Molecular Assessment of Dried Blood Spot Quality during Development of a Novel Automated *in situ* TREC qPCR Assay for SCID Screening

J Bai, T Henry, J Benfer, S Berberich, T Kreman, and L DesJardin

State Hygienic Laboratory at the University of Iowa

Assay Background

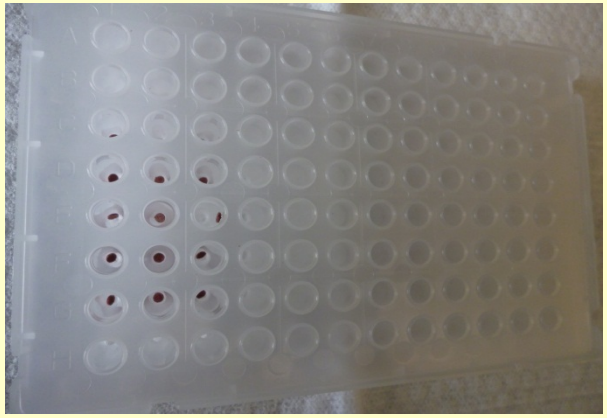
On-card/ *in situ* format adapted from CDC – DBS punched directly into 96-well PCR plate for processing and amplification

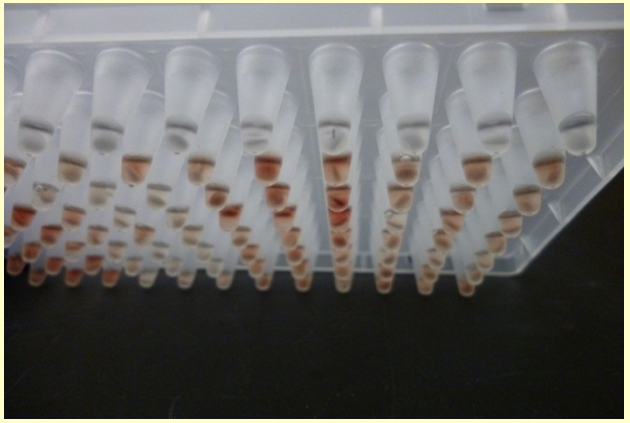
Automated wash of DBS to reduce PCR inhibitors

Automated addition of PCR master mix

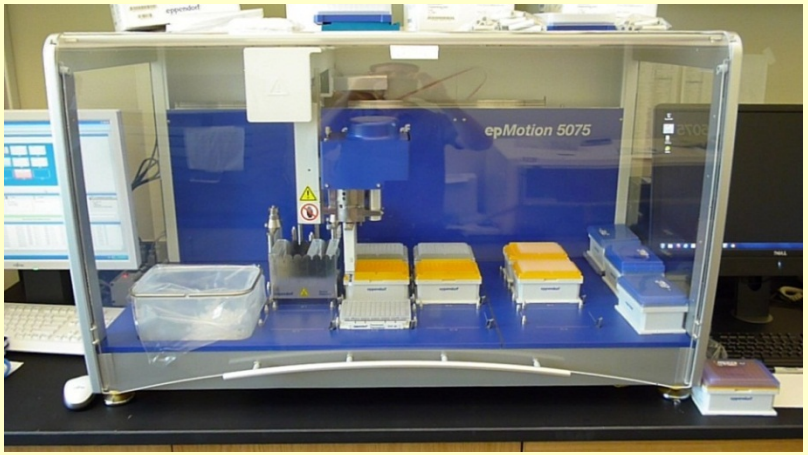
Lab prepared DBS calibrators and TREC plasmid added for QA-QC

For more detail, visit our poster "A Novel, Automated Cost Effective *in situ* TREC qPCR Assay for Newborn SCID Screening".

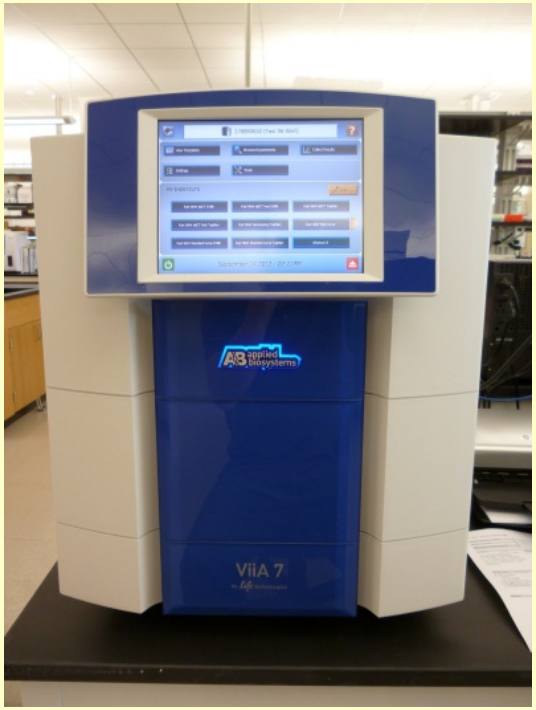




Assay Optimization



DNA Extraction/DBS Wash
Separate Extraction Step
Current PHL Methods
Commercial Products
LifeTech Sample to SNP
Qiagen Generation
CDC on-card/in situ Method
epMotion 5075 LH automated DBS wash
novel buffer



PCR Amplification
Master Mix*
Primers
Probes
Fluorophores
RNP kits

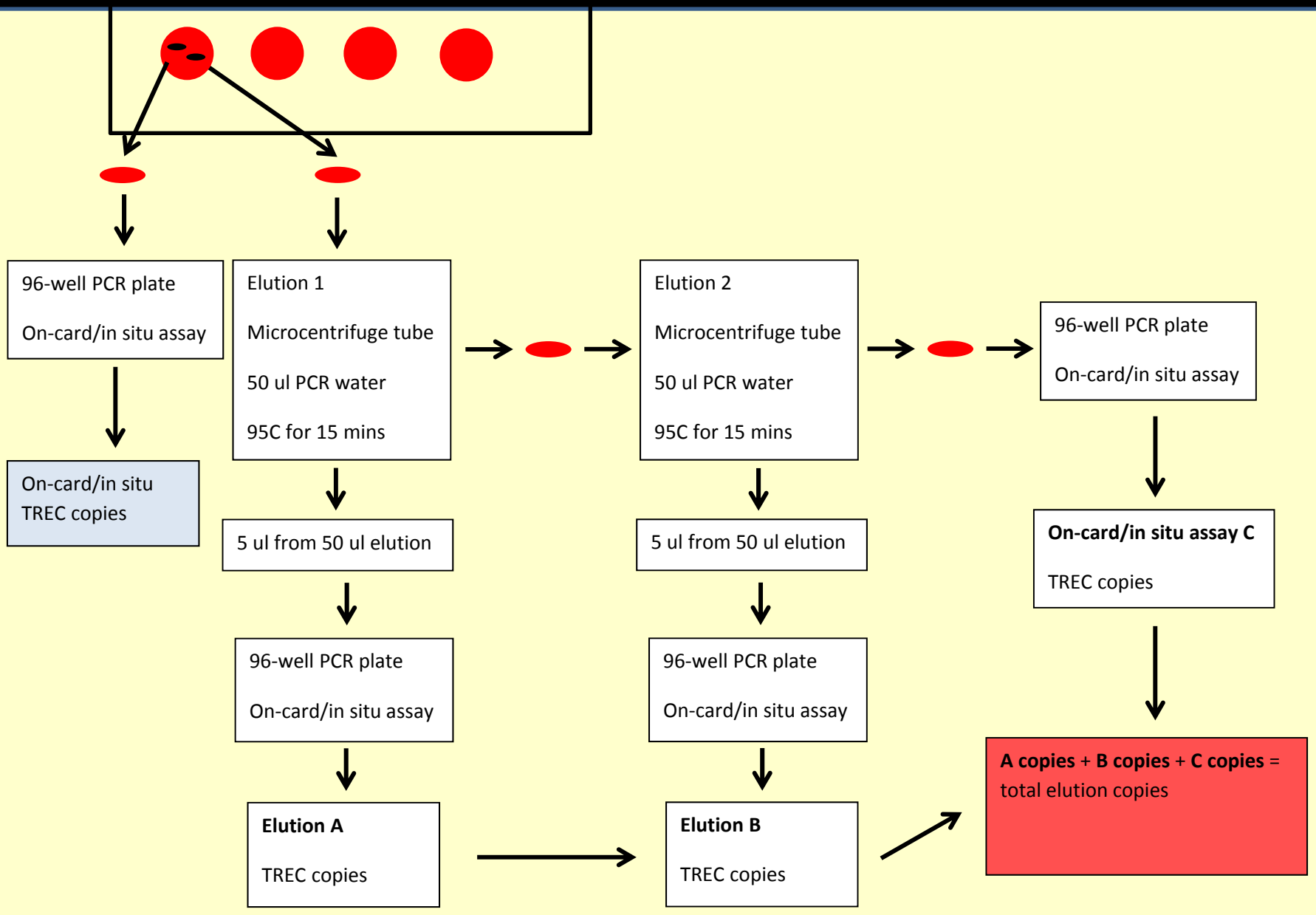
Cycling Parameters
Standard vs Fast Ramp
Extension Times
Pre-PCR 95^{o}C Incubation Time

Specimen Types

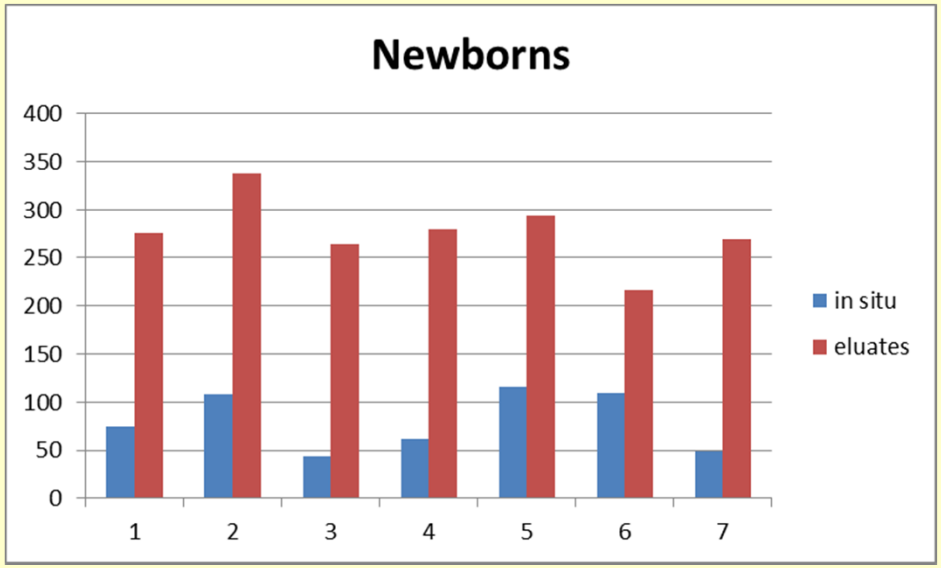
- Early *in situ* assay development experiments suggested minimal DBS washing needed; however, these experiments were performed with laboratory prepared cord-blood derived calibrators.

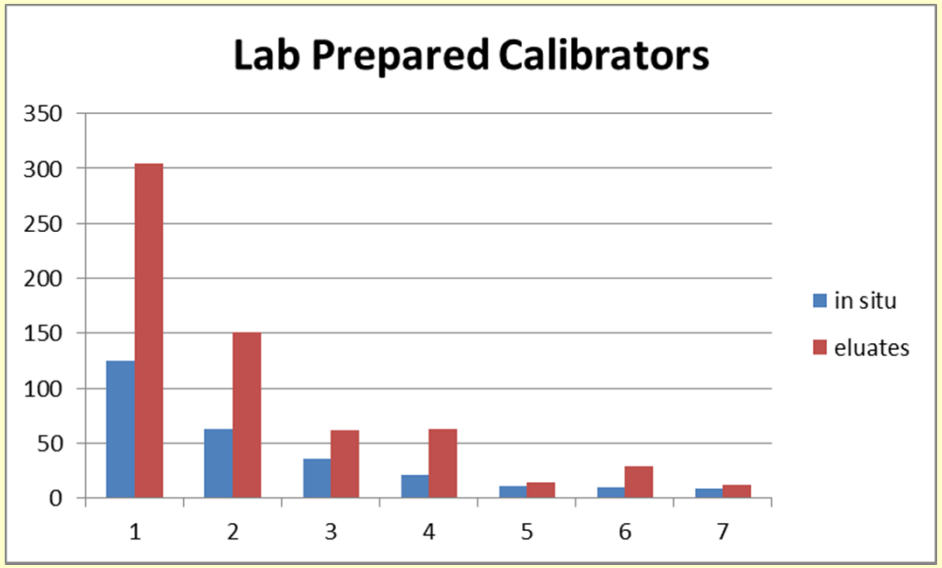
- Subsequent experiments with newborn DBS revealed significant differences between newborn DBS and lab prepared calibrators which directly impacted DNA extraction/DBS wash procedures.

DNA Recovery and Access Comparison



DNA Recovery and Access Comparison



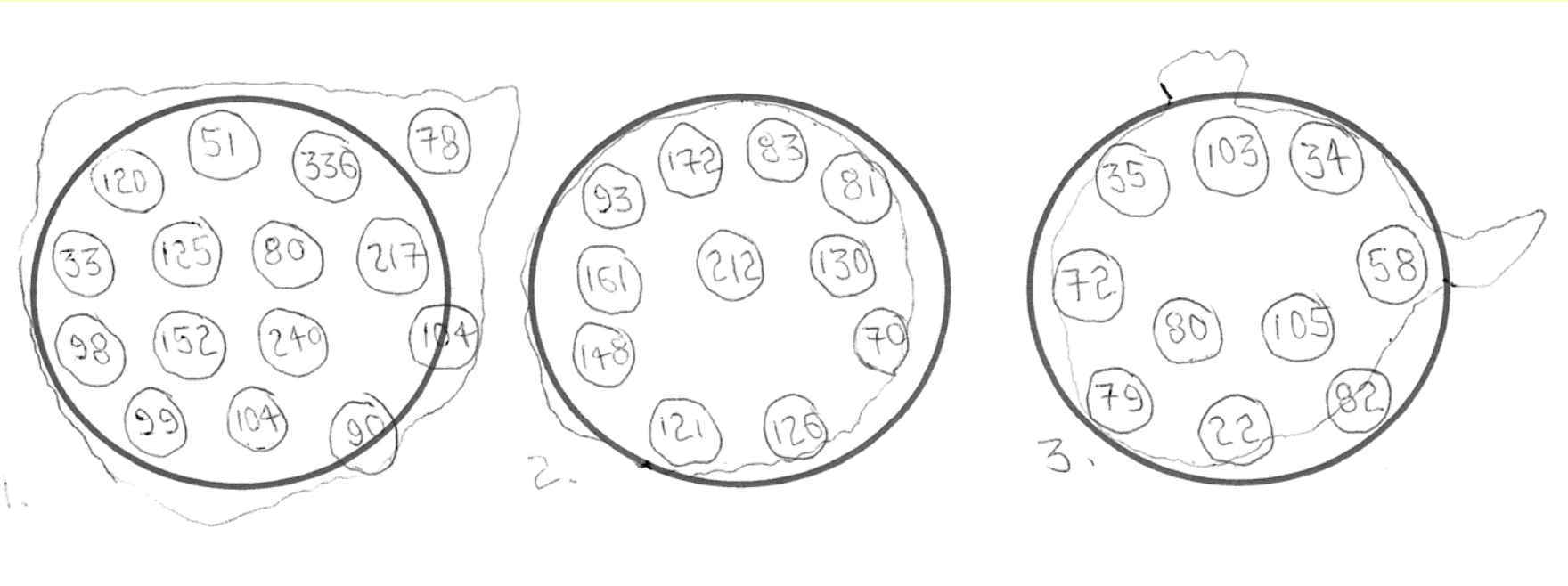


Newborn DBS	1	2	3	4	5	6	7
in situ	75	109	44	62	115	109	49
eluates	276	337	264	279	294	217	269
percent *in situ* access	27.16%	32.18%	16.57%	22.06%	39.27%	50.51%	18.17%
Lab Prepared Cals	1	2	3	4	5	6	7
in situ	126	63	36	21	11	10	9
eluates	305	151	62	63	14	29	12
percent *in situ* access	41.17%	41.71%	58.68%	33.26%	76.15%	34.48%	69.62%

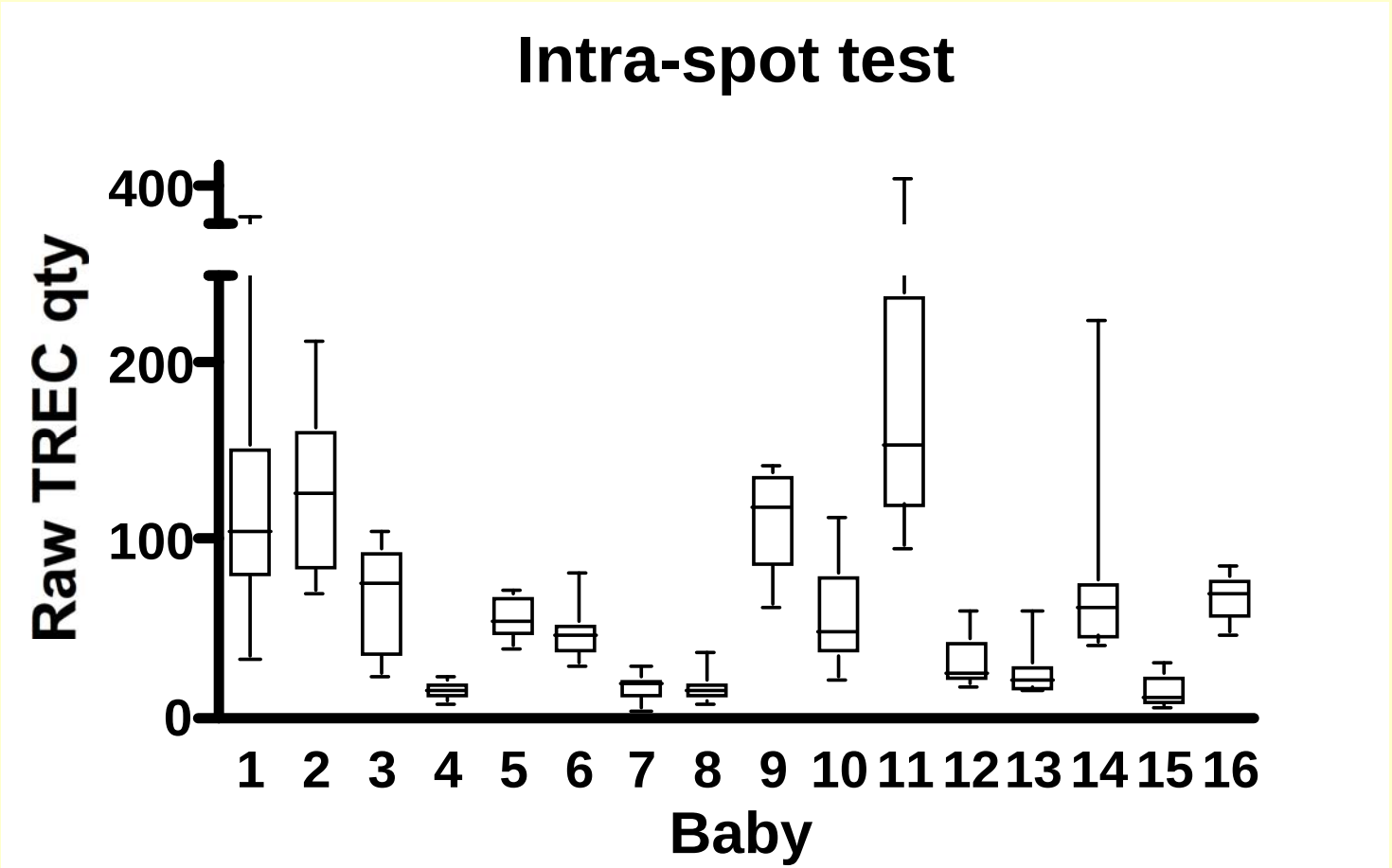
	Newborns	Lab Prepared Calibrators
Average recovery	29.42%	50.72%

Intra-DBS TREC Variation

Multiple 2mm punches sampled from 16 newborn DBS to determine potential variation in TREC amount across the DBS.



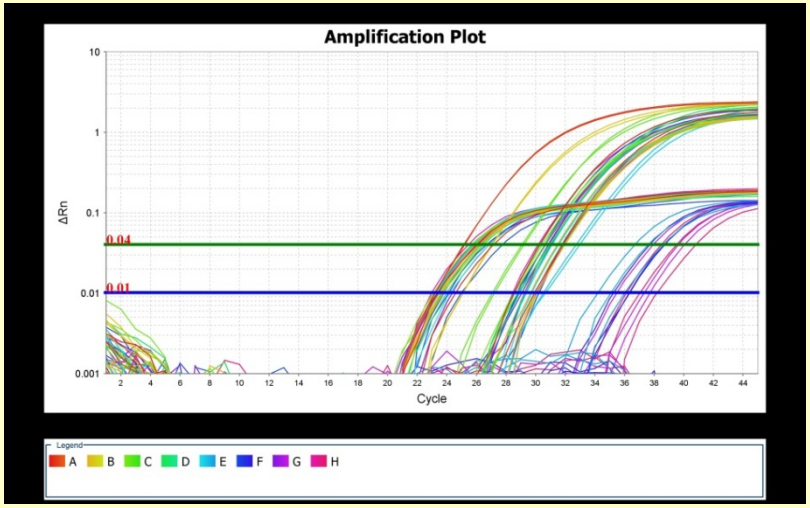
Intra-DBS TREC Variation

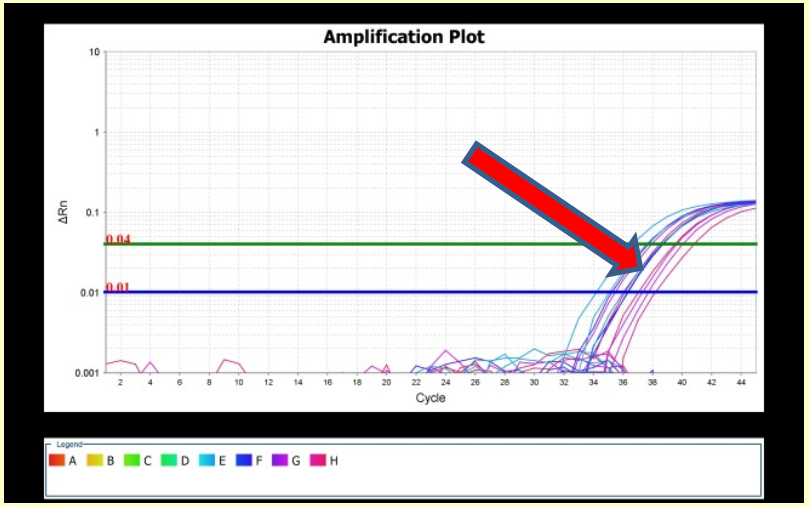


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Punches	15	11	10	13	14	11	12	11	12	13	11	15	10	15	12	11
Median	104	126	75	15	55	45	18	16	119	49	153	25	20	62	12	69
Std. Dev.	80	44	29	4	12	14	7	9	27	26	94	13	13	46	9	13

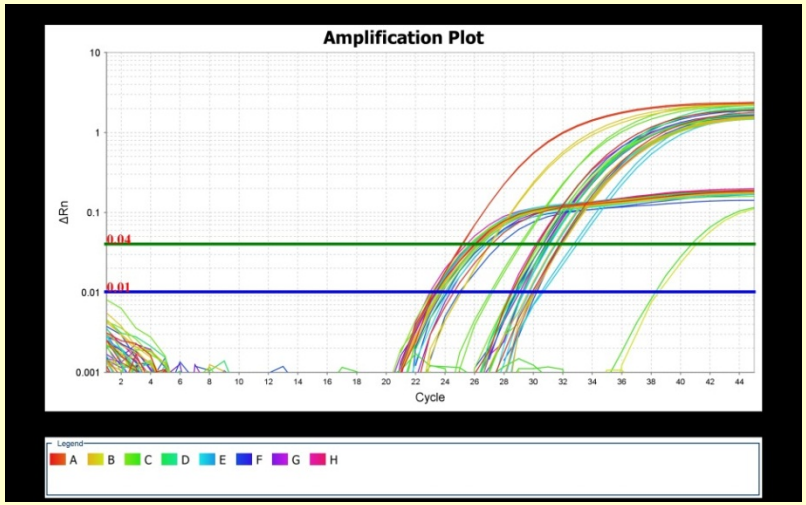
DNA Carryover during DBS Punching

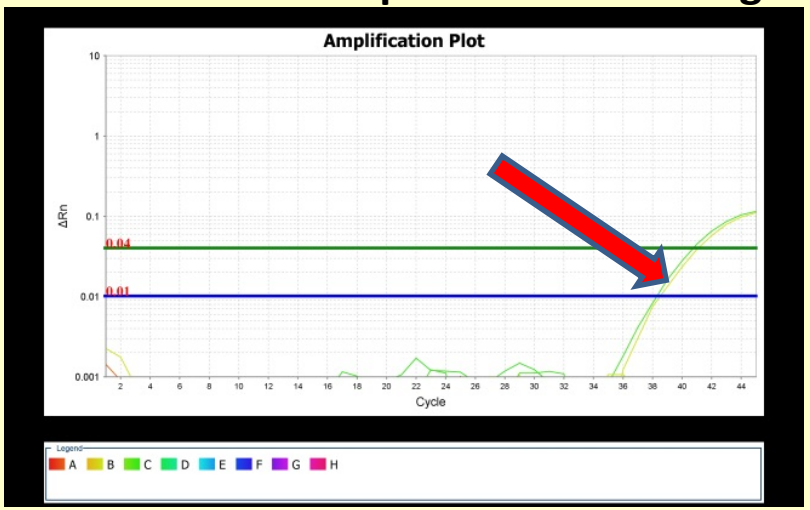
Hand punch used to punch blank Guthrie card after newborn samples without bleaching



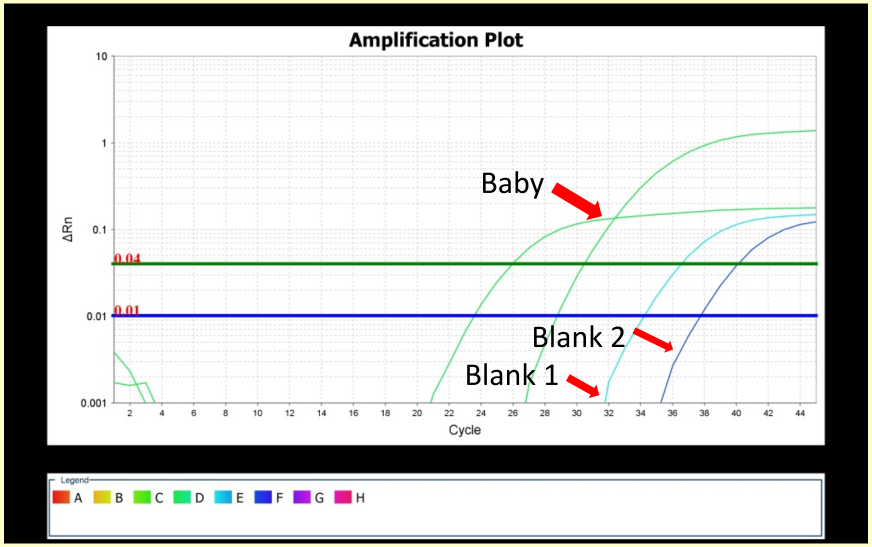


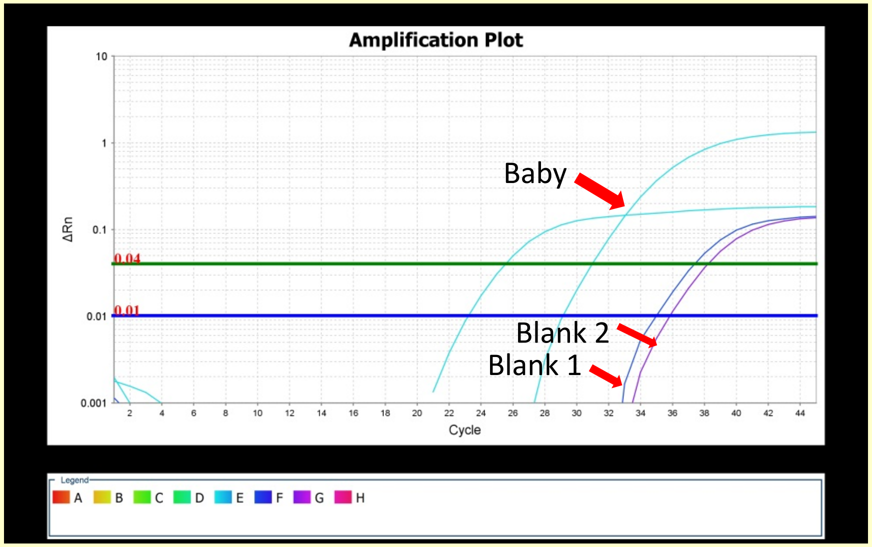
Hand punch used to punch blank Guthrie card after newborn samples with bleaching

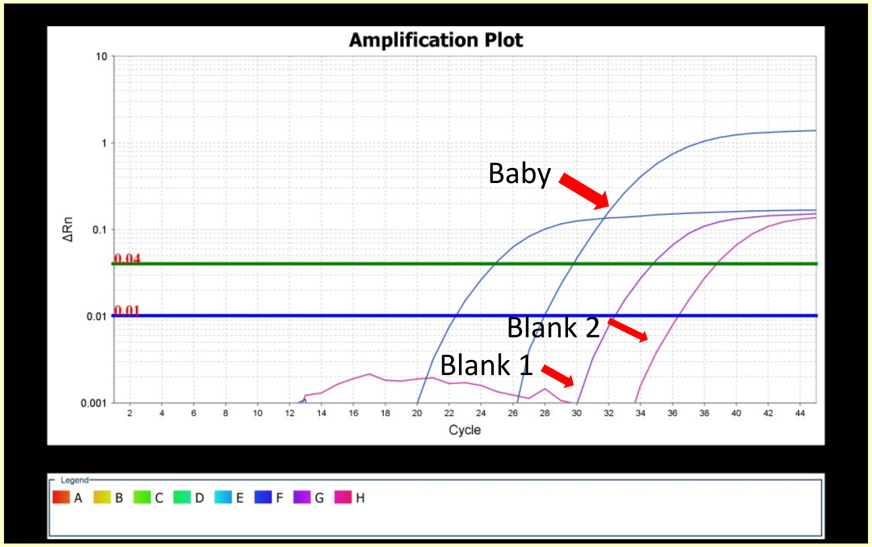


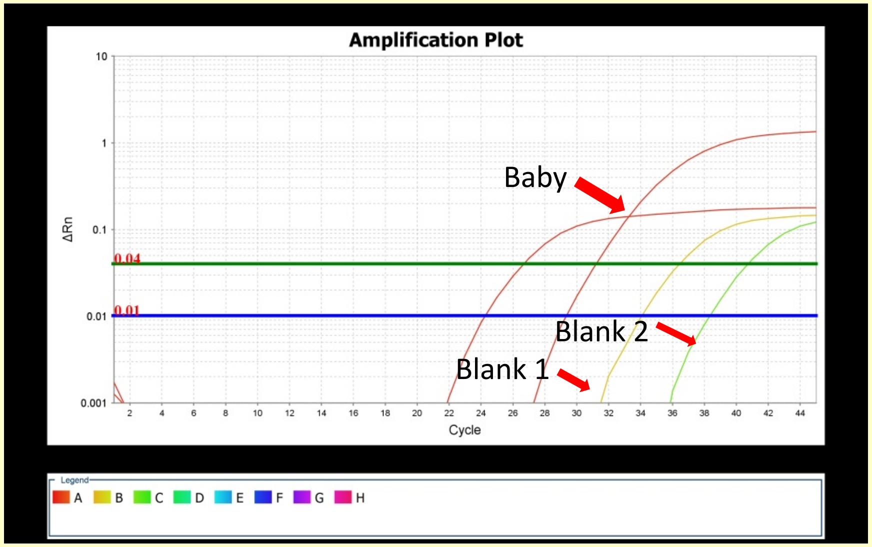


DNA Carryover



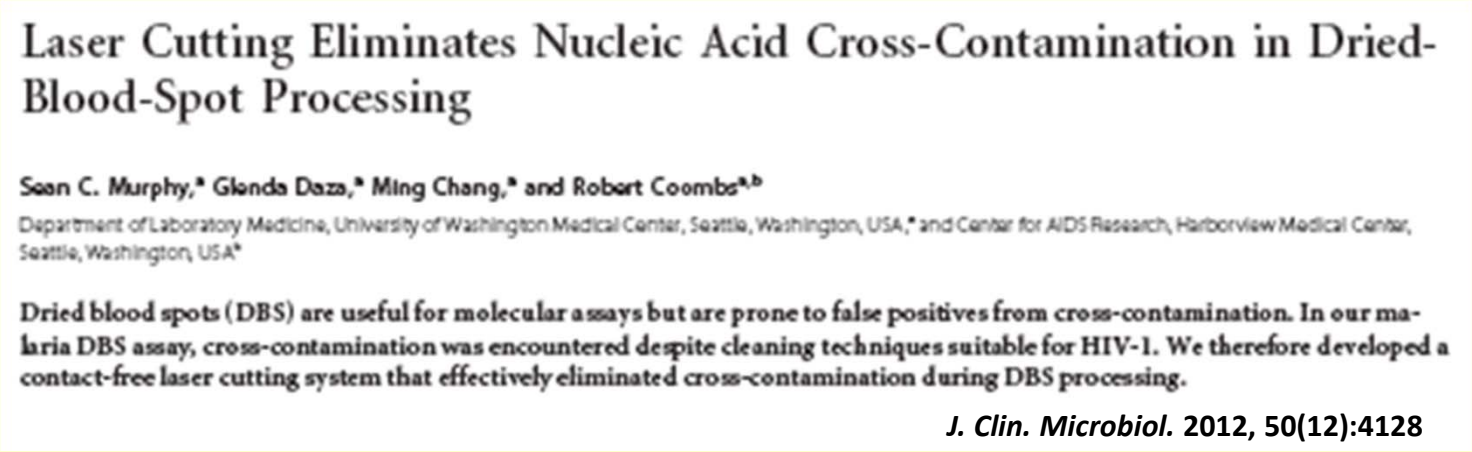






DNA Carryover

- Low level RNP contamination of blank punches occurs when using a multi-puncher. The Ct values range from ~31 to 44. TREC contamination was observed in ~5% of the blank punches, with a Cq value of range from ~36 to 44.
- SCID screens which use separate DNA extraction step may not detect DNA carryover.



Laser Cutting Eliminates Nucleic Acid Cross-Contamination in Dried-Blood-Spot Processing

Sean C. Murphy,[a] Glenda Daza,[a] Ming Chang,[a] and Robert Coombs[a,b]

Department of Laboratory Medicine, University of Washington Medical Center, Seattle, Washington, USA,[a] and Center for AIDS Research, Harborview Medical Center, Seattle, Washington, USA[b]

Dried blood spots (DBS) are useful for molecular assays but are prone to false positives from cross-contamination. In our malaria DBS assay, cross-contamination was encountered despite cleaning techniques suitable for HIV-1. We therefore developed a contact-free laser cutting system that effectively eliminated cross-contamination during DBS processing.

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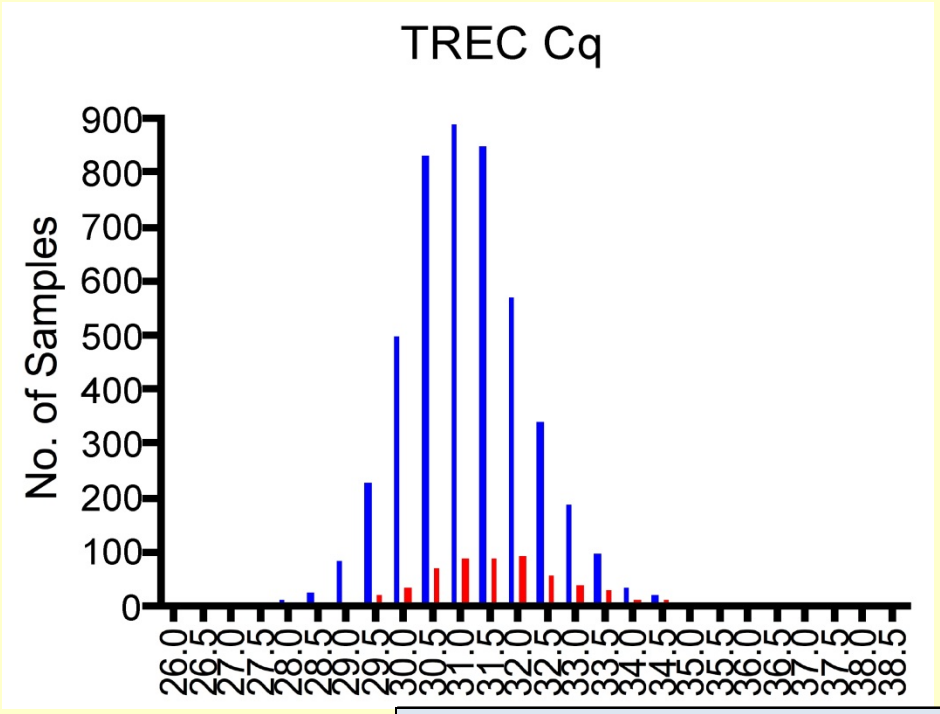
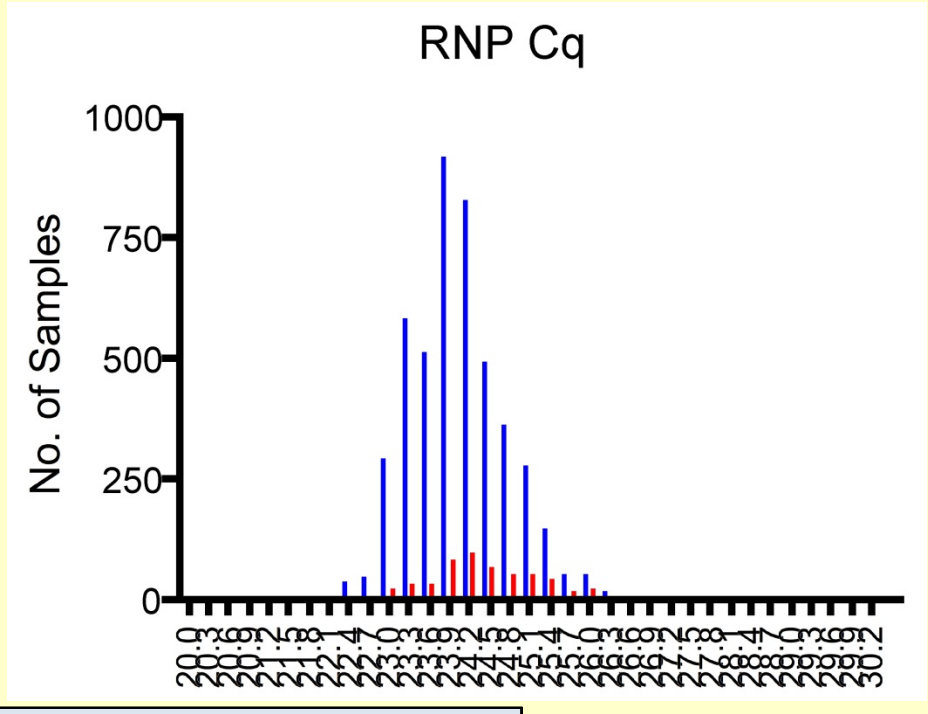
Newborn Data Overview

	TREC Cq		RNP Cq	
	ALL NEWBORN	PRE-TERM	ALL NEWBORN	PRE-TERM
Number of values	4644	537	4644	537
Minimum	27.68	27.80	20.96	22.38
Median	31.13	31.56	24.03	24.32
Maximum	38.45	36.90	30.27	27.40
Std. Deviation	1.065	1.249	0.739	0.860

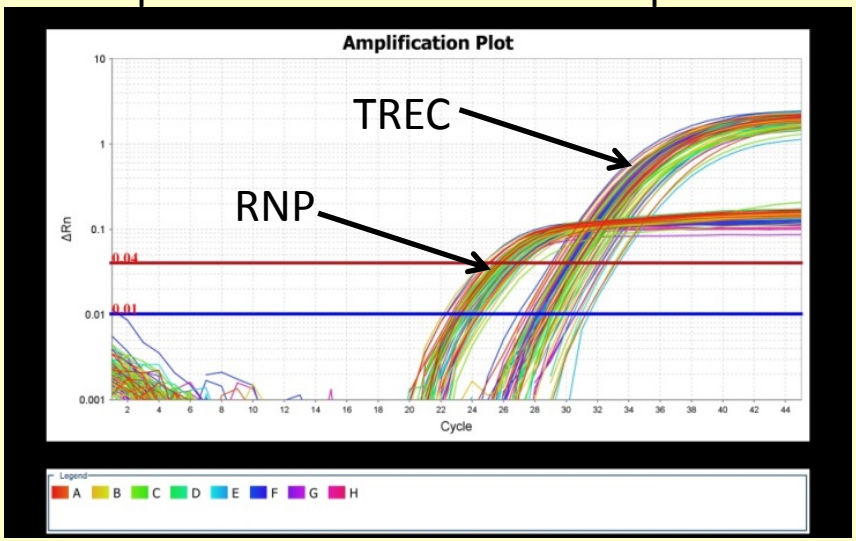




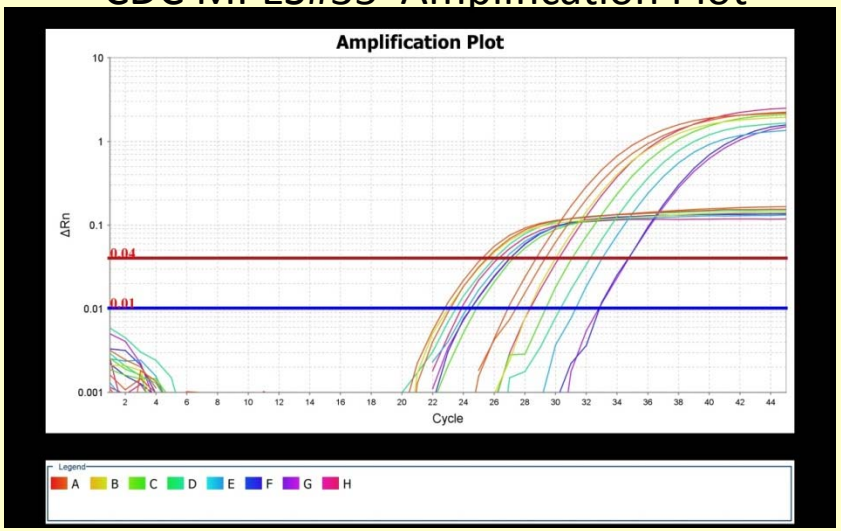
Current Blinded Pre-Pilot Results		
TREC below cut-off	39 of 4644	0.84%
TREC below cut-off with valid RNP	4 of 4644	0.09%

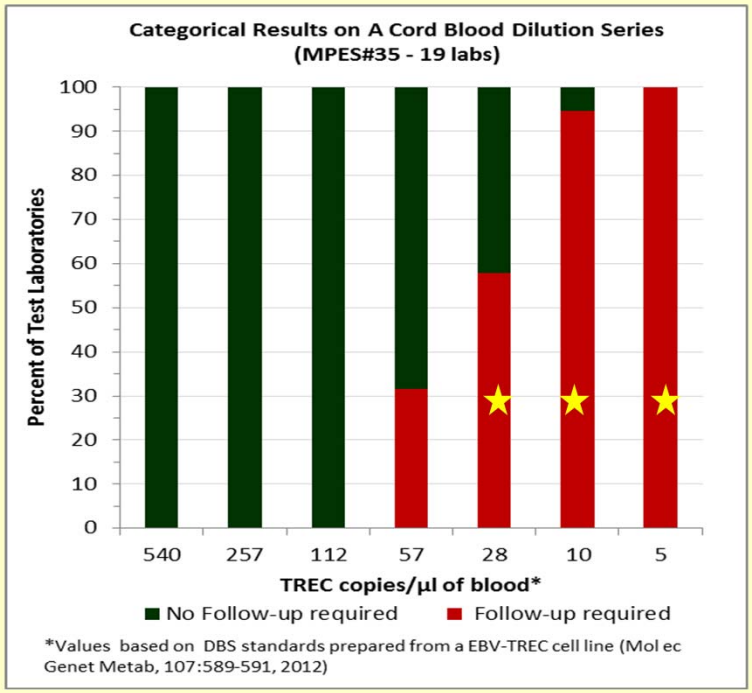
Model Performance Evaluation Surveys

Representative Newborn Amp Plot



CDC MPES#35 Amplification Plot

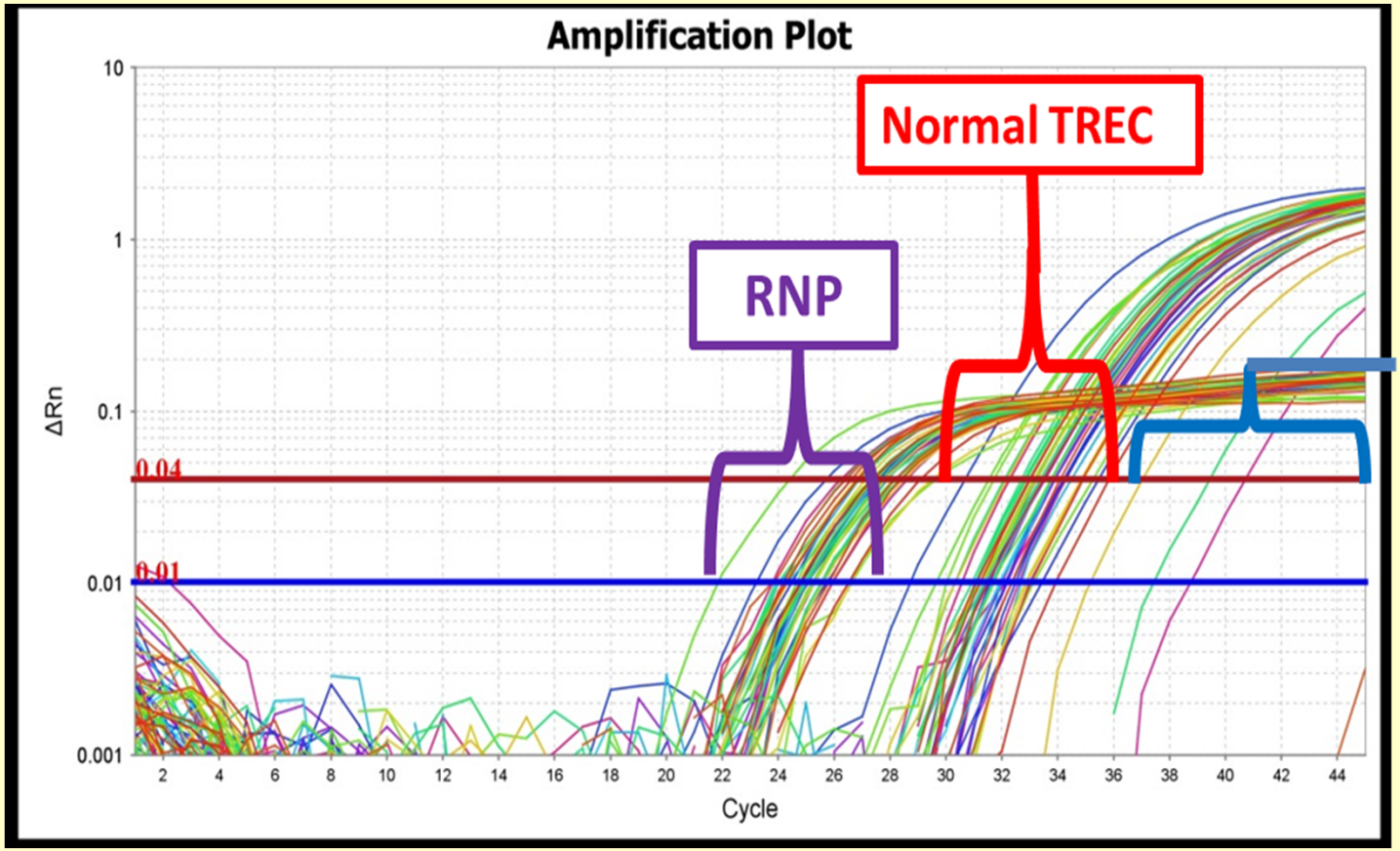




★ Survey sample identified by our assay as needing clinical follow-up*

Graph provided by Francis Lee,
Centers for Disease Control and Prevention

Primary Immunodeficiency Patient Samples



DBS from babies with known T Cell lymphopenia or SCID

Diagnosis	No. of cases	TREC Cqs
SCID	2	UNDET, UNDET
Omenn Syndrome	2	UNDET, UNDET
22q11 del	4	39, 38.5, 37, UNDET
hypogammaglobulinemia	1	32

Critical Parameters

Critical Findings and Parameters

- **epMotion Instrument settings must be customized: Program available from SHL upon request**
- Manufacturer's instrument-to-instrument variation had to be overcome by calibrating the instruments to perform identically. There were slight variations in volumes pipetted and X-Y-Z parameters.
- Customized dispense and aspiration parameters were needed to remove all buffer from the wells.
- It is necessary to optimize wash well depth, volume, speed, incubation time, number times mixed during each wash step, and number of washes.

PCR on ViiA7

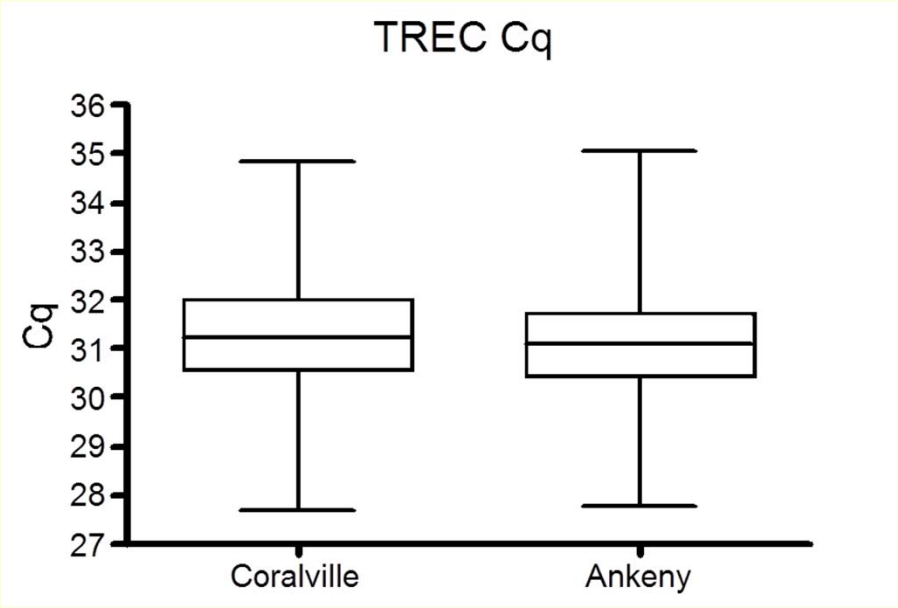
- A 2mm spot, not 3mm, must be used for *in situ* PCR in 96-well Fast plate.
- Use separate threshold settings for RNP and TREC appropriate for qPCR.
- Of the Master Mixes tested, Fast Advanced had the best performance for efficiency and background.
- Volume of master mix is critical for *in situ* PCR.

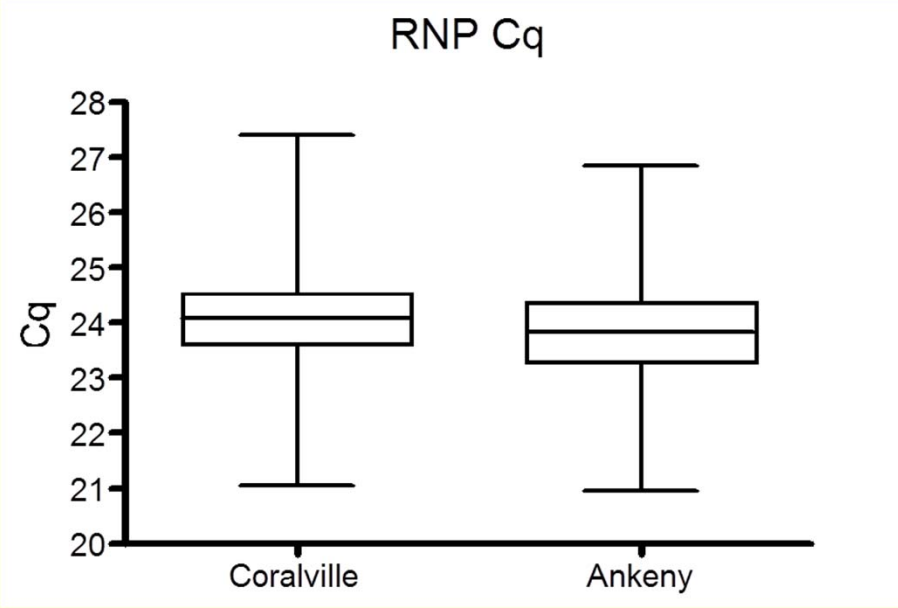
Key Features

Automated and easily adapted to laboratories with limited molecular capacity

- Low hands-on time reduced throughput time compared to manual methods
- No storage issues for extracted nucleic acid
- Streamlined workflow – no heating, shaking, centrifugation
- Use of a novel buffer found to be more effective and substantially less expensive than Generation DNA Purification Solution and Generation DNA Elution Solution (Qiagen), Limited disposables (only one PCR plate)
- Low cost (reagents and consumables)

SCID *in situ* Assay Portability



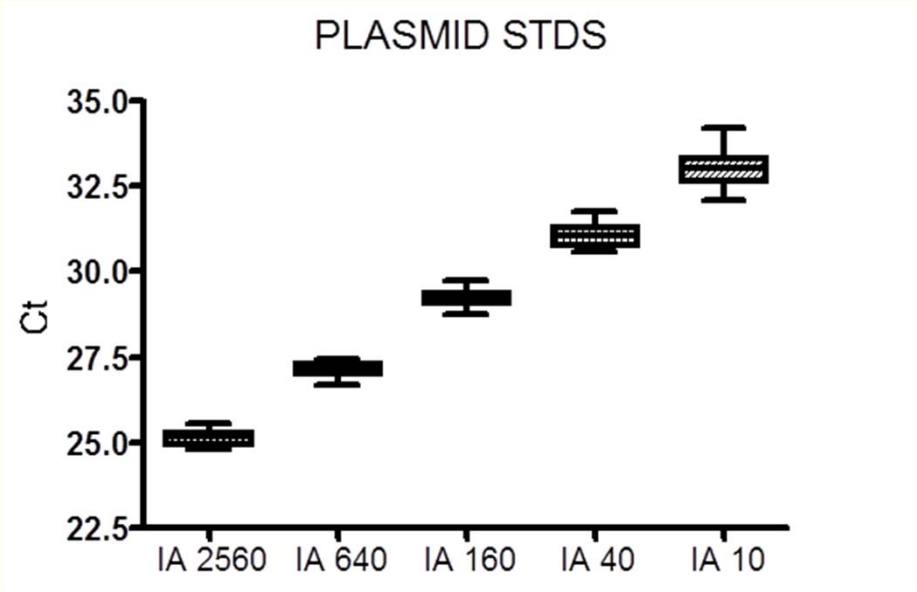


TREC Cq	Coralville	Ankeny
Minimum	27.68	27.78
Median	31.24	31.11
Maximum	34.86	35.06
Std. Deviation	1.137	1.084

RNP Cq	Coralville	Ankeny
Minimum	21.05	20.99
Median	24.07	23.84
Maximum	27.4	26.85
Std. Deviation	0.7698	0.8113

Quantification and Standard Curve Performance

- Screen positive criteria based on TREC quantity are highly variable (3.9 vs 40 vs 250).
- SCID TREC assays are based on the absence of detection using a molecular method which has the potential to detect as low as 1 copy. However, reliable detection of 1 copy is based on Poisson distribution.
- Therefore, any attempt to quantify as the copy number approaches 0 results in additional noise due to Poisson distribution.



References and Acknowledgements

- The in situ protocol was adapted from a procedure developed by the Centers for Disease Control and Prevention.
- Primer and probe sequences are from Douek, et al. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. Lancet, 355 (2000), pp. 1875–1881
- Scott D. Rose Ph.D., Integrated DNA Technologies, designed the modified, dual quenched TREC probe.
- The TREC plasmid used in the standard curve was graciously provided by Jackie Gerstel-Thompson and Ann Comeau, New England Newborn Screening Program, University of Massachusetts Medical School.
- TREC plasmid was transformed and purified at the Roy J. Carver Center for Genomics (CCG), University of Iowa.
- Funding was provided by the Iowa Department of Public Health