Case Study of Molecular Assay Validation:
TREC Copy Number Assessment on NBS Dried Blood Specimens Using Real-Time PCR

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Definitions

- **Analytic accuracy**: Closeness of an individual measurement to the "true" value, as determine by the reference method.
- **Analytic precision**: Closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.
- **Analytic reproducibility**: Closeness of agreement between test results obtained with the same method on identical test material within short intervals of time.

Definitions

- **Analytic sensitivity** (limits of detection): The lowest target that can be detected by a test system with a stated probability.
- **Clinical sensitivity**: Ability of a test to correctly identify disease.

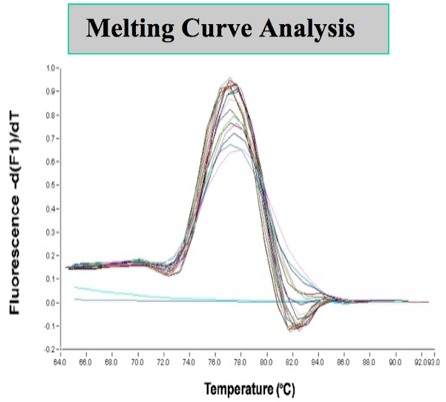
- **Analytic specificity**: Ability of a method to detect only the analyte it was designed to measure.
- **Clinical specificity**: Ability of a test to correctly exclude disease

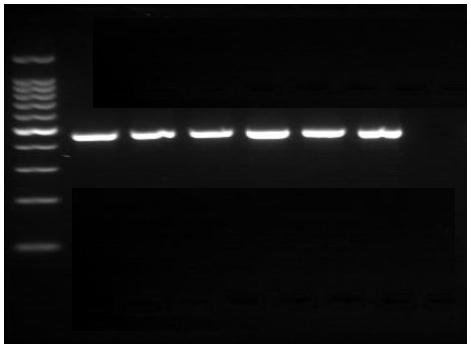
- **Validation:** The process of documenting that the previously verified analytic procedure employed for a specific test is suitable for its intended use and repeatedly gives expected results over a period time.

- **Verification**: The process of documenting that a test method performs as expected.

Verification of Amplification

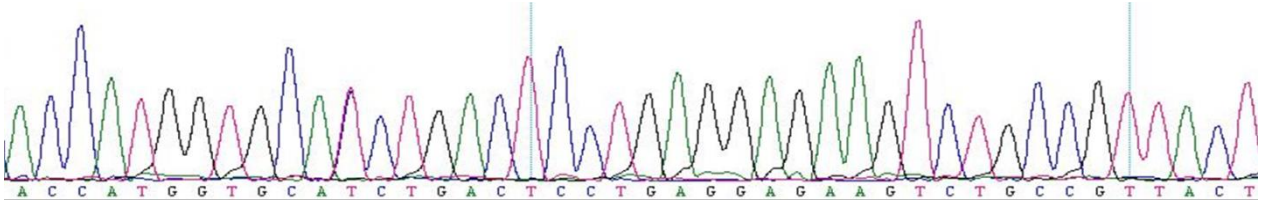
- Single peak with melt-curve analysis or single band with gel electrophoresis





Anne Atkins

- Match the expected size by gel electrophoresis and/or match the expected sequence by sequencing analysis



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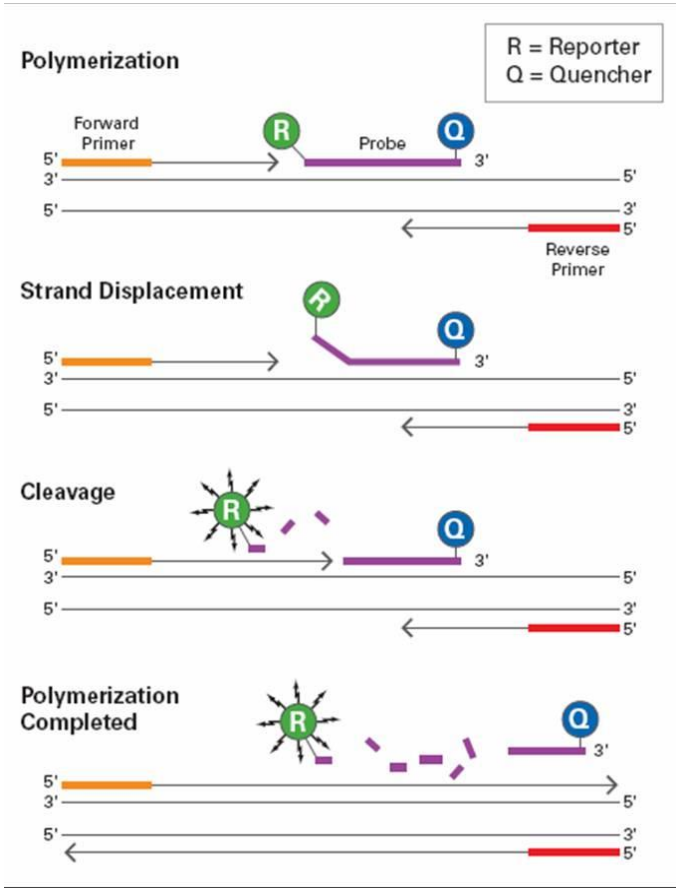
TaqMan Real-Time PCR



Quantification Strategies

- **Comparative method**

  A relative quantification that determines the changes of steady-state transcription of a gene. A relative standard curve consists of a dilution series created with a calibrator with arbitrary units.

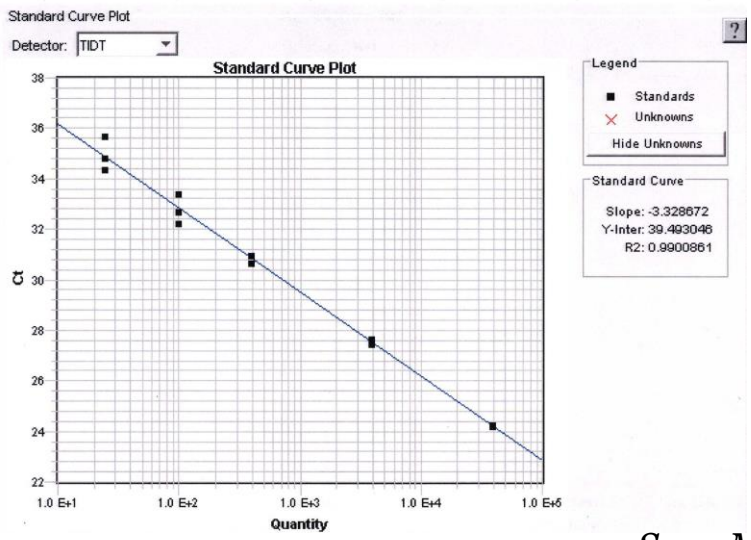
- **Standard curve method**

  An absolute quantification that allows the exact determination of copy number per sample matrix. Absolute quantification requires the construction of a standard curve, plotting the Ct values against the logarithm of the initial copy numbers of known concentration.

RT-PCR Characteristics

- Slope

  Between -3.1 to -3.6 (90-110% efficiency)
- Coefficient of correlation: $0.99 \leq r^2 \leq 0.999$
- Y-Intercept



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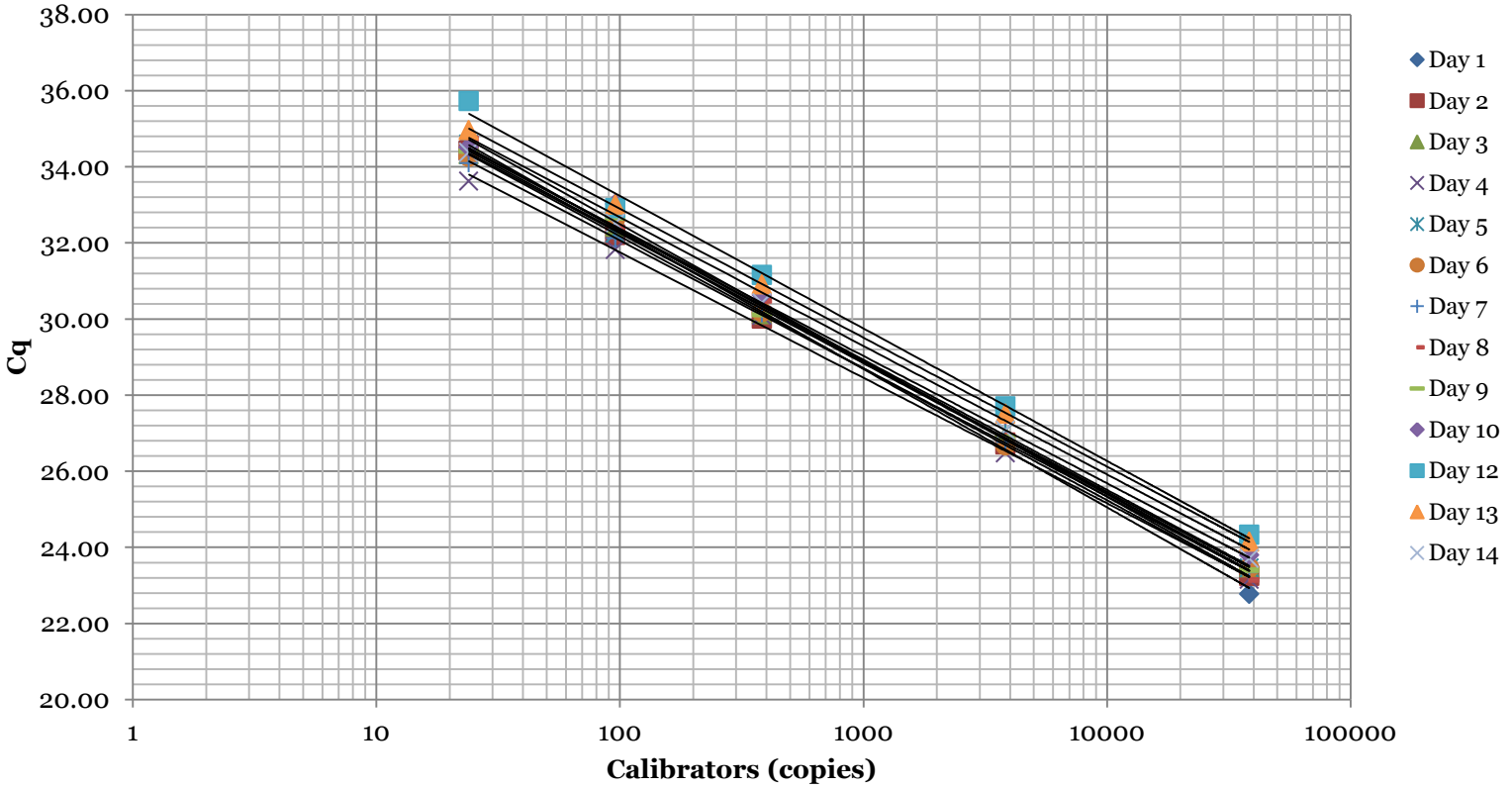
Analytical Validation

Calibrators



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Analytical Validation
Calibrators

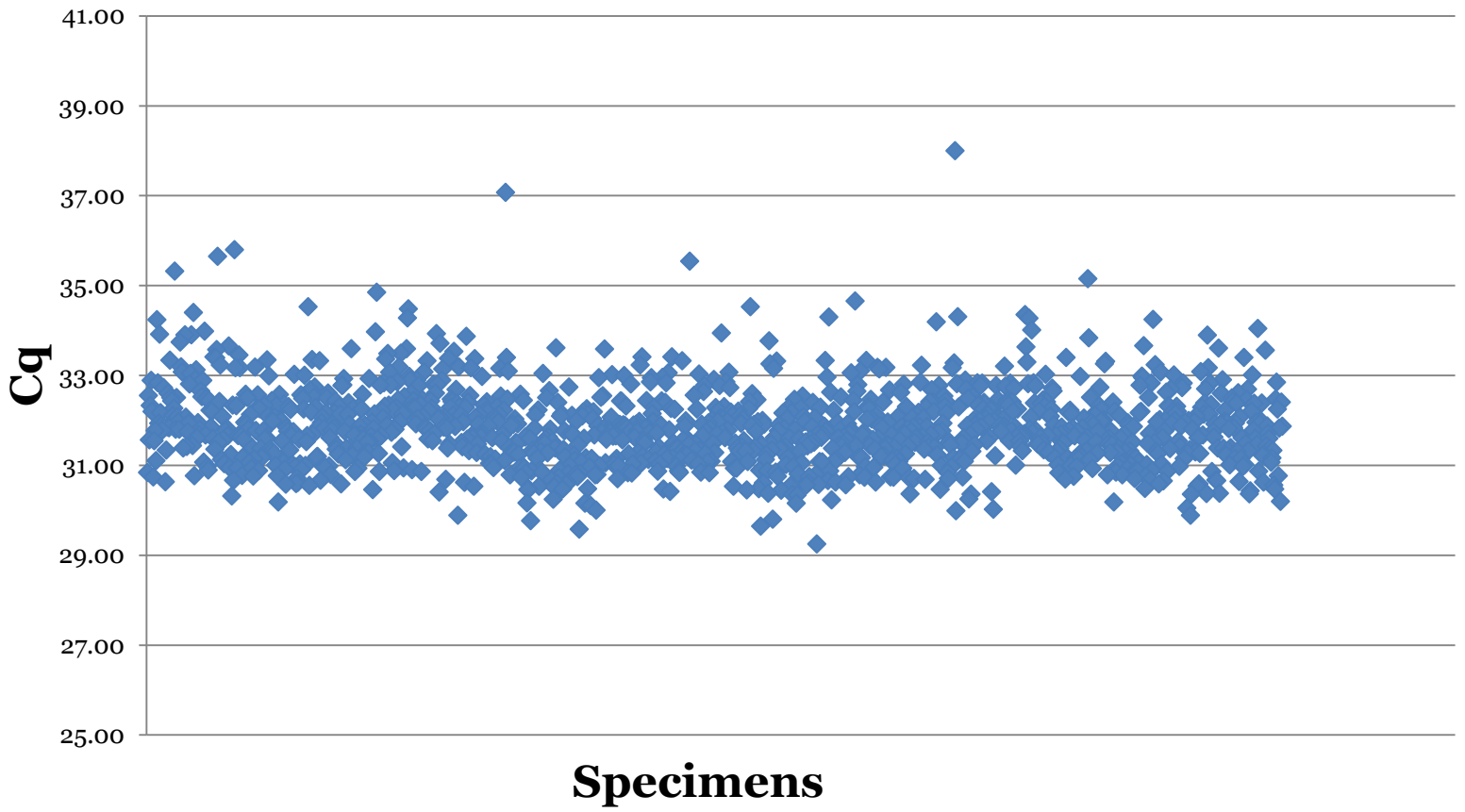
Copies	38400	3840	384	96	24	Slope	Y-Intercept	R2
Ct Mean	23.54	27.04	30.41	32.52	34.56	-3.44	39.31	0.99
STDEV	0.44	0.37	0.36	0.40	0.49	0.09	0.48	0.003
CV (%)	1.86	1.38	1.18	1.24	1.43	-2.67	1.22	0.26

Analytical Validation

Dried Blood Spot Specimens

ID	Day 1	Day 2	Day 3	MEAN	STDEV	CV (%)
CDC1_856	931	1485	962	1126	311	28
CDC1_Ct	31.11	30.50	31.06	30.89	0.34	1.09
CDC3_254	262	390	315	323	64	20
CDC3_Ct	32.97	32.66	32.65	32.76	0.19	0.57
CDC5_55	58	114	56	76	33	43
CDC5_Ct	35.23	34.50	35.44	35.06	0.49	1.40
CDC7_16	27	24	27	26	2	6
CDC7_Ct	36.41	36.92	36.30	36.54	0.33	0.91

Screening Test Cutoff Determination



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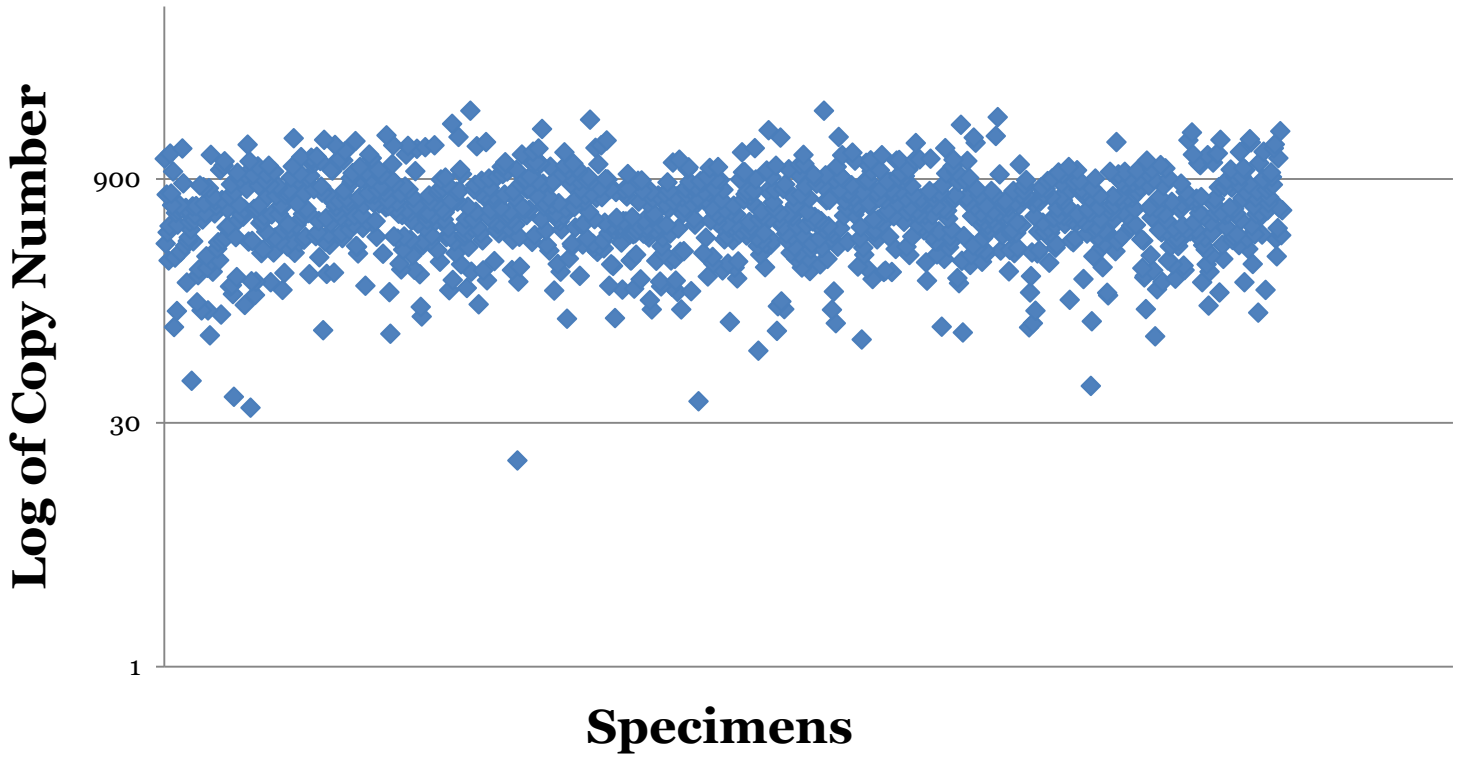
Screening Test Cutoff Determination



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Clinical Validation

- **Clinical intended question**
- **Correlation to disease**
  - Positive predictive value
  - Negative predictive value

What Else….

- **Manual vs. Automation**
- **Multiple instruments**
- **Interference**
- **Quality control**
  - Amplification (positive) control
  - Negative control
  - Statistical follow-up of a positive control
- **Proficiency testing**

Wisconsin SCID Screening

2014 Summary

	Flow Cytometry Full Term	Flow Cytometry Preterm
SCID	1	
22q11	1	
Lymphopenia	7	1
Expired	1	
Normal Newborn	13	3
Total	**23**	**4**
PPV	**41%**	**25%**

Takeaway…

- Are you confident that the assay fits intended use?
- Are you satisfied with the analytic and clinical performance?
- Have you documented EVERYTHING?