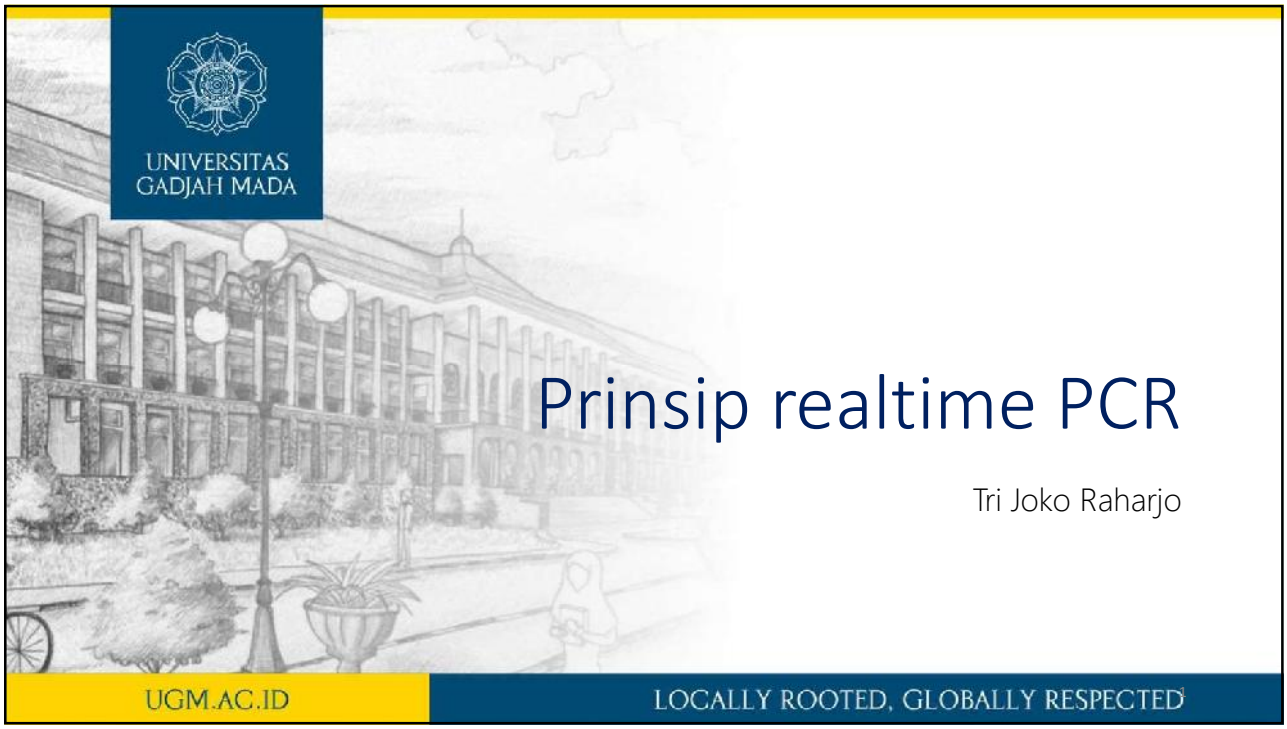

PCR and RT-PCR

PCR, or the Polymerase Chain Reaction, is a process for the amplification of specific fragments of DNA → tell us what

Real-Time PCR a specialized technique that allows a PCR reaction to be visualized "in real time" as the reaction progresses → tell us how much

As we will see, Real-Time PCR allows us to measure minute amounts of DNA sequences in a sample!

2

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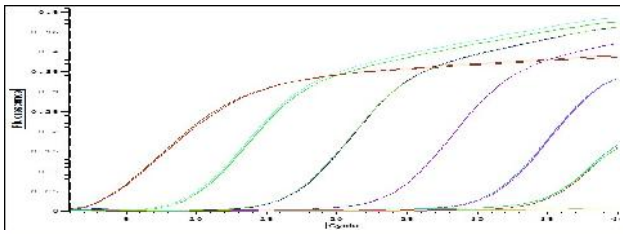
Definition

Real-time PCR is the continuous collection of fluorescent signal from one or more polymerase chain reactions over a range of cycles.

Quantitative real-time PCR is the conversion of the fluorescent signals fromeach reaction into a numerical value for each sample



PCR amplification of DNA

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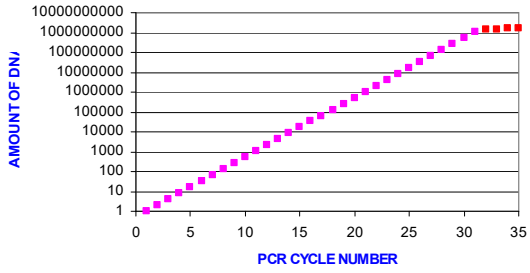

CYCLE #	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,550,000,000
34	1,600,000,000

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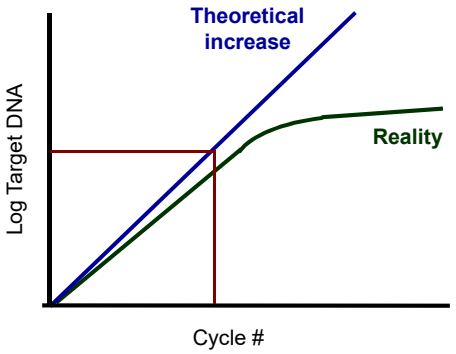
PCR, Theory vs reality

- In theory, PCR reactions amplify exponentially, doubling every cycle and grow forever.
- In reality, the reactant for polymeration is limited



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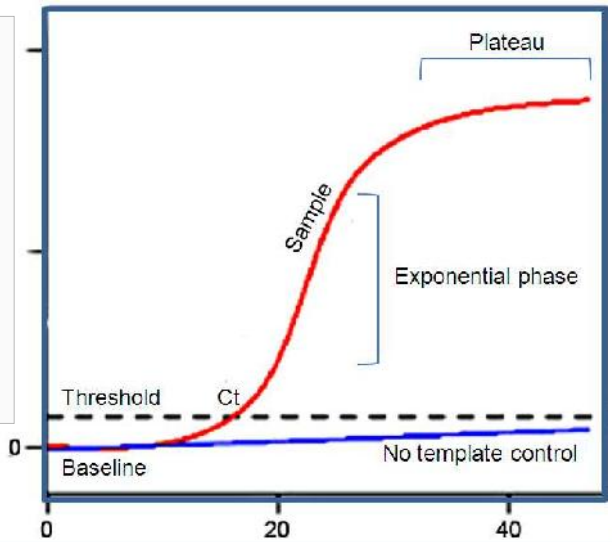
Threshold Cycle

- **threshold cycle 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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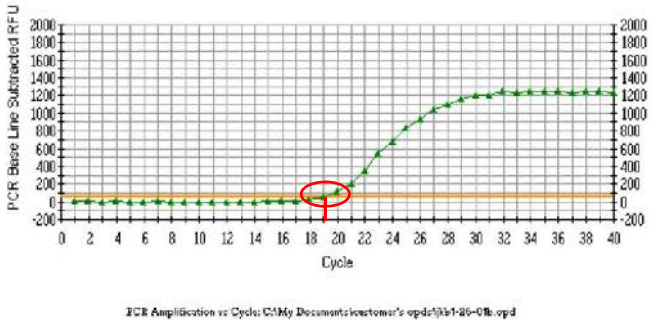
Threshold Cycle, C_T

The point at which the fluorescence rises appreciably above background



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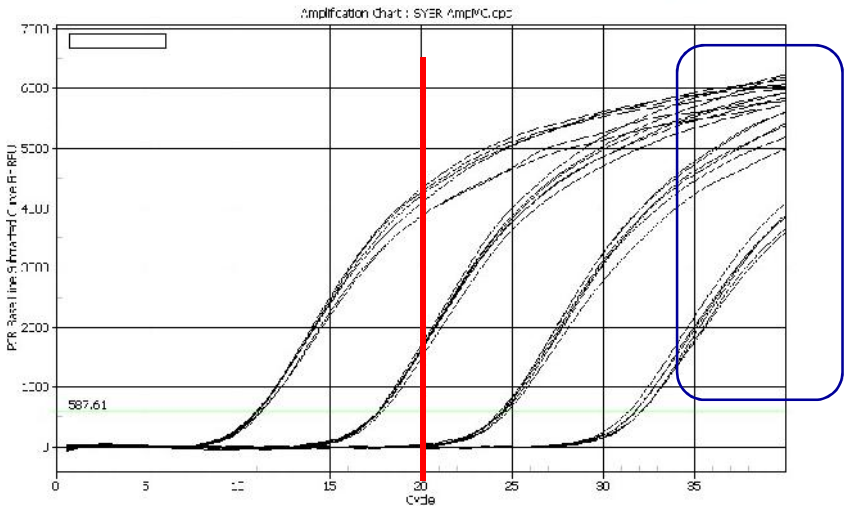

100 fold dilution series

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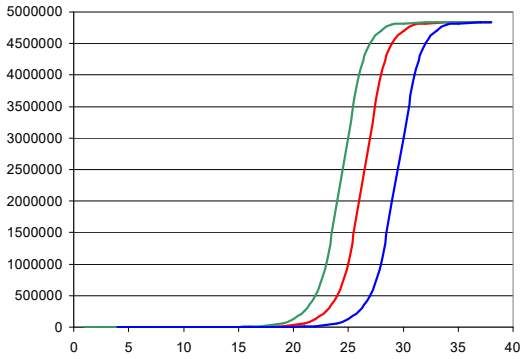
We can easily see that the left-right shift in the curves is related to the starting quantity of DNA!

Cq ("Cycle Quantity) values identify the curve positions, based on where they cross a threshold.

DNA Quantity and Cq value are related as:

$$\text{Quantity} \sim 2^{Cq}$$



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How do we detect and measure DNA?

1. Non-specific fluorescent dyes:
- intercalation with any dsDNA
- SYBR green, EVAGREEN

2. **Sequence specific primers & DNA probes**:
- oligonucleotides labeled with fluorescent reporter

(a) Hydrolisis probe: TaqMan, Molecular Beacon, Scorpion

(b) Hybridizing probe: FRET

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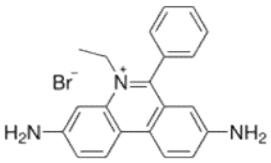
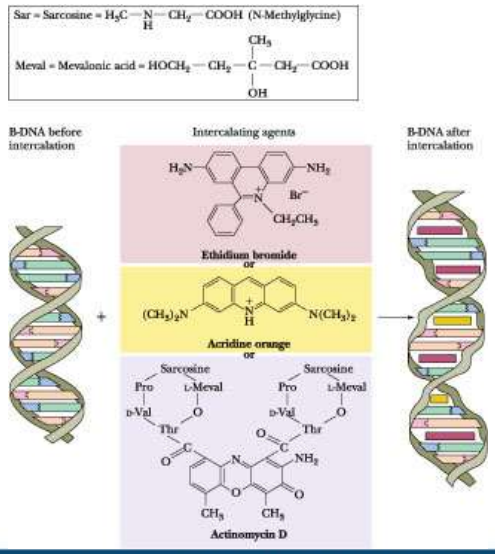
Ethidium Bromide

+ = common and well known

- = toxic, not very bright





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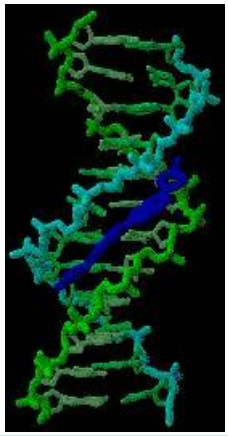
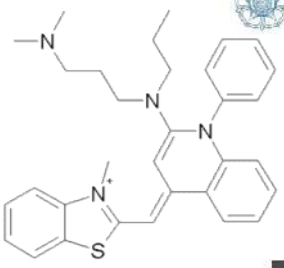
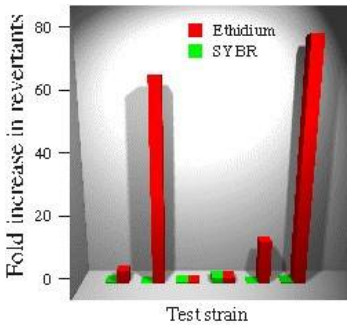
SYBR Green I

+ = Bright fluorescence!

+ = Low toxicity!







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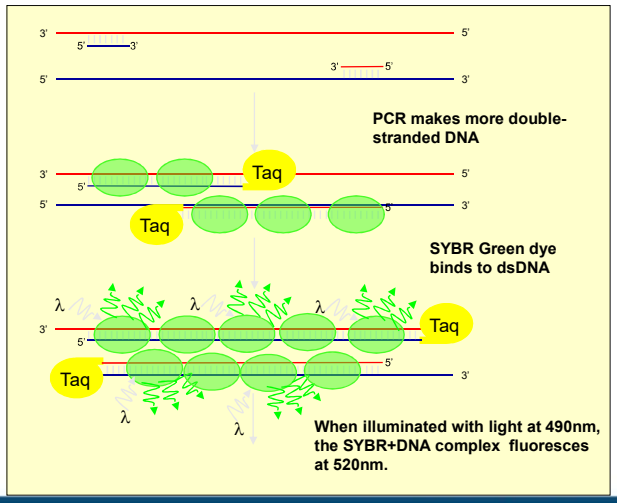
SYBR Green in Action



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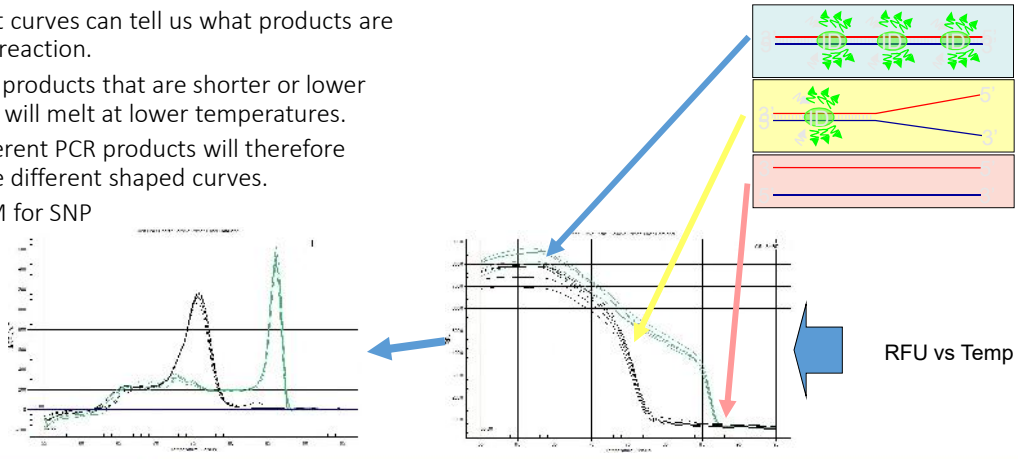
The advantage of SYBR Green

- Melt curve analysis
  - Melt curves can tell us what products are in a reaction.
  - PCR products that are shorter or lower G+C will melt at lower temperatures.
  - Different PCR products will therefore have different shaped curves.
  - HRM for SNP



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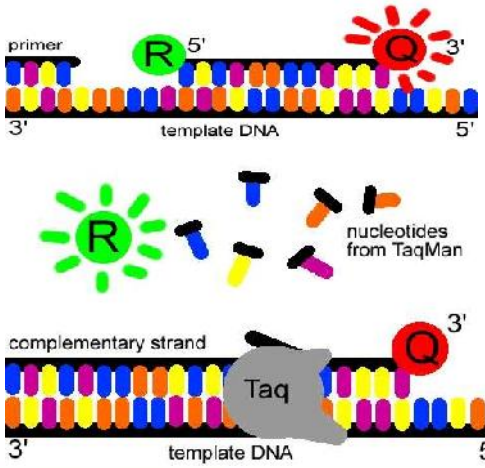
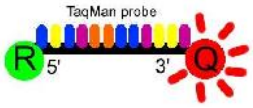
TaqMan **PROBE**





R=reporter
Q= quencher

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Detecting Multiple dyes

Multiplexing from an instrumen perspective

Dye	Excitation(nm)	Emission(nm)	
SYBR	497	520	
FAM	495	520	1
TET	521	536	2
JOE	520	548	
VIC		~555	
HEX	535	556	
R6G	524	557	
Cy3	550	570	3
TAMRA	555	576	
NED		~576	
Cy3.5	581	596	4
ROX	575	602	5
Texas Red	583	603	
Cy5	649	670	6
Cy5.5	675	694	7

Singleplex - FAM
Duplex- FAM, VIC
Triplex - FAM,VIC,

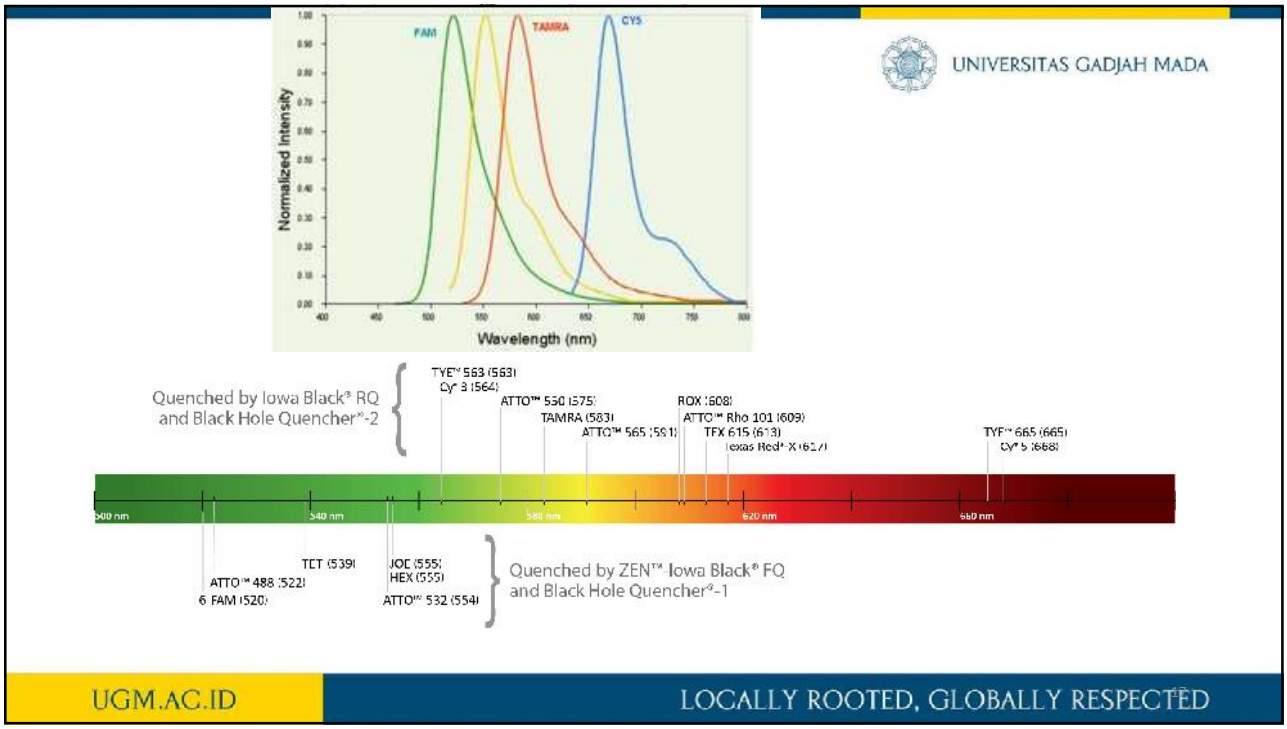
Choosing a Flourophore & Quencher

Fluorophores:

- FAM
- TET, VIC
- HEX
- JOE
- CY3
- TAMRA
- CY3.5, Redmond Red
- Texas Red, ROX
- CY5 LC640
- CY5.5 LC705

Quenchers:

- BHQ-1
- DABCYL
- Eclipse
- TAMRA
- QSY-7
- BHQ-2
- BHQ-3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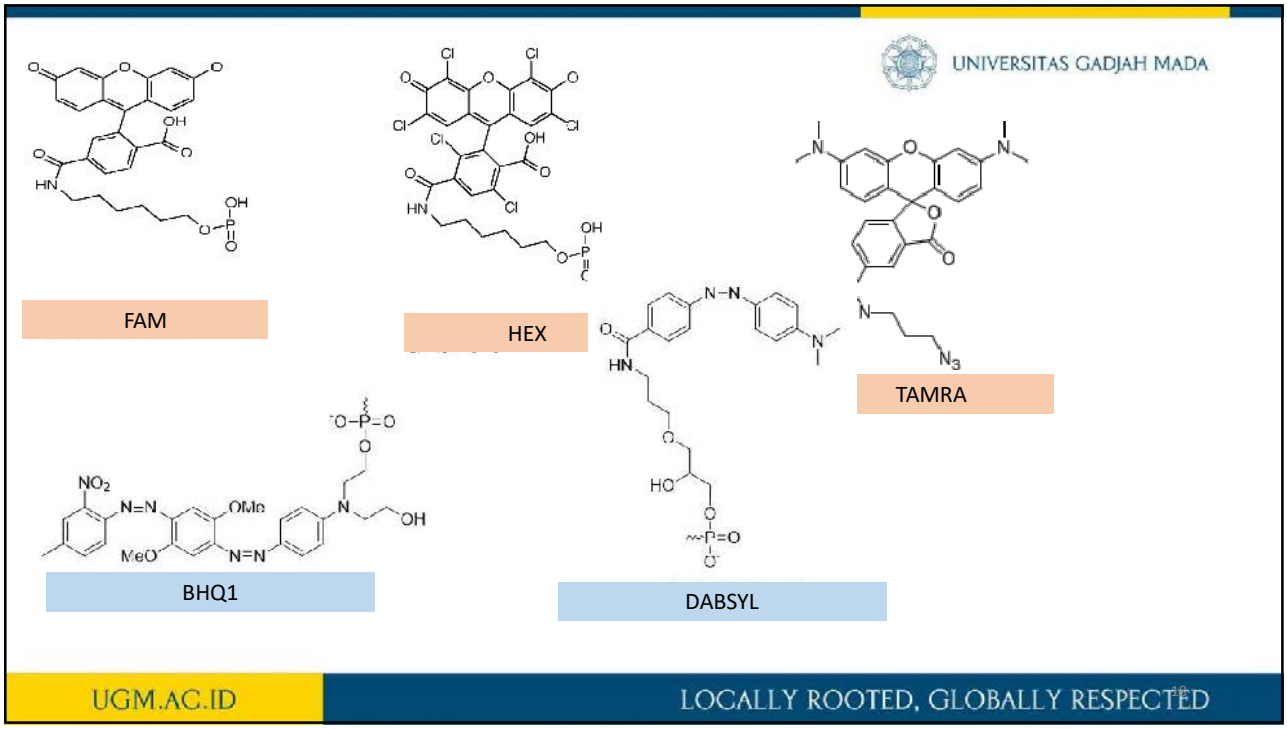
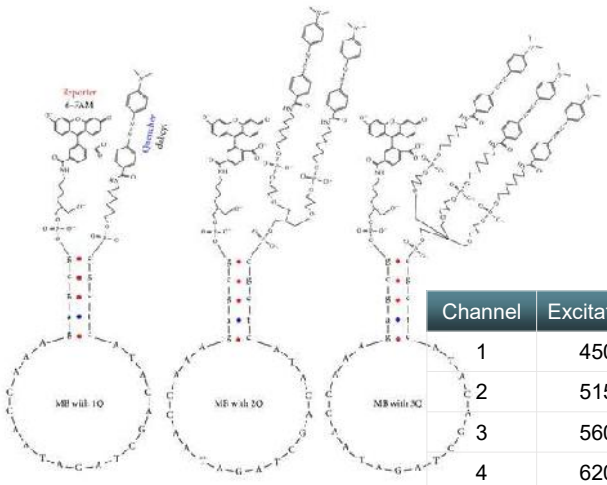
FAM
TAMRA
CY5
Normalized Intensity
Wavelength (nm)
Quenched by Iowa Black® RQ and Black Hole Quencher™-2
TYE™ 563 (563)
Cy® 3 (564)
ATTO™ 530 (575)
TAMRA (583)
ATTO™ 565 (591)
ROX (608)
ATTO™ Rho 101 (609)
TFX 615 (613)
Texas Red® X (617)
TYE™ 665 (665)
Cy® 5 (668)
500 nm
540 nm
580 nm
620 nm
660 nm
TET (539)
JOE (555)
HEX (555)
ATTO™ 488 (522)
6-FAM (520)
ATTO™ 532 (554)
Quenched by ZEN™, Iowa Black® FQ and Black Hole Quencher®-1
UGM.AC.ID
LOCALLY ROOTED, GLOBALLY RESPECTED


UNIVERSITAS GADJAH MADA
FAM
HEX
TAMRA
BHQ1
DABSYL
UGM.AC.ID
LOCALLY ROOTED, GLOBALLY RESPECTED

UNIVERSITAS GADJAH MADA



Molecular beacon

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

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Other system

- Dual hybridization probes (FRET)
- Eclipse probes
- Amplifluor assays
- Scorpions PCR primers
- LUX PCR primers
- QZyme PCR primers

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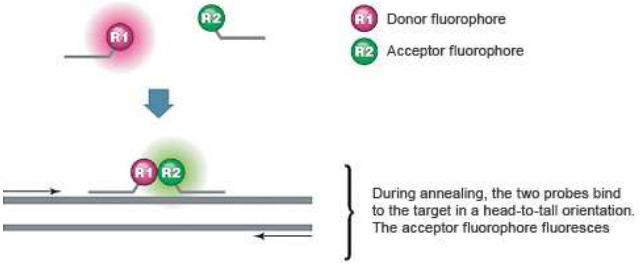
FRET



- Use two sequence-specific oligonucleotide probes designed to bind to adjacent sequences in the target
- The probes are labeled with a pair of dyes that exhibit fluorescence resonance energy transfer (FRET). The donor dye is attached to the 3' end of the first probe, while the acceptor dye is attached to the 5' end of the second probe.
- At the annealing step, the probes hybridize to their target sequences in a head-to-tail arrangement. This annealing brings the donor and acceptor dyes into proximity, allowing FRET to occur, resulting in fluorescent emission by the acceptor.
- The increasing amount of acceptor fluorescence is proportional to the amount of PCR product present.

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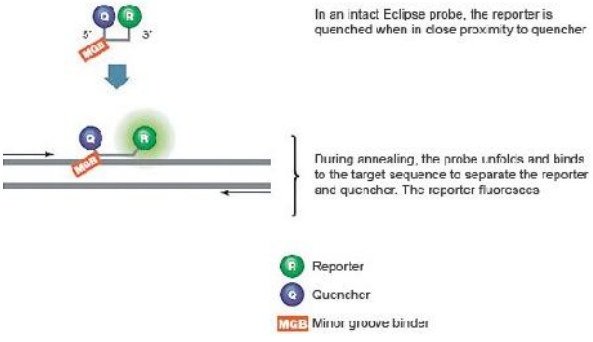
Eclipse probes



- The Eclipse probe is complementary to a sequence within the target and has a fluorescent reporter molecule at the 3' end combined with a quencher and a DNA minor-groove binder at the 5' end.
- The unhybridized probe is quenched.
- During the annealing the probe thus becomes linearized, resulting fluorescence signal is proportional to the amount of amplified product in the sample.

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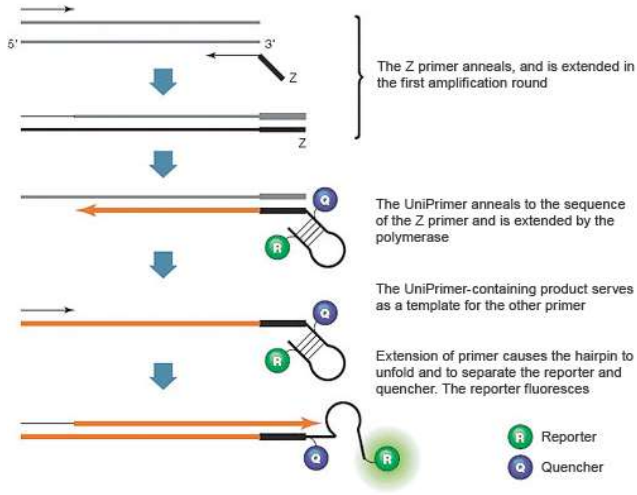
Amplifluor assays



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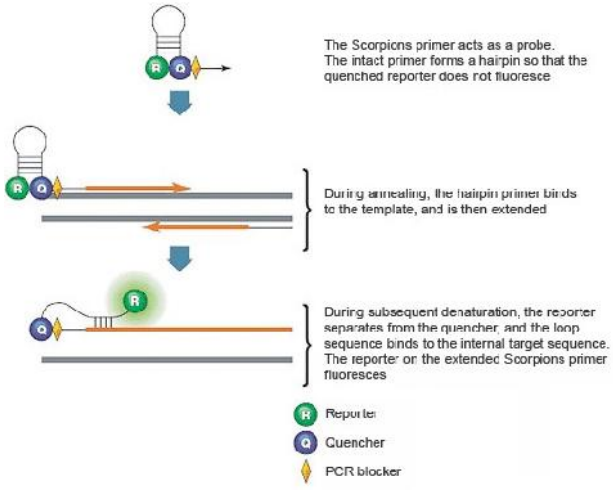
Scorpions PCR primers



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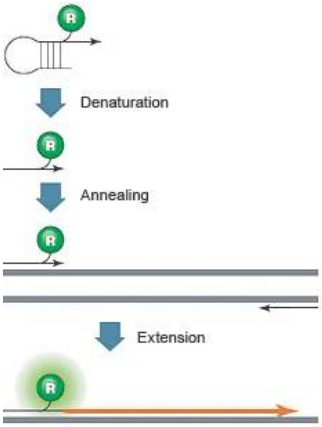
LUX PCR primers



The LUX primer forms a hairpin so that the quenched reporter does not fluoresce

The primer becomes single-stranded

During extension, the primer is extended, and the reporter fluoresces in the double-stranded DNA product

R Reporter

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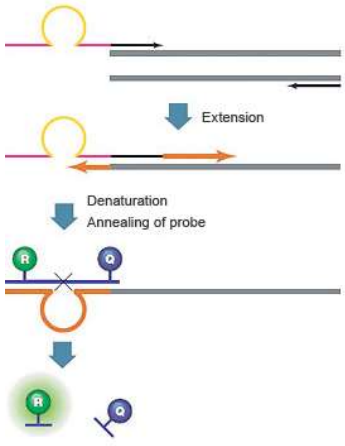
QZyme PCR primers



The Qzyme primer encodes the catalytic DNA. During annealing, the Qzyme primer anneals to the target

During extension, the catalytic DNA is made

During the next annealing step, the catalytic DNA binds the probe and cleaves the reporter from the quencher

The free reporter fluoresces

R Reporter
Q Qunecher

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Designing primer and probe guideline

Primer specificity

- Any gene of sequence of a species could be as target of primer design
- Single copy gene represent (haploid genome equivalent-HPE)
- Non-specific amplification is one of the greatest challenges → prevent mispriming
- comparing sequence similarity between the primers and all other template sequences in the design space (software available)

Primer length

- 17–22 nucleotides long
- Too short lead to non specific primer
- Too long to secondary structure

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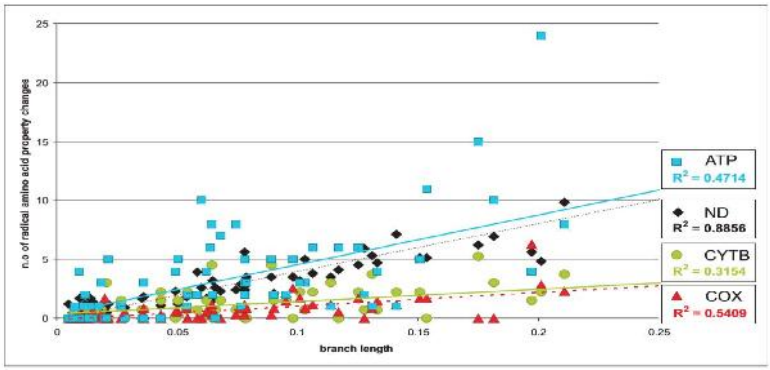
An example targeting a gene



ATPase genes allow the discrimination of even closely related species, hence, allowing ATPase genes to have sufficient degree of intra-species regions and inter-species polymorphism which simplified the species detection procedure

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Designing primer and probe guideline (Cont)

Primer GC content

- 35% to 65%
- Too low → poor annealing
- Too high → mispriming

Primer 3' end stability (entrophy calculation)

- To stabile → mispriming

Primer sequence complexity

- Low-complexity sequences should be discarded → mispriming, difficult to synthesis

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Designing primer and probe guideline

Primer melting temperature $\mathrm{Tm} = 4 * (G + C) + 2 * (A + T).$

- forward and reverse primers should have similar Tm values

Primer location in the sequence

- Importance for expression study

Amplicon size

- Typically the size range is 60–150 bp
- Affect the efficiency

Primer and template sequence secondary structure

- secondary structures can retard primer annealing

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Taqman Probe

- The probe Tm should be ~10ºC higher than the primers
- Do not put G at the 5’ end of the probe as this will quench reporter fluorescence
- GC content should be 35–65%
- The probe should not self-anneal to form secondary structure
- The probe should be selected from gene-specific regions (the primer could be general)
- The probe should be as close to the forward and reverse primers as possible, without overlapping the primer sequences

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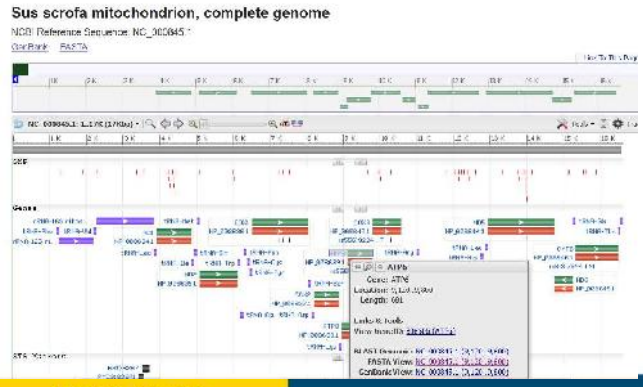
Example: Design of porcine (*Sus scrofa*) specific TaqMan probe and primers

Sus scrofa mitochondrion, complete genome (NC_000845)

http://www.ncbi.nlm.nih.gov/



Sus scrofa mitochondrion, complete genome

NCBI Reference Sequence: NC_000845.1

GenBank Graphics

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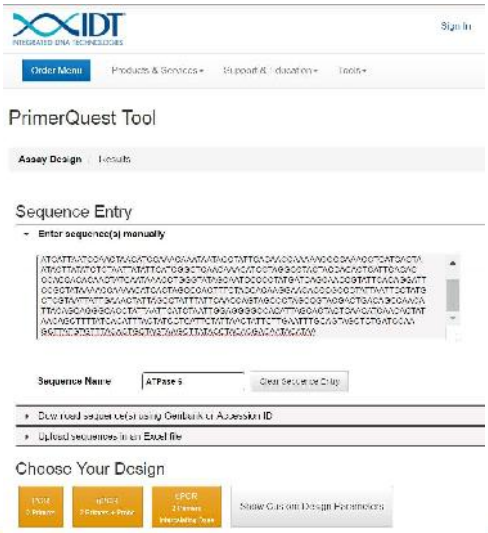
http://sg.idtdna.com/PrimerQuest



The design parameters:
amplicon size : 70 – 150 bp

- Primers
- size : 18 – 30 bp
- the melting temperature: 59 – 65 ℃
- GC content : 35 – 60 %
- Probe
- size : 20 – 30 bp
- the melting temperature : 64 – 72 ℃
- GC content : 40 – 60 %.

List of primers and probe set

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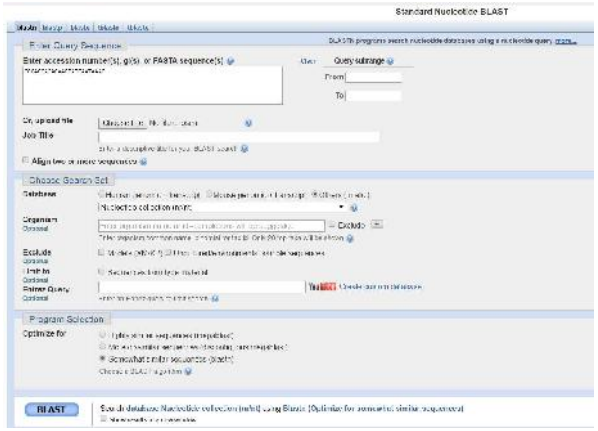
Primers and probe set



http://blast.ncbi.nlm.nih.gov/Blast.cgi

% homology of the primers and probe to species

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Microorganism	Target gene	Application	Test characteristics	Food matrix
qPCR studies				
Salmonella spp.	*invA*	Detection	Enrichment – qPCR – TaqMan®, IAC[a] DL[b]: ≤2.5 CFU/25 g salmon and minced meat, 5 CFU/25 g chicken meat, 5 CFU/25 ml milk	Artificially contaminated chicken meat, minced meat, salmon, raw milk
Salmonella spp.	*invA*	Detection	Enrichment – qPCR – LightCycler® hybridization probes, IAC DL: <5 cells/25 g	Artificially contaminated fish, minced beef, raw milk Naturally contaminated raw milk and meat
Salmonella spp.	*invA*	Detection	Enrichment – qPCR – TaqMan® DL: 0.08 or 0.2 CFU/g (24 h-enrichment or 48 h enrichment)	Artificially contaminated mashed potatoes, soft cheese, chilli powder, chocolate, eggs, sprouts, apple juice, fish, shrimp, ground beef, ground chicken
Salmonella spp.	*sstA*	Detection	Enrichment – qPCR – TaqMan®, IAC DL: 1–10 CFU/cm^2	Artificially contaminated fresh meat carcasses
Salmonella spp.	*iagA*	Detection	Enrichment – qPCR – Molecular Beacon DL: 4 CFU/25 g	Artificially contaminated cantaloupe, mixed-salad, cilantro, alfalfa sprouts
Salmonella enterica	*invA*	Detection	Enrichment – qPCR – TaqMan® DL: <3 CFU/25 g	Artificially contaminated chicken carcass rinses, ground beef, ground pork, raw milk Naturally contaminated chicken carcass rinses, raw milk
Salmonella enterica	*ssaN*	Detection	Enrichment – qPCR – TaqMan®, IAC DL: 1 CFU/10 g	Artificially contaminated chicken, liquid egg, peanut butter

Food Microbiology, 2011, 28, (5) : 848-861

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Microorganism	Target gene	Application	Test characteristics	Food matrix
qPCR studies				
Salmonella spp.	*invA*	Detection	Enrichment – qPCR – TaqMan®, IAC[a] DL[b]: ≤2.5 CFU/25 g salmon and minced meat, 5 CFU/25 g chicken meat, 5 CFU/25 ml milk	Artificially contaminated chicken meat, minced meat, salmon, raw milk
Salmonella spp.	*invA*	Detection	Enrichment – qPCR – LightCycler® hybridization probes, IAC DL: <5 cells/25 g	Artificially contaminated fish, minced beef, raw milk Naturally contaminated raw milk and meat
Salmonella spp.	*invA*	Detection	Enrichment – qPCR – TaqMan® DL: 0.08 or 0.2 CFU/g (24 h-enrichment or 48 h-enrichment)	Artificially contaminated mashed potatoes, soft cheese, chilli powder, chocolate, eggs, sprouts, apple juice, fish, shrimp, ground beef, ground chicken
Salmonella spp.	*sstA*	Detection	Enrichment – qPCR – TaqMan®, IAC DL: 1–10 CFU/cm^2	Artificially contaminated fresh meat carcasses
Salmonella spp.	*iagA*	Detection	Enrichment – qPCR – Molecular Beacon DL: 4 CFU/25 g	Artificially contaminated cantaloupe, mixed-salad, cilantro, alfalfa sprouts

Sequences of primers and probes used in design and development of the *sstA Salmonella* assay.

Name	Sequence	Size (bp)	T_m (°C)
Entero-tm F	5′-GCGGCTGATTCTGCATTCGA-3′	20	63.6
Entero-tm R	5′-TGGTGGAGCTGGCGGGA-3′	17	67.5
FPF	5′-CCTCGTAAAAAGCCGCA-3′	17	59.1
FPR	5′-GAGTTGAACCCGCGTC-3′	16	59.0
SAM 1	FAM-CAAACGACGAAACCTACGCTTTAGC-BBQ	25	68.8
SAM 2	FAM-AGACTAGCCTGATTCGTTTTAACGCT-BBQ	26	68.6
IAC-Entero	ROX-TCAAACCCAAAAGAGATCCCGTGGA-BHQ2	25	68.9

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Listeria monocytogenes	*prfA*	Detection	Enrichment + qPCR – TaqMan®, IAC DL: 7.5 CFU/25 ml milk, 9 CFU/15 g salmon, 1 CFU/15 g pâté and cheese	Artificially contaminated raw milk, salmon, paté, green-veined cheese Naturally contaminated fish, meat, meat products, and dairy products
Listeria monocytogenes and other species	*ssrA*	Detection	Enrichment + qPCR – hybridization probes, IAC DL: 1–5 CFU/25 g	Artificially contaminated soft cheese, meat, milk, vegetables (coleslaw), smoked salmon
Listeria monocytogenes and other species	*ssrA*	Detection	Enrichment + qPCR – LightCycler hybridization probes, IAC DL: 1–5 CFU/25 g	Milk and milk products, meat and meat products, fish and fishery products
Listeria monocytogenes	*16S rRNA*	Detection Quantification	Enrichment + qPCR – SYBR® Green DL: 1–5 CFU/50 g	Artificially and naturally contaminated collard green, cabbage, lettuce, mixed parsley and spring onion bunches, Chinese cabbage, arugula, chicory, wild chicory, spinach, watercress
Staphylococcus aureus	*nuc*	Detection Quantification	qPCR – SYBR® Green, TaqMan® QL: 20–300 CFU/2 g (depending on the type of cheese)	Artificially contaminated cheese
Staphylococcus aureus	*nuc*	Detection Quantification	qPCR – SYBR® Green, TaqMan® DL: 5×10^2 CFU/g	Artificially contaminated beef Natural fresh meat products, salads, cheese, smoked salmon, pâté, entrails, prepared egg, ready to serve dishes, ice cream, dry-cured meat products, fresh salmon
Staphylococcus aureus	*nuc*	Detection Quantification	qPCR – TaqMan®	Milk from cows with intramammary infection
Staphylococcus aureus	*hlyA*	Detection	Enrichment + qPCR – SYBR® Green DL: 1 CFU/g; 10^3 CFU/g without enrichment	Artificially contaminated milk, pork
Enterobacteriaceae	*lacZ*	Detection Quantification	Enrichment + qPCR – SYBR® Green DL: 1 cell/ml	Artificially contaminated cheese
Escherichia coli	*uidA*	Detection Quantification	Enrichment + qPCR – TaqMan® DL/QL: 1 CFU/g; 10^3 CFU/g without enrichment	Artificially contaminated minced beef, tuna, raw oyster
Escherichia coli O157:H7	*eae*	Detection	Enrichment + qPCR – Scorpion D/QL: 10^3 CFU/ml (without enrichment)	Artificially contaminated milk Natural samples of raw milk, pasteurized milk, ice cream, kulfi (frozen dessert), paneer (soft cheese), infant foods

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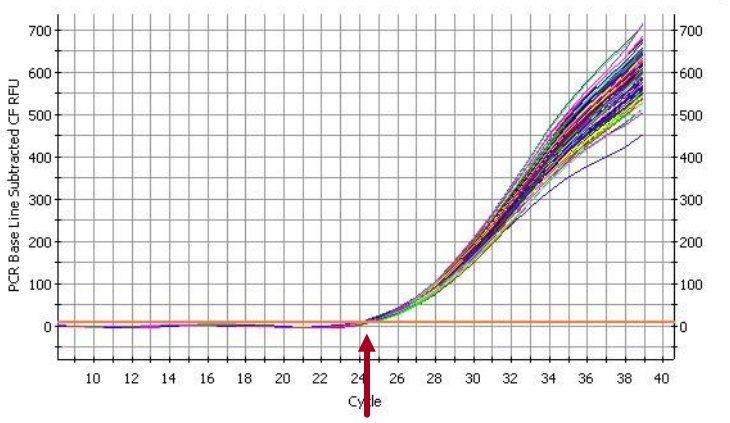
Interpretasi Data Real Time PCR

Tri Joko Raharjo

Ct dan ΔRn

- The Ct is defined as the cycle when sample fluorescence exceeds a chosen threshold above background fluorescence. Cp or crossing point.
- Rn, the normalized reporter signal
  - reporter signal is normalized to a reference dye by dividing the raw fluorescence of the reporter by the fluorescence of the passive reference, to compensate for minor well-to-well variation
- ΔRn, normalized fluorescence value corrected for the background

96 identical reactions will have almost identical C_T values



3

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Ideal PCR

$$Product_T=(Template_0)2^n$$

Where n=Number of Cycles

- **1 C_T Difference = 2 fold difference in starting template amount**
- **3.3 C_T Difference = 10 fold difference in starting template amount**

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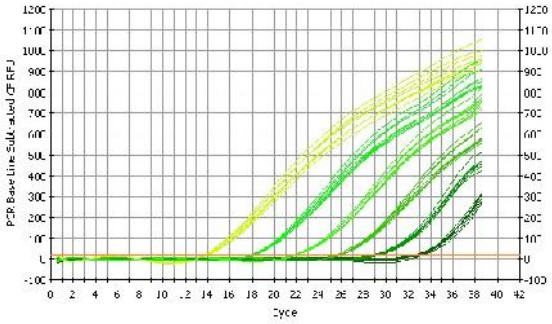
Ct Correlates strongly with the starting copy number

$2^n = 10$ fold

$n \ln 2 = \ln 10$

$n = \frac{\ln 10}{\ln 2}$

$n = 3.32$



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Standard curves

- To determine if an assay is optimal
- Determine the concentration of template (OD 260 nm)
- Convert to molecule number (copy number)

$$\frac{\text{Mass (in grams)} \times \text{Avogadro's Number}}{\text{Average mol. wt. of a base} \times \text{template length}} = \text{molecules of DNA}$$

- Example

$$\frac{0.8 \times 10^{-12}\ \text{gm} \times 6.023 \times 10^{23}\ \text{molecules/mole}}{330\ \text{gm/mole/base} \times 75\ \text{bases}}$$

$$= 2.0 \times 10^{7}\ \text{molecules (copies) of DNA}$$

- For double-stranded templates, use 660 gm/mole/base

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Standard curves

- Prepare seven 10-fold dilutions, starting with 1×10^7 copies to 10 copies
- Perform the realtime PCR, use a optimized primer/probe concentration that gives the highest ΔRn and the lowest Ct
- Draw xy plots with the log template amount as the x value and the cycle threshold (Ct) as the y value

$$y = m x + b$$

where $y = Ct$, m = slope, $x = log10$template amount, and b = y-intercept.
r^2, coefficient of determination

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Efficiency

- Efficiency is primarily an indication of how well the PCR reaction has proceeded

$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1$$

- r^2, measure of the accuracy of the dilutions and precision of pipetting
- y-intercept is an indication of the sensitivity of the assay and how accurately the template has been quantified.

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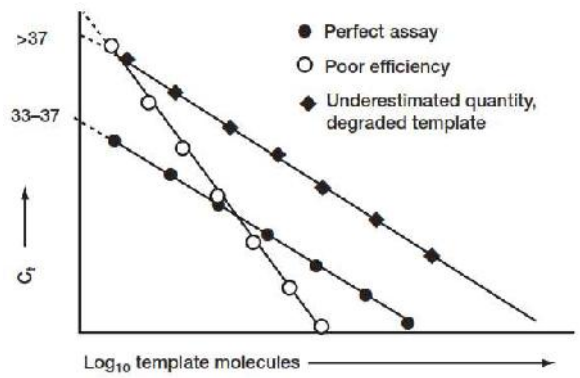

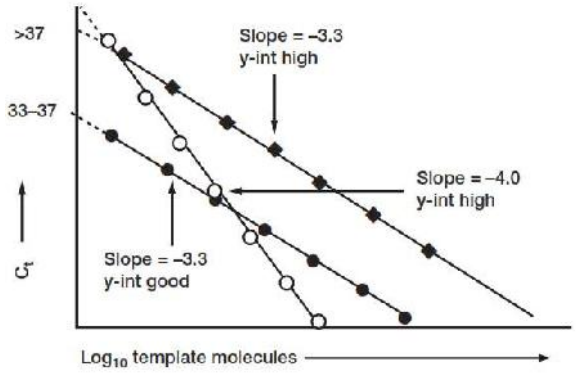

$Nn = (No)\ (1+E)^n$ $n = Log(Nn) - log(No)/log(1+E)$

Nn= number of copies of thentemplate after n cycles,
No= number of copies of the template at cycle 0,
E = efficiency
n = number of cycles.

- At 100% PCR efficiency, a single copy of template should be detected between 33.3–36.5 cycles.
- Therefore, a perfect assay would have a slope of –3.32 (100% efficiency), a y-intercept between 33 and 37 cycles and an r of 1.00.
- An assay can have an apparently acceptable efficiency of 95–100% but if the y-intercept is substantially higher than 37 or lower than 33, it indicates that the amount of template has not been correctly determined

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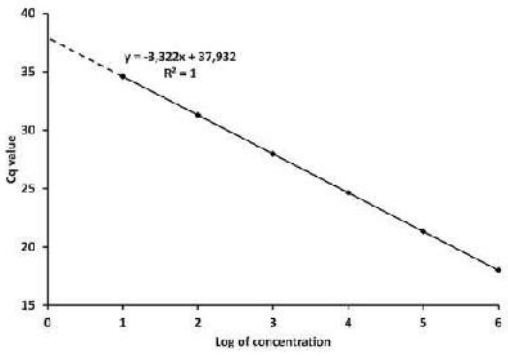

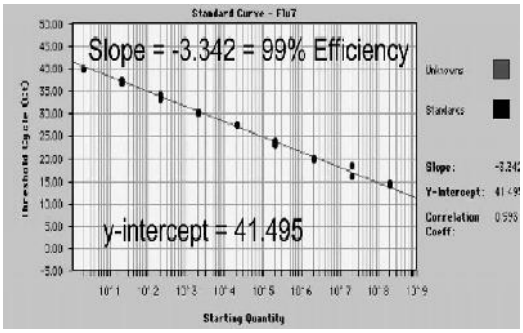

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- In the case of a high y-intercept, deterioration of the template is likely *Degradation* of template is a common result of too many freeze–thaw cycles of the standard or storing the template at too low a concentration without a carrier.

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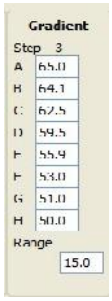
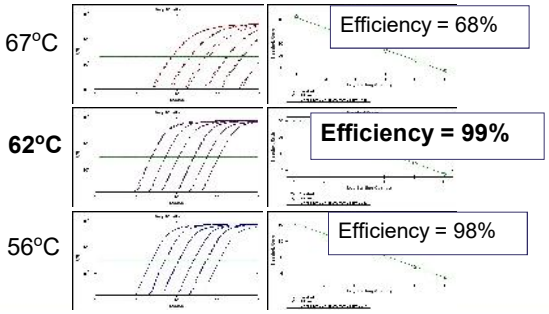
Annealing temperature is critical



- Specificity
- Reproducibility
- PCR Reaction Efficiency
- Sensitivity
- Reliable data

- Serial dilutions
  8 temps from 55°C to 68°C
- 62°C is optimal
  -low Cts and highest reaction efficiency



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	Default analysis settings		Adjusted analysis settings		Difference	
Instrument	Efficiency	y-Intercept	Efficiency	y-Intercept	Δ Efficiency	Δ y-Intercept
ABI Prism® 7500	128%	33.3	99%	35.9	−29.0%	−2.67
ABI Prism® 7700	106%	35.8	103%	35.9	−3.0%	−0.10
BioRad iCycler®	100%	34.8	98%	35.9	−1.3%	−1.12
Cepheid SmartCycler I®	94%	38.5	96%	36.9	2.7%	1.62
Corbett Rotorgene™	101%	36.3	101%	37.4	0.3%	−1.04
Roche LC 1.2®	95%	36.8	94%	38.1	−0.8%	−1.32
Roche LC 2®	99%	34.5	98%	35.9	−1.7%	−1.41
Stratagene Mx3000p®	102%	37.8	103%	35.9	0.6%	1.83
Stratagene Mx4000®	101%	36.9	102%	35.9	0.5%	0.97
Mean	**103%**	**36.1**	**99%**	**35.9**	**−3.5%**	**0.21**
Range	**94%–128%**	**33.3–38.5**	**94%–103%**	**35.90–38.11**	**0.3%–29%**	**0.21–2.67**

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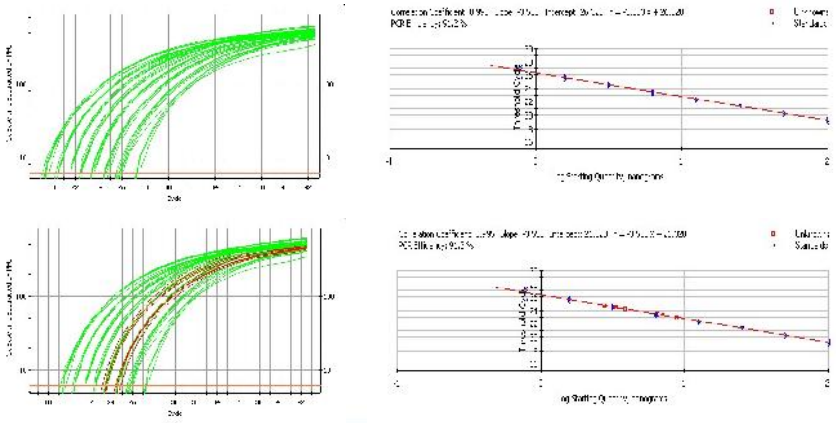
Relative quantitative analysis

- From C_T values, we can determine the initial copy number



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Data Analysis:

- Basic delta Ct
- Delta-delta Ct
- Pfaffl delta-delta Ct

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Calculating for relative quantitation

- Basic delta Ct method:
  - *(no normalization to reference gene)*

	Primer set #2
Tissue #1:	22
Tissue #2:	24
Delta Ct:	24-22 = 2

Fold induction = $2^2 = 4$

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- Delta-delta Ct method: *(assumes same efficiencies for each primer set)*

		Reference Primer set	GOI Primer set
	Tissue #1:	21	22
	Tissue #2:	20	24
1st Delta	Delta Ct:		22-21 = 1
	Delta Ct:		24-20 = 4
2nd Delta	Delta Ct:		4-1 = 3

Fold induction = $2^3 = 8$

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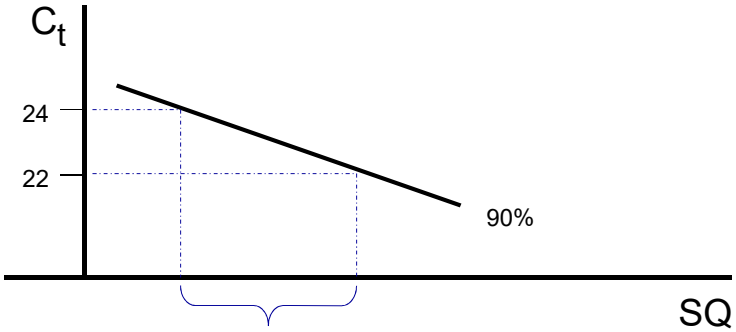
- Problems of delta-delta Ct method:



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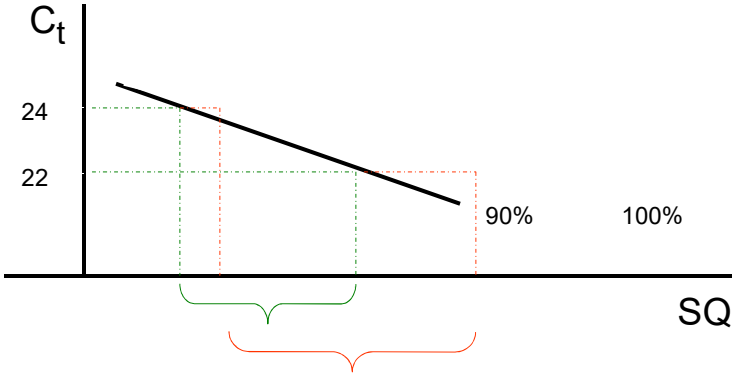
- Problems of delta-delta Ct method:



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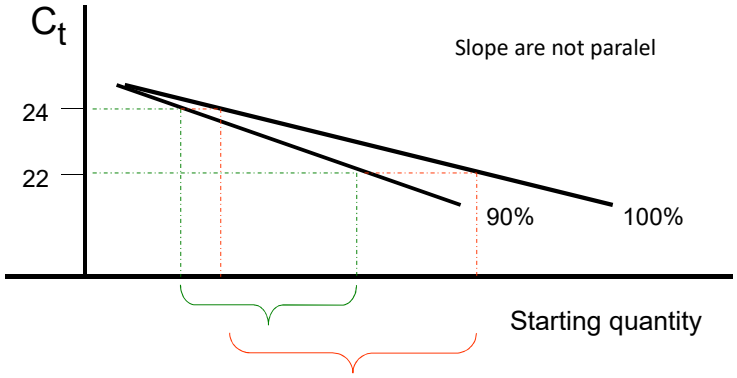
- Problems of delta-delta Ct method:



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- Pfaffl method:

$$\text{Fold induction} = \frac{\text{Efficiency}_{\text{target}}^{\text{deltaCt }_{\text{target}}\text{ (control-sample)}}}{\text{Efficiency}_{\text{reference}}^{\text{deltaCt }_{\text{reference}}\text{ (control-sample)}}}$$

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

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Pfaffl method:
(efficiencies are normalized)

		Primer set #1Reference	Primer set #2 GOI
	Tissue #1:	21	22
	Tissue #2:	20	24
(From Standard curve)	Efficiency:	90% = 1.9	100% = 2
	Delta Ct:	20-21 = -1	24-22 = 2

$$\text{Fold induction} = \frac{2_{\text{target}}^{\text{deltaCt }_{\text{target}}\ (\mathbf{24\text{-}22 = 2})}}{1.9_{\text{reference}}^{\text{deltaCt }_{\text{reference}}\ (\mathbf{20\text{-}21 = -1})}} = \frac{4}{0.53} = 7.5$$

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Comparison of methods for relative quantitation calculations

- Basic delta Ct method: (no reference gene)
  - Fold induction : 4
- Delta-delta Ct method: (reference gene)
  - Fold induction : 8
    - Ideal for primer pairs with an E ≥ 90% AND large fold changes in expression (10 fold or more)
- Pfaffl method: (reference gene and efficiency)
  - Fold induction : 7.5

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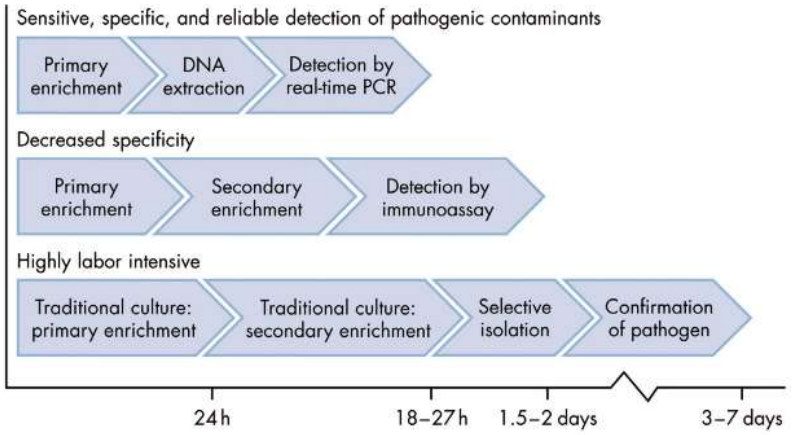
Characteristic of pathogen microbes in food

- Small numbers and difficult to detect amid large numbers of harmless background microflora in a complex sample matrix.
- Traditional culture techniques are time-consuming and laborious
- More rapid methods, such as DNA hybridization, enzyme immunoassays, and real-time PCR (RTi-PCR).
- However, at best, these methods detect 10^3 to 10^4 CFU/g of target pathogens.
- Culture enrichment steps are still necessary, as is confirmation of presumptively positive results



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Separating bacteria from a food matrix

- Filtration and centrifugation → Buoyant density centrifugation (BDC)
- The benefits of BDC are
  - the possibility of separating biological matrix particles and microorganisms with different buoyant densities;
  - elimination of parts of the PCR-inhibiting food substances ;
  - prevention of false-positive results due to DNA originating from dead cells, which has limited the use of quantitative PCR (qPCR) ;
  - the possibility of direct quantification of target organisms in the presence of a large background flora;
  - maintenance of cell viability, which allows isolation and analysis of the microorganisms
  - speed and easy handling

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Combination of the separation method

Bacterium (no. of strains) or food homogenate(s)	Strains	Buoyant density (g/ml)
Bacteria		
Escherichia coli	EC-2736, EC-2649, EC-3515, EC-4725, EC-4131, SE-02005, SE-02025, SE-02027	1.064-1.083
Salmonella spp.	Sal-2339, Sal-2340, Sal-2341	1.075-1.085
Yersinia enterocolitica	Pa177, Pa241, Pa2718, Pa9346, Pa12986	1.082-1.084
Providencia alcalifaciens	ATCC 9886, 112, 118	1.080-1.083
Campylobacter jejuni	SC009, SC010, SC011, SC012	1.075-1.098
Vibrio cholerae	ATCC 14035, NIID63-93, NIID169-68, SVP84	1.052-1.066
TDH-positive *V. parahaemolyticus*	SVP02, SVP03, SVP04, NIIDK4	1.050-1.058
Vibrio vulnificus	SVV1526, SVV04001, SVV04003	1.031-1.035
Aeromonas hydrophila	ATCC 7966, M25	1.045-1.058
Staphylococcus aureus	SS 05, FB0501, FB0601	1.109-1.120
Bacillus cereus	127, 128, 129, 130, 131, 132, 133, 135, 136, 137	1.085-1.092
Clostridium perfringens	CW2, H2	1.082
Food homogenates		
Minced beef, bovine liver, minced chicken, processed cheese, scrambled egg, tofu, Chinese noodle, bread, raw chopped jack horse mackerel, short-neck clam		≦1.025
Minced pork, ready-to-eat hamburger steak		≦1.033
Milk		≦1.049

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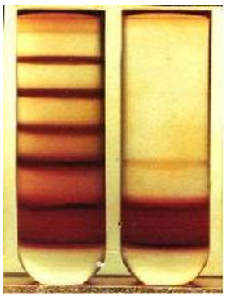
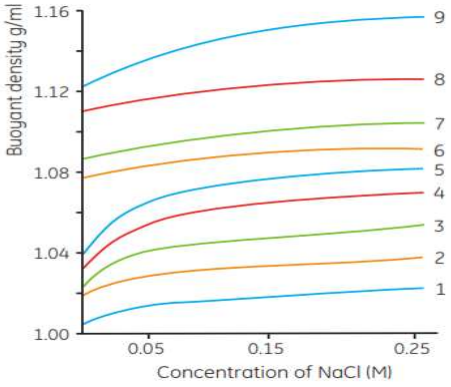
Percoll consists of colloidal silica particles of 15–30 nm diameter (23% w/w in water) which have been coated with polyvinylpyrrolidone (PVP).





Detail about percoll BDC

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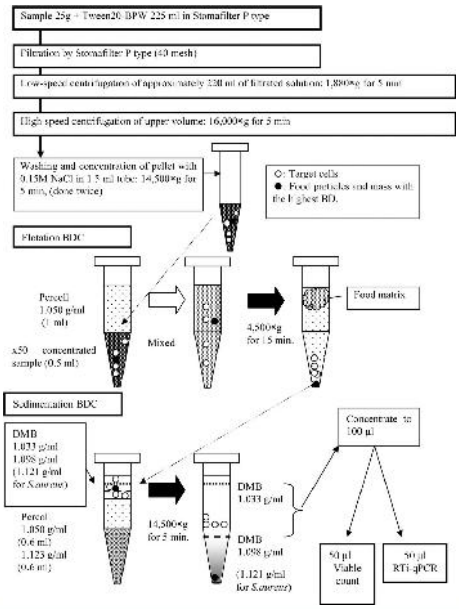

Appl. Environ. Microbiol. 2007 vol. 73 no. 1 92-100
J Biochem Biophys Methods. 2000 Jul 10;44(1-2):1-30

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Food sample	Inoculum (log_{10} CFU/25 g) and concn of cells (log_{10} CFU/25 g) as determined by viable-cell counting and RTi-qPCR[a]																	
	S. enterica			*E. coli* O157:H7			*Y. enterocolitica*			*P. alcalifaciens*			*C. jejuni*			*C. perfringens*		
	Inoculum	VC	qPCR	Inoculum	VC	qPCR	Inoculum	VC	qPCR	Inoculum	VC	qPCR	Inoculum	VC	qPCR	Inoculum	VC	qPCR
Beef	5.9	5.2	5.3	5.0	4.7	4.9	5.9	4.9	5.8	5.9	4.5	3.9	5.7	5.2	4.6	6.3	4.3	4.5
Bovine liver	5.9	5.0	5.8	5.0	3.9	4.5	5.9	4.7	4.5	5.9	5.4	4.8	5.7	3.9	3.1	6.3	4.7	4.2
Pork	5.9	5.0	5.3	5.0	4.2	4.5	5.9	4.5	4.9	5.9	5.3	4.6	5.7	4.9	3.8	6.3	3.7	3.9
Chicken	5.9	5.1	6.0	5.0	4.7	5.1	5.9	4.9	5.0	5.9	4.7	4.4	5.7	4.2	3.2	6.3	4.0	4.6
Processed cheese	5.9	4.9	5.8	5.0	4.2	5.3	5.9	4.5	5.5	5.9	5.1	4.4	5.7	4.5	4.0	6.3	3.5	4.3
Scrambled egg	5.9	5.2	5.8	5.0	4.5	5.2	5.9	5.0	6.0	5.9	5.1	4.5	5.7	3.9	4.0	6.3	4.7	5.0
Tofu	5.9	4.9	5.4	5.0	4.7	4.6	5.9	5.2	4.3	5.9	4.9	4.1	5.7	3.2	3.7	6.3	3.8	4.6
Chinese noodle	5.9	5.5	5.0	5.0	4.2	5.2	5.9	5.2	5.1	5.9	5.0	4.7	5.7	2.9	3.0	6.3	3.7	4.9
Bread	5.9	4.5	5.2	5.0	3.9	5.1	5.9	4.2	4.3	5.9	5.1	4.0	5.7	3.9	3.3	6.3	3.0	4.8
Jack horse mackerel	5.9	5.2	5.4	5.0	3.9	4.6	5.9	4.5	4.7	5.9	5.0	4.0	5.7	4.8	3.5	6.3	4.2	4.7
Short-neck clam	5.9	5.2	5.6	5.0	4.2	4.8	5.9	5.0	5.7	5.9	4.5	3.5	5.7	3.9	3.8	6.3	2.5	3.0
Hamburger steak	5.9	5.1	5.8	5.0	4.4	4.2	5.9	4.3	4.7	5.9	5.0	5.1	5.7	5.0	5.1	6.3	4.0	5.3
Whole milk	5.9	5.2	5.6	5.0	4.7	5.1	5.9	5.7	4.7	5.9	4.5	4.6	5.7	3.7	3.8	6.3	4.7	5.3

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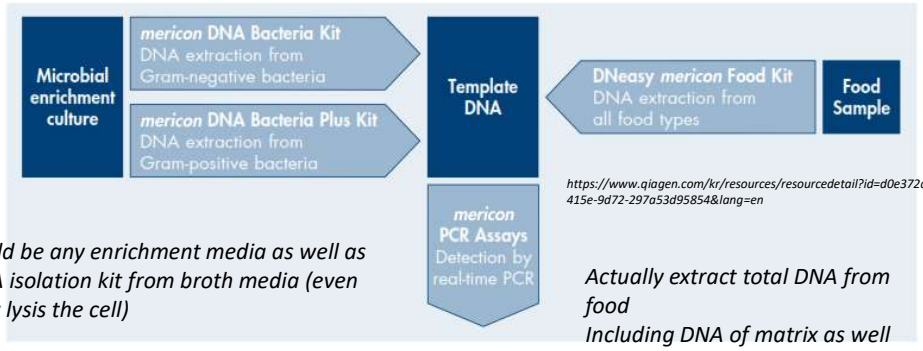
Qiagen



https://www.qiagen.com/kr/resources/resourcedetail?id=d0e372d7-6f6a-415e-9d72-297a53d95854&lang=en

Could be any enrichment media as well as DNA isolation kit from broth media (even only lysis the cell)

Actually extract total DNA from food
Including DNA of matrix as well dead microbe

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- Enterobacteriaceae, *Staphylococcus aureus, Bacillus cereus were cultured overnight at* 37°C (or 30°C for *Yersinia)* in buffered peptone water (BPW) (BBL)
- Members of the *Vibrionaceae were cultured overnight at 37°C in alkali peptone water (Nissui)*
- *C. jejuni was cultured overnight at 42°C using microaerobic incubation* in Preston medium (Oxoid)
- *Clostridium perfringens was cultured overnight at* 37°C using anaerobic incubation in thioglycolate medium

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Comparison of Bacterial DNA isolation kit

xtraction Method	Manufacturer	Principle	Costs per sample [€]	Completion time (hands-on-time)	Cell count	Yield [µg] (SD)	Purity [A260/280] (SD)
Genomic-tip 20/G	Qiagen	anion-exchange column (gravity)	8.1	8 h (45 min)	4×10^9	9.8 (3.5)	1.77 (0.06)
MagAttract HMW DNA Kit	Qiagen	DNA-binding magnetic beads, silica-based	4.4	2 h 40 min (1 h)	2×10^9	10.3 (6.6)	1.83 (0.05)
MasterPure DNA Purification Kit	Epicentre	salting-out	1.1	2 h 10 min (35 min)	0.4×10^9	3.3 (1.0)	1.82 (0.03)
Wizard Genomic DNA Purification Kit	Promega	salting-out	2.0	3 h (35 min)	4×10^9	18.1 (7.5)	1.58 (0.01)
DNeasy Blood & Tissue Kit	Qiagen	silica-membrane column (spin)	3.2	3 h (45 min)	2×10^9	10.9 (1.3)	1.72 (0.05)
Plasmid Mini Kit	Qiagen	alkaline lysis, anion-exchange column (gravity)	5.2	3 h 50 min (40 min)	18×10^9	0.50 (0.2)	1.67 (0.03)

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Comparison of Food DNA isolation kit

E. coli

Food	Target gene	C_t/log CFU (mean ± SE) determined by:			
		Prepman Ultra	Bugs'n Beads	Nucleospin food kit	Wizard magnetic DNA purifica- tion system for food
TSB	*stx*1	10.2 ± 0.4	10.7 ± 0.4	9.8 ± 0.4	10.1 ± 0.3
	*stx*2	10.4 ± 0.3	11.1 ± 0.4	10.3 ± 0.3	10.7 ± 0.3
Bread	*stx*1	9.8 ± 0.2	11.0 ± 0.2	10.1 ± 0.5	9.8 ± 0.3
	*stx*2	9.4 ± 0.3	10.9 ± 0.4	9.8 ± 0.4	9.7 ± 0.4
Ground beef	*stx*1	10.3 ± 0.3	12.2 ± 0.2	11.1 ± 0.5	9.7 ± 0.3
	*stx*2	10.3 ± 0.3	12.5 ± 0.4	11.6 ± 0.5	9.7 ± 0.2
Salad greens	*stx*1	10.0 ± 0.1	10.9 ± 0.2	10.0 ± 0.0	10.1 ± 0.2
	*stx*2	10.0 ± 0.2	10.9 ± 0.0	10.0 ± 0.2	10.4 ± 0.2
Salad dressing	*stx*1	10.0 ± 0.3	10.3 ± 0.4	10.0 ± 0.8	9.4 ± 0.3
	*stx*2	10.0 ± 0.3	10.3 ± 0.5	9.5 ± 0.3	10.1 ± 0.6

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Comparison of Food DNA isolation kit

E. coli

Kit	Method	Ease of use
Prepman Ultra	Proprietary solution	Very simple, minimum manipulation of sample
Bugs'n Beads	Magnetic beads	Simple, centrifuge not absolutely necessary
NucleoSpin food kit	Spin columns	Considerable hands-on time
Wizard magnetic DNA purification system for food	Magnetic beads	Considerable hands-on time, centrifuge not absolutely necessary

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Key parameter in validation

- Accuracy
- Precision
- Limit of Detection and Limit of Quantification

Reference on RT-PCR validation

> *"**ISO 22174:2005:** Microbiology of food and animal feeding stuffs -- Polymerase chain reaction (PCR) for the detection of food-borne pathogens -- General requirements and definitions"*

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Validation parameter (Broeder et al, 2014)

Parameter	Quantitative qPCR method	Qualitative qPCR method
Method acceptance parameters		
Applicability	+	+
Practicability	+	+
Specificity	+	+ (no false pos/neg)
Sensitivity (LOD)	–	+ (≤20 HGE)[a]
Sensitivity (LOQ)	+ (0.9%[b] or 0.1%[c])	–
PCR efficiency (ε)	+ (90–110%)[d]	Only for multiplex (80–120%)
Linearity (R^2)	+ ($R^2 \geq 0.98$)	Only for multiplex ($R^2 \geq 0.98$)
Accuracy	+	
Trueness	+ (±25%)	–
Precision (repeatability)	+ ($RSD_r \leq 25\%$)	–
Robustness	+ (≤30%)	+ (correct pos/neg classification)
Method performance parameters		
False positive/ negative rate	–	+ (correct pos/neg classification)
Precision (reproducibility)	+ ($RSD_R \leq 25\%$)	–
Measurement uncertainty	+ (≤50%)	–

Trends in Food Science & Technology 37 (2014) 115e126

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Applicability

- Applicability of the method with different matrix
- Prove the method is able to overcome PCR inhibitors from the matrix
- warnings on the interference with other analytes and its inapplicability to certain matrices and conditions should be included when identified (i.e. special treatment during isolation or enrichment)

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Practicability

- to check if the new method can easily be combined with other methods that are already used in the laboratory and can be run under the same condition
- blind samples can for example
- Inter-laboratory study

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Specificity

- Sequence confirmation
  - Perform PCR to amplify targeted gene (i.e. *ssrA* for Salmonella) using primer of RT-PCR assay
  - Clone the fragment to TA plasmid (optional fot internal positive control the PCR)
  - Sequence PCR fragment or cloning product
  - BLAST to confirm the identity
- Specificity to other species (closely related or high possibility to be co presented in the matrix)
- Specificity to matrix
  - Using spike and unspike matrix sampel

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Example: closely related to Salmonella or high possibility to be co presented in the meat matrix

Salmonella and non *Salmonella* serovars/strains used in real time based assay design and validation.

Salmonella Serovar	Strain	Non *Salmonella* Organism	Strain
Agona	NCTC 11377[a]	*Acinetobacter calcoaceticus*	ATCC 23055[b]
Anatum	SARB 2[c]	*Aeromonas hydrophila*	ATCC 35654[b]
Braenderup	NCTC 05750[a]	*Arthrobacter globiformis*	ATCC 8010[b]
Bredeney	NCTC 05731[a]	*Bacillus cereus*	NCTC 07464[a]
Derby	SARB 31[c]	*Citrobacter diversus*	CCFRA[d] 1198
Dublin	NCTC 05676[a]	*Citrobacter freundii*	NCTC 8090[a]
Enteritidis	ATCC 13076[b]	*Citrobacter freundii*	NCTC 09750[a]
Enteritidis PT4	NCTC 13349[a]	*Citrobacter koseri*	NCTC 10768[a]
Gallinarum	NCTC 423,287/91[a]	*Enterobacter aerogenes*	NCTC 10006[a]
Gallinarum	NCTC 13346[a]	*Enterobacter agglomerans*	NCTC 09381[a]
Goldcoast	NSRL[d]	*Enterobacter cloacae*	NCTC 11933[a]
Hadar	Uljub MI 2[e]	*Enterobacter intermedius*	NDC 427[f]
Heidelberg	NCTC 5717[a]	*Enterobacter sakazaki*	NCTC 11467[a]
Infantis	Uljub VF 25/04[e]	*Enterococcus faecalis*	NCTC 12697[a]
Kentucky	NCTC 05735[a]	*Enterococcus faecium*	ATCC 35667[b]
Livingstone	NCTC 05125[a]	*Escherichia coli*	ATCC 25922[b]
London	NCTC 05777[a]	*Escherichia coli*	NDC 544[f]
Manhattan	NCTC 06245[a]	*Escherichia coli*	NCTC 09001[a]
Newport	SARB 26[c]	*Klebsiella oxytoca*	ATCC 43086[b]
Nottingham	NCTC 07832[a]	*Klebsiella pneumoniae*	ATCC 13883[b]
Panama	SARB 40[c]	*Lactobacillus plantarum*	ATCC 8014[b]
Saint-Paul	Uljub VF S-13/95[e]	*Leuconostoc mesenteroides*	ATCC 8293[b]
Senftenberg	SARB 59[c]	*Proteus mirabilis*	DSM 4479[g]
Stanley	SARB 63[c]	*Pseudomonas aeruginosa*	NCTC 12903[a]
Typhimurium	ATCC 14028[b]	*Pseudomonas fragi*	DSM 3456[g]
Typhimurium LT 2	NCTC 12416[a]	*Pseudomonas putida*	ATCC 49128[b]
Typhimurium DT 104	NCTC 13348[a]	*Staphylococcus epidermidis*	AFRC[f]
Uganda	NCTC 06015[a]	*Staphylococcus haemolyticus*	ATCC 29970[b]
Virchow	NCTC 05742[a]	*Staphylococcus saprophyticus*	ATCC 15305[b]
Caminara	NCTC 5797[a]	*Streptococcus lactis*	NCDO 2003[h]

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Internal positive control

- Clone the fragment to TA plasmid (optional)
- Certain correct copy number can be obtained
- Useful during finding of PCR optimum condition

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Specificity to other species (pure culture)

- 100 mL of BPW was inoculated with 1 CFU for targeted species (final concentration of 0.01 CFU/ml) and 1000 CFU for non targeted species/strains (final concentration of 10 CFU/ml).
- Approximate cell density of each test strain was established by plate counts
- Following 18 h enrichment in BPW, reference (TPC) and alternative methods (RT-PCR) were performed in parallel
- Six replicates fro precision study

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Specificity to matrix

- Matrix tested for the level of the background
- Matrix were inoculated with targeted species at five inoculation levels (1, 10, 100, 1000, 5000 CFU). 6 replicates
- Let the culture grow (condition according to specification of the species)
- The spiked samples were then tested for the presence of the species based TPC and RT-PCR method .

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Accuracy (for quatitative)

- Trueness (quantitative)
  - How close the result to the true value

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Precision

- Repeatability
  - 6 replicate of the sample tested using the method
  - RSD of Ct value are evaluated for qualitative
  - RSD Copy number for are evaluated for quantitative

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False positive and false negative

$$\text{False neg rate} = \frac{100 \times \text{number misclassified known pos samples}}{\text{tot number known pos samples}}$$

$$\text{False pos rate} = \frac{100 \times \text{number misclassified known neg samples}}{\text{tot number known neg samples}}$$

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LoD and LoQ

- LoD =the lowest amount of analyte (measurand) in a sample that can be detected with (stated) probability, although perhaps not quantified as an exact value.
- LoQ = the lowest amount of measurand in a sample that can be quantitatively determined with (stated) acceptable precisionand stated, acceptable accuracy, under stated experimentalconditions

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LoD and LoQ

- Serial dilution (100 to 0.1 copies)
- 6 biological replicates, 3 technical replicates
- Evaluate the RSD of Ct or copy number result

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Robustness

- Different experimental conditions can be slightly changed and their impact on the results studied
- Identify the critical condition that could change randomly
  - Annealing temp.
  - Pipetting error (from pipet calibration)
  - Etc
- Run the method at the deviation condition
- The robust method is not affected by the condition

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Linearity (R^2)

- Prepare seven 10-fold dilutions, starting with 1×10^7 copies to 10 copies
- Perform the realtime PCR, use a optimized primer/probe concentration that gives the highest ΔRn and the lowest Ct
- Draw xy plots with the log template amount as the x value and the cycle threshold (Ct) as the y value

$$y = mx + b$$

where $y = Ct$, m = slope, $x = log10$template amount, and b = y-intercept.
r^2, coefficient of determination

r^2 should be less than 0.98

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Efficiency

- Efficiency is primarily an indication of how well the PCR reaction has proceeded

$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1$$

- Efficiency accepted value are 80-120%

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