OPCR COUTSE

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What do you want to know?

Data Analysis and interpretation

Statistical analysis of gPCR



Application of gPCR



Normalization

Troubleshooting

More

Everything

Results of the quiz

1. Did you learn anything new from the lecture

A lot	yes	a bit
2	10	4

2. If yes, do you think you will remember it tomorrow?

no,	unless	I refresh it	I hope	yes
		2	5	9

3. Would you prefer to have more information in the lecture, keep it the same, reduce it?

Keep the same	More information	concerning the prints:
15	1	how bad do you want them?

4. Were the tasks too easy, ok, or too hard?

too hard	ok	too easy
3	11	2
		comment: the tasks were diffuse

8. Would you suggest any changes?

it was a bit too fast

the course website info

warm up questions from Lecture 1

Intro with definition of technique and application DNA replication should be summarised on one slide

Mg2+ in the active center of pol



Nakamura et al., 2012. Nature

from the Lecture 1

what is DNA made of?

is it water soluble?

will it dissolve in 70% Ethanol?

what are 3' and 5' ends?

how does DNA pol add nucleotides to the growing strand?

what is 3 step PCR?

Reverse transcription?



quantitative

OPCK

end point PCR vs real time PCR

End point PCR

mix PCR

Real time PCR

mix PCR together with fluorescent dye

> run PCR and make detection after EACH cycle

run PCR until the very

last cycle

mix with fluorescent dye (EtBr/GelRed etc)

use UV Lamp and your eyes to see fluorescence on a gel

what is fluorescence?

"glowing under UV light", George Stokes 19th century

what is fluorophore?

φορος (-phoros, "bearing", "carrying")

detection in end point PCR



detection in end point PCR



detection in end point PCR

mix PCR

run PCR until the very last cycle

mix with fluorescent dye

use UV Lamp to induce fluorescence





what is "real time" in the RT-PCR



mix PCR together with fluorescent dye

run PCR and make detection after EACH cycle threshold level of fluorescence (fluorescence level is above background => detectable)



 C_t = threshold cycle = C_q = quantitative cycle

Ct value represents how many PCR cycles are required for the sample fluorescence to reach the threshold level.

C

quantitative

Ct value is determined by initial amount of a template in a sample

Lower Ct => higher amount of the template



Higher Ct => Lower amount of the template

two types of quantification

Absolute

Relative

how many copies

how much more/less

is estimated using a standard curve

requires use of reference genes

Mhy ofcr?

- It is accurate (readings during exponential phase)
- It is quantitative
- Is much more sensitive than regular PCR, (detection at a few pg)
- Broad dynamic range (> 9 orders of magnitude)
- Fully integrated (amplify->detect and calculate) => allows sample throughput

For what ofc?

- Transcription research/verification of RNAseq and microarray
- Diagnostic research: how much virus is swimming in your
 blood?
- Forensics: is it your DNA on the crime scene?
- Detect amount of GMO in stuff
- Detection of gene duplication or deletion
- Allelic discrimination assay
- Detection of % methylation of specific regions
- High resolution melt curve (SNP detection)
- etc.

Different types of aPCR

I'm teaching you yesterdays high tech

- · Based on DNA-binding fluorescent dyes
- · Based on fluorescent primers/probes:
 - · TaqMan hydrolysis probe
 - · Molecular beacons hairpin probe
- Digital PCR
- etc.

MIQE guidelines

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^a All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If primers are from RTPrimerDB, information on qPCR target, oligonucleotides, protocols, and validation is available from that source.

^b FFPE, formalin-fixed, paraffin-embedded; RIN, RNA integrity number; RQI, RNA quality indicator; GSP, gene-specific priming; dNTP, deoxynucleoside triphosphate. ^c Assessing the absence of DNA with a no–reverse transcription assay is essential when first extracting RNA. Once the sample has been validated as rDNA free, inclusion of a no–reverse transcription control is desirable but no longer essential.

^d Disclosure of the probe sequence is highly desirable and strongly encouraged; however, because not all vendors of commercial predesigned assays provide this information, it cannot be an essential requirement. Use of such assays is discouraged.

efficiency of PCR

if PCR works ideally (E=100%) then with each cycle the amount of amplicon is doubled, i. e.



1 cycle of PCR





Efficiency of PCR

if PCR works ideally (E=100%) then with each cycle the amount of amplicon is doubled, i. e.



1 template gives rise to 2 copies

grey is the newly synthesised strand

efficiency of PCR

if PCR works ideally (E=100%) then with each cycle the amount of amplicon is doubled, i. e.

N-number of template copies n-number of PCR cycles

happens in an ideal world

Nfinal = Ninitial X 2ⁿ

this is the data you get in a form of the threshold fluorescence

this is what you want to know

Efficiency is estimated by running PCR with template dilution series

- template concentration is hard to get ->
 use dilutions
- The efficiency of a qPCR reaction is 100%
 when the DNA doubles with each cycle.
- adding twice less template will increase
 the Ct value by 1 cycle



 $E = 10^{-1/slope}$ % Efficiency = (E - 1) × 100%





what is Log?

Logabee => aceb

109101000=? $10^3 = 1000$ => $10^2 = 100$ 10910100=? => 1091010=? $10^{1} = 10$ => $10^{\circ} = 1$ Log101=? => $10^{1.2} = 16$ log1016=? => 100.9=8 109108=? => $10^{0.6} = 4$ => 109104=? 100.3=2 Log102=? =>





What can mess up efficiency of amplification?

E > 100%

Low primer specificity (melt curve) primer dimers (melt curve) inhibitors (dilution series)

E < 100%:

bad primers/primer concentration dead polymerase



coefficient of determination

= how well does the model predict future outcomes

should I really trust this?

if R² is > 0.98, then you can trust it

R2=1-(SSerr/SStot)

you want \mathbb{R}^2 to be as close to 1 as possible.

what will happen to R² if you use 2 dilutions for your curve?

3 basic methods to quantify relative gPCR data

Normalized expression = E-act

- Pfaffl method
- @ ACE

AX AX X AY = ? $A \times / A = ?$ $A \times / B = ?$

A gene control sample A gene treated sample Reference gene control sample Reference gene treated sample

what are the units for this?

what is this?

why does this look so bad?

what's this?

what are the units for this?

cles

what are these? DCEAC CLAtCERC CERE

27.5

N-number of template copies n-number of PCR cycles

happens in an ideal world

Nfinal = Ninitial X 2m

this is the data you get in a form of the threshold fluorescence

this is what you want to know

Ninitial = N_{final} / E^n

how is this expressed in the qPCR data? How do you see amount of DNA?

A gene control sample A gene treated sample Reference gene control sample Reference gene treated sample

where are Nfinal and Ninitial?

cycles

Ninitial control = Nfinal control / ECt control

Ninitial treatment = Nfinal treatment / ECt treatment

how much more/less DNA we have in the treatment?



Normalized expression = E-(ct-ct) = E-act



A gene control sample A gene treated sample Reference gene control sample Reference gene treated sample

20 2527 CEAC CEAE CERC CERC

cles

Pfaffl method (most reliable)

1. Normalize expression of gene of interest in control and treatment

2. Normalize expression of reference gene control and treatment

3. Calculate expression ratio of genA and reference gene

AACE

2-Dack method, Livak method

is the same as Pfaffl method, but requires 100% efficiency,

or equal efficiency of all primers you use (you put it instead of 2)

1. normalize gene of interest to the reference gene

 $\Delta C_{t} control = C_{t} A c - C_{t} R c$ $\Delta C_{t} t reatment = C_{t} A t - C_{t} R t$

2. normalize test to control

 $\Delta\Delta C_t = \Delta C_t treatment - \Delta C_t control$ 3. expression ratio

Normalized expression ratio = 2-DACE

why can't you use Livak's method if E for genes are not the same?

act method

no reference gene is used, instead samples were equilibrated using another parameter (DNA concentration, amount of cells etc.)

Normalized expression ratio = Eact = E(Ct control - Ct treatment)

MELL CLIVE



Macle Curve



MELL CUTVE



Homework will be uploaded on the site