PyroMAT™ Software Validation Summary

The life science business of Merck KGaA, Darmstadt, Germany operates as MilliporeSigma in the U.S. and Canada.
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1 Introduction

This summary documents the validation of the PyroMAT™ Software protocols.

The PyroMAT™ software has been developed by MilliporeSigma and comprises a range of different protocols for different methods of analysis to be used in connection with the proprietary software Gen5™ from BioTek Instruments Inc. All versions of Gen5™ software have been fully validated by BioTek Instruments Inc.

The PyroMAT™ software is used to analyze the results of a Monocyte Activation Test performed with PyroMAT™ System, according to the criteria and specifications described in the European Pharmacopoeia 07/2017:20630 - Monocyte Activation Test.

The PyroMAT™ software has been fully validated to operate per written product specifications. This validation was conducted utilizing several controlled test protocols, which challenged the data analysis, data reporting and instrument interface functionality.

All the PyroMAT™ software documentation, including the specifications, requirements and validation documentation, are managed on a Jama Server under the Project Key P169.

The PyroMAT™ software documentation represents more than 1,000 items including more than 500 test cases.

The detailed documentation is available upon request for review by authorized agents of a customer by a scheduled on-site audit.

2 Principle of PyroMAT™ Assay

The PyroMAT™ System is a set of reagents needed to perform a Monocyte Activation Test (MAT) using a cell line as a source of monocytes. It is based on the innate immune response of the Mono-Mac-6 cells.

Mono-Mac-6 cells respond to endotoxin and non-endotoxin pyrogens by a rapid and strong production of cytokines (Interleukin-6) which are detected in an immunological assay (ELISA).

3 References

The following chapters of the European Pharmacopoeia were used to develop the PyroMAT™ software protocols:

- European Pharmacopoeia 07/2017:20630 - Monocyte Activation Test
- European Pharmacopoeia 07/2016:50300 corrected 9.2 - 5.3 Statistical analysis of results of biological assays and tests

4 Validation Purpose & Tests Summaries

Aim of the present validation is to verify that the product software complies with its requirements and specifications.

The validation activities follow good engineering practices for the creation of quality Software.

This includes the following:

- Requirements & Specifications Verification in order to verify the completeness, correctness, and testability of the software
- Code Verification in order to verify the compliance with Coding Standards and code Writing Good Practices
- Validation of Technical Specifications by running Unit Test Cases
- Validation of Functional Specifications by running Integration Test Cases
- Validation of the User Requirement Specifications by running Application Test Cases
- Configuration management in order to ensure the right environment for the software to function as expected
- Risk Assessment including a Failure Mode and Effect Analysis
• Plan of Action resulting from the Risk Assessment
• Defects Logging and Change management in order to improve and maintain the software
• Traceability Matrix in order to keep track of the link between the Requirements/Specifications and the tests

A snapshot of the project in Jama illustrates all these items were covered during the software development and validation:

All validation activities were performed with the following materials:

<table>
<thead>
<tr>
<th>Material/Software</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dell Latitude E5470/Intel® Core™ i5-6300U <a href="mailto:CPU@2.40GHz">CPU@2.40GHz</a> / OS 64 bits</td>
<td>Dell Inc.</td>
</tr>
<tr>
<td>Windows® 7 Enterprise - 64 bits</td>
<td>Microsoft Corp.</td>
</tr>
<tr>
<td>Gen5™ Secure V3.03.09</td>
<td>Biotek Instruments, Inc</td>
</tr>
<tr>
<td>ELx808™ Microplate reader</td>
<td>Biotek Instruments, Inc</td>
</tr>
</tbody>
</table>

PyroMAT™ Software protocols validated:

<table>
<thead>
<tr>
<th>Protocol file name</th>
</tr>
</thead>
<tbody>
<tr>
<td>PyroMAT_Method_A.prt</td>
</tr>
<tr>
<td>PyroMAT_Method_B.prt</td>
</tr>
<tr>
<td>PyroMAT_Method_C.prt</td>
</tr>
<tr>
<td>PyroMAT_PSV_I_II_III_A.prt</td>
</tr>
<tr>
<td>PyroMAT_PSV_I_II_III_B.prt</td>
</tr>
<tr>
<td>PyroMAT_PSV_IV.prt</td>
</tr>
</tbody>
</table>

5 Results

Requirements & Specifications Verification:

The Requirements & Specifications Verification was performed by Reviews made by the project team before implementation and testing.

A special attention was given to the specification of the logistic model to fit the Endotoxin Standard curves and the 2 acceptance criteria requested by the Pharmacopoeia.

As these specifications were identified as the main source of errors and as explained in the European Pharmacopoeia 9.2 - 5.3 Statistical analysis, ‘the logistic model raises a number of statistical problems which may require different solutions for different types of assays, and no simple summary is possible. A wide variety of possible approaches is described in the relevant literature. Professional advice is therefore recommended for this type of analysis’. Therefore, these specifications were defined with the support of a professional statistician, who is Senior Lecturer in the Applied Mathematics Department from the University of Strasbourg.

The model that best suits the MAT application using Mono-Mac-6 Cell Line is a 5 parameters curve model. The 5 parameters (5P) curve is nearly identical to the 4 parameters (4P) except for an additional parameter ‘e’ (which is equal to 1 in a 4P curve and makes the curve symmetrical). With ‘e’, the 5P curve fit is better able to model asymmetric experiment results.

The acceptance criteria, which verifies the P-value for regression (p < 0.01), uses the ‘No Effect’ statistical test.

The acceptance criteria, which verifies the P-value for linearity (p > 0.05), uses the ‘Lack of fit’ statistical test.

Code Verification:

The first Code Review identified some dead code and errors due to floating point number used for numerical tests:

“2.7. Is there any floating point number used for numerical tests? If yes, is there some tolerance set in order to avoid numerical errors, because floating point numbers don’t match exactly the value (ex. of floating point number: 1.0 can be 0.9999997, ex. of tolerance: ((myFloatValue – 17.0D0) < 10E-4)…)?”.

The defects were logged and the code was changed accordingly in the next Release Candidate to be tested.
Validation of Technical Specifications:
Main defect was on ‘[EEU/mL]xDIL_LIMITS’ in PyroMAT_Method_A.prt protocol where a ‘<’ sign was expected, while the software displayed nothing. The defect was corrected in the next Release Candidate.

A strict equality was found between two floating point numbers for the CheckMVD_B tested in the PyroMAT_Method_B.prt protocol.

Validation of Functional Specifications:
A wrong dilution in the plate layout in the PyroMAT_Method_B protocol was detected. The defect was corrected in the next Release Candidate.

Validation of Requirements Specifications:
The date language was wrong in both PyroMAT_Method_A.prt and PyroMAT_Method_B.prt protocols. These defects were corrected in the next Release Candidate.

Change Requests:

- Development phase: Main change requests improved the curve fit and added an Additional Criteria (Not required by European Pharmacopoeia) to verify that the curves reach a minimal reactivity. This improvement was applied to PyroMAT_Method_A, PyroMAT_PSV_I_II_III_A and PyroMAT_PSV_I_II_III_B.

- Change request from October 2018 was to improve the calculation of the Spike recovery when the Sample Values are below 0.05 EU/mL and when at least one value of Sample is masked (e.g. due to suspicion of outlier). This improvement led to PyroMAT_Method_A.prt (V1.4.3.0) & PyroMAT_PSV_I_II_III_A.prt (V1.3.3.0)."

Configuration management:
Validation was performed on Gen5™ Software 3.03 installed on Windows® 7 64 bits.

The printer was configured to print the report in A4, landscape.

The released software files integrity can be verified by checking the MD5 or SHA1 cryptographic hash of the content of the *.prt files.

Risk Assessment & PoA
The 3 main risks are given below with their Risk Probability Number (RPN):

<table>
<thead>
<tr>
<th>ID</th>
<th>Name</th>
<th>Description</th>
<th>RPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>P169-BIOM_RA-2</td>
<td>EP-2 acceptance criteria</td>
<td>Risk of not taking the right statistical test to perform the 2 acceptance criteria given in the EP</td>
<td>729</td>
</tr>
<tr>
<td>P169-BIOM_RA-6</td>
<td>Printing - Latest Data are not updated in the Report</td>
<td>Risk of having Data Views not reported in the Report</td>
<td>378</td>
</tr>
<tr>
<td>P169-BIOM_RA-8</td>
<td>EP-MVD</td>
<td>Risk of having a miscalculation of the MVD due to wrong CLC units</td>
<td>343</td>
</tr>
</tbody>
</table>

These risks have the following PoA:

<table>
<thead>
<tr>
<th>ID</th>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>P169-BIOM_POA-1</td>
<td>GEP - EP-2 acceptance criteria</td>
<td>Use the consultancy of professional statistician from the University of Strasbourg</td>
</tr>
<tr>
<td>P169-BIOM_POA-6</td>
<td>GEP- Build Report @RC</td>
<td>Report is built with Data View before making the Release Candidate</td>
</tr>
<tr>
<td>P169-BIOM_POA-8</td>
<td>User Guide - MVD Calculation</td>
<td>Add a note in the User Guide regarding the input prompt of CLC and indicates the unit of CLC in the prompt (EU/mL)</td>
</tr>
</tbody>
</table>

Acronyms:
- RA: Risk Assessment
- PoA: Plan of Action
- GEP: Good Engineering Practices
- EP: European Pharmacopoeia
- RC: Release Candidate
- CLC: Contaminant Limit Concentration
- MVD: Maximum Valid Dilution

The consultancy was performed, the good engineering practice to build the report before releasing the software was followed and the User Guide gives an indication that the unit of CLC shall be Endotoxin Unit per milliliter (EU/mL).

6 Conclusion
The conclusion of the Software Validation is that all versions of protocols were fully validated and released to operate per written product specifications.
PyroMAT™ Software protocols were developed for data analysis of Monocyte Activation Test performed with PyroMAT™ System. Protocols are available for analysis of tests results performed following Method A, B or C from European Pharmacopoeia, Chapter 2.6.30.

PyroMAT™ Software protocols are to be used in connection with Gen5™ software from Biotek® and can be downloaded, free-of-charge on our website: EMDMillipore.com/pyromat

In case of question related to the use of PyroMAT™ Software protocols or data analysis from PyroMAT™ assay, please contact our technical service: EMDMillipore.com/techsersvice

In case of question regarding the use of Gen5™ Software, please contact the technical assistance center of Biotek®: TAC@biotek.com
PyroMAT™ System related products

<table>
<thead>
<tr>
<th>Product</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PyroMAT™ cells</td>
<td>PyrOMATCELLS</td>
</tr>
<tr>
<td>PyroMAT™ kit</td>
<td>PyrOMATKIT</td>
</tr>
<tr>
<td>Reference Standard Endotoxin</td>
<td>1.44161.0001</td>
</tr>
<tr>
<td>NEP Control HKSA</td>
<td>MATHKSA</td>
</tr>
<tr>
<td>NEP Control Flagellin</td>
<td>MATFLAGELLIN</td>
</tr>
<tr>
<td>IL-6 Control</td>
<td>PyrOMATIL6</td>
</tr>
</tbody>
</table>

PyroMAT™ System related Services

We provide additional services to support you in the use of PyroMAT™ System (training) and in the data analysis of the data generated with PyroMAT™ System. Please refer to our Service webpage from EMDMillipore.com/biomonitoring.