Challenges in Method Calibration

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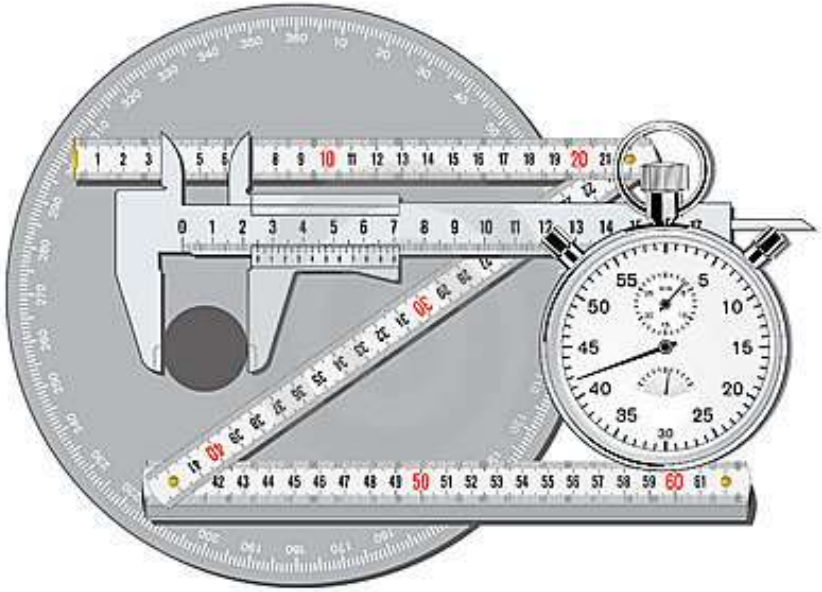
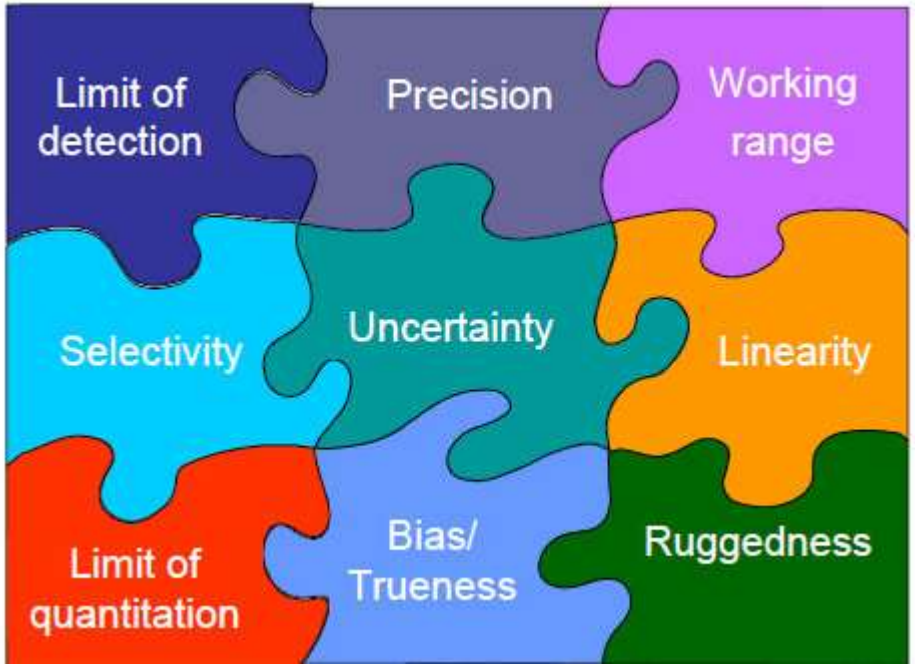
Method Calibration

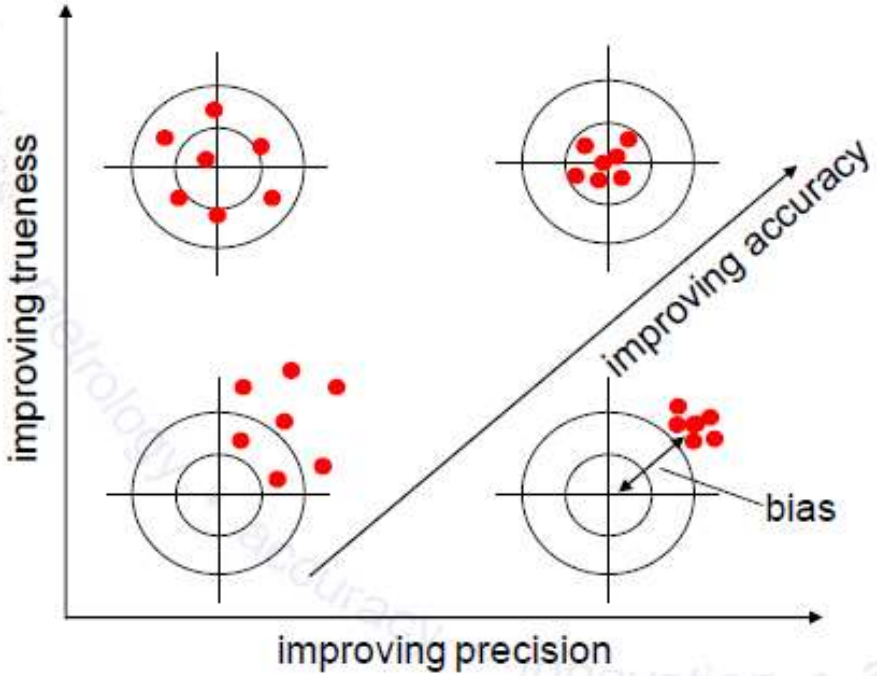
- to determine, check, rectify a measurement by comparison with a **standard**
- Good method calibration is required to produce meaningful data



First, we need a Method



Sensitive, Specific, Accurate, Rugged



Sensitivity & Specificity

- Sensitive – detects signal when target is present (true positive)
- Specific - does not detect signal when target is not present (no false positive)
- No assay is perfectly sensitive or specific
- There is a chance for both false positives and false negatives

idealized 2x2 contingency table

		True Result	
		+	-
Test Result	+	**100%**	**0%**
	-	**0%**	**100%**

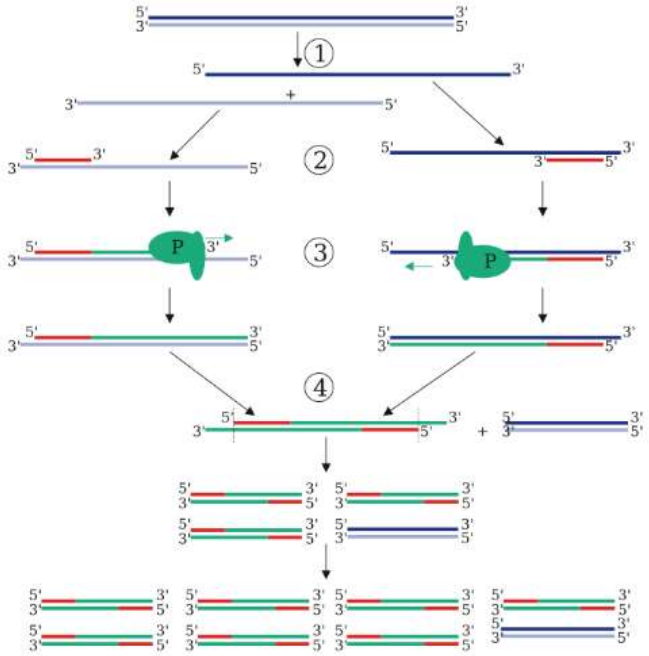
The Source Identification Pilot Project (SIPP) & California Microbial Source Identification Manual

Study → Validate → Apply

- 41 MST methods
- Tested by 27 laboratories
- 12 possible fecal sources
- 64 blind samples (in PBS)
- Assessed for sensitivity & specificity
  - >80% for all labs needed
  - Assays that passed criteria had SOPs included in the Manual

qPCR = quantitative PCR

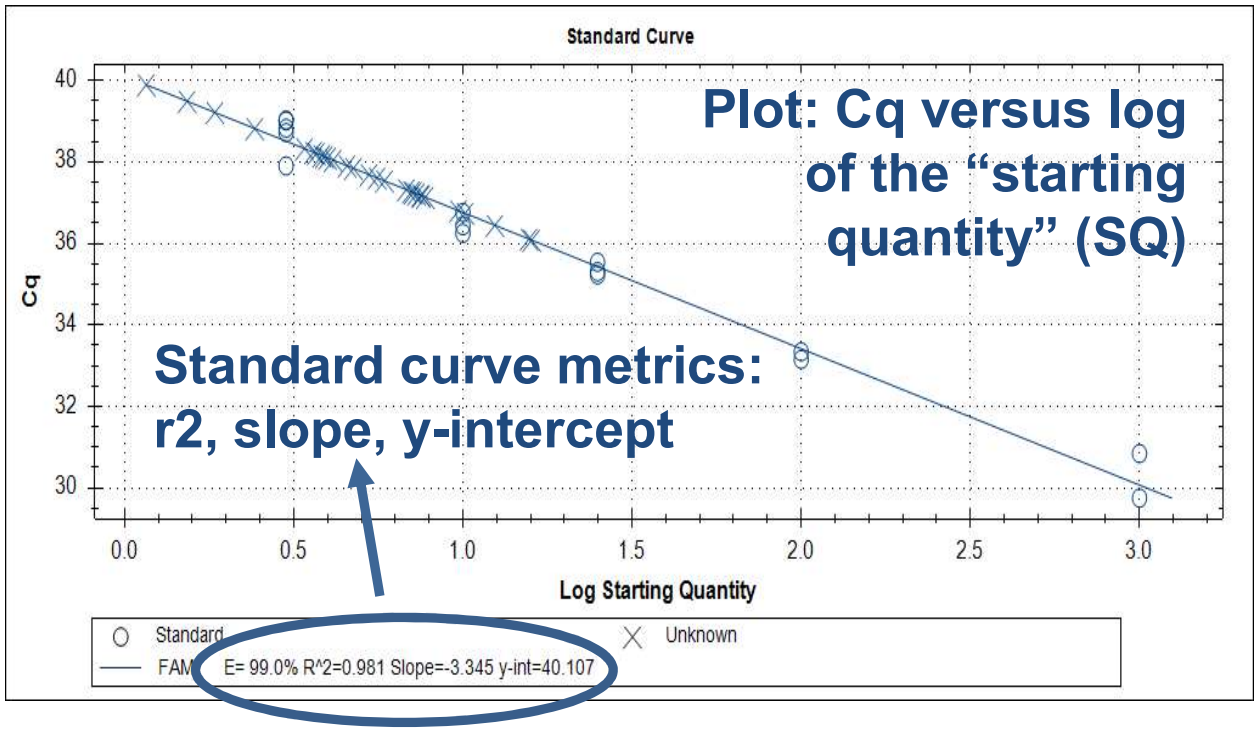
The promise **and** the challenge of qPCR is the "q"



Once an assay is in hand,
a standard curve needs to be established.

✓ assays are available

For typical qPCR, the quantity of DNA target in a sample is calculated from a standard curve



$y = mx+b$: $Cq = logSQ * slope + y_{intercept}$

$x = (y-b)/ m$: $log\ SQ = (Cq - y_{intercept})/slope$

$SQ = 10 \wedge ((Cq - y_{intercept})/slope)$

"absolute quantification"

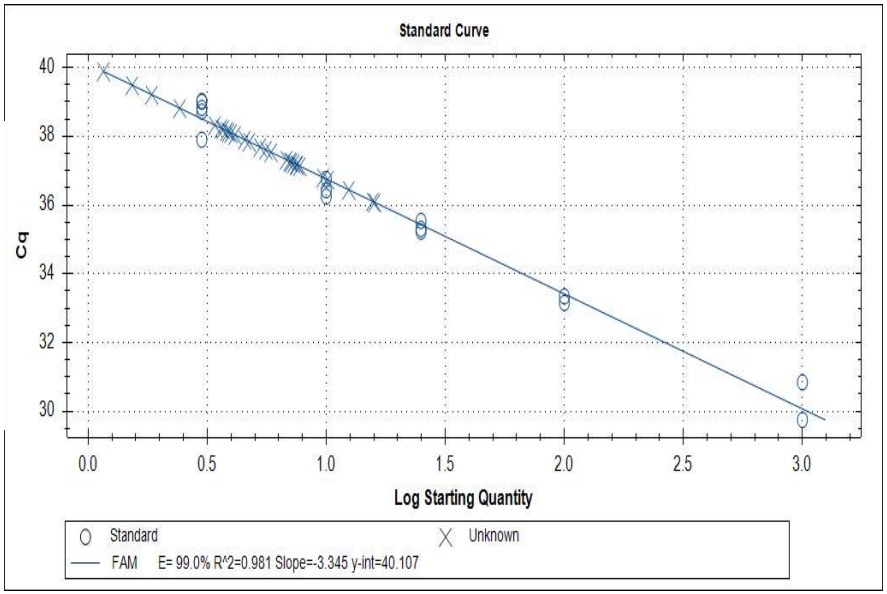
To ensure method precision, criteria are set on for standard curve quality

AF = amplification factor = 10 ^ (-1/slope)

%Efficiency = (AF-1) * 100

(also: % Efficiency = (E-1)*100)

E = %Efficiency = ((**10^(-1/slope)) -1)*100**

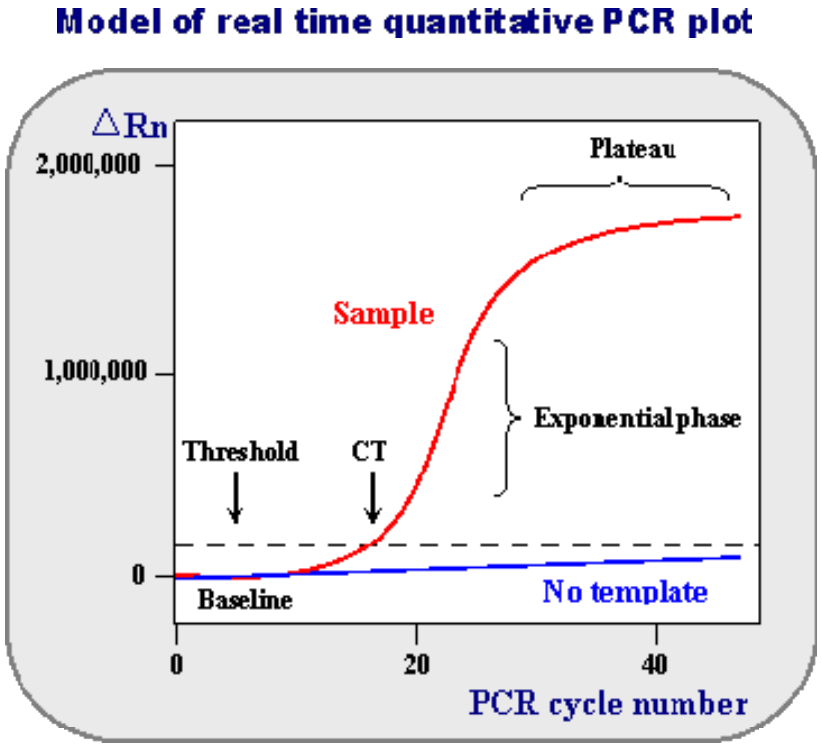


Requirements differ, typical criteria:

- Efficiency between 90-110%
- R^2 greater than 0.98
- Boundaries are rarely set for the y-intercept, although quantification is sensitive to y.

✓ criteria can be met

A source of quantified DNA is needed



There is no true gold standard for DNA quantitation.
There are a several common methods.

DNA Quantification

- UV Spectrophotometer (e.g., NanoDrop)

The pattern of DNA UV absorbance allows for DNA quantification. At 260nm, 1 A = 50 µg/ml of double-stranded DNA

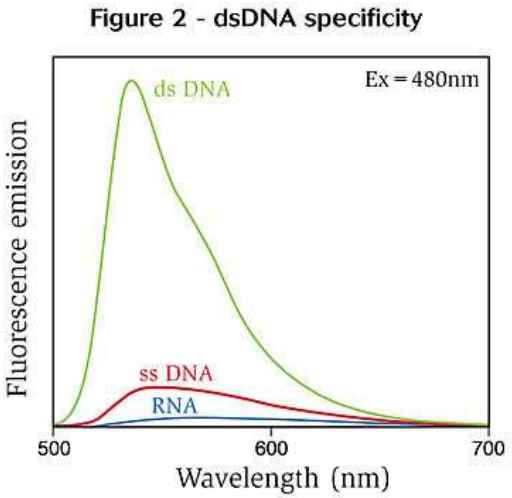
Pros: convenient, can use small amount of DNA, gives information about purity based on 260;280 ration

Cons: need pure DNA (not crude lysate), can't differentiate double-stranded DNA from single stranded DNA, RNA, or nucleotides. This can lead to overestimation of reading and inaccuracy in the results. Sensitive to pH. Tends to overestimate at low concentrations. *Known to not be strictly accurate.*

DNA Quantification

- Fluorescent Dyes (e.g., Qubit)

For example, PicoGreen is a DNA intercalating dye that fluoresces when bound to double stranded DNA. The dye is excited and emits at a particular wavelength.



Fluorescence enhancement of the PicoGreen® Quantitation Reagent upon binding dsDNA, ssDNA and RNA.

Pros: The dye is specific for double-stranded DNA. More sensitive than UV spec.

Cons: Provides no information on purity. Has a more narrow linear range. Uses more DNA. Requires a single-point standard curve. *Known to not be strictly accurate*.

DNA Quantification

- Bioanalyzer

Uses a intercalating fluorescent dye – basically works like an agarose gel, but on a microfluidic scale so that only 1 ul of DNA is needed.

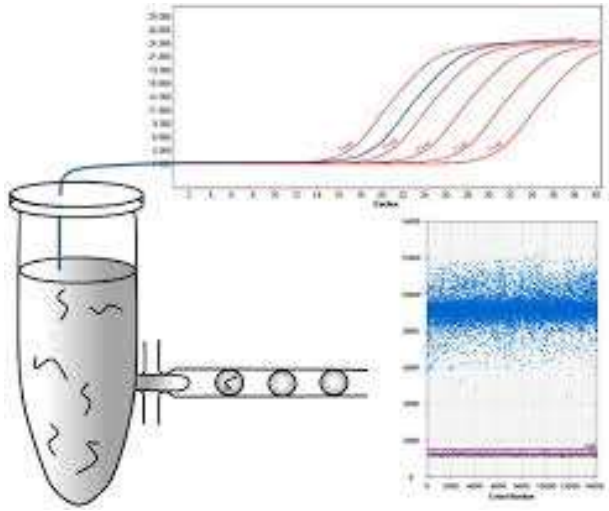
Pros: Provides information on DNA fragmentation. Specific for dsDNA. Sensitive.

Cons: Expensive.

DNA Quantification

- Digital PCR

Uses many parallel PCR reactions so that quantification is analogous to MPN analysis rather than based on a calibration curve



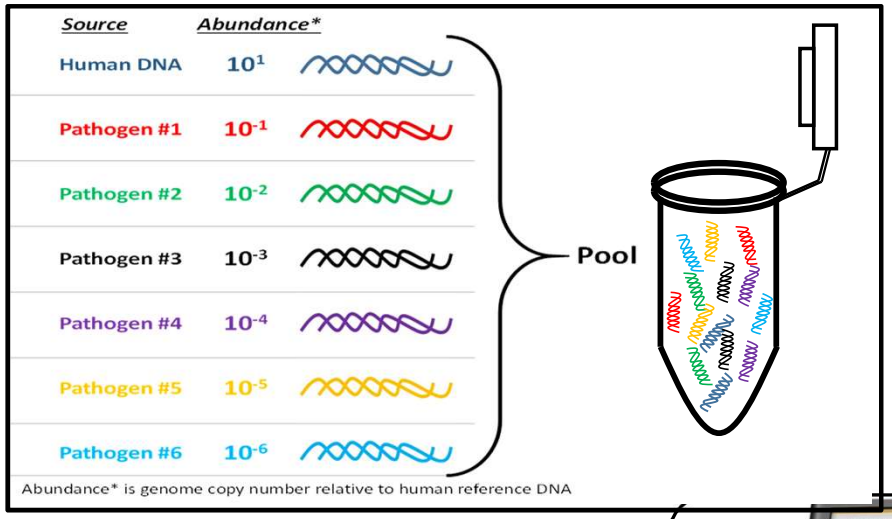
Pros: No reliance on standards. Uses similar mechanism to qPCR.

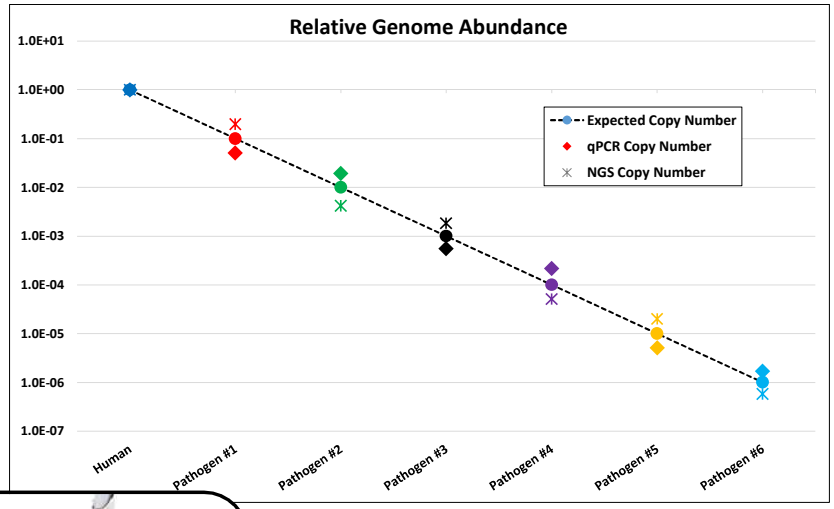
Cons: Uses similar mechanism to qPCR, which could hide PCR bias (i.e., ddPCR can't fix a bad assay). Expensive.

✓ ddPCR is helping solve quantification of reference materials

DNA Reference Materials

Digital PCR may become the “gold standard” for quantification, but not everyone will have one**. Reference materials are needed** so that individual labs can calibrate DNA stocks.





DNA Reference Materials

Some progress is about to be made in this area. NIST is creating DNA reference materials for a set of bacterial pathogens, and some will be of value for environmental microbiology applications.

Furthermore, EPA-available DNA standards (plasmids) may soon be NIST-certified. ☺

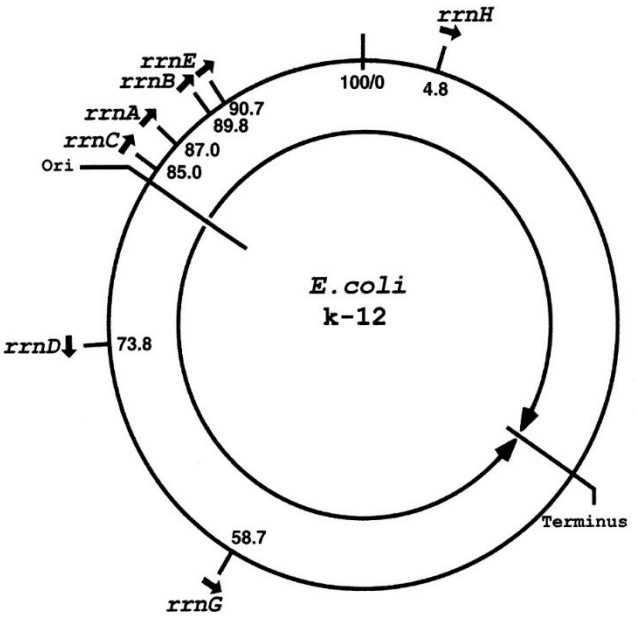
#NISTPathogen

✓ NIST may help solve the reference material issue

DNA Reference Materials

Progress in genome sequencing is improving estimates of copy number.

This information is used to turn copies into “target sequence copies (TSC)” (sometimes known as genome copies or cell equivalents – nomenclature usage varies), to account for multiple target copies per cell.



E. faecalis: $\frac{3.6 \times 10^7 \text{ fg/ul}}{3.6 \text{ fg/genome}} * \frac{4 \text{ copies}}{\text{genome}} = 4.04 \times 10^7 \text{ TSC/ul}$

B. dorei: $\frac{3.6 \times 10^7 \text{ fg/ul}}{6.1 \text{ fg/genome}} * \frac{7 \text{ copies}}{\text{genome}} = 4.04 \times 10^7 \text{ TSC/ul}$

✓ plasmid vs. genomic standards is still an issue

DNA Reference Materials

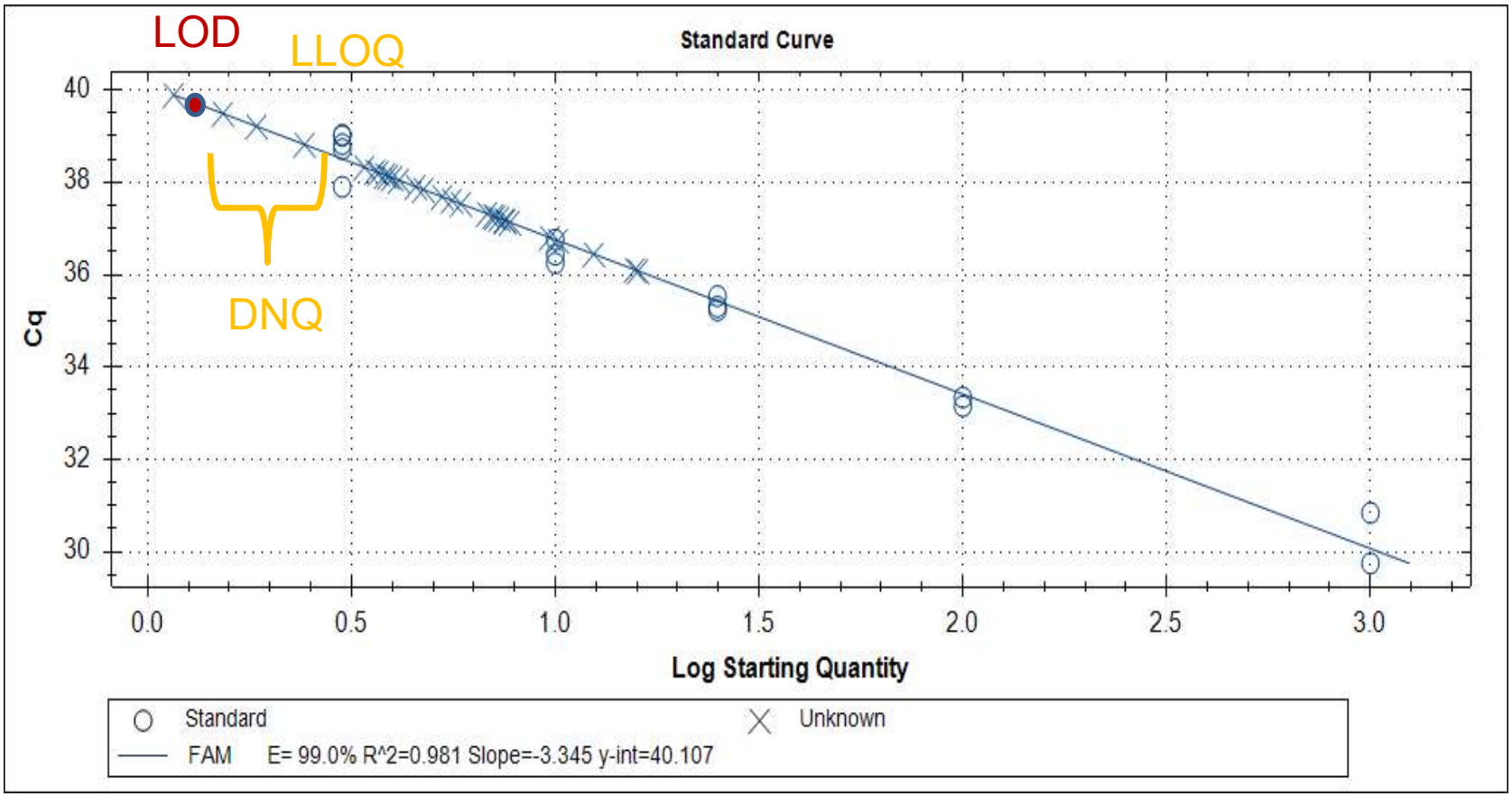
E. faecalis DNA is used as the standard, but the assay detects multiple species of enterococci (as do culture methods), do all species have the same number of gene copies?

– no. However, the variation is in the same order of magnitude.

How do plasmid standards translate into TSC (aka cell equivalents)?

– they don't. Absolute quantification of plasmid vs. genomic standards are similar but distinct.

✓ issues remain with absolute quantification



Reference materials can be used to calibrate DNA standard curves, improving their accuracy.

This is an important FIRST STEP for obtaining overall method calibration.

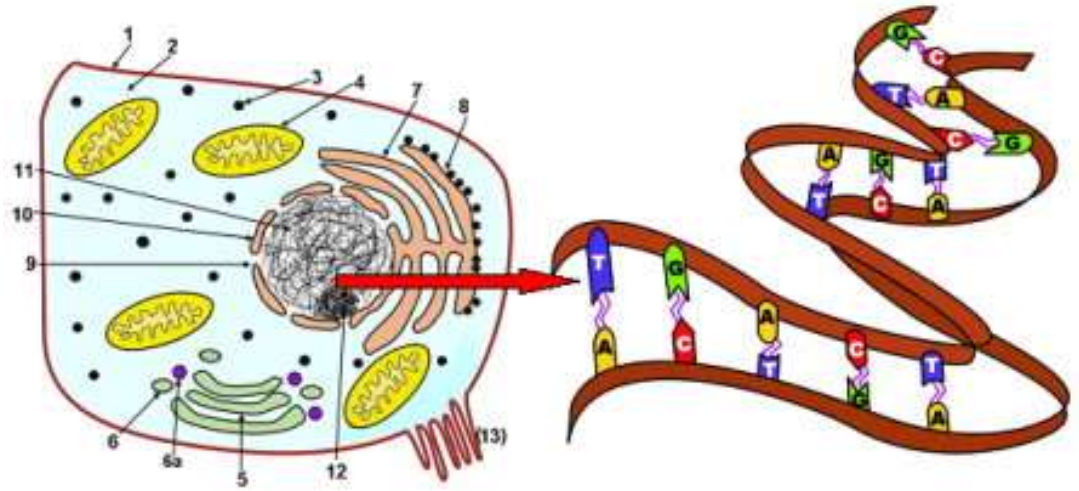
Calibration to Cells

Method calibration could be done solely in terms of DNA.

Risk, too, could have been assessed in these units, but ultimately it was not.

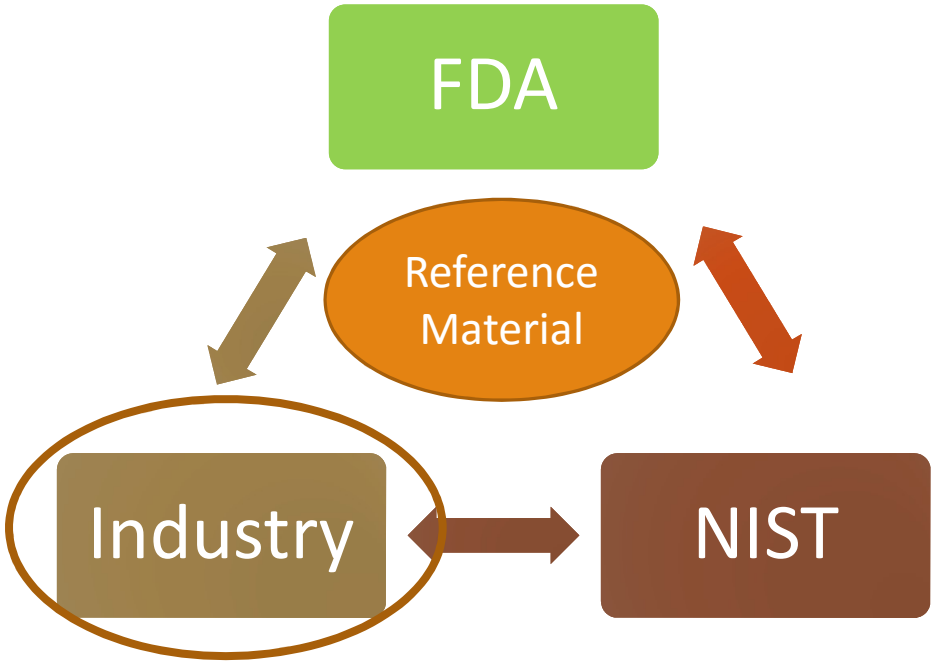
Furthermore, DNA is not the unit in which most people tend understand the problem.

Therefore, method calibration in terms of cells is needed.



Cell Reference Materials (?)

NIST has not volunteered to extend this exercise to calibrating the reference materials in terms of cell numbers, an indication of the challenge of the task. However, an industry partner has volunteered to do so.



Cell Reference Materials

Availability of the reference materials in terms of DNA and cell number would be helpful.

Otherwise, we will remain without a gold standard for determining extraction efficiency (DNA recovery).

Calibration to Cells

Calibration to cells is an issue.

EPA's acceptance criteria demonstrates.

EPA Acceptance Criteria for 550 CFU Enterococci Spike in PBS			
Spike Type	%CV (RSD)	% Recovery Ongoing	In terms of CFU
Lab-prepared	126	Detect-3,064	>0 - 16,852
BioBall™	104	Detect-256	121 - 1,408

Required weekly.

Calibration to Cells

The problem tends to be worse in ambient waters.

EPA Acceptance Criteria for 550 CFU Enterococci Spike in Marine Water			
Spike Type	%CV (RSD)	% Recovery Ongoing (OPR)	In terms of CFU
Lab-prepared	126	Detect-13,513	>0 - 74,322
BioBall™	104	Detect-181	>0 - 996

Required for 1/20 samples.

If ambient water contained ≥550 CFU per filter, result would have to be redone.

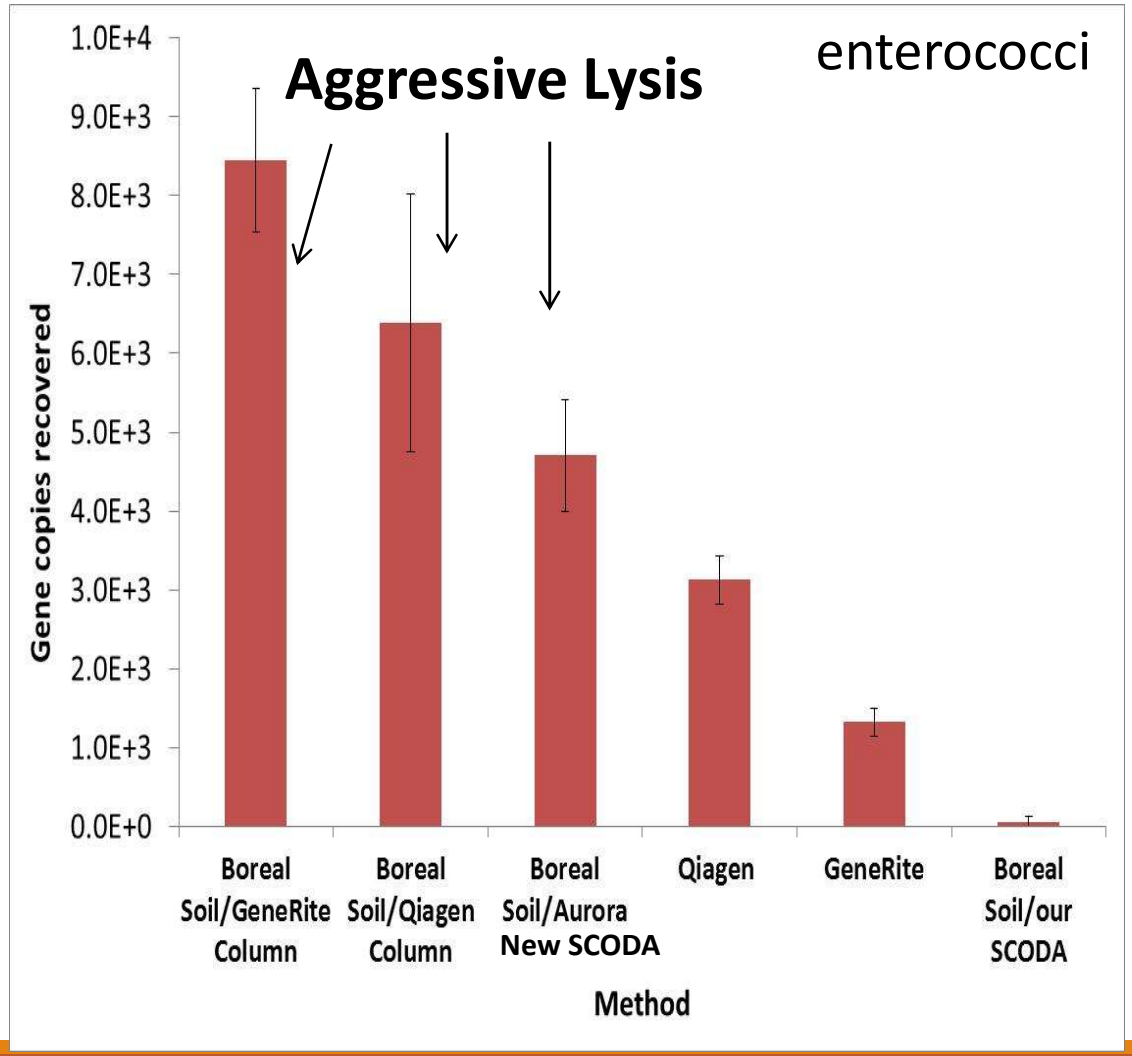
Calibration to Cells

There are a number of issues contributing to the problem.

- Difficulty quantifying cells. There are different methods (spec, culture) that provide various results and no gold standard – as seen for DNA quantification.
- DNA extraction variability. Recovery can be poor, inconsistent, concentration dependent, and vary by cell type (G+ vs. G-).
- Inhibition of DNA amplification. There are different modes of inhibition – e.g., DNA can be bound so that it is unavailable or the polymerase chemistry can experience direct interference.

Variable Extraction Efficiency

Copies recovered, PCR-inhibited seawater



Lactobacillus

sample	% recovery	mean % recovery	%CV
2a	17%	20%	20
2b	22%		
3a	10%	20%	71
3b	30%		
4a	27%	31%	18
4b	35%		
5a	6%	11%	69
5b	17%		
6a	1550%	889%	105
6b	228%		

Inhibition

The extraction method for MST worked well to remove inhibition, but not the crude lysate method for enterococci

Enterococci from PCR-inhibited seawater (PMACS concentrate) **Inhibition Factor (IF): $copies_{undil}/copies_{1:10}$** **IF ≥ 0.81 not inhibited; < 0.81 inhibited; 0 = fully inhibited**	
Method	**IF ± STD**
GeneRite DNA-EZ ST1 Kit	0.85 ± 0.21
MO BIO PowerSoil®	0.80 ± 0.10
MO BIO PowerWater®	0.73 ± 0.26
Qiagen DNeasy Blood and Tissue	0.43 ± 0.07
Bead beating (crude lysis)	0.32 ± 0.17
Bead beating/GeneRite column	0.51 ± 0.25
Boström lysis with tRNA	0.00 ± 0.00

A.M. Cox and K.D. Goodwin. Environmental Pollution Bulletin, 73(1), 47-56, 2013

Strategies to Address Issues

The EPA uses several strategies to address DNA recovery and inhibition issues.

- Calibrators: enterococci cells added to filters
- Sample Process Controls (SPC): salmon sperm DNA added to samples (and calibrators) during extraction
- Internal Amplification Controls (IAC): synthetic piece of DNA added to each sample that primes with the same primer set.

Controls – strategies to calibrate

The attempt to combat these issues has led to an onerous list of quality controls.

- Media sterility
- NTCs (no template controls)
- Method Blanks (3 kinds: field, lab, extraction)
- Standard curves (initial & ongoing)
- Positive control (every sample day – they are calibrators)
- Calibrators (initial & ongoing)
- Precision Recovery (initial- Each analyst & ongoing- weekly)
- Matrix spikes (initial & ongoing -1/20 samples)
- Sample Processing Controls (every sample)
- Internal Amplification Controls (every sample)

Strategies to Calibrate - issues

- Cell Calibrators
- Sample Processing Controls (SPC)
- Internal Amplification Control (IAC)

The results are not entirely satisfying nor do they have universal consensus, particularly EPA's relative calibration method (ΔΔC) that adjusts final values based on cell calibrators (versus flagging values).

The IAC is based on competitive inhibition – this is tricky

The calculations are complicated, particularly for the average stakeholder.

These strategies add significant expense and effort to the process.

Strategies to Calibrate

A source of trusted reference materials could help resolve concerns and **streamline methods**.

Such a development appears key to advancing a *successful* lab accreditation program.

Accreditation : third-party conformity assessment attesting to demonstration of competent completion of assessment tasks.
Certification : third-party attestation related to products, processes, systems or persons.

THANK YOU!