Cell Counting Validation

NIST-FDA Cell Counting Workshop
Gaithersburg, MD

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Outline

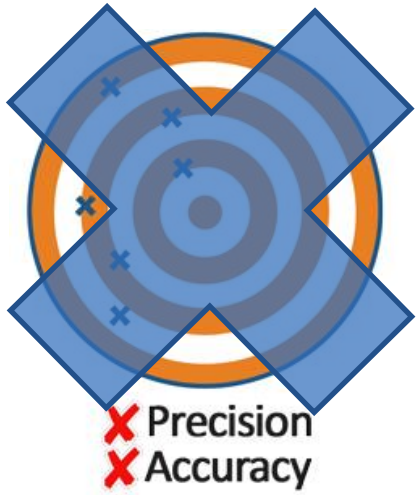
- Background
- Development (Instrument, Assay)
- Validation
- Summary

BACKGROUND

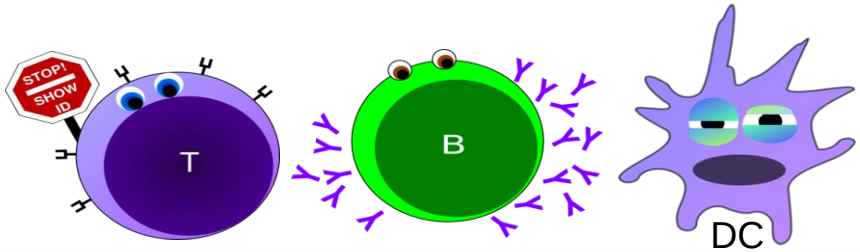
A Cell Counting Problem

Ideal cell count

Typical cell count due to lack of cell reference standard



Unacceptable cell count



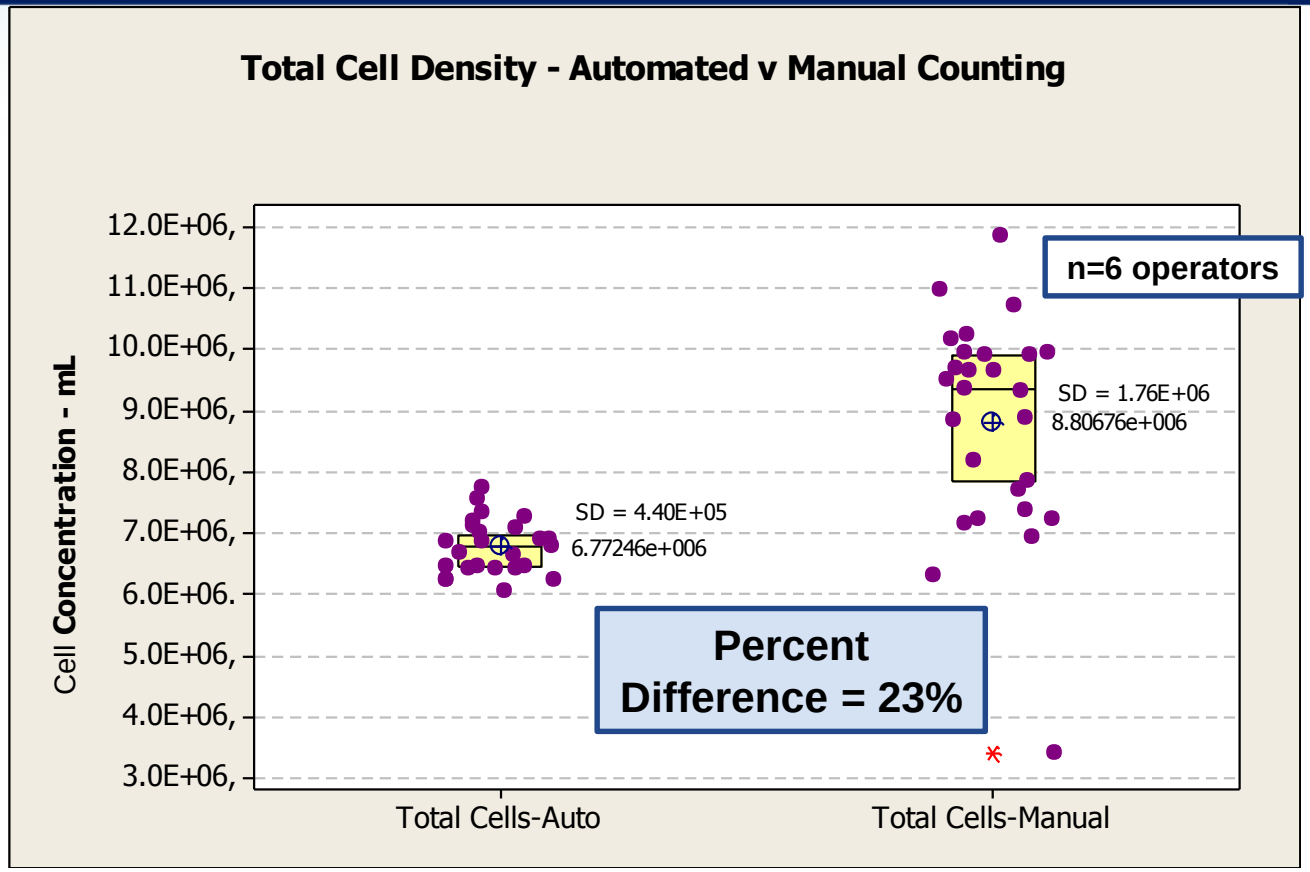
http://www.clker.com/

http://wonderwalsh.net/unit-1---science-and-measurement.html

Counting Methods Evaluated by Celgene for PDACs (2010)

	Hemacytometer	Nuclear staining	Image-based	Coulter method	Flow cytometry
Cell counting	✓	✓	✓	✓	✓
Automated sampling	X	X	✓	✓	✓
Automated analysis	X	✓	✓	✓	✓
Instrument differences	✓	✓	✓	✓	✓
Other parameters					
Viability	✓	✓	✓	X	✓
Cell size	X	X	✓	✓	X
Identity	X	X	X	X	✓

Manual vs. Automated Image-Based Cell Counting



- Main factors that affected manual cell counting:
  1. Operator
  2. Sample preparation
- Automated cell counting increased precision
- Difficult to gage accuracy

Factors to Consider for Cell Counting

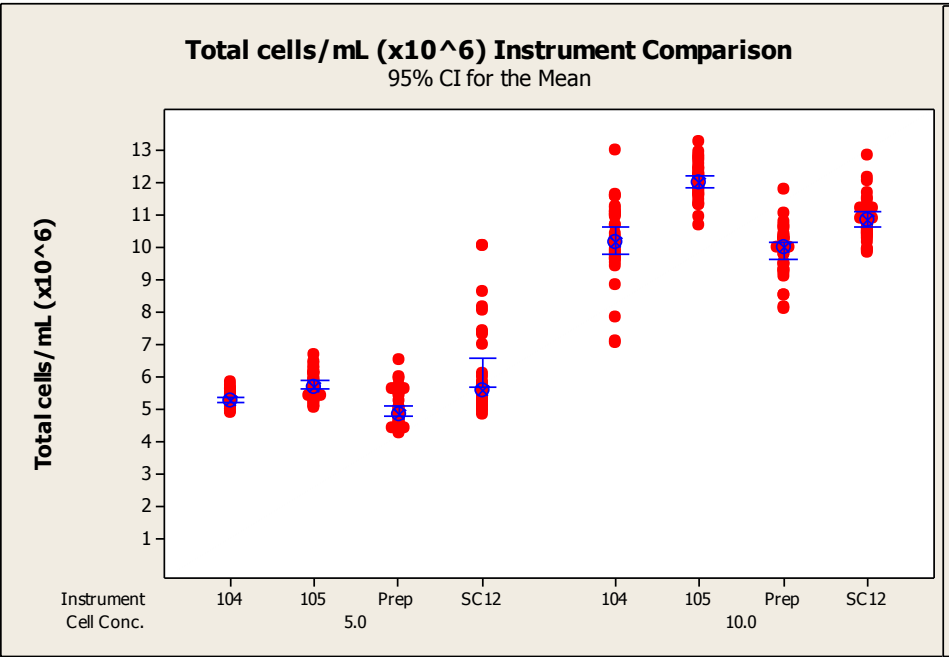
Instrument	Assay
▪ Instrument-to-instrument differences ▪ Controls: beads or cells? ▪ Objective data analysis/gating	▪ Cell concentration ▪ Diluent, dilution factor ▪ Cell matrix ▪ Cell type ▪ Operators ▪ Controls: beads or cells? - difficult to gage accuracy

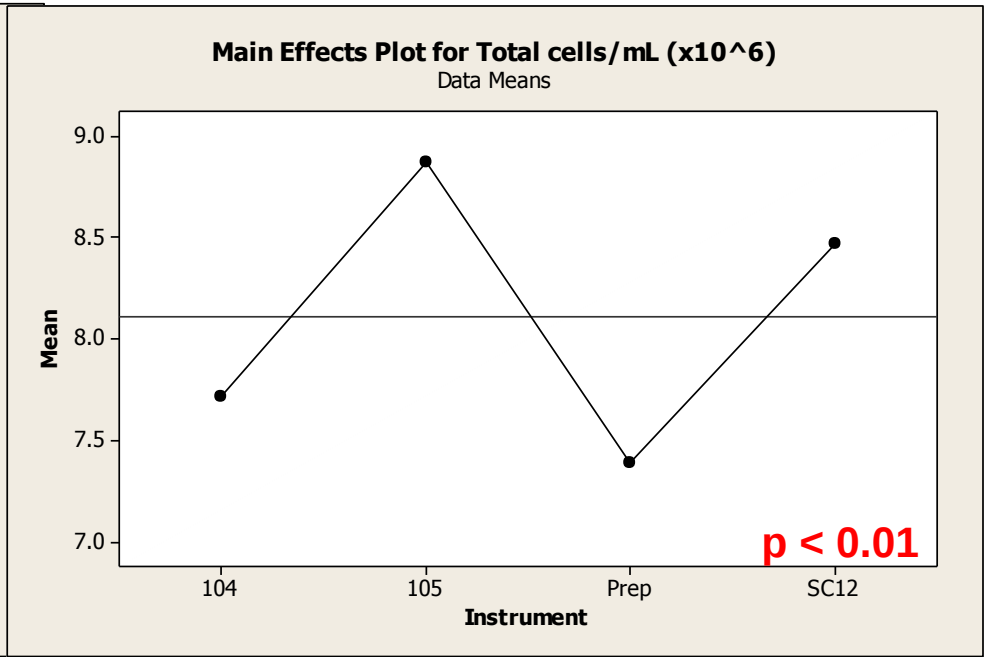
DEVELOPMENT

(Instrument)

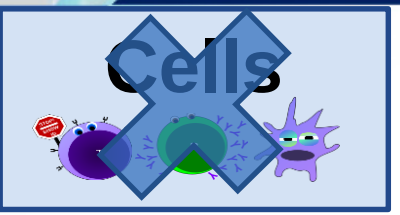
Instrument Differences

Instrument differences are evident





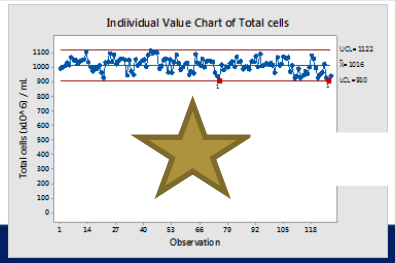
Controlling the Instrument



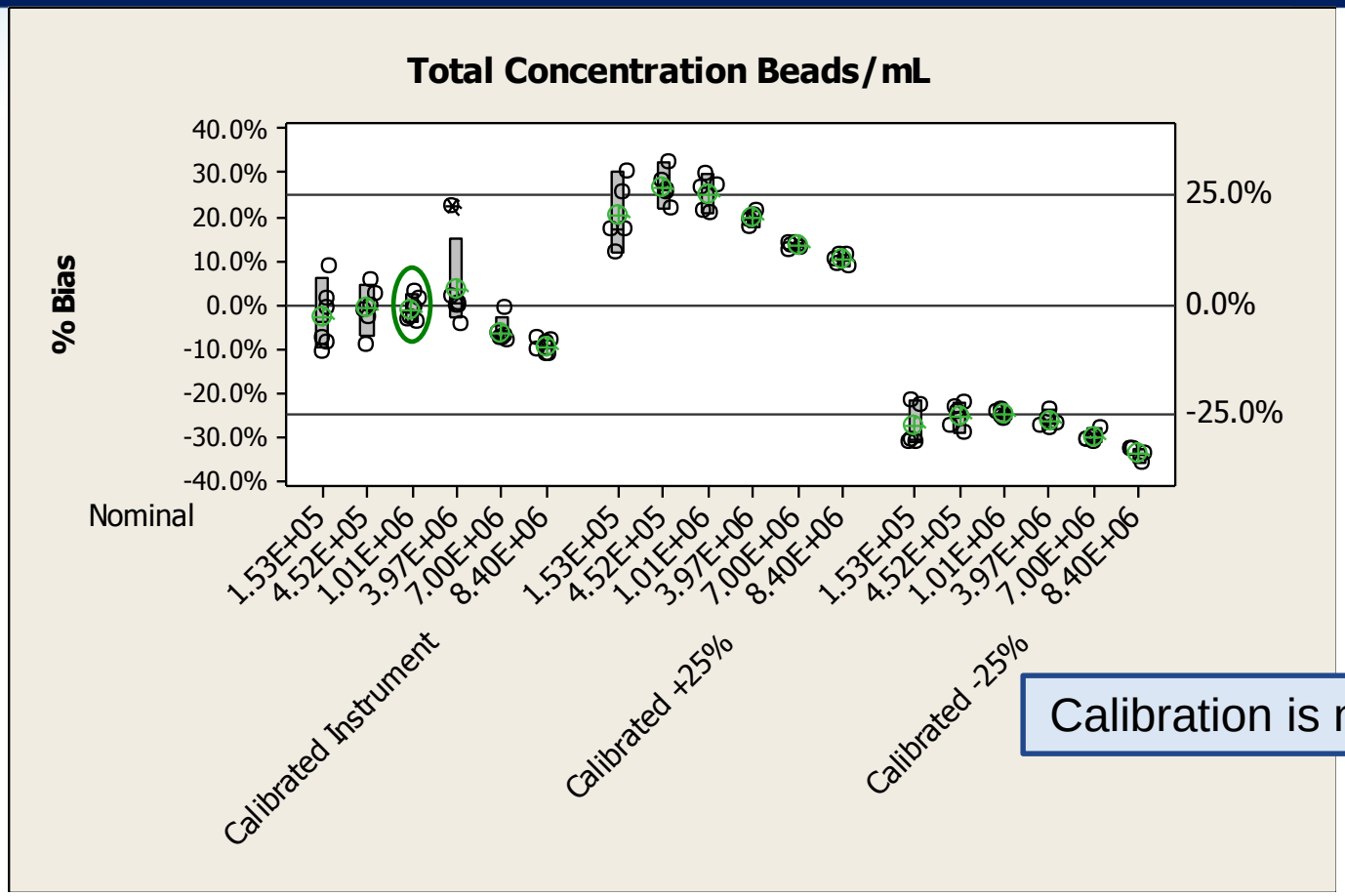
≠

Concentration beads

Advantages	Disadvantages
Wide range of concentrations (off-the-shelf and custom-made)	Beads ≠ cells
Ease of use (i.e. single use vials, no dilution)	Actual concentration may not always match with C of A
Can be used for instrument control/calibration	Possible vial to vial variability
Helps identify the concentration range of the instrument	Settling of beads
Typically precise results	



Instrument Calibration with Concentration Beads



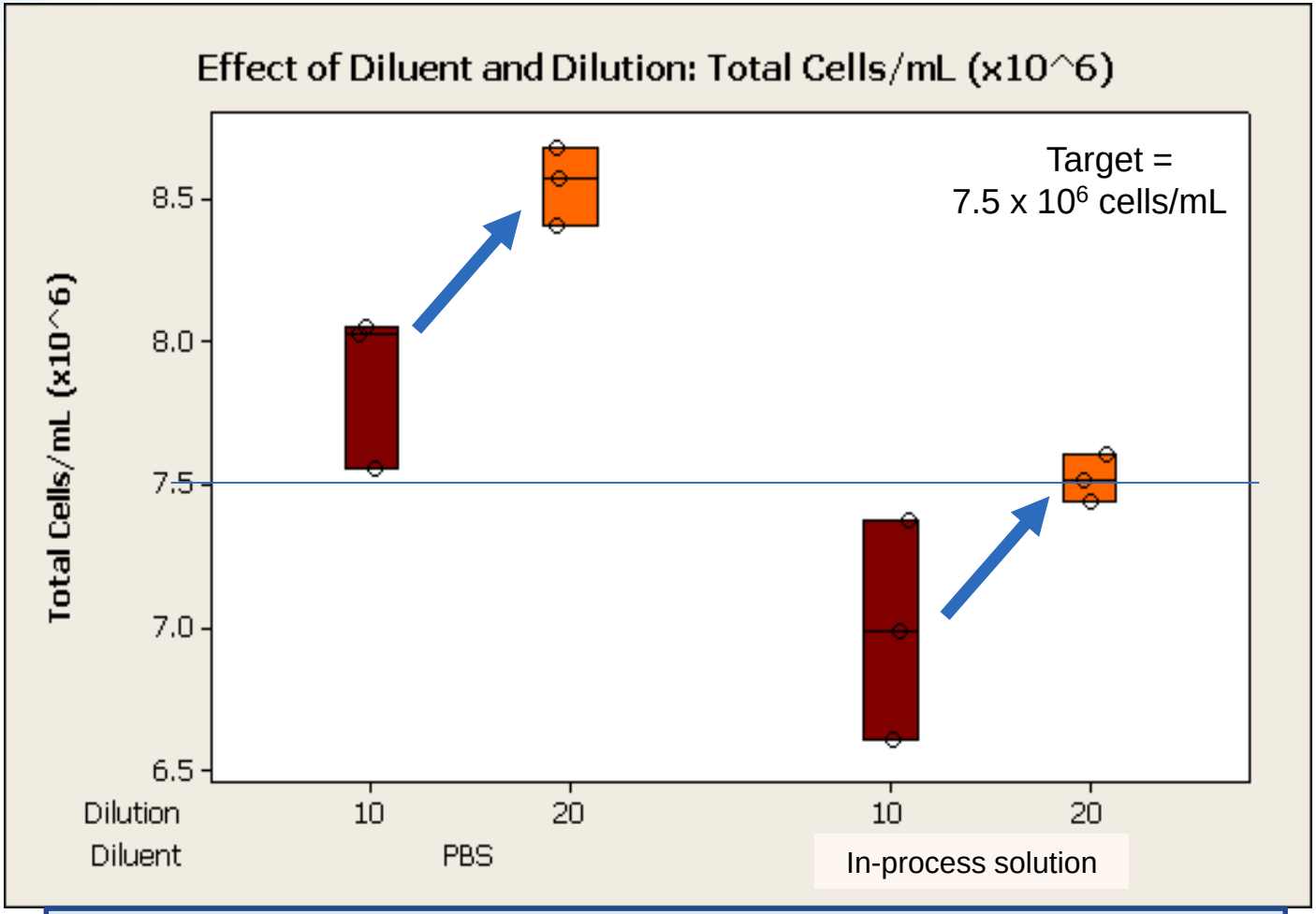
Calibration is necessary!

- Intentional skewing of calibration demonstrated how sound the calibration method was
- Implemented bead control to ensure instrument is working as expected
- Used one bead concentration to serve as a pseudo system suitability control

DEVELOPMENT

(Assay)

Diluents and Dilutions



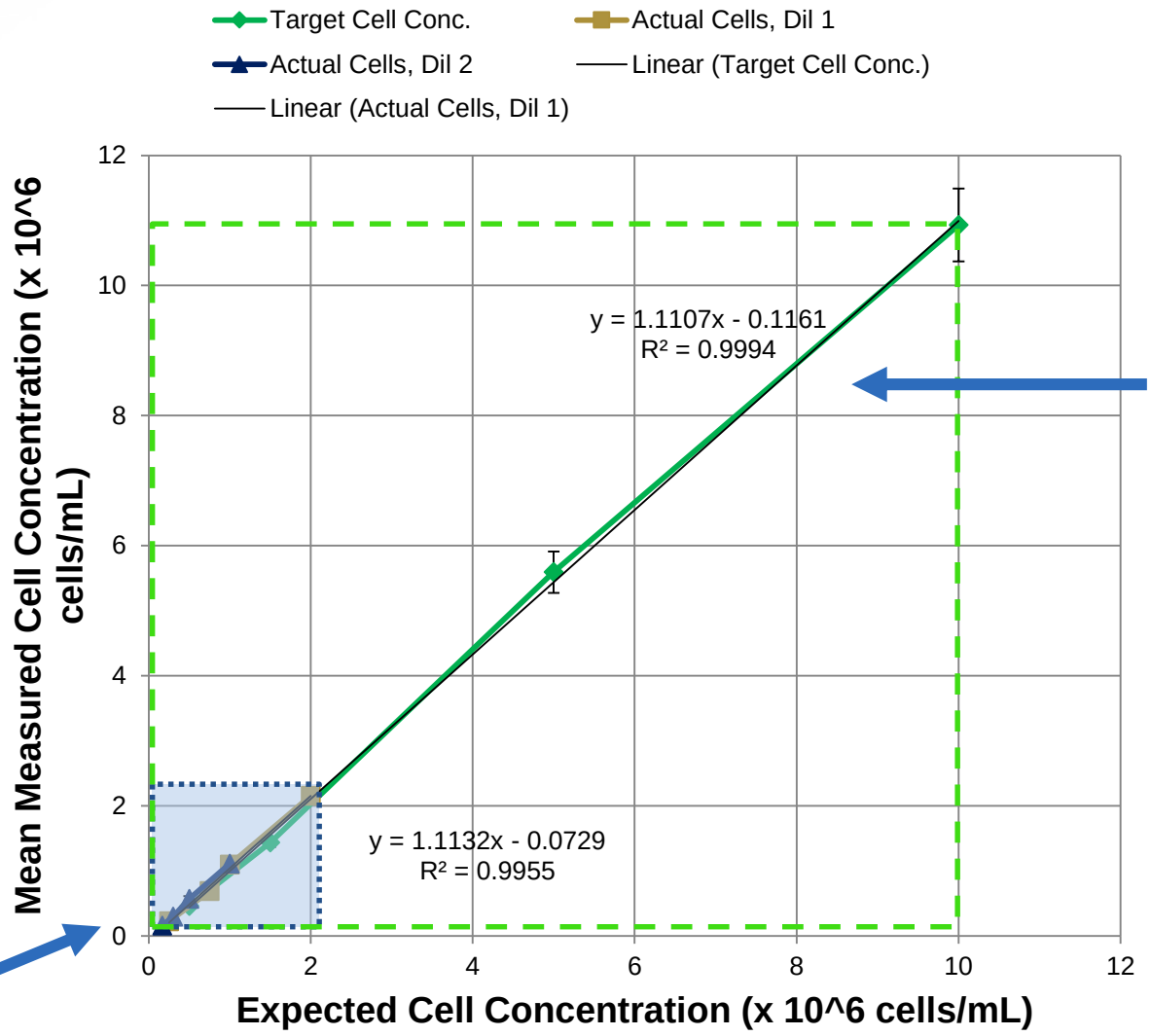
- Choice of diluents and dilution factor impact cell counting measurements
- Highlights that accuracy is unknown

Cell Matrix Interference

Cell matrix	Total events/100 images
PBS	1
Culture medium	4
Trypsin solution	4
Cryopreservation solution	24
In-process solution	67

- Cell matrix can interfere with cell counting
- Selected a common matrix to dilute samples in with the least number of events
- Identified best dilution in this matrix

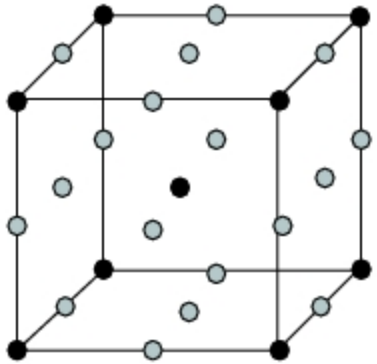
Instrument Range vs. Target Cell Range



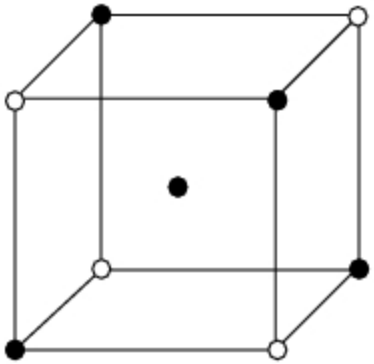
Target Cell Concentration (undiluted)

Actual Cell Concentration counted (diluted)

Statistical Design of Experiments (DOE)

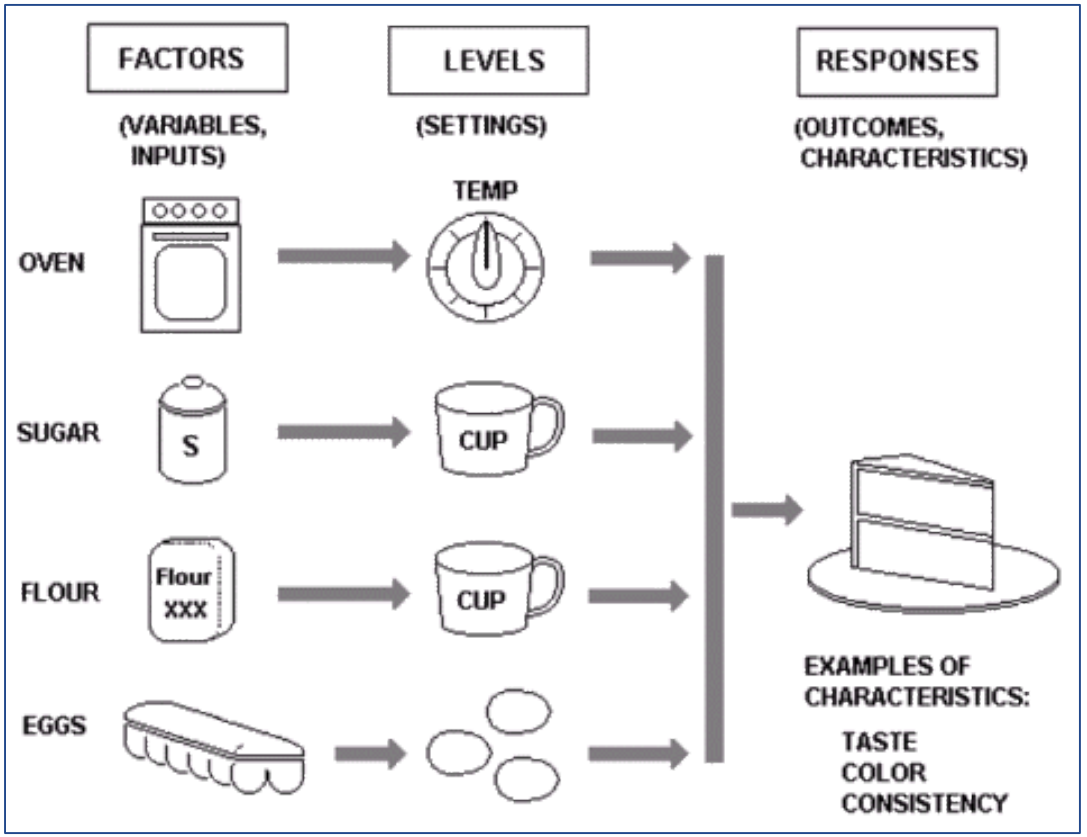


Full factorial



Fractional factorial

Recipe for DOE



https://www.moresteam.com/toolbox/pics/4081.gif

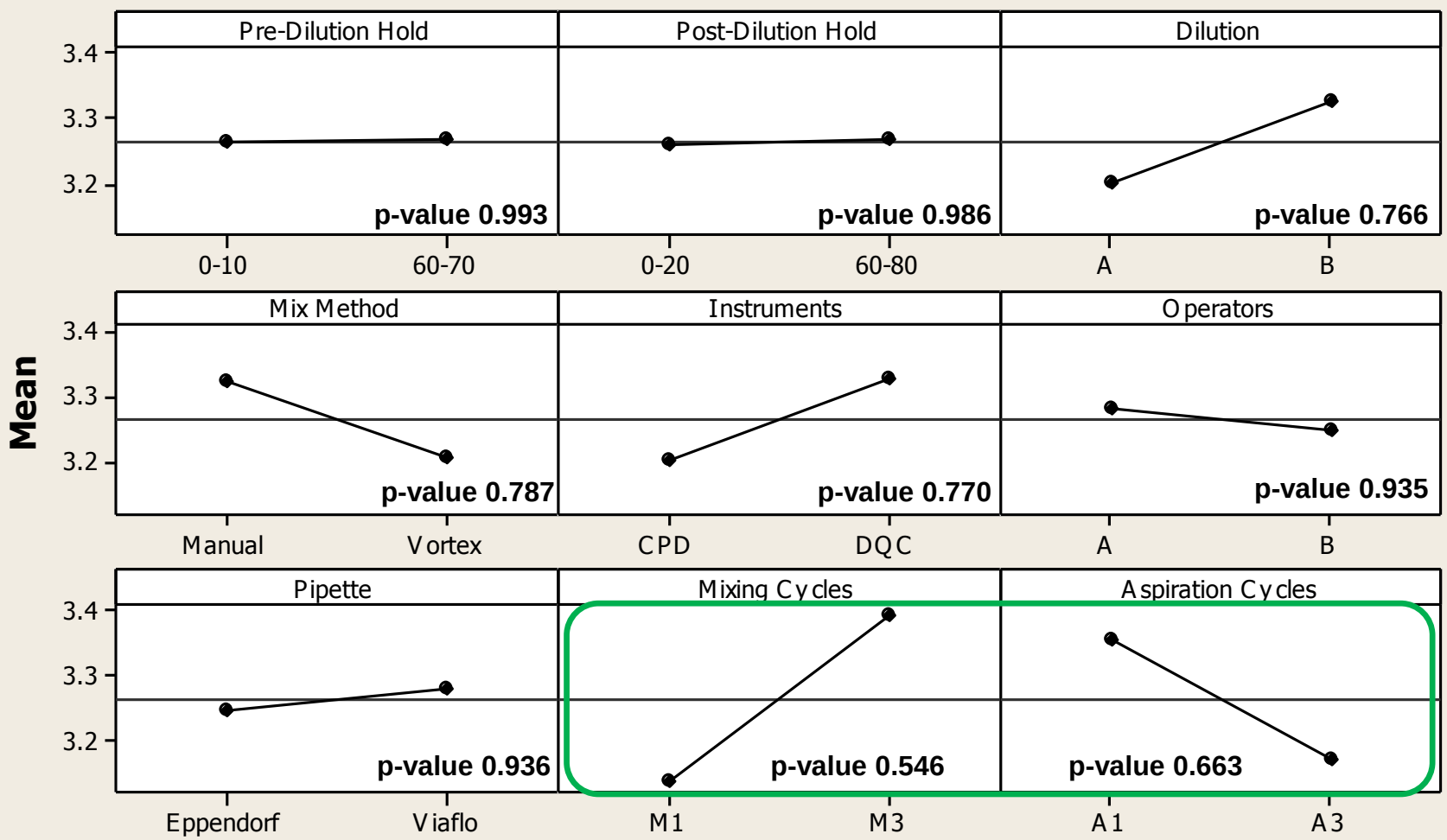
Pre-validation DOE

Plackett-Burman design:

a. Factors = 9
b. Assay runs = 24
c. Cell concentrations = 3

Factors	Level 1	Level 2
Pre-Dilution Hold Time	0-10	60-70
Post Dilution Hold Time	0-20	60-80
Dilution	2X	10X
Mix Method	Manual	Vortex
Instrument	1158185	1362331
Operator	A	B
Pipette	Manual	Electronic
Mixing Cycles	1	3
Aspiration Cycles	1	3

Pre-Validation Results



VALIDATION STRATEGY

Validation Parameters

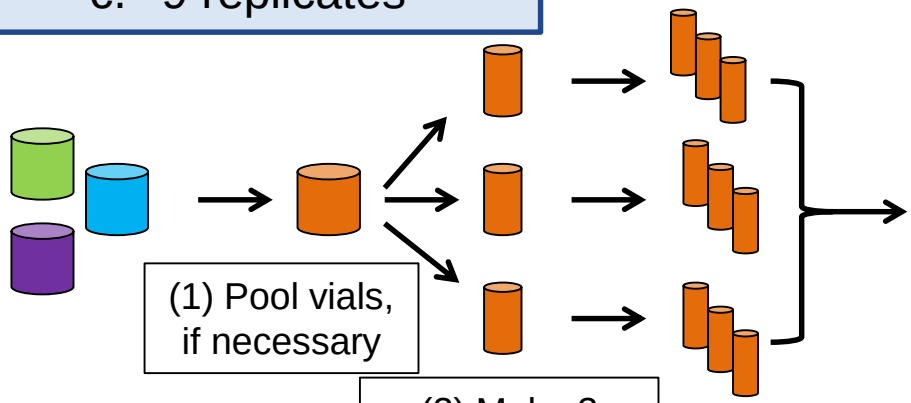
Parts of Measurement System:	Instrument (beads)	Assay (cells)
Accuracy	✓	X
Precision	✓	✓
Linearity	✓	✓
Range	✓	✓
Specificity	X	✓
Dilution Integrity	X	✓

Experimental Design

2 operators

Beads:
- a. 5 concentrations
- b. 5 replicates

Cells:
- a. 4 concentrations
- b. 2 dilution factors
- c. 9 replicates



(1) Pool vials, if necessary

(2) Make 3 dilutions from pool/sample vial

(3) Aliquot diluted sample into 3 cups

2 instruments:
- a. 3 replicate assays per instrument

N = 582 data points

http://www.beckman.com/particle/instruments/cell-sizing-and-processing/vi-cell-xr

Validation Results (Concentration Beads)

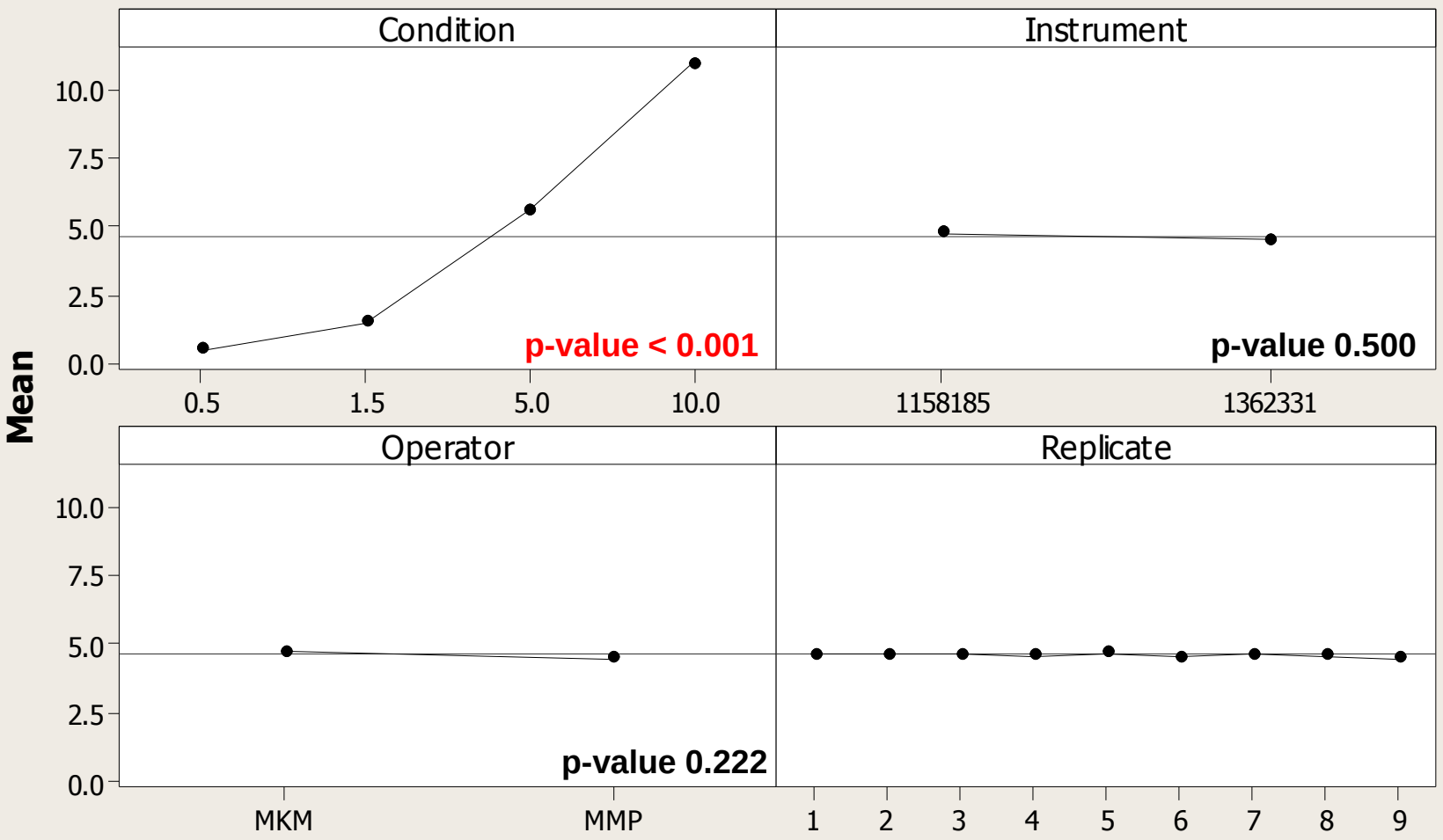
	Nominal Bead Conc.	Accuracy	Repeatability	Intermediate Precision	Linearity	Range
	(x 10^6 beads/mL)	Mean Bias (%)	CV	CV	R^2	(x 10^6 beads/mL)
Concentration Beads	**0.15**	-3.7	1.5 to 5.8%	1.2%	0.999	0.15 to 3.86
	0.52	-7.2	2.0 to 4.3%	1.0%		
	1.00	-1.0	4.9 to 7.2%	2.4%		
	2.02	-9.0	1.2 to 2.2%	1.2%		
	3.86	-4.8	3.3 to 5.9%	0.9%		

Validation Results (Cells)

	Conc.	Dil.	Repeatability	Intermediate Precision	Linearity	Range	Specificity	Dilution Integrity
	(x 10^6 cells/mL)		CV	CV	R^2	(x 10^6 cells/ mL)	Mean total cells/100 images	% Difference
Cells	**0.5**	1	2.6-8.2%	2.0%	0.999	**Actual:**	**Sample Matrix:**	3.5
	0.5	2	4.4-8.2%	2.3%				
	1.5	1	3.1-5.4%	2.6%		0.17 to 2.0	1.2	8.3
	1.5	2	3.5-6.5%	1.4%				
	5.0	1	3.8-5.2%	2.7%		**Target:**	**Assay Matrix:**	3.9
	5.0	2	2.8-8.7%	7.3%				
	10	1	2.1-3.9%	1.6%		0.50 to 10	0.9	3.3
	10	2	2.1-5.6%	6.9%				

Precision ≠ Accuracy!

Main Effects Plots



Summary

- Precise ***and*** accurate cell counting = necessary, but not truly achievable without a cell reference standard
- Instrument and assay controls will help to minimize variability in the cell counting method
- Use of DOE/statistics to understand factors that affect the cell counting method facilitates development and eventual validation

Acknowledgements:
C. Wiwi
Analytical Development
Quality Control