UNIVERSITY OF TARTU

FACULTY OF SCIENCE AND TECHNOLOGY

Quantitative analysis of Mitochondrial DNA (mtDNA) copy number in yeast *S.cerevisiae* strain W303 α

MASTER THESIS

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1. List of abbreviations

mtDNA - Mitochonarial DNA

qPCR - real-time polymerase chain reaction

S.cervisiae - *Saccharomyces cerevisiae*

C. albicans - *Candida albicans*

CsCl - cesium chloride

(EtBr) - ethidium bromide

Ct – threshold cycles

CN – copy number

ddH_2O - Deionized distilled water

EtOH - ethanol

KoAC - Potassium acetate

MgCl2 - Magnesium chloride

UV-VIS – Ultraviolet-visible spectroscopy

i-PrOH – *iso*-propanol

2. Introduction

Mitochondrion is a double phospholipids bilayer membrane-enclosed organelle

found in most eukaryotic cells, contains DNA, and carries out oxidative phosphorylation, The mitochondria are the sites for much of the metabolism necessary for the production of enrgy in the form of ATP[1,2].

Mitochondrial DNA (mtDNA) copy number (molecules or genomes per cell) is believed to be relatively constant. Early reports demonstrated that the control of mtDNA synthesis is independent of the mechanisms controlling mtDNA replication during S phase of the cell cycle. Mitochondrial DNA encodes for a number of respiratory chain peptides and RNA molecules for protein biosynthesis in mitochondria. Therefore, stability of mtDNA copy number is essential to produce fully functional cells.

The regulation of mtDNA synthesis, the defects in oxdative phosphorylation enzymatic machinery during human disease and different fundamentals questions concerning DNA cis acting elements can only be understood when the data on correct copy number of mtDNA will be available[3].

mtDNA copy number has only been measured in a population of cells and quantitative mtDNA copy number in single cell had not been published. The budding yeast *Saccharomyces cerevisiae* which is studied as organism model in our lab has been widely used to analyze the mechanisms of mtDNA replication and stability, There are few reports on proteins that could play a role in mtDNA copy number control, mtDNA binding protein Abf2p has been proposed to be a key character in these processes (Zelenaya-Troitskaya et al., 1998); Pif1p is a conserved DNA helicase which occurs both in the nucleus and mitochondria in S.cerevisiae (Foury and Lahaye, 1987; Schulz and Zakian, 1994), and previous research postulated that Pif1p plays a role in the repair or tolerance of oxidative mtDNA damage, perhaps by governing the rate of mtDNA replication or regulating mtDNA copy number[4]. However, the fundament of mechanism for maintaining mtDNA copy number has remained a mystery.

The main goal for this thesis was to develop reliable quantitative analysis method to measure mtDNA copy number in yeast *S. cerevisae* cells. qPCR based system was next used to ask questions that have biological relevance. Gene copy number for

different mitochondrial genomic loci was analyzed. We also discuss the possibilities to use the method for single cell analysis.

3. Literature overview

3.1 Basic concept of yeast

Yeast Serves as a Minimal Model Eukaryote

In order to analyze the internal workings of the eukaryotic cell without the additional problems of multi-cellular development, we usually use a species that is unicellular and as simple as possible. Therefore, the yeast species is a popular choice for this role. *S.cerevisiae* is a small, single-celled member of the kingdom of fungi (Figue 3.1) and thus, according to modern views, at least as closely related to animals as it is to plants[5].

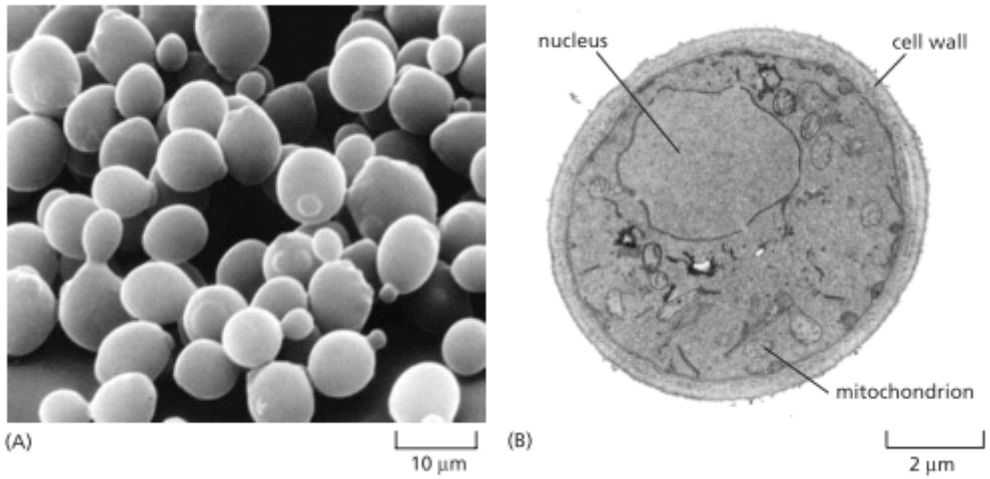


Figure 3.1 The yeast *Saccharomyces cerevisiae.* (A) A scanning electron micrograph of a cluster of the cells (B) A transmission electron micrograph of a cross section of a yeast cell, showing its nucleus, mitochondrion and thick cell wall. (Alberts. Molecular Biology Of The Cell.5th.Ed)

3.2 Structure of yeast mitochondrial DNA

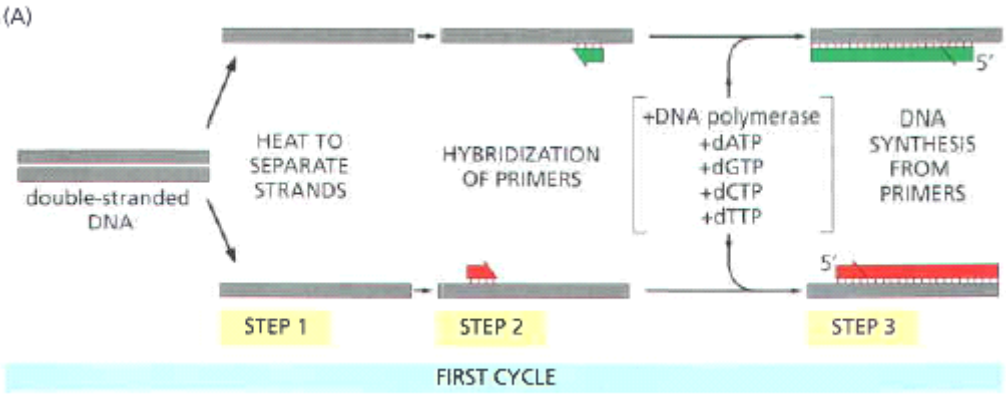
The mtDNA of strain FY1679, an isogenic derivative S288C, is a 85779 kb double-stranded molecule, which contains the following genes: 2 rRNA genes (15s rRNA and 21s rRNA), cytochrome c oxidase subunit I, II and III (cox1, cox2 and

cox3), ATP synthase subunits 6, 8 and 9 (atp6, atp8, and atp9), apocytochrome b (cytb), a ribosomal protein (var1) and several intron-related open reading frames (ORFs)[6,7,8].

Yeast mtDNA has often been considered as a circle form during past 40 years. However, this view is probably not correct and instead of circular molecules the mtDNA consists of a phage T4 like network, our previous research found that the *C. albicans* mtDNA forms a complex branched network that does not contain detectable amounts of circular molecules by using conventional and pulsed-field gel electrophoresis, two-dimensional gel electrophoresis and DNA sequence analysis[9]. The aerobic *C. albicans* is a suitable model organism to study mtDNA maintenance in yeast, Unfortunately there are currently no sufficientdata on mtDNA isoforms in *S.cerevisiae.*wild type cells

3.3 Theory of DNA amplification

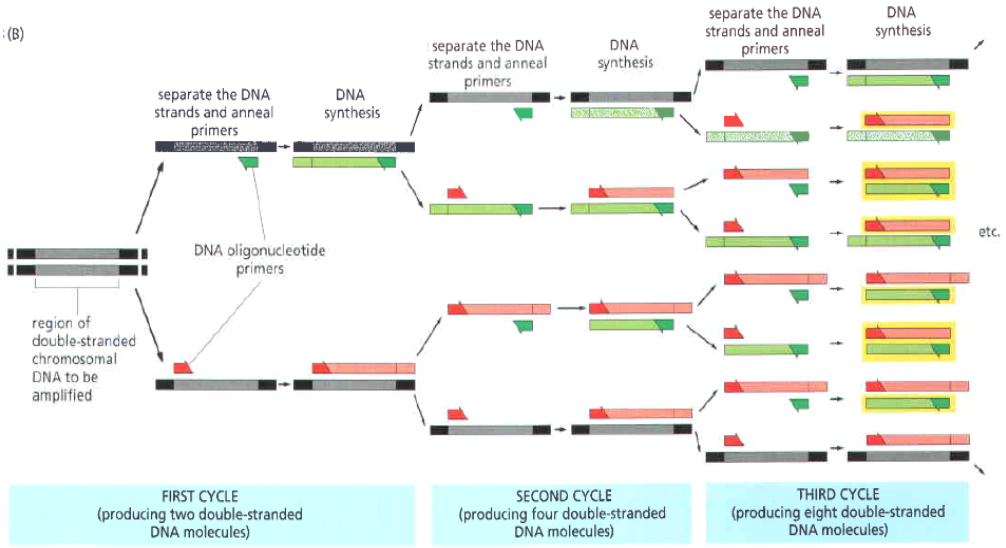
Genes can be selectively amplified by polymerase chain reaction (PCR). Conventionally, the PCR amplification reaction is described as at first stochastic, then exponential and finally stagnant[10]. The PCR method is extremely sensitive, and even can detect the single DNA molecule.



Scheme 3.2 First cycle of PCR amplification process(Molecular Biology Of The Cell.5th.Ed)

In the first cycle, double-stranded DNA is separated to single strands by heating. Then the pair of DNA oligonucleotides which are chosen to flank the desired nucleotide sequence of the gene are used to prime DNA synthesis on single strands.

The newly synthesize double-stranded DNA is produced in a reaction catalyzed in vitro by a purified DNA polymerase[5].



Scheme 3.3 PCR amplification overview (Molecular Biology Of The Cell.5th)

In principle, every cycle doubles the amount of DNA synthesized in the previous cycle. After strand separation, the two primer DNA oligonucleotides allowed to hybridize to complementary sequences in the two DNA strands by cooling the reaction mixture. The synthesize direction is from 3'-OH terminus to 5'terminus on template strand DNA.

3.3.1. Principle of design primer

Good primer design is essential for successful PCR reactions. The important design has to follow several key principles which are described below.

Primer length: the common length of PCR primers is 18-27bp. It is not suitable to use primers longer than 38bp. Too long primers can lead annealing temperatures higher than 74℃ and this is not suitable for TAQ DNA polymerase reaction.

Primer annealing temperature: The primer melting temperature is the estimate of DNA hybrid stability and is a critical parameter of the system. Too high annealing temperature will produce insufficient primer template hybridization, leading low PCR product yield. Too low temperature may lead to non-specific products. Therefore, it is necessary to run melting curve within qPCR experiment and afterwards possibly to check the product by gel electrophoresis.

GC content: the optimal content should be 40% - 60%. Furthermore, there should be no large melting temperature differences between the upstream and the downstream primer.

Avoid non-specificity amplification: It is necessary to avoid regions of homology, in order to improve specificity of primers. Therefore primers designed for a particular sequence must not amplify other genes in the target DNA sequence. Commonly, we tested the primers by BLAST on Saccharomyces GENOME DATABASE (http://www.yeastgenome.org/).

3.4 Theory of Plasmid DNA

3.4.1.Principle of recombination plasmid DNA

Plasmids are widely used as vectors to carry the genes into bacterial cells, Plasimds are small circular molecules of double-stranded DNA. In this research work, the pGEM-7Zf which produced by Promega Corporation (USA) has been used as cloning vectors to insert the standard gene fragment that used as reference material for create of the calibration curve. To use as a vector, the purified plasmid DNA has to be cleaved with restriction nuclease to create a liner DNA molecule. The insert gene fragment of interest was amplified with PCR and primers with added the same restriction nuclease sites and after cleavage with restriction enzymes the fragments of the gene of the interest to be cloned are then added into the liner plasmid DNA by annealingvia their cohesive ends to form recombinant DNA circles. These recombinant DNA containing interest fragment inserts are then covalently linked with enzyme DNA ligase[5]. The Figure 3.4 demonstrates the process of introducing foreign DNA fragment into plasmid DNA.

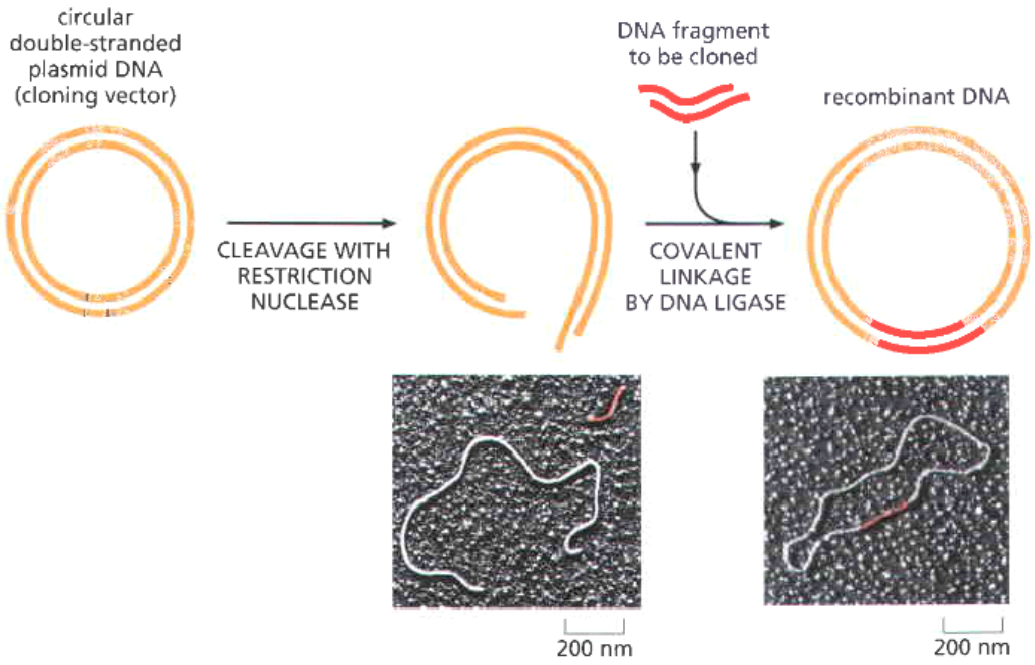


Figure 3.4 the process of introducing foreign DNA fragment into plasmid DNA (Molecular Biology Of The Cell.5th)

3.4.2. The type of restriction end

The recombinant plasmid DNA was transformed into bacterial cells (E.coli), in this work competent strain DH5 alpha competent cell were used as transform host cells to obtain colonies for screening the .the recombinant plasmids and for large-scale plasmid purification. Traditional methods to introduce DNA into bacterial cells involve incubating bacterial cells in calcium salt solution that makes the cell membrane leaky, permeable to the plasmid DNA

3.4.3.Plasmid Isolation

To use plasmid DNA which contains a target gene as a reference material, high purity plasmid must be obtained. Generally, in order to obtain plasmid DNA, the cells are harvested, the bacteria are lysed, and plasmid DNA recovered by alcohol precipitation. The best method for further DNA purificationis cesium chloride (CsCl)/ethidium bromide (EtBr) centrifugation[11]. Although many techniques have been invented for plasmid isolation, Cesium Chloride (CsCl) Density Gradient Centrifugation technique has always being considered as the best method to obtain the highest purity plasmid[12]. Therefore Cesium Chloride (CsCl) Density Gradient

Centrifugation technique was applied in this work.

CsCl is highly soluble in water, under high centrifugal force, CsCl molecules will dissociate and the heavy Cs^+ atoms will be forced towards the outer end of the tube, thus forming a shallow density gradient. DNA molecules placed in this gradient will migrate to the point where they have the same density as the gradient (the neutral buoyancy or isopycnic point). The gradient is sufficient to separate different types of DNA with slight difference in density due to differing G+C content, different isotopic content or due to different EtBr binding. The latter is the basis for separationof plasmid and chromosomal DNA. or physical form. In this work, after 15hours centrifugagation, plasmid DNA separated from chromosomal DNA, was detected under the UV light as a separate heavier band in the CsCl density gradient.

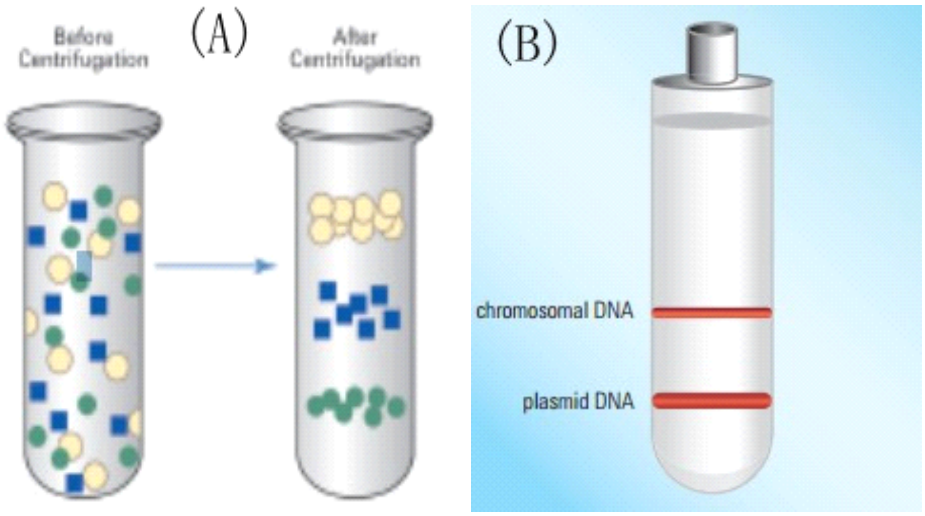


Figure 3.8 The procedure of CsCl Density Gradient Centrifugation. (A) position comparison between initial and final procedure; (B) the position of plasmid DNA after centrifugation.

3.5 Suggestion for plasmid DNA purification

Impure plasmid DNA may impact qPCR amplification, leading inaccurate initial copy number estimation in the reference plasmid material. Hence,in this work the plasmids were treated with 2mg/mL RNase and purified on CsCl Density Gradient Centrifugation to remove large size RNA. However, there was trace amount of RNA in plasmid DNA preparations. Therefore urther purification of DNA was performed with gel filtration.

The technique of gel filtration chromatography is used to separate molecules of different sizes and shapes[13]. The process of gel filtration involves letting the mixture of RNA and DNA be carried by an elution buffer through a filtration matrix contained in a glass column. The matrix consists of microscopic beads which are porous[13]. Compact molecules which used in this work were Sephacryl S-400 HR tend to get trapped in the pores for awhile before passing through the matrix[13]. Therefore, a heavier molecule will pass through the matrix faster than a lighter one, meaning the plasmid DNA should pass through the matrix faster than RNA. Hence, plasmid DNA allows to be collect and RNA stays remain in matrix after forcing brief micro-centrifugation.

3.6 Suggestion for plasmid copy number calculation

As the molecular weight of the plasmid and insert gene are know, it is possible to calculate the copy number as follow[14]:

$$\text{copy number} = \frac{6.02\times10^{23}\,(\text{copy/mol})\times\text{DNA amount(g)}}{\text{DNA length(bp)}\times 660\,(\text{g/mol/bp})} \qquad (1)$$

(where: bp = base pairs, weight in Daltons(g/mol)=(bp size of dsDNA product)(330 Da × 2nt/bp, ds = double-stranded, nt = nucleotides)

3.7 Description of used analytical methods

3.7.1.Gel Electrophoresis

Gel electrophoresis separates molecules according to their size and shape, but also according to their charge (JAC Biology NYA Laboratory Outlines, 2001). The samples to be analyzed are placed into a gel bed and when a voltage is applied across the gel bed, the molecules will migrate towards the pole they are attracted to according to their charge. In the case of DNA and RNA the charge-to-mass ratio on the two types of nucleic acids is not a factor, because there are always two negatively charged phosphate groups for each base pair charge (JAC Biology NYA Laboratory Outlines, 2001). Both types of nucleic acid will migrate towards the positive pole, however the RNA will move faster through the gel bed because it is smaller[15].In this

work, gel electrophoresis method mainly used to identify specific DNA fragments.

3.7.2. UV-Vis Spectrophotometer

Spectrophotometer is the instrument for quantitative measurement of the reflection or transmission properties of a material as a function of wavelength[16]. The most common spectrophotometers are used in the UV and visible region of the spectrum.

Analysis of nucleic acids is regularly performed to determine the average concentrations of DNA or RNA present in a mixture, as well as their purity. The Beer-Lambert law is used to determine unknown concentrations without the need for standard curves. In essence, the Beer Lambert Law makes it possible to relate the amount of light absorbed to the concentration of the absorbing molecule.

Usually A260 (ultraviolet light absorption at the wavelength of 260 nm) is used as a quantitative measure for nucleic acids.

The secondary benefit of using spectrophotometric analysis for nucleic acid quantitation is the possibility to determine sample purity using the 260 nm/280 nm ratio calculation. A pure DNA sample will yield an A260/280 of approximately 1.8. The pure RNA should have an A260/280 ratio of approximately 2.0. These ratios are commonly used to assess the amount of protein contamination that is left from the nucleic acid isolation process since proteins absorb at 280 nm[17].

The spectrophotometer which used in this study was “NanoDrop 2000” produced by Thermo scientific company.

3.7.3.Real-time polymerase chain reaction

Real-time polymerase chain reaction also called real-time quantitative PCR (qPCR) offers fast and reliable quantification of any target sequence in a DNA sample. The quantity can be either an absolute copy number or relative amount of target DNA when the housekeeper gene is being used as a reference in mRNA quantifications performed on cDNA[18.] Nowadays, many qPCR assays have been widely used in several applications such as environmental, clinical, forensic analysis. Real-time qPCR assays are being used in those applications due to high levels of sensitivity, specificity and precision. The technique allows for the continuous collection

fluorescence signal of PCR products during the reaction process. Ideally, these products will be exponentially amplidied, which means their quantities double with each thermal reactions cycles. Hence, qPCR can be applied to determine a threshold where the accumulation of amplified product is first significantly visible in the data. The fractional cycle number where PCR product accumulation passes this fixed threshold is called the threshold cycles (Ct)[19].A basic PCR run can be broken up into three phases: Exponential, Linear and Plateau.

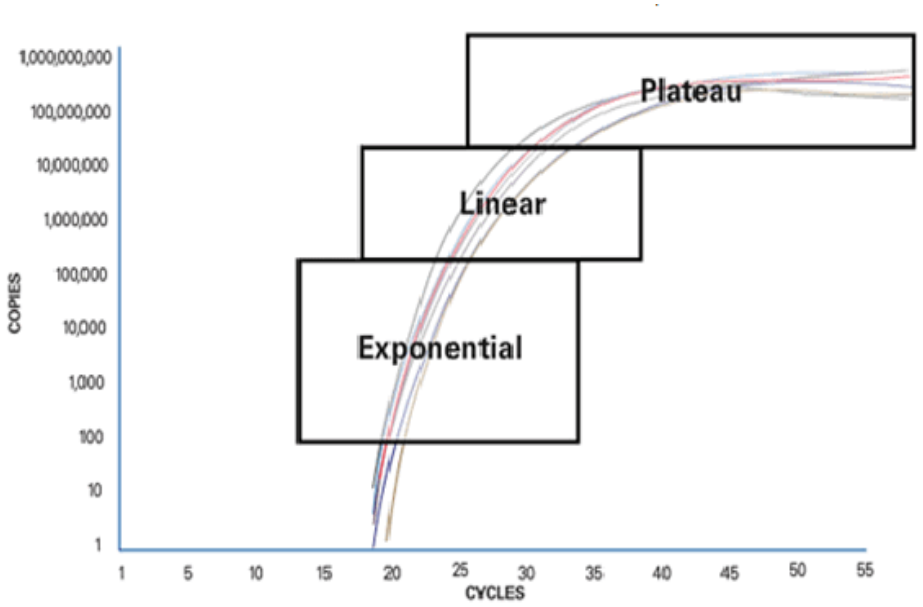


Figure 3.8 PCR phase （source：http://www.invitrogen.com）

At the linear phase, some of reagents are being consumed up as a result of amplification. The reactions start to slow down and the PCR product is no longer being doubled at each cycle, which means this phase generates high variable copy number. By contrast, the reaction in the exponential phase is very specific and precise, because all of the reagents are fresh and available, the kinetics of the reaction lead the reaction to favor doubling of the amplicon. Therefore, the noise baseline has to be set up as lower as possible, the threshold line should cross the exponential phase.

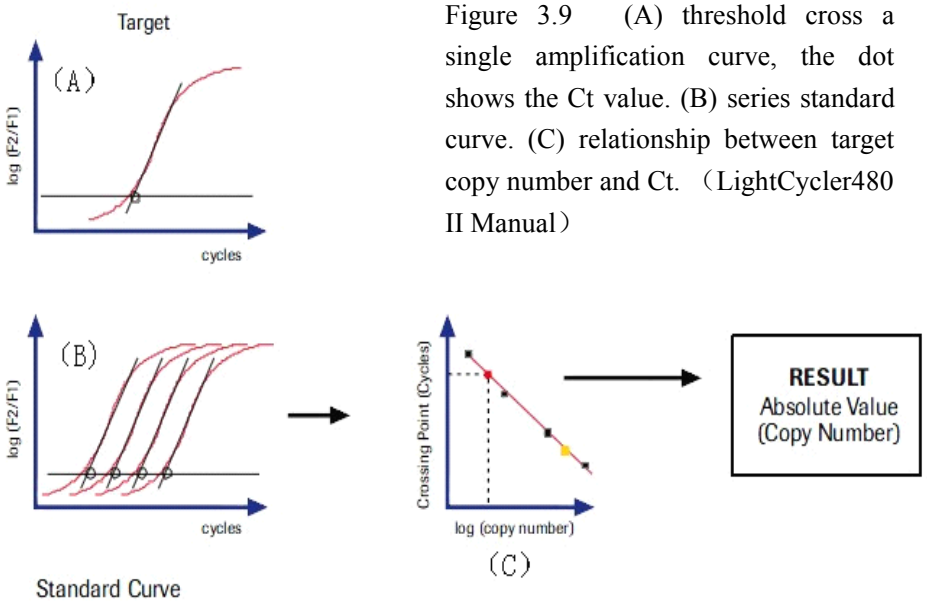


Figure 3.9 (A) threshold cross a single amplification curve, the dot shows the Ct value. (B) series standard curve. (C) relationship between target copy number and Ct. （LightCycler480 II Manual）

In an Absolute Quantification analysis, a standard curve is used to determine the concentration of unknown samples. In a standard curve, the concentrations of standard samples are plotted against the Ct of the samples. There is a log-linear relationship between the initial amount of DNA target in the reaction and the Ct value that is obtained.

Real-time PCR employs fluorescent dyes or probes that interact with the PCR products. The two primary types of fluorescent detection are DNA binding dyes, such as SYBR Green, or fluorescently tagged sequence specific probes, such as TaqMan or Molecular Beacon probes[20]. SYBR Green dye was used in this study. SYBR Green is a dye that binds the Minor Groove of double stranded DNA. When SYBR Green dye binds to double stranded DNA, the intensity of the fluorescent emissions increases. As more double stranded amplicons are produced, SYBR Green dye signal will increase. SYBR Green dye will bind to any double stranded DNA molecule, Since detection of fluorescent signal from these dyes is not sequence specific, melting temperature analysis must be performed to ensure the production of a single PCR product[21].

4. Experiment part

4.1 Reference plasmid preparation

Plasmid which contains specific sequence of analyte was used in qPCR

measurement to set up the calibration curve to calculate sample's copy number.

pGEM-7Zf plasmid vector (Promega, Madison, Wisconsin, USA) was used in this study. Specific fragments of yeast mitochondrial DNA were amplified with PCR. The oligonucleotides used for amplification were also used to introduce restriction sites for cloing at the fragment end. The fragments were purified from agarose gel by UltraClean®15 DNA purification kit (MO BIO, Carlsbad, CA USA). Fragments of 15S rRNA, COX3, COX1-exon1, COX1-exon4, SceI and ATP9 genes were inserted between the restriction sites XbaI and EcoRI. A fragments of COB4 was inserted between XbaI and SmaI, SmaI was used to create a blunt ends, because the inserter COB4 fragment sequence contains a restriction site EcoRI (comfirmed by WebCutter program). Therefore COB4 could not be cloned by using EcoRI.

The list of oligonucleotides used for amplification of mitochondrial DNA fragments is presented in the Table 4.1.

Table 4.1 Oligonucleotides for amplification of mitochondrial DNA fragments

Name	Sequences (5'→3')	Product size (bp)	Unavailable enzyme
15S rRNA	L-Xba: CTATCTAGA TCAAGCCAATAATGGTTTAGGT R-EcoRI: CTGAATTC AACTGGATCAATCTTTCGATCA	169	
COX3	L-Xba: CTATCTAGA TCCATTCAGCTATGAGTCCTGA R-EcoRI: CTGAATTC TGAACCATAAACACCATCAGAGA	281	
COX1-exon1	L-Xba: CTATCTAGA TGATTATATTCAACAAATGCAAAAGA R-EcoRI: CTGAATTC CCATGTAACACTTAACTCCACCT	325	
COX1-exon4	L-Xba: CTATCTAGA CTACACGTGTTGCACCCATT R-EcoRI: CTGAATTC GGTCCTGAATGTGCCTGAAT	266	
ATP9	L-Xba: CTATCTAGA GCAGCTAAATATATTGGAGCAGGTA R-EcoRI: CTGAATTC TGAAACCATTAAACAGAATAAACCTG	192	
COB4	L-Xba: CTATCTAGA AAATAGGATTAAGATATAGTCCGAACA	246	EcoRI

	R-EcoRI: CTGAATTC TGAATGCATTGGAATTCTATCTAAA		
SceI	L-Xba: CTATCTAGA ATTTACCCCCTTGTCCCATT R-EcoRI: CTGAATTC CCTCCATCATCTATAAATCAATATGC	736	HindIII

The list of constructed plasmid is presented in Table 4.2.

Table 4.2 List of constructed plasmid

Name	Upstream site	Downstream site	Plasmid size(bp)
15S rRNA	Xbal	EcoRI	3166
COX3	Xbal	EcoRI	3278
COX1-exon1	Xbal	EcoRI	3322
COX1-exon4	Xbal	EcoRI	3263
ATP9	Xbal	EcoRI	3189
COB4	Xbal	SmaI	3230
SceI	Xbal	EcoRI	3733
(Plasmid size = pGEM7/Zf size + inserter size)			

The reference plasmids were purified from 100-200ml bacterial cultures by alkaline lysis, Ribonouclease A treatment (20 mkg RNAse A in 1 ml, 56 ^{o}C, for 30 min) and CsCl Density Gradient Centrifugation. The traces of low-molecular RNA were removed by additional purification step on Sephacryl S-400 HR gel filtration column. The plasmid preparations were inspected by agarose gel electrophoreses.

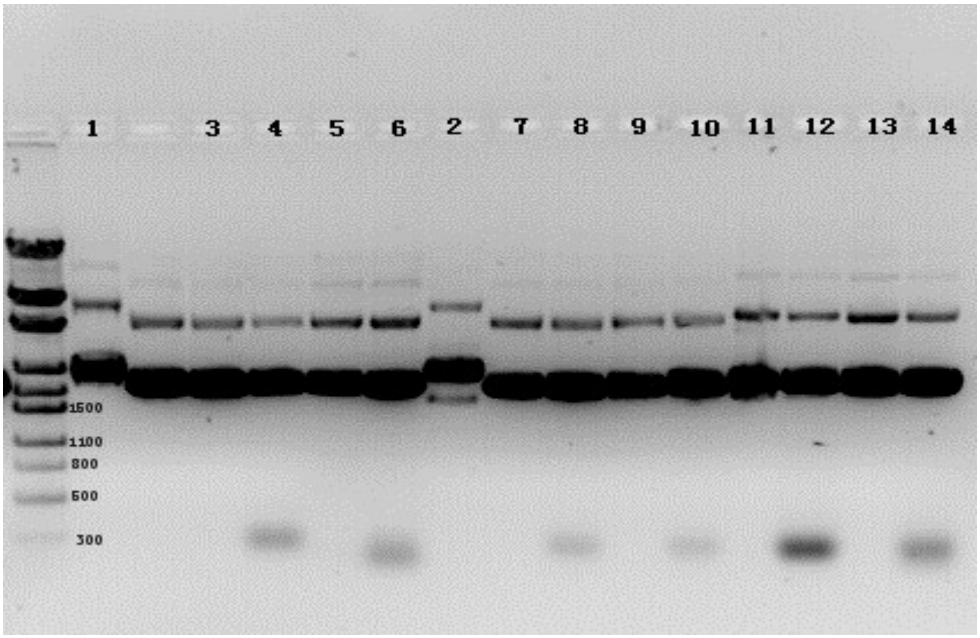


Figure 4.1 Inspection of plasmid purification of plasimd by agarose gel electrophoreses. Lane 1,3,5,7,9,11,13 corresponds SceI,cox3,cox1-1,ATP9,Cob4,15s,cox1-4 after gel filtration treatment, respectively. Column 2,4,6,8,10,12,14 corresponds SceI,cox3,cox1-1,ATP9,Cob4,15s,cox1-4 before gel filtration, respectively. The result demonstrated that there was no rsidual RNA remains in the Plasmid preparations.

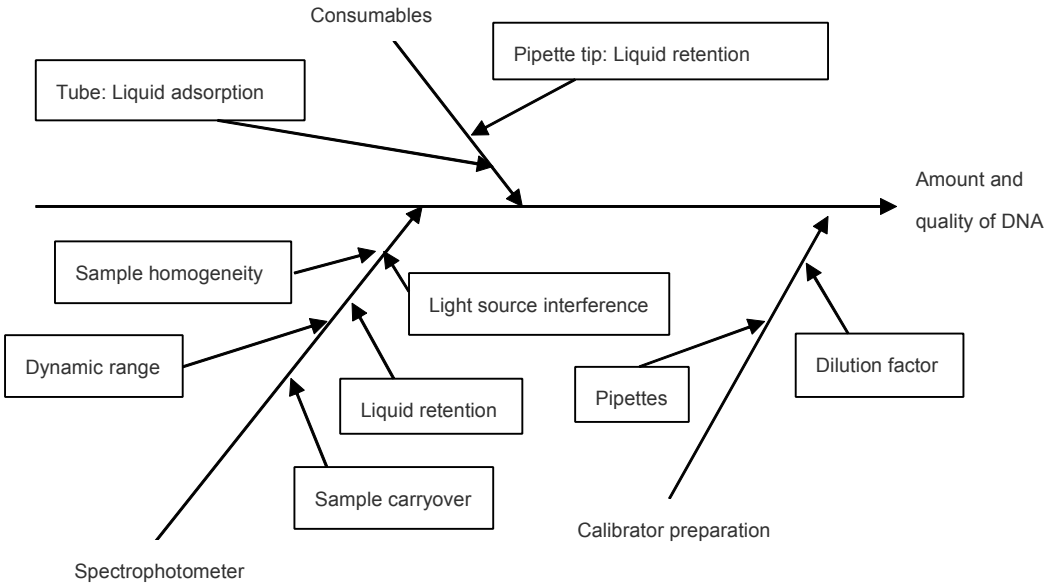
Chromosome reference gene was FIT2. FIT2 was amplified by PCR, and then fragment purified from agarose gel by UltraClean®15 DNA purification kit (MO BIO, Carlsbad, CA USA).

The stocks of reference plasmid were stored in $T_{10}E_{0.1}$ at +4 ^{o}C.

4.2 Reference plasmid copy number calculation and uncertainty estimate

The copy number calculation formula was given by (1). In order to calculate the copy number of plasmid, concentration of plasmid was measured by Nanodrop 2000 spectrophotometer by 5 replications. The uncertainty source flow is presented in Figure 4.2. If the DNA is not of sufficient quality, the downstream PCR efficiency may be affected. The quality of DNA can be affected by its size, stability, level of degradation, structural integrity, and presence of inhibitors and modifications, which may be inherent in the sample matrix[22]

Fig4.2 uncertainty source flow



It is important to ensure that the sample was mixed well before quantification, and that the spectrophotometer has been cleaned to ensure no sample carryover between redings. Based on manufacturer's guidelines, a 2μl drop is used for t measurements. The blank sample was $T_{10}E_{0.1}$ which was also used to dissolve plasmid DNA.

The plasmid DNA was diluted to a proper concentration, keep in $T_{10}E_{0.1}$ at +4 ^{o}C for routine use.

The series dilution process is presented in Table 4.3

Table 4.3 Series dilution process

Name	Initial concentration		Dilution No.1 concentration		Dilution No.2 concentration			Final concentration	
15S	147.82ng/μl	5μl	68.91μlTE	10ng/μl	5μl	45μlTE			1ng/μl
COX1-1	970.18 ng/μl	5μl	43.509μlTE	100 ng/μl	5μl	45μlTE	5μl	45μlTE	1ng/μl

COX1-4	2394.16 ng/µl	5µl	114.71µlTE	100 ng/µl	5µl	45µlTE	5µl	45µlTE	1ng/µl
COX3	1705.12 ng/µl	5µl	80.25µlTE	100 ng/µl	5µl	45µlTE	5µl	45µlTE	1ng/µl
ATP9	1474.4 ng/µl	5µl	68.72µlTE	100 ng/µl	5µl	45µlTE	5µl	45µlTE	1ng/µl
COB4	2473.5 ng/µl	5µl	118.68µlTE	100 ng/µl	5µl	45µlTE	5µl	45µlTE	1ng/µl
SCEI	2173.14 ng/µl	5µl	103.66µlTE	100 ng/µl	5µl	45µlTE	5µl	45µlTE	1ng/µl
FIT2	18.52 ng/µl	10µl	8.52µlTE	10 ng/µl	5µl	45µlTE			1ng/µl

The pipette was set at the proper volume to calculate dilution uncertainty by 5 replications. The measurement data is presented in Appendices Table 4.

Uncertainty is associated with dilution procedures prior the calibrator using in the work. The exactly uncertainty of the dilution factor depends on the series dilution process. The numerator and denominator in the formula for calculating the dilution factor are correlated (same a). Therefore, it is not completely correct to apply the rules of combining independent components of uncertainty when estimating the uncertainty of a dilution factor[23]. The uncertainty variance of a dilution step is obtained from:

$$u_f^2 = \frac{a^2 u_b^2 + b^2 u_a^2}{a^4} = \frac{u_b^2}{a^2} + \frac{b^2 u_a^2}{a^4} \qquad (2)$$

And the RSD squared from:

$$w_f^2 = \frac{1}{(a+b)^2}\left[\frac{a^2u_b^2 + b^2u_a^2}{a^2}\right] = \frac{b^2}{(a+b)^2}(w_b^2 + w_a^2) \qquad (3)$$

a = suspension transfer volume

b = diluton blank volume

u_a = standard uncertainity of a

u_b = standard uncertainity of b

w_a = relative standard uncertainty of a

w_b = relative standard uncertainty of b

If the total dilution contains of k similar steps the combined RSD squared of the dilution factor is calculated from:

$$w_F^2 = kw_f^2 \qquad (4)$$

If the total dilution contains several different steps, like each time different volume to perform dilution, the relative uncertainty of each step should be separately estimated, the combined RSD squared of the dilution factor is calculated from:

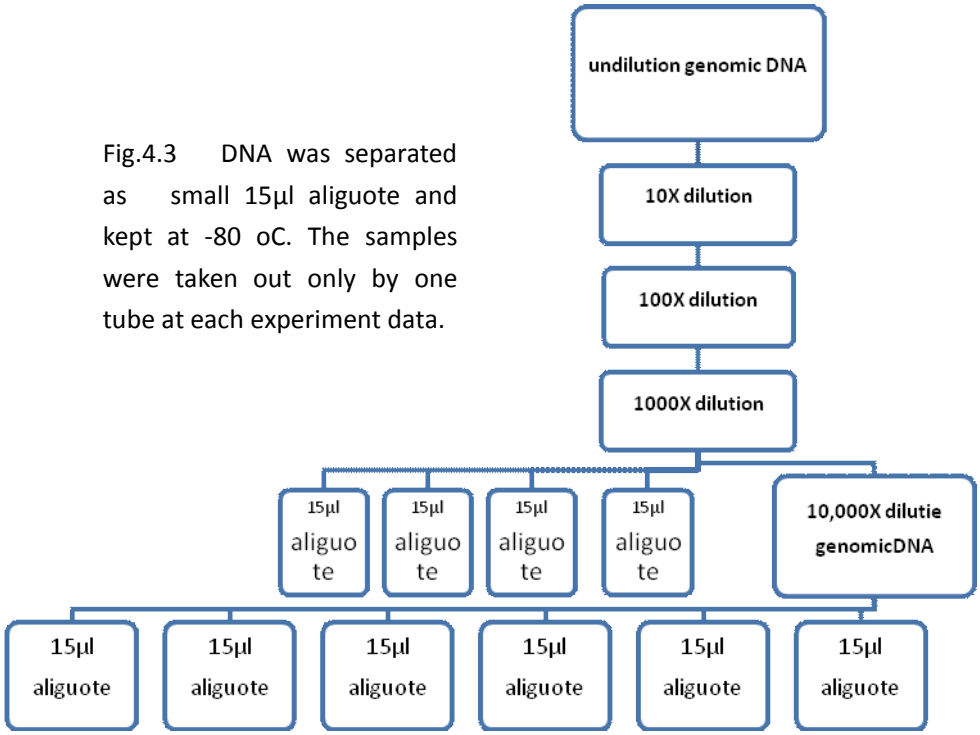
$$w_F^2 = w_{f_1}^2 + w_{f_2}^2 + \ldots + w_{f_k}^2 \qquad (5)$$

4.3 Total DNA preparation

The cultures of yeast *S.cerevisiae* strain W303 α were grown in 12ml YPG at 25 ^{o}C to OD600 = 0.70 (logarithmic growth phase), then transformed to 70ml YPG and grown to OD600 = 1.02, 10ml of the culture was spun down at that moment and used for DNA preparation.YPG contains yeast extract, peptone and glycerol($C_3H_8O_3$).

DNA was extracted with the following protocol that minimizes damage to high molecular weight molecules. And then keep DNA at -80 ^{o}C in small aliguotes. Once DNA was resuspended in the $T_{10}E_{0.1}$ (PH7.5), tips with cut end were used to handle DNA solution. The concentration of total DNA was 147.8ng/µl. Then DNA was diluted to 1000 and 10,000 times, separated in 6 tubes (15µl per tube). The protocol details are presented in Appendices 3.

Fig.4.3 DNA was separated as small 15µl aliguote and kept at -80 oC. The samples were taken out only by one tube at each experiment data.



4.4 Real-time polymerase chain reaction experiment

The calibration curves consisted each 5 calibration points: 10^5, 10^4, 10^3, 10^2, 10^1 copies of target gene per PCR assay. The copies of calibration plasmid were calculated based on formula (1). qPCR measurements were performed in MicroAmp® Optical 96-Well Reaction plates (Applied Biosystems, Lennik, Belgium) on a Roche LightCycler® 480 Real-Time PCR System using DNA binding dyes SYBR Green PCR MasterMix. Briefly, PCR cycling conditions were as follows: activation of DNA polymerase and initial denaturation for 15 mins at 95 °C; 50-60 cycles of denaturation for 10 s at 95 °C, annealing for 10s at 53 °C and extension for 15s at 72 °C, followed by a melting curve program, 1s quick denaturation at 95 °C, cooling down to 53 °C for 30s, followed by continued detection at 95 °C. All PCR assays were performed in triplicate using 10µl reaction system.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A1	A1	B1	B1	B1	C1	C1	C1	D1	D1	D1
B	A2	A2	A2	B2	B2	B2	C2	C2	C2	D2	D2	D2
C	A3	A3	A3	B3	B3	B3	C3	C3	C3	D3	D3	D3
D	A4	A4	A4	B4	B4	B4	C4	C4	C4	D4	D4	D4
E	A5	A5	A5	B5	B5	B5	C5	C5	C5	D5	D5	D5
F	X	X	X	X	X	X	X	X	X	X	X	X
G	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
H	N	N	N	N	N	N	N	N	N	N	N	N

Fig. 4.4 Schematic representation of the PCR plate design used in this study. The wells are marked with the following numeric coding for the respective series dilution of plasmid DNA (A = plasmid A, B = plasmid B, C = plasmid C, D = plasmid D). The rectangles represent triplicates for each point of the different calibration curves. The wells labelled X contain the genomic DNA samples, Y was stochastic variables, ie, 10x dilution of previously loaded genomic DNA, spiked plasmid DNA etc. The wells were marked N is negative control point (there was no DNA in it).

qPCR reaction components		
Chemical	volume per reaction (µl)	final concentration
10X homemade PCR buffer	1	1x
2mM dNTPs	1	0.2mM
20µM primers	0.3+0.3	600nM
20mM $MgCl_2$	0/0.25-0.5	3-4mM
1:10,000 SYBR Green dye	1	1/10 of reaction volume
Genomic DNA	1	
Hot TAQ	0.1	1/10 of reaction volume
Deionized distilled water	4.8-5.3	

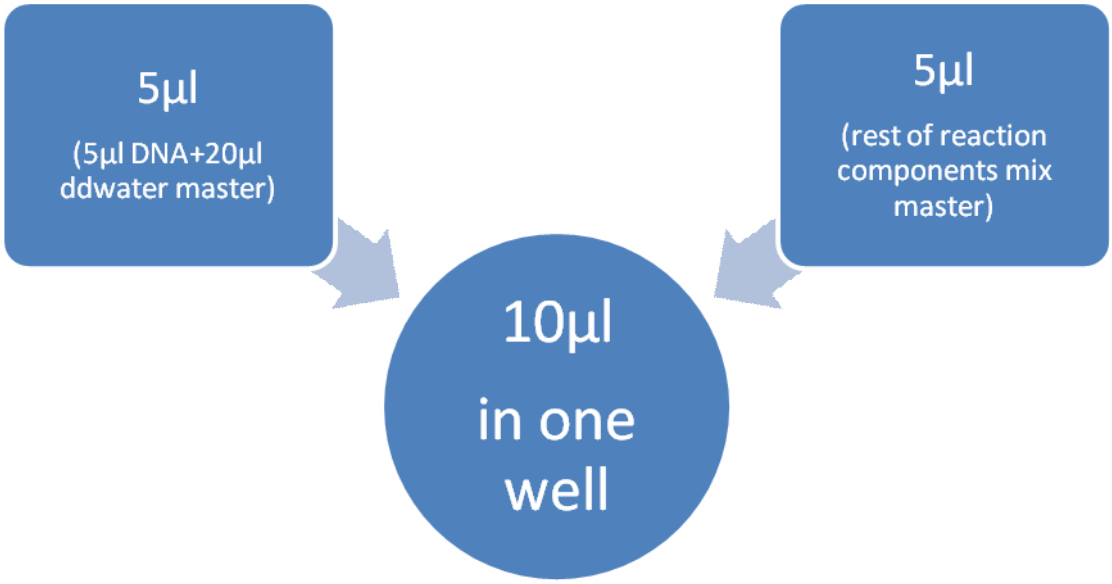
◆ 10x Homemade Core PCR Buffer consists 30mM $MgCl_2$, 200mM KCl,100mM Tris HCl (pH8.5), 1% Triton X-100

◆ $MgCl_2$ concentration was fluctuated for different genes, decision made by

reaction efficiency, As a rule, higher $MgCl_2$ concentration can significantly raise efficiency, but can also lead to non-specificity amplification[24].

10 fold series plasmid dilution made from 1ng/µl stock solution to 0.0001pg/µl by a standard scheme (5µl DNA solution mix with 45µl ddwater).

Fig. 4.5 A example for mixing reaction components



★ The loading sample for each individual well was 1µl.

Suggestion for avoiding contamination

△ **Keep your stock solution in the safe place**

△ **Use new tips for each experiment**

△ **Prepare several gloves for experiment, change them frequently**

△ **Keep working environment sterile**

5. Results and discussion

5.1 Absolute quantitative analysis of 15S rRNA

Uncertainty estimate

15S rRNA copy number was measured 4 times at 2 different days. The concentration of stock plasmid calibrator was 147.82ng/µl with 0.25% relative

uncertainty. According to dilution process, following the formula (1), (3) and (5), the 5 series 10 fold calibration points had 1.24%, 1.38%, 1.51%, 1.63% and 1.74% relative uncertainty, respectively.

However, the concentration or copy number of the calibrator does not correspond to the Ct value, but the $\log_{10}$(copy number) was used to establish the calibration curve with Ct. The uncertainty of $\log_{10}$(copy number) was calculated from formula:

$$f_{(x)} = \log_{10}(CN) \qquad (6)$$

$$u_{f_{(x)}} = \sqrt{\left(\frac{\partial_{f_{(x)}}}{\partial_{CN}} \times u_{CN} \right)^2} \qquad (7)$$

$$\frac{\partial_{f_{(x)}}}{\partial_{CN}} = \frac{1}{x \times In10} \qquad (8)$$

CN = copy number

$u_{f_{(x)}}$ = uncertainty of $\log_{10}$copy number

Hence, the uncertainty of $\log_{10}$copy number of plasmid standard DNA was 0.0054, 0.0060, 0.0066, 0.0071, and 0.0076, respectively.

The uncertainty of the Ct was observed by calculating the standard deviation of mean of triplicates reading.

The directive result from the calibration curve was sample initial copy number in logarithmic form; uncertainty of this result was estimated by Kragten approach. The formula is presented as following:

$$\log_{10}(CN) = \frac{Ct - b}{a} \qquad (9)$$

CN = copy number

Ct = threshold cross value

b = the intercept of the calibration function

a = the slope of the calibration function

The sample initial copy number and its uncertainty were calculated by following formula:

$$CN_{IN-run} = f_{(Ct)} = 10^{\frac{Ct-b}{a}} \quad (10)$$

$$CN_{IN-run} = 10^{\log_{10}(CN)}$$

$$STDVEV_{In-run} = \sqrt{(\frac{\partial f_{(y)}}{\partial_{\log_{10}(CN)}} \times STDVEV_{\log_{10}(CN)})^2} \quad (11)$$

$$\frac{\partial f_{(y)}}{\partial_{\log_{10}(CN)}} = In10 \times 10^{\log_{10}(CN)}$$

The recovery factor was measured by spiking same amount of plasmid DNA. The result was 1.03, which was close to 1, and considering this it is not necessary to correct the result.

The the copy number was converted to its undiluted a smple copy number by correcting with the dilution factor:

$$CN_{undiluted} = CN_{IN-run} \times d \quad (12)$$

$$u_{CN_{undiluted}} = CN_{undiluted} \times \sqrt{\left(\frac{u_{CN_{IN-run}}}{CN_{IN-run}}\right)^2 + \left(\frac{u_d}{d}\right)^2}$$

$$d = dilution\ factor$$

$$CN_{IN-run} = copy\ number\ of\ loading\ sample$$

$$CN_{undiluted} = copy\ number\ of\ undiluted\ sample$$

The result are presented in following table:

Gene ID	No.	Data	Copy_undiluted	u_rel	R^2	E
15s RNA	1	1-1	241851756	11.64%	0.9872	99.51%
	2	1-2	229206668	13.17%	0.9832	96.89%
	3	2	260983230	12.47%	0.9961	97.73%
	4	3	208288676	27.74%	0.9945	99.21%
E: efficiency of amplification; $E = 10^{(-1/slope)} - 1$						

Linearity

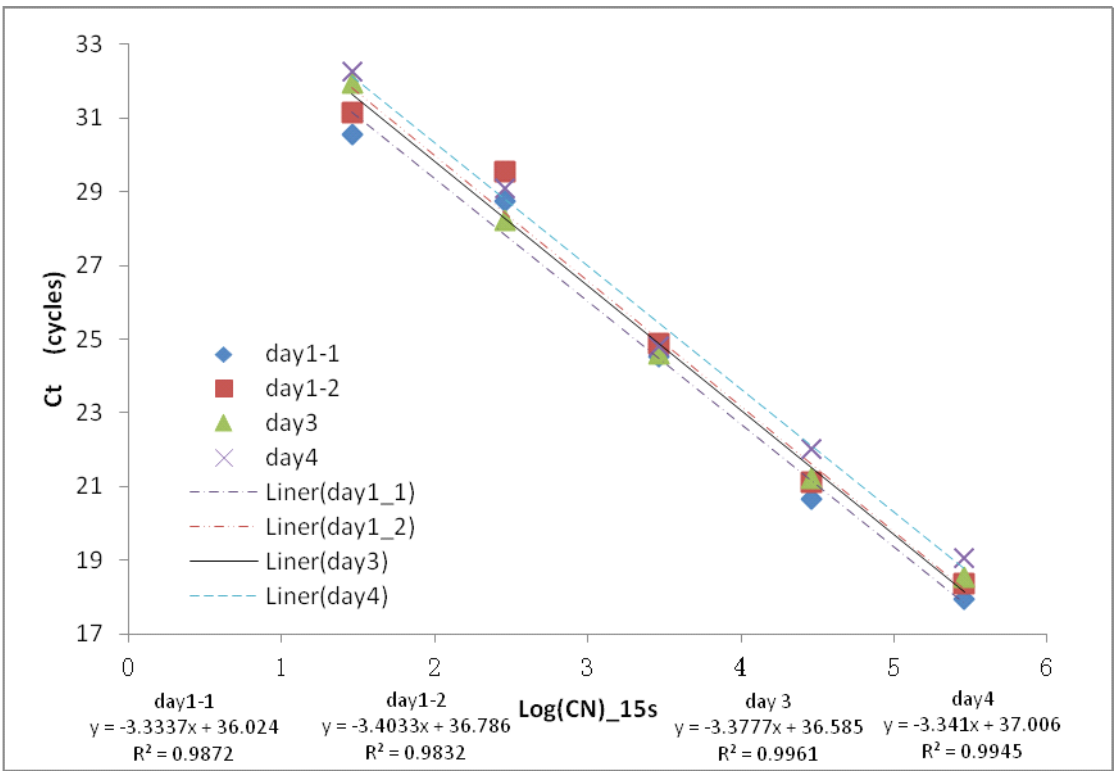


Fig.5.1 15S rRNA calibration curve comparison in different days. R^2 was less than 0.99, the linearity should be caution.

For each of the 4 platforms independently, a two-way analysis of variance (ANOVA, "GraphPad Prism 5") was used to test for significant differences. The results from both platforms showed that at the 95% level of confidence, there was a non-significant interaction (P>0.005) between the each calibration samples points and Ct value within different platforms. However, the P value of Ct less than 0.05. Considering it is significant. The calibration curve also was compared by 95% confidence interval as following Fig.5.1.2. There are no extreme result which is out of 95% CI occurred . (The statistical result form is presented in Appendices)

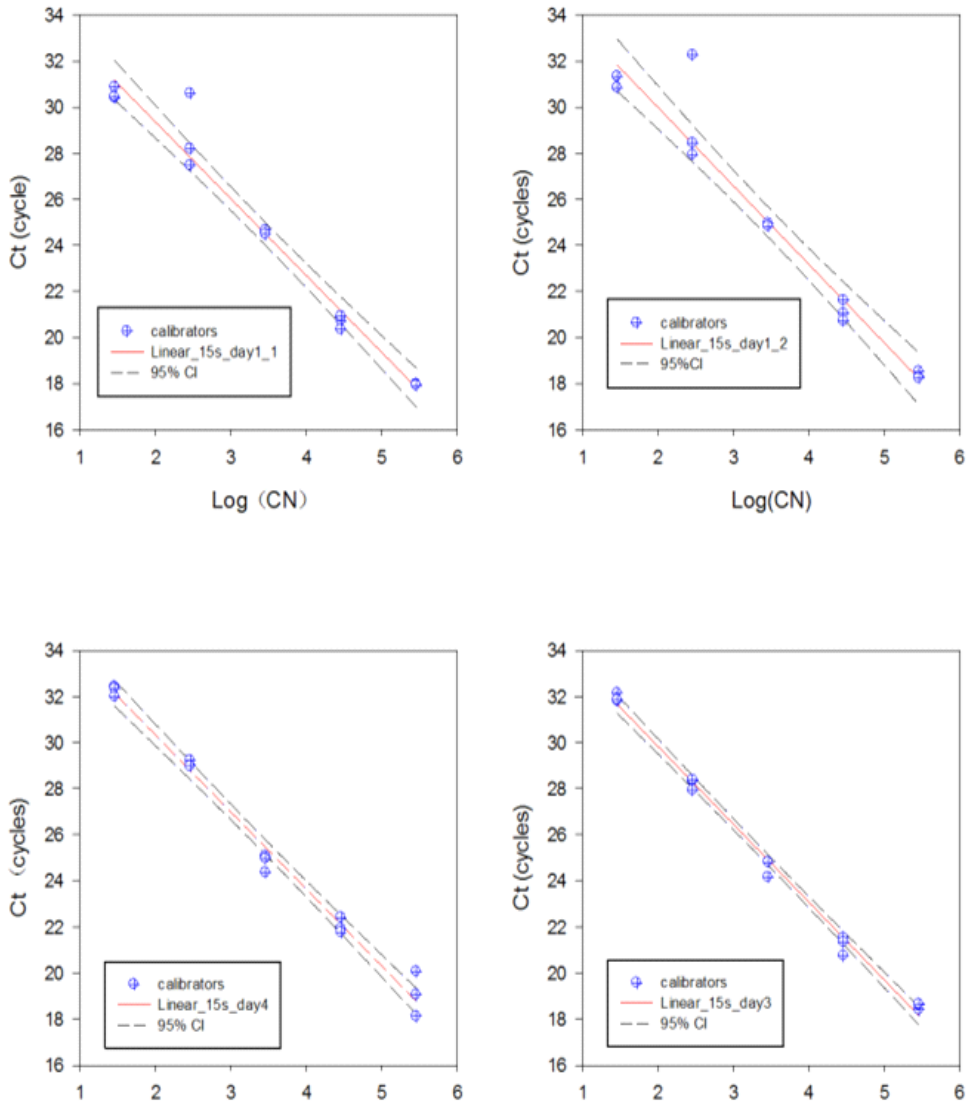


Fig.5.1.2　95% confidence interval test

Specificity

For each run the melting curve detection procedure was applied. The specific gene amplification products are homogeneous molecules that have identical melting temperature, therefore if the melting curve demonstrates only one peak, the specificity can be concluded[25].

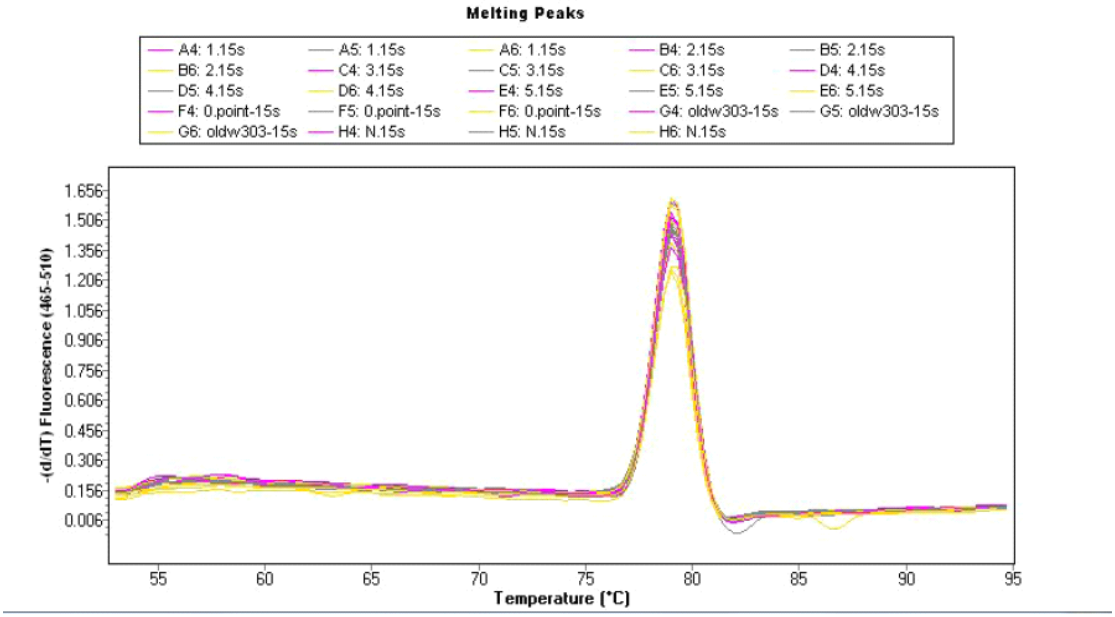


Fig.5.1.3 The melting curve at day 3. Because the running cycle was set up at 60 cycles, the single gene copy amplification should end before the 40th cycle by using those primers. The negative control samples present specific amplification from contaminating mtDNA traces and the singal. In these samples demonstrates the extreme sensitivity of the method.

5.2 Absolute quantitative analysis of Cox3

Cox3 copy number was measured 2 times at 2 different days. The concentration of stock plasmid calibrator was 1707.12ng/μl with 0.45% relative uncertainty. According to dilution process, the uncertainty of $\log_{10}$copy number of five 10-fold plasmid standard DNA was 0.0062, 0.0068, 0.0073, 0.0077 and 0.0082, respectively.

Uncertainty of the Ct was derived by calculating the standard deviation of mean of triplicates reading.

The directive result from the calibration curve was sample initial copy number in logarithmic form; uncertainty of this result was estimated by Kragten approach, and then transformed to absolute copy number by formula (10), (11), (12).

The recovery factor was measured by spiking same amount of plasmid DNA. The result was 1.01, which was close to 1, and considering this it is not necessary to correct the result.

The result are presented in thefollowing table:

Gene ID	No.	Data	Copy_undiluted	u_rel	R^2	E
Cox3	1	2	108060821	29.49%	0.9987	89.34%
	2	5	147951996	8.04%	0.9964	93.26%
E: efficiency of amplification; $E = 10^{(-1/slope)} - 1$						

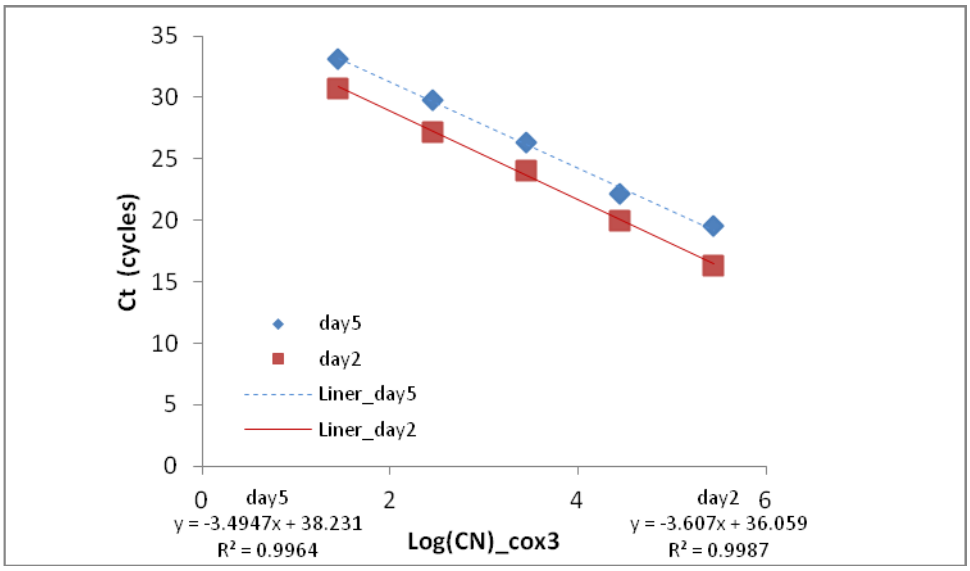


Fig.2 cox3 calibration curve comparison in different days. R^2 was greater than 0.99, the linearity was acceptable. The graph demonstrated the slopes are not significantly different. If the overall elevations were identical, intercepts are not significantly different. There was a non-significant interaction (P>0.005) between the each calibration samples points and Ct value within different platforms. However, the P value of Ct less than 0.05 and F-ratio was greater. Considering it is significant.

5.3 Absolute quantitative analysis of COX1-exon1

Cox1-exon1 copy number was measured 3 times at 3 different days. The concentration of stock plasmid calibrator was 970.18ng/μl with 0.44% relative uncertainty. According to dilution process, the uncertainty of $\log_{10}$copy number of five 10-fold plasmid standard DNA was 0.0056, 0.0062, 0.0068, 0.0073 and 0.0077, respectively.

Uncertainty of the Ct was derived by calculating the standard deviation of mean of triplicates reading.

The directive result from the calibration curve was sample initial copy number in

logarithmic form; uncertainty of this result was estimated by Kragten approach, and then transformed to absolute copy number by formula (10), (11), (12).

The recovery factor was measured by spiking same amount of plasmid DNA. The result was 1.03, which was close to 1, and considering this it is not necessary to correct the result.

The result are presented in the following table:

Gene ID	No.	Data	Copy_undiluted	u_rel	R^2	E
Cox1_exon1	1	2	159588837	19.33%	0.9901	94.28%
	2	5	143168448	23.60%	0.9982	73.97%
	3	6	85824549	12.27%	0.9906	76.17%
E: efficiency of amplification; $E = 10^{(-1/slope)} - 1$						

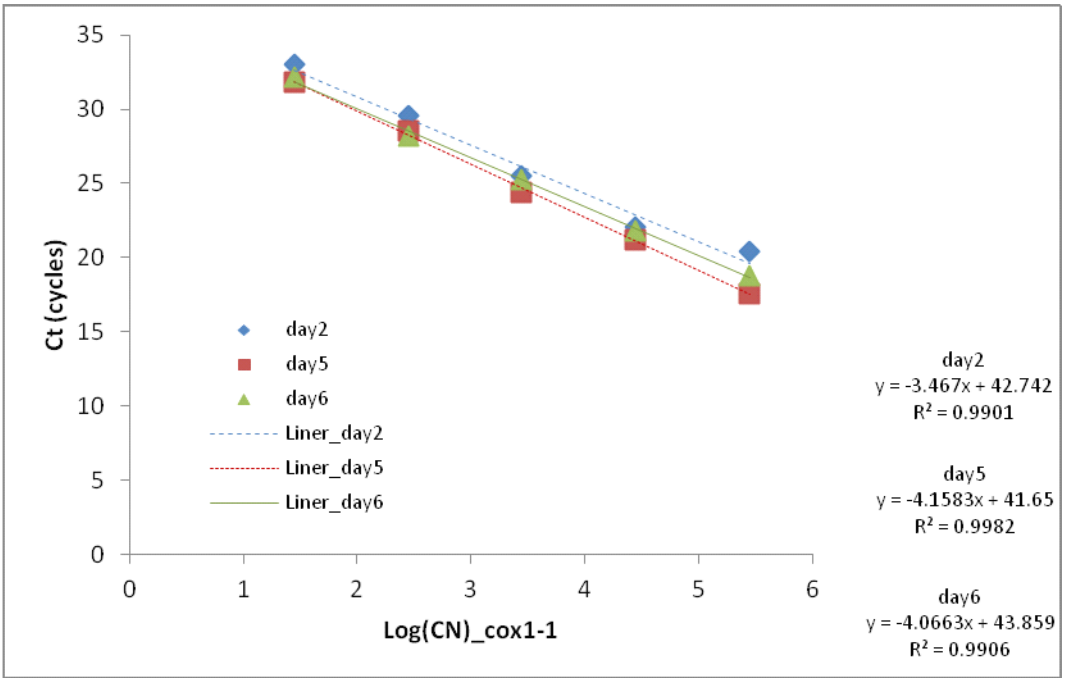


Fig.5.3 cox1_1 calibration curve comparison in different days. R^2 was greater than 0.99, the linearity was acceptable. There wasn't a non-significant interaction ($P<0.005$) between the each calibration samples points and Ct value within different platforms. The P value of Ct less than 0.05 and F-ratio was greater. Considering it is significant.

5.4 Absolute quantitative analysis of Cox1_exon4

Cox1_4 copy number was measured 3 times at 3 different days. The

concentration of stock plasmid calibrator was 2394.2ng/μl with 0.56% relative uncertainty. According to dilution process, the uncertainty of log_{10}copy number of five 10-fold plasmid standard DNA was 0.0064, 0.0070, 0.0074, 0.0079 and 0.0083, respectively.

Uncertainty of the Ct was derived by calculating the standard deviation of mean of triplicates reading.

The directive result from the calibration curve was sample initial copy number in logarithmic form; uncertainty of this result was estimated by Kragten approach, and then transformed to absolute copy number by formula (10), (11), (12).

The result is presented in following table:

Gene ID	No.	Data	Copy_undiluted	u_rel	R^2	E
Cox1_exon4	1	2	155989983	20.39%	0.9826	102.23%
	2	5	140773979	18.28%	0.9986	100.65%
	3	6	96891310	8.31%	0.9978	90.10%

E: efficiency of amplification; $E = 10^{(-1/slope)} - 1$

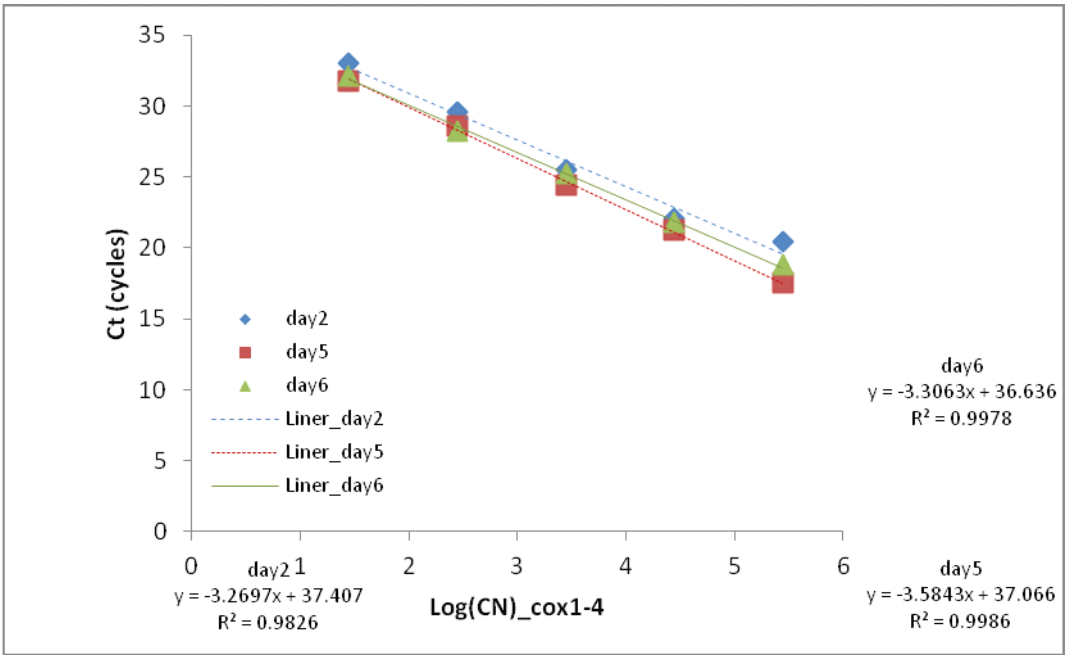


Fig.5.4 cox1_4 calibration curve comparison in different days. R^2 was greater than 0.99, the linearity was acceptable. There wasn't a non-significant interaction ($P<0.005$) between the each calibration samples points and Ct value within different platforms. The P value of Ct less than 0.05 and F-ratio was greater. Considering it is significant.

5.5 Absolute quantitative analysis of ATP9

ATP9 copy number was measured 3 times at 2 different days. The concentration of stock plasmid calibrator was 1474.4ng/μl with 0.27% relative uncertainty. According to dilution process, the uncertainty of $\log_{10}$copy number of five 10-fold plasmid standard DNA was 0.0060, 0.0066, 0.0071, 0.0076 and 0.0080, respectively.

Uncertainty of the Ct was derived by calculating the standard deviation of mean of triplicates reading.

The directive result from the calibration curve was sample initial copy number in logarithmic form; uncertainty of this result was estimated by Kragten approach, and then transformed to absolute copy number by formula (10), (11), (12).

The result are presented in the following table:

Gene ID	No.	Data	Copy_undiluted	u_rel	R^2	E
ATP9	1	2	171712693	29.29%	0.9807	89.38%
	2	7	119323099	43.41%	0.9812	83.36%
	3	7	134358399	30.90%	0.9816	83.20%
E: efficiency of amplification; $E = 10^{(-1/slope)} - 1$						

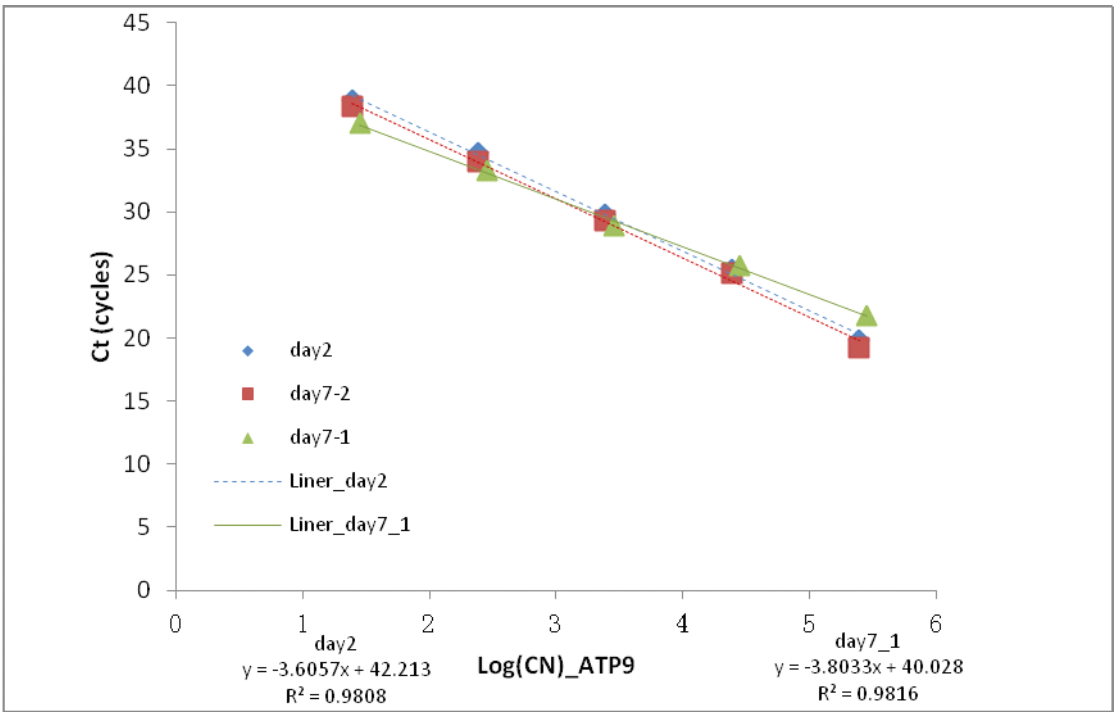


Fig. 5.5 ATP9 calibration curve comparison in different days. R^2 was greater than 0.98, the linearity was acceptable. There was a non-significant interaction ($P > 0.005$) between the each calibration samples points and Ct value within different platforms. The P value of Ct less than 0.05 and F-ratio was greater. Considering it is significant.

5.6 Absolute quantitative analysis of Cob4

Cob4 copy number was measured 3 times at 2 different days. The concentration of stock plasmid calibrator was 2473.5ng/μl with 0.55% relative uncertainty. According to dilution process, the uncertainty of $\log_{10}$copy number of five 10-fold plasmid standard DNA was 0.0063, 0.0069, 0.0074, 0.0070 and 0.0083, respectively.

Uncertainty of the Ct was derived by calculating the standard deviation of mean of triplicates reading.

The directive result from the calibration curve was sample initial copy number in logarithmic form; uncertainty of this result was estimated by Kragten approach, and then transformed to absolute copy number by formula (10), (11), (12).

The result are presented in the following table:

Gene ID	No.	Data	Copy_undiluted	u_rel	R^2	E
Cob4	1	2	103537584	23.55%	0.9918	96.50%
	2	7	178176340	24.42%	0.9983	84.48%
	3	7	123255941	31.56%	0.09981	83.47%

E: efficiency of amplification; $E = 10^{(-1/slope)} - 1$

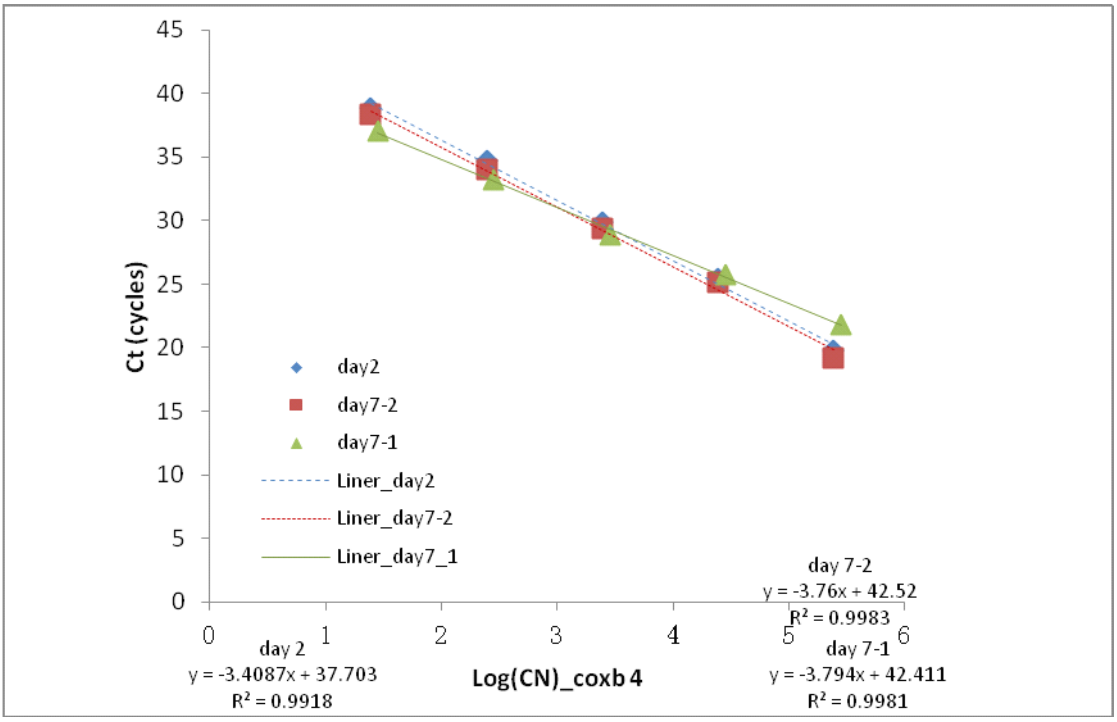


Fig.5.6 cob4 calibration curve comparison in different days. R^2 was greater than 0.98, the linearity was acceptable. There wasn't a non-significant interaction ($P<0.005$) between the each calibration samples points and Ct value within different platforms. The P value of Ct less than 0.05 and F-ratio was greater. Considering it is significant.

5.7 Absolute quantitative analysis of SceI

SceI copy number was measured 4 times at 3 different days. The concentration of stock plasmid calibrator was 2173.14ng/μl with 0.28% relative uncertainty. According to dilution process, the uncertainty of $\log_{10}$copy number of five 10-fold plasmid standard DNA was 0.0060, 0.0066, 0.0071, 0.0076 and 0.0080, respectively.

Uncertainty of the Ct was derived by calculating the standard deviation of mean of triplicates reading.

The directive result from the calibration curve was sample initial copy number in logarithmic form; uncertainty of this result was estimated by Kragten approach, and then transformed to absolute copy number by formula (10), (11), (12).

The result are presented in the following table:

Gene ID	No.	Data	Copy_undiluted	u_rel	R^2	E
SceI	1	8	117698439	12.71%	0.9985	60.67%

	2	5	100346614	27.30%	0.9661	69.31%
	3	7	111995901	9.40%	0.9961	62.50%
	4	7	96504569	14.43%	0.9976	63.05%
E: efficiency of amplification; $E = 10^{(-1/slope)} - 1$						

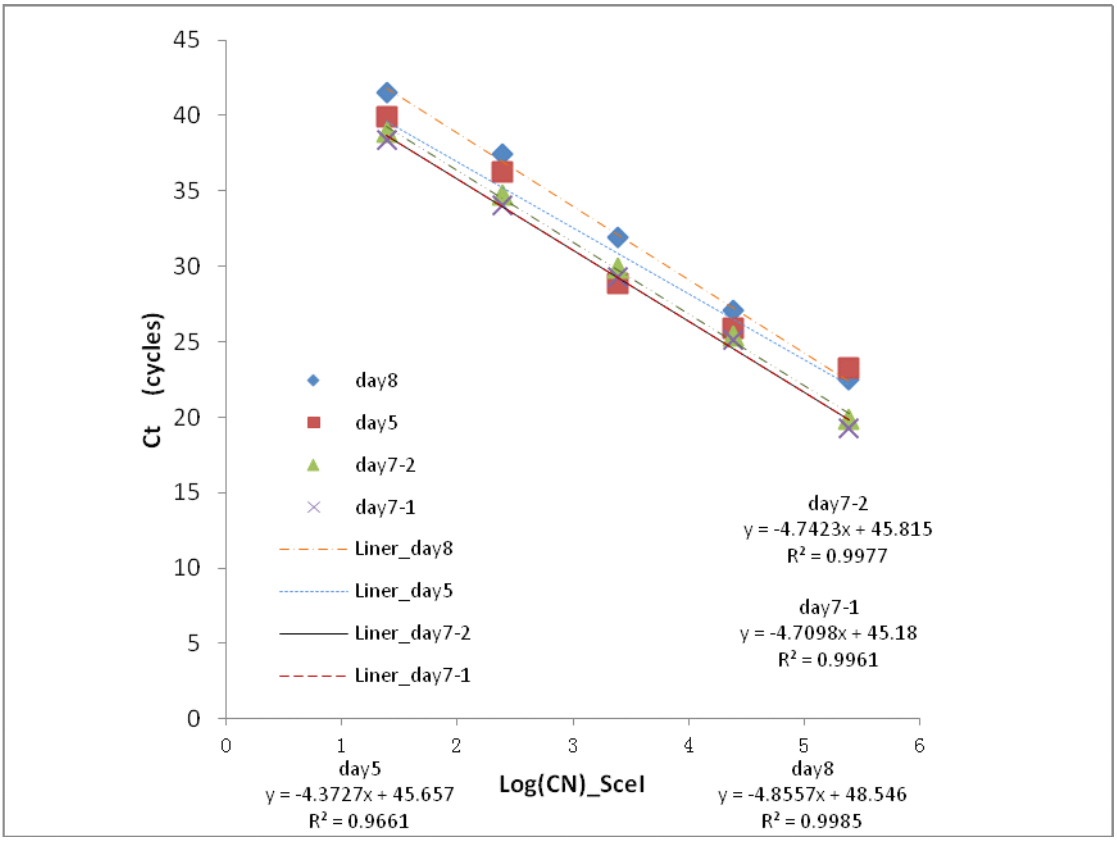


Fig.5.7 SceI calibration curve comparison in different days, At day 5, the R^2 was 0.96 and associated with higher relative uncertainty, therefore the result was eliminated from final comparison.

5.8 Absolute quantitative analysis of FIT2

FIT2 copy number was measured 5 times at 4 different days. The concentration of stock plasmid calibrator was 18.5ng/µl with 0.47% relative uncertainty. According to dilution process, the uncertainty of $\log_{10}$copy number of five 10-fold plasmid standard DNA was 0.0062, 0.0068, 0.0073, 0.0074 and 0.0082, respectively.

Uncertainty of the Ct was derived by calculating the standard deviation of mean of triplicates reading.

The directive result from the calibration curve was sample initial copy number in logarithmic form; uncertainty of this result was estimated by Kragten approach, and

then transformed to absolute copy number by formula (10), (11), (12).

The result is presented in following table:

Gene ID	No.	Data	Copy_undiluted	u_rel	R^2	E
FIT2	1	2	24360613	21.82%	0.9989	78.21%
	2	3	21578152	18.50%	0.9923	76.04%
	3	5	27712454	17.90%	0.9951	77.26%
	4	8	45093626	9.56%	0.9966	74.00%
	5	8	50287795	11.61%	0.9968	73.67%

E: efficiency of amplification; $E = 10^{(-1/slope)} - 1$

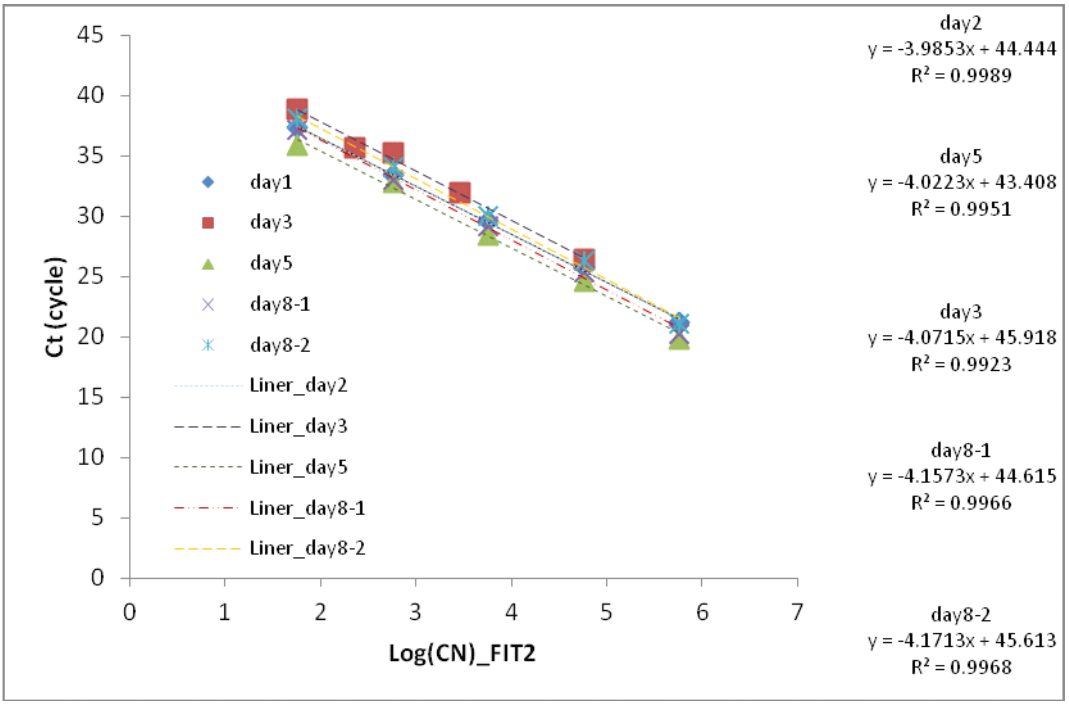


Fig.5.8 cob4 calibration curve comparison in different days. R^2 was greater than 0.99, the linearity was acceptable. There was a non-significant interaction ($P > 0.005$) between the each calibration samples points and Ct value within different platforms. The P value of Ct less than 0.05 and F-ratio was greater. Considering it is significant.

5.9 mtDNA copy number in single cell

The chromosome gene FIT2 is single copy chromosomal gene, that is present as a single copy in haploid cells during most of the cell cycle. Therefore the ratio of mtDNA gene to FIT2 is approximates to the mitochondrial genome copy number in a single cell.

Each gene was observed several independent copy number result from different days. The result was treated with box and whisker plot (presented below), using the description outlined above where the whiskers encompass 5% to 95% of the range of results. An outlier can be defined as a data point that does not follow the typical distribution of the rest of the data set and can be regarded as an irregular observation[27].There was no significant outlier, hence the mean value were able to be a true value as used to compare with FIT2 copy number. The pooled standard deviation was used to calculate uncertainty in this case.

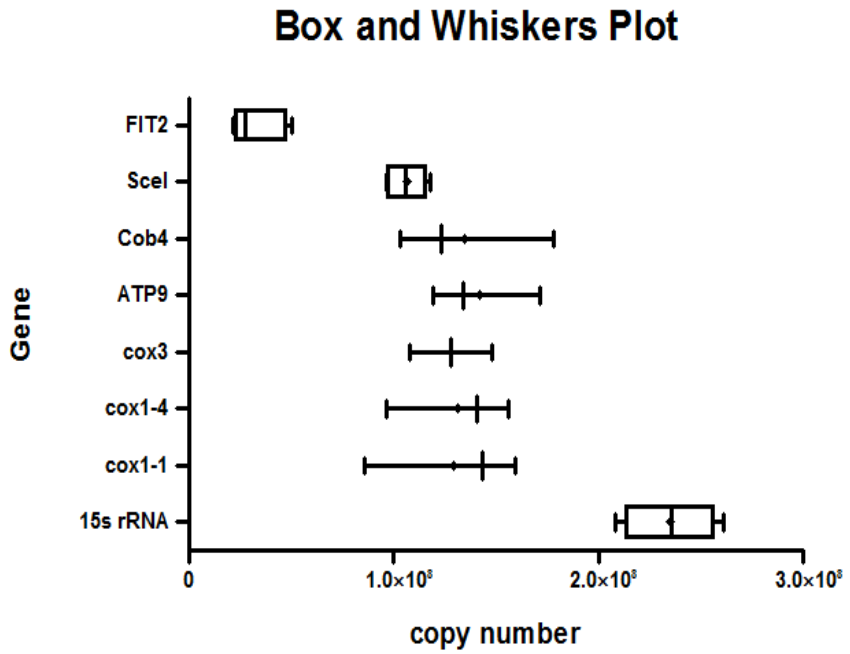


Table 5.9 mtDNA genes ratio			
Gene	Ratio		Relative Uncertainty
15s	8.81	±	29.48%
cox1-1	4.67	±	29.76%
cox1-4	4.73	±	28.63%
cox3	4.62	±	27.62%
ATP9	5.12	±	40.96%
Cob4	4.45	±	37.49%

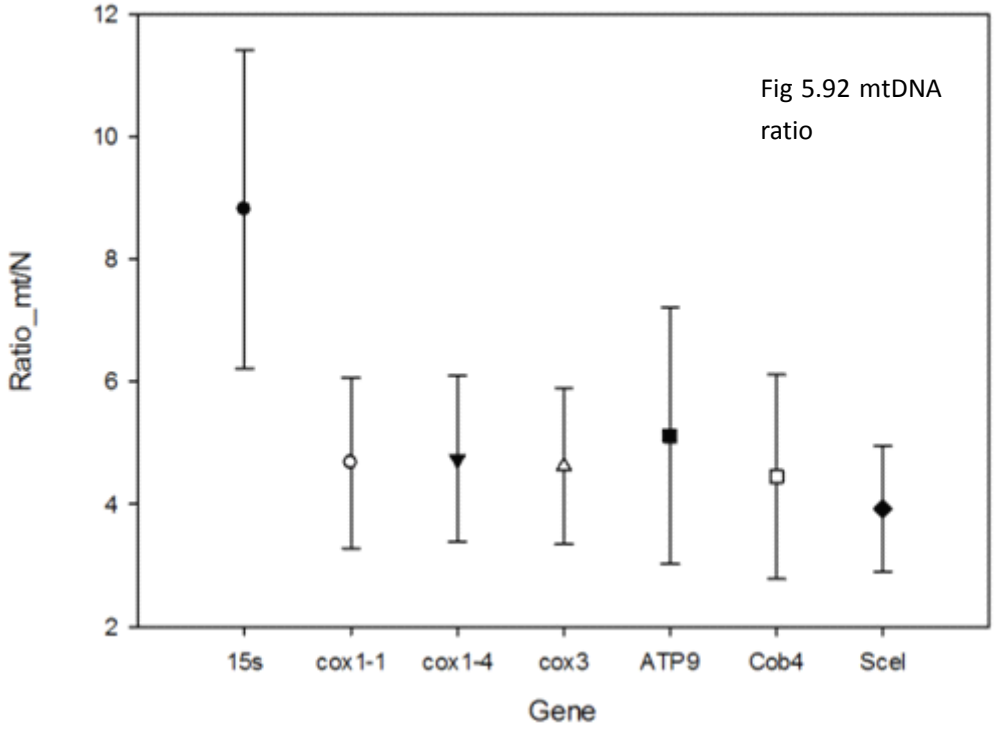


Fig 5.92 mtDNA ratio

6. Summary

The main goal of this work was developing the qPCR method for yeast mitochondrial DNA analysis , and to estimate the ratio of between mitochondrial and nuclear genes.

This study revealed that the mtDNA copy number in yeast S. cerevisiae stain w303 α does not have a constant value for each mitochondrial genetic elements.The analyzed protein encoding genes, cytochrome c oxidase subunit I, III (cox1_ exon1, cox1-exon4 and cox3), had roughly the same copy number. However, the copy number of the mitochondrial RNA gene, 15S, is approximately twofold higher. Hence, based on this study, we can speculate that the mitochondrial gene copy number depends on the gene function and obviously can be regulated independently, according to the metabolic needs of the organelle. It will be therefore interesting to test how the changes of copy number of individual mitochondrial genes during different growth conditions, for example on different carbon sources. It will also be interesting to analyze other yeast strains to confirm the detected copy number difference in other genetic

background.

7. Summary in Estonian

Töö peamiseks eesmärgiks oli qPCR-l baseeruvate mõõtmismeetodite väljaarendamine, mille abil oleks võimalik kvantitatiivselt iseloomustada mitokondriaalse DNA koopiaarvu pärmi *S. cerevisiae* rakkudes.

Töö käigus läbiviidud analüüs näitas, et *S. cerevisiae* tüves w303 on erinevate mitokondriaalsete geenide koopiaarv erinev. Valke kodeerivad tsütokroomi oksidaasi subühiku I (eksonid 1 ja 4) ja III geenide koopiaarv on ligikaudu võrdne, ent mitokondriaalse ribosoomi 15S rRNA geeni koopiaarv on kaks korda kõrgem. Seega on mitokondriaalse DNA koopiaarv sõltuv konkreetselt analüüsitavast geenist ja võib olla individuaalselt reguleeritud vastavalt metabolismi vajadustele. Huvitav oleks analüüsida, kuidas muutub koopiaarv erinevates kasvutingimustes, näiteks erinevaid süsinikuallikaid kasutades ning kuidas sõltub mitokondriaalse DNA koopiaarv tüve tuuma genoomi erisustest.

8. Acknowledgments

Hereby, I would like to express my sincere gratitude to all the persons who have helped during my studies in Estonia.

I want to thank Professor Juhan Sedman for accepting me as a research student in his group and for guidance during these years.

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Last, but not the least, I would like to thank my family for support, understanding and true motivation during hard time.

9. Reference

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10. Appendices

Appendice1. ANOVA statistical analysis result

- **15s**

Two-way RM ANOVA	Matching by rows	
Source of Variation	% of total variation	P value
Interaction	0.44	0.2832
Plate	97.56	< 0.0001
Ct	0.50	0.0032

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	12	6.220	0.5183	1.275
Plate	4	1365	341.3	391.0
Ct	3	6.972	2.324	5.715
Subjects (matching)	10	8.729	0.8729	2.146
Residual	30	12.20	0.4067	

- **Cox3**

Two-way RM ANOVA	Matching by rows	
Source of Variation	% of total variation	P value
Interaction	0.11	0.8094
Ct	92.26	< 0.0001
plate with different calibration opint	5.98	< 0.0001
Subjects (matching)	0.9286	0.3473

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	4	0.9281	0.2320	0.3927
Ct	4	757.6	189.4	248.4
plate with different calibration opint	1	49.10	49.10	83.10
Subjects (matching)	10	7.625	0.7625	1.290
Residual	10	5.909	0.5909	

- **COX1-excon1**

Two-way RM ANOVA	Matching by rows	
Source of Variation	% of total variation	P value
Interaction	1.08	0.0013
Plate	91.64	< 0.0001
Ct	6.46	< 0.0001
Subjects (matching)	0.3037	0.3610

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	8	16.06	2.008	5.210
Plate	4	1369	342.2	754.5
Ct	2	96.52	48.26	125.2
Subjects (matching)	10	4.535	0.4535	1.177
Residual	20	7.708	0.3854	

- **COX1-excon4**

Two-way RM ANOVA	Matching by rows	
Source of Variation	% of total variation	P value
Interaction	0.55	0.0088
Ct	97.44	< 0.0001
plate with different calibration points	1.47	< 0.0001
Subjects (matching)	0.1725	0.5333

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	8	5.819	0.7274	3.659
Ct	4	1035	258.8	1412
plate with different calibration points	2	15.58	7.788	39.18
Subjects (matching)	10	1.833	0.1833	0.9220
Residual	20	3.976	0.1988	

- **SceI**

Two-way RM ANOVA	Matching by rows	
Source of Variation	% of total variation	P value
Interaction	1.04	0.2217
Ct_secI	93.06	< 0.0001
plates with differents calibrator points	3.28	< 0.0001
Subjects (matching)	0.7640	0.3110

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	12	27.80	2.316	1.396
Ct_secI	4	2492	622.9	304.5
plates with differents calibrator points	3	87.69	29.23	17.61
Subjects (matching)	10	20.46	2.046	1.233
Residual	30	49.78	1.659	

• ATP9

Two-way RM ANOVA	Matching by rows	
Source of Variation	% of total variation	P value
Interaction	0.55	0.5690
Ct_ATP9	89.17	< 0.0001
Plates with different calibration points	5.75	< 0.0001
Subjects (matching)	2.9094	0.0071

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	8	7.881	0.9851	0.8536
Ct_ATP9	4	1273	318.2	76.62
Plates with different calibration points	2	82.10	41.05	35.57
Subjects (matching)	10	41.53	4.153	3.599
Residual	20	23.08	1.154	

• Cob4

Source of Variation	% of total variation	P value
Interaction	0.40	0.0101
Ct_cbo4	89.60	< 0.0001
Plates with different calibration points	9.10	< 0.0001
Subjects (matching)	0.6092	0.0027

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	8	5.419	0.6774	3.557
Ct_cbo4	4	1204	300.9	367.7
Plates with different calibration points	2	122.3	61.14	321.0
Subjects (matching)	10	8.184	0.8184	4.297
Residual	20	3.809	0.1904	

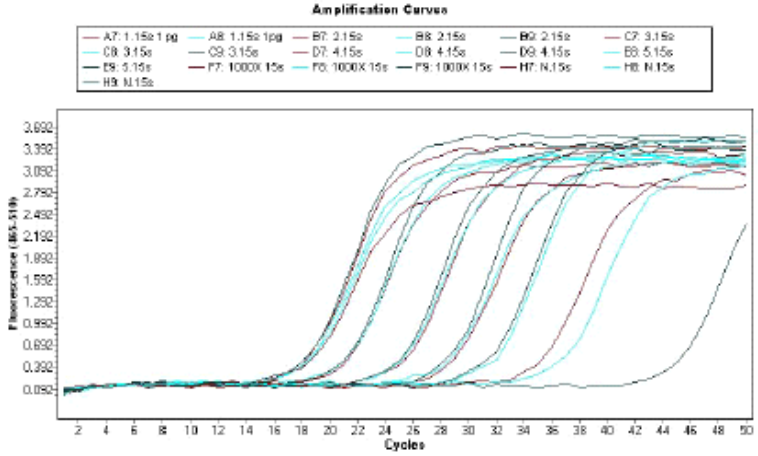
• FIT2

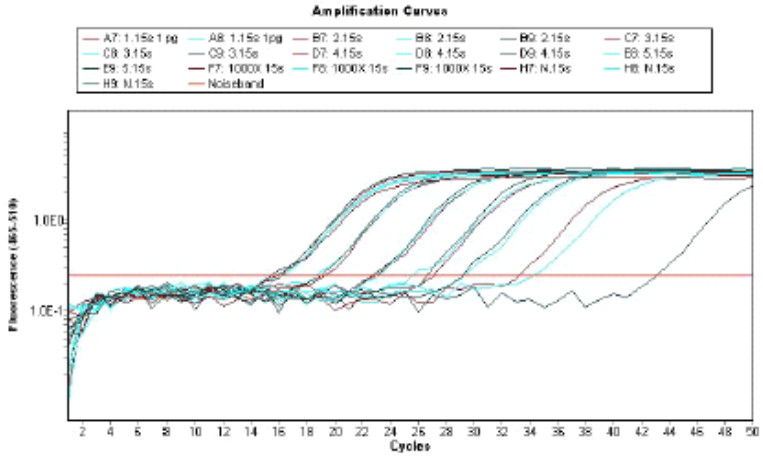
Two-way RM ANOVA	Matching by rows	
Source of Variation	% of total variation	P value
Interaction	0.10	0.9626
Ct_FIT2	98.03	< 0.0001
Plates with different calibration points	1.08	< 0.0001
Subjects (matching)	0.1522	0.7033

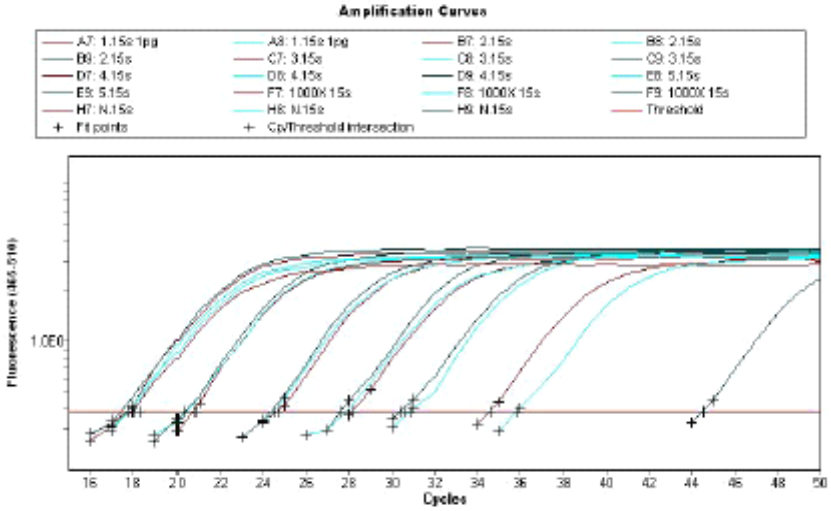
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	12	1.958	0.1632	0.3747
Ct_FIT2	4	2007	501.7	1610
Plates with different calibration points	3	22.20	7.399	16.99
Subjects (matching)	10	3.116	0.3116	0.7156
Residual	30	13.06	0.4355	

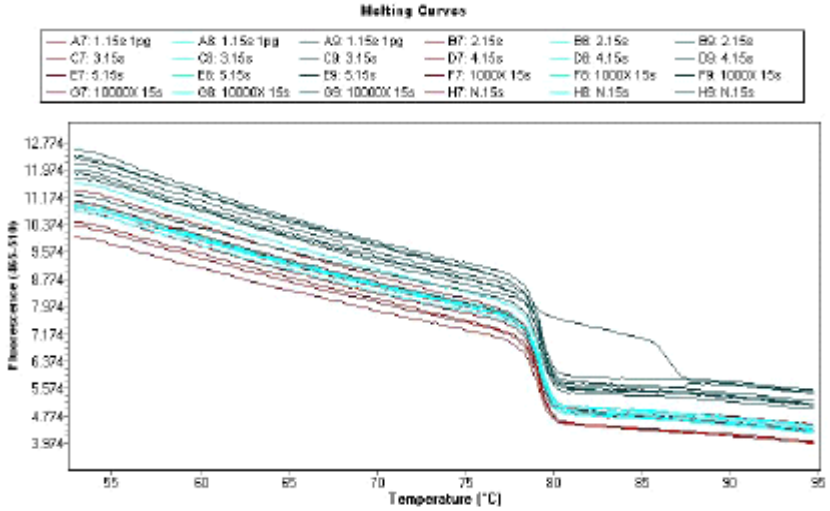
Appendice2. qPCR amplify curve and melting curve

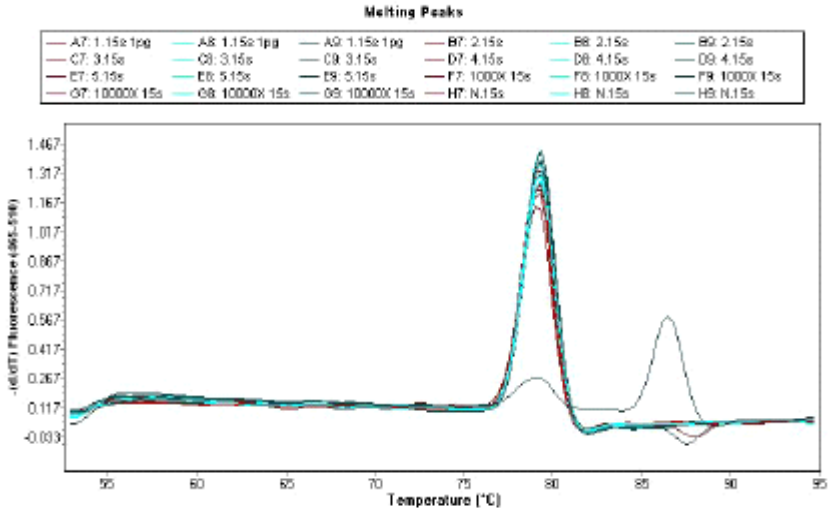
15s


Amplification Curves
A7: 1.15s 1 pg
A8: 1.15s 1pg
B7: 2.15s
B8: 2.15s
B9: 2.15s
C7: 3.15s
C8: 3.15s
C9: 3.15s
D7: 4.15s
D8: 4.15s
D9: 4.15s
E8: 5.15s
E9: 5.15s
F7: 1000X 15s
F8: 1000X 15s
F9: 1000X 15s
H7: N.15s
H8: N.15s
H9: N.15s
Fluorescence (465-510)
Cycles

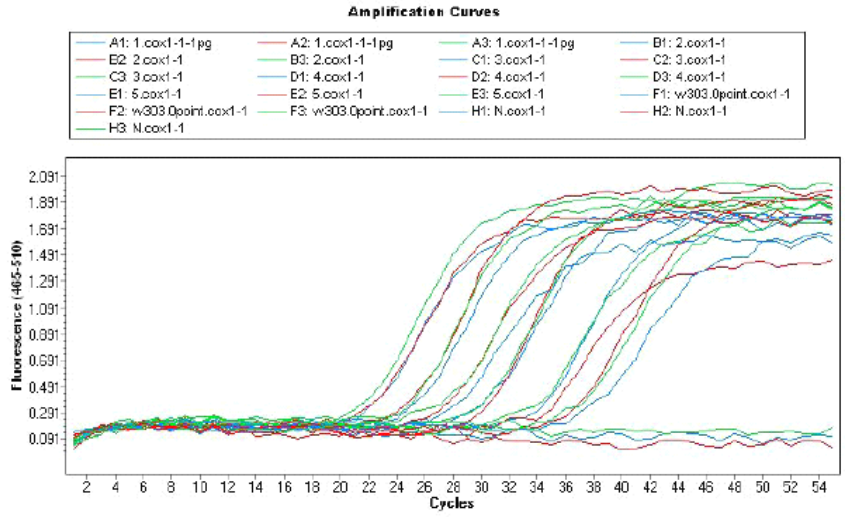

Amplification Curves
A7: 1.15s 1 pg
A8: 1.15s 1pg
B7: 2.15s
B8: 2.15s
B9: 2.15s
C7: 3.15s
C8: 3.15s
C9: 3.15s
D7: 4.15s
D8: 4.15s
D9: 4.15s
E8: 5.15s
E9: 5.15s
F7: 1000X 15s
F8: 1000X 15s
F9: 1000X 15s
H7: N.15s
H8: N.15s
H9: N.15s
Noiseband
1.0E0
1.0E-1
Fluorescence (465-510)
Cycles

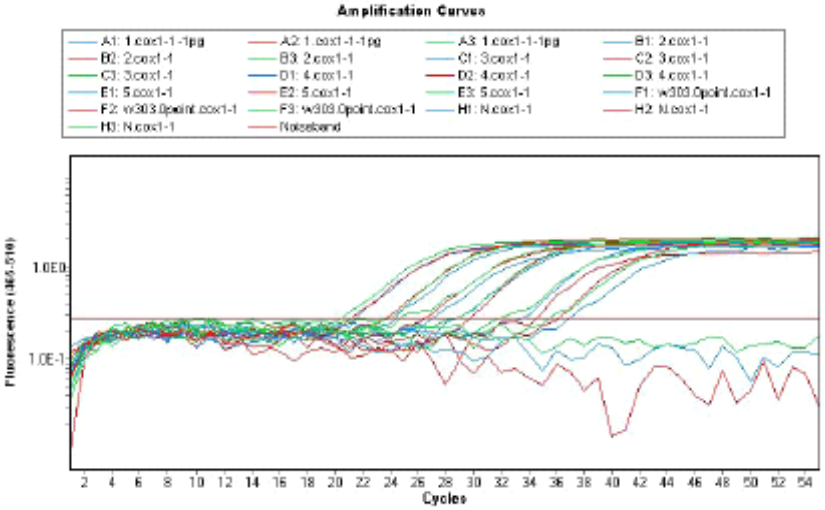

Amplification Curves
A7: 1.15s 1pg
A8: 1.15s 1pg
B7: 2.15s
B8: 2.15s
B9: 2.15s
C7: 3.15s
C8: 3.15s
C9: 3.15s
D7: 4.15s
D8: 4.15s
D9: 4.15s
E8: 5.15s
E9: 5.15s
F7: 1000X 15s
F8: 1000X 15s
F9: 1000X 15s
H7: N.15s
H8: N.15s
H9: N.15s
Threshold
Fit points
Cp/Threshold Intersection
1.0E0
Fluorescence (465-510)
Cycles

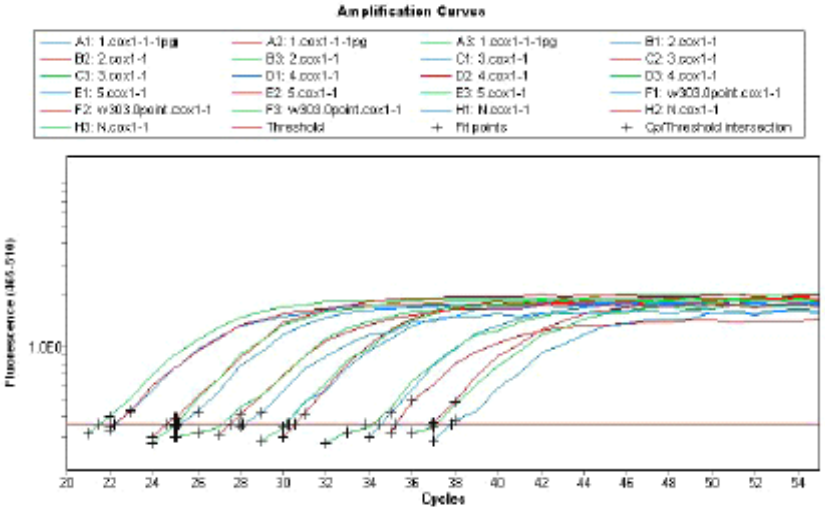

Melting Curves
A7: 1.15s 1pg
A8: 1.15s 1pg
A9: 1.15s 1pg
B7: 2.15s
B8: 2.15s
B9: 2.15s
C7: 3.15s
C8: 3.15s
C9: 3.15s
D7: 4.15s
D8: 4.15s
D9: 4.15s
E7: 5.15s
E8: 5.15s
E9: 5.15s
F7: 10000X 15s
F8: 10000X 15s
F9: 10000X 15s
G7: 100000X 15s
G8: 100000X 15s
G9: 100000X 15s
H7: N.15s
H8: N.15s
H9: N.15s
Fluorescence (465-510)
12.774
11.974
11.174
10.374
9.574
8.774
7.974
7.174
6.374
5.574
4.774
3.974
55
60
65
70
75
80
85
90
95
Temperature (°C)

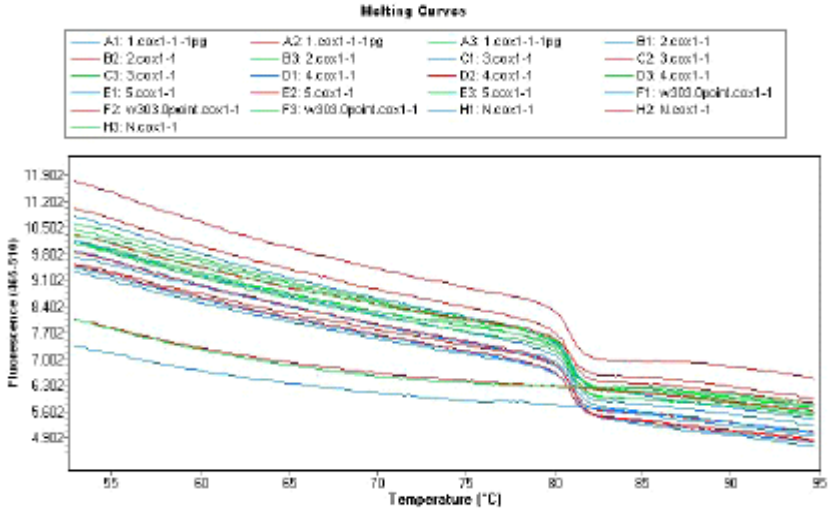

Melting Peaks
A7: 1.15s 1pg
A8: 1.15s 1pg
A9: 1.15s 1pg
B7: 2.15s
B8: 2.15s
B9: 2.15s
C7: 3.15s
C8: 3.15s
C9: 3.15s
D7: 4.15s
D8: 4.15s
D9: 4.15s
E7: 5.15s
E8: 5.15s
E9: 5.15s
F7: 10000X 15s
F8: 10000X 15s
F9: 10000X 15s
G7: 100000X 15s
G8: 100000X 15s
G9: 100000X 15s
H7: N.15s
H8: N.15s
H9: N.15s
-(d/dT) Fluorescence (465-510)
1.467
1.317
1.167
1.017
0.867
0.717
0.567
0.417
0.267
0.117
-0.033
55
60
65
70
75
80
85
90
95
Temperature (°C)

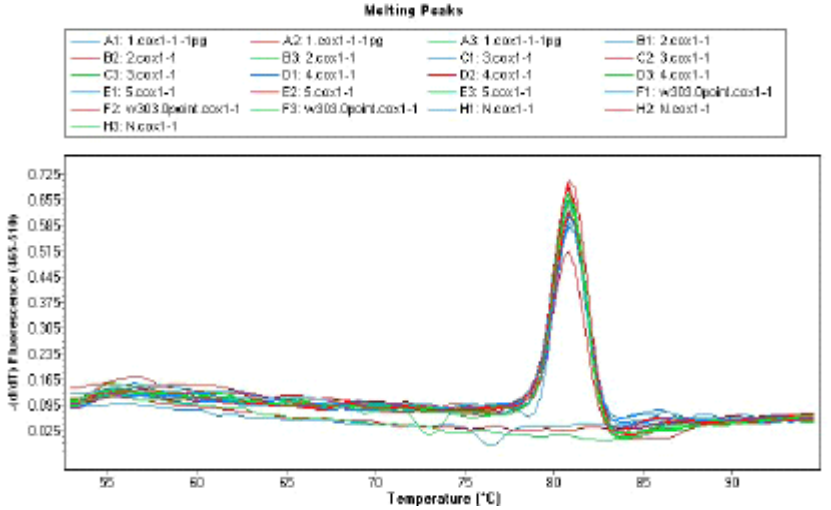
Cox1_excon1


Amplification Curves
A1: 1.cox1-1-1pg
A2: 1.cox1-1-1pg
A3: 1.cox1-1-1pg
B1: 2.cox1-1
B2: 2.cox1-1
B3: 2.cox1-1
C1: 3.cox1-1
C2: 3.cox1-1
C3: 3.cox1-1
D1: 4.cox1-1
D2: 4.cox1-1
D3: 4.cox1-1
E1: 5.cox1-1
E2: 5.cox1-1
E3: 5.cox1-1
F1: w303.0point.cox1-1
F2: w303.0point.cox1-1
F3: w303.0point.cox1-1
H1: N.cox1-1
H2: N.cox1-1
H3: N.cox1-1
Fluorescence (465-510)
Cycles

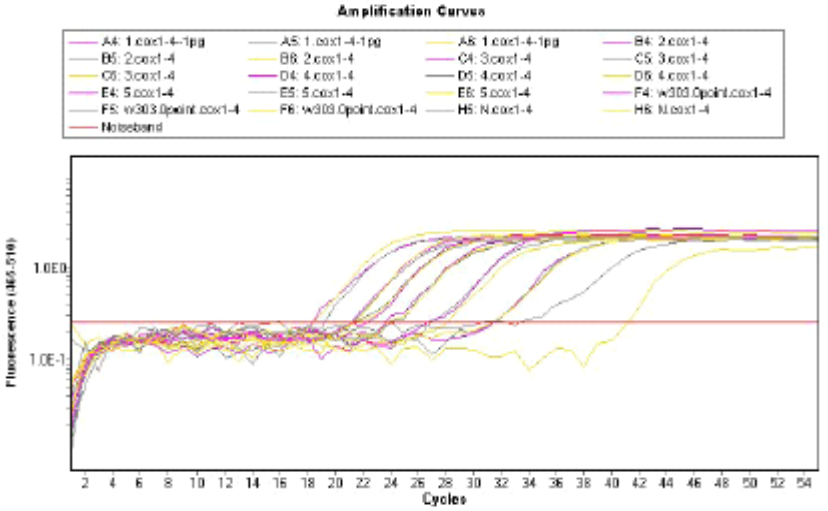

Amplification Curves
A1: 1.cox1-1-1pg
A2: 1.cox1-1-1pg
A3: 1.cox1-1-1pg
B1: 2.cox1-1
B2: 2.cox1-1
B3: 2.cox1-1
C1: 3.cox1-1
C2: 3.cox1-1
C3: 3.cox1-1
D1: 4.cox1-1
D2: 4.cox1-1
D3: 4.cox1-1
E1: 5.cox1-1
E2: 5.cox1-1
E3: 5.cox1-1
F1: w303.0point.cox1-1
F2: w303.0point.cox1-1
F3: w303.0point.cox1-1
H1: N.cox1-1
H2: N.cox1-1
H3: N.cox1-1
Noiseband
Cycles

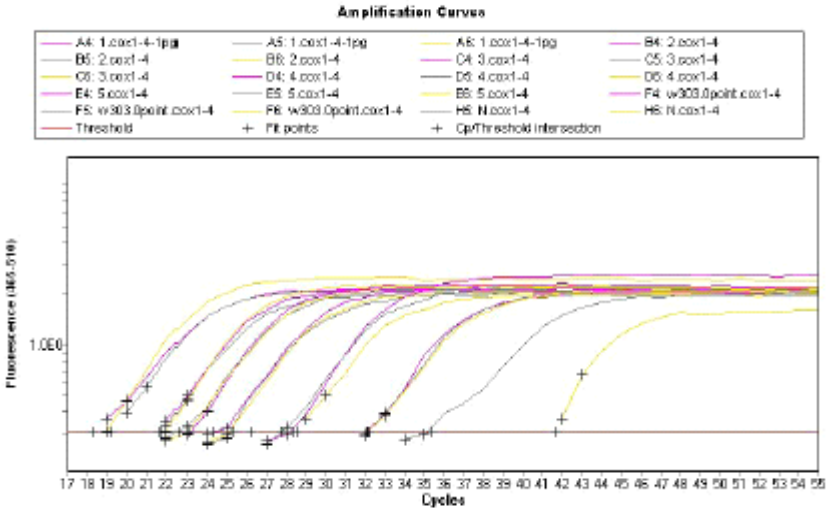

Amplification Curves
A1: 1.cox1-1-1pg
A2: 1.cox1-1-1pg
A3: 1.cox1-1-1pg
B1: 2.cox1-1
B2: 2.cox1-1
B3: 2.cox1-1
C1: 3.cox1-1
C2: 3.cox1-1
C3: 3.cox1-1
D1: 4.cox1-1
D2: 4.cox1-1
D3: 4.cox1-1
E1: 5.cox1-1
E2: 5.cox1-1
E3: 5.cox1-1
F1: w303.0point.cox1-1
F2: w303.0point.cox1-1
F3: w303.0point.cox1-1
H1: N.cox1-1
H2: N.cox1-1
H3: N.cox1-1
Threshold
Fit points
Cp/Threshold intersection
Cycles


Melting Curves
A1: 1.cox1-1-1pg
A2: 1.cox1-1-1pg
A3: 1.cox1-1-1pg
B1: 2.cox1-1
B2: 2.cox1-1
B3: 2.cox1-1
C1: 3.cox1-1
C2: 3.cox1-1
C3: 3.cox1-1
D1: 4.cox1-1
D2: 4.cox1-1
D3: 4.cox1-1
E1: 5.cox1-1
E2: 5.cox1-1
E3: 5.cox1-1
F1: w303.0point.cox1-1
F2: w303.0point.cox1-1
F3: w303.0point.cox1-1
H1: N.cox1-1
H2: N.cox1-1
H3: N.cox1-1
Fluorescence (465-510)
11.902
11.202
10.502
9.802
9.102
8.402
7.702
7.002
6.302
5.602
4.902
55
60
65
70
75
80
85
90
95
Temperature (°C)


Melting Peaks
A1: 1.cox1-1-1pg
A2: 1.cox1-1-1pg
A3: 1.cox1-1-1pg
B1: 2.cox1-1
B2: 2.cox1-1
B3: 2.cox1-1
C1: 3.cox1-1
C2: 3.cox1-1
C3: 3.cox1-1
D1: 4.cox1-1
D2: 4.cox1-1
D3: 4.cox1-1
E1: 5.cox1-1
E2: 5.cox1-1
E3: 5.cox1-1
F1: w303.0point.cox1-1
F2: w303.0point.cox1-1
F3: w303.0point.cox1-1
H1: N.cox1-1
H2: N.cox1-1
H3: N.cox1-1
-(d/dT) Fluorescence (465-510)
0.725
0.655
0.585
0.515
0.445
0.375
0.305
0.235
0.165
0.095
0.025
55
60
65
70
75
80
85
90
Temperature (°C)

Cox1_excon4

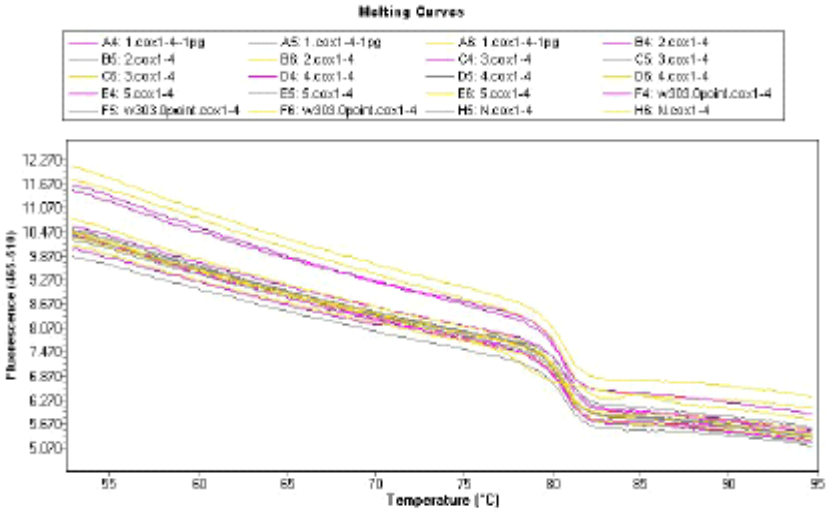


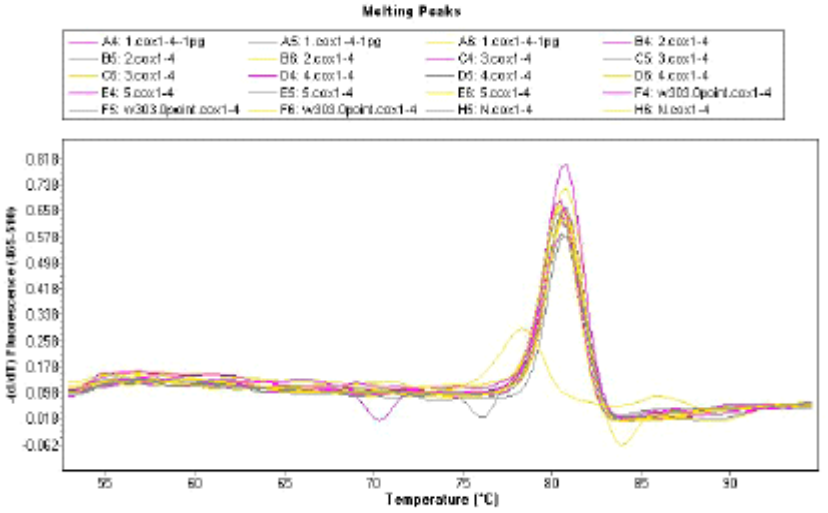


Settings

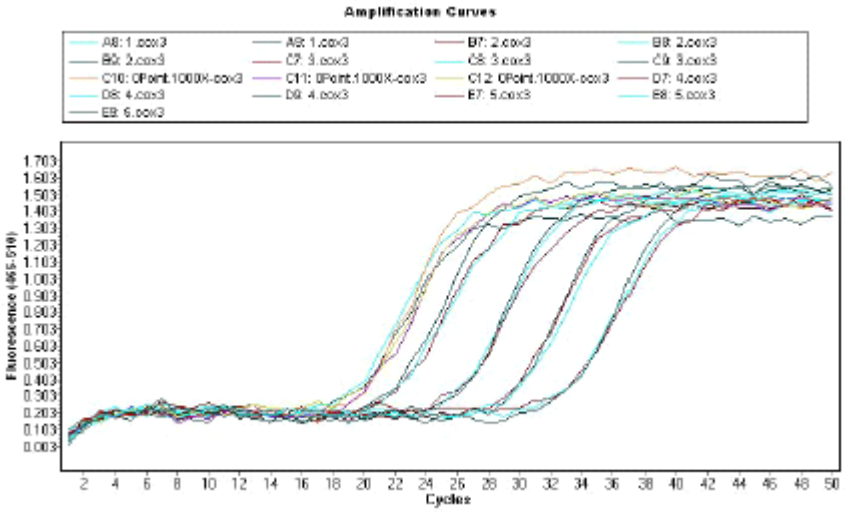
Channel	465-510				
Color Compensation	Off				
Standard Curve	In-run				
Program	Program			Units	
First Cycle	1	Last Cycle	55	Background	2-6
Noiseband Method	Noiseband (Fluor)	Noiseband	0.2542	STD Dev Multiplier	3.6906
# of Fit Points	2	Threshold Method	Auto	Threshold	0.3050

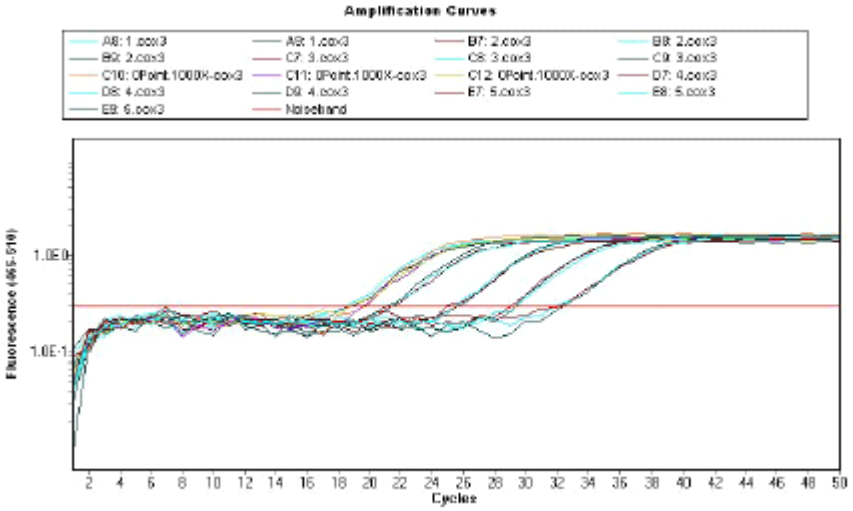
Subset Name	cox1-4 22

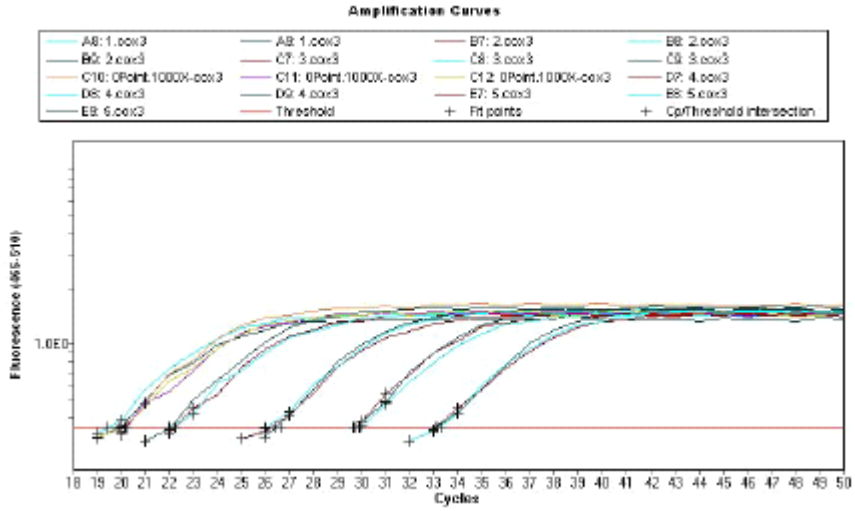

Melting Curves
A4: 1.cox1-4--1pg
A5: 1.cox1-4-1pg
A6: 1.cox1-4-1pg
B4: 2.cox1-4
B5: 2.cox1-4
B6: 2.cox1-4
C4: 3.cox1-4
C5: 3.cox1-4
C6: 3.cox1-4
D4: 4.cox1-4
D5: 4.cox1-4
D6: 4.cox1-4
E4: 5.cox1-4
E5: 5.cox1-4
E6: 5.cox1-4
F4: w303.0point.cox1-4
F5: w303.0point.cox1-4
F6: w303.0point.cox1-4
H5: N.cox1-4
H6: N.cox1-4
Fluorescence (465-510)
12.270
11.670
11.070
10.470
9.870
9.270
8.670
8.070
7.470
6.870
6.270
5.670
5.070
55
60
65
70
75
80
85
90
95
Temperature (°C)

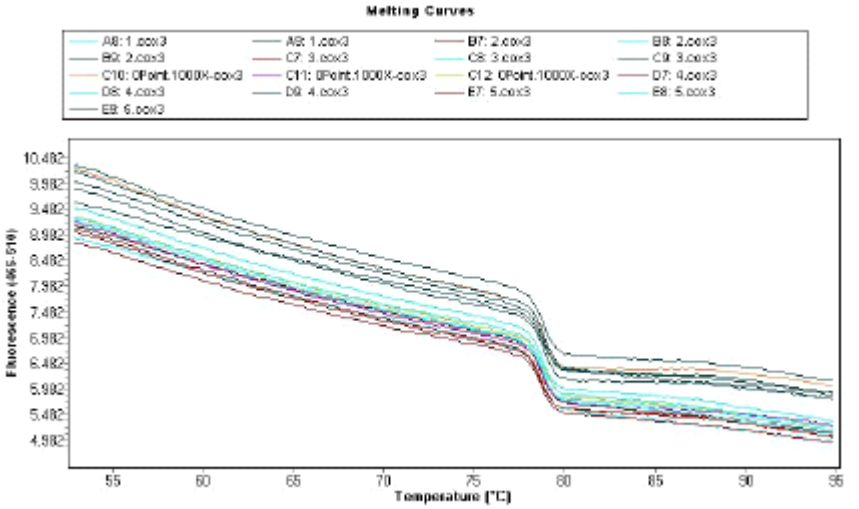

Melting Peaks
A4: 1.cox1-4--1pg
A5: 1.cox1-4-1pg
A6: 1.cox1-4-1pg
B4: 2.cox1-4
B5: 2.cox1-4
B6: 2.cox1-4
C4: 3.cox1-4
C5: 3.cox1-4
C6: 3.cox1-4
D4: 4.cox1-4
D5: 4.cox1-4
D6: 4.cox1-4
E4: 5.cox1-4
E5: 5.cox1-4
E6: 5.cox1-4
F4: w303.0point.cox1-4
F5: w303.0point.cox1-4
F6: w303.0point.cox1-4
H5: N.cox1-4
H6: N.cox1-4
-(d/dT) Fluorescence (465-510)
0.818
0.738
0.658
0.578
0.498
0.418
0.338
0.258
0.178
0.098
0.018
-0.062
55
60
65
70
75
80
85
90
Temperature (°C)

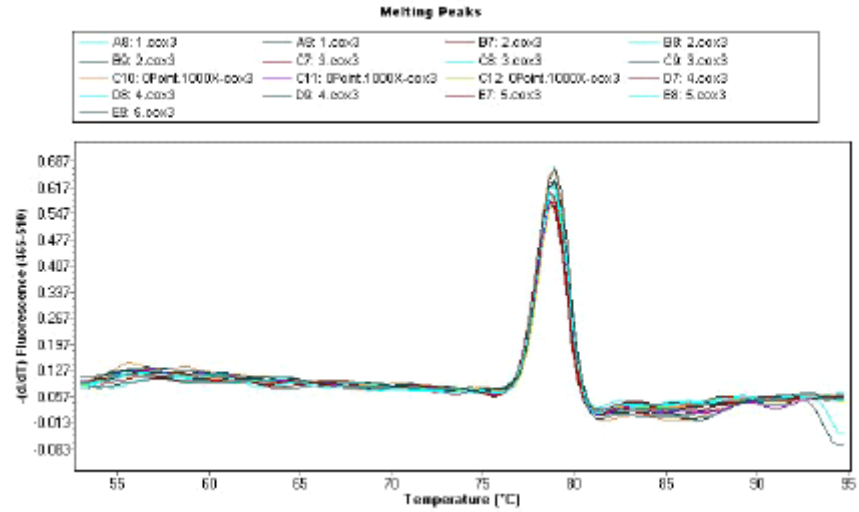
Cox3


Amplification Curves
A8: 1.cox3
A9: 1.cox3
B7: 2.cox3
B8: 2.cox3
B9: 2.cox3
C7: 3.cox3
C8: 3.cox3
C9: 3.cox3
C10: 0Point.1000X-cox3
C11: 0Point.1000X-cox3
C12: 0Point.1000X-cox3
D7: 4.cox3
D8: 4.cox3
D9: 4.cox3
E7: 5.cox3
E8: 5.cox3
E9: 6.cox3
Fluorescence (465-510)
Cycles

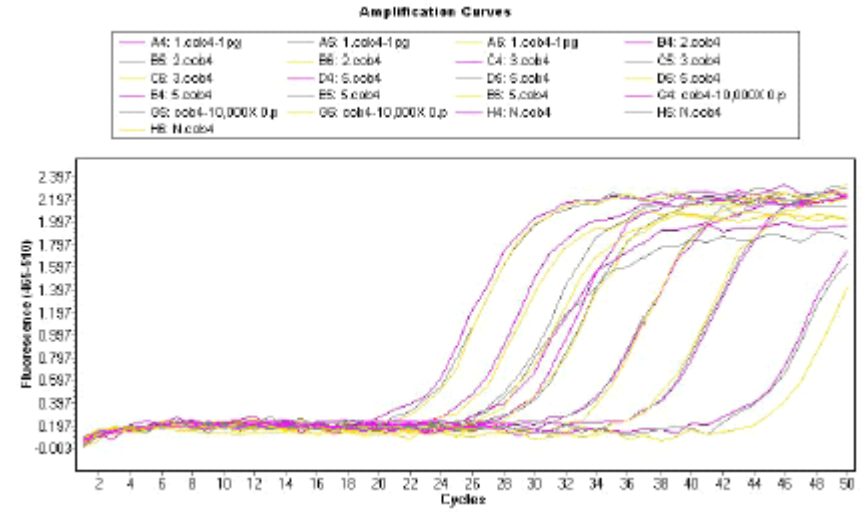

Amplification Curves
A8: 1.cox3
A9: 1.cox3
B7: 2.cox3
B8: 2.cox3
B9: 2.cox3
C7: 3.cox3
C8: 3.cox3
C9: 3.cox3
C10: 0Point.1000X-cox3
C11: 0Point.1000X-cox3
C12: 0Point.1000X-cox3
D7: 4.cox3
D8: 4.cox3
D9: 4.cox3
E7: 5.cox3
E8: 5.cox3
E9: 6.cox3
Noisband
Fluorescence (465-510)
1.0E0
1.0E-1
Cycles

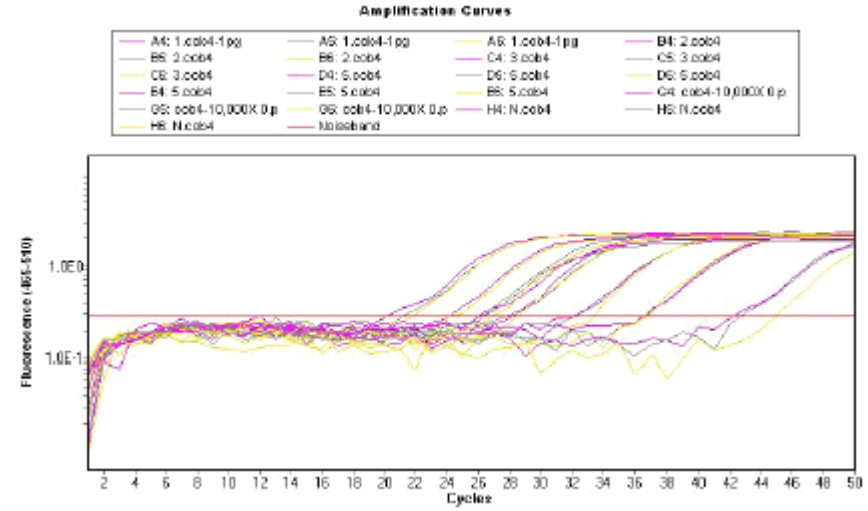

Amplification Curves
A8: 1.cox3
A9: 1.cox3
B7: 2.cox3
B8: 2.cox3
B9: 2.cox3
C7: 3.cox3
C8: 3.cox3
C9: 3.cox3
C10: 0Point.1000X-cox3
C11: 0Point.1000X-cox3
C12: 0Point.1000X-cox3
D7: 4.cox3
D8: 4.cox3
D9: 4.cox3
E7: 5.cox3
E8: 5.cox3
E9: 6.cox3
Threshold
Fit points
Cp/Threshold intersection
Fluorescence (465-510)
1.0E0
Cycles

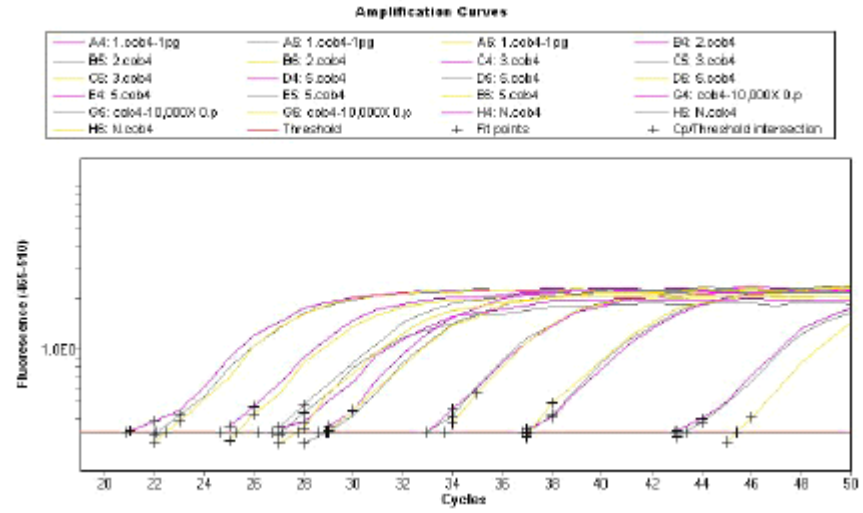

Melting Curves
A8: 1.cox3
A9: 1.cox3
B7: 2.cox3
B8: 2.cox3
B9: 2.cox3
C7: 3.cox3
C8: 3.cox3
C9: 3.cox3
C10: 0Point.1000X-cox3
C11: 0Point.1000X-cox3
C12: 0Point.1000X-cox3
D7: 4.cox3
D8: 4.cox3
D9: 4.cox3
E7: 5.cox3
E8: 5.cox3
E9: 5.cox3
Fluorescence (465-510)
Temperature (°C)

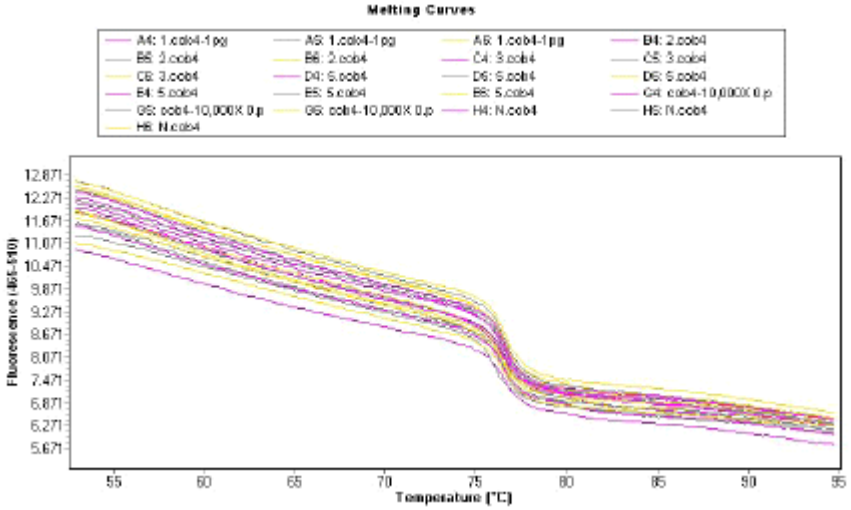

Melting Peaks
A8: 1.cox3
A9: 1.cox3
B7: 2.cox3
B8: 2.cox3
B9: 2.cox3
C7: 3.cox3
C8: 3.cox3
C9: 3.cox3
C10: 0Point.1000X-cox3
C11: 0Point.1000X-cox3
C12: 0Point.1000X-cox3
D7: 4.cox3
D8: 4.cox3
D9: 4.cox3
E7: 5.cox3
E8: 5.cox3
E9: 5.cox3
-(d/dT) Fluorescence (465-510)
Temperature (°C)

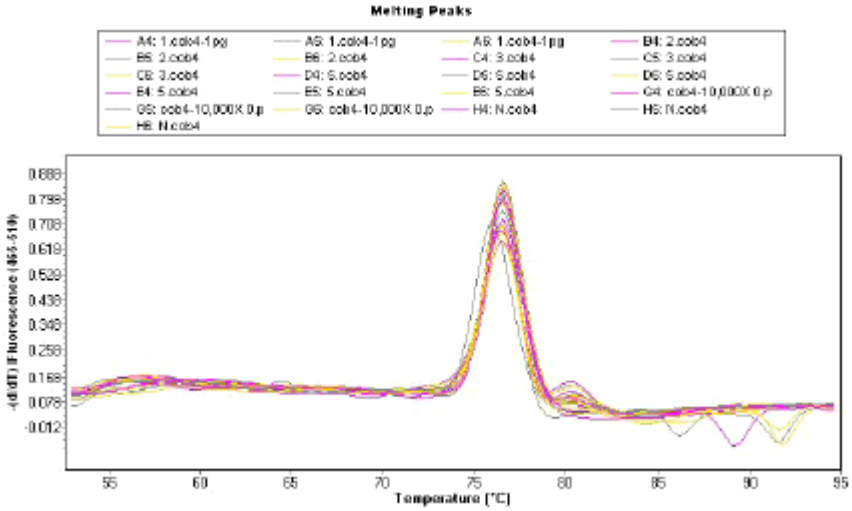
Cob4


Amplification Curves
A4: 1.cob4-1pg
A5: 1.cob4-1pg
A6: 1.cob4-1pg
B4: 2.cob4
B5: 2.cob4
B6: 2.cob4
C4: 3.cob4
C5: 3.cob4
C6: 3.cob4
D4: 5.cob4
D6: 5.cob4
D5: 5.cob4
E4: 5.cob4
E5: 5.cob4
B5: 5.cob4
G4: cob4-10,000X 0.p
G5: cob4-10,000X 0.p
G6: cob4-10,000X 0.p
H4: N.cob4
H5: N.cob4
H6: N.cob4
Fluorescence (465-510)
Cycles

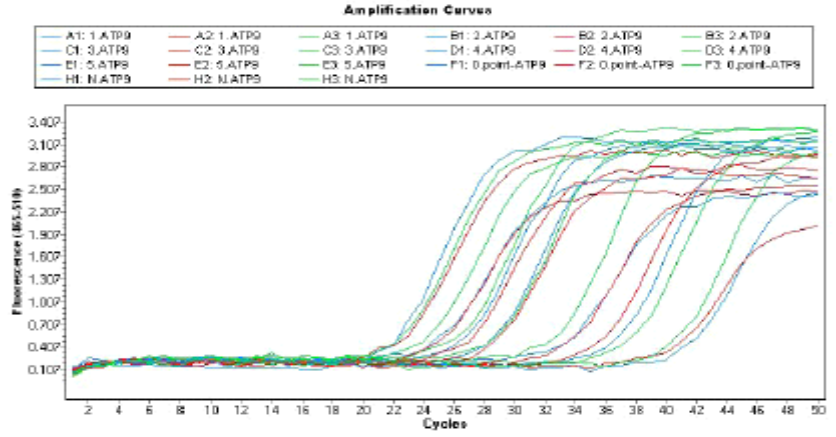

Amplification Curves
A4: 1.cob4-1pg
A5: 1.cob4-1pg
A6: 1.cob4-1pg
B4: 2.cob4
B5: 2.cob4
B6: 2.cob4
C4: 3.cob4
C5: 3.cob4
C6: 3.cob4
D4: 5.cob4
D6: 5.cob4
D5: 5.cob4
E4: 5.cob4
E5: 5.cob4
B5: 5.cob4
G4: cob4-10,000X 0.p
G5: cob4-10,000X 0.p
G6: cob4-10,000X 0.p
H4: N.cob4
H5: N.cob4
H6: N.cob4
Noiseband
Fluorescence (465-510)
Cycles

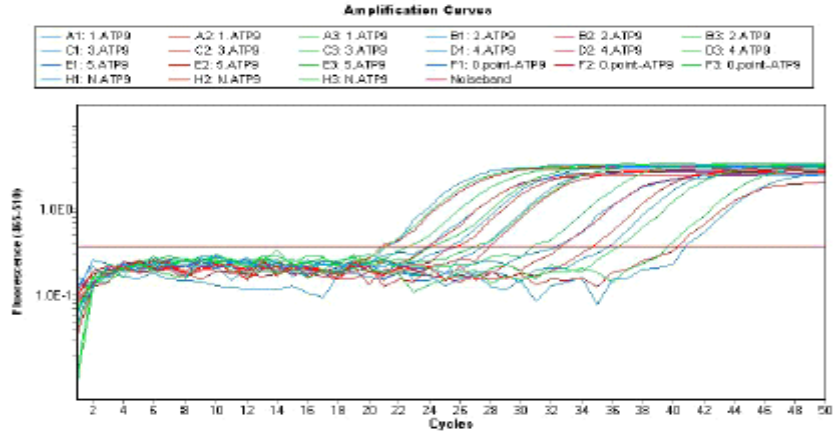

Amplification Curves
A4: 1.cob4-1pg
A5: 1.cob4-1pg
A6: 1.cob4-1pg
B4: 2.cob4
B5: 2.cob4
B6: 2.cob4
C4: 3.cob4
C5: 3.cob4
C6: 3.cob4
D4: 5.cob4
D6: 5.cob4
D5: 5.cob4
E4: 5.cob4
E5: 5.cob4
B5: 5.cob4
G4: cob4-10,000X 0.p
G5: cob4-10,000X 0.p
G6: cob4-10,000X 0.p
H4: N.cob4
H5: N.cob4
H6: N.cob4
Threshold
Fit points
Cp/Threshold intersection
Fluorescence (465-510)
Cycles

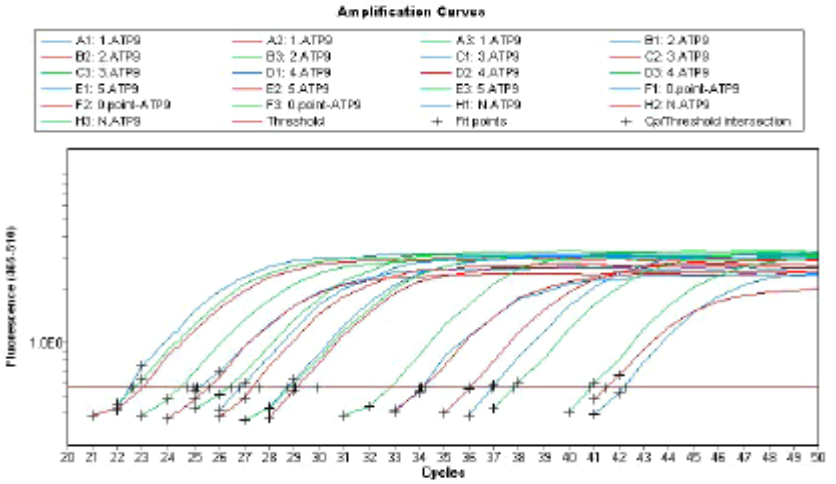

Melting Curves
A4: 1.cob4-1 pg
A5: 1.cob4-1 pg
A6: 1.cob4-1 pg
B4: 2.cob4
B5: 2.cob4
B6: 2.cob4
C4: 3.cob4
C5: 3.cob4
C6: 3.cob4
D4: 5.cob4
D5: 5.cob4
D6: 5.cob4
E4: 5.cob4
E5: 5.cob4
E6: 5.cob4
G4: cob4-10,000X 0.p
G5: cob4-10,000X 0.p
G6: cob4-10,000X 0.p
H4: N.cob4
H5: N.cob4
H6: N.cob4
Fluorescence (465-510)
12.871
12.271
11.671
11.071
10.471
9.871
9.271
8.671
8.071
7.471
6.871
6.271
5.671
55
60
65
70
75
80
85
90
95
Temperature (°C)


Melting Peaks
A4: 1.cob4-1 pg
A5: 1.cob4-1 pg
A6: 1.cob4-1 pg
B4: 2.cob4
B5: 2.cob4
B6: 2.cob4
C4: 3.cob4
C5: 3.cob4
C6: 3.cob4
D4: 5.cob4
D5: 5.cob4
D6: 5.cob4
E4: 5.cob4
E5: 5.cob4
E6: 5.cob4
G4: cob4-10,000X 0.p
G5: cob4-10,000X 0.p
G6: cob4-10,000X 0.p
H4: N.cob4
H5: N.cob4
H6: N.cob4
-(d/dT) Fluorescence (465-510)
0.888
0.798
0.708
0.619
0.529
0.439
0.349
0.259
0.169
0.079
-0.012
55
60
65
70
75
80
85
90
95
Temperature (°C)

ATP9


Amplification Curves
A1: 1.ATP9
A2: 1.ATP9
A3: 1.ATP9
B1: 2.ATP9
B2: 2.ATP9
B3: 2.ATP9
C1: 3.ATP9
C2: 3.ATP9
C3: 3.ATP9
D1: 4.ATP9
D2: 4.ATP9
D3: 4.ATP9
E1: 5.ATP9
E2: 5.ATP9
E3: 5.ATP9
F1: 0.point-ATP9
F2: 0.point-ATP9
F3: 0.point-ATP9
H1: N.ATP9
H2: N.ATP9
H3: N.ATP9
Fluorescence (465-510)
Cycles


Amplification Curves
A1: 1.ATP9
A2: 1.ATP9
A3: 1.ATP9
B1: 2.ATP9
B2: 2.ATP9
B3: 2.ATP9
C1: 3.ATP9
C2: 3.ATP9
C3: 3.ATP9
D1: 4.ATP9
D2: 4.ATP9
D3: 4.ATP9
E1: 5.ATP9
E2: 5.ATP9
E3: 5.ATP9
F1: 0.point-ATP9
F2: 0.point-ATP9
F3: 0.point-ATP9
H1: N.ATP9
H2: N.ATP9
H3: N.ATP9
Noiseband
1.0E0
1.0E-1
Fluorescence (465-510)
Cycles

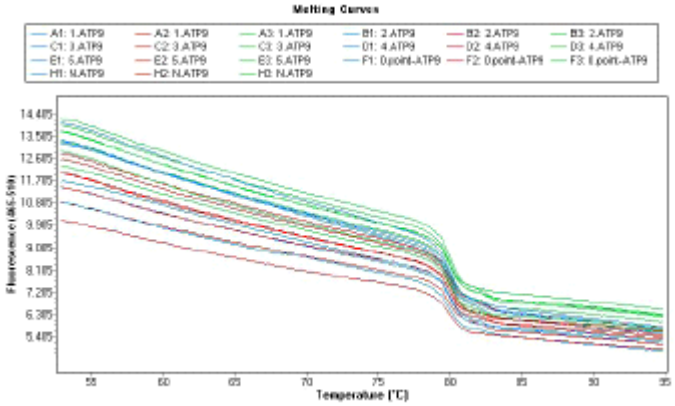

Amplification Curves
A1: 1.ATP9
A2: 1.ATP9
A3: 1.ATP9
B1: 2.ATP9
B2: 2.ATP9
B3: 2.ATP9
C1: 3.ATP9
C2: 3.ATP9
C3: 3.ATP9
D1: 4.ATP9
D2: 4.ATP9
D3: 4.ATP9
E1: 5.ATP9
E2: 5.ATP9
E3: 5.ATP9
F1: 0.point-ATP9
F2: 0.point-ATP9
F3: 0.point-ATP9
H1: N.ATP9
H2: N.ATP9
H3: N.ATP9
Threshold
Fit points
Cp/Threshold intersection
1.0E0
Fluorescence (465-510)
Cycles

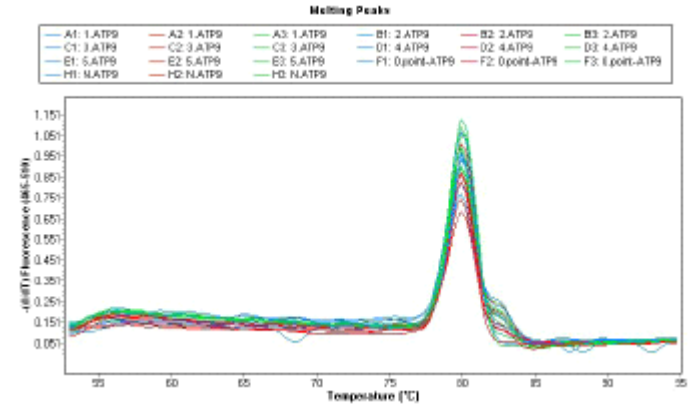
Abs Quant/Fit Points for ATP9 (Abs Quant/Fit Points)

Settings

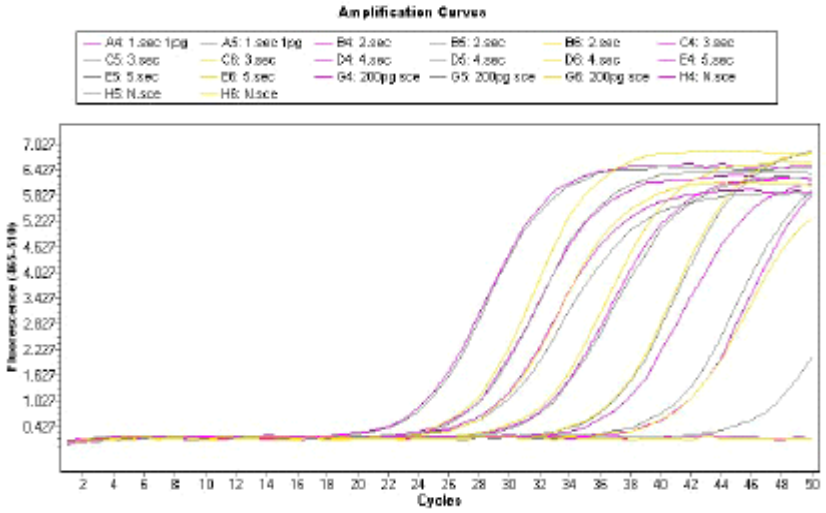
Channel	465-510				
Color Compensation	Off				
Standard Curve	In-run				
Program	Program			Units	
First Cycle	1	Last Cycle	50	Background	2-6
Noiseband Method	Noiseband (Fluor)	Noiseband	0.3675	STD Dev Multiplier	4.6816
# of Fit Points	2	Threshold Method	Auto	Threshold	0.5566

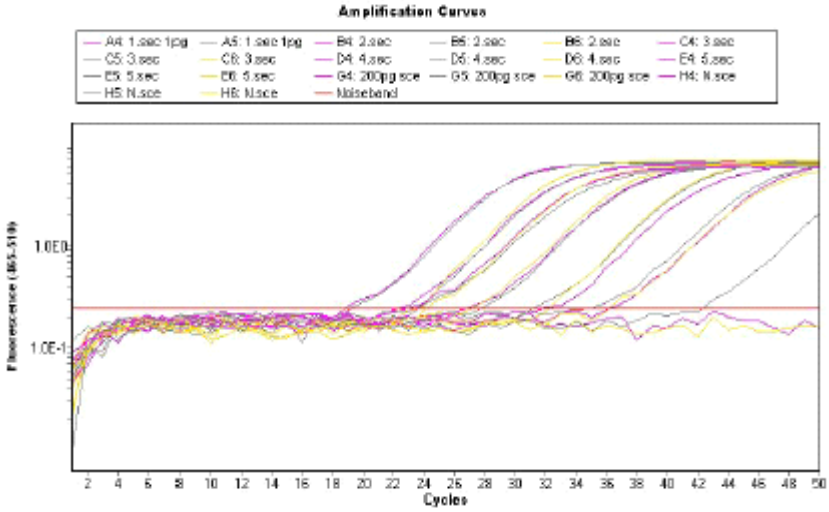
Subset Name	ATP9

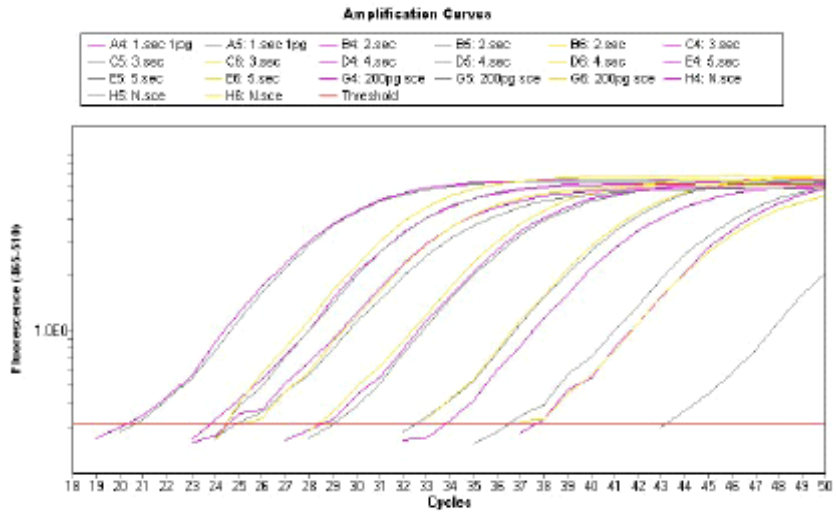




SceI


Amplification Curves
A4: 1.sec 1pg
A5: 1.sec 1pg
B4: 2.sec
B5: 2.sec
B6: 2.sec
C4: 3.sec
C5: 3.sec
C6: 3.sec
D4: 4.sec
D5: 4.sec
D6: 4.sec
E4: 5.sec
E5: 5.sec
E6: 5.sec
G4: 200pg sce
G5: 200pg sce
G6: 200pg sce
H4: N.sce
H5: N.sce
H6: N.sce
Fluorescence (465-510)
Cycles

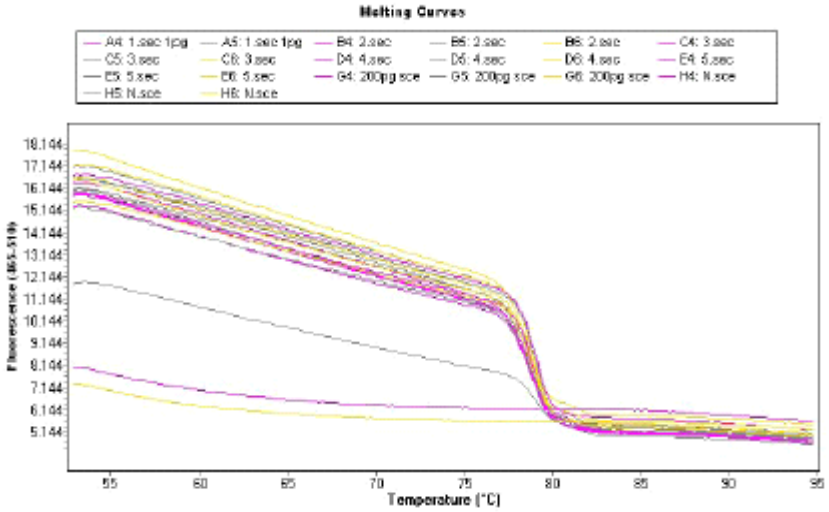

Amplification Curves
A4: 1.sec 1pg
A5: 1.sec 1pg
B4: 2.sec
B5: 2.sec
B6: 2.sec
C4: 3.sec
C5: 3.sec
C6: 3.sec
D4: 4.sec
D5: 4.sec
D6: 4.sec
E4: 5.sec
E5: 5.sec
E6: 5.sec
G4: 200pg sce
G5: 200pg sce
G6: 200pg sce
H4: N.sce
H5: N.sce
H6: N.sce
Noisebend
Fluorescence (465-510)
1.0E0
1.0E-1
Cycles

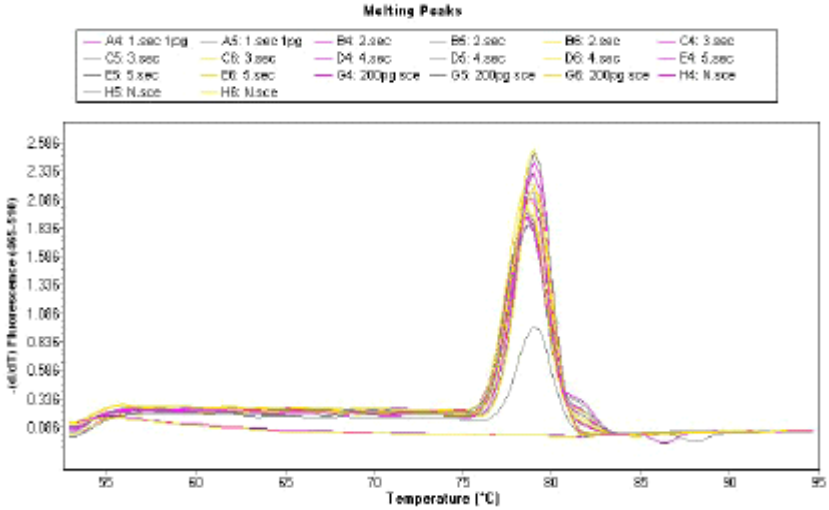

Amplification Curves
A4: 1.sec 1pg
A5: 1.sec 1pg
B4: 2.sec
B5: 2.sec
B6: 2.sec
C4: 3.sec
C5: 3.sec
C6: 3.sec
D4: 4.sec
D5: 4.sec
D6: 4.sec
E4: 5.sec
E5: 5.sec
E6: 5.sec
G4: 200pg sce
G5: 200pg sce
G6: 200pg sce
H4: N.sce
H5: N.sce
H6: N.sce
Threshold
Fluorescence (465-510)
1.0E0
Cycles

Abs Quant/Fit Points for sce (Abs Quant/Fit Points)

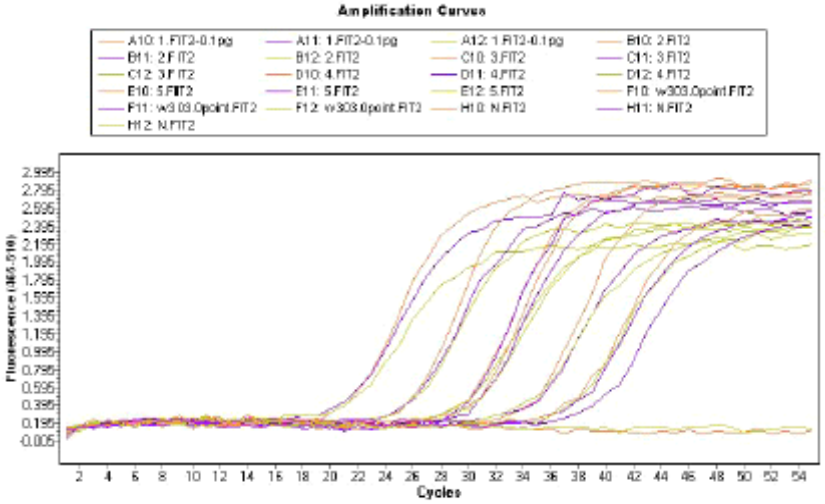
Settings

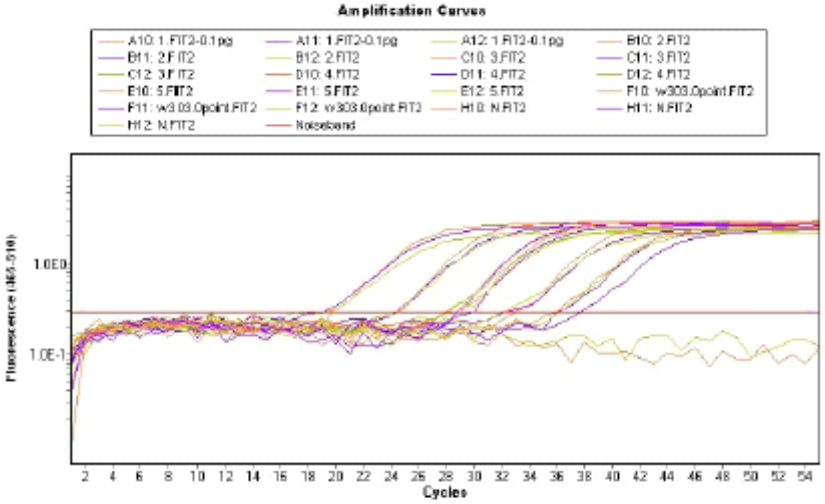
Channel	465-510				
Color Compensation	Off				
Standard Curve	In-run				
Program	Program			Units	
First Cycle	1	Last Cycle	50	Background	2-6
Noiseband Method	Noiseband (Fluor)	Noiseband	0.2407	STD Dev Multiplier	2.5602
# of Fit Points	2	Threshold Method	Auto	Threshold	0.3145

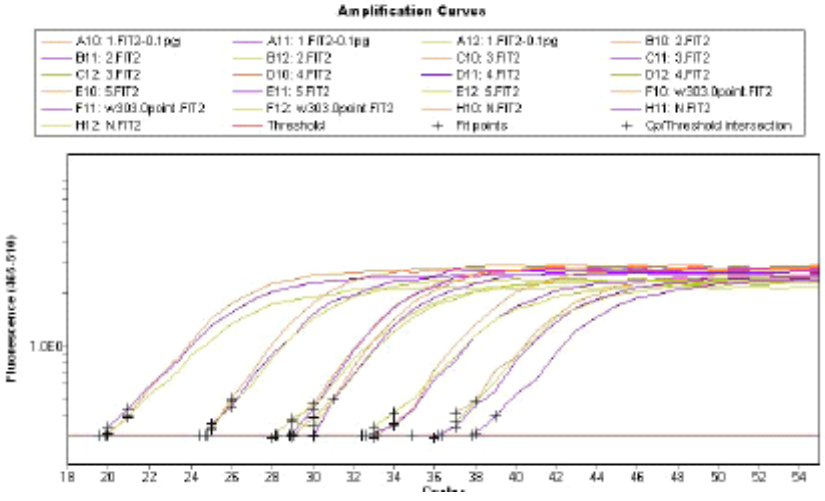




FIT2


Amplification Curves
A10: 1.FIT2-0.1pg
A11: 1.FIT2-0.1pg
A12: 1.FIT2-0.1pg
B10: 2.FIT2
B11: 2.FIT2
B12: 2.FIT2
C10: 3.FIT2
C11: 3.FIT2
C12: 3.FIT2
D10: 4.FIT2
D11: 4.FIT2
D12: 4.FIT2
E10: 5.FIT2
E11: 5.FIT2
E12: 5.FIT2
F10: w303.0point.FIT2
F11: w303.0point.FIT2
F12: w303.0point.FIT2
H10: N.FIT2
H11: N.FIT2
H12: N.FIT2
Fluorescence (465-510)
Cycles


Amplification Curves
A10: 1.FIT2-0.1pg
A11: 1.FIT2-0.1pg
A12: 1.FIT2-0.1pg
B10: 2.FIT2
B11: 2.FIT2
B12: 2.FIT2
C10: 3.FIT2
C11: 3.FIT2
C12: 3.FIT2
D10: 4.FIT2
D11: 4.FIT2
D12: 4.FIT2
E10: 5.FIT2
E11: 5.FIT2
E12: 5.FIT2
F10: w303.0point.FIT2
F11: w303.0point.FIT2
F12: w303.0point.FIT2
H10: N.FIT2
H11: N.FIT2
H12: N.FIT2
Noiseband
1.0E0
1.0E-1
Fluorescence (465-510)
Cycles

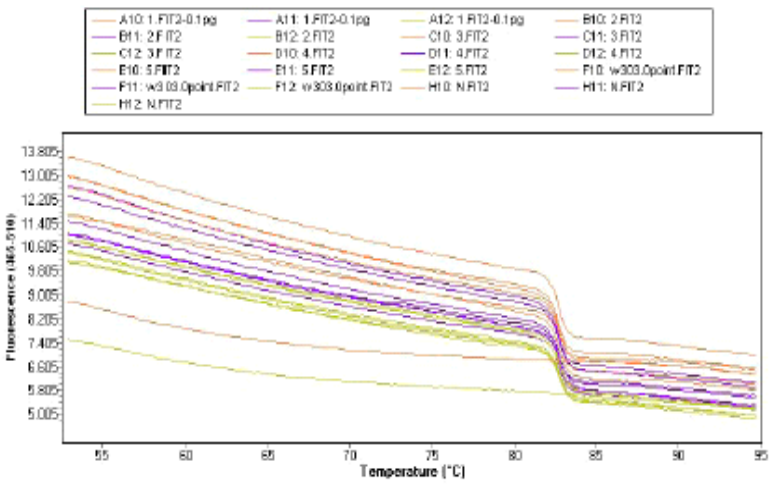

Amplification Curves
A10: 1.FIT2-0.1pg
A11: 1.FIT2-0.1pg
A12: 1.FIT2-0.1pg
B10: 2.FIT2
B11: 2.FIT2
B12: 2.FIT2
C10: 3.FIT2
C11: 3.FIT2
C12: 3.FIT2
D10: 4.FIT2
D11: 4.FIT2
D12: 4.FIT2
E10: 5.FIT2
E11: 5.FIT2
E12: 5.FIT2
F10: w303.0point.FIT2
F11: w303.0point.FIT2
F12: w303.0point.FIT2
H10: N.FIT2
H11: N.FIT2
H12: N.FIT2
Threshold
Fit points
Cp/Threshold intersection
1.0E0
Fluorescence (465-510)

Abs Quant/Fit Points for FIT 22 (Abs Quant/Fit Points)

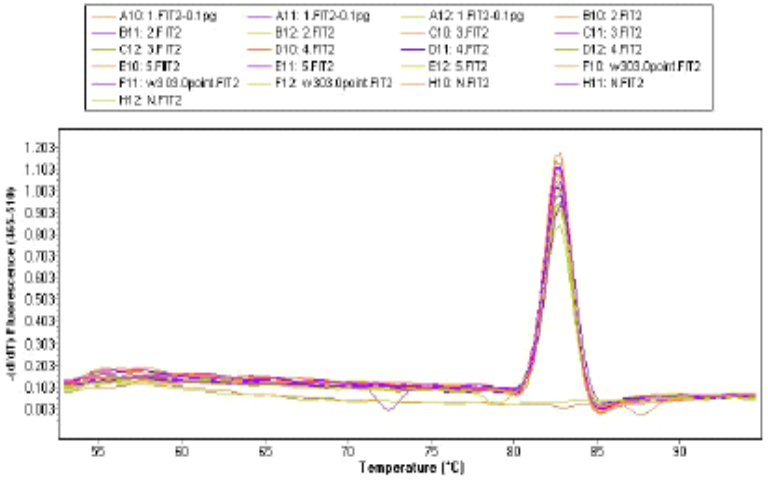
Settings

Channel	465-510				
Color Compensation	Off				
Standard Curve	In-run				
Program	Program			Units	
First Cycle	1	Last Cycle	55	Background	2-6
Noiseband Method	Noiseband (Fluor)	Noiseband	0.2914	STD Dev Multiplier	3.7402
# of Fit Points	2	Threshold Method	Auto	Threshold	0.3037

Melting Curves



Melting Peaks



Appendice3. DNA extraction protocol

1. Pour out 10ml YPG culture ,spin 5min at 3500rpm (+4℃)

2. Discard the liquid solution, resuspend pellet into 0.5 ml sorbitol buffer(1M sorbitol; 10mM Tris Ph8.0; 1mM EDTA Ph8)

3. weight empty epp-tube

4. tranfer resuspend solution into epp-tube, spin 1min at 5000 rpm

5. Discard solution ,weight epp-tube again, get the weight of pellet(cells)

6. Add Zymolyase 20T(4mg/per 1g of cell),add o.5ml sorbitol buffer, incubate 30mins at 37℃ (shake the tube during this time)

7. spin 1min at 5000rpm, dicard suspention

8. resuspend pellet in 0.5ml of $T_{50}E_{20}$

9. Add 50µl of 10%SDS,mix it well

10. Incubate 20mins at 65℃

11. transfer tubers on ice

12. Add 200µl 5M KoAC, mix it, incubate on ice for 40mins

13. spin 5mins at the max speed ,+4℃

14. Transfer suspention to the fresh tube

15. Add 1vol of the isopropanaol

16. Mix it, let it stay 5mins at the room temperature

17. Spin 1 min at max speed ,dicard sup.

18. wash pellet with 80% EtoH

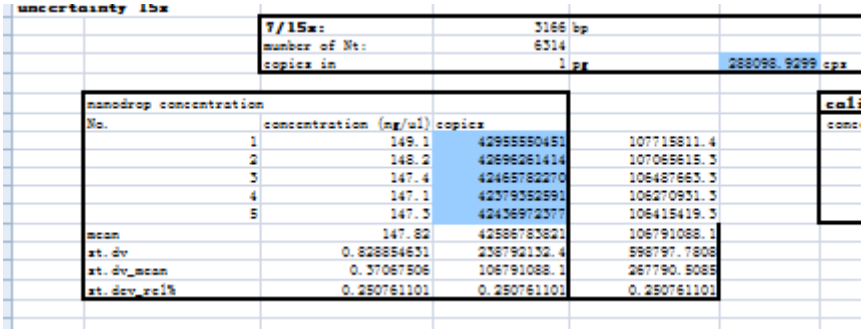
19. Keep it in the $T_{10}E_{0.1}$

20. storage in -80℃

Appendice4. Calculation example

15s

Estimate copy number from plasmid concentration



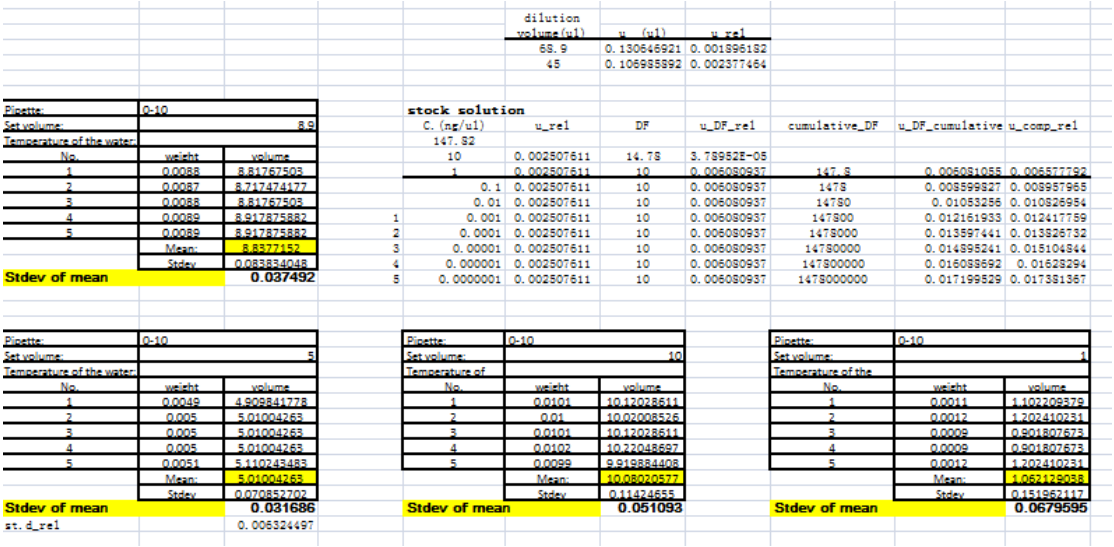
uncertainty 15s

7/15s:		3166	bp		
number of Nt:		6514			
copies in		1	pg	288098.9299	cps

nanodrop concentration

No.	concentration (ng/ul)	copies		
1	149.1	42955550451	107715811.4	
2	148.2	42696261414	107065615.3	
3	147.4	42465782270	106487665.3	
4	147.1	42379352591	106270931.3	
5	147.3	42436972377	106415419.3	
mean	147.82	42586783821	106791088.1	
st. dv	0.828854631	238792132.4	598797.7808	
st. dv_mean	0.37067506	106791088.1	267790.5085	
st. dev_rel%	0.250761101	0.250761101	0.250761101	

Uncertaity of Dilution factor:



	dilution volume(ul)	u (ul)	u_rel
	68.9	0.130646921	0.001896182
	45	0.106985892	0.002377464

Pipette: 0-10
Set volume: 8.8
Temperature of the water:

No.	weight	volume
1	0.0088	8.81767503
2	0.0087	8.717474177
3	0.0088	8.81767503
4	0.0089	8.917875882
5	0.0089	8.917875882
Mean:		8.8377152
Stdev		0.083834048
Stdev of mean		0.037492

stock solution

	C (ng/ul)	u_rel	DF	u_DF_rel	cumulative_DF	u_DF_cumulative	u_comp_rel
	147.82						
	10	0.002507611	14.78	3.78952E-05			
	1	0.002507611	10	0.006080937	147.8	0.006081055	0.006577792
	0.1	0.002507611	10	0.006080937	1478	0.008599827	0.008957965
	0.01	0.002507611	10	0.006080937	14780	0.01053256	0.010826954
1	0.001	0.002507611	10	0.006080937	147800	0.012161933	0.012417759
2	0.0001	0.002507611	10	0.006080937	1478000	0.013597441	0.013826732
3	0.00001	0.002507611	10	0.006080937	14780000	0.014895241	0.015104844
4	0.000001	0.002507611	10	0.006080937	147800000	0.016083692	0.01628294
5	0.0000001	0.002507611	10	0.006080937	1478000000	0.017199529	0.017381367

Pipette: 0-10
Set volume: 5
Temperature of the water:

No.	weight	volume
1	0.0049	4.909841778
2	0.005	5.01004263
3	0.005	5.01004263
4	0.005	5.01004263
5	0.0051	5.110243483
Mean:		5.01004263
Stdev		0.070852702
Stdev of mean		0.031686
st. d_rel		0.006324497

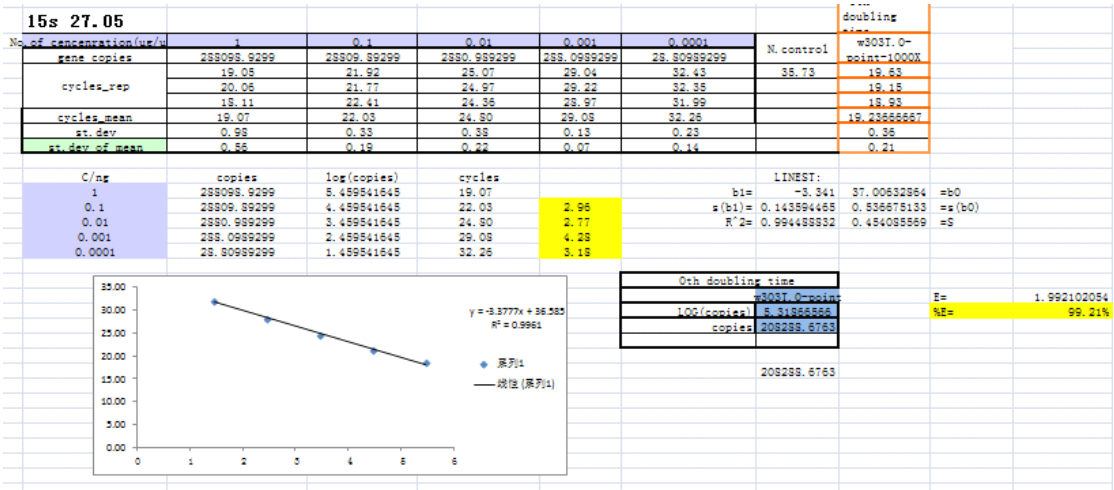
Pipette: 0-10
Set volume: 10
Temperature of

No.	weight	volume
1	0.0101	10.12028611
2	0.01	10.02008526
3	0.0101	10.12028611
4	0.0102	10.22048697
5	0.0099	9.919884408
Mean:		10.08020577
Stdev		0.11424655
Stdev of mean		0.051093

Pipette: 0-10
Set volume: 1
Temperature of the

No.	weight	volume
1	0.0011	1.102209379
2	0.0012	1.202410231
3	0.0009	0.901807673
4	0.0009	0.901807673
5	0.0012	1.202410231
Mean:		1.062132038
Stdev		0.151962117
Stdev of mean		0.0679595

Uncertainty estimate by Kragten method:



15s 27.05

No. of concenration(ul/u	1	0.1	0.01	0.001	0.0001	N. control	doubling w303T. 0-point-1000X
gene copies	288098.9299	28809.99299	2880.999299	288.0999299	28.80999299		
cycles_rep	19.05	21.92	25.07	29.04	32.43	35.73	19.62
	20.06	21.77	24.97	29.22	32.35		19.15
	18.11	22.41	24.36	28.97	31.99		18.93
cycles_mean	19.07	22.03	24.80	29.08	32.26		19.23666667
st. dev	0.98	0.33	0.38	0.13	0.23		0.36
st. dev of mean	0.56	0.19	0.22	0.07	0.14		0.21

C/ng	copies	log(copies)	cycles				
1	288098.9299	5.459541645	19.07				
0.1	28809.99299	4.459541645	22.03	2.96	b1=	-3.341	37.00632964 =b0
0.01	2880.999299	3.459541645	24.80	2.77	s(b1)=	0.143594465	0.536675133 =s(b0)
0.001	288.0999299	2.459541645	29.08	4.28	R^2=	0.994488832	0.454085569 =S
0.0001	28.80999299	1.459541645	32.26	3.18			

LINEST:

0th doubling time	
w303T. 0-point	
LOG(copies)	5.31588888
copies	208288.6763
	208288.6763

E= 1.992102054
%E= 99.21%

No.	alue(logcopies	u_log
1	5.459541645	5.39E-03
2	4.459541645	6.00E-03
3	3.459541645	6.56E-03
4	2.459541645	7.07E-03
5	1.459541645	7.55E-03
A1	19.07	0.56
A2	22.03	0.19
A3	24.80	0.22
A4	29.08	0.07
A5	32.26	0.14
A_Sample	19.23666667	0.21

5.464934609	5.459541645	5.459541645	5.459541645	5.459541645	5.459541645	5.459541645	5.459541645	5.459541645	5.4595416	5.459541645
4.459541645	4.465546519	4.459541645	4.459541645	4.459541645	4.459541645	4.459541645	4.459541645	4.459541645	4.4595416	4.459541645
3.459541645	3.459541645	3.466101596	3.459541645	3.459541645	3.459541645	3.459541645	3.459541645	3.459541645	3.4595416	3.459541645
2.459541645	2.459541645	2.459541645	2.466613236	2.459541645	2.459541645	2.459541645	2.459541645	2.459541645	2.4595416	2.459541645
1.459541645	1.459541645	1.459541645	1.459541645	1.467090277	1.459541645	1.459541645	1.459541645	1.459541645	1.4595416	1.459541645
19.07333333	19.07333333	19.07333333	19.07333333	19.07333333	19.64	19.07333333	19.07333333	19.07333333	19.073333	19.07333333
22.03333333	22.03333333	22.03333333	22.03333333	22.03333333	22.03333333	22.23	22.03333333	22.03333333	22.033333	22.03333333
24.8	24.8	24.8	24.8	24.8	24.8	24.8	25.02	24.8	24.8	24.8
29.07666667	29.07666667	29.07666667	29.07666667	29.07666667	29.07666667	29.07666667	29.07666667	29.15	29.076667	29.07666667
32.25666667	32.25666667	32.25666667	32.25666667	32.25666667	32.25666667	32.25666667	32.25666667	32.25666667	32.39	32.25666667
19.23666667	19.23666667	19.23666667	19.23666667	19.23666667	19.23666667	19.23666667	19.23666667	19.23666667	19.236667	19.44

Components	5.321843885	5.32096309	5.319747538	5.318884999	5.31742607	5.418392923	5.341117119	5.331948261	5.318978933	5.3117623	5.256807933
Components^2	0.003178225	0.00229743	0.001081878	0.000219339	-0.00123959	0.099727263	0.022451459	0.013282601	0.000313273	-0.006903	-0.061857727
sum(Com^2)	1.01011E-05	5.27818E-06	1.17046E-06	4.81098E-08	1.53658E-06	0.009945527	0.000504068	0.000176428	9.81398E-08	4.766E-05	0.003826378
u_c_logCN	0.01451829										
indexes	0.12049186										
	0.07%	0.04%	0.01%	0.00%	0.01%	68.50%	3.47%	1.22%	0.00%	0.33%	26.36%

Result was correct by dilution factor:

								u_rel_d=	1.22%	12.16187416	
								u_rel_C=	27.74%	57788.18935	
	C_Sample_uncorrect=	5.31866566	log(copies)								
Result											
	C_Sample_uncorrect=(| 208289 | ± | 115576.3787 |)copies/ul,k=2			C_undilute=	208288676.3	±	57788744.57			27.74%

Appendice5. Determination uncertainty of Pipette

Pipette:	0-10	
Set volume:		5
Temperature of the water:		
No.	weight	volume
1	0.0049	4.909841778
2	0.005	5.01004263
3	0.005	5.01004263
4	0.005	5.01004263
5	0.0051	5.110243483
	Mean:	5.01004263
	Stdev	0.070852702
Stdev of mean		0.031686

Pipette:	0-10	
Set volume:		10
Temperature of the water:		
No.	weight	volume
1	0.0101	10.12028611
2	0.01	10.02008526
3	0.0101	10.12028611
4	0.0102	10.22048697
5	0.0099	9.919884408
	Mean:	10.08020577
	Stdev	0.11424655
Stdev of mean		0.051093

Pipette:	0-10	
Set volume:		8.9
Temperature of the water:		
No.	weight	volume
1	0.0088	8.81767503
2	0.0087	8.717474177
3	0.0088	8.81767503
4	0.0089	8.917875882
5	0.0089	8.917875882
	Mean:	8.8377152
	Stdev	0.083834048
Stdev of mean		0.037492

Pipette:	0-10	
Set volume:		8.52
Temperature of the water:		
No.	weight	volume
1	0.0083	8.316670767
2	0.0083	8.316670767
3	0.0086	8.617273324
4	0.0085	8.517072472
5	0.0086	8.617273324
	Mean:	8.476992131
	Stdev	0.151962117
Stdev of mean		0.06796

Pipette:	0-10	
Set volume:		3.509
Temperature of the water:		
No.	weight	volume
1	0.0034	3.406828989
2	0.0035	3.507029841
3	0.0036	3.607230694
4	0.0035	3.507029841
5	0.0035	3.507029841
	Mean:	3.507029841
	Stdev	0.070852702
Stdev of mean		0.031686

Pipette:	0-10	
Set volume:		6.6
Temperature of the water:		
No.	weight	volume
1	0.0065	6.51305542
2	0.0066	6.613256272
3	0.0066	6.613256272
4	0.0065	6.51305542
5	0.0065	6.51305542
	Mean:	6.553135761
	Stdev	0.054882267
Stdev of mean		0.024544

Pipette:	0-10	
Set volume:		1
Temperature of the water:		
No.	weight	volume
1	0.0011	1.102209379
2	0.0012	1.202410231
3	0.0009	0.901807673
4	0.0009	0.901807673
5	0.0012	1.202410231
	Mean:	1.062129038
	Stdev	0.151962117
Stdev of mean		0.06796

Pipette:	0-10	
Set volume:		7.02
Temperature of the water:		
No.	weight	volume
1	0.007	7.014059683
2	0.0071	7.114260535
3	0.007	7.014059683
4	0.007	7.014059683
5	0.007	7.014059683
	Mean:	7.034099853
	Stdev	0.044811184
Stdev of mean		**0.02004**

Pipette:	0-200	
Set volume:		68.9
Temperature of the water:		
No.	weight	volume
1	0.0693	69.43919086
2	0.0685	68.63758404
3	0.0691	69.23878915
4	0.0692	69.33899001
5	0.0689	69.03838745
	Mean:	69.1385883
	Stdev	0.316862918
Stdev of mean		**0.141705**

Pipette:	0-200	
Set volume:		80.256
Temperature of the water:		
No.	weight	volume
1	0.0798	79.96028038
2	0.0799	80.06048123
3	0.0798	79.96028038
4	0.0797	79.86007953
5	0.0796	79.75987868
	Mean:	79.92020004
	Stdev	0.11424655
Stdev of mean		**0.051093**

Pipette:	0-200	
Set volume:		114.708
Temperature of the water:		
No.	weight	volume
1	0.1139	114.1287711
2	0.1133	113.527566
3	0.1148	115.0305788
4	0.1146	114.8301771
5	0.1145	114.7299762
	Mean:	114.4494139
	Stdev	0.615236499
Stdev of mean		**0.275142**

Pipette:	0-200	
Set volume:		118.675
Temperature of the water:		
No.	weight	volume
1	0.1174	117.635801
2	0.1173	117.5356001
3	0.1174	117.635801
4	0.118	118.2370061
5	0.1182	118.4374078
	Mean:	117.8963232
	Stdev	0.410701281
Stdev of mean		**0.183671**

Pipette:	0-200	
Set volume:		103.6
Temperature of the water:		
No.	weight	volume
1	0.1021	102.3050705
2	0.1027	102.9062756
3	0.1026	102.8060748
4	0.1029	103.1066773
5	0.103	103.2068782
	Mean:	102.8661953
	Stdev	0.351417976
Stdev of mean		**0.157159**