

Dasar-Dasar Real-Time PCR

dr. Ahmad Hamim Sadewa, PhD

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Topik Bahasan

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

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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).

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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)

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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

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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.

BRCA1

DNA

mRNA

Protein

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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.

Virus RNA

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RT-PCR for diagnosis of SARS-CoV-2

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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

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Tantangan Analisis Real-time PCR

- * **Memerlukan skill yang tinggi**
- * **Harga alat mahal**
- * **Reagen mahal**
- **Sangat sensitif dengan kontaminasi***
- **Kalau rusak perlu waktu perbaikan lama***

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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))

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Perbandingan one step dan two steps RT PCR

	Advantages	Disadvantages
One-step	<ul style="list-style-type: none"> •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 	<ul style="list-style-type: none"> •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	<ul style="list-style-type: none"> •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 	<ul style="list-style-type: none"> •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

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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

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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.

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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.

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III. Sintesis cDNA

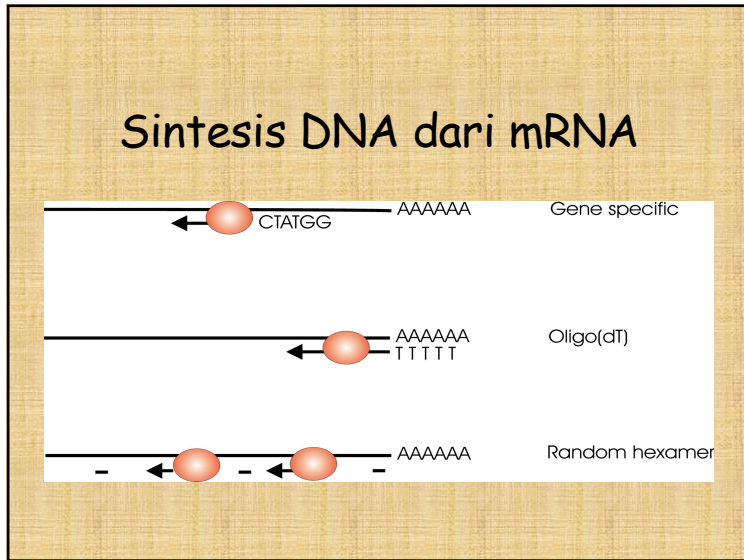
Sample atau specimen yang berupa RNA atau miRNA harus dikonversi menjadi DNA terlebih dahulu.

Reaksi ini adalah reaksi konversi, tidak ada amplifikasi

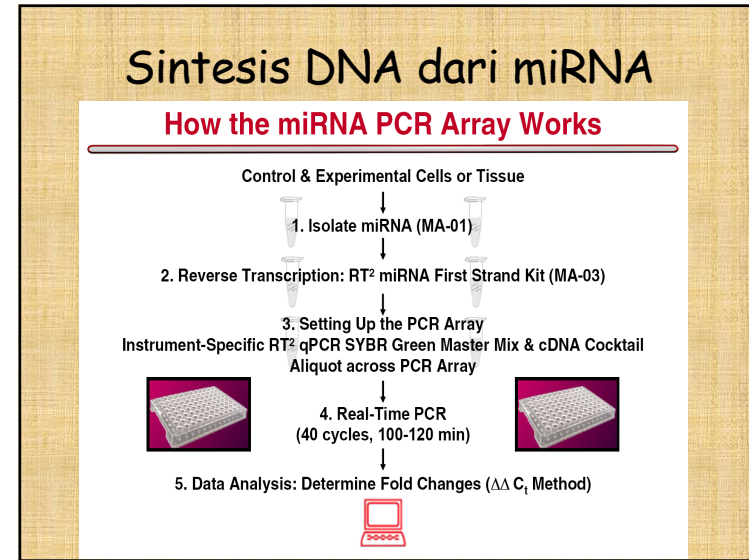
Bahan yang diperlukan adalah template RNA atau miRNA, primer oligo-dT, random primer, dNTP, buffer dan enzim Reverse Transcriptase.

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

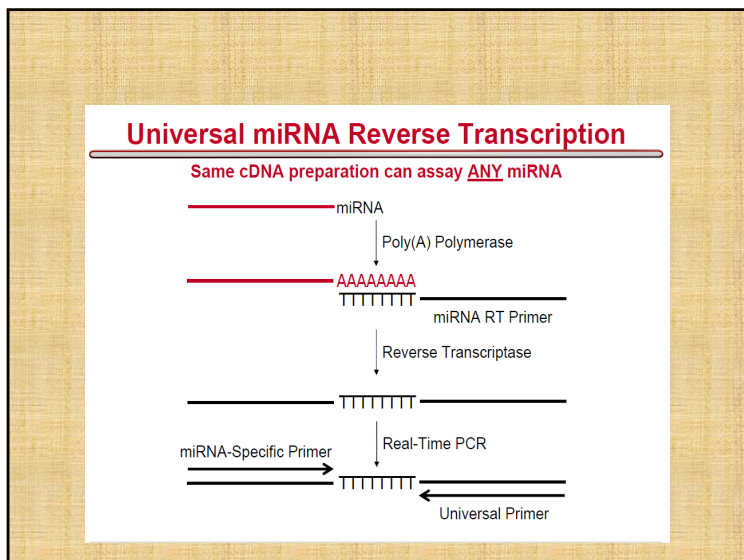
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Contoh protocol first strand cDNA synthesis

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	1-5 μ l
d(T) ₂₃ /VN (50 μ M)	2 μ l
nuclease-free H ₂ O	variable
Total Volume	8 μ l

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 μ l
M-MuLV Enzyme Mix	2 μ l

To the negative control tube, add the following:

M-MuLV Reaction Mix	10 μ l
H ₂ O	2 μ l

4. Incubate the 20 μ l 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 50°C for 5 minutes. Dilute reaction to 50 μ l with 30 μ l H₂O for PCR. The cDNA product should be stored at -20°C. For downstream PCR amplification, the volume of cDNA product should not exceed 1/10 of the PCR reaction volume.

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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 (1 μL))	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

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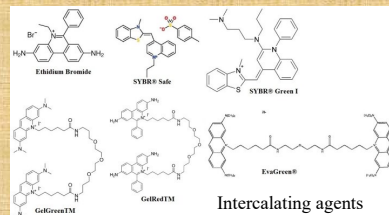
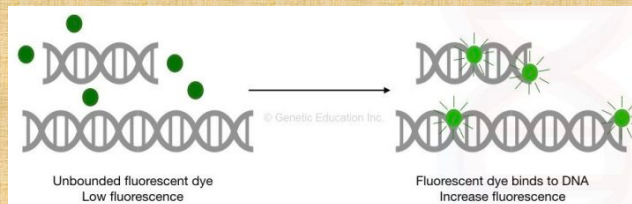
IV. Quantitative PCR

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).

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Double Strand DNA Binding Dyes



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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

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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.

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Contoh kondisi qPCR

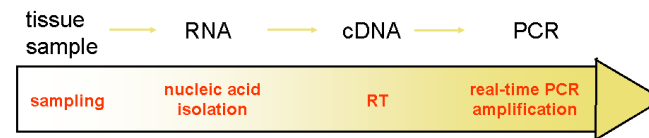
Program	PCR	SYBR Green-based real-time PCR
Pre-incubation	95°C, 6 min	95°C, 10 min
Amplification	35 cycles	35 cycles
	95°C, 30 s	95°C, 5 s
	62°C, 15 s	62°C, 10 s
	72°C, 30 s	72°C, 20 s (Single)
Melting	N/A ^a	95°C, 0 s
		65°C, 10 s
		95°C, 0 s
		0.2°C/s (Continuous)
Cooling	N/A ^a	40°C, 30 s

^a Not applicable

Ket. : Amplification disetting oleh peneliti
 Melting analysis dilakukan secara otomatis (default)

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



- | | | | |
|---|--|---|---|
| <p>Sampling method:</p> <ul style="list-style-type: none"> • Biopsy • Fixed material • Fresh blood • Tissue storage • Liquid Nitrogen • RNA Later • 1st extraction buffer • RNA storage -80°C ⇒ native RNA | <p>Extraction method:</p> <ul style="list-style-type: none"> • total RNA <ul style="list-style-type: none"> • mRNA • microRNA • liquid-liquid • columns • Robot vs. hand made • RNA integrity: <ul style="list-style-type: none"> • Bioanalyzer 2100 • Experion • Nano-Drop • mFold algorithm | <p>Efficiency of RT:</p> <ul style="list-style-type: none"> • RT enzyme type • RT temperature • Primers: <ul style="list-style-type: none"> • poly-T Primer • Random-hexamers • Specific primer • Primer mixtures • one-step qRT-PCR • two-step RT-qPCR | <p>PCR Efficiency / Specificity:</p> <ul style="list-style-type: none"> • Primer design <ul style="list-style-type: none"> • Primer specificity • Consensus Primer • mRNA abundance • RNA / cDNA input • Polymerase types • Polymerase Mixtures • PCR Inhibitors & Enhancers • Robot vs. hand made |
|---|--|---|---|

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Cycle Treshold (Ct) atau Cycle Quantification (Cq)

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

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

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Cycle Treshold (Ct) atau Cycle Quantification (Cq)

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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

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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
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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

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Imagine Real-Time PCR

started with different amount of DNA, calculate at particular cycle

Cycle 25 → 

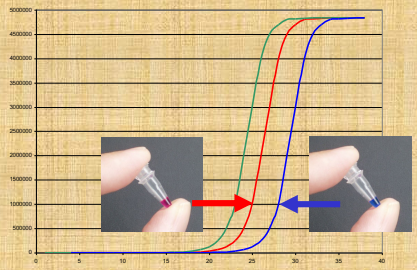
Cycle	1,000,000	125,000
25	1,000,000	125,000
26	2,000,000	250,000
27	4,000,000	500,000
28	8,000,000	1,000,000

Measuring Quantities

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Imagining Real-Time PCR

Measuring Quantities



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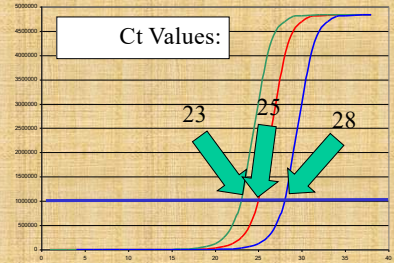
Imagining Real-Time PCR

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:

$$\text{Quantity} = 2^{\text{Ct}}$$

Measuring Quantities



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Imagining Real-Time PCR

Measuring Quantities



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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

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

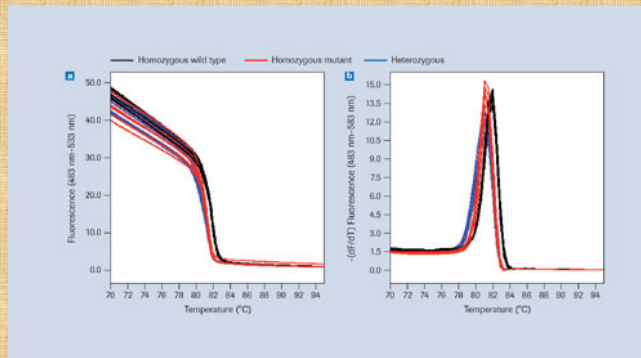
Kurva leleh adalah analisis post PCR untuk mengetahui spesifitas produk PCR (amplikon) berdasar karakter leleh (karakter denaturasi). Satu jenis amplikon akan menghasilkan 1 jenis kurva leleh dan puncak leleh.

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.

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

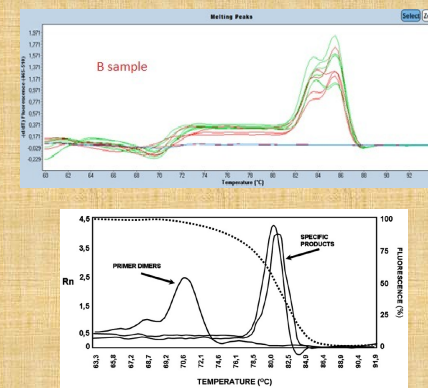


Diperlukan optimasi yang intensif untuk mendapat hasil spesifik.

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Contoh amplikon yang tidak spesifik



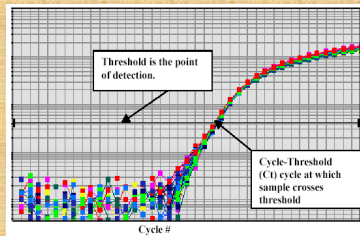
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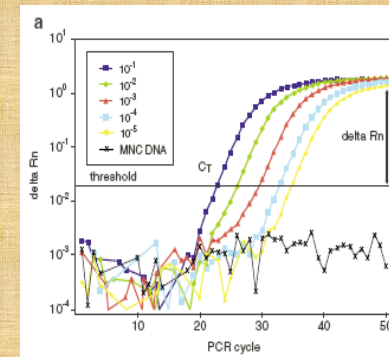
V. Analisis Data qPCR

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



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•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

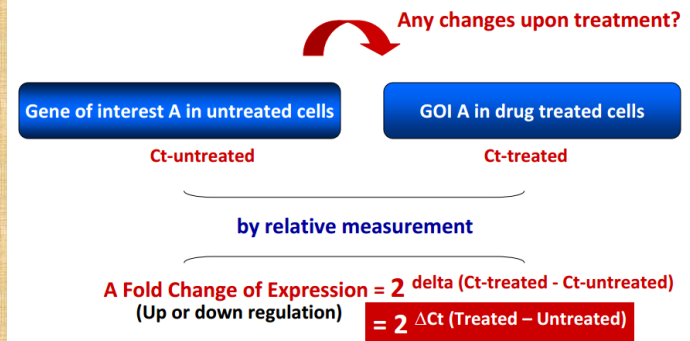
- Mathematical determination
- Calibrator sample used as a 1x standard
- Best used when particular ratios are expected or to verify trends



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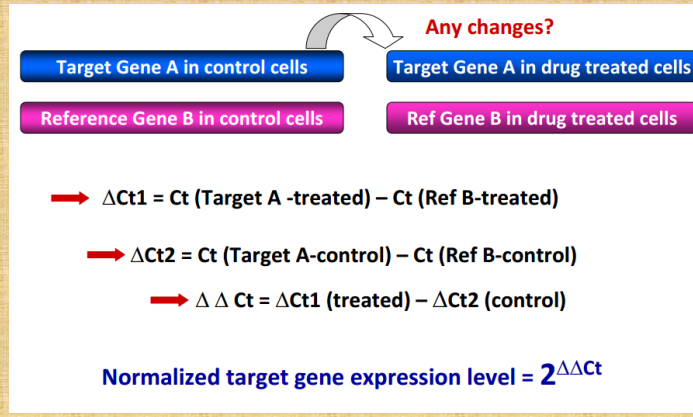
Quantification

Gene Expression Profiling Analysis Focused on Changes in Gene Expression Across Samples



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Quantification



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Metode Livak

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

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

Hasil perhitungan adalah fold change (perubahan kelipatan)

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Sample	Housekeeping gene			Gene of Interest			ΔCt	$\Delta \Delta Ct$	$2^{-\Delta \Delta Ct}$
	Ct1	Ct2	Average Ct	Ct1	Ct2	Average Ct			
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		

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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:

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

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

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

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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, qPCR)

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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 possible 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

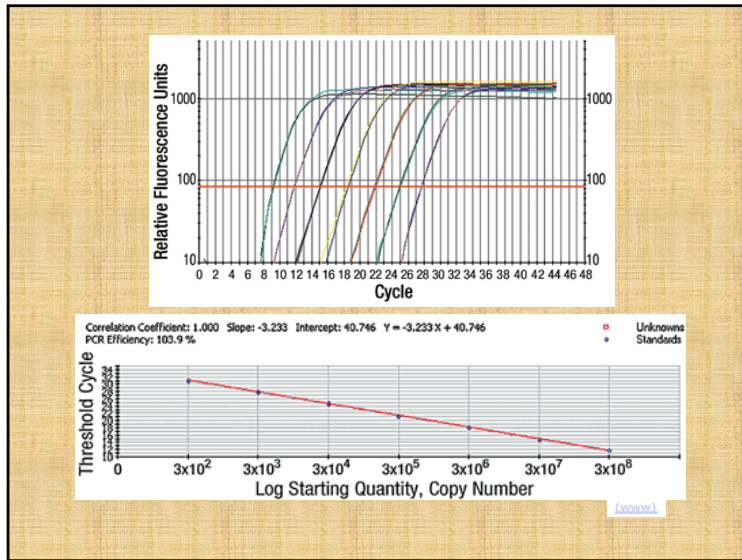
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Internal standards = housekeeping genes

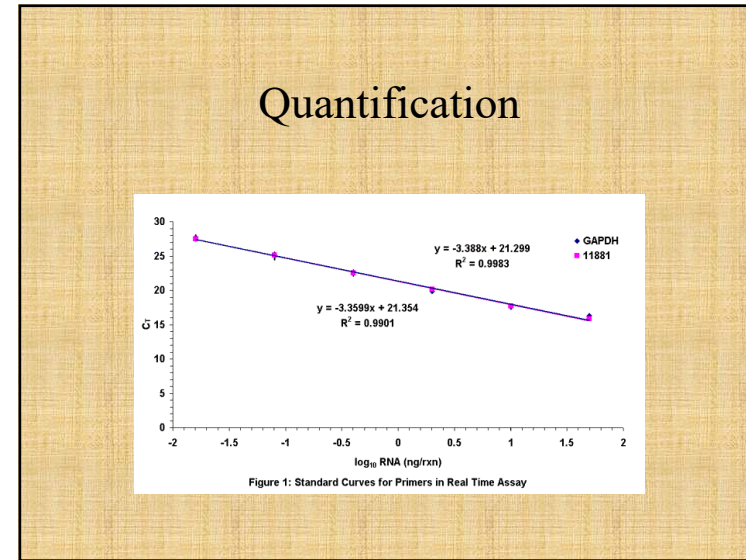
- Commonly used standards
 - Glyceraldehyde-3-phosphate dehydrogenase mRNA
 - Beta-actin mRNA
 - MHC I (major histocompatibility complex I) mRNA
 - Cyclophilin mRNA
 - 28S or 18S rRNA
 - Untuk miRNA : U6RNA

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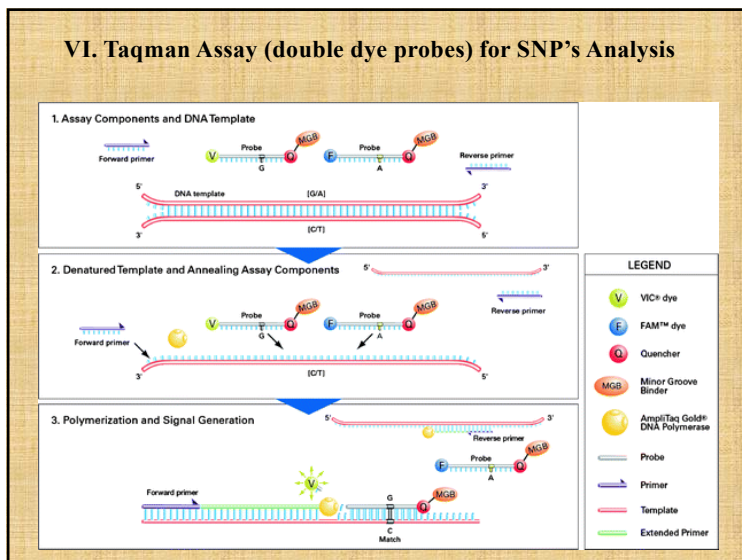
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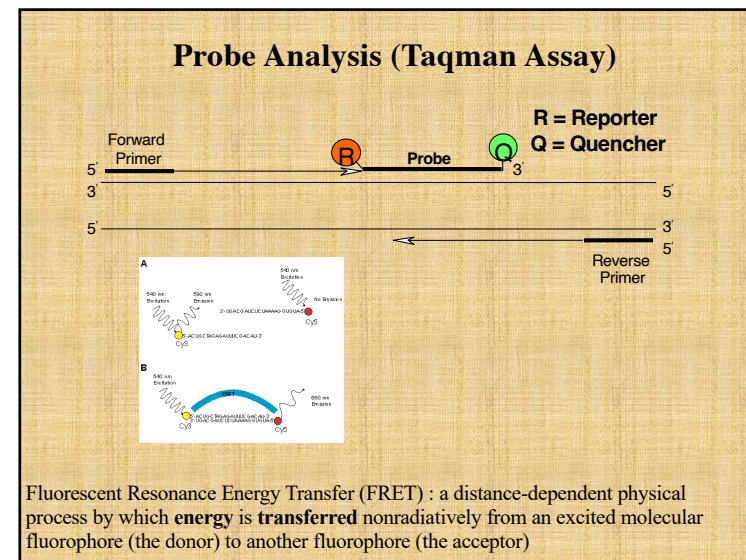
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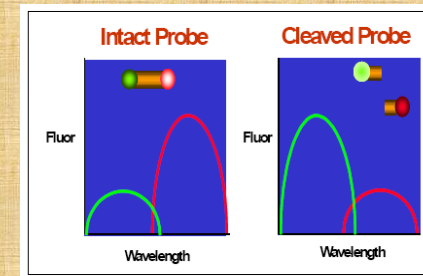
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For Real Time PCR we need a specific probe with a fluorescent reporter.



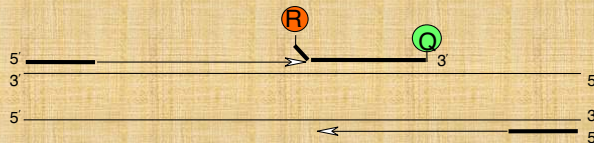
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When in close contact with the reporter, the quencher absorbs its emission.



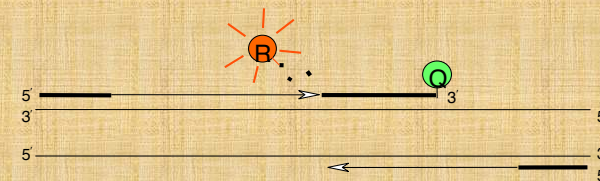
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Strand Displacement

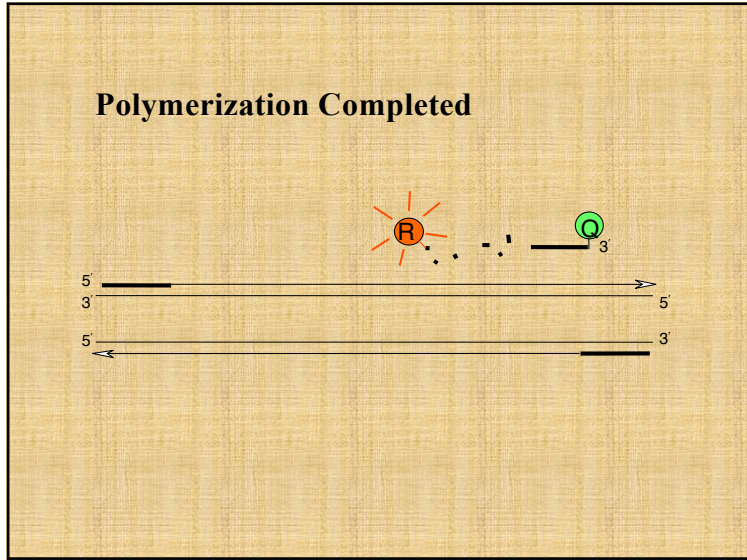


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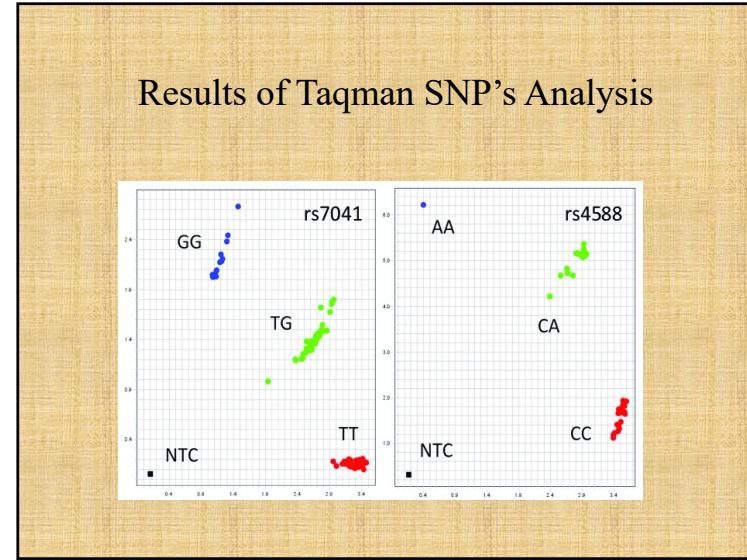
Cleavage



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