Dasar-Dasar Real-Time PCR

dr. Ahmad Hamim Sadewa, PhD

Topik Bahasan

I. Pendahuluan
II. Isolasi RNA
III. Sintesis cDNA
IV. qPCR
V. Analisis Data
VI. Analisis TaqMan

I. Pendahuluan

Real-Time PCR adalah satu teknik PCR yang dapat mendeteksi dan menyajikan perkembangan reaksi PCR secara kontinyu (real time) sehingga jumlah DNA di awal reaksi dapat dihitung secara kuantitatif.

Hal ini dapat dilakukan karena adanya senyawa yang berikatan dengan DNA untai ganda dan melepaskan fluoresen setelah terjadinya ikatan tersebut.

Selain itu, teknik RT PCR dapat mengukur jumlah DNA yang sangat kecil (kurang dari 1 ng).

Nomenklatur

RT-PCR = Reverse Transcriptase PCR (reaksi pengubahan RNA menjadi cDNA dengan menggunakan enzim Reverse Transcriptase)

RT-PCR = Real-Time PCR atau Quantitative PCR (qPCR)

Aplikasi Real-Time PCR

1. Mengukur ekspresi gen (mRNA)
2. Analisis gene copy number
3. Mengukur jumlah virus (viral load)
4. Deteksi patogen (SARS-Covid-2)
5. Mengukur ekspresi miRNA
6. Analisis variasi genetik

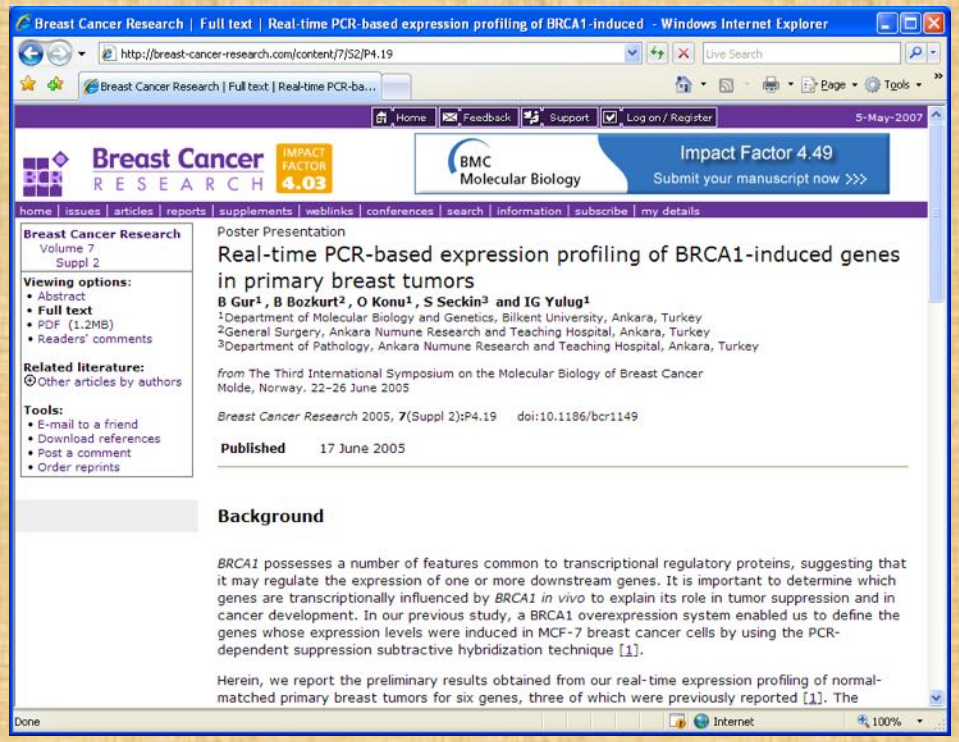
Real-Time PCR in Gene Expression Analysis

Example: BRCA1 Expression Profiling

BRCA1 is a gene involved in tumor suppression.

BRCA1 controls the expression of other genes.

In order to monitor level of expression of BRCA1, real-time PCR is used.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Poster Presentation

Real-time PCR-based expression profiling of BRCA1-induced genes in primary breast tumors

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Background

BRCA1 possesses a number of features common to transcriptional regulatory proteins, suggesting that it may regulate the expression of one or more downstream genes. It is important to determine which genes are transcriptionally influenced by *BRCA1 in vivo* to explain its role in tumor suppression and in cancer development. In our previous study, a BRCA1 overexpression system enabled us to define the genes whose expression levels were induced in MCF-7 breast cancer cells by using the PCR-dependent suppression subtractive hybridization technique [1].

Herein, we report the preliminary results obtained from our real-time expression profiling of normal-matched primary breast tumors for six genes, three of which were previously reported [1]. The

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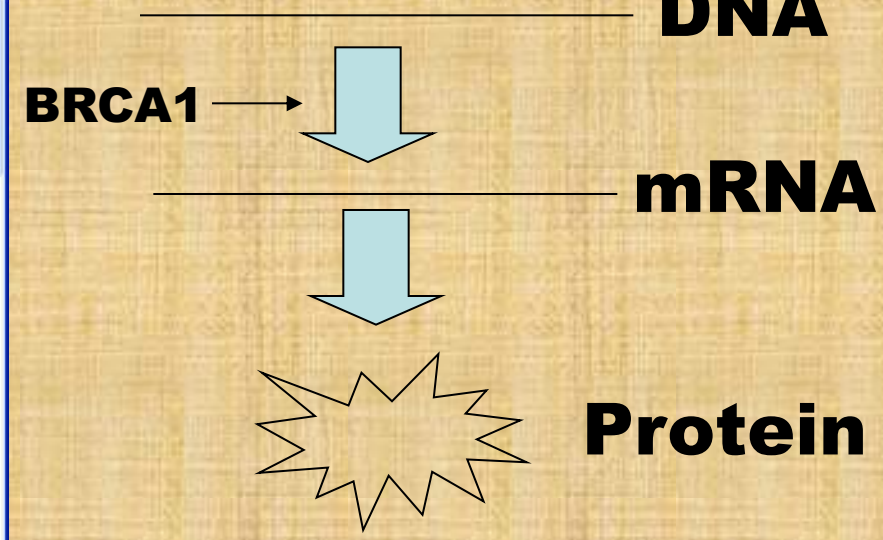
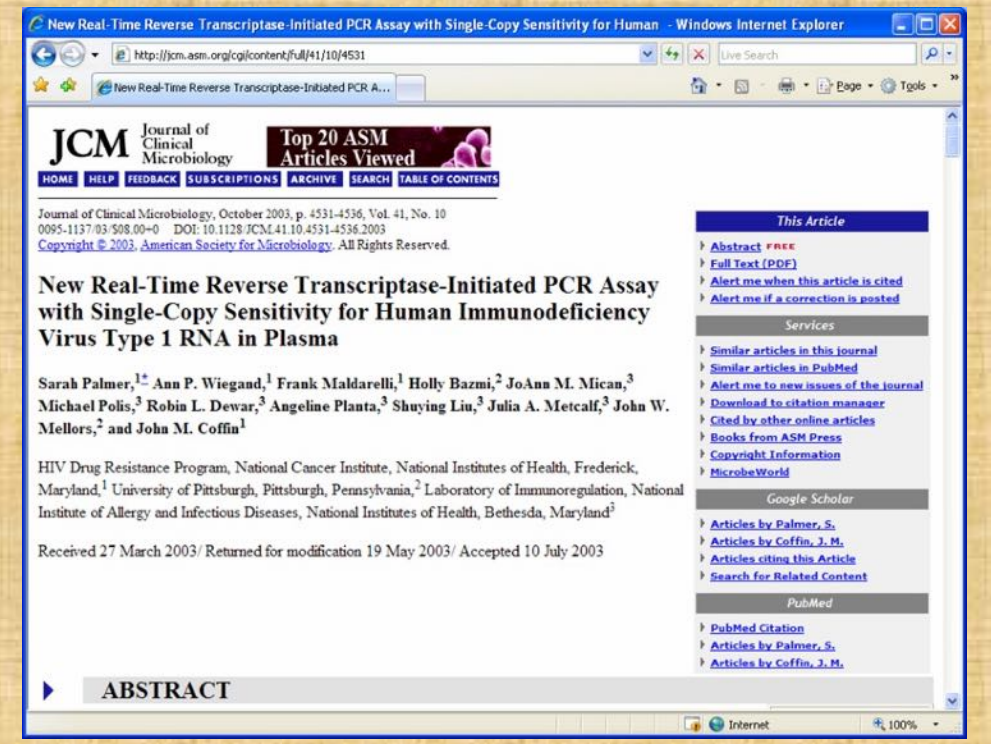


Real-Time PCR in Disease Management

Example: HIV Treatment

Drug treatment for HIV infection often depends on monitoring the "viral load".

Real-Time PCR allows for direct measurement of the amount of the virus RNA in the patient.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New Real-Time Reverse Transcriptase-Initiated PCR Assay with Single-Copy Sensitivity for Human Immunodeficiency Virus Type 1 RNA in Plasma

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ABSTRACT

RT PCR for diagnosis of SARS-CoV-2

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RT-qPCR Testing of SARS-CoV-2: A Primer

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Abstract

Go to:

Testing for the presence of coronavirus is an essential diagnostic tool for monitoring and managing the current COVID-19 pandemic. The only reliable test in current use for testing acute infection targets the genome of SARS-CoV-2, and the most widely used method is quantitative fluorescence-based reverse transcription polymerase chain reaction (RT-qPCR). Despite its ubiquity, there is a significant amount of uncertainty about how this test works, potential throughput and reliability. This has resulted in widespread misrepresentation of the problems faced using this test during the current COVID-19 epidemic. This primer provides simple, straightforward and impartial information about RT-qPCR.

Keywords: COVID-19, SARS, pandemic, reverse transcription, real-time fluorescence PCR

1. Introduction

Go to:

There can be little doubt that worldwide governmental and public health organisation responses to the current COVID-19 outbreak have been far from ideal. There have been huge differences in the pursuit of the most appropriate policies for, and effective methods of, testing potential carriers, their contacts, health workers and other emergency service workers. Given that on 9th January 2020 SARS-CoV-2 was

Mengukur Ekspresi Gen

DNA→mRNA→protein

RT PCR

- Kuantitatif (=memerlukan ketrampilan tinggi)
- Objective (= diukur oleh sistem/software yang ada di komputer)
- Spesifik (=menggunakan primer yang spesifik, tidak ada reaksi silang)
- Sensitif (= dapat mengukur kada DNA yang sangat kecil (< 1 ng)
- Tidak memerlukan perlakuan post-qPCR

Tantangan Analisis Real-time PCR

* Memerlukan skill yang tinggi

* Harga alat mahal

* Reagen mahal

•Sangat sensitif dengan kontaminasi*

•Kalau rusak perlu waktu perbaikan lama*

Tahap-Tahap Analisis RT PCR

- Isolasi RNA (atau miRNA)
- Sintesis cDNA (Reverse Transcription-PCR)
- qPCR

Apabila sample sudah dalam bentuk DNA, maka langsung ke tahap qPCR (misalnya mengukur mtDNA atau DNA copy number)

Dapat dilakukan satu tahap/satu kali selesai (One Step RT PCR) atau dalam 2 tahap (Two Steps qPCR; isolasi RNA + sintesis cDNA – stop – qPCR))

Perbandingan one step dan two steps RT PCR

	Advantages	Disadvantages
One-step	•Less experimental variation since both reactions take place in the same tube •Fewer pipetting steps reduces risk of contamination •Suitable for high throughput amplification/screening •Fast and highly reproducible	•Difficult to optimize the two reactions separately •Less sensitive than two-step because the reaction conditions are a compromise between the two combined reactions •Detection of fewer targets per sample
Two-steps	•A stable cDNA pool is generated that can be stored for long periods of time and used for multiple reactions •The target and reference genes can be amplified from the same cDNA pool without multiplexing •Optimized reaction buffers and reaction conditions can be used for each individual reaction •Flexible priming options	•The use of several tubes and pipetting steps exposes the reaction to a greater risk of DNA contamination Time consuming •Requires more optimization than one-step

II. Isolasi RNA: Trizol

Trizol : campuran guanidine thioacyanate dan phenol, secara efektif memisahkan DNA dan RNA setelah homogenasi jaringan atau lisis sel.

Setelah penambahan kloroform, sampel disentrifugasi sehingga terbentuk 3 lapisan. Lapisan paling atas dan jernih adalah lapisan yang mengandung RNA.

Setelah itu dilakukan presipitasi dan pencucian RNA (precipitation and washing).

Pada akhir isolasi didapatkan Total RNA

Kadar RNA diukur dengan Nanodrop

Larutan RNA (dalam buffer) tidak terkontaminasi oleh reagen isolasi EDTA atau buffer isolasi) karena dapat menghambat kerja *Taq* polymerase atau mengubah konsentrasi buffer.

Isolasi RNA sangat penting karena RNA tidak stabil, kualitas dan kuantitas RNA sangat menentukan tahap reaksi selanjutnya. Semua sampel diperlakukan sama sehingga menjamin kuantitas dan kualitas RNA juga sama.

Setelah isolasi, RNA diukur kadarnya sebelum disimpan pada suhu -80 °C.

Tambahan reagen yang digunakan pada isolasi RNA

- **Diethyl pyrocarbonate** (**DEPC**), adalah senyawa untuk menginaktivasi enzim RNAase yang terdapat dalam larutan atau alat laboratorium.
- DEPC bekerja dengan cara membentuk ikatan kovalen dengan asam amino histidine (paling kuat), lisine, sistein, dan tirosin.
- DEPC-treated water digunakan untuk handling RNA di laboratorium untuk mengurangi risiko degradasi RNA oleh RNAase.

III. Sintesis cDNA

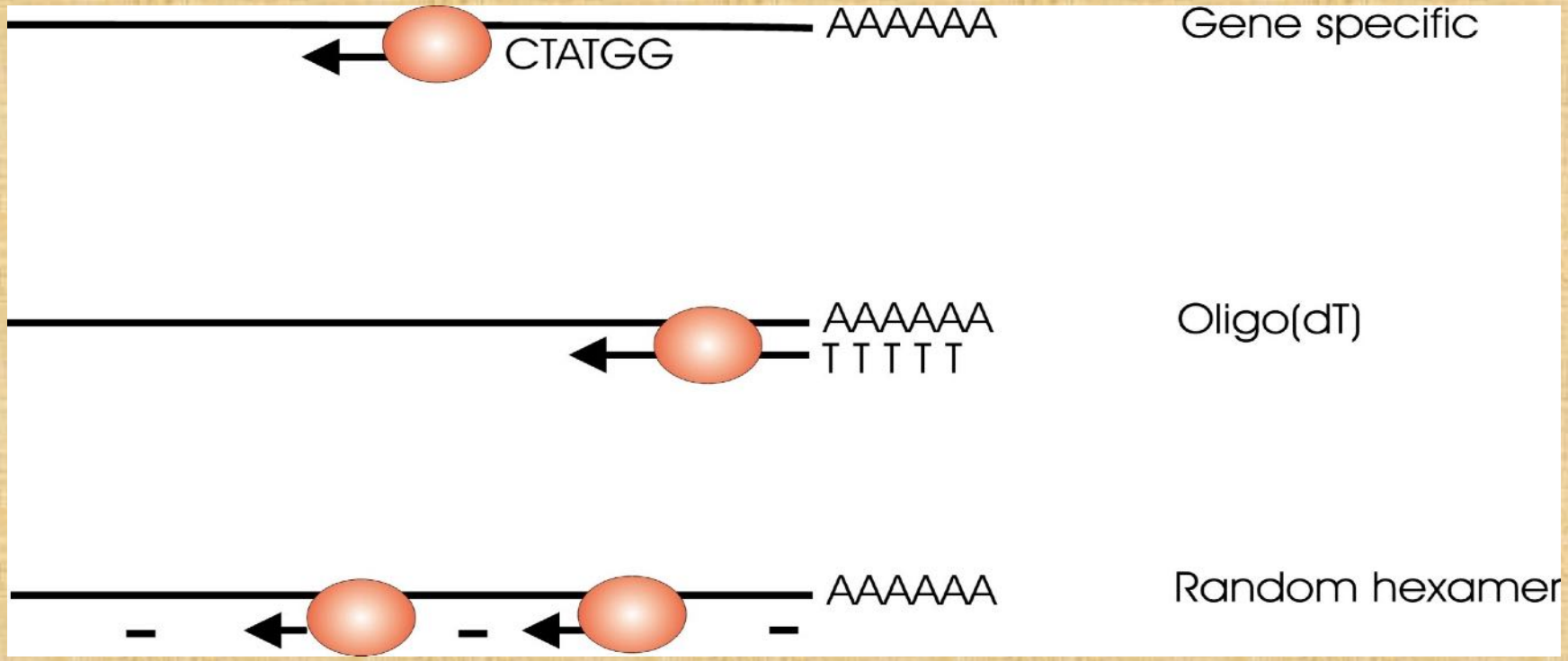
Sample atau specimen yang berupa RNA atau miRNA harus dikonversi menjadi DNA terlebih dahulu.

Reaksi ini adalah reaksi konversi, tidak ada amplifikasi

Bahan yang diperlukan adalah template RNA atau miRNA, primer oligo-dT, random primer, dNTP, buffer dan enzim Reverse Transcriptase.

RNAase H : enzim yang mendegradasi ikatan DNA-RNA dupleks sehingga meningkatkan efisiensi sintesis cDNA

Sintesis DNA dari mRNA



Sintesis DNA dari miRNA

How the miRNA PCR Array Works

Control & Experimental Cells or Tissue

↓

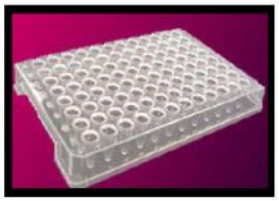
1. Isolate miRNA (MA-01)

↓

2. Reverse Transcription: RT² miRNA First Strand Kit (MA-03)

↓

3. Setting Up the PCR Array
Instrument-Specific RT² qPCR SYBR Green Master Mix & cDNA Cocktail
Aliquot across PCR Array



↓

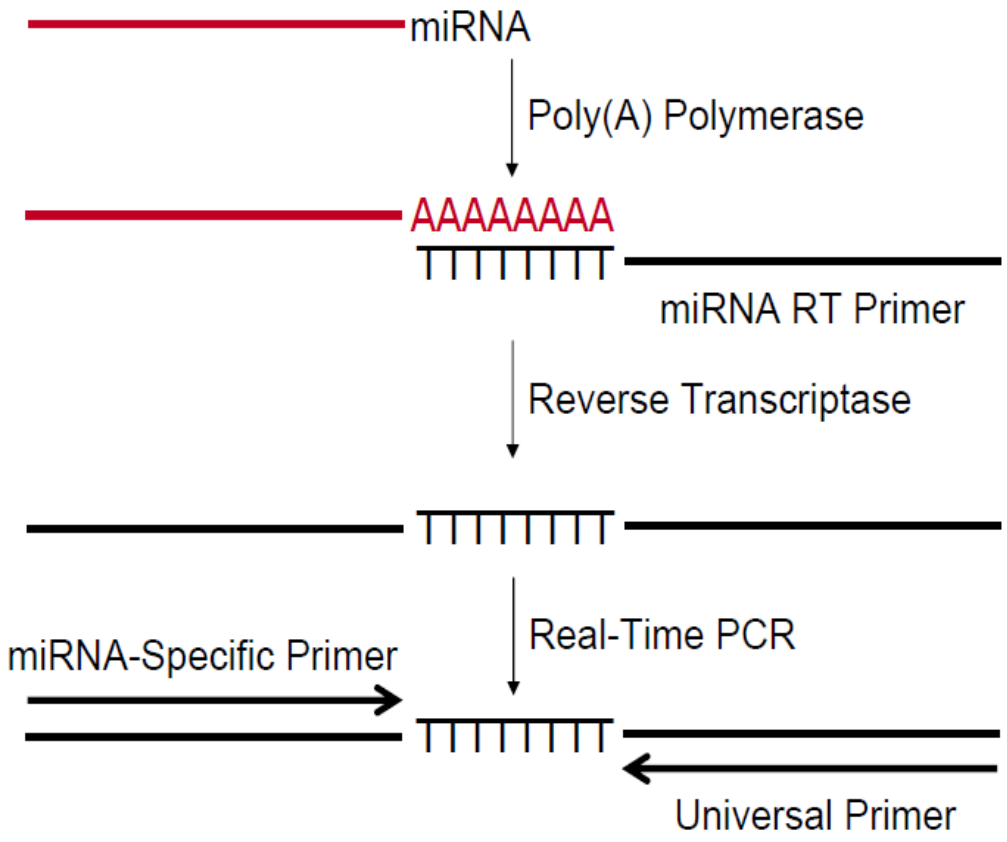
4. Real-Time PCR
(40 cycles, 100-120 min)

↓

5. Data Analysis: Determine Fold Changes ($\Delta\Delta C_t$ Method)

Universal miRNA Reverse Transcription

Same cDNA preparation can assay ANY miRNA



Contoh protocol first strand cDNA synthesis

First Strand cDNA Synthesis Protocols (E6300)

Introduction

Thaw system components and put on ice. A control reaction without reverse transcriptase is recommended to examine the DNA contamination in the samples.

Protocol

1. Mix RNA sample and primer $d(T)_{23}VN$ in two sterile RNase-free microfuge tubes.

Total RNA	1–6 µl
$d(T)_{23}VN$ (50 µM)	2 µl
nuclease-free H_20	variable
Total Volume	**8 µl**

2. Denature RNA for 5 minutes at 70°C. Spin briefly and put promptly on ice. This step is optional. However, it improves the cDNA yield for long messenger RNAs and GC-rich RNA regions.
3. Add the following components to one tube.

M-MuLV Reaction Mix	10 µl
M-MuLV Enzyme Mix	2 µl

To the negative control tube, add the following:

M-MuLV Reaction Mix	10 µl
H_2O	2 µl

4. Incubate the 20 µl cDNA synthesis reaction at 42°C for one hour. **If Random Primer Mix is used, an incubation step at 25°C for 5 min is recommended before the 42°C incubation.**
5. Inactivate the enzyme at 80°C for 5 minutes. Dilute reaction to 50 µl with 30 µl H_2O for PCR. The cDNA product should be stored at -20°C. For downsteam PCR amplification, the volume of cDNA product should not exceed 1/10 of the PCR reaction volume.

Contoh protocol first strand cDNA synthesis

Reagents	Volume 1X (µL)
Reaction buffer 5X	4
$MgCl_2$ 2.5 mM	6
Reverse Transcriptase	1
dNTP	4
Test sample (RNA (2 µg) + DEPC + oligodT (1µL))	5
Total	20

(B)

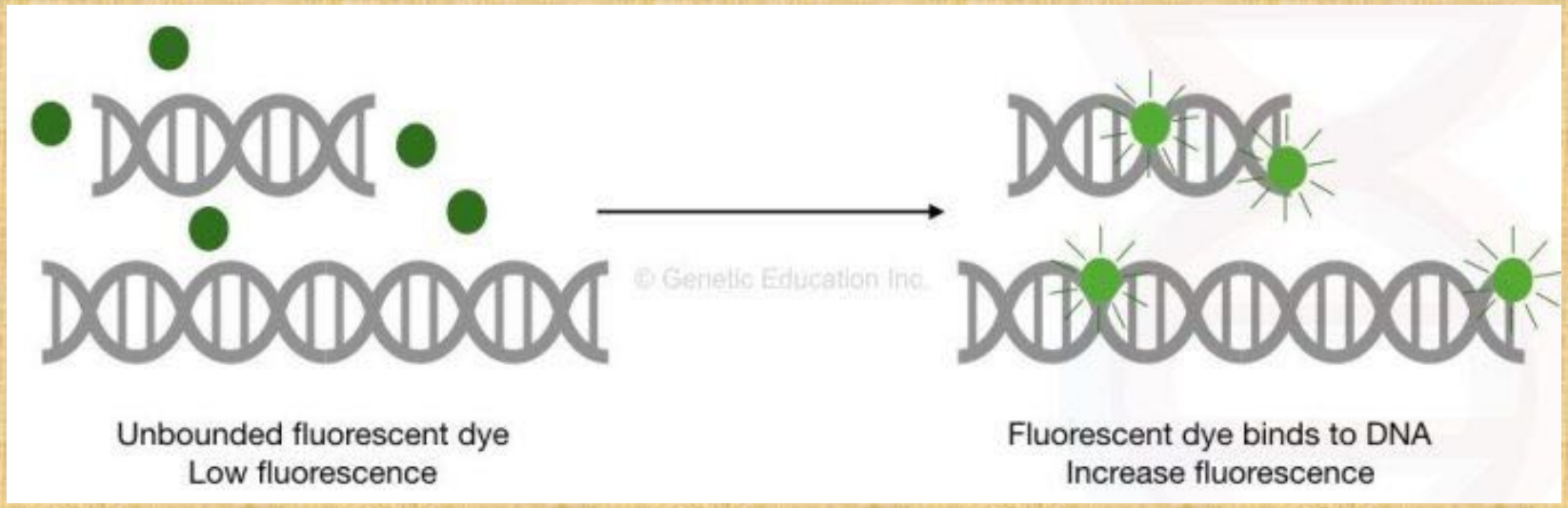
Experimental Step	Condition
Annealing	25 °C for 5 min
Extension	42 °C for 2 h
Reverse transcriptase inactivation	75 °C for 10 min
Storage	4 °C for 30 min

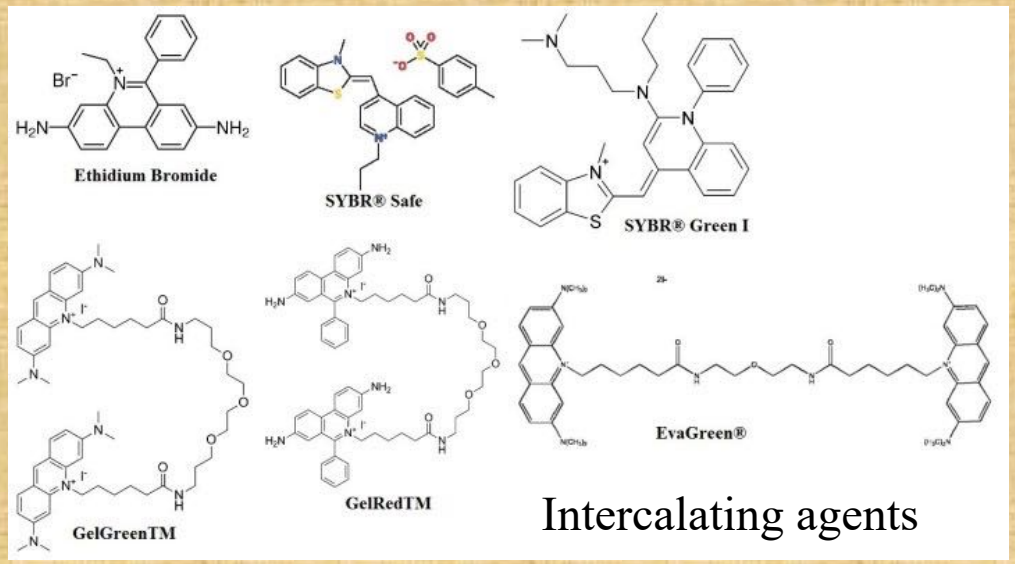
IV. Quantitative PCR

Pada prinsipnya, qPCR mirip dengan PCR konvensional hanya ditambahkan senyawa yang dapat melepaskan fluoresen apabila senyawa tersebut berikatan dengan DNA untai ganda (double strand DNA binding dye).

Campuran reaksi ditambahkan dengan akurat sehingga semua reaksi mempunyai kandungan yang sama (→ memerlukan skill yang tinggi).

Double Strand DNA Binding Dyes





Intercalating agents

SYBR Green/Evagreen (double-stranded DNA binding dye)

* Mengemisikan (melepaskan) sinyal fluoresen yang kuat setelah berikatan dengan dsDNA

•Tidak spesifik, berikatan dengan semua dsDNA yang ada dalam reaksi

* Memerlukan optimasi yang ekstensif

•Amplikon yang lebih panjang akan menghasilkan sinyal fluoresen yang lebih tinggi

SYBRgreen atau Evagreen

RT PCR tube

Contoh campuran reaksi qPCR

Component	Volume/reaction	50× master mix	Final concentration
2× reaction mix	12.5 µl	625 µl	1×
Forward primer	0.5 µl	25 µl	1 µM
Reverse primer	0.5 µl	25 µl	1 µM
RNase-free water	Variable (bring to 25 µl per reaction)	—	—
Template cDNA (to be added at step 4 to each reaction tube)	Variable (1-5 µl if using preparation described above)	—	<500 ng/reaction
Total	25 µl	1250 µl	

Semua bahan harus dipipet dengan akurasi tinggi
Dapat dibuat master mix untuk mengurangi peluang perbedaan antar tube, tetapi lebih boros reagen.

Contoh kondisi qPCR

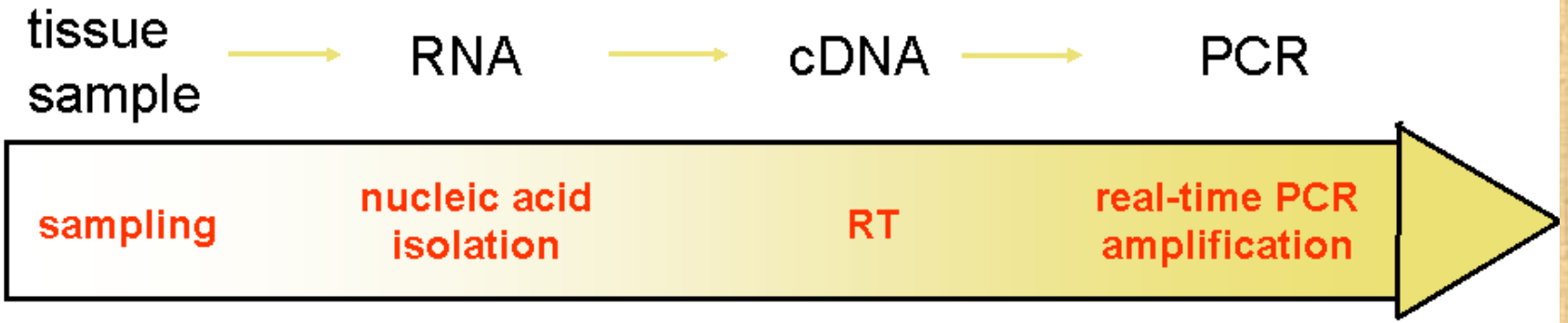
Program	PCR	SYBR Green-based real-time PCR
Pre-incubation	95°C, 6 min	95°C, 10 min
Amplification	35 cycles	35 cycles
	95°C, 30 s	95°C, 5 s
	62°C, 15 s	62°C, 10 s
	72°C, 30 s	72°C, 20 s (Single)
Melting	N/A[a]	95°C, 0 s
		65°C, 10 s
		95°C, 0 s
		0.2°C/s (Continuous)
Cooling	N/A[a]	40°C, 30 s

[a] Not applicable

Ket. : Amplification disetting oleh peneliti

Melting analysis dilakukan secara otomatis (default)

Steps and variables of a successful mRNA quantification using real-time RT-PCR (1)



Sampling method:

- Biopsy
- Fixed material
- Fresh blood
- Tissue storage
- Liquid Nitrogen
- RNA Later
- 1st extraction buffer
- RNA storage –80°C

  => native RNA

Extraction method:

- total RNA
  - mRNA
  - microRNA
- liquid-liquid
- columns
- Robot vs. hand made
- **RNA integrity:**
  - Bioanalyzer 2100
  - Experion
  - Nano-Drop
  - mFold algorithm

Efficiency of RT:

- RT enzyme type
- RT temperature
- **Primers:**
  - poly-T Primer
  - Random-hexamers
  - Specific primer
  - Primer mixtures
- **one-step qRT-PCR**
- **two-step RT-qPCR**

PCR Efficiency / Specificity:

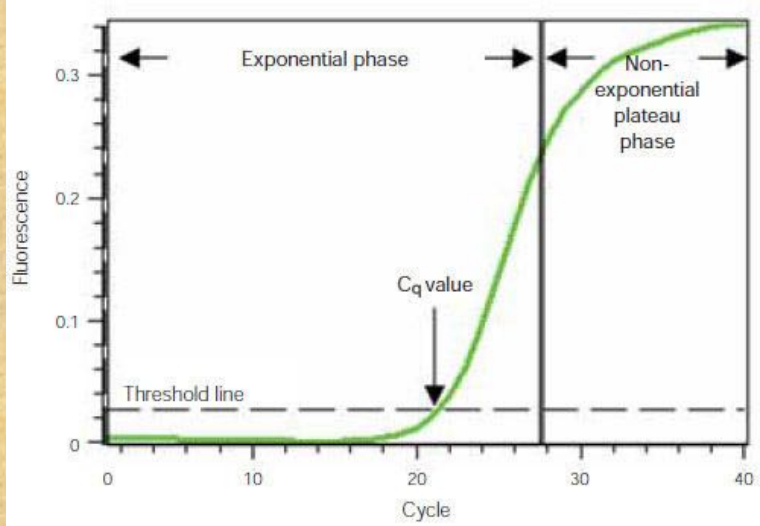
- Primer design
  - Primer specificity
  - Consensus Primer
- mRNA abundance
- RNA / cDNA input
- Polymerase types
- Polymerase Mixtures
- PCR Inhibitors & Enhancers
- Robot vs. hand made

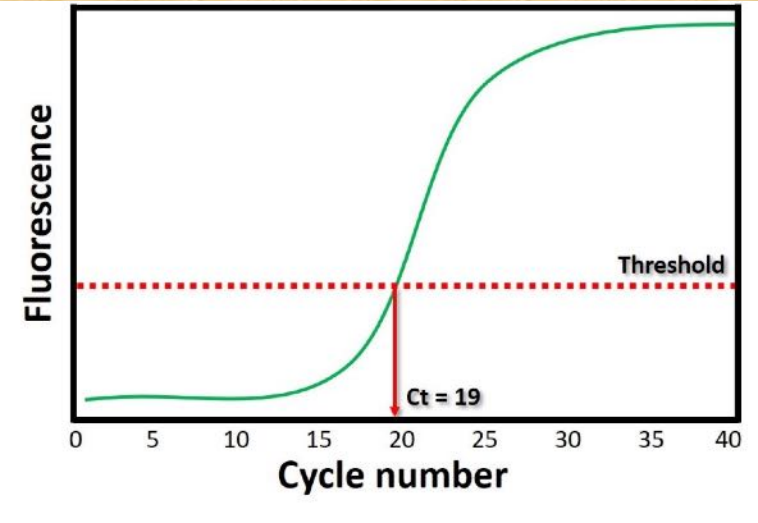
Cycle Treshold (Ct) atau Cycle Quantification (Cq)

Cycle treshold (Ct) atau juga disebut Cycle quantification (Cq) adalah siklus pada saat fluoresen yang dihasilkan melewati ambang batas fluoresen yang ditentukan.

Pada Ct, sejumlah amplikon dapat terdeteksi pada fase exponensial reaksi PCR. Ct ini berbanding terbalik dengan level ekspresi gen target.

Cycle Treshold (Ct) atau Cycle Quantification (Cq)

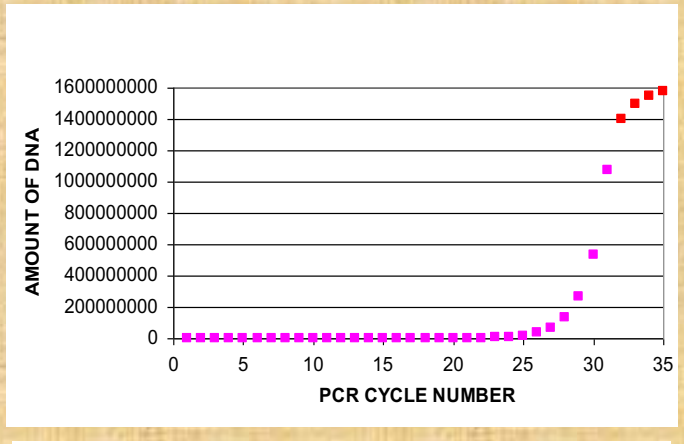


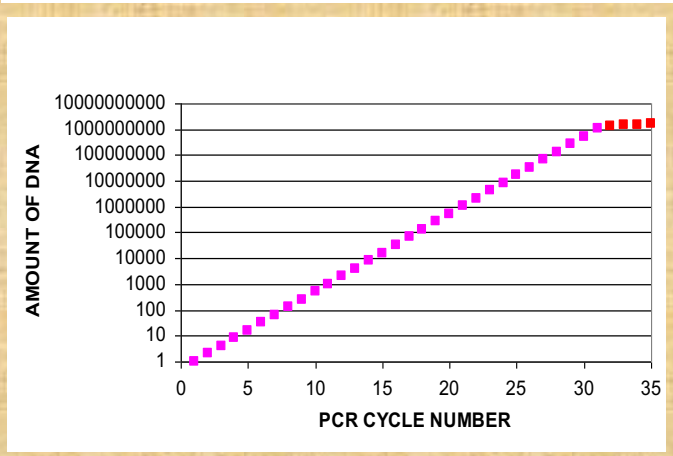


Dasar Perhitungan qPCR

0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	2,147,483,648
32	4,294,967,296
33	8,589,934,592
34	17,179,869,184

CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,550,000,000
34	1,580,000,000





Mengapa ada fase eksponensial dan non eksponensial?

1. Aktivitas Taq Polymerase berkurang
2. Jumlah reagen berkurang
3. Penurunan kapasitas buffer

Imagine Real-Time PCR

Measuring Quantities

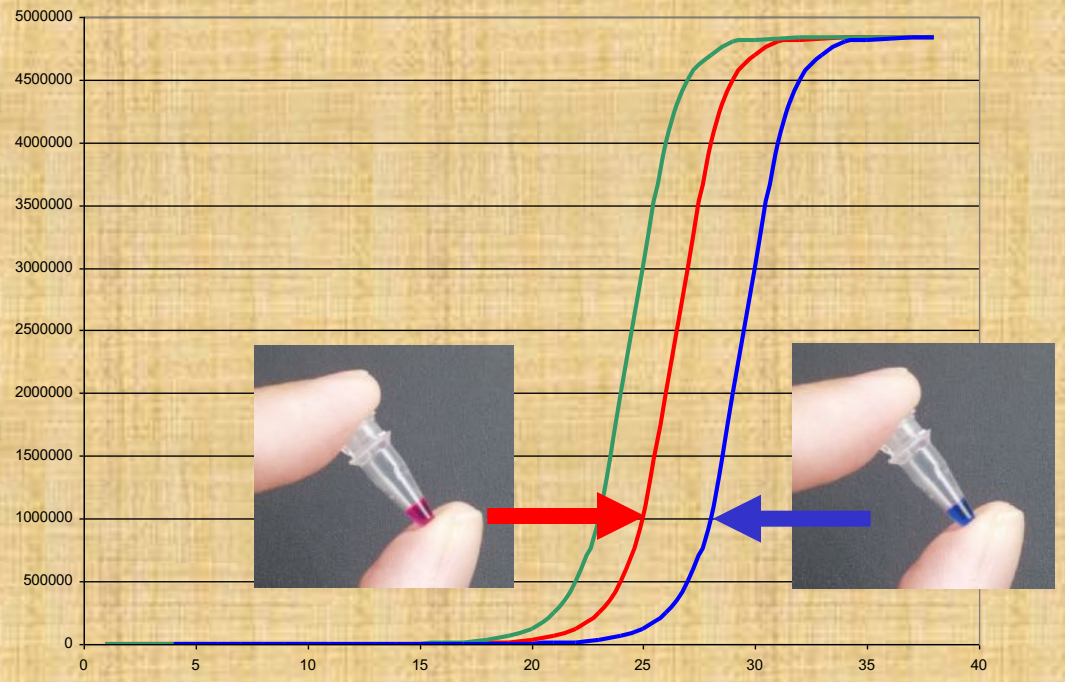
started with different amount of DNA, calculate at particular cycle

Cycle 25 →

Cycle		
25	1,000,000	125,000
26	2,000,000	250,000
27	4,000,000	500,000
28	8,000,000	1,000,000

Imagining Real-Time PCR

Measuring Quantities



Imagining Real-Time PCR

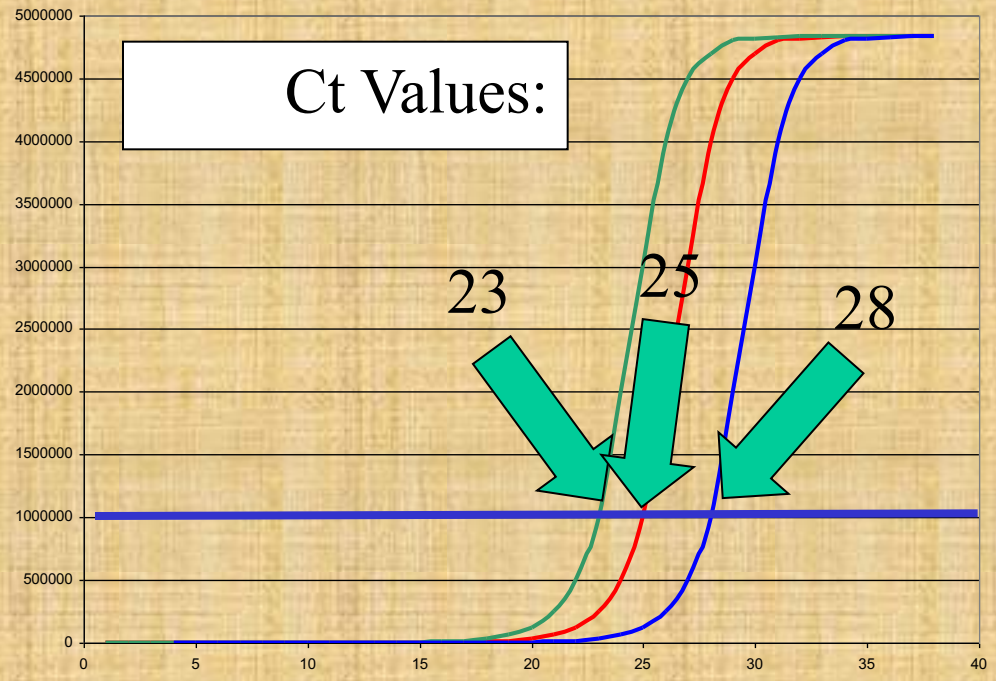
Measuring Quantities

We describe the position of the lines with a value that represents the cycle number where the trace crosses an arbitrary threshold.

This is called the "Ct Value".

Ct values are directly related to the starting quantity of DNA, by way of the formula:

Quantity = 2^Ct



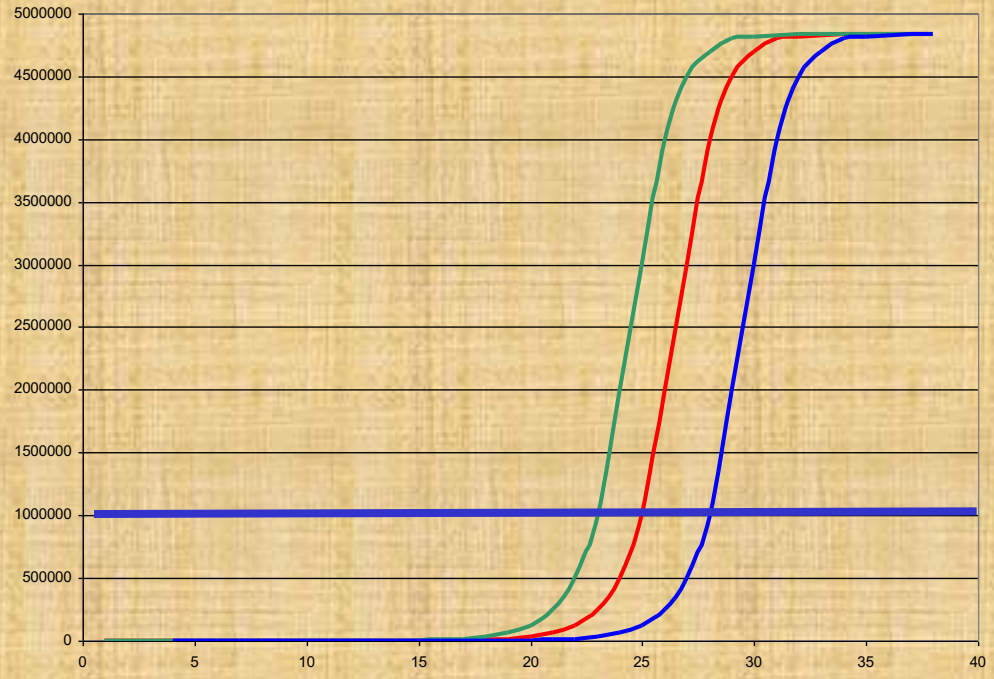
Imaging Real-Time PCR

Measuring Quantities

4 units
Ct=23

1 unit
Ct=25

1/8 unit
Ct=28



Cycle threshold pada diagnosis COVID-19

Semakin rendah Ct maka semakin banyak jumlah virus yang ada di dalam tubuh

Ct < 25 merupakan risiko untuk terjadi gejala yang berat, meskipun pada saat diperiksa gejala ringan

Ct > 35 merupakan indikasi jumlah virus dalam tubuh rendah

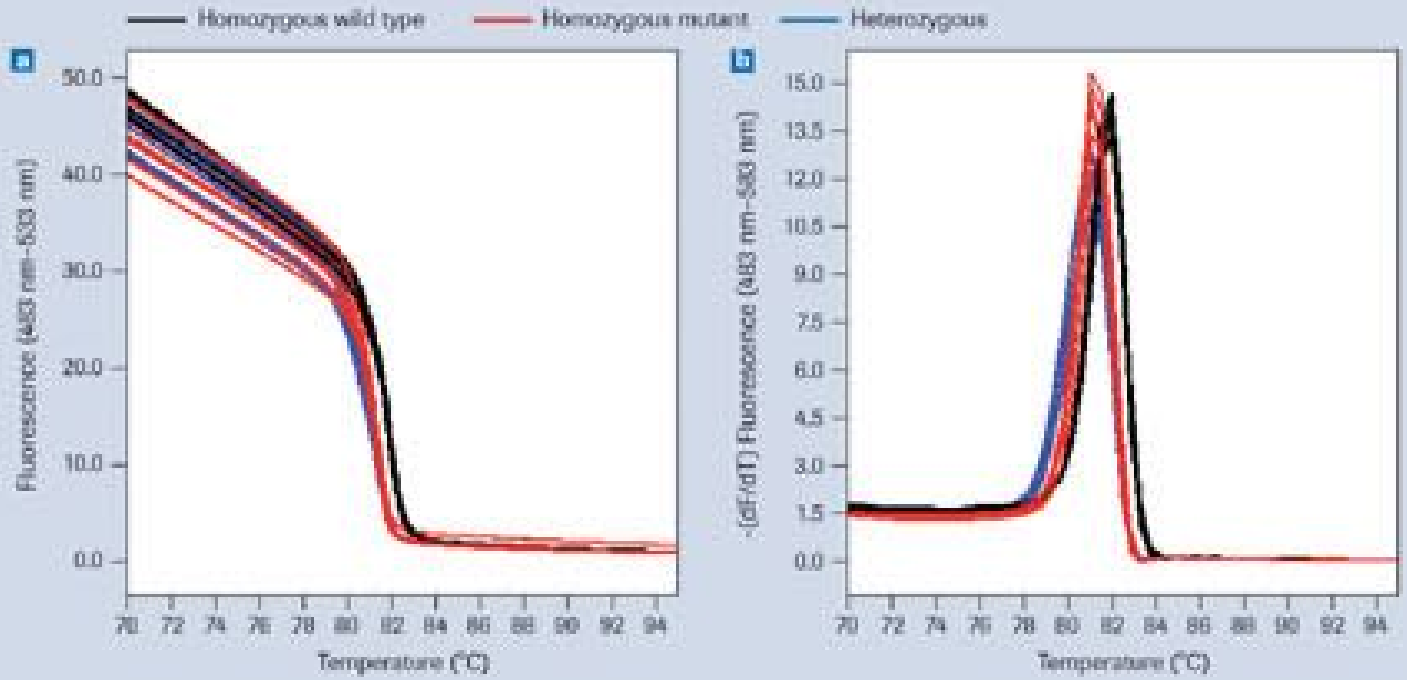
Melt Curve (Kurva Leleh) dan Melt Peak (Puncak Leleh)

Kurva leleh adalah analisis post PCR untuk mengetahui spesifitas produk PCR (amplikon) berdasar karakter leleh (karakter denaturasi). Satu jenis amplikon akan menghasilkan 1 jenis kurva leleh dan puncak leleh.

Ketika mencapai suhu meningkat, maka produk PCR akan terdisosiasi sehingga terbentuk DNA untai tunggal yang tidak mengikat fluoresen yang menyebabkan sinyal fluoresens turun.

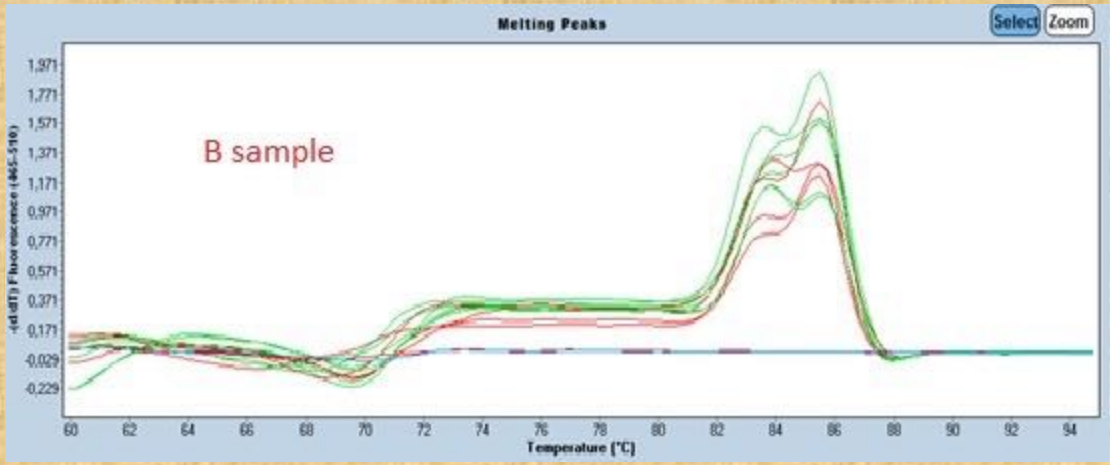
Temperatur puncak leleh tergantung pada panjang amplikon, konten G:C dan kondisi buffer. Puncak leleh menunjukkan suhu pada saat ~50% of the DNA dalam keadaan untai tunggal.

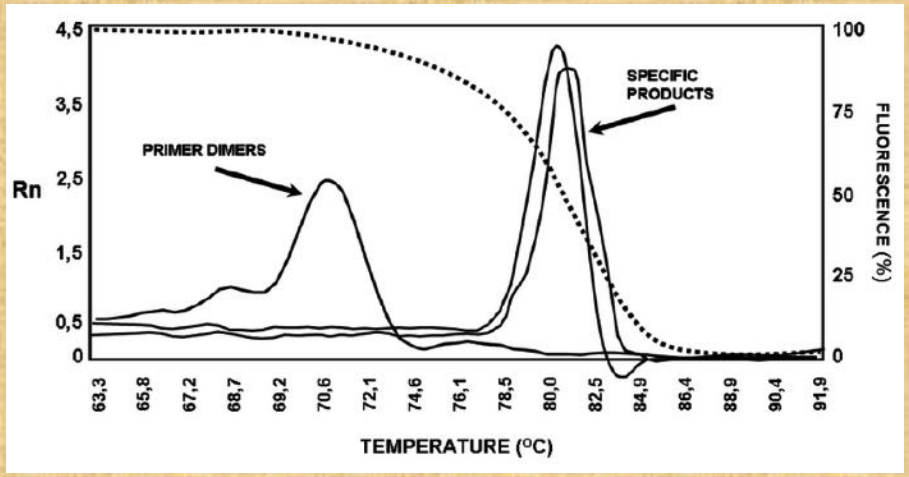
Melting Curve (kurva leleh) dan Melting Peak (puncak leleh)



Diperlukan optimasi yang intensif untuk mendapat hasil spesifik.

Contoh amplikon yang tidak spesifik





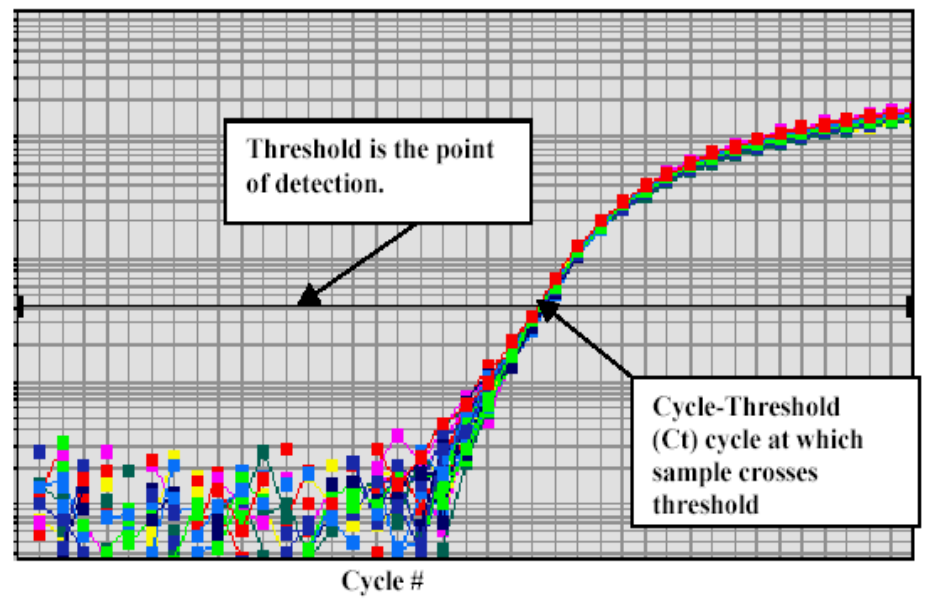
V. Analisis Data qPCR

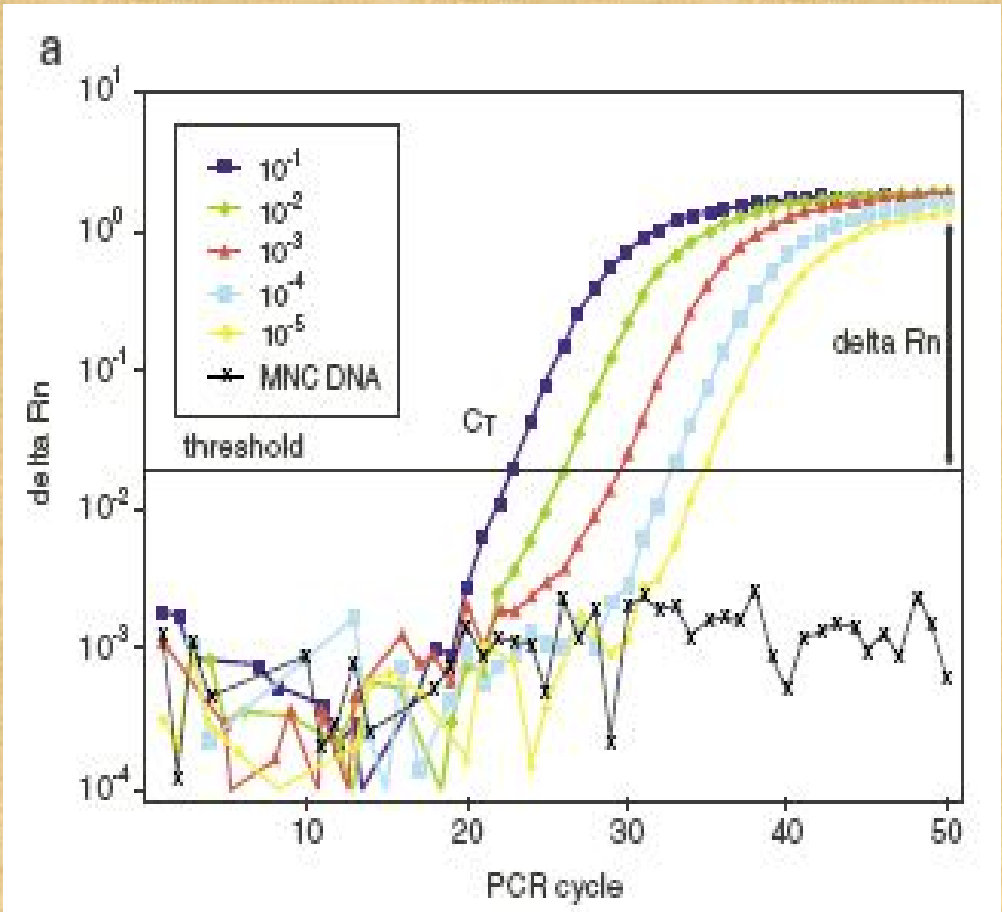
Cycle Threshold

* cycle threshold or the C_T value is the cycle at which a significant increase in ΔRn is first detected

* it is the parameter used for quantitation

* C_T value of 40 or more means no amplification and cannot be included in the calculations





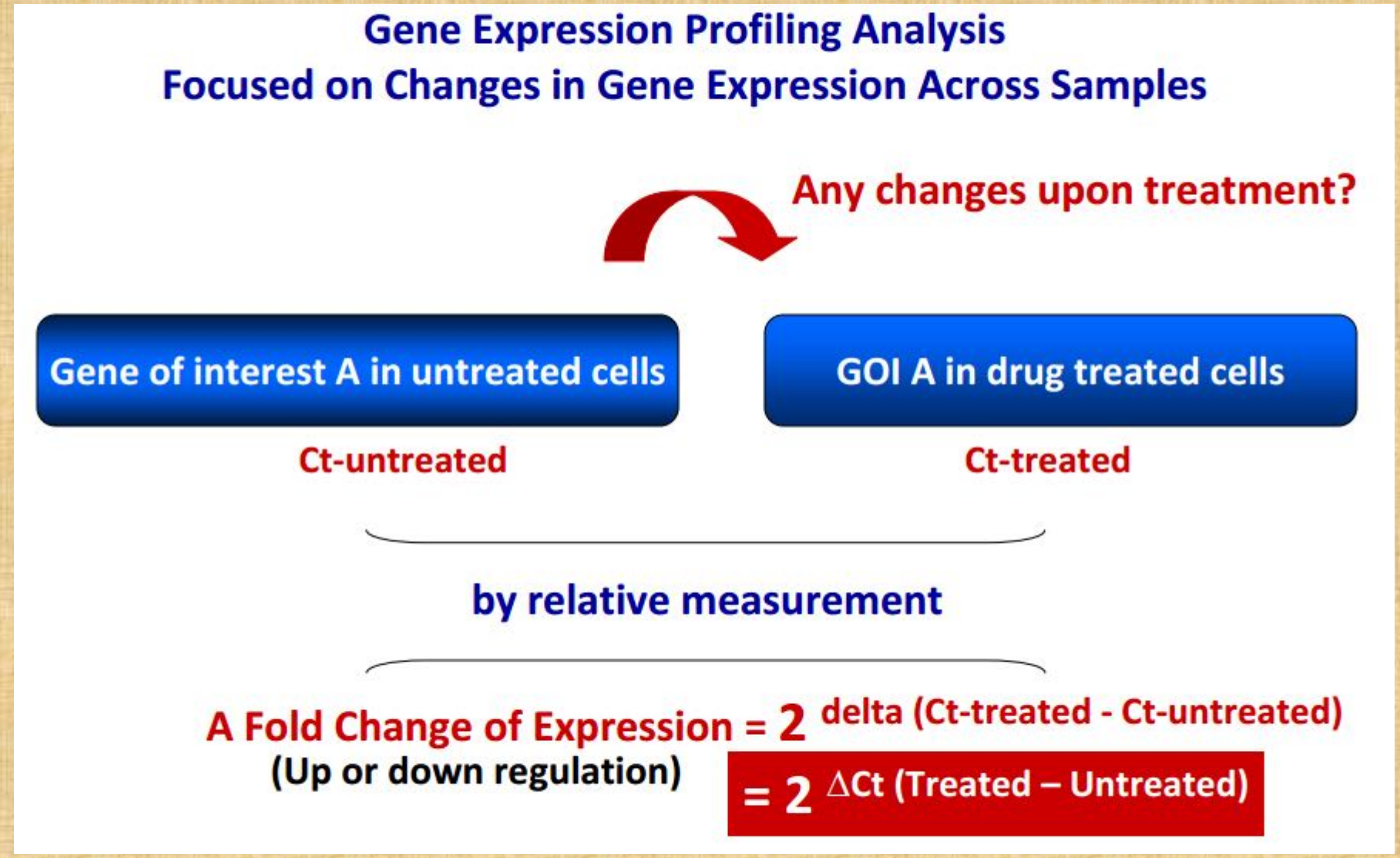
Van der Velden. Leukemia 2003 (www)

- Absolute quantitation
  - Standard curve
  - Standards must be accurately quantitated
  - Best used for viral load determination
- Relative quantitation
  - Standard curve
  - Standards are serial dilutions of a calibrator template
  - Best used for gene expression studies
- Comparative quantitation
  - Mathematical determination
  - Calibrator sample used as a 1x standard
  - Best used when particular ratios are expected or to verify trends

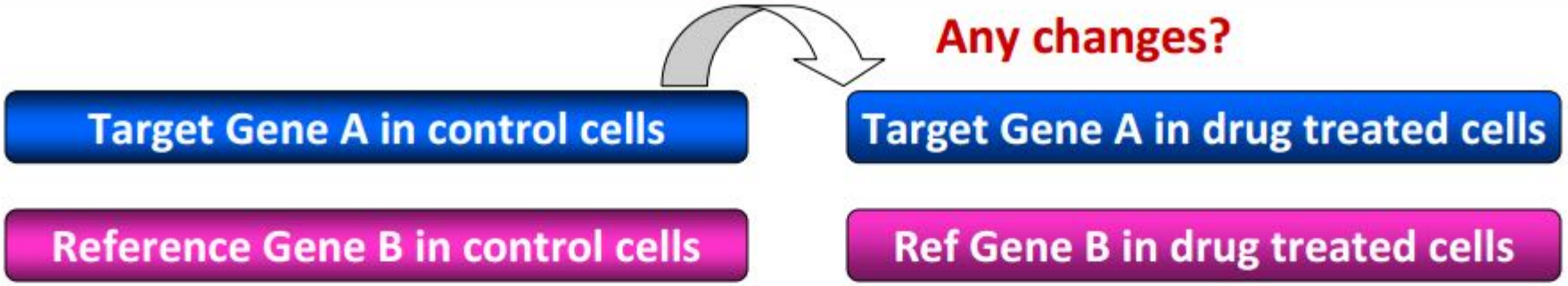
Ambion
THE RNA COMPANY®

(www)

Quantification



Quantification



→ $\Delta Ct1 = Ct\ (Target\ A\ -treated) - Ct\ (Ref\ B\text{-}treated)$

→ $\Delta Ct2 = Ct\ (Target\ A\text{-}control) - Ct\ (Ref\ B\text{-}control)$

→ $\Delta\Delta Ct = \Delta Ct1\ (treated) - \Delta Ct2\ (control)$

Normalized target gene expression level = $2^{\Delta\Delta Ct}$

Metode Livak

Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method

Kenneth J. Livak* and Thomas D. Schmittgen†,[1]

Hasil perhitungan adalah fold change (perubahan kelipatan)

	Housekeeping gene			Gene of interest					
Sample	Ct1	Ct2	Average Ct	Ct1	Ct2	Average Ct	ΔCt	ΔΔCt	2^-(ΔΔCt)
Control 1	17.19	17.16	17.18	30.57	30.53	30.55	13.38	-0.18	1.13
Control 2	16.96	16.95	16.96	30.73	30.37	30.55	13.60	0.04	0.97
Control 3	17.07	17.15	17.11	30.76	30.82	30.79	13.68	0.13	0.91
Treated 1	18.04	17.95	18.00	26.11	25.54	25.83	7.83	-5.72	52.71
Treated 2	17.99	17.91	17.95	25.70	25.56	25.63	7.68	-5.87	58.49
Treated 3	17.90	17.86	17.88	25.64	25.74	25.69	7.81	-5.74	53.45
Control average							13.55		

The Pfaffl Method

- The Livak method for calculating relative gene expression is valid only when the amplification efficiencies of the target and reference genes are similar.
- If the amplification efficiencies of the two PCR products are not the same, an alternative formula must be used to determine the relative expression of the target gene in different samples. To determine the expression ratio between the test sample and calibrator for a target normalized to a reference (ref), use the following equation:

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

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

E_{target}	is the amplification efficiency of the target gene.
E_{ref}	is the amplification efficiency of the reference gene.
ΔCq, ref (calibrator - test)	is the Cq of the reference gene in the calibrator minus the Cq of the reference gene in the test sample.
ΔCq, target (calibrator - test)	is the Cq of the target gene in the calibrator minus the Cq of the target gene in the test sample.

Internal Standards/Reference gene/Internal control

Syarat-syarat internal standard

- Gen dengan jumlah copy number sama di semua sel
- Gen yang terekspresi di semua sel
- Gen dengan jumlah copy number medium with (2 copies)

Fungsi internal standard :

1. Menormalkan hasil qPCR (karena perbedaan kualitas specimen)
2. Menyamakan kondisi qPCR karena adanya berbagai inhibitor dalam reaksi

→ Dipastikan kondisi perlakuan sama (handling sample, isolasi, cDNA synthesis, qPRC)

Internal Standard : Housekeeping gene

- Knowing the amount of mRNA in one sample from one specific gene does not tell us alot
- You don´t know the total amount of mRNA in your sample
- You also dont know how much the mRNA level has changed compared to other mRNA levels

Example:

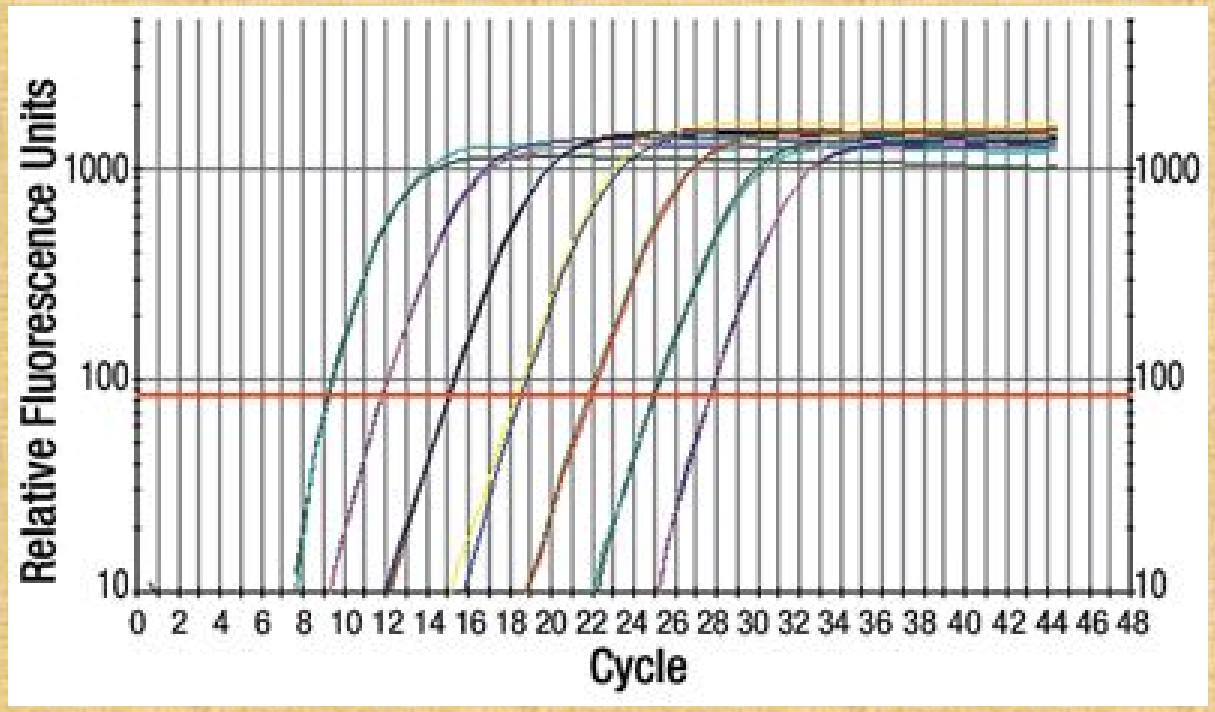
mRNA levels increase 2x after induction

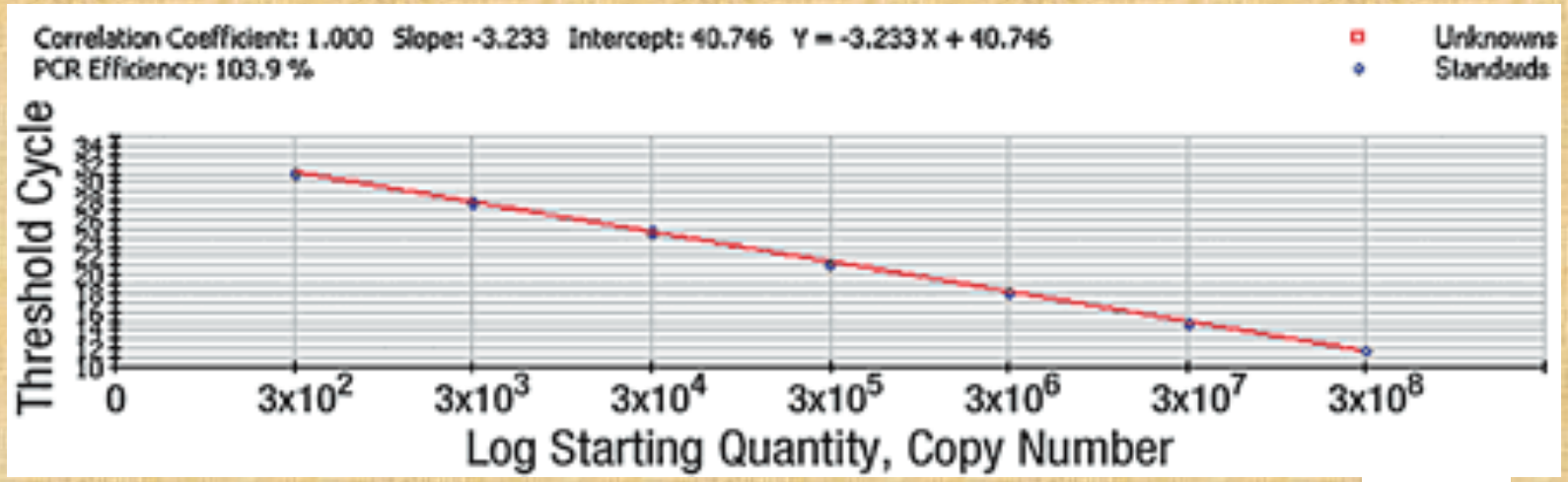
It is possable that all gene expression in the cell has increased

→ compare the expression of our gene to another gene which expression is normally constant, a housekeeping gene

Internal standards = housekeeping genes

- Commonly used standards
  - Glyceraldehyde-3-phosphate dehydrogenase mRNA
  - Beta-actin mRNA
  - MHC I (major histocompatability complex I) mRNA
  - Cyclophilin mRNA
  - 28S or 18S rRNA
  - Untuk miRNA : U6RNA


Relative Fluorescence Units
1000
100
10
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48
Cycle


Correlation Coefficient: 1.000 Slope: -3.233 Intercept: 40.746 Y = -3.233 X + 40.746
PCR Efficiency: 103.9 %
Unknowns
Standards
Threshold Cycle
0
3x10^2
3x10^3
3x10^4
3x10^5
3x10^6
3x10^7
3x10^8
Log Starting Quantity, Copy Number

(www)

Quantification

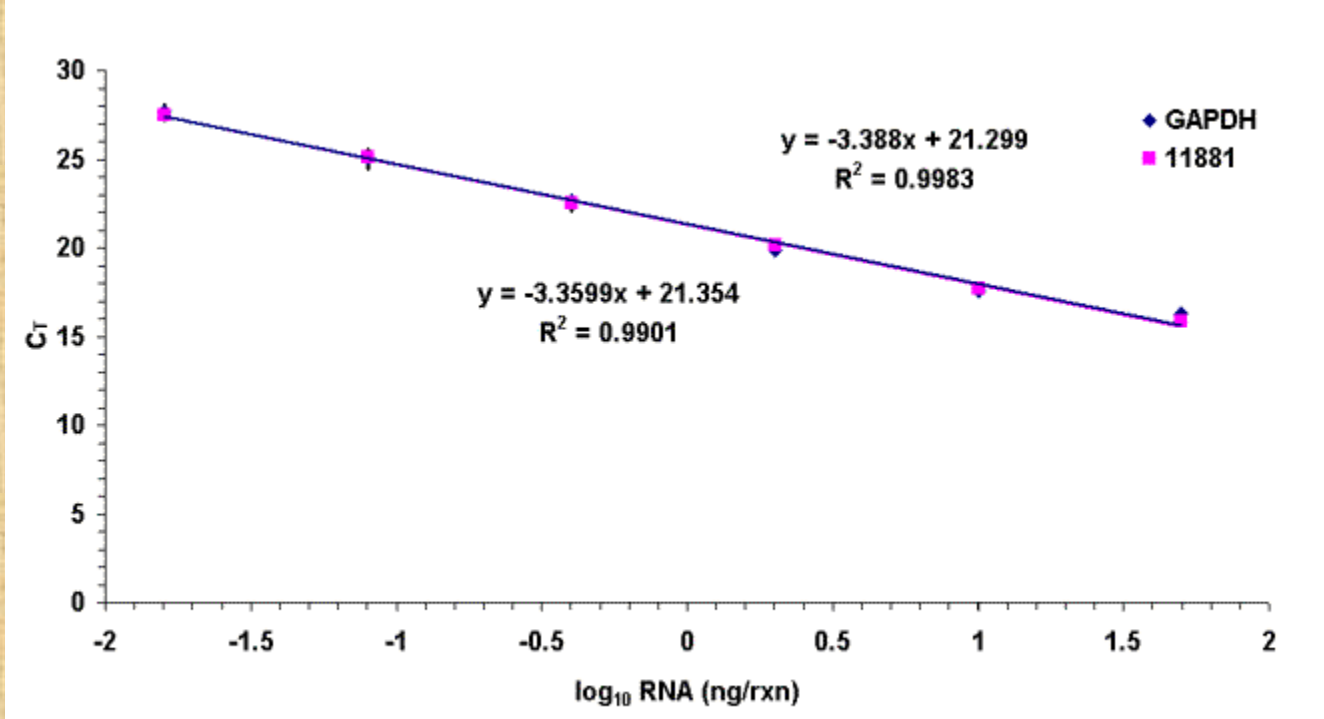
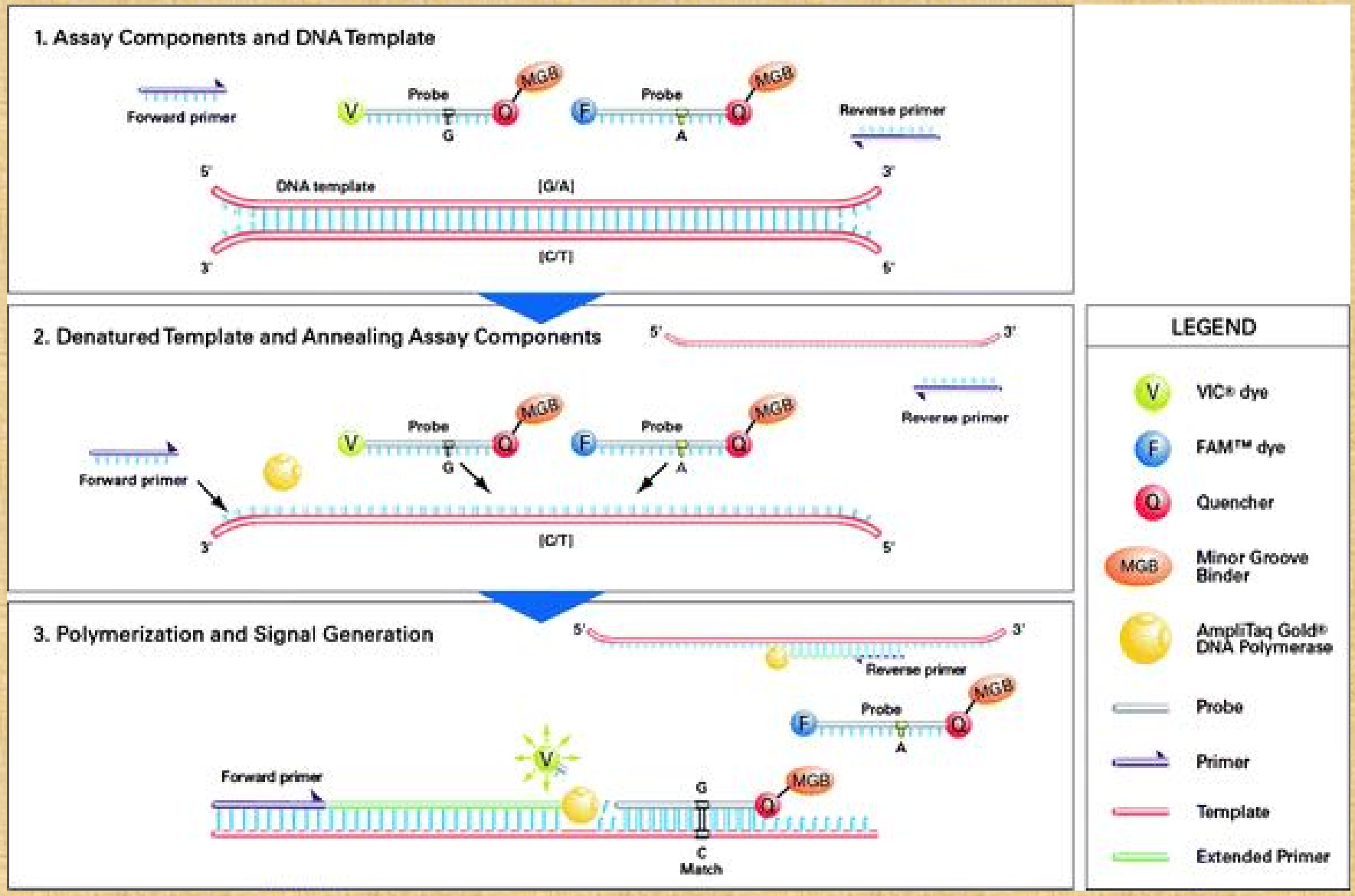
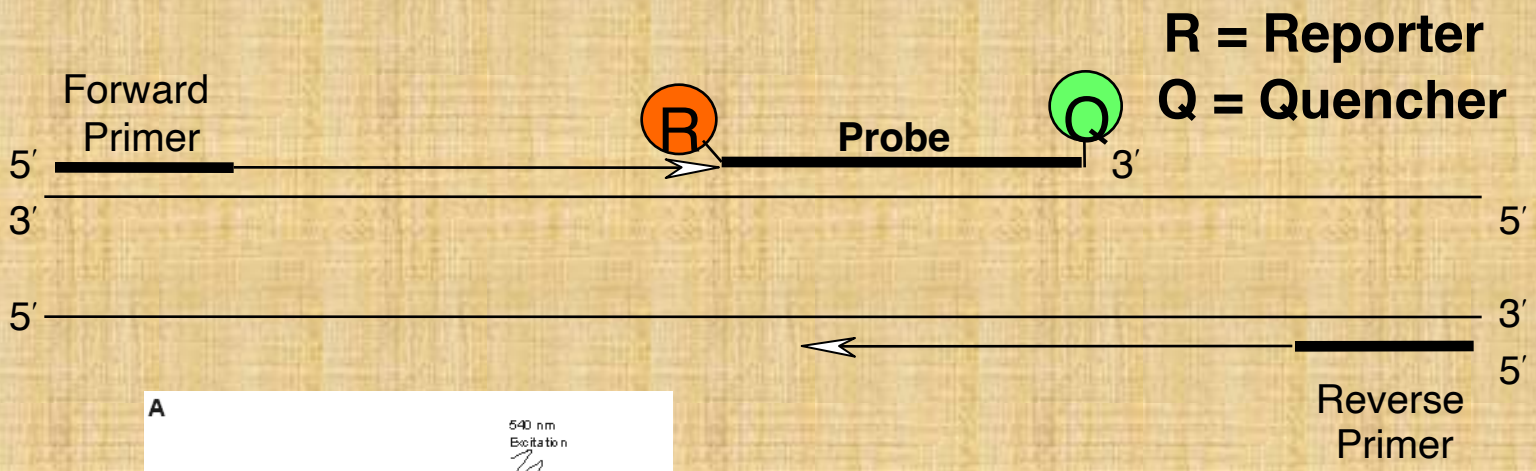


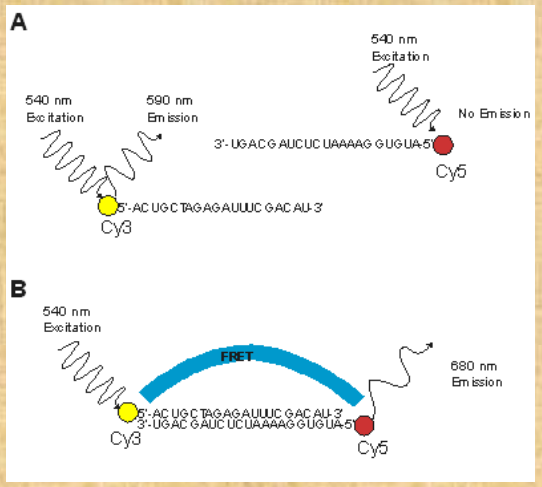
Figure 1: Standard Curves for Primers in Real Time Assay

VI. Taqman Assay (double dye probes) for SNP's Analysis



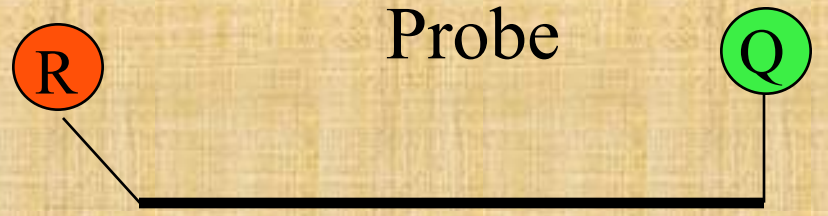
Probe Analysis (Taqman Assay)



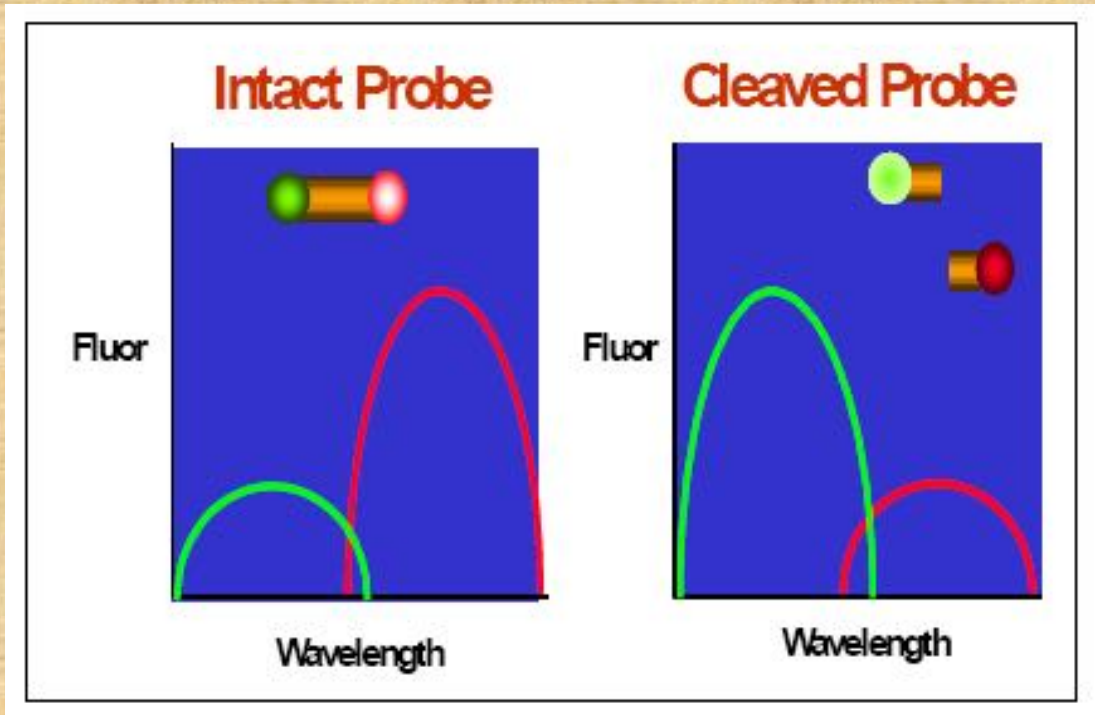


Fluorescent Resonance Energy Transfer (FRET) : a distance-dependent physical process by which **energy** is **transferred** nonradiatively from an excited molecular fluorophore (the donor) to another fluorophore (the acceptor)

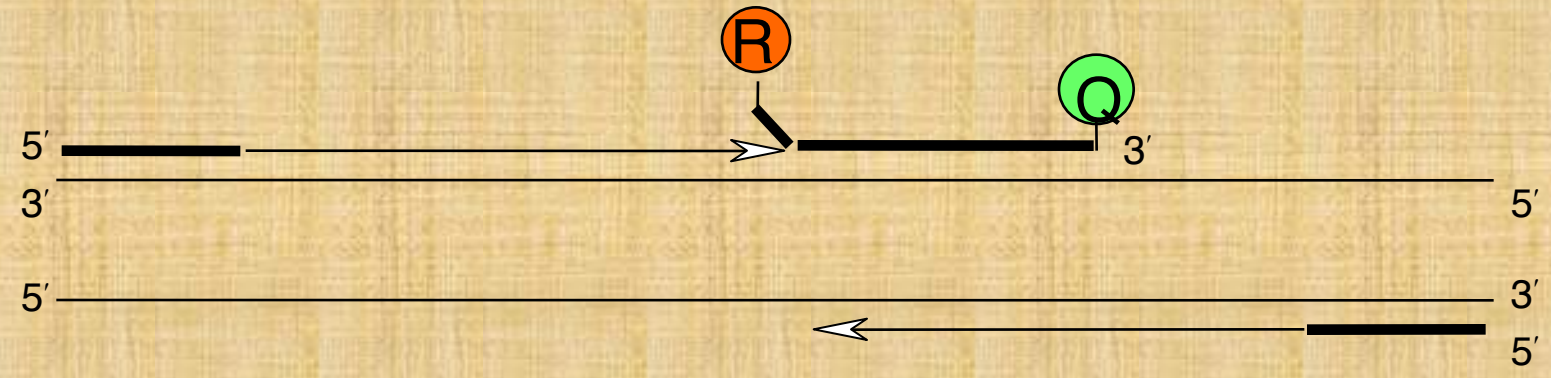
For Real Time PCR we need a a specific probe with a fluorescent reporter.



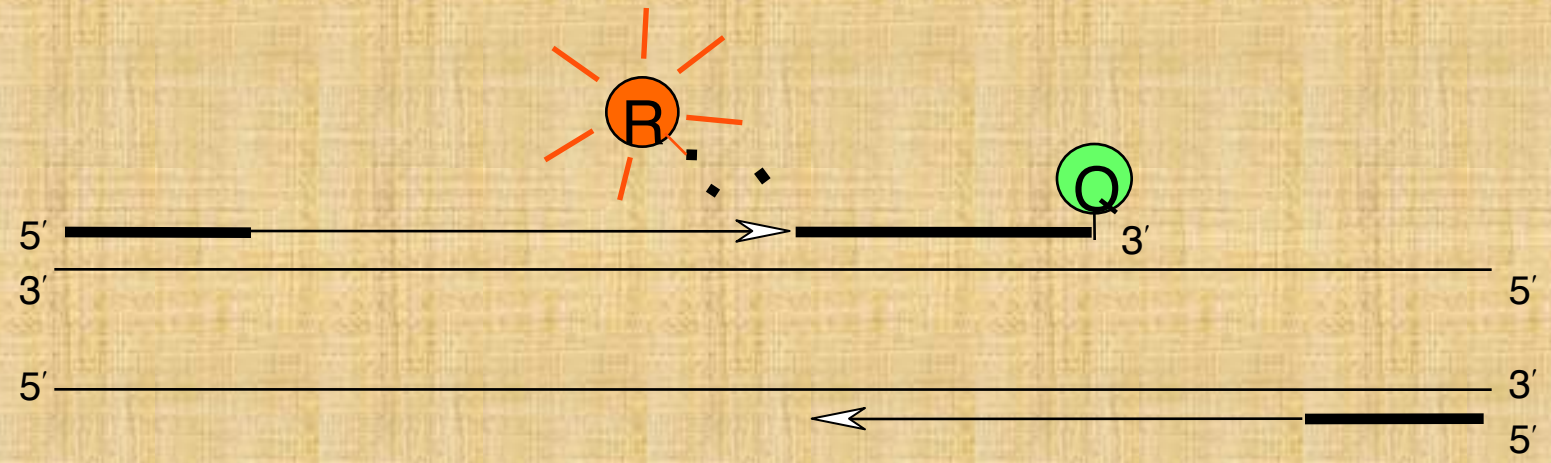
When in close contact with the reporter, the quencer absobes its emission.



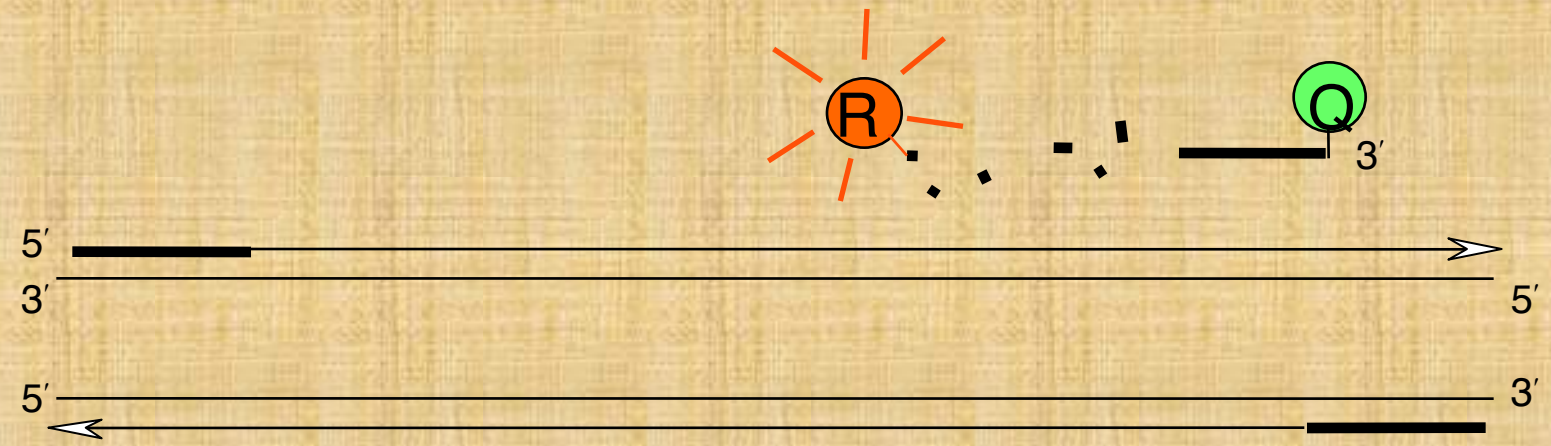
Strand Displacement



Cleavage



Polymerization Completed



Results of Taqman SNP's Analysis