Lonza

# It's All Mixed Up

## Vortex Mixing vs. Pipette Mixing in a Robotic BET Assay System

by Allen L. Burgenson, John Cooley, Lonza Walkersville, Inc.

**The PyroTec® PRO System with automated pipette mixing delivers equivalency to manual preparation of bacterial endotoxin testing standards using vortex mixing.**

In order for proper assay performance in any technique, mixing to a homogeneous level is critical. Vortex mixing has been the standard for mixing samples, standards, and product dilutions in the long history of the Bacterial Endotoxins Test (BET) (USP<85>, EP 2.6.30, and JP 4.01). Manual vortex mixing is accepted as thorough, and will yield the desired homogeneous mixture. In the case of an automated robotic platform, where the use of a vortex mixer is impractical, a suitable alternative must be used. The use of the pipettes on the robotic arm will draw the liquid up and down multiple times to achieve a level of mixing equivalent to the use of a vortex mixer.

Our laboratories performed a study to demonstrate that endotoxin standard preparation by automated pipette mixing on the PyroTec® PRO System is equivalent to manual preparation of standards using vortex mixing.

Lonza's Kinetic-QCL® Kinetic Chromogenic LAL Assay Kit provided the necessary endotoxin standard and LAL reagents needed to complete this testing. The CSE standard was reconstituted with the required amount of LAL Reagent Water (LRW) indicated on the Certificate of Analysis for the KQCL kit. The CSE vial was vigorously mixed for 15 minutes on a vortex mixer, and a set of standards prepared by dilution with LRW by both the PyroTec® PRO System and manually by a skilled analyst. The PyroTec® PRO system dilutes and mixes by repeated aspirating and dispensing of 700 µL from a 1 mL total volume seven times for each concentration. Manual dilutions were mixed by vortexing for one minute, as described in the current LAL Kinetic-QCL® Kit Insert.

The goal in mixing dilutions was to obtain homogeneous solutions at each concentration. Pipette mixing was successful if the standards produced exhibited similar characteristics to those of the manually mixed standards using a vortex mixer. Each standard curve derived by either manual mixing with a vortex mixer, or pipette mixing on the robot had to meet the system suitability criteria to be included in the final analysis.

The system suitability criteria that must be met in all Lonza kinetic chromogenic assays were the following:

- Standard Curve with correlation coefficient between -1.000 to 0.980, Slope -0.400 to -0.100, and Y-Intercept between 2.500 to 3.500
- Positive Product Control (PPC) with % recovery between 50% to 200%, and endotoxin prediction of a 0.5 EU/mL PPC between 0.25 EU/mL to 1.0 EU/mL.
- Endotoxin standards with % CV of < 10 %
- Run temperature maintained between 36 – 38°C

This study was comprised of twelve separate comparison tests of CSE standard dilutions with concentrations from 50 EU/mL to 0.005 EU/mL and blank controls, LRW samples, and LRW samples plus PPC.

The PyroTec® PRO System followed commands from a WinKQCL® Software template to instruct the analyst where to place dilution tubes and the pre-prepared 50 EU/mL CSE on the deck. While the instrument made the automated dilutions for the standard curve, the analyst manually diluted and vortex mixed the standards.

The analyst manually loaded standards from each method, blanks, and test samples to the assay plate. The PyroTec® PRO System produced standard was loaded into the first two columns with blank and test sample. The analyst loaded the PPC spike from this 5.0 EU/mL standard into the sample PPC wells. The manually produced standard, blank, sample, and sample plus PPC spike were loaded into the third and fourth columns.

Separate templates for the automation standard with sample and the manual standard with sample were run as a Merged Plate template in each assay run.

Assay reports, generated automatically at the completion of each assay run, were evaluated and the data saved for analysis at the completion of all twelve tests.

The protocol acceptance criteria were as follows:

- Calculate the means and standard deviations of the slope and Y-intercept for the 12 replicates of the automated curve and for the 12 replicates of the manual curve
- For the 12 runs combined, analyze the means of the automated and manual slope values via a two-tailed T-test with $\alpha$= 0.05. The p-value must be > 0.05
- For the 12 runs combined, analyze the means of the automated and manual Y-intercept values via a two-tailed T-test with $\alpha$ = 0.05. The p-value must be > 0.05

# Results

In the course of running the protocol, a single blank replicate from the manual preparation in Run 7 reacted before the last standard. Per the study protocol, the invalid run was omitted from the final results. A full assay run, Run 13, replaced Run 7 and was included in the analysis.

All system suitability criteria were met in the analyzed runs. Run temperatures were verified to be within 36 – 38°C for all runs. In all runs the lowest standard reaction time was less than the blank reaction time.

The initial data analysis considered the method overlay of all 12 standard curves from each of the two preparation methods. These graphs show little to no variability from run to run. See Figure 1 below.

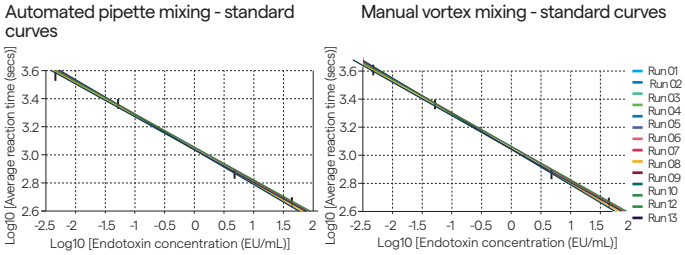


**Figure 1.**
Composite Standard Curves by Method.

The standard curves were averaged by each mixing method, and the data plotted as a composite. In Figure 2 below, it is difficult to distinguish the differences between the standards by method. It does appear that the automated pipette mixing method has slightly faster reaction times at the ends of the curve (50 and 0.005 EU/mL concentrations). Average reaction times for the standards are shown in Table 1. Values shown are the averages for 24 individual wells of each standard from 12 combined runs.

| | Automated | | | Manual | | | |
|---|---|---|---|---|---|---|---|
| **Standard (EU/mL)** | Avg. rxn time (sec) | Standard deviation | % CV | Avg. rxn time (sec) | Standard deviation | % CV | Auto vs man. % RPD |
| 0.005 | 3798 | 113 | 3.0 | 3919 | 95 | 2.4 | 3.1 |
| 0.05 | 2413 | 31 | 1.3 | 2455 | 37 | 1.5 | 1.7 |
| 0.5 | 1275 | 22 | 1.8 | 1289 | 24 | 1.9 | 1.1 |
| 5 | 746 | 15 | 1.9 | 752 | 14 | 1.9 | 0.8 |
| 50 | 451 | 15 | 3.2 | 466 | 12 | 2.5 | 3.3 |

**Table 1.**
Average Reaction Times (seconds) for standards in the study

## Comparison of standard curves by mixing method

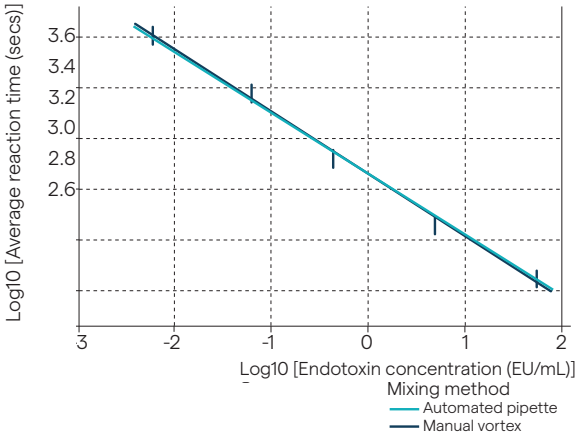


**Figure 2.**
Composite of All Standard Curves by Mixing Method

The analysis of the %CV of the assay runs shows that each method met the system suitability criteria of <10 %. See Figure 3 below.

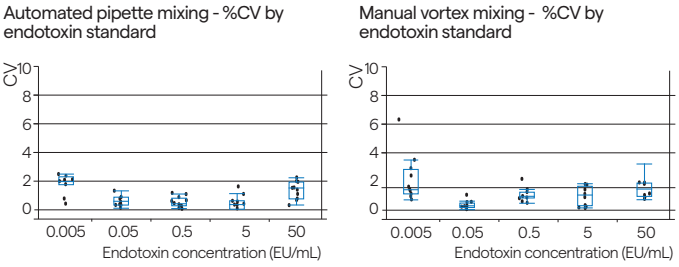


**Figure 3.**
%CV of standard concentrations by method

These graphs show comparable distribution of the %CV of each dilution per mix method.

The analysis of the Correlation Coefficient of the assay runs shows that each method met the system suitability criteria of -1.000 to -0.980. See Figures 4 and 5 below.

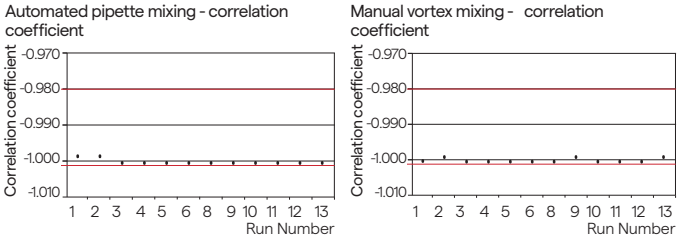


**Figure 4.**
Correlation coefficient by method

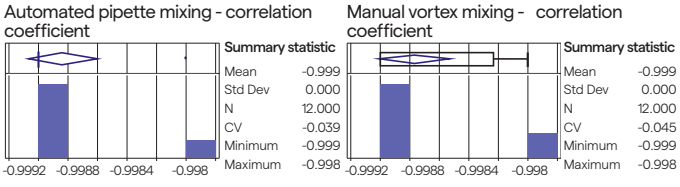


**Figure 5.**
Correlation coefficient summary statistics by method

The analysis of the Slope of the assay runs shows that each method met the system suitability criteria of -0.400 to -0.100. See Figures 6 and 7 below.

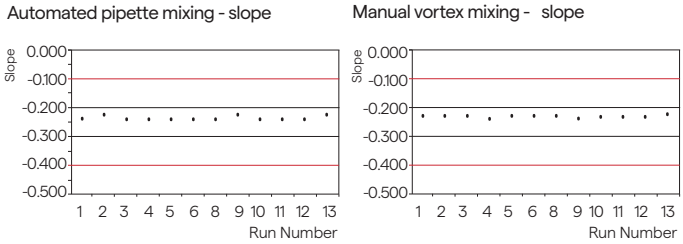


**Figure 6.**
Slope by method

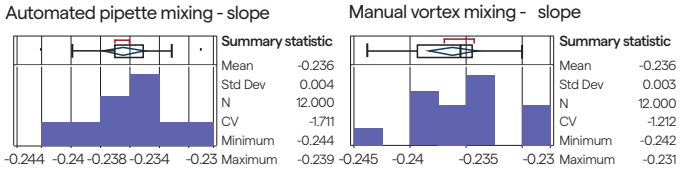


**Figure 7.**
Slope statistics by method

Further analysis of the slope included an equal variance T-test analysis, which is performed when there is a comparison of two independent samples with equal variance. The null hypothesis of the T-test is that there is no difference between the two mean populations of data.

Figure 8 below, shows this analysis.

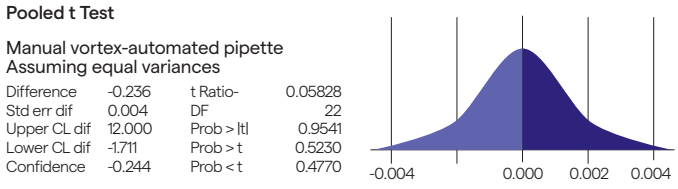


**Figure 8.**
Slope statistics by method

In this data, the Prob > |t| shows the p-value for the two-tailed test is equal to 0.9541. This p-value is not significant at a confidence level of 0.95 ($\alpha = 0.05$). Therefore, the null hypothesis can be accepted to say that there is no statistically significant difference between the automation pipette mixing and the manual vortex mixing for slope.

The analysis of the Y-Intercept of the assay runs shows that each method met the system suitability criteria of 2.500 to 3.500. See Figures 9 and 10 below.

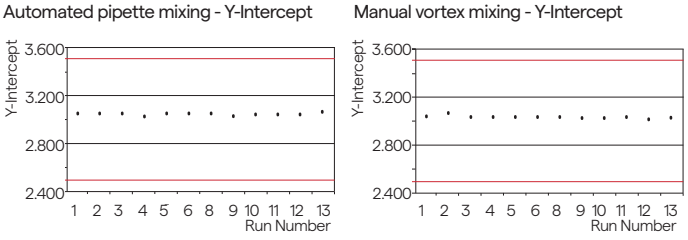


**Figure 9.**
Y-Intercept analysis by method

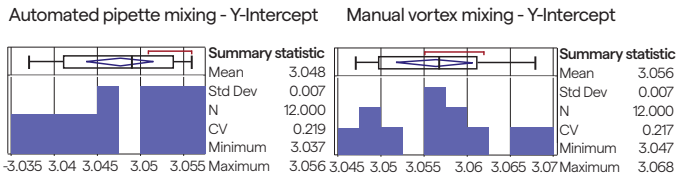


**Figure 10.**
Y-Intercept statistics by method

The T-test analysis for the Y-Intercept was performed as described for the Slope previously, with the null hypothesis that there is no difference between the two mean populations of data.

Figure 11 below, shows this analysis.

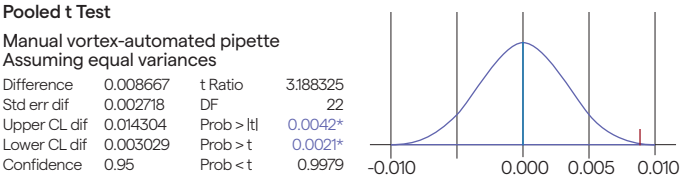


**Figure 11.**
Y-Intercept pooled T-test analysis

In this data, the Prob > |t| shows the p-value for the two-tailed test is equal to 0.0042. This p-value is significant at a confidence level of 0.95 ($\alpha = 0.05$). Therefore, the null hypothesis is rejected and there is a statistically significant difference between the automation pipette mixing and the manual vortex mixing for the Y-Intercept.

The analysis of the %PPC Recovery of the assay runs shows that each method met the system suitability criteria of 50 – 200 %. See Figures 12 and 13 below.

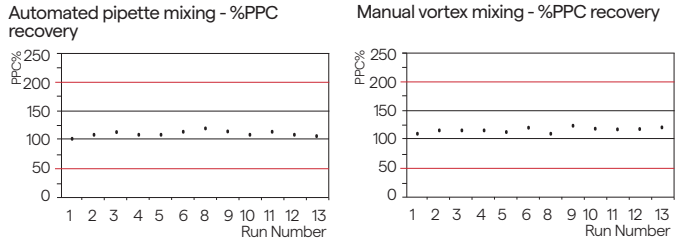


**Figure 12.**
%PPC recovery by method

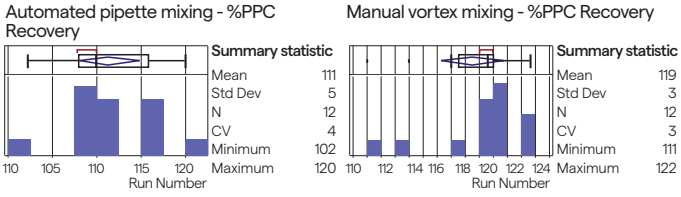


**Figure 13.**
%PPC recovery statistics by method

The analysis of the PPC Sample Back Prediction of the assay runs shows that each method met the system suitability criteria of 0.25 EU/mL to 1.0 EU/mL. See Figures 14 and 15 below.

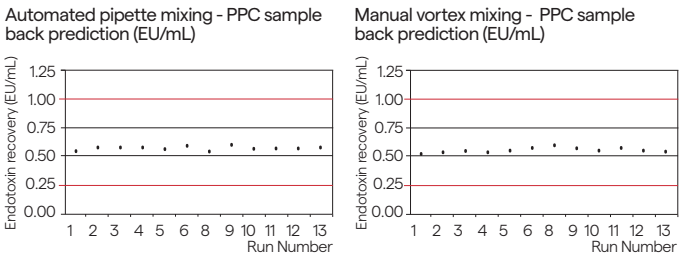


**Figure 14**.
PPC sample back prediction by method

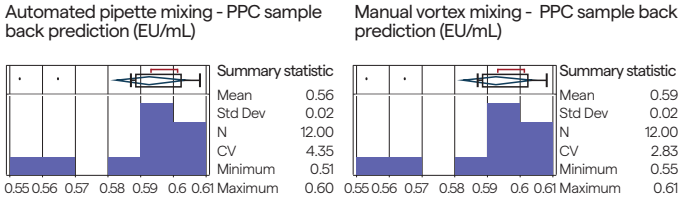


**Figure 15**.
PPC sample back prediction statistics by method

The PPC for this protocol was LRW spiked with 0.5 EU/mL endotoxin. The reaction times of the PPC spike and the standard curve parameters were used to back calculate the amount of endotoxin spiked into the PPC test sample. Figure 16 below shows that both methods back calculate a similar amount of PPC. The automated pipette mixing is slightly more accurate (closer to the nominal value of 0.5 EU/mL) but less precise than the manual vortex mixing.

Comparison of PPC sample back prediction (EU/mL) by mixing method

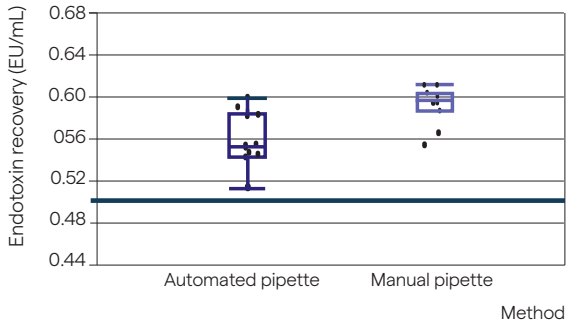


**Figure 16**.
PPC back prediction comparison

## Conclusion

While equivalency of the Y-Intercept parameter stated in the protocol was not confirmed, the difference seen between mixing methods for the Y-intercept had no effect on the ability of the PyroTec® PRO System to recover an endotoxin spike in the positive product control (PPC) or meet all assay performance criteria.

Therefore, the automated pipette mixing on the PyroTec® PRO System is comparable to the manual preparation of endotoxin standards using vortex mixing.

Finally, the interpretation of this mixing data is only applicable to the use of automated pipette mixing of standards associated with the PyroTec® PRO System and is not intended to imply the use of pipette mixing as a substitute for vortexing dilutions when running assays manually.

## Contact Us


### North America
Customer Service: + 1 800 638 8174 (toll free)
order.us@lonza.com
Scientific Support: + 1 800 521 0390 (toll free)
scientific.support@lonza.com

### Europe
Customer Service: + 32 87 321 611
order.europe@lonza.com
Scientific Support: + 32 87 321 611
scientific.support.eu@lonza.com

### International
Contact your local Lonza Distributor
Customer Service: + 1 301 898 7025
Fax: + 1 301 845 8291
scientific.support@lonza.com

Lonza Walkersville, Inc. – Walkersville, MD 21793





RT-SP023 11/22