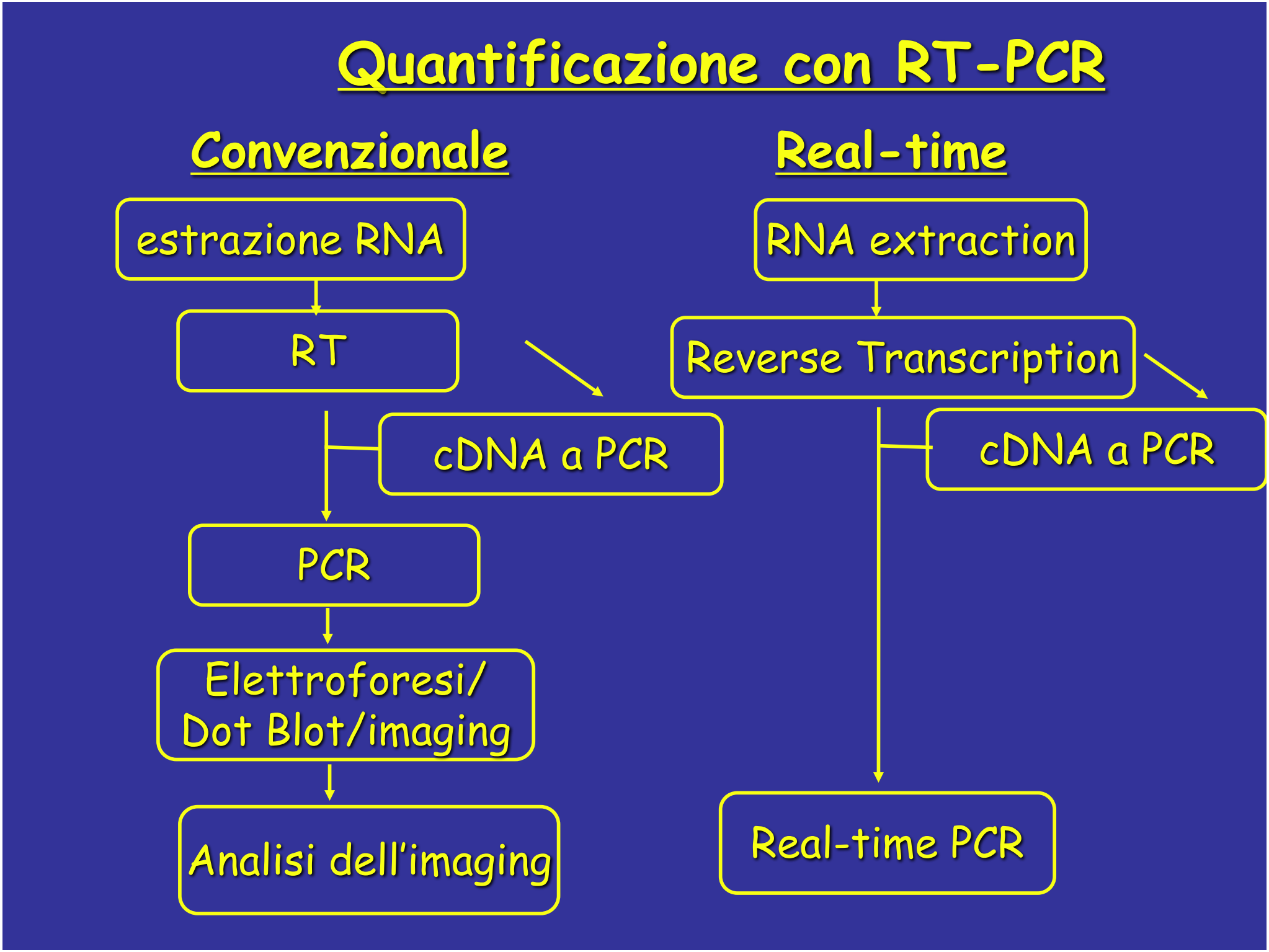
Quantitative Real-Time RT-PCR (QPCR)

Metodi in uso comune per rilevare/quantificare trascritti

- Northern blotting e ibridazione in situ
2. RNAse protection assays
3. cDNA microarrays
4. RT-PCR in end-point o real-time

Quantificazione con RT-PCR



Possibilità per un saggio RT-PCR

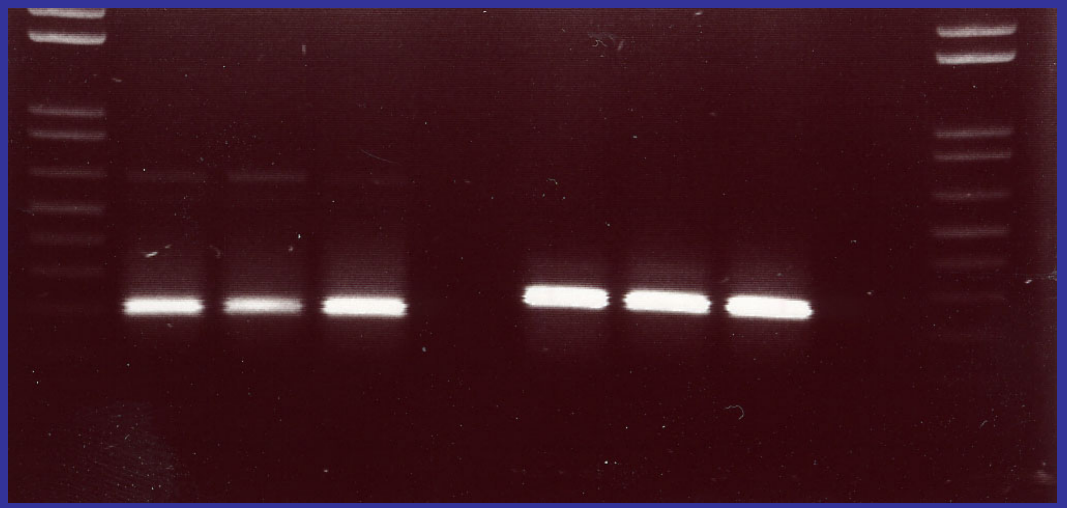
1. 1 enzyme-1 tube. *Tth* polymerase with RT activity (Roche; uses Mn^{2+})

2. 2 enzymes-1 tube. (Stratagene 1-step QRT-PCR Master Mix; Qiagen QuantiTect Probe RT-PCR Kit

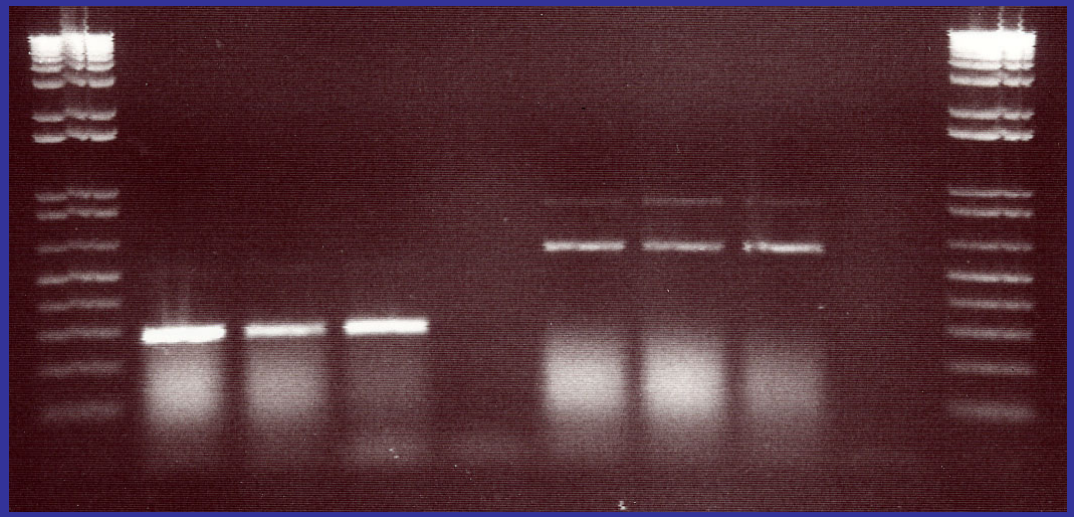
3. 2 enzymes-2 tubes. AMV-RT o MMLV-RT H^-, Taq polymerase. (Stratagene Brilliant SYBR Green QPCR Master Mix; Qiagen QuantiTect SYBR Green PCR Kit; ABI SYBR Green PCR Core Reagents

Specificity with dsDNA dyes depends on RNA preps

DNase treatment



w/o DNase



real-time PCR advantages

- Fast
- > sensitivity
- Reduced variability
- multiplexing
- No manipulation of post amplified products
- Absolute or relative quantification

QUANTIFICATION METHODS

Absolute quantification determines the **exact number** of copies generally by referring the PCR product to a standardization curve. The **relative quantification** quantifies in **relation to a control**. The relative quantitative analysis of gene expression is faster

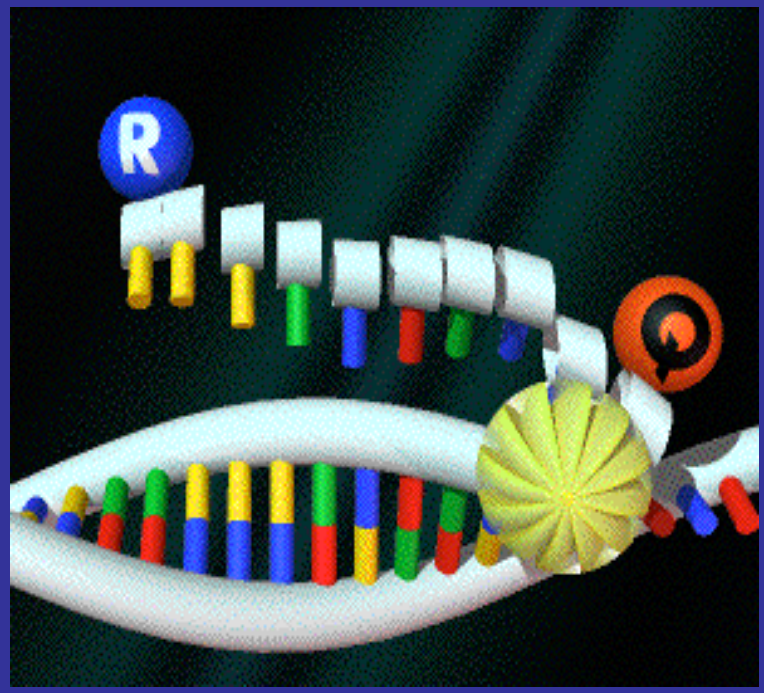
TOOLS

Thermocycler interfaced with a system capable of inducing and reading fluorescence. Fluorescence emission stimulation is induced by a laser. The detection of λ 500 to 660 nm allows multiplex PCR analysis with the use of multiple fluorophores in a single tube.

DETECTION CHEMISTRY

SYBR Green I

TaqMan



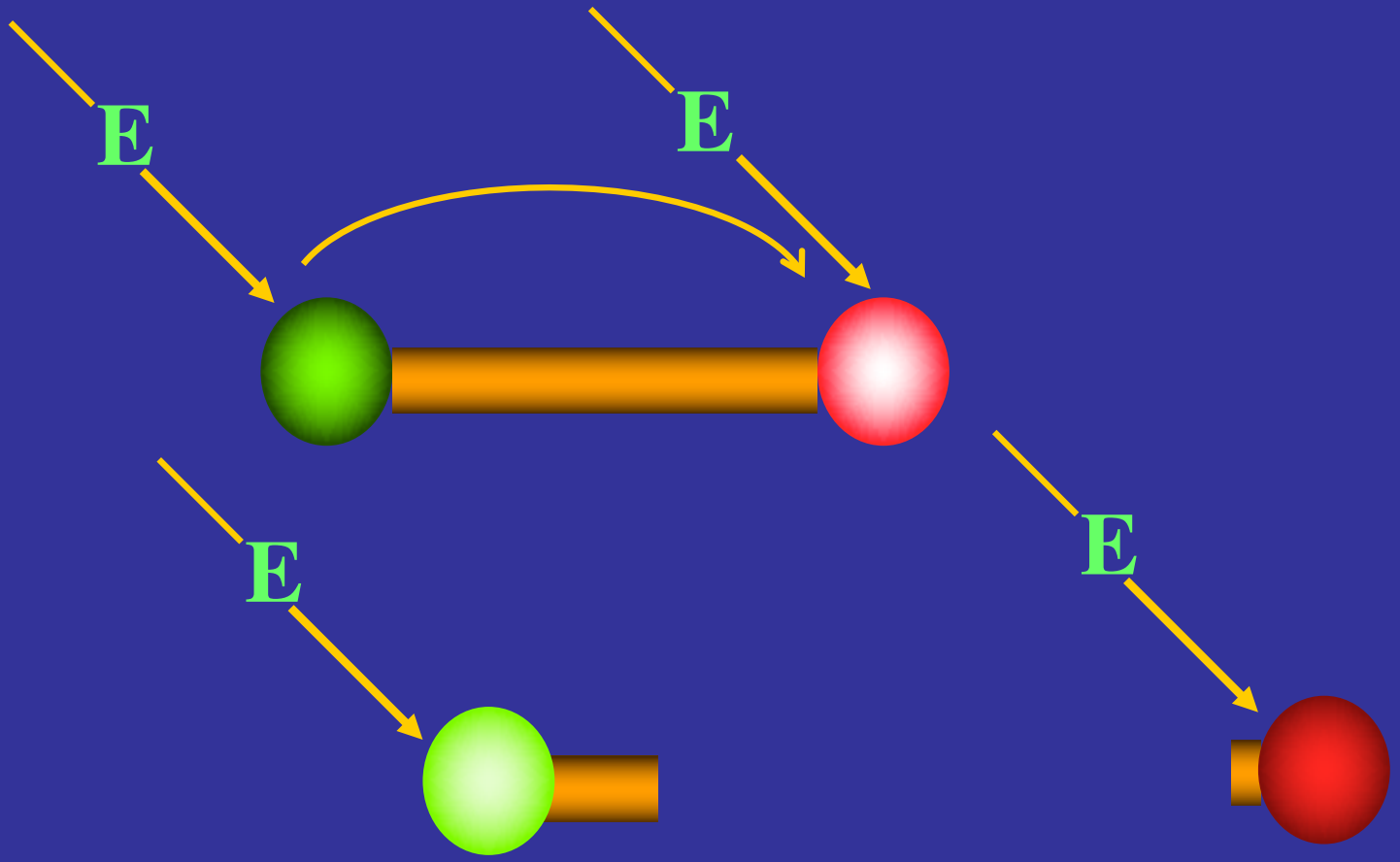
SYBR Green

it is a DNA dye that is incorporated in the double strand. It does not emit in fluorescence when it is free, but only when bound to DNA. It has the advantage of being able to be used with any primer set. However, it also reveals non-specific products. The use of the melting curve allows setting the software so that the system acquires only the fluorescence relative to that curve.

TaqMan

3 oligos for each PCR run: primer up, a primer dw and a probe, all specific for the target. The probe carries in 5 'a reporter (fluorescent dye) and in 3' a quencer (fluorescent dye). When the probe is intact the quencer absorbs the reporter's fluorescence due to their proximity. When Taq polymerase (with 5 'exonuclease activity) meets the probe, it hydrolyzes the probe dividing quencer from reporter. In this way the reporter, separated from the quencer, changes its fluorescent signal emission. During the PCR, if in the sample there is a target, the probe will reach it. Taq cuts the probe, allowing fluorescent emission. The increment in fluorescence is cycle after cycle and is a direct consequence of the amplification process.

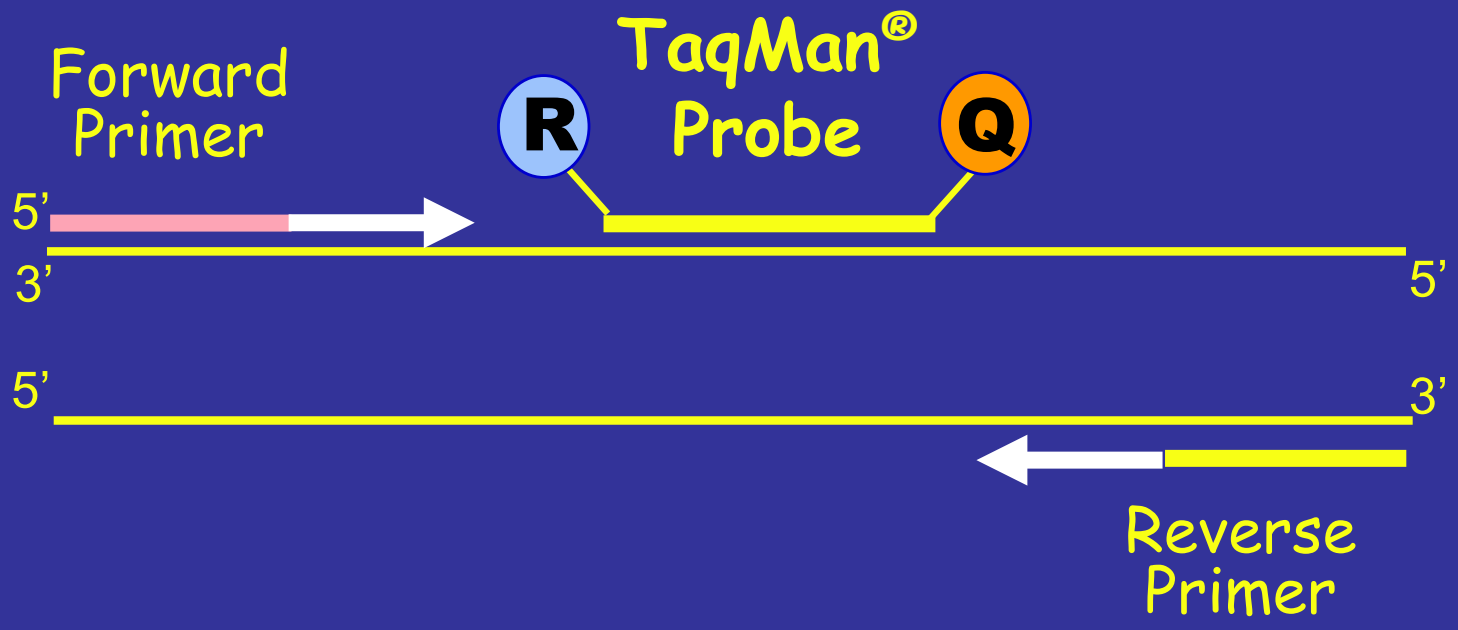
Fluorescence Resonance Energy Transfer (FRET)



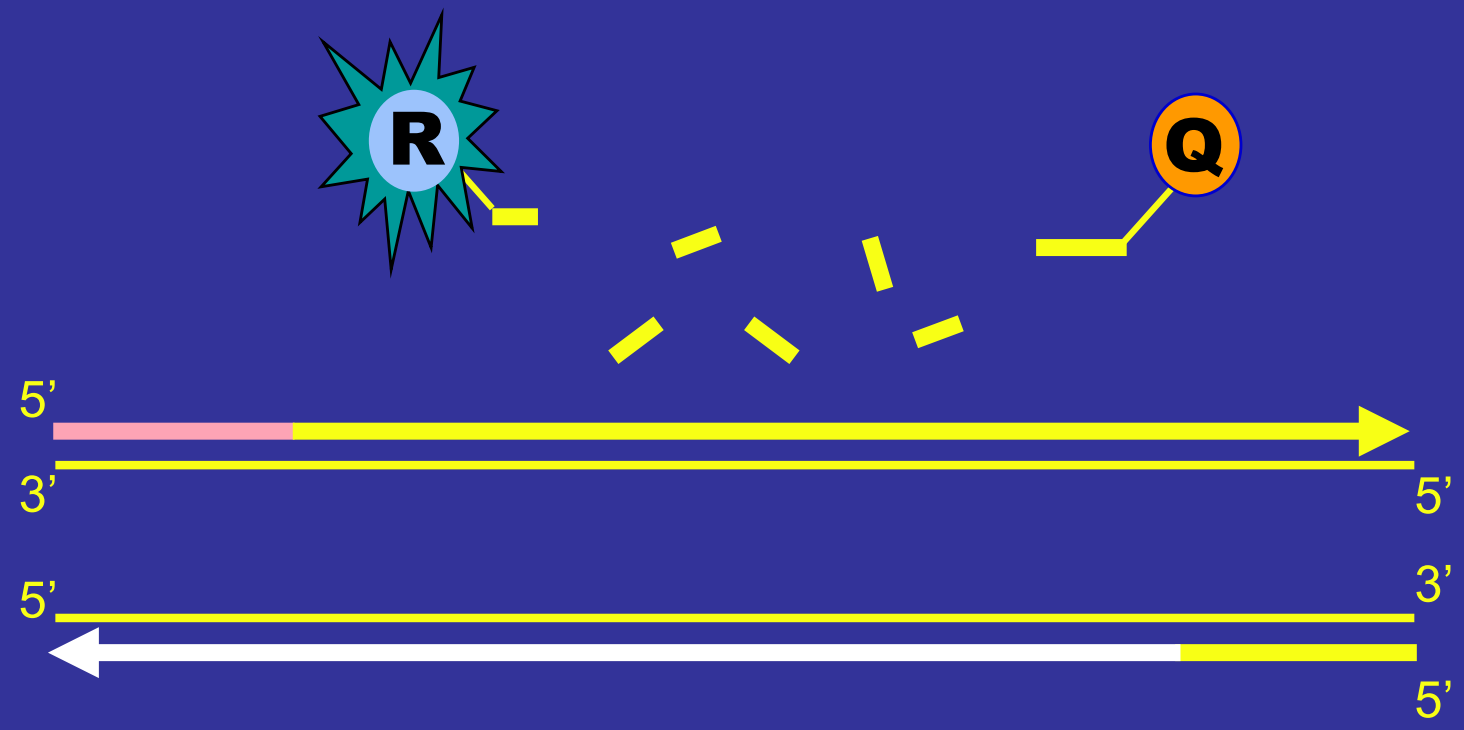
Taqman probes

Hydrolysis probes

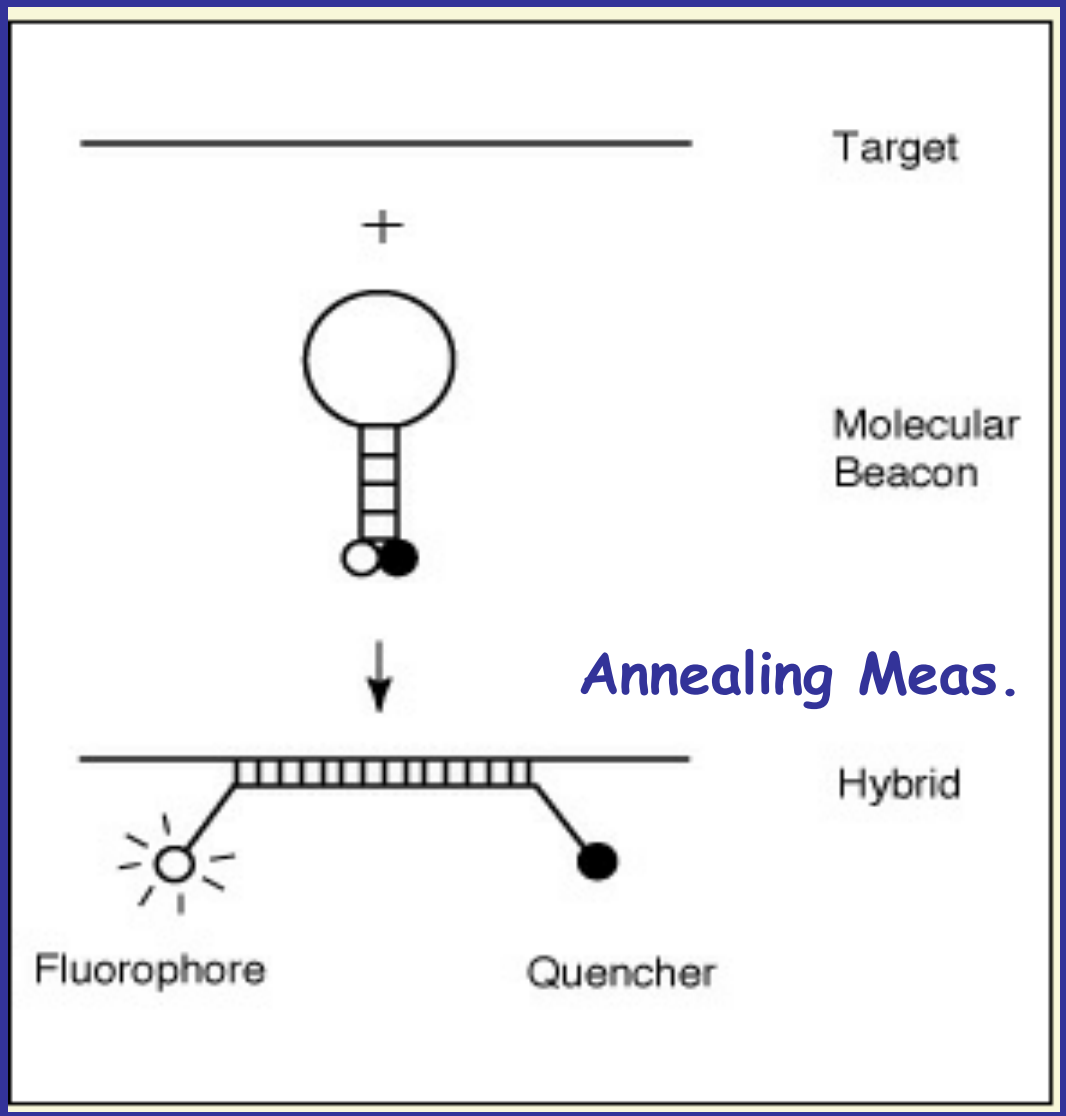
Polimerization



Mechanism



Molecular Beacons –hybridization probes



When using SYBR Green

1. Assays in which no specificity is required
2. General screening of transcripts before using specific probe
3. When the PCR is optimized: no primer dimers or non-specific amplicons from genomic DNA.

When not to use SYBR Green

multiplex reactions, detection of rare transcripts, low levels of pathogen, allelic discrimination assays (now exceptions to this rule).

Primer, Probe and Amplicon Design

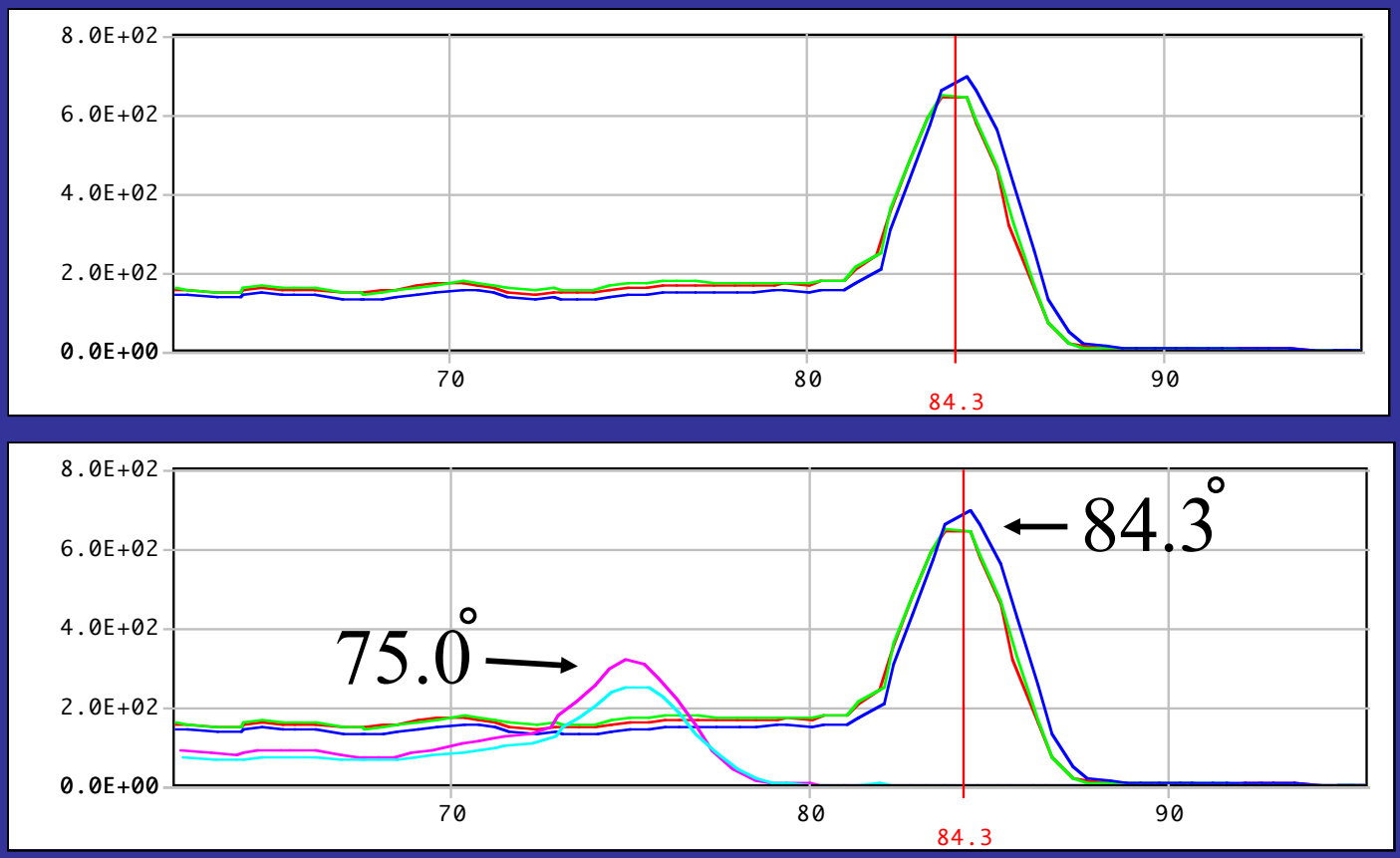
Primer Design

- Amplicon 50-150 bp; better if short- SHORT AMPLICONS ALLOW SHORT EXTENSION TIMES (AS SHORT AS 15 SEC), REDUCING POSSIBILITY OF AMPLIFYING GENOMIC DNA
- 3' less stable than (GC : Not more than 2 G and C in the last 5 bases). No complementarity in 3'.

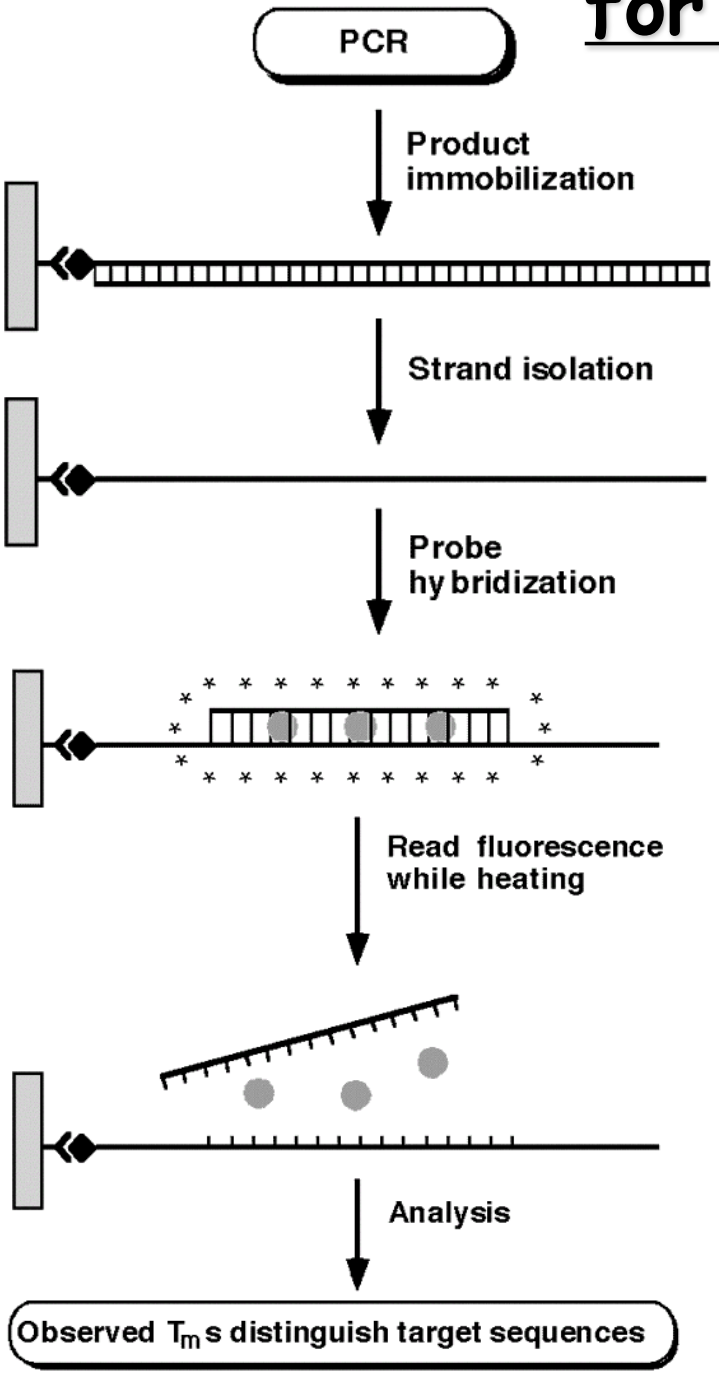
Probe Design

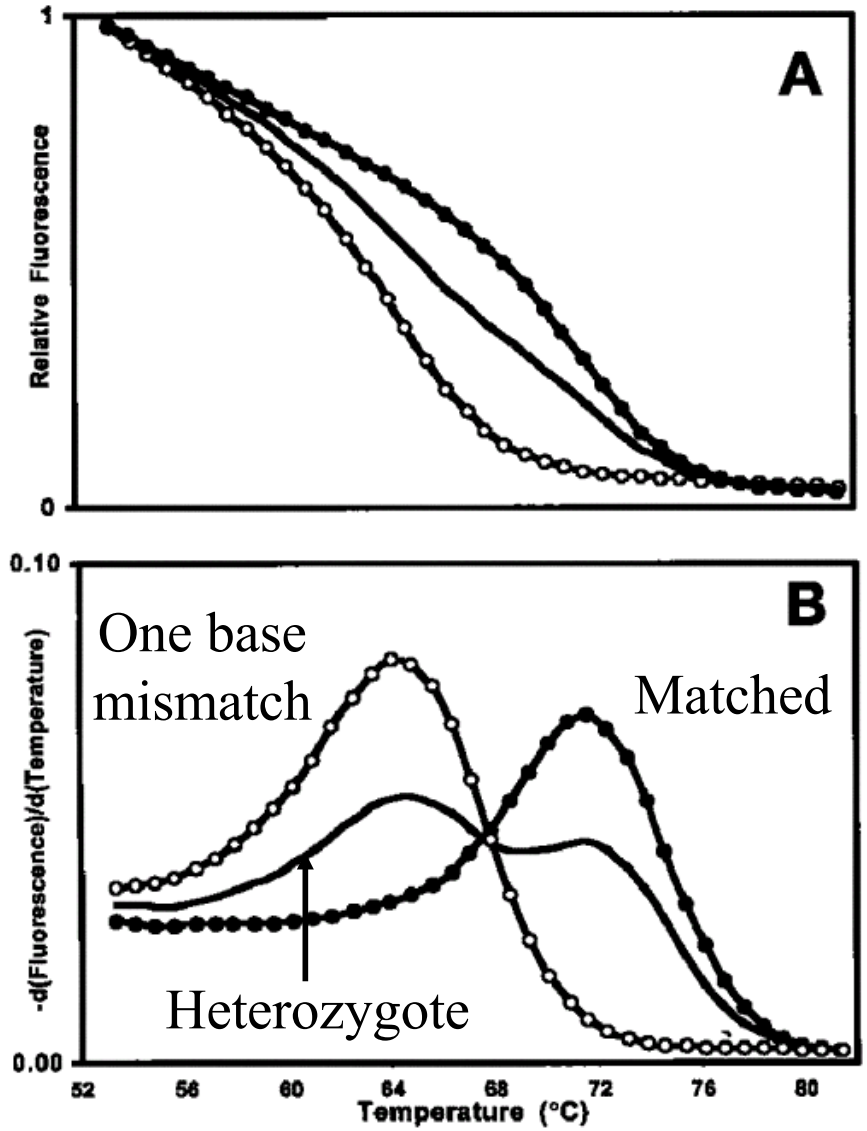
- Tm ~10° C > primers (~68-70°C)
- No G in 5' end
- amplification fragments with more C than G

Melting Curve Analysis can return reaction specificity



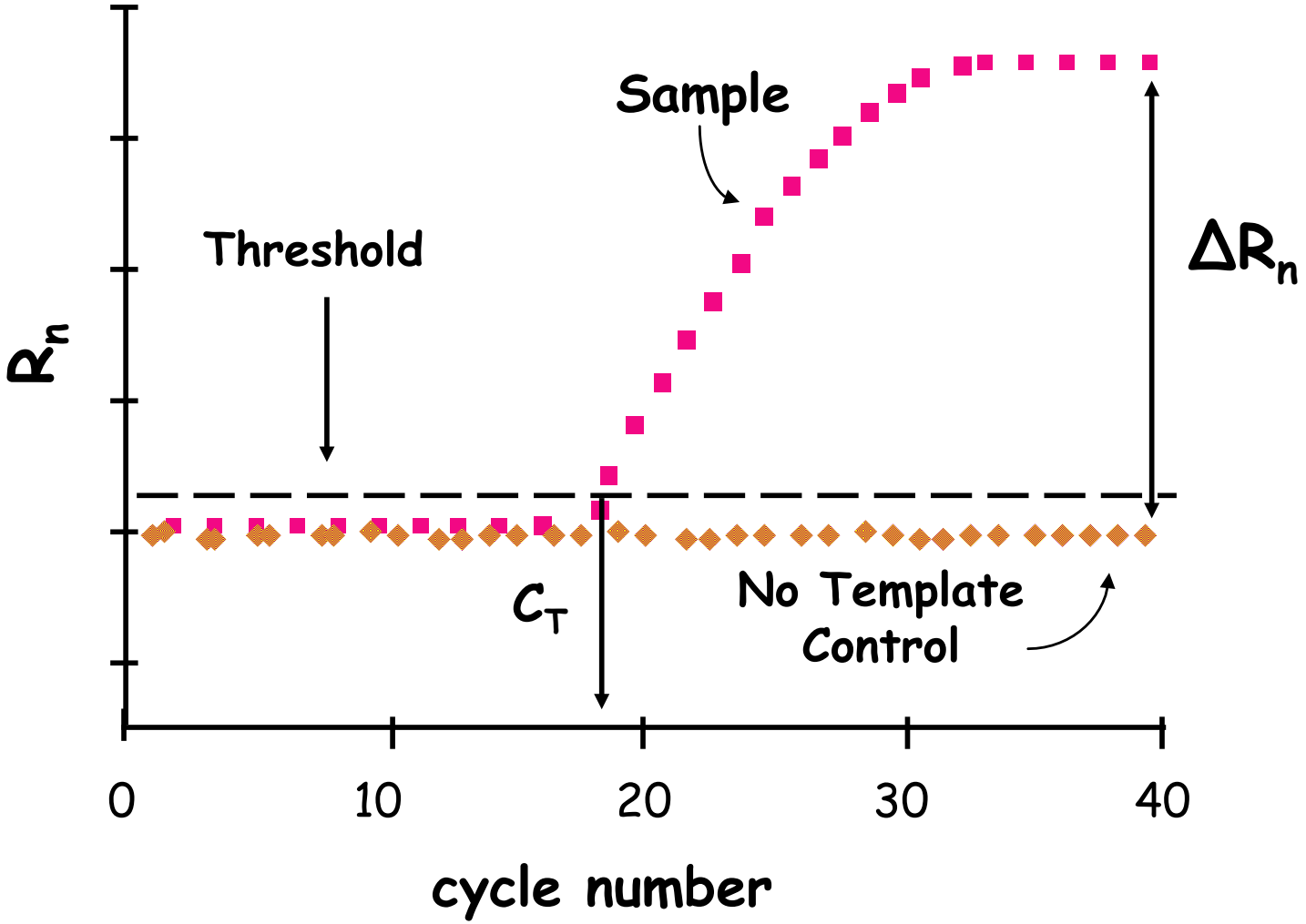
Melting Curve Analysis used in 7700 for SNP Genotyping



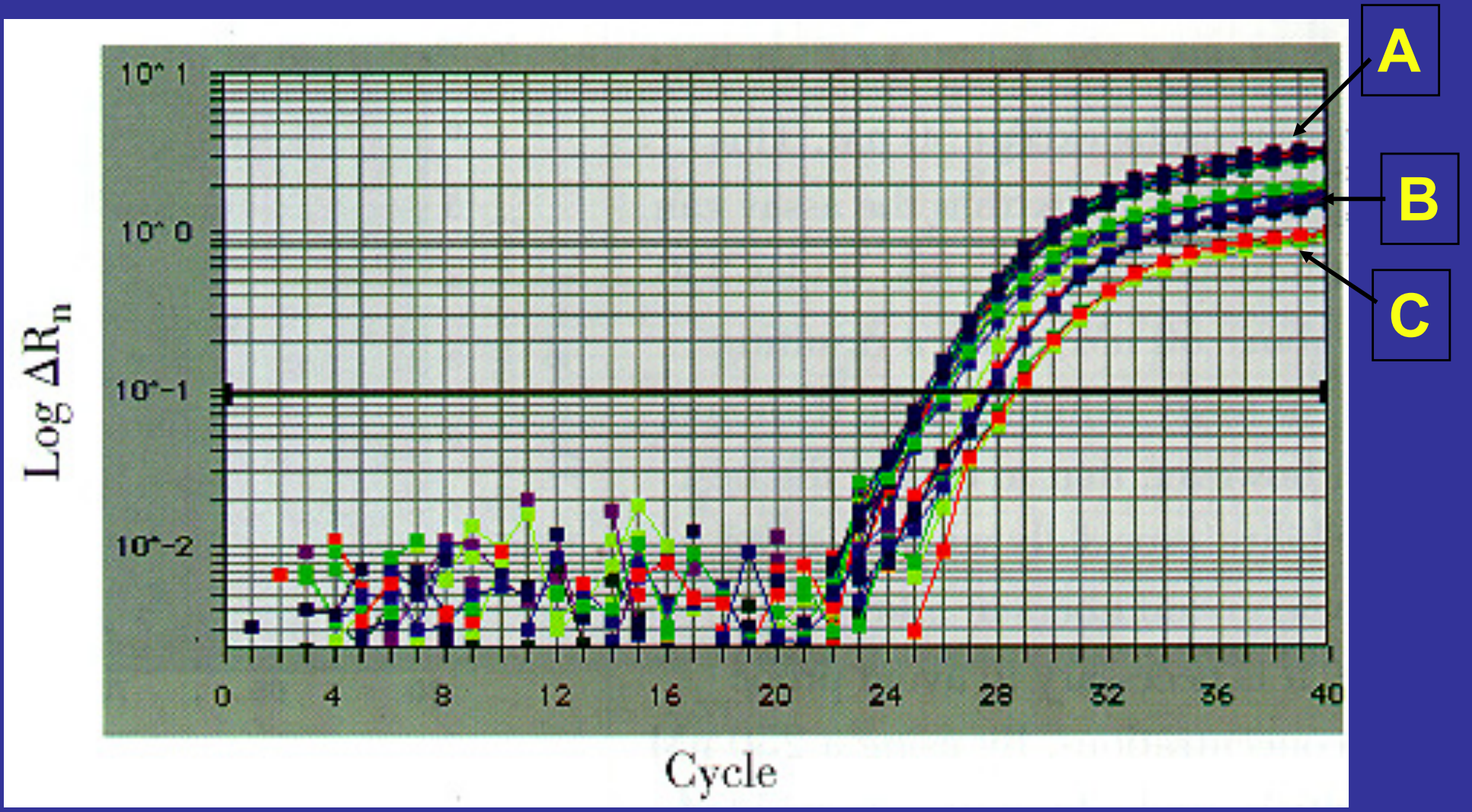


Nature Biotech. 17: 87, 1999

Amplification plot



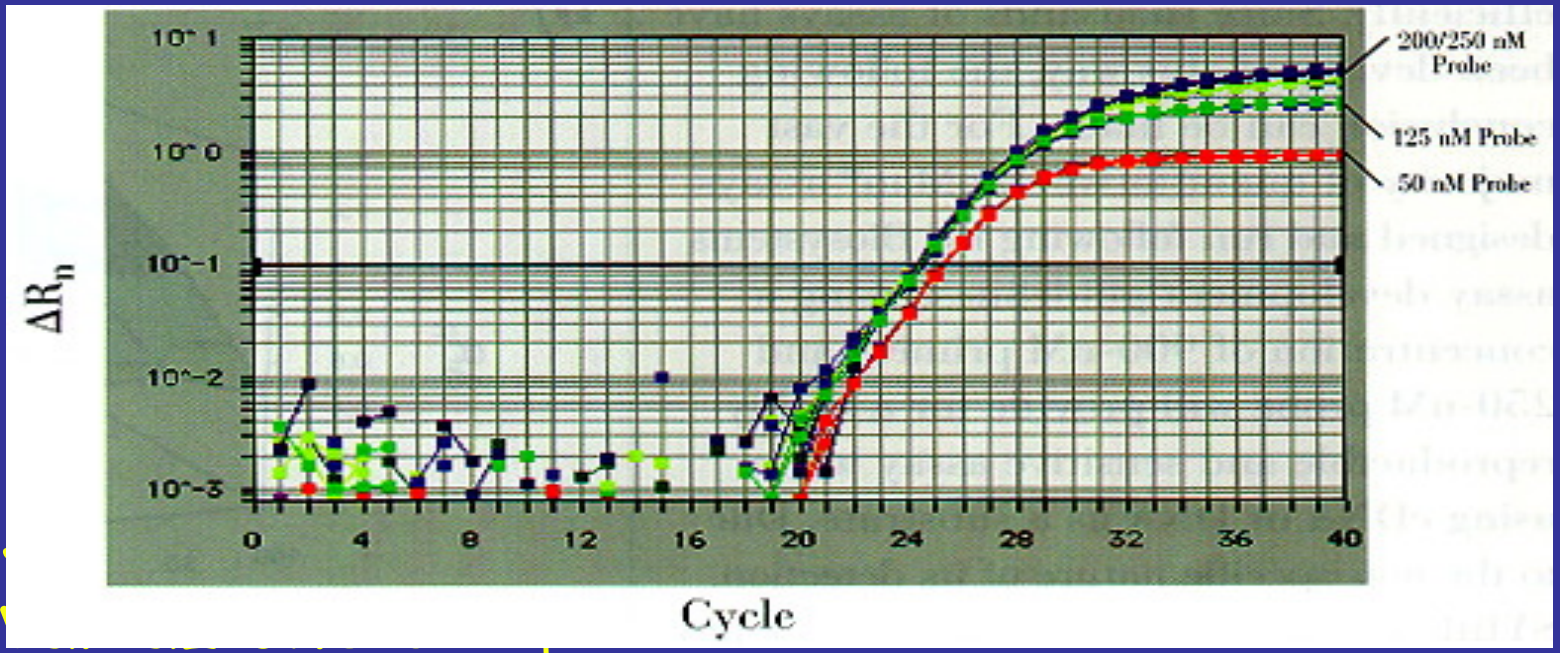
Primer Matrix



- Search for minor C_t with higher ΔR_n

Probe Titration

- Probe @ 25, 50, 75, 100, 125, 150, 175, 200, 225 & 250nM



Standard curves

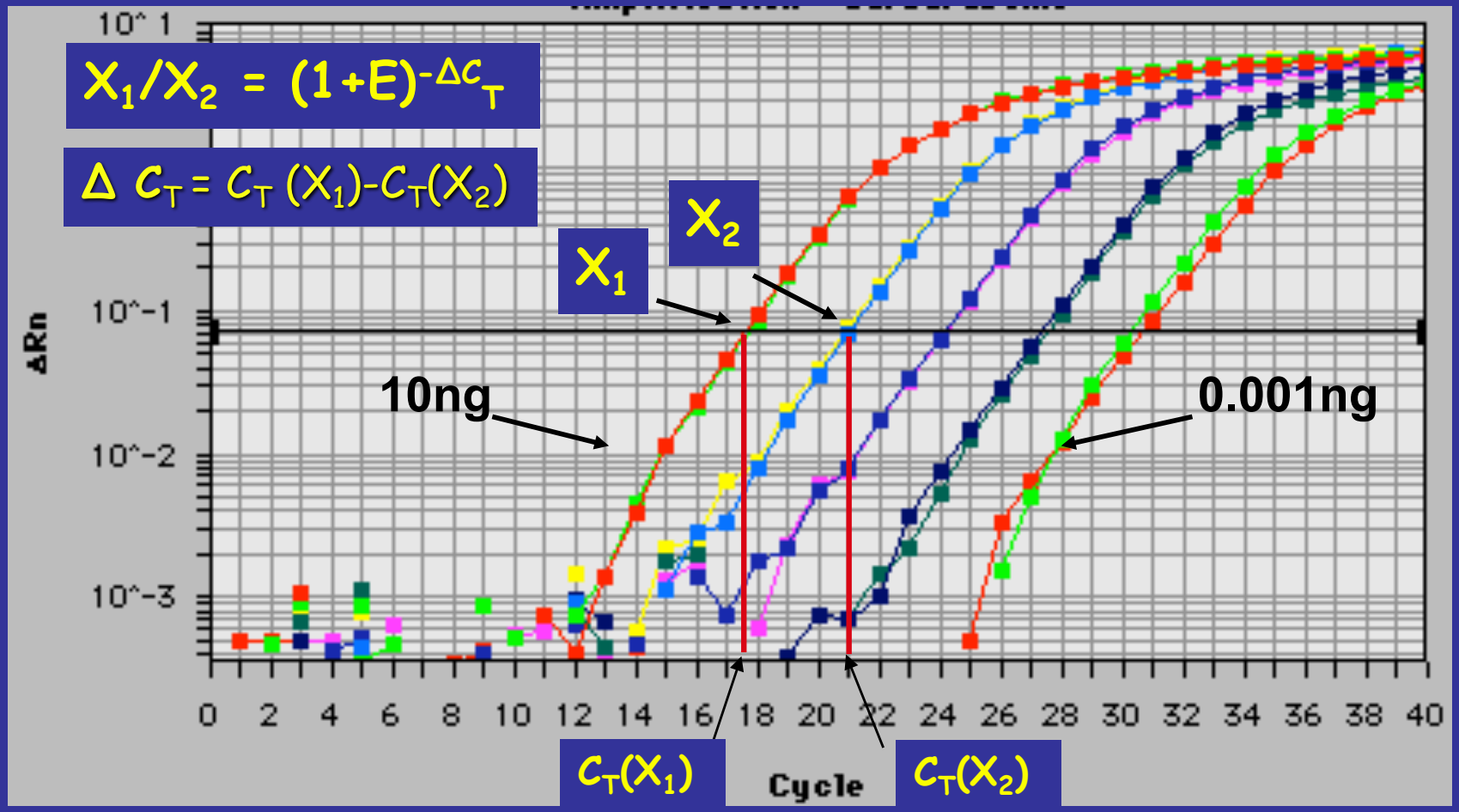
a sample with known concentration is used to construct the curve. The concentration of the standard (DNA or RNA) is evaluated by spectrophotometer at 260 nm, and converted to number of copies using the PM of the DNA or RNA (NB stability of the standards).

Relative analysis using Ct

Arithmetic forms are used to calculate the relative expression levels in relation to a calibrator which can be a control, for example. The amount of normalized target on the level of expression housekeeping gene and related to a control is given by : $2^{exp(-\Delta\Delta C_T)}$ where $\Delta\Delta C_T = \Delta C_T$ sample- ΔC_T calibrator and ΔC_T is tareget C_T - housekeeping Ct. The equation thus represents the normalized expression of the target gene in the unknown sample relative to the normalized expression of the calibrator. The method is applicable if the PCR efficiency for the target gene is similar to that of housekeeping. For each target efficincy must be evaluated by considering how the CT sample and the CT calibrator vary with the dilution of the template.

Comparative C_T Method: $\Delta\Delta$ C_T

Range dinamico del saggio



- Template titration; 10, 1, 0.1, 0.01, 0.001 ng

Effetto dell'efficienza di amplificazione

$$X_n = X_0(1+E)^n$$

Case 1: E = 0.9

$X_n = 100\ (1+0.9)^{30}$

$X_n = 2.3 \times 10^{10}$

Case 2: E = 0.8

$X_n = 100\ (1+0.8)^{30}$

$X_n = 4.6 \times 10^{9}$

Result: a difference of 0.1 in the amplification efficiency generates a 5-fold difference in the final ratio of PCR products after 30 cycles.

Comparative C_T Method: $\Delta\Delta$ C_T

C_T (Target gene, control) – C_T (Endog. refer. gene, control) = $\Delta C_{T,cont}$ (Control tissue)

C_T (Target gene, exp.) – C_T (Endog. refer. gene, exp.) = $\Delta C_{T,exp}$ (Experimental tissue)

$$\Delta C_{T,cont} - \Delta C_{T,\,exp} = \Delta\Delta\, C_T$$

$$\frac{\text{Target gene exp}}{\text{Target gene calib}} = 2^{-(\text{ave. } \Delta\Delta\, C_T)}$$

Meglio……..

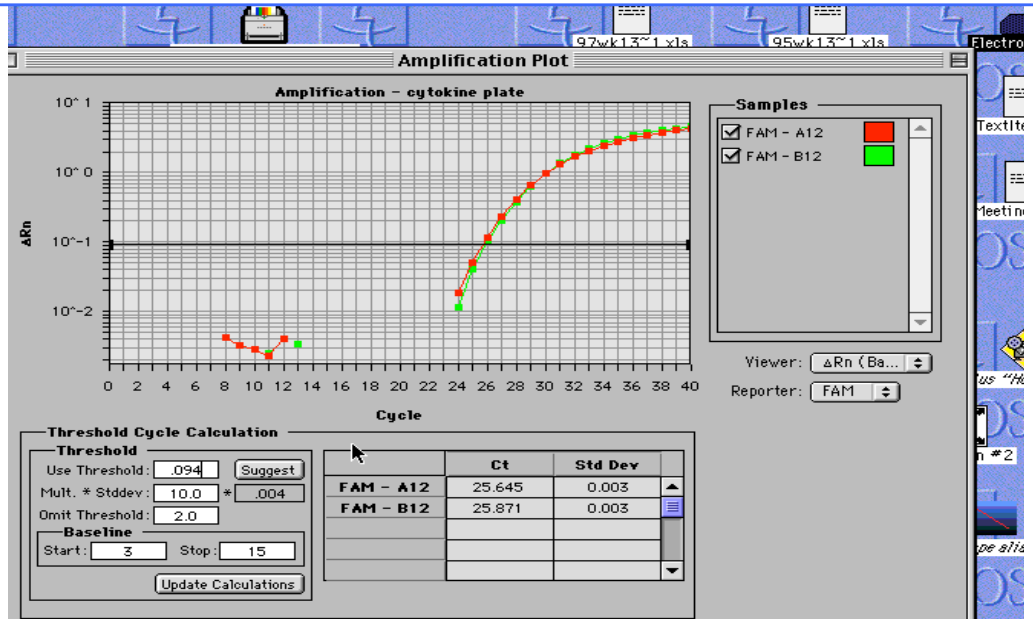
$$\text{Ratio} = \frac{(E_{\text{tareget gene}})^{\Delta Ct \text{ target gene}}}{(E_{\text{reference}})^{\Delta Ct \text{ reference}}}$$

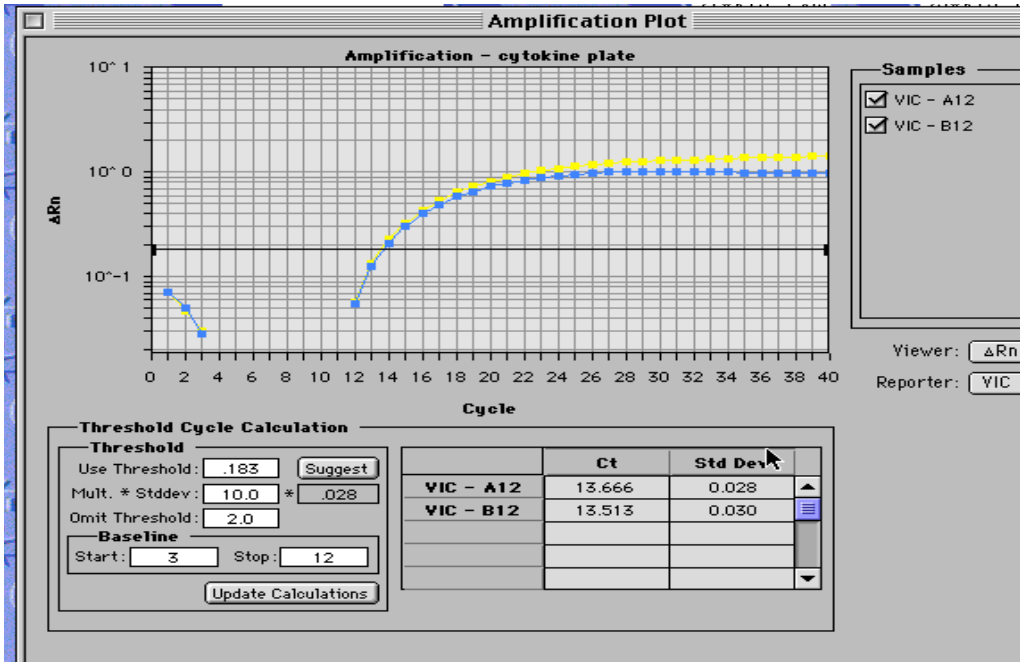
Dove:

$E = 10^{-1/\text{slope}}$

$\Delta Ct_{\text{target gene}} = (Ct \text{ control} - Ct \text{ sample})_{\text{target gene}}$

$\Delta Ct_{\text{ref gene}} = (Ct \text{ control} - Ct \text{ sample})_{\text{ref gene}}$





Target RNA

TNFα in Control tissue

C_Ts - 25.645 - A12

25.971 - B12

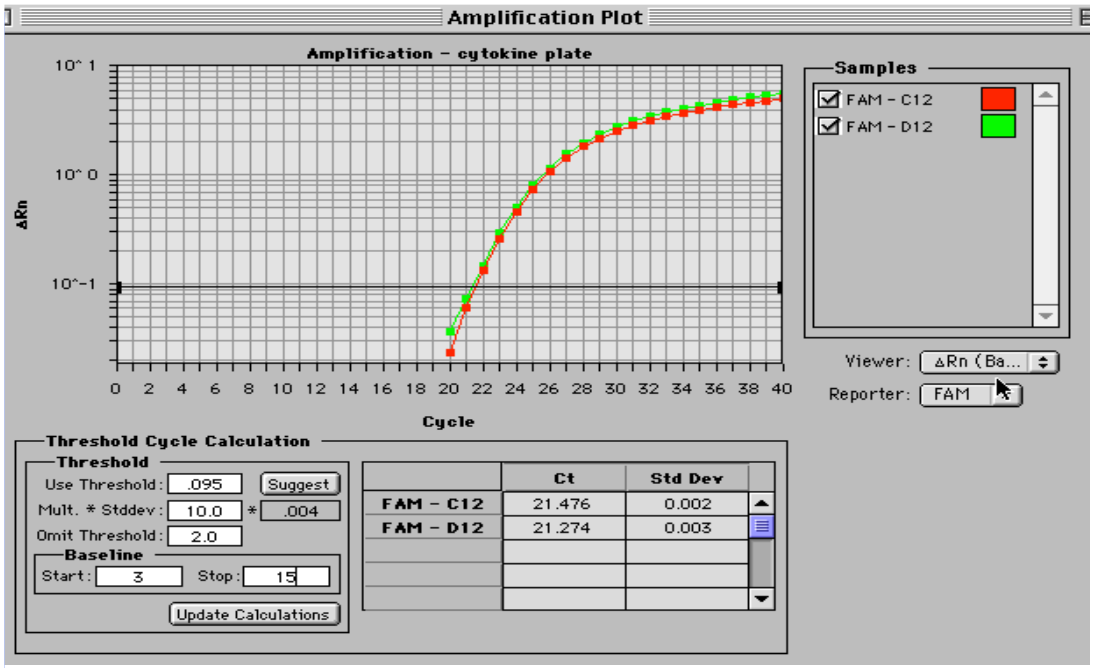
Endogenous reference

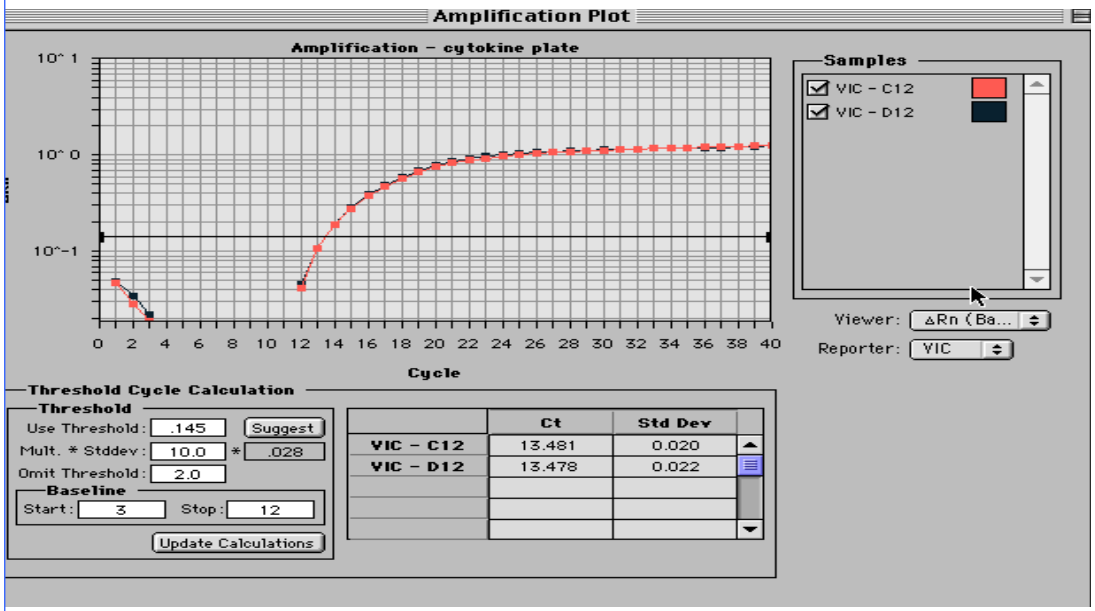
18s rRNA in Control tissue

C_Ts -13.666 - A12

13.513 - B12

Ave ΔC_T Control = C_T(target)-C_T(end.ref.) = 12.218





Target RNA

TNF-α Experimental tissue

C_Ts = 21.476 - C12

21.274 - D12

Endogenous reference RNA

18s rRNA Experimental tissue

C_Ts = 13.481 - C12

13.478 - D12

Ave. ΔC_T(Exper.) = C_T (target) - C_T (end.ref.) = 7.89

Quantificazione relativa con $\Delta\Delta C_T$

ΔC_T (Control) = 12.218

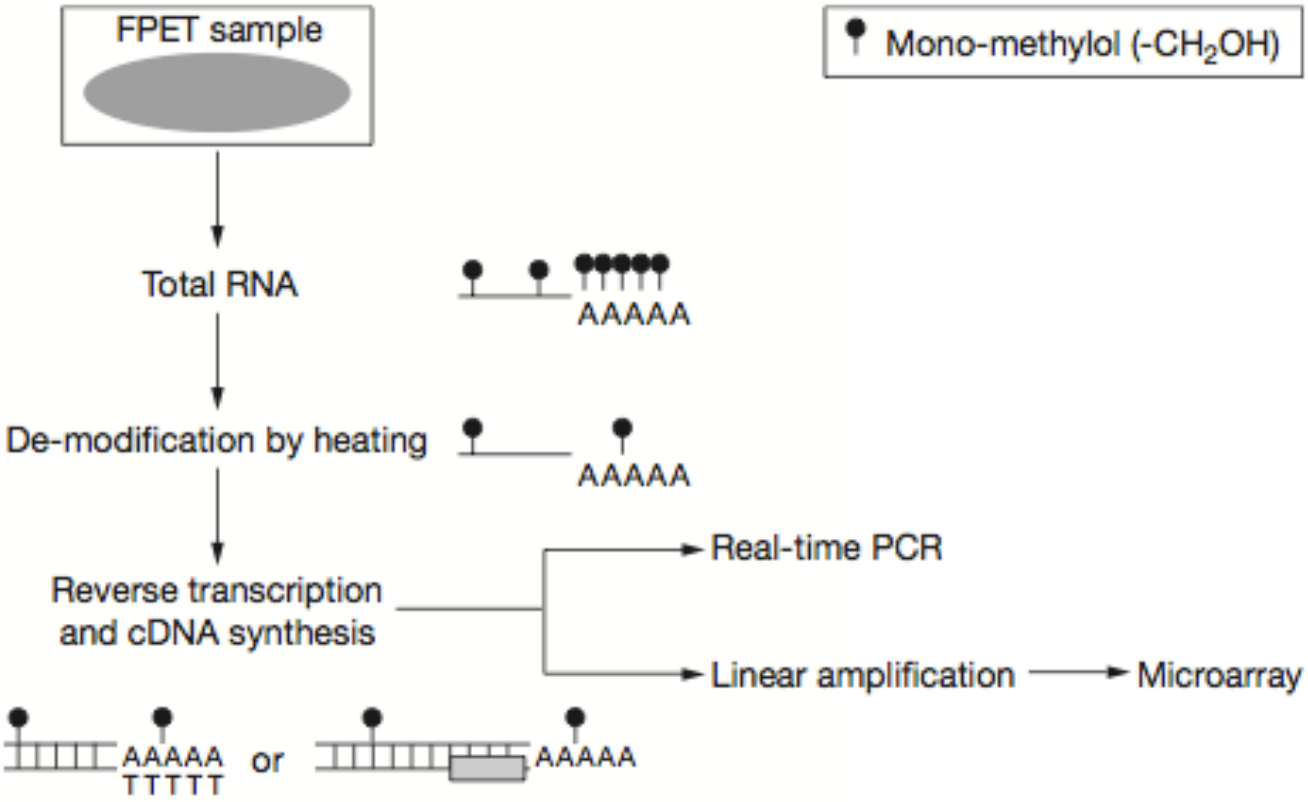
ΔC_T (experimetal) = 7.895

$\Delta\Delta C_T = \Delta C_T$(Cont.) - Δ exp.)= 4.323 $\Delta\Delta C_T = \log_2(\text{Target}_{exp}/\text{Target}_{cont})$

Relative expression of TNF-α in experimental sample in comparison to control sample

$= 2^{\Delta\Delta C_T} = 2^{4.323}$ = 20-fold increase

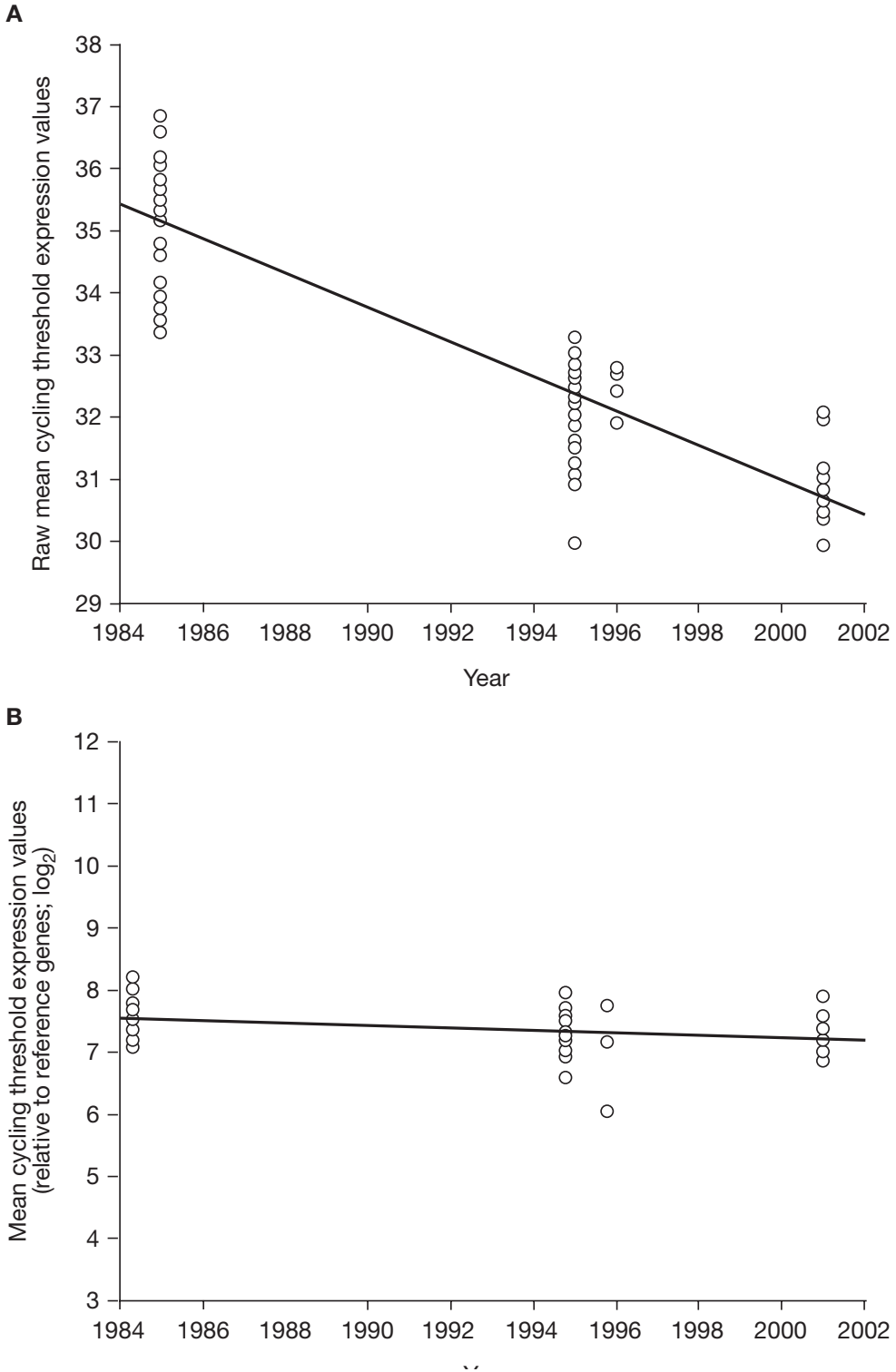
GENERAL STEPS INVOLVED IN GENE EXPRESSION PROFILING OF FFPE TISSUE



Paik S Nature Clinical Practice Oncol 2005

RELATIVE QUANTIFICATION

- ✓ Absolute quantification is not possible in FFPE specimens because the level of degradation varies among different samples.
- ✓ Relative quantification against the expression level of one or more housekeeping genes is the most popular method.



Which reference gene?

Gene-expression levels from qRT-PCR requires normalization of target genes to reference genes to remove degradation effect.

Reference genes are ideally constitutively expressed in every cell, but many genes used for normalization has been shown to vary with tissue type, cellular proliferation, cancer progression, and degradation of nucleic acids.

Genes	Protein Name	Cellular Function
ACTB	Actin, β	Major constituent of the contractile apparatus and cytoskeleton
B2M	β-2-microglobulin	Association with major histocompatibility complex (MHC) class I heavy chain
CALM2	Calmodulin 2 (phosphorylase kinase, Δ)	Phosporylase kinase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Carbohydrate metabolism
GUSB	Glucuronidase, β	Degradation of glycosaminoglycans
HMBS	Hydroxymethylbilane synthase	Catalyzation of part of the heme biosynthetic pathway
IPO8	Importin 8	Nuclear import of proteins
MRPL19	Mitochondrial ribosomal protein L19	Protein synthesis within the mitochondrion
OAZ1	Ornithine decarboxylase antizyme 1	Regulation of polyamine synthesis
PSMC4	Proteasome (prosome, macropain) 26S subunit, ATPase, 4	Multicatalytic proteinase complex
PUM1	Pumilio homolog 1 (Drosophila)	Translational regulation of embryogenesis, cell development and differentiation
RPL37A	Ribosomal protein L37a	Component of the 60S subunit of the ribosomes that catalyze protein synthesis
RPLP0	Ribosomal protein, large, P0	Component of the 60S subunit of the ribosomes that catalyze protein synthesis
SF3A1	Splicing factor 3a, subunit 1, 120 kDa	Belongs to the SURP protein family that are thought to mediate RNA binding
TBP	TATA box binding protein	General transcription factor
TFRC	Transferrin receptor (p90, CD71)	Carrier protein for transferrin

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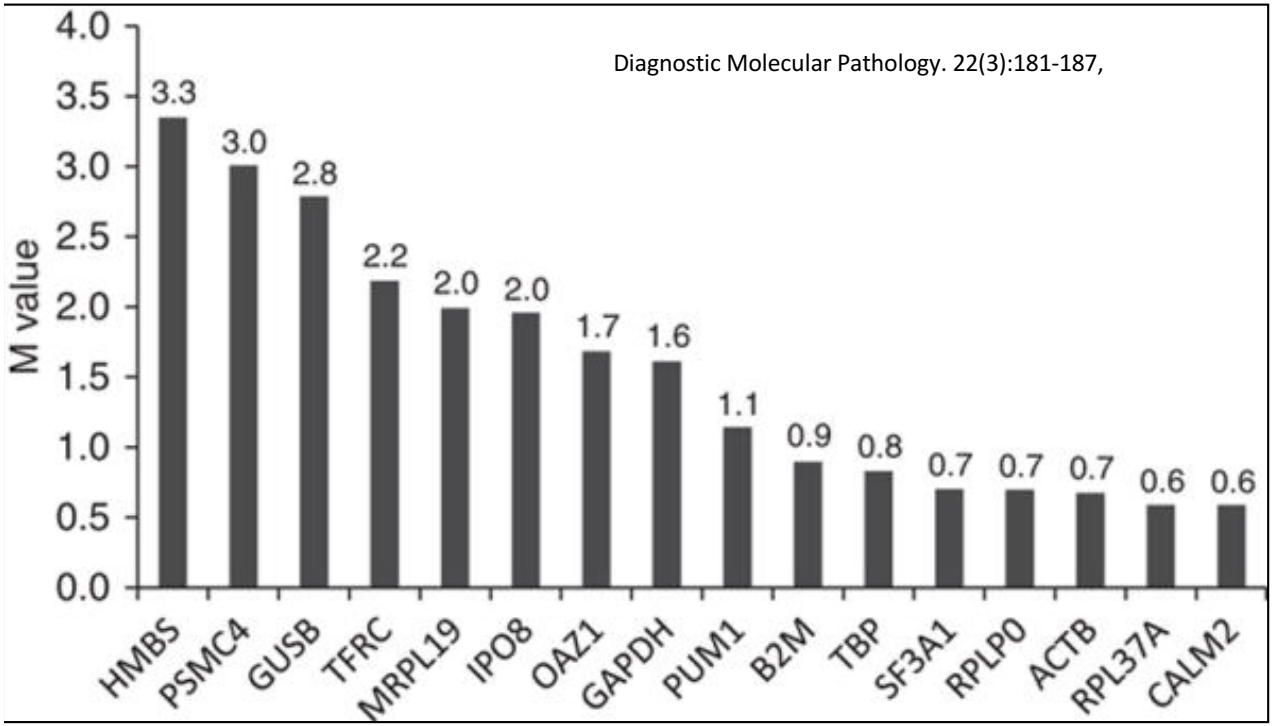
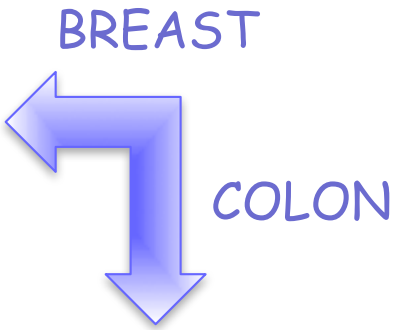
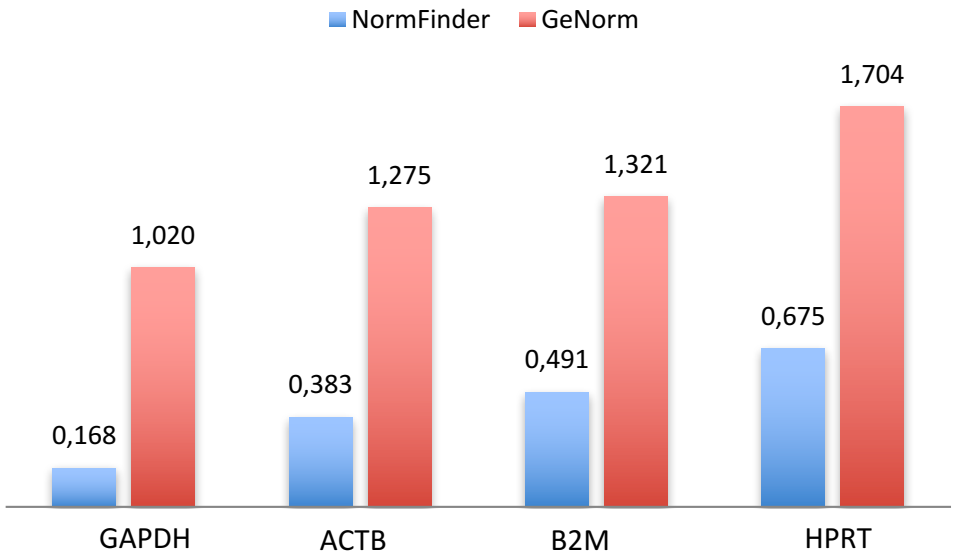
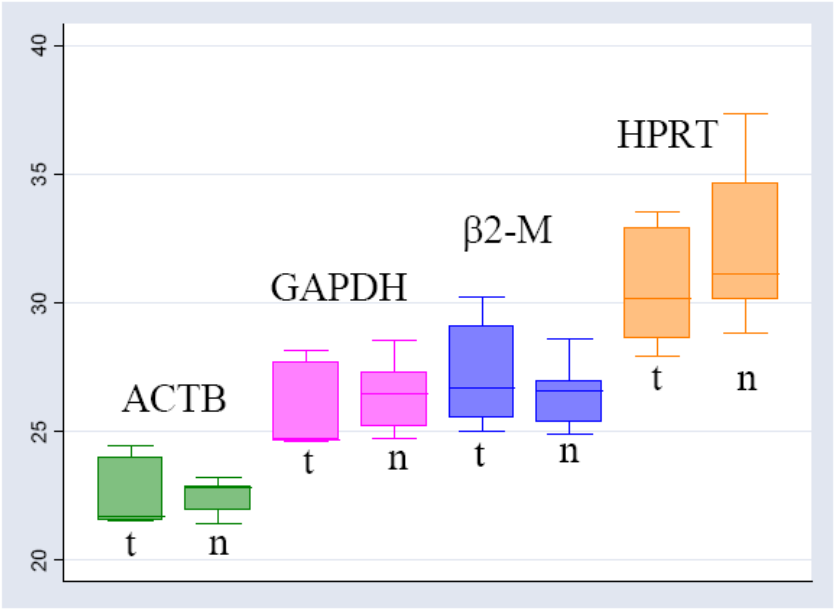
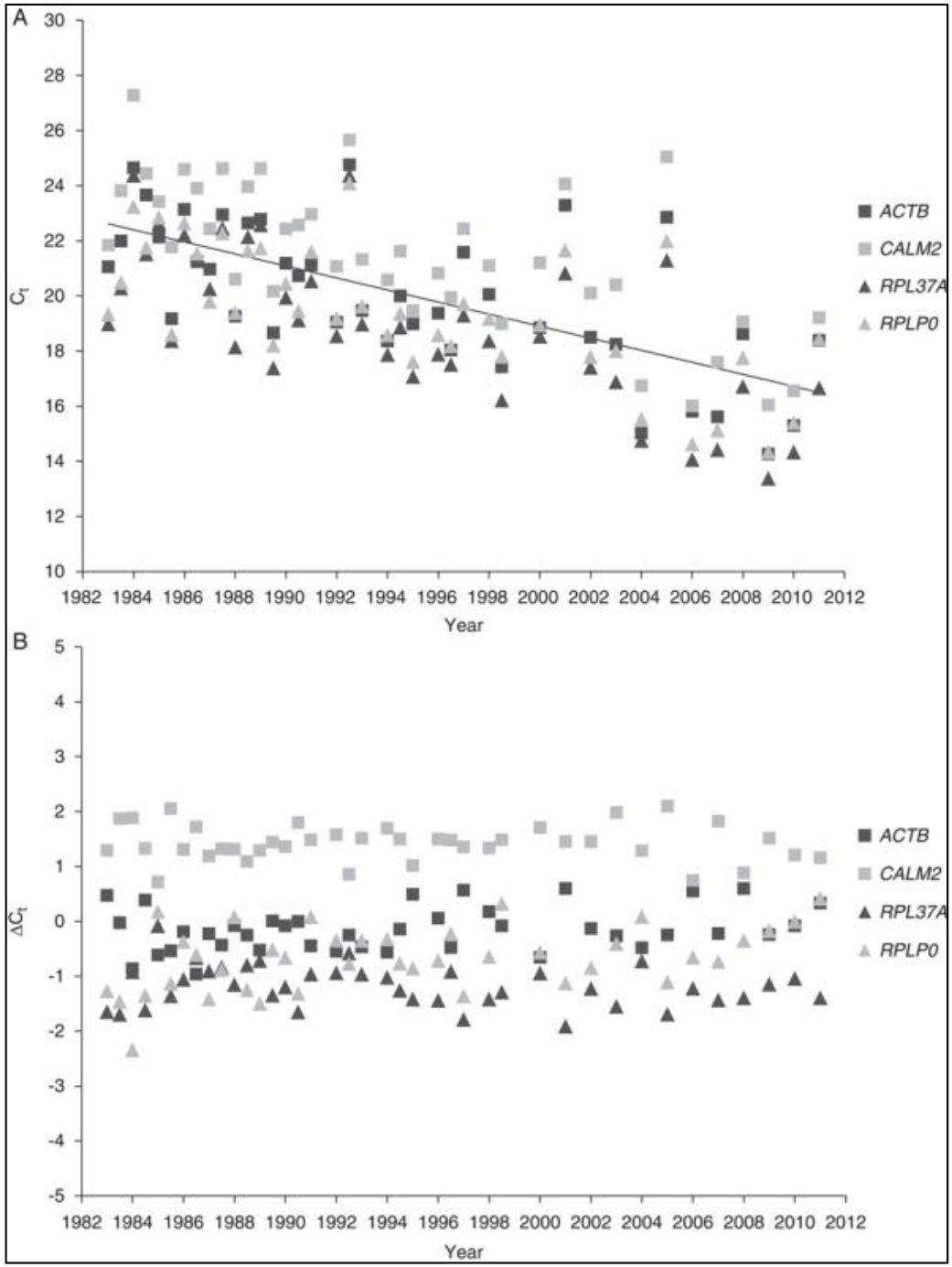




Expression stability values (M values by GeNorm) arranged after decreasing values. Lower values indicate higher stability.







Reference genes should be selected for each specific study cohort and specific organ and/or disease

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