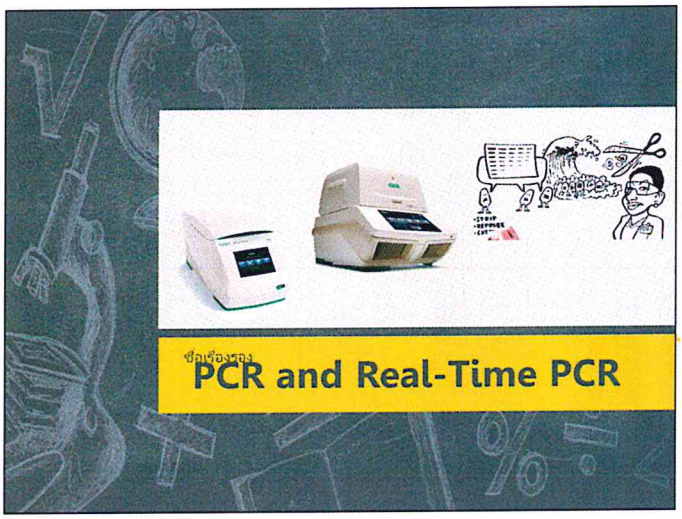
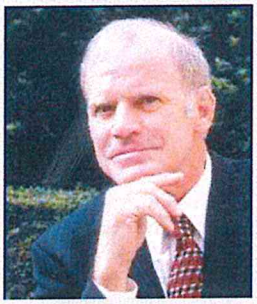
5.1.5.8



ชื่อเรื่องรอง

PCR and Real-Time PCR

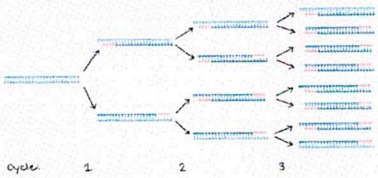
The Amplification of DNA
Polymerase Chain Reaction (PCR)



- One of the most powerful tools in molecular biology
- Invented by Kary Mullis in 1983, resulting in his Nobel Prize in Chemistry in 1993
- In essence, this process acts as a "copying machine" for DNA

About Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. It is an easy, cheap, and reliable way to repeatedly replicate a focused segment of DNA, a concept which is applicable to numerous fields in modern biology and related sciences.



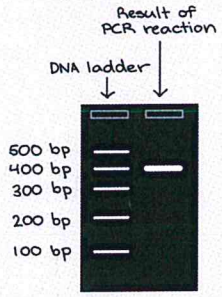
The Amplification of DNA
Polymerase Chain Reaction (PCR)

José Luis www.joseluisphotographer.com

- Generate very large quantities of specific DNA sequences, aiding in cloning
- Genomic sequencing or editing
- DNA "fingerprinting"-->forensic science
- Disease diagnosis
- Paternity determination
- Don't forget cloning dinosaurs (Jurassic Park)
- Many more to come....

About Polymerase Chain Reaction (PCR)

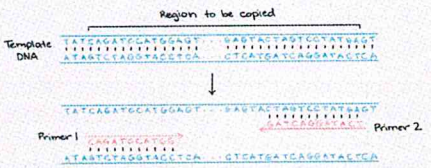
PCR amplifies a specific region of a DNA strand (the DNA target). Most PCR methods amplify DNA fragments of between 0.1 and 10 kilo base pairs (kbp), although some techniques allow for amplification of fragments up to 40 kbp in size.
The amount of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses



What are these things doing in my PCR reaction?

Primers are short pieces of DNA that are made in a laboratory. Since they're custom built, primers can have any sequence of nucleotides you'd like.

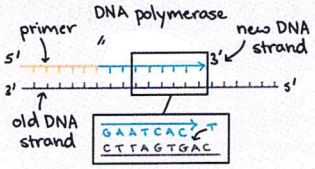
In a PCR experiment, two primers are designed to match to the segment of DNA you want to copy. Through complementary base pairing, one primer attaches to the top strand at one end of your segment of interest, and the other primer attaches to the bottom strand at the other end.



What are these things doing in my PCR reaction?

Heat-resistant DNA Polymerase is a naturally occurring complex of proteins whose function is to copy a cell's DNA before it divides in two. When a DNA polymerase molecule bumps into a primer that's base-paired with a longer piece of DNA, it attaches itself near the end of the primer and starts adding nucleotides. (In nature, these primers are made by an enzyme called primase).

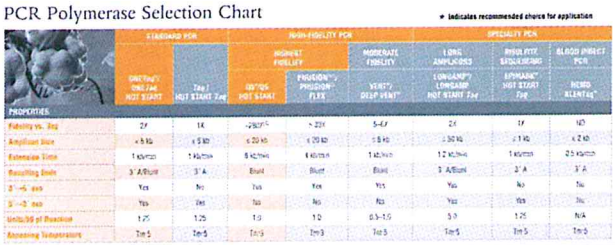
Buffer solution is a suitable chemical environment for optimum activity and stability of the DNA polymerase



What are these things doing in my PCR reaction?

PCR Polymerase Selection Chart

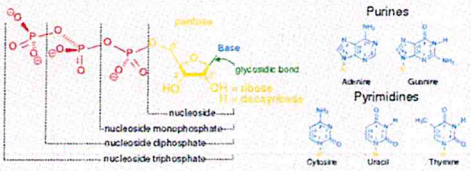
* indicates recommended choice for application



What are these things doing in my PCR reaction?

Nucleotides (deoxynucleoside triphosphates, or dNTPs) are the building blocks that DNA molecules are made of. You add a mixture of four types of nucleotides to your PCR reaction - A's, C's, G's and T's. DNA polymerase grabs nucleotides that are floating in the liquid around it and attaches them to the end of a primer.

DNA template that contains the DNA target region to amplify

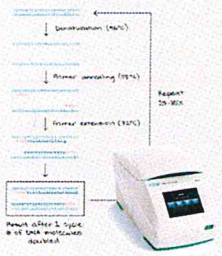


The steps of PCR

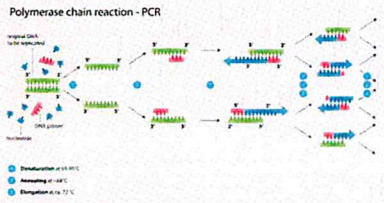
The key ingredients of a PCR reaction are Taq DNA polymerase, primers, template DNA, and nucleotides (DNA building blocks). The ingredients are assembled in a tube, along with cofactors needed by the enzyme, and are put through repeated cycles of heating and cooling that allow DNA to be synthesized.

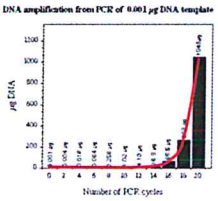
The basic steps are:

- **Denaturation** (96°C): Heat the reaction strongly to separate, or denature, the DNA strands. This provides single-stranded template for the next step.
- **Annealing** (55 - 65°C): Cool the reaction so the primers can bind to their complementary sequences on the single-stranded template DNA.
- **Extension** (68 - 72°C): Raise the reaction temperatures so DNA polymerase extends the primers, synthesizing new strands of DNA.

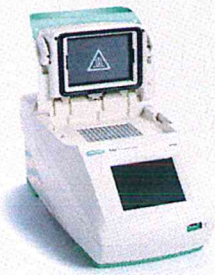


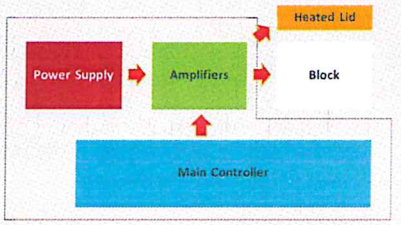
The steps of PCR



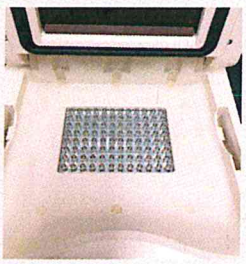


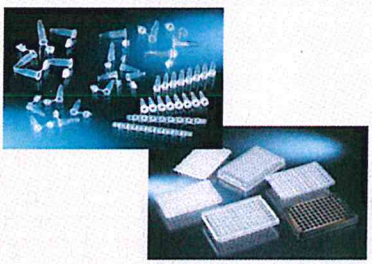
PCR Thermal Cycler



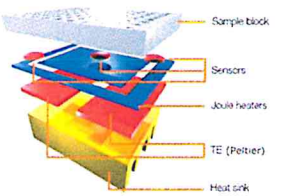


Thermal Cycler: 96-wells Sample Block

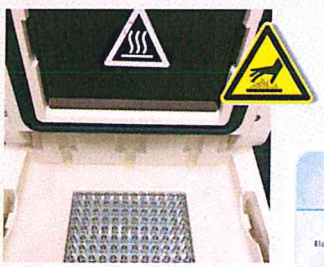


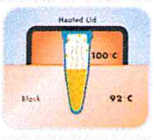


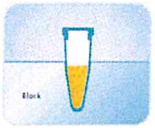
Thermal Cycler: 96-wells Sample Block

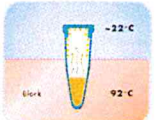


Thermal Cycler: Heated Lid





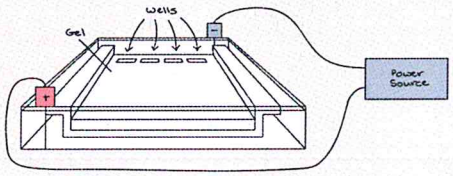




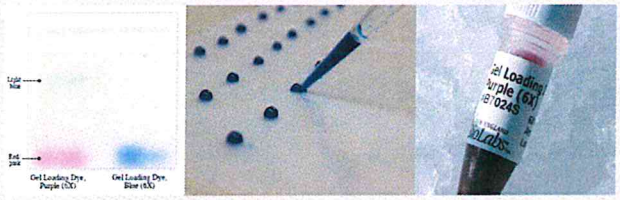
How to Set Up a PCR

Gel electrophoresis

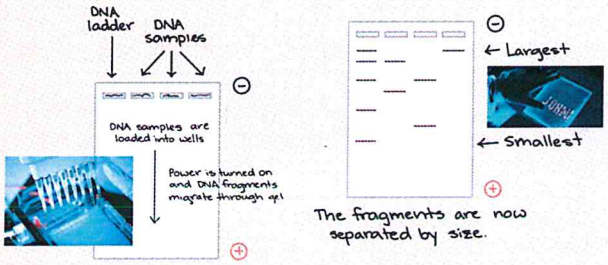
Gel electrophoresis is a technique used to separate DNA fragments (or other macromolecules, such as RNA and proteins) based on their size and charge. Electrophoresis involves running a current through a gel containing the molecules of interest. Based on their size and charge, the molecules will travel through the gel in different directions or at different speeds, allowing them to be separated from one another.



Gel Loading Dye

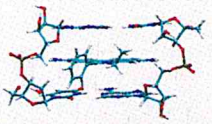


How do DNA fragments move through the gel?

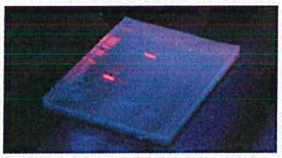


Visualizing the DNA fragments

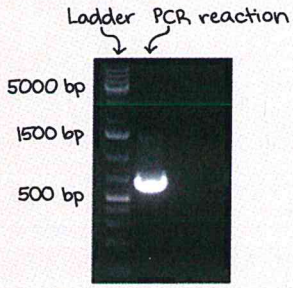
Once the fragments have been separated, we can examine the gel and see what sizes of bands are found on it. When a gel is stained with a DNA-binding dye such as EtBr or SYBR Green and placed under UV or blue light, the DNA fragments will glow, allowing us to see the DNA present at different locations along the length of the gel.



Ethidium bromide intercalated between two adenine-thymine base pairs.



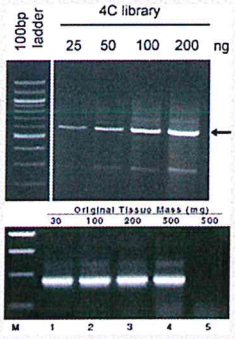
Visualizing the DNA fragments



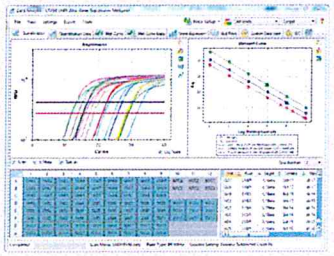
By comparing the bands in a sample to the DNA ladder, we can determine their approximate sizes. For instance, the bright band on the gel above is roughly 700 base pairs (bp) in size.

Limitations of standard PCR (End-Point PCR)

- Size-based discrimination only
- Low sensitivity
- Poor precision
- DNA-binding dye staining is not very quantitative
- Short dynamic range < 3 logs
- Results are not expressed as numbers



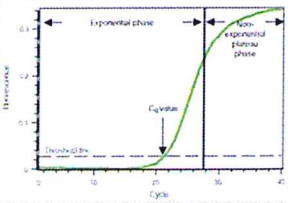
What is Real-Time PCR (qPCR)?



End-Point PCR vs Real-Time PCR

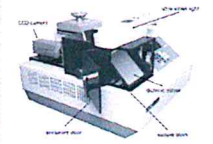
In conventional PCR, the amplified DNA product, or amplicon, is detected in an end-point analysis.

In real-time PCR, the accumulation of amplification product is measured as the reaction progresses, in real time, with product quantification after each cycle.



History of Real-Time PCR

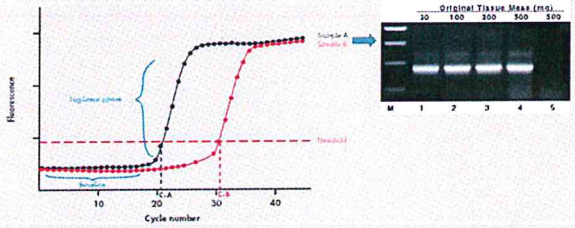
- The Pioneer: Russell Higuchi
- This "real-time" system includes
  - Intercalator Ethidium Bromide
  - Thermal cycler with ultraviolet light
  - Cooled CCD camera with computer-controlled
- By plotting the increase in fluorescence versus cycle number, the amplification produces increasing amounts of double-stranded DNA, which binds ethidium bromide, resulting in an increase in fluorescence.



How Real-Time PCR is Good?

Real-time Kinetic

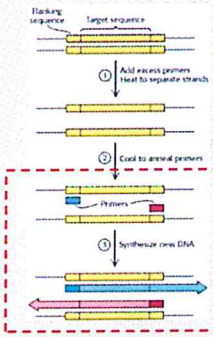
- Detection of "amplification-associated fluorescence" at each cycle during PCR.



What Is Real-Time PCR?

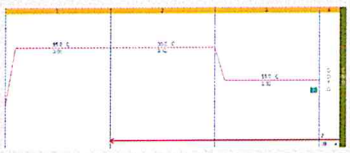
The qPCR workflow below delineates the steps in real-time PCR. First, amplification reactions are set up with PCR reagents and unique or custom primers. Reactions are then run in real-time PCR instruments and the collected data is analyzed by proprietary instrument software

How Real-Time PCR is Good?



The Real-Time PCR-reaction is subdivided into only two steps

- Denaturation
- Annealing with Synthesizing



How Real-Time PCR is Good?

More Sensitivity and Specificity

- Detects 1 copy of target sequence in genomic DNA
- Melting curve and Specific probe

Easy to analysis

- Computer base analysis with real-time analytical software

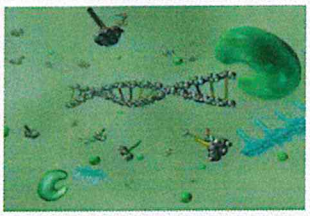
More advantages

- Relative and Comparative Quantitation, Melting Curves analysis, Genotyping analysis (Allelic Discrimination and SNP) and EndPoint analysis

DNA binding dyes in qPCR

EvaGreen and SYBR Green are fluorescent dye that non-specifically binds to double-stranded DNA.

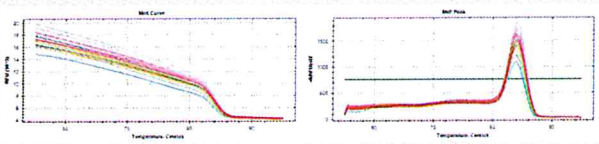
Thus amplicon production is measured by the increase in fluorescence intensity of this DNA binding dye in a non-sequence specific manner.



- **Advantages:**
  - Used in singleplex reactions
  - Essential tool for optimization of primer pairs when used with Melt Curve Analysis
- **Disadvantages:**
  - Detection of non-specific amplification

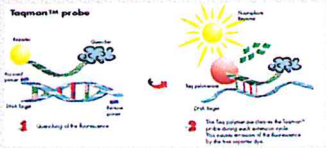
DNA binding dyes in qPCR

Because EvaGreen/SYBR Green binds to all dsDNA, it is necessary to check the specificity of your qPCR assay by analyzing the reaction product(s). To do this, use the meltcurve function on your real-time instrument and also run products on an agarose gel.



Hydrolysis Probe (TaqMan probes)

- Hydrolysis Probe (TaqMan probes) are oligonucleotides that contain a fluorescent dye on the 5' base (typically) and a quenching dye on the 3' end.



- While the probe is intact, the close proximity of the reporter and the quencher prevent the emission of fluorescence.
- TaqMan probes anneal to an internal region of a PCR product, thus when the Taq polymerase with exonuclease activity replicates a template on which TaqMan is bound, the probe is cleaved, releasing the signal.

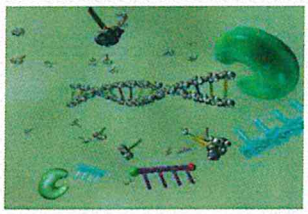
Hydrolysis Probe (TaqMan probes)

Advantages:

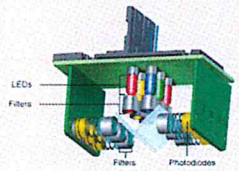
- Detects only amplification of specific product
- Uses standard PCR protocols
- Hybridization and cleavage does not interfere with accumulation of the product

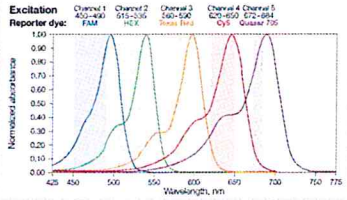
Disadvantages:

- Requires that specific probes be generated for each template



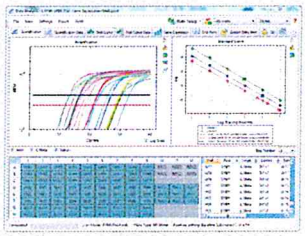
Multiplex Real-Time PCR assay



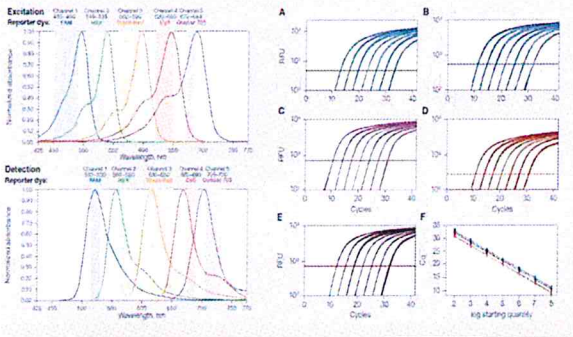


Applications: Absolute quantification

Absolute quantification is achieved by comparing the Cq values of the test samples to a standard curve. The result of the analysis is quantity of nucleic acid (copy number, µg) per given amount of sample (per cell, per µg of total RNA).



Applications: Absolute quantification



Detection of *Tomato black ring virus* by real-time one-step RT-PCR

Scott J. Harper, Catia Delmiglio, Lisa I. Ward*, Gerard R.G. Clover

Plant Health and Environment Laboratory, Investigation and Diagnostic Centre, MAF Biosecurity New Zealand, 231 Morrin Road, P.O. Box 2095, Auckland 1140, New Zealand

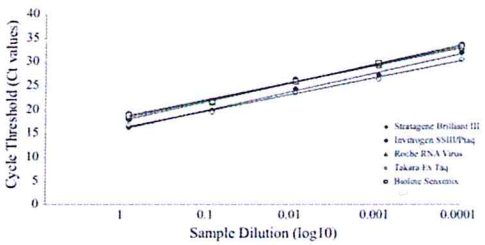
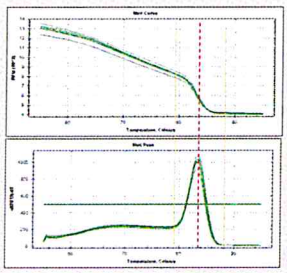


Fig. 1. A comparison of the standard curves of a ten-fold dilution series of TBRV-infected RNA amplified by real-time RT-PCR using five different reagent sets.

Melt Curve Analysis

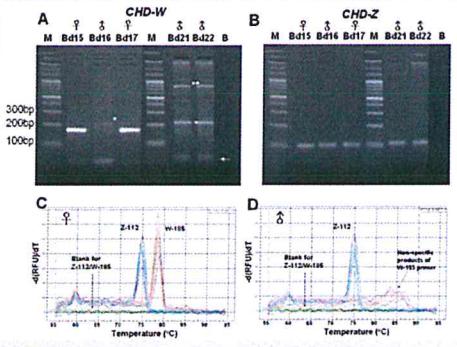
Melting curve analysis is an assessment of the dissociation-characteristics of double-stranded DNA during heating. The information gathered can be used to infer the presence and identity of single-nucleotide polymorphisms



Methodology article — Open Access

High-throughput avian molecular sexing by SYBR green-based real-time PCR combined with melting curve analysis

Hsueh-Wei Chang*[1,2], Chun-An Cheng[3], De-Leung Gu[3], Chia-Che Chang[4], San-Hua Su[3], Cheng-Hao Wen[3], Yii-Cheng Chou[3], Ta-Ching Chou[5], Cheng-Te Yao[2,6], Chi-Li Tsai[7,8] and Chien-Chung Cheng*[1]



High-resolution melting curve analysis to establish CYP2C19*2 single nucleotide polymorphism: Comparison with hydrolysis SNP analysis

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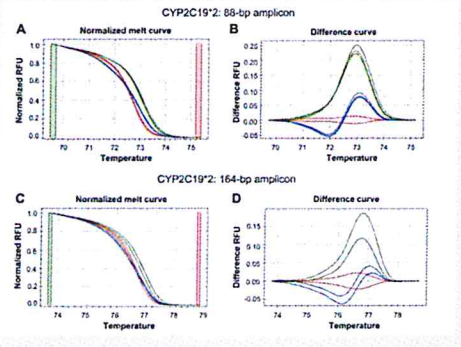
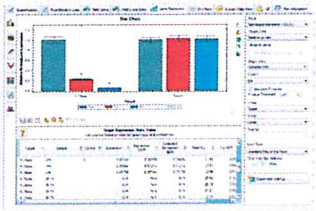


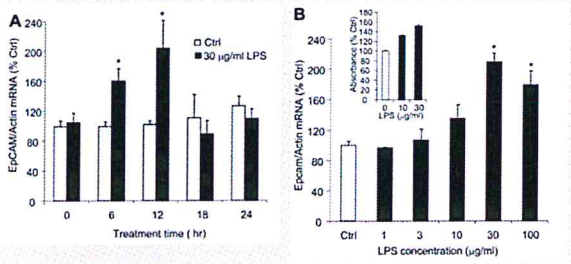
Applications: Relative quantification

Relative quantification, the analysis result is a ratio: the relative amount (fold difference) of a target nucleic acid for equivalent amounts of test and control sample A vs. B. Both cases need to address the question of what the "amount of sample" is, and in relative quantification, to ensure that equivalent amounts of samples are compared.



A newly identified epithelial cell adhesion molecule (EpCAM) from grass carp (*Ctenopharyngodon idellus*): Cloning, tissue distribution and lipopolysaccharide-induced expression in head kidney leucocytes

Xinyan Wang, Yanan Wang, Mu Yang, Hong Zhou*

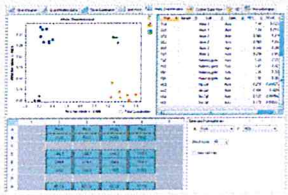


Applications: End Point analysis

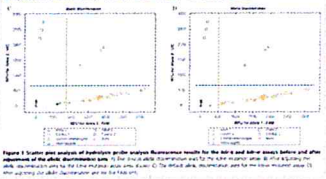
The End Point analysis shows the average RFU (relative fluorescence unit) values to determine whether or not the target amplified by the last (end) cycle.

Use these data to determine if a specific target sequence is present (positive) in a sample. Positive targets have higher RFU values than the cutoff level you define.

Applications: Allelic Discrimination for Genotyping Analysis



A comparison of DNA sequencing and the hydrolysis probe analysis (TaqMan assay) for knockdown resistance (*kdr*) mutations in *Anopheles gambiae* from the Republic of the Congo



More information

- https://en.wikipedia.org/wiki/Polymerase_chain_reaction
- http://learn.genetics.utah.edu/content/labs/pcr/
- https://www.khanacademy.org/science/biology/biotech-dna-technology/dna-sequencing-pcr-electrophoresis/a/polymerase-chain-reaction-pcr
- https://www.khanacademy.org/science/biology/biotech-dna-technology/dna-sequencing-pcr-electrophoresis/a/gel-electrophoresis
- https://en.wikipedia.org/wiki/Ethidium_bromide
- http://www.sci.sdsu.edu/~smaloy/MicrobialGenetics/topics/in-vitro-genetics/PCR.html
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- https://en.wikipedia.org/wiki/Real-time_polymerase_chain_reaction