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Assay: Accuracy and Precision with Serial Dilution

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Sample Mixing Efficiency with the Bravo™ Liquid Handling Platform

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Many laboratory protocols require the serial dilution of reagents or compounds. IC50 assays, commonly used to evaluate drug efficacy, and assay development procedures, as well as standard-curve generation, involve the serial dilution of compounds, proteins, or detection agents. These processes can be streamlined by utilizing automated liquid-handling equipment with serial dilution capabilities.

Serial dilution processes face two major challenges. The first is error propagation across columns or rows. With each sequential serial dilution step, transfer inaccuracies lead to less accurate and less precise dispensing. The result is that the highest dilutions will have the most inaccurate results. To compensate for this error possibility, longer mixing times are required, which then increases the time required to perform the serial dilution. These challenges greatly limit the throughput capacity of an automated serial dilution system.

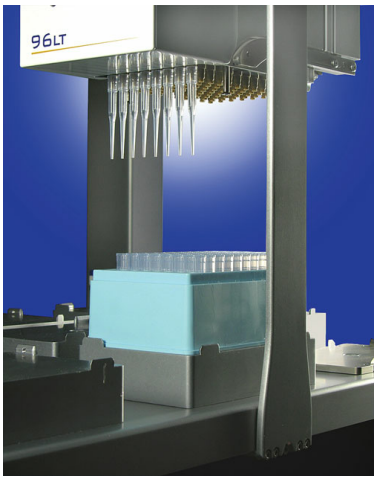

Figure 1

To overcome these challenges, the effects of various mixing parameters of a serial dilution protocol were explored. **Velocity11's** (www.velocity11.com) Bravo™ Liquid Handling Platform performed serial dilution with the same pipette head as a full plate dispenser (Figure 1). With the platform's VWorks™ software, the application allowed the total control of liquid transfer and mixing heights and speeds, which allowed efficient exploration of mixing parameters. The goals were to determine which parameters had the greatest effect on mixing and to reduce the time required to perform a serial dilution.

Serial Dilution Mix Cycles

The basic experiment diluted fluorescein across the columns of a 96-well plate, from A1 to A10 (A11 and A12 were blank wells). The starting volume was 300 µL, and 200 µL tips were utilized for the transfer (150 µL, a 1:2 dilution) and mixing steps (190 µL). There are two main components of an accurate and precise serial dilution: the accuracy and precision of the transfer and the efficiency of mixing. Transfers were previously determined to have a precision and accuracy of >99% at this volume; any observed deviations in precision and accuracy were due to error propagation from ineffective mixing.

Two measures were used to evaluate mixing efficiency. The Coefficient of Variance (CV) of each column indicated the precision of the mixing step. The CV also provided information on the propagation of error across a plate—the CV increased sharply across the plate if mixing was incomplete.

The second indication was the accuracy of the transfer. A calibration curve was prepared, and each experimental dilution concentration was plotted against the standard curve to determine the real concentrations in each column. The first experiment varied the number of mixing cycles between 3 and 20. The average precision (averaging CVs for columns 1–10) improved asymptotically as the number of mix cycles increased. Three mixes before each transfer yielded an average CV of 11.8%, while 20 mixes gave a considerably better CV of 1.7%.

The precision in all cases generally worsened as the serial dilution proceeded across the plate; this was expected as the error in the earlier columns propagated with each transfer.

The accuracy ratio improved as the number of mix cycles increased. The accuracy ratio is an average of the concentration of the diluted column compared to the previous column—a perfect serial dilution has an accuracy ratio of 1:2.00 across the entire plate. The accuracy ratio of the plate improved with more mix cycles, improving from 1:1.85 to 1:2.01.

While the precision and accuracy with 20 mix cycles is close to a perfect serial dilution, the length of time required might be considered impractical. The 20-mix cycle protocol required 20 minutes per plate, while a three-mix cycle protocol required less than six minutes. Efforts were then focused on the factors that could improve the three-mix cycle protocol to produce accuracy and precision results consistent with the 20-mix cycle protocol.

Mix Tip Height

The mix tip height was modified in order to determine the effect of distributing the liquid at different locations in the well. As the mix tip height was raised, the average precision improved. At a height of 3 mm from the bottom of the well, the average precision was 3.9%. The precision worsened as the tip distance from the bottom of the well decreased, reaching a CV of 15% at a height of 0.1 mm. Accuracy tracked with precision, and the higher mix height also improved the accuracy ratio to 1.95. This trend is possibly because the higher dispense height ensures that more of the sample was circulated by the mix cycle.

In a mix roughly in the middle of the well volume, dispensed liquid is forced toward the well bottom while dispensing, and aspirated liquid is pulled from the center of the well. If the mix occurs close to the bottom of the plate, the dispensed liquid is pulled back into the tip during the aspiration. Mixing in the center allows the dispensed liquid to be more evenly distributed in the sample, thus increasing the likelihood of efficient mixing.

Mix Liquid Class Setting

The VWorks software controlling the Bravo platform allows the creation of liquid classes, which allows the operator to modify the velocity and acceleration for aspirating, dispensing, and mixing tasks. The original liquid class settings for the mix were 100 µL/s velocity and 500 µL/s2 acceleration. Precision and accuracy improved as the mix velocity increased. This effect plateaus; above 300 µL/s, there is no appreciable improvement in increasing the speed. The cause of this is likely due to the creation of more turbulent mixing, which in turn distributed the fluorescein dye more quickly throughout the solution.

Dynamic Tip Retraction/Extension

Finally, the effect of dynamic tip retraction and extension was explored. This function moved the tips deeper into the well during each aspirate step, and retracted them during each dispense step. This allowed a larger volume of the well to be effected by the mix step by adding the movement of the tip into the mix task. There was a marginal improvement (less than 0.5% improvement in CV/accuracy) observed in using this technique.

Additionally, no effect was observed by utilizing another mix standard, which involved aspirating close to the bottom of the well and dispensing near the top of the solution. This mixing method caused no improvement once the other parameters described above had been optimized. These experiments mixed homogenous solutions; there may be an improvement with this technique if the solutions are expected to have different viscosities.

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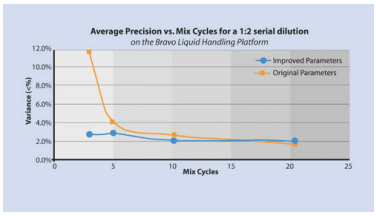

Figure 2

Conclusion

Based on these experiments, the parameters that had the largest impact on efficient mixing were (in decreasing order):

• Speed of the mixing step

• Height of the tip during the mix

• Tip-retraction capabilities

To verify this conclusion, the first experiment (varying the number of mix cycles) was repeated with the improved mix parameters. The new parameters provided increased precision and accuracy, and improved the accuracy and precision of the 3-mix cycle operation to a level comparable with the 20-mix cycle operation (Figure 2). More importantly, the new parameters also decreased the time required to run an effective serial dilution protocol from 20 minutes to just under 5 minutes. This has tremendous potential in automating a serial dilution assay and ensuring accurate and precise results.

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