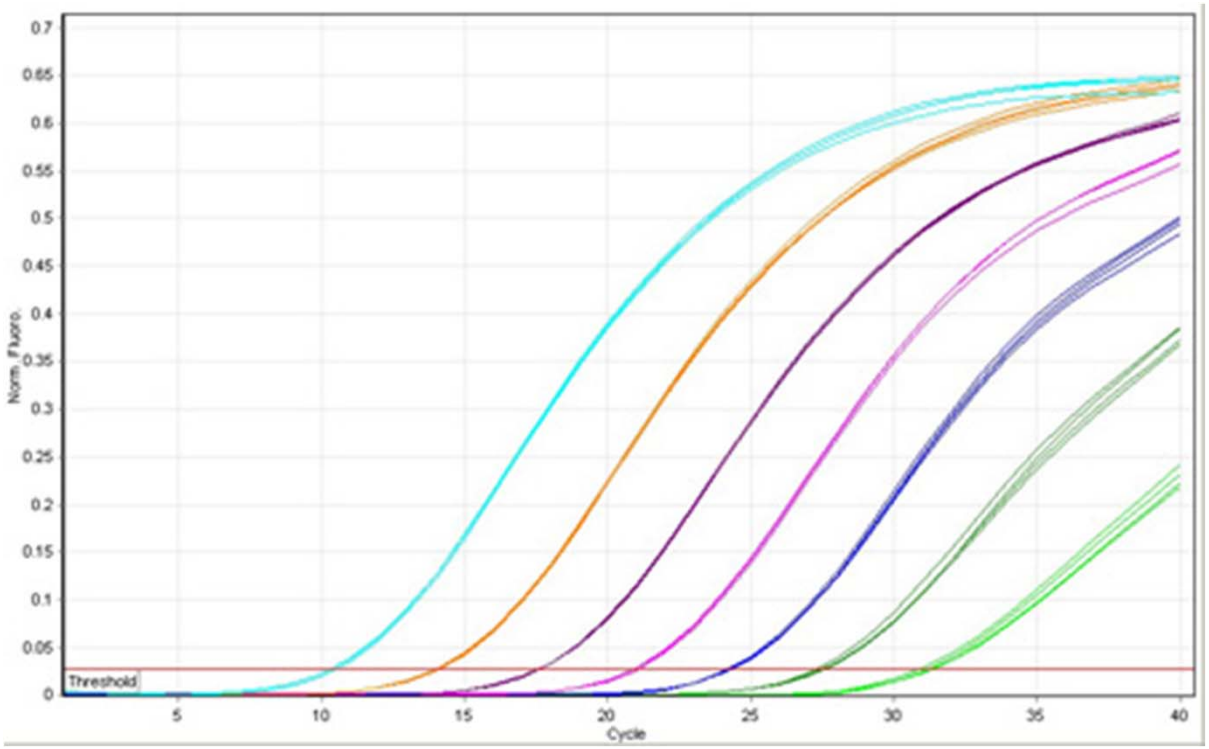
What Can You Do With qPCR?

Applications and Uses



(adapted from Roche RealTime PCR Application Manual)

What is qPCR?

Real-time PCR - also known as quantitative PCR (qPCR) - measures PCR amplification as it occurs, so that it is possible to determine the starting concentration of nucleic acid.

Every real-time PCR contains a fluorescent reporter molecule—a TaqMan® probe or SYBR® Green dye, for example—to monitor the accumulation of PCR product.

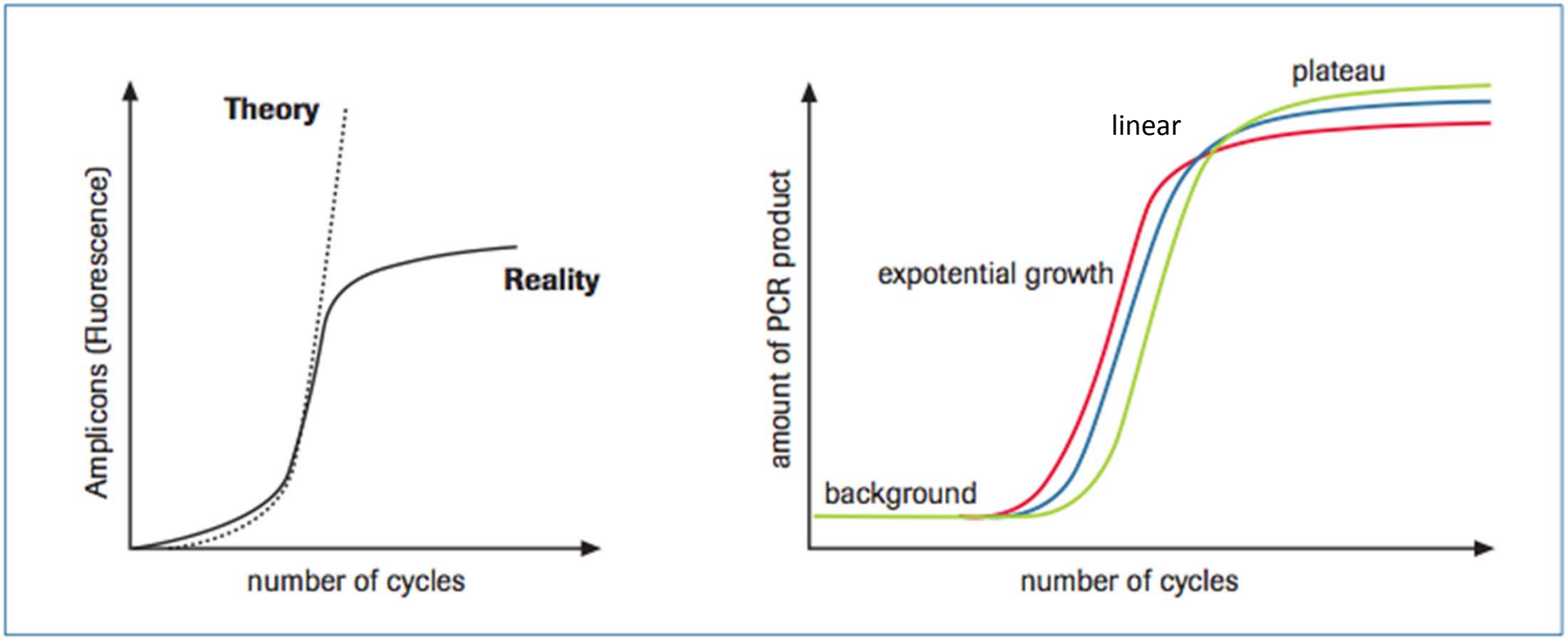
As the quantity of target amplicon increases, so does the amount of fluorescence emitted from the fluorophore.

Advantages of real-time PCR include:

- Generation of accurate quantitative data
- Increased dynamic range of detection
- Elimination of post-PCR processing
- Detection down to one copy
- Increased precision to detect smaller fold changes
- Increased throughput

There are three phases in a basic PCR run:

- Exponential
- Linear (High Variability)
- Plateau (End-Point: Gel detection for traditional methods)



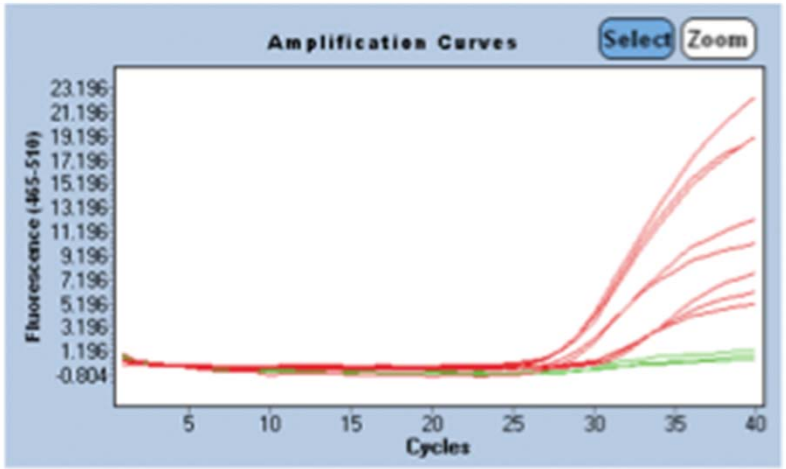
What Can I Do with qPCR?

- Gene Detection
  - Qualitative Analysis
  - Quantification
    - Absolute Quantification
    - Relative Quantification
- Genetic Variation Analysis
  - SNP allele detection
  - Endpoint Genotyping
  - Melting Curve Genotyping

Gene Detection

Qualitative Analysis

- Provides ‘presence/absence’ data
- Does not quantify the amount of DNA
- Standards are not required
- Positive and negative controls should be included

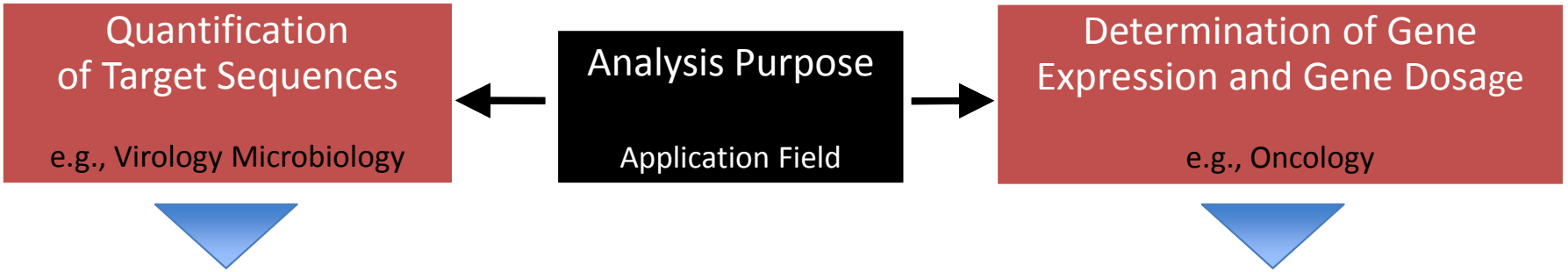


Samples				Results		
Include	Color	Pos	Name	Cp	Concentration	Standard
☑		B4	Sample 28			
☑		B5	Sample 29			
☑		B6	Sample 30			
☑		B7	Sample 31	29.61		
☑		B8	Sample 32	30.78		
☑		B9	Sample 33	30.21		
☑		B10	Sample 34	28.27		
☑		B11	Sample 35	28.15		
☑		B12	Sample 36	28.19		
☑		B13	Sample 37	27.96		
☑		B14	Sample 38	28.18		

Quantification

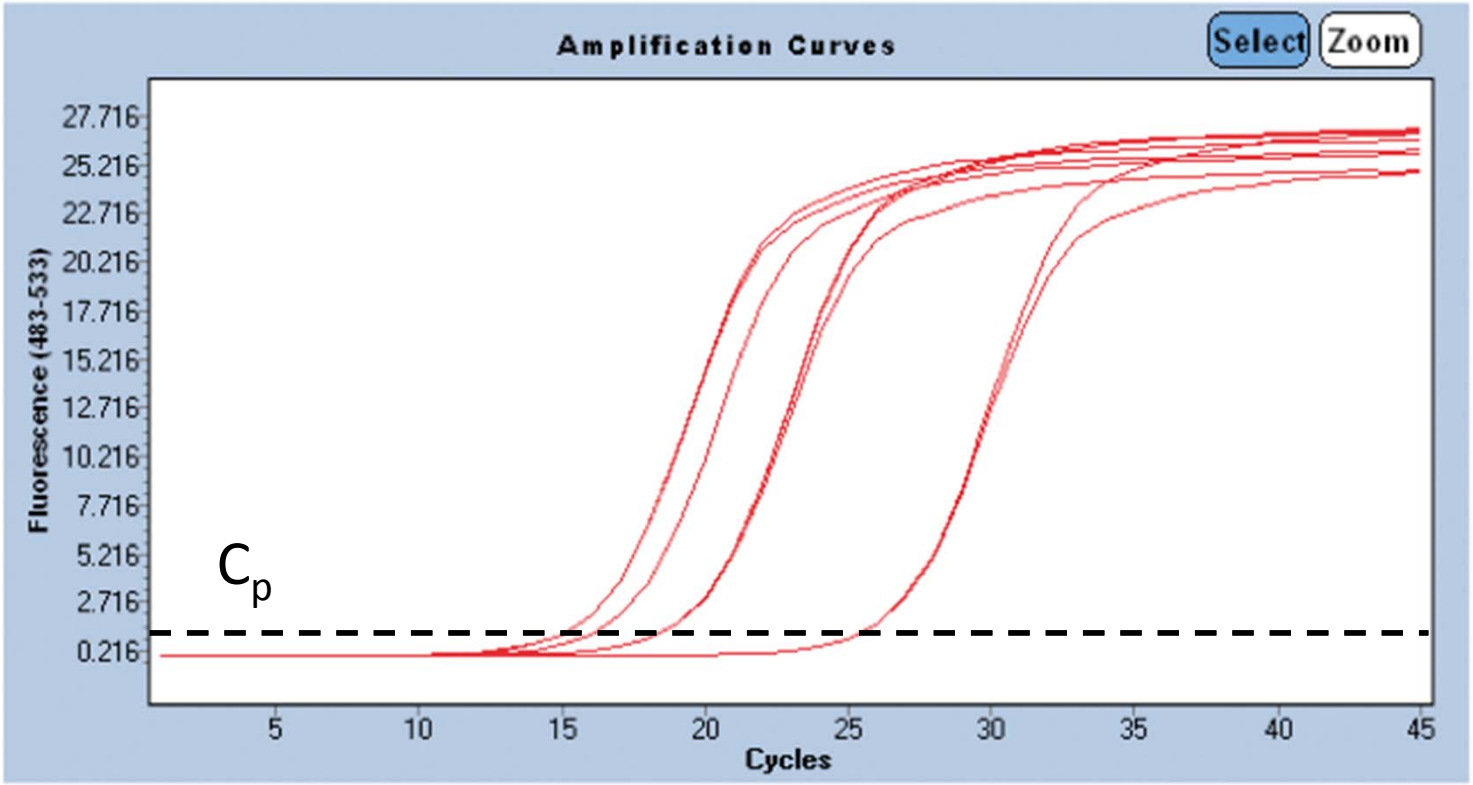
- **Absolute quantification** allows you to quantify a single target sequence and express the final result as an absolute value.

- **Relative quantification** compares the levels of two different target sequences in a single sample.



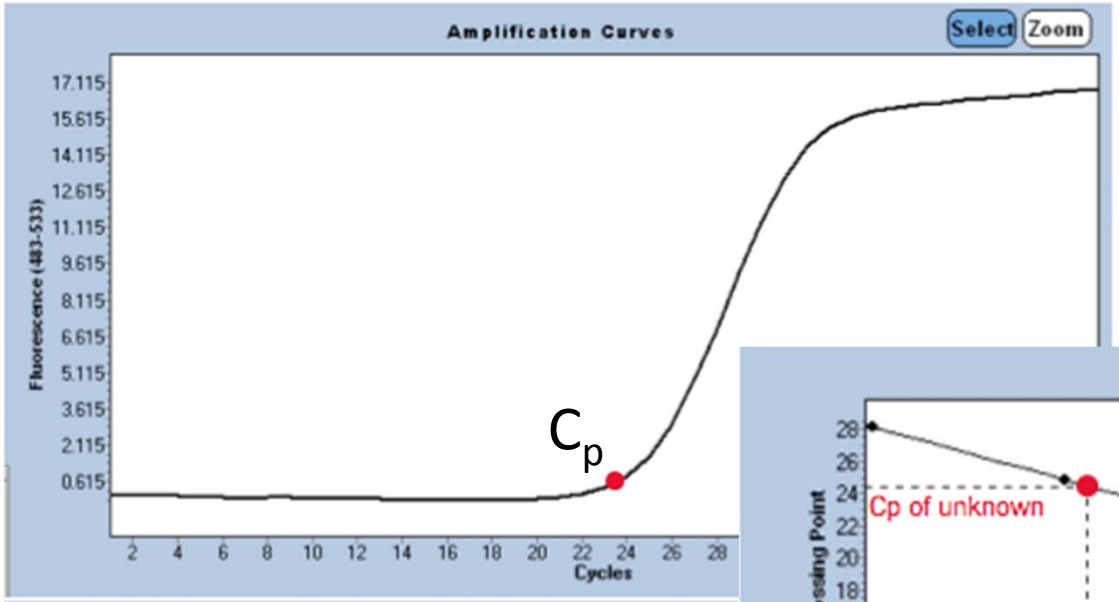
Typical Application Fields:	Typical Application Fields:
Detection of specific DNA/RNA (*e.g.,* oncology research)	Studies on minimal residual diseases (MRD)
Identification of species (*e.g.,* bacteria, virus)	Determination of mRNA expression levels (*e.g.,* cytokines, chemokines)
Pathogen detection (*e.g.,* legionella, anthrax)	Gene dosage quantification (*e.g.,* chromosomal aberrations)
Antibiotic resistance screening (*e.g.,* MSRA, VRE)	GMO detection
Water quality monitoring	

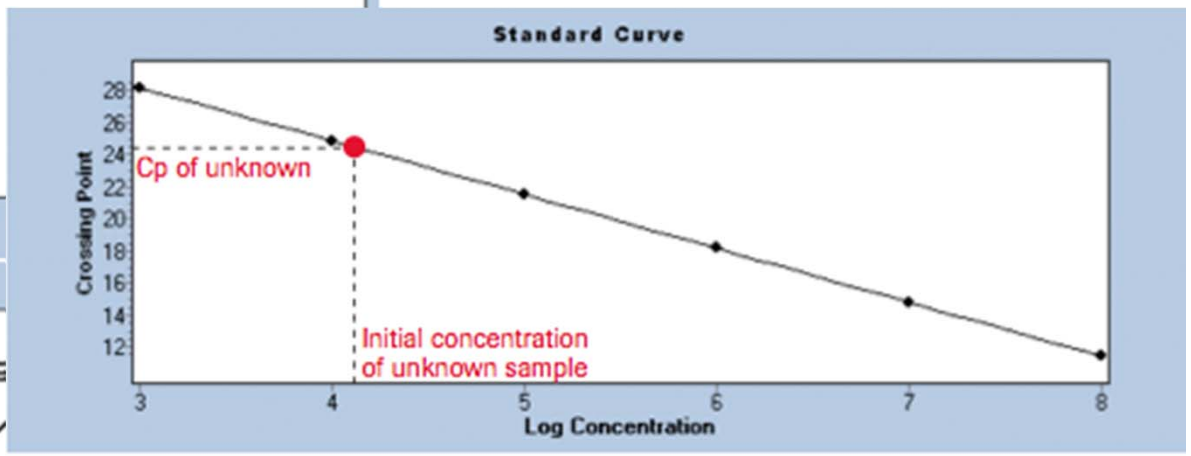
Absolute Quantification

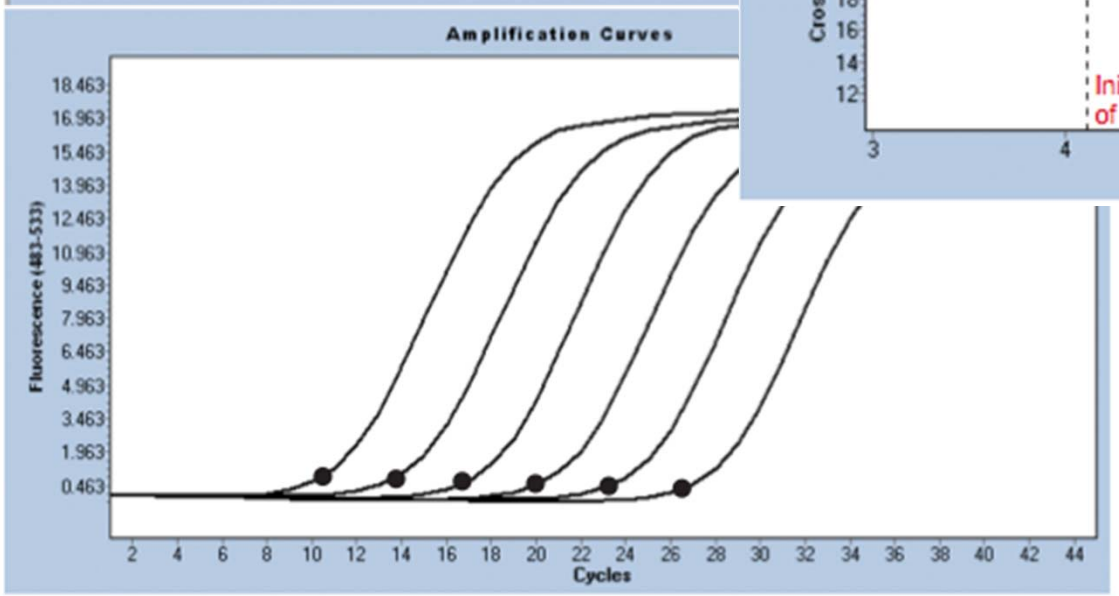


Samples				Results	
Include	Color	Pos	Name	Cp	Concentration
☑		E24	Sample 6	16.14	9.47E4
☑		F11	Sample 2	19.60	9.14E3
☑		F13	Sample 5	26.61	9.68E1
☑		H1	Sample 8	19.66	8.77E3
☑		J13	Sample 5	26.72	9.03E1
☑		P1	Sample 6	16.16	9.30E4
☑		P3	Sample 7	17.17	4.69E4
☑		P7	Sample 8	19.68	8.68E3

Absolute Quantification with External Standards







Plot C_p vs. Concentration

Relative Quantification

Relative quantification compares the levels of two different target sequences in a single sample (e.g., target gene of interest and a reference gene) and expresses the final result as a ratio of these gene levels.

Concept of Relative Quantification

$$\text{relative ratio} = \frac{\text{concentration of target}}{\text{concentration of reference}}$$

Reference Genes

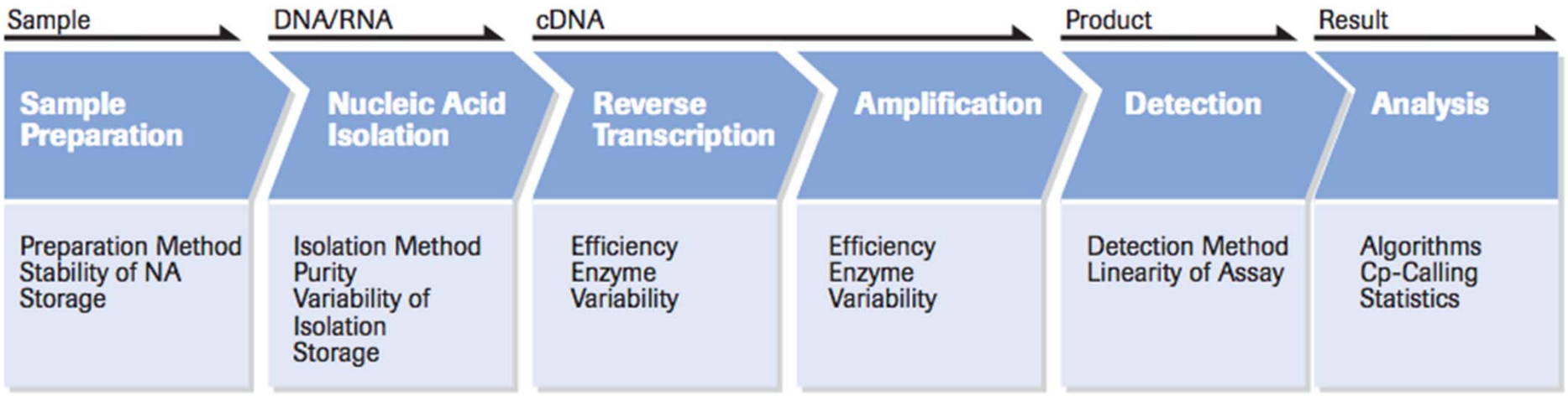
- Reference genes (aka ‘housekeeping genes’) are stably expressed and should not be affected by the different experimental conditions.
- Examples are ß-actin, GAPDH, rRNA
- If no single reference gene is suitable for all conditions, consider using more than one housekeeping gene and averaging their assay levels to form a single reference value.

Reference Gene Normalization

Normalization to a reference gene corrects for qualitative and quantitative differences in the sample, such as those caused by:

- Variations in initial sample amount or nucleic acid recovery
- Possible RNA degradation in sample material
- Differences in sample and/or nucleic acid quality
- Variations in cDNA synthesis efficiency
- Variations in sample loading or pipetting errors
- PCR inhibitors and other factors influencing PCR

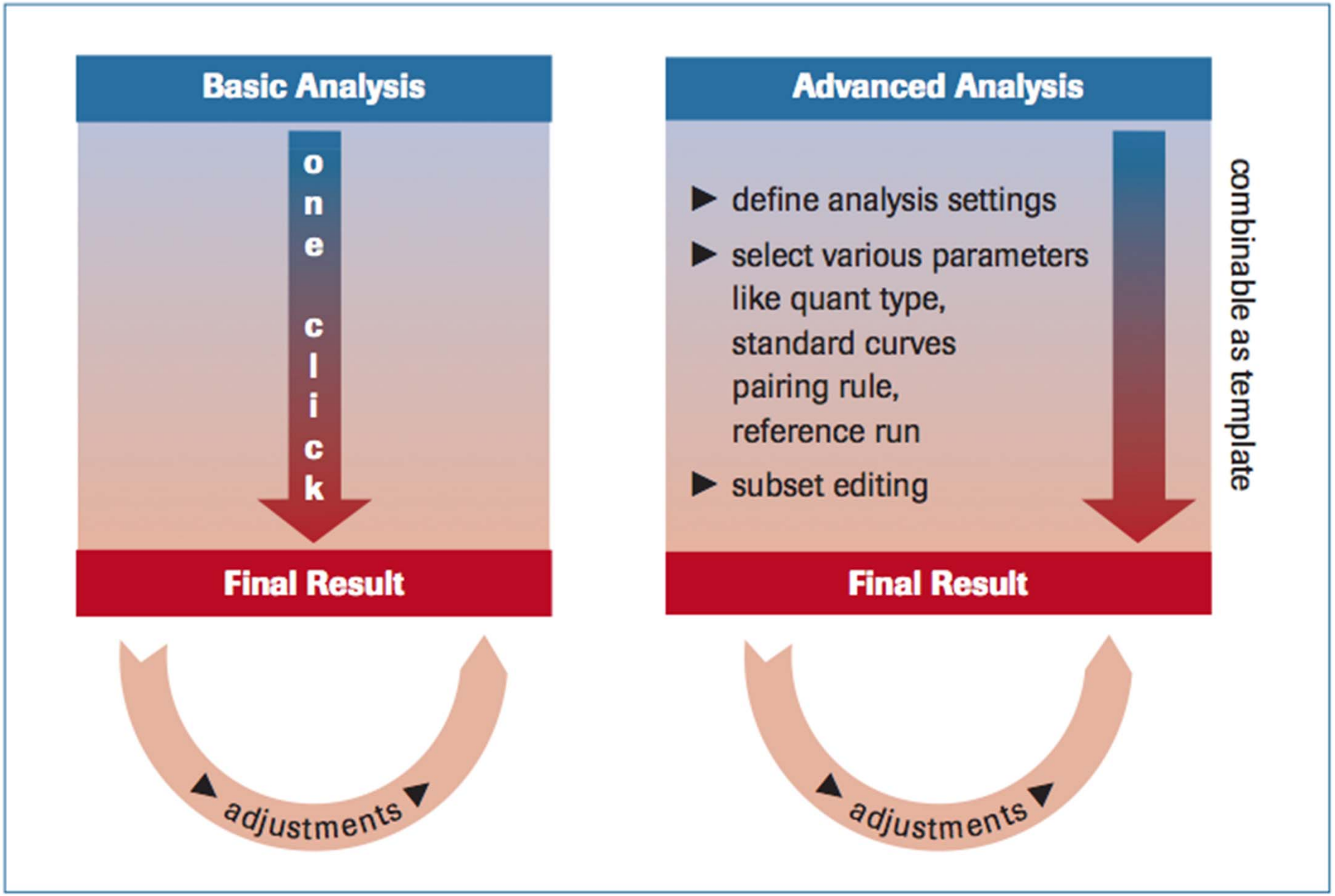
Optimization of Reaction Conditions



Specificity, sensitivity, efficiency, and reproducibility are the important criteria to consider when optimizing a quantitative assay.

Should be little or no test-to-test variations in C_p and fluorescent signal intensity.

Relative Quantification Analysis Methods



Analysis options for the Basic and Advanced Analysis methods

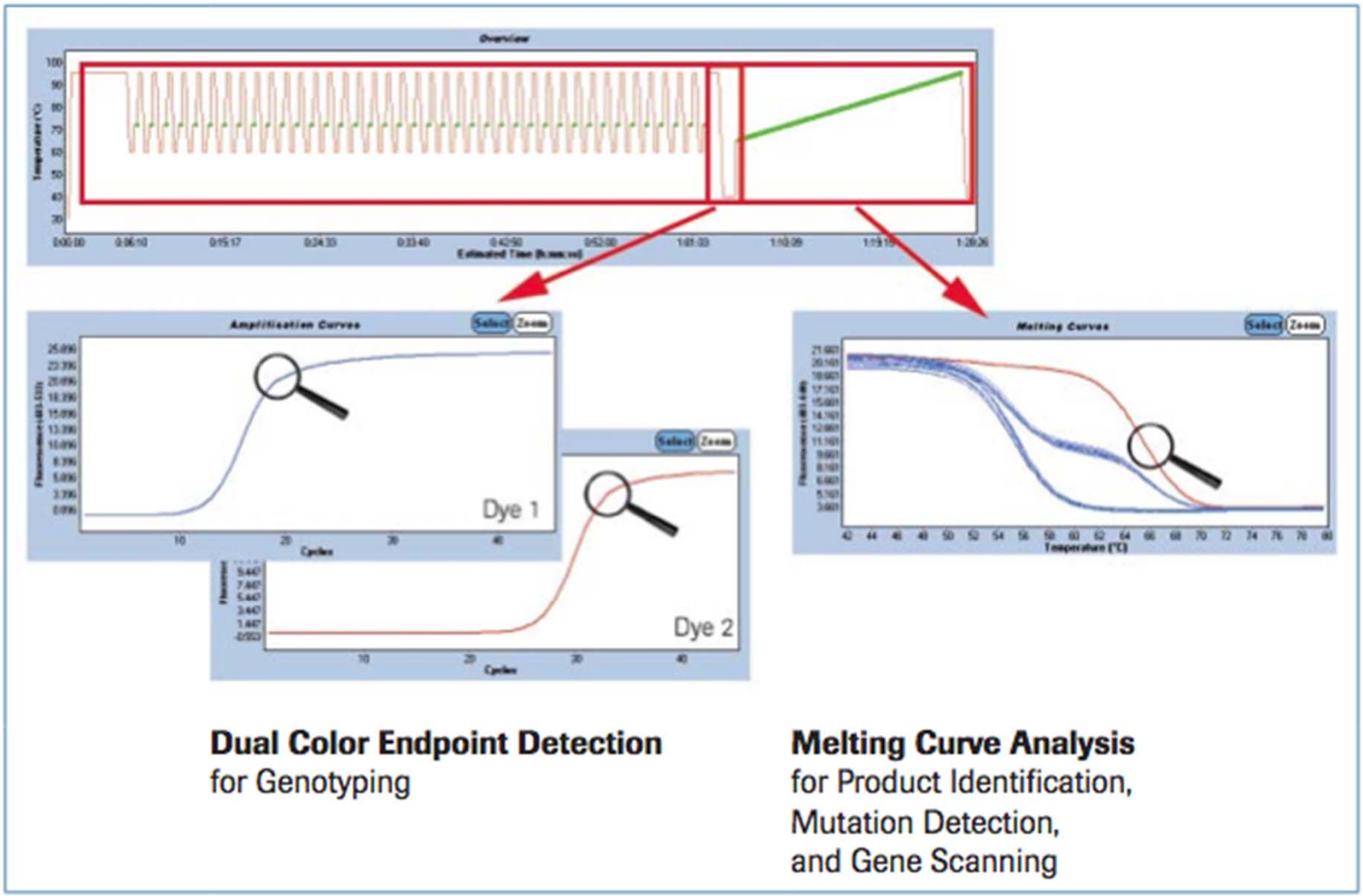
	Basic	Advanced
Target Gene	one / many	one / many
Reference Gene	one / many	one / many
Pairing Target-to-Reference	All-to-mean	One-to-One All-to-mean All-to-All Mean-to-All
Calibrator	with / without	with / without
Assay Set-up on MWP	Target & Reference on same plate Full plate analyzed	Target & Reference on same plate Target & Reference on different plates Full plate, and/or Subsets analyzed
Cp Analysis	Fit Points Method	Fit Points Method 2nd Der. Max. Method
Efficiency	$E = 2 / \neq 2$	$E = 2 / \neq 2$ standard curves: linear or non-linear fit
Multiplex	monocolor / dual color	monocolor / dual color

Genetic Variation Analysis

Single-nucleotide polymorphisms (SNPs) account for more than 90% of all genome sequence differences between individuals.

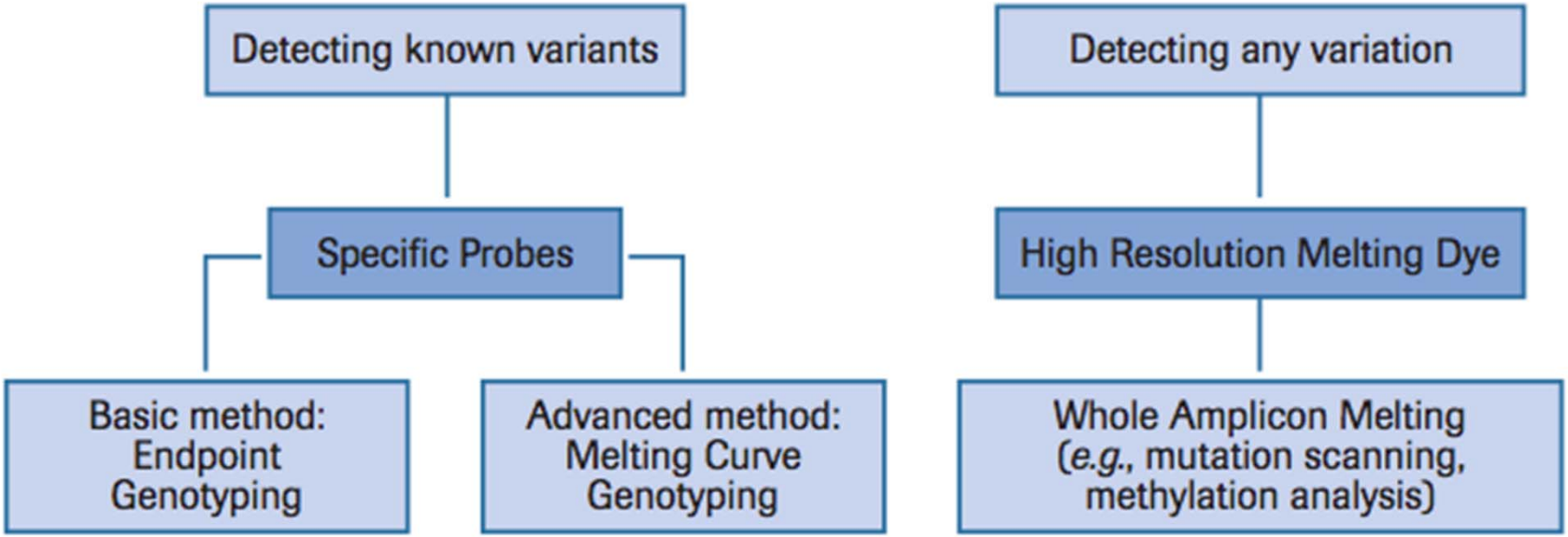
In all genetic variation studies, a large number of individuals must be genotyped, in order to characterize a large number of markers. Alleles of known SNPs must be identified and called correctly, and the presence of newly arising variants must be detected.

Genetic Variation Analysis



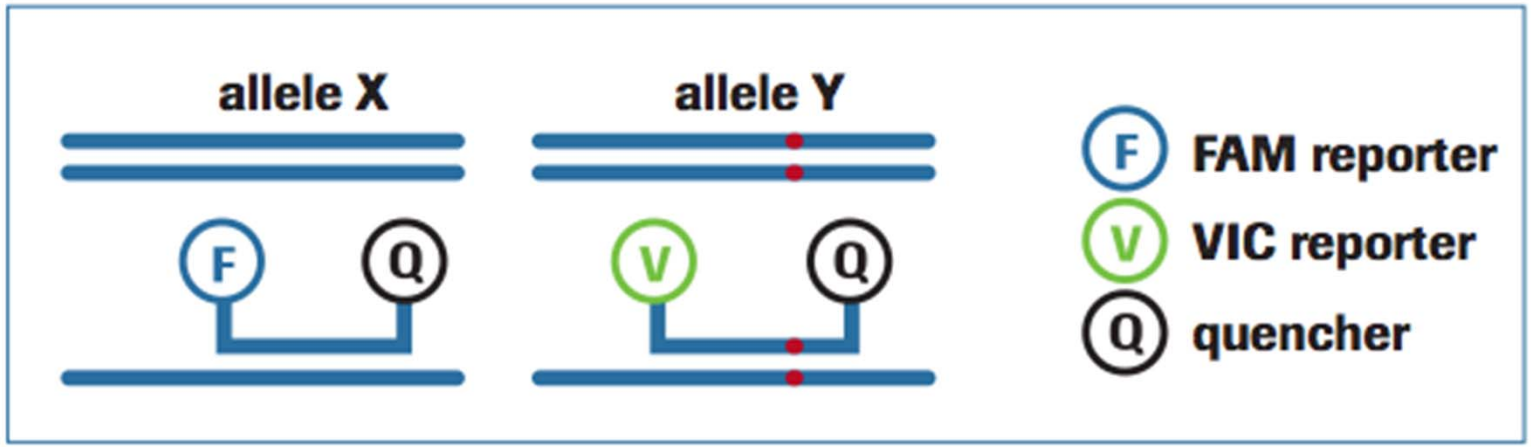
Different genotyping methods analyze fluorescent signals from different parts of a Real-Time PCR run.

Genetic Variation Analysis



SNP analysis methods for the detection of known or unknown variants

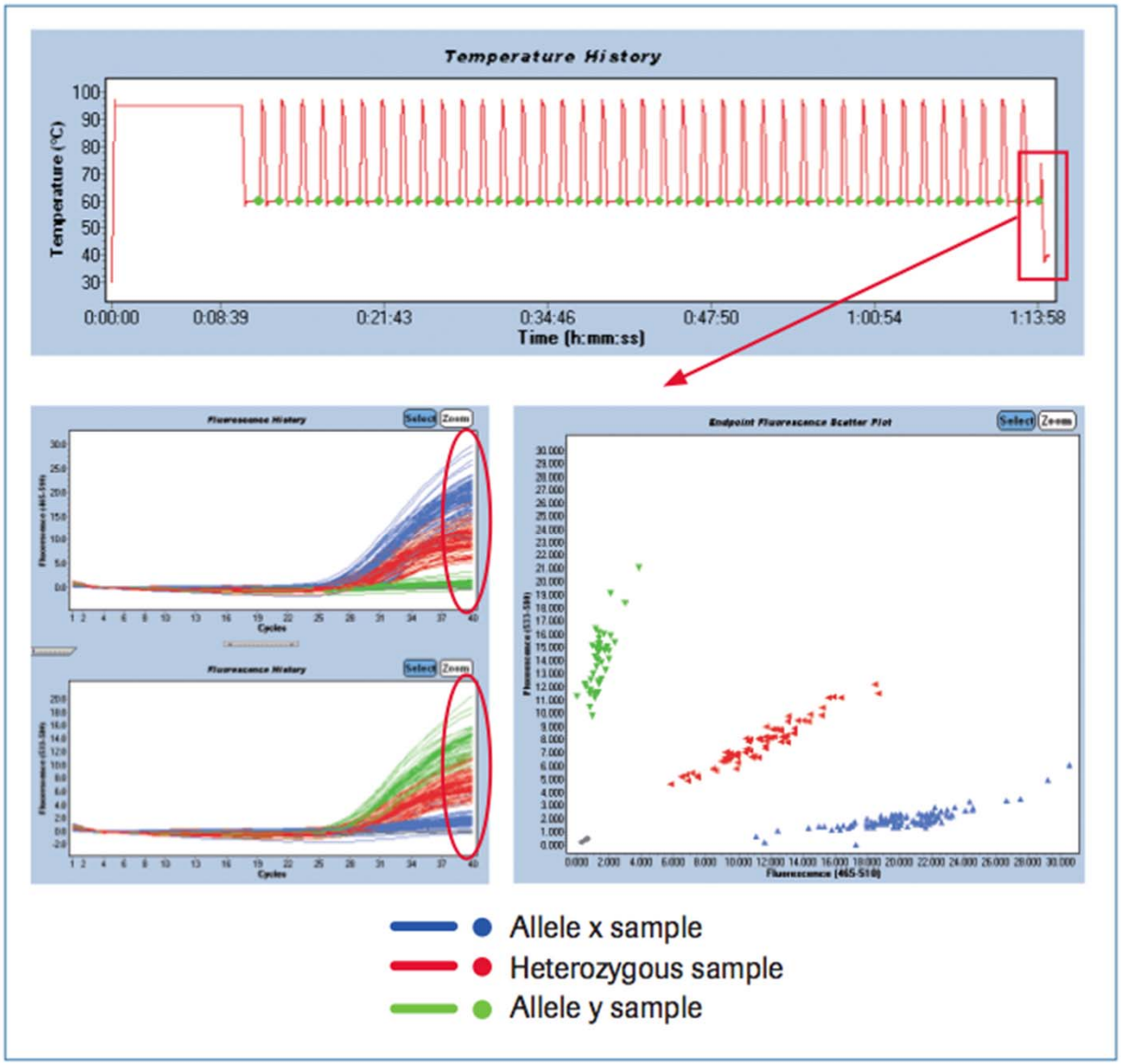
Detecting Known Variants



FAM dye detects samples that are homozygous for allele X.

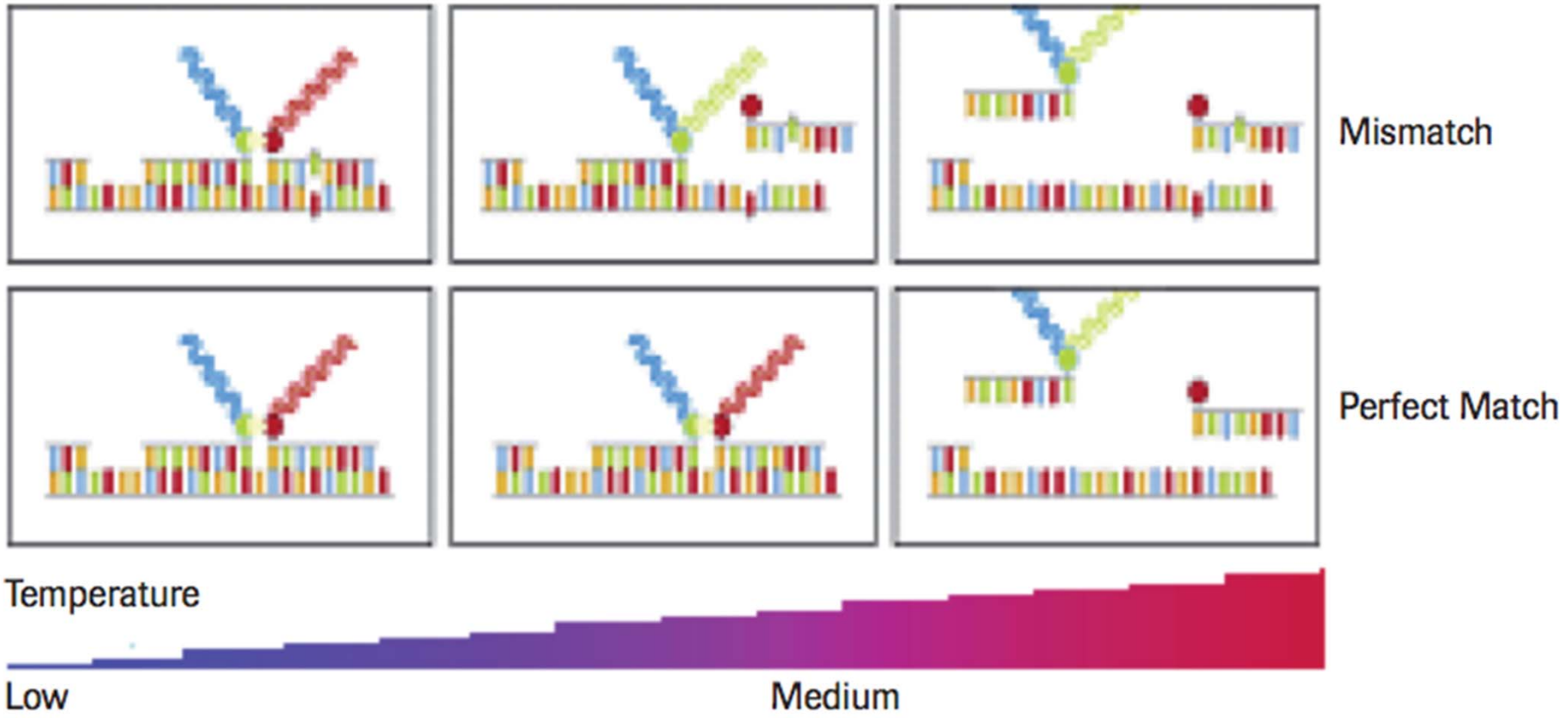
VIC/HEX dye detects samples that are homozygous for allele Y.

Endpoint genotyping is based on a dual color approach.



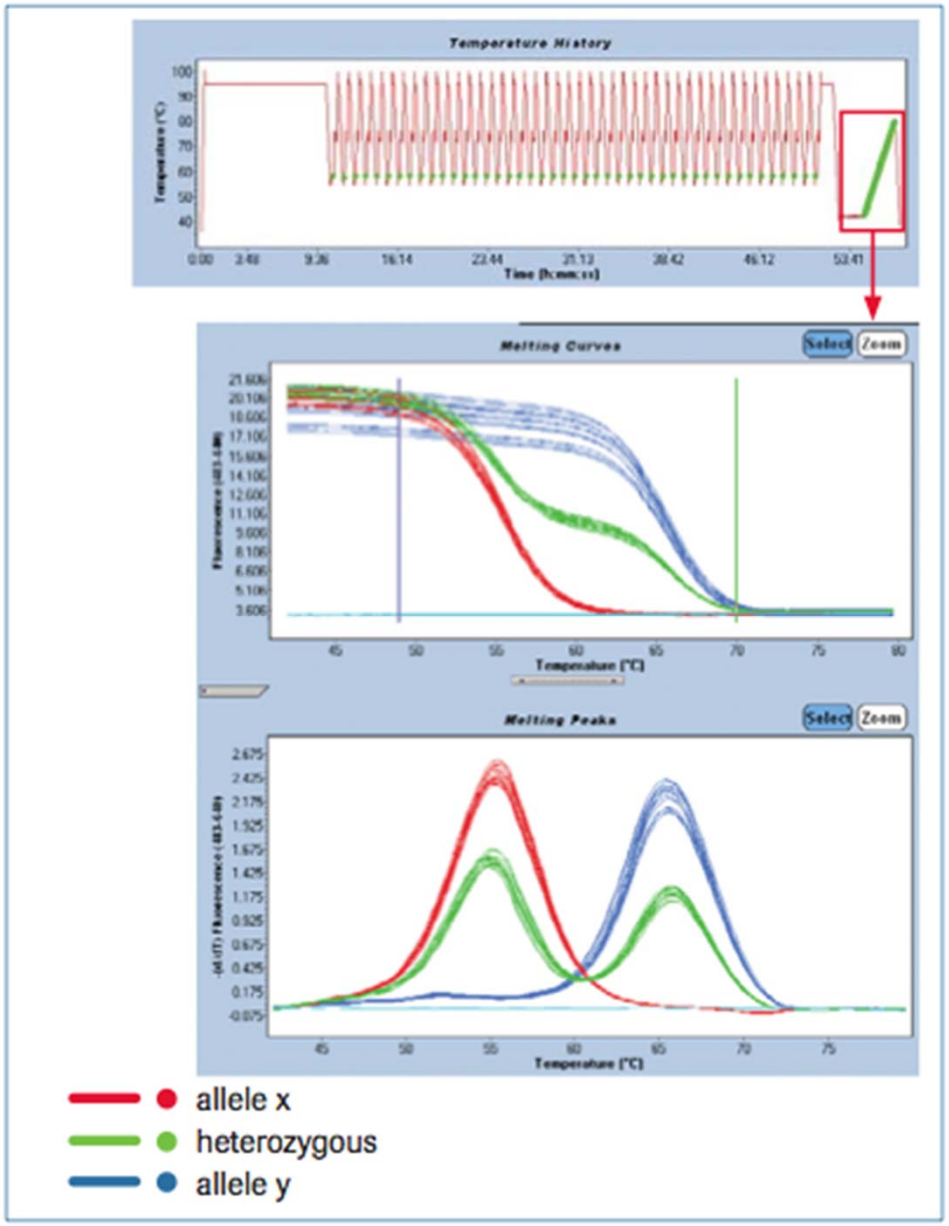
The Endpoint Genotyping module in the LightCycler® 480 software groups samples with similar intensity distributions together and identifies each group as a genotype.

Advanced Method: Melting Curve Genotyping



SNP variation is detected by binding sequence-specific anchor and and sensor probes next to each other and a signal is generated by FRET.
A single base change will lead to an earlier melting temperature of the probe-target complex. The melting temperatures (Tms) will be different for amplicons with sequence differences (SNP alleles).

Advanced Method: Melting Curve Genotyping



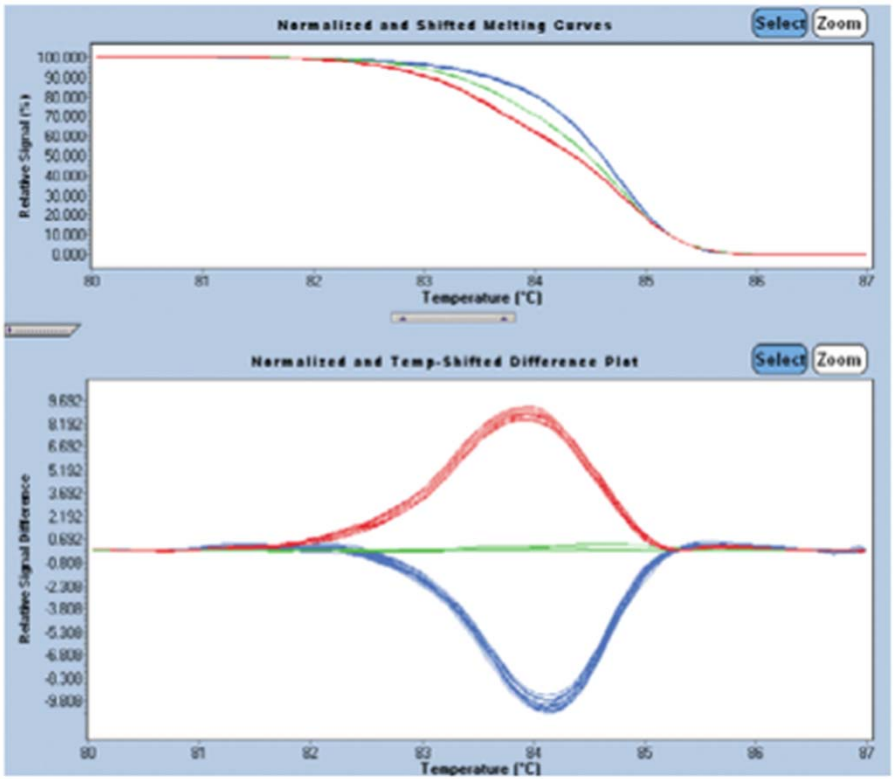
The Melting Curve Genotyping module in the LightCycler® 480 Software groups samples with similar melting profiles together and identifies each group as a genotype.

Melt Curve genotyping allows analysis of several variable sites in combination (*e.g.* haplotypes).

It requires careful design to make sure that the probe sequence covers at least one SNP, and optimization of each assay.

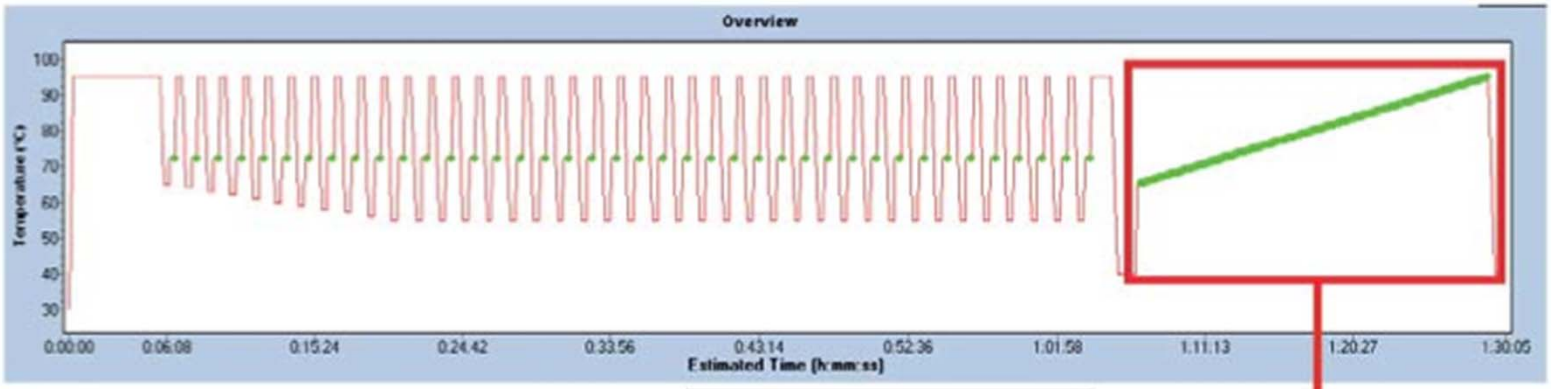
Detecting Unknown Variants--

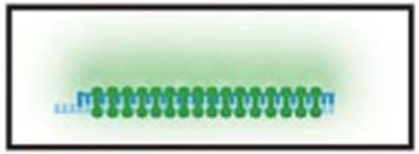
Gene Scanning by High Resolution Melting - HRM



HRM analysis with non-specific, saturating DNA dyes allows differentiation of homo- and heterozygotes.

Gene Scanning by High Resolution Melting - HRM

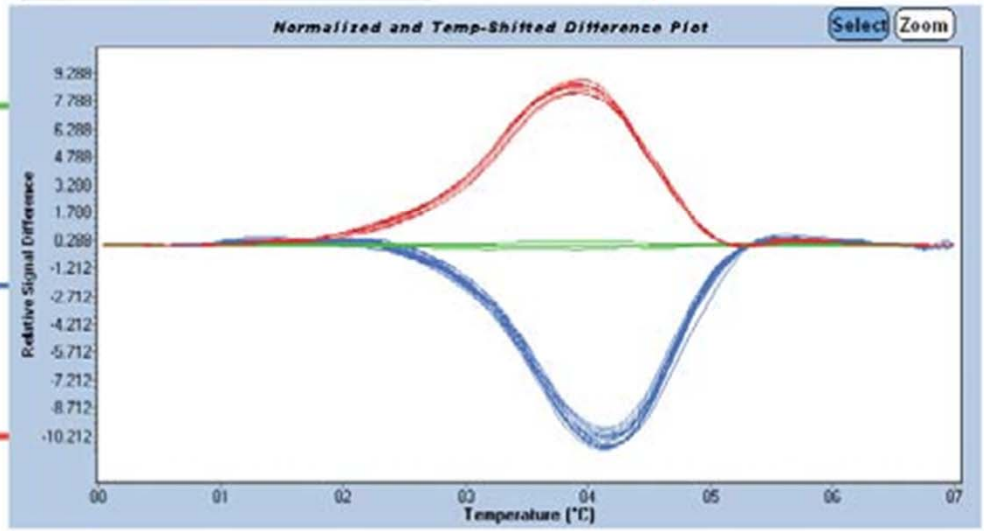




homozygous mutant (homoduplexes)

homozygous wildtype (homoduplexes)

heterozygous (homo- and heteroduplexes)



Difference Plot

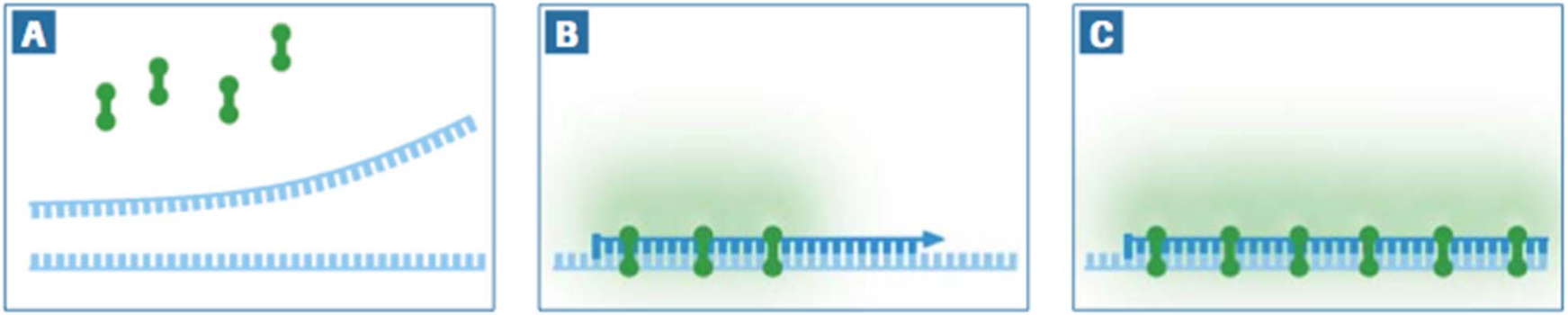
Four major types of analysis are available

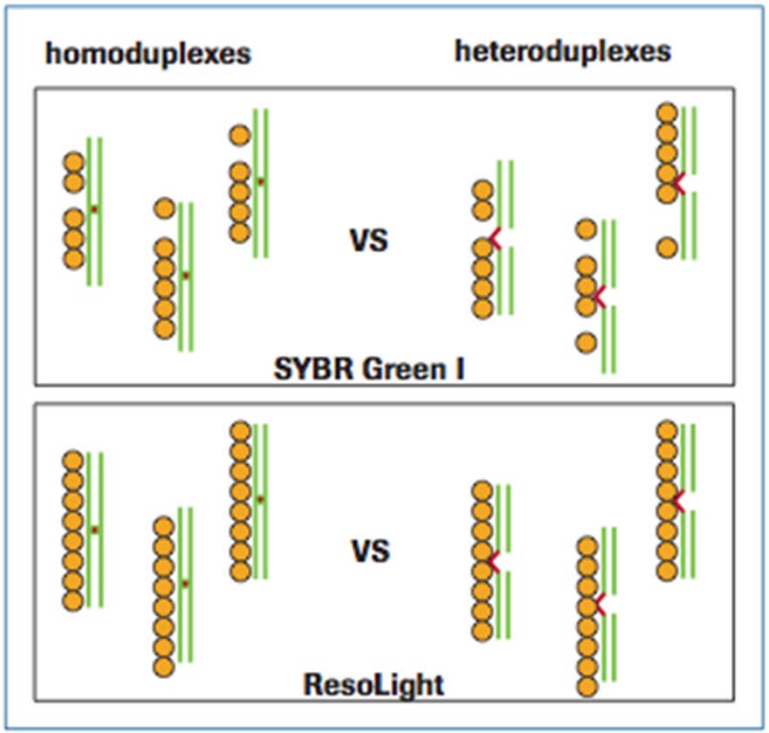
- *Summary* – available for both Absolute and Relative Quantification.
- *Trend* – displays data trends for different research samples and/or targets, *e.g.,* changes in RNA expression that depend on incubation with an active compound.
- *Genotyping* – used to display the genotypes of research samples in different analyses and to calculate the allele frequency of variants.
- *Haplotyping* - used to display and count the distribution patterns for variations on the same chromosome.

Analysis Mode	Input	Derived from LC480 SW 1.5 Analysis	Output into Excel	
AbsQuant Summary	C_p values or concentrations	AbsQuant/2nd Derivative or AbsQuant/Fit Points	overview summary analysis box plot analysis t-Test	analysis comparison criteria comparison criteria t-Test sample comparison
AbsQuant Trend	C_p values or concentrations	AbsQuant/2nd Derivative or AbsQuant/Fit Points	overview trend statistics	criteria comparison sample comparison
RelQuant Summary	ratio or normalized ratio	RelQuant Basic or Advanced	overview summary analysis box plot analysis t-Test	analysis comparison criteria comparison criteria t-Test sample comparison
RelQuant Trend	ratio or normalized ratio	RelQuant Basic or Advanced	overview trend reference analysis	statistics criteria comparison sample comparison
Genotyping	genotype	Endpoint Genotyping Melt Curve Genotyping Gene Scanning	overview results group names	genotyping Hardy-Weinberg criteria comparison
Haplotyping	genotype	Endpoint Genotyping Melt Curve Genotyping Gene Scanning	overview results group names	haplotyping criteria comparison

Detection Formats

SYBR Green

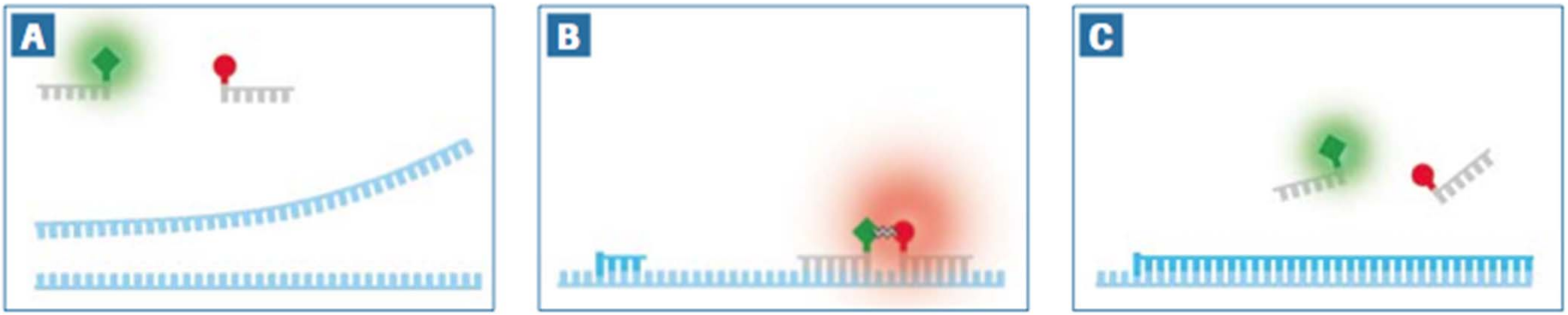




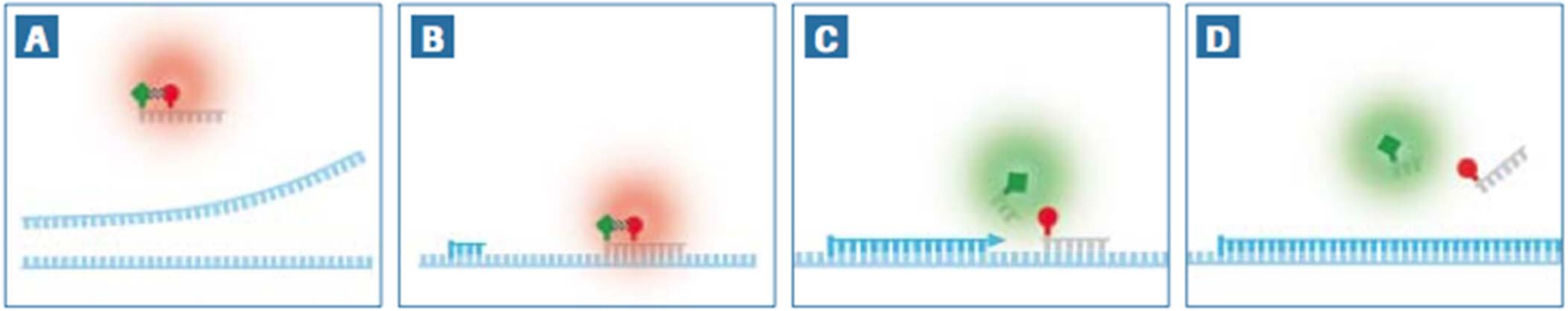
Saturating dyes for HRM, e.g. ResoLight

Detection Formats

Hybridization Probes, e.g. HybProbe

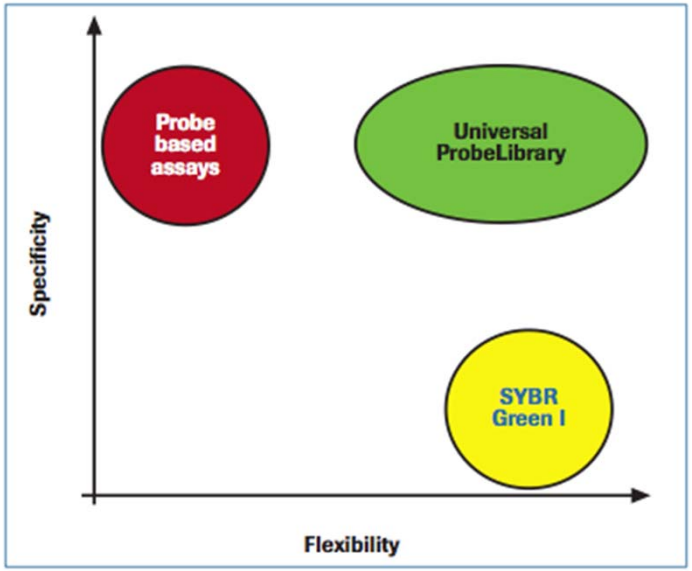


Hydrolysis Probes, e.g. TaqMan probe



Detection Formats

Universal Probe Library



These short LNA-modified probes detect a specific PCR amplicon, but also bind to more than one site in the transcriptome.

However, their combination with suitable target-specific primers results in a target- specific assay.

SimpleProbe Probes