Clinical Chemistry Trainee Council
Pearls of Laboratory Medicine
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TITLE: Quantitative Real-Time PCR

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Slide 1:
Hello, my name is Katie Bennett. I am an Assistant Professor of Molecular Pathology at Texas Tech University Health Sciences Center (TTUHSC), in Lubbock, Texas, and clinical Laboratory Director of the TTUHSC Laboratory for Molecular Diagnostics. Welcome to this Pearl of Laboratory Medicine on "Quantitative Real-Time PCR."

Real-time PCR is a molecular diagnostic method that is widely used in the clinical laboratory. I will outline the differences between traditional PCR and real-time PCR, discuss the principles of DNA quantification using real-time PCR, and point out some of the clinical applications of real-time PCR.

Slide 2: What is Real-Time PCR?
Real-time PCR is a DNA amplification method that monitors the PCR reaction as it happens, in "real-time." When real-time PCR is used to quantify nucleic acid, it is also known as qPCR. Real-time PCR may be used clinically to detect disease, quantify a molecular target, or for genotyping.

Slide 3: Endpoint PCR vs. Real-Time PCR
Traditional endpoint PCR is an exponential reaction in which the copy number of DNA approaches 2^n, where n is equal to the number of PCR cycles. Endpoint PCR requires analysis at the conclusion of 30-45 cycles. This is typically done by gel electrophoresis or hybridization detection. Because PCR is a saturable reaction in which primers, enzyme, and nucleotides may be depleted, endpoint analysis may not represent the doubling of PCR product during the exponential phase of PCR.

Slide 4: Endpoint PCR vs. Real-Time PCR (cont.)
During real-time PCR, the accumulation of DNA product is monitored using fluorescence. By detecting fluorescence levels at each cycle, instead of only at the end of the reaction, the exponential period of amplification can be observed. This capability is important for accurate quantification of nucleic acid. Real-time PCR is essentially a "normal" PCR reaction with fluorescent chemistry added in, analyzed throughout the entire reaction.

Slide 5: Fluorescence vs. Cycle Number

This slide shows a typical graph of a real-time PCR reaction. The fluorescence, which is proportional to the amount of amplicon, is plotted at each cycle of the PCR reaction. Because data is collected throughout the reaction, no gel electrophoresis analysis is necessary

Slide 6: Threshold and C_T value

For analysis of real-time PCR data, a threshold is set at a point within the exponential region of the amplification. The cycle at which the fluorescent signal of the sample crosses the threshold is called the threshold cycle, or C_T. The C_T of a reaction is determined by the amount of starting DNA template at the beginning of the PCR reaction. Large amounts of template will require fewer amplification cycles to give detectable fluorescent signal, while small amounts of template will require more PCR cycles to generate signal above background. Thus, low C_T values indicate the presence of more target DNA, while high C_T values indicate less DNA target in the sample. This concept, along with the theoretical doubling of amplicon at each cycle, is the basis for quantification in real-time PCR.

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The next several slides show an example of five DNA samples that were analyzed by real-time PCR. Sample A has the highest amount of DNA at the beginning of the reaction. Samples B-E are 10-fold dilutions from Sample A, with E having the lowest amount of starting DNA template. On this real-time PCR graph, note that Sample A shows fluorescence levels that meet the threshold first, around 16 cycles (C_T of ~16). Sample E takes much longer for fluorescent signal to cross the threshold (C_T of ~29), indicating the lower starting quantities of DNA.

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If the same samples were assayed by traditional endpoint PCR, they would be analyzed by electrophoresis at the end of the reaction, such as at 35 cycles. Notice that although Samples A-E differ by a factor of 10, at 35 cycles there is little difference in the final amount of product.

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This will result in nearly equivalent results on the less-sensitive electrophoresis gel, despite the large differences in original DNA quantity. Therefore, endpoint PCR is only semi-quantitative at best. Real-time PCR tracks the reaction as it occurs and the differences in DNA quantity between samples can be easily determined by observing amplification earlier in the reaction before the reaction plateaus.

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This slide shows the real-time PCR data table for Samples A-E (performed in triplicate). Note the C_T values for each sample and the inverse correlation with the DNA quantity in the far right column.

Slide 11: Real-time PCR Chemistries

There are a number of PCR chemistries available to monitor the amplification of the target sequence in real-time PCR. They can be placed into two major categories, DNA binding dyes and probes. SYBR green is the most common DNA binding dye. It binds double-stranded DNA and provides high sensitivity.

However, it can result in non-specific signal from inappropriate primer hybridization or from primer-dimer. Real-time PCR probes bind specific nucleotide sequences within the target, which increases specificity of fluorescent signal. In a process called multiplexing, multiple probes that target several unique DNA sequences can be used together within one reaction. There are a number of probes available, including 5’ nuclease probes (TaqMan), hybridization probes, molecular beacons, and Scorpion probes.

Slide 12: Quantification Methods

There are two major quantification methods in real-time PCR: absolute quantification and relative quantification. Absolute quantification makes use of standard curves developed from a dilution series of known DNA template. Unknowns can be quantified according to an absolute copy number. In relative quantification, mathematical methods are used to calculate the fold-change in DNA target of a sample from that of a control sample. Both methods may be used with either DNA or RNA targets.

Slide 13: Absolute Quantification

The absolute quantification method is the most common approach used clinically.This figure shows an example of a standard curve, also known as a calibration curve. The standard DNA samples are amplified in the same run as the unknown samples. The C_T values are determined for the standards and then plotted against the log of the known starting DNA quantity. Note that this figure uses the term “crossing point” or C_P, which is the same as C_T. The standard curve should be a straight line with a negative slope. Remember that the C_T (or C_P) value is inversely related to the quantity of DNA template. Once a standard curve is generated, the unknown samples can be plotted on the curve to calculate the amount of target DNA. Some quantitative real-time PCR systems may be optimized for use with only 2-3 calibration controls or have internal calibration curves saved within the system.

Slide 14: Relative Quantification

Relative quantification is a method that is used to compare fold-changes in target DNA from a control sample. This method is most commonly used in research to compare changes in gene expression from a control or untreated sample, but it may also be useful to quantify gene duplications or deletions for clinical analysis. In this method, the target DNA sequence and a reference sequence are assayed simultaneously. The amount of starting target template is normalized against the reference sequence (housekeeping gene). Then, the target/reference ratio for both control and unknown samples are compared to determine a fold-change in target template from control. The delta delta C_T ($\Delta\Delta C_T$) formula is a common mathematical method used to calculate relative quantities of DNA. This figure shows an example of relative quantification.

Slide 15: Applications of Real-Time PCR

Real-time PCR has many applications in both research and clinical laboratories. The quantitative capabilities may be used for applications such as viral load monitoring or minimal residual disease analysis of tumor burden in cancer. The high sensitivity and fast run times also make real-time PCR useful for qualitative detection of disease, such as in microbial detection. The technique can also be used to perform genotyping for mutation or polymorphism analysis.

Slide 16: Advantages and Disadvantages

Advantages of real-time PCR include quantification of DNA, fast turnaround times, high sensitivity, and multiplexed reactions. Real-time PCR also reduces the potential for amplicon contamination of the laboratory, because post-amplification manipulation of samples is typically unnecessary. Disadvantages of real-time PCR include the higher cost of reagents and instrumentation as well as the need for more complex data analysis.

Slide 17: Advances in Real-Time PCR Technology

A number of recent advances have been made in real-time PCR technology. Assays have been developed that can analyze gene expression profiles in single cells using reverse transcriptase-qPCR. A number of rapid tests are utilized clinically that go from sample to result in less than an hour, allowing real-time PCR technology to function as a point-of-care test. This progress is based on the optimization of microfluidic technology. Other advances include digital PCR, which is a modification of real-time PCR that has even higher sensitivity and can be used for absolute quantification.

Slide 18: Clinical Real-Time PCR Tests

The chart on this slide shows just a few examples of real-time PCR assays that are FDA-approved for clinical use in the United States. Many clinical laboratories also choose to use laboratory-developed tests, or “home brews.”

Slide 19: Summary

In sum, real-time PCR is a molecular amplification method that has many applications in both research and clinical diagnostics. The use of fluorescent probes or dyes allows for accurate quantification of the target DNA during the exponential phases of amplification. Real-time PCR is rapidly becoming the method of choice for many clinical laboratories, with applications in microbiology, oncology, genetics, and other laboratory departments.

Slide 20: References and Resources

Slide 21: Disclosures

Slide 22: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine on “Quantitative Real-Time PCR.” I am Katie Bennett.