Newborn Screening for Severe Combined Immunodeficiency (SCID) and Spinal Muscular Atrophy (SMA) by Real-Time PCR

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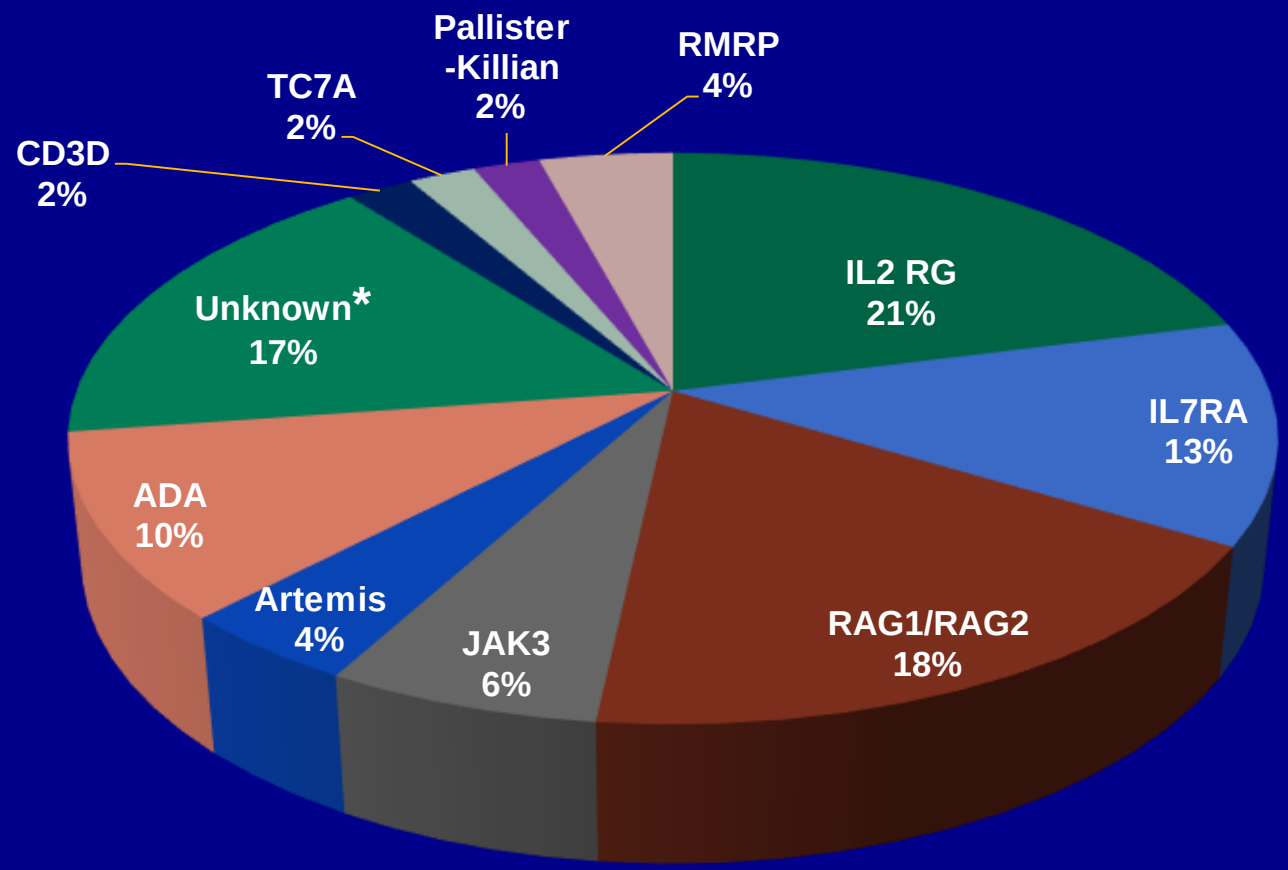
National Center for Environmental Health
Division of Laboratory Sciences

What Is SCID?

- A heterogeneous group of inherited disorders caused by single gene defects resulting in a combined immune deficiency
- Prevalence: ~ 1:58,000
- Over 30 different genes: hundreds of variants
- Profound defects in T lymphocyte differentiation and function
- End result: patients cannot fight viral, bacterial, or fungal infections

Major SCID Genetic Types



Results based on 3 million US newborn babies screened for SCID

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

* No molecular defect in known SCID genes

Newborn Screening Test for SCID

TREC Assay

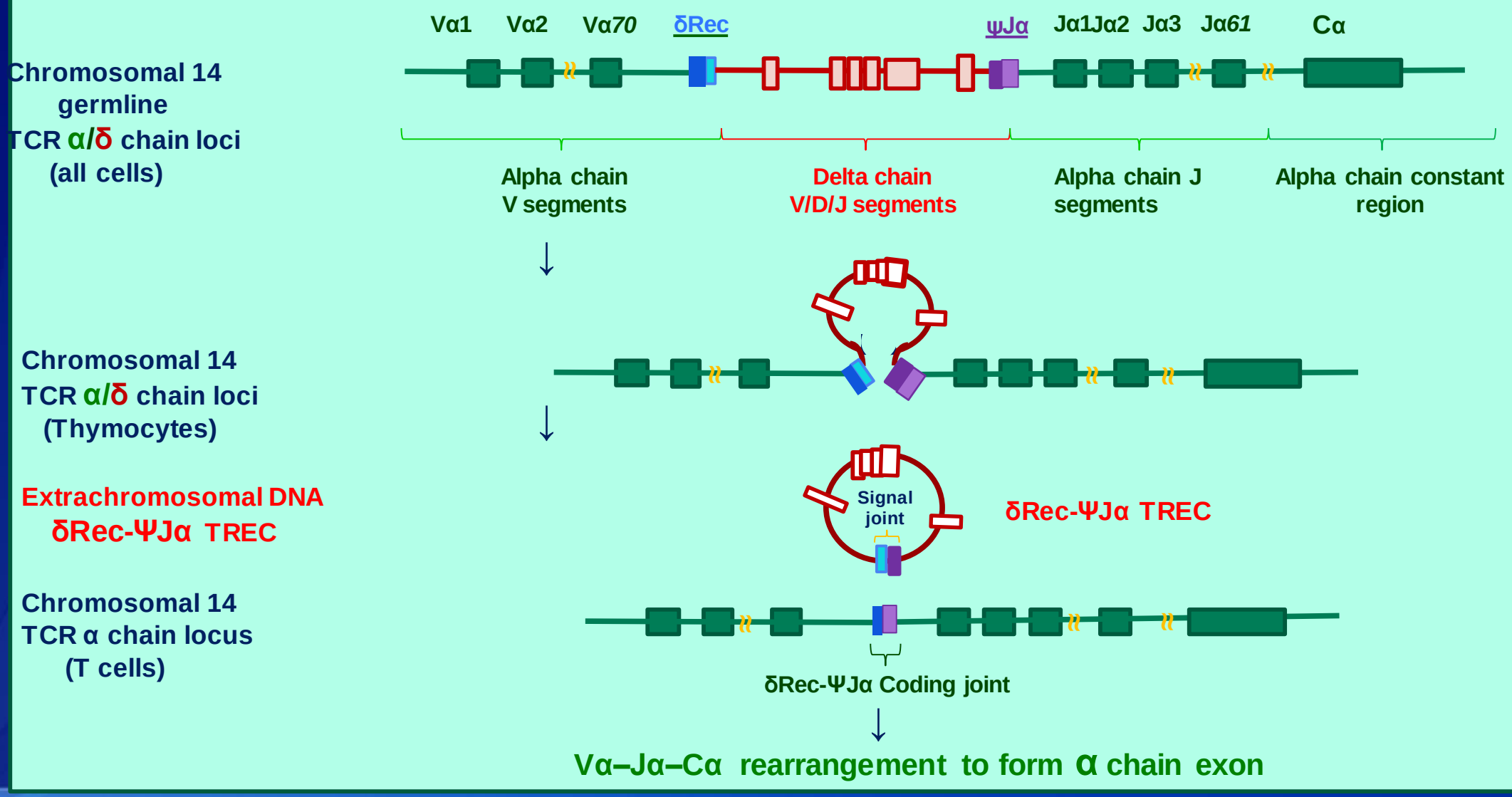
measuring T Cell Receptor Excision Circles using DNA from dried blood spots collected routinely on all newborns

- TREC - extrachromosomal DNA produced during rearrangement of V-D-J regions in TCR gene: essential step for producing T cells
- Any genetic 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

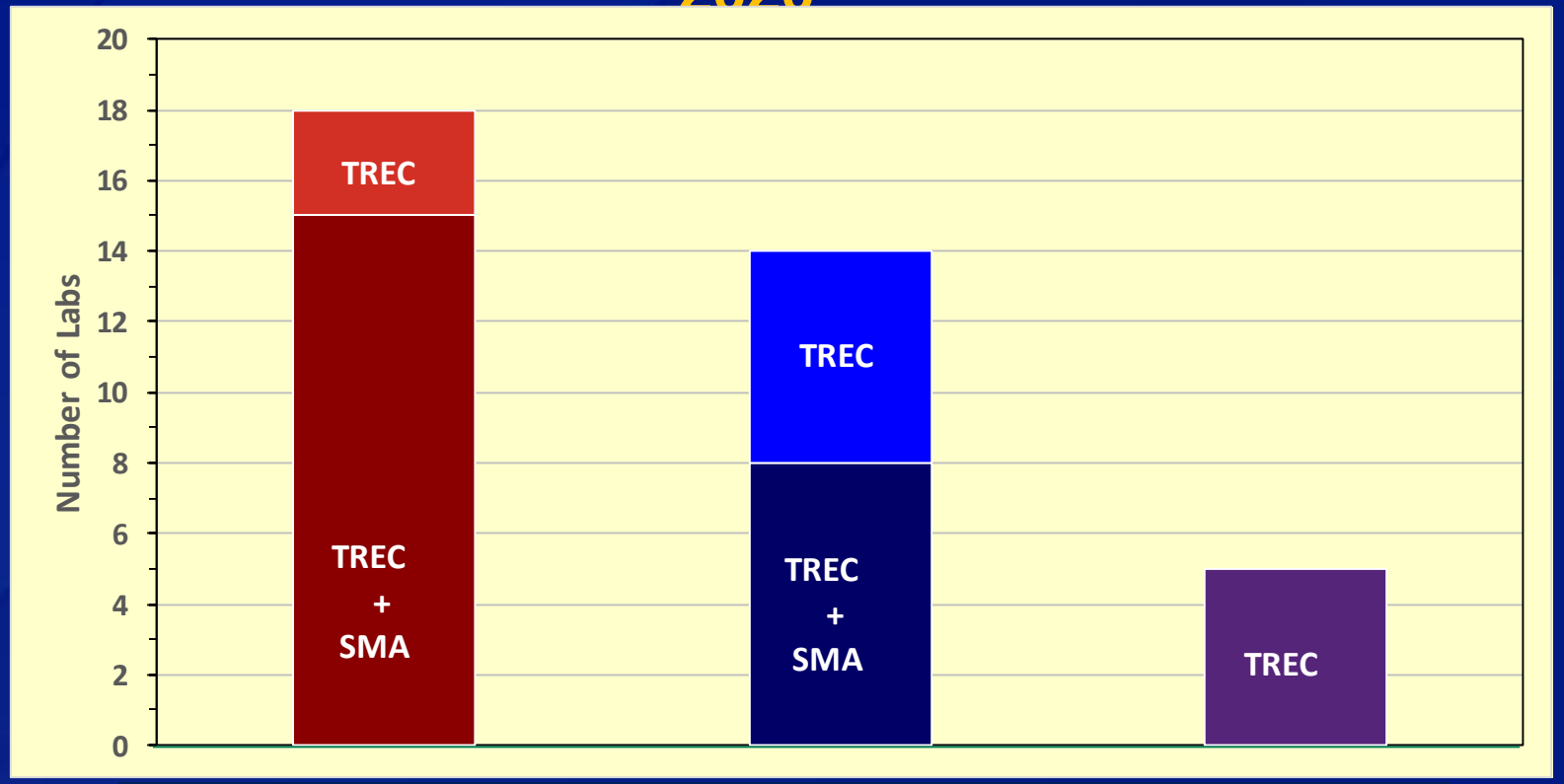
Formation of δRec-ΨJα TREC

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



TREC Quantitative PCR Assay Platforms

used by US state newborn screening laboratories, September 2020



Real time PCR with extracted DNA

In-situ Real time PCR

PE EnLite Neonatal TREC Kit

What is Spinal Muscular Atrophy (SMA)?

- A neuro-muscular disease resulting in the progressive degeneration of spinal anterior horn neurons
- Symptoms include progressive loss of normal motor function, muscle weakness, and respiratory failure
- Three clinical types based on age of onset and severity
  - Type I: Birth – 6 mos (most common form)
  - Type II: 6 mos. – 2 years
  - Type III: 18 mos. – 3+ years

SMA Is The Leading Genetic cause of Infant Death

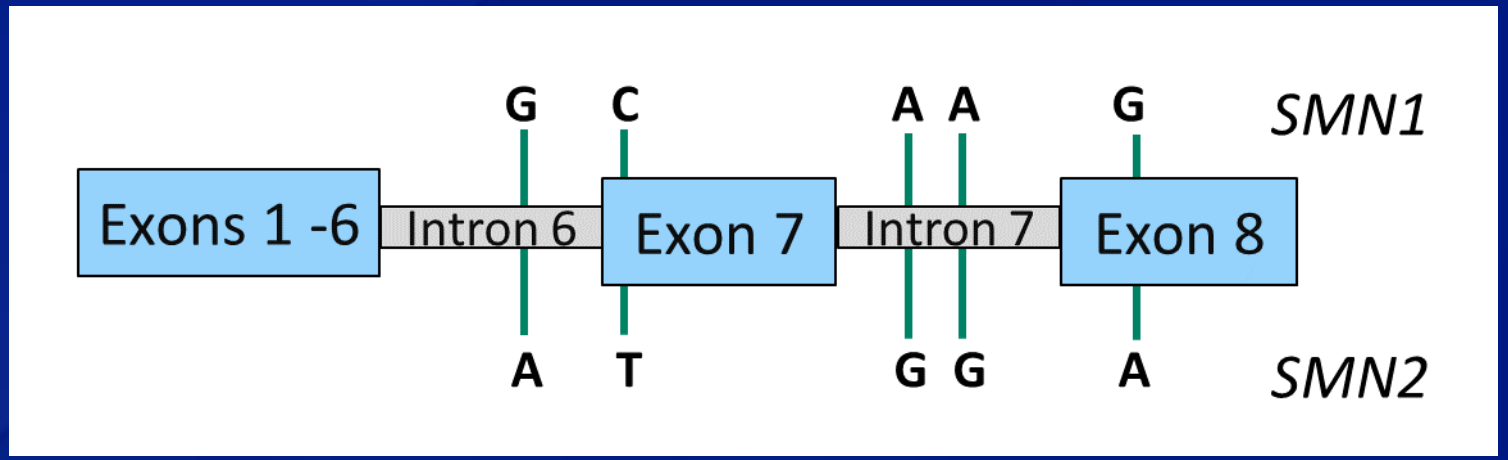
- The birth prevalence is approx. 1 in 10,000
- Majority of children with Type I SMA do not survive beyond 2 years without effective therapy
- FDA approved first SMA therapeutic (intrathecal antisense oligonucleotide) in Dec. 2016
- In July 2018, SMA was added to the Recommended Uniform Screening Panel (RUSP) for newborns
- Second therapeutic (gene therapy) approved by FDA in 2020
- Third drug (oral liquid pyridazine derivative) approved by FDA also in 2020 for patients 2 months or older

Genetic Characterization of SMA

- **Autosomal recessive inheritance (carrier rate 1:50)**
- **>95% of SMA cases are caused by pathogenic variants of the *SMN1* gene**
- **Most involve the loss of *SMN1* exon 7 (on both chromosomes) by deletion or gene conversion**
  - **Loss of this gene region results in a non-functional SMN protein**
  - ***SMN2*, a paralog gene of *SMN1*, may moderate the disease severity**
    - *SMN2* can only produce 10% of the SMN protein normally produced by *SMN1*

Wirth B., *Human Mutation* (2000), 15:228-37

>95% SMA cases caused by loss of *SMN1* exon 7 in both chromosomes

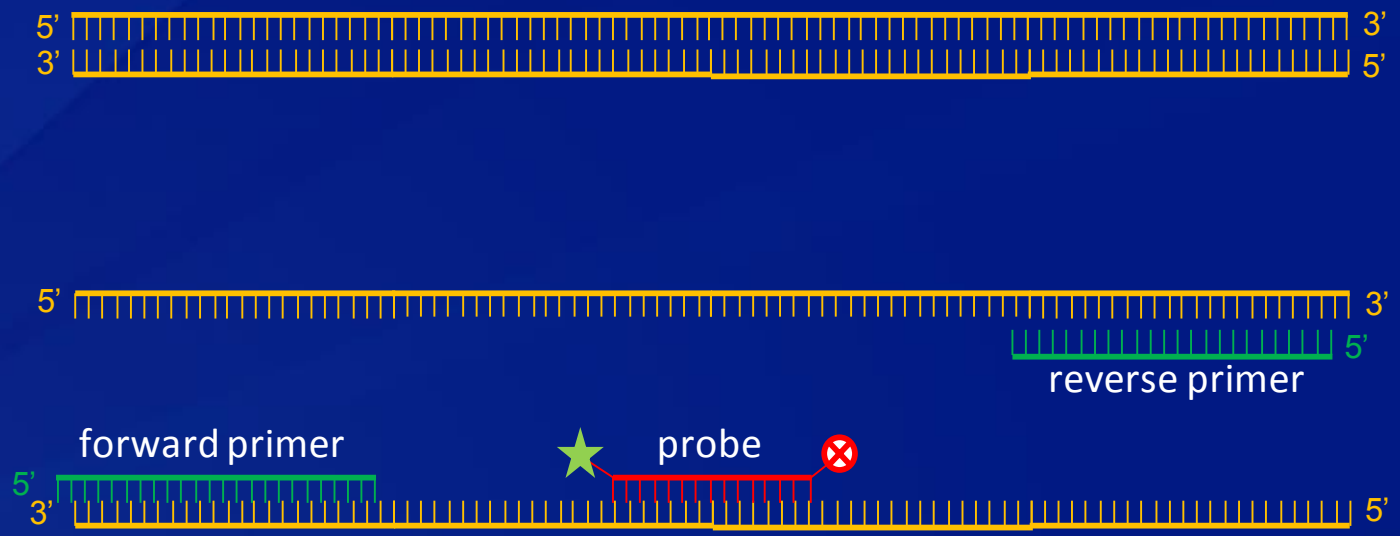


Both SCID and SMA can be detected by a multiplex real-time PCR assay

What Happens During Real-time PCR?

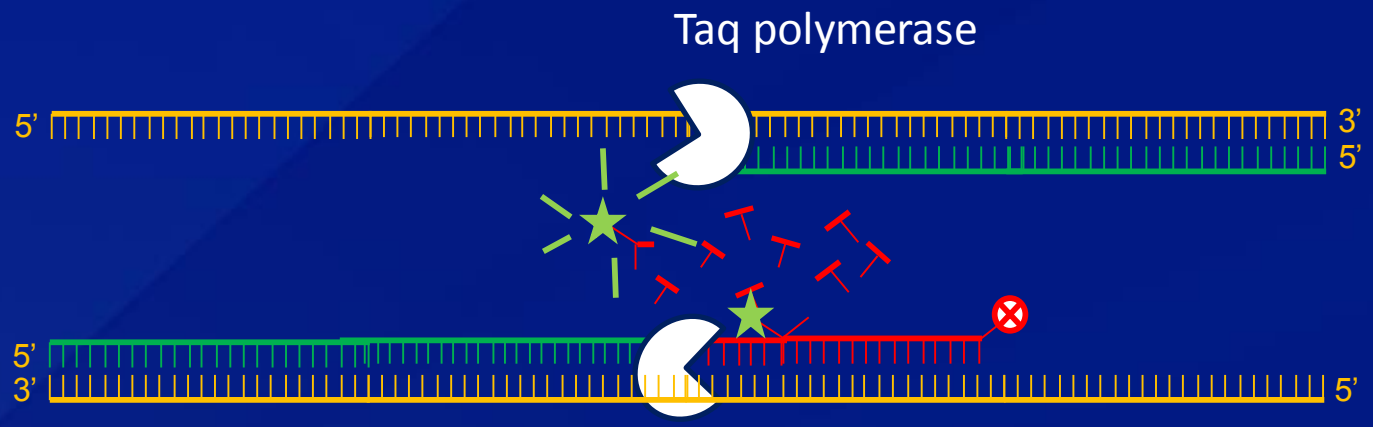
Step 1. Denaturation and Annealing

The two strands of dsDNA are separated (denaturation), allowing the primers and probe to anneal to their complementary sequence



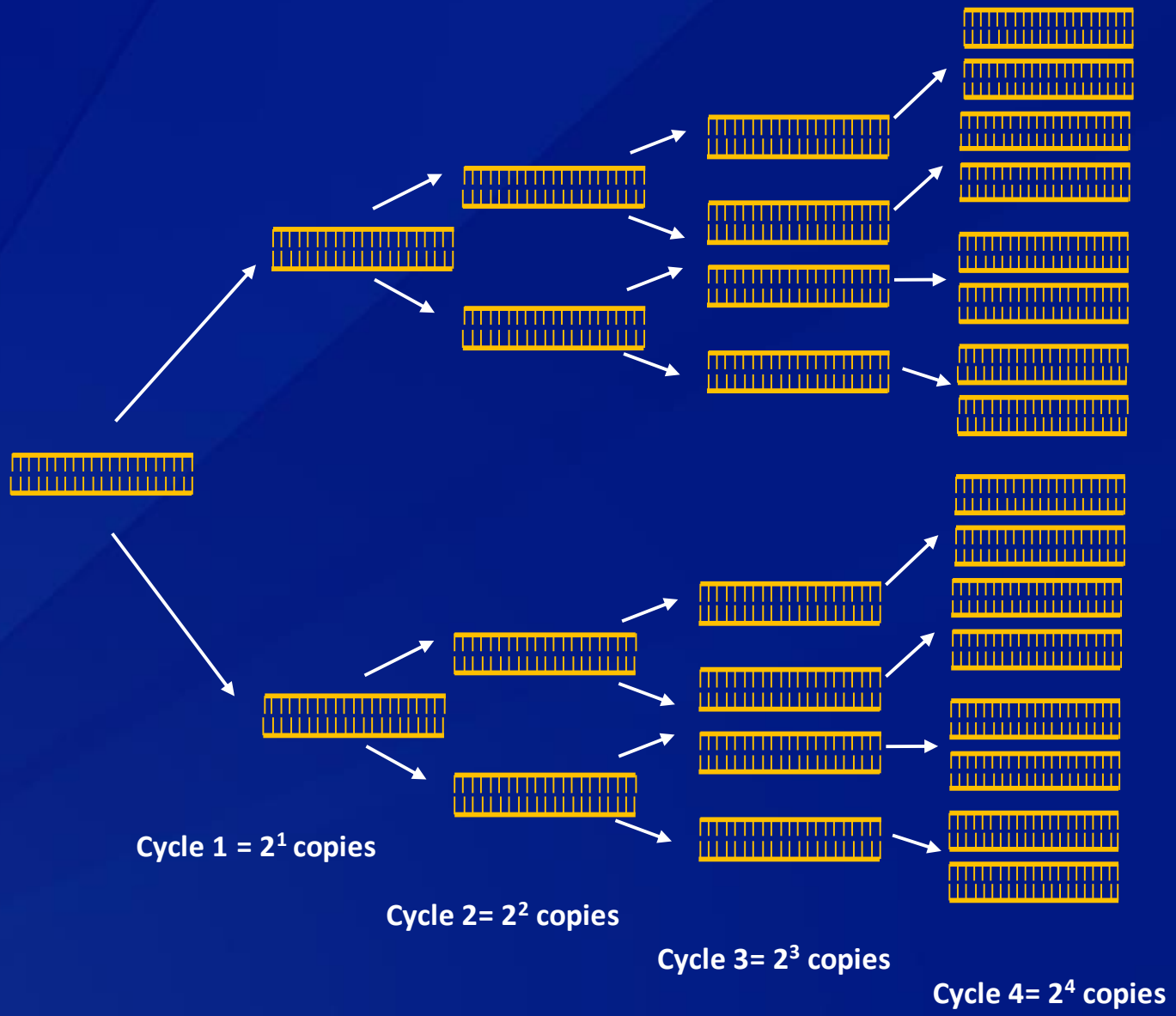
Step 2. Extension:

The polymerase incorporates complementary dNTPs, producing two new molecules of double stranded DNA during each cycle

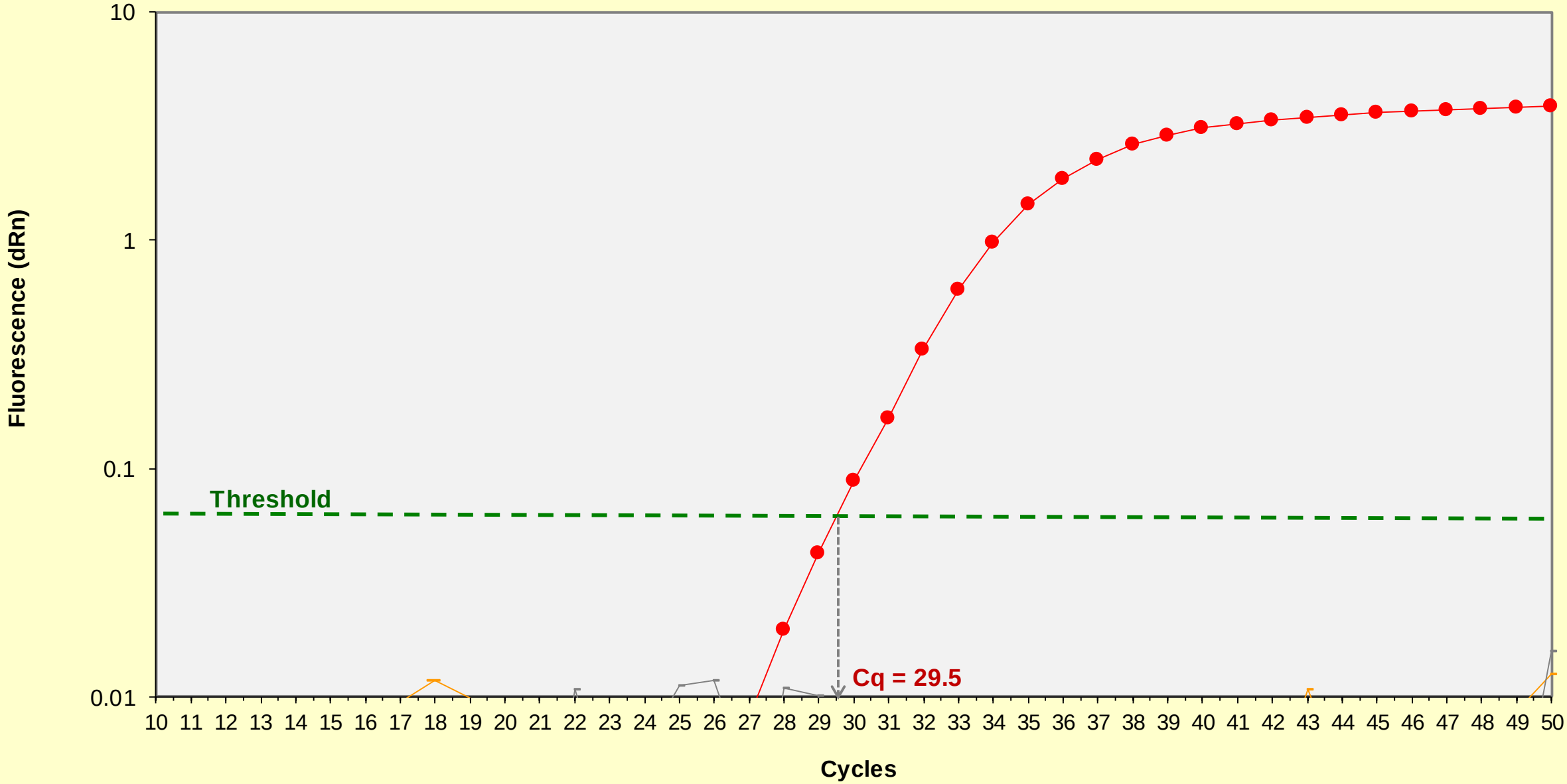


The Taq polymerase has exonuclease activity that cleaves the nucleotides of the probe when it is encountered during extension. When the probe is cleaved, the fluorophore is separated from the quencher and will then produce a fluorescent signal that is measured and recorded.

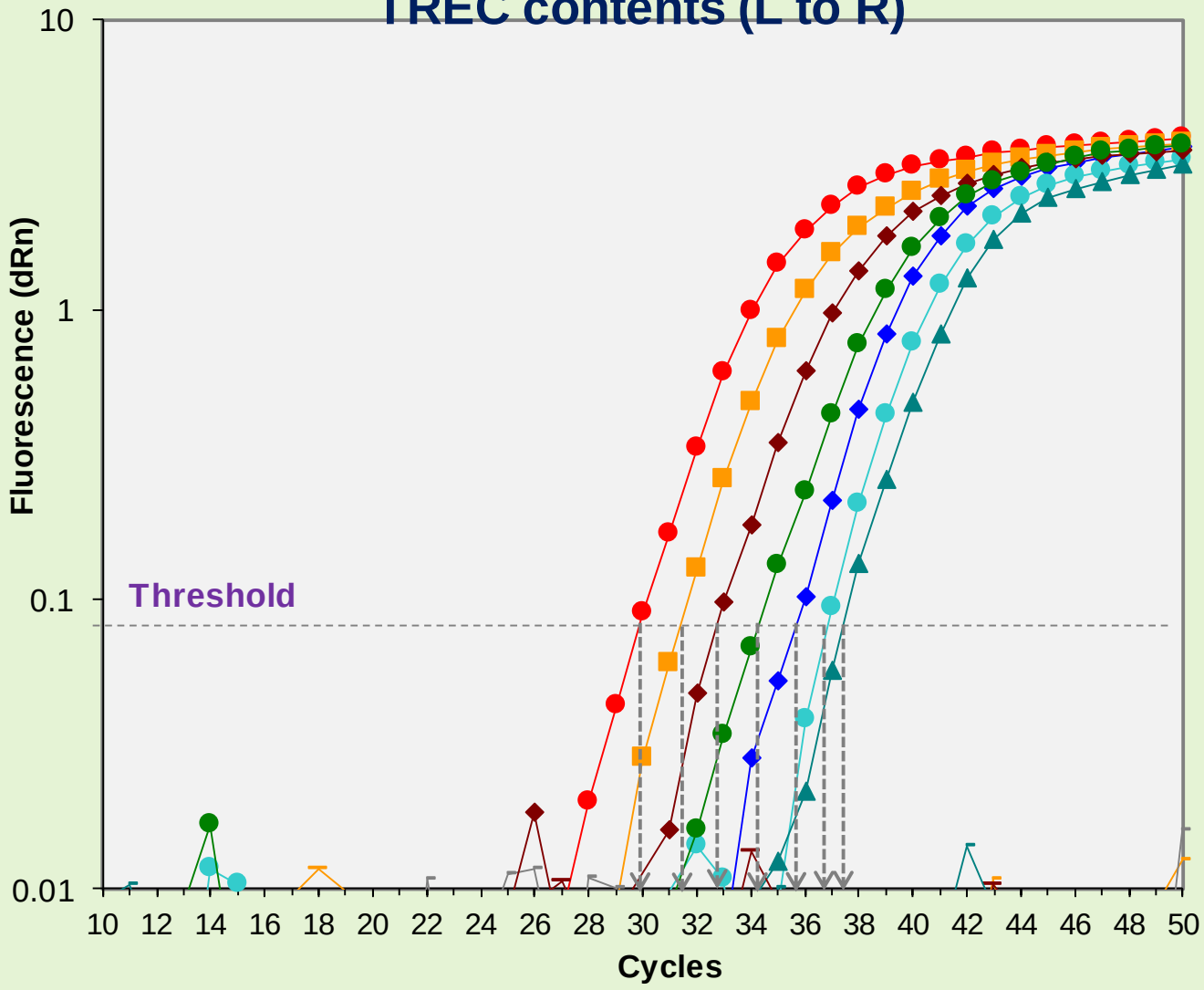
The amount of DNA doubles during each PCR cycle



TREC Real-time PCR Amplification Profile

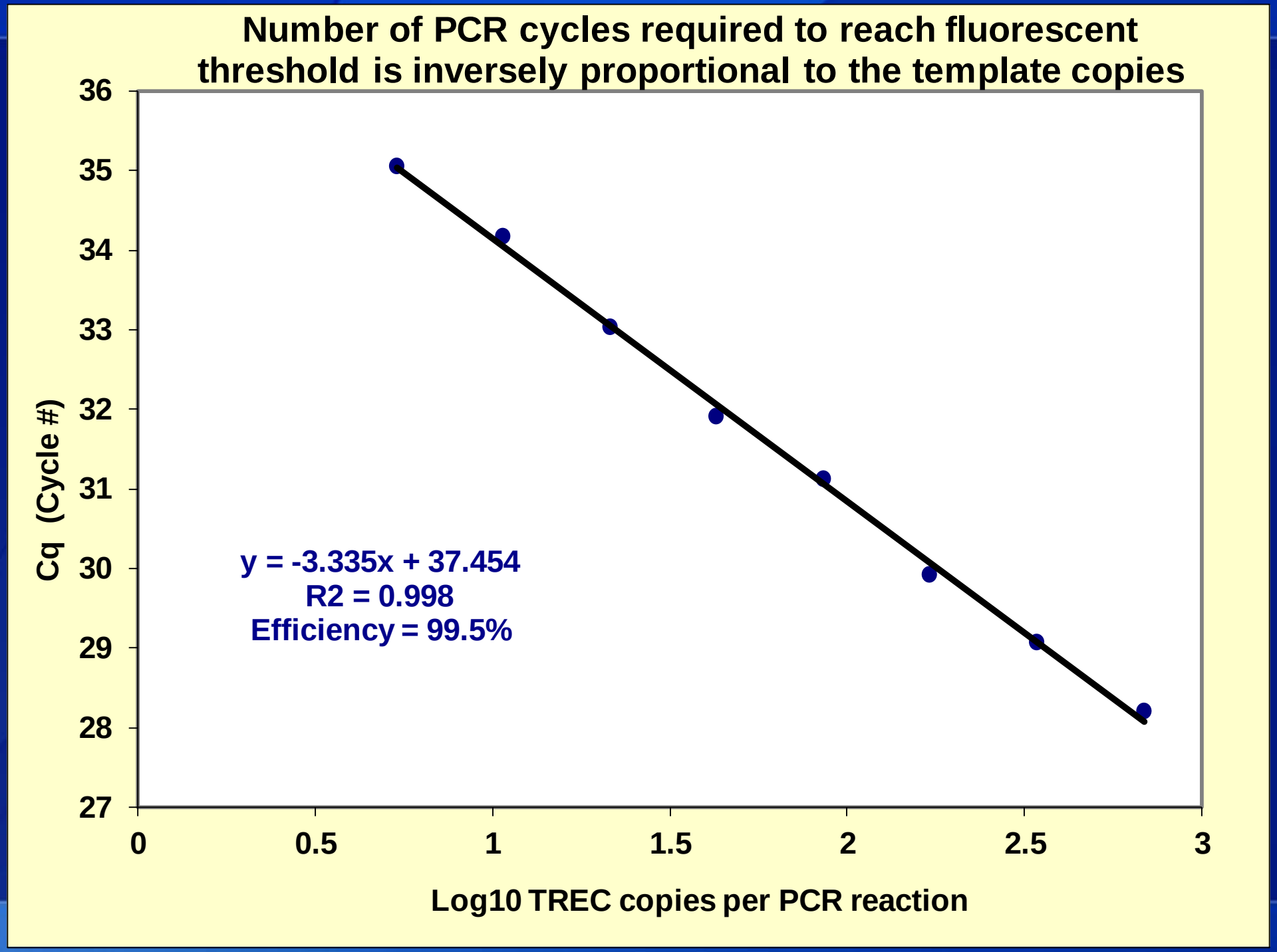


Amplification curves of samples with decreasing TREC contents (L to R)



The number of PCR cycles to reach a threshold depends on the initial concentration of target

Copy of amplicon doubles with each cycle	Number of amplification cycles required to reach 1 million copies		
	Sample with 1 copy	Sample with 16 copies	Sample with 128 copies
1	1		
2	2		
4	3		
8	4		
16	5	1	
32	6	2	
64	7	3	
128	8	4	1
256	9	5	2
512	10	6	3
1024	11	7	4
2048	12	8	5
4096	13	9	6
8192	14	10	7
16384	15	11	8
32768	16	12	9
65536	17	13	10
131072	18	14	11
262144	19	15	12
524288	20	16	13
1048576	21	17	14
2097152	22	18	15


Number of PCR cycles required to reach fluorescent threshold is inversely proportional to the template copies
Cq (Cycle #)
36
35
34
33
32
31
30
29
28
27
y = -3.335x + 37.454
R2 = 0.998
Efficiency = 99.5%
0
0.5
1
1.5
2
2.5
3
Log10 TREC copies per PCR reaction

Analysis of Data

Result of real-time PCR assay can be expressed as

1. **Cq**
2. **Copy number**
3. **Multiple of median: sample to population median ratio**
4. **Z-score: (sample – population mean) / standard deviation**

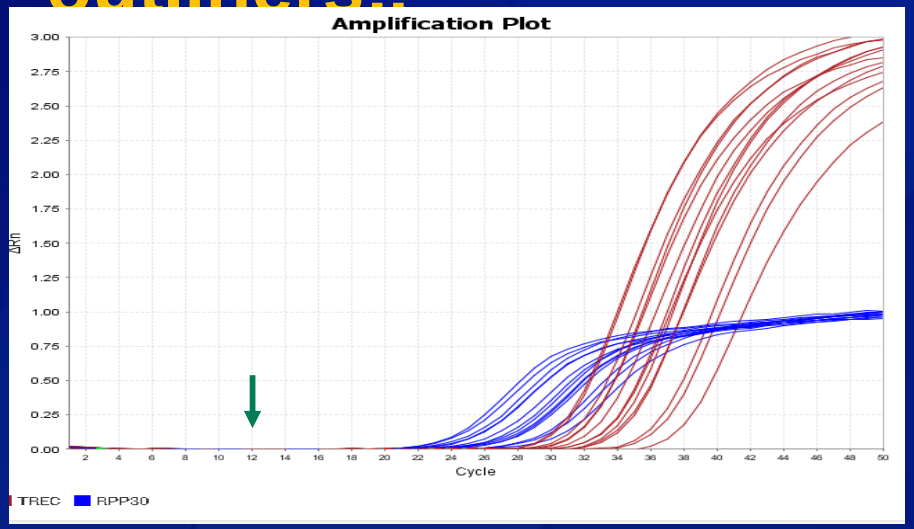
Cutoff value for positive result can be based on any of the above

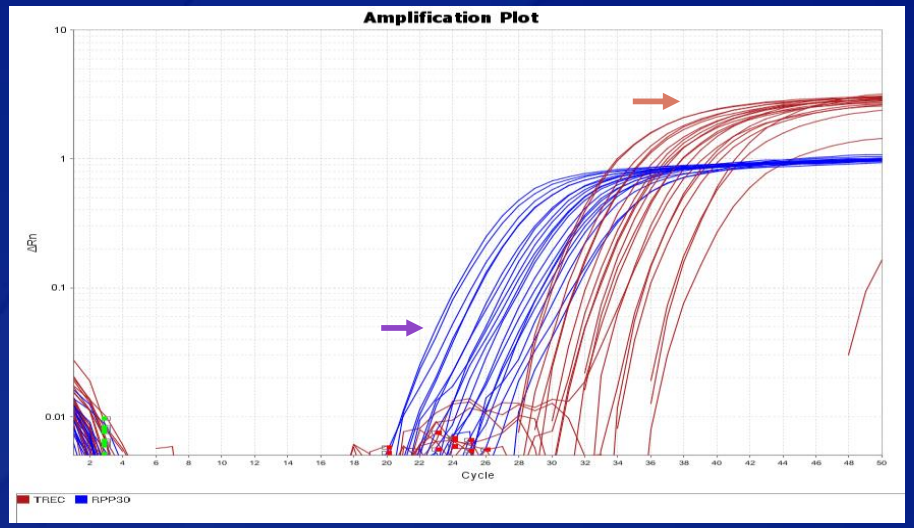
“Anybody can run real-time PCR”

. . . . . most of the time

Some useful tips for real time PCR assays

Always check amplification curves after a run to look for outliners!!





Good curves

On linear scale for Y axis:

- Smooth sigmoidal shape
- Uniform baseline ( )

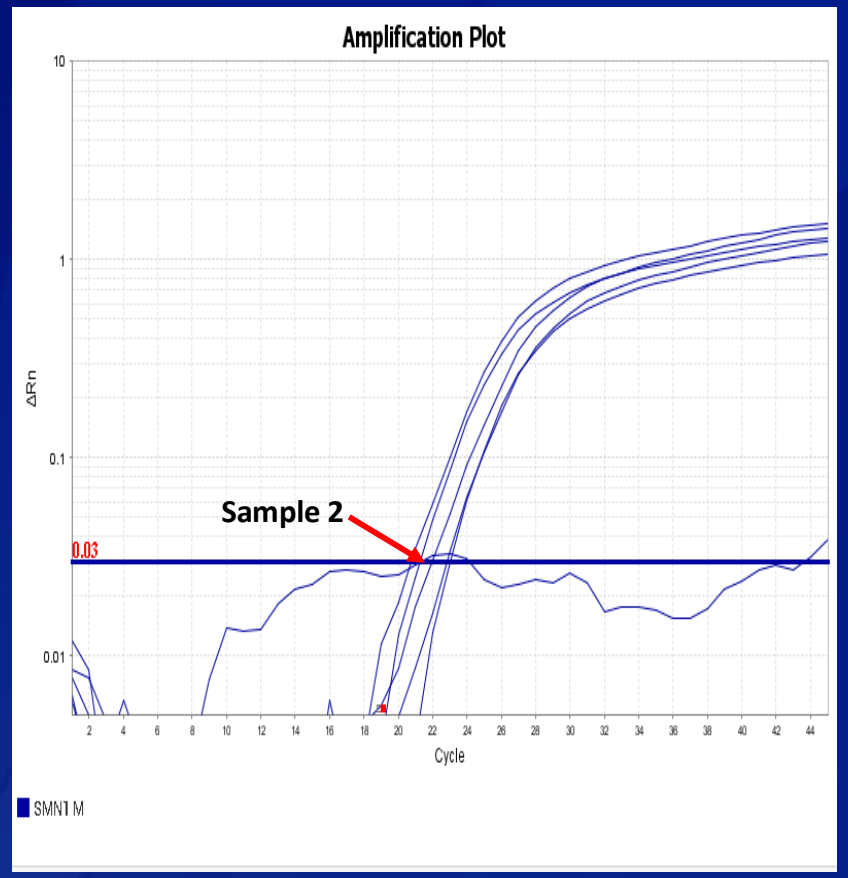
On log scale for Y axis:

- Uniform plateau for most samples ( )
- Parallel exponential phase ( )

But sometimes things can go wrong . . .

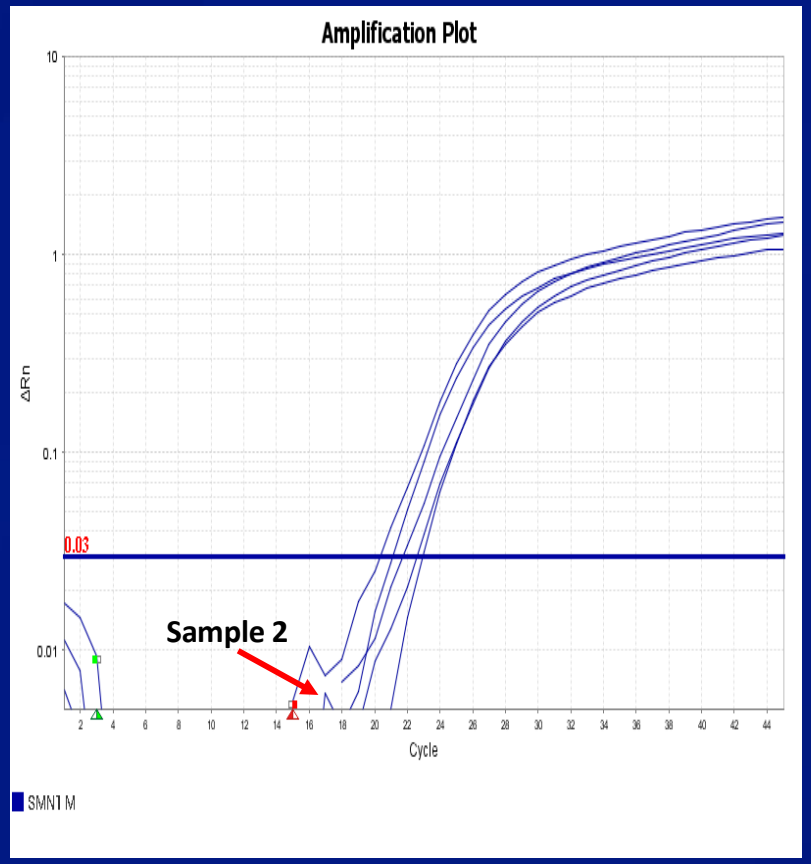
Amplification curve that doesn't fit the pattern …

Automatic baseline setting



	SMN1 Cq	Dx
Sample 1	20.90	normal
Sample 2	20.87	false negative
Sample 3	21.11	normal
Sample 4	21.20	normal
Sample 5	23.36	normal
Sample 6	22.24	normal

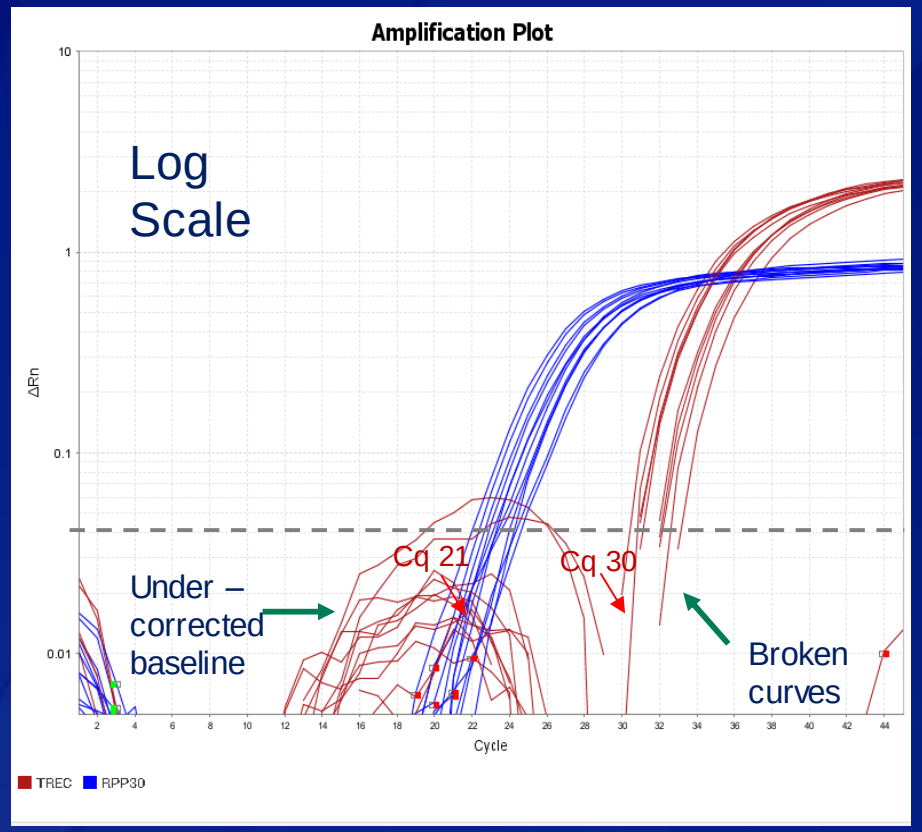
Manual baseline setting



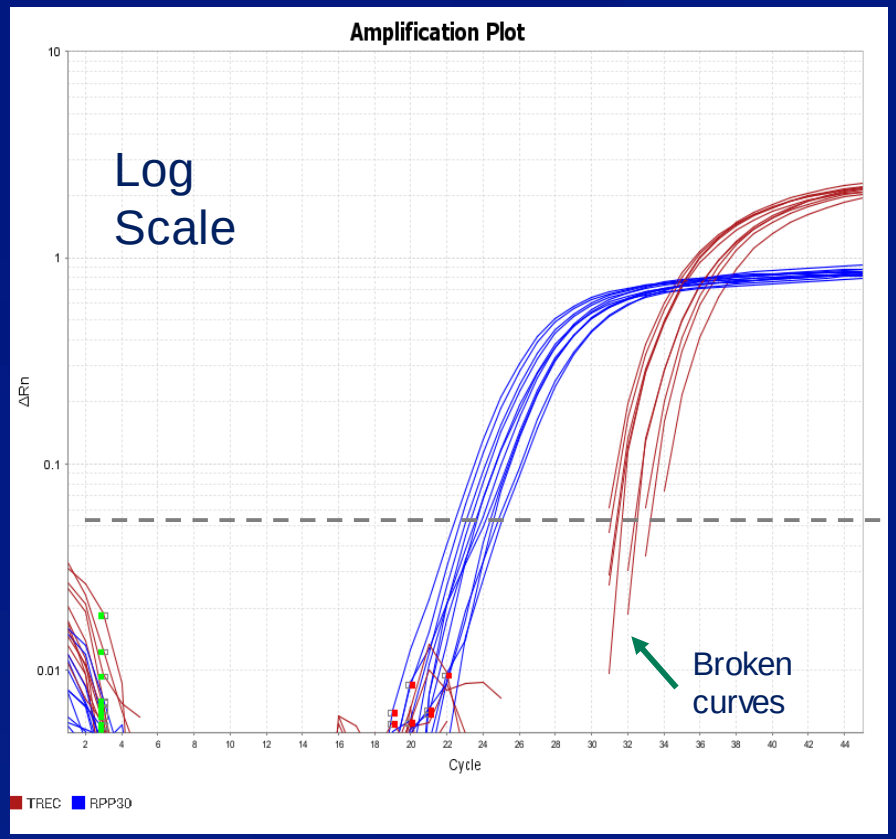
Sometimes baseline set automatically by software may result in odd-looking amplification curve, which can be corrected by using manual-set baseline (note: sample 2 was a positive SMA sample with no amplification for *SMN1*)

Multiple Setting Errors

Software automatic baseline



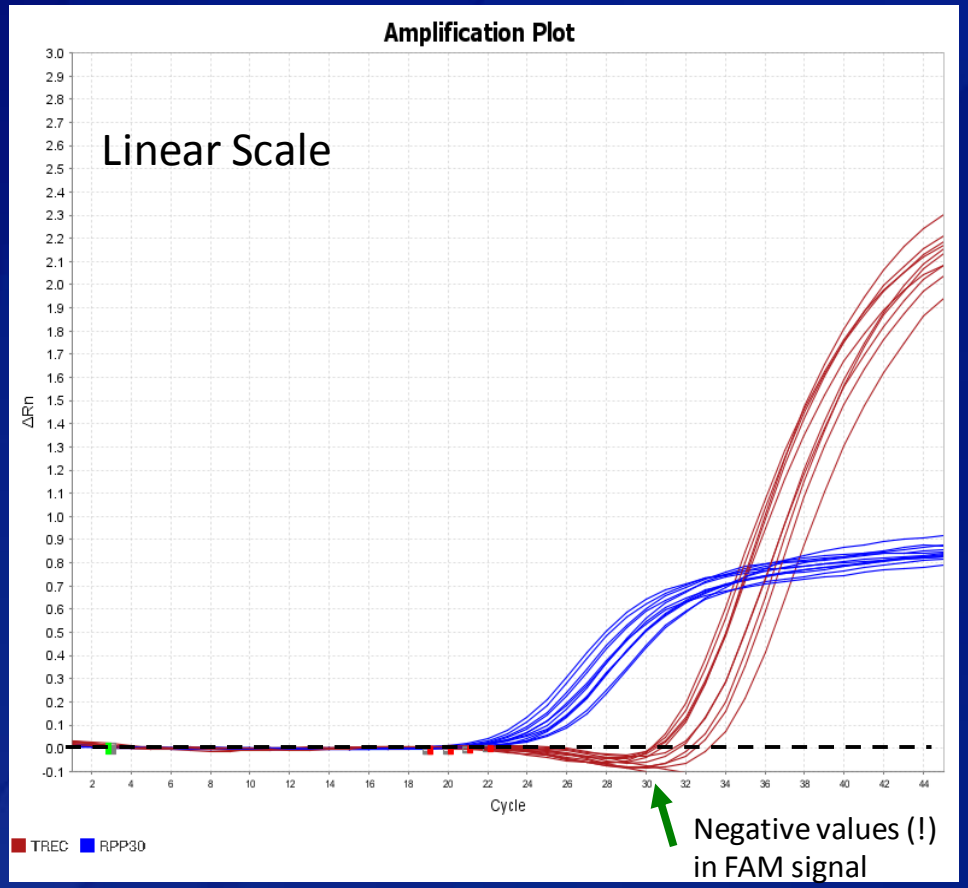
Manual set baseline (cycle 3 – 17)



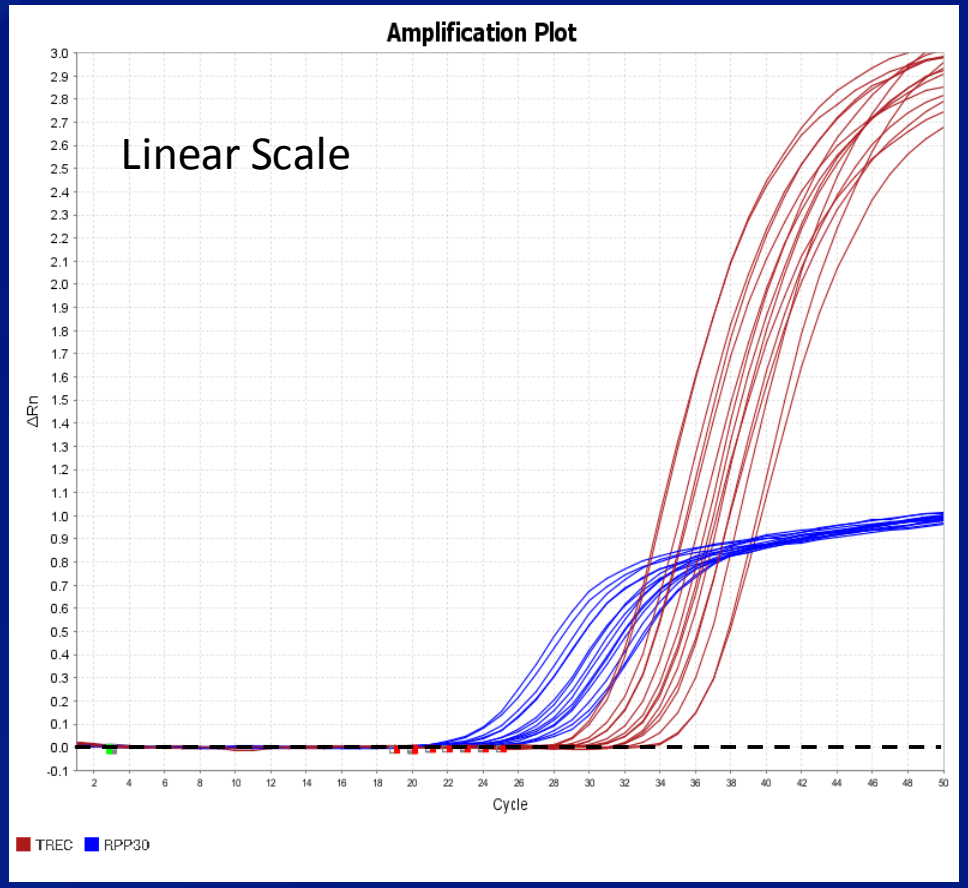
This test run had two problems: 1) high background due to incorrect baseline setting and 2) broken curves because of fluorescence over-compensation

Incorrect Fluorescence Compensation for Multiple Dyes

HEX probe using VIC channel

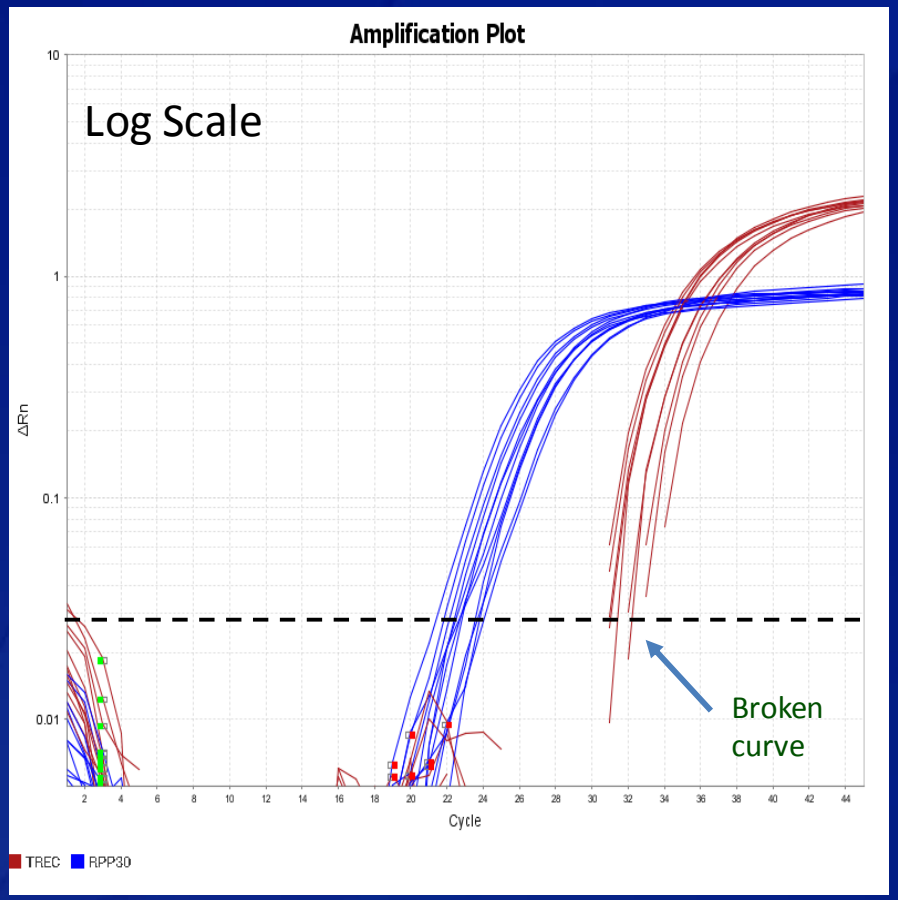


HEX probe using HEX channel

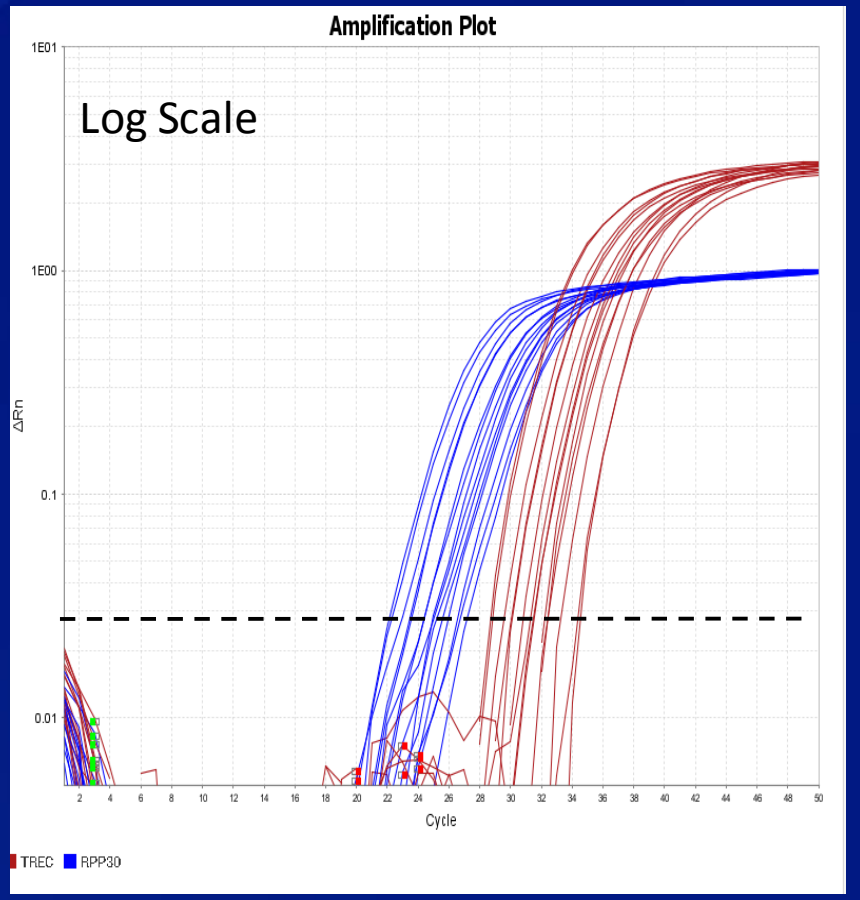


Using factory-installed VIC channel to detect HEX signal caused fluorescence over-compensation in the FAM (TREC) channel (left panel), which was corrected by customized calibration of Quantstudio real-time PCR instrument with HEX dye (right panel)

HEX probe using VIC channel



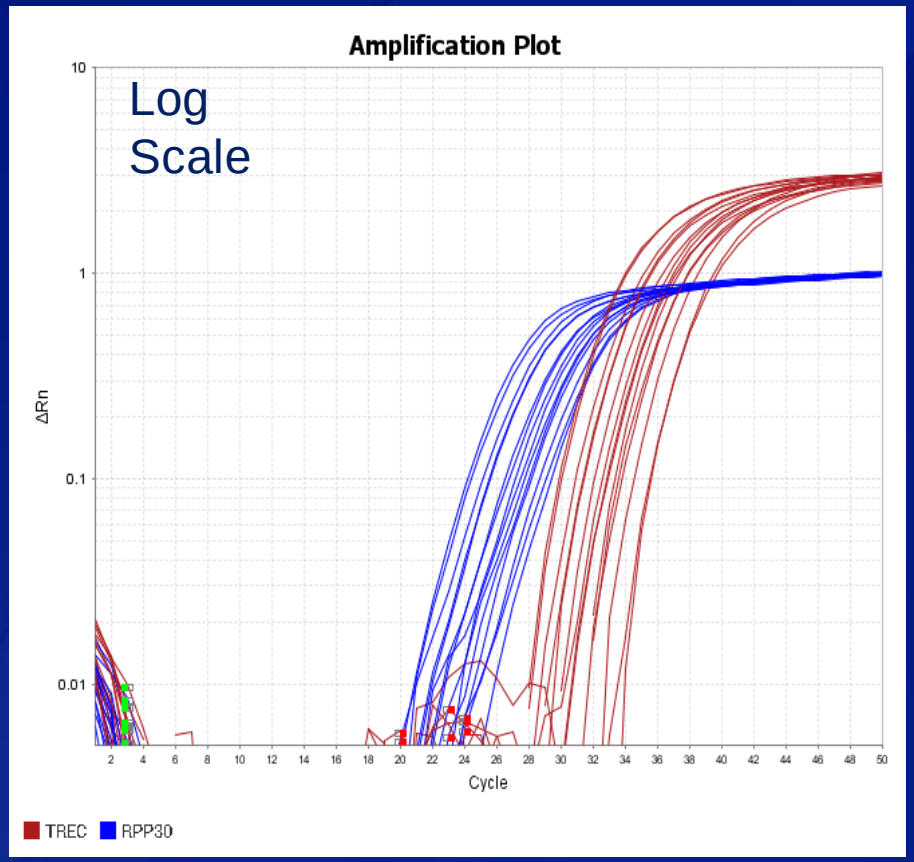
HEX probe using HEX channel



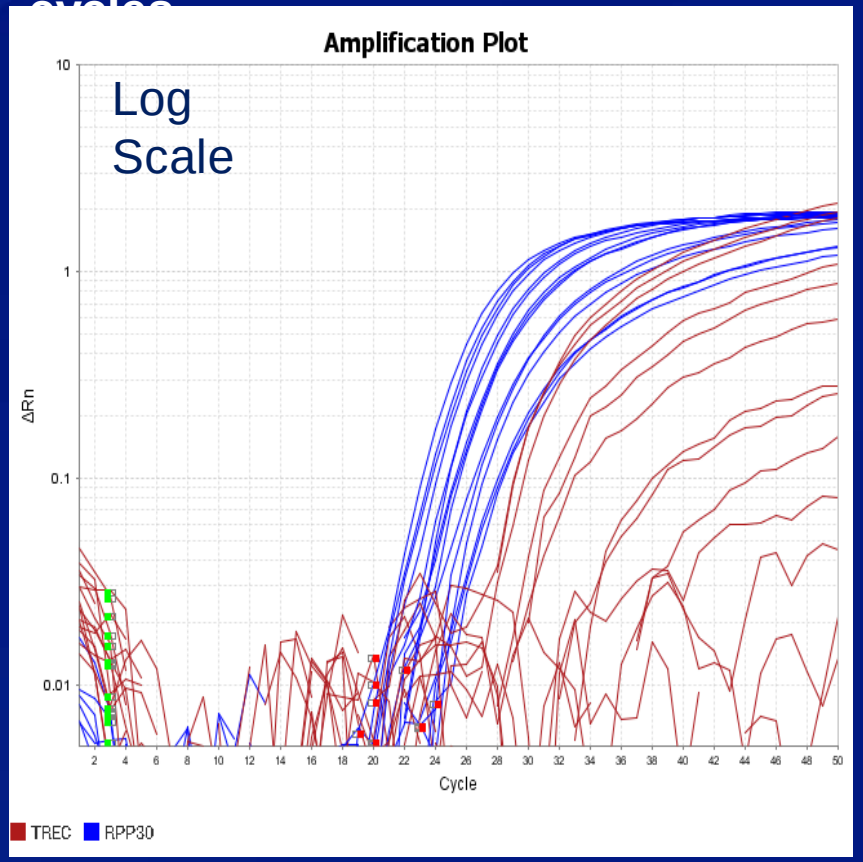
Broken amplification curves (left panel) have been corrected by customized calibration of instrument for the specific HEX dye (right panel)

More Trouble Shooting . .

Good PCR efficiency



Decreasing PCR efficiency in later cycles



Need to check primers, probe, polymerase (mastermix) … multiple possible causes

Conclusion

Real-time PCR is a powerful and versatile tool

- High throughput
- Relatively simple to run
- Modest cost

Better understanding of the mechanics of the technology can avoid unexpected issues

Thank you for your attention!

Newborn Screening

***Saving* Lives.**

***Promoting* Healthier Babies.**

***Protecting* our Future.**

For more information please contact Centers for Disease Control and Prevention

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E-mail: cdcinfo@cdc.gov Web: www.cdc.gov

CDC stock image