Newborn Screening for Severe Combined Immunodeficiency (SCID) in the Laboratory

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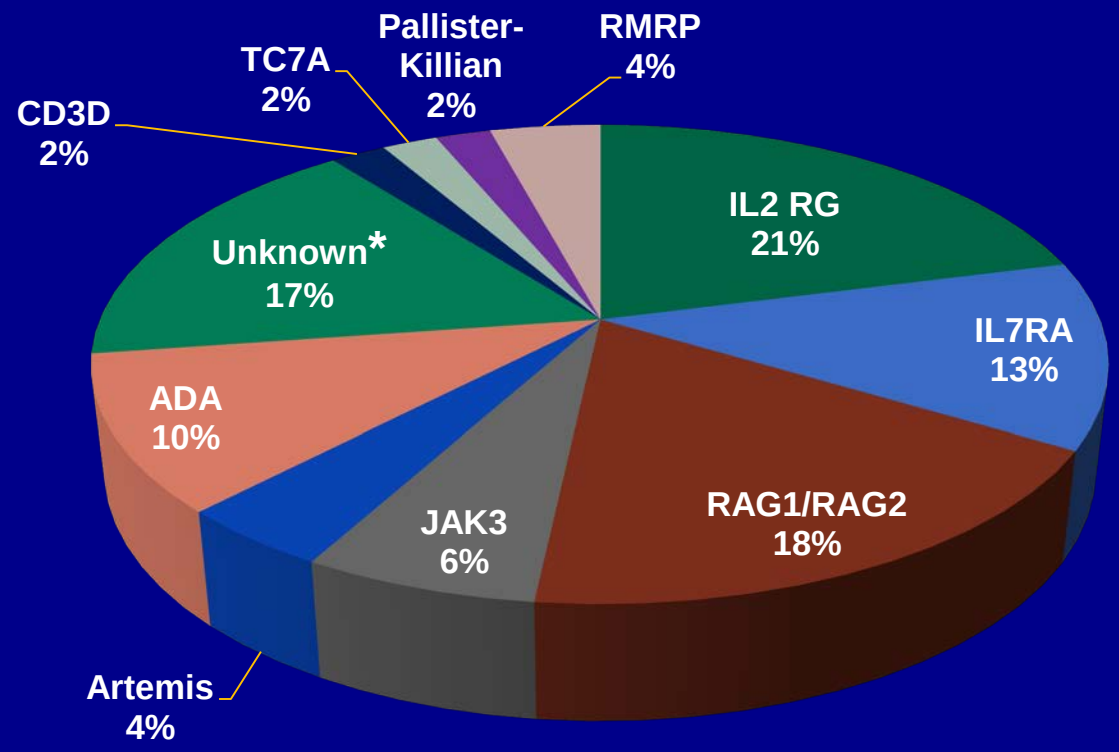
APHL Severe Combined Immunodeficiency (SCID) National In-Person Meeting
Washington, DC, August 8 – August 9, 2017

National Center for Environmental Health
Division of Laboratory Sciences

What is SCID?

- A heterogeneous group of inherited disorders caused by single gene defects resulting in a combined immune deficiency
- *Prevalence: ~ 1:50,000*
- *Over 20 different genetic forms: hundreds of mutation sites*
- All have profound defects in T lymphocyte differentiation and function
- Some (not all) have defects in B cell and/or NK cell differentiation as well
- End result: patients cannot fight viral, bacterial, fungal or opportunistic infections

Genetic Types



US Newborn Screening

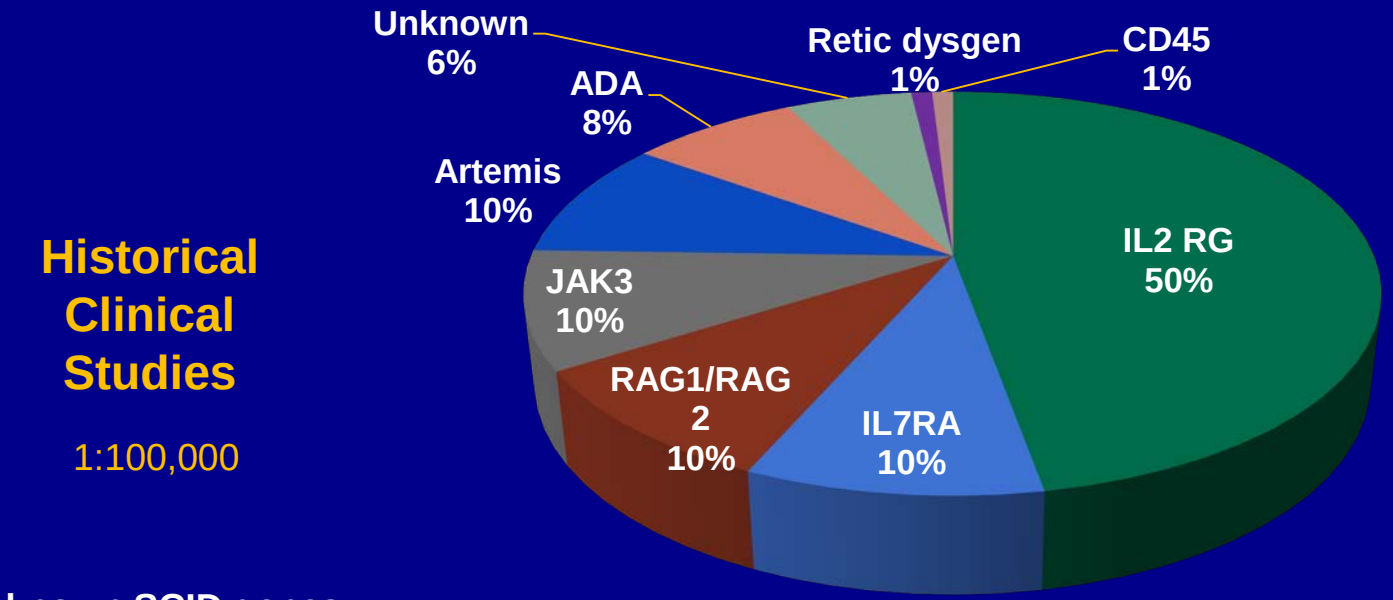
3 million babies in 11 NBS programs

1:58,000

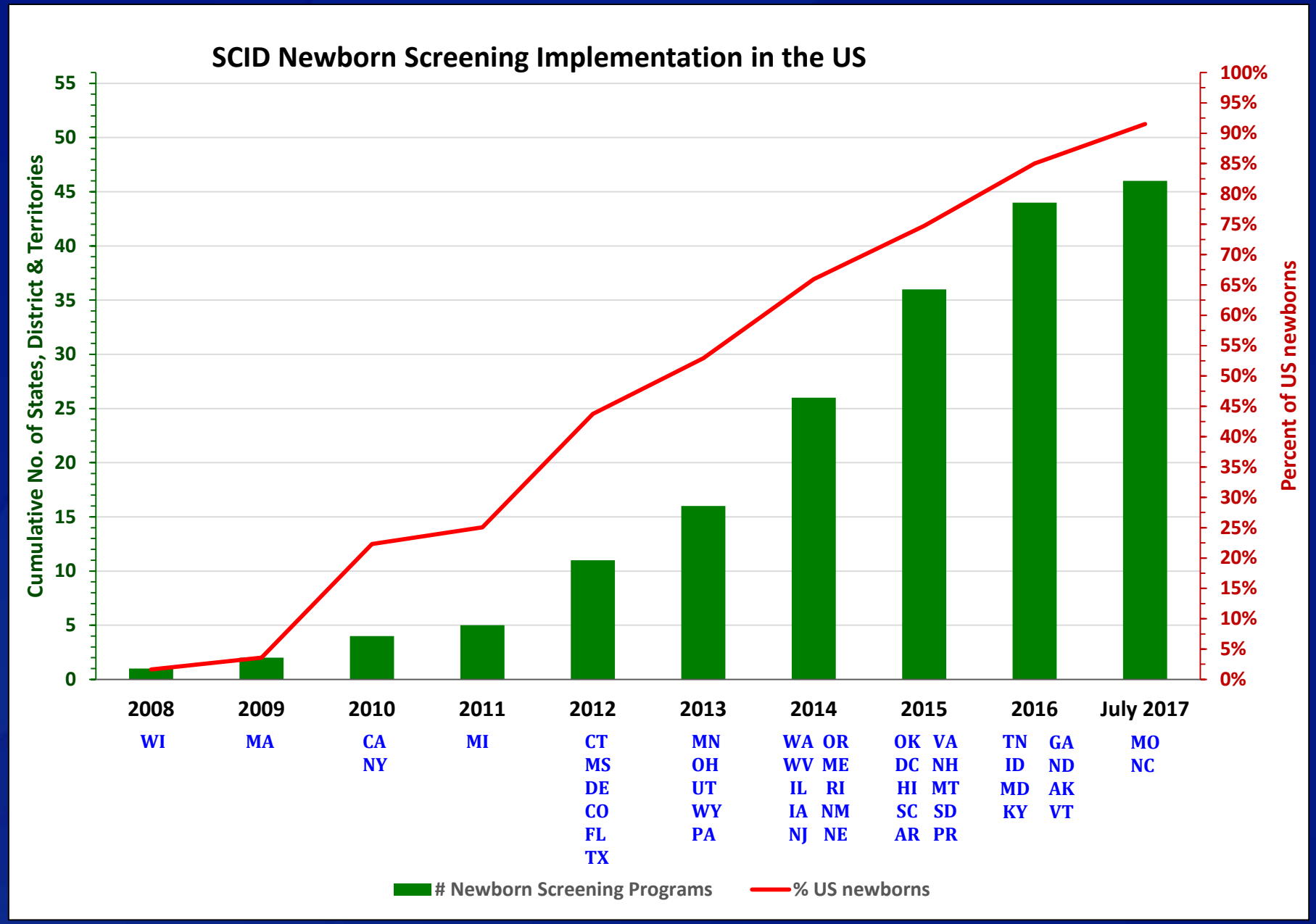
Kwan A etal, JAMA. 2014;312,:729-738

Historical Clinical Studies

1:100,000



* No molecular defect in known SCID genes


SCID Newborn Screening Implementation in the US
Cumulative No. of States, District & Territories
Percent of US newborns
0
5
10
15
20
25
30
35
40
45
50
55
0%
5%
10%
15%
20%
25%
30%
35%
40%
45%
50%
55%
60%
65%
70%
75%
80%
85%
90%
95%
100%
2008
WI
2009
MA
2010
CA
NY
2011
MI
2012
CT
MS
DE
CO
FL
TX
2013
MN
OH
UT
WY
PA
2014
WA OR
WV ME
IL RI
IA NM
NJ NE
2015
OK VA
DC NH
HI MT
SC SD
AR PR
2016
TN GA
ID ND
MD AK
KY VT
July 2017
MO
NC
Newborn Screening Programs
% US newborns

Newborn Screening Test for SCID

TREC Assay

measuring T Cell Receptor Excision Circles using DNA from dried blood spots collected routinely on all newborns

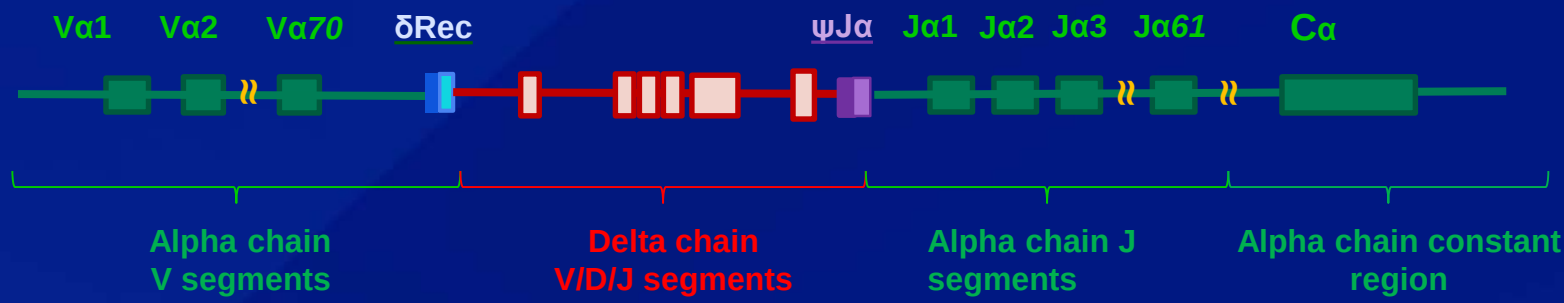
- **TREC - extrachomosomal DNA produced during rearrangement of V-D-J regions in TCR gene – essential step for the production of T cells**
- **Any immune defect that affects T cell production or destruction will cause a decrease in TREC**
- **Phenotypic assay (not genotypic)**

*** T cell receptors are protein molecules on T cells surface, responsible for recognition of antigens**

Formation of δRec-ΨJα TREC

during *Delta segment* deletion in rearrangement of T cell receptor gene

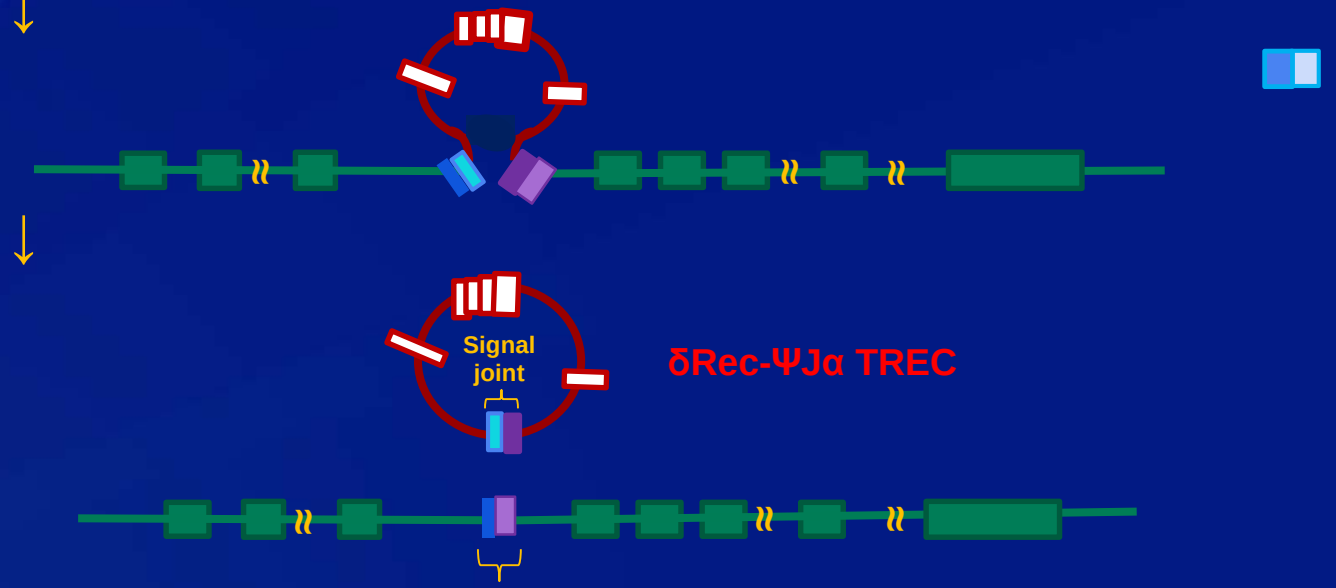
Chromosomal 14
germline
TCR α/δ chain loci
(all cells)



Chromosomal 14
TCR α/δ chain loci
(T cells)

Extrachromosomal DNA
δRec-ΨJα TREC

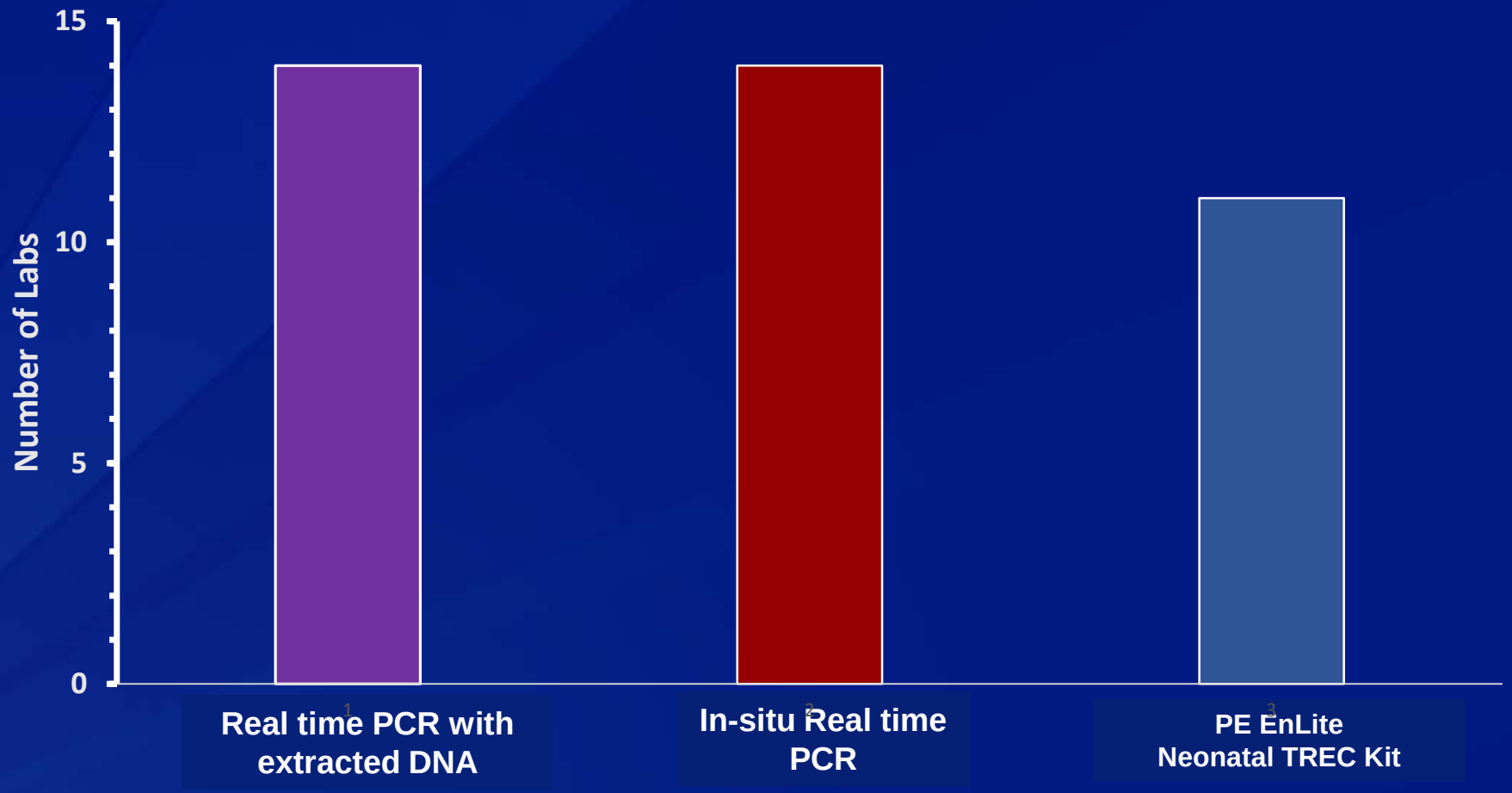
Chromosomal 14
TCR α chain locus



Vα–Jα–Cα rearrangement to form α chain exon

TREC Quantitative PCR Assay Platforms

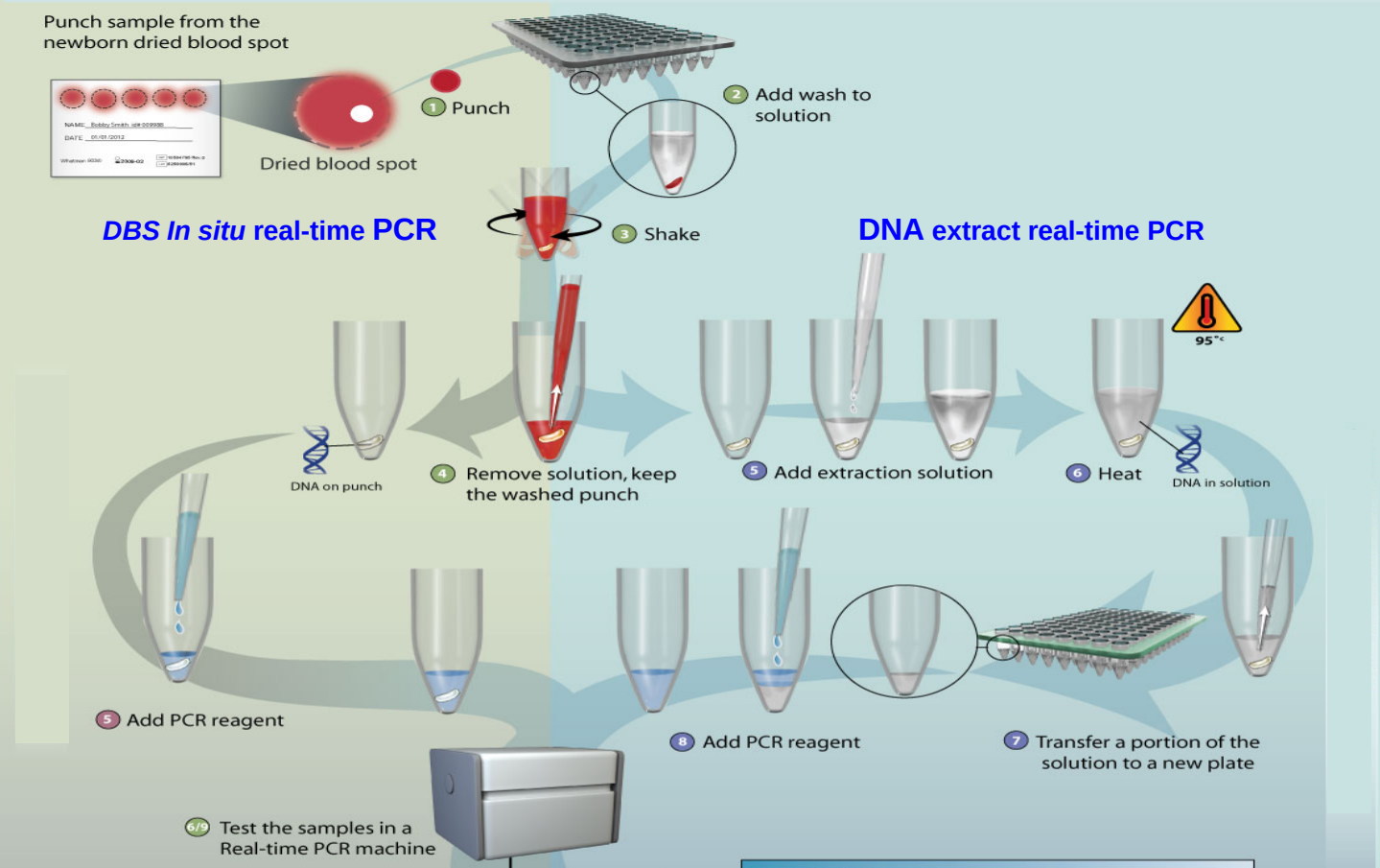
Selected by US newborn screening laboratories

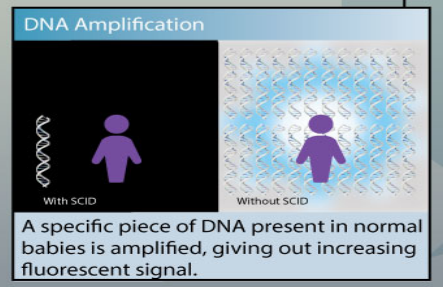


Real time PCR TREC Assay

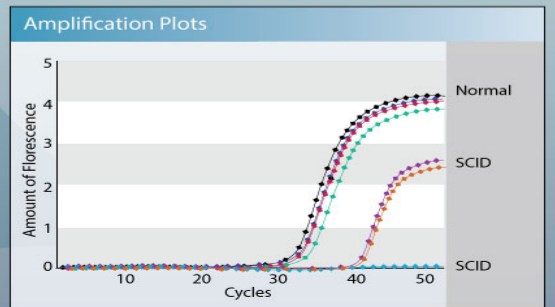
(with TaqMan probes)

SEVERE COMBINED IMMUNODEFICIENCY





A specific piece of DNA present in normal babies is amplified, giving out increasing fluorescent signal.



DNA from babies with Severe Combined Immunodeficiency produces delayed signal or no signal at all.

Centers for Disease Control and Prevention
National Center for Environmental Health

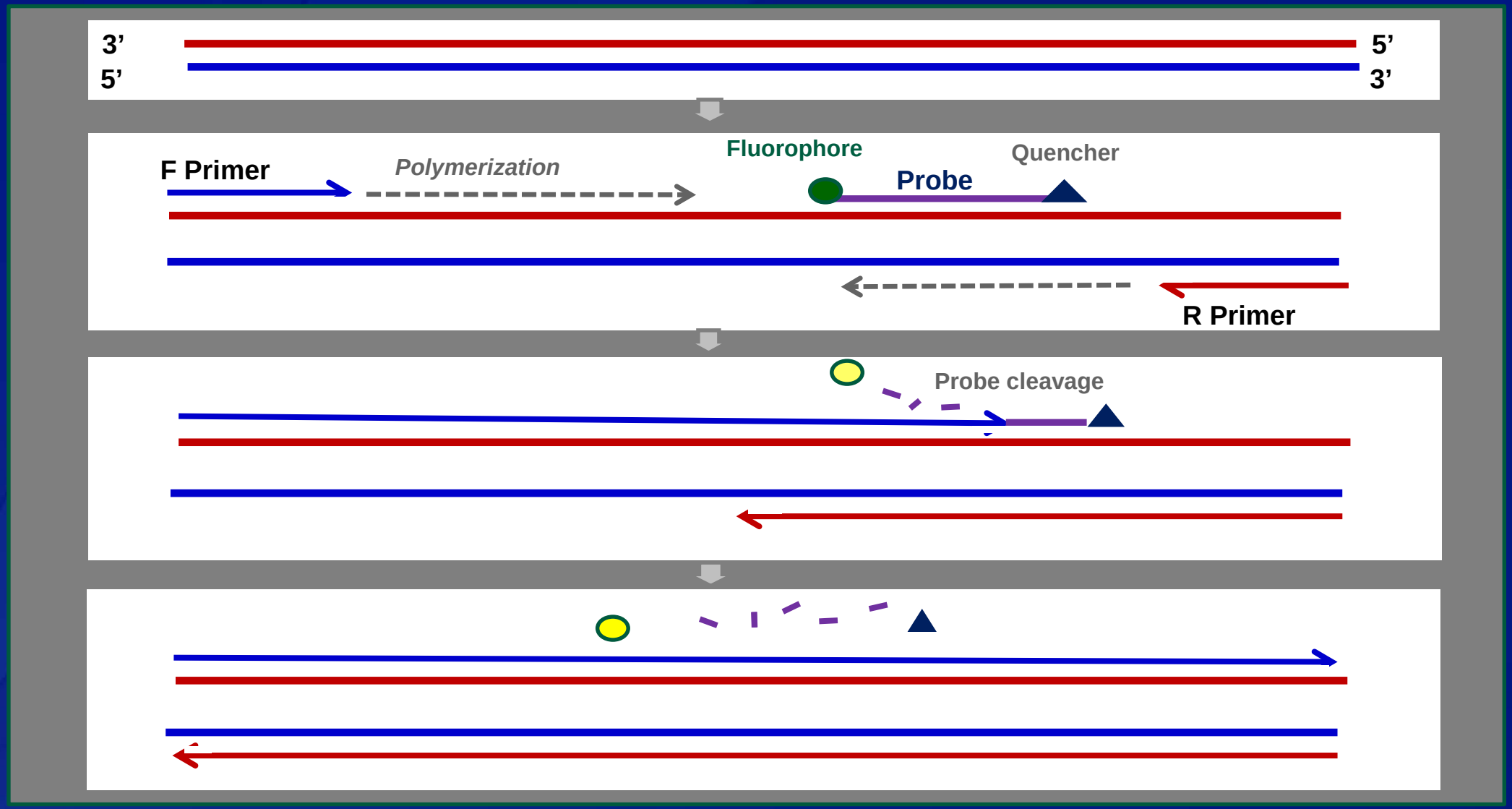
CS235346-G

TCRD TREC Sequence: 376 bp (out of 85Kb) flanking the **δRec-ΨJα signal joint**

AAAGAGGGCAGCCCTCTCCAAGGCAAAATGGGGCTCCTGTGGGGAAAGAGGGGTGCCTCTGTC
AACAAAGGTGATGCCACATCCCTTTCAACCATGCTGACACCTTTGGTT**TTTGTAAAGGTGCCCACT**
CCTGTG^CA**CGGTGATGCATAGGCACCT**CACCCCGTGCCTAAACCCTGCAGCTGGCACGGGCCC
TGTCTGCTCTTC**ATTCACCGTTCTCACGAGTTGCAATA**AGTTCAGCCCTCCATGTCACACTGTGTT
TTCCATCCTGGGGAGTGTTTCACAGCTATCCCAAGCCCCACGCTGACGATCACGGCCGAAAACAC
ACTCTGATGCCAGCACAGACCACGGAGCAAATGTCAGACAAGATCAGCCT

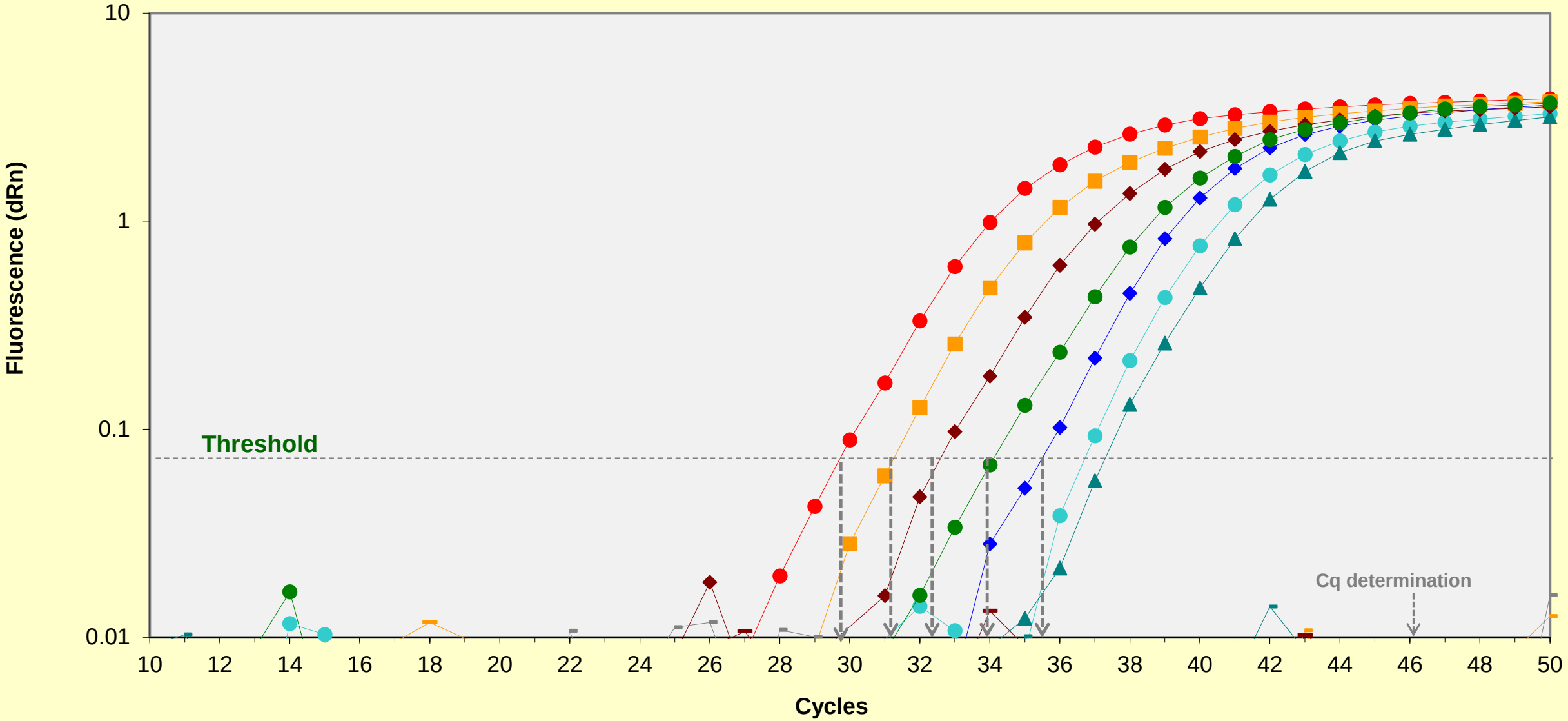
Blue – CDC forward primer, Green – CDC reverse primer binding site, Red – Taqman probe, **^ -** signal joint position

Real-time PCR with TaqMan Probes



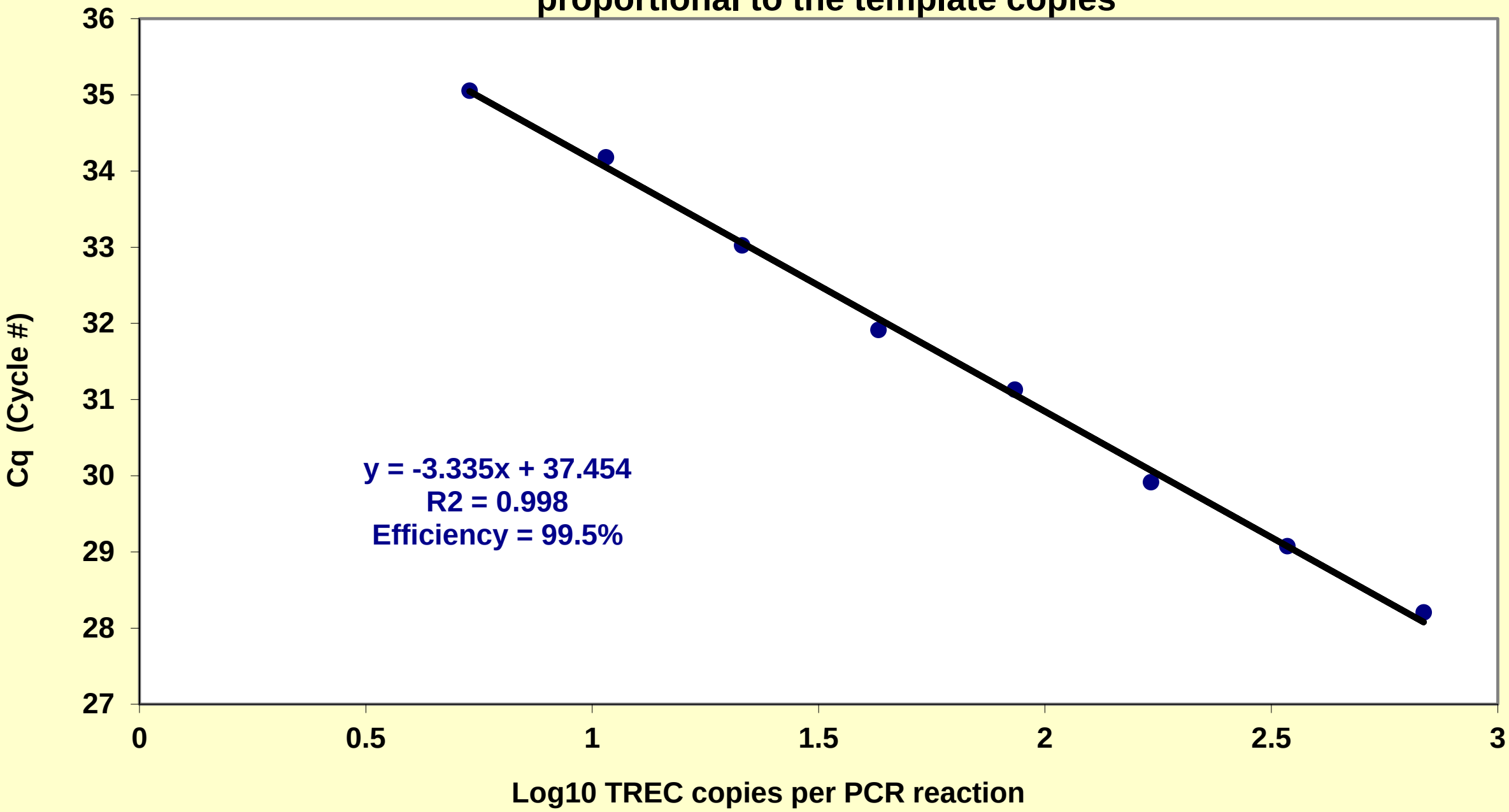
TREC Real-time PCR Amplification Profile

Amplification curves of samples with decreasing TREC content (L to R)



Cycle of Quantification (Cq) inversely correlates with template concentration

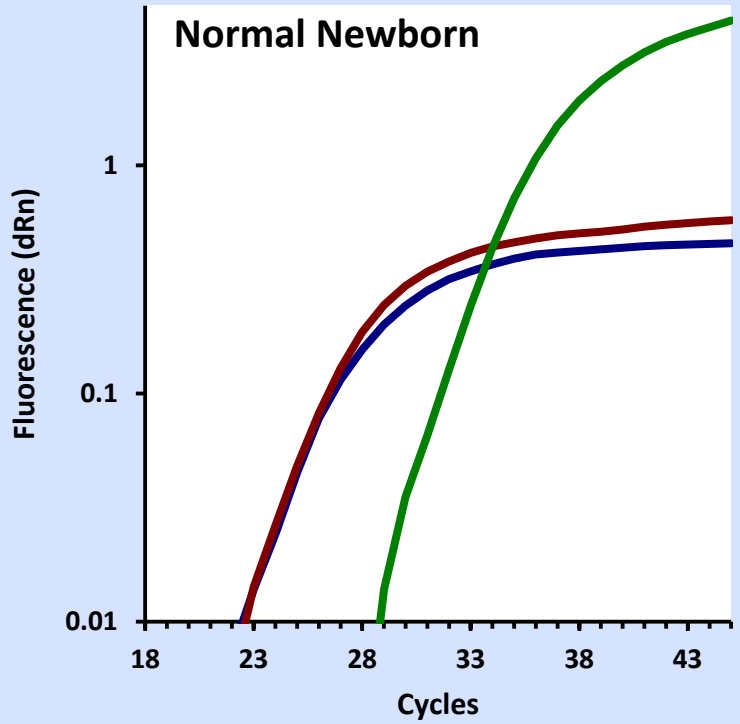
Number of PCR cycles required to reach fluorescent threshold is inversely proportional to the template copies

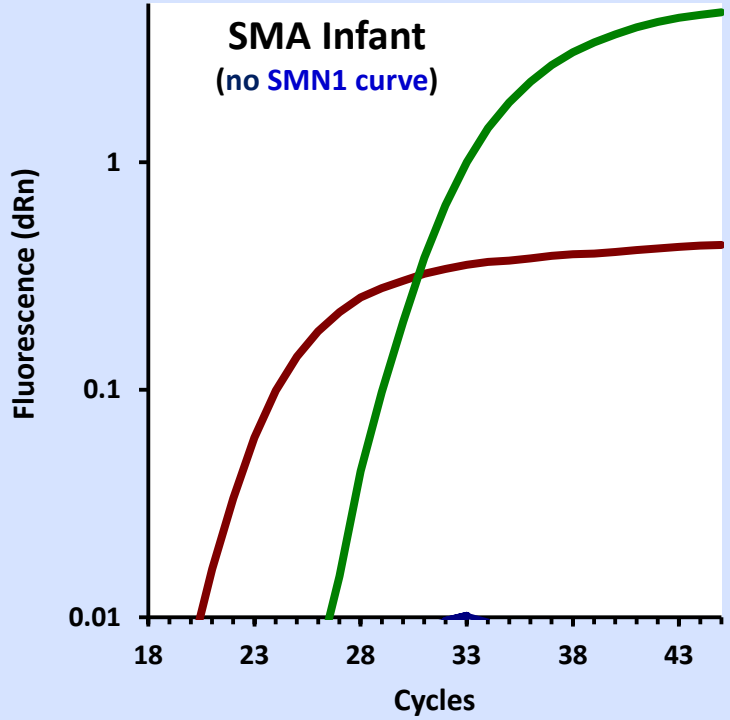


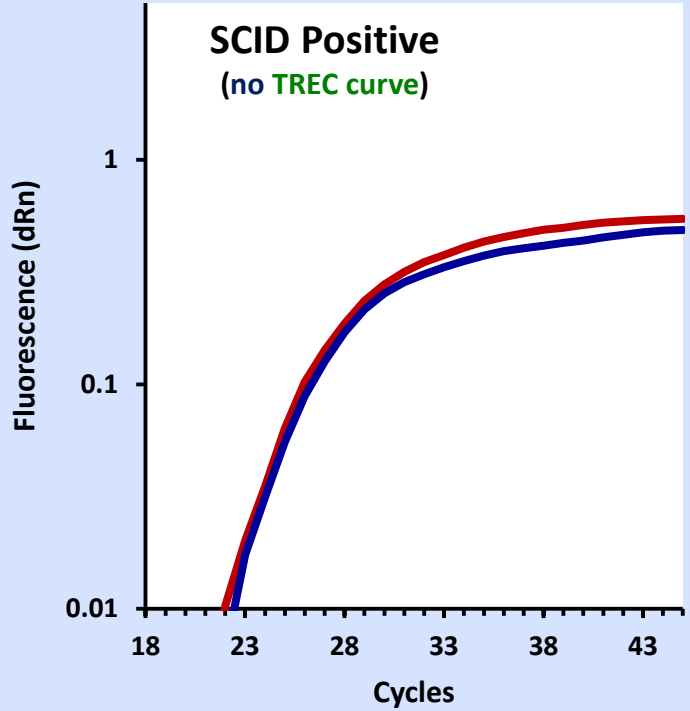
Multiple targets can be measured in a single real-time PCR test

Multiplex TREC/*SMN1*/RNaseP Assay

In Newborn Screening for SCID and Spinal Muscular Atrophy (SMA)



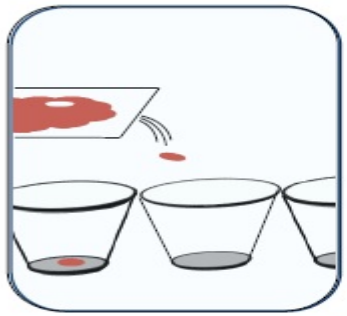




End-point PCR TREC Assay

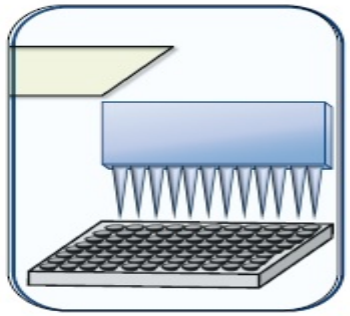
(PE EnLite Assay)

PE EnLite Neonatal TREC Assay



1. PUNCHING

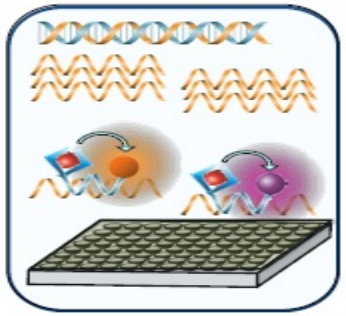
- 1.5 mm DBS disc
- Into PCR-plates directly



2. DISPENSING & SEALING

- 10 µL of elution buffer
- Sealing
- Spin the plates
- DNA elution
  45 min at 98°C
  2 min at 4° C

SPIN THE PLATES

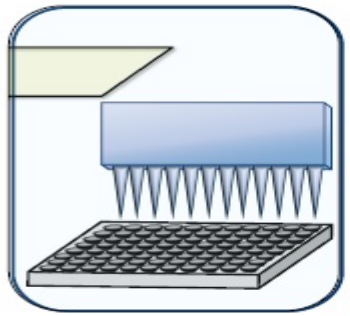


4. AMPLIFICATION & HYBRIDIZATION

- Amplification and hybridization
  In thermocycler

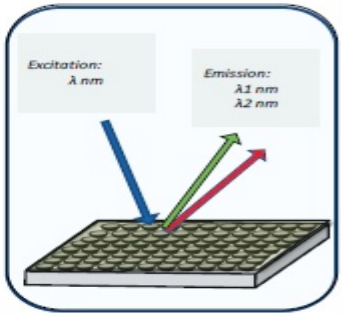
PCR 37 cycles: ~1 hr 40 min
35°C x 1 hr
24°C x 5 min

SPIN THE PLATES



3. DISPENSING & SEALING

- Unsealing
- 20 µL of PCR mix
- Sealing



5. MEASUREMENT

- TREC and β-actin
- Two signals from each well

Time-resolve Fluorometer

Analysis of data

EnLite Assay

Copy number from standard curve

Real time PCR Assay

1. Cq
2. Copy number
3. Multiple of median

Cutoff value determination can be based on any of above

Cq: Cycle of Quantification

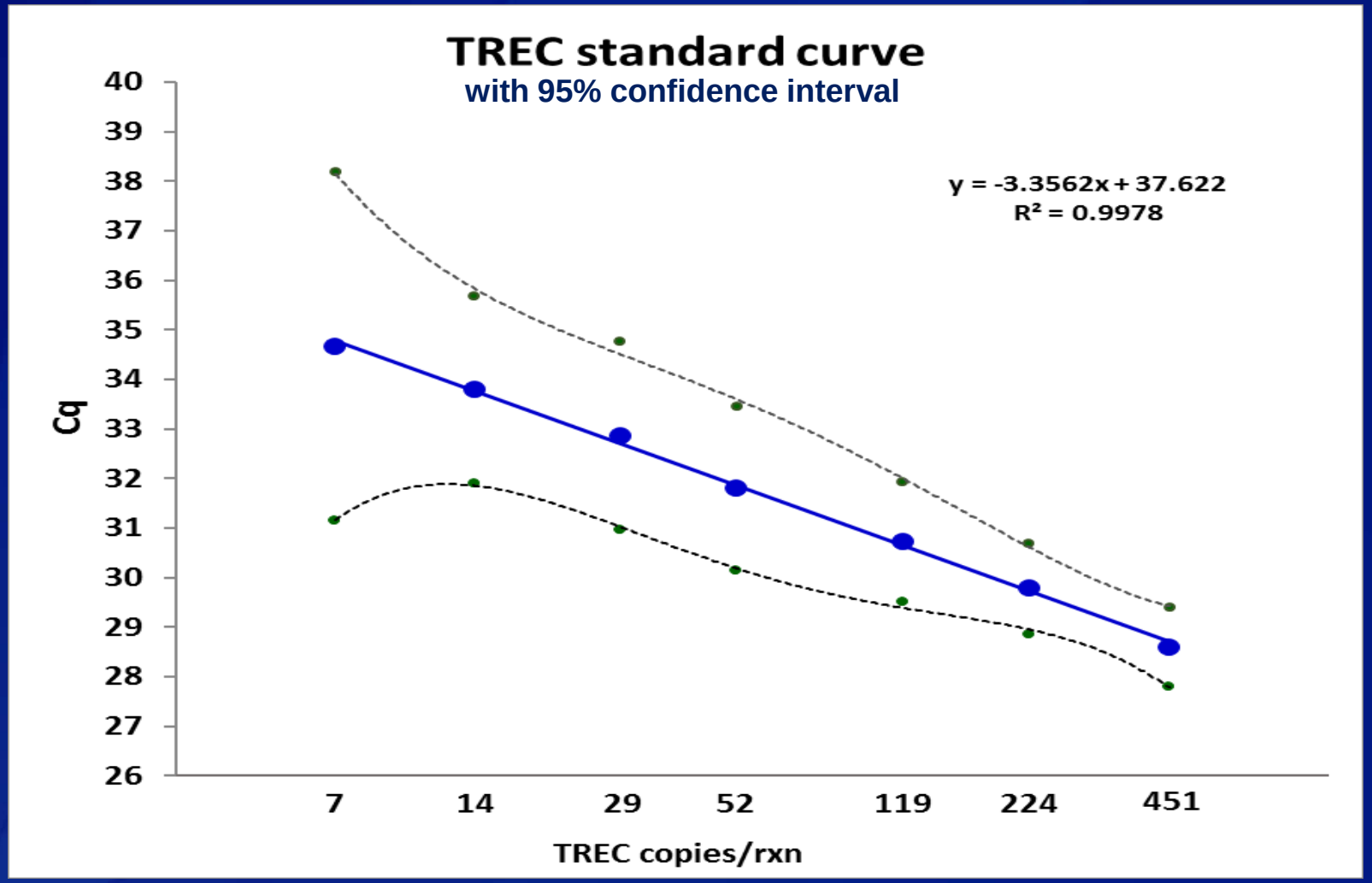
- The number of PCR cycles at which an amplification curve meets a predefined threshold of fluorescence
- Inversely proportional to TREC copies in sample

- Advantages
  - Direct read out from machine software
  - Does not require standard curve
  - Comparatively consistent reproducibility for same sample

- Limitations
  - Unfamiliar to most non-molecular biologists
  - No ‘normalization’ mechanism to compensate for reagent and instrument variations
  - Requires
    - Titrating each new reagent lot and adjusting concentration
    - Calibrating real time PCR instruments at regular intervals
    - QC at several TREC levels within each run – meeting predetermined acceptable Cq range for each QC result

TREC copy number

- Converting Cq into copy number through a standard curve
- Advantages
  - Easy to explain results
  - Normalized results
- Limitations
  - No universal standard calibrator available
  - Plasmid calibrators may vary in reactivity (circular or linearized)
    Alternative: synthetic ds DNA gene fragment (gBlock, geneStrands)
  - Plasmid calibrators do not undergo DBS DNA extraction process
    Alternative: cell-based calibrator (B-TREC)
  - Standard curve necessarily includes very low level of TREC; large difference in statistical variance at top and bottom levels of standard curve challenges validity of linear regression model and causes inconsistent slope



Mean Cq	28.6	29.8	30.7	31.8	32.9	33.8	34.7
CV%	1.40	1.50	2.00	2.60	2.90	2.80	5.10
Variance	0.18	0.21	0.37	0.69	0.89	0.90	3.11

Problem of using simple linear regression model near limit of detection:

Significant variance difference over the range covered by curve
→ inconsistent slope and intercept

Multiple of Median (MoM)

- A measure of how far an individual test result deviates from the population median
- Expressed as a ratio (TREC copies in sample / Median TREC)

Advantages:

- Easy to understand
- Normalized results
- Does not require standard curve – can be calculated from Cq

Limitations:

- Needs a reasonable number of samples to obtain reliable population median (usually not a problem with NBS 1st assays)

Calculating MoM from Cq: formula derivation

MoM = TREC copies in sample / Median TREC copies for population

PCR doubles the copies with each cycle. If a sample contains "*S*" copies of TREC initially, after *n* cycles the level of TREC will be (*S* x 2 x 2 x 2….*n*), or $\boldsymbol{S \times 2^n}$

By definition, Cq is the number of PCR cycles when the level of amplification product (TREC) reached a certain pre-defined threshold. If the threshold is reached at cycle number Cq_S for sample S, the number of TREC copies at threshold is

$$S \times 2^{Cq_s}$$

Similarly, for a sample containing population median *M*, the amount of TREC copies at threshold, which is reached at cycle number Cq_m , is

$$M \times 2^{Cq_m}$$

Since the threshold for Cq determination is the same for both samples

$$S \times 2^{Cq_s} = M \times 2^{Cq_m} \quad or \quad S / M = 2^{Cq_m} / 2^{Cq_s} = 2^{(Cq_m - Cq_s)}$$

$$\mathbf{MoM} = 2^{(Cq_m - Cq_s)}$$

where Cq_m and Cq_s *represent median Cq and sample Cq respectively*

MoM calculation

Example 1

The Cq for sample A is 32, and the median Cq for population is 29

MoM for sample A = $2^{(29-32)} = 2^{-3} = 1/2^3 = 1/8 = 0.125$

Sample A contains 12.5% of the population median in TREC

Example 2

The Cq for sample B is 28.5, and the median Cq is 29

MoM for sample B = $2^{(29-28.5)} = 2^{0.5} = 1.41$

Sample B contains 141% of the population median in TREC

Note: In a previous survey, it was found that the cutoff value in copy numbers used by majority of labs to be around 10–15 % of their population median values, which corresponds to 0.10 – 0.15 MoM.

Reporting Newborn Screening Results for SCID

1. Categorical:

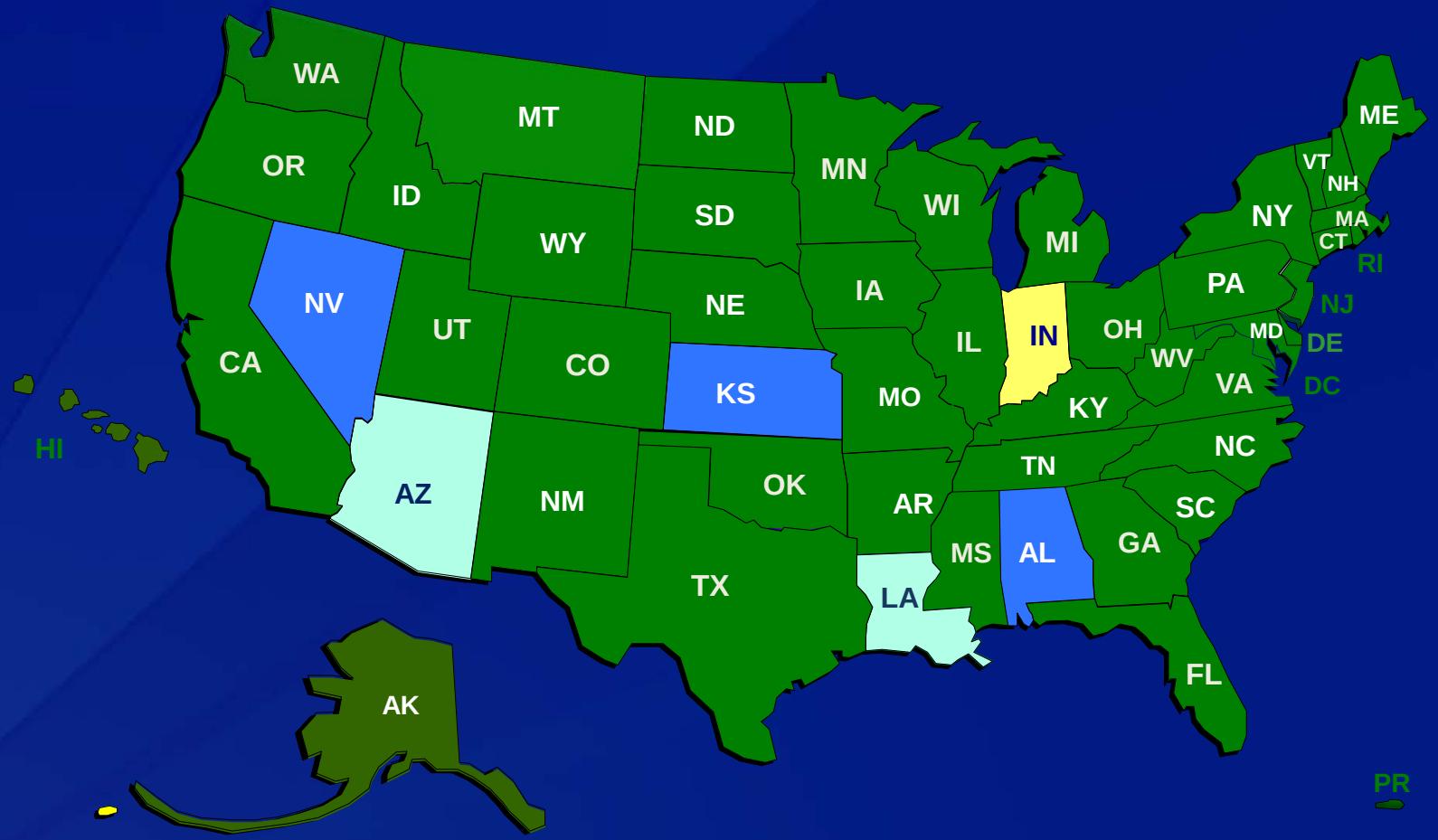
    a. Within reference range (Normal)

    b. Below reference range (TREC , follow-up required)

    c. Inconclusive (internal reference gene , repeat sample required)

2. Quantitative: copy number of TREC or Cq

August, 2017 Newborn Screening Status for SCID – US States and Territories



Screening for SCID
(91% of all US newborns)

At assay validation stage

At planning & procurement stage

Others

Thank you for your attention!

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