of a product (where pyrogen-negative (<CLC) batches = below the contaminants limit concentration (CLC) for the product and pyrogen-positive (>CLC) = above the CLC).

(ii) to generate independent GMP method validation data to facilitate the wider acceptance of the MAT in the regulatory community and to help it become *the* compendial method for testing for pyrogenic and pro-inflammatory contaminants in countries where it is currently "an alternative method" (to the RPT).

## Materials and methods

Material and Methods were as described previously (Daniels et al., 2022).

### *Critical reagents*

MAT Cell Set (Essange Reagents, REF: M2017), using the cryo-PBMC from the kit (pMAT Cells) and human AB serum as culture media supplement; Iscove's Modified Dulbecco's Media (IMDM; 40 mL from a new, unopened bottle) from Lonza (REF: BE12 722F); Endotoxin reference standard that has been calibrated against the International Standard, endotoxin standard Biological Reference Preparation (BRP) from the *Ph. Eur.* (EDQM, REF: E0150000); sterile, non-pyrogenic distilled or deionised water to reconstitute endotoxin standard (Charles River REF: W120); PGN from *Staphylococcus aureus* (PGN-Sandi Ultrapure) from Invivogen (REF: tlrl-sipgn); PeliKine compact human IL-6 kit (Essange Reagents REF: M1916); PeliKine tool set 1 (additional reagents for application in PeliKine compact ELISA kits) (Essange Reagents, REF: M1980); Distilled water for ELISA buffers (WFI from Gibco, REF: A1287301 (500 mL)).

### *Cell culture and IL-6 ELISA*

Cell culture and IL-6 ELISA were as described previously with all MATs carried out using a qualified lot of PBMC (lot #3, see Daniels et al., 2022).

## Theory/calculation

### *Product-specific fit for purpose and validation testing*

The product-specific fit for purpose testing and validation were carried out using *Ph. Eur.* MAT Method A Quantitative Test. Standard endotoxin was added to samples of 3 different batches of a therapeutic monoclonal antibody (mAb) to generate deliberately contaminated samples of the mAb containing either 25 % of the CLC or 125 % of the CLC. The non-endotoxin pyrogen (NEP) peptidoglycan (PGN-$EC_{50}$, 5 μg/mL) was also added to the mAb at either 25 % CLC or 125 % CLC. MATs were carried out by 2 different operators on 5 different days with 1 lot of (qualified) cryo-preserved PBMC comprising cells from 4 different donors (PBMC lot #3, see Daniels et al., 2022), the evaluation comprising a total of 21 plates. Even though the method had been validated previously (Daniels et al., 2022), a second, independent, set of validation data was collected by independently assessing: limit of detection (LOD), basal release of IL-6 (using the cut-off (OD)) as well as accuracy, precision, linearity, confirmation of the limit of quantification (LOQ), confirmation of the range, specificity (see PDA tech report no 33, 2013; ICH Q2 R1, 1994, 1996 and US General Chapter <1223>, 2013). Also, it was determined whether or not the criteria for the endotoxin standard curve were satisfied for the standard curve on each plate.

The MVD (maximum valid dilution) for the mAb was calculated and rounded down as CLC/LOD = (23.33 EE/mL)/(0.03 EE/mL) = 777. It should be noted that the *Ph. Eur.* definition of LOD is less stringent than defining LOQ in the MVD calculation as CLC/LOQ. The MVD calculation as CLC/LOQ was chosen for this study to ensure that the necessarily large dilutions of samples spiked with 125 % of the product CLC remained within the MVD and were therefore valid for testing. Three different batches of the mAb were deliberately contaminated with standard endotoxin at 25 % of the CLC (5.83 EE/mL) and at 125 % of the CLC (29.16 EE/mL). Each sample was additionally spiked with standard endotoxin at the middle point of its standard curve, $EC_{50}$ = 0.16 EU/mL, and tested at dilutions f, fx2, and fx4 (see Figs. 1 and 2 for values for f) to permit the calculation of % recovery of the added spike. The *Ph. Eur.* (chapter *2.6.30*, 2017) requires the recovery of this spike to be 50–200 % for the related unspiked sample to be deemed valid for inclusion in further analysis, with at least one valid dilution (from f, fx2, and fx4) required to calculate the contaminant concentration. All samples, standards, controls and blanks were tested in 4 replicates. The reportable results were based on the worst-case scenario from 3 plates. In order to estimate correctly the repeatability (i.e., short-term precision of the assay), it was required to generate at least 2 results (i.e., 6 plates) within a single series. Two operators generated the 6 required plates (2 reportable results) in one single day that was considered as the series.

### *Data analysis*

Data analysis was described previously (Daniels et al., 2022). The results for the 3 batches of the mAb were found to be very similar and so were pooled for the data analysis except where data for individual batches was required, e.g., for reportable results.

## Results

### *Assay acceptance criteria*

The endotoxin standard curves on all 21 plates were S-shaped and met all acceptance criteria: *p*-values for regression ($p < 0.01$) and non-linearity ($p > 0.05$), basal IL-6 release OD minimum = 0.029, maximum = 0.054, mean = 0.041 and standard deviation = 0.008, and LOD (EE/mL) minimum = 0.01, maximum = 0.05, mean = 0.03 and standard deviation = 0.001. All 21 plates passed the acceptance criteria for the basal IL-6 release OD (<0.100) and LOD (<0.08 EE/mL). The LOQ of the MAT was calculated as 0.08 EE/mL using values back-calculated from the endotoxin standard curves, consistent with previous data for this MAT (Daniels et al., 2022). The acceptance criteria and outcomes are summarised in Table 1.

### *Recovery of standard endotoxin $EC_{50}$ from samples of mAb < CLC and > CLC*

The samples of 3 batches #1, #2, and #3 of mAb to which had been added 25 % of the CLC were tested at dilutions of 32, 64 and 128 and these dilutions gave endotoxin $EC_{50}$ (0.16 EU/mL) spike recoveries of 50–200 % for 14/21, 10/21 and 21/21 plates, respectively (STable 1). The samples of 3 batches of mAb to which had been added 125 % of the CLC were tested at dilutions of 64, 128 and 256 and these dilutions gave endotoxin $EC_{50}$ spike recoveries of 50–200 % for 14/21, 18/21 and 21/21 plates, respectively (STable 2).

### *Discrimination of samples of mAb < CLC from samples of mAb > CLC*

The MAT correctly identified samples of 3 batches of mAb to which had been added 25 % of the CLC as "pyrogen-negative batches" (<CLC) on 21/21 plates = 7/7 reportable results since a reportable result is derived from the worst-case scenario value from 3 plates (Fig. 1, mAb batch #1 tested on plates 1–9, mAb batch #2 tested on plates 10–12, mAb batch #3 tested on plates 13–21). The reported values ranged from 6.59 to 11.71 EE/mL, mean = 8.8 EE/mL, all below the CLC of 23.33 EE/mL but all above the 5.83 EE/mL nominal concentration in the

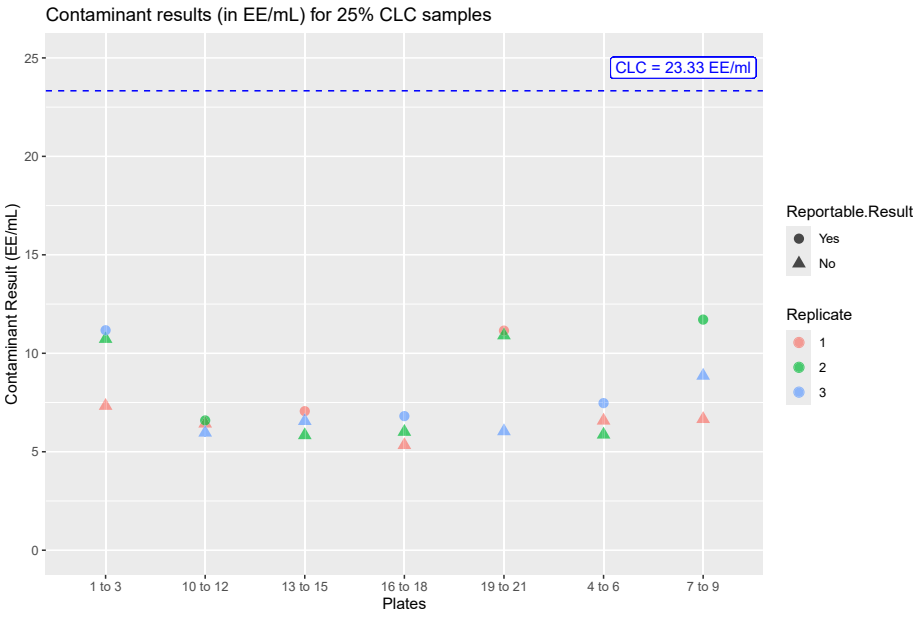


**Fig. 1.** Reportable results (circles) 25 % CLC sample highest result and the mean back calculated recoveries for 25 % CLC spiked mAb. When the result is < LOQ for a valid dilution (meaning an acceptable spike recovery), the EE/mL is calculated based on 32x dilution which had invalid spike recovery in fit for purpose and further validation testing, but a valid recovery in the product specific study. This is performed for statistical analysis only. Dashed line represent the CLC.

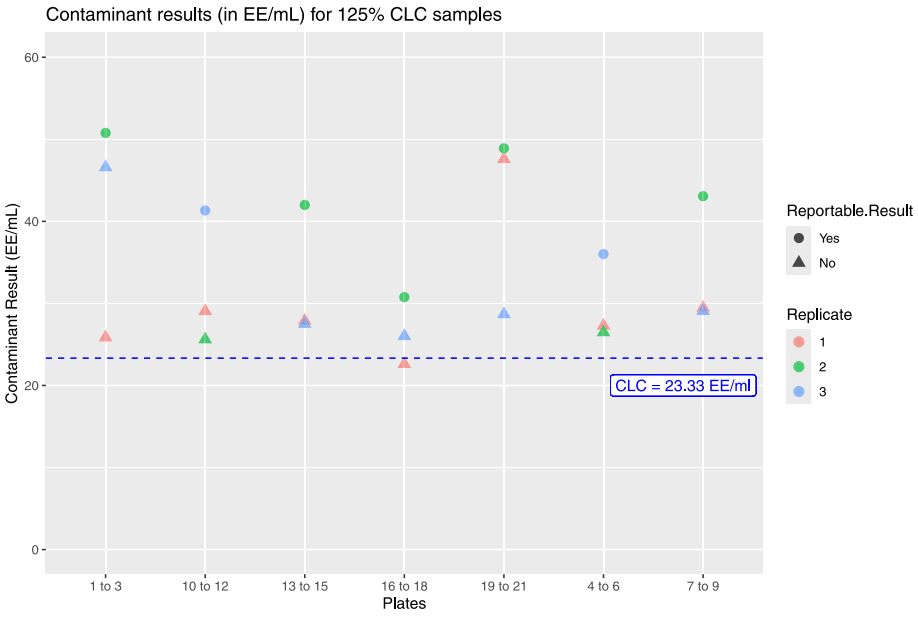


**Fig. 2.** Reportable results (circles) 125% CLC sample highest result and the mean back calculated recoveries for 125% CLC spiked mAb. Dashed line represent the CLC.

"pyrogen-negative (<CLC)" sample. Similarly, the MAT correctly identified samples of 3 batches of mAb to which had been added 125 % of the CLC as "pyrogen-positive" (>CLC) on 21/21 plates = 7/7 reportable results (Fig. 2, mAb batch #1 tested on plates 1–9, mAb batch #2 tested on plates 10–12, mAb batch #3 tested on plates 13–21). The reported results ranged from 30.77 to 50.78 EE/mL, mean = 43.1 EE/mL, all above the CLC of 23.33 EE/mL and all above the 29.16 EE/mL nominal concentration in the "pyrogen-positive (>CLC)" sample.

*Accuracy*

Method accuracy was assessed from the 25 % CLC and 125 % CLC samples of 3 batches of the mAb. When the accuracy is exactly 100 % the measured concentration is exactly the same as the spiked concentration of endotoxin. The reportable results, the average concentration and the accuracy of the reportable results as well as the 90 % confidence intervals (CIs) are shown in Fig. 1 for mAb with 25 % CLC and in Fig. 2 for mAb with 125 % CLC. Fig. 3 combines the data from Figs. 1 and 2, the corresponding values for the individual data points are show. As can be seen, the 90 % CIs for the mean recovery for both concentrations lie within 50 %–200 % and passed the criteria for accuracy (Table 1 and Fig. 3).

*Precision*

Precision of the assay was assessed from the data generated for the

**Table 1**
Summary of MAT data for 25% CLC and 125% CLC spiked mAb, PGN spiked medium, PGN spiked in 25% CLC and 125% CLC spiked mAb and endotoxin standard curve.

| | Data | Acceptance criteria | Outcome | Comment | Reference |
|---|---|---|---|---|---|
| Accuracy | 25 % CLC, 125 % CLC spiked mAb | 90 % CI between 50 and 200 % Recovery value = Mean back calculated concentration (EE/mL) compared with the theoretical 25 % and 125 % CLC | ≤155 % 25 % CLC: 154 % (90 % CI between 131 and 182 %), 125 % CLC: 151 % (90 % CI between 127 and 180 %) | Upwards bias worst-case reportable result | Figs. 1 and 2 and 3 |
| Repeatability | 25 % CLC, 125 % CLC spiked mAb | <25 % (GCV%) | GCV ≤ 17 % | | |
| Intermediate precision | 25 % CLC, 125 % CLC spiked mAb | <25 % (GCV%) | GCV ≤ 23 % | | |
| Linearity (linear regression) | 25 % CLC, 125 % CLC spiked mAb; endotoxin standard curve | $R^2$ above 0.9 | 25 %: Intercept = 0.453 Slope = 0.989 125 %: Intercept = 0.012 slope = 0.970 $R^2$ = 1.0 | 90 % CI Intercept not zero worst-case reportable result | |
| Confirmation Range of quantification | 25 % CLC, 125 % CLC spiked mAb; endotoxin standard curve | 70 % β-expectation tolerance limits on the relative error between −50 and + 100 (corresponds to 50 %-200 % recovery Ph. Eur.) | 25 % CLC (5 %; 105 %), 125 % CLC (17 %; 76 %) | upper limit 25 % CLC greater + 100 % worst-case reportable result | Figs. 4 and 5 |
| Confirmation of LOQ | endotoxin standard curve | NA | range 0.08 EE/mL to 0.32 EE/mL, LOQ 0.08 EE/mL | | Fig. 5 |
| Specificity – positive controls | 25 % CLC, 125 % CLC spiked mAb; PGN spiked medium; PGN spiked in 25 % CLC, 125 % CLC spiked mAb | Above 0.08 EE/mL (LOQ) | above 0.08 EE/mL (LOQ) or above upper asymptote | | Figs. 1 and 2 and STable 3 |
| Cut-off (OD) | Blank | Below 0.1 OD | Maximum 0.05 | | |
| LOD | Blank | Below 0.08 EE/mL (LOQ) | Maximum 0.05 EE/mL | | |

GCV = Geometric Coefficient of Variation.
Intercept and Slope, Confirmation of LOQ are for information only.

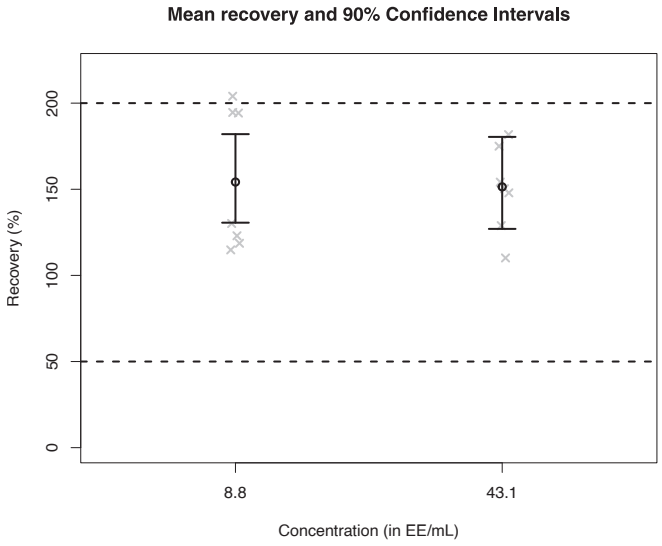


**Fig. 3.** Mean recoveries (circles) of the reportable results and 90% confidence intervals (intervals) for 25% CLC and 125% CLC spiked mAb, against the acceptance criterion for mean recovery (50% to 200 %, dashed line). Crosses represent the individual data points.

accuracy study. The repeatability and intermediate precision for mAb with 25 % CLC added were 2.5 % geometric coefficient of variation (GCV) (90 % CI: 1.3 %–42.0 %) and 27.4 % GCV (90 % CI: 18.2 %–60.8 %), respectively. The intermediate precision was higher than the acceptance criterion of 25 % due to the high day-to-day (assay date) variability. In addition, there were 4 values that were < LOQ since the lower dilutions had invalid spike recoveries. Where values < LOQ were obtained for dilutions of mAb with 25 % CLC, the reportable result was calculated from a lower dilution, albeit one with an invalid endotoxin spike recovery though this 'invalid dilution' had given 50–200 % spike recovery in the preliminary testing of pyrogen-negative (<CLC) product spiked with $EC_{50}$ endotoxin. Repeatability and the intermediate precision were similar for mAb with 125 % CLC added since the day-to-day variability was estimated to be zero. The repeatability and intermediate precision for mAb with 125 % CLC were both 17.4 % GCV (90 % CI: 12.0 %–34.0 %). The overall repeatability, obtained by combining the data for mAb to which had been added 25 % CLC or 125 % CLC, was 17.2 % GCV (90 % CI: 12.1 %–31.1 %). The intermediate precision was 22.9 % GCV (90 % CI: 16.6 %–38.6 %). The overall intermediate precision and repeatability (which is specified for the combined data for both 25 % CLC and 125 % CLC) was based upon the larger body of data and so is to be preferred over the analyses that were conducted for 25 % and 125 % CLC considered separately. Thus, the overall coefficient of variation (GCV) values of 17 % and 23 % for the repeatability and intermediate precision respectively, satisfy the specified criteria for precision (Table 1).

### *Linearity*

For linearity of the dose–response curve, the relationship between the assumed concentrations of endotoxin and the measured concentration was determined by linear regression. Linearity was evaluated with the data set for accuracy. Linearity of the responses for mAb with 25 % CLC and 125 % CLC, corresponding to the linearity (on log–log scale) of the obtained concentration versus the spiked concentration was assessed

by fitting a mixed model. For the standard curve, the linearity characteristics, i.e. $R^2$, slope and intercept were 1.0, 0.970 and 0.012, respectively. For the samples of mAb with 25 % CLC and 125 % CLC (combined data), the linearity characteristics, i.e. $R^2$, slope and intercept were 1.0, 0.989 and 0.453, respectively. The confidence intervals around the intercept for mAb with 25 % and 125 % CLC samples did not include zero: this was expected due to the use of worst-case approach for the reportable results. For example, the intercept = 0.453 for the 25 % CLC and 125 % CLC model means that the reportable result that is measured by the assay equals 1.573 (i.e. exp(0.453)) EE/mL when the true values equals 0 EE/mL (conservative approach). Further, the intercept for the endotoxin standard curve does not include zero. For both linear regressions, the $R^2$ was 1.0, i.e. above the acceptance criterion of 0.9. Thus, the MAT satisfies the criterion for linearity (Table 1).

*Range*

The relative β-expectation tolerance limits of spike recovery (US Pharmacopeia General Chapter <1210>, 2018), with the 70 % level of confidence for 25 % CLC to 125 % CLC added to mAb and for concentrations of standard endotoxin between 0.08 EU/mL and 0.32 EU/mL are shown in Figs. 4 and 5, respectively. Confirmation that the range used in this study was suitable was based on 25 % CLC to 125 % CLC added to mAb and the values obtained that were back calculated from endotoxin standard curves (range 0.08 EU/mL to 0.32 EU/mL) and was carried out using 70 % β-expectation tolerance limits and the corresponding values for the individual data points are show (Fig. 4). The upper limit of the 70 % β-expectation tolerance limits of the relative error for 25 % CLC was greater than the upper limit of the acceptance criteria for relative error. In contrast, the relative 70 % β-expectation tolerances limits with the 70 % level of confidence for the recovery on the range of the dose response curve from 0.08 EE/mL to 0.32 EE/mL were within 50 % to 200 % and confirms that the range for the standard curve shows smaller variability (Fig. 5). The relative 70 % β-expectation tolerance limits with the 70 % level of confidence for the recovery outside the range of the dose response curve from 0.08 EE/mL to 0.32 EE/mL, such as 0.01 EE/mL and 0.02 EE/mL, were not within 50 % to 200 % (Fig. 5). Fig. 5 also shows that data in the range from 0.08 EE/mL to 0.32 EE/mL for the standard curve shows smaller variability.

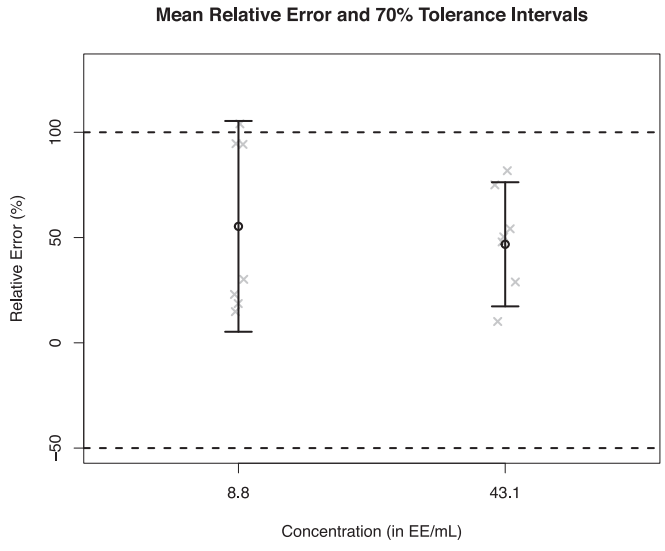


**Fig. 4.** Illustration of the 70 % β-expectation tolerance limits for the relative errors. Total error for the levels 25 % CLC and 125 % CLC spiked in mAb. Dashed lines represent the −50 % and + 100 % interval (corresponding to the 50 % − 200 % spike recovery required by the *Ph. Eur.*). Crosses represent the individual data points.

*Specificity*

Samples of the 3 mAb batches to which had been added 25 % and 125 % of the CLC gave 40/42 responses (2 per plate) to PGN $EC_{50}$ above the upper asymptote of the standard curve. The other 2 responses, one for 25 % CLC and one for 125 % CLC, from 2 different plates, were 2.14 and 1.95 EE/mL, respectively, i.e. all 42 responses were well above their specificity requirement of > LOQ (0.08 EE/mL, Table 1 and STable 3).

## Discussion

This study describes fit for purpose testing and the collection of independent GMP validation data for comparison with validation data obtained in an earlier study using cryo-preserved PBMC with IL-6 as the readout (Daniels et al., 2022).

Verification of the monocyte activation test (*Ph. Eur.* chapter *2.6.30*, 2017) for pyrogens and pro-inflammatory contaminants of medicines and vaccines requires the testing of doubling dilutions of test products spiked with a fixed concentration of standard endotoxin ($EC_{50}$) to show that products being tested do not cause inhibition or enhancement in the test. While this procedure is appropriate for testing for interference in the test, it does not mimic what happens in 'real world' testing where contaminants are diluted as the test product is diluted. Thus, the procedure does not show that the test is fit for purpose by reliably discriminating pyrogen-negative (<CLC) from pyrogen-positive (>CLC) batches of product and ensuring that the test generates very few false positives (pyrogen-negative (<CLC) testing as pyrogen-positive (>CLC) and – especially – very few false negatives (pyrogen-positive (>CLC) testing as pyrogen-negative (<CLC)). So, the first objective of the present study was to determine whether or not the chosen MAT set-up can reliably discriminate pyrogen-negative (<CLC) from pyrogen-positive (>CLC) batches of a product.

The data presented above shows that 3 batches of a mAb deliberately contaminated with standard endotoxin to 25 % of the product's CLC correctly tested as pyrogen-negative (<CLC) 100 % of the time: 21/21 plates and 7/7 reportable results (each reportable result being based on worst-case data from 3 plates). Similarly, 3 batches of a mAb deliberately contaminated with standard endotoxin to 125 % of the product's CLC correctly tested as pyrogen-positive (>CLC) 100 % of the time: 21/21 plates and 7/7 reportable results (each reportable result being based on data from 3 plates). Thus, there were no false positives and no false negatives for any of the samples of the 3 batches of mAb to which had been added 25 % and 125 % of the CLC. Being a quantitative test, the MAT was able to measure the (added) contamination, rather than simply report results as pass/fail. For samples of mAb deliberately contaminated with 25 % and 125 % of CLC, mean recoveries of the (added) contamination were a little over 150 % of the nominal concentrations. That accuracy was not 100 % but a little over 150 % is due to the approach of selecting the worst-case for reportable results, i.e., the largest value from 3 plates. The worst-case approach likely also accounts for the upper limit of the 70 % β-expectation tolerance limits of the relative error for 25 % CLC being greater than the upper limit of the acceptance criteria for relative error. Such a worst-case approach inevitably leads to an upward bias of the measured concentrations and therefore of the values obtained for accuracy. The overestimation of contaminants by some 50 % means that this implementation of the MAT errs on the side of patient safety while increasing the risk of identifying a pyrogen-negative (<CLC) batch = < CLC as pyrogen-positive (>CLC). Obviously, this is to be preferred to the opposite situation where accuracy would be less than 100 %.

The *Ph. Eur.* stipulates 50–200 % recovery of standard endotoxin $EC_{50}$ from all dilutions of all samples tested so this stipulation was applied to samples to which 25 % or 125 % of the product's CLC had already been added. Unfortunately, this "double-spiking" inevitably pushes measured values outside the linear range of the standard curve (0.08–0.32 EE/mL) which inevitably leads to invalid values for spike

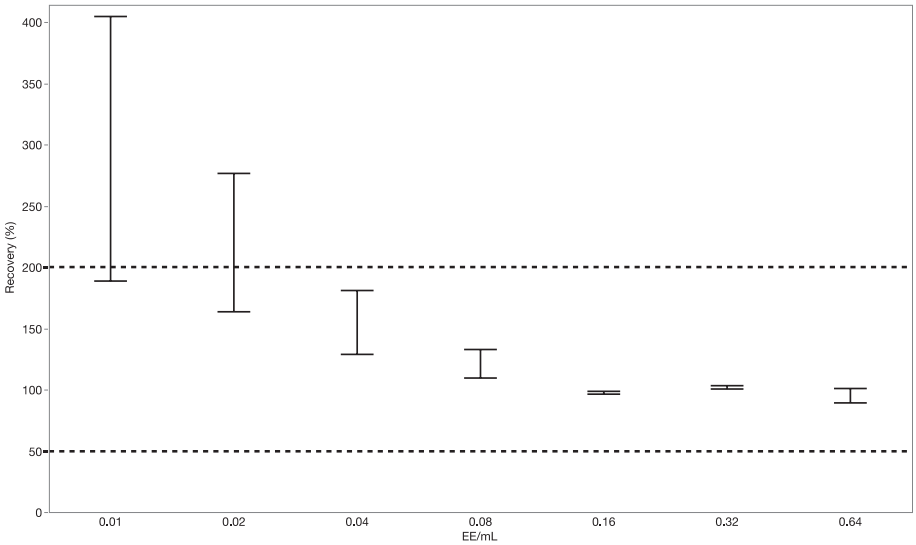


**Fig. 5.** Limits of the 70% β-expectation tolerance limits on recoveries obtained from the back-calculated concentrations from the 21 standard endotoxin curves.

recovery. If these "invalid dilutions" – minus the added standard endotoxin to 25 % and 125 % of the product's CLC – had already been shown in the PREPARATORY TESTING (*Ph. Eur.* chapter *2.6.30*, 2017) to give 50–200 % recovery of standard endotoxin $EC_{50}$ then repeating the spiking in the presence of, say, 25 % and 125 % of the product's CLC, i.e., "double-spiking", is surely not necessary and, as can be seen here, can cause issues with the MAT.

The non-endotoxin positive control PGN had a synergistic effect with the tested mAb to which had been added standard endotoxin at 25 % and 125 % CLC, so much so that with just 2 exceptions responses to PGN ($EC_{50}$) were all above the upper asymptote of the standard curve. This finding was to be expected since the mAb itself synergises with PGN to some extent (STable 3), and endotoxin + other non-endotoxin pyrogens are known to synergise to give large IL-6 responses from PBMC (Solati et al., 2022).

The second objective of the present study was to generate independent robust data from a GMP validation study to facilitate the wider acceptance of the MAT in the regulatory community and to help it become *the* compendial method for testing for pyrogenic and pro-inflammatory contaminants in countries where it is currently "an alternative method" (to the RPT). In this regard, independent data was obtained in this GMP study for accuracy, precision, linearity, confirmation of the limit of quantification (LOQ), confirmation of the range, and specificity (see PDA TR33, 2013, ICH Q2 R1, 1994, 1996 and US General Chapter <1223>, 2013). The data for all of these parameters met all of the acceptance criteria set out in Table 1, was entirely consistent with the data for the MAT GMP validation that was published previously (Daniels et al., 2022) and so adds to the existing body of work that shows that the MAT could replace the RPT for many products.

For any generalization across product types, the data published previously (Daniels et al., 2022) comprised a general validation and product-specific validations with 3 therapeutic mAbs. Currently, the total number of validations carried out is for 13 mAbs and 2 non-mAbs and represents a diversity of pharmaceutical formulations, suggesting the MAT's applicability across different types of products. The data presented in the current study was obtained with 3 batches of selected mAbs and one lot of the cryo-preserved PBMC which were qualified in the previous study. We acknowledge that the fit for purpose testing with the focus on specific product batches does not represent the diversity of pharmaceutical formulations, nor is it intended to. It is that diversity of product types and pharmaceutical formulations that necessitates a product-specific validation for every product. Noteworthy, the study did not involve an inter-laboratory comparison, and this variant of this MAT has not been validated in ring trials.

The basis of the MAT is the activation of human monocytes or monocytic cells to release endogenous mediators such as pro-inflammatory cytokines, e.g., tumor necrosis factor alpha, interleukin-1 beta (IL-1β), and IL-6 upon exposure to pro-inflammatory and pyrogenic contaminants. The released cytokines have a role in fever pathogenesis and other inflammatory responses. Consequently, the MAT detects the presence of pyrogenic/pro-inflammatory contaminants in the test sample. The reliance of the MAT assay on IL-6 as a marker for pyrogenic activity may not capture all pro-inflammatory responses, limiting the test's sensitivity to a particular pyrogen or product or product/pyrogen combinations, anti-IL-6 mAbs or soluble IL-6 receptors being obvious examples. Nonetheless, IL-6 is broadly accepted for this purpose. Although the role of IL-6 in fever production is not fully understood, IL-6 clearly functions as a circulating pyrogenic/pro-inflammatory mediator (Cartmell et al., 2000) and, as a readout in MATs with various cell sources: "*IL-6 was produced most sensitively in response to traces of the pyrogens and detected in the largest quantities in the culture medium*" (Nakagawa et al., 2002). Further, an MAT using PBMC (fresh not cryo-preserved) with IL-6 as readout was able to detect non-endotoxin pyrogens that were not detected in MATs using other readouts (United States Patent No.: US 9,023,647 B2, May 15, 2015).

Daniels et al., 2022 showed that there were differences in the responses of the different PBMC lots to PAM, PGN and HKSA. The reactivity to FLA was more consistent among the different PBMC lots. All 4 NEPs tested were detected in MATs with all 3 PBMC lots. Therefore, per internal practice, each new PBMC lot is qualified with the 4 NEPs, each at 4 different concentrations to account for the variability of the different PBMC lots. In addition, at least one NEP is used as positive control in each plate of the MAT assay to monitor the reactivity of the PBMC lot used.

The technical and operational challenges when implementing MAT in a routine quality control environment might pose challenges related to the standardization of protocols, training of personnel, and ensuring consistent test conditions but these challenges are not different from conditions applying to the introduction of other tests in a routine quality control environment.

## Conclusions

The first objective of the present study to determine whether or not the chosen MAT set-up can reliably discriminate pyrogen-negative (<CLC) from pyrogen-positive (>CLC) batches of a product was met.

The second objective to generate independent robust data from a GMP validation study that was consistent with the previous GMP validation study was also achieved. Thus, there is now consistent robust data from two independent GMP validation studies that will hopefully facilitate the wider acceptance of the MAT in the regulatory community and to help it become *the* compendial method for testing for pyrogenic and pro-inflammatory contaminants in countries where it is currently "an alternative method" (to the RPT). It was also found that the non-endotoxin positive control PGN had a synergistic effect with a therapeutic antibody to which had been added standard endotoxin, illustrating the complexity of product/endotoxin/non-endotoxin interactions that need to be taken into account when testing products in the MAT.

## Math formulae

Relative bias (%) = [(GM (Measured potency))/(Target potency)-1] × 100 %.

## CRediT authorship contribution statement

**Ruth Daniels:** Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Writing – original draft. **Wim Van der Elst:** Data curation, Formal analysis, Software, Visualization, Writing – review & editing. **Chi K. So:** Writing – review & editing. **Liesbeth Voeten:** Writing – review & editing. **Philip Breugelmans:** Writing – review & editing, Resources. **Marijke W.A. Molenaar-de Backer:** Data curation, Software, Writing – review & editing. **Stephen Poole:** Conceptualization, Methodology, Writing – original draft. **Mehul Patel:** Conceptualization, Methodology, Resources, Writing – review & editing.

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## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Sanquin Diagnostic Services (MMB) performs MAT service testing for customers. All other authors have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2024.100206.

## Data availability

Data will be made available on request.

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