Serial Dilution with the Agilent Bravo Automated Liquid Handling Platform

Technical Overview

Summary

- Accurate serial dilution with a full channel head
- Efficient mixing with as few as three mix cycles
- No tip changes necessary
- No wash steps necessary

Introduction

Many laboratory protocols require the serial dilution of reagents or compounds. IC_{50} assays are commonly used to evaluate drug efficacy, and assay development procedures as well as standard curve generation involve the serial dilution of proteins, compounds, or other detection agents. These processes can be streamlined with automated liquid handling equipment with serial dilution capabilities.

The Bravo Automated Liquid Handling Platform from Agilent Automation Solutions can be utilized to perform serial dilutions. It has nine standard deck positions that can hold any SBS standard microplate. These deck positions can also be configured for heating, cooling, shaking, and tip washing. The design of the head, along with various tip and head options, allows serial dilutions to be performed by column or row, from 300 nL to 200 µL dilutions.

Operators can easily write protocols with the included Agilent VWorks software package. One key feature is the Serial Dilution Wizard. The Serial Dilution Wizard lets the operator easily program the instrument for making serial dilutions. The wizard walks the operator through the steps of the serial dilution task: tip changes, mix parameters, and volume transfers. The operator can either determine the transfer based on initial volumes (transfer by volume) or by a concentration gradient (transfer by concentration).

The Agilent Bravo has nine deck positions and can be configured with interchangeable 8-, 16-, 96-, or 384-fixed and disposable tip heads.

This technical overview explores the parameters that lead to an efficient, precise, and accurate serial dilution protocol. The goal is to produce good precision and accuracy for each dilution in the least amount of time. The mix parameters are essential for a robust serial dilution task. The parameters explored include:

- The number of mix cycles
- Mix height
- Liquid class settings
- Tip retraction/extension

Materials Used

- Bravo with a 96-Channel LT Disposable Tip Head
- Agilent 96LT 200 µL tips (product no. 06880-102)
- Agilent 96-well manual fill reservoirs (product no. 08105-001)
- 96-well polystyrene, black flat clear bottom plates (Greiner 655087)
- Fluorescein solution (3 µM in 50 mM Tris-HCl, pH 8.0)
- SPECTRAFluor Fluorescence Spectrophotometer (Tecan)

Method

A reservoir containing 60 mL of fluorescein solution is placed on position 2 of the Agilent Bravo (fluorescein reservoir for serial dilution by Row is placed on position 7). A reservoir containing 100 mL of 50 mM Tris-HCl is placed on position 3. A 96-well polystyrene plate is placed on position 5. 200 µL tip boxes are placed on positions 6 and 9. An Agilent Works liquid class for 51-200 µL dispense is utilized.

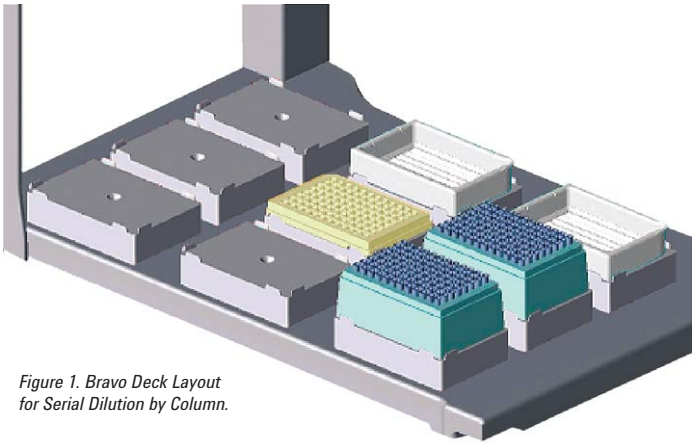

Figure 1. Bravo Deck Layout for Serial Dilution by Column.

1. Tips are pressed onto the head from position 9.
2. 150 µL Tris-HCl solution is transferred from position 3 to position 5. Aspirate parameters are 6 mm from the bottom of the reservoir with a 2 µL pre-aspirate volume. Dispense parameters are 2 mm from the bottom of the plate, with a 2 µL blowout volume.
3. Tips are unloaded back to position 9.
4. Eight tips are pressed onto the last column of the head from position 6.
5. Fluorescein solution is mixed at position 2. Mix parameters are 150 µL, 3 mix cycles 6 mm from the bottom of the plate, with a 2 µL air gap.
6. 150 µL fluorescein solution is transferred from position 2 to Column 1 of the plate at position 5. Aspirate parameters are 6 mm from the bottom of the reservoir with a 2 µL pre-aspirate volume. Dispense parameters are 2 mm from the bottom of the plate, with a 2 µL blowout volume.
7. 190 µL was mixed in Column 1 of the 96-well plate. Mix parameters vary on each experiment performed throughout the study as described below.
8. A 1:2 serial dilution (150 µL) was performed from Column 1-10. All aspirations have a 2 µL pre-aspirate volume at 2 mm from the bottom of the plate. All dispenses occur 2 mm from the bottom of the plate, with a 2 µL blowout volume. Mix parameters vary on each experiment performed throughout the study as described below.
9. 150 µL of excess volume Column 10 is transferred to waste.
10. Tips are unloaded back to position 6.
11. Plates are centrifuged at 1800 rpm for 60 seconds to ensure consistent well menisci.
12. Fluorescence absorption is read at 485 nm excitation and 535 nm emission wavelength with a gain of 63, 5 flash cycles, and a 40 ms integration time.

A similar serial dilution method by row is performed, with the exception that the fluorescein reservoir is placed on position 7.

Fluorescence measurements from each well are used to determine the precision of the transfer. Coefficient of Variance (CV) calculations are made by dividing the standard deviation by the mean for each column/row. Accuracy is calculated based on an equation derived from a fluorescein/Tris-HCl calibration curve consisting of data points at 2, 1.5, 1, 0.5, 0.2, 0.1, 0.05, 0.02, 0.01 and 0.005 µM, compared to the actual fluorescence value in each well.

Results may vary, depending on individual experimental methods and liquid class optimization.

Results

Number of mix cycles

The first experiment was to determine the number of mixes required to produce a precise and accurate 1:2 serial dilution. The volume of the mix was just under the maximum volume of the tip, and the number of mixes was varied from 3-20 cycles. Table 1 shows the resulting CV of each column for each mix cycle protocol. The average precision (averaging CVs for columns 1-10) shows that the precision improves asymptotically as the number of mix cycles is increased (Figure 2). Three mixes yielded an average CV of 11.8%, while 20 mixes had a considerably better CV of 1.7%. The precision in all cases worsens as the serial dilution proceeds across the plate; this is expected as the error in the earlier columns is propagated with each transfer.

# of Mixes	CV/Column										Avg. Precision	Time (sec/plate)	Acc. Ratio
	1	2	3	4	5	6	7	8	9	10			
3	0.8%	6.1%	6.1%	3.9%	9.7%	11.5%	20.1%	18.7%	19.2%	22.4%	11.8%	340	1:1.85
5	0.4%	2.0%	4.1%	3.9%	2.6%	4.7%	4.5%	6.3%	8.1%	5.9%	4.2%	440	1:1.92
10	0.6%	1.2%	1.4%	1.6%	2.2%	3.1%	3.2%	3.6%	5.0%	5.3%	2.7%	690	1:1.97
20	0.5%	0.6%	1.0%	1.3%	1.5%	1.7%	2.4%	2.6%	2.4%	3.4%	1.7%	1200	1:2.01

Table 1. Precision, accuracy, and serial dilution time with varying number of mix cycles.

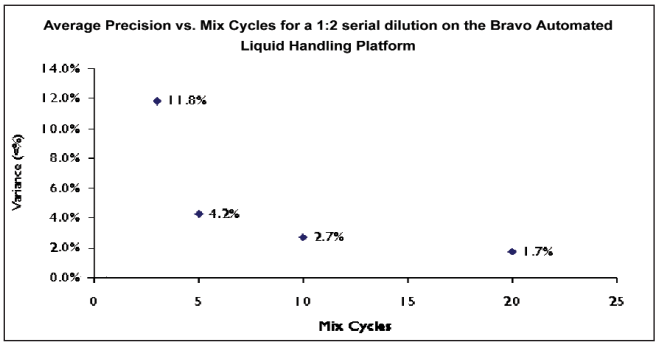

Figure 2. Average precision improves as the number of mix cycles increases.

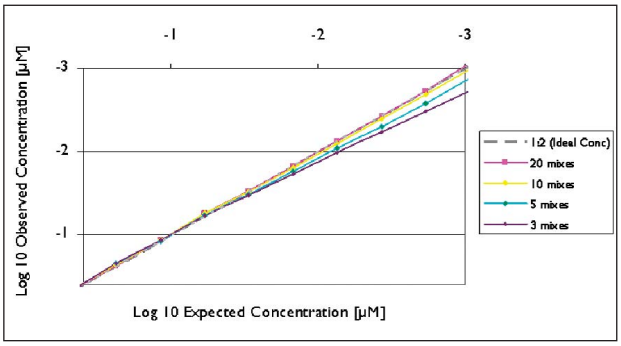

Figure 3. As the number of mix cycles increases, the observed concentrations approach the idealized 1:2 serial dilution.

In addition, the accuracy ratio improves as the number of mix cycles increases (Figure 3). The accuracy ratio is an average of the concentration of the diluted column compared to the previous column—a perfect serial dilution would have an accuracy ratio of 1:2.00 across the entire plate. The accuracy ratio of the plate improves with more mix cycles, varying from 1:1.85 to 1:2.01.

This is expected as the fluorescein would be more equally distributed in the well the more the well contents are mixed, ensuring that transfers are made from a uniformly mixed solution.

While the precision and accuracy with 20 mix cycles is very close to a perfect serial dilution, it also undoubtedly takes too much time for an automated process. The 20 mix cycle protocol takes 20 minutes to perform, while a 3 mix cycle protocol takes less than six minutes. Efforts were then focused on the factors that can improve the 3 mix cycle protocol to produce accuracy and precision results that were closer to the achieved 20 mix cycle protocol.

Mix Height

The mix height was adjusted in order to determine the effect of distributing the liquid at a different location in the well. As the mix height was increased, the average precision improved. Figure 4 shows that at a height of 3 mm, the average precision is 3.9%, significantly lower than 15% at a height of 0.1 mm. Following the same trend as previously observed, accuracy tracks with precision, so the higher mix height also improves our accuracy ratio to 1.95 (Table 2). This trend is observed because the higher dispense height at this dispense speed ensures that more of the sample is circulated by the mix cycle: in a mix far from the bottom of the well, dispensed liquid is forced closer to the bottom while dispensing, and aspirated liquid is pulled from the top of the well. If the mix occurs closer to the bottom of the plate, the dispensed liquid is pulled back into the tip during the aspiration.

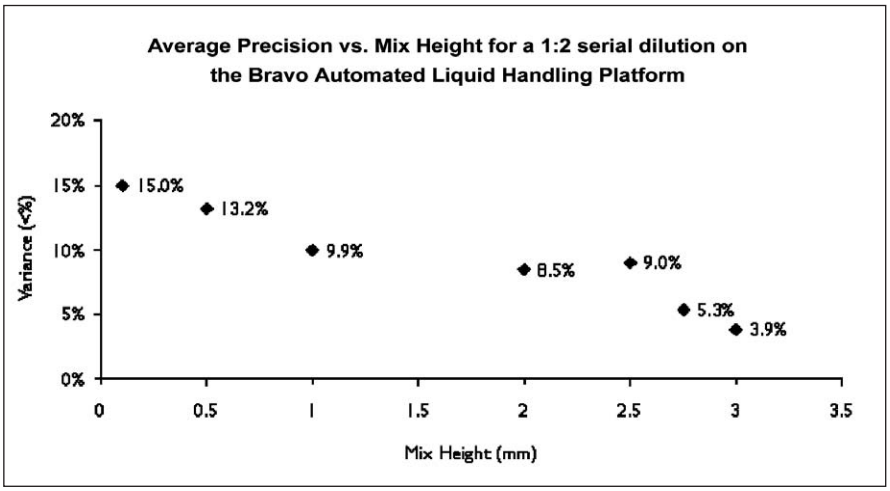

Figure 4. Average precision improves as mix height increases.

Mix Height	CV/Column										Avg. Precision	Acc. Ratio
	1	2	3	4	5	6	7	8	9	10		
0.1	7.9%	8.5%	11.2%	11.5%	11.8%	19.8%	15.8%	17.5%	25.1%	21.1%	15.0%	1:1.78
0.5	6.6%	4.7%	6.9%	16.3%	6.9%	12.3%	13.5%	21.9%	22.7%	20.0%	13.2%	1:1.74
1	6.3%	4.3%	6.3%	8.8%	14.2%	9.7%	12.1%	15.0%	11.1%	11.4%	9.9%	1:1.82
2	1.8%	4.3%	7.5%	6.8%	5.2%	4.6%	10.4%	13.8%	16.6%	14.1%	8.5%	1:1.86
2.5	2.2%	2.1%	3.5%	8.2%	8.5%	8.7%	11.1%	18.9%	15.2%	11.6%	9.0%	1:1.90
2.75	3.7%	4.1%	4.9%	3.8%	3.8%	5.2%	6.2%	9.2%	4.7%	7.4%	5.3%	1:1.92
3	1.8%	2.5%	2.8%	2.4%	2.1%	3.1%	4.2%	6.4%	5.8%	7.9%	3.9%	1:1.95

Table 2. Precision and accuracy with varying mix heights.

Mix Liquid Class Setting

The next parameter explored was the mix liquid class settings. Each liquid class has a set of velocities and accelerations for the aspirate and dispense. This experiment was to determine if increasing the aspirate and dispense speeds of the mix would result in improved serial dilution results.

The original liquid class speed for the mix was 100 µL/s velocity and 500 µL/s^2 acceleration. Based on the average precision of the data (Table 3), mixing became more effective as the mix speed increased. Accuracy is also improved as the mix speed increases, because of consistency of the mixes across the plate. Under these conditions, a mix liquid class setting of a velocity 300 µL/s and an acceleration 1000 µL/s^2 was utilized. There was no appreciable difference in time or performance by increasing the velocity from 300 µL/s to 500 µL/s.

Liquid Class Speed Vel. (µL/s)	Acceler. (µL/s^2)	CV/Column 1	2	3	4	5	6	7	8	9	10	Avg. Precision	Acc. Ratio
100	500	5.6%	3.7%	5.6%	10.3%	10.1%	19.0%	21.1%	17.8%	14.5%	15.7%	12.4%	1:1.90
300	1000	2.1%	1.9%	1.9%	1.9%	1.6%	1.8%	1.7%	2.9%	3.0%	6.7%	2.6%	1:1.98
500	1000	2.0%	1.3%	1.4%	2.6%	2.2%	2.3%	3.6%	4.0%	4.9%	5.1%	2.9%	1:1.97

Table 3. Precision and accuracy with varying mix liquid class settings.

Dynamic Tip Retraction/Extension

To further explore improving precision and accuracy, dynamic tip retraction/extension was used on the mix parameter. Utilizing this particular parameter in the mix cycle, the tip moves into the well during each aspiration and retracts during each dispense. The following retraction/extension test heights were calculated based on the optimal mix height on experiment 2 to give an effective mixing. Mix area was also considered on this test (Figure 5). This will show whether retracting/extending tips during the mixing gives a noticeable change in the CV and amount transferred.

In this experiment, the average precision improved slightly as mix area increased (Table 4). Mixing in half the well was marginally worse than mixing in the entire well, and mixing in the lower half of the well was marginally worse than mixing in the top half. In fact, mixing in half the well was worse than not moving the tips during the mix at all. There was little to no variation in accuracy as the tip retraction changed, suggesting that this parameter did not have an effect, and/or the mix parameters were optimized to the best degree possible.

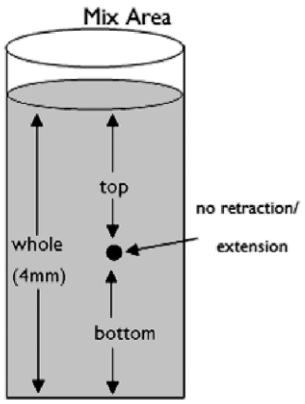

Figure 5. Mix area schematic.

Dynamic Tip Retraction/ Extension (mm/µL)	Mix Height (mm)	Mix Area	CV/Column 1	2	3	4	5	6	7	8	9	10	Avg. Precision	Acc. Ratio
no retraction/ extension	2	no retraction/ extension	2.1%	1.9%	1.9%	1.9%	1.6%	1.8%	1.7%	2.9%	3.0%	6.7%	2.6%	1:1.98
0.02	0.1	whole	2.4%	1.9%	1.7%	2.2%	1.3%	1.4%	1.4%	1.9%	2.6%	6.1%	2.3%	1:1.97
0.01	0.1	bottom	1.4%	1.6%	2.5%	2.5%	2.4%	3.5%	3.8%	4.6%	5.1%	6.4%	3.4%	1:1.98
0.01	2	top	2.6%	1.8%	2.8%	2.4%	2.3%	2.8%	3.3%	2.8%	3.5%	5.3%	3.0%	1:1.98

Table 4. Precision and accuracy with varying mix tip retraction/extension.

Number of mix cycles with the improved mix parameters

Based on the experiments above, the conclusion was that the best mix parameters should have the following characteristics:

- Three mix cycles
- 2 mm mix height
- 300 µL/s velocity and 1000 µL/s^2 acceleration
- 0.02 mm/µL tip retraction

To verify this conclusion, the first experiment (varying the number of mix cycles) was repeated with the improved mix parameters. Table 5 compares the old mix parameters with the improved mix parameters. The improved mix parameters (Figure 6) provides increased precision and accuracy because the mix was more effective. Additionally, the improved mix parameters decreased the time required to run a serial dilution protocol by nearly a minute. Most of this improvement was due to increased liquid class speeds. Accuracy also improved in comparison to the original mix settings.

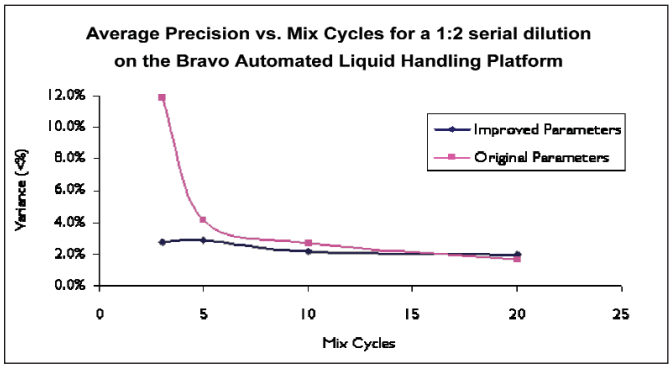

Figure 6. Average precision of improved vs. original parameters.

	Mix Parameters					
	Improved			Original		
Number of mix cycles	Average Precision	Serial Dilution Time (seconds/plate)	Accuracy Ratio	Average Precision	Serial Dilution Time (seconds/plate)	Accuracy Ratio
3	2.8%	285	1:2.01	11.9%	340	1:1.85
5	2.9%	365	1:2.02	4.2%	440	1:1.92
10	2.2%	545	1:2.04	2.7%	690	1:1.97
20	2.0%	900	1:2.04	1.7%	1200	1:2.01

Table 5. Precision, accuracy, and serial dilution time utilizing improved vs. original mix parameters.

Serial Dilution by Row

The Agilent Bravo can perform serial dilutions by column and by row. While all of the previous experiments were serial dilution by column, it was necessary to confirm that the serial dilution by row would work equally well. Table 6 shows that by applying the improved mix parameters, high precision and accuracy were possible in another serial dilution method. Average precision was improved mostly due to the fact that there were seven dilutions instead of 10; error was propagated over a fewer number of dilutions. With fewer dilutions, the overall serial dilution time decreased as well.

Plate Number	CV/Row							Average Precision	Serial Dilution Time (sec/plate)	Accuracy Ratio
	1	2	3	4	5	6	7			
1	1.4%	1.6%	0.9%	1.1%	1.1%	2.2%	2.5%	1.5%	225	1:1.97
2	1.0%	1.3%	1.0%	1.4%	1.5%	1.8%	1.3%	1.3%	225	1:1.97
3	0.6%	1.1%	1.6%	1.6%	1.7%	2.2%	2.8%	1.6%	225	1:1.95
4	0.9%	1.7%	1.2%	1.3%	1.4%	1.6%	2.8%	1.5%	225	1:1.95

Table 6. Precision and accuracy of serial dilution by row.

Conclusion

This study shows that the Agilent Bravo can quickly and reliably serial dilute across columns or rows of a plate. Based on these experiments, the following settings gave us low CV and an accurate overall dispense. It is possible to achieve accurate and precise serial dilution results in a short process time with:

- No tip changes
- A minimum number of mix cycles
- No wash steps

The ability of the Bravo to do serial dilution with a 96- or 384-channel disposable tip head means that serial dilution can be performed with one instrument with one head type. A head swap is not necessary. A parameter setting in the protocol allowed a change from transferring liquid with the entire head to transferring with a single row or column.

Serial dilution experiments can be optimized through a number of different parameter changes, and based on the results shown here, the keys to obtaining accurate and precise mixing are the following, in order of decreasing contribution to overall mixing efficiency:

- Maximize volume of mixing
- Maximize mixing speed
- Maximize the area traversed by tip retraction/extension.

Please contact your sales representative or Agilent Applications Support if you have particular questions regarding your specific application. Supplemental information (protocol files and data analysis spreadsheets) are also available upon request.

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