Clinical Chemistry
Trainee Council

PEARLS OF LABORATORY MEDICINE

Quantitative Real-Time PCR

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What is Real-Time PCR?

- Polymerase chain reaction (PCR)
- Real-Time /Quantitative PCR
  - Monitors the PCR reaction in “real-time”
  - qPCR
- Clinical laboratory applications
  - Detect infectious organisms
  - Identify mutations/genotyping
  - Quantify a molecular target sequence

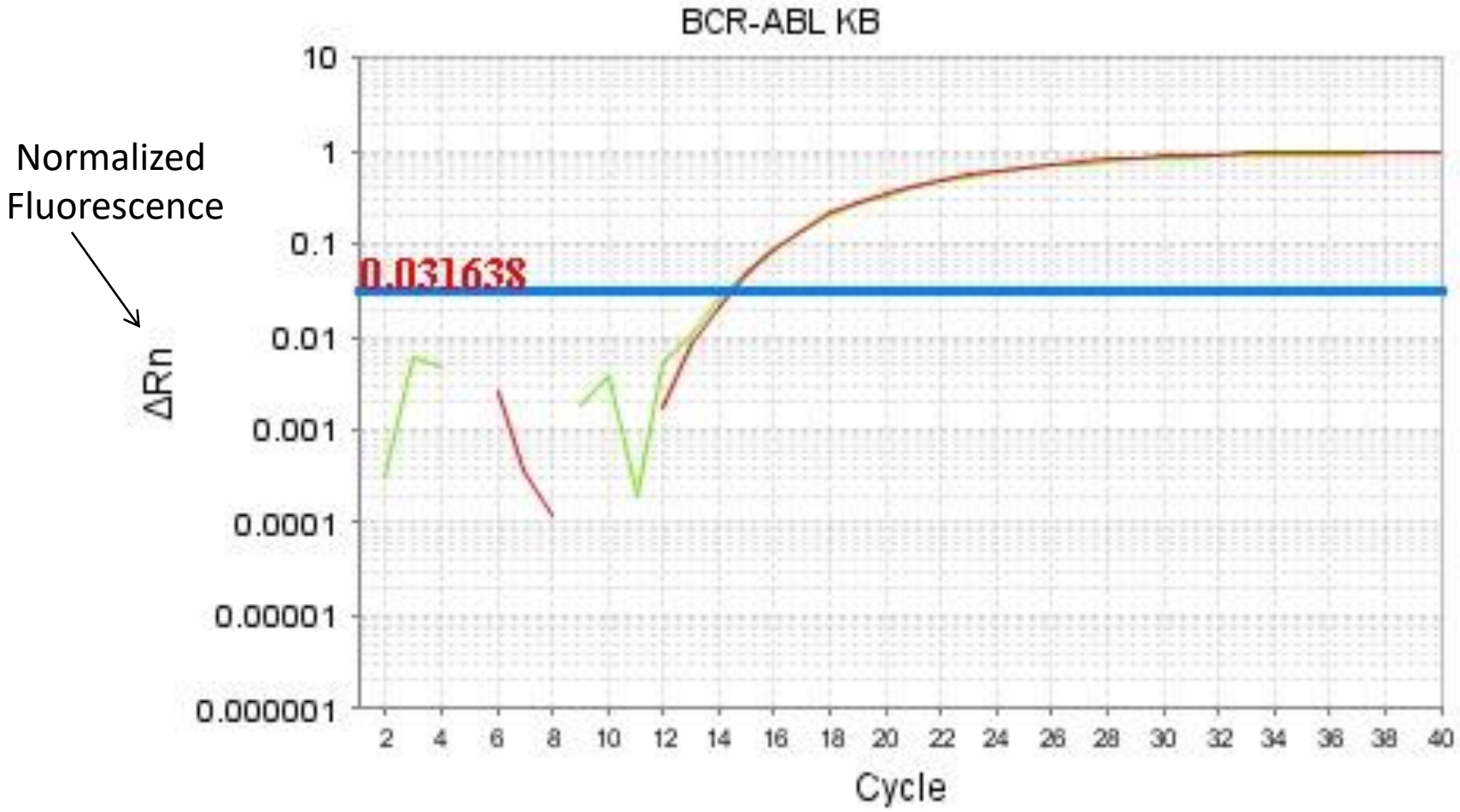
Endpoint PCR vs. Real-Time PCR

- Traditional PCR (endpoint PCR)
  - Amplifies nucleic acid
  - DNA doubles at each cycle (2^n)
  - Requires post-reaction analysis
    - Gel electrophoresis
    - Hybridization reactions
  - Only semi-quantitative
    - Reaction analyzed after exponential phase is over

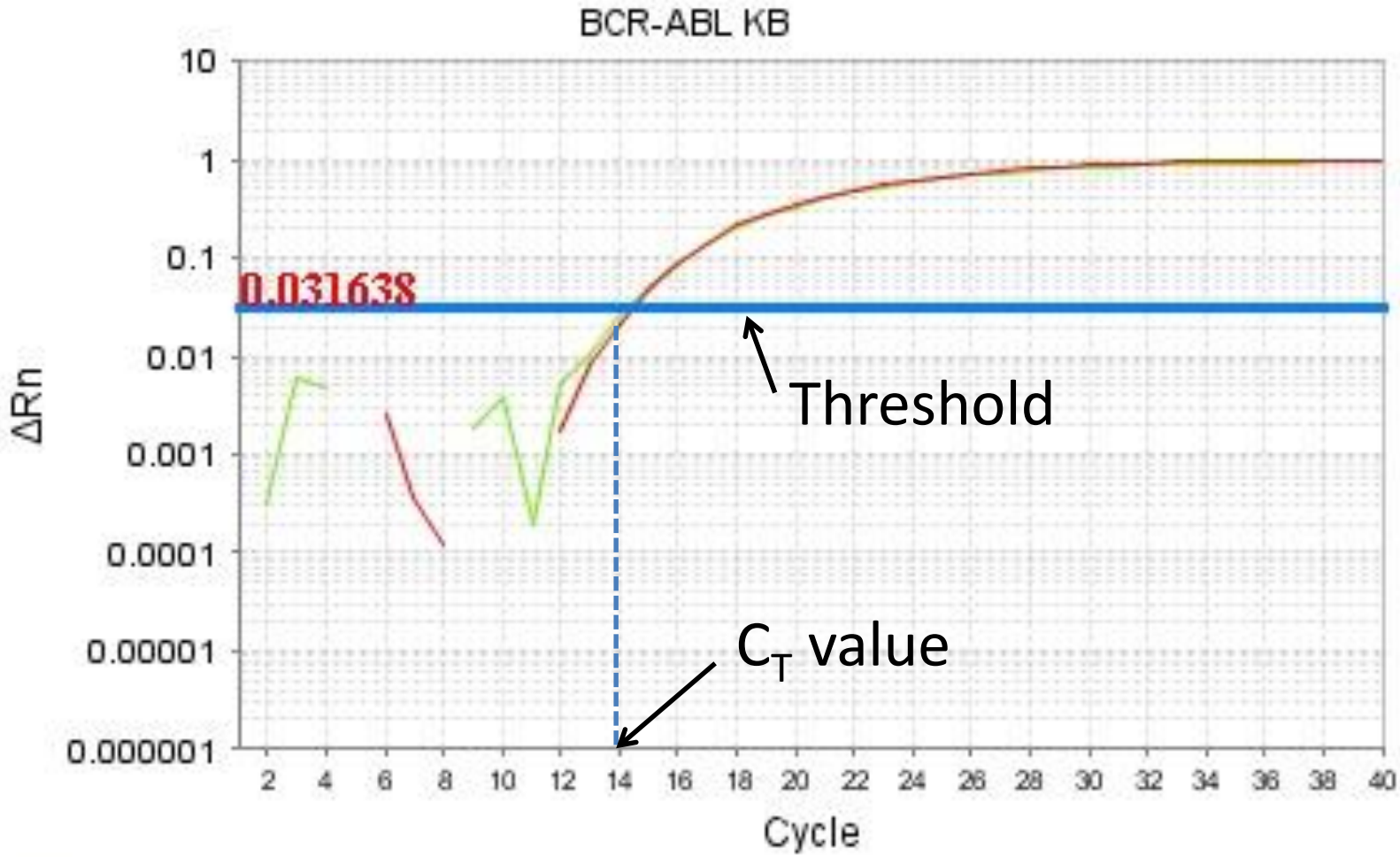
Endpoint PCR vs. Real-Time PCR

- Real-Time PCR
  - Amplicon accumulation tracked by fluorescence detection
  - Fluorescence is measured at each cycle
    - Allows data collection during exponential phase of reaction
  - Like “normal” PCR, with fluorescence chemistry that is monitored during entire reaction

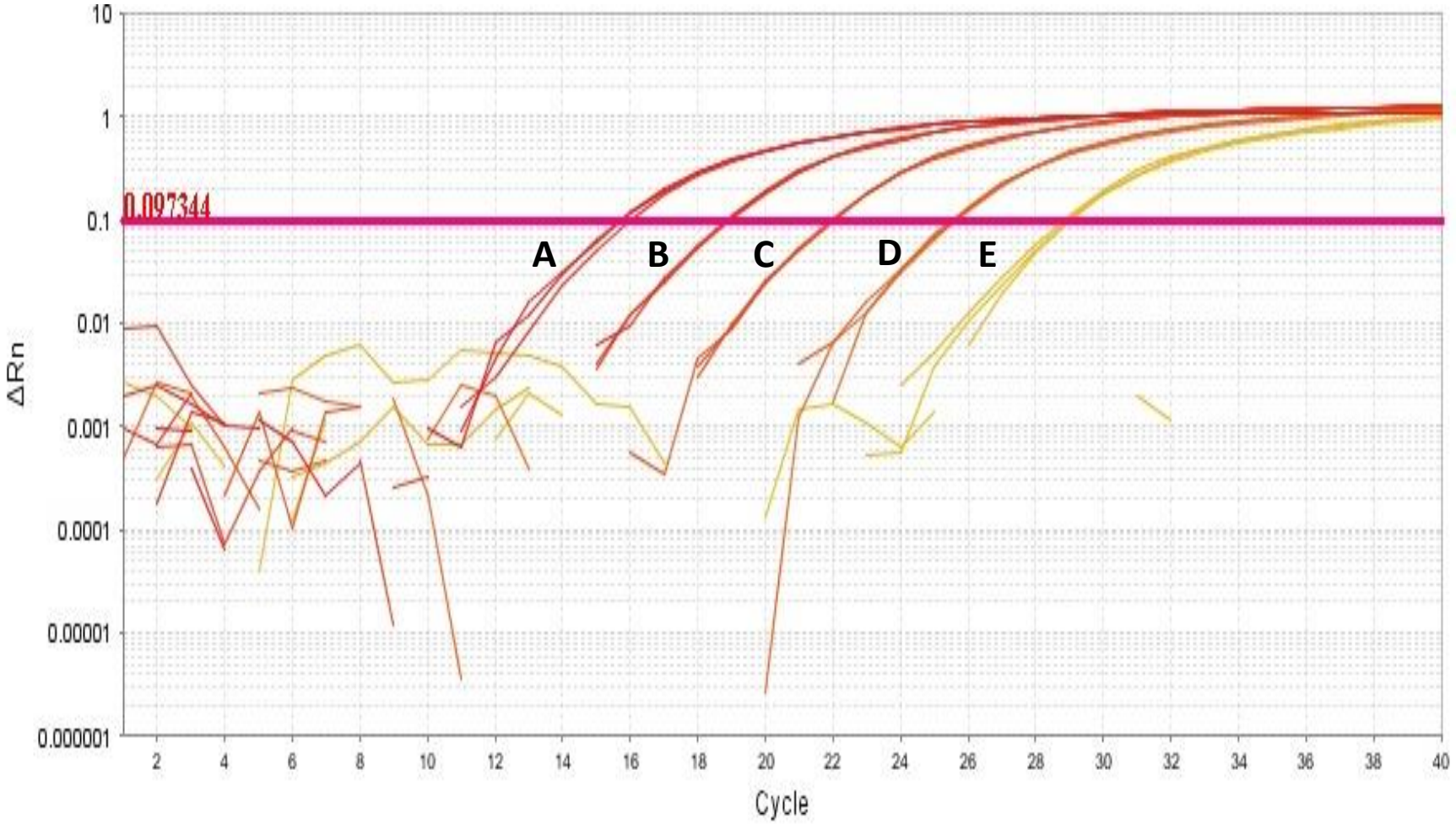
Fluorescence vs. Cycle Number



Threshold and C_T value



Real-Time PCR 10-fold dilution series



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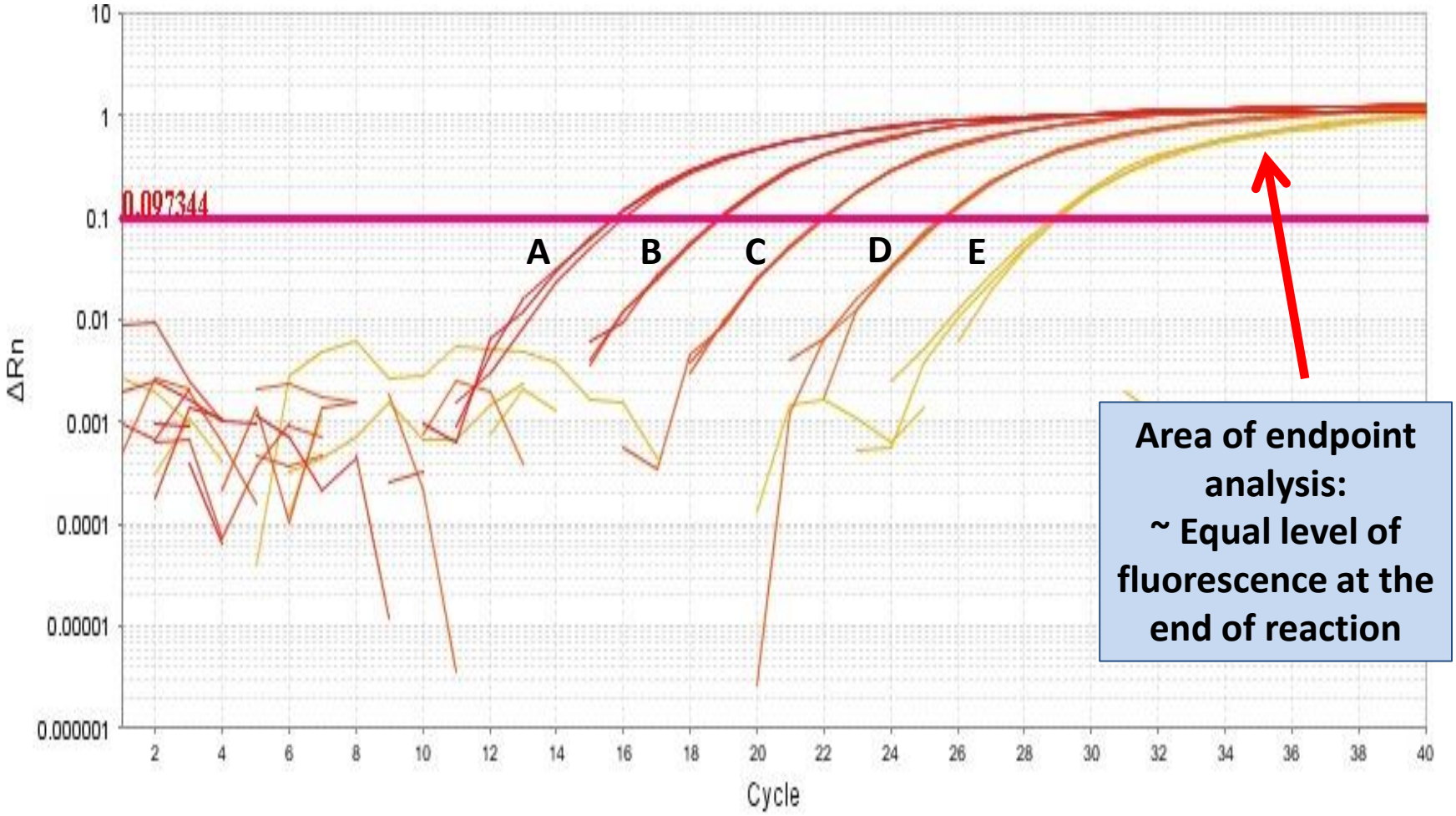
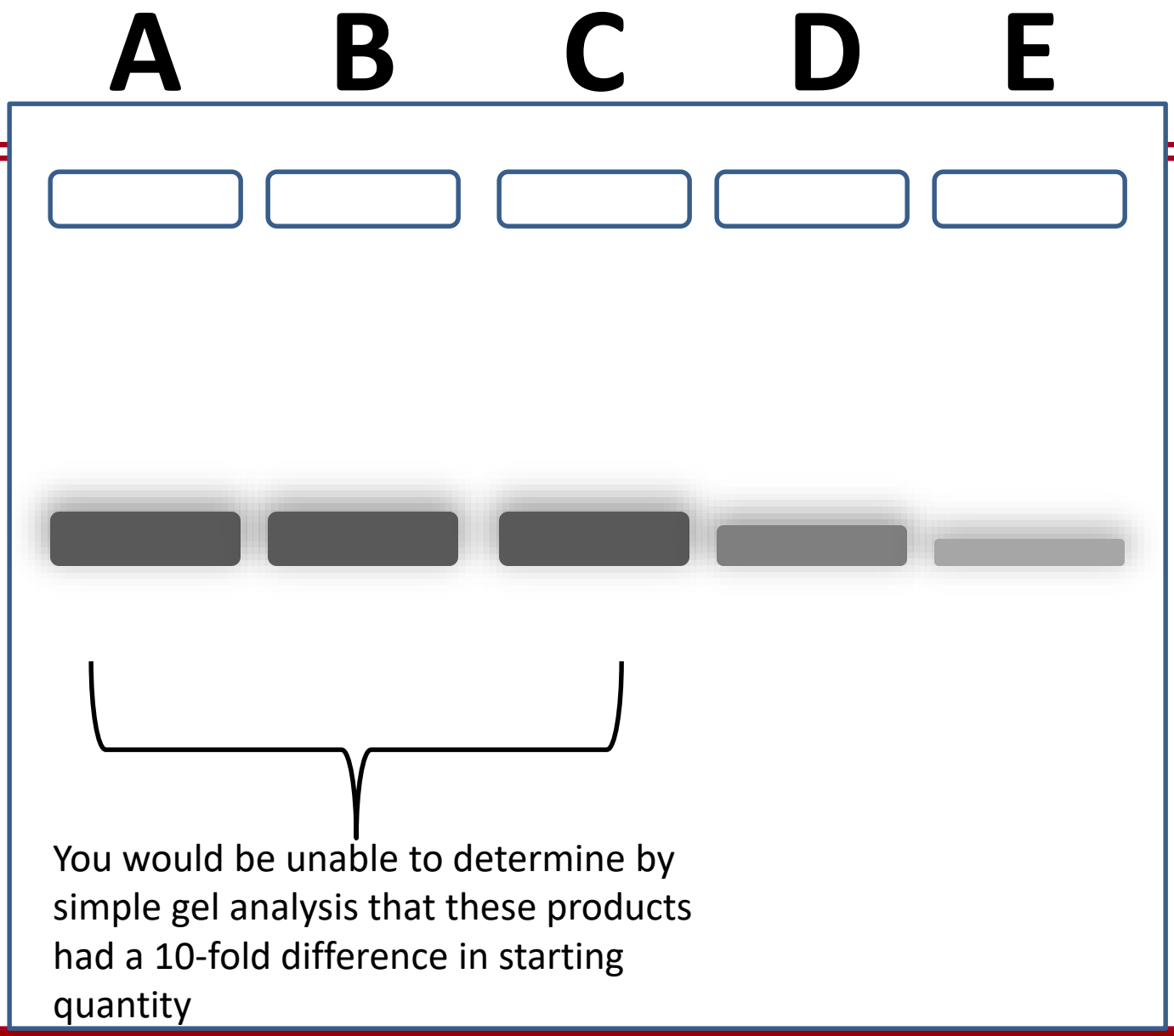



A
B
C
D
E
If gel run after 35 cycles of PCR…
You would be unable to determine by simple gel analysis that these products had a 10-fold difference in starting quantity

Real-Time PCR Data Table

#	Sample ...	Target N...	Task	Dyes	Ct	Ct Mean	Ct SD	Quantity
1		KB1/KB2	STANDARD	SYBR-None	15.650	15.739	0.152	100,000
2	A	KB1/KB2	STANDARD	SYBR-None	15.653	15.739	0.152	100,000
3		KB1/KB2	STANDARD	SYBR-None	15.915	15.739	0.152	100,000
4		KB1/KB2	STANDARD	SYBR-None	18.787	18.8	0.05	10,000
5	B	KB1/KB2	STANDARD	SYBR-None	18.758	18.8	0.05	10,000
6		KB1/KB2	STANDARD	SYBR-None	18.856	18.8	0.05	10,000
7		KB1/KB2	STANDARD	SYBR-None	21.923	21.922	0.021	1,000
8	C	KB1/KB2	STANDARD	SYBR-None	21.943	21.922	0.021	1,000
9		KB1/KB2	STANDARD	SYBR-None	21.901	21.922	0.021	1,000
10		KB1/KB2	STANDARD	SYBR-None	25.438	25.492	0.053	100
11	D	KB1/KB2	STANDARD	SYBR-None	25.544	25.492	0.053	100
12		KB1/KB2	STANDARD	SYBR-None	25.495	25.492	0.053	100
13		KB1/KB2	STANDARD	SYBR-None	28.747	28.86	0.099	10
14	E	KB1/KB2	STANDARD	SYBR-None	28.926	28.86	0.099	10
15		KB1/KB2	STANDARD	SYBR-None	28.908	28.86	0.099	10
16		KB1/KB2	NTC	SYBR-None	Undetermi...			
17								
18								

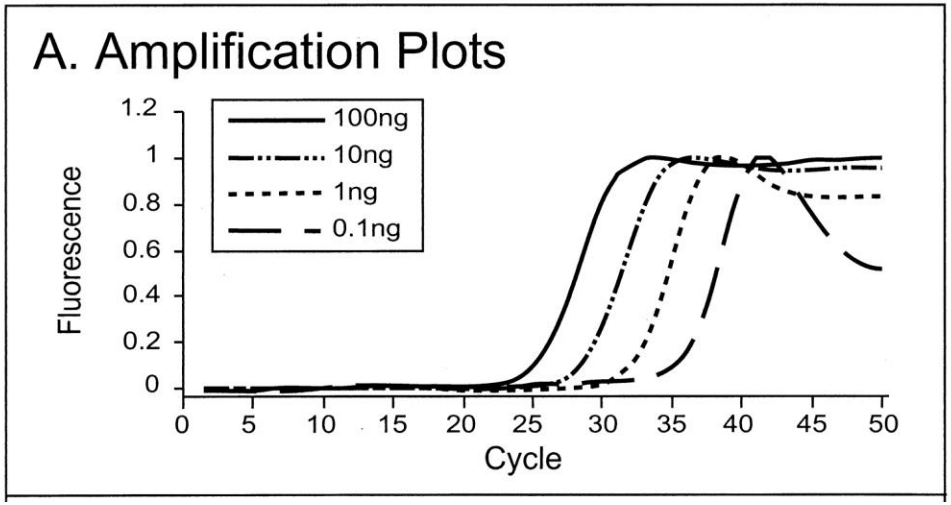
Real-Time PCR Chemistries

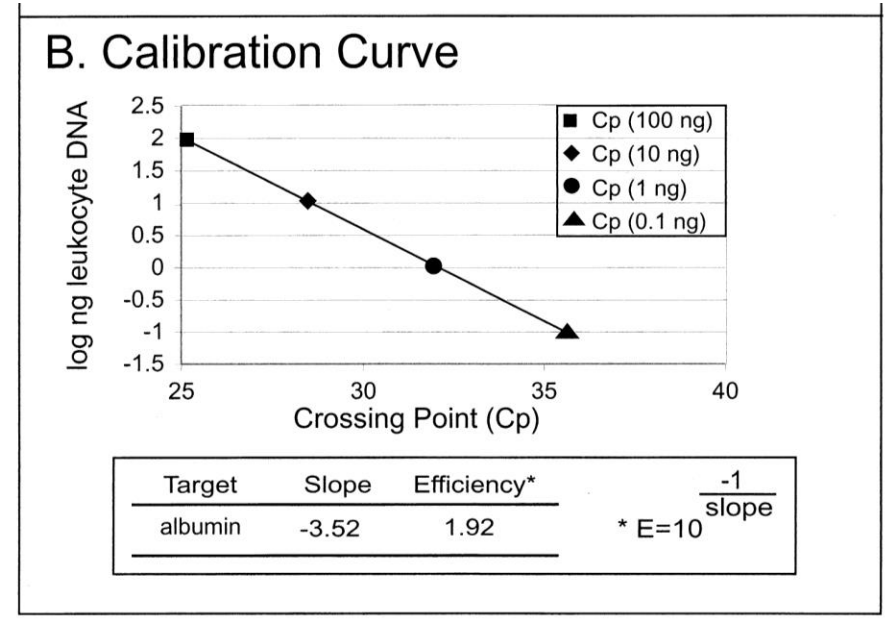
- DNA Binding Dyes
  - SYBR Green
  - LC Green
- Probes
  - 5’ Nuclease (TaqMan)
  - Hybridization probes
  - Molecular Beacons
  - Scorpion probes

Quantification Methods

- Absolute Quantification
  - Uses a standard/calibration curve
  - Results are given as copy number or DNA mass
- Relative Quantification
  - Uses mathematical formulas
    - Delta delta C_T method ($\Delta \Delta C_T$)
  - Requires a reference gene ("housekeeping gene")
  - Compares to a control sample
  - Fold-change is calculated

Absolute Quantification





Bernard PS and Wittwer CT. Real-time PCR technology for cancer diagnostics. Clin Chem 2002;48:1178-85.
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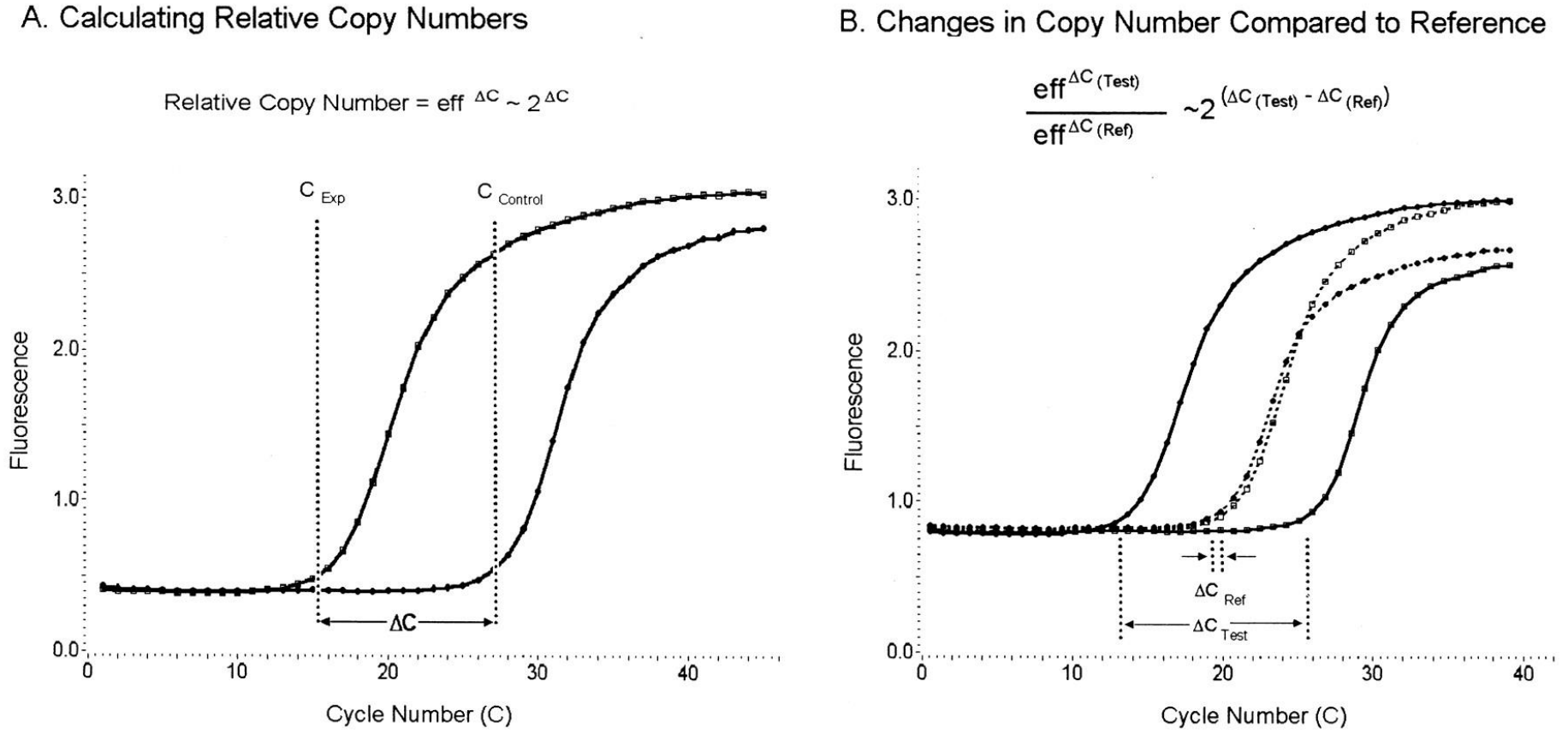
Relative Quantification



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Applications of Real-Time PCR

- Quantitative Applications
  - Viral load monitoring
  - Minimal residual disease testing
  - Copy number variations
- Qualitative Applications
  - Infectious disease detection
    - Bacteria and viruses
    - Fungi, parasites
  - Genotyping
    - Mutation detection (genetic or cancer)
    - Polymorphism analysis

Advantages and Disadvantages

- Advantages
  - Quantitative
  - Fast turnaround times
  - High sensitivity
  - Multiplexed reactions
  - Less risk of amplicon contamination in the lab
- Disadvantages
  - Higher cost than endpoint PCR
  - May require more complex data analysis

Advances in Real-Time PCR Technology

- Point-of-care applications
  - Sample to result in less than a hour
- Single cell amplification
  - For heterogenous specimens
- Microfluidic technology
  - GeneXpert
- Digital PCR
  - QuantStudio 3D Digital PCR System

Clinical Real-Time PCR Tests

Test	Manufacturer	Test Name
Clostridium difficile	Cepheid	Xpert C. difficile Test
Chlamydia trachomatis/Neisseria gonorrhoeae	Abbott	Abbott Real-time CT/NG m2000
Group B *Streptococci*	BD Diagnostics - GeneOhm	BD Max GBS Assay
Respiratory Panel	Biofire Diagnostics, Inc.	FilmArray RP Test
Influenza virus panel	Qiagen Inc	Artus Infl A/B RG RT-PCR Kit
HCV quantification	Roche Molecular Diagnostics	COBAS TaqMan HCV Test
Colorectal Cancer, KRAS	Roche Molecular Diagnostics	COBAS KRAS Mutation Test
Melanoma, BRAF V600 Mutation	Roche Molecular Diagnostics	COBAS 4800 BRAF V600 Mutation Test
Drug metabolizing enzymes	ParagonDx, LLC	Gentris Rapid Genotyping Assay-CYP2C9 & VKORC1
Factor V Leiden	Roche Molecular Diagnostics	Factor V Leiden kit

These FDA-approved tests are listed at http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm330711.htm

Summary

- Real-Time PCR is a valuable tool in the clinical laboratory
- Use of fluorescence allows for accurate quantification
- Common method used in molecular laboratories
- Many applications
  - Microbiology
  - Oncology
  - Genetics

References and Resources

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Disclosures/Potential Conflicts of Interest

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- **Patents:** None declared

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