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Chapter 2

Molecular Science
Assay Design and Validation

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Assay Design Considerations

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- Sample preparation
- Sample extraction method
- Preparation of template
- Experiment setup
- Optimization of primers and probes

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Sample Preparation

- Is it a DNA or RNA sample?
- Source of sample
  - Examples: whole blood, body fluids
- Homogeneity of sample
- Preparation time to extract nucleic acids
- Sample degradation

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Sample Extraction

- DNA/RNA
  - Determine the best extraction method based on whether it's DNA or RNA and the source of the sample
    - Organic phase extraction
    - Liquid phase extraction
    - Solid phase extraction
- Possible contaminants
  - Starches
  - Lipids
  - Metals
- Contaminants as a result of extraction
  - Phenol chloroform (organic extraction)
  - Salts (inorganic extraction)
- Possible sample degradation as a result of extraction

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Preparing Template DNA

- Genomic DNA
  - A restriction enzyme that does not cut within the amplicon is preferred
  - Boil genomic DNA for approximately 10 minutes and place on ice
- Plasmid DNA
  - Use restriction enzyme that does not cut within the amplicon
- Complementary DNA (cDNA)
  - A DNA copy (cDNA) of mature mRNA can be made by reverse transcription of mRNA
    - Treat RNA with RNAse-free DNAse before reverse transcription
    - Select an enzyme that is able to digest RNA from RNA:DNA hybrid after making cDNA

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Template Preparation: RNA

- Extract and analyze RNA
- Careful quantification is necessary
- RiboGreen Assay - Quantification
- NanoDrop – Quantification and purity
- Experion™ - Quality and quantification

Source: Bio-Rad (www.biorad.com/genomics/pcrsupport)

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Experiment Setup

- Replicates
  - Need for both technical replicates and biological replicates
  - Number of replicates will depend on level of differences that are being presented.
  - Lower expression genes tend to require more replicates to establish statistical validity of small differences.

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Optimization of Primers And Probes

- Characteristics of an optimized PCR Assay
  - One specific product
  - Efficient PCR
  - Accurate reproducibility of results
  - Wide range of sensitivity
- Each of the mentioned characteristics can be tested experimentally
- Spending more time on designing an assay can result in less time achieving validated results

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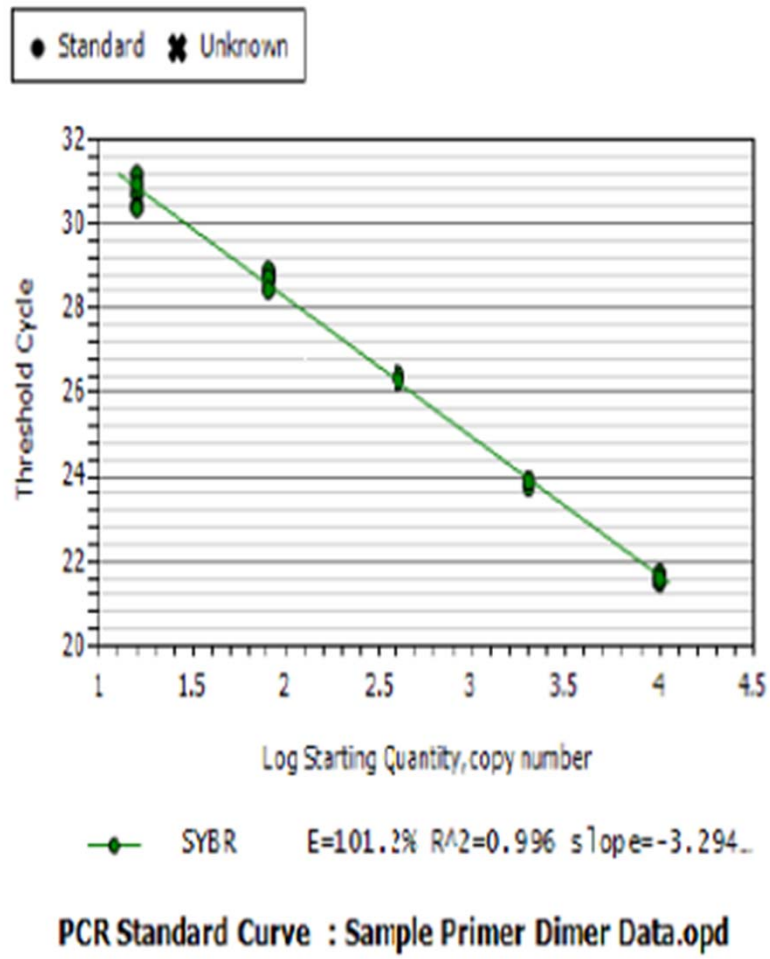
Optimization of Primers and Probes

- A PCR Reaction efficiency is 100% if the product doubles
  - Efficiency should be 100+/-10%
- Efficiency can be measured by using a serial dilution of the template
- Calculate efficiency using a standard curve slope



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Optimization of Primers and Probes

- Limit the production of secondary structures
- Make sure primers and probes have no more than a 50 to 60% overall GC content
- Limit stretches of G or C's in a sequence longer than 3 bases
- Make sure there are no G's on the 5' end of sequence
- Place C's and G's on the end of primers, but limit this to no more than 2 in the last five bases on the 3' end

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Guidelines for Selecting Primers

- Avoid choosing primers that have a tendency to anneal to themselves or to other primers
- Choose primers that are specific to your target sequence
- Avoid primers containing sequences that are complementary to the internal sequences of the intended final product, especially at the 3' end of primers
- Use primers between 18 and 25 bases that are matched in melting temperature to each other
- Select a desired product length that is lees than 500 bases
  - Shorter products tend to amplify with greater efficiency than lon products
- Perform a BLAST search to search for sequences that are similar to the selected primers (http://www.ncbi.nlm.nih.gov/BLAST)

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Assay Design: General Guidelines

- CLIA regulations (42 CFR Section 493.1253):
  - Laboratory developed assays, or "home brew" assays must have the following requirements documented:
    - Analytical sensitivity
    - Analytical specificity
    - Precision
    - Accuracy
    - Measuring range of test results
    - Reference intervals

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Criteria for Assessment of Test Performance

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- The clinical sensitivity of an assay equals:

$$\frac{TP}{TP+FN} \times 100$$

- The clinical specificity of an assay equals:

$$\frac{TN}{TN+FP} \times 100$$

TP = true positive, TN = true negative,
FN = false negative, FP = false positive

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Criteria for Assessment of Test Performance

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- The clinical accuracy of an assay equals:

$$\frac{TN + TP}{TN+TP+FN+FP} \times 100$$

TP = true positive, TN = true negative,
FN = false negative, FP = false positive

- The precision of an assay is the reproducibility of test results

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Process for Validating a Molecular Assay

- Assay Design
  - Target gene identification and database search
  - Primer and probe optimization
  - Mastermix and MgCl+ optimization
  - Sensitivity
  - Specificity
  - Ease of test reproducibility

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Validation of Lab Developed Assays ("Home Brews")

- Compare results to FDA approved assay of the same type
- "Spike" clinical samples and compare to expected results
- 30 positives samples-10 must be close or no more than 10x limit of detection (LOD)
- Test must be performed in a clinical sample (example: blood). If no clinical samples available, seeded specimens can be utilized

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Controls

- Controls are samples of known type or amount that are treated like and run with patient specimens.
- With qualitative tests, a positive, negative, and in some cases, a sensitivity control, are required.
- In quantitative methods, high-positive, low-positive, and negative controls are included.
- For amplification procedures, amplification controls are required to avoid false-negative results.

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Controls

- Quantitative PCR methods that automatically analyze results require a standard curve or dilution series of the positive control.
- In methods requiring detection of a target-specific product or relative amounts of target, internal controls are run simultaneously, preferably in the same reaction mix as the test specimen.
  - *e.g.*, housekeeping genes, centromere probes, amplification controls

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Use of Appropriate Controls

- Extraction controls- Lysis/Extraction
  - Bacteria or virus preferred, if unavailable can use DNA Seeded at a low level
- Amplification controls (positive, no template control (NTC))
- Inhibition testing control
  - Control that many labs do not test for or test for improperly
  - Ideally used as internal control in every sample
  - For well established method can submit data from 100 samples per specimen type at a low level

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Quality Assurance

- Periodic review and documentation of test results is required.
- Molecular quantitative methods should have a defined dynamic range, sensitivity level, and accuracy.
- Assay levels that distinguish positive from negative results (cut-off values) must also be well defined and verified at regular intervals.

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Summary

- Designing an assay involves the following considerations:
  - Sample preparation
  - Sample extraction
  - Template preparation
  - Experiment setup
  - Optimization of primers and probes
- Laboratory designed assays must meet the following FDA standards:
  - Analytical sensitivity
  - Analytical specificity
  - Precision
  - Accuracy
  - Measuring range of test results
  - Reference intervals

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