The Key to Successfully Automating Your NGS Workflow

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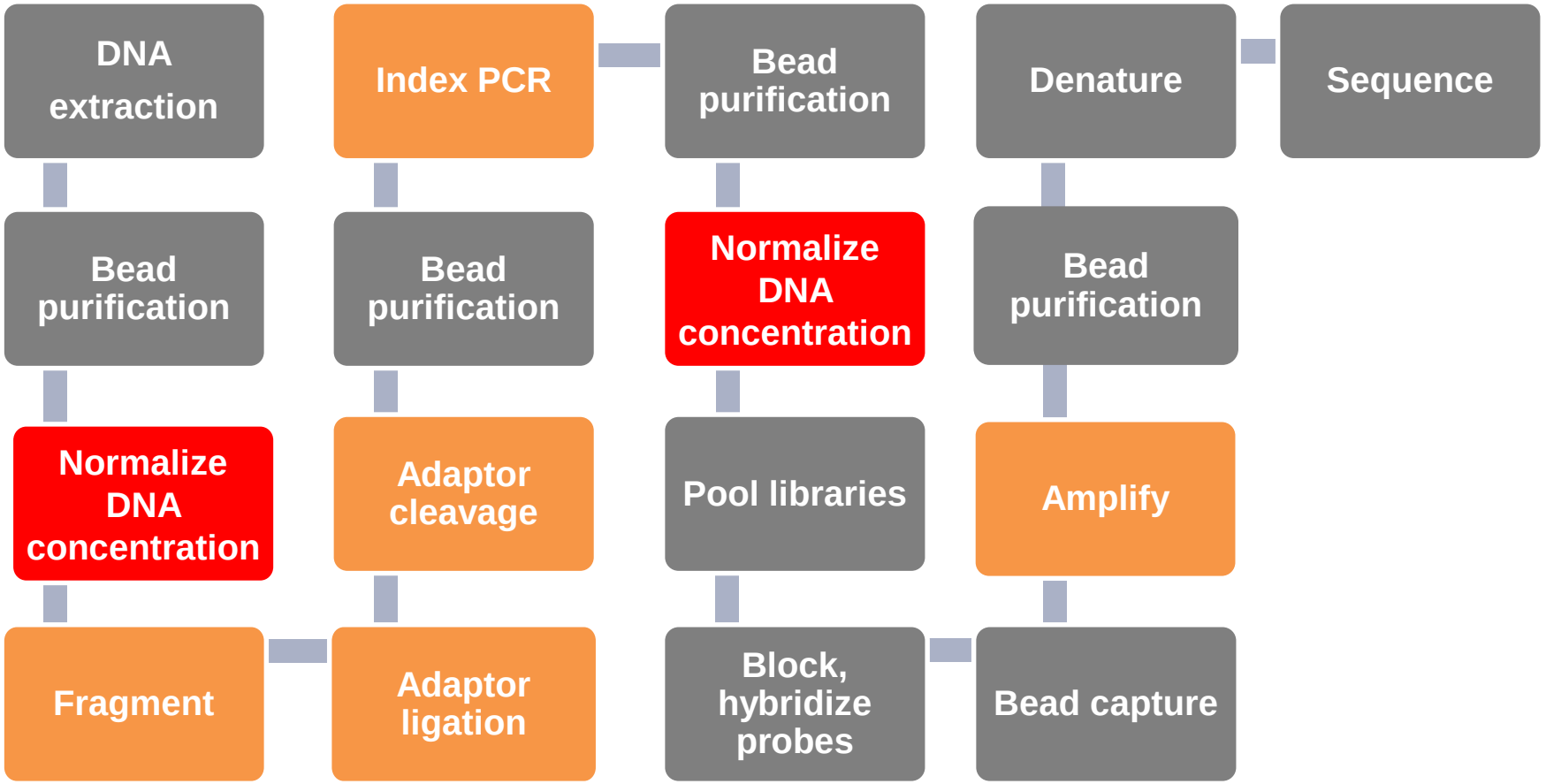
Agenda

- Review typical NGS workflow
  - Understand critical sources of assay variability
  - Discuss solutions to address the workflow challenges

Example Next Generation Sequencing Workflow



NGS Workflow QC Tools

Method	Instrument	QC Attribute
Spectrophotometry (260/280)	• NanoDrop • Take3	DNA concentration and purity
Fluorimetry	• Qubit	DNA concentration
Electrophoretic	• BioAnalyzer • TapeStation • Fragment Analyzer	DNA concentration and size distribution
Quantitative PCR (qPCR)	• NEBNext Library Quant Kit • Roche hgDNA Quantification/QC Assay	DNA concentration and quality

These are the typical NGS workflow QC tools, but they are ***reactive***.

By verifying performance or optimizing key steps in a ***proactive*** manner, the success rate of the QC steps can be improved.

Importance of DNA Concentration in NGS

Library Preparation > Cluster Generation

- Optimal amount of adapter-ligated fragments are required for cluster generation; narrow range (e.g., 6-10 pM for MiSeq platform)
- If multiple libraries are pooled, an equal amount of each library is needed for **even sequence coverage**

Determining DNA concentration is important, but that's only part of the solution. Dilution and normalization steps require liquid handling. If this is imprecise and inaccurate, then final concentrations will be not be correct!

Importance of DNA Concentration in NGS

Using the familiar dilution equation:

$$C_1V_1 = C_2V_2$$

If we assume the DNA quantitative technique is perfect (C_1), but either volume of DNA (V_1) or the diluent (V_2) are off, then C_2 will not be correct!

The liquid handling part of NGS steps are indeed a vital component to success!!

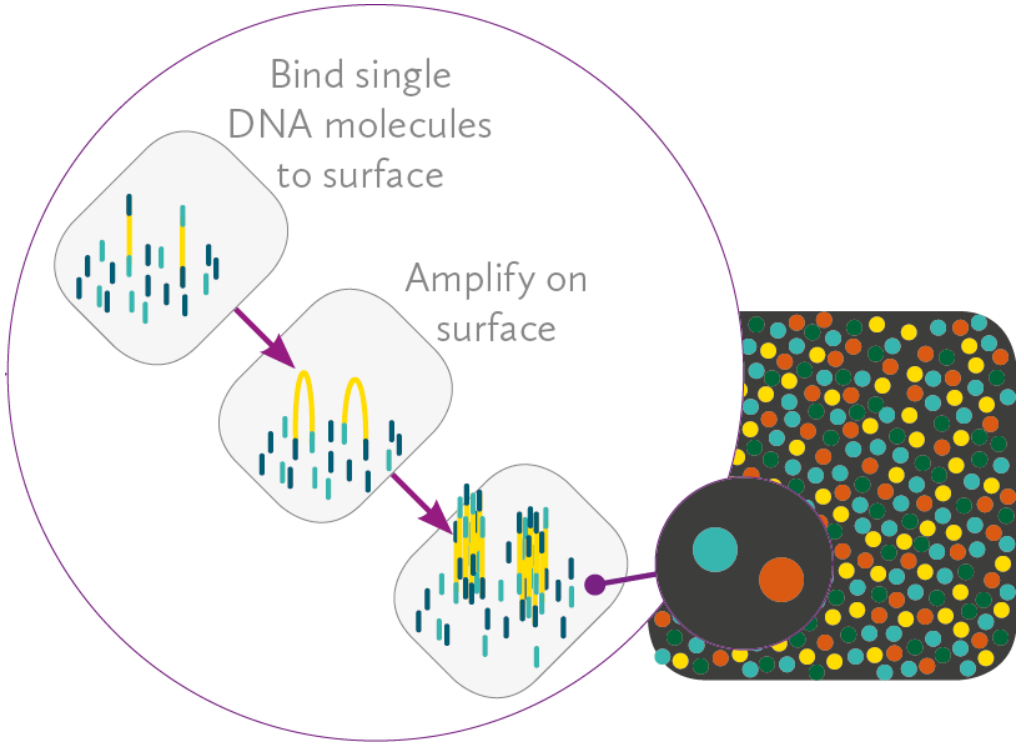
Effect of Concentration on Read Depth

High concentration = overrepresentation in flow cell: increases read depth but wastes capacity.
Low concentration = underrepresentation in flow cell: might result in poor read depth and unreliable data, which wastes capacity and precious sample, and increases repeat.

Amount of DNA on Flow Cell Affects Clonal Amplification

- The indexed libraries are combined to create one pool of DNA, representing multiple HLA loci of multiple samples. After quantification, the library is denatured and loaded into the MiSeq instrument. Clonal amplification (or bridge PCR) is used to generate clusters of identical DNA sequences.

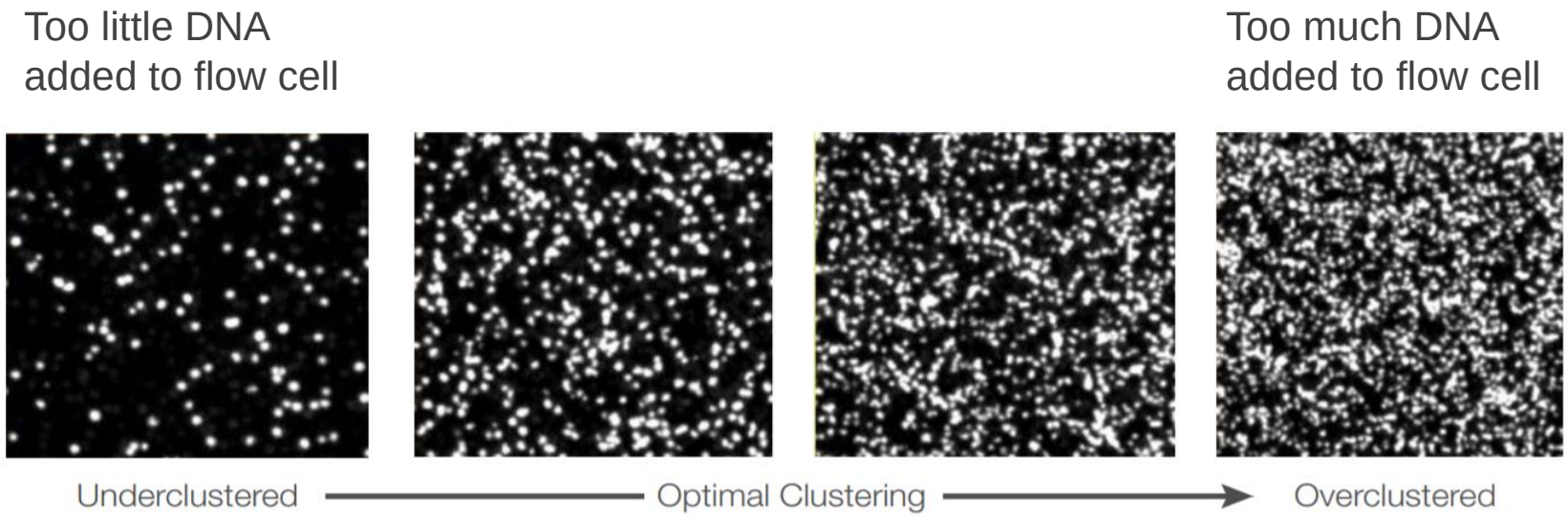
Different amounts of DNA that are pooled can affect the amplification efficiency, which ultimately affects base calling accuracy.



Source: *GenDx (website accessed 09/19/2019)*

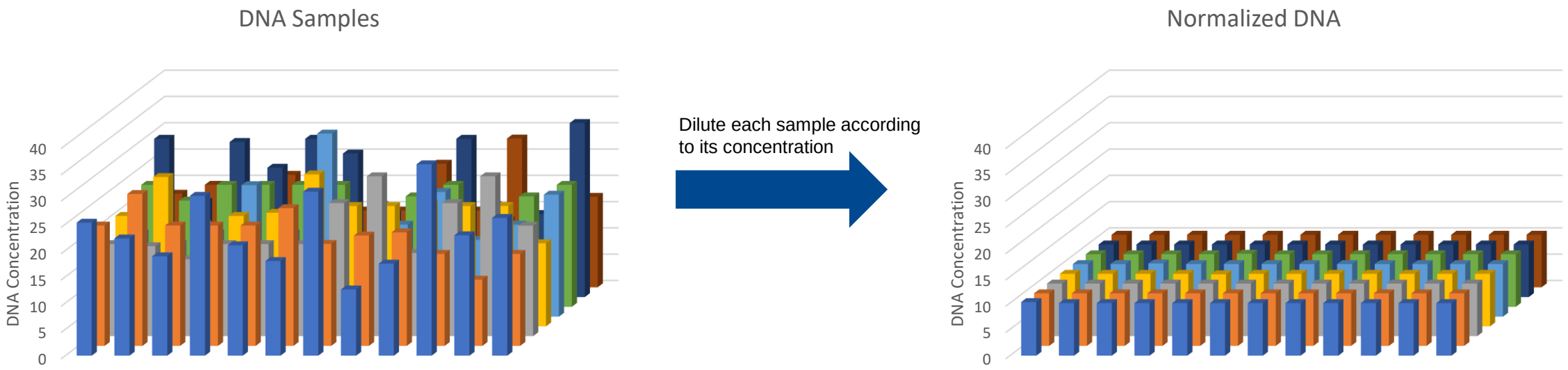
Amount of DNA on Flow Cell Affects Clonal Clustering

- Under-clustering results in lower data output; Over-clustering results in poor image resolution (focus failure), analysis problems (base calling ambiguity, lower Q score, poor base calling), poor template generation.



Source: *Optimizing Cluster Density on Illumina Sequencing Systems*, Illumina (2016)

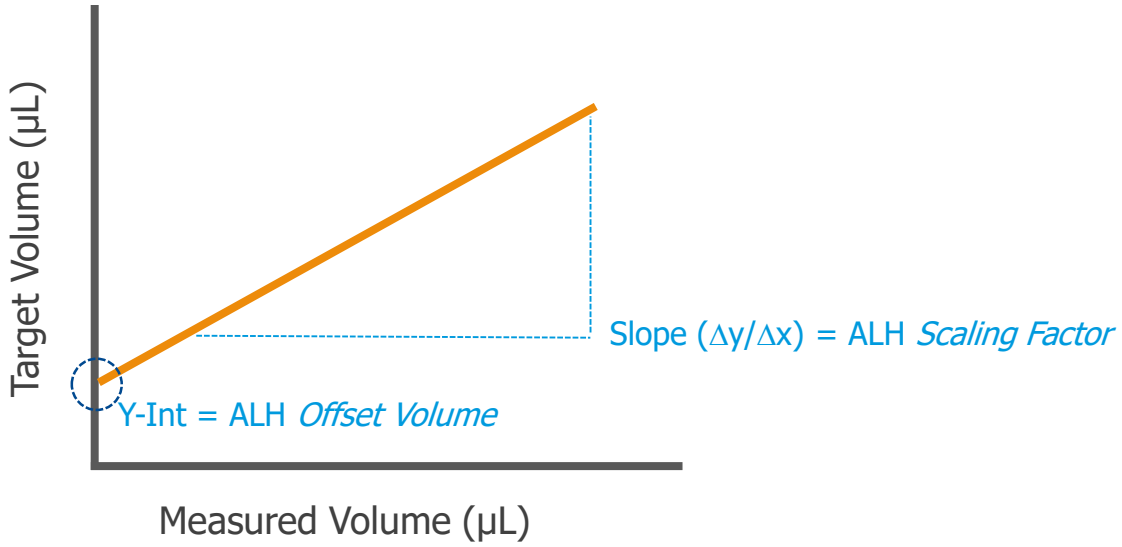
The Key to DNA Normalization is Low Volume Accuracy



Typical volumes for automated DNA normalization steps are 0.5 – 10 µL

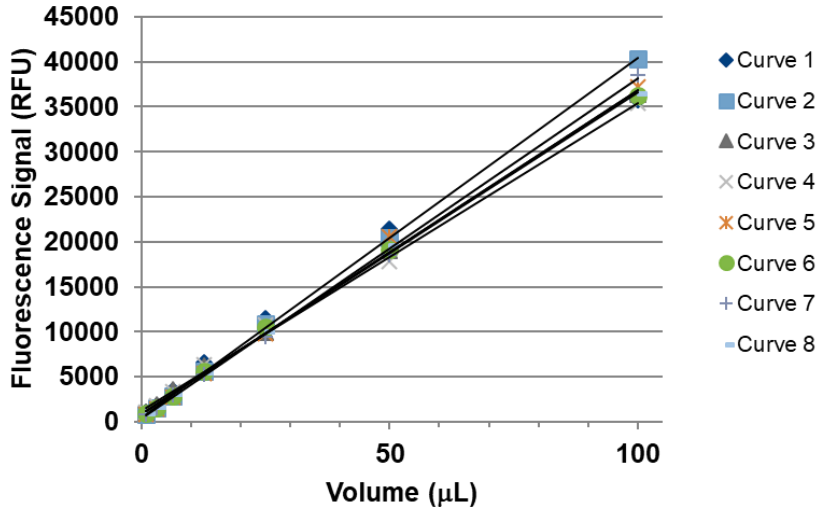
How to Achieving Low Volume Accuracy

- Select at least three volumes around your target range
- Dispense target volumes
- Measure dispensed volumes
- Plot *Target* volume against *Measured* volume
- Calculate slope and y-intercept
- Input slope value into the ALH *scaling factor*
- Input Y-intercept value into ALH *offset value*
- Save new ALH method



Example (n = 96)	**Pre-adjust**	**Post-Adjust**
Target volume (µL)	2.5	2.5
Measured volume (µL)	2.83	2.55
Rel. inaccuracy (%)	13.34	2.04
Precision (%CV	2.91	4.14

Accuracy is Absolutely Essential



1 volume measurement applied to 8 calibration curves

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Calculated Volume (µL)	49.81	47.04	51.25	52.72	50.16	51.56	50.12	51.09
Inaccuracy	-0.37%	-5.92%	2.50%	5.44%	0.32%	3.12%	0.23%	2.19%

Artel MVS ensures consistent volume accuracy from day to day, person to person!

Implications of Library Normalization Variability

- Cost
  - Wasted capacity
  - Repeat library preparation
- Application
  - Sequence ambiguity will lead to experimental repeat…assuming there is enough patient sample
- Patient Outcome
  - A rare allele or single nucleotide variation (SNV) might be missed; preventing the patient from a more appropriate treatment plan.

Equimolar DNA ratios before pooling helps ensure that all libraries are represented equally on the flow cell.

Other Sources of Assay Variability

- ALH liquid class development/optimization
- Assay component volume variability
- Reagent mixing
- Disposable tips for ALH
- Training

Master Mix Additions

Typical NGS Enzymes

- DNA polymerase (*Taq*, T4, Klenow, etc.)
- Fragmentase
- DNA ligase (e.g., T4, *Taq*, etc.)
- Klenow DNA polymerase I
- T4 polynucleotide kinase
- Klenow exonuclease

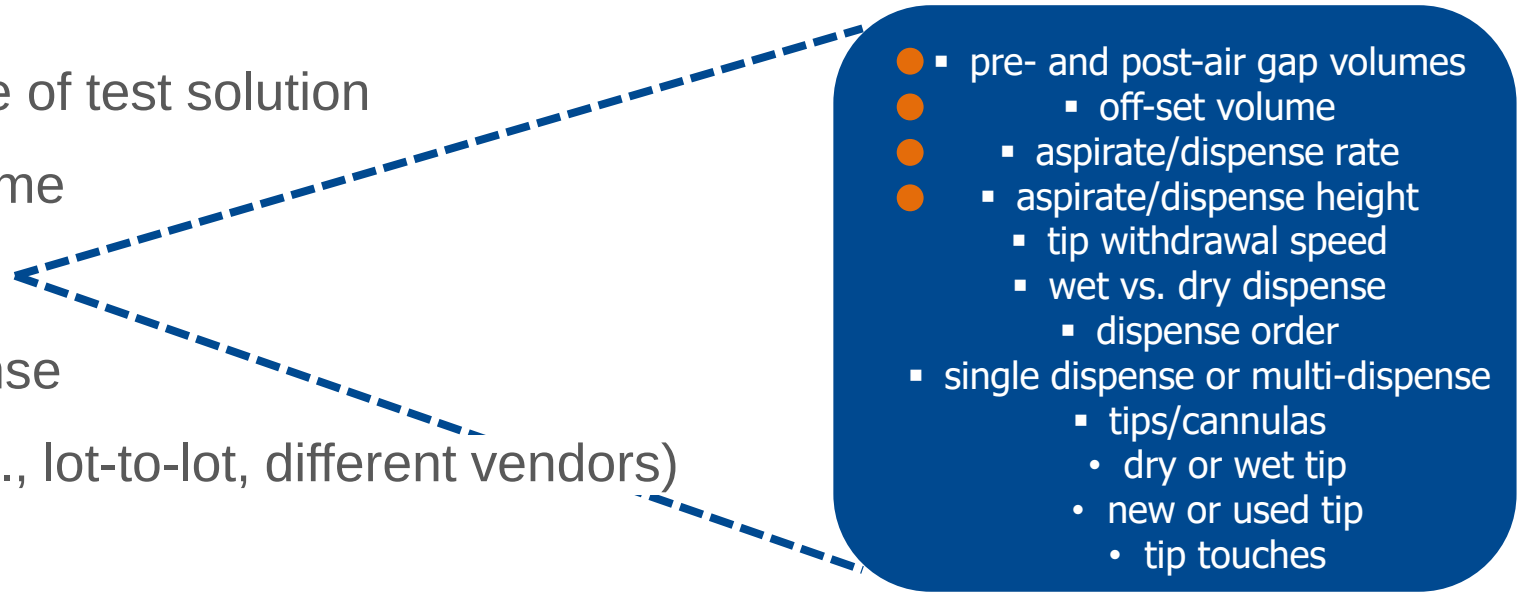
Typical Enzyme Solution Constituents

- Glycerol (2.5 – 75%)
- Sucrose (2.5 – 45%)
- DMSO (2.5 – 10%)
- PEG6000 (<10%)
- Triton (<0.2%)
- Combinations of the above

These solutions do not behave like ideal aqueous solutions and therefore must be considered differently when optimizing ALH liquid classes. Properties such as density, viscosity, temperature, vapor pressure, specific volume/weight/gravity, and surface tension all affect how fluids are dispensed.

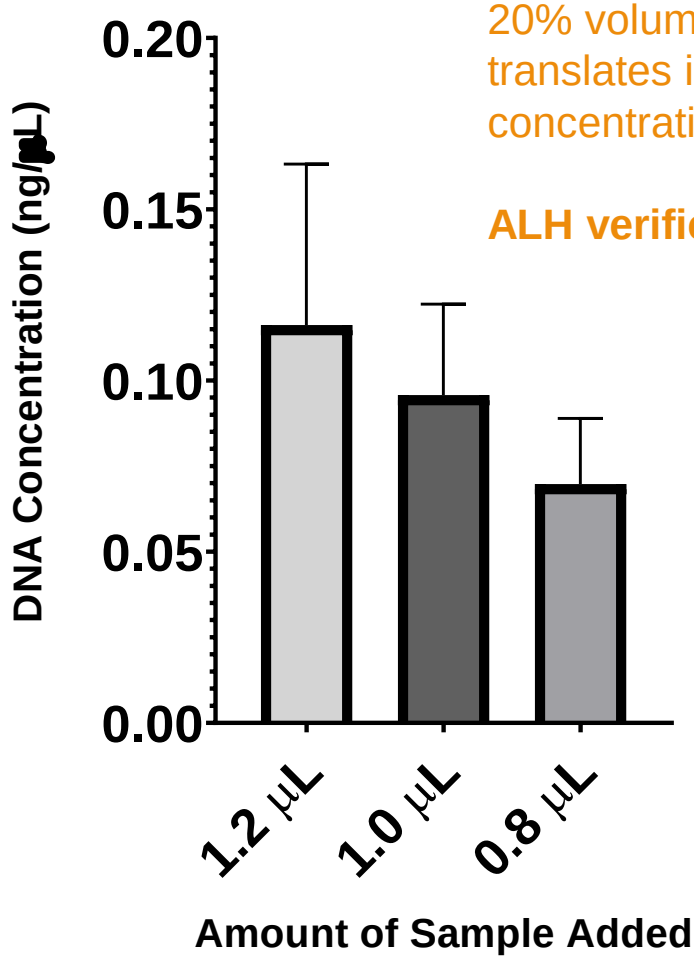
Key to Liquid Class Development

- Fluid properties of master mix solutions in various NGS steps are not the same as DNA solutions or diluents!
- Rapid liquid class development for your automated liquid handler
  - Begin by selecting your ALH's default liquid class, such as "PCR", "viscous", "glycerol", etc.
  - Dispense desired volume of test solution
  - Measure dispensed volume
  - Optimize default settings
- Evaluate cold reagent dispense
- Evaluate disposable tips (e.g., lot-to-lot, different vendors)



Effect of Sample Volume on qPCR Results

- DNA source: lambda phage (Nu1 gene)
- Automation: Eppendorf EPMotion
- qPCR Method: SYBR Green
- Experimental Set-up
  - 10 µL master mix (BioRad, xxx)
  - 0.5 µL forward primer
  - 0.5 µL reverse primer
  - **1 µL (control), 1.2 µL and 0.8 µL DNA sample**
  - 8 µL water
  - n = 9 replicates
  - Mixing = 4x asp/disp cycles

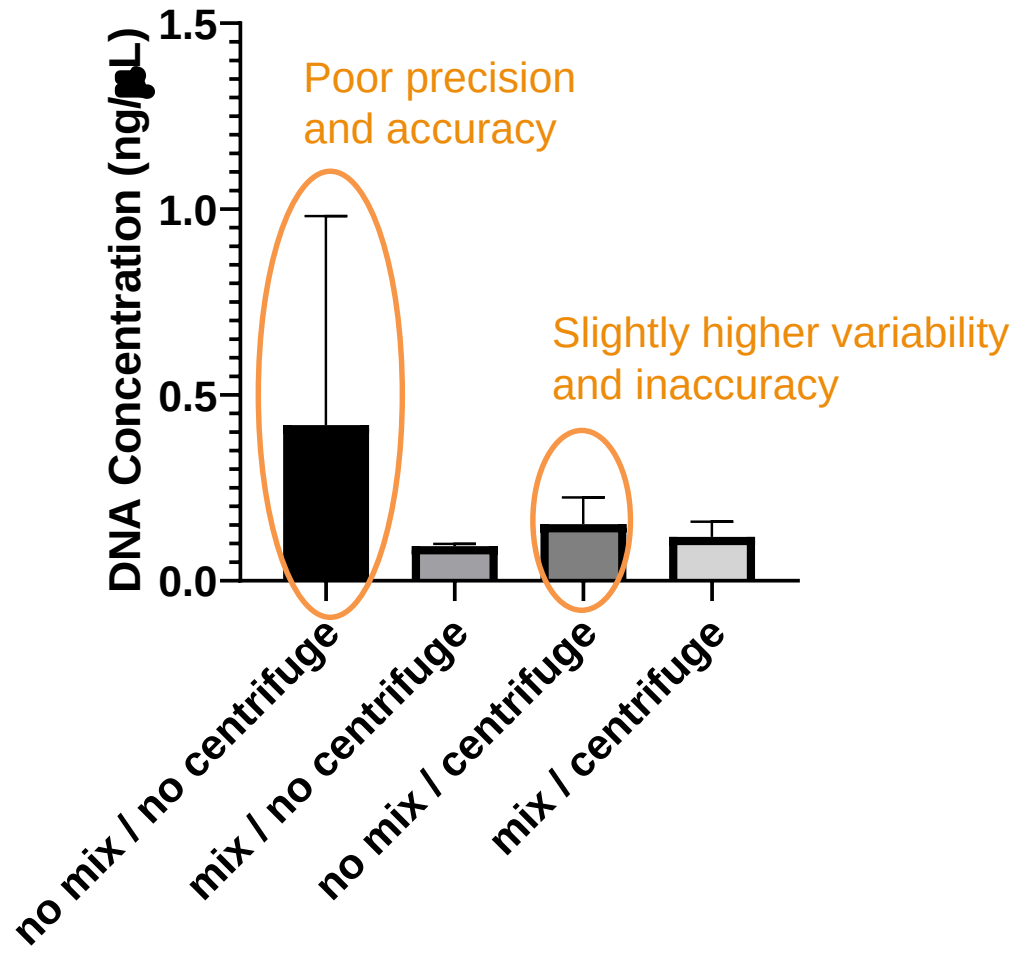


20% volume inaccuracy translates into a ~20% concentration inaccuracy.

ALH verification is very critical!

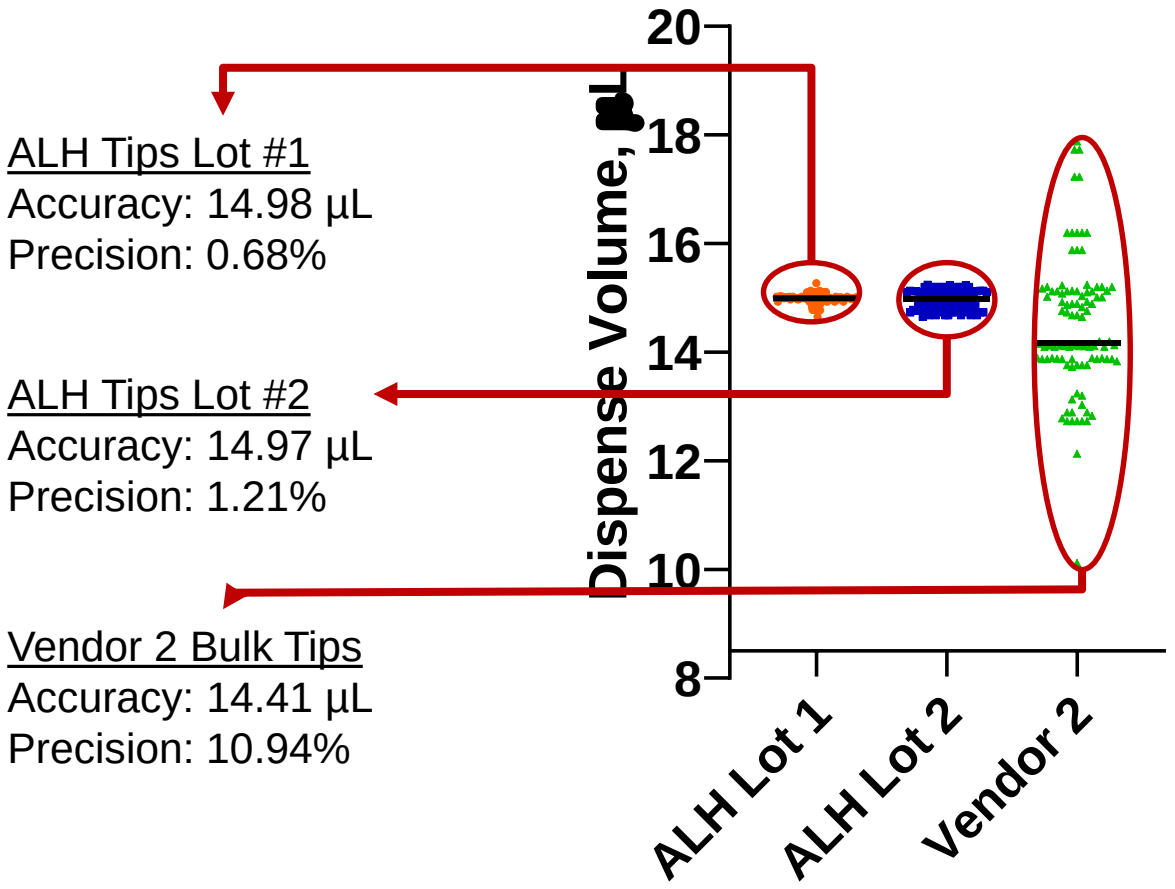
Effect of Mixing on qPCR Results

- DNA source: lambda phage (Nu1 gene)
- Automation: Eppendorf EPMotion
- qPCR Method: SYBR Green
- Experimental Set-up
  - 10 µL master mix (BioRad, xxx)
  - 0.5 µL forward primer
  - 0.5 µL reverse primer
  - 1 µL DNA sample (0.1 ng/µL target concentration)
  - 8 µL water
  - n = 9 replicates
- Mixing
  - **4x asp/disp cycles, with and without centrifugation**
  - **No mix, with and without centrifugation**

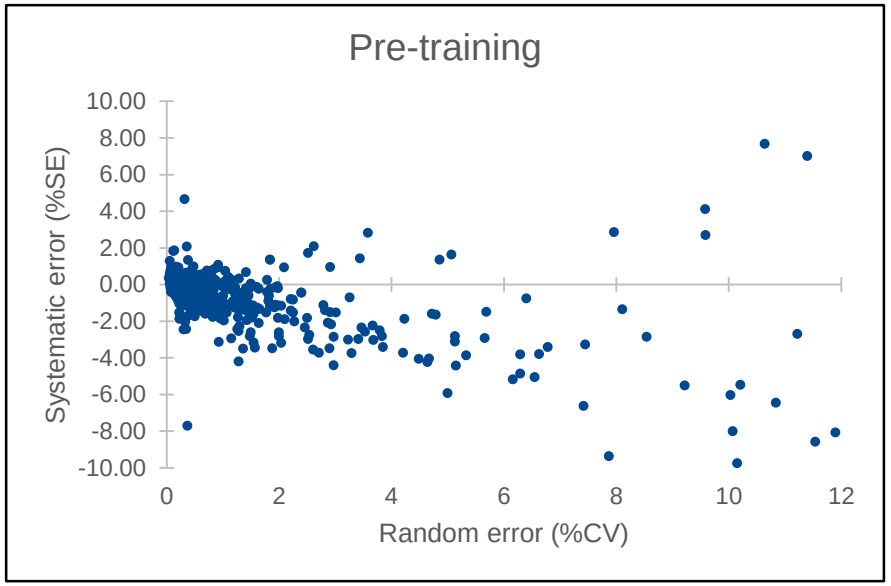


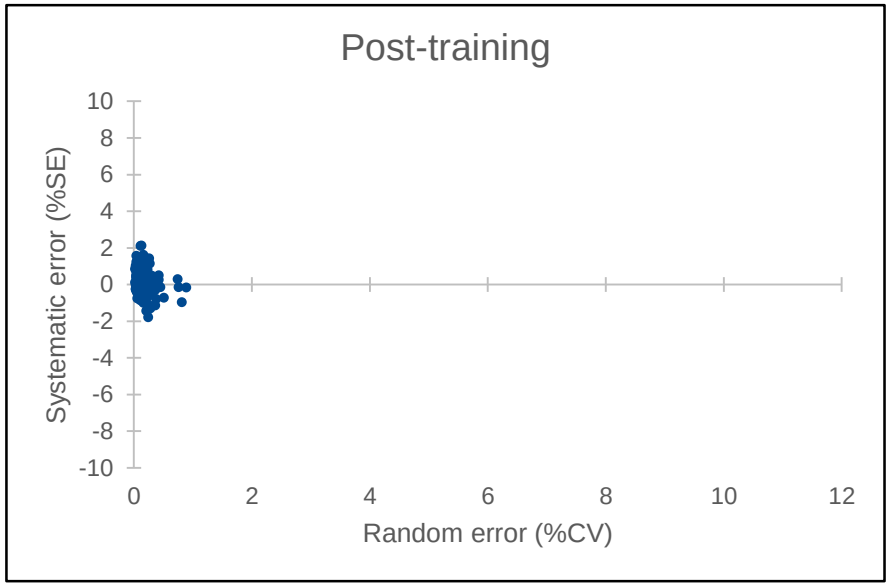
Disposable Pipette Tip Evaluation

- Target volume = 15 µL
- Disposable tips from ALH vendor and two different sources of bulk tips
- N = 96
- *Motivation: compare lot-to-lot variability and other sources*



Training is Fundamental for Assay Success



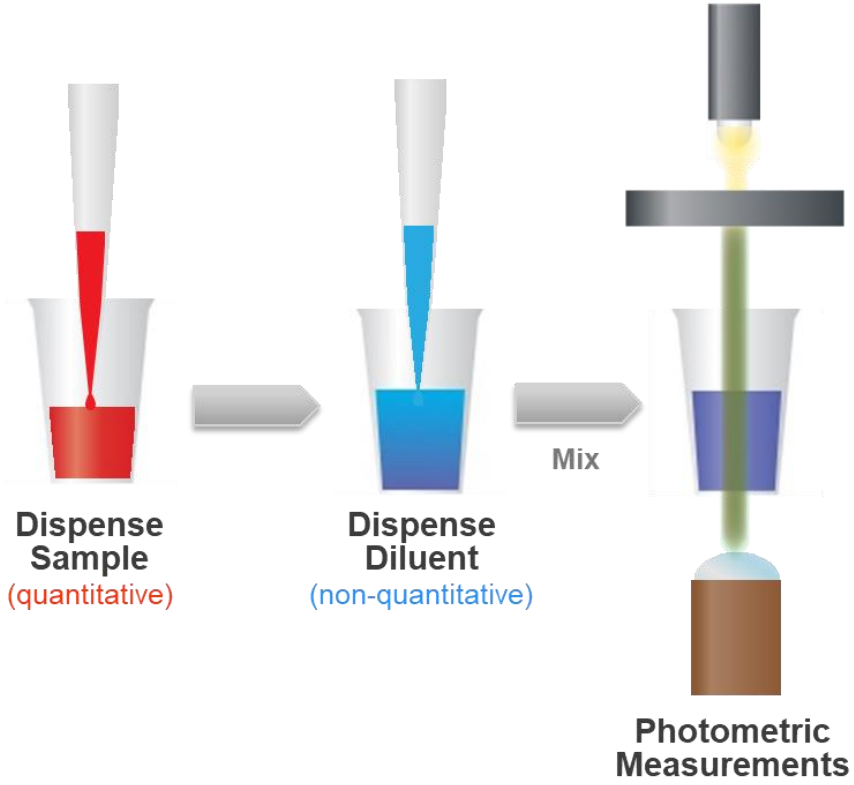


475 operators shown; each data point represents 5 replicates of 20 µL

Training allows adherence to regulatory requirements and minimizes downtime due to operator-to-operator inconsistencies.

The Solution: MVS (and PCS) Technology

- Employs a dual-dye, dual-wavelength, ratiometric absorbance-based measurement method for calculating the dispense volume.
- The results of the verification tests are traceable to national and international standards.
- How it works: dyes of known concentration are dispensed into well-characterized microtiter plate. The plate is mixed on a plate shaker to ensure solution homogeneity. Absorbance readings are taken at 520 nm and 730 nm.



$$V_S = V_T \left(\frac{a_b}{a_r} \right) \left(\frac{A_{520}}{A_{730}} \right)$$

Sources of Variability for Liquid Handling Calibration Measurements

- Liquid handler
- ✓ Reagents
- Tips
- ✓ Temperature
- ✓ Operator

contribute directly to the liquid ***dispensing variability***

- ✓ Mixing
- ✓ Detector
- ✓ Plate type

affect the ***measurement process***

Sources of Variability for Liquid Handling Calibration Measurements

Artel MVS removes all contributors to variability in liquid handling calibration… except the liquid handler and the tips.

Leaving you with exactly the two things you want to measure!

Where Can Artel Help?

- PCR master mix addition
  - ALH liquid class optimization
  - *for steps involving other enzymes as part of your NGS workflow*
- DNA Normalization
  - Ensure your ALH is delivering the low volume (0.5-10 µL) sample or diluent with the precision and accuracy you expect
- Training to ensure operator consistency
- Custom services to get you going faster

Thank You