Review of PCR Technologies

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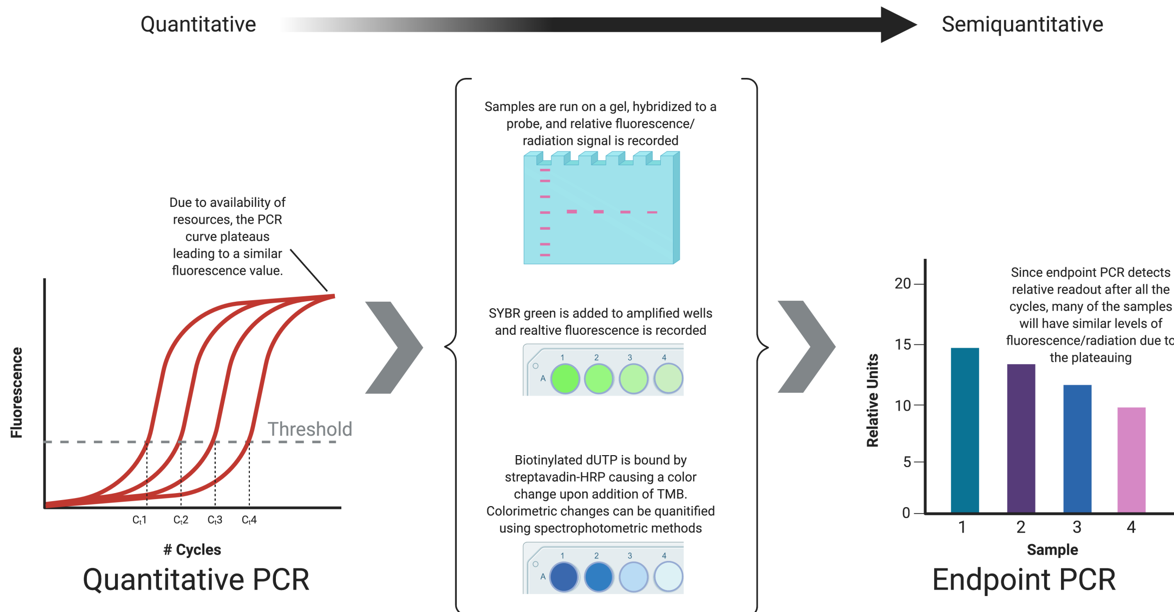
- **PCR basics**
  - Process:
    - **Denaturation**: sample heated to 95°C to fully melt all dsDNA
    - **Annealing**: Sample is cooled to allow the primers to anneal to their complementary target sequence on the DNA sample
    - **Extension:** sample is heated to 75°C (or the ideal temperature for the polymerase being used, usually Taq, but Pfu is often used as well)
  - What is required (5 things)
    - dNTPs, Mg++, Polymerase, Primers, DNA template
- **End-point PCR**
  - Detection methods
    - Colorimetric detection
      - A biotin dUTP is used during the PCR reaction to label amplicons.
      - Streptavidin/avadin is then conjugated to the HRP and when the biotin-dUTP is present, the avadin-HRP complex binds and creates a color change when the TMB substrate is added.
    - Fluorescent detection
      - After PCR is complete, incubate DNA with SYBR green and measure fluorescence and compare level of fluorescence
    - Radioactive detection
      - After PCR is complete, run the amplicon products on a gel, incubate the gel with a radiolabeled probe, process the film, compare film darkening
      - Some may use this methodology without a radioactive probe and opt for use of EtBr instead. This technique would yield relative fluorescence instead of radioactivity and can be completed on the gel imager
  - This method is good at detecting amplification, but it does not detect concentrations or any other specific information.
    - Relative amounts can be detected is serial dilutions are performed. After a certain amount of cycles, the relative amounts of amplicon are relatively uniform, so the serial dilutions help to differentiate these.
- **Quantitative PCR (qPCR)**
  - The basic chemistry
    - qPCR can determine relative amounts of amplicon present throughout the reaction
    - Either a probe or a fluorescent dye is added to the reaction, and the excitation wavelength is applied to the sample after each cycle.
    - When the fluorophore is excited, an emission spectra is released and detected.
      - Emission spectra are always lower in energy than the excitation spectra.
    - The relative amplicon amounts are compared by analysis of the cycle threshold values

- **Cycle Threshold** (Ct) values are numbers representing the point at which the fluorescence



- **FRET**: Fluorescence Resonance Energy Transfer
  - Two fluorophores are used. One fluorophore has a unique excitation wavelength, but the other fluorophore has an excitation wavelength similar to that of the emission wavelength of the previous fluorophore.
  - If the two fluorophores are next to each other, the excitation wavelength of the first fluorophore will result in an emission (and consequently a detection) at the wavelength of the second fluorophore.
- **Quenchers**:
  - Light comes in, but energy is released as heat (no emission spectra)

- Five main types of qPCR
  - **SYBR Green**
    - SYBR Green is a fluorophore that binds to double stranded DNA by intercalating in between the bases in the minor groove.
    - SYBR Green fluorescence increases as amplicon copy increases.
      - SYBR Green does not fluoresce until bound to DNA.
    - This method is not sequence or amplicon specific.
    - Easy to adapt to new assays, but not as sensitive as other methods.
      - Easy and cheap (all you need is primers)
    - Melt curve analysis
      - While not as sensitive as Hybridization Probe Chemistry, SYBR Green melt curves can help detect DNA contamination if the

amplicon is intron spanning (in this example DNA would have a higher melting point because the amplicon length is longer)

- **TaqMan Hydrolysis Probe Chemistry**
    - The chemistry:
        - There is a probe that has a fluorophore attached to it that has sequence specificity between the two primers.
        - When the probe is annealed to the DNA, the fluorophore emits red light due to FRET.
        - When the DNA gets amplified, however, the polymerase acts by hydrolyzing the probe through 5'-3' exonuclease activity.
        - When the probe is degraded, the fluorophore is released, and FRET no longer acts of the secondary fluorophore causing green emission.
    - The polymerase must have 5' to 3' exonuclease activity
- **Hybridization Probe Chemistry**
    - Two probes are used that anneal adjacent to each other. When they anneal adjacent to each other, the donor and acceptor fluorophore experience FRET (unlike when they are in solution)
    - This polymerase just knocks the probe off during extension
    - Measurement is taken during annealing step
    - You can take melting curve analysis with this method
        - This method is so sensitive that it can detect a single nucleotide polymorphism by analyzing the derivative of the melt curve (bringing the DNA to a high temp slowly).
        - Perfect matches between probe and template yield a high melting point while even single nucleotide difference result in lower melting temps (about 5 degrees per nucleotide).
    - Polymerase should not have 5' to 3' exonuclease activity
- **Molecular Beacon Chemistry**
    - The probe consists of a stem-loop structure.
    - The 5' end has a fluorophore, but the 3' end has a quencher
    - When the stem-loop structure is active, no fluorescence is emitted
    - The stem-loop has preferential equilibrium toward binding to DNA, so when the DNA is present, the loop can flatten and the fluorophore can emit light at its excitation wavelength
- **Scorpion Chemistry**
    - There is a stem-loop structure similar to that of the molecular beacon technology with a 5' fluorophore and a 3' quencher; however, there is an addition of a primer next to the quencher that actually initiates the PCR
    - After the extension of DNA, there is an additional denaturation and annealing that allows the scorpion loop to come back down on the PCR product it just initiated causing the quencher and fluorophore to separate allowing the fluorophore to emit light.

- Types of controls in qPCR reactions

PathElective

- **Internal controls** (IC): help determine inhibition of the reaction (run within the test reaction and differentiated from target by probe design)
  - **Endogenous IC** (housekeeping or found in sample): endogenous ICs can be used to calculate relative gene expression (if RT-qPCR is being utilized) and control for cell lysis/extraction
    - Found in the test sample
    - If IC is too high, it can compete with test sequencing for amplification leading to altered
    - Do not allow differentiation between extraction and amplification failure
  - **Exogenous IC** (addition of outside DNA source, must be spiked in either during extraction or before PCR): an exogenous IC can help control for
    - **Homologous exogenous IC**: a synthetically formulated DNA sequencing with the same primer binding sites but different probe binding site (could lead to competition with test sequence and must design a new IC with each reaction)
    - **Heterologous exogenous IC**: exogenous template that has its own set of primers and probes (less competition and can be implemented into different assays)

Table 1: Comparison of internal control

	Exogenous Homologous IC	Exogenous Heterologous IC	Endogenous IC
Universality between assays		x	
Purification control	x	x	x
Differentiates purification vs amp	x	x	
Consistent quantities	x	x	
Non-competitive		x	x

  - IC design considerations
    - Should not compete with amplification of target
    - Should not be too strong (IC is able to overcome inhibition while target is not) to avoid false negatives
    - Should be a little weaker than target system. If the IC system is too weak (IC is more affected by inhibition than target) true negatives will need to be re-tested.
    - Should be versatile in order to be used in multiple assays (saving time in assay implementation)
  - Probe for control is unique from sequence of interest
- **Negative Controls**: help determine false positive results due to contamination
  - **No Template Control (NTC)**: no DNA/RNA present. An NTC determines if there is contamination in the master mix and primer-dimer formation. NTCs

PathElective

should be composed of the same solution the nucleic acid samples are suspended in (i.e.: diH_2O).
  - **No RT control (-RT)**: no reverse transcriptase is added (for RT-qPCR runs only). The -RT will help determine if there is DNA contamination
  - **Negative extraction control**:
  - **Negative amplification control**: no polymerase is added. This control helps control for background noise and stability of the probe
- **Positive Controls**: help determine if the reaction is valid
  - **PCR positive control**: a DNA sample with a known positive result that can be added to a run, in order to assess overall functionality of the run. This validates the master mix and primer/probe set. Multiple PCR positive reactions could be used in multiplex to validate each of the primer/probe sets.
  - **Positive external control**: a clinical isolate with known result. This sample is treated in parallel to the test samples in its own reaction.
  - **Internal positive control** (see IC section above)
  - **Standards** and dilutions can also be added to a run as a positive control in order to address copy number (see section below)
- Two types of standards
  - **Quantitative Standard**
    - Synthetic RNA/DNA target molecule is added to the master mix in a known concentration
    - The synthetic molecule has the same primer sequence with unique amplicon sequence. This allows the sample and control probe to be unique, and both can be detected
    - Determined using difference between QS Ct
      - This method helps control for pipetting inconsistencies and differences between reactions
  - **Concentration Standards**
    - Specific concentrations of a nucleic acid are added at various concentrations so that a standard curve can be extrapolated
    - When the experimental samples are run, the samples can be plotted on the standard curve, and the exact copy number from the initial sample can be identified
- Controlling for contamination
  - **Elimination**
    - Limit number of personnel into and out of the molecular lab
  - **Substitution**
    - Use **AmpErase** during PCR setup
      - Using dUTP in the PCR instead of dTTP, AmpErase protects your samples from contamination from previous PCRs
      - Uracil-N-Glycosylase (UNG) is added to the master mix. UNG selectively destroys samples with dUTP protecting samples from contamination

- New amplicons are not degraded because UNG is inactivated at temperatures above 55 degrees (all temperatures during PCR are above this temperature)

- **Engineering controls**
  - Use a PCR hood to limit airflow around PCR set-up
  - Use dedicated equipment
  - Use filtered tips
- **Administrative controls** (standard operating procedures)
  - One-way workflow from dirty to clean
  - Cleaning benches with bleach, UV, or DNA/RNA degradation solutions
- **Personal protective equipment**
  - Change gloves often and use a dedicated lab coat

- Types of qPCR assays (questions you can address with qPCR)
  - **Qualitative**: is a pathogen present? (positive/negative)
  - **Informative**: What type of pathogen is present? (multiplex)
  - **Quantitative**: How much pathogen is present? (qPCR)

Nested PCR

- Meant to reduce the non-specific binding in products due to amplification of unexpected primer binding sites.
- This type of PCR requires the use of two primers and two separate PCR runs
- The first reaction is run as normal with the primers binding and amplifying DNA, but if there seems to be non-specific binding, a second reaction is run with a new set of primers nested within the desired amplicon sequence cutting out contamination

Multiplex PCR

- Multiple DNA primers are added to the reaction tube all optimized to melt and anneal at the same temperature but recognize different target sequences
- The two main ways of distinguishing between the various amplicons is by size (if the samples are different enough to visualize on a gel), with fluorescently tagged primers, or with probes
- Applications:
  - Microbial/pathogen identification
  - High throughput SNP genotyping
  - Mutation analysis
  - Gene deletion analysis
  - Template quantitation
  - Linkage analysis
  - RNA detection
  - Forensics
  - Diet analysis

Reverse Transcriptase PCR

- RNA is reverse transcribed into cDNA using the protein reverse transcriptase from retroviruses; however, this process does not amplify the sample.

- RNA samples must be reverse transcribed before being analyzed or amplified by any other PCR-based method. The only exception thus far is **NanoPore** sequencing.
    - NanoPore sequencing could yield more reliable expression data because no reverse transcription is required
- Once the RNA is reverse transcribed, the sample can be amplified and analyzed via end-point PCR or quantitative PCR. The latter method (termed RT-qPCR) can be used to detect RNA viruses or determine gene expression
- Gene expression
    - In order to determine gene expression, the target Ct value must be compared to an endogenous control. The endogenous control should be uniformly expressed across the cell types being studied.
    - A quantification standard may also be added to the master mix in a known concentration to control for pipetting variability
- Difference between cDNA and gDNA
    - gDNA has introns, so if you have an intron-spanning primer-pair, gDNA will have a higher melting point because of the increased length
- Special considerations with RNA
    - RNases are ubiquitous in nature, so their stability is significantly reduced when compared to DNA
    - Reverse transcriptase can fall off the extracted mRNA and could possibly exhibit RNA species discrimination

- **Digital PCR (dPCR)**
    - The process
        - The DNA and master mix (including primers) are emulsified in an oil solution which partitions the DNA into individual liquid droplets. Each droplet has, on average, 0-1 copy of target.
        - The tube containing all liquid bubbles is incubated using a modified PCR protocol. In the end, each bubble is counted as positive or negative based on amplification.
        - dPCR yields an exact quantification of the number of target DNA copies