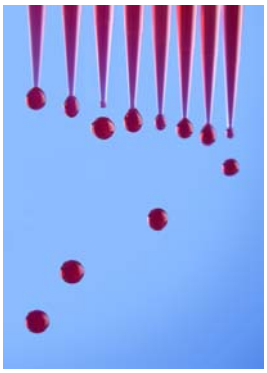
Determining Dilution Accuracy in Microtiter Plate Assays

Using the Artel MVS® Multichannel Verification System



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- **Title:** **Determining Dilution Accuracy in Microtiter Plate Assays Using the Artel Multichannel Verification System (MVS®)**
- Presenter: John Thomas Bradshaw, Ph.D.
- Description: Many critical assays are based upon conducting accurate dilutions of key reagents. Accurately knowing sample concentration is critical for properly interpreting experimental results, which can only be obtained if the experimental dilution ratio is known and controlled. Herein we present an application of the MVS which provides a NIST traceable, dual-dye absorbance method for determining the accuracy of each step in a dilution process. The measurements provided by this method allow for testing both single and multiple point dilutions, and cover a testable dilution range of up to 1/2000. Both theory and experimental validation of this method will be discussed.

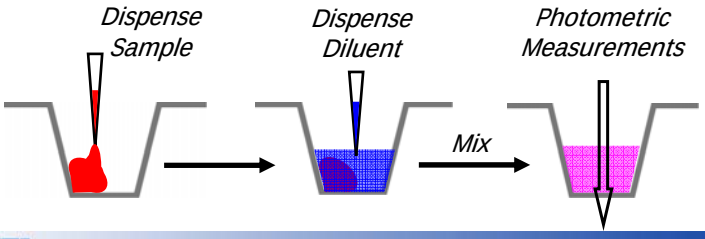
Workshop Outline

- Artel
- Dual-Dye Photometry and the Artel MVS
- Dilution Application using the MVS
- Conclusions

- Leader: Liquid handling quality assurance
- Focus: Laboratory quality & productivity
- Core Technology: Ratiometric Photometry™

Ratiometric Photometry

- Technology used for both the ARTEL PCS® Pipette Calibration System and the ARTEL MVS® Multichannel Verification System:
  - Photometric measurement of liquid volume
  - Two specially-formulated dyes measured at two wavelengths
  - Ratiometric measurements and calculation of results
  - Simultaneous measurement of accuracy & precision *per channel*



MVS® Multichannel Verification System

- Verifies liquid handlers from any manufacturer
- Standardized measurement platform
- Traceable results — repeatability and standardization
- Accuracy and precision per channel

MVS Components

Characterized Microtiter Plates

Sample + Diluent Solutions

Calibrator Plate

Plate Shaker

Notebook Computer w/ System Software & Barcode Reader

Microtiter Plate Reader

MVS Performance

Calibrator Plate

Characterized Microtiter Plates

Software

Sample Solutions

- Optimizes liquid handling instrumentation
- Complete, integrated, mobile system

MVS – Features

- Extremely accurate and precise
- Exceptional for low volumes – down to 0.03 µL
- Easy to use and convenient
- Fast – 5-10 minutes
- Traceable to national standards (NIST)
- ISO-approved method
- Compliance with 21 CFR
- Helps maintain experimental integrity and confidence

MVS is the Standard Platform

- Components of the Artel MVS work together to provide accuracy and precision assessment for target volumes dispensed into microtiter plates.
- Standardization between liquid handlers and/or laboratories *really* means…

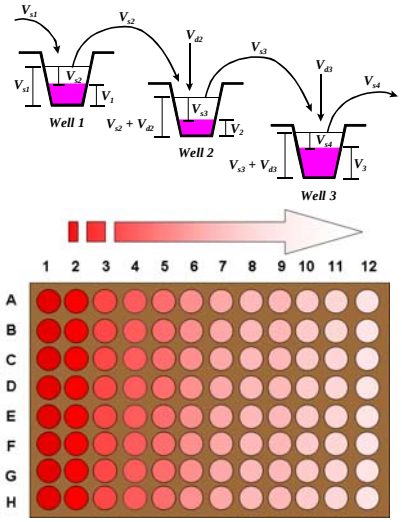
2 µL dispensed by a Tecan Evo in Harlow, UK on Tuesday	=	2 µL dispensed by a Beckman FX in Collegeville, PA on Thursday	=	2 µL dispensed by a PerkinElmer JANUS in Research Triangle Park, NC on Friday

MVS Summary

- The MVS ratiometric absorbance method provides an easy way to assess the volume delivered from a liquid handler
- Traceability of MVS results = standardization across multiple laboratories and equipment
- Fast & Easy
- Accurate & Precise
- Automation-ready

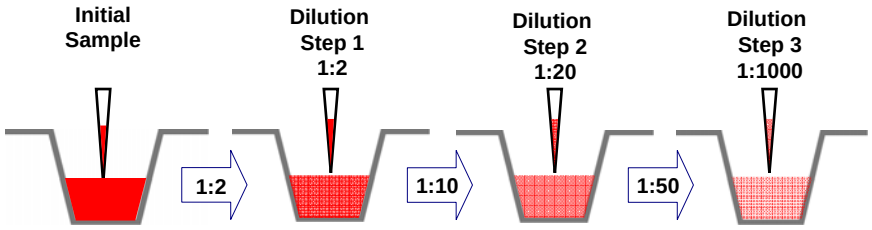
- *and now*, MVS measures dilution accuracy

Dilution Application using MVS

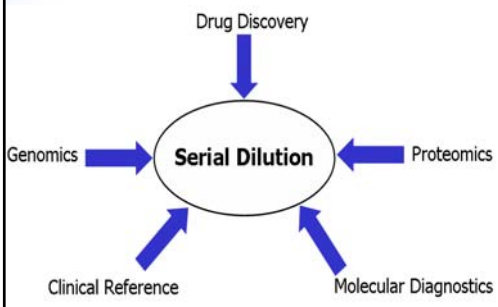


What are Dilutions?

- Controlled reduction in concentration of an analyte of interest
- Critically important process in most laboratories
- Accuracy is imperative at each step
- A common dilution protocol involves multiple serial dilution steps over a defined range



Applications for Serial Dilutions



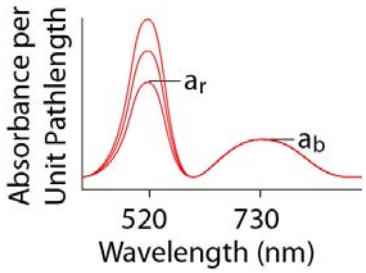
- Primary screening/compound management
- Secondary screening/ADME-Tox
- Dose response
- Viral loading
- HIV testing
- Other clinical applications
- Bacteria isolation
- And many others…

- The accuracy of many of these experiments is dependent upon knowing the ***final concentration*** of some analyte(s).

Dilution Testing with the MVS:
Review of MVS Components

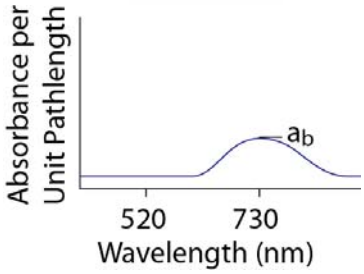
MVS Sample Solutions

- Contain 2 dyes, red and blue
- Distinct absorbance maxima (520 & 730 nm)
- Different concentrations of red dye for different volume ranges
- Blue dye at the same concentration for all ranges
- Stable and traceable to national standards

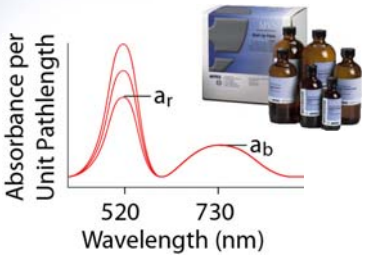


MVS Diluent

- Contains blue dye only
- Absorbance maximum at 730 nm
- Concentration of blue dye same as in sample solutions
- Used to fill wells to working volume
- Stable and traceable to national standards



Diluting MVS Sample Solutions with Diluent



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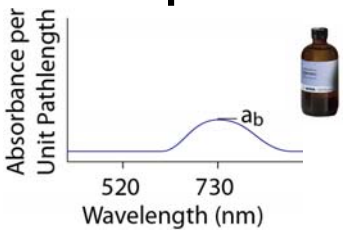

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- Variable Red Dye concentration
- **Uniform Blue Dye concentration**
  - *any* dilution made results in no change to the blue dye concentration

MVS Dilution Foundation: Beer's Law

- MVS Dilution calculation uses a foundation based on the Beer-Lambert Law:

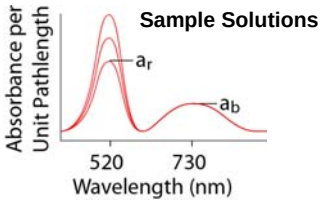
$$A_\lambda = \left(\varepsilon_\lambda C\right)\, l = a_\lambda \cdot l$$

- a_λ is the absorbance per centimeter of a solution with *known* dye concentration

Absorbance per Pathlength for MVS Dyes

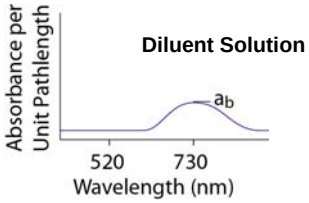
- The absorbance per pathlength for Red dye, which is in Sample Solution only:

$$a_r = \varepsilon_{520} \cdot C_r$$



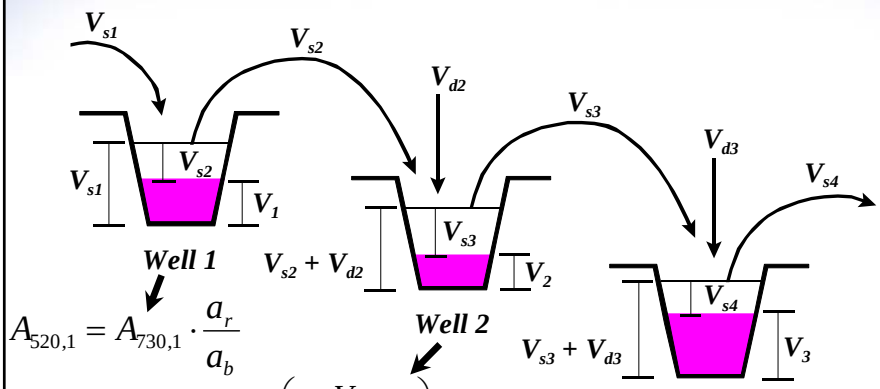
- The absorbance per pathlength for Blue dye, which is the same for Sample and Diluent Solutions:

$$a_b = \varepsilon_{730} \cdot C_b$$



MVS Dilution Theory

(JALA, October 2007, vol 12, pp 260-266)



$$A_{520,1} = A_{730,1} \cdot \frac{a_r}{a_b}$$

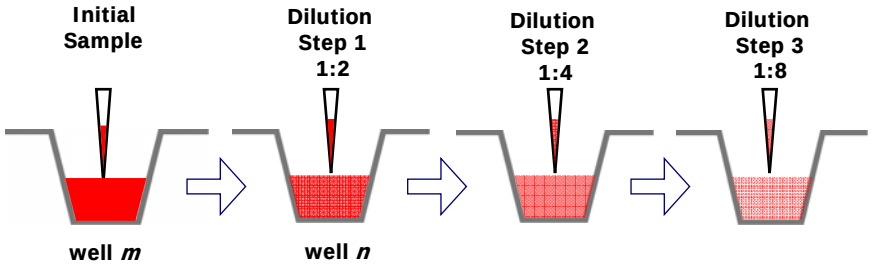
$$A_{520,2} = A_{730,2} \cdot \frac{a_r}{a_b} \cdot \left(\frac{V_{s2}}{V_{s2} + V_{d2}} \right)$$

$$A_{520,3} = A_{730,3} \cdot \frac{a_r}{a_b} \cdot \left(\frac{V_{s2}}{V_{s2} + V_{d2}} \right) \cdot \left(\frac{V_{s3}}{V_{s3} + V_{d3}} \right)$$

Accuracy of Dilution Steps

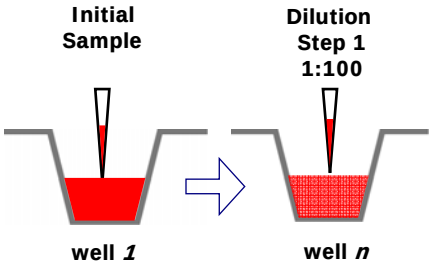
- When dilution steps result in measurable absorbance values for both red and blue dyes, then:

$$R_{mn} = \frac{A_{520,m}}{A_{520,n}} \cdot \frac{A_{730,n}}{A_{730,m}}$$



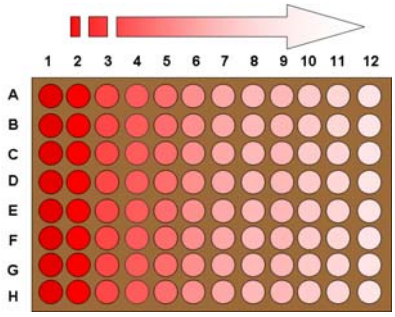
Large Dilution Steps

- When large dilution steps are performed, highly concentrated red dyes are used.
- Plate reader cannot measure the absorbance in well 1.
- Absorbance per pathlength values known for red & blue dyes



$$R_{1n} = \frac{a_r}{a_b} \cdot \frac{A_{730,n}}{A_{520,n}}$$

MVS Dilution Measurement
Example 1: An 11-Step Serial 1:2 Dilution



- Starting with neat solution in column 1, serial 1:2 dilutions will be made across a microtiter plate, to a final dilution of 1:2048

Conducting a 1:2 Dilution

- Column 2: 100 µL Sample + 100 µL Diluent = 200 µL Total
- Mix
- Aspirate 100 µL from the 200-µL mixture
- Dispense 100 µL into next column
- In column 2, red dye is now ½ as concentrated
- Repeat…in column 3, the dye is now ¼ as concentrated

- After multiple steps, the red dye becomes too dilute to measure……so the process is repeated with a different (more concentrated) starting red dye solution

Dilution Range for MVS Sample Solutions

Sample Solution	Red dye absorbance per pathlength (cm^{-1}) $(a_r = \varepsilon_{520} \cdot C)$	Dilution Range
Range A	3.75	**1 – 1:4**
Range B	15	**1:4 – 1:20**
Range C	75	**1:20 – 1:100**
Range D	185	**1:100 – 1:400**
Range E	740	**1:400 – 1:2000**

- To cover all 11 steps of a 1:2 serial dilution protocol, ***all*** range solutions will be used

Parameters of 1:2 Serial Dilution Example

- 96-well plate
- 8-channel liquid handler, dispensing left to right
- 1:2 dilution across plate, 11 steps
- Starting with 200 µL in first column (with *neat* reagent, *i.e.*, Range A, B, C…)
- Transfer volume = 100 µL
- Columns 2-12 contain 100 µL Diluent

Plate 1 (Range A): Set up

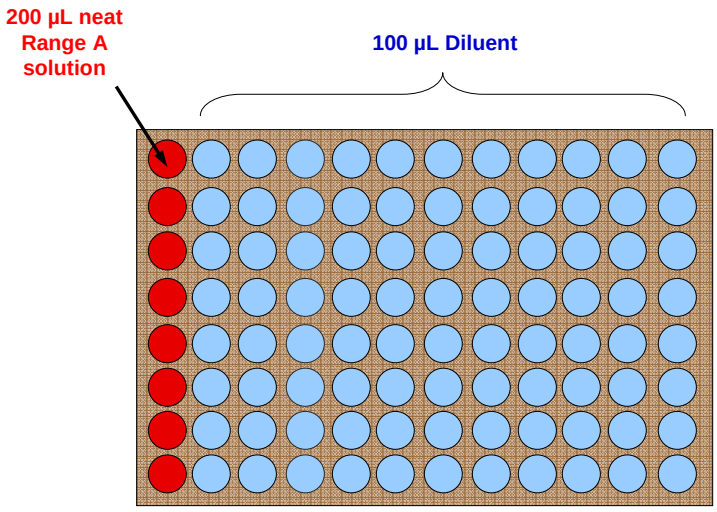


Plate 1 (Range A): First Step

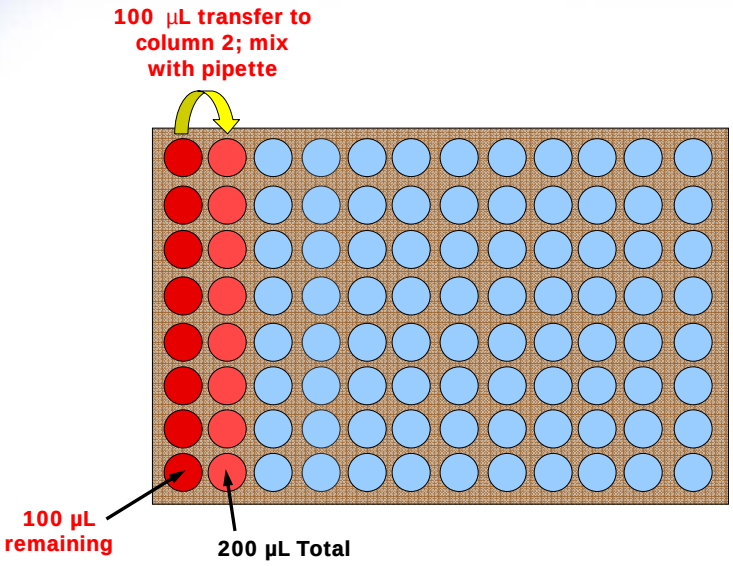


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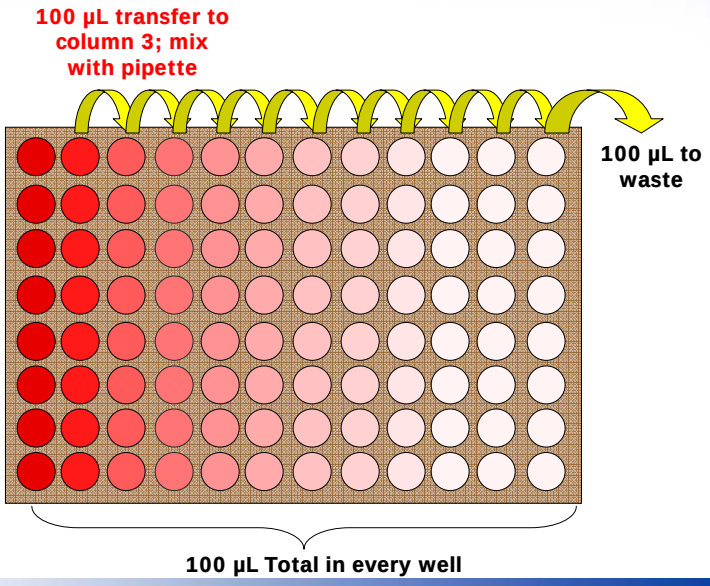
Plate 1 (Range A): Remaining Steps



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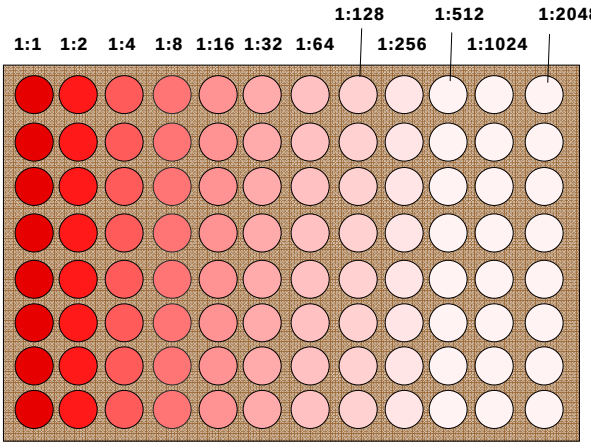
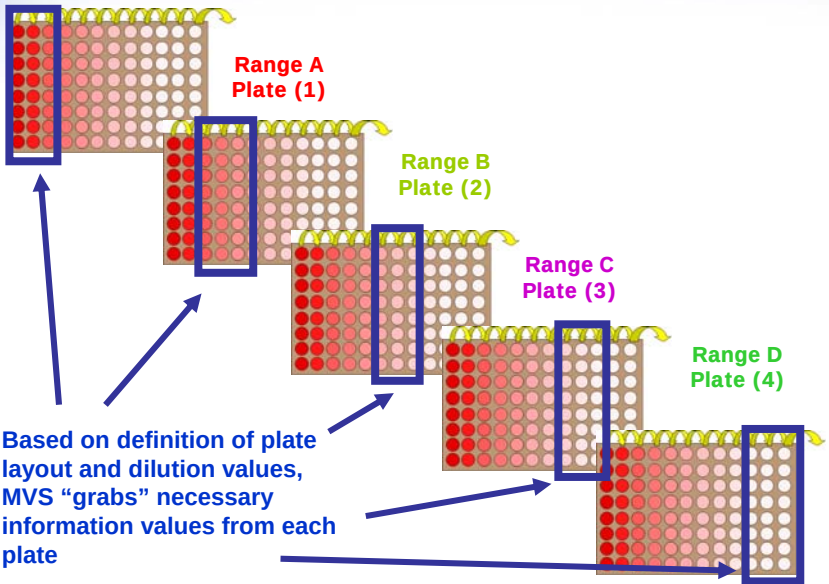
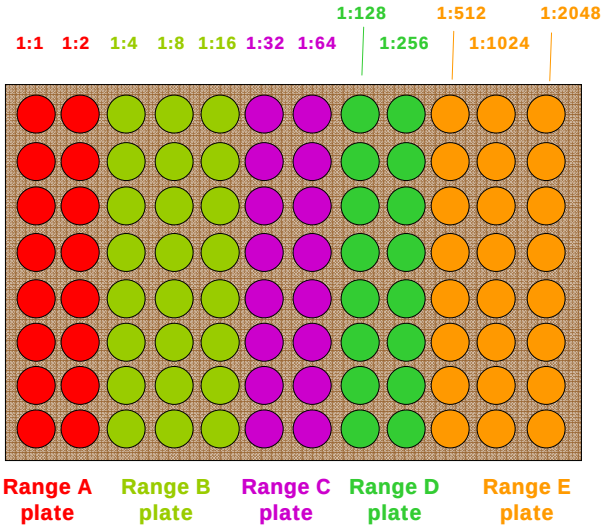
Dilution IDs for 11-step 1:2 Serial Dilution



Repeat Serial 1:2 Dilution Steps for Each Range Solution – 5 plates required



Dilution IDs for Each Range Solution: 5 plates required



Other Step Dilution Examples

- 1:2 –
  - 150 uL into 150 uL (300 uL total), remove 150 uL
  - 50 uL into 50 uL (100 uL total), remove 50 uL
- 1:3 –
  - 50 uL into 100 uL (150 uL total), remove 50 uL
  - 100 uL into 200 uL (300 uL total), remove 100 uL
- 1:5 –
  - 20 uL into 80 uL (100 uL total), remove 20 uL
  - 40 uL into 160 uL (200 uL total), remove 40 uL
- 1:10 –
  - 10 uL into 90 uL (100 uL total), remove 10 uL
  - 30 uL into 270 uL (300 uL total), remove 30 uL

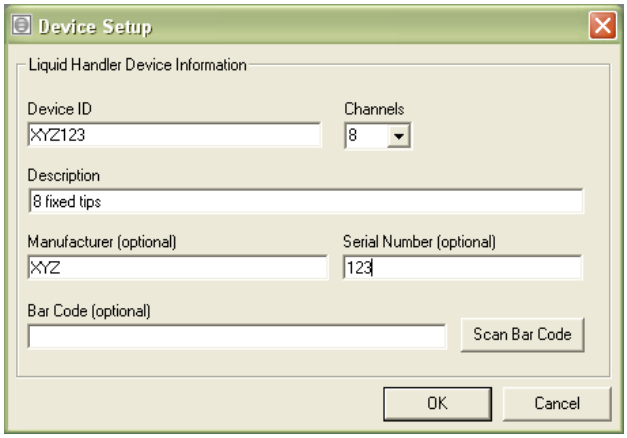
Critical Influences on Dilution Accuracy

- **Accurate transfer** of volume at each step (!!!!)
- **Mixing** (!!!!!)
    - Before transferring, if each well **is not optimally mixed**, **errors will propagate** with each successive step, compounding the error.
- Proper methodology or system to measure dilution steps

Dilution Guide: MVS Data Manager Software

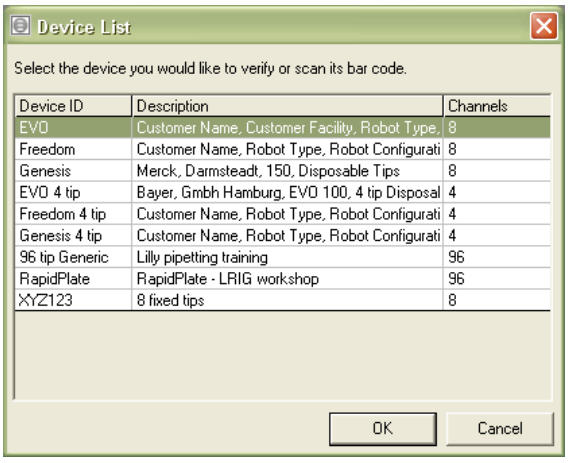
- MVS Data Manager software controls the volume verification and dilution testing processes
- Immediate output reports with pass/fail results
- Manual or auto-exporting (HTML, XML)
- Data bases can be networked
- 21 CFR Part 11 compliance ready
- Automation compatible for in-process volume checks

Defining the Liquid Handler



- 8 or 12 channels
- Use general or specific descriptors

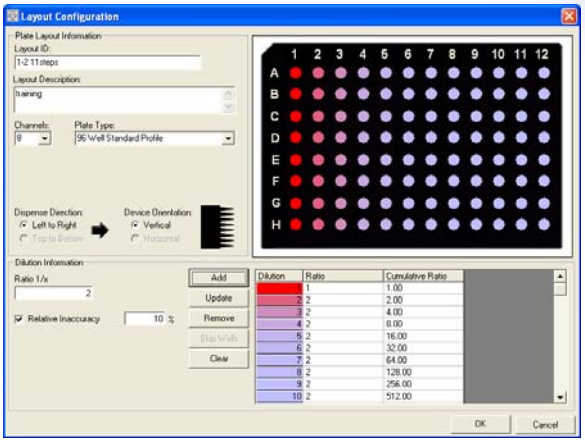
Defining the Liquid Handler



- Liquid handler IDs are stored for repetitive testing

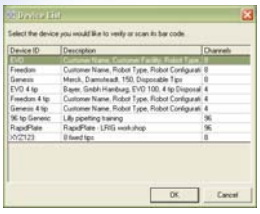
Plate Layouts Define Dilution Protocol

- Define the test parameters (plate format, target dilutions, no. of channels & reps, tolerances)
- Each dilution value may have different tolerance specifications for accuracy and precision



- Plate layout templates are stored for repetitive testing

MVS Software Guides User During Verification

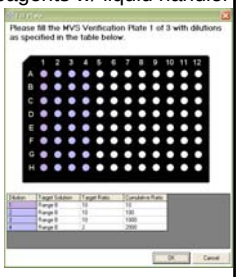


Select device to test

Select plate layout and plate type

Scan all required materials

Dispense reagents w/ liquid handler



Shake plate for uniform meniscus

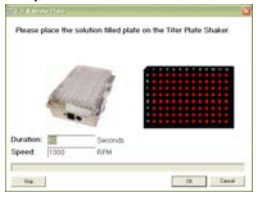
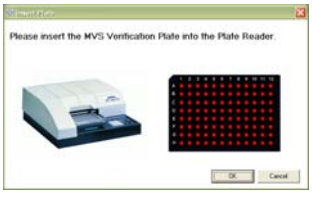
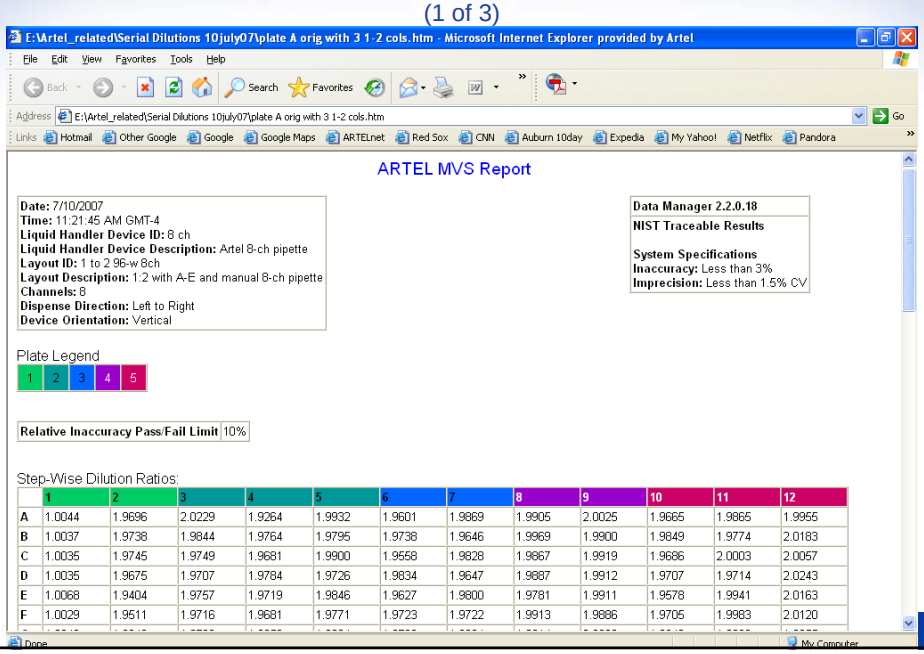


Plate Reader measurements



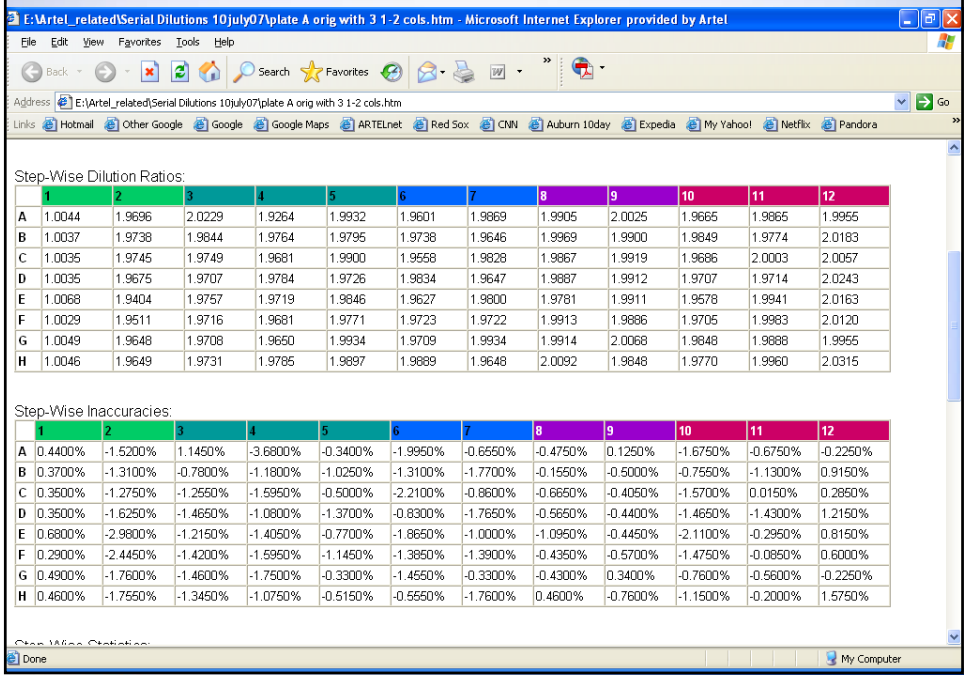
After Measuring 5 plates…MVS Output Report

(1 of 3)



	1	2	3	4	5	6	7	8	9	10	11	12
A	1.0044	1.9696	2.0229	1.9264	1.9932	1.9601	1.9869	1.9905	2.0025	1.9665	1.9865	1.9955
B	1.0037	1.9738	1.9844	1.9764	1.9795	1.9738	1.9646	1.9969	1.9900	1.9849	1.9774	2.0183
C	1.0035	1.9745	1.9749	1.9681	1.9900	1.9558	1.9828	1.9867	1.9919	1.9686	2.0003	2.0057
D	1.0035	1.9675	1.9707	1.9784	1.9726	1.9834	1.9647	1.9887	1.9912	1.9707	1.9714	2.0243
E	1.0068	1.9404	1.9757	1.9719	1.9846	1.9627	1.9800	1.9781	1.9911	1.9578	1.9941	2.0163
F	1.0029	1.9511	1.9716	1.9681	1.9771	1.9723	1.9722	1.9913	1.9886	1.9705	1.9983	2.0120

(2 of 3)

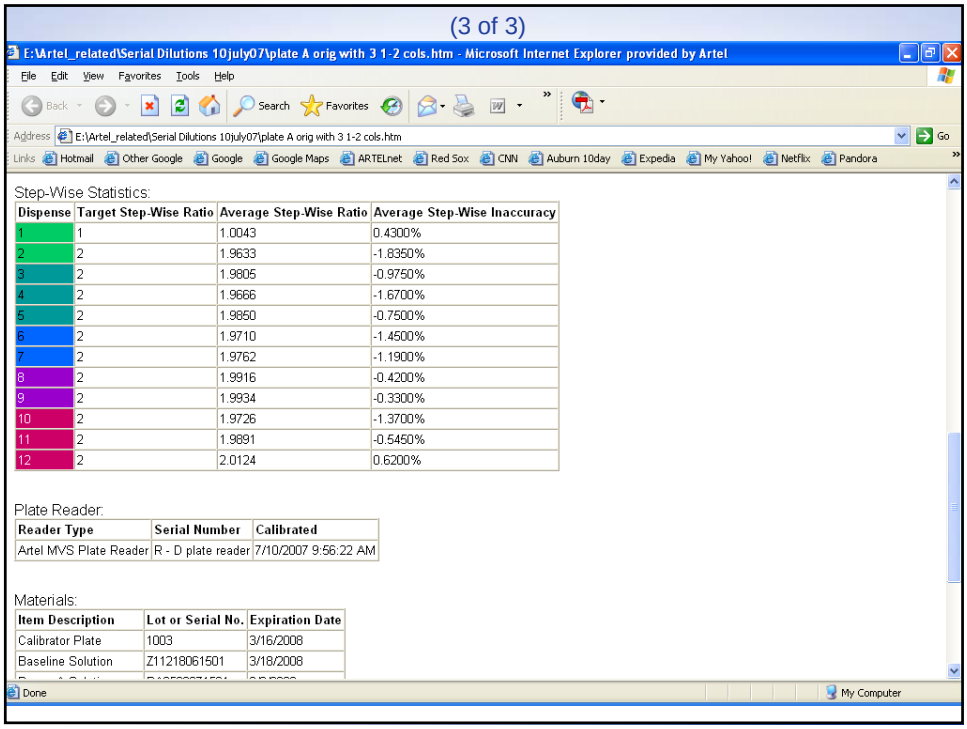


	1	2	3	4	5	6	7	8	9	10	11	12
A	1.0044	1.9696	2.0229	1.9264	1.9932	1.9601	1.9869	1.9905	2.0025	1.9665	1.9865	1.9955
B	1.0037	1.9738	1.9844	1.9764	1.9795	1.9738	1.9646	1.9969	1.9900	1.9849	1.9774	2.0183
C	1.0035	1.9745	1.9749	1.9681	1.9900	1.9558	1.9828	1.9867	1.9919	1.9686	2.0003	2.0057
D	1.0035	1.9675	1.9707	1.9784	1.9726	1.9834	1.9647	1.9887	1.9912	1.9707	1.9714	2.0243
E	1.0068	1.9404	1.9757	1.9719	1.9846	1.9627	1.9800	1.9781	1.9911	1.9578	1.9941	2.0163
F	1.0029	1.9511	1.9716	1.9681	1.9771	1.9723	1.9722	1.9913	1.9886	1.9705	1.9983	2.0120
G	1.0049	1.9648	1.9708	1.9650	1.9934	1.9709	1.9934	1.9914	2.0068	1.9848	1.9888	1.9955
H	1.0046	1.9649	1.9731	1.9785	1.9897	1.9889	1.9648	2.0092	1.9848	1.9770	1.9960	2.0315

Step-Wise Inaccuracies:

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.4400%	-1.5200%	1.1450%	-3.6800%	-0.3400%	-1.9950%	-0.6550%	-0.4750%	0.1250%	-1.6750%	-0.6750%	-0.2250%
B	0.3700%	-1.3100%	-0.7800%	-1.1800%	-1.0250%	-1.3100%	-1.7700%	-0.1550%	-0.5000%	-0.7550%	-1.1300%	0.9150%
C	0.3500%	-1.2750%	-1.2550%	-1.5950%	-0.5000%	-2.2100%	-0.8600%	-0.6650%	-0.4050%	-1.5700%	0.0150%	0.2850%
D	0.3500%	-1.6250%	-1.4650%	-1.0800%	-1.3700%	-0.8300%	-1.7650%	-0.5650%	-0.4400%	-1.4650%	-1.4300%	1.2150%
E	0.6800%	-2.9800%	-1.2150%	-1.4050%	-0.7700%	-1.8650%	-1.0000%	-1.0950%	-0.4450%	-2.1100%	-0.2950%	0.8150%
F	0.2900%	-2.4450%	-1.4200%	-1.5950%	-1.1450%	-1.3850%	-1.3900%	-0.4350%	-0.5700%	-1.4750%	-0.0850%	0.6000%
G	0.4900%	-1.7600%	-1.4600%	-1.7500%	-0.3300%	-1.4550%	-0.3300%	-0.4300%	0.3400%	-0.7600%	-0.5600%	-0.2250%
H	0.4600%	-1.7550%	-1.3450%	-1.0750%	-0.5150%	-0.5550%	-1.7600%	0.4600%	-0.7600%	-1.1500%	-0.2000%	1.5750%

(3 of 3)



Conclusion

- MVS aides in optimizing performance of automated liquid delivery systems
- Fast, easy, accurate and precise methodology for productivity, repeatability and data integrity
- Standardized system and reagents allows for dilution testing up to a dilution of 1:2000
- The new MVS **serial dilution capability** supports critical liquid handling processes in life science laboratories

Thank You

Please visit us in Booth **#551** for a demonstration of our products.