正茂生物科技股份有限公司
Genmall Biotechnology Co., Ltd.

BIO-RAD

CFX Opus

Specialist ：蔡鼎諾（Tim）

Genmall

qPCR Operation Training

Outline

PCR

DNA

Principles

Elements

Procedure

What is Polymerase Chain Reaction ?

A common laboratory technique which is used to "amplify" - copy - small segments of DNA (In Vitro) via DNA polymerase and a pair of specific primers .

PCR

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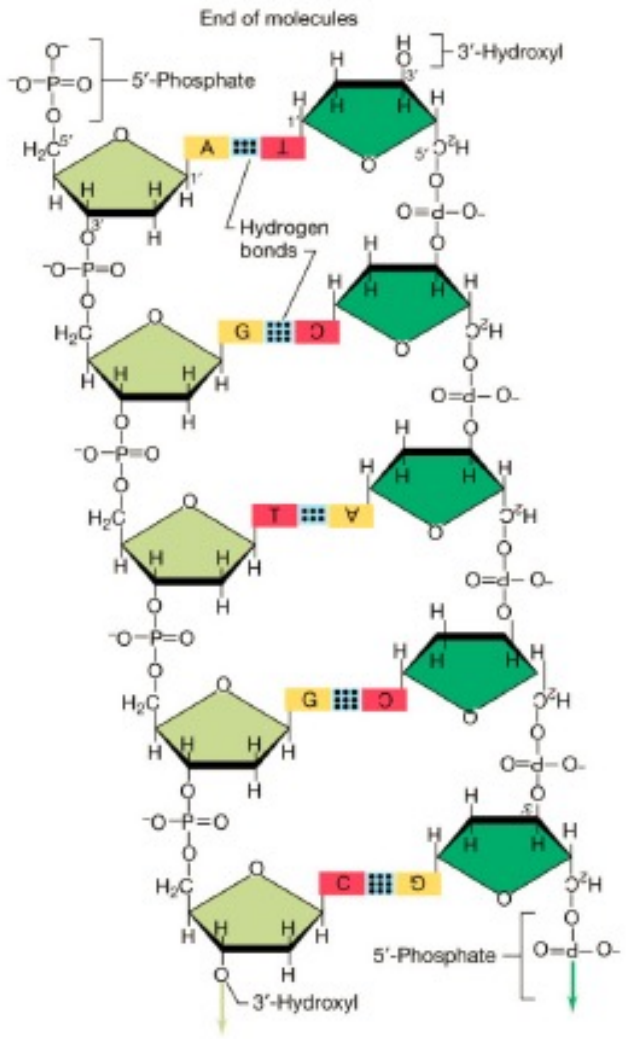
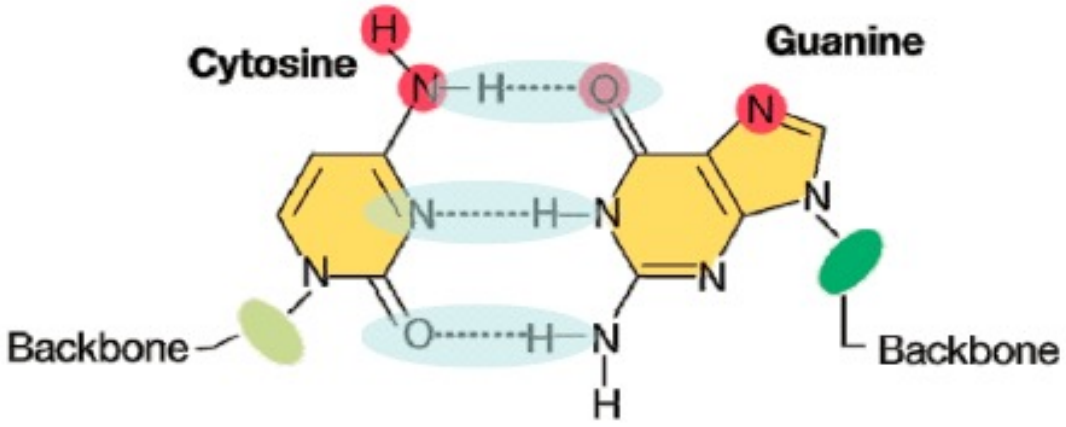
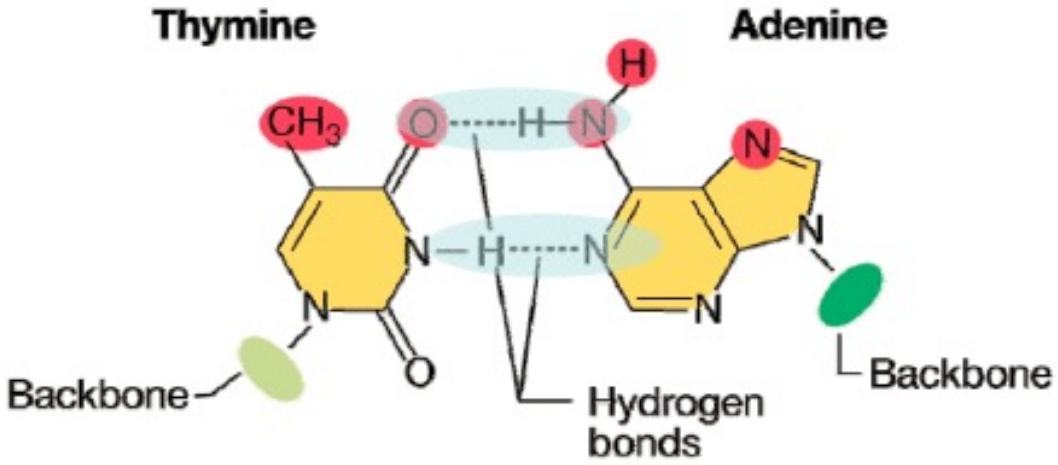




GC pair > AT pair



PCR

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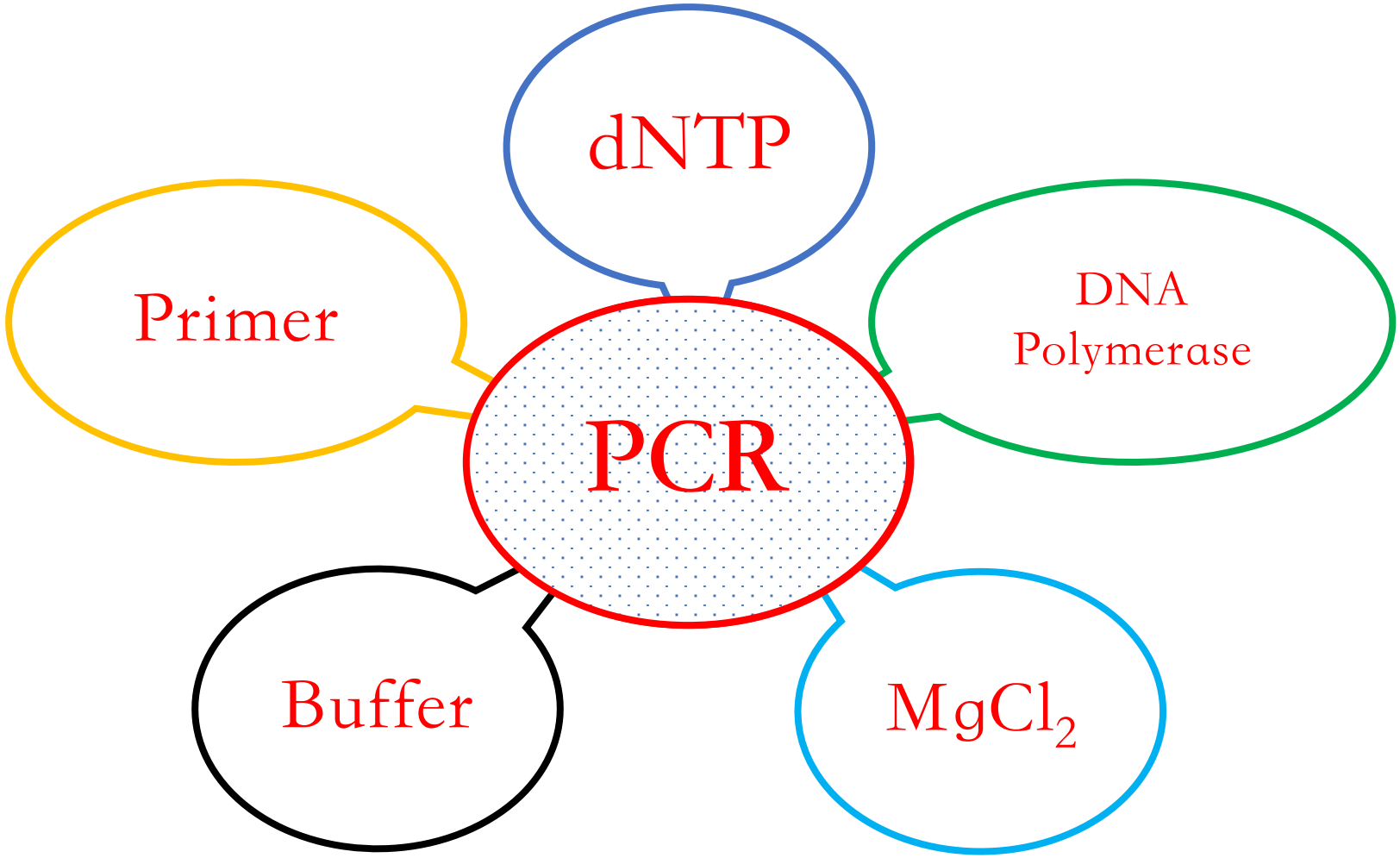

PCR

DNA

Principles

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Procedure

- ✓ DNA template : A segment of DNA which can be used as a model for duplication.
- ✓ Primer : Two primers (forward& reverse primer) are used in each PCR reaction, and they are designed so that they flank the target region (region that should be copied).
- ✓ DNA polymerase : Synthesizes - builds - two new strands of DNA, using the original strands as templates.

PCR

DNA

Principles

Elements

Procedure

- ✓ **Deoxynucleotide triphosphate** (dNTP)： There are four types of dNTP 「dATP、dGTP、dCTP、dTTP」 in PCR is to expand the growing DNA strand with the help of DNA polymerase. 。

- ✓ **Magnesium chloride** ($MgCl_2$) ： Magnesium ion (Mg^{2+}) functions as a cofactor for activity of DNA polymerases by enabling incorporation of dNTPs during polymerization.

- ✓ **Buffer** ： Provides a suitable chemical environment for activity of DNA polymerase. The buffer pH is usually between 8.0 and 9.5 (KCl) and is often stabilized by Tris-HCl, which promotes primer annealing.

PCR

DNA

Principles

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Procedure

核酸抽取

DNA isolation kits
RNA isolation kits

PCR反應管

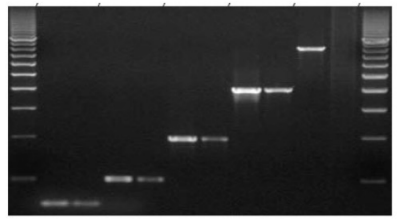
iTaq DNA polymerase
iProof DNA polymerase

PCR進行

T100
Thermocycler

電泳分析

Horizontal
Sub-Cell GT



染色 & 分析

EtBr

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qPCR

qPCR

Comparison

Principle

C_T value

DNA binding dye

Hydrolysis probe

1. **Real-Time PCR :** divide into two parts : 「Real-time」 and 「PCR」
2. **Real-Time PCR :** It is also known as 「quantitative PCR」
3. Abbreviation : 「Real Time RT-PCR」 or 「qPCR」
4. **Method :** Add 「fluorescent DNA biding dye」 or 「probe」 for detection during DNA amplification (PCR)
5. **Advantage :** qPCR is sensitive, specific detection (via fluorescence) and quantification of nucleic acid targets during PCR cycle (so-called Real time) .

qPCR

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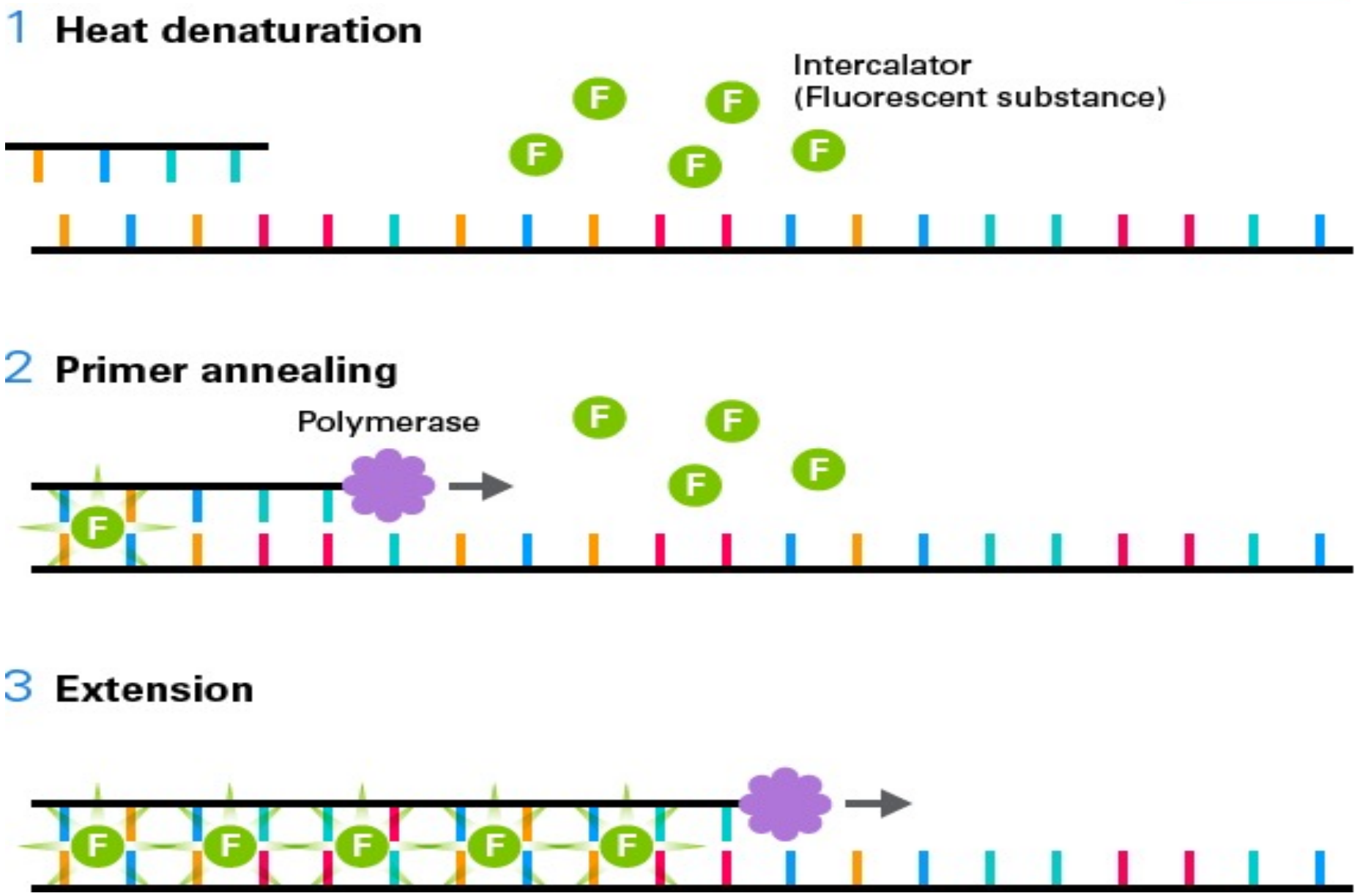

PCR V.S. qPCR

qPCR

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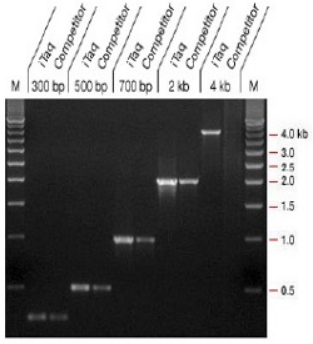
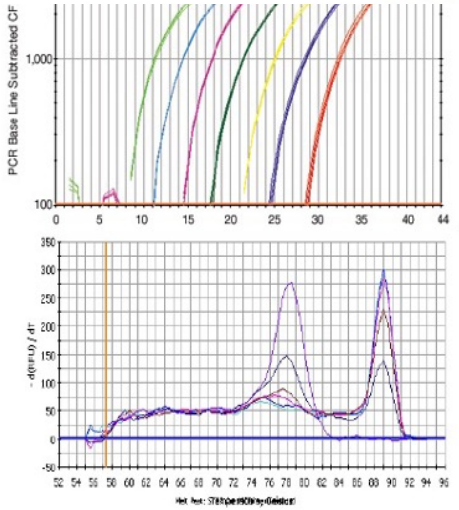
+Taq
+dNTP
+$MgCl_2$
+H_2O
+Buffer
+Template
+Primer
(On ice)



- End point
- Fuzzy data
- Labor intensive

3hrs (1.5 hrs labor)

+2x Master Mix
+H_2O
+Template
+Primer
(Room Temp)



- Online monitoring
- More sensitive
- Automated

<1 hrs (20 min labor)

qPCR

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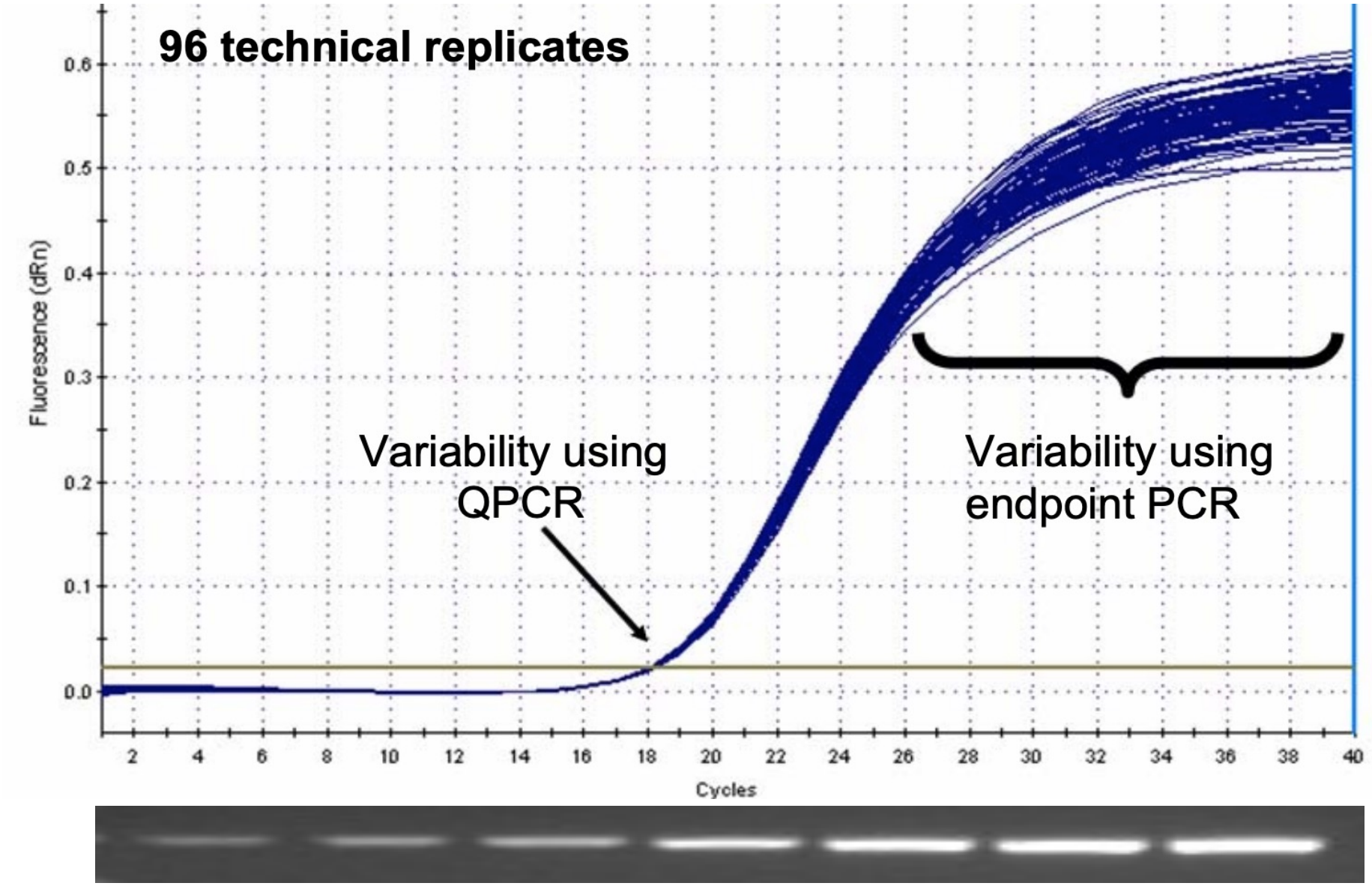

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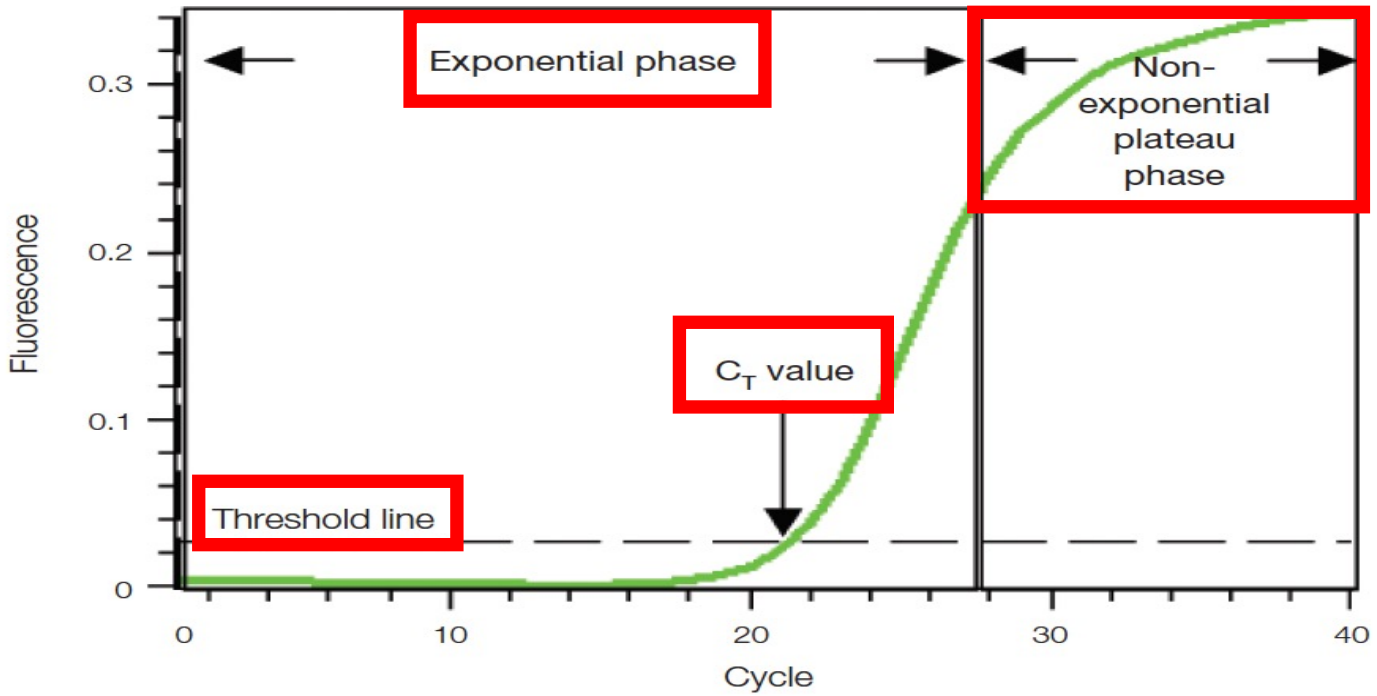

Fig. 1.1. Amplification plot. Baseline-subtracted fluorescence is shown.

✓ Threshold line (background or baseline) : At the start of the run, when the amount of PCR product is low, this produces very little fluorescence.

✓ Exponential phase : Once the amount of PCR product is amplified enough, it will enter the exponential phase. This is when the amount of PCR product doubles for every PCR cycle. 「C_T」 can be used and be defined as quantitative marker during this phase.

✓ Non-exponential Plateau phase : Once all of the reagents, such as nucleotides, have been used up in the PCR reaction, the amplification will slow and ultimately plateau. This is the region where no more PCR products cannot be produced.

qPCR

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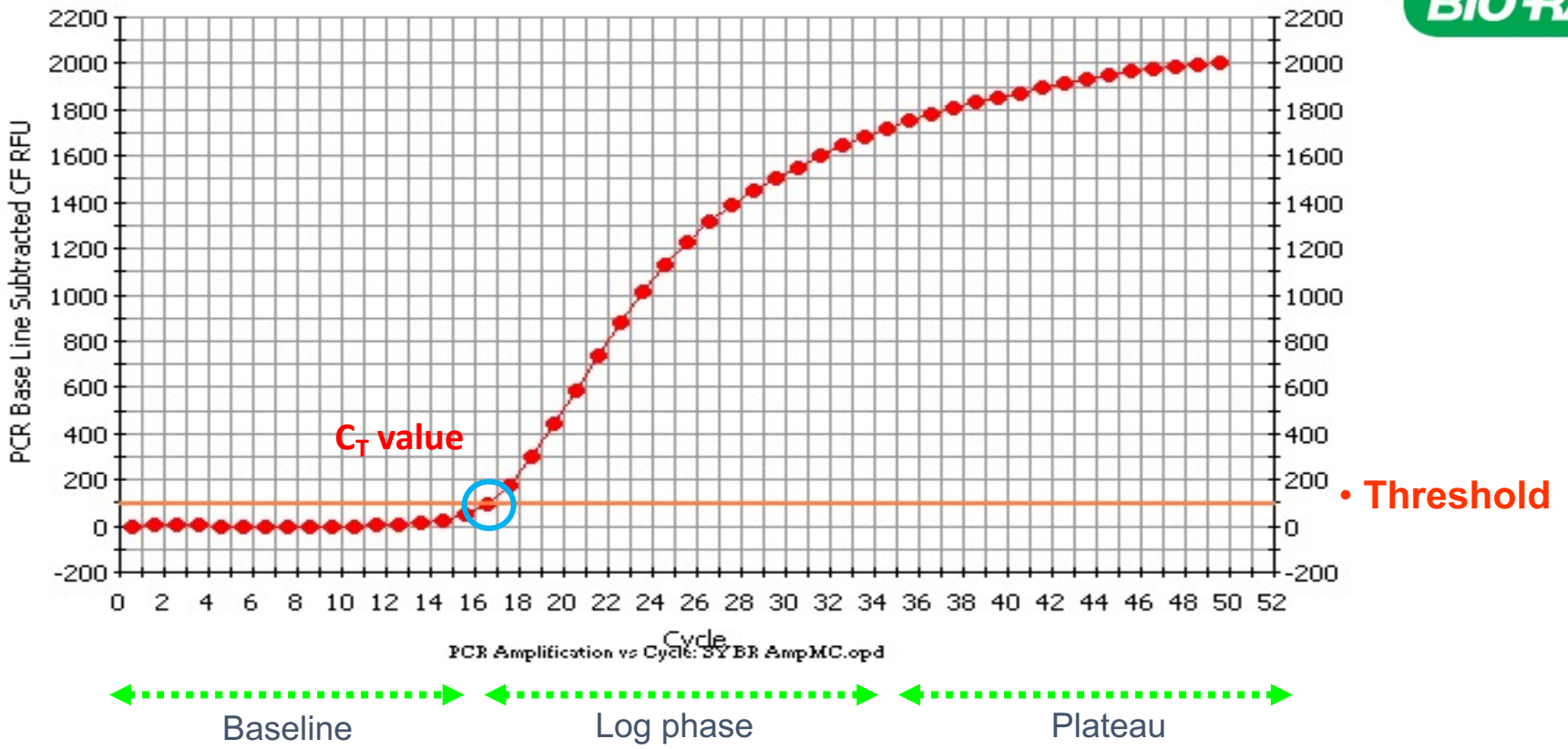

✓ Threshold : Basic reaction line of background which is derived from fluorescent reagents.

✓ C_T (cycle threshold) / Cq (cycle quantification) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level).

qPCR

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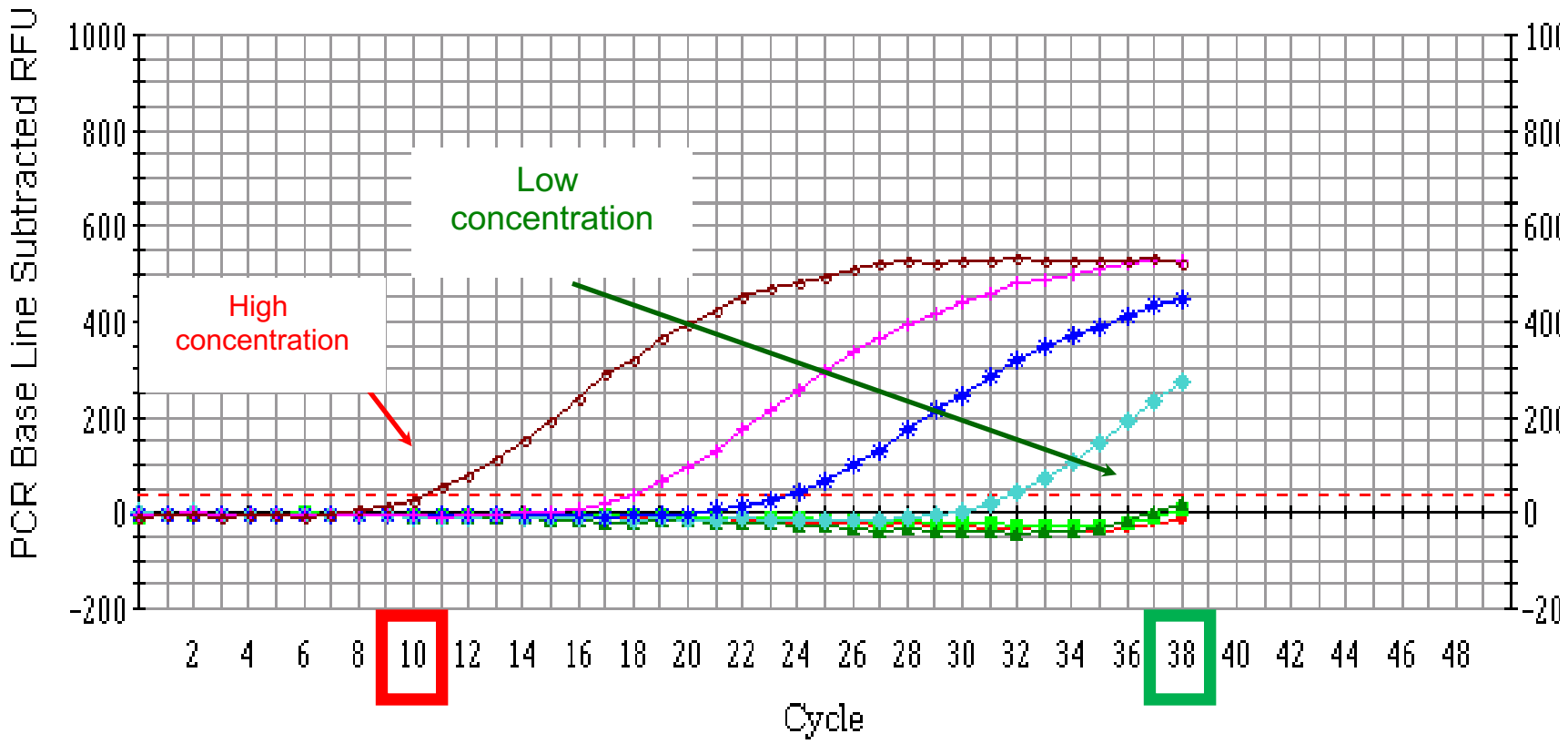
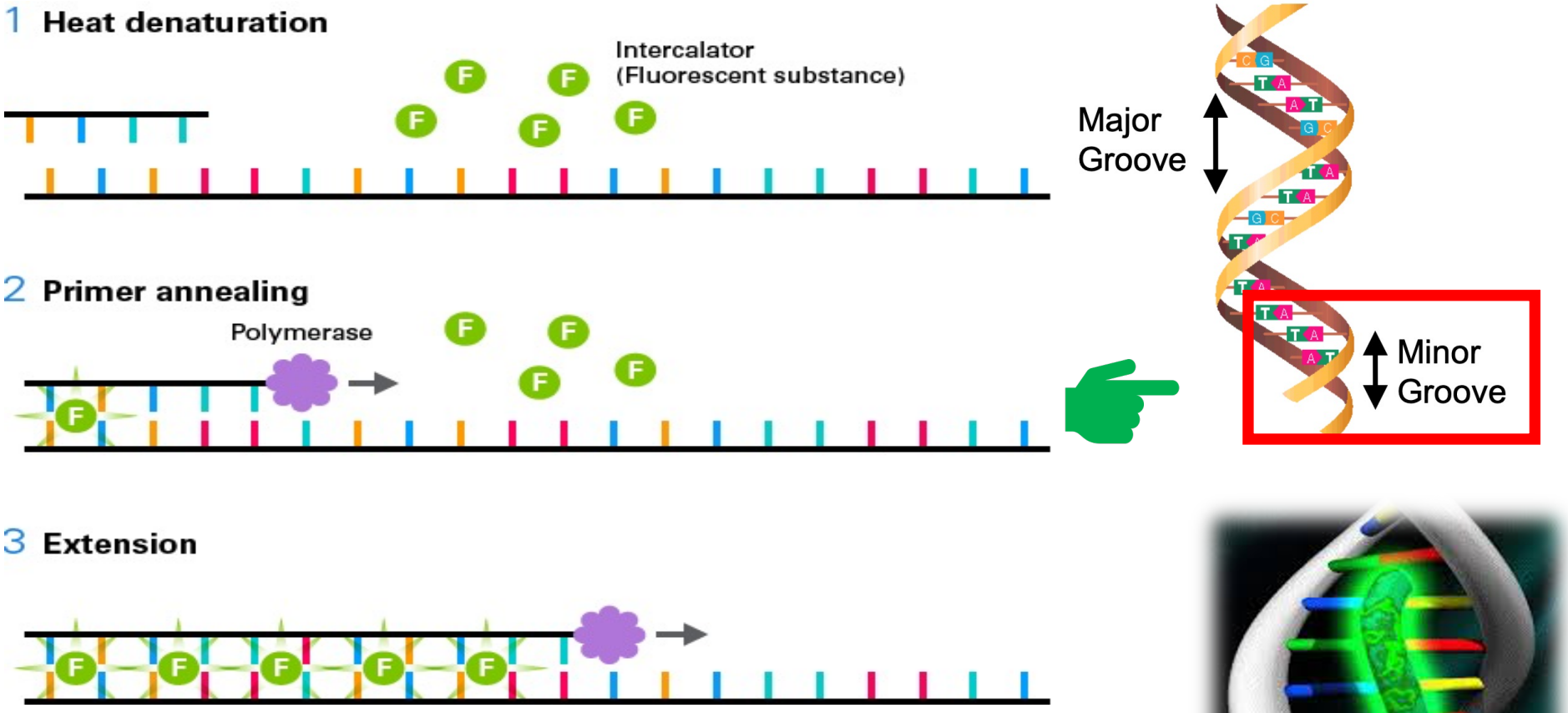
Hydrolysis probe



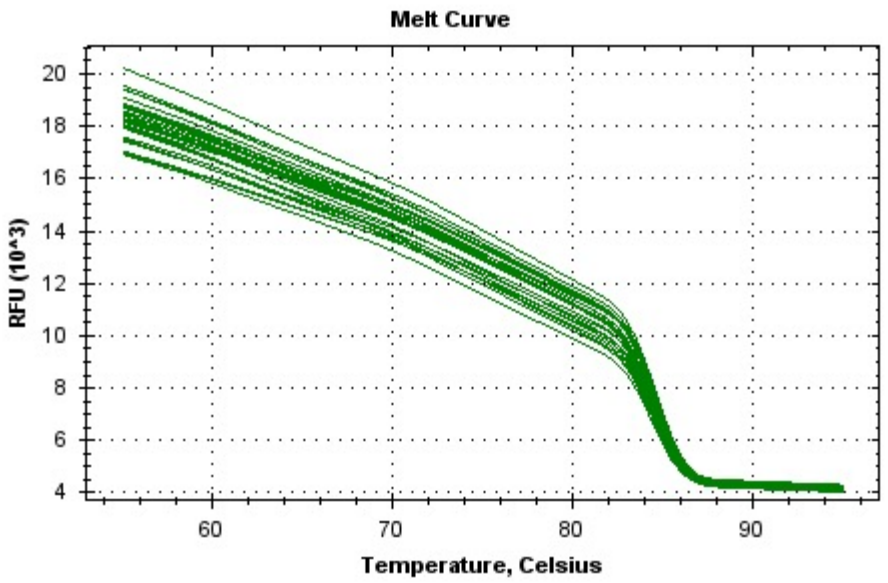
C_T value are inversely proportional to the **amount** of target nucleic acid

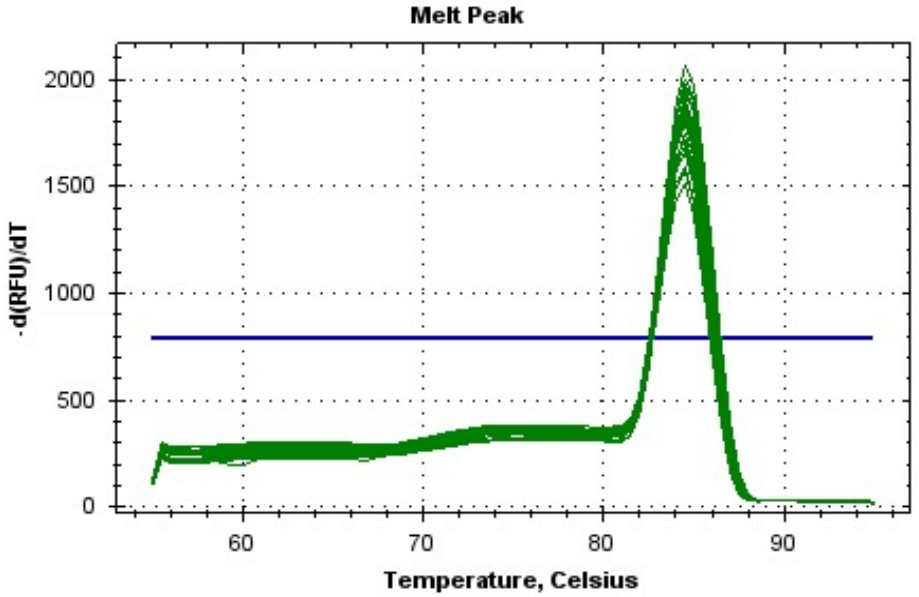
DNA binding dye – SYBR Green I



SYBR Green I is a dsDNA binding dye, which can be used to quantify amplicon amount during the course of the PCR by tracking overall fluorescence emission.

DNA binding dye – SYBR Green I





How to confirm the 「specificity」 of PCR products when performing SYBR Green qPCR experiments?

1. Method: Use the characteristics of DNA Tm value to ensure.
2. Analytical method ：

① Melt Curve （ The upper left ）

② Melt Peak （ The upper right ）

qPCR

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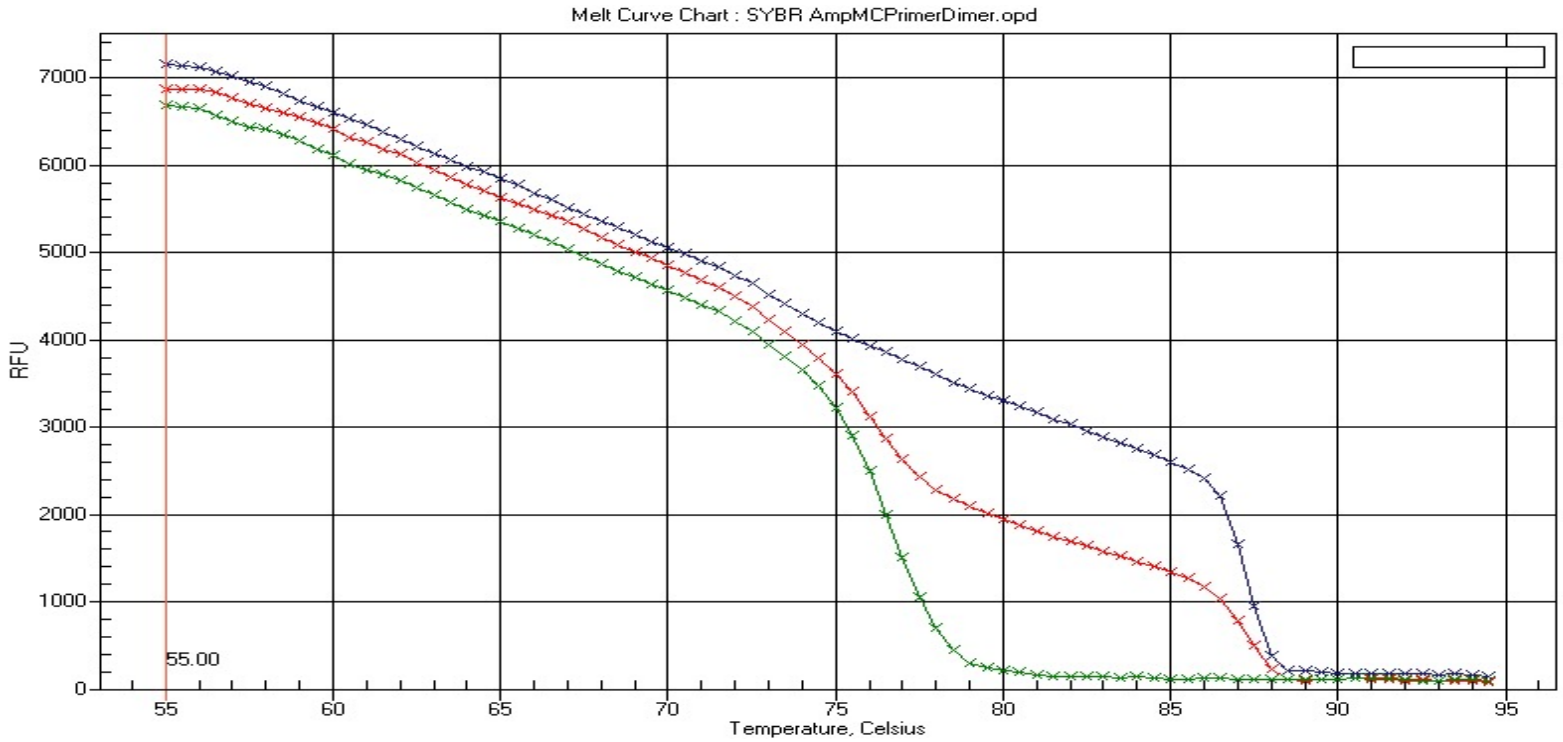
Melting Curve - Tm value

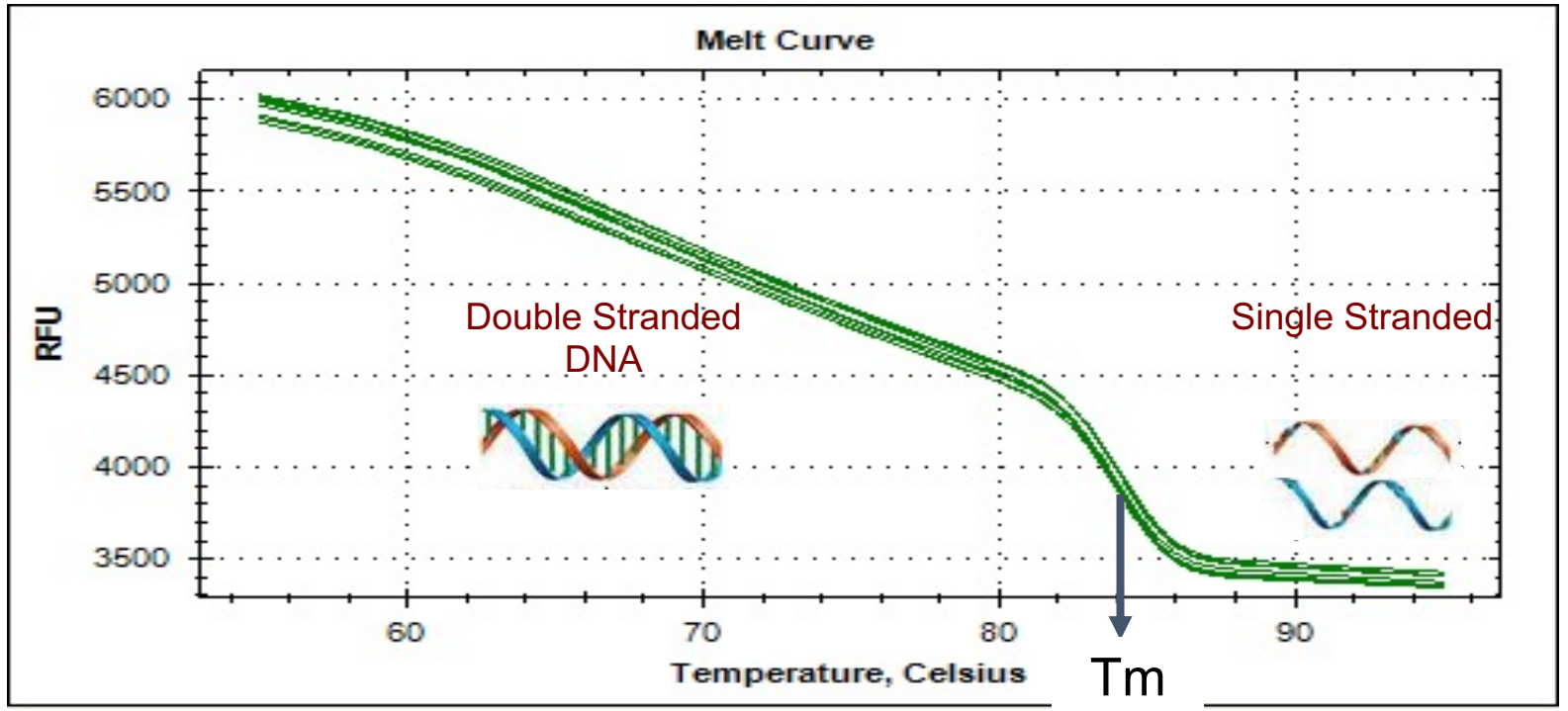


➢Melting temperature (Tm)

① The temperature at which one half of the DNA duplex will dissociate to become single stranded and indicates the duplex stability. ◦

② Depends on nucleotide content (GC ratio) and length

Melting Curve - Tm value



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Melting Peak – Tm value

qPCR

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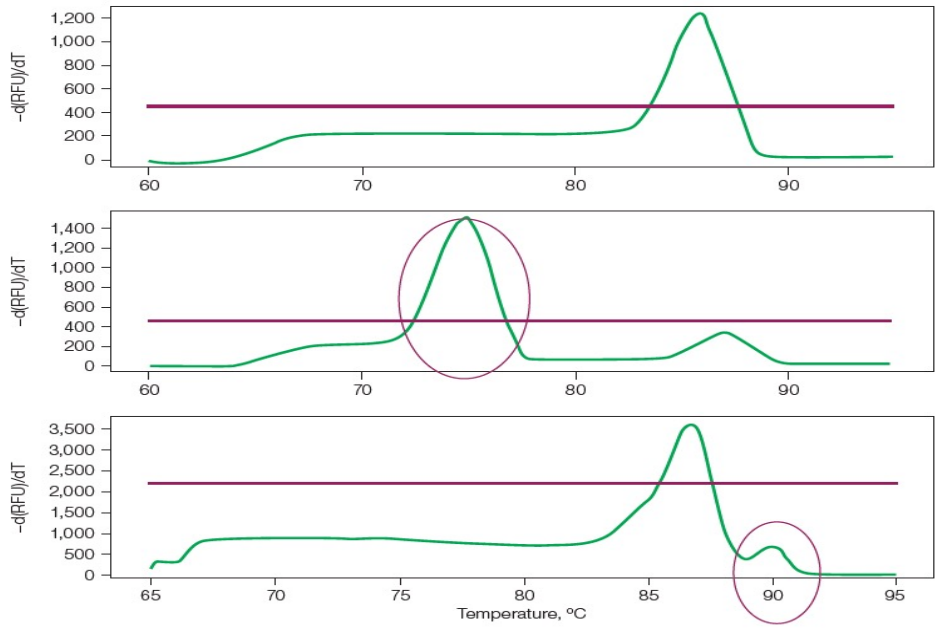

Target Product

Primer-dimers

Mispriming

Fig. 6. Melt curve profiles. A, a single well-defined peak indicates a single specific product. **B**, two or more peaks indicate poor specificity. A typical primer-dimer (in red) is due to excess final primer concentrations. Primer-dimers typically amplify in the mid-70°C range. **C**, two or more peaks indicate poor specificity. A typical mispriming (in red) is shown.

➢Melting temperature (Tm)

① The temperature at which one half of the DNA duplex will dissociate to become single stranded and indicates the duplex stability. ◦

② Depends on nucleotide content (GC ratio) and length

Melting Peak – Tm value

qPCR

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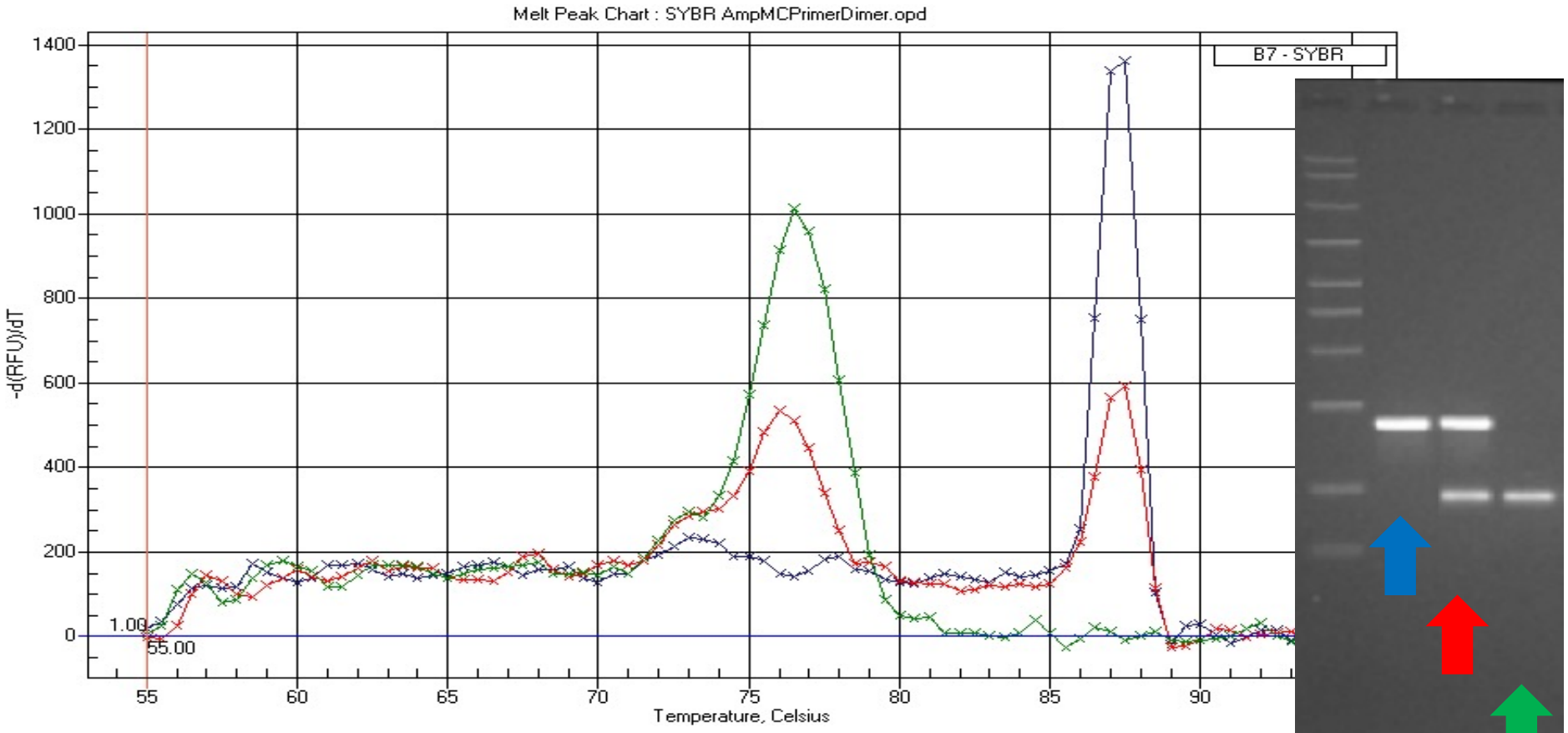
Principle

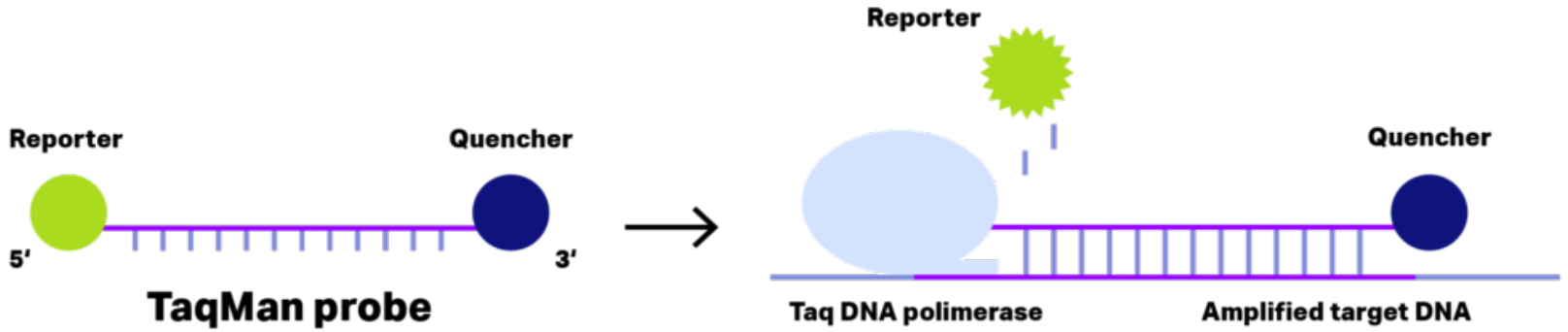
C_T value

DNA binding dye

Hydrolysis probe



Hydrolysis probe – TaqMan probe



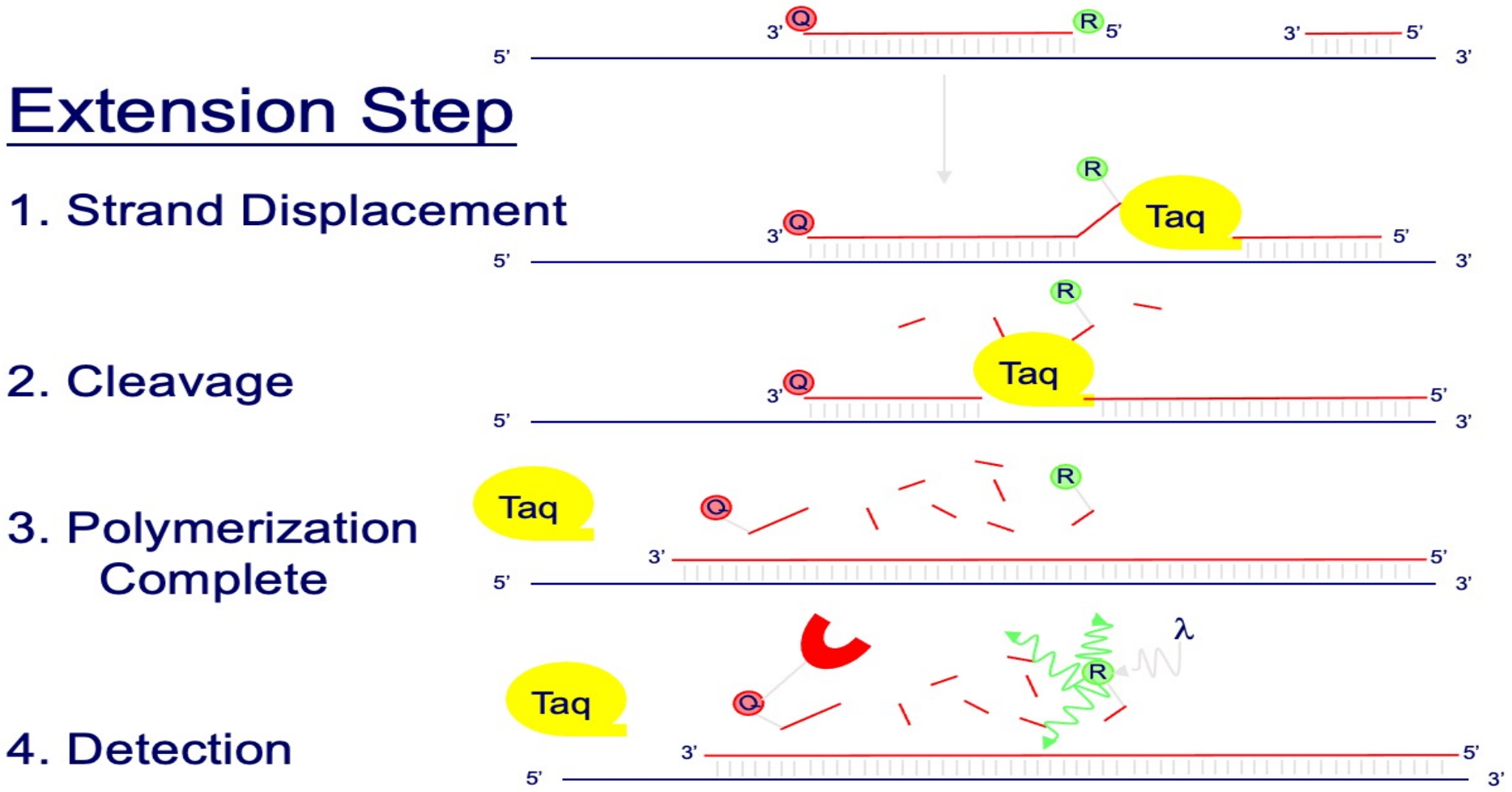
① **Structure** : Labeled with a fluorescent **reporter at the 5' end** and a **quencher at the 3' end**.

② **Enzyme** : The dsDNA-specific 5' → 3' exonuclease activity of *Taq*

③ **Principle** : The **reporter** is separated from the **quencher**, resulting in a fluorescence signal that is proportional to the amount of amplified product in the sample.

④ **Advantages** : High specificity and the ability to perform **multiplex** reactions.

Hydrolysis probe – TaqMan probe



Binding dye v.s. Probe

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➢ SYBR Green I Dye

① Singleplex

② Non-specific

③ Binding site：dsDNA

④ Sensitivity： Middle (10 - 100 copies)

⑤ Notice：Avoid Primer-dimer formation

⑥ Cost: Inexpensive

➢ TaqMan Probe

① Multiplex

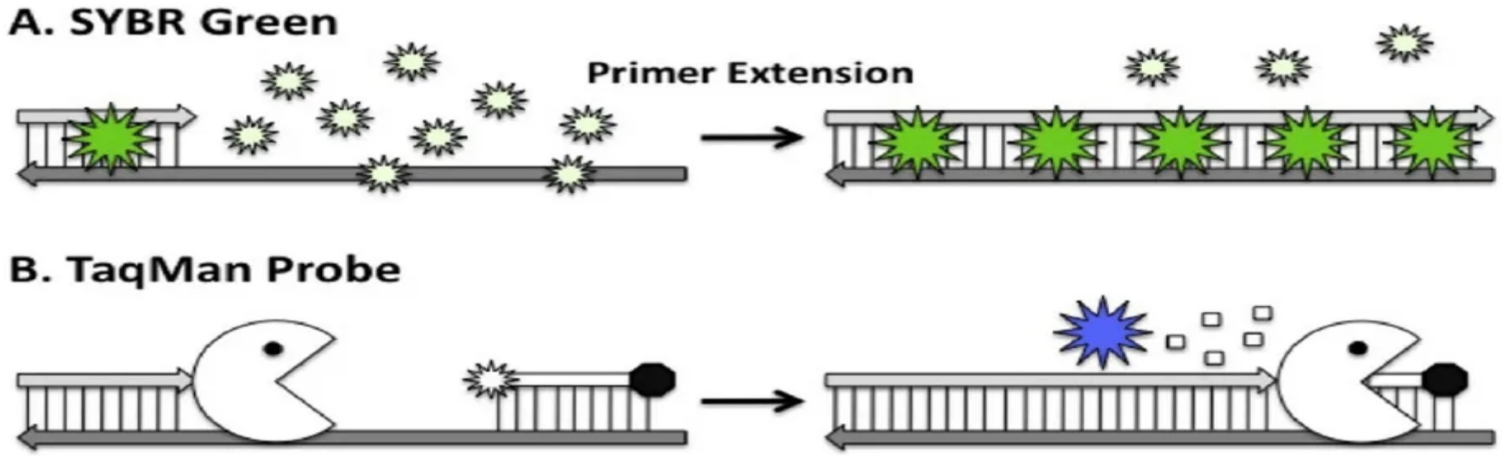
② Highly Specific

③ Binding site ：probe-specific region

④ Sensitivity ：High （1-10 copies）

⑤ Notice： Specific fluorescent probe is necessary.（Must be less than 30 bp）

⑥ Cost: Expensive



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Application

Standard Curve

- **Linear standard curve：** Coefficient of Correlation, $R^2 > 0.980$
- **PCR Efficiency：** > 90 - 110%
- **Equation ：**

① Amplification efficiency, $E = 10^{-1/slope}$

② % Efficiency = (E - 1) x 100%

- When E < 90% ： Might be interfered by PCR inhibitors in your sample or solution.
- When E > 110% ： Might be influenced by pipetting error or primer-dimer.

Standard Curve

Essential

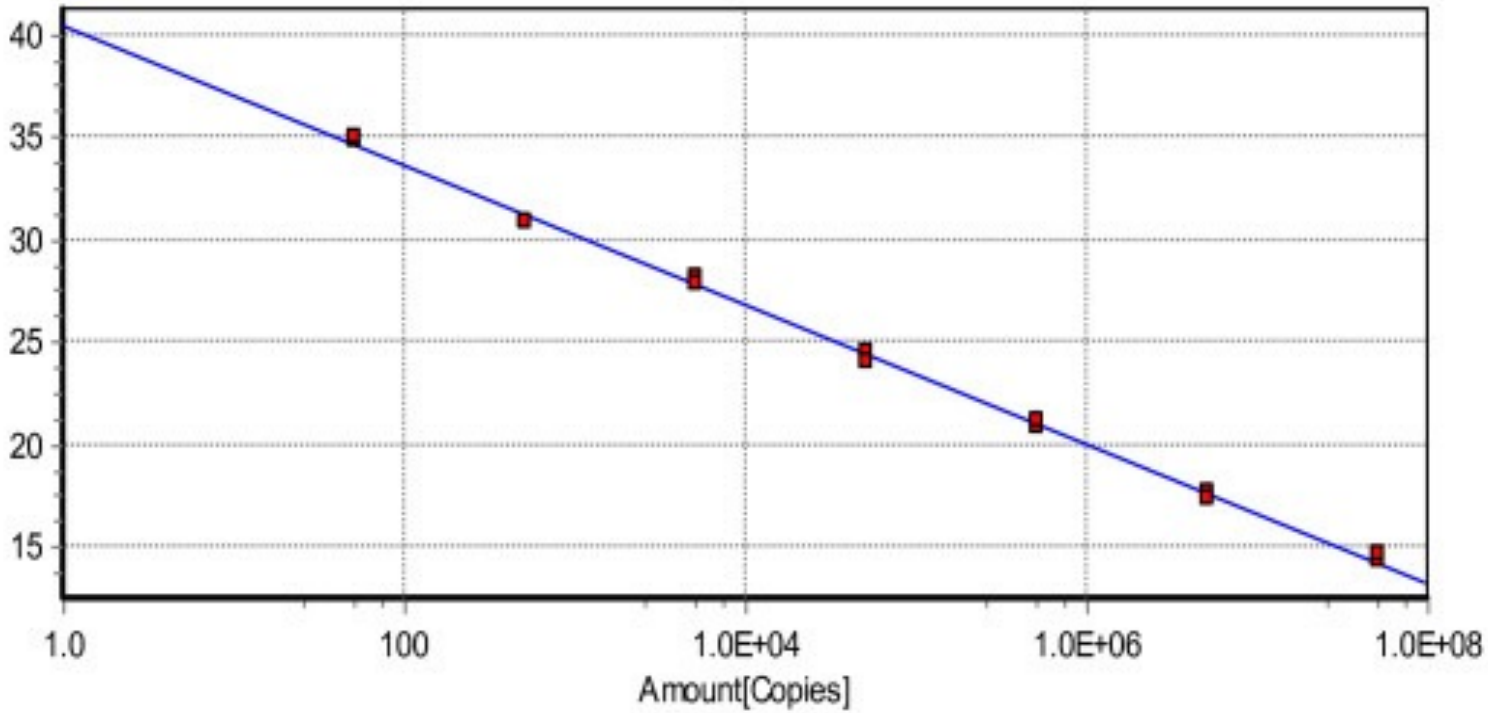
Absolute Qu.

Relative Qu.

Application

MIQE

Notice



Slope:	-3.395
Y-Intercept:	40.47
Efficiency:	0.97
R^2:	0.998

✓「Absolute quantification」is performed by constructing a standard curve for each gene of the genes of interest (GOI).

✓Plotting the quantification cycle (Cq) values against log[quantity] of a dilution series of known GOI amount. (Interpolation)

✓According to the different standard products, it is usually expressed in the form of common units as follows:

① Concentration unit ： ng/ml、μg/ml

② Measurement unit： ng、pg、copy number

✓Note for absolute quantification ： The slope of the standard curve and the correlation coefficient (R^2)

① Slope：「3.0 ~ -3.6」，(Correspond to PCR efficiency from 90%~110%)

② Correlation coefficient ：「$R^2 > 0.980$」

Essential

Absolute Qu.

Relative Qu.

Application

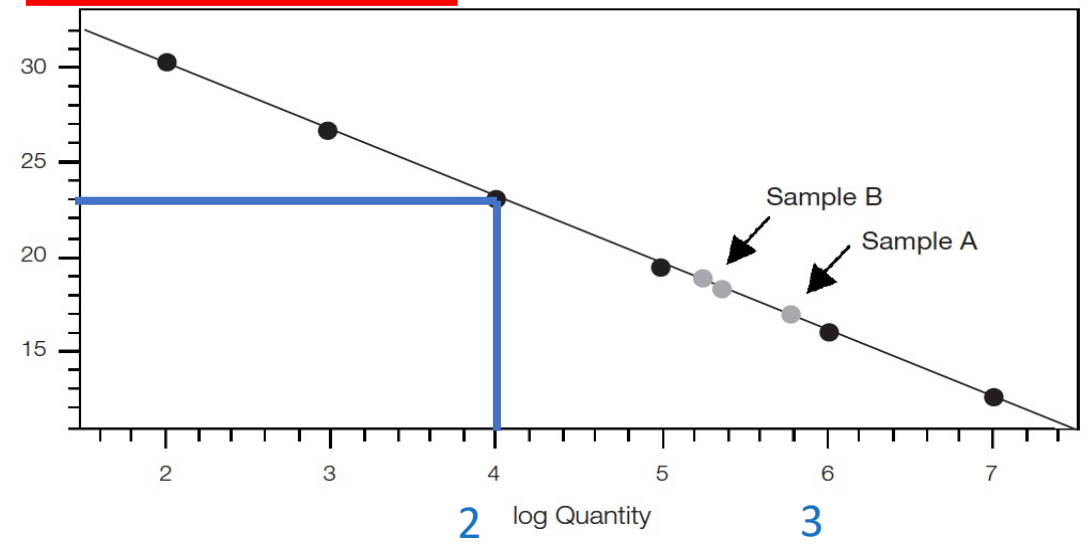
MIQE

Notice

1

$y = mx + b$

$y = -3.50x + 37.20$; $r^2 = 0.999$



2

3

Sample	Replicate	C_T	Copies
A	1	18.61	204,577
A	2	18.41	234,115
A	3	18.87	172,300
Average			203,664 ± 30,917
B	1	17.06	569,789
B	2	17.07	563,823
B	3	17.00	591,173
Average			574,928 ± 14,381

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➢ $\Delta\Delta C_T$ (Livak)

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		Reference	Target
Tissue #1 (Control):		21	22
Tissue #2 (Test) :		20	24
1st Delta	ΔC_T #1:		22-21 = 1
	ΔC_T #2:		24-20 = 4
2nd Delta	$\Delta\Delta C_T$:		1-4 = -3

Fold induction = $2^{-\Delta\Delta Ct} = 2^{-(-3)} = 8$

Essential

Absolute Qu.

Relative Qu.

Application

MIQE

Notice

Gene Expression

① Drug reaction

② Tumor marker

③ Gene regulation （siRNA/miRNA）

④ Gene therapy

⑤ Microarray

⑥ GMO detection

Pathogen detection

① Multiplex detection

② Multiple specimen detection

③ Synchronous analysis：Qualitative / Quantitative

④ Drug Safety and Effectiveness Monitoring

SNP Genotyping Analysis

① Evaluate the identified position of SNP

② Use for drug design

③ Genetic disease detection

Essential

Absolute Qu.

Relative Qu.

Application

MIQE

Notice

BIO-RAD

a blueprint for successful qPCR assay design

miqe

Clinical Chemistry 55:4
611–622 (2009)

Special Report

The MIQE Guidelines: *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments

Stephen A. Bustin,[1*] Vladimir Benes,[2] Jeremy A. Garson,[3,4] Jan Hellemans,[5] Jim Huggett,[6] Mikael Kubista,[7,8] Reinhold Mueller,[9] Tania Nolan,[10] Michael W. Pfaffl,[11] Gregory L. Shipley,[12] Jo Vandesompele,[5] and Carl T. Wittwer[13,14]

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Essential

Absolute Qu.

Relative Qu.

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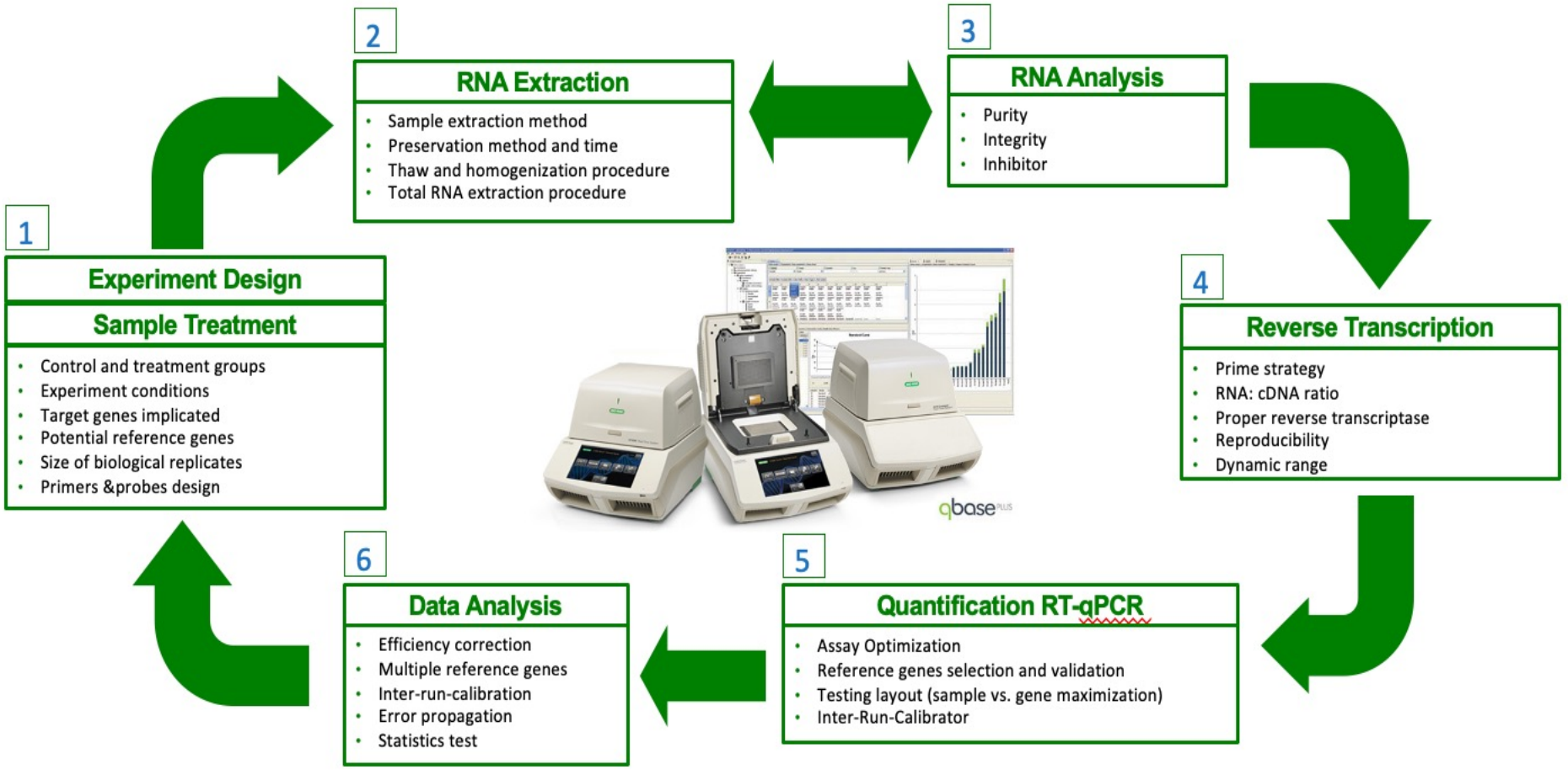
MIQE

Notice



MIQE guideline

BIO-RAD

1

檢核項目	重要性	說明
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	**E**	敘述實驗設計組別、樣本數以及執行的實驗室等
Number within each group	**E**	
Assay carried out by core lab or investigator's lab?	D	
Acknowledgement of authors' contributions	D	

1

SAMPLE		
Description	**E**	敘述樣本來源(例如組織或細胞)、細胞數(量)、處理方式(多少時間內完成)、保存環境(溫度)
Volume/mass of sample processed	D	
Microdissection or macrodissection	**E**	
Processing procedure	**E**	
If frozen - how and how quickly?	**E**	
If fixed - with what, how quickly?	**E**	
Sample storage conditions and duration (especially for FFPE samples)	**E**	

2 3

NUCLEIC ACID EXTRACTION		
Procedure and/or instrumentation	**E**	提供萃取的方法或儀器
Name of kit and details of any modifications	**E**	提供 **Kit** 的名稱/型號/批號/廠牌
Source of additional reagents used	D	是否有額外添加的試劑，有則註明型號廠牌
Details of DNase or RNase treatment	**E**	敘述對 **DNase (for RNA** 實驗**)or RNase (for DNA** 實驗**)** 的處理
Contamination assessment (DNA or RNA)	**E**	以尚未進行反轉錄的 **RNA** 樣本進行 **qPCR**，確認是否有產物
Nucleic acid quantification	**E**	核酸定量**:**使用的儀器廠牌/方法，**OD260/OD280** 比值，計算濃度和總量
Instrument and method	**E**	
Purity (A260/A280)	D	
Yield	D	
RNA integrity method/instrument	**E**	確認 **RNA** 品質**:** 求 **RIN/RQI** 值[1]，跑膠確認 **RNA** 是否降解以及測試樣品內是否有抑制物質(效應)[2]
RIN/RQI or Cq of 3' and 5' transcripts	**E**	
Electrophoresis traces	D	
Inhibition testing (Cq dilutions, spike or other)	**E**	

4

REVERSE TRANSCRIPTION		
Complete reaction conditions	**E**	敘述反轉錄反應條件
Amount of RNA and reaction volume	**E**	**RNA** 量與反應體積
Priming oligonucleotide (if using GSP) and concentration	**E**	**Primer** 種類(廠牌)和濃度
Reverse transcriptase and concentration	**E**	反轉錄酵素(廠牌)和濃度
Temperature and time	**E**	溫度/時間/**cycle** 數
Manufacturer of reagents and catalogue numbers	D	試劑廠牌和型號
Cqs with and without RT	D	**RT** 試劑測試
Storage conditions of cDNA	D	記錄保存條件

資料來源：https://ntuhmc.ntuh.gov.tw/epaper-25th.htm

MIQE guideline

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5

qPCR TARGET INFORMATION		
If multiplex, efficiency and LOD of each assay.	E	於同一試管做多基因定量才需要確定該項目
Sequence accession number	E	先以資料庫或軟體確定基因序列、比對、二級結構分析、**Primer** 對應於 **exon** 位置等資訊
Location of amplicon	D	
Amplicon length	E	
In silico specificity screen (BLAST, etc)	E	
Pseudogenes, retropseudogenes or other homologs?	D	
Sequence alignment	D	
Secondary structure analysis of amplicon	D	
Location of each primer by exon or intron (if applicable)	E	
What splice variants are targeted?	E	

5

qPCR OLIGONUCLEOTIDES		
Primer sequences	E	**Primer** 或 **Probe** 合成的序列、廠商、純化方法等
RTPrimerDB Identification Number	D	
Probe sequences	D[3]	
Location and identity of any modifications	E	
Manufacturer of oligonucleotides	D	
Purification method	D	

5

qPCR PROTOCOL		
Complete reaction conditions	E	敘述定量 **PCR** 實驗反應條件
Reaction volume and amount of cDNA/DNA	E	**cDNA** 量與反應體積
Primer, (probe), Mg++ and dNTP concentrations	E	**Primer**、鎂離子濃度、聚合酶濃度、緩衝液組成、其他添加物等
Polymerase identity and concentration	E	
Buffer/kit identity and manufacturer	E	
Exact chemical constitution of the buffer	D	
Additives (SYBR Green I, DMSO, etc.)	E	
Manufacturer of plates/tubes and catalog number	D	分析盤(管)廠牌和型號
Complete thermocycling parameters	E	反應條件(溫度/時間/**cycles**)
Reaction setup (manual/robotic)	D	反應設定
Manufacturer of qPCR instrument	E	儀器廠牌機型

6

qPCR VALIDATION		
Evidence of optimisation (from gradients)	D	是否有測試 **annealing** 溫度梯度
Specificity (gel, sequence, melt, or digest)	E	確認反應專一性
For SYBR Green I, Cq of the NTC	E	是否 **NTC** 控制組有產物
Standard curves with slope and y-intercept	E	建立標準曲線[4]:由斜率推算 **PCR** 效率、計算信賴區間與標準差以及 r2
PCR efficiency calculated from slope	E	
Confidence interval for PCR efficiency or standard error	D	
r2 of standard curve	E	
Linear dynamic range	E	求得線性關係範圍[5]: 計算信賴區間、最低極限時的 **Cq** 變異數以及是否確認 **LOD** 偵測極限
Cq variation at lower limit	E	
Confidence intervals throughout range	D	
Evidence for limit of detection	E	
If multiplex, efficiency and LOD of each assay.	E	於同一試管做多基因定量計算效率和偵測極限

DATA ANALYSIS		
qPCR analysis program (source, version)	E	分析程式/工具(版本)、分析方法 **(threshold** 或 **regression)**以及敘述樣本盤排列方式等
Cq method determination	E	
Outlier identification and disposition	E	
Results of NTCs	E	**NTC** 控制組是否有產物
Justification of number and choice of reference genes	E	**Reference gene** 的數目和選擇
Description of normalisation method	E	敘述標準化方法
Number and concordance of biological replicates	D	生物樣本數
Number and stage (RT or qPCR) of technical replicates	E	單一生物樣本在儀器偵測的 **n** 值
Repeatability (intra-assay variation)	E	計算重複性、再現性、使用何種統計方法、統計軟體(版本)
Reproducibility (inter-assay variation, %CV)	D	
Power analysis	D	
Statistical methods for result significance	E	
Software (source, version)	E	
Cq or raw data submission using RDML	D	以 **RDML** 格式提供原始數據

Essential

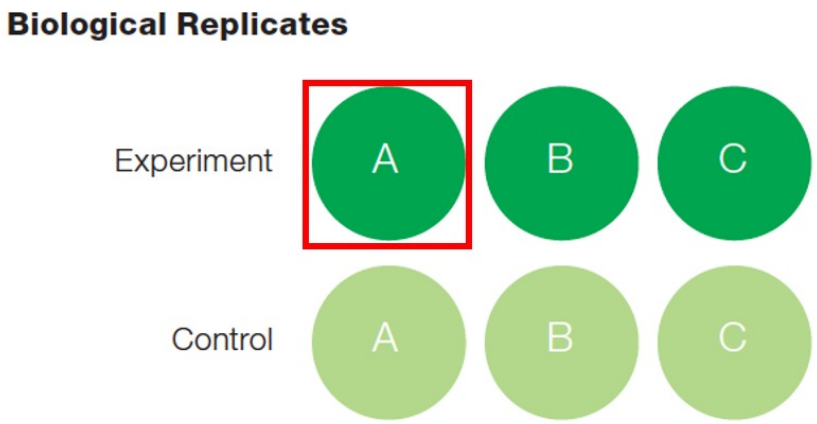
Absolute Qu.

Relative Qu.

Application

MIQE

Notice



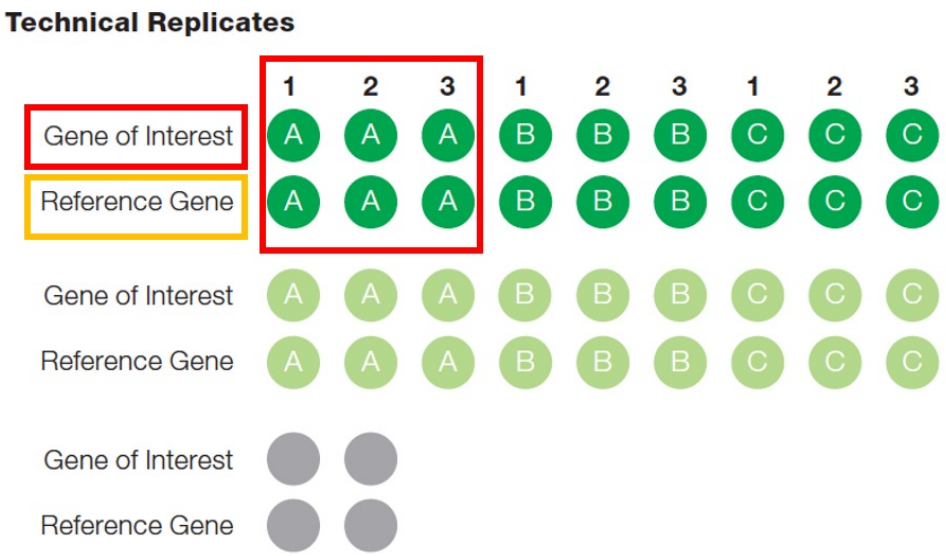


Fig. 6. Experimental replicates. All experiments should be designed with a combination of biological and technical replicates. This illustrates a simple experiment with triplicate biological samples from control (■) and treatment/experimental (■) conditions. For each biological sample, three technical replicates are recommended for the gene of interest as well as for the reference gene(s). This results in a total of at least 36 samples plus the duplicate NTC (■) for a total of >40 wells.

Essential

Absolute Qu.

Relative Qu.

Application

MIQE

Notice

Common 「PCR inhibitor」

Sample	Assay
Bile salts	Cholic acid
	Deoxycholic acid
Polysaccharides	Polygalacturonic acid
Lipids	Cholesterol hydrochloride
Algae	Alginic acid
Clay	Montmorillonite
Blood	Heparin
	Hematin
	EDTA
	Serum
Soil	Humic acid
Textile	Indigo
Wine	Tannic acid
Plants	Cellulose
	Pectin (for fiber control)
Hair, tissues	Melanin
Bones, teeth	$CaCl_2$

Sample	Assay
Cell lysates	MEM+FBS
	PBS
	Trypsin
Sample preparation	NaAc
	NaCl
	EtOH
	Isopropanol
	TRIzol
	InstaGene Matrix
Miscellaneous	Green tea
	Chocolate
	Dust
	SDS
	DMSO
	DTT
Body fluids	Spermidine
	Urea

★Red：From 「sample」

★Green： From 「reagent / solution」

Essential

Absolute Qu.

Relative Qu.

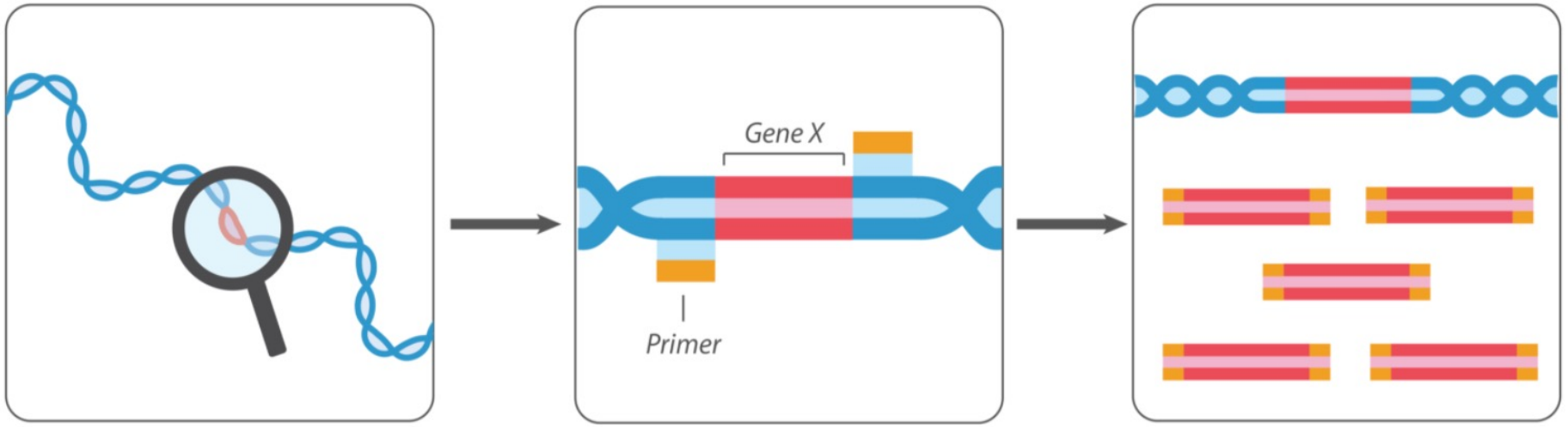
Application

MIQE

Notice

Amplicon

1. Recommended length ：75 - 200bp
2. Regional structure ： Should be free of strong secondary structures .
3. Repeats：Prevent quadruple repeats of G or C appear in designed region.
4. CG % ：40 - 60%



Essential

Absolute Qu.

Relative Qu.

Application

MIQE

Notice

Primer design

1. Recommended length：18 - 25bp

2. Regional structure ： Should be free of strong secondary structures and self-complementarity 「Primer-dimer」

3. Repeats：Prevent triple repeats of G or C appear in designed region.

4. CG %：40 - 60%

5. Tm value：50 - 65℃ ，Formula：Tm = 4℃ (G + C)+ 2℃ (A + T)

(q)PCR - 產線支援

Essential

Absolute Qu.

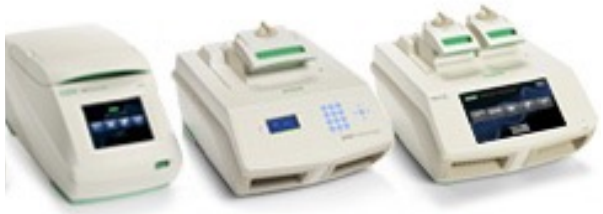
Relative Qu.

Application

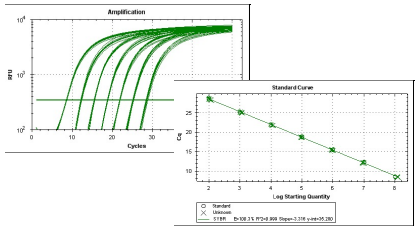
MIQE

Notice

PCR

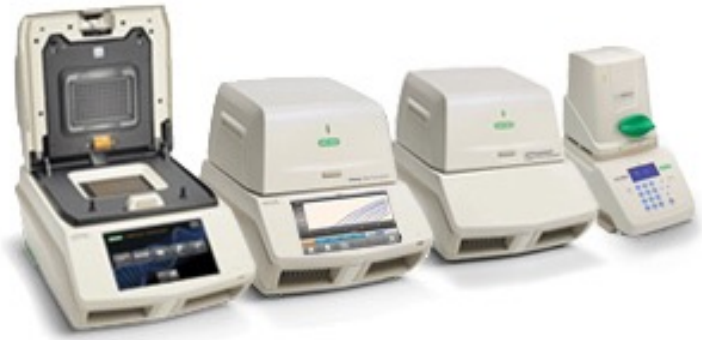


CFX Manager Software



(q)PCR Reagents

Real-Time PCR (qPCR)



Automation options

(q)PCR consumables

2022 寅

Operation

System Overview - Front View

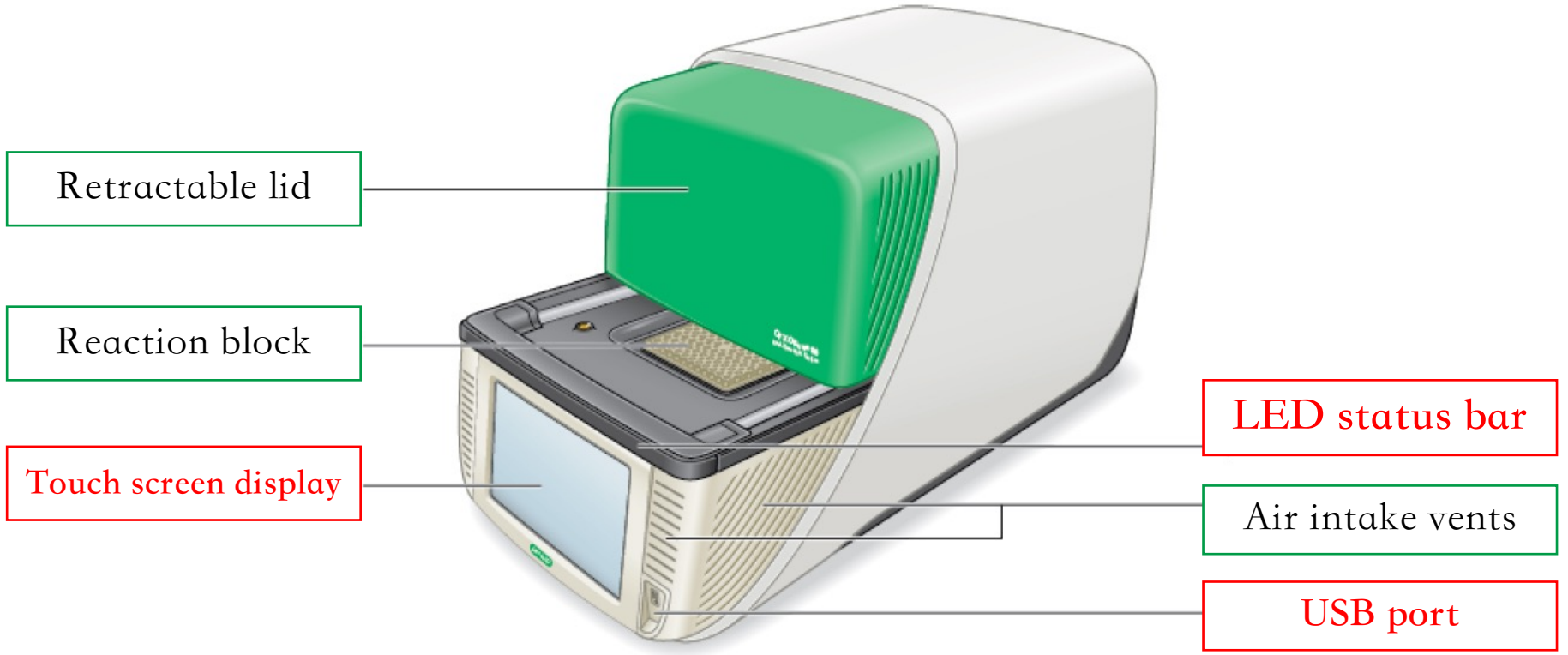
Appearance

Device Boot

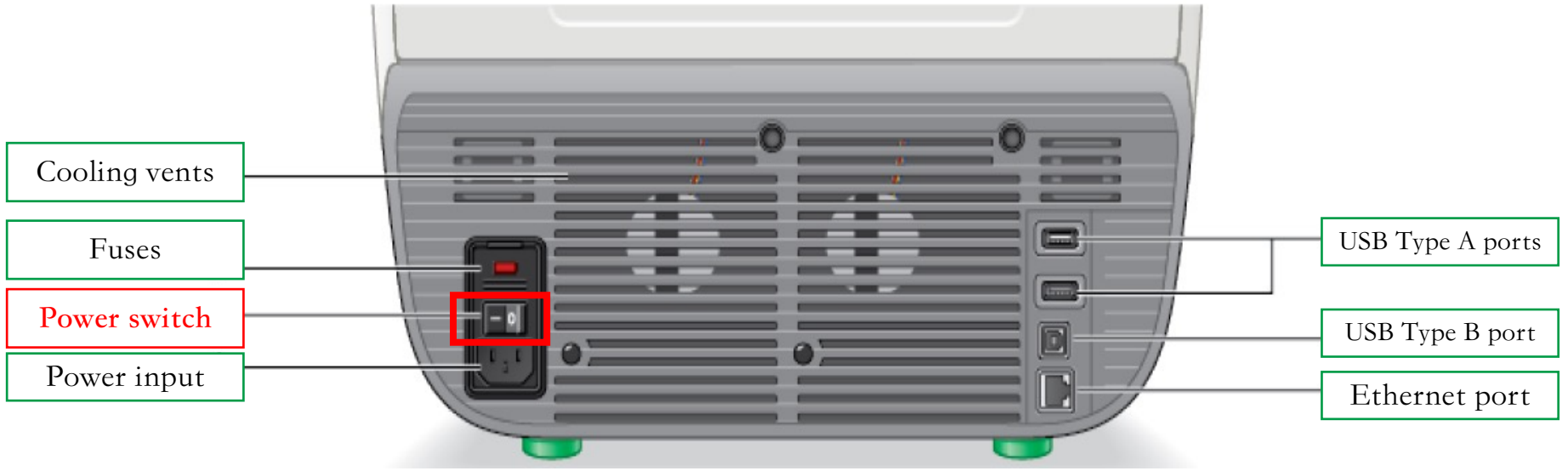
Consumables

Software

Optics



System Overview - Back View



Touch Screen Overview - Open lid

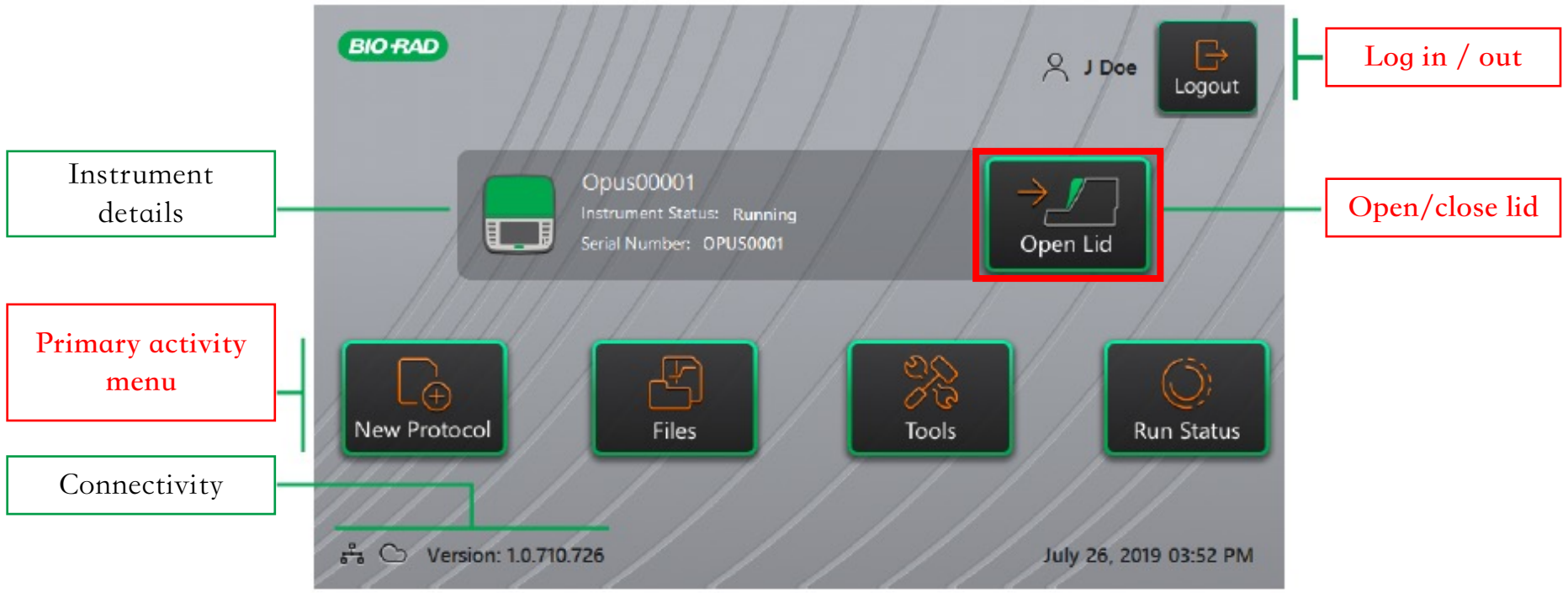
Appearance

Device Boot

Consumables

Software

Optics



Creating a Protocol

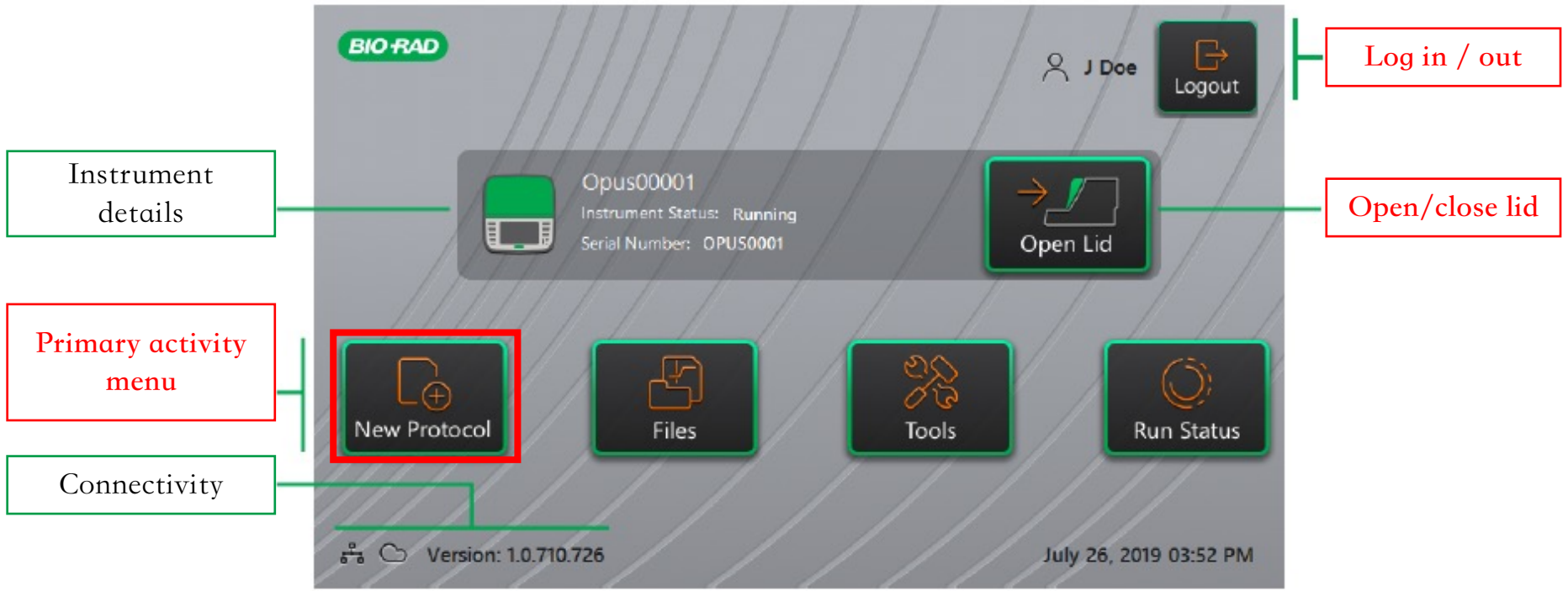
Appearance

Device Boot

Consumables

Software

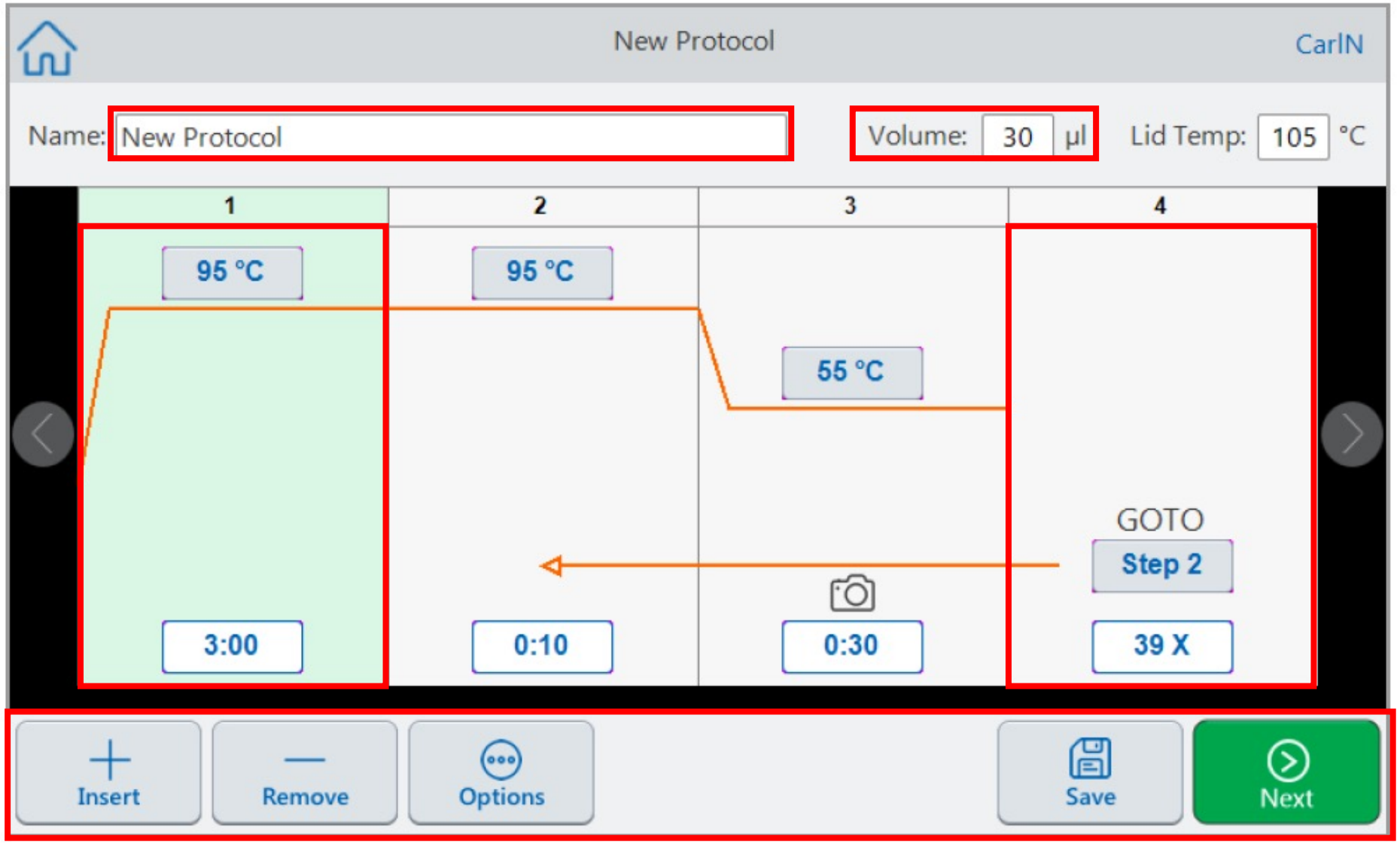
Optics



Modifying the Settings in a Protocol Step

BIO-RAD

- Appearance
- Device Boot
- Consumables
- Software
- Optics



Modifying the Settings in a Protocol Step

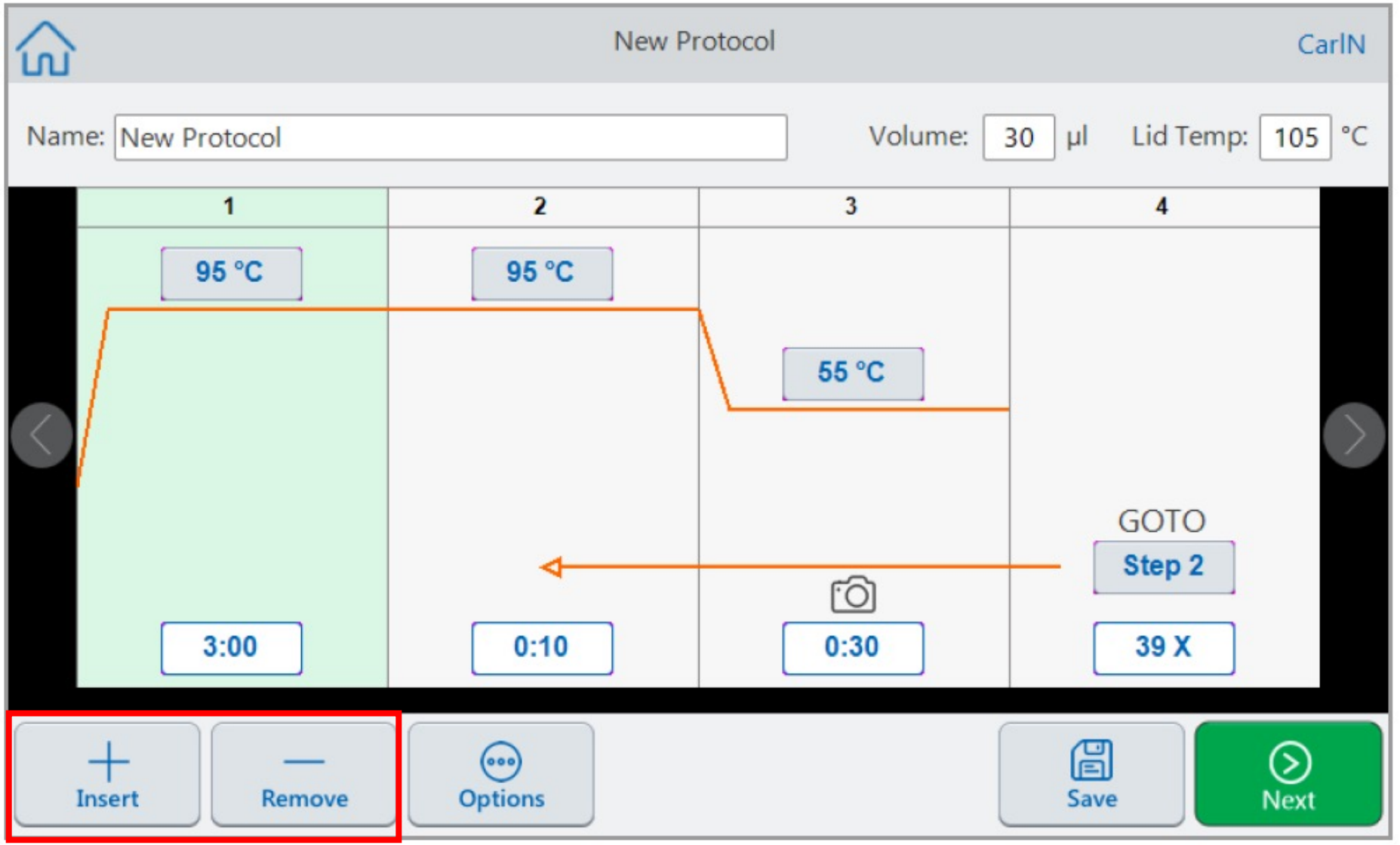
Appearance

Device Boot

Consumables

Software

Optics



Modifying the Settings in a Protocol Step

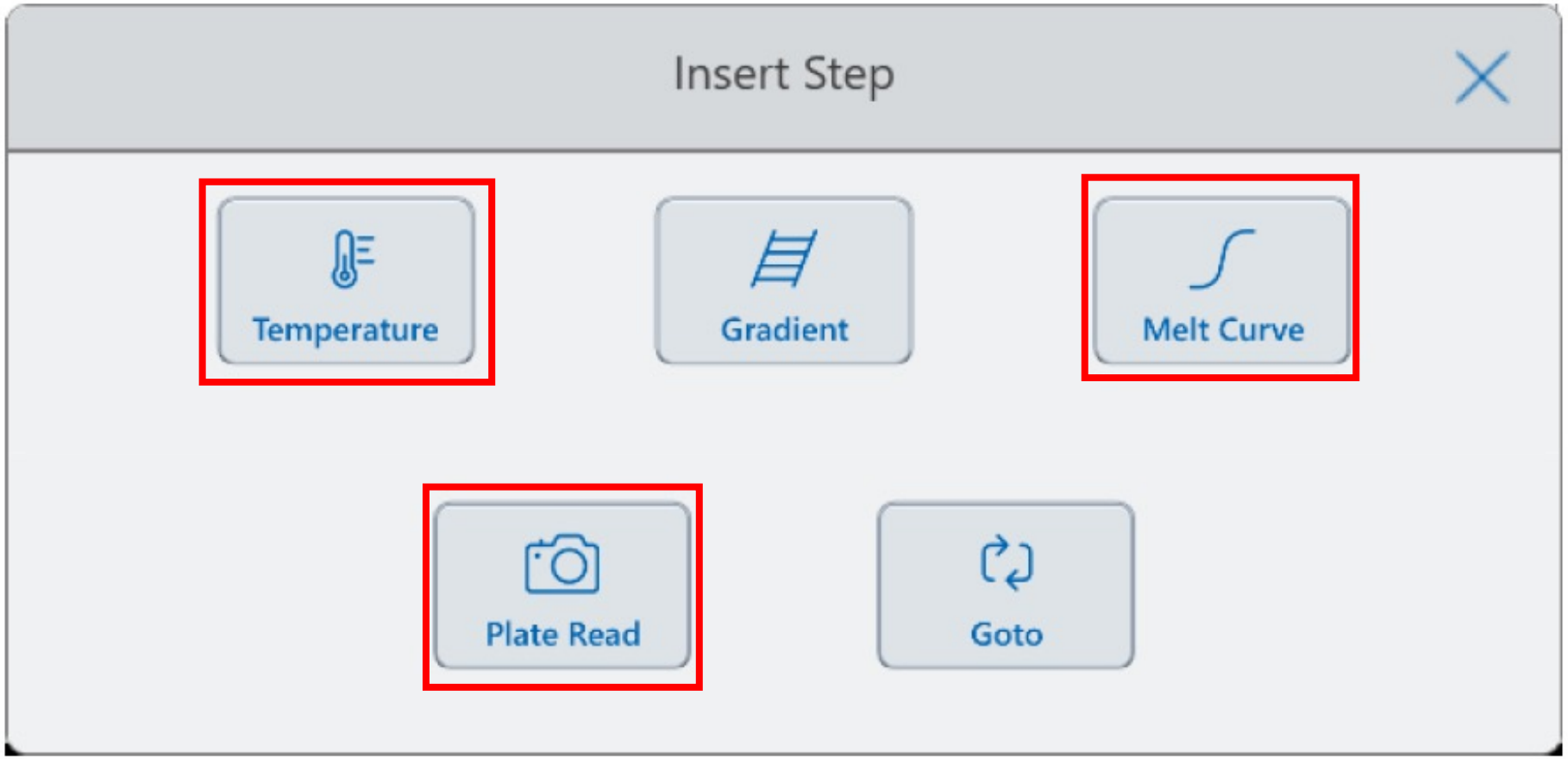
Appearance

Device Boot

Consumables

Software

Optics



Changing Target Gradient and Ramp Rate

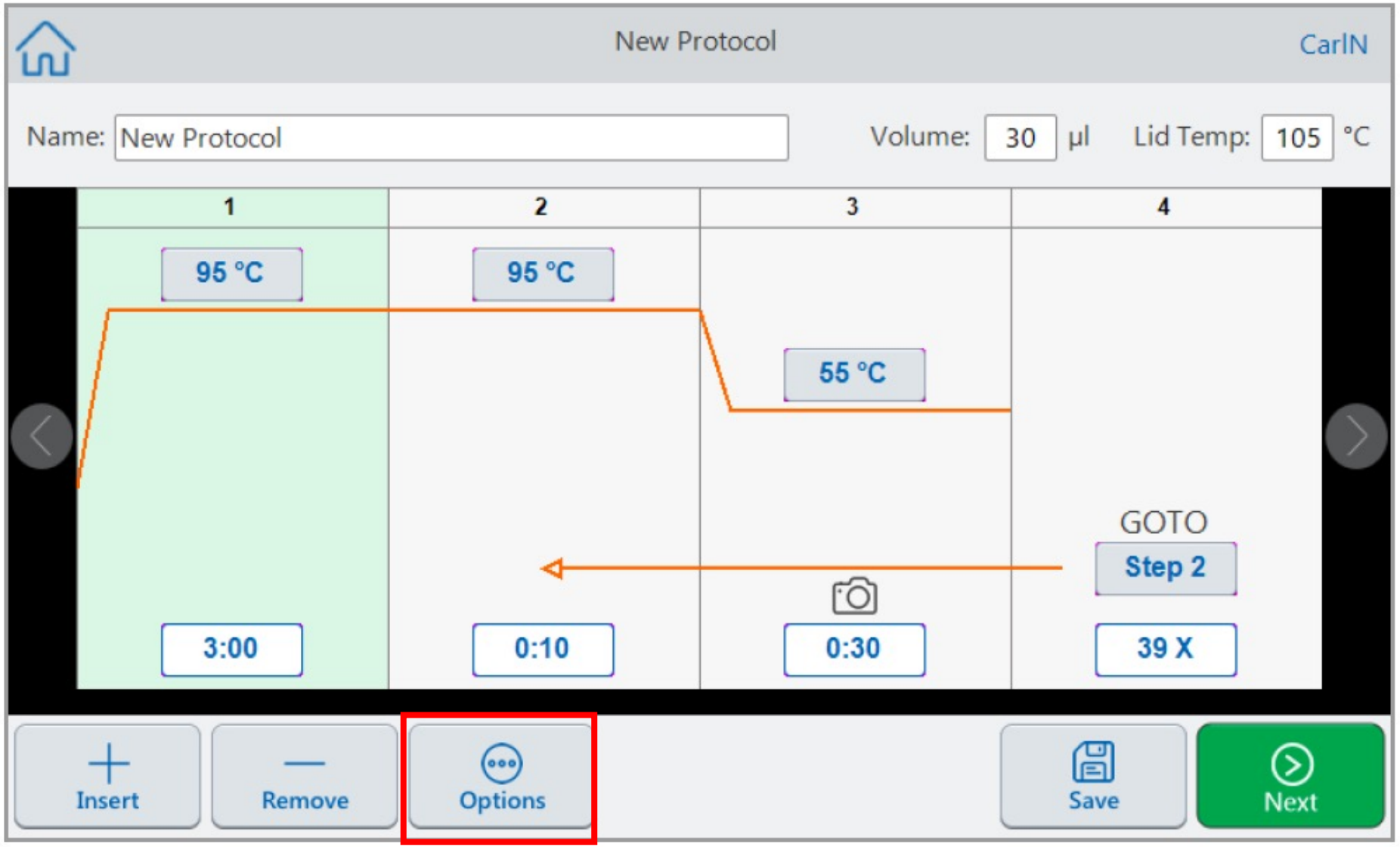
Appearance

Device Boot

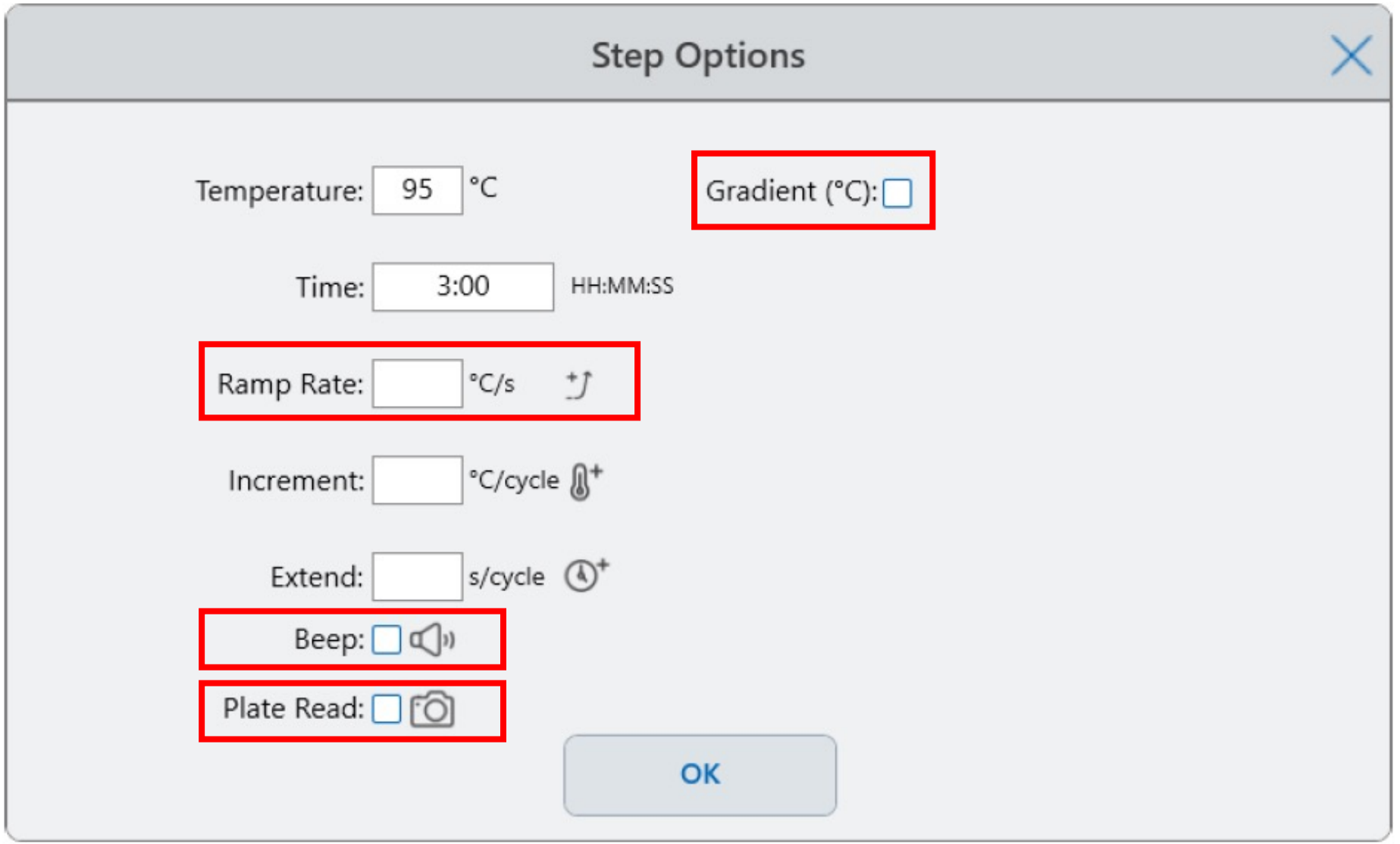
Consumables

Software

Optics



Changing Target Gradient and Ramp Rate



Adding or Removing a Temperature Gradient

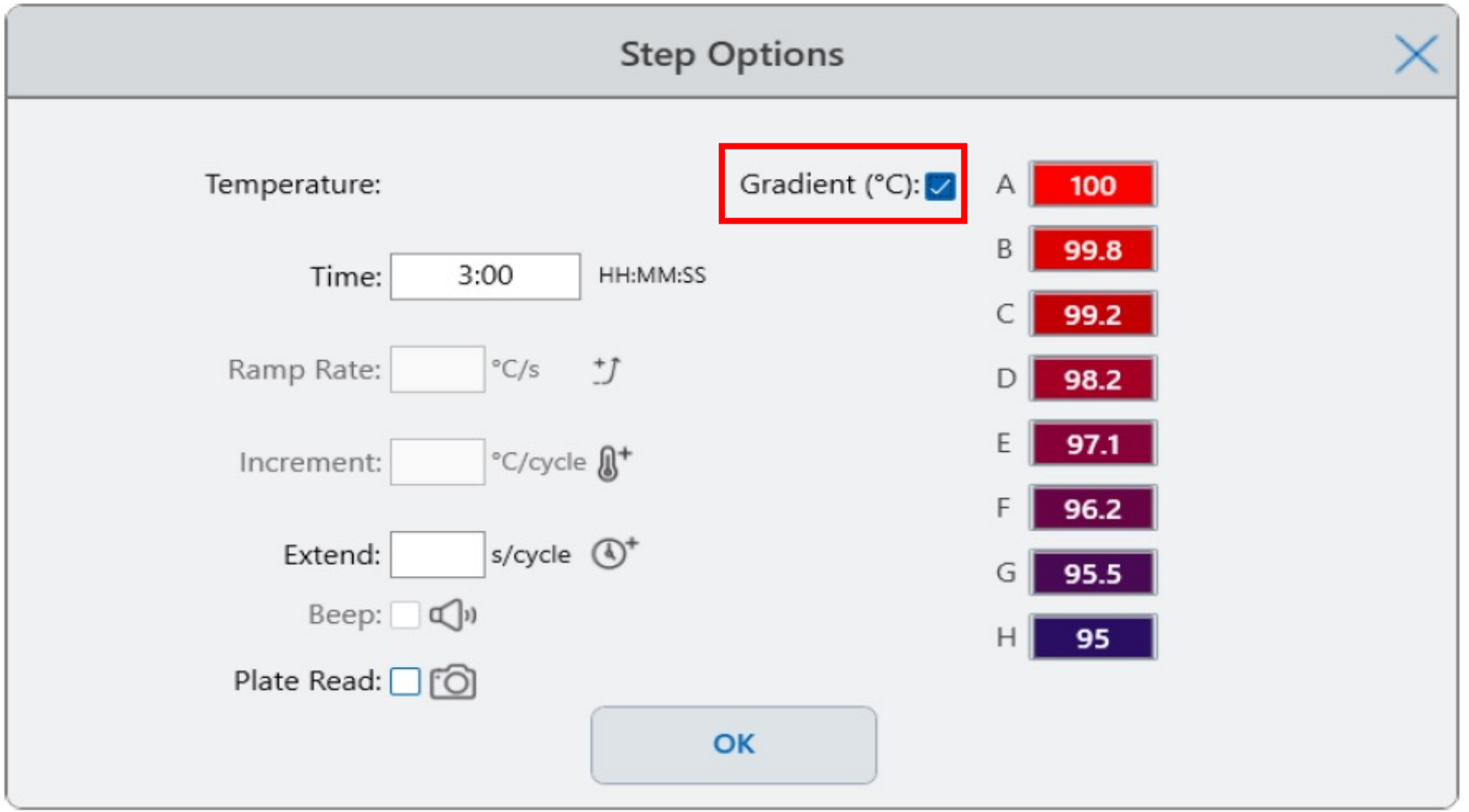
Appearance

Device Boot

Consumables

Software

Optics



Saving a Protocol

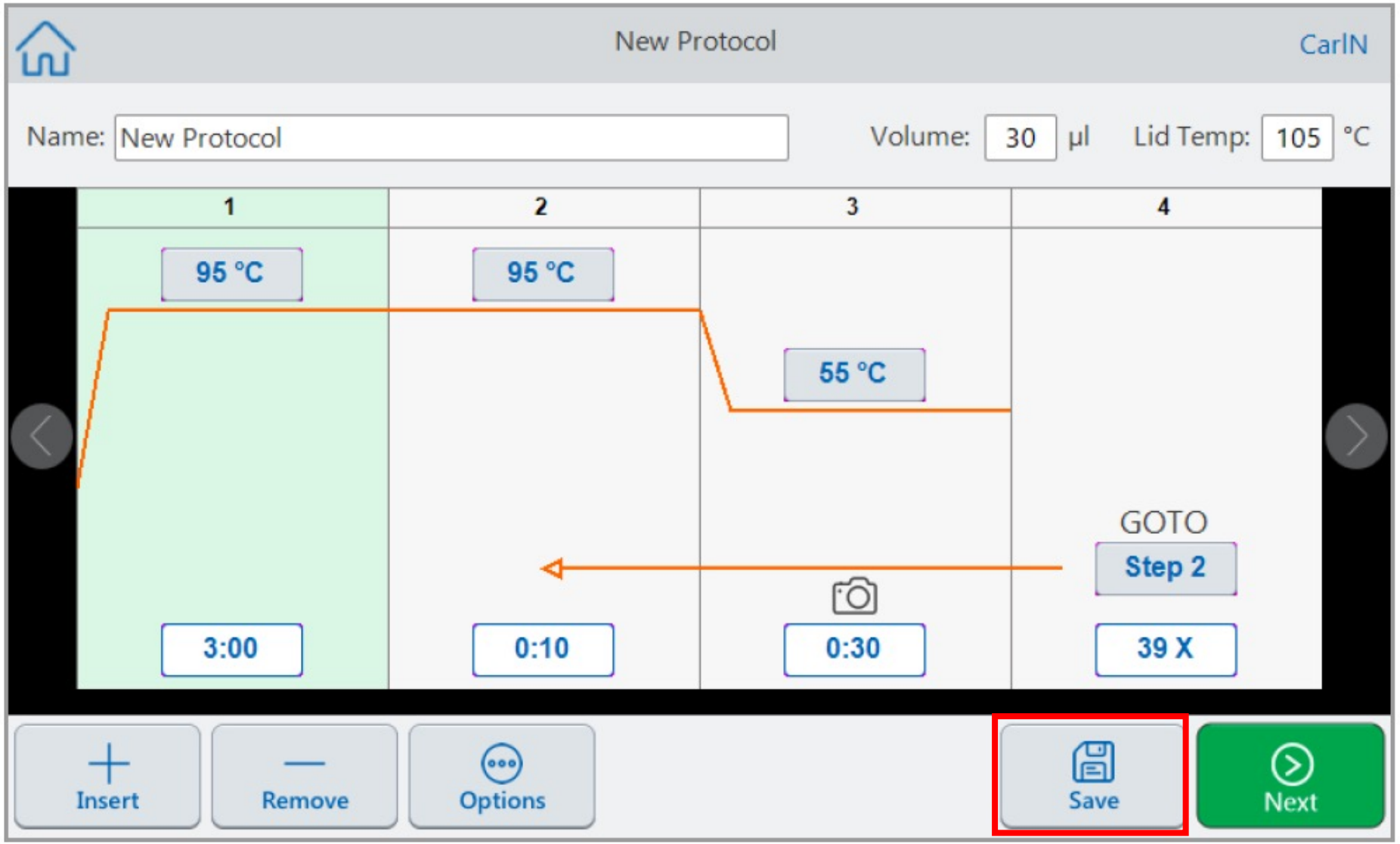
Appearance

Device Boot

Consumables

Software

Optics



Saving a Protocol

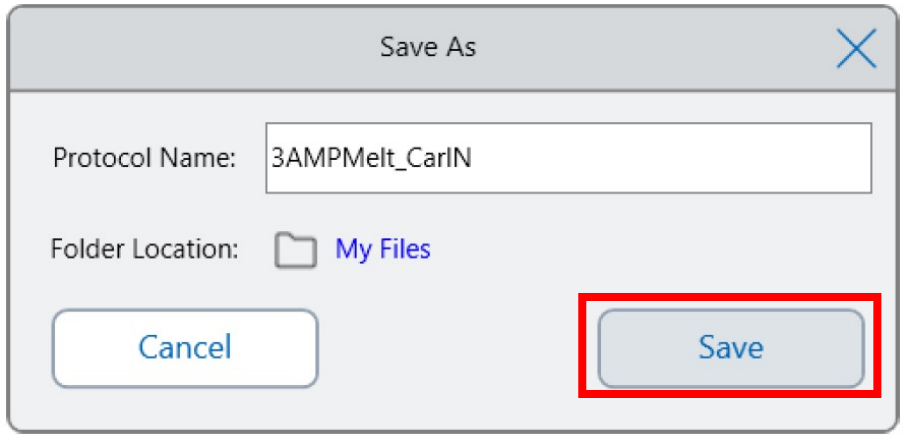
Appearance

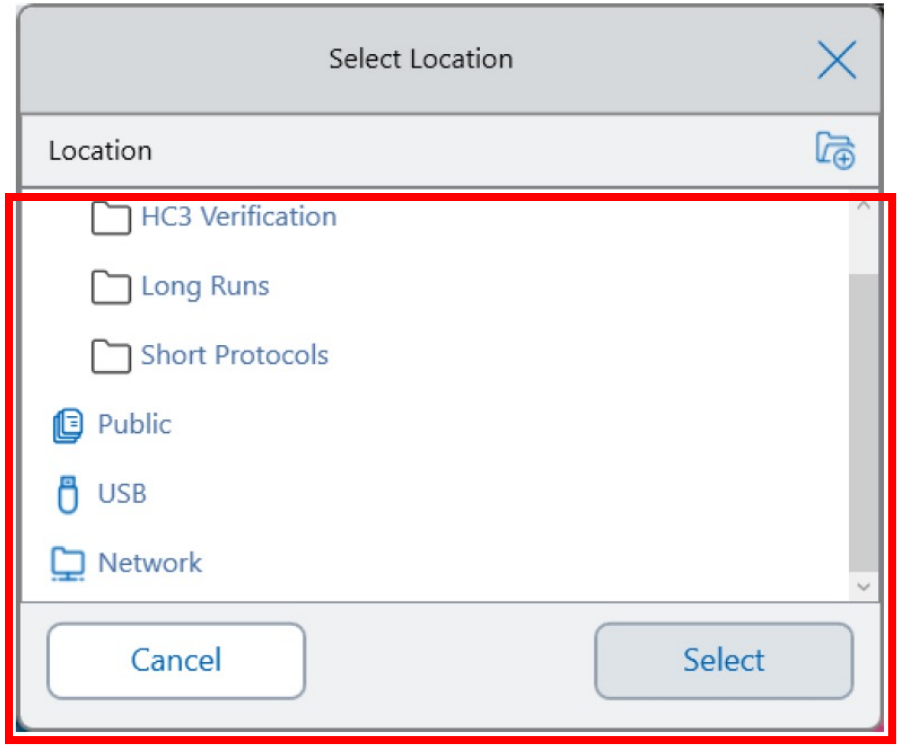
Device Boot

Consumables

Software

Optics





Run Setup

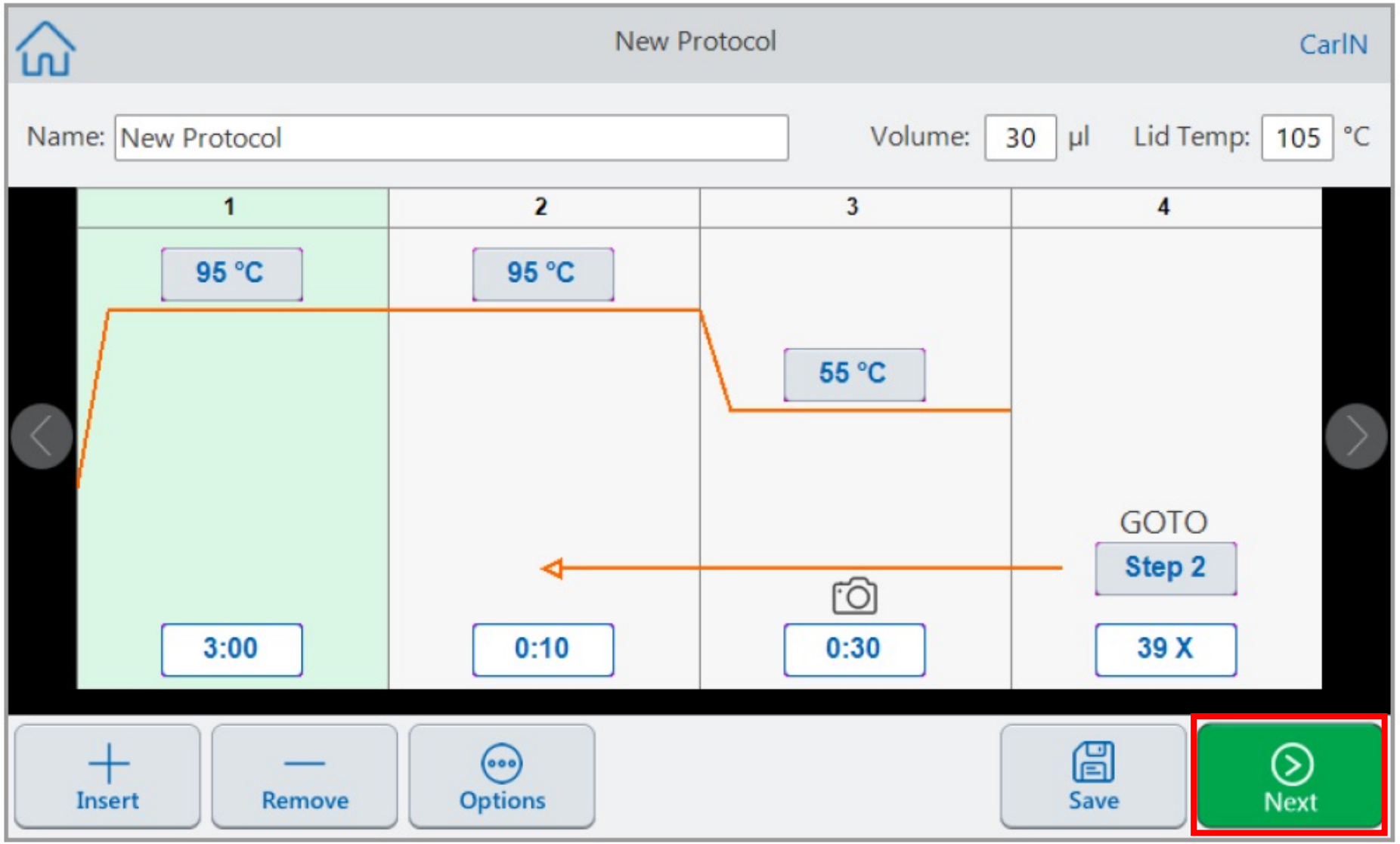
Appearance

Device Boot

Consumables

Software

Optics



Run Setup - Start Run

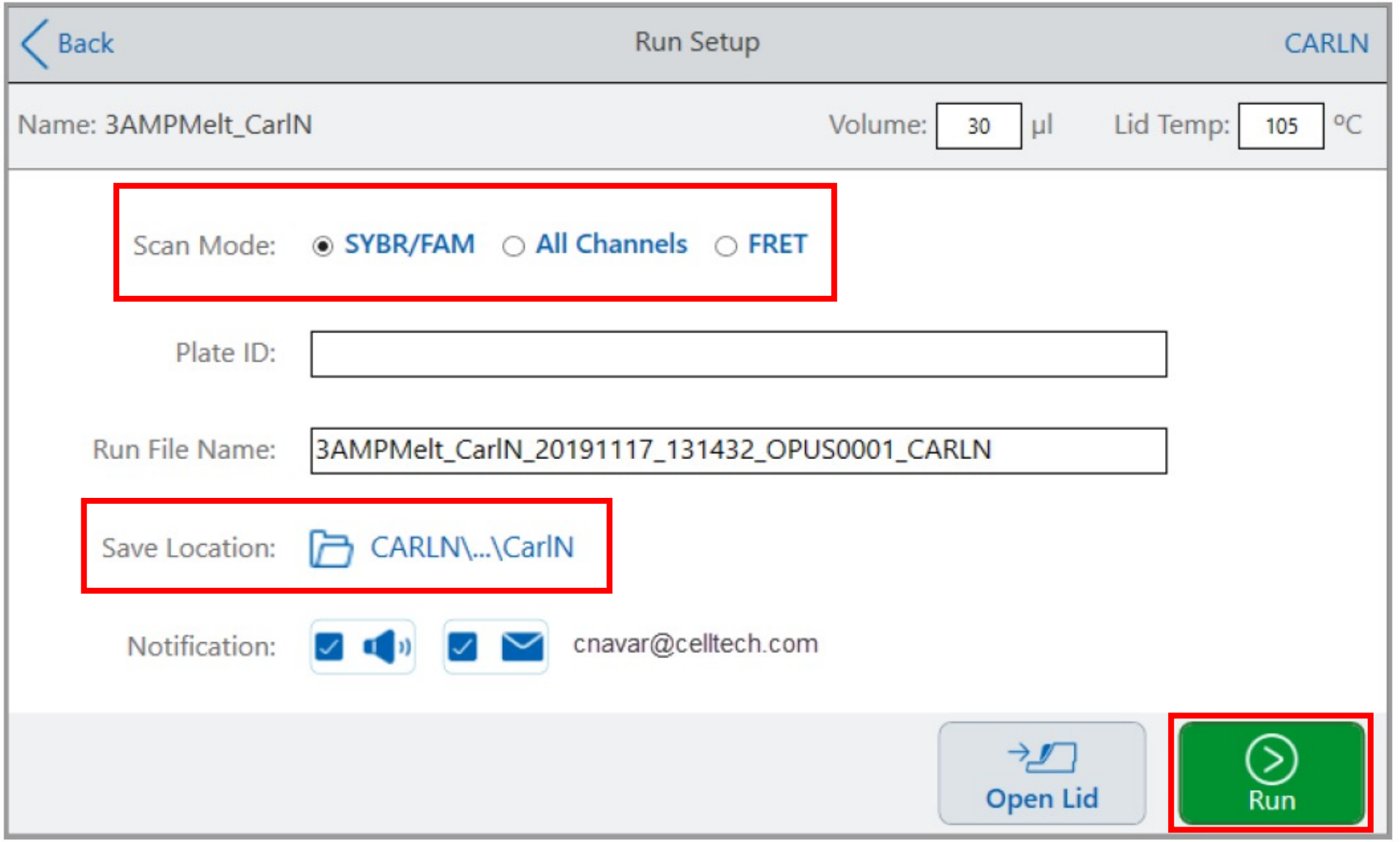
Appearance

Device Boot

Consumables

Software

Optics



Touch Screen Overview - Proceeding

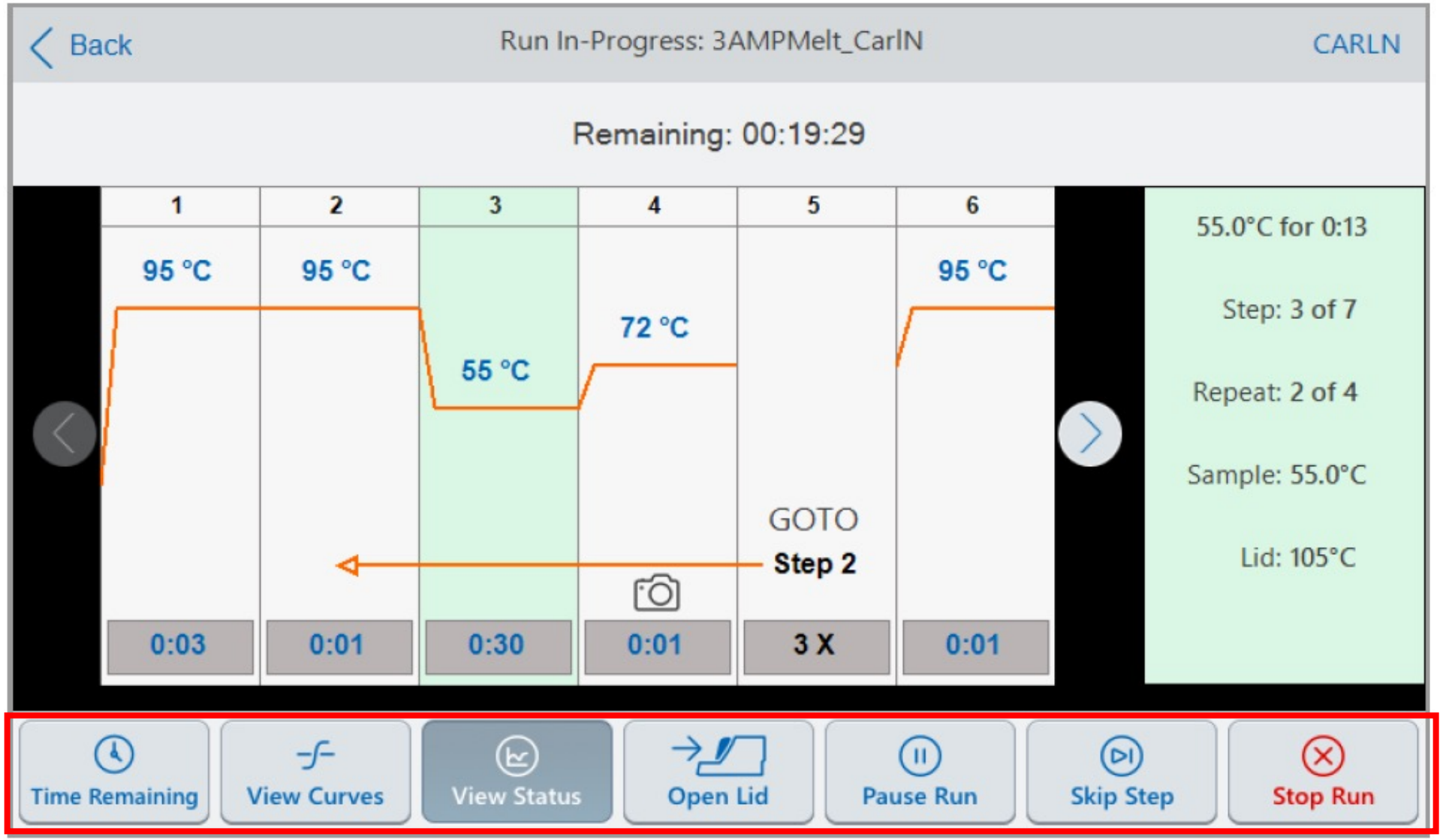
Appearance

Device Boot

Consumables

Software

Optics



Touch Screen Overview - File

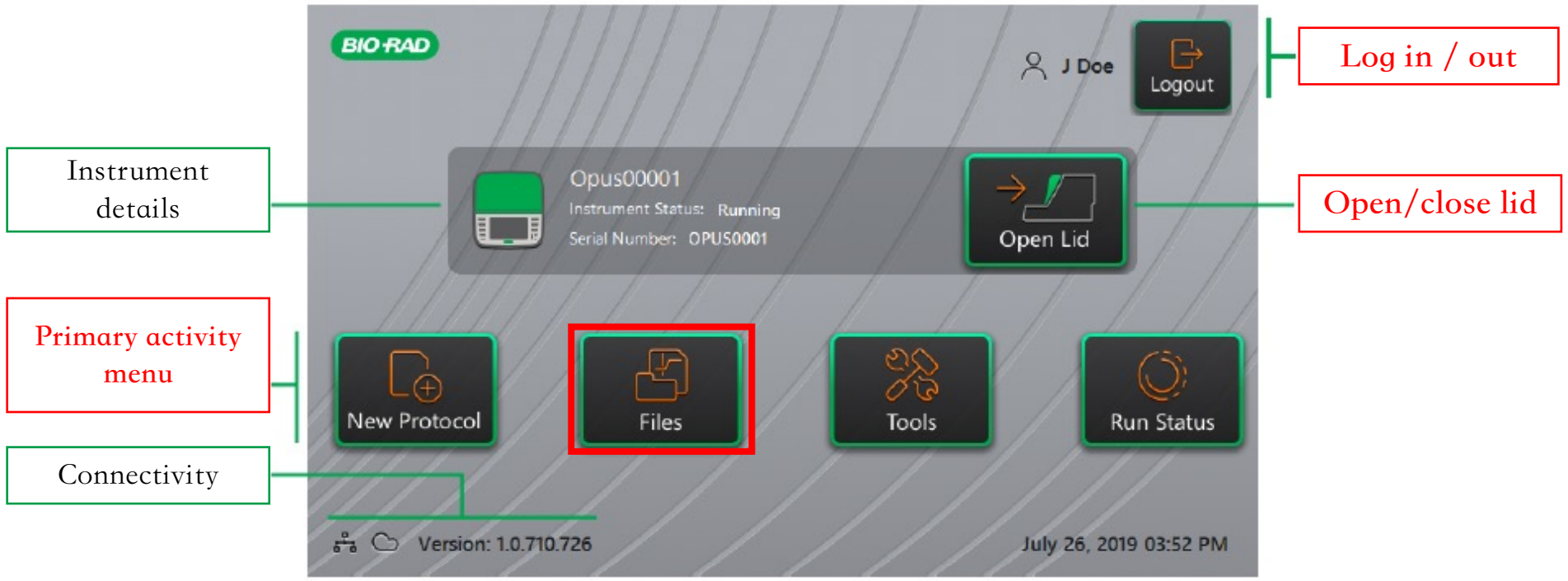
Appearance

Device Boot

Consumables

Software

Optics



The File Browser Screen

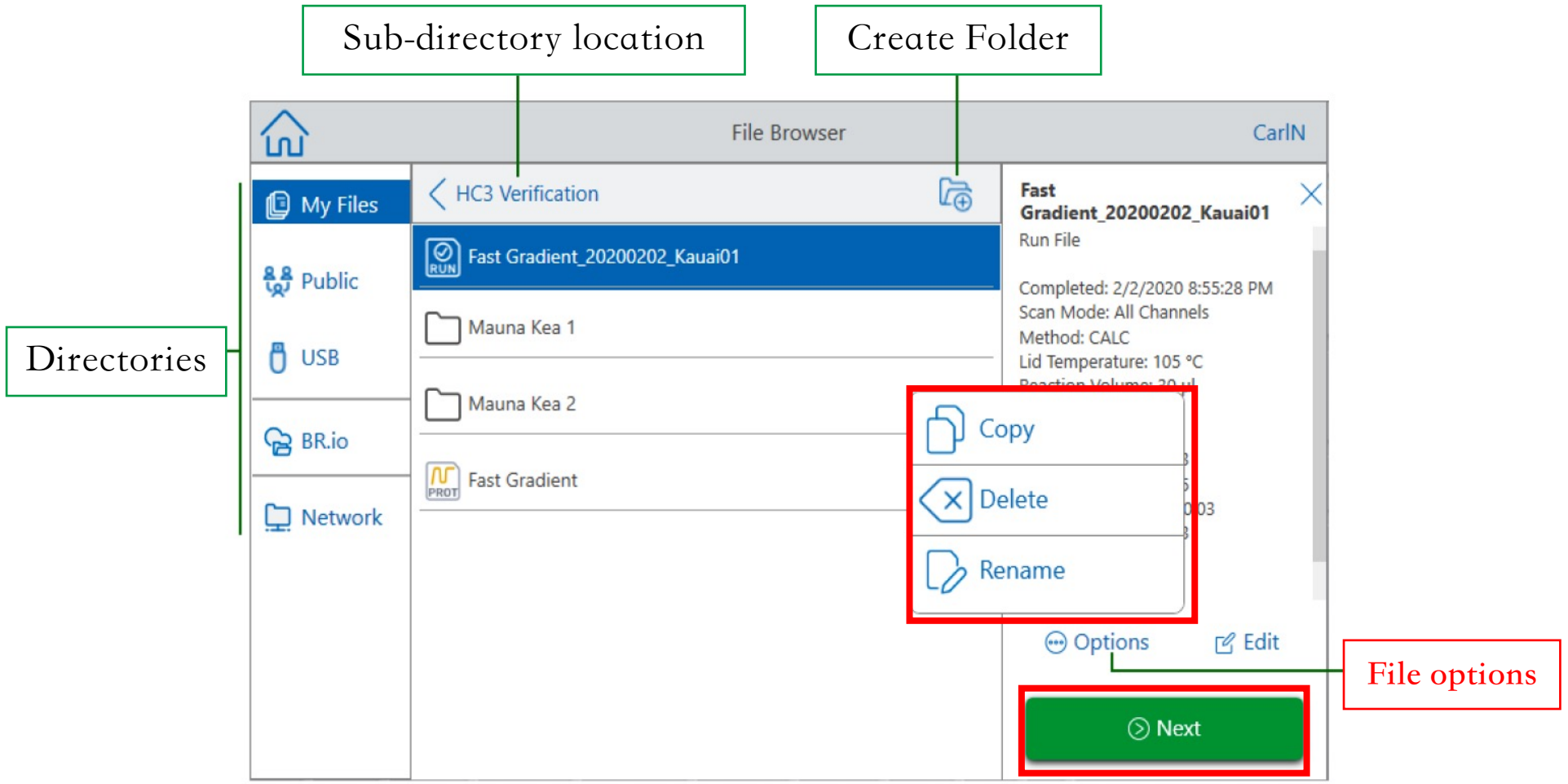
Appearance

Device Boot

Consumables

Software

Optics



Extracting and Editing a Protocol from a Run

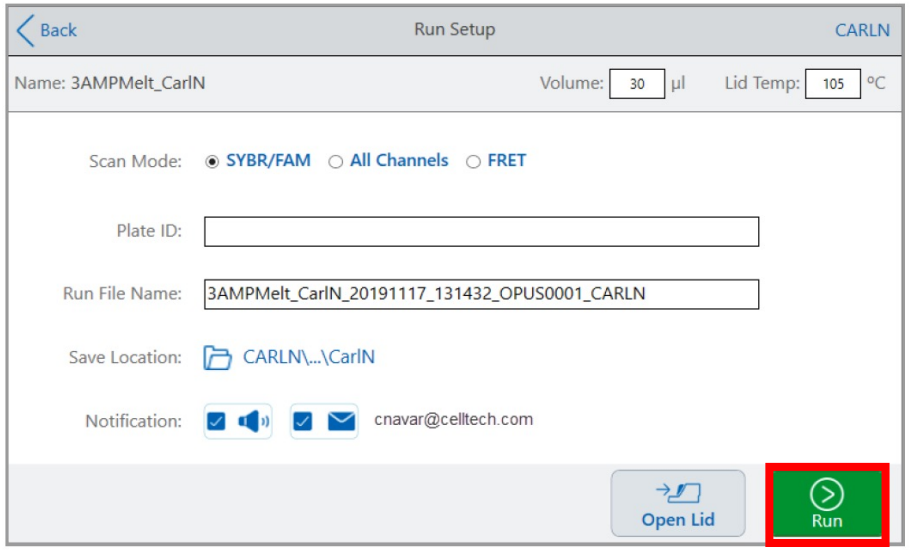
Appearance

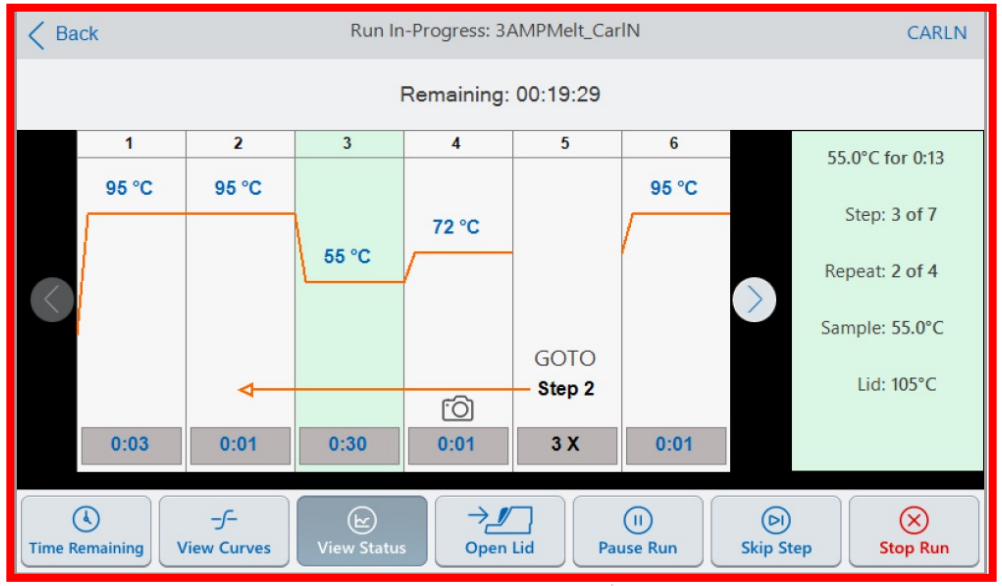
Device Boot

Consumables

Software

Optics





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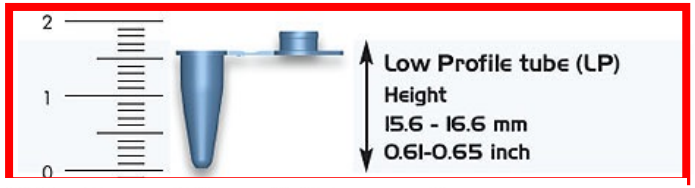
Genmall Biotechnology Co., Ltd.

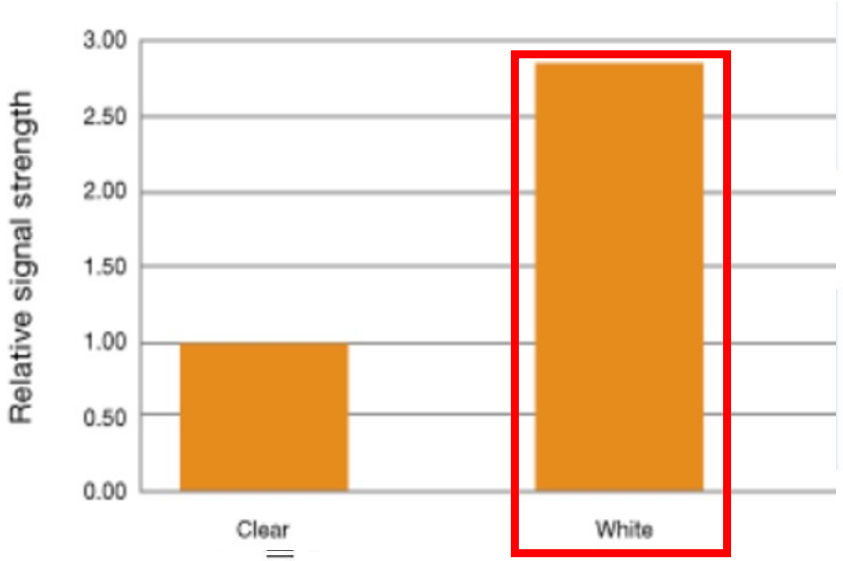
Consumables - Specification

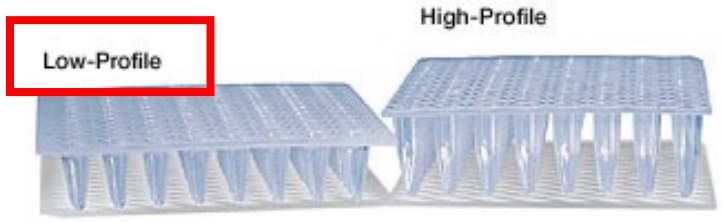
- Appearance
- Device Boot
- Consumables
- Software
- Optics

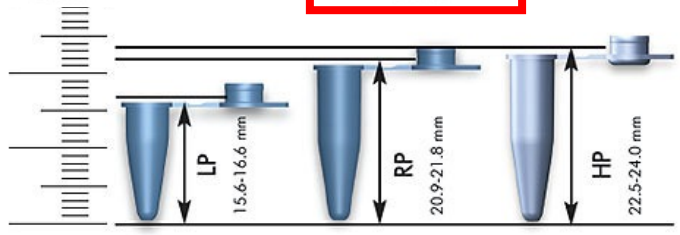
Low – Profile Only !!



Well Color Effect on Signal Strength

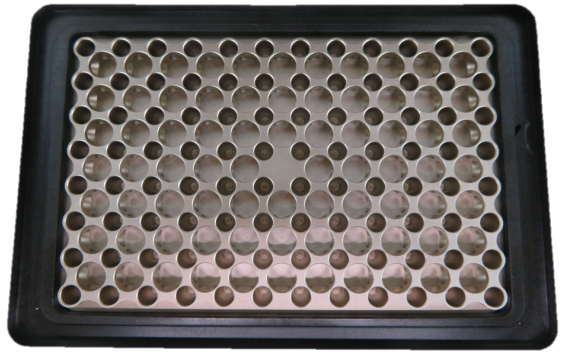


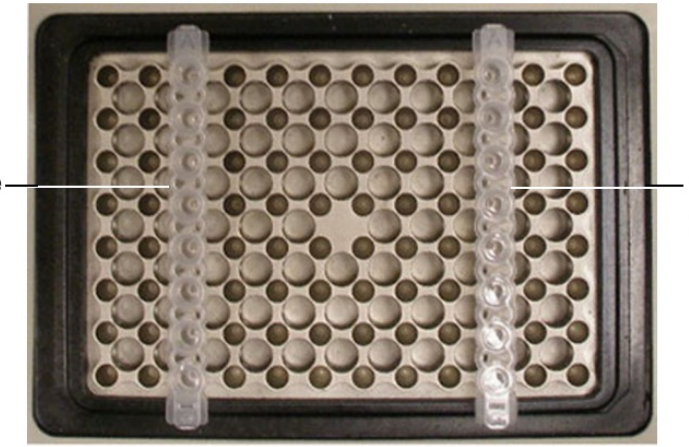




Notice for usage

- Appearance
- Device Boot
- Consumables
- Software
- Optics





Connection with Opus 96 - Overview

Appearance

Device Boot

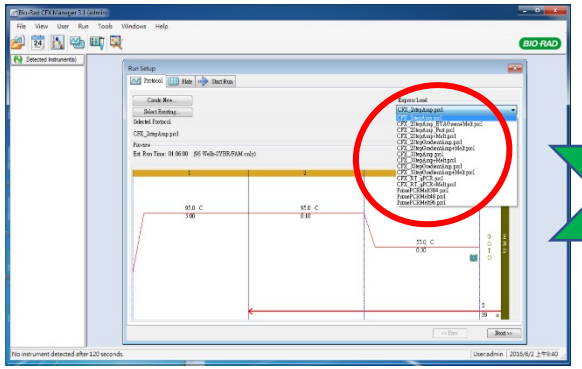
Consumables

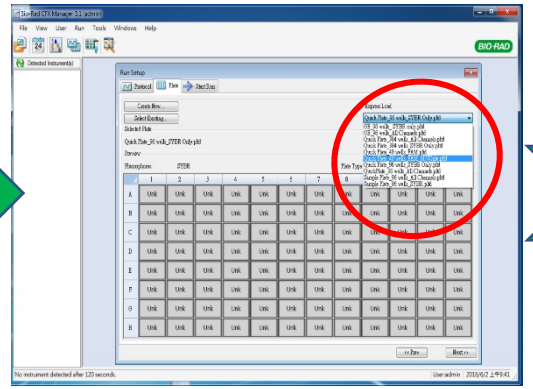
Software

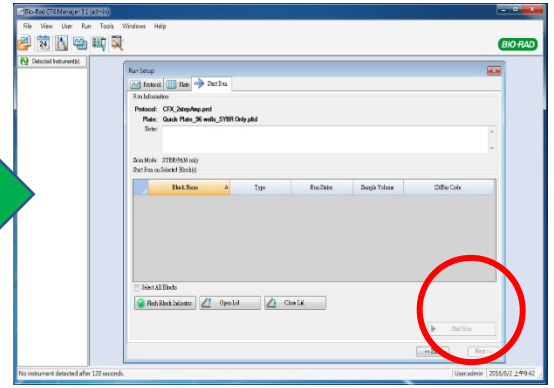
Optics

BIO RAD CFX Maestro

Protocol → **Plate** → **Start Run**







Start Run !

正茂生物科技股份有限公司
Genmall Biotechnology Co., Ltd.

Operation setting - Overview

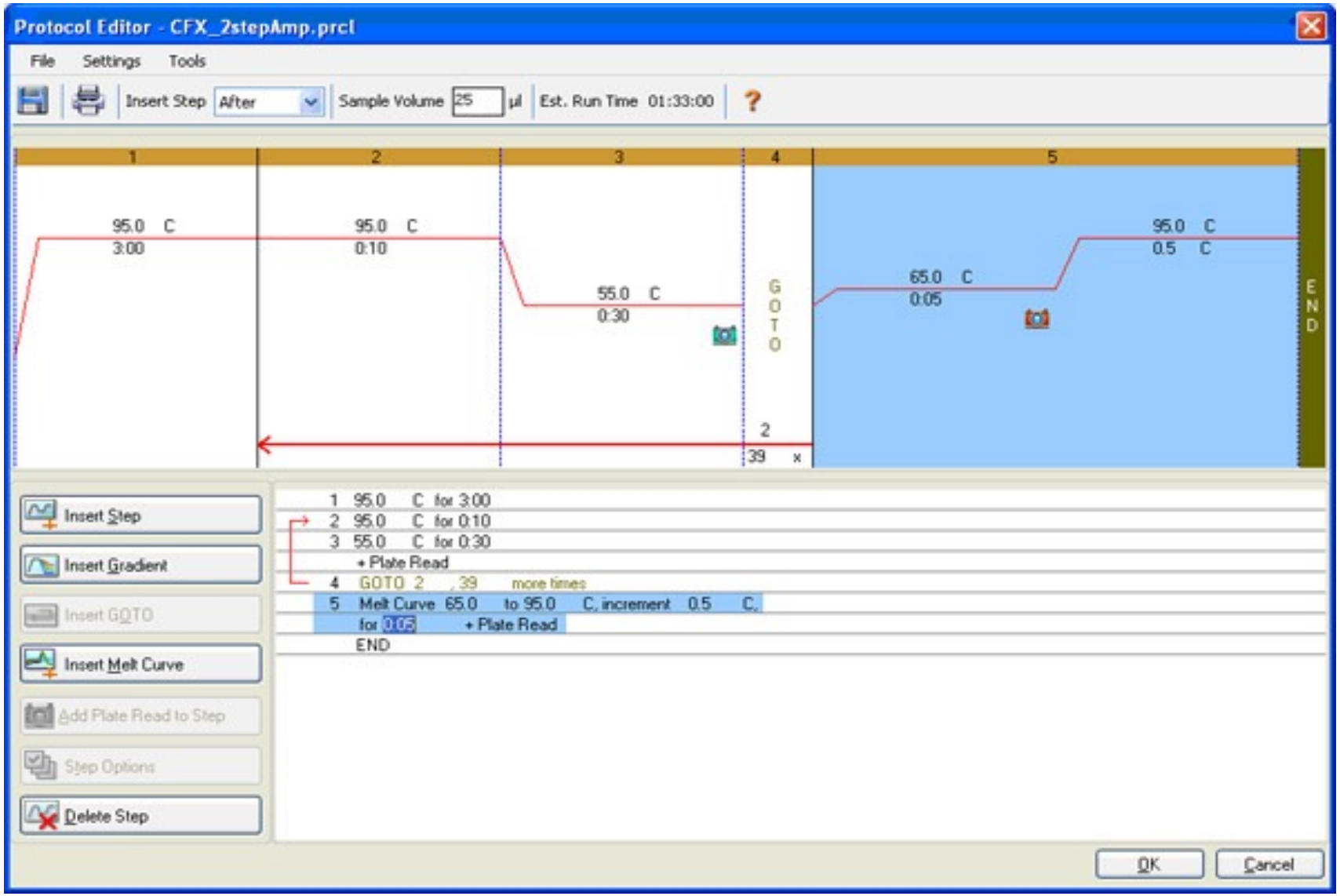
Appearance

Device Boot

Consumables

Software

Optics



正茂生物科技股份有限公司
Genmall Biotechnology Co., Ltd.

Optical design for detection

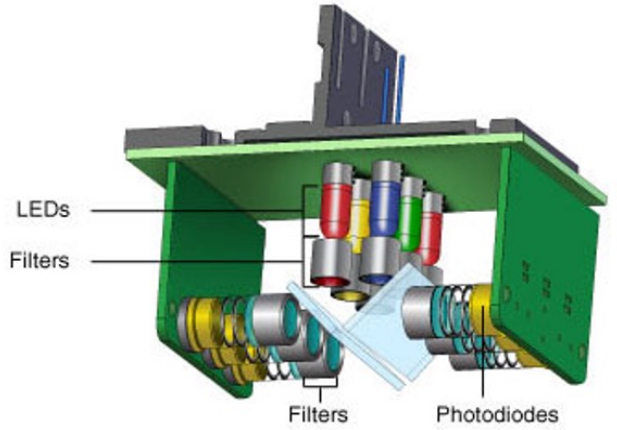
Appearance

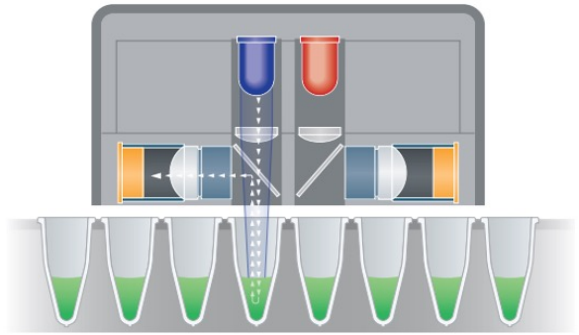
Device Boot

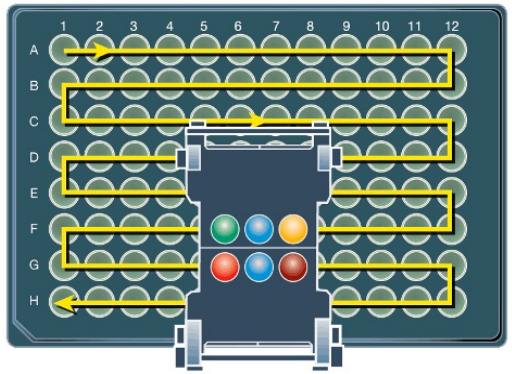
Consumables

Software

Optics

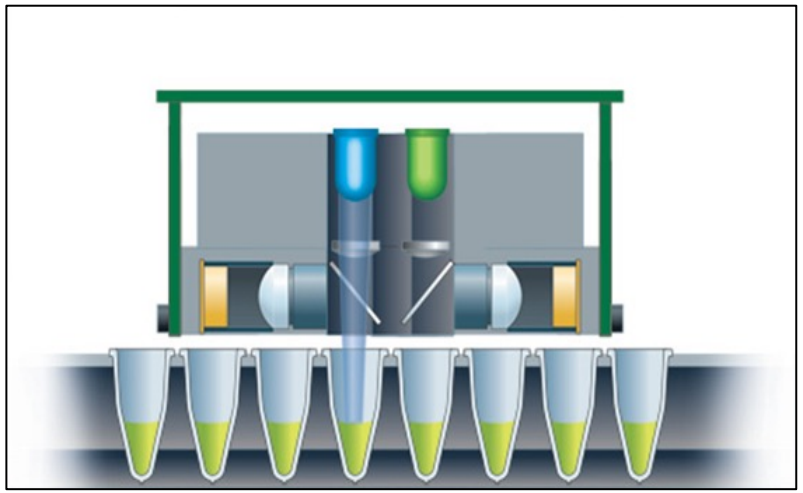


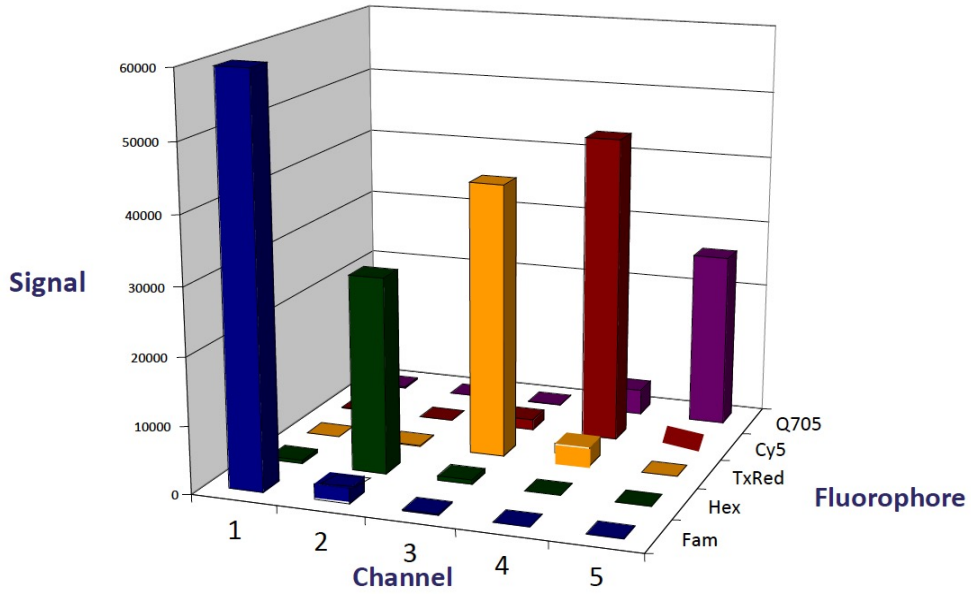


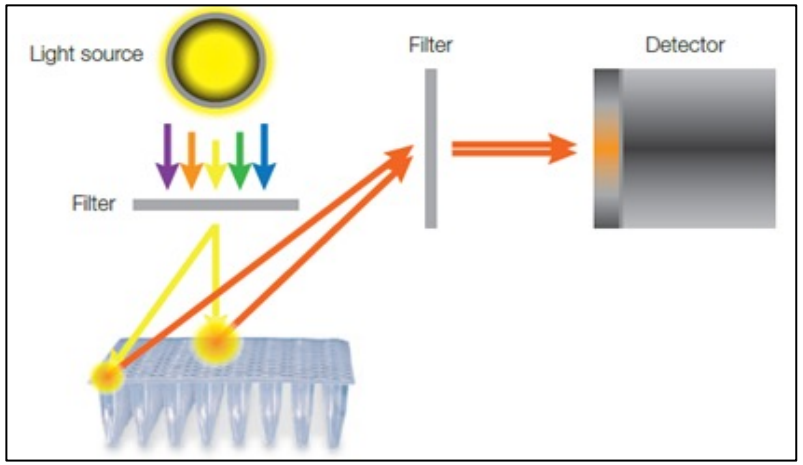


Optical design for detection

- Appearance
- Device Boot
- Consumables
- Software
- **Optics**







Competitor

Vertical light path ： 「Rox dye」 calibration is unnecessary

Fluorescence : 「high strength / specificity 」

Light source : LEDs with long lifespan, allowing you to use them right away

Optical application - Dye / Wave band

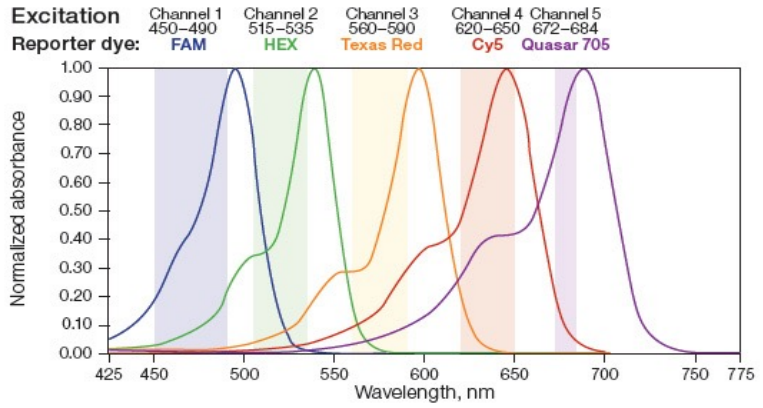
Appearance

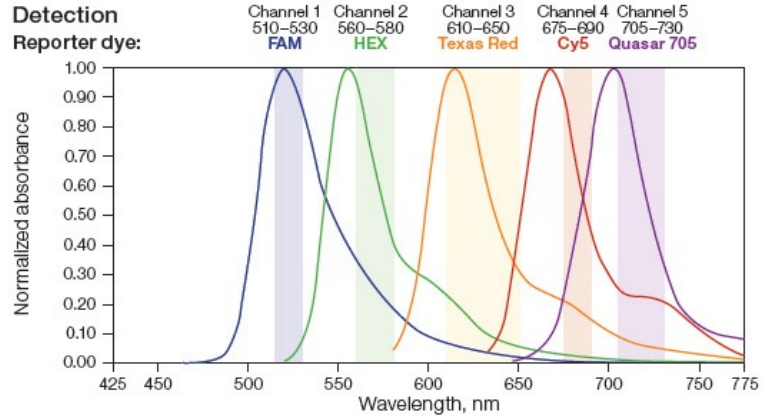
Device Boot

Consumables

Software

Optics





Channel	Excitation (nm)	Detection (nm)	Calibrated Fluorophores
1	450-490	515-530	FAM™, SYBR Greem I™, EvaGreen™
2	515-535	560-580	VIC®, HEX™, TET™, Cal Gold 540™
3	560-590	610-650	ROX™, Texas Red®, Cal Red 610™
4	620-650	675-690	Cy5, Quasar 670™
5	672-684	705-730	Quasar 705™
6	450-490	560-580	Accommodates FRET Chemistry

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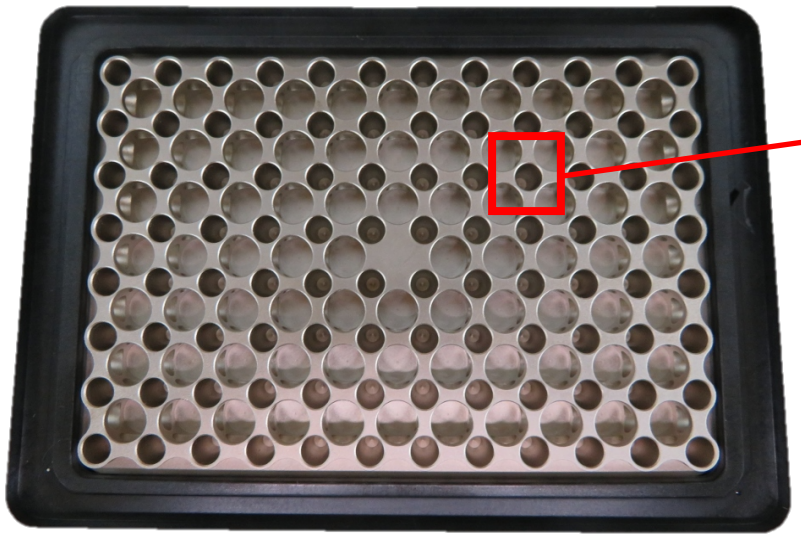
Supplement

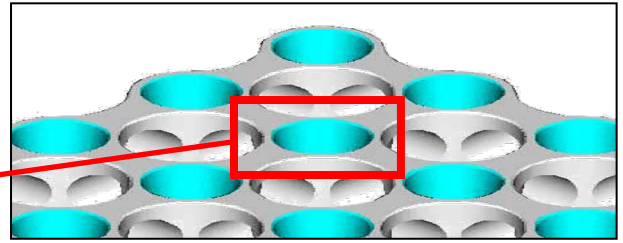
Blocks

Gradient

Application



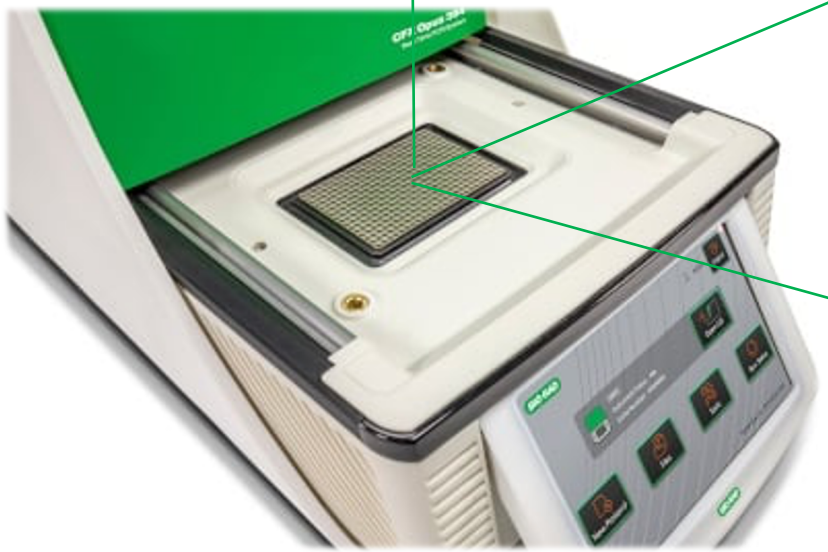
Mass-reduced sample block



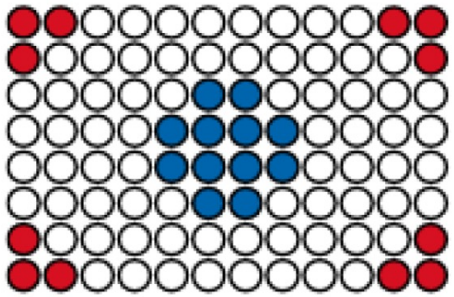
Uniform / Rapid heating

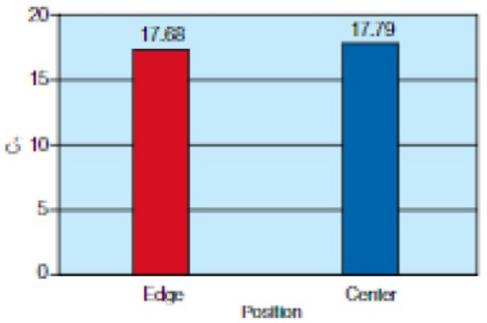
Max ramp rate	5°C / sec
Temp Accuracy	± 0.2°C
Temp Uniformity	± 0.3°C

Accurate temperature control



Uniformity ↑



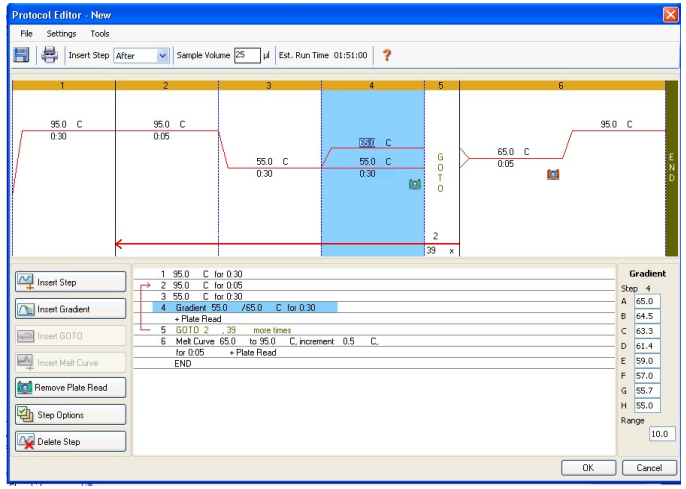


Supreme「 uniformity 」in any position

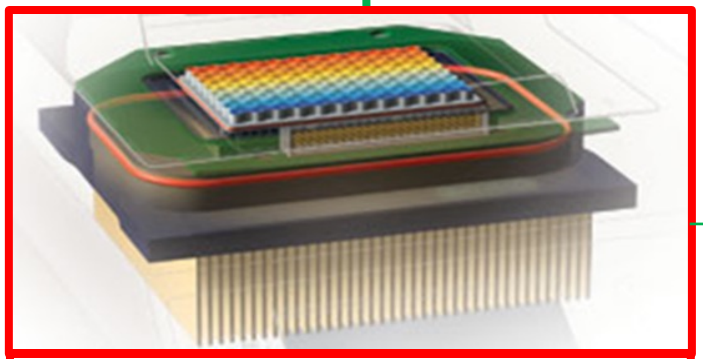
Blocks

Gradient

Application



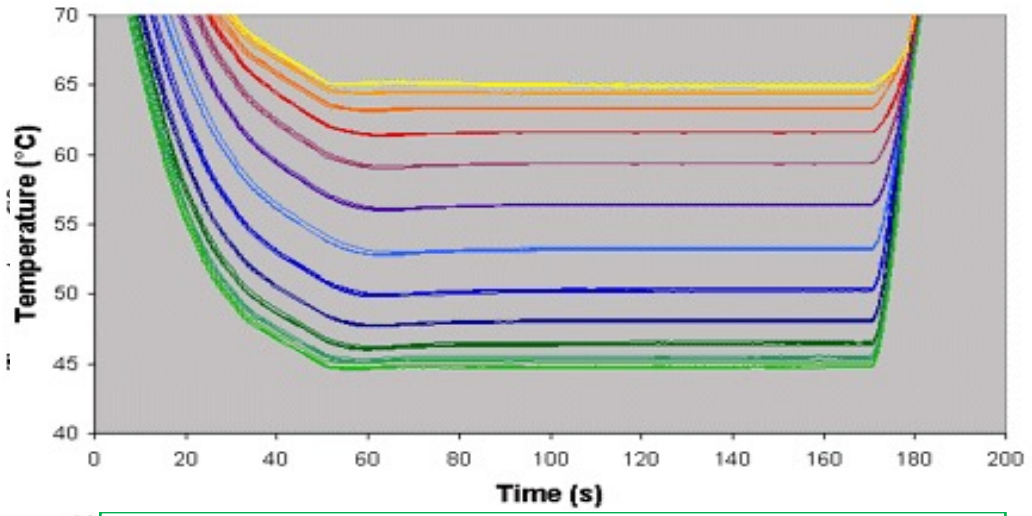
Graphical interface : friendly operation and setting



Peltier Thermal System

		1	2	3	4	5	6	7	8	9	10	11	12
A	70.0	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk
B	69.5	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk
C	68.4	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk
D	66.4	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk
E	64.0	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk
F	62.0	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk
G	60.7	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk
H	60.0	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk

Gradient temperature setting : 「1 - 24°C 」



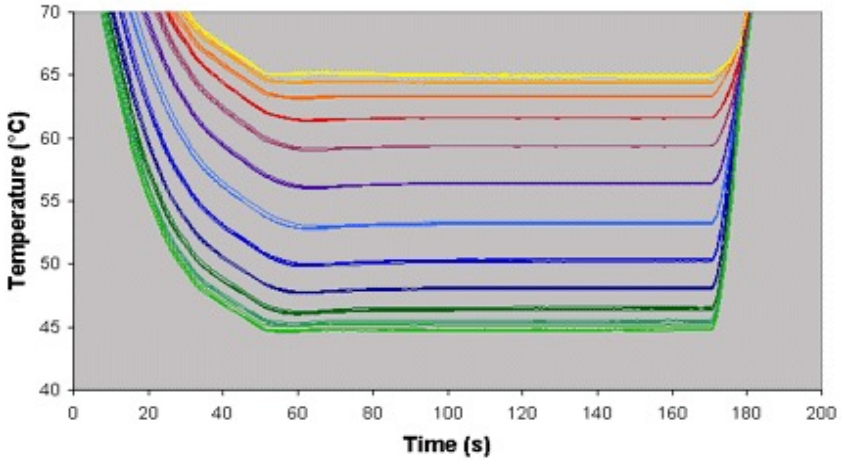
Same starting point / Reaction is accurate

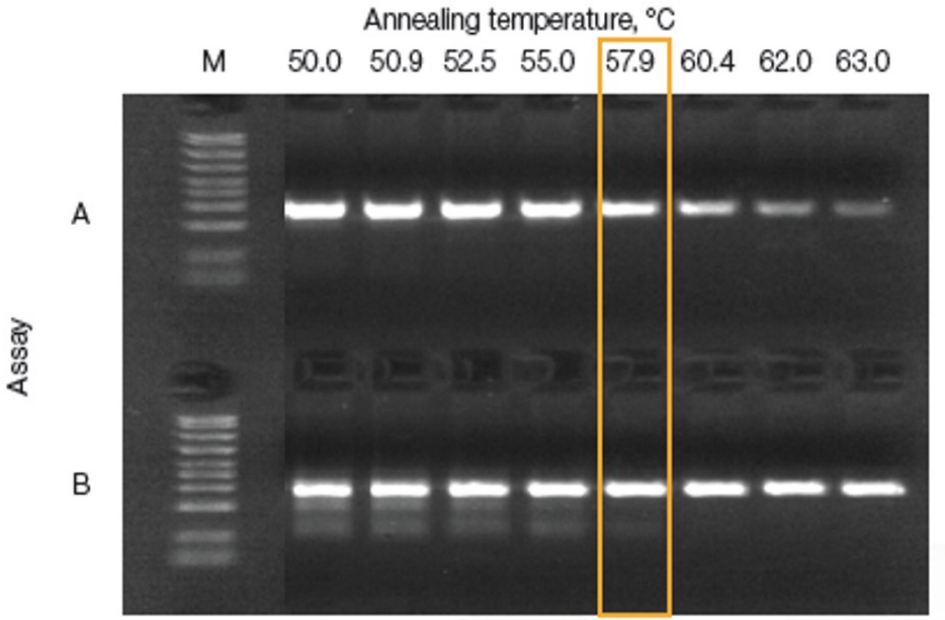
30s

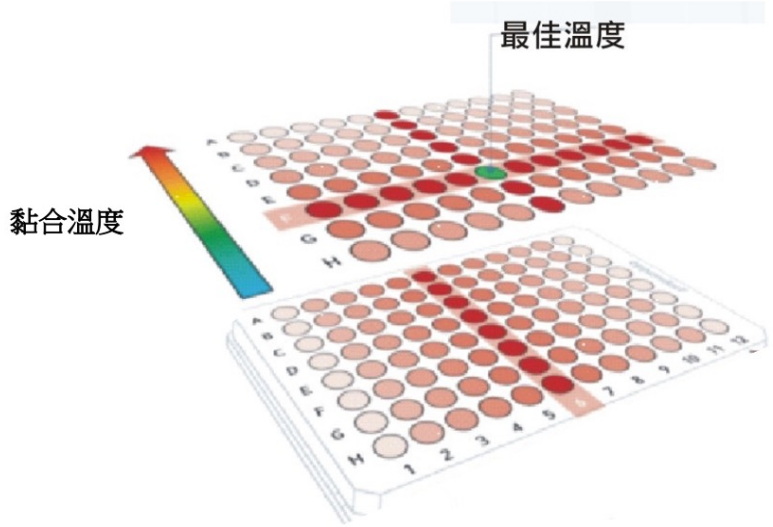
Blocks

Gradient

Application







Optimization of an assay results in better yields and specificity. Results show that assays A and B can be run at an annealing temperature of 57.9°C on the same plate. Higher temperatures result in a reduced yield in assay A while lower temperatures result in nonspecific products in assay B. M, markers.

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Thank You!