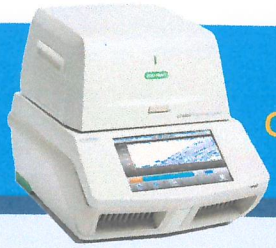


Real-Time PCR : Current technology and Application

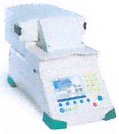
Mr. Pakorn Pengin
[Product Specialist]
THEERA TRADING CO., LTD.

31 Years of Innovation

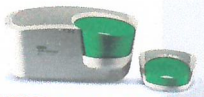
From the introduction of the PTC100 in 1988 to the launch of the QX200 droplet digital PCR in 2013, Bio-Rad Laboratories has led the way in thermal cycler innovation for 30 years.

Bio-Rad's unique family of products for PCR research gives you the power to do the experiments you want to do, the way you want to do them.

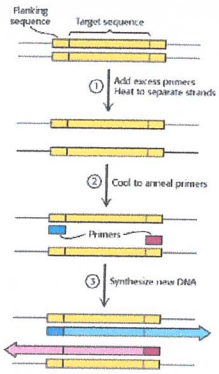

PTC100


iCycler iQ


CFX96 Touch qPCR

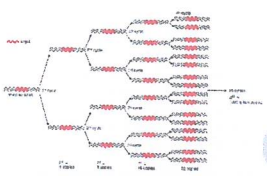

QX200 droplet digital PCR

PCR-reaction



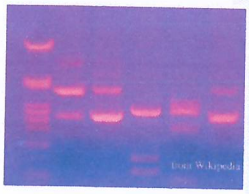
The PCR-reaction is subdivided into three steps

- **Denaturation**
- **Annealing**
- **Synthesizing**



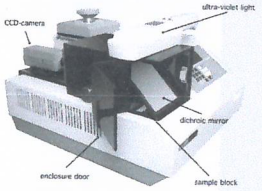
What is Wrong with Agarose Gels?

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



History of Real-Time PCR

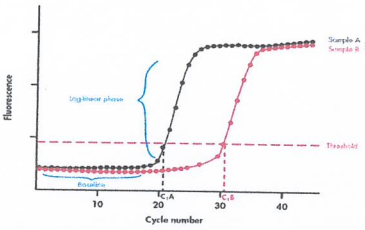
- The Pioneer: Russell Higuchi
- This "real-time" system includes
  - Intercalator Ethidium Bromide
  - Thermal cycler with ultraviolet light
  - Cooled CCD camera with computer-controlled
- By plotting the increase in fluorescence versus cycle number, the amplification produces increasing amounts of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

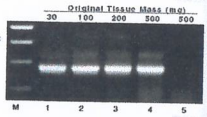


What Is Real-Time PCR?

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.



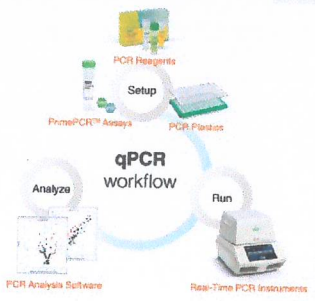


What Is Real-Time PCR?

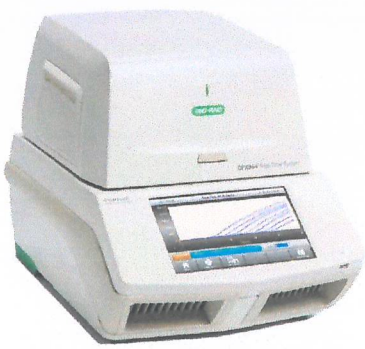
The qPCR workflow below delineates the steps in real-time PCR.

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

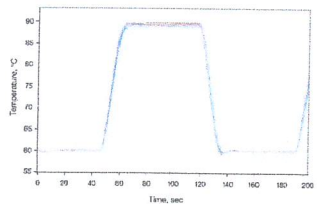


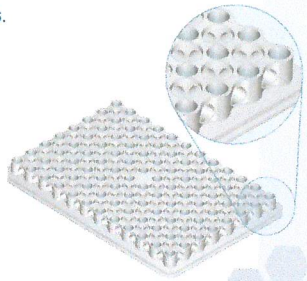
CFX96 Touch Real-Time PCR



Next Generation Block Design

- 6 independently controlled thermal electric modules
- Reduced-mass sample block
  - Heats and cools more quickly than traditional standard blocks.
  - Short settling time.

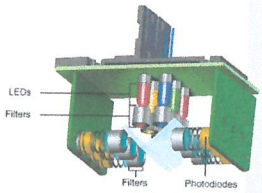


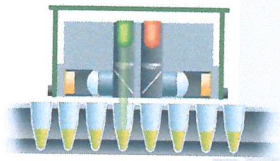


* Bio-Rad Patent Pending

Next Generation Optical Technology

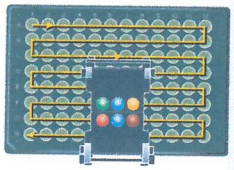
- CFX96 uses a scanning shuttle
  - 6 filtered LEDs for excitation
  - 6 filtered photodiodes for detection
- True Five-Target Multiplexing
- Maximal excitation of dyes with a fixed optical path for all wells and no cross talk for better multiplexing

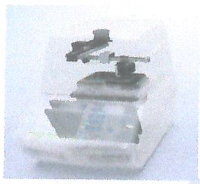




Worry Free Performance

- Hassle free maintenance as the instrument is factory calibrated and will not require recalibration
- Don't worry about making errors in plate setup, data is always acquired from all wells
- In a power failure, the instrument and computer will shut down. If the power failure is not long time, then the instrument will resume running a protocol, but the Application log will note the power failure.



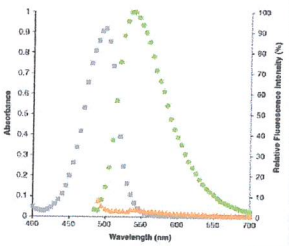


DETECTION TECHNOLOGY

Fluorescence Dye and Energy transfer technique

DNA binding dyes in qPCR

- **SYBR Green I**
- **EvaGreen**
- SYBR Gold
- YO (Oxazole Yellow)
- TO (Thiazole Orange)
- PG (PicoGreen)



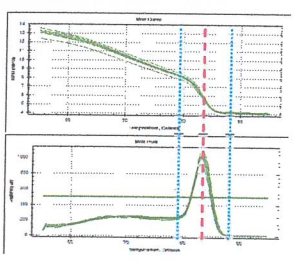
EvaGreen/SYBR Green I

- 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

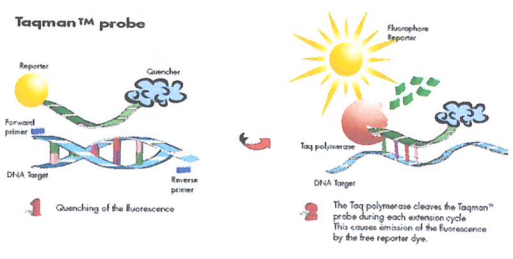
EvaGreen/SYBR Green I

- 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

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



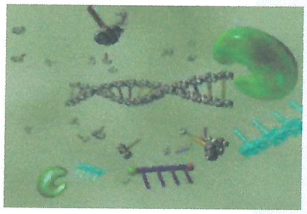
Hydrolysis Probe

Advantages:

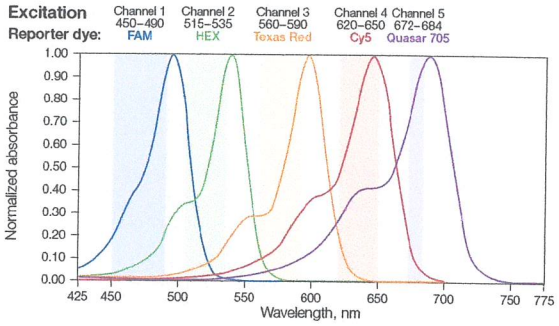
- Detects only amplification of specific product
- Uses standard PCR protocols
- Hybridization and cleavage does not interfere with accumulation of the product

Disadvantages:

- Requires that specific probes be generated for each template



Multiplex Real-Time PCR



Range of excitation/emission

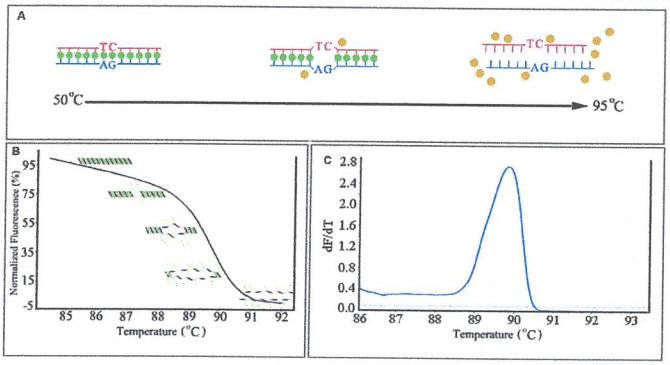
Channel	Excitation (nm)	Detection (nm)	Fluorophores
1	450-490	515-530	FAM, SYBR Green I, EvaGreen
2	515-535	560-580	VIC, HEX, TET, Cal Gold 540 and Cal Fluor Orange 560
3	560-590	610-650	ROX, TEXAS RED, Cal Red 610 and TEX 615
4	620-650	675-690	CY5 and Quasar 670
5	672-684	705-730	CY5.5 and Quasar 705
6	450-490	560-580	Accommodates FRET Chemistry

High Resolution Melt (HRM) Analysis

High Resolution Melt (HRM) analysis is a powerful technique in molecular biology for the detection of mutations, polymorphisms and epigenetic differences in double-stranded DNA samples. It was discovered and developed by Idaho Technology and the University of Utah. It has advantages over other genotyping technologies, namely:

- It is cost effective vs. other genotyping technologies such as sequencing and TaqMan SNP typing. This makes it ideal for large scale genotyping projects.
- It is fast and powerful thus able to accurately genotype many samples rapidly.
- It is simple. With a good quality HRM assay, powerful genotyping can be performed by non-geneticists in any laboratory with access to an HRM capable real-time PCR machine.

How does HRM analysis work?



Basic Principles of HRM

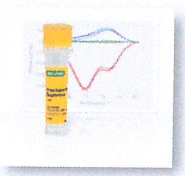
HRM is different from a standard SYBR Green I dye melt curve analysis:

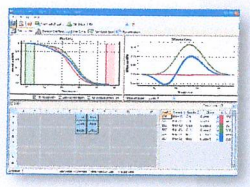
- Chemistry: Uses saturating or "release-on-demand" dsDNA binding dyes such as LC Green and LC Green Plus, ResoLight, EvaGreen, Chromofy and SYTO 9
- qPCR Instrument: More melting data points
- Software: Difference fluorescent normalization algorithms

Basic Principles of HRM

Release-on-demand dsDNA binding dye
Precision Melt Supermix

HRM calibrated Real-Time
CFX96 Real-Time System

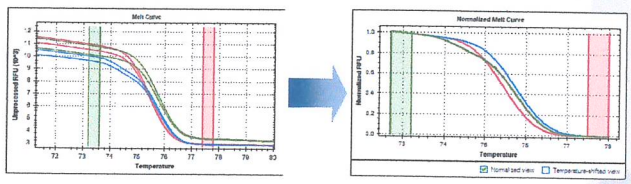




HRM analysis software
Precision Melt Analysis™ Software

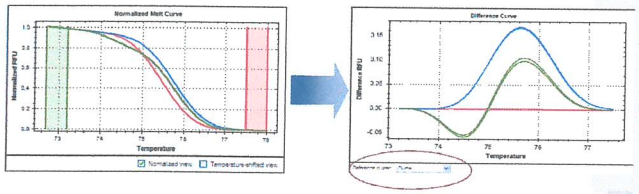
Normalization

- **Pre-melt (initial) and post-melt (final) fluorescence signals of all samples are normalized to relative values of 100% and 0%**
- **Eliminates differences in background fluorescence between curves**



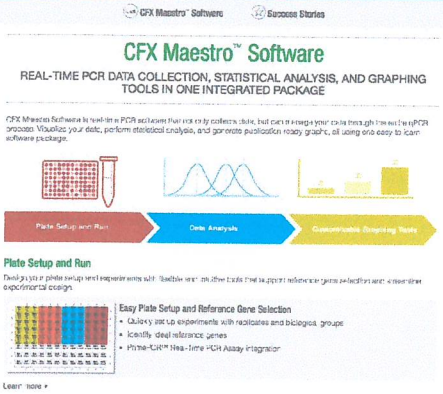
Difference Plot

- Magnify curve differences by subtracting each curve from the most abundant type or from a user-defined reference
- Sets a baseline, so small differences become visible

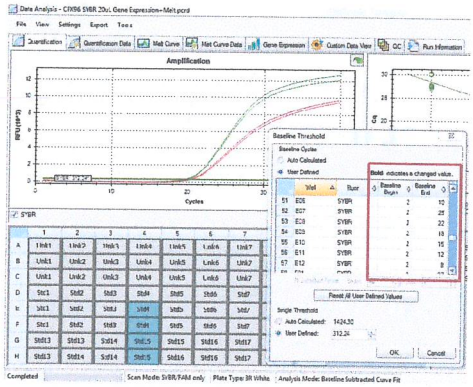


QPCR SOFTWARE AND APPLICATION

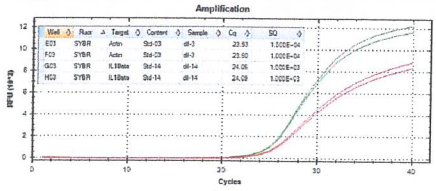
A Powerful Software



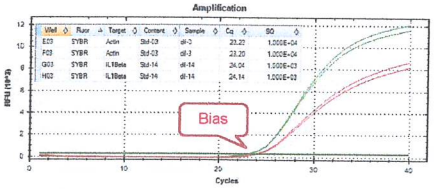
Individual base line correction



Cq Determinations

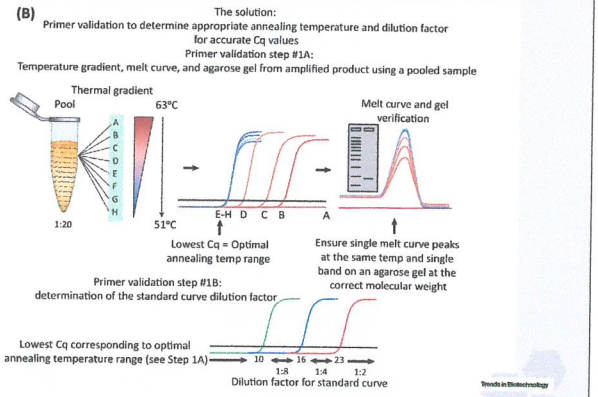


Regression mode



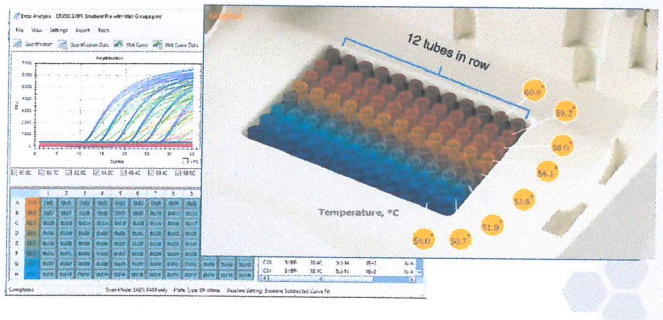
Single Threshold mode

Primer Validation



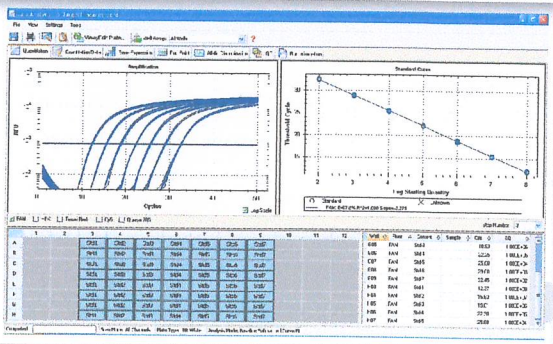
Gradient Analysis

- **Only CFX Maestro software can automatically analysis and display qPCR efficiencies for individual thermal gradient standard curves simultaneously**

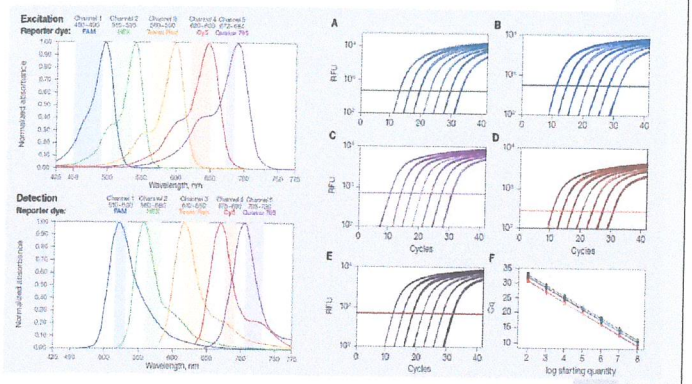


Absolute quantification

Absolute quantification is achieved by comparing the C_T 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). In



Multiplex quantification



Absolute Quantification Using a Standard Curve

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

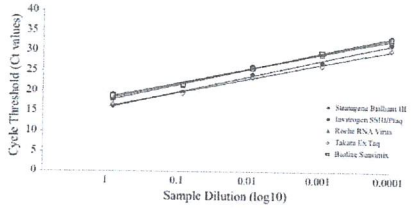
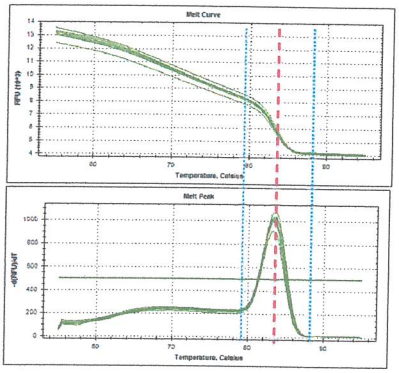


Fig. 1. A comparison of the standard curves of a ten-fold dilution series of TBRV-infected RNA amplified by real-time RT-PCR using five different reagent sets.

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

Melt Curve Analysis

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[1], Chia-Che Chang[4], San-Hua Su[1], Cheng-Hao Wen[1], Yii-Cheng Chou[5], Ta-Ching Chou[6], Cheng-Te Yao[7,8], Chi-Li Tsai[7,9] and Chien-Chung Cheng*[1]

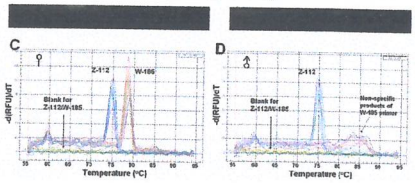
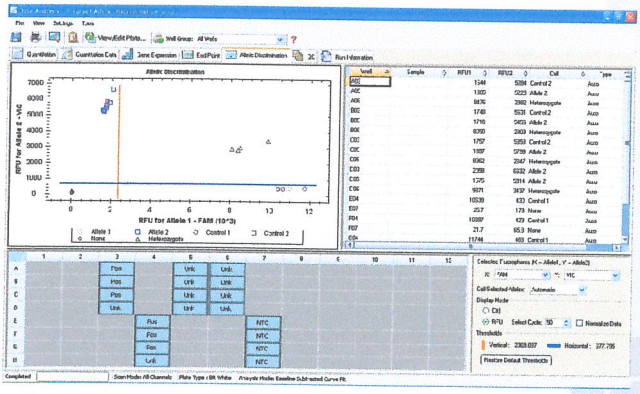


Figure 4
Representative gel view and MCA using sex-specific primers of *S. c. haya*. PCR products with 185- and 112-bp amplified by (A) CHD-W-F/R and (B) CHD-Z-F/R primers in different PCR-wells were run in 4% agarose gel, respectively. The arrow of Fig. 4A indicates the remaining primers or primer-dimers of PCR. Non-specific products of W-185 indicated with star symbols were only occurred in male samples. (C, D) demonstration for high-throughput molecular sexing of multiple *S. c. haya* samples using MCA. All data are performed in duplicate. (C) 7 females (Bds 12, 14, 15, 17, 19, 20, and 23) and (D) 6 males (Bds 13, 16, 18, 21, 22, and 24) were included. Z-112 and W-185 represent the primer mixtures for CHD-Z-F/CHD-Z-R (112-bp) and CHD-W-F/CHD-W-R (185-bp). The Tm value for Z-112 and W-185 is 75.0°C and 79°C, respectively. The Tm of non-specific products of W-185 is greatly larger than 79°C.

Allelic Discrimination for Genotyping Analysis



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

Kweng Shik Choi[1,2], Belinda L Spillings[1,2], Maureen Coetzee[1,*], Richard H Hunt[1,3], Lizette L Koekemoer[1,2]

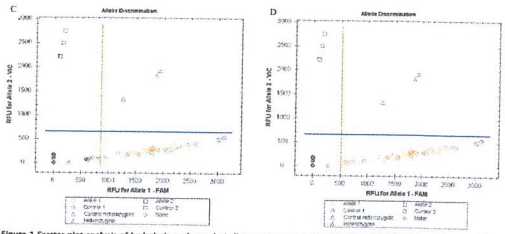
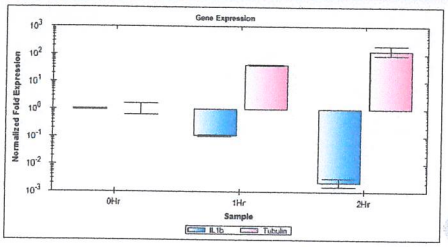


Figure 1 Scatter plot analysis of hydrolysis probe analysis fluorescence results for the *kdr-e* and *kdr-w* assays before and after adjustment of the allelic discrimination axes. A) The default allelic discrimination axes for the *kdr-e* mutation assay. B) After adjusting the allelic discrimination axes for the *kdr-e* mutation assay were shown. C) The default allelic discrimination axes for the *kdr-w* mutation assay. D) After adjusting the allelic discrimination axis for the FAM only.

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.



Relative Quantification Normalized to a Reference Gene

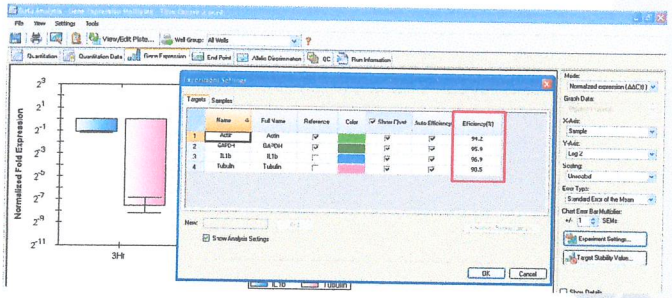
The Pfaffl Method

- **The $2^{-\Delta\Delta Cq}$ method for calculating relative gene expression is only valid when the amplification efficiencies of the target and reference genes are similar.**
- **If the amplification efficiencies of the two amplicons are not the same, an alternative formula must be used to determine the relative expression of the target gene in different samples..**

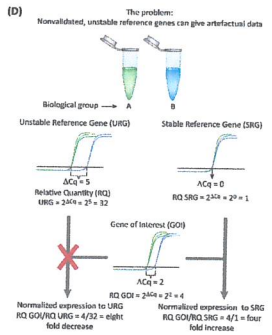
$$\text{Ratio} = \frac{(E_{target})^{\Delta C_T,\ target\ (calibrator\ -\ test)}}{(E_{ref})^{\Delta C_T,\ ref\ (calibrator\ -\ test)}}$$

Gene Expression Analysis

- Delta Cq and Delta-Delta Cq
- Multiple reference genes (vandesompele method)
- Individual reaction efficiencies using the and Pfaffl methods



Reference Gene



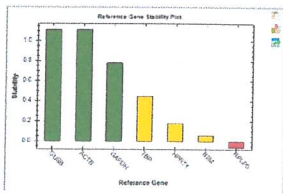
- Consequence of using poorly validated reference genes.
- qPCR data can change dramatically when normalization is performed using a stable versus unstable reference gene.

Taylor SC et al (2019). The ultimate qPCR experiment: Producing publication quality, reproducible data the first time. Trends Biotechnol [published ahead of print Jan 14, 2019] Accessed April 23, 2019

Validation and Selection Tool

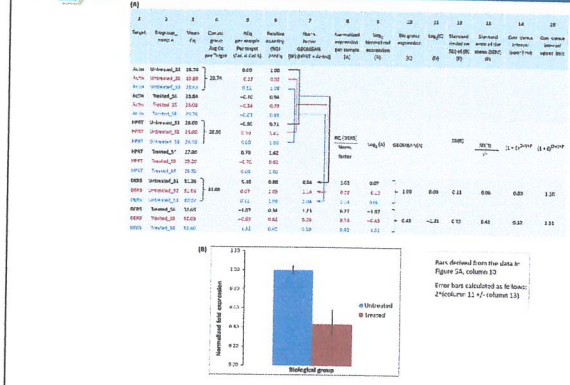
6	Reference gene validation	Survey literature for potential reference genes	Use the search term 'qPCR reference gene [GeNorm or NormFinder or BestKeeper] [Organism and Tissue of interest]' using Google Scholar. Pick seven to ten targets from the articles, and validate the primers (Figure 4B,C). A spike-in RNA or DNA sample into all test samples can help assess reference gene stability and may also be a useful normalization target [47].
		Confirm target stability	Test the validated reference gene primers against three cDNA samples from each biological/treatment group. Test stability using GeNorm, NormFinder and BestKeeper software. Normalize to multiple reference genes (Figure 4D).

Trends in Biotechnology



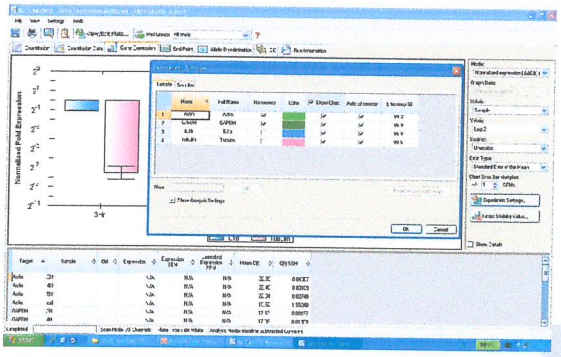
Order	Gene Name	Evaluation	Avg M Value	Stability (Ln(1/AvgM))	# Samples
1	GUSB	Ideal	0.328	1.113	4
2	ACTB	Ideal	0.329	1.113	4
3	GAPDH	Ideal	0.459	0.779	4
4	TBP	Acceptable	0.641	0.445	4
5	HPRT1	Acceptable	0.640	0.174	4
6	B2M	Acceptable	0.945	0.057	4
7	RPLP0	Unstable	1.080	-0.058	4

Multiple reference genes



Multiple reference genes

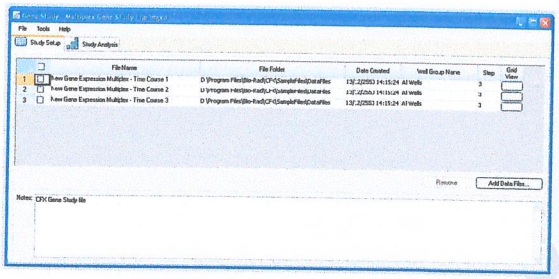
Multiple reference genes (Vandesompele method)



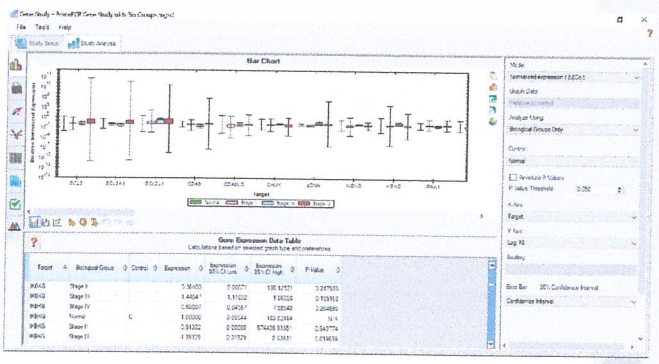
Gene Study

Gene Study function

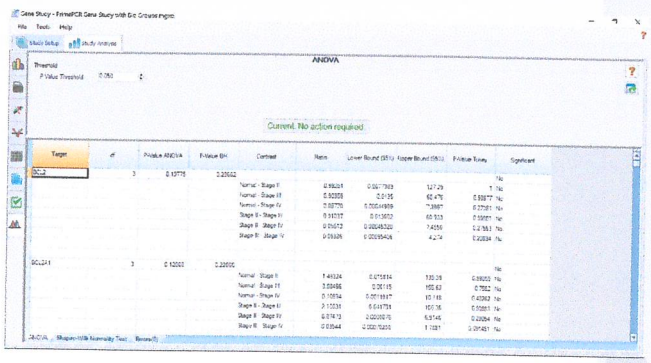
Create a Gene Study to compare gene expression data from one or more real-time PCR experiments using an inter-run calibrator to normalize between the experiments. Create a Gene Study by adding data from one or more data files to the Gene Study, the software groups them into a single file.



T-test



One-Way ANOVA and Tukey HSD Test

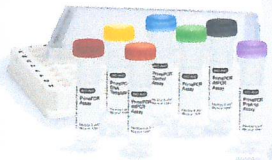


PrimePCR™ PCR

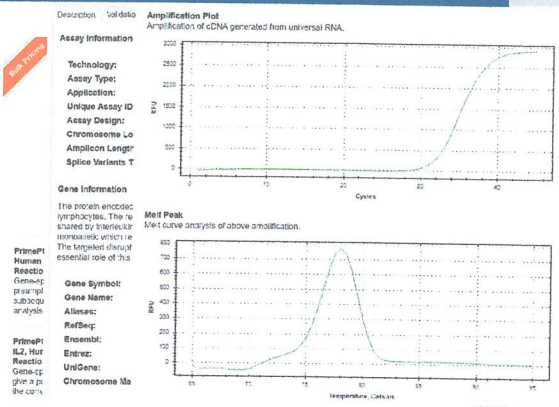
PrimePCR™ Assays and Panels

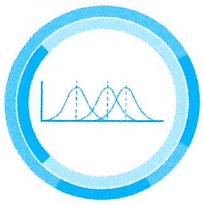
We focused on the details so you can focus on what really matters — your results.

- Expertly designed PCR primer and probe assays for quantitative PCR (qPCR) and Droplet Digital™ PCR (ddPCR™)
- Experimentally validated for guaranteed performance
- Assays for gene expression analysis, copy number variation, and mutation detection



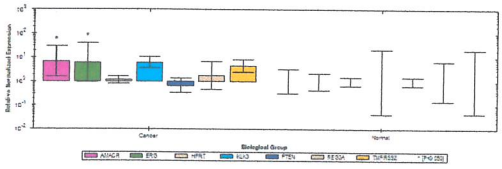
PrimePCR™ PCR





Push-Button Data Analysis and Statistics

- Automatically calculate Cq values and reaction efficiency
- Easily perform statistical analysis including *t*-tests, and one-way ANOVA
- Efficiently analyze multi-plate studies

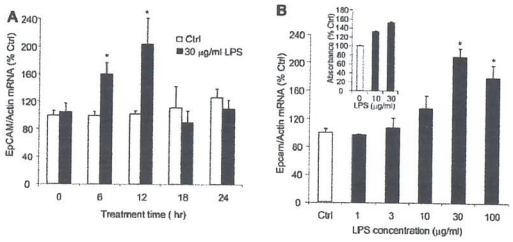


Gene Expression

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*

School of Life Science and Technology, University of Electronic Science and Technology of China, Chengdu 610054, People's Republic of China



HRM Applications

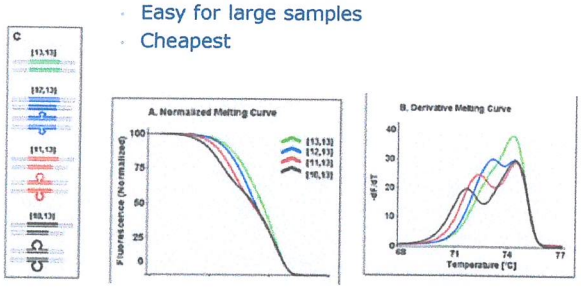
- **Mutation discovery/gene scanning**
- **SNP genotyping**
- **DNA methylation analysis**
- **Species identification**
- **DNA fingerprinting**

95% of all applications

- **Screening for loss of heterozygosity**
- **Allelic prevalence in a population**
- **Characterization of haplotype blocks**
- **HLA compatibility testing**
- **Identification of candidate predisposition genes**

Mutation discovery/gene scanning

- Before and After sequencing
- Easy for large samples
- Cheapest

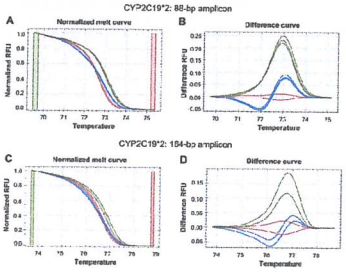


HRM Applications

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

Manna Temesvári [a,1], József Paulik [b,1], László Kóbori [c], Katalin Monostory [a,*]

[a] Chemical Research Center, Hungarian Academy of Sciences, Pusztaszeri 59-67, H-1025 Budapest, Hungary
[b] Bio-Rad Laboratories Hungary Ltd, Futó u. 47-53, H-1082 Budapest, Hungary
[c] Transplantation and Surgical Clinic, Semmelweis University, Baross 23-25, H-1082 Budapest, Hungary



Software Comparison

Papers in Press. Published April 9, 2014 as doi:10.1373/clinchem.2013.220160
The latest version is at http://twinsaint.clinchem.org/cgi/doi/10.1373/clinchem.2013.220160

Clinical Chemistry 60:6
000–000 (2014)

Molecular Diagnostics and Genetics

Genotyping Accuracy of High-Resolution DNA Melting Instruments

Mei Li,[1,2] Luming Zhou,[1] Robert A. Palais,[3] and Carl T. Wittwer[1]*

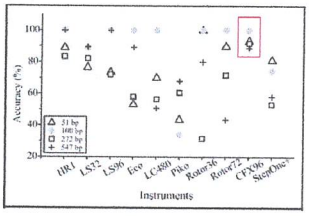


Fig. 4. Accuracy of homozygous genotyping on 10 instrument configurations with 4 PCR product lengths.

The instrument melting rates decrease from left to right as given in Table 1. The manufacturers' recommended melting conditions were used with custom analysis software.

Software Comparison

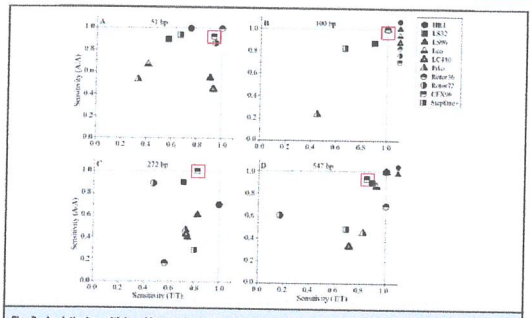


Fig. 3. Analytical sensitivity of homozygote detection across 10 instrument configurations and 4 PCR product sizes. By plotting the sensitivity of detecting the T/T genotype against the A/A genotype, each panel correlates genotype sensitivities in 2 dimensions. Instruments with 100% accuracy appear at the top right corner. All samples were melted under manufacturers' recommended conditions and analyzed with the same custom software.

HRM Applications

Advances in molecular sexing of birds: a high-resolution melting-curve analysis based on *CHD1* gene applied to *Coturnix* spp.

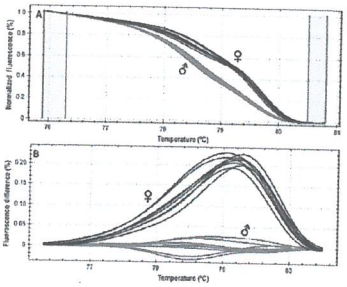


Fig. 1. HRM analysis of PCR products amplified with *CHD1* universal primer pair (P2/P8). (A) normalized and temperature-shifted melting curves, and (B) fluorescence differences for sex genotyping of common and Japanese quails. Males (♂) and females (♀) from both subspecies showed similar melting curve patterns.