Development, Verification and Validation of Manufacturer Pre-Designed and In-House Designed Molecular Assays

Newborn Screening
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I have no conflicts to disclose.
Use of commercial names in this presentation is a reflection of our experience.

Molecular Testing of Nucleic Acids

There are an increasing number of molecular assays available for many applications for testing multiple analytes on different platforms and of varying complexities. There are very few kits available that can be directly applied to newborn screening.

Nucleic Acid Testing	Examples
Applications	Genetic mutations, viral load, viral subtyping, gene dosage
Multiple Analytes	Mutation analysis for genetic conditions, viral detection, oncogenes
Different Platforms	PCR, RT PCR, bead arrays, sequencing, high-resolution melt (HRM) profiling
Varying Complexity	Single- or multi-plex, Laboratory Developed Test, ASR, FDA-approved

Molecular Testing of Nucleic Acids

What all these have in common is that they are molecular tests that must be developed, verified and validated prior to implementation for clinical reporting.

Nucleic Acid Testing	Examples
Development	Accuracy, precision, reproducibility, sensitivity, specificity, robustness
Verification	Identify samples or reference materials of known quantitative value to verify the test
Validation	Identify analytic and clinical performance characteristics and test limitations

Molecular Testing of Nucleic Acids

The focus of this discussion will be on assay development, verification and validation of Luminex-based assays and quantitative real-time PCR.

Nucleic Acid Testing	Examples
Luminex-based assays	Traditional PCR followed by post-amplification analysis. Detection of the final product is after the amplification is complete. *CFTR, galactosemia, MCADD*
Quantitative real-time PCR	Simultaneous amplification and quantification in a closed system measuring the increase in fluorescence produced by a reporter molecule with each reaction cycle: *SCID*

Multiplex PCR

- The simultaneous detection of two or more target sequences from the same sample in a single reaction.
- Diagnostic capacity of a test is improved by acquiring additional information from a single test-run.
- Multiplexing allows addition of internal controls, such as a positive reaction control for amplification, and can be used as an indicator of template quantity or quality.
- Improves time and cost efficiency that otherwise would require more reagents, multiple independent assays and more time to perform.
- Essential technique if the amount of sample is very limited.

Luminex xTAG™ Product Line

A qualitative traditional PCR platform that facilitates the simultaneous detection and identification of multiple targets in a single tube.

- Pre-designed “off the shelf” assays - xTAG® Cystic Fibrosis Assay

- MagPlex Microspheres for custom designed assays: internally labeled with fluorescent dyes and are pre-coupled with complementary oligonucleotide sequences that *allow for flexible genetic assay development*

Luminex xTAG™ Genotyping Assay

Polymerase Chain Reaction

(Multiplex amplification of mutation and variant regions)

↓

Exonuclease I / Shrimp Alkaline Phosphatate

(Removal of unincorporated oligonucleotides and dNTPs)

↓

Allele-Specific Primer Extension

(Multiplex Tag-GSO primer extension, incorporation of Biotin-dCTP)

↓

xTAG™ Bead Hybridization

(Anti-tags attached to colored bead, SA-PE binds to Biotin as reporter)

↓

Luminex® xMAP™ System

(2 color lasers, 1 laser counts beads, theother looks for SA-PE reporter)

Distinguishing Features of Luminex Assay – the Allele Specific Primer Extension reaction

- ASPE is a solution based, sequence specific enzymatic reaction that can be used to determine genotype by targeting multiple loci in a single tube.
- The ASPE primer has a 3’ end that is specific to the gene and its targeted allele and a 5’ end that is specific to a complementary anti-TAG sequence on the xTAG bead.
- Following perfect annealing with the allele, the primer is extended and during extension a Biotin-labelled dCTP is incorporated.
- The ASPE product is then captured using xTag beads, each having a complementary anti-Tag sequence, and taking advantage of solution phase kinetics for sorting and identification.

Distinguishing Features of Luminex Assay – bead hybridization

- Attached to each differently colored bead is an anti-TAG sequence that binds to the complementary TAG sequence attached to the ASPE primers.
- Universal hybridization conditions – no optimization required.
- The Tag portion of the allele-specific extension products are hybridized with the corresponding bead anti-Tag using a universal temperature.

Verification and Validation of the Pre-designed, FDA-Approved Luminex CFTR Assay

- xTAG® Cystic Fibrosis 39 Assay tests for the 23 CFTR mutations recommended by the ACMG/ACOG plus an additional 16 mutations, with reflex testing of dF508 polys and intron 8 poly T.
- Moving from an FDA-approved version 1 to an FDA-approved version 2 assay still requires assay verification and validation.
- CLSI recommends that the DNA extraction method is included and all alleles for which the assay was designed to detect be verified before the introduction of the assay for routine use.
- Used DNA eluate from previously detected specimens with known CFTR genotype and CDC CFTR proficiency test samples, and synthetic controls for those relatively rare mutations for which there is no control (SeraCare ACCURUN® or Maine Molecular INTROL™ Quality Controls).

Previously Extracted DNA from Known Genotype

Known genotype →

TDAS CFTR (version 2.01) - Summary view

File View Sample Variation Admin Help

File name: ...\Copy of copy CF Tag-It V2 Validation L2 1.25.12_20120125_1 Date and time cre
Batch name: CF Tag-It V2 Validation L2 1.25.12 Analyzed by:

Lo...	Sample	Mu D Calls	HET Calls	Variant Ca...
1(1...	dF508/dF508	dF508		
2(1...	dI507/+		dI507	
3(1...	G542X/G542X	G542X		
4(1...	N1303K/+		N1303K	
5(1...	R347P/G551D		R347P, G551D	
6(1...	621+1G>T/3120+1G>G		621+1G>T, 3120+1G>A	
7(1...	394delTT/2184delA		394delTT, 2184delA	
8(1...	621-1G>T/R1162X		621+1G>T, R1162X	
9(1...	dF508/+		dF508	
10(...	ND			
11(...	R117H/+		R117H	7T
12(...	1717-1G>A/+		1717-1G>A	
13(...	621-1G>T/dF508		621+1G>T, dF508	
14(...	W1282X/W1282X	W1282X		
15(...	dF508/R553X		dF508, R553X	
16(...	ND			
17(...	G85E/+		G85E	
18(...	1078delT/+		1078delT	
19(...	3849+10kbC>T/+		3849+10kbC>T	
20(...	S549N/+		S549N	
21(...	Blank			
22(...	dF508		dF508	
23(...	Neg			
24(...	Neg			

→ Result concordant with genotype

Variation	Call	Raw Signal (MFI)		Background (MFI)		Net Signal (MFI)		Allelic Ratio		AR Thresholds		
		Wt Allele	Mut Allele	Wt Allele	Mut Allele	Wt Allele	Mut Allele	Wt Allele	Mut Allele	WT Call	Wt Present	Mut Present
G85E	WT	2552.5	61.0	9.0	21.0	2543.5	40.0	0.98	0.02	0.88	0.33	0.23
394delTT	WT	3843.0	261.0	9.0	11.0	3834.0	250.0	0.94	0.06	0.73	0.30	0.37
R117H	WT	3958.5	44.0	12.0	7.0	3946.5	37.0	0.99	0.01	0.85	0.25	0.35
Y122X	WT	2385.0	65.0	9.0	10.0	2376.0	55.0	0.98	0.02	0.85	0.30	0.30
621+1G>T	WT	2295.0	78.0	4.0	3.0	2291.0	75.0	0.97	0.03	0.85	0.25	0.35
711+1G>T	WT	2416.0	26.0	17.0	5.0	2399.0	21.0	0.99	0.01	0.85	0.30	0.30
1078delT	WT	4482.0	144.0	7.0	7.5	4475.0	136.5	0.97	0.03	0.85	0.33	0.27
R334W	WT	3282.0	84.0	6.0	18.0	3276.0	66.0	0.98	0.02	0.85	0.30	0.30
R347P	WT	4181.5	108.0	9.0	9.0	4172.5	99.0	0.96	0.02	0.85	0.27	0.30
└ R347H			66.5		5.0		61.5		0.01			0.35
A455E	WT	4233.0	23.0	11.0	9.0	4222.0	14.0	1.00	0.00	0.85	0.30	0.30
dI507	HET (dF50...	2228.0	227.0	11.0	12.0	2217.0	215.0	0.45	0.04	0.87	0.30	0.35
└ dF508			2508.5		11.0		2497.5		0.51			0.23
V520F	WT	6806.5	206.0	10.0	9.0	6796.5	197.0	0.97	0.03	0.85	0.30	0.30
1717-1G>A	WT	3214.5	115.0	5.5	10.0	3209.0	105.0	0.97	0.03	0.85	0.30	0.30
G542X	WT	3277.0	88.0	7.0	1.5	3270.0	86.5	0.97	0.03	0.85	0.30	0.30
S549N	WT	6366.0	59.0	7.0	8.0	6359.0	51.0	0.99	0.01	0.85	0.30	0.30
S549R(T>G)	WT	4317.5	94.0	7.0	6.0	4310.5	88.0	0.98	0.02	0.85	0.30	0.30
G551D	WT	3650.0	31.0	8.0	6.0	3642.0	25.0	0.99	0.01	0.85	0.28	0.32
R553X	WT	2388.0	22.5	7.0	10.0	2381.0	12.5	0.99	0.01	0.85	0.23	0.37
A559T	WT	2573.5	59.0	9.0	8.0	2564.5	51.0	0.98	0.02	0.85	0.30	0.30
R560T	WT	3165.0	38.0	9.0	9.0	3156.0	29.0	0.99	0.01	0.90	0.35	0.25
1898+1G>A	WT	2516.5	55.5	8.0	7.5	2508.5	48.0	0.98	0.02	0.85	0.25	0.35
1898+5G>T	WT	3180.5	145.0	9.0	9.0	3171.5	136.0	0.96	0.04	0.85	0.30	0.30
2183AA>G	WT	2800.0	32.0	6.5	11.0	2793.5	21.0	0.96	0.01	0.85	0.30	0.35
└ 2184delA			106.0		8.0		98.0		0.03			0.27
2307insA	WT	3307.0	145.5	9.5	5.5	3297.5	140.0	0.96	0.04	0.85	0.30	0.30
2789+5G>A	WT	3850.0	21.0	6.0	9.0	3844.0	12.0	1.00	0.00	0.85	0.30	0.30
3120+1G>A	WT	4050.0	92.0	6.5	10.0	4043.5	82.0	0.98	0.02	0.85	0.30	0.30
Y1092X-C>G	WT	5510.0	42.0	7.0	11.0	5503.0	31.0	0.96	0.01	0.85	0.30	0.30
└ Y1092X...			191.0		4.0		187.0		0.03			0.30
M1101K	WT	2242.5	109.0	2.0	7.5	2240.5	101.5	0.96	0.04	0.82	0.30	0.33
R1162X	WT	5289.0	98.0	1.0	8.0	5288.0	90.0	0.98	0.02	0.85	0.30	0.30
3659delC	WT	3538.0	108.0	7.0	9.0	3531.0	99.0	0.97	0.03	0.85	0.30	0.30
S1255X(ex...	WT	4612.0	116.5	11.0	6.0	4601.0	110.5	0.98	0.02	0.85	0.30	0.30
S1255X(ex...	WT	3807.0	21.0	5.5	0.0	3801.5	21.0	0.99	0.01	0.85	0.25	0.35
3849+10k...	WT	3454.0	64.0	3.0	1.0	3451.0	63.0	0.98	0.02	0.85	0.30	0.30
3876delA	WT	5167.0	219.0	1.0	0.0	5166.0	219.0	0.96	0.04	0.85	0.30	0.30
3905insT	WT	5491.0	145.0	6.0	0.0	5485.0	145.0	0.97	0.03	0.85	0.30	0.30
W1282X	WT	7308.0	243.0	6.0	0.5	7302.0	242.5	0.97	0.03	0.85	0.30	0.30
N1303K	WT	2145.0	5.0	4.0	0.0	2141.0	5.0	1.00	0.00	0.85	0.30	0.30
5T	CH				10.0							0.30
├ 7T					9.0							0.30
└ 9T					12.0							0.30
I506V	ND		44.0		10.0		34.0					
├ I507V			54.0		14.0		40.0					
└ F508C			44.5		12.0		32.5					

dF508/+ Complete Data View

Genotype is determined by the ratio of the MFI signals compared to allelic ratio threshold values.

Variation	Call	Raw Signal (MFI)		Background (MFI)		Net Signal (MFI)		Allelic Ratio		
		Wt Allele	Mut Allele	Wt Allele	Mut Allele	Wt Allele	Mut Allele	Wt Allele	Mut Allele	WT
G85E	HET	1429.0	1234.5	9.0	21.0	1420.0	1213.5	0.54	0.46	0.8
394delTT	HET	2488.0	1955.0	9.0	11.0	2479.0	1944.0	0.56	0.44	0.7
R117H	HET	2331.0	3767.0	12.0	7.0	2319.0	3760.0	0.38	0.62	0.8
Y122X	HET	1591.0	1659.0	9.0	10.0	1582.0	1649.0	0.49	0.51	0.8
621+1G>T	Mu D	49.0	1805.0	4.0	3.0	45.0	1802.0	0.02	0.98	0.8
711+1G>T	Mu D	23.0	1357.0	17.0	5.0	6.0	1352.0	0.00	1.00	0.8
1078delT	Mu D	186.5	2399.0	7.0	7.5	179.5	2391.5	0.07	0.93	0.8
R334W	Mu D	205.0	1511.0	6.0	18.0	199.0	1493.0	0.12	0.88	0.8
R347P	HET (P/H)	65.0	2126.0	9.0	9.0	56.0	2117.0	0.01	0.48	0.8
└ R347H			2286.0		5.0		2281.0		0.51	
A455E	Mu D	21.0	3044.0	11.0	9.0	10.0	3035.0	0.00	1.00	0.8
dI507	Mu D (dF508)	989.0	1462.0	11.0	12.0	978.0	1450.0	0.16	0.24	0.8
└ dF508			3578.5		11.0		3567.5		0.60	
V520F	Mu D	41.0	1870.0	10.0	9.0	31.0	1861.0	0.02	0.98	0.8
1717-1G>A	Mu D	45.0	1553.0	5.5	10.0	39.5	1543.0	0.02	0.98	0.8
G542X	HET	2451.5	2195.0	7.0	1.5	2444.5	2193.5	0.53	0.47	0.8
S549N	HET	2934.0	3309.0	7.0	8.0	2927.0	3301.0	0.47	0.53	0.8
S549R(T>G)	HET	3126.0	2294.0	7.0	6.0	3119.0	2288.0	0.58	0.42	0.8
G551D	HET	3091.0	2109.0	8.0	6.0	3083.0	2103.0	0.59	0.41	0.8
R553X	Mu D	321.0	1725.0	7.0	10.0	314.0	1715.0	0.15	0.85	0.8
A559T	Mu D	82.5	1144.0	9.0	8.0	73.5	1136.0	0.06	0.94	0.8
R560T	Mu D	516.0	1167.0	9.0	9.0	507.0	1158.0	0.30	0.70	0.9
1898+1G>A	Mu D	175.0	2004.0	8.0	7.5	167.0	1996.5	0.08	0.92	0.8
1898+5G>T	HET	2289.0	1174.0	9.0	9.0	2280.0	1165.0	0.66	0.34	0.8
2183AA>G	HET (2183AA>G/2184delA)	128.0	1519.0	6.5	11.0	121.5	1508.0	0.04	0.44	0.8
└ 2184delA			1775.0		8.0		1767.0		0.52	
2307insA	Mu D	98.0	3092.0	9.5	5.5	88.5	3086.5	0.03	0.97	0.8
2789+5G>A	Mu D	34.0	1639.5	6.0	9.0	28.0	1630.5	0.02	0.98	0.8
3120+1G>A	Mu D	84.0	2292.0	6.5	10.0	77.5	2282.0	0.03	0.97	0.8
Y1092X-C>G	HET (C>G/C>A)	56.0	1370.5	7.0	11.0	49.0	1359.5	0.02	0.47	0.8
└ Y1092X...			1474.0		4.0		1470.0		0.51	
M1101K	Mu D	131.0	2025.5	2.0	7.5	129.0	2018.0	0.06	0.94	0.8
R1162X	Mu D	69.0	1686.5	1.0	8.0	68.0	1678.5	0.04	0.96	0.8
3659delC	HET	840.0	1643.5	7.0	9.0	833.0	1634.5	0.34	0.66	0.8
S1255X(ex...	Mu D	23.0	1114.0	11.0	6.0	12.0	1108.0	0.01	0.99	0.8
S1255X(ex...	HET	1630.0	2113.0	5.5	0.0	1624.5	2113.0	0.43	0.57	0.8
3849+10k...	Mu D	251.0	3492.0	3.0	1.0	248.0	3491.0	0.07	0.93	0.8
3876delA	HET	1962.5	2754.5	1.0	0.0	1961.5	2754.5	0.42	0.58	0.8
3905insT	HET	1357.0	2689.0	6.0	0.0	1351.0	2689.0	0.33	0.67	0.8
W1282X	Mu D	114.0	3729.0	6.0	0.5	108.0	3728.5	0.03	0.97	0.8
N1303K	Mu D	20.0	2229.5	4.0	0.0	16.0	2229.5	0.01	0.99	0.8
5T	5T/9T D		2614.0		10.0		2604.0		0.46	
├ 7T			665.0		9.0		656.0		0.12	
└ 9T			2448.0		12.0		2436.0		0.43	
I506V	I506V,I507V,F508C D		1026.0		10.0		1016.0			
├ I507V			1601.0		14.0		1587.0			
└ F508C			1084.0		12.0		1072.0			

SeraCare ACCURUN® Complete Data View

Synthetic, multiplex control that covers all of the mutations in the Luminex CFTR panel

Development, Verification and Validation of the Homebrew Luminex Assay

- Galactosemia 9-plex, MCADD single-plex
- Moving from an existing assay using the xTAG microspheres to the MagPlex microspheres requires new assay development, verification and validation.
- CLSI recommends that the DNA extraction method is included and all alleles for which the assay was designed to detect be verified before the introduction of the assay for routine use.
- Used DNA eluate from previously detected specimens with known genotypes and synthetic controls for those relatively rare mutations for which there is no control (Synthetic oligonucleotides – sequences complementary to the ASPE primers).

Luminex Home-brew Assay Development

Multiplex PCR

- Select which allelic variants are commonly associated with the condition of interest.
- Design and optimize the multiplex PCR (as previously discussed):
  - Locus must be within the amplified region with sufficient sequence to allow for later ASP extension.
  - More than one locus can be located with in the amplicon.
  - Include a minimum of 3 G-residues in the direction of extension to allow for incorporation of Biotin-dCTP.

Luminex Home-brew Assay Development

ASPE reaction primer design

- Choose the GSO sequence complementary to either the forward **or** reverse strand directly adjacent to and including the locus.
- Add a 5' TAG to the GSO sequence.
  (http://smartnote.miraibio.com/, PrimerPlex by Premier Biosoft)
- Only one primer per allelic variant
- Each ASP pair in the same direction.

Luminex Home-brew Assay Development

Optimizing MFI signal strength and allelic ratios

- Change the direction of the allele-specific primers.
- Lengthen or shorten one or both ASP.
- Select a hybridization bead with a different Hybridization Dose Response (HDR) value.
- Oligonucleotides complementary to the GSO region can be used to relatively evaluate signal strengths (also useful in evaluating new ASP and bead mix preparations).

Luminex Home-brew Assay Development

Additional considerations

- MagPlex microsphere bead mixes contain only those beads that are complementary to the ASP TAGs.
- Exonuclease I / Shrimp Alkaline Phosphatase (ExoSAP) is critical for removing residual primers and dNTPs'
- Heterozygote genotypes are essential for development and validation (homozygotes for both wt and mut are useful).
- Establish threshold values for determining genotype.

Allele Not Present	Heterozygote	Homozygote
0 to 0.24	0.25 to 0.74	0.75 to 1.00

Homebrew Assay Luminex® output.csv

Program	Luminex 100 IS												
Build	2.3												
Date	4/7/2008	4:11:50 PM											
SN	LX10004131305												
Session	Gal 9-variant assay												
Operator													
Samples	4	Min Even	0										
Results													
DataType	Median												
Location	Sample	01: Q188	02: Q188	03: K285	04: K285	05: S135	06: S135	07:F171S	08: F171	09: L195	10: L195	11: Y209	12: Y2
1	Syn Oligos	1774	2582	4773	9003	5602	7269	2211.5	5961	7497.5	1749	3792	365
2	+/+	1776	84	2589	68	5287	192	2012	143.5	5265.5	19	2605	
3	Q188R/+	1242	2273	1677.5	35	5769	171	2110	110	5110	18	2596	
4	Neg	13	6	10	18	12	7	9.5	5.5	11	8.5	7	14
DataType	Resul												
Location	Samp					S135		07:F171S	08: F171	09: L195	10: L195	11: Y209	12: Y20
1	Syn Oligos												
2	+/+												
3	Q188R/+												
4	Neg												
DataType	Count												
Location	Sample	01: Q188	02: Q188	03: K285	04: K285	05: S135	06: S135	07:F171S	08: F171	09: L195	10: L195	11: Y209	12: Y20
1	Syn Oligos	143	129	130	100	117	143	132	126	104	121	119	12
2	+/+	166	159	124	144	141	164	143	146	100	125	138	145
3	Q188R/+	142	135	113	132	110	145	141	134	113	100	138	129
4	Neg	131	107	117	102	124	124	130	116	100	112	121	132

Wt RawMFI

Mut RawMFI

Signal Detected

No Signal Detected

No Template/Background

Bead Count ≥ 100 Events

Galactosemia 9-Plex Assay

MFI Signal for Clinical Samples and Synthetic Controls

Sample	Allele	Q188R	K285N	S135L	F171S	L195P	Y209C	N314D	T138M	IVS2-2A>G
synth.oligo#1	Normal	2026	2194	5398	3900	2155	1789	3267	1753	1021.5
	Variant	1966	1967	4134	2494	1058	1249.5	1710	2800	789
synth.oligo#2	Normal	1928	2265.5	5435	3836	2058.5	1768	3401	1725.5	1029
	Variant	1879	2018	4001	2335.5	1062.5	1223	1628	2901.5	741.5
Gal501#1	Normal	2333	2341	7155.5	5180	4477	4029	3430	5257	3682.5
	Variant	3243.5	26	89	65	32	24	2836	90	71
Gal501#2	Normal	2380	2799	7166	4796.5	4383	4337	3669	5483.5	3796
	Variant	3411	34.5	95.5	54	37	24.5	2983.5	98	73
Reagent Blank	Normal	11	3	8.5	7.5	10	6	4	7.5	18
	Variant	8.5	7	12	14	12	2	4	0.5	9
+/+	Normal	3247	2684	7451	5036	4427	4254	5174	5792.5	3958.5
	Variant	112	39	92	49	31	32	57	131	75
Q188R/+	Normal	2563.5	2478	7304	4409.5	4200	3991.5	4861	5755.5	3813
	Variant	2763.5	35.5	89.5	53	24.5	29	57	110	78
No Template Control	Normal	12.5	8	8	12	16	9	11	11	6
	Variant	17	8.5	15	9	8	6	12	13	15.5

Galactosemia 9-Plex Assay

Assay Development for the MagPlex Microspheres

- Selection of the Luminex technology:
  - Offered user flexibility to add/remove variants as needed or to detect variants previously not available with other assays.
  - Ability to multiplex (or not).
  - Computer-generated data facilitating QA/QC and for LIMS-based reporting.
  - A single-platform assay for the detection of numerous conditions, some of which might be grouped into single reactions.
- Luminex CFTR version 2 assay has a shortened protocol using T*fi* DNA polymerase. This did not work with for our homebrew assays. So, even though we are using the same technology, the CFTR and homebrew assays will be somewhat different.

Galactosemia 9-Plex Assay

Assay Development for the MagPlex Microspheres

Galactosemia-9 Assay	Tot Bases	%GC full Seq	Tm Full Seq	GSO Bases	%GC GSO	ºC Tm GSO	Bead Lot Number	Bead HDR value	HDR Ratio	MFI Result (Taq,Tsp)	Allelic Ratio
F171S(R)-Mut(73)	52	38.5	65.4	28	50	62.1	B23357	8602	0.51	1390.5	0.47
F171S(R)-Wt(77)	52	36.5	65.6	28	46.4	61.9	B23743	8235	0.49	1558.5	0.53
IVS2-2A>G(F)-Mut(62)	42	38.1	62.1	19	47.4	51.9	B23355	6009	0.47	2435.5	0.60
IVS2-2A>G(F)-Wt(14)	43	34.9	62.3	18	47.4	51.9	B25386	6817	0.53	1592.5	0.40
K285N(F)_Mut(48)	48	31.3	62.8	24	37.5	54.5	B25420	7829	0.52	4168.5	0.52
K285N(F)_Wt(67)	47	34	62.7	22	43.5	54.9	B23353	7234	0.48	3817	0.48
L195P(R)-Mut(56)	48	43.8	65.4	24	62.5	64.1	B23352	7533	0.50	2246	0.51
L195P(R)-Wt(64)	44	38.6	65.2	20	55	59.6	B23354	7416	0.50	2194.5	0.49
N314D(R)-Mut(25)	48	41.7	65.5	24	58.3	64.3	B245418	7711	0.47	1027.5	0.38
N314D(R)-Wt(66)	48	39.6	65.8	25	54.2	64	B23174	8586	0.53	1673	0.62
Q188R(R)-Mut(22)	50	46	67.6	26	65.4	66.2	B24429	7968	0.53	3633	0.54
Q188R(R)-Wt(28)	52	46.2	67.7	28	64.3	68.2	B23509	7019	0.47	3127	0.46
S135L(R)-Mut(74)	44	38.6	64.6	20	55	57.5	B23740	7595	0.44	1610	0.30
S135L(R)-Wt(45)	44	40.9	64.2	20	60	58.5	B23176	9733	0.56	3839.5	0.70
T138M(R)-Mut(78)	43	39.5	64.2	19	57.9	59.5	B23744	6637	0.52	3641.5	0.63
T138M(R)-Wt(43)	43	41.9	64.1	19	63.2	60.6	B23178	6062	0.48	2173	0.37
Y209C(F)-Mut(52)	44	43.2	64.9	20	65	60.5	B23502	8122	0.51	3044	0.50
Y209C(F)-Wt(65)	44	40.9	64.4	20	60	58.9	B25763	7899	0.49	3057	0.50

Galactosemia 9-Plex Assay

Assay Development for the MagPlex Microspheres

- Adding or removing bases from the gene-specific region to try improving the signal intensity of the S135L mutation

ASPE Primer	Bead Number	Bead Lot Number	Bead HDR value	HDR Ratio	MFI Result (Taq,Tsp)	Allelic Ratio
S135L(R)-Mut(74)	74	B23740	7595	0.44	1610	0.30
S135L(R)-Wt(45)	45	B23176	9733	0.56	3839.5	0.70
S135L(R)-Mut(74)+5	74	B23740	7595	0.44	1742	0.35
S135L(R)-Wt(45)-2	45	B23176	9733	0.56	3214.5	0.65
S135L(R)-Mut(74)	74	B23740	7595	0.44	940.5	0.26
S135L(R)-Wt(45)-2	45	B23176	9733	0.56	2634.00	0.74
S135L(R)-Mut(74)+5	74	B23740	7595	0.44	1814	0.31
S135L(R)-Wt(45)	45	B23176	9733	0.56	4074	0.69
S135L(R)-Mut(30)+5	30	B23511	9862	0.63	4637	0.62
S135L(R)-Wt(36)+7	36	B25412	5748	0.37	2847	0.38

Summary of xTAG Home-brew Assay Development

- PCR primers have a few rules specific to performing an ASPE reaction
- ASPE primers have unique design guidelines
- QA/QC of signal data performance evaluation
- Allele signal ratios are used to determine genotype
- Strategies to improve MFI signals and resulting allelic ratio
- MFI signals, “no allele present” background, and allelic ratios must be reviewed for each set of results and compared with previous data periodically

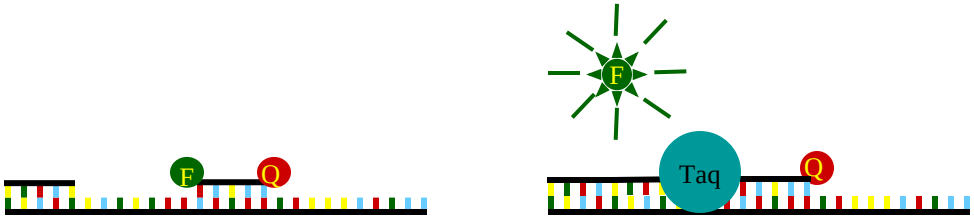
Luminex xTAG® Technology

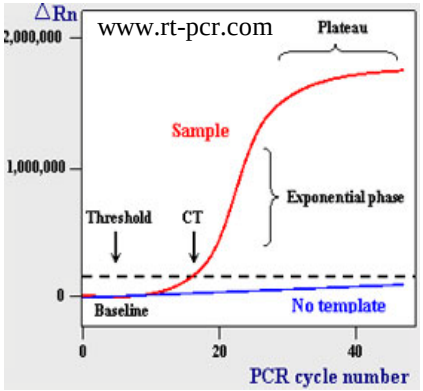
Advantages	Disadvantages
• Significantly reduces cost and labor with high multiplexing capability and assay flexibility.	• Cost per assay compared to real-time PCR (which has limited multiplexing capability).
• Can combine and multiplex any set of 100 DNA tests simultaneously in a single reaction.	• End-point assay - longer amount of time from sample-in to results-out compared to real-time PCR.
• Liquid bead reaction kinetics assure fast and easy assay optimization under universal hybridization conditions assuring more reproducible results.	
• Multiplexing requires smaller sample starting material.	
• Easy to develop, optimize, and expand assays.	• Changes in xTAG bead HDR values from lot to lot can affect allelic signal ratios (MagPlex®-TAG™ beads have HDR ±2.5x range).
• Easy to troubleshoot.	

Absolute Quantitative Real-Time PCR

Absolute Quantitative Real-Time PCR (qPCR) using Taqman Technology

- qPCR - The detection and simultaneous quantification of one or more target sequences.
- Quantification using DNA Taqman (hydrolysis) probes labeled with a fluorescent reporter and quencher. (FRET = Förster or fluorescence resonance energy transfer.)
- After specific hybridization of the primers and probe with the target, polymerization releases the fluor and the fluorescent signal increases in direct proportion to the amount of PCR product





qPCR Application for Population-based Newborn Screening

- Severe Combined Immunodeficiency – an ideal example
  - There are multiple loci
  - Some genes have yet to be identified
  - All SCID have low or absent autologous T cells
  - There is a surrogate marker for recent thymic emigrants, the T-cell receptor excision circle (TREC)
- To date, all SCID NBS applications use Absolute Real-Time qPCR.

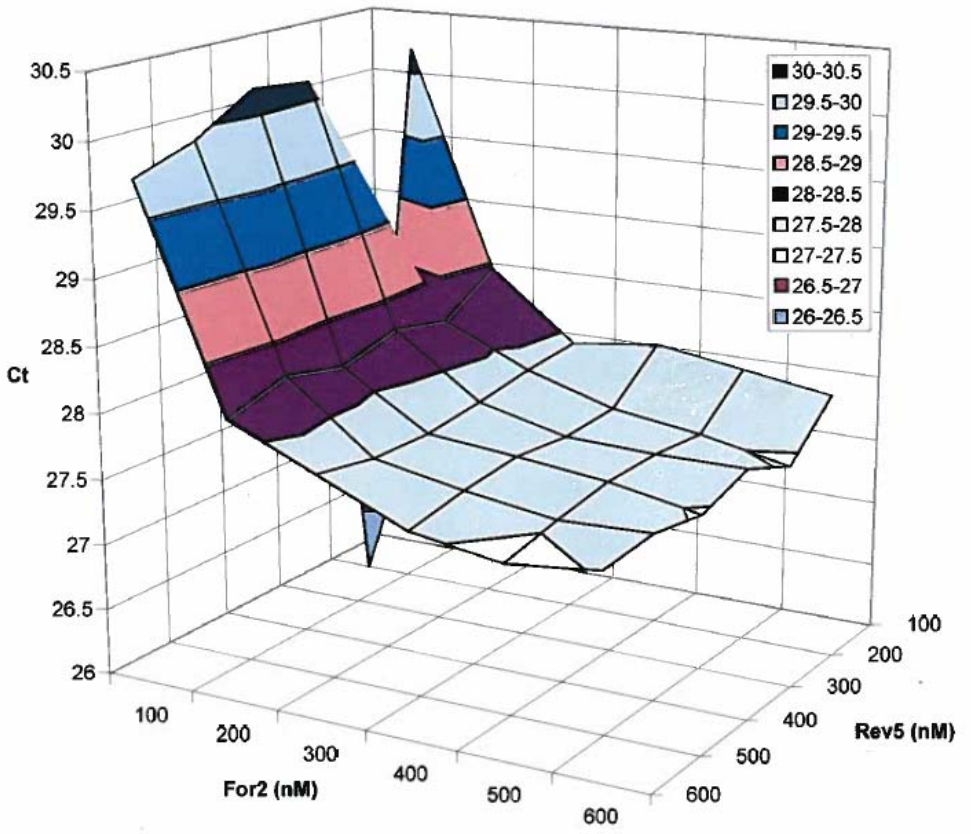
Rationale for Design of Massachusetts' Multiplexed Absolute qPCR for SCID NBS

- We are looking for absent to low TREC:
  - need for contamination control
  - Need calibration curve with reliable low limit of quantification.
- Application is to dried blood spot:
  - need for measures of consistency
  - Need measures of DNA quality – internal control
- Molecular assays are expensive:
  - look to the future, one extraction for multiple assays (lower reagent and labor costs).

Development, Verification and Validation of the Homebrew SCID Assay

- Design of primer/probe set against published target sequence.
- Selecting an appropriate reference gene (RNase P, B-Actin, etc.).
- Accurately quantitated calibrators for each target.
- Slope –3.1 to –3.6 (ideal –3.323), Correlation Coefficient (R^2) $\geq$0.99.
- Accuracy, Precision, Sensitivity, and Dynamic Range.
- Each primer/probe set should be optimized in single-plex.
- Primers/probes are tested in the multiplex to assure accuracy, precision, specificity and dynamic range for each target is maintained.
- Both target fluorescence should cross the Cq, adjusting concentrations to prevent the more abundant gene from consuming limited reagents.

Primer Limiting Matrix for Multiplex qPCR



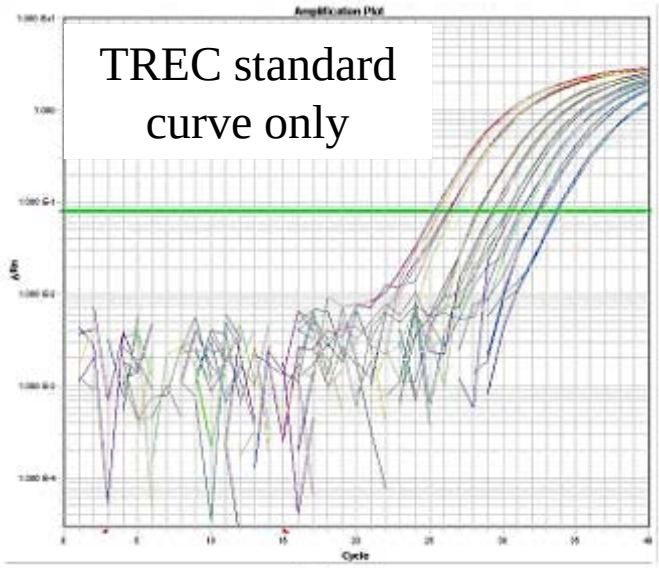
- The goal of primer-limiting the assay is to find the primer concentration that gives the lowest (earliest) possible Ct value for the more abundant target without distorting the Ct value of the less abundant target.[1]
- Limiting the primer concentration for the more abundant target has the effect of lowering its ΔRn; however, the Ct should remain unchanged under primer-limited conditions. [1]

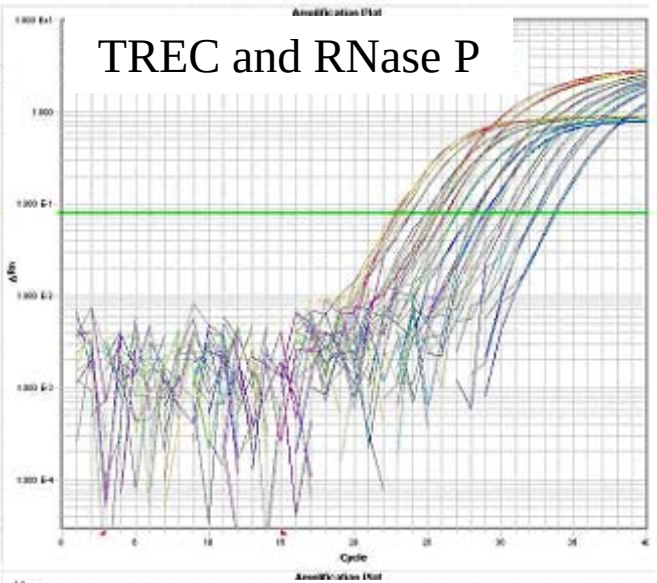
[1] Life Technologies Application Note: Factors Influencing Multiplex Real-Time PCR

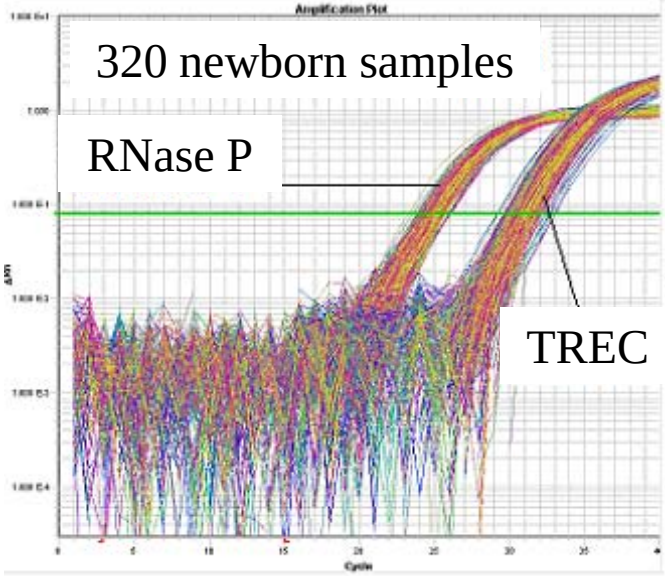
Development, Verification and Validation of the Homebrew SCID Assay

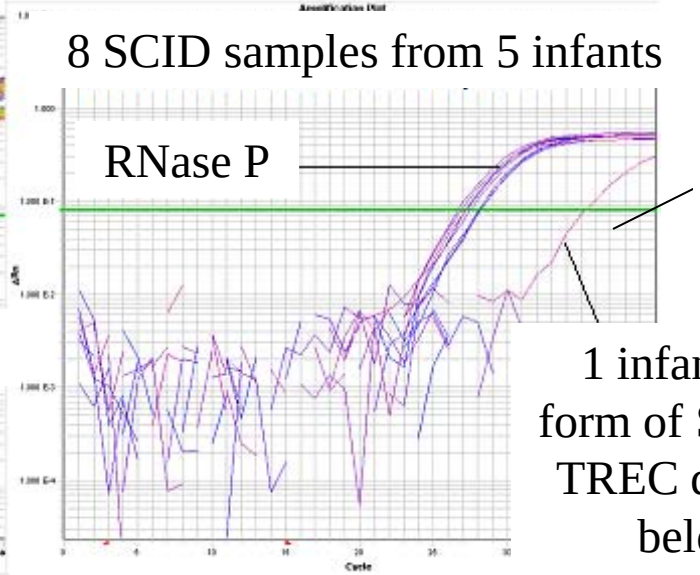
- Once the assay was been developed and verified, validation was done using unlinked newborn samples.
- 4960 samples were tested for assay validation.
- Known SCID babies were included to demonstrate accuracy.
- Samples were tested multiple times and across instruments to show reproducibility.

Amplification Curves for the Calibrators and Samples







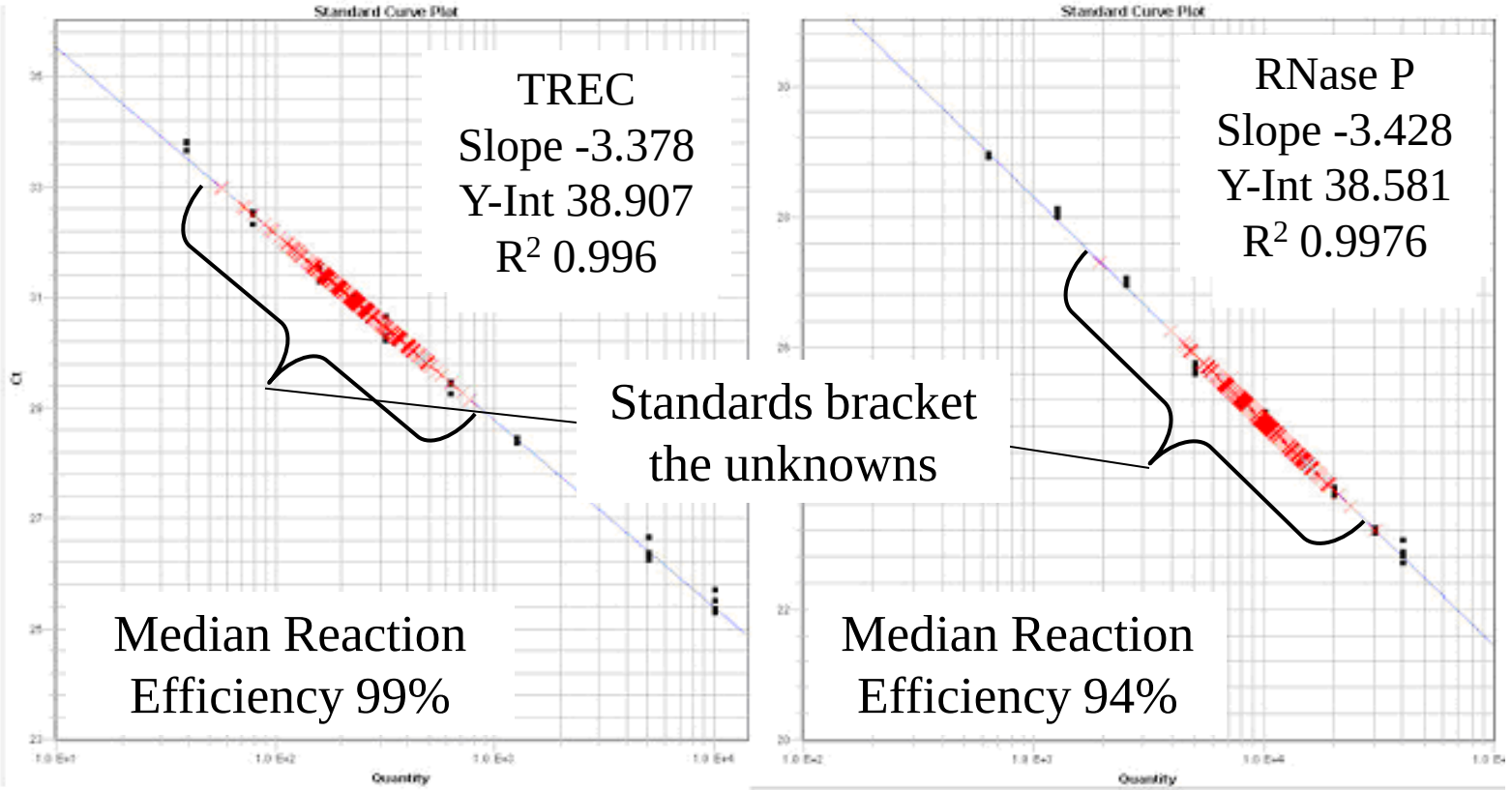


7 samples from 4 infants with no TRECs detected

1 infant with PNP* form of SCID showing TREC detection well below cutoff

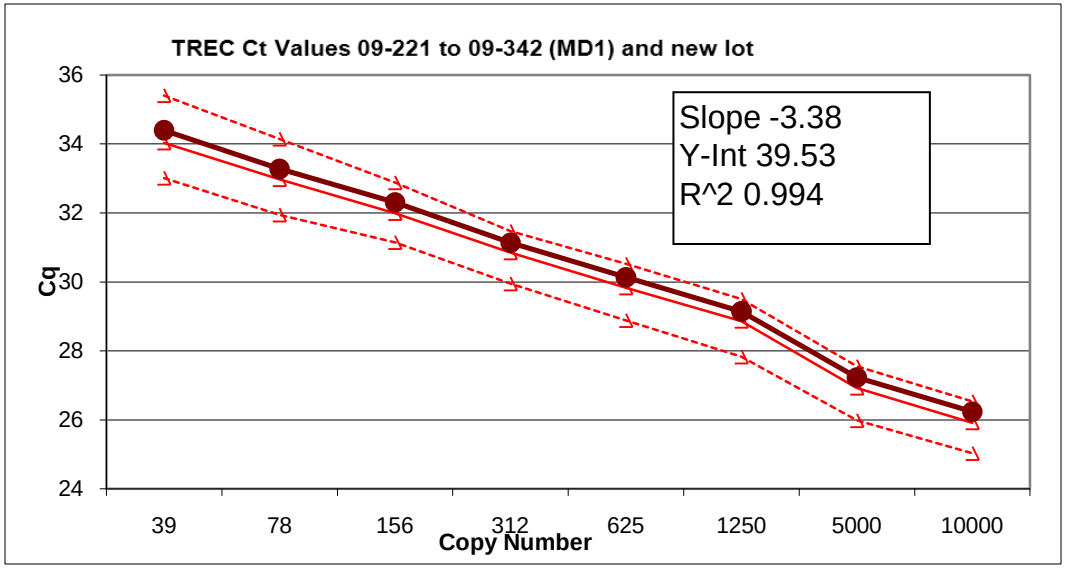
*PNP = purine nucleoside phosphorylase deficiency

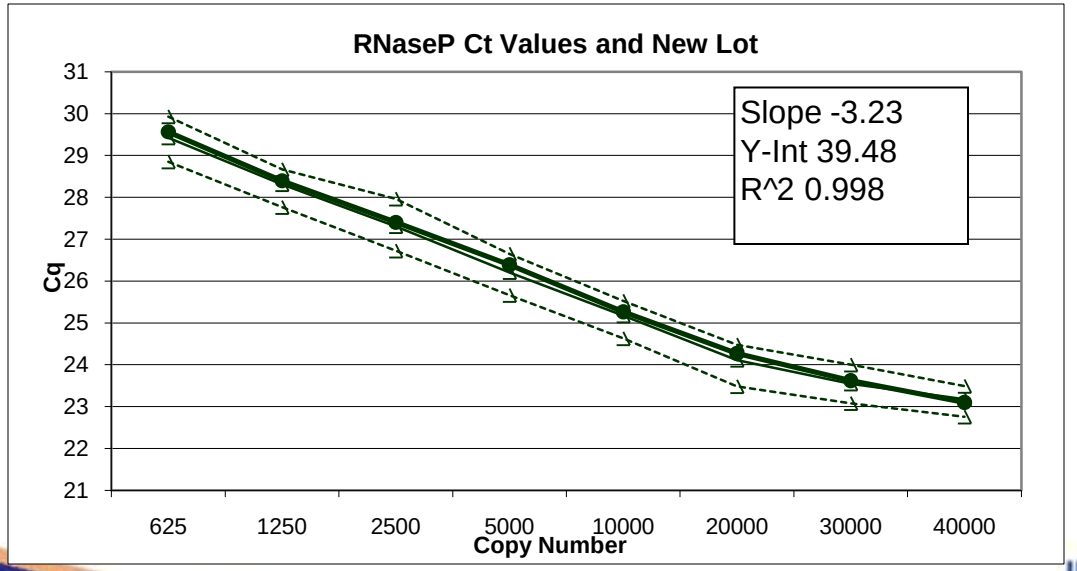
Calibrator Regression Plots and Sample Data



- 8-point standard curve run in quad (32 datapoints)
- Up to 6 datapoints may be dropped (at least one 39 copy must remain) to achieve a TREC slope nearing –3.323 (100% Efficiency)
- Slope –3.100 –3.600, R^2 $\geq$0.97, Y-Intercept $\geq$36 to $\leq$41

Quality Control of the qPCR Calibration Curve





- The Cq values from Daily qPCR runs are recorded for monitoring quality control.
- These values are used to monitor new calibration curve preparations.
- New calibration curve preparations are plotted on graphs from daily runs that were done when the assay was first started.
- Assures that there is no Cq drift over time.

Quality Control of the qPCR Calibration Curve

Cq Values of the 4 Common Datapoints				
TREC	**625**	**1250**	**5000**	**10000**
1	29.36	28.08	26.41	25.41
2	29.50	28.28	26.35	24.02
3	29.60	28.49	26.44	25.50
4	29.43	28.33	26.27	25.26
average	29.47	28.30	26.37	25.05
RNaseP	**625**	**1250**	**5000**	**10000**
1	28.97	28.12	25.90	25.06
2	28.88	27.77	25.82	24.89
3	29.38	28.19	26.24	25.34
4	29.26	27.94	25.91	25.12
average	29.12	28.00	25.97	25.10
Absolute Difference of the 4 Common dp				
average	0.35	0.29	0.40	0.06

- The NENSP multiplex SCID assay calibration curve has four common datapoints (input copy numbers).
- In a qPCR that is running at or near 100% efficiency the amount of amplified product doubles after each cycle.
- Each doubling results in a 1 Cq increase.
- In a 100% efficient reaction, the same input copy numbers should have Cq values within 1 Cq of each other.
- For each two-fold dilution the Cq value should differ by one Cq.

Real-Time Quantitative PCR

Advantages	Disadvantages
Quantitative results in real-time	Limited multiplexing capability
Closed tube, reduced risk of contamination	Can be complex to set up, particularly for multiplexed reactions
Rapid cycling time (30 minutes to 2 hours)	
Highly sequence specific	Intra- and inter-assay variation, hence the need for an internal monitoring control

Thank you!