Introduction to PCR Methodology and Current State of the Art

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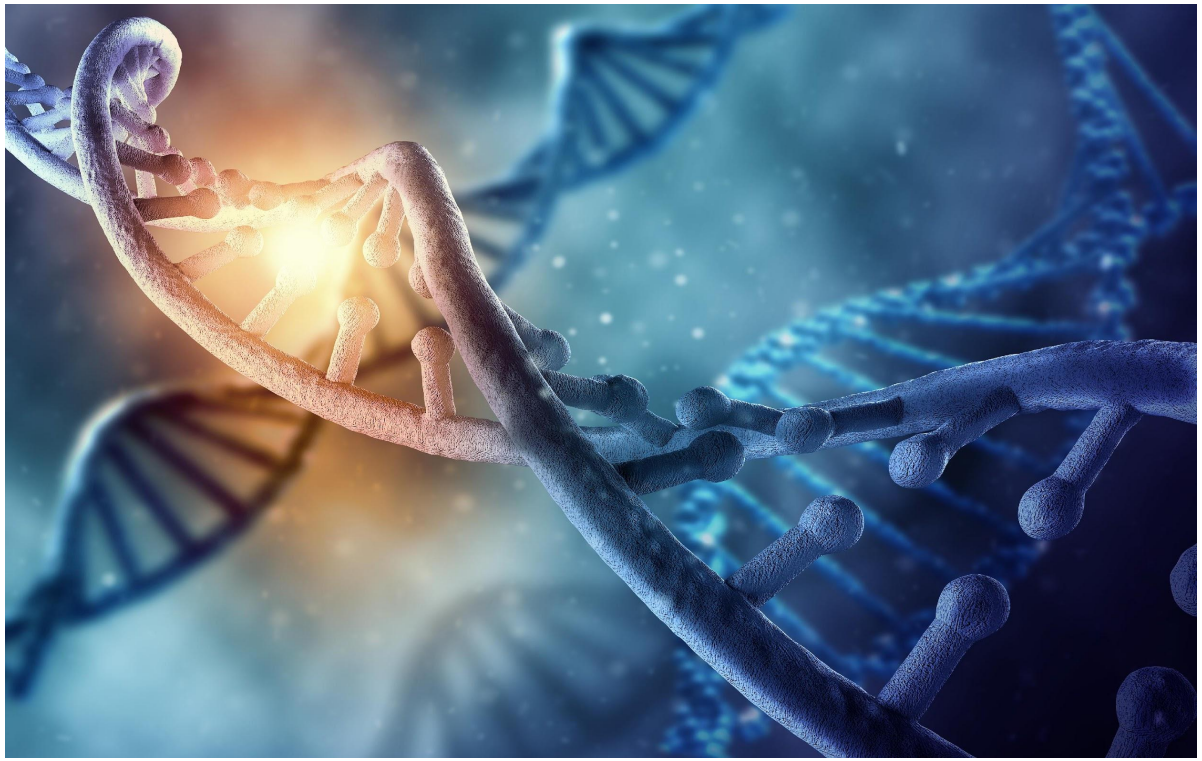
Introduction to Polymerase Chain Reaction (PCR) Methodology and Current State of the Art

By Balaji Boovaraghan, James Haudenshield, Md. Shofiqul Islam, Ray Shillito, and Sherry Whitt



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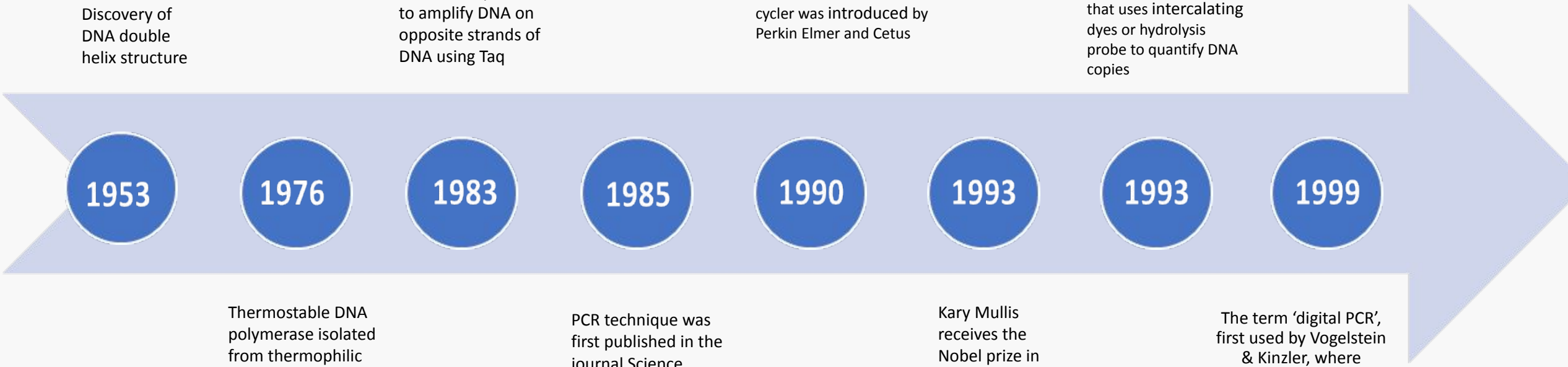
Why should you be interested in PCR?

PCR has revolutionized the areas of medicine, biology, chemistry, and forensics allowing scientists to amplify DNA sequence for various applications.

PCR is a critical tool in agriculture for plant breeding and biotechnology and can also be used in the identification and detection of infectious diseases of plants, animals and humans.

PCR has wide applicability in the areas of medicine especially for drug development and research in cancer detection and treatment.

History of PCR



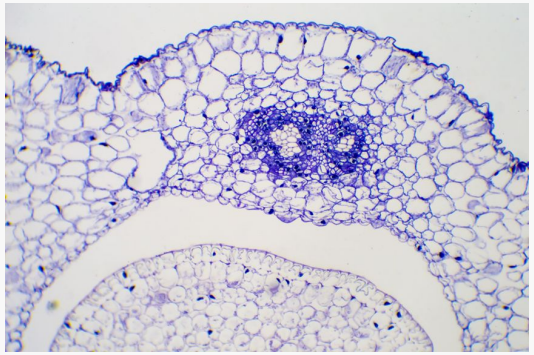
Definition and process of PCR

Polymerase Chain Reaction is a laboratory process to make millions of copies of a specific segment of DNA in a matter of hours through iterative heating and cooling steps.

PCR mimics the DNA replication process that naturally occurs in organisms in a targeted way.

PCR reactions need a catalyst or enzyme (DNA polymerase) to drive the reaction.

Sampling considerations



The first step in conducting a PCR experiment is obtaining a sample. Remember - sampling is destructive.

A sample by definition should be representative of the whole.

The sample must be appropriate for the method being utilized. If the method includes a limit of detection or threshold, then the sample must be taken and prepared to account for those measures.

Some considerations include: Size, Type, Labeling, Conditions (ambient vs dry ice), Transport, and Timing.

If the potential for contaminants exist, that needs to be properly

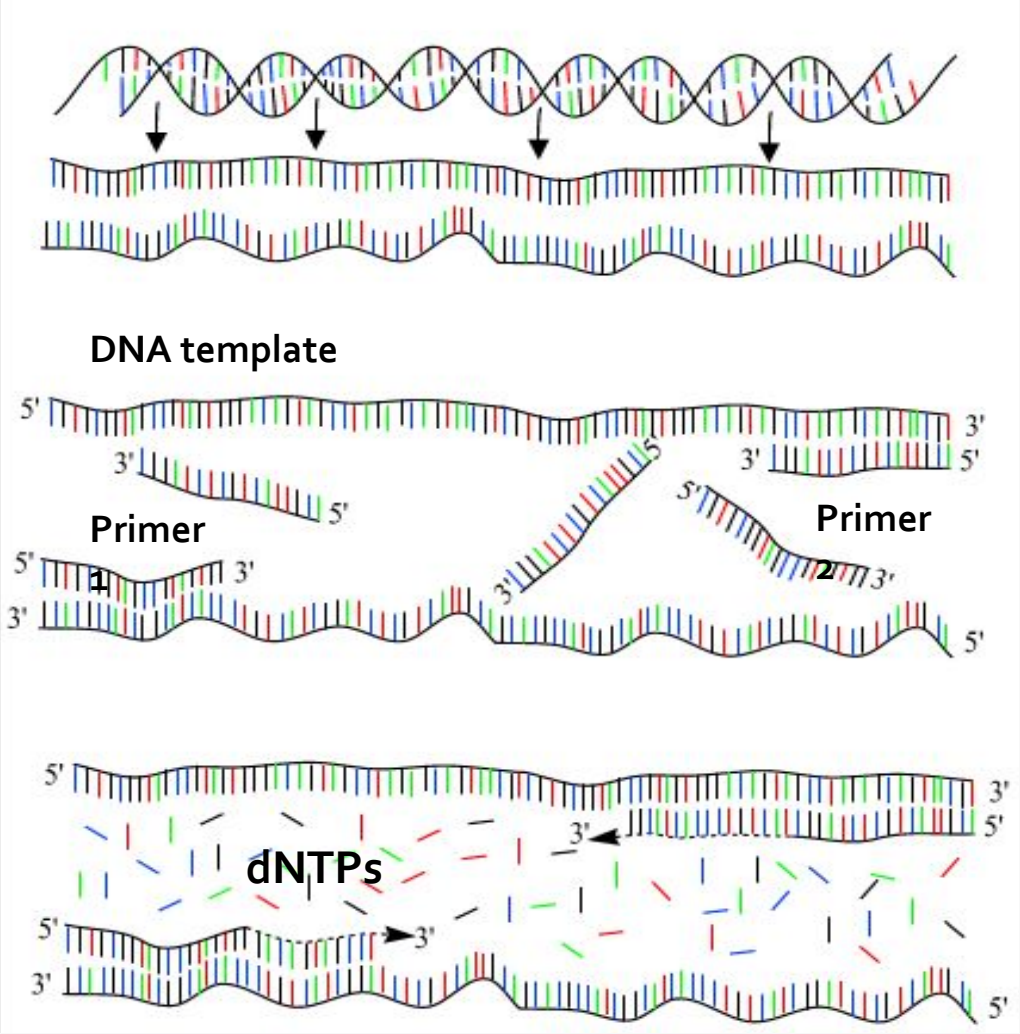
Conventional PCR

www.thermofisher.com

www.bio-rad.com

www.eppendorf.com

Conventional PCR: How does it work?



Adapted from 'The Steps of PCR Amplification' (source: Vierstraete, 1999)

The following steps are repeated many times during the cycling process:

Denaturation occurs at ~96 ^{0}C and causes the two strands of the double helix to separate.

Annealing enables the primers to hybridize at the melting temperature (55-75 ^{0}C) to the complementary region of the template DNA strands.

Extension (70-72 ^{0}C) of the template strand is aided by the DNA polymerase and free

Conventional PCR: Chemistry and Components

The following reagents are needed to perform a PCR experiment:

- A set of primers (forward and reverse) for the DNA segment to be amplified
- *Taq* DNA polymerase
- Buffer for the specific Taq DNA polymerase
- Deoxynucleotides (dNTPs)
- DNA template
- Sterile water

Additional reagents: typical ranges provided below.

- Magnesium salt Mg2+ (at a final concentration of 1.5-4.0 mM)
- Potassium salt K+ (at a final concentration of 35-100 mM)
- Dimethylsulfoxide (DMSO, at a final concentration of 1-10%)
- Formamide (at a final concentration of 1.25-10%)
- Bovine serum albumin (at a final concentration of 10-100 µg/ml)
- Betaine (at a final concentration of 0.5-2.5M)

Primer Design for (any) PCR

The following guidelines should be adhered to for maximizing PCR efficiency and yield:

1) Primer length should be 18-30 nucleotide bases.
2) G-C content should range between 40-60%.
3) The 3’ prime end of primers should contain a G or C to clamp the primer increasing priming efficiency.
4) The 3’ ends of a primer set should not be complementary to each other. This results in the formation of primer dimer and the formation of hairpin loop structure.
5) Melting temperatures for primers range between 50-65^{o}C with an optimum range between 52-58^{o}C. The Tm for both primers should not differ by more than 5^{o}C.
6) Di-nucleotide repeats or single base runs should be avoided as they can create a hairpin loop structure. If unavoidable due to the repeat region in the DNA template, a maximum of 4 bases can be included in the primer from the repeat or single base region.

Conventional PCR: An example protocol

PCR set up: The following reagents have been calculated for a total PCR volume of 25 µL per reaction.

Reagents	Volume/sample (µL)	Final concentration
5x PCR buffer (without $MgCl^2$)	5	1x
*$MgCl^2$, 25 mM	1.5	1.5 mM
dNTPs, 10 mM	2.5	1 mM
Primer-F, 10 µM	1.25	0.5 µM
Primer-R, 10 µM	1.25	0.5 µM
Taq DNA polymerase, 5 U/µL	1	0.2 U/µL
Sample DNA, 10 to 50 ng/µL	2	variable
Sterile water	upto final reaction volume	

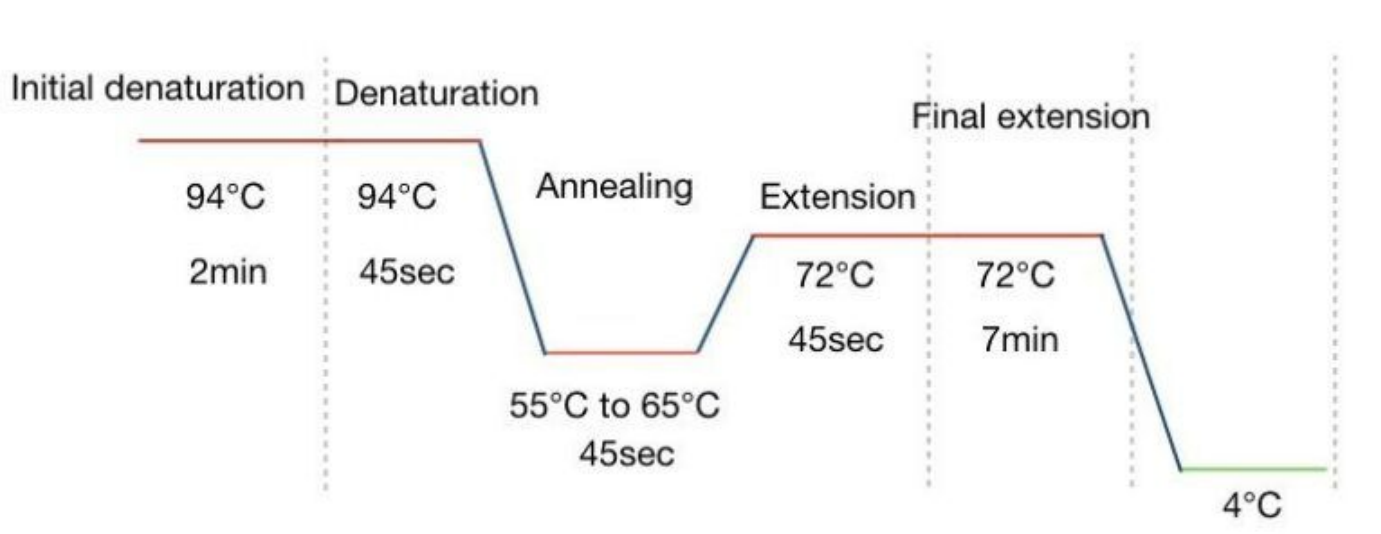
*If the PCR buffer contains $MgCl^2$, the final concentration of $MgCl^2$ in the reaction mix should be adjusted to 1.5 mM.

Conventional PCR: An example protocol

PCR control: As a positive and negative control, DNA from certified reference materials and no template control should include in the PCR reaction plate.

PCR program for cycling conditions

Step	Temperature	Time
Enzyme activation	94-96°C	1-5 min
Denaturation	94-96°C	30 sec
Annealing (25-36 cycles)*	55°C to 67°C*	30 sec
Extension	72°C	30 Sec/Kb
Final extension	72°C	7-10 min
	4°C	hold



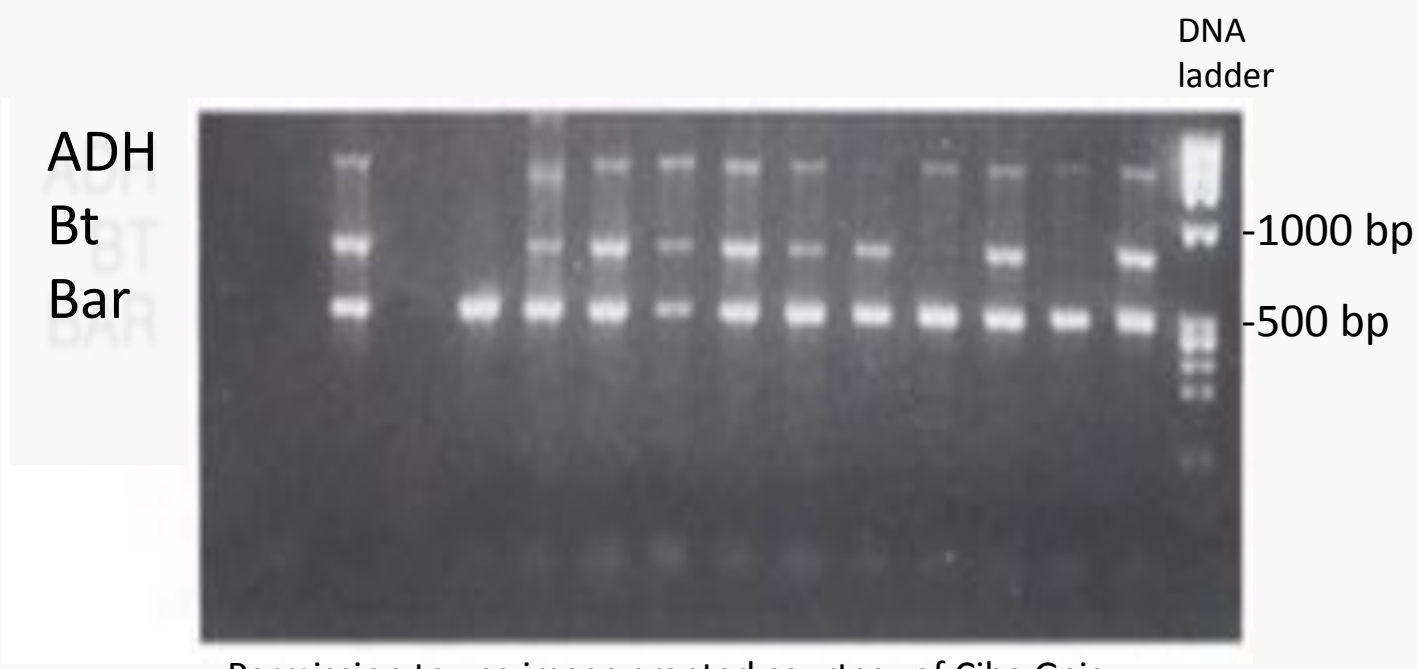
geneticeducation.co.in/function-of-taq-dna-polymerase-in-pcr/

*Annealing temperature to be determined by user based on primers Tm. Time to be determined based on PCR assay mix.

Conventional PCR: Visualization

www.atlantisbioscience.com.sg

Gel apparatus



Permission to use image granted courtesy of Ciba Geigy.

Visualization of PCR product on agarose gel electrophoresis.

Quantitative PCR

https://www.bio-rad.com

https://www.thermofisher.com

https://azurebiosystems.com

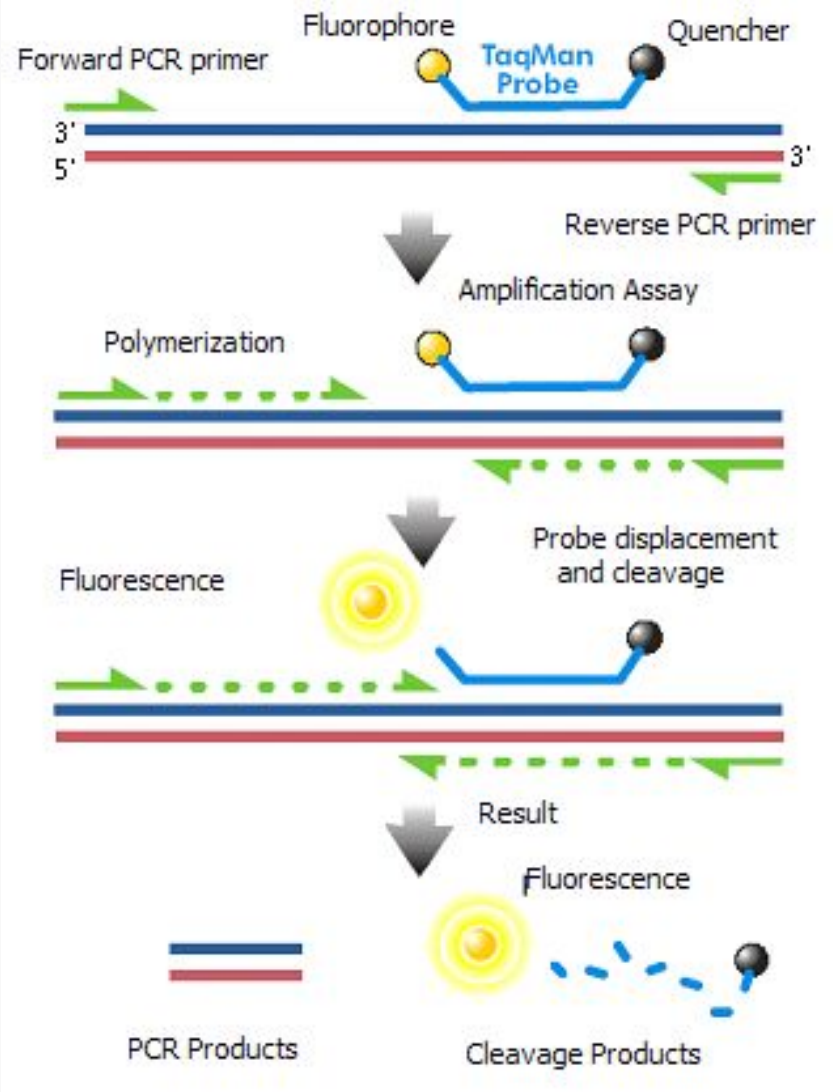
https://www.escolifesciences.com

Real-time Quantitative PCR (qPCR): How does it work?

Real-time PCR measures the amount of PCR product at each amplification cycle.

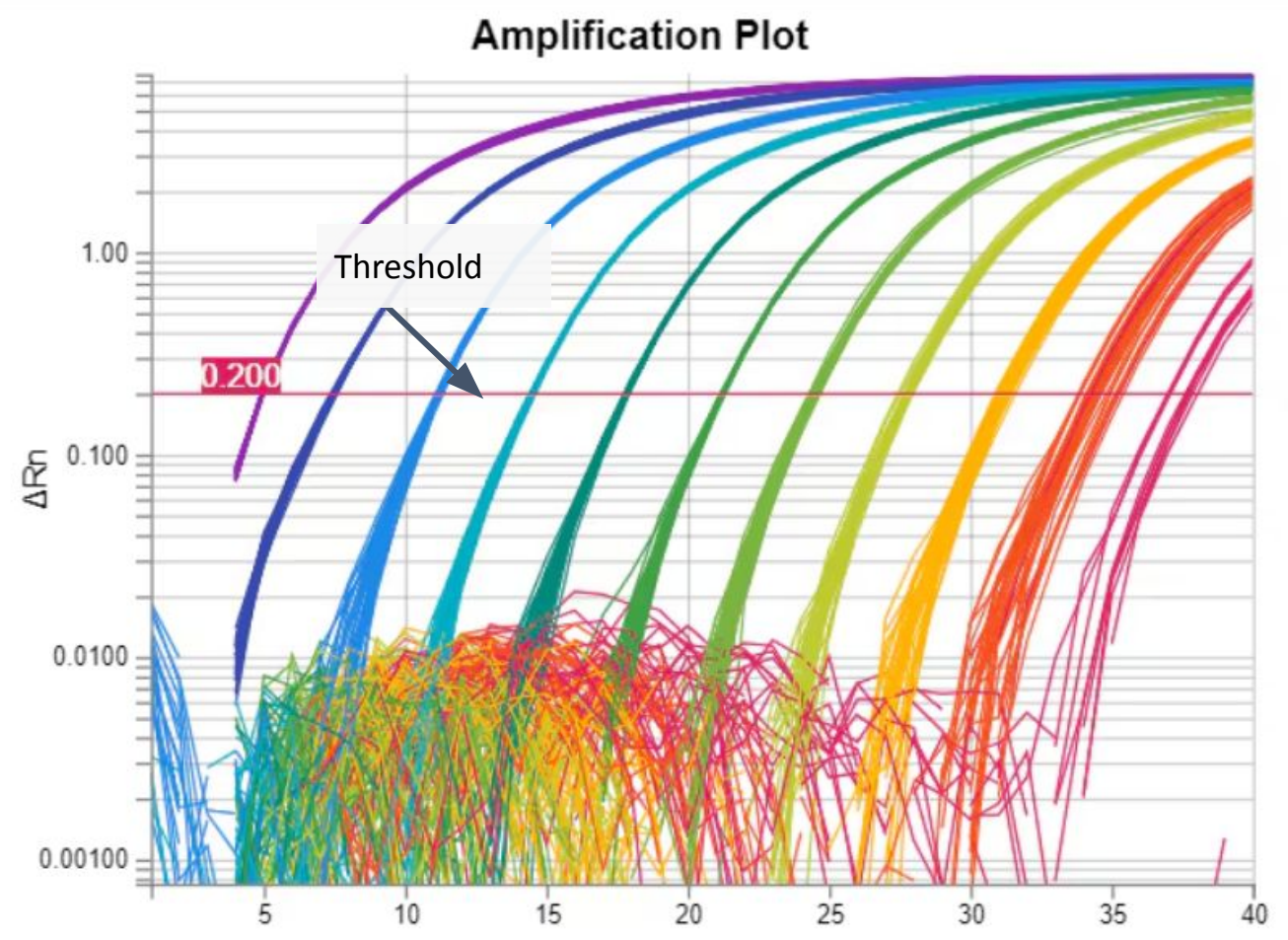
The amount of PCR product is an accumulation of the fluorescent signal emitted by a specialty primer called a probe over the duration of the PCR reaction.

The PCR amount can be quantitated by comparing the signals to a known amount of starting DNA using a standard curve.



Open source: https://commons.wikimedia.org/wiki/File:Taqman.png

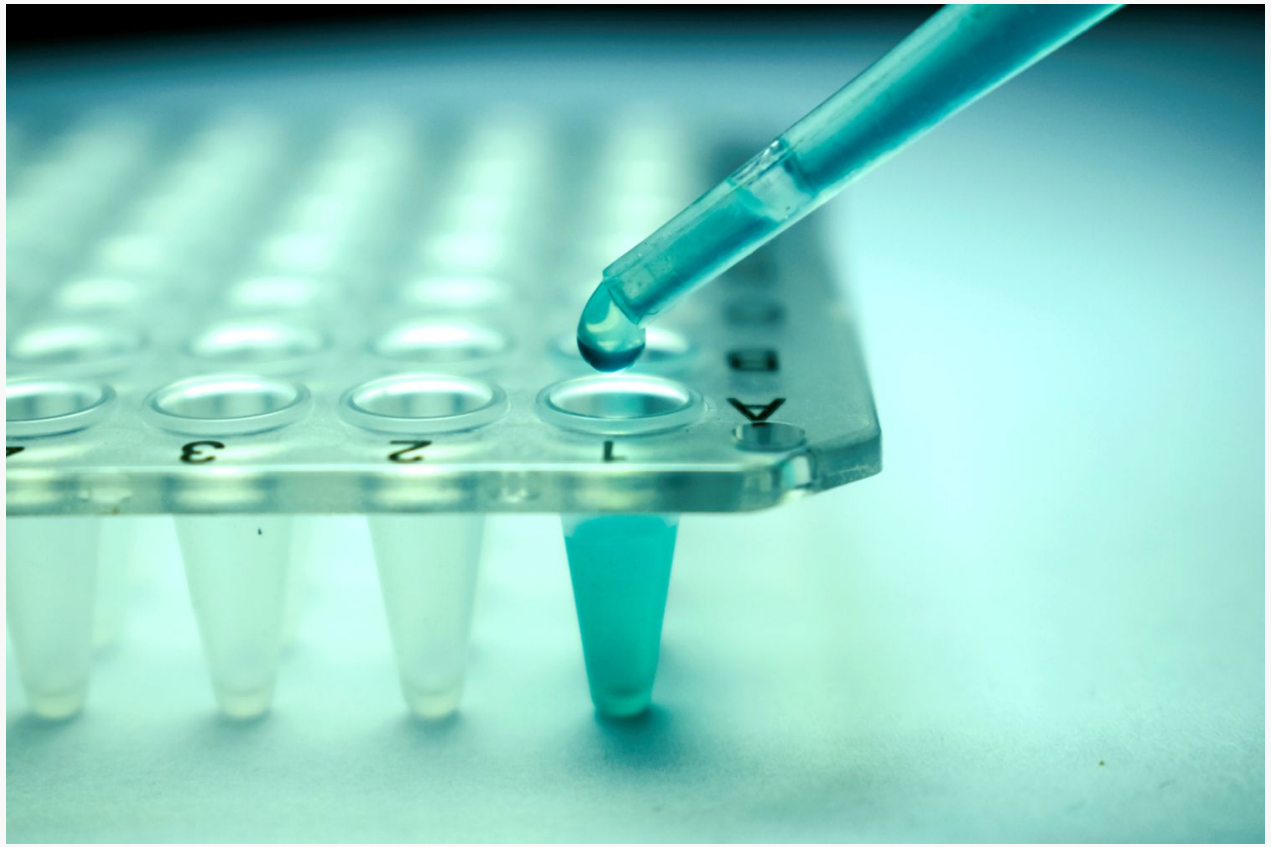
Real-time Quantitative PCR (qPCR): How does it work cont'd?



Understanding Ct Values in Real-Time PCR (thermofisher.com)

The threshold is an arbitrary line where the signal crosses at a particular cycle above the baseline or background signal. The measurement is taken during the exponential phase of the PCR cycle.

qPCR: Chemistry and components



The primary components are identical to endpoint PCR including: DNA or RNA, forward and reverse primers, deoxynucleotide triphosphates (dNTPs), Magnesium chloride, polymerase and water.

Due to the inclusion of a taqman probe, there is a fluorophore label at the 5’ (eg. FAM, HEX) end and a quencher (eg. TAMRA, Blackhole quencher or BHQ) at the 3’ end.

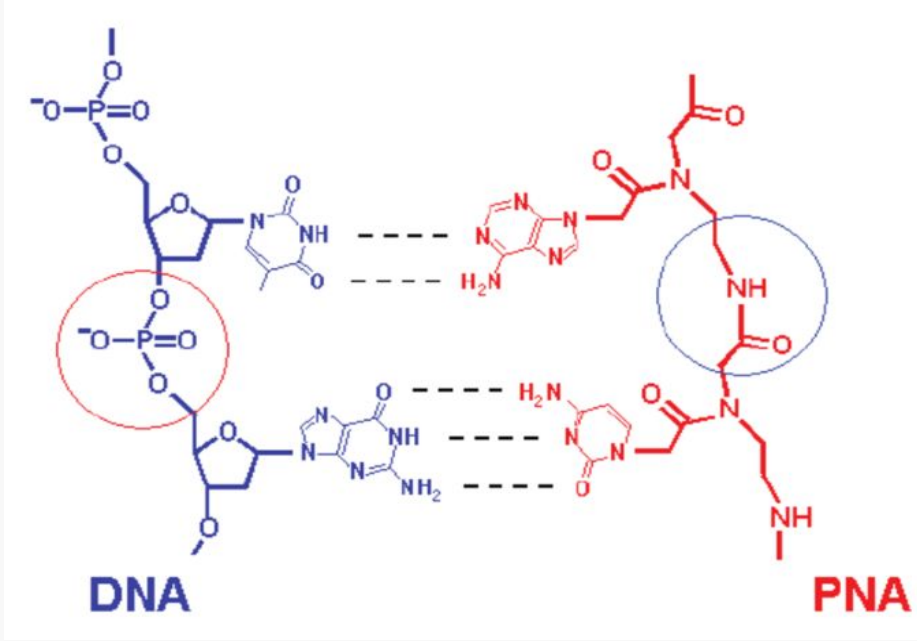
Probes can be designed for increased specificity and sensitivity by including a chemical moeity.

qPCR: Chemistry and components

SNP genotyping requires specialty probes that utilize chemical modifications.

The minor groove binder (MGB) or locked nucleic acid (LNA) enable the increase of the melting temperature for enhanced specificity.

Peptide nucleic acids (PNA) are DNA mimics that can attach to the phosphate backbone of DNA. The effect is to create a blocking or clamping mechanism which prevents amplification of a specific target DNA.



DOI: 10.5772/7160

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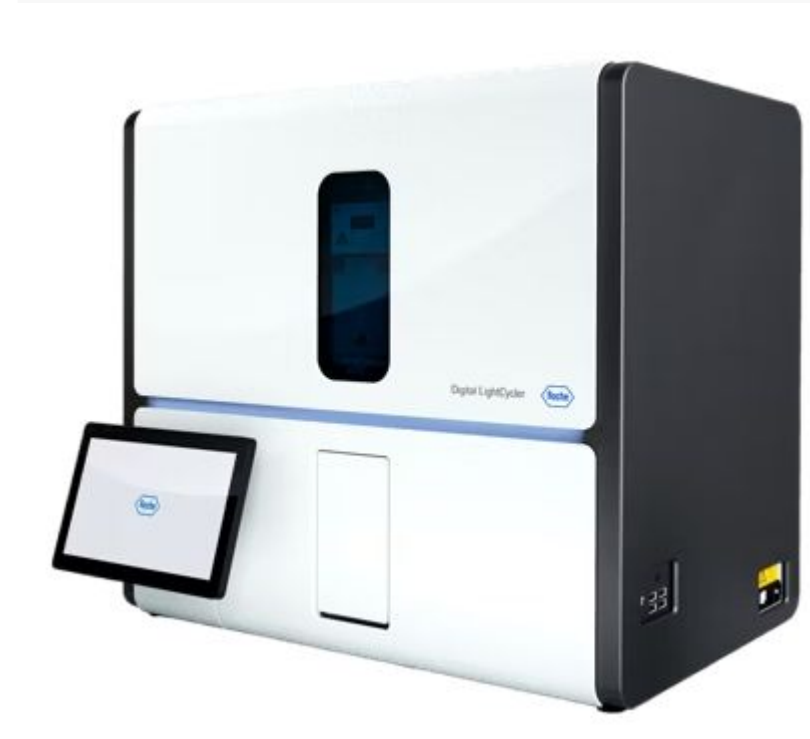
Digital PCR

https://www.qiagen.com/us/products/instruments

QX600 Droplet Digital PCR System – Bio-Rad



Digital LightCycler® System (roche.com)

Digital PCR: How does it work?

Digital PCR (dPCR) is the third generation PCR in which specialized equipment partitions reaction mix and subsequently amplifies to an endpoint

Poisson distribution modeling then calculates the number of copies in a reaction to provide an end user absolute abundance of copies in the reaction

dPCR is used across many applications such as rare event mutations, copy number variations (CNVs) and gene detection where absolute quantification is needed

Digital PCR: General Description

During dPCR, fluorescent probes are used to detect the positive partition (or droplet) and negative partition (or droplet)

After analyzing the number of positive and negative reactions, the “absolute” number of molecules present in the sample can be calculated using Poisson statistics.

dPCR does not depend on standard curves or Controls for nucleic acid quantification. This makes it an ideal technology to detect rare events or precise copy number in complex backgrounds.

Digital PCR: Results interpretation

Poisson statistics are based on a discrete probability distribution that expresses the probability of a given number of events occurring in a fixed interval of time or space if these events occur with a known constant mean rate and independently of the time since the last event.

For example, if you toss or flip a two-sided coin a given number of times (events) you could calculate the likelihood of obtaining a range of outcomes.

How many heads do you expect to get if you flip a coin 10, 100 or 1000 times? The more flips the closer you will get to the 50% mark with a tighter range of the distribution.

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Isothermal PCR (isoPCR)

Isothermal PCR (isoPCR) is a targeted DNA amplification method

Isothermal PCR methods are a class of DNA sequence amplifications methods that are distinguished from traditional PCR in that they are performed at a single temperature, rather than requiring a series of repeating high temperature phases to denature DNA, followed by DNA synthesis phases.

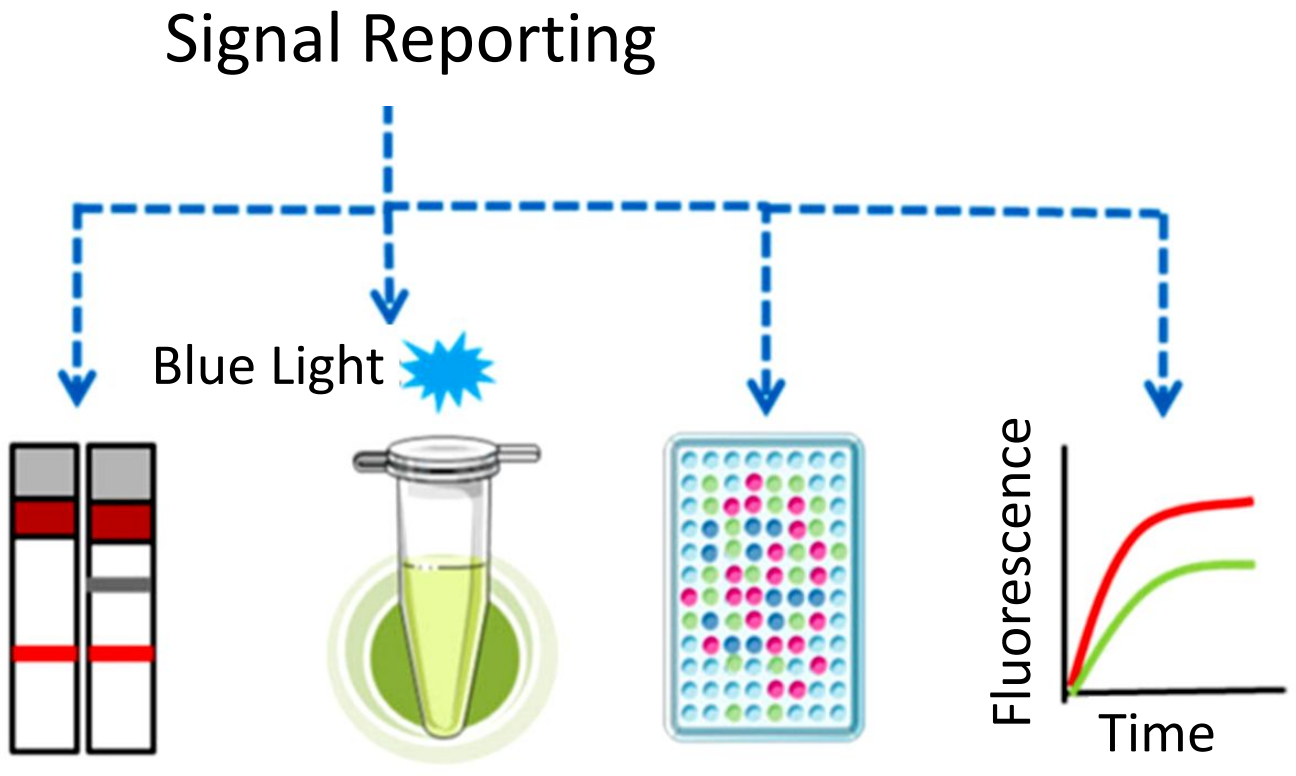
These methods rely on a priming sequence (RNA or DNA) and DNA polymerases that have a high strand displacement activity; they can directly unzip the DNA substrate as they synthesize the complementary strand, rather than needing denaturation first.

In addition isoPCR is in almost every case quicker to provide a result than other PCR methods, and can often be carried out on unpurified DNA samples.

Detection of the isoPCR product can be carried out via a number of means

Lateral flow device, Colorimetric, Fluorescent and Electrochemically are some of the visualization techniques used to evaluate amplicons.

Each tool has advantages and disadvantages related to time to answer, instrumentation cost and complexity, and ability to provide quantitation.



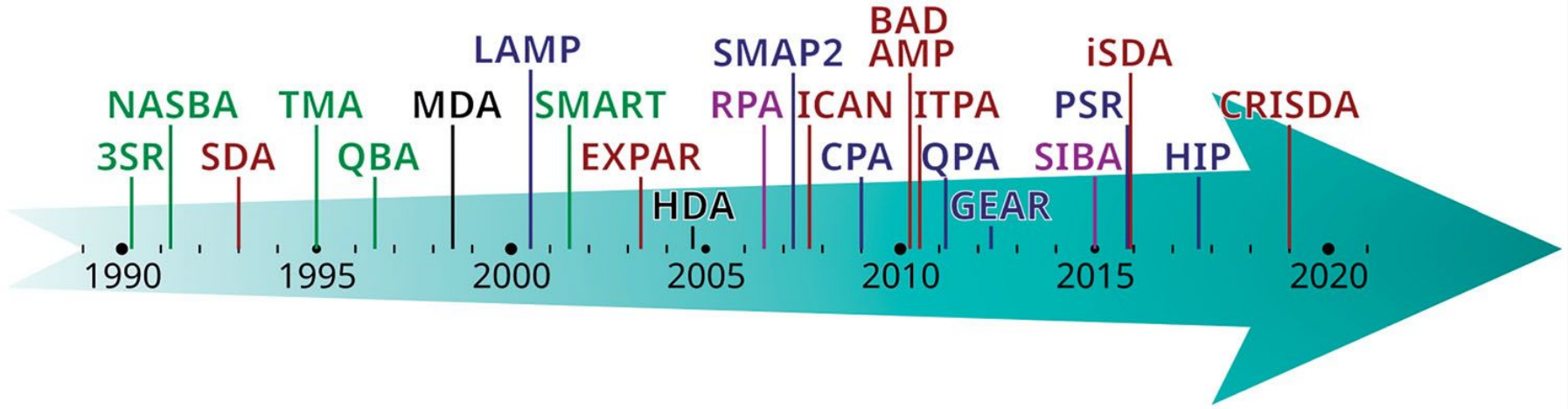
He et al., The CRISPR/Cas System: A Customizable Toolbox for Molecular Detection. Genes 2023, 14, 850. https://doi.org/10.3390/genes14040850

Isothermal PCR continues to develop

There are more than 20 varieties of Isothermal PCR. The first were developed in the 1990's. (Glokler et al., 2021 https://doi.org/10.1080/10409238.2021.1937927)

Some require an initial high temperature step. However, after that they do not require temperature cycling.

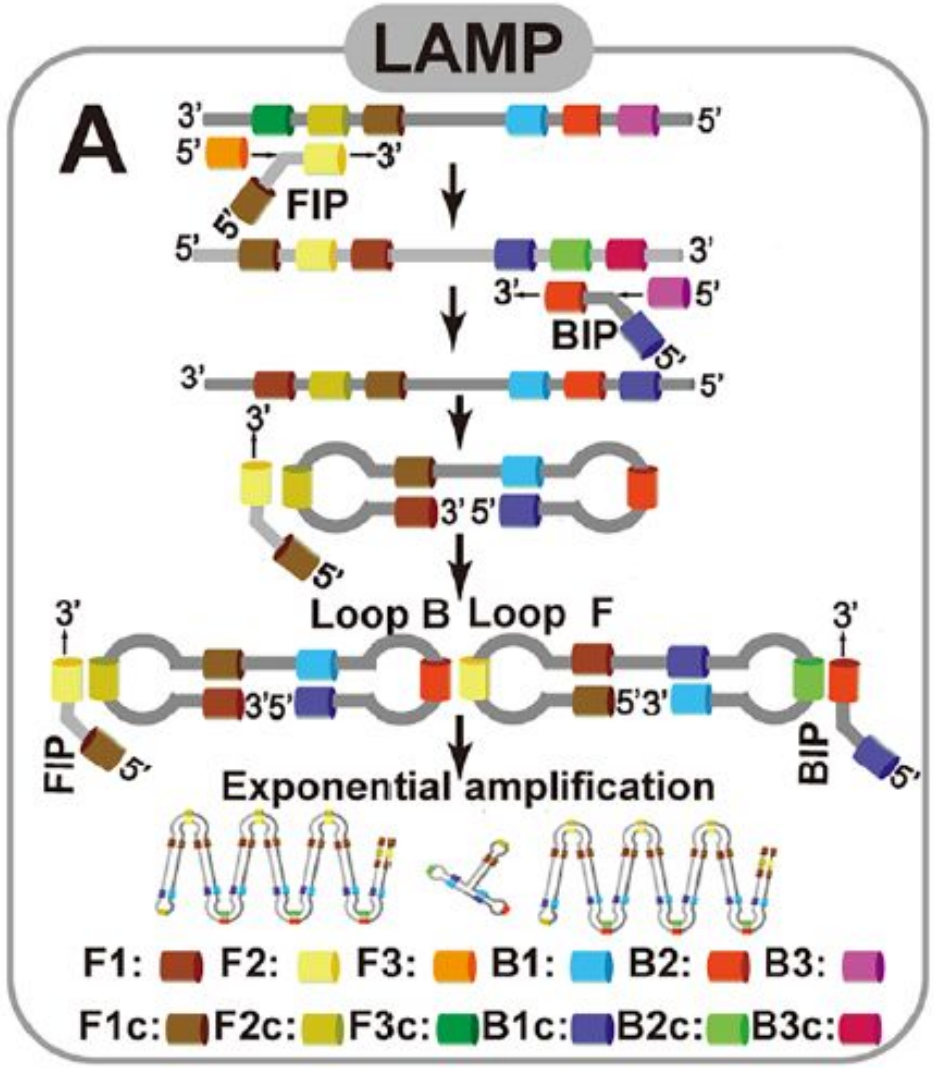
The most popular methods are LAMP, and RCA. Others that are used less often are HCR, RPA, NASBA, SDA and TMA (Oliviera et al. 2021 doi: 10.3389/fsens.2021.752600)



LAMP is the most commonly used method

Many isoPCR methods are protected by patent rights. This appears to have reduced their use, particularly in the research field.

Loop-mediated Isothermal Amplification (LAMP) requires 4-6 carefully designed primers.



Qiao et al., Recent advances of food safety detection by nucleic acid isothermal amplification integrated with CRISPR/Cas, Critical Reviews in Food Science and Nutrition, 2023. DOI:10.1080/10408398.2023.2246558.

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ISO 22942-1:2021(E) **Molecular biomarker analysis — Isothermal polymerase chain reaction (isoPCR) methods — Part 1: General requirements**

Qiao Jiali, Zhiying Zhao, Yaru Li, Minghui Lu, Shuli Man, Shengying Ye, Qiang Zhang & Long Ma 2023. **Recent advances of food safety detection by nucleic acid isothermal amplification integrated with CRISPR/Cas.** Critical Reviews in Food Science and Nutrition, DOI:10.1080/10408398.2023.2246558.

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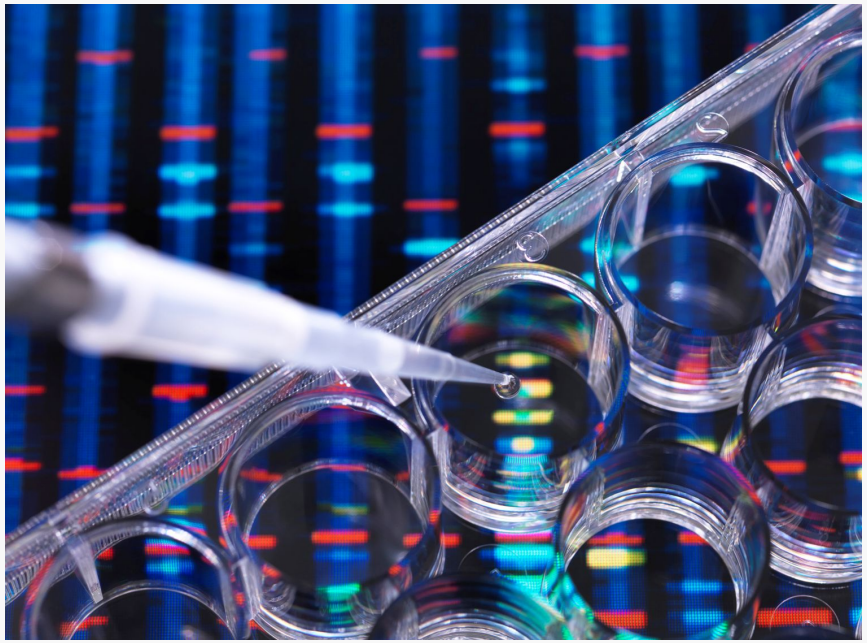
PCR Characteristics

Several factors are considered during the design, execution and interpretation of PCR experiments.

Proper validation of a method is required to ensure the method meets the stakeholders' established criteria.

Experimental controls are required to have confidence in method performance and verify results.

Performance Criteria of PCR



Limit of detection - The lowest concentration of target that can be identified. A PCR method should be able to amplify at least 0.1 ng DNA extracted from any kind of sample.

Sensitivity - The ability of detecting a tiny amount of DNA present in a sample e.g., forensic DNA, adventitious presence of GMO trait in crop species.

Specificity - The PCR assay is to be highly specific to the target DNA and shall not amplify any non-specific DNA fragment.

Repeatability - If the PCR assay is highly accurate and optimized, the participatory labs are able to generate similar results using DNA extracted from the certified reference materials.

Performance Criteria of PCR

Size of amplicon- The size of the amplicon should be chosen to match the molecular weight present in the sample under study, e.g. for denatured DNA from processed foodstuffs, the size of the amplicon should ideally be in the range of 50 to 150 bp. For raw foods, a broader range of amplicons up to e.g. 250 bp is applicable.

PCR primer validation- Primer validation should include theoretical and experimental evaluation. The theoretical evaluation of primer sequences is to perform a BLASTn search against nucleic acid databases such as EMBL and GenBank. The experimental evaluation tests the primer specificity to discriminate between the target and closely related species DNA sequences and negative control DNA.

Controls- Due to the risk of obtaining false positive and/or false negative results, appropriate controls shall be included in each diagnostic PCR assay. These shall at a minimum include a positive DNA target control, a negative DNA target control and an amplification reagent control, see prEN ISO 24276 (general).

Results interpretation

PCR is a molecular biology tool used to amplify the target DNA fragment.

The PCR results are interpreted based on the presence and absence of the target DNA fragment, visualized either by agarose gel electrophoresis, high resolution melting or fluorescent reporters.

In a qualitative method, the results can be either detected or not detected. The detected (positive) result indicates a target DNA fragment is present in the sample and the not detected result indicates a target DNA fragment is absent or below the limit of detection of the method.

In a quantitative method, the results are interpreted by counting the number of amplified target DNA molecules at the end of the PCR cycles divided by the number of DNA molecules present at the beginning of PCR i.e., the endogenous single copy gene.

For confirmation of a PCR result, the amplified DNA nucleotide sequence shall be identical with the target DNA nucleotide sequence and can be verified by direct sequencing or cloning and sequencing.

PCR Do's and Dont's

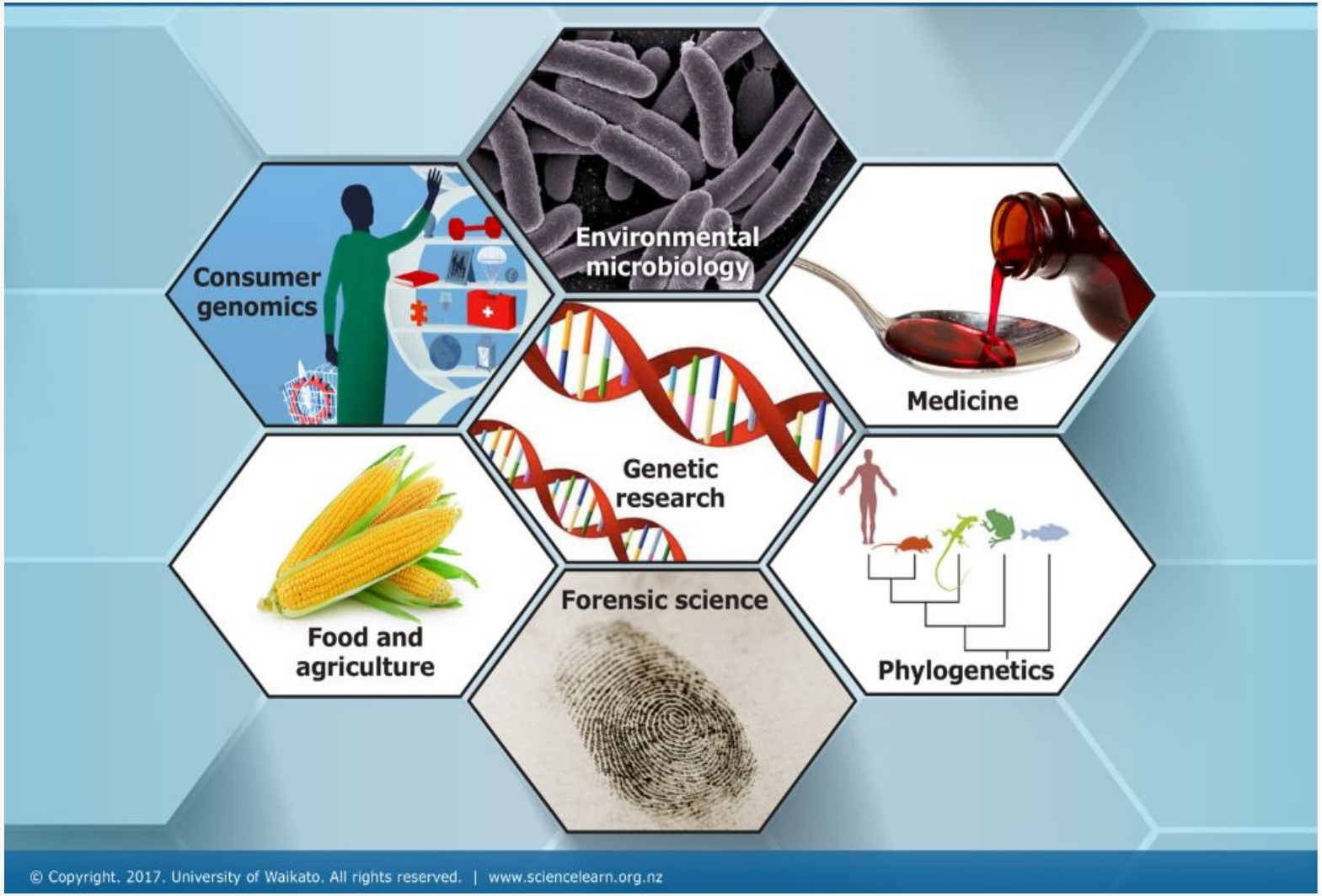
Always include the proper controls in every batch or run. Typical controls are listed in ISO 24276. Minimally, known DNA positive and negative samples are needed.

The most critical aspect of clean PCR is ensuring separation of space to contain post PCR accumulated products!

This is particularly problematic for downstream applications such as agarose gel electrophoresis and sequencing that require processing the post PCR product.

The amplified product can be easily aerosolized and become a contaminant in pre PCR reactions.

PCR Applications



PCR is used in numerous scientific disciplines including pharmaceuticals, medicine, forensics, agriculture, and animal husbandry.

There are 37 different types of PCR.

PCR can be:
Qualitative (yes/no) .

Semi-quantitative (copy number, threshold for bulk grain).

Quantitative (% or ug of unintended material present).

Image Source: Science Learning Hub – Pokapū Akoranga Pūtaiao, The University of Waikato Te Whare Wānanga o Waikato, www.sciencelearn.org.nz

PCR type	Principle	Application
Conventional or Endpoint	Standard endpoint reaction using two primers targeting a specified region of ~100bp-3000bp.	Amplicon size detection to confirm gene of interest components and/or vector backbone or exogenous DNA absence.
Long-range	Standard endpoint reaction using two primers targeting a specified region of ~3000bp-15000bp.	Amplicon size detection to confirm gene of interest components and/or vector backbone or exogenous DNA absence.
Real-time	Exponential product reaction using three to six primers where target analyte accumulation is above or below an arbitrary threshold.	SNP genotyping, Gene of interest copy number determination. Adventitious presence and pathogen testing.
Digital	Random partitioning of sample molecules with subsequent amplification of one or more analyte targets to enable absolute quantification.	Quantify rare allele or target analyte presence in individual or bulk samples.
Isothermal	Time dependent reaction using two-three primers where target analyte accumulation occurs at a single temperature.	SNP genotyping, Gene of interest copy number determination. Adventitious presence and pathogen testing.
Multiplex	Standard endpoint reaction using more than two primers targeting more than one specified region of ~100bp-3000bp.	Amplicon size detection to confirm gene of interest components and/or vector backbone or exogenous DNA absence.
Nested	Standard endpoint reactions using more than two primers targeting a primary specified region in a first round of PCR. Then using the 1st round PCR as template in a second round of PCR, targeting a region contained within the primary region. ~100bp-3000bp.	Amplicon size detection to confirm gene of interest components and/or vector backbone or exogenous DNA absence.
Allele specific (ARMS or ASPE)	Standard endpoint reaction using more than two primers targeting individual alleles contained within a region of ~100bp-3000bp.	SNP genotyping, Confirmation of polymorphic sites for identity preservation.

Applications utilizing PCR amplicons as the template

The products of PCR are used in numerous downstream applications where high sensitivity and specificity are needed.

Sanger sequencing, Genotyping by Sequencing and Whole Genome sequencing can benefit from large scale amplification of genomic DNA in order to interrogate alleles or identify parental/varietal genetic differences.

Iso-PCR can be combined with CRISPR tools to detect nucleic acids at very low concentrations in the attomolar range!

Applications in Agricultural Biotechnology

Maintaining the quality and integrity of the traited seed is critical for stakeholders along the value chain.

Testing occurs at various points from the field to the elevator and may involve the use of protein and/or DNA based methods and tools.

Some examples include:

-qPCR for adventitious presence or low level presence of unintended material in seedlots and bulk grain.

-SNP genotyping for varietal identification and confirmation.

-Sequencing of *in vivo* genome edits and the selection individuals with the intended edit.

PCR Applications in Ag Biotechnology

Why do we test?	How do we test?
To ensure that the correct DNA / editing template is used	Sequencing, PCR
Confirm expected genotype in our crop of interest	Sequencing, PCR, dPCR
Select the intended events for further testing	End-point/Real-time qPCR
Control of field trials – the right edit/event is in the right place	End-point/Real-time qPCR, dPCR
Quality monitoring for cross contamination	End-point/Real-time qPCR
Regulatory submission of a dossier to an authority	qPCR event specific (bulk)
Trade in agricultural goods and commodities	End-point PCR /qPCR
Regulatory compliance and labeling for export and import	End-point PCR /qPCR

PCR Challenges

Highly dependent on quality of samples (templates) used for amplification in PCR.

Presence of PCR inhibitors complicate the interpretation of results.

Increased risk of false positive or false negatives results.

PCR amplification of GC (Guanidine and Cytosine) rich DNA sequences.

Secondary structure hindering complete denaturation and primer annealing.

Prone to contamination from other sources of nucleic acids (DNA and RNA).

Presence of non-specific products and the presence of primer dimers.

PCR Advantages

Most valuable technique currently used in biosciences, diagnostic and forensic science.

Most sensitive technique even when the amount of DNA is limited.

PCR can quickly and efficiently amplify to million copies in just a few hours.

Cost effective, throughput can be scaled according to needs.

Potential to increase the –plex (duplex, triplex, multiplex) for simultaneously targeting more than one gene at a time.