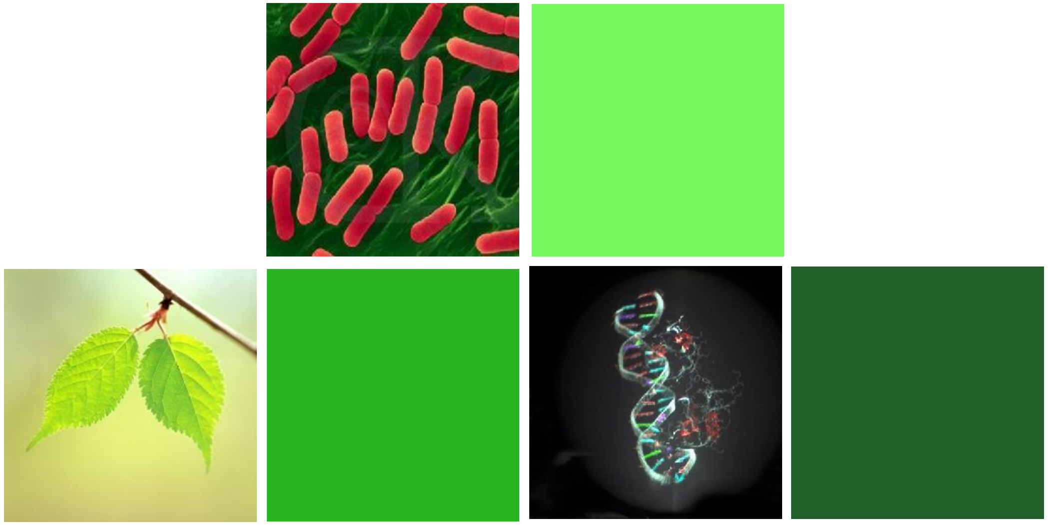


MIQE - 实时荧光定量PCR实验流程 及数据处理的国际化标准

定量PCR存在问题

实验前：

如何合理设计实验？

选择什么样的试剂耗材，仪器？

预实验怎么开展，实验条件如何优化？

实验后：

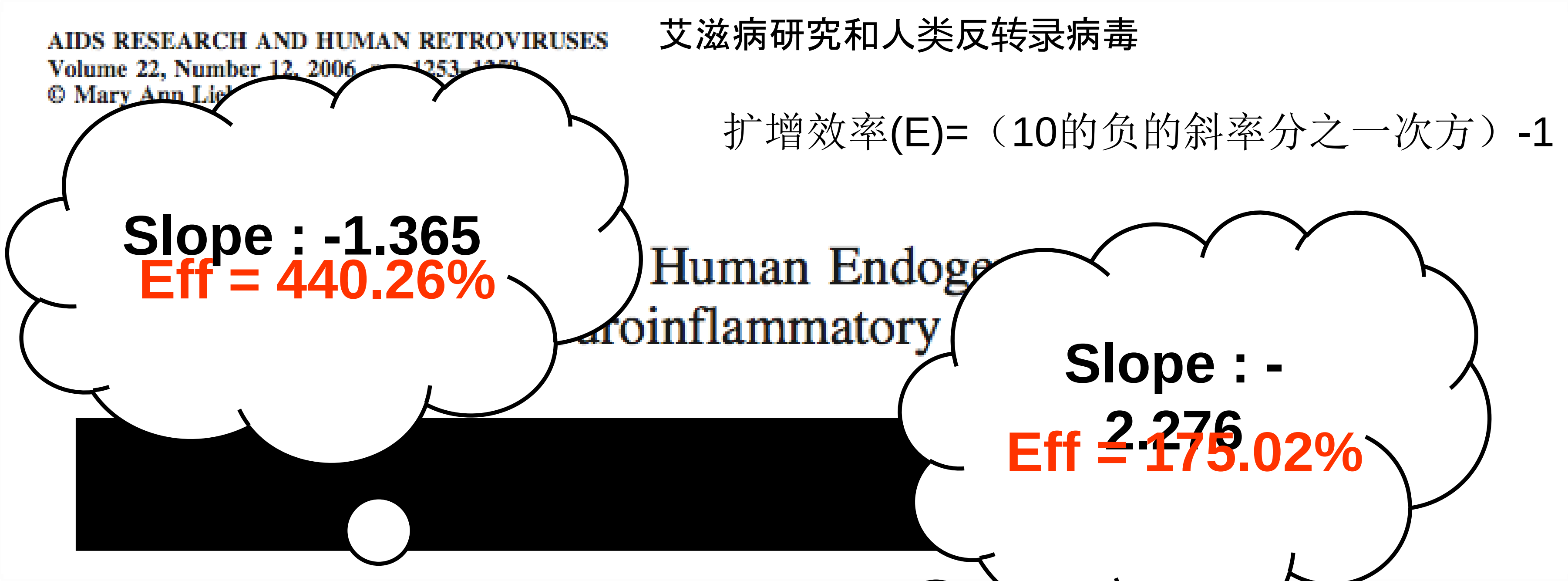
实验数据如何分析，结果如何描述？

投稿时提供哪些实验信息？

已有的实验结果是否能够很好的重复？

参考文献中是否存在一些不可靠或前后矛盾的数据？

Questionable Data 问题数据



AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 22, Number 12, 2006 pp. 1253–1259
© Mary Ann Lie

Human Endoge
roinflammatory

艾滋病研究和人类反转录病毒

扩增效率(E)=（10的负的斜率分之一次方）-1

Slope : -1.365
Eff = 440.26%

Slope : -2.276
Eff = 175.02%

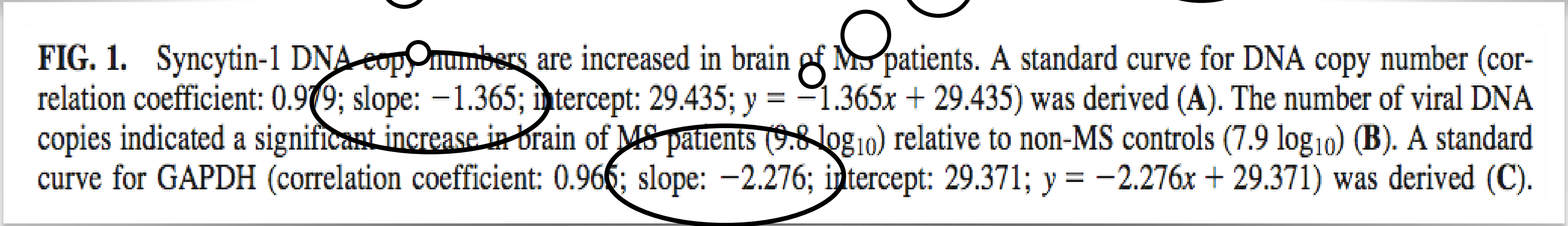
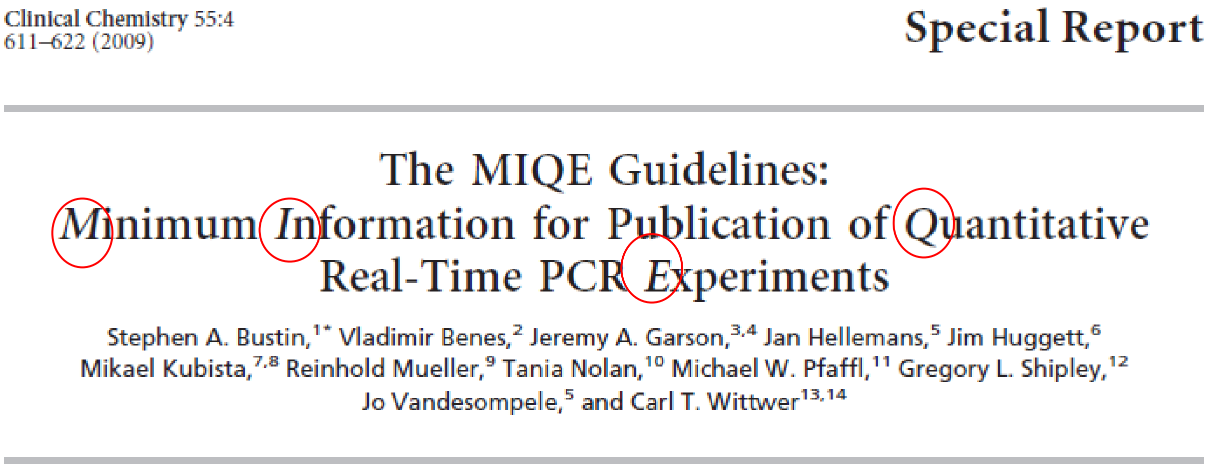


FIG. 1. Syncytin-1 DNA copy numbers are increased in brain of MS patients. A standard curve for DNA copy number (correlation coefficient: 0.979; slope: −1.365; intercept: 29.435; $y = -1.365x + 29.435$) was derived (**A**). The number of viral DNA copies indicated a significant increase in brain of MS patients (9.8 $\log_{10}$) relative to non-MS controls (7.9 $\log_{10}$) (**B**). A standard curve for GAPDH (correlation coefficient: 0.966; slope: −2.276; intercept: 29.371; $y = -2.276x + 29.371$) was derived (**C**).

MIQE—qPCR的国际标准

qPCR的国际标准：就评价qPCR实验和发表文章时所必需的实验信息提出了最低限度的标准。



Clinical Chemistry 55:4
611–622 (2009)

Special Report

The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

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]

MIQE has been frequently cited by other researchers: => 5000 times

http://Scholar.Google.com/

What is MIQE?
It’s a Checklist (检查清单) !

Table 1. MIQE checklist for authors, reviewers, and editors.[a]

Item to check	Importance	Item to check	Importance
Experimental design		qPCR oligonucleotides	
Definition of experimental and control groups	E	Primer sequences	E
Number within each group	E	RTPrimerDB identification number	D
Assay carried out by the core or investigator's laboratory?	D	Probe sequences	D[d]
Acknowledgment of authors' contributions	D	Location and identity of any modifications	E
Sample		Manufacturer of oligonucleotides	D
Description	E	Purification method	D
Volume/mass of sample processed	D	qPCR protocol	
Microdissection or macrodissection	E	Complete reaction conditions	E
Processing procedure	E	Reaction volume and amount of cDNA/DNA	E
If frozen, how and how quickly?	E	Primer, (probe), Mg^{2+}, and dNTP concentrations	E
If fixed, with what and how quickly?	E	Polymerase identity and concentration	E
Sample storage conditions and duration (especially for FFPE[b] samples)	E	Buffer/kit identity and manufacturer	E
Nucleic acid extraction		Exact chemical composition of the buffer	D
Procedure and/or instrumentation	E	Additives (SYBR Green I, DMSO, and so forth)	E
Name of kit and details of any modifications	E	Manufacturer of plates/tubes and catalog number	D
Source of additional reagents used	D	Complete thermocycling parameters	E
Details of DNase or RNase treatment	E	Reaction setup (manual/robotic)	D
Contamination assessment (DNA or RNA)	E	Manufacturer of qPCR instrument	E
Nucleic acid quantification	E	qPCR validation	
Instrument and method	E	Evidence of optimization (from gradients)	D
Purity (A_{260}/A_{280})	D	Specificity (gel, sequence, melt, or digest)	E
Yield	D	For SYBR Green I, C_q of the NTC	E
RNA integrity: method/instrument	E	Calibration curves with slope and *y* intercept	E
RIN/RQI or C_q of 3′ and 5′ transcripts	E	PCR efficiency calculated from slope	E
Electrophoresis traces	D	CIs for PCR efficiency or SE	D
Inhibition testing (C_q dilutions, spike, or other)	E	r^2 of calibration curve	E
Reverse transcription		Linear dynamic range	E
Complete reaction conditions	E	C_q variation at LOD	E
Amount of RNA and reaction volume	E	CIs throughout range	D
Priming oligonucleotide (if using GSP) and concentration	E	Evidence for LOD	E
Reverse transcriptase and concentration	E	If multiplex, efficiency and LOD of each assay	E
Temperature and time	E	Data analysis	
Manufacturer of reagents and catalogue numbers	D	qPCR analysis program (source, version)	E
C_qs with and without reverse transcription	D[c]	Method of C_q determination	E
Storage conditions of cDNA	D	Outlier identification and disposition	E
qPCR target information		Results for NTCs	E
Gene symbol	E	Justification of number and choice of reference genes	E
Sequence accession number	E	Description of normalization method	E
Location of amplicon	D	Number and concordance of biological replicates	D
Amplicon length	E	Number and stage (reverse transcription or qPCR) of technical replicates	E
In silico specificity screen (BLAST, and so on)	E	Repeatability (intraassay variation)	E
Pseudogenes, retropseudogenes, or other homologs?	D	Reproducibility (interassay variation, CV)	D
Sequence alignment	D	Power analysis	D
Secondary structure analysis of amplicon	D	Statistical methods for results significance	E
Location of each primer by exon or intron (if applicable)	E	Software (source, version)	E
What splice variants are targeted?	E	C_q or raw data submission with RDML	D

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

[b] FFPE, formalin-fixed, paraffin-embedded; RIN, RNA integrity number; RQI, RNA quality indicator; GSP, gene-specific priming; dNTP, deoxynucleoside triphosphate.

[c] Assessing the absence of DNA with a no–reverse transcription assay is essential when first extracting RNA. Once the sample has been validated as DNA free, inclusion of a no–reverse transcription control is desirable but no longer essential.

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

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- **Experimental Design （实验设计）**
- **Sample Information （样本信息 ）**
- **Nucleic Acid Extraction （核酸提取）**
- **Reverse Transcription （反转录）**
- **qPCR Target Information （目的基因信息）**
- **qPCR Oligonucleotides （qPCR 寡核苷酸）**
- **qPCR Protocol （qPCR 程序）**
- **qPCR Validation （qPCR验证）**
- **Data Analysis （数据分析）**

MIQE 指南好处

□ **规范专业术语和概念**

- 术语: reference genes, qPCR, Cq (quantification cycle)
- 概念: sensitivity, specificity, accuracy, repeatability, reproducibility
- 灵敏度，特异性，准确度，重复性，重现性

□ **规范操作标准**

- 样品获取、 处理和制备
- RNA 或 DNA质控
- 反转录
- qPCR 过程(酶,、耗材、 特异性、引物序列、 二级结构,、对照、校正)
- 数据分析(均一化, 生物重复和技术重复)

- **评审可以更好的评估研究者所用的实验方案的有效性**
- **确保文献的完整性和可靠性**

OXFORD JOURNALS
OXFORD UNIVERSITY PRESS

JNCI
Journal of the National Cancer Institute

Nucleic Acids Research

Clinical Chemistry

BMC
Cell Biology

BMC
Molecular Biology

BMC
Developmental Biology

qPCR进行基因表达分析

实验设计

↓

核酸提取

↓

反转录

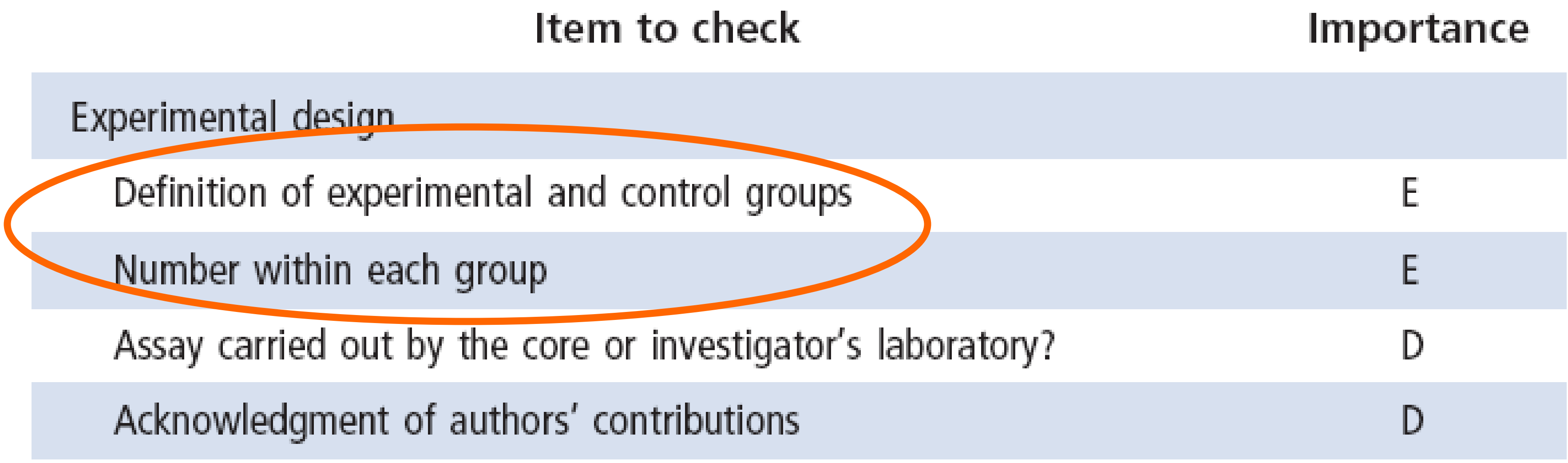
↓

qPCR实验

↓

数据分析

实验设计



Item to check	Importance
Experimental design	
Definition of experimental and control groups	E
Number within each group	E
Assay carried out by the core or investigator's laboratory?	D
Acknowledgment of authors' contributions	D

- 实验样品的分类，处理和对照都要具备并明确定义
- 重复数

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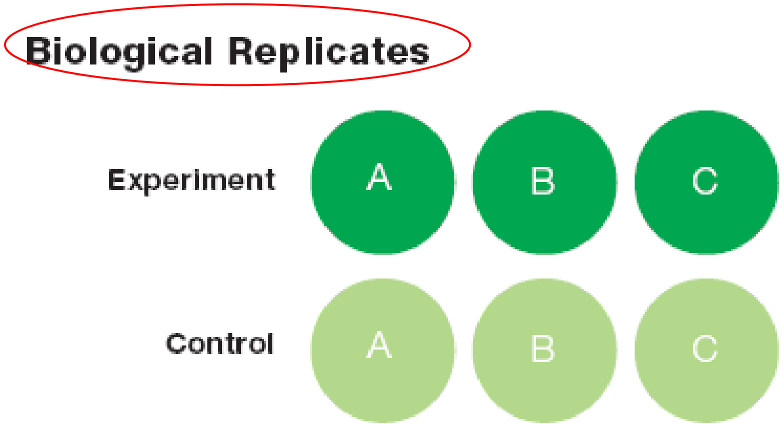
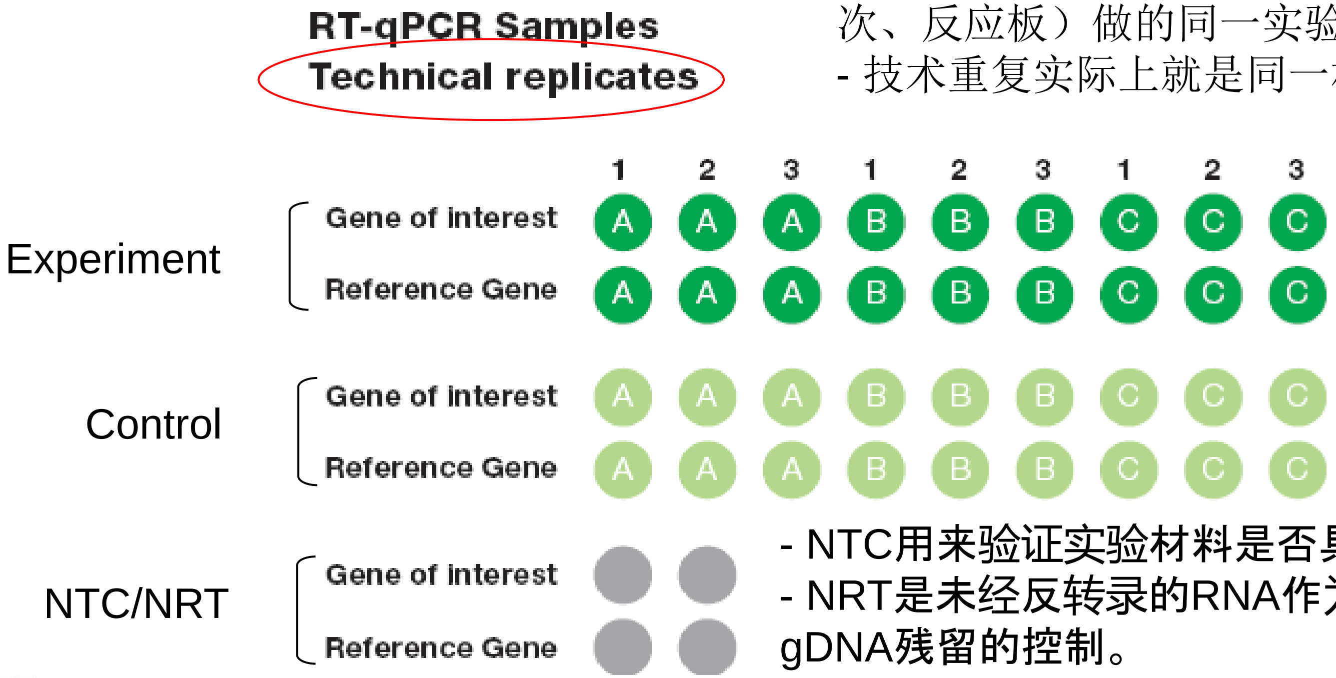
样品重复数

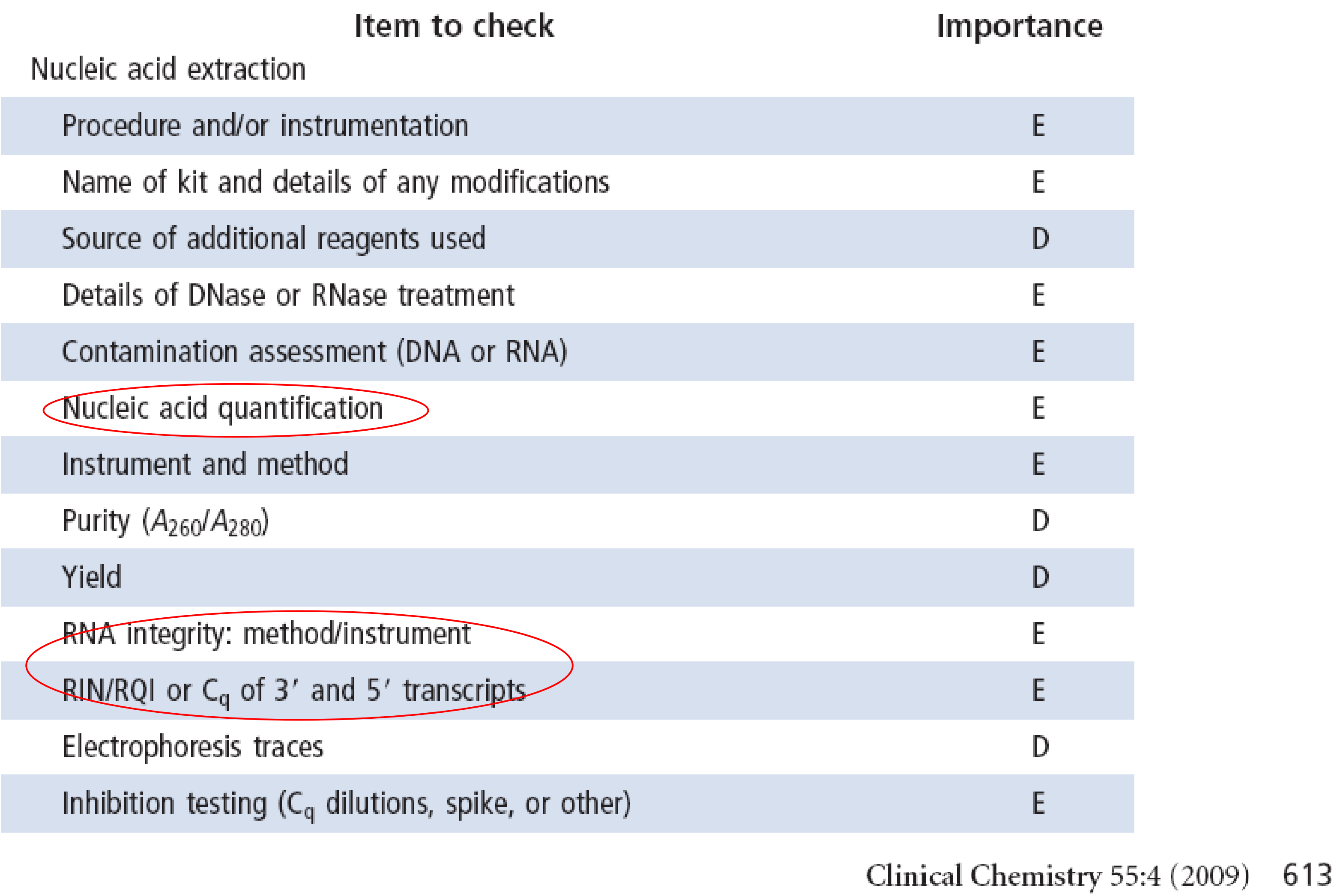


- 生物性重复：不同的材料（时间、植株、批次、反应板）做的同一实验
- 技术重复实际上就是同一材料包括的复孔



- NTC用来验证实验材料是否具有污染。
- NRT是未经反转录的RNA作为阴性对照。对gDNA残留的控制。

Nucleic Acid Extraction （核酸提取）



Item to check	Importance
Nucleic acid extraction	
Procedure and/or instrumentation	E
Name of kit and details of any modifications	E
Source of additional reagents used	D
Details of DNase or RNase treatment	E
Contamination assessment (DNA or RNA)	E
Nucleic acid quantification	E
Instrument and method	E
Purity (A_{260}/A_{280})	D
Yield	D
RNA integrity: method/instrument	E
RIN/RQI or C_q of 3′ and 5′ transcripts	E
Electrophoresis traces	D
Inhibition testing (C_q dilutions, spike, or other)	E

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核酸提取

RT-qPCR要求高质量的RNA：

•使用高纯度（不含DNA和蛋白质及抑制物）和高度完整（无降解）的RNA进行实验是整个RT-qPCR实验流程中最关键的环节！

整个RNA提取过程中严格避免Rnase污染造成的RNA降解。

- 仔细分析所得RNA 的质量，包括浓度和完整性

- **RNA质控的重要性：**

- RNA不纯将导致对下游PCR反应的抑制，Cq将右移，产生数据偏差。

•若RNA发生降解，将导致不正确的结果。

RNA的纯度和完整性是不相关的，都需要进行检测！

RNA定量和完整性分析

- 分析RNA纯度和完整性

•UV 吸光度

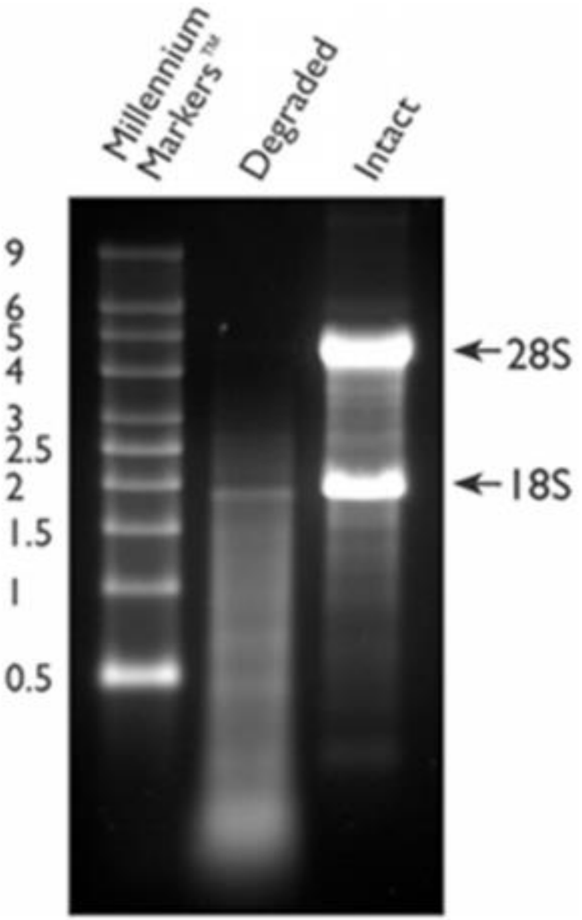
– A260/A280=2.0,

– A260: 0.15~1

– A260/A230=2.5

– 凝胶电泳分析

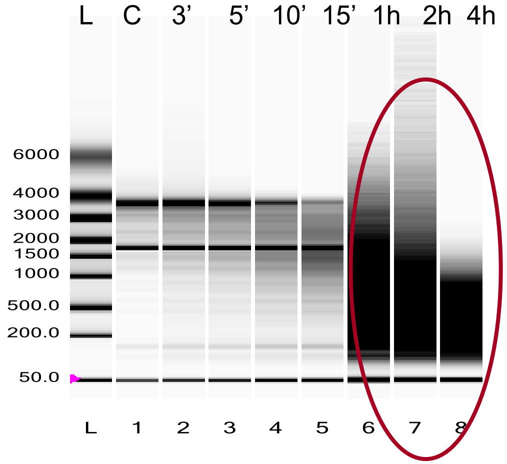
28S rRNA，18S rRNA



28S:18S≈2:1

RNA 纯度和完整性

Experion Virtual Gel



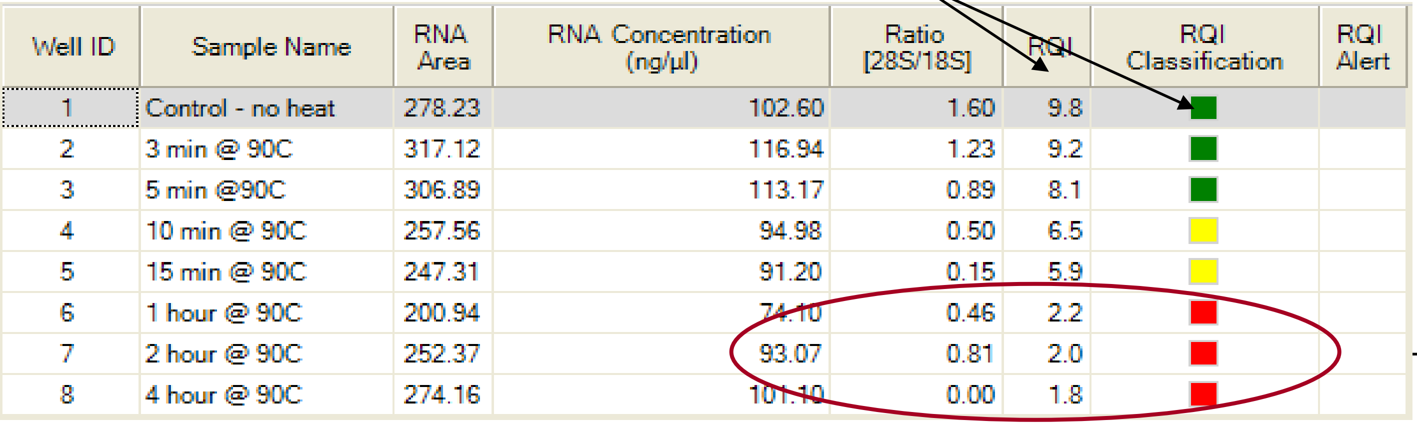
Samples 6,7,8 are highly degraded.

Corresponding Nanodrop Readings

Sample	Conc (ng/ul)	A260/280*	A260/230*
Control-no heat	115	1.90	2.44
3 min @ 90C	114	1.93	2.40
5 min @ 90C	115	2.06	2.37
10 min @ 90C	115	2.03	2.37
15 min @ 90C	116	2.02	2.31
1 hr @ 90C	109	1.99	2.18
2hr @ 90C	117	2.00	2.32
4 hr @ 90C	118	1.89	2.23

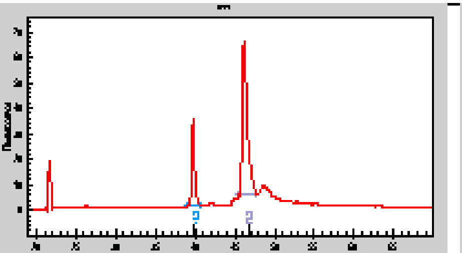
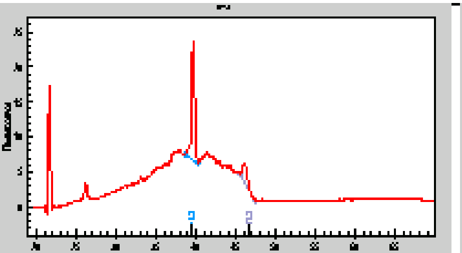
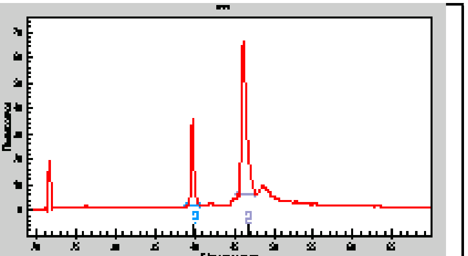
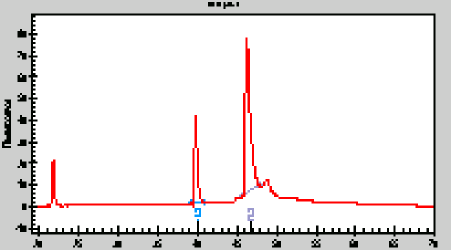
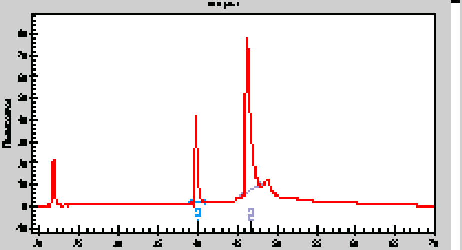
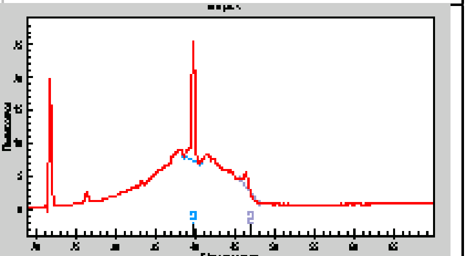
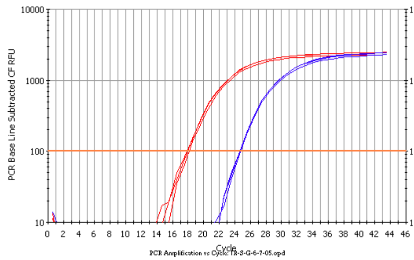
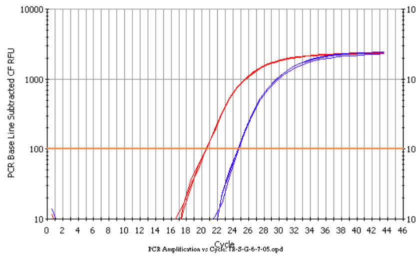
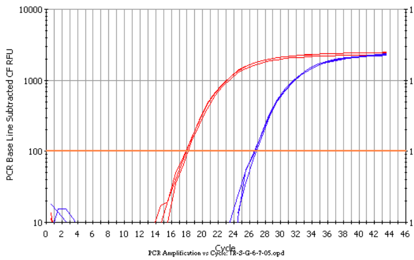
*Generally accepted ratios (A260/280 and A260/230) for good quality RNA are > 1.8.

RQI Value & Color Coded Classification

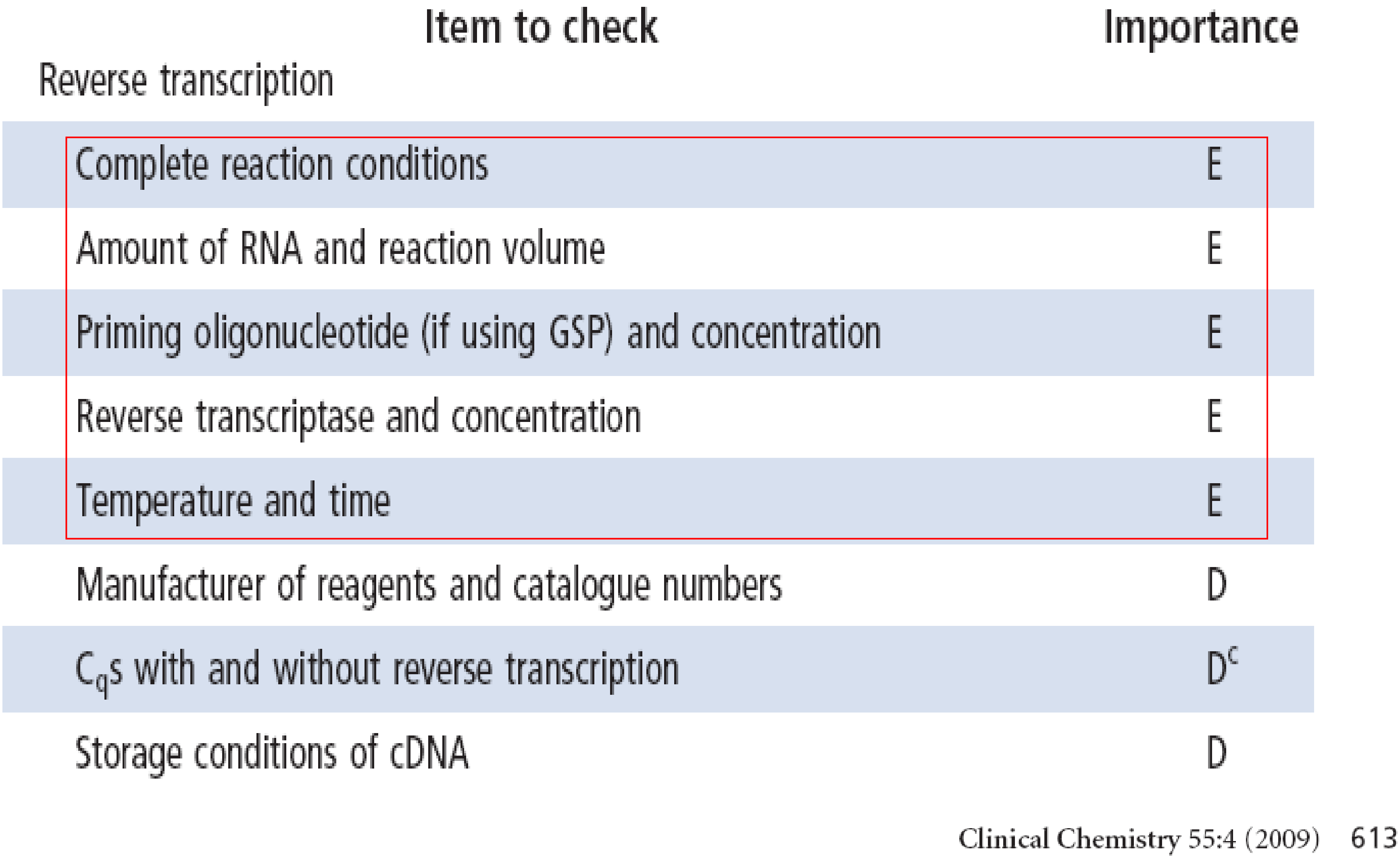


Well ID	Sample Name	RNA Area	RNA Concentration (ng/µl)	Ratio [28S/18S]	RQI	RQI Classification	RQI Alert
1	Control - no heat	278.23	102.60	1.60	9.8	🟩	
2	3 min @ 90C	317.12	116.94	1.23	9.2	🟩	
3	5 min @90C	306.89	113.17	0.89	8.1	🟩	
4	10 min @ 90C	257.56	94.98	0.50	6.5	🟨	
5	15 min @ 90C	247.31	91.20	0.15	5.9	🟨	
6	1 hour @ 90C	200.94	74.10	0.46	2.2	🟥	
7	2 hour @ 90C	252.37	93.07	0.81	2.0	🟥	
8	4 hour @ 90C	274.16	101.10	0.00	1.8	🟥	

Experion Automated Electrophoresis System

GOI			
Reference Gene			
RT-qPCR			
C_T GOI	**17.9**	**20.5**	**17.9**
C_T Ref Gene	**24.7**	**24.7**	**26.7**
ΔC_T	**6.8**	**4.3**	**8.8**

反转录



Item to check	Importance
Reverse transcription	
Complete reaction conditions	E
Amount of RNA and reaction volume	E
Priming oligonucleotide (if using GSP) and concentration	E
Reverse transcriptase and concentration	E
Temperature and time	E
Manufacturer of reagents and catalogue numbers	D
C_qs with and without reverse transcription	D^c
Storage conditions of cDNA	D

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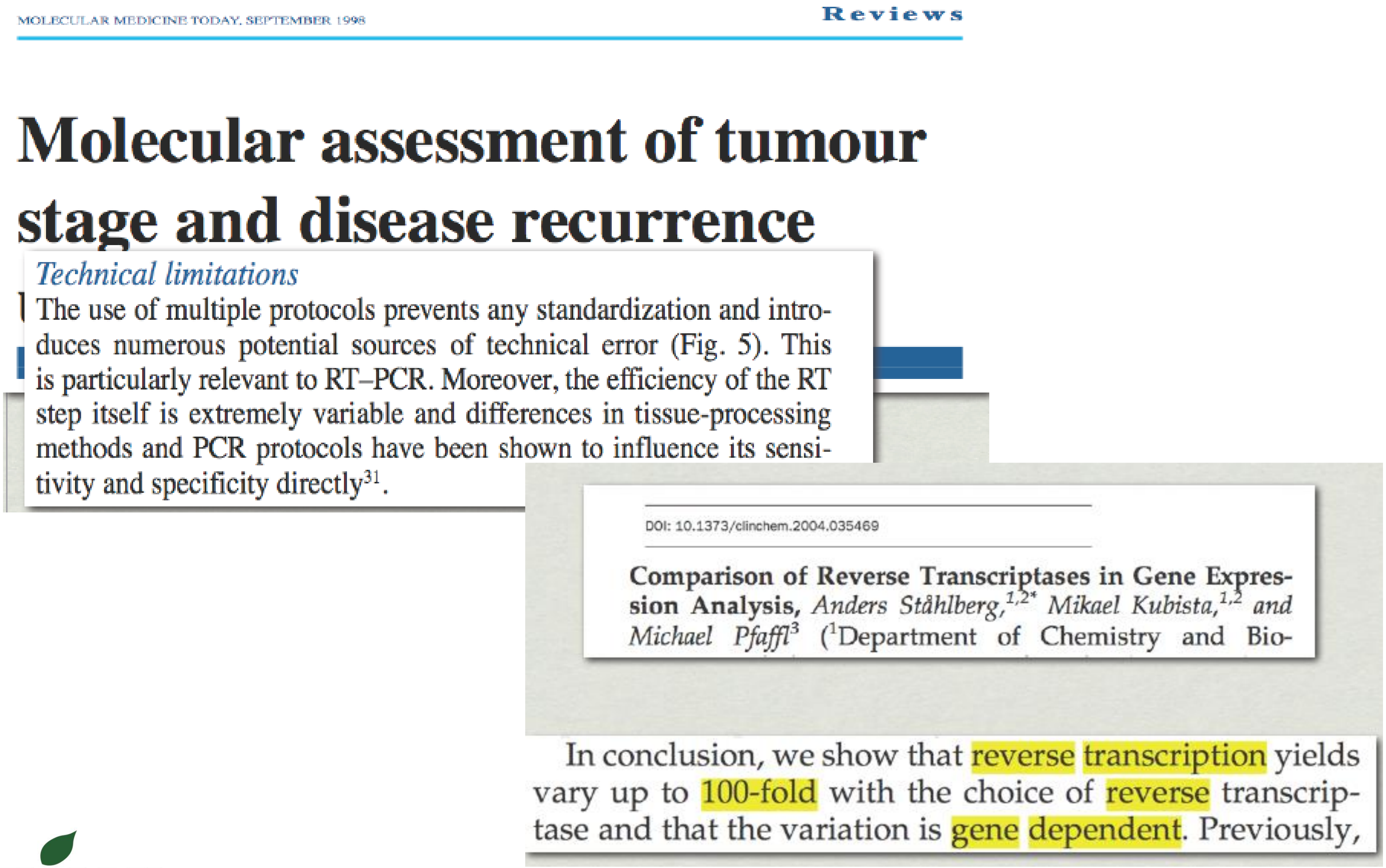
反转录效率



MOLECULAR MEDICINE TODAY, SEPTEMBER 1998

Reviews

Molecular assessment of tumour stage and disease recurrence

Technical limitations

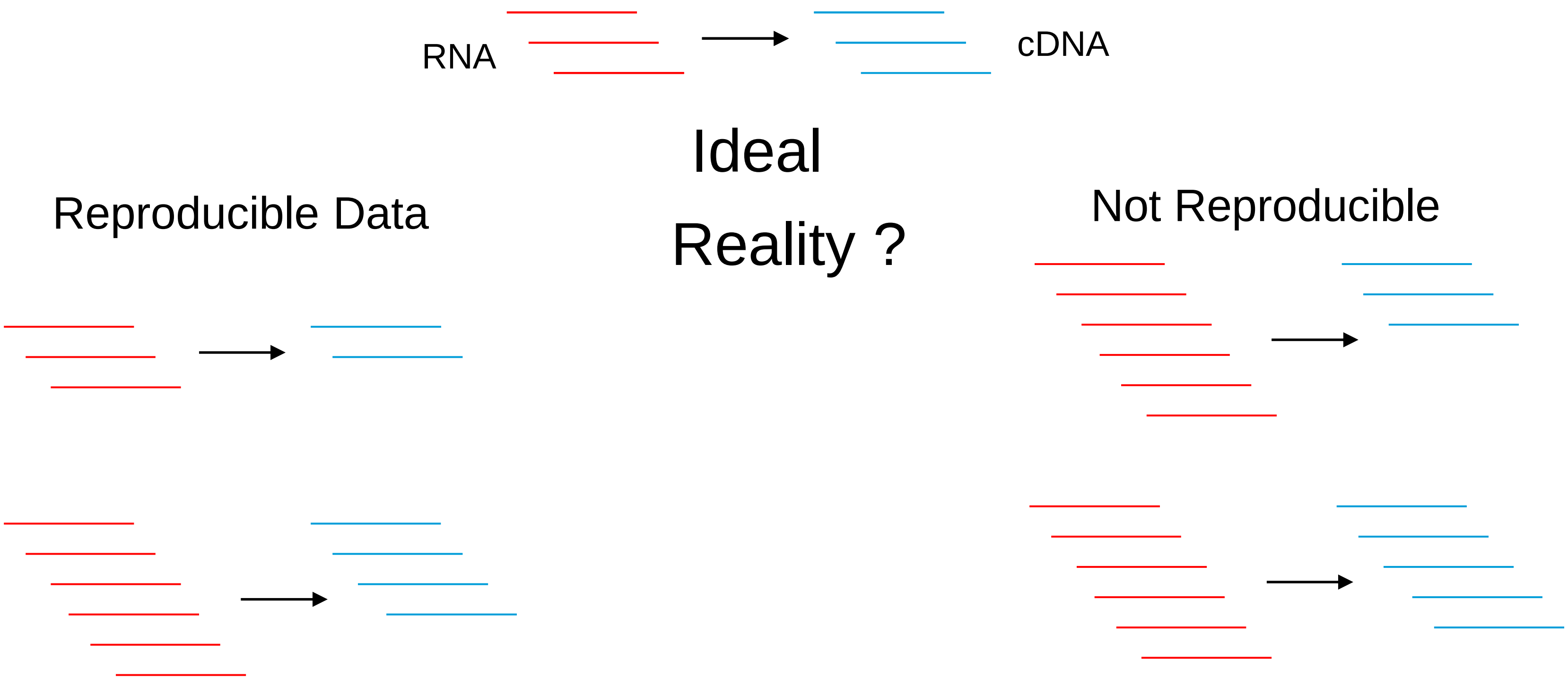
The use of multiple protocols prevents any standardization and introduces numerous potential sources of technical error (Fig. 5). This is particularly relevant to RT–PCR. Moreover, the efficiency of the RT step itself is extremely variable and differences in tissue-processing methods and PCR protocols have been shown to influence its sensitivity and specificity directly[31].

DOI: 10.1373/clinchem.2004.035469

Comparison of Reverse Transcriptases in Gene Expression Analysis, *Anders Ståhlberg,*[1,2*] *Mikael Kubista,*[1,2] *and Michael Pfaffl*[3] ([1]Department of Chemistry and Bio-

In conclusion, we show that reverse transcription yields vary up to 100-fold with the choice of reverse transcriptase and that the variation is gene dependent. Previously,

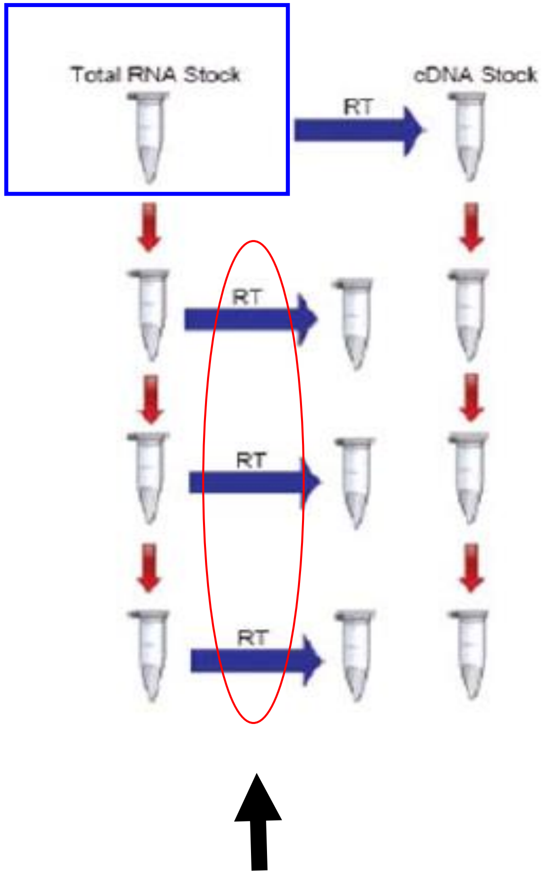
反转录效率一致性



不管target gene的丰度如何、起始的RNA输入量如何，反转录的效率都是一致的 **→** 确保cDNA的差异能够真实反映 RNA i.e. 基因表达的差异。 不影响表达差异的判定。

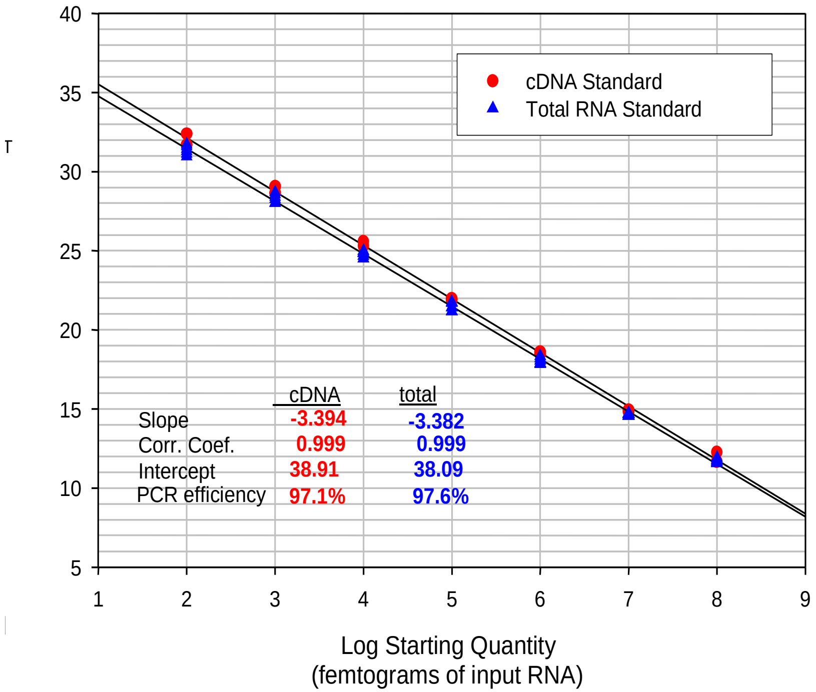
反转录效率一致性可以通过预实验来判定

- 实验: 梯度稀释RNA及cDNA



iScript qRT-PCR Standard Curve Comparison:

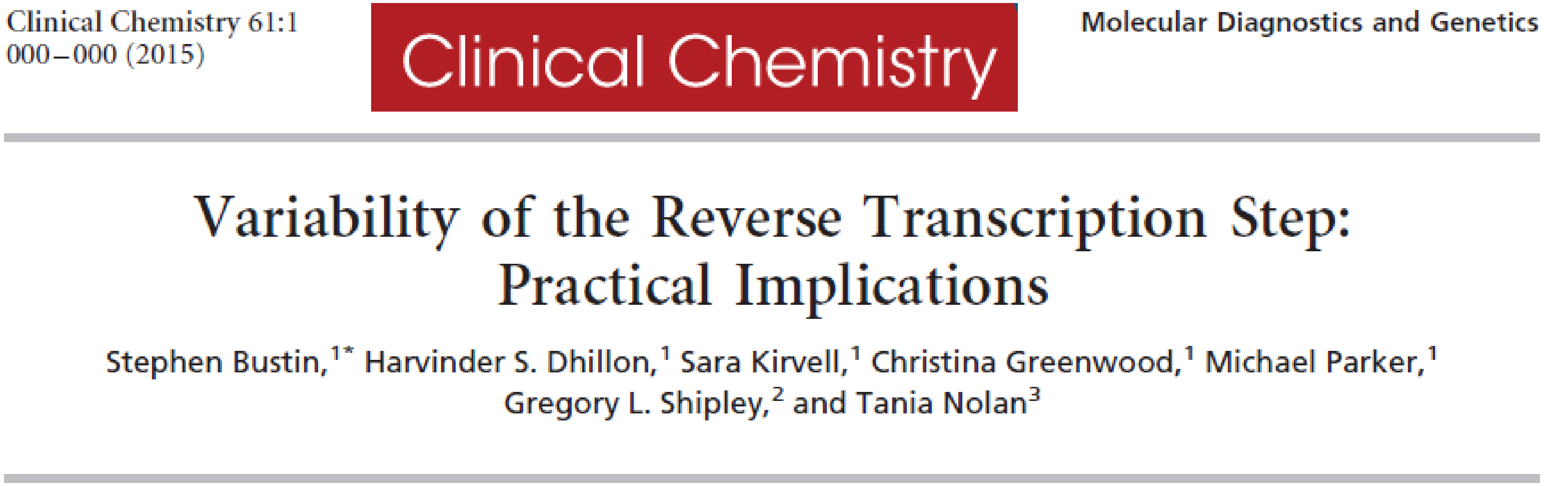
cDNA serial dilution vs. total RNA serial dilution



	cDNA	total
Slope	-3.394	-3.382
Corr. Coef.	0.999	0.999
Intercept	38.91	38.09
PCR efficiency	97.1%	97.6%

Note: 1/10th of cDNA reaction used for PCR

Published Evidences



Clinical Chemistry 61:1
000–000 (2015)

Clinical Chemistry

Molecular Diagnostics and Genetics

Variability of the Reverse Transcription Step: Practical Implications

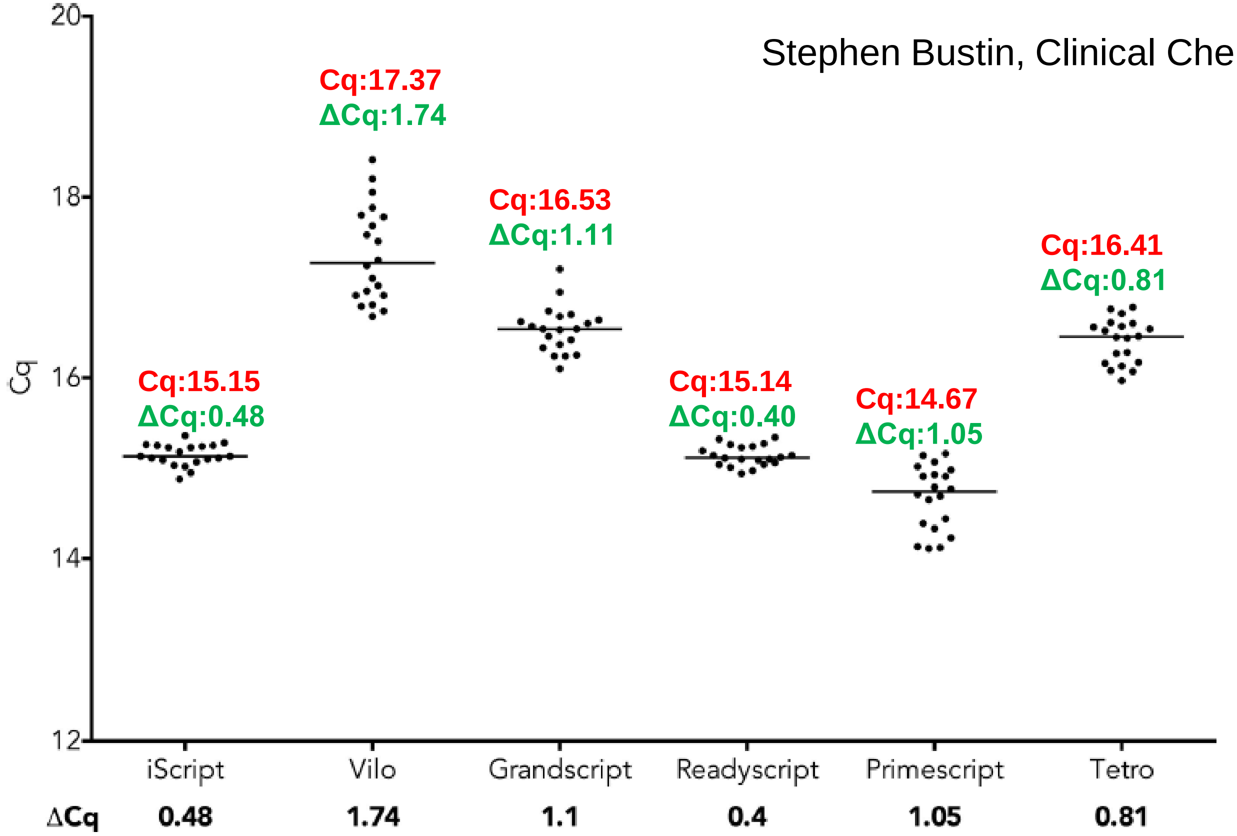
Stephen Bustin,[1*] Harvinder S. Dhillon,[1] Sara Kirvell,[1] Christina Greenwood,[1] Michael Parker,[1]
Gregory L. Shipley,[2] and Tania Nolan[3]

BACKGROUND: cDNA is a necessary first step for numerous research and molecular diagnostic applications. Although RT efficiency is known to be variable, little attention has been paid to the practical implications of that variability.

RESULTS: RT efficiency is enzyme, sample, RNA concentration, and assay dependent and can lead to variable correlation between mRNAs from the same sample. This translates into relative mRNA expression levels that generally vary between **2- and 3-fold**, although **higher levels** are also observed.

Steven Bustin was involved in the development of the MIQE guidelines for qPCR experiments. Image courtesy of Steven Bustin.

Published Evidences



Stephen Bustin, Clinical Chemistry 61:1 (2015)

Cq:15.15
ΔCq:0.48

Cq:17.37
ΔCq:1.74

Cq:16.53
ΔCq:1.11

Cq:15.14
ΔCq:0.40

Cq:14.67
ΔCq:1.05

Cq:16.41
ΔCq:0.81

Variability exemplified by qPCR results of GAPDH#1 reverse-transcribed by iScript, Vilo, Grandscript, Readyscript, Primescript, or Tetro.
Scatterplots show the Cqs obtained from individual RT replicates (n = 10) carried out for each RTase and subjected to qPCR analysis (n = 2), i.e., on the basis of Cqs recorded from the 20 qPCR reactions (10 RT X 2 qPCR replicates). The horizontal line shows the median Cq values, and the ΔCqs record the range between the lowest and highest Cqs for each RTase.

什么是好的cDNA合成？

- 转录效率一致性 — 真实反应样品中RNA的表达量
- 反转录效率 — 灵敏度
- GC含量高的RNA模板
- 避免转录mRNA 5’ ,3’ 区域序列不均一
- 操作方便，快速
- 反转录的RNA模板容量

qPCR 实验

引物和探针的设计

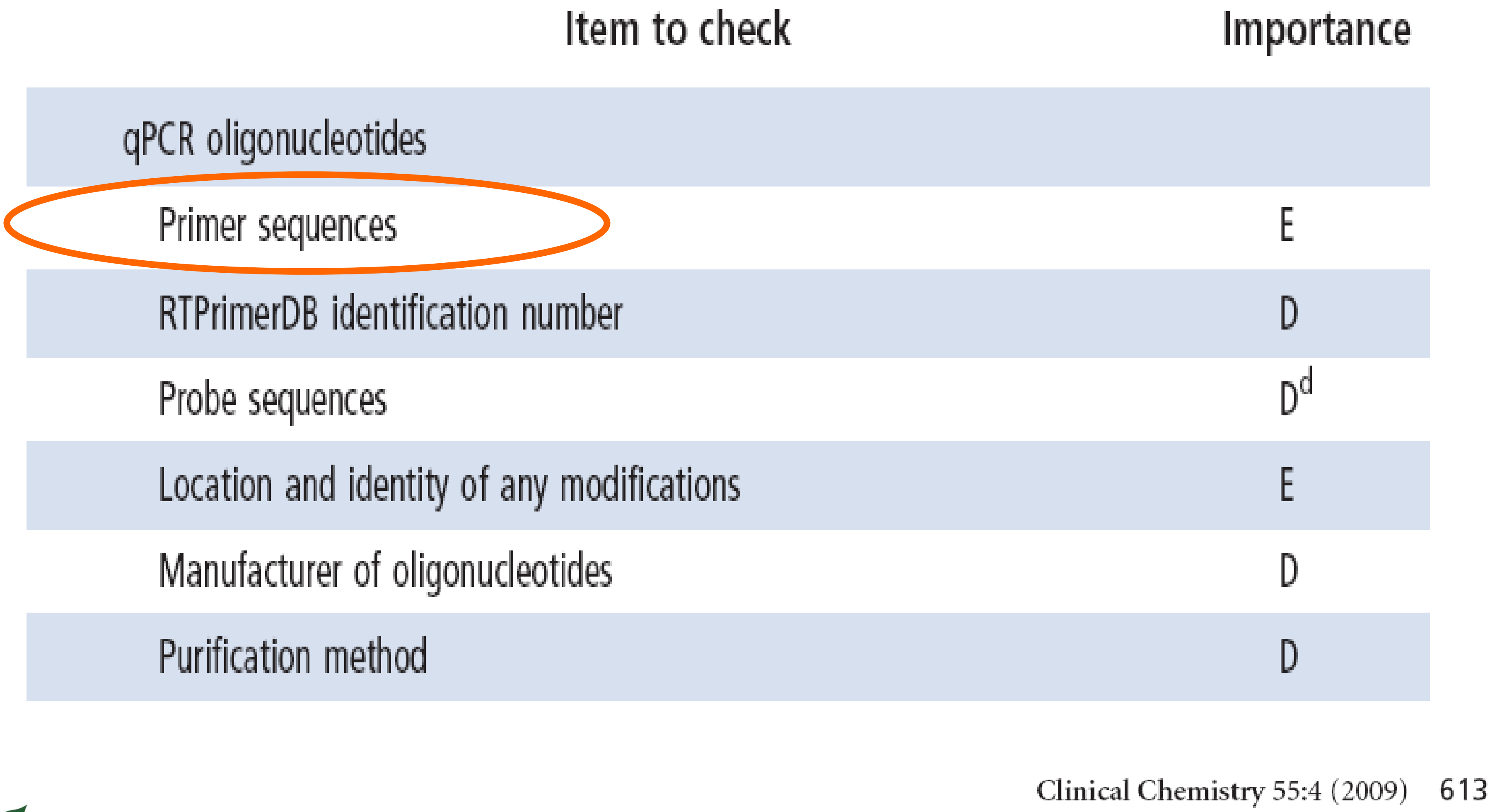
反应体系的优化

标准曲线

内参基因的选择

Clinical Chemistry 55:4 (2009) 613

qPCR Oligonucleotides （引物和探针）



Item to check	Importance
qPCR oligonucleotides	
Primer sequences	E
RTPrimerDB identification number	D
Probe sequences	D[d]
Location and identity of any modifications	E
Manufacturer of oligonucleotides	D
Purification method	D

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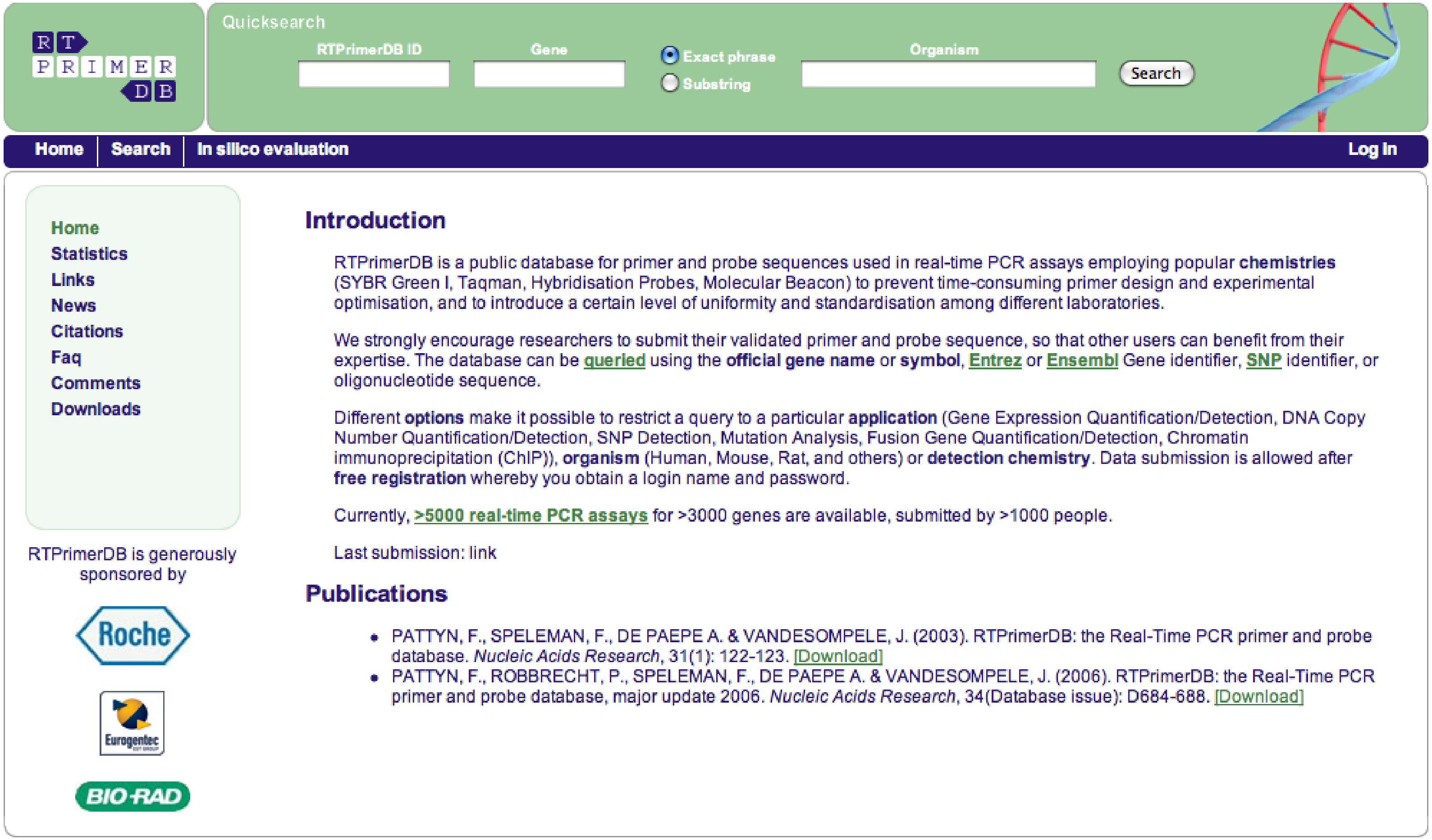
引物探针相关资源

- Workflow for PCR primer design. The following web sites are pertinent:
- PrimerBlast: https://www.ncbi.nlm.nih.gov/tools/primer-blast;
- DINAmelt: http://unafold.rna.albany.edu/?q=DINAMelt;
- Mfold: http://unafold.rna.albany.edu/?q=mfold;
- BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi;
- Ensembl: http://www.ensembl.org/index.html;.

RTPrimerDB - Primer & Probe Database （引物/探针资源共享）



Introduction

RTPrimerDB is a public database for primer and probe sequences used in real-time PCR assays employing popular **chemistries** (SYBR Green I, Taqman, Hybridisation Probes, Molecular Beacon) to prevent time-consuming primer design and experimental optimisation, and to introduce a certain level of uniformity and standardisation among different laboratories.

We strongly encourage researchers to submit their validated primer and probe sequence, so that other users can benefit from their expertise. The database can be queried using the **official gene name** or **symbol**, Entrez or Ensembl Gene identifier, SNP identifier, or oligonucleotide sequence.

Different **options** make it possible to restrict a query to a particular **application** (Gene Expression Quantification/Detection, DNA Copy Number Quantification/Detection, SNP Detection, Mutation Analysis, Fusion Gene Quantification/Detection, Chromatin immunoprecipitation (ChIP)), **organism** (Human, Mouse, Rat, and others) or **detection chemistry**. Data submission is allowed after **free registration** whereby you obtain a login name and password.

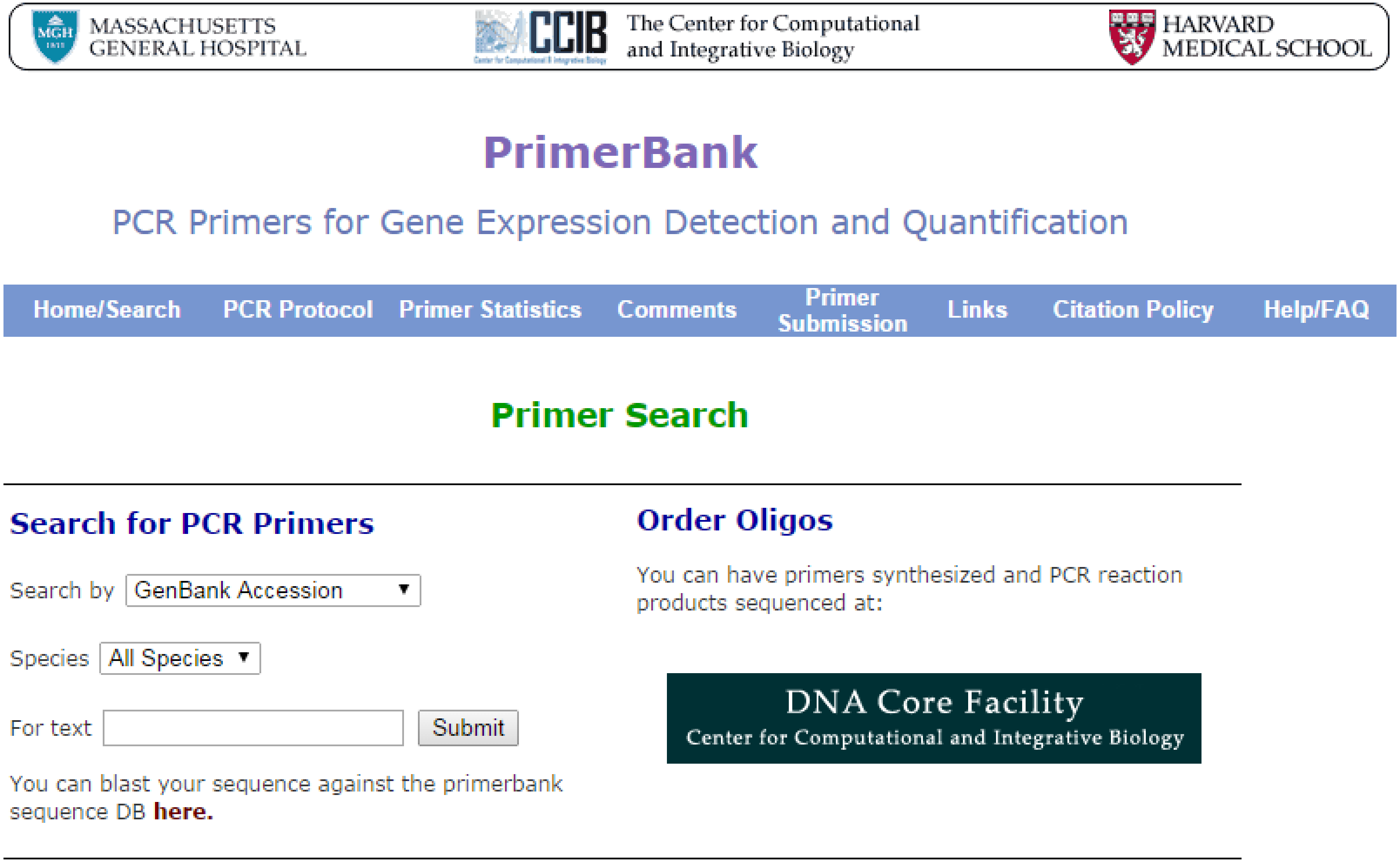
Currently, **>5000 real-time PCR assays** for >3000 genes are available, submitted by >1000 people.

Last submission: link

Publications

- PATTYN, F., SPELEMAN, F., DE PAEPE A. & VANDESOMPELE, J. (2003). RTPrimerDB: the Real-Time PCR primer and probe database. *Nucleic Acids Research*, 31(1): 122-123. [Download]
- PATTYN, F., ROBBRECHT, P., SPELEMAN, F., DE PAEPE A. & VANDESOMPELE, J. (2006). RTPrimerDB: the Real-Time PCR primer and probe database, major update 2006. *Nucleic Acids Research*, 34(Database issue): D684-688. [Download]

RTPrimerDB is generously sponsored by



MASSACHUSETTS GENERAL HOSPITAL

CCIB The Center for Computational and Integrative Biology

HARVARD MEDICAL SCHOOL

PrimerBank

PCR Primers for Gene Expression Detection and Quantification

Primer Search

Search for PCR Primers

Search by GenBank Accession ▼

Species All Species ▼

For text

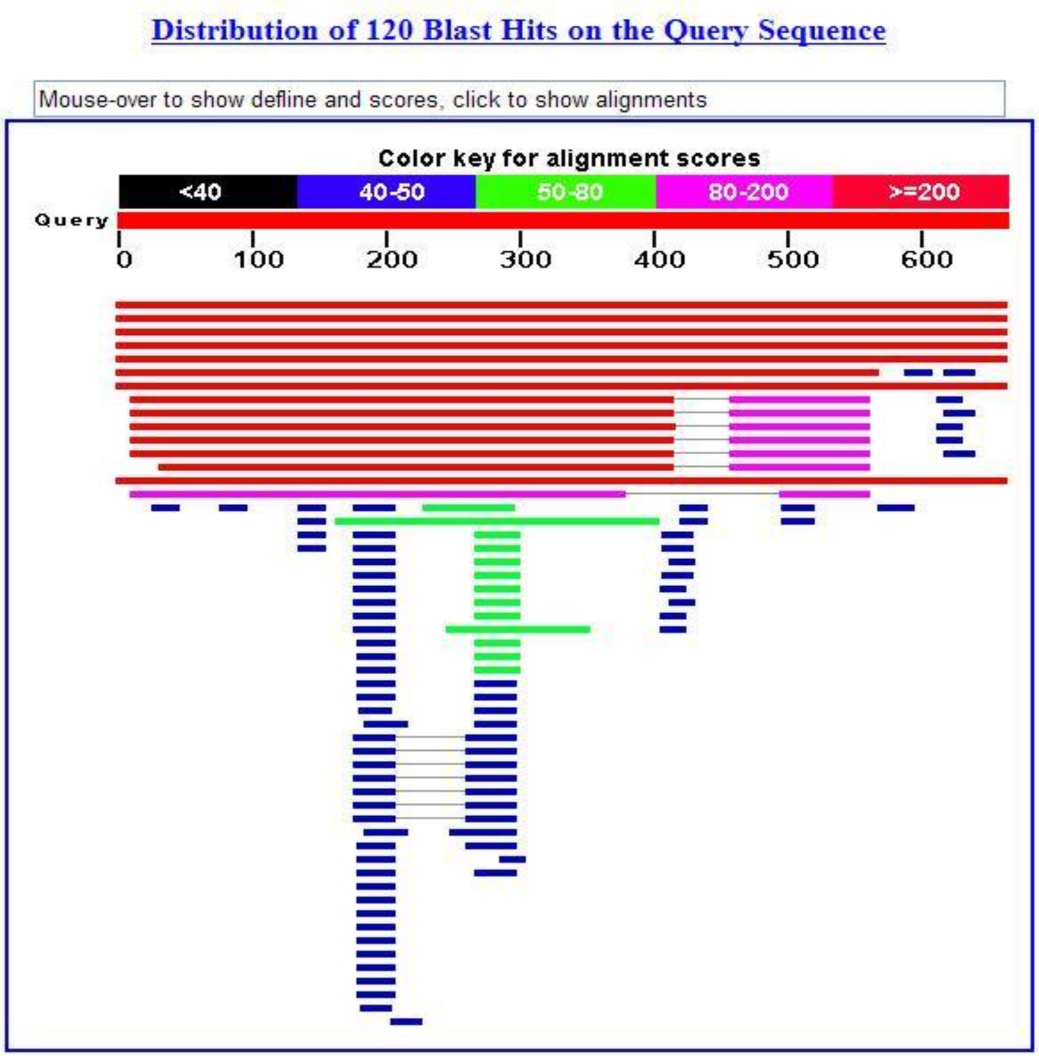
You can blast your sequence against the primerbank sequence DB **here.**

Order Oligos

You can have primers synthesized and PCR reaction products sequenced at:

DNA Core Facility
Center for Computational and Integrative Biology

序列同源性分析(BLAST)



Distribution of 120 Blast Hits on the Query Sequence

Mouse-over to show defline and scores, click to show alignments

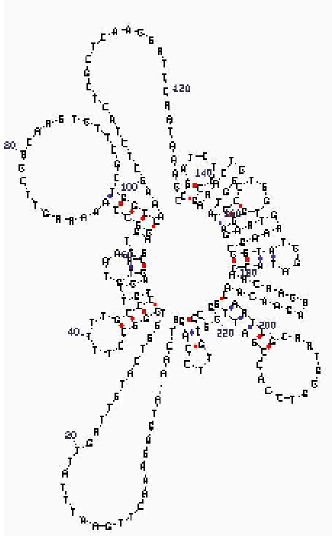
Color key for alignment scores

http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi

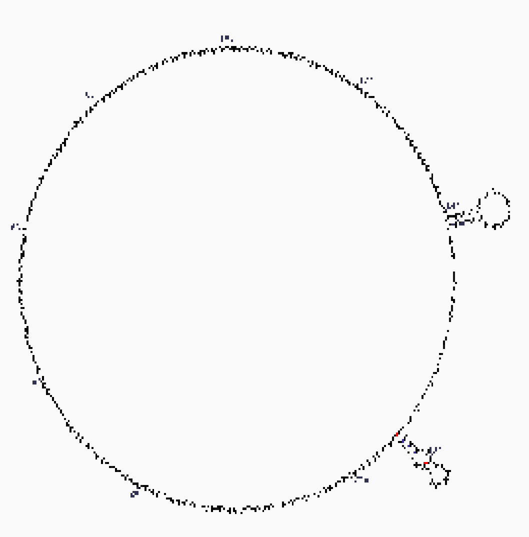
Amplicon Secondary Structures

http://mfold.rna.albany.edu/?q=mfold

- Bad location for primers
- Good location for primers



55° C



60° C

引物的设计

- 在公共资源或数据库中寻找已经发表的或经过实验验证的引物和探针，比如 http://www.rtprimerdb.org
- GC含量在30%~80%(理想范围为40~60%)，引物长度在18~30bp，Tm在55~65℃；上下游引物ΔTm≤4 ℃.
- 避免引物和模板间错配，特别是引物3`端的错配。
- 限制二级结构。
- 避免引物位于模板的二级结构丰富区（茎环结构）。
- 限制连续的G 或C长度超过3个碱基。
- 将C或G置于引物的3`端，但是3`端最后5个碱基中不要超过2个C或G。
- 避免3’端最后碱基为T(可能产生错配）。
- 避免引物间的互补，以避免产生引物二聚体，特别注意引物间3`端互补不能超过2个碱基。
- 在NCBI数据库上比对引物、探针、扩增子序列，确保特异性。
- 同时检查引物退火位置的是否存在SNP位点。
- 产物长度在75~200bp之间，最大不超过250bp。
- 设计退火位置在不同外显子上的引物，如果必要引物可跨外显子/外显子连接点。

Optimization and Validation
优化与验证

qPCR validation Item to check	Importance
Evidence of optimization (from gradients)	D
Specificity (gel, sequence, melt, or digest)	E
For SYBR Green I, C_q of the NTC	E
Calibration curves with slope and y intercept	E
PCR efficiency calculated from slope	E
CIs for PCR efficiency or SE	D
r^2 of calibration curve	E
Linear dynamic range	E
C_q variation at LOD	E
CIs throughout range	D
Evidence for LOD	E
If multiplex, efficiency and LOD of each assay	E

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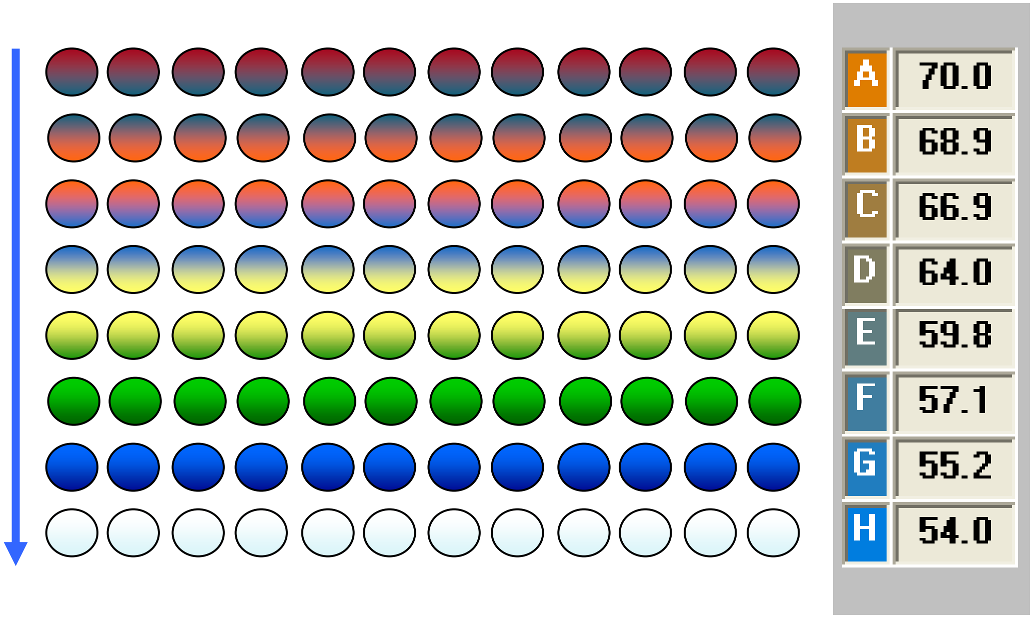
欲速则不达！！！

利用温度梯度优化体系

dynamic thermal gradient

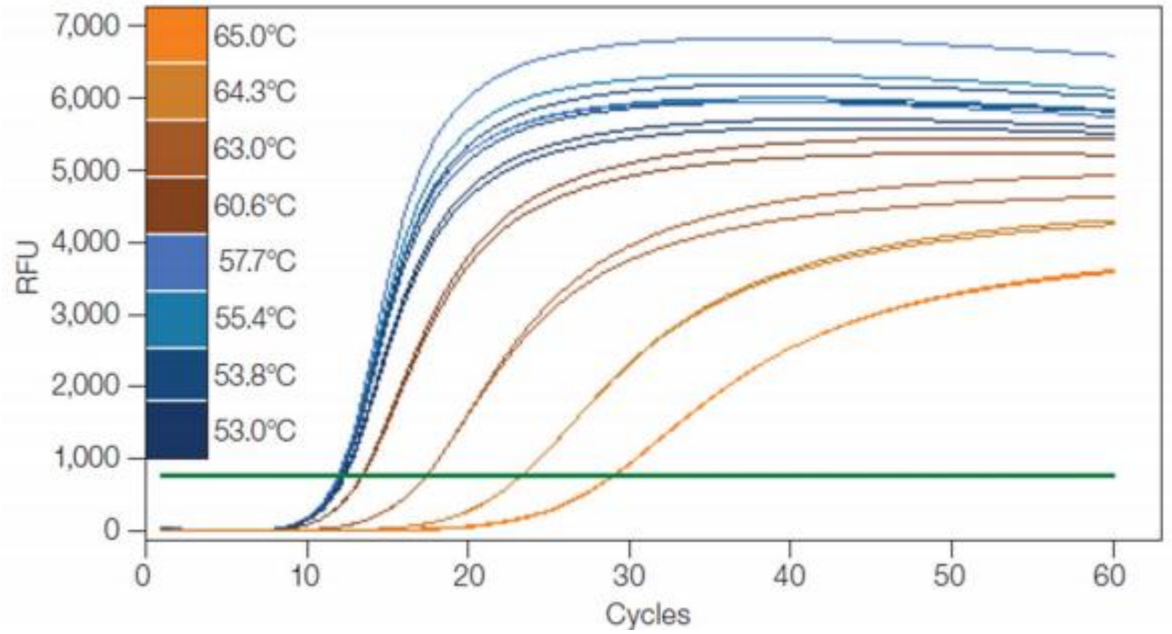


A	70.0
B	68.9
C	66.9
D	64.0
E	59.8
F	57.1
G	55.2
H	54.0

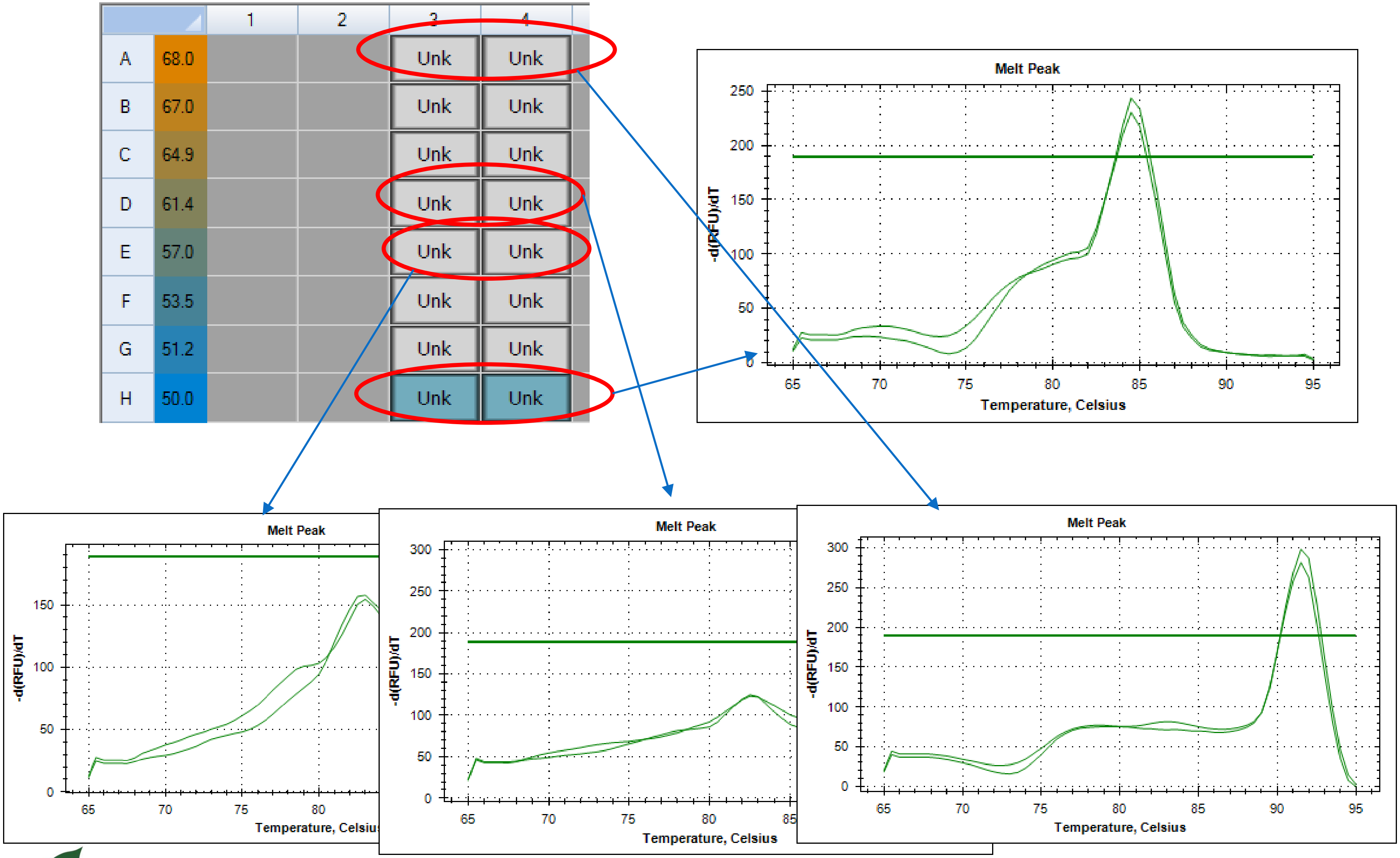
10°C Above

6°C Below

利用温度梯度优化体系

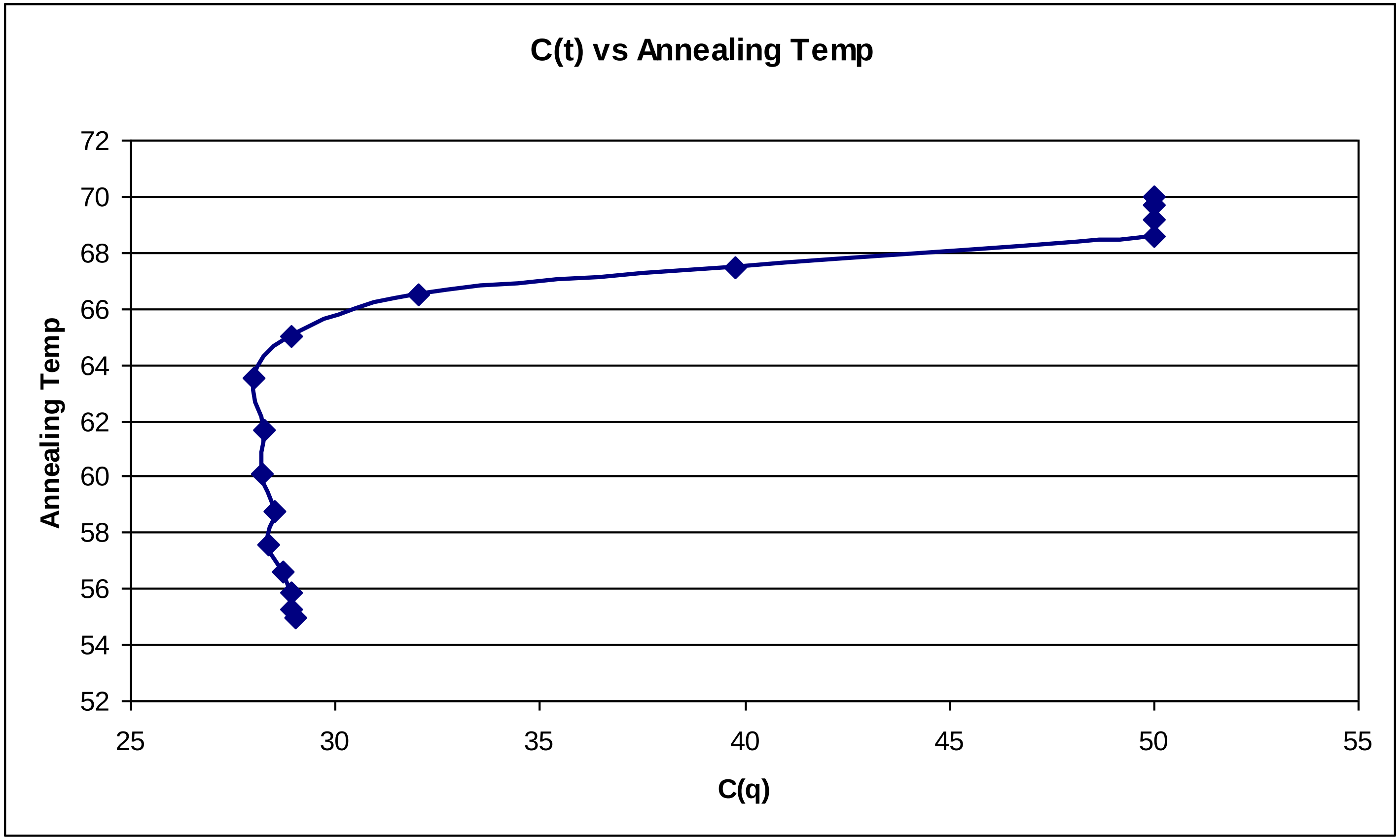


- 退火温度的优化通常是qPCR体系优化的第一个参数
- 软件计算的引物Tm并不准确。
- 在最适退火温度下进行过PCR扩增是很关键的。
- 选择兼容性好的supermix



		1	2	3	4
A	68.0			Unk	Unk
B	67.0			Unk	Unk
C	64.9			Unk	Unk
D	61.4			Unk	Unk
E	57.0			Unk	Unk
F	53.5			Unk	Unk
G	51.2			Unk	Unk
H	50.0			Unk	Unk

Effect of Annealing Temp on C(t)
退火温度影响Cq值

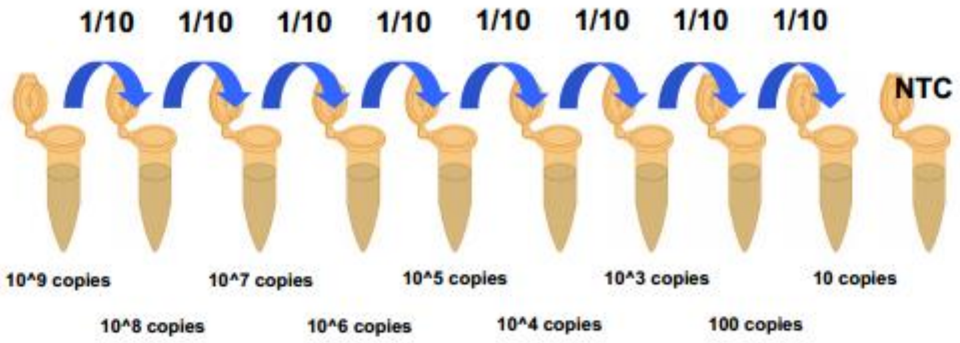


CCl26 amplified using Bio-Rad iQ SYBR Green Supermix: 5ul Assay 95ºC 60sec / 50x95ºC 10 sec, 55-70ºC 60 sec / melt analysis

标准曲线

利用标准曲线检验验证下列因素

- ✓ 扩增效率（90%≤E≤110%）
- ✓标准品的动态范围
- ✓ PCR抑制剂的存在与影响



1/10 1/10 1/10 1/10 1/10 1/10 1/10 1/10

NTC

10^9 copies

10^8 copies

10^7 copies

10^6 copies

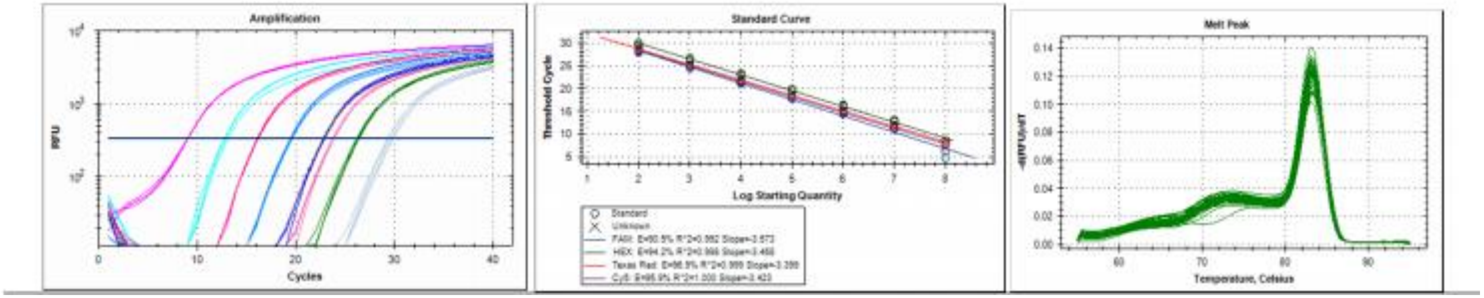
10^5 copies

10^4 copies

10^3 copies

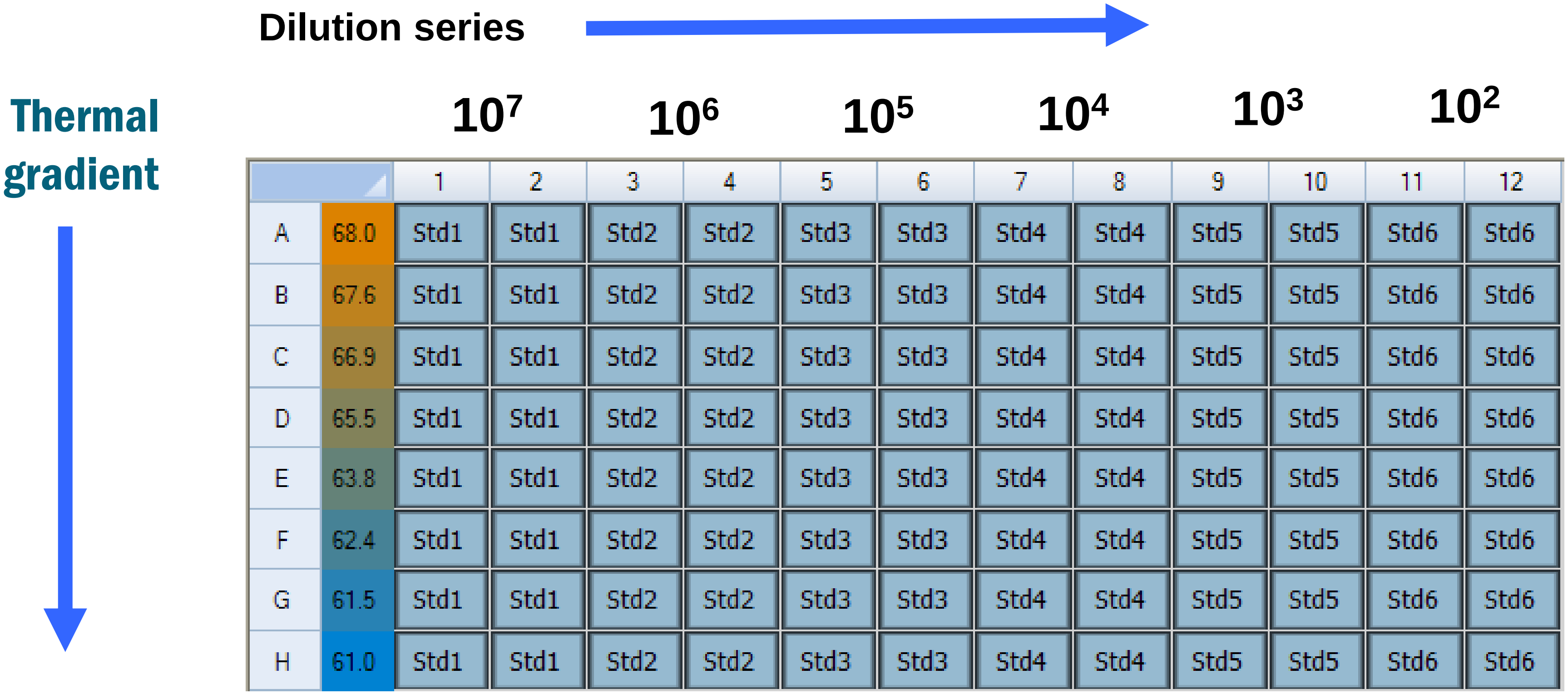
100 copies

10 copies



利用温度梯度优化体系

Single Plate Optimisation of Annealing Temperature and PCR Efficiency via Slope of Calibration Curve 一次试验完成引物退火温度和扩增效率的优化



Dilution series

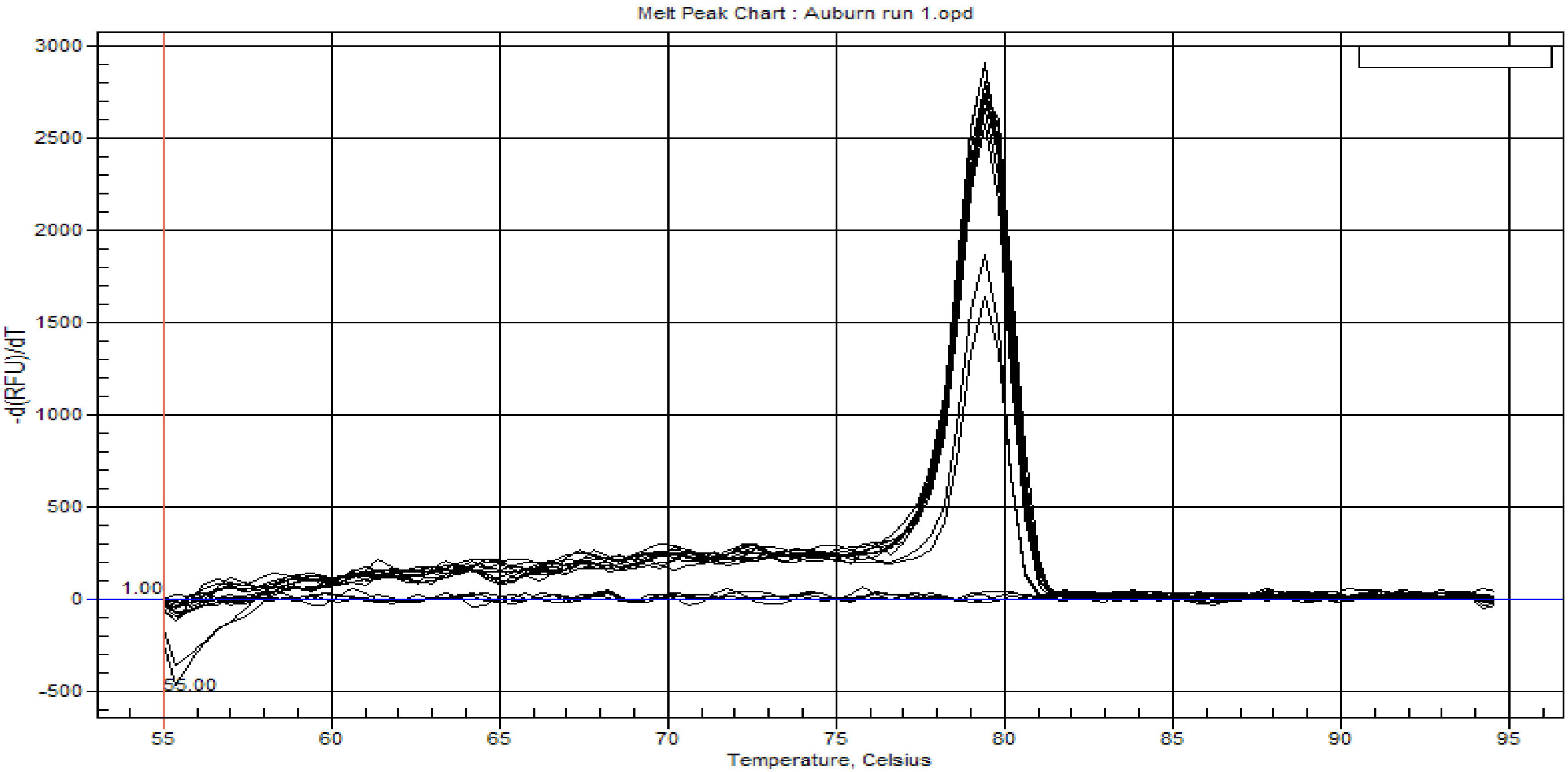
Thermal gradient

		1	2	3	4	5	6	7	8	9	10	11	12
		10^7		10^6		10^5		10^4		10^3		10^2	
A	68.0	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
B	67.6	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
C	66.9	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
D	65.5	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
E	63.8	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
F	62.4	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
G	61.5	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
H	61.0	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6

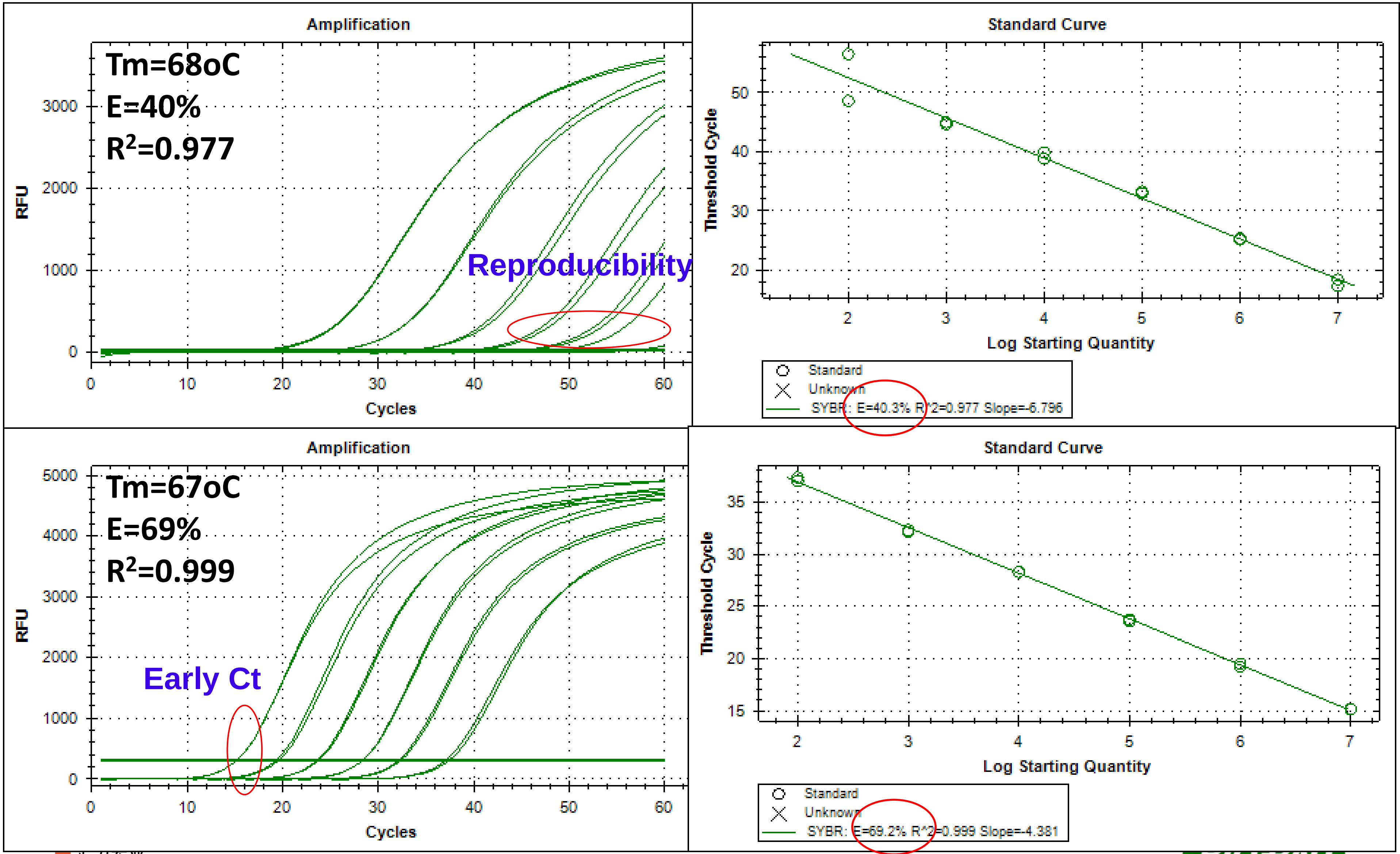
一对引物在每一个温度下，做一个模板稀释序列 (每个稀释两个重复），引物浓度固定。

利用温度梯度优化体系

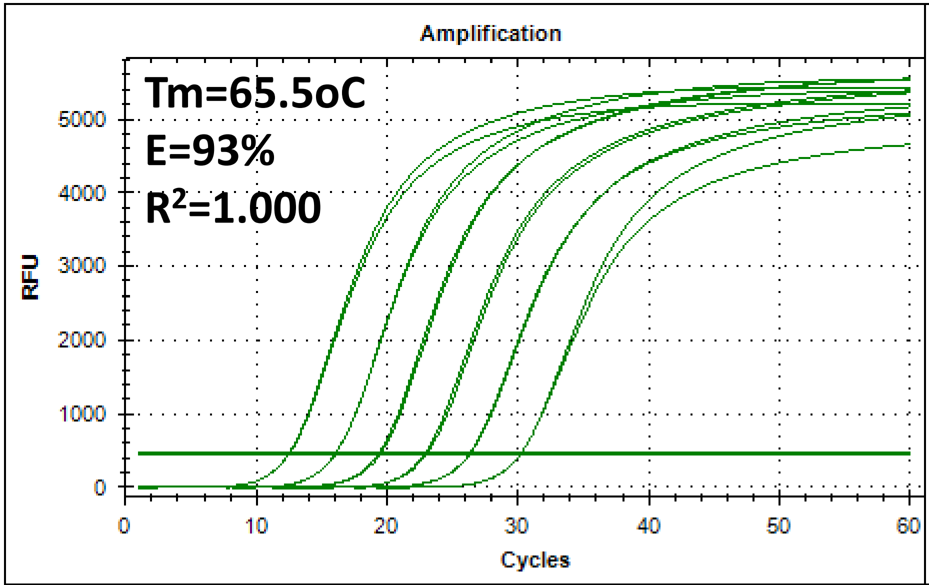
检查特异性



利用温度梯度优化退火温度



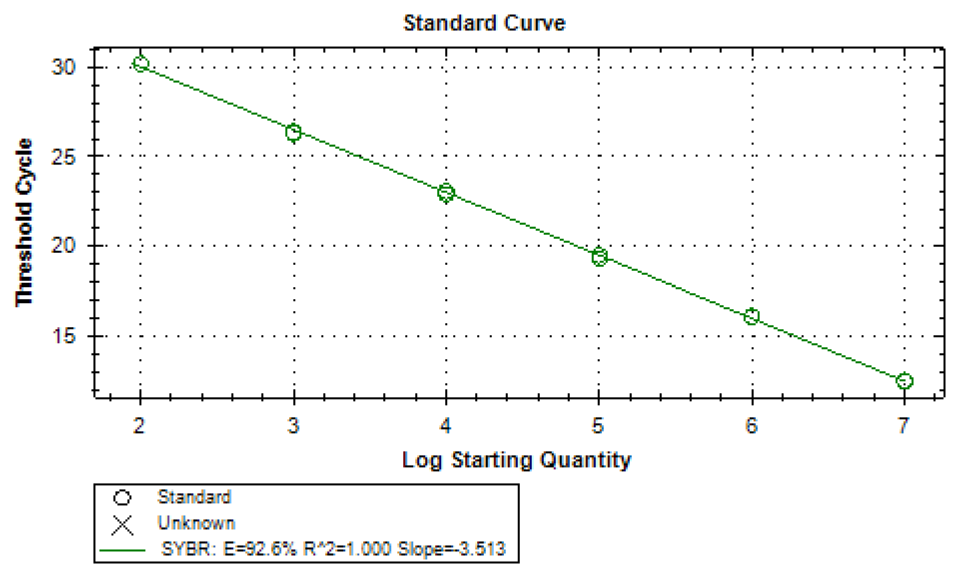
利用温度梯度优化退火温度

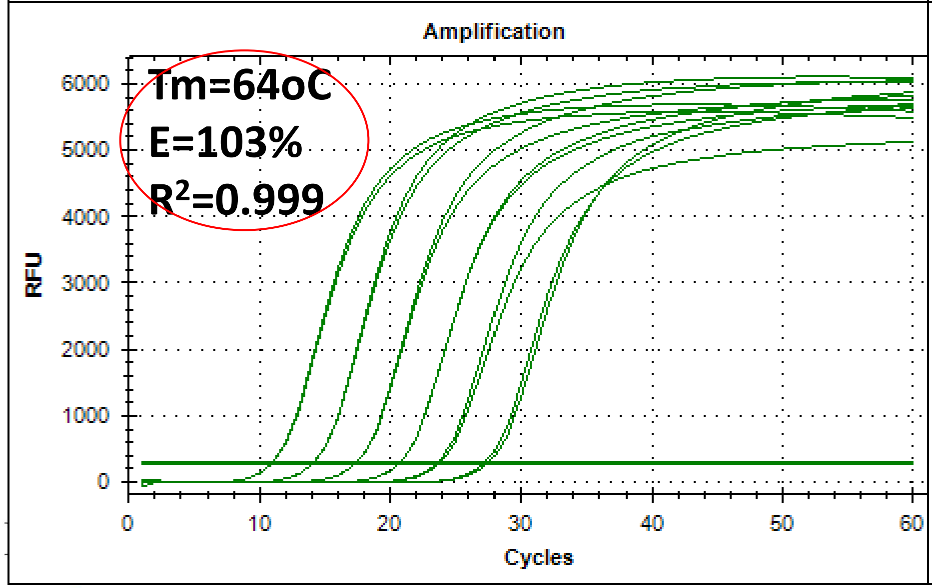


Tm=65.5oC

E=93%

R^2=1.000

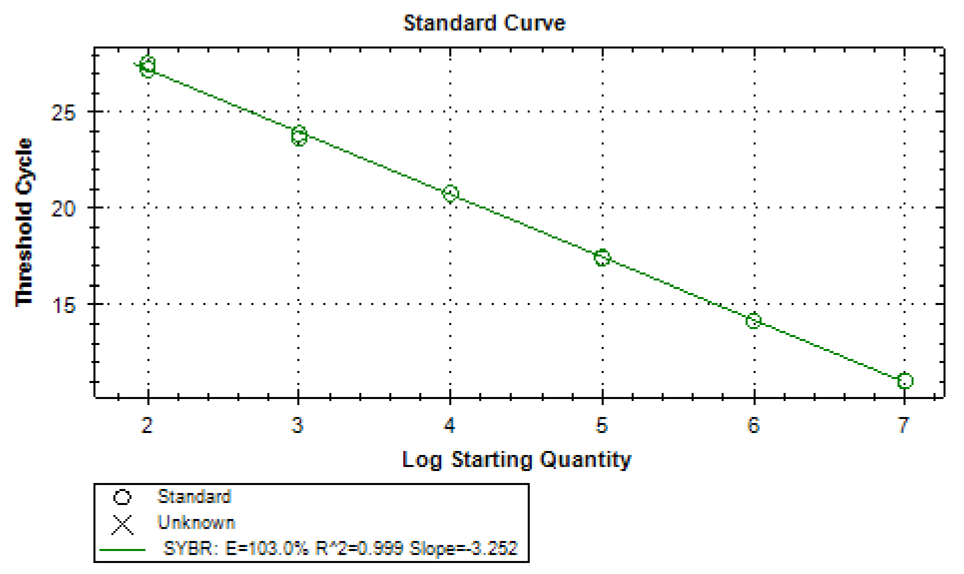




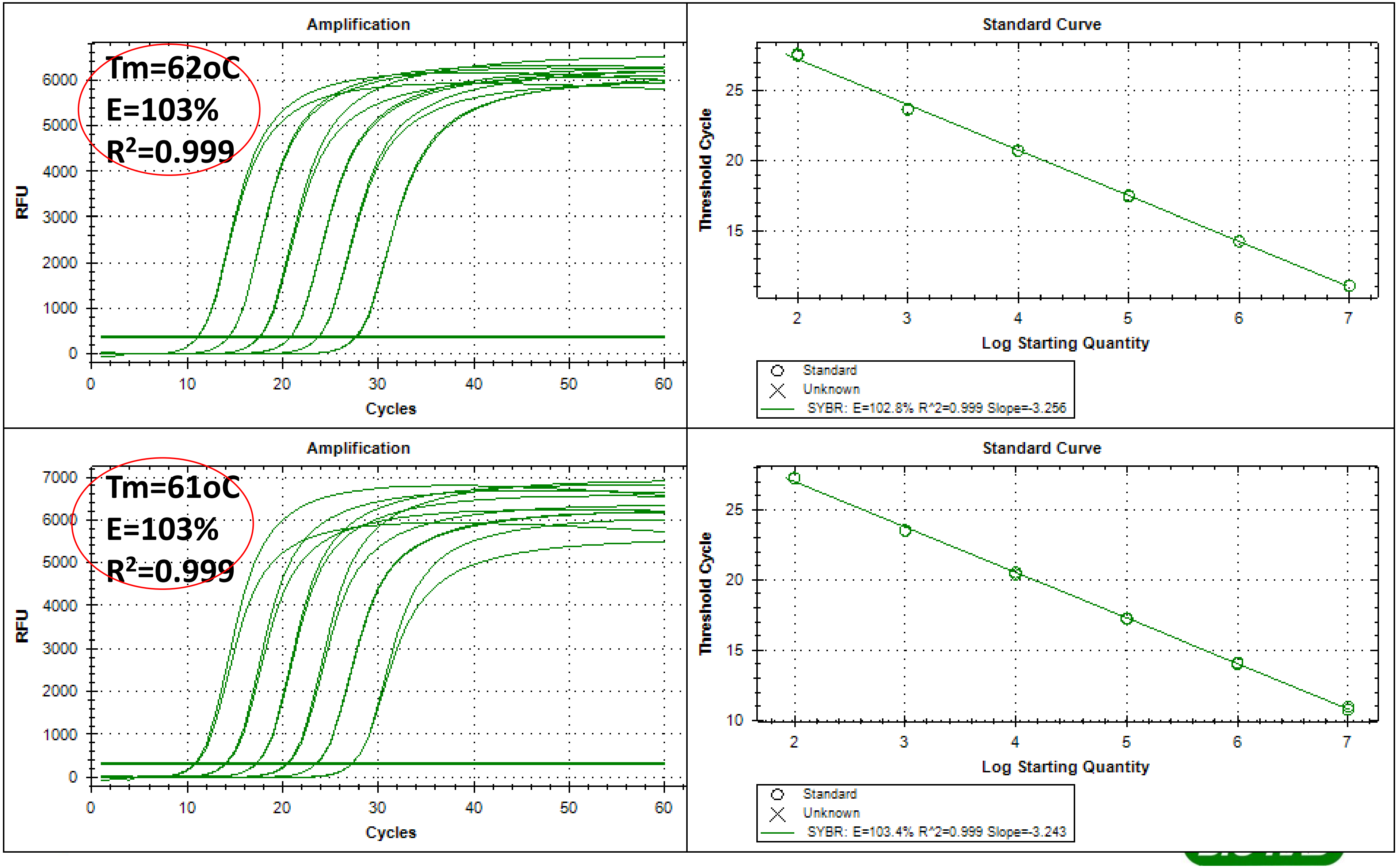
Tm=64oC

E=103%

R^2=0.999



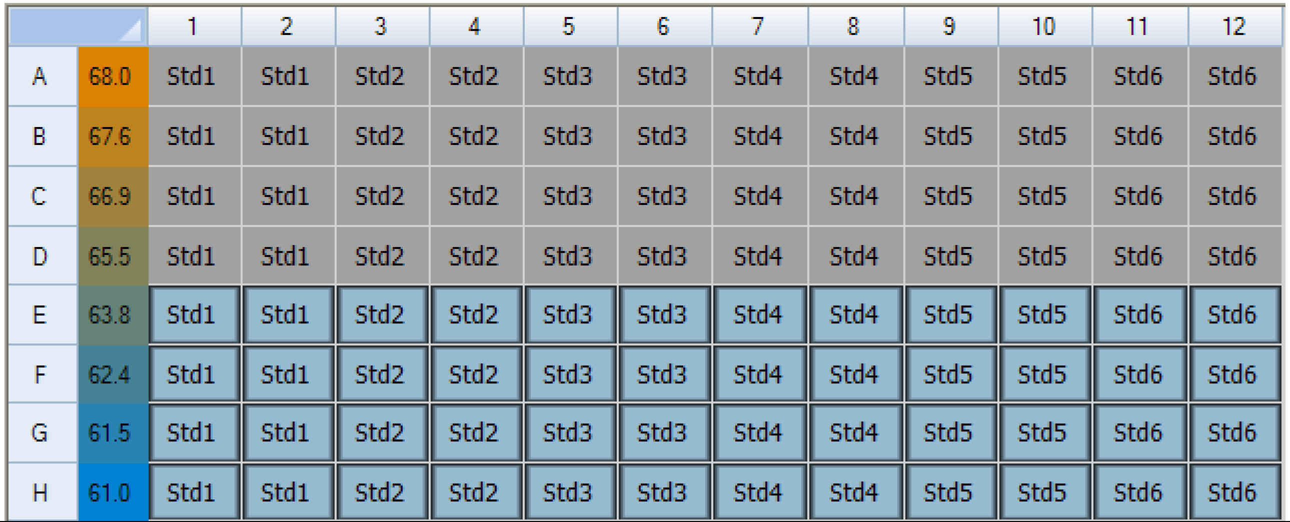
利用温度梯度优化体系


Tm=62oC
E=103%
R^2=0.999

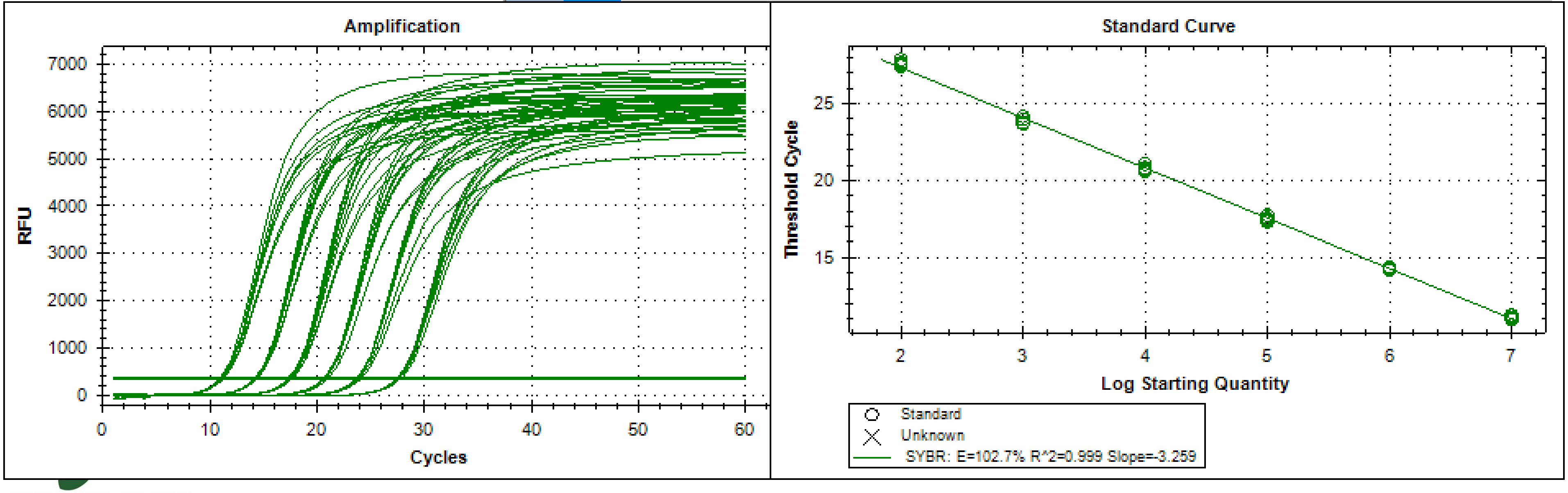
Tm=61oC
E=103%
R^2=0.999

利用温度梯度优化体系

<u>Optimal Conditions</u>
TA = 61 - 64C
E=103%
R^2=0.999



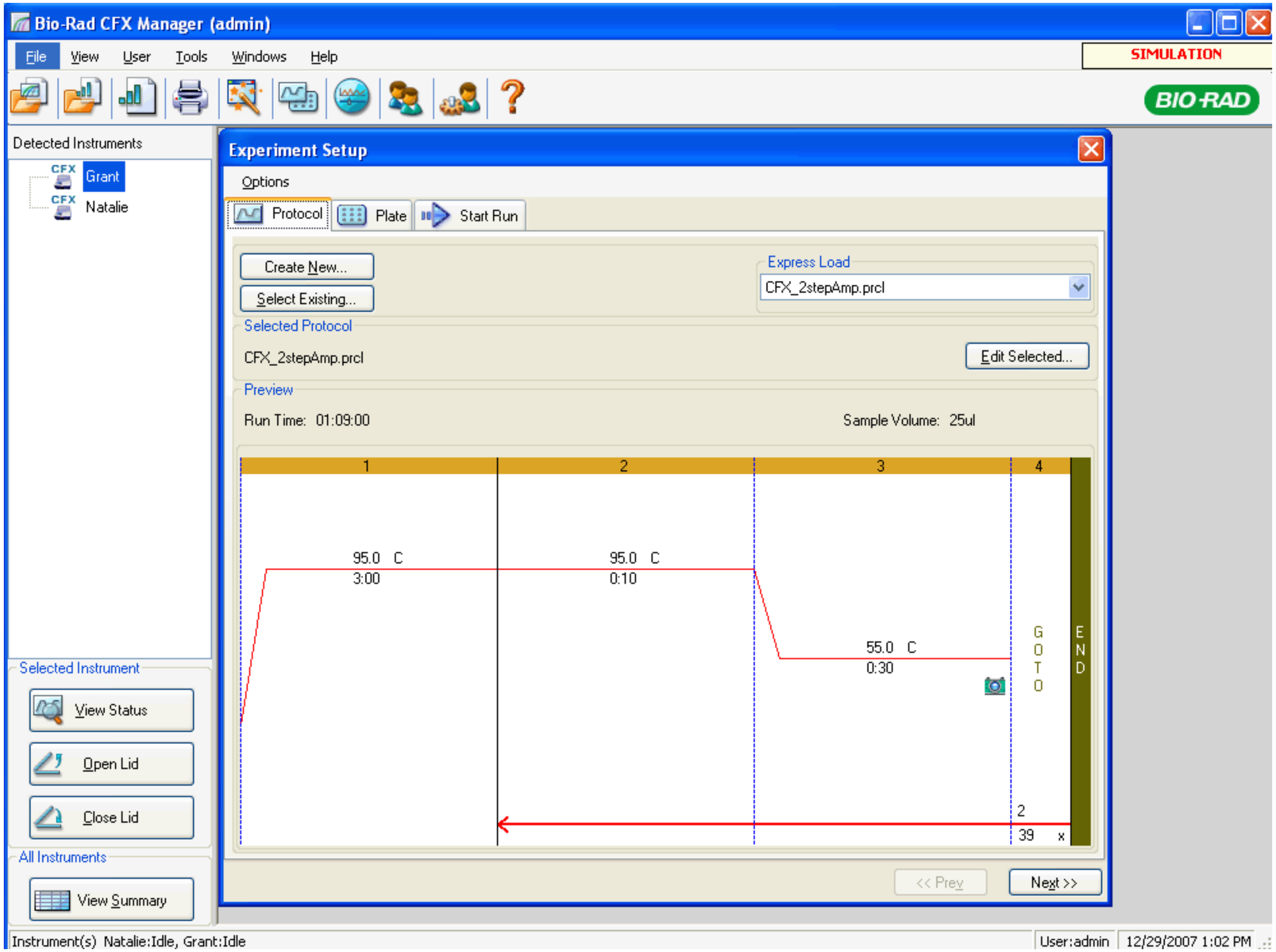
		1	2	3	4	5	6	7	8	9	10	11	12
A	68.0	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
B	67.6	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
C	66.9	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
D	65.5	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
E	63.8	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
F	62.4	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
G	61.5	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6
H	61.0	Std1	Std1	Std2	Std2	Std3	Std3	Std4	Std4	Std5	Std5	Std6	Std6



什么是好的qPCR supermix?

- 高灵敏度，特异性，重复性
- 高扩增效率（数据才可靠）
- 适用难扩增的基因，如GC含量高
- 对模板中的抑制剂耐受（如血液、植物、土壤等复杂样品中提取的核酸，或使用Trizol抽提的核酸
- 试剂稳定（小体积反应，放置较长时间）
- 兼容广泛的扩增条件，减少优化
  - 引物浓度
  - 退火延伸温度
- 速度更快

CFX maestro——符合国际标准的分析软件



Express Load: CFX_2stepAmp.prcl

Selected Protocol: CFX_2stepAmp.prcl

Run Time: 01:09:00

Sample Volume: 25ul

Instrument(s) Natalie:Idle, Grant:Idle | User:admin | 12/29/2007 1:02 PM

CFX maestro——符合国际标准的分析软件

- 内置基因表达功能，有多种基因分析模式，如多参照基因分析 **(Vandesompele Model)**，根据每个基因的扩增效率分析（**Pfaffl Model**），选择内参基因
- 多板合并功能，能够同时分析多次实验的数据
- 批次间差异校正

符合MIQE理念的逻辑运算算法

PCR扩增效率
Normalization
Reference gene

△△ Ct

数据分析——归一化

- Relative Quantity (△Cq)

– Not normalized

– Normalization accomplished via equal loading of samples

– Post analysis normalization

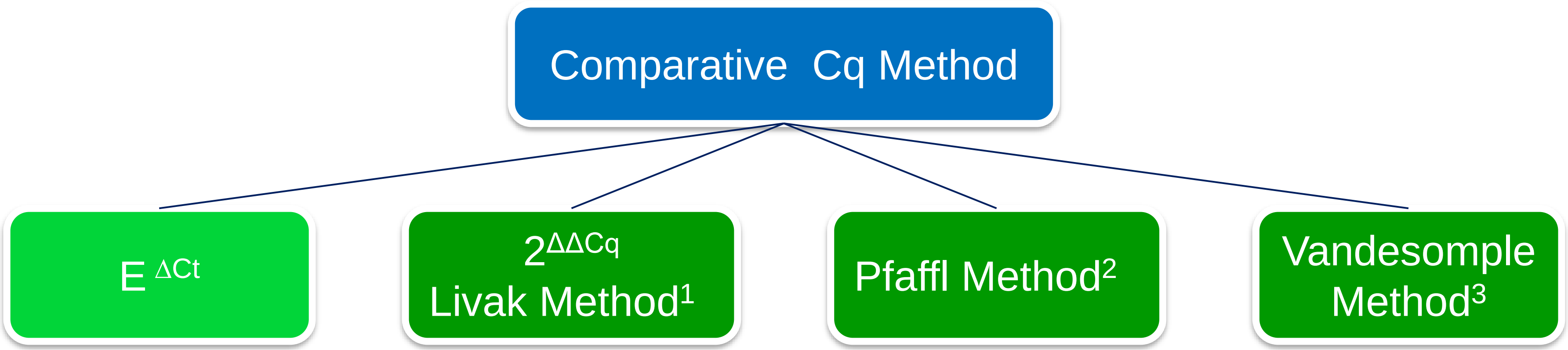
- Normalized Expression (△△Cq)

– Accounts for loading differences

– Usually normalize to reference gene

– Relative quantity of Target is normalized by the relative quantity of the reference genes

基因表达分析（相对定量）的计算方法



Comparative Cq Method

$E^{\Delta Ct}$

□ No normalized, equal loading of samples

$2^{\Delta\Delta Cq}$ Livak Method[1]

- Hypothesis of 100% PCR efficiency, 1 reference gene

$$NRQ = 2^{\Delta\Delta Cq}$$

Pfaffl Method[2]

- adjusted PCR efficiency, 1 reference gene

$$NRQ = \frac{E_{goi}^{\Delta Cq,goi}}{E_{ref}^{\Delta Cq,ref}}$$

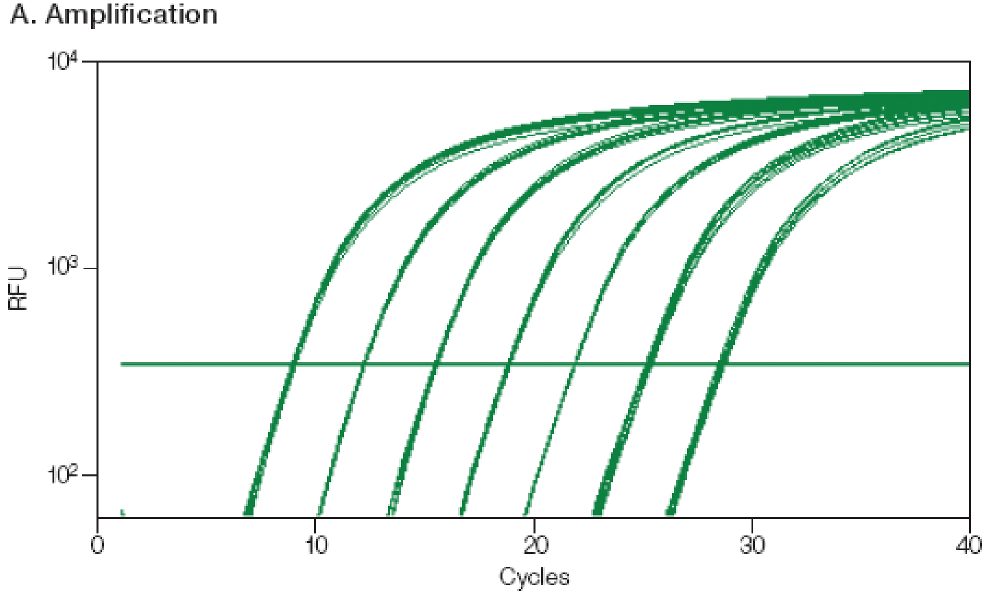
Vandesomple Method[3]

- adjusted PCR efficiency, multiple reference genes

$$NRQ = \frac{E_{goi}^{\Delta Cq,goi}}{\sqrt[n]{\prod_{i}^{n} E_{ref}^{\Delta Cq,ref}}}$$

CFX Maestro Software provides all mathematical models.

数据分析——归一化


A. Amplification

$$X_n = X_0 \times (1 + E_X)^n,$$

↓

E靶基因 = E内参

$$\frac{X_{N,q}}{X_{N,cb}} = \frac{K \times (1 + E)^{-\Delta C_{T,q}}}{K \times (1 + E)^{-\Delta C_{T,cb}}} = (1 + E)^{-\Delta\Delta C_T}.$$

↓

E=100%

$$2^{-\Delta\Delta C_T}.$$

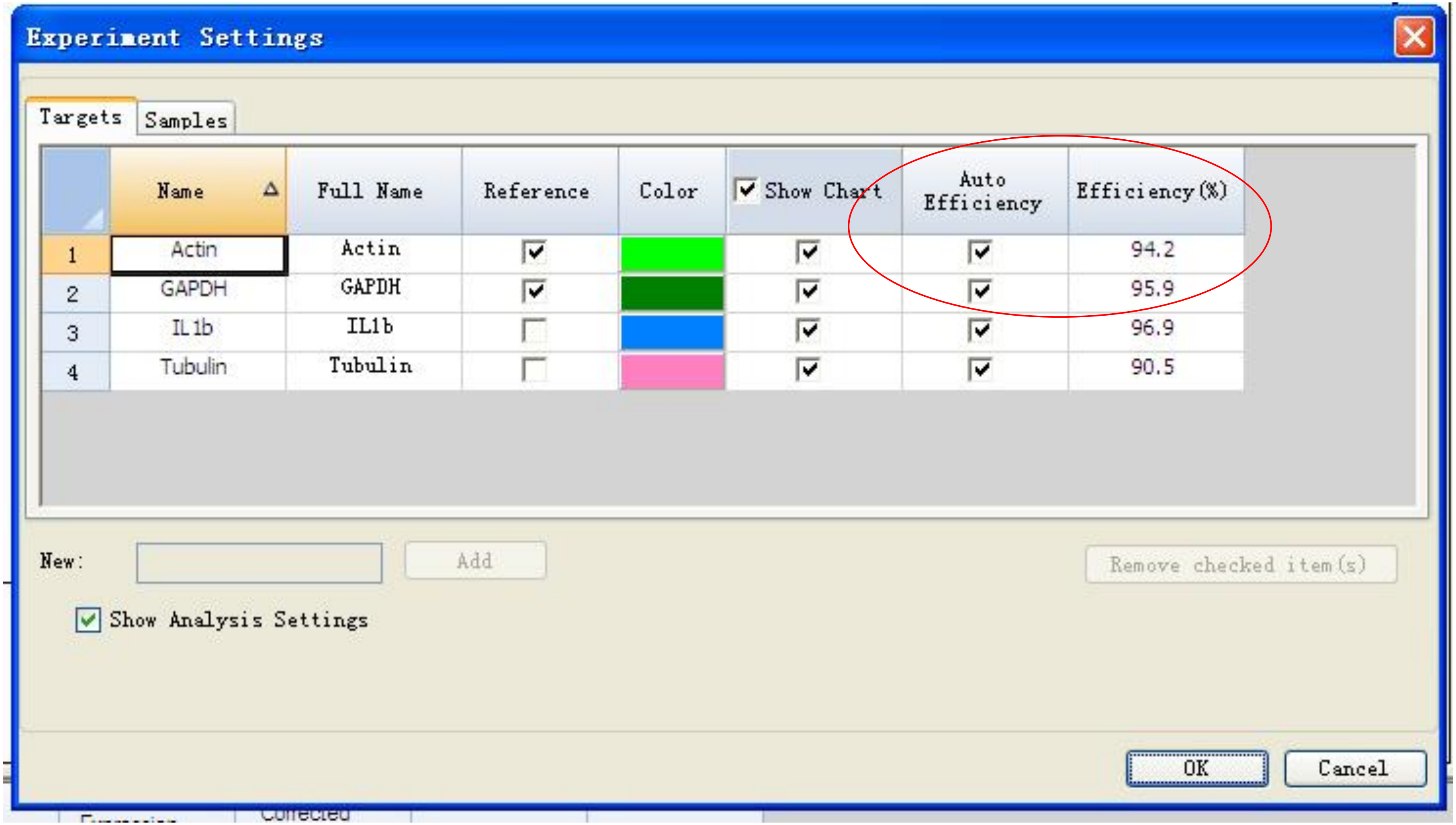
相对定量 - Pfaffl modifcation

	Reference Cq	Target gene Cq
Tissue #1: (sample)	21	22
Tissue #2: (control)	20	24

(From Standard curve)	Efficiency:	90% = 1.9	100% = 2
	Delta Cq:	20-21 = -1	24-22 = 2

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

相对定量的数据处理法选择



Experiment Settings

Targets | Samples

	Name	Full Name	Reference	Color	☑ Show Chart	Auto Efficiency	Efficiency(%)
1	Actin	Actin	☑	🟩	☑	☑	94.2
2	GAPDH	GAPDH	☑	🟩	☑	☑	95.9
3	IL1b	IL1b	☐	🟦	☑	☑	96.9
4	Tubulin	Tubulin	☐	🟪	☑	☑	90.5

New:

☑ Show Analysis Settings

内参基因的选择

选择正确的内参基因对于qPCR实验是必不可少的

- 由于生物系统的复杂性，没有任何一个内参基因对于所以的实验都是适用的。
- 正确的内参基因必须保证在实验系统中恒定表达（通常是组织特异的）。
- 使用未经验证的单内参基因将导致明显的偏差(25%的例子中这种偏差超过3倍，而10%的例子中多达6倍)
- **使用通过验证的多内参基因作为校正子是最适当且普遍适用的校正方法**。

内参基因验证

关键词：源文献，GeNorm

内参基因选择：

Google Scholar搜索

‘qPCR reference gene genorm [your organism/tissue of interest]

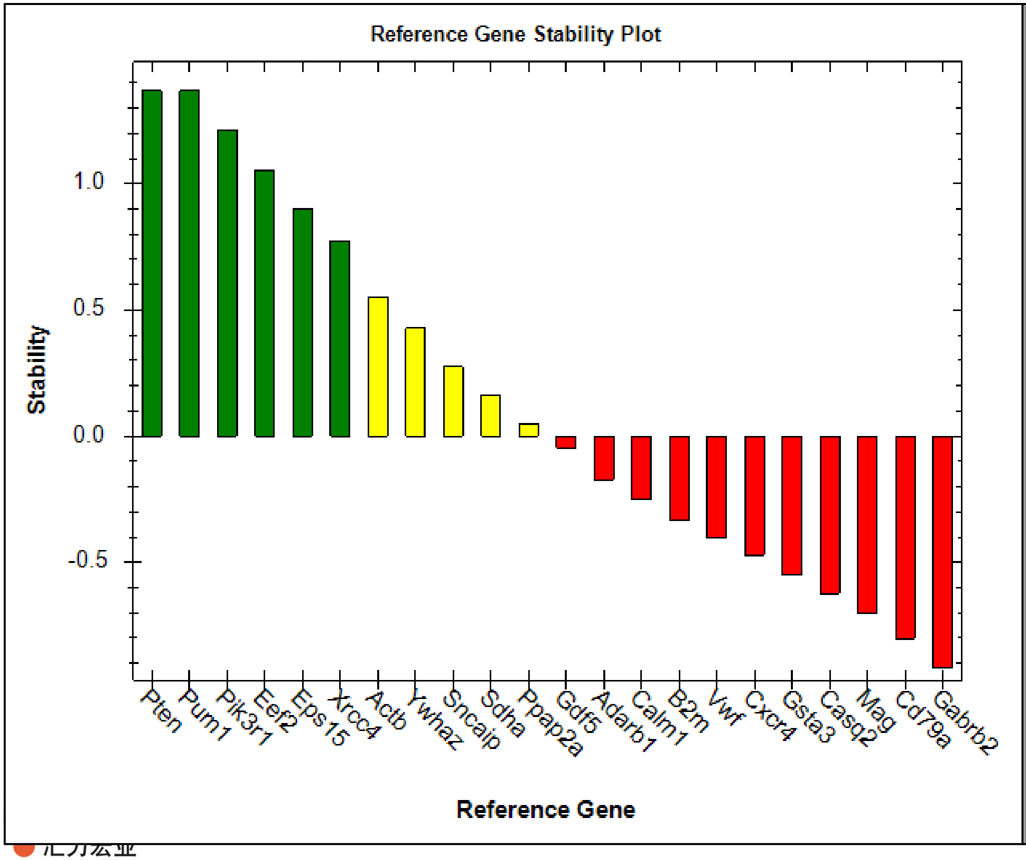
选取文献中报道的6-10个在靶生物/组织中稳定表达的内参基因

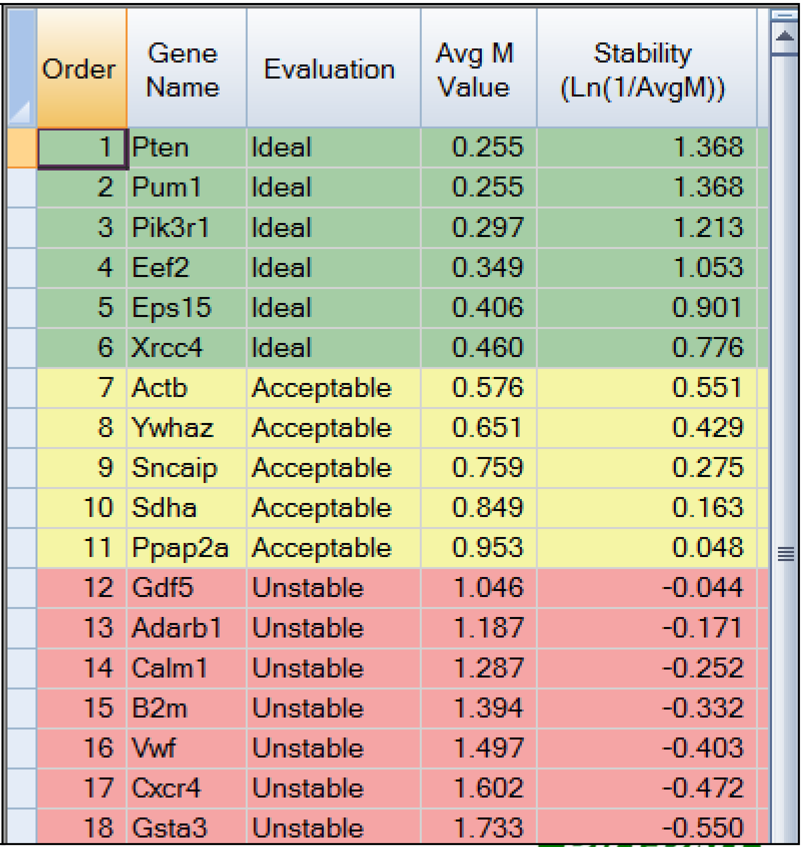
□ 如何筛选和验证某研究中适合的参考基因?

- 选择8-10个备选参考基因，以及每种处理下~10个代表性样本
- 备选参考基因应代表不同的生物学途径，具有不同的表达水平
- qPCR测定每个样本中每个备选参考基因的相对含量（以表达量最高的样本为对照）
- **CFX™ Maestro software**可自动计算每个备选参考基因的M值 (参考基因稳定值，reference gene stability value).
  - M值越小，对应的参考基因表达越稳定.
  - 多数情况下，2-3个参考基因有助于提高数据的可靠性

内参基因的选择和验证

- CFX Maestro 软件自动筛选某研究的最适参考基因
  - ideal (**Green**): 可任意选择，用作本研究的参考基因
  - Acceptable(**Yellow**): 选择至少3个或更多参考基因用于该研究.
  - Unstable(**Red**): 均不可用作本研究的参考基因





Order	Gene Name	Evaluation	Avg M Value	Stability (Ln(1/AvgM))
1	Pten	Ideal	0.255	1.368
2	Pum1	Ideal	0.255	1.368
3	Pik3r1	Ideal	0.297	1.213
4	Eef2	Ideal	0.349	1.053
5	Eps15	Ideal	0.406	0.901
6	Xrcc4	Ideal	0.460	0.776
7	Actb	Acceptable	0.576	0.551
8	Ywhaz	Acceptable	0.651	0.429
9	Sncaip	Acceptable	0.759	0.275
10	Sdha	Acceptable	0.849	0.163
11	Ppap2a	Acceptable	0.953	0.048
12	Gdf5	Unstable	1.046	-0.044
13	Adarb1	Unstable	1.187	-0.171
14	Calm1	Unstable	1.287	-0.252
15	B2m	Unstable	1.394	-0.332
16	Vwf	Unstable	1.497	-0.403
17	Cxcr4	Unstable	1.602	-0.472
18	Gsta3	Unstable	1.733	-0.550

多板合并分析与批间差校正

- 什么是批间校正（inter-run calibration）？
  - 修正相同基因在不同qPCR检测批次之间的Cq值差异的数据处理过程

- 为什需要inter-run calibration?
  - qPCR仪器通量有限，必须多次运行
  - 随机误差 (PCR block, lamp, filters, detectors, and so on),
  - 数据分析设置 (扩增曲线拟合、基线扣除、阈值线设置),
  - 试剂耗材的批间差 (反复冻融，荧光素衰减等)

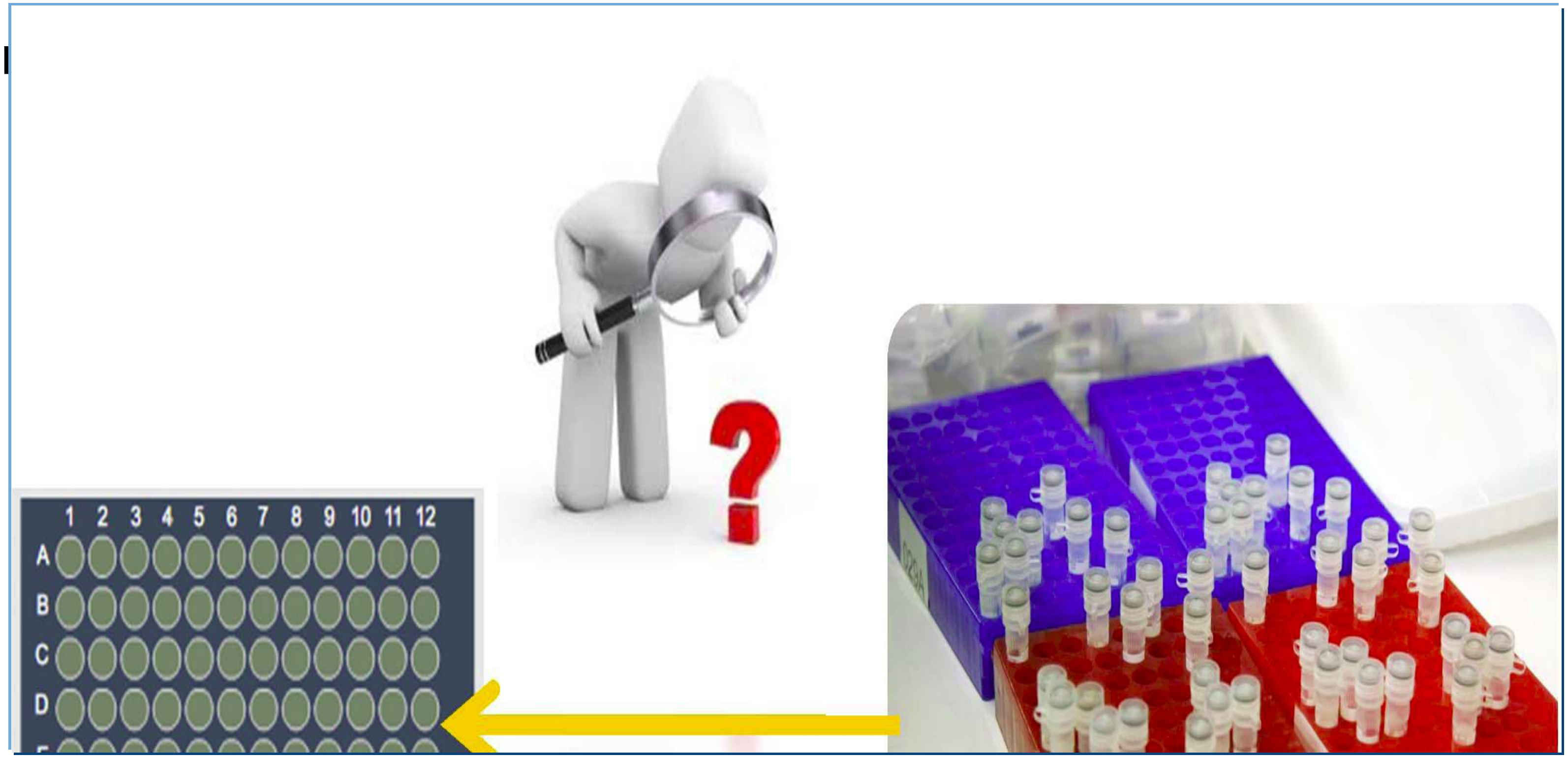
- 如何修正批间差（with inter-run calibrators, IRCs)?
  - 在各运行批次间分别测试1个或多组完全相同的样本。
  - 这些相同的样本在不同Plate上的Cq值会不一样

- How does it work?
  - For example……

关于IRC（inter-run calibration）

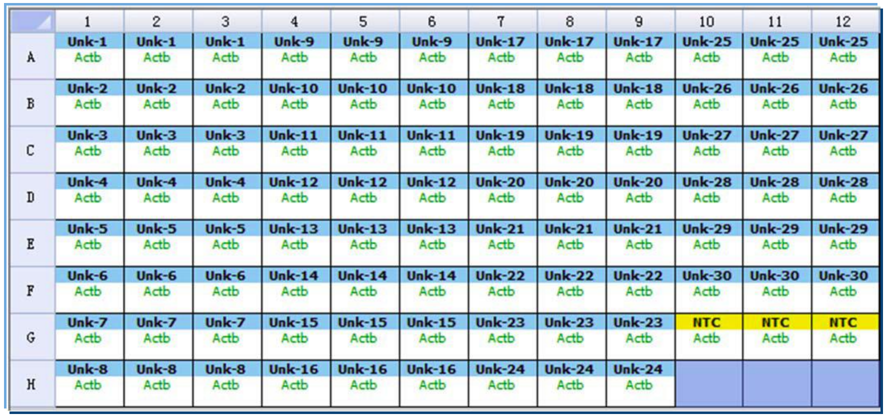
例如有一个基因表达分析实验，包括4个不同基因(Actb, GAPDH, P53, EGFR)以及30个不同的cDNA样品。

- 两种样品检测布局策略: Gene maximization and Sample

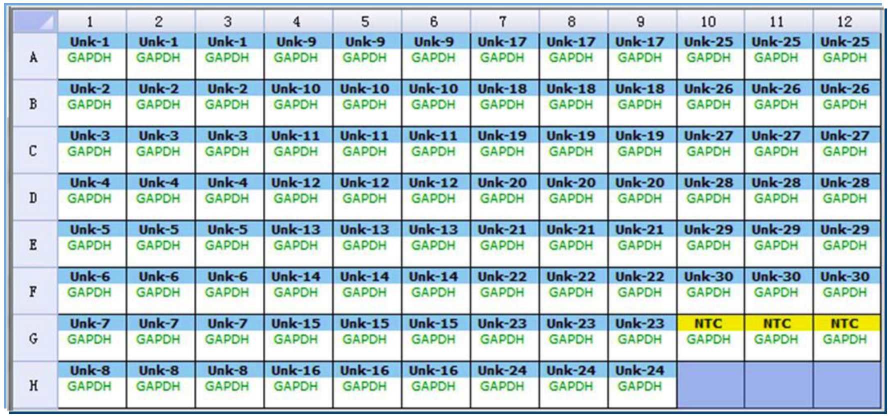


关于IRC（inter-run calibration）

Sample maximization method



	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk-1 Actb	Unk-1 Actb	Unk-1 Actb	Unk-9 Actb	Unk-9 Actb	Unk-9 Actb	Unk-17 Actb	Unk-17 Actb	Unk-17 Actb	Unk-25 Actb	Unk-25 Actb	Unk-25 Actb
B	Unk-2 Actb	Unk-2 Actb	Unk-2 Actb	Unk-10 Actb	Unk-10 Actb	Unk-10 Actb	Unk-18 Actb	Unk-18 Actb	Unk-18 Actb	Unk-26 Actb	Unk-26 Actb	Unk-26 Actb
C	Unk-3 Actb	Unk-3 Actb	Unk-3 Actb	Unk-11 Actb	Unk-11 Actb	Unk-11 Actb	Unk-19 Actb	Unk-19 Actb	Unk-19 Actb	Unk-27 Actb	Unk-27 Actb	Unk-27 Actb
D	Unk-4 Actb	Unk-4 Actb	Unk-4 Actb	Unk-12 Actb	Unk-12 Actb	Unk-12 Actb	Unk-20 Actb	Unk-20 Actb	Unk-20 Actb	Unk-28 Actb	Unk-28 Actb	Unk-28 Actb
E	Unk-5 Actb	Unk-5 Actb	Unk-5 Actb	Unk-13 Actb	Unk-13 Actb	Unk-13 Actb	Unk-21 Actb	Unk-21 Actb	Unk-21 Actb	Unk-29 Actb	Unk-29 Actb	Unk-29 Actb
F	Unk-6 Actb	Unk-6 Actb	Unk-6 Actb	Unk-14 Actb	Unk-14 Actb	Unk-14 Actb	Unk-22 Actb	Unk-22 Actb	Unk-22 Actb	Unk-30 Actb	Unk-30 Actb	Unk-30 Actb
G	Unk-7 Actb	Unk-7 Actb	Unk-7 Actb	Unk-15 Actb	Unk-15 Actb	Unk-15 Actb	Unk-23 Actb	Unk-23 Actb	Unk-23 Actb	NTC Actb	NTC Actb	NTC Actb
H	Unk-8 Actb	Unk-8 Actb	Unk-8 Actb	Unk-16 Actb	Unk-16 Actb	Unk-16 Actb	Unk-24 Actb	Unk-24 Actb	Unk-24 Actb			



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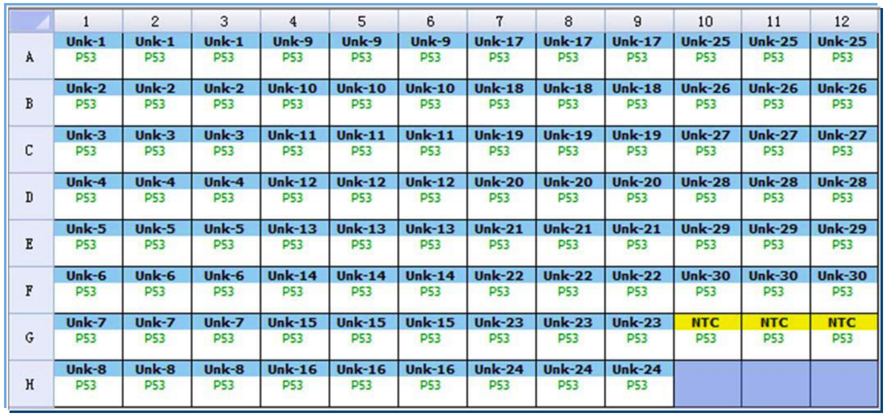
A	Unk-1 GAPDH	Unk-1 GAPDH	Unk-1 GAPDH	Unk-9 GAPDH	Unk-9 GAPDH	Unk-9 GAPDH	Unk-17 GAPDH	Unk-17 GAPDH	Unk-17 GAPDH	Unk-25 GAPDH	Unk-25 GAPDH	Unk-25 GAPDH
B	Unk-2 GAPDH	Unk-2 GAPDH	Unk-2 GAPDH	Unk-10 GAPDH	Unk-10 GAPDH	Unk-10 GAPDH	Unk-18 GAPDH	Unk-18 GAPDH	Unk-18 GAPDH	Unk-26 GAPDH	Unk-26 GAPDH	Unk-26 GAPDH
C	Unk-3 GAPDH	Unk-3 GAPDH	Unk-3 GAPDH	Unk-11 GAPDH	Unk-11 GAPDH	Unk-11 GAPDH	Unk-19 GAPDH	Unk-19 GAPDH	Unk-19 GAPDH	Unk-27 GAPDH	Unk-27 GAPDH	Unk-27 GAPDH
D	Unk-4 GAPDH	Unk-4 GAPDH	Unk-4 GAPDH	Unk-12 GAPDH	Unk-12 GAPDH	Unk-12 GAPDH	Unk-20 GAPDH	Unk-20 GAPDH	Unk-20 GAPDH	Unk-28 GAPDH	Unk-28 GAPDH	Unk-28 GAPDH
E	Unk-5 GAPDH	Unk-5 GAPDH	Unk-5 GAPDH	Unk-13 GAPDH	Unk-13 GAPDH	Unk-13 GAPDH	Unk-21 GAPDH	Unk-21 GAPDH	Unk-21 GAPDH	Unk-29 GAPDH	Unk-29 GAPDH	Unk-29 GAPDH
F	Unk-6 GAPDH	Unk-6 GAPDH	Unk-6 GAPDH	Unk-14 GAPDH	Unk-14 GAPDH	Unk-14 GAPDH	Unk-22 GAPDH	Unk-22 GAPDH	Unk-22 GAPDH	Unk-30 GAPDH	Unk-30 GAPDH	Unk-30 GAPDH
G	Unk-7 GAPDH	Unk-7 GAPDH	Unk-7 GAPDH	Unk-15 GAPDH	Unk-15 GAPDH	Unk-15 GAPDH	Unk-23 GAPDH	Unk-23 GAPDH	Unk-23 GAPDH	NTC GAPDH	NTC GAPDH	NTC GAPDH
H	Unk-8 GAPDH	Unk-8 GAPDH	Unk-8 GAPDH	Unk-16 GAPDH	Unk-16 GAPDH	Unk-16 GAPDH	Unk-24 GAPDH	Unk-24 GAPDH	Unk-24 GAPDH			



	1	2	3	4	5	6	7	8	9	10	11	12
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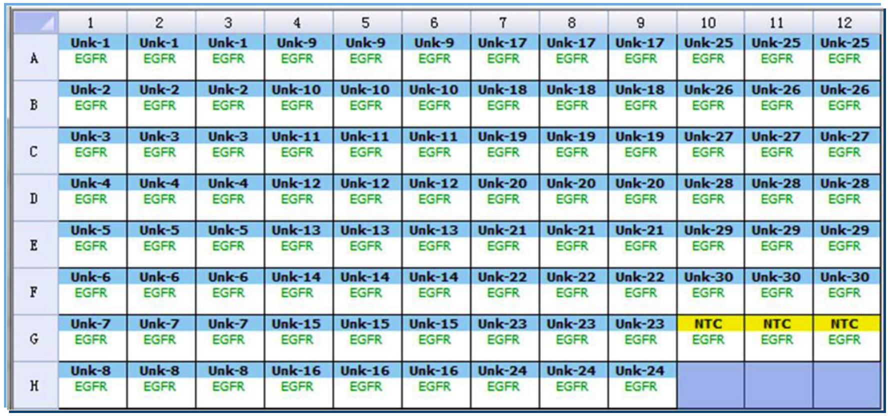
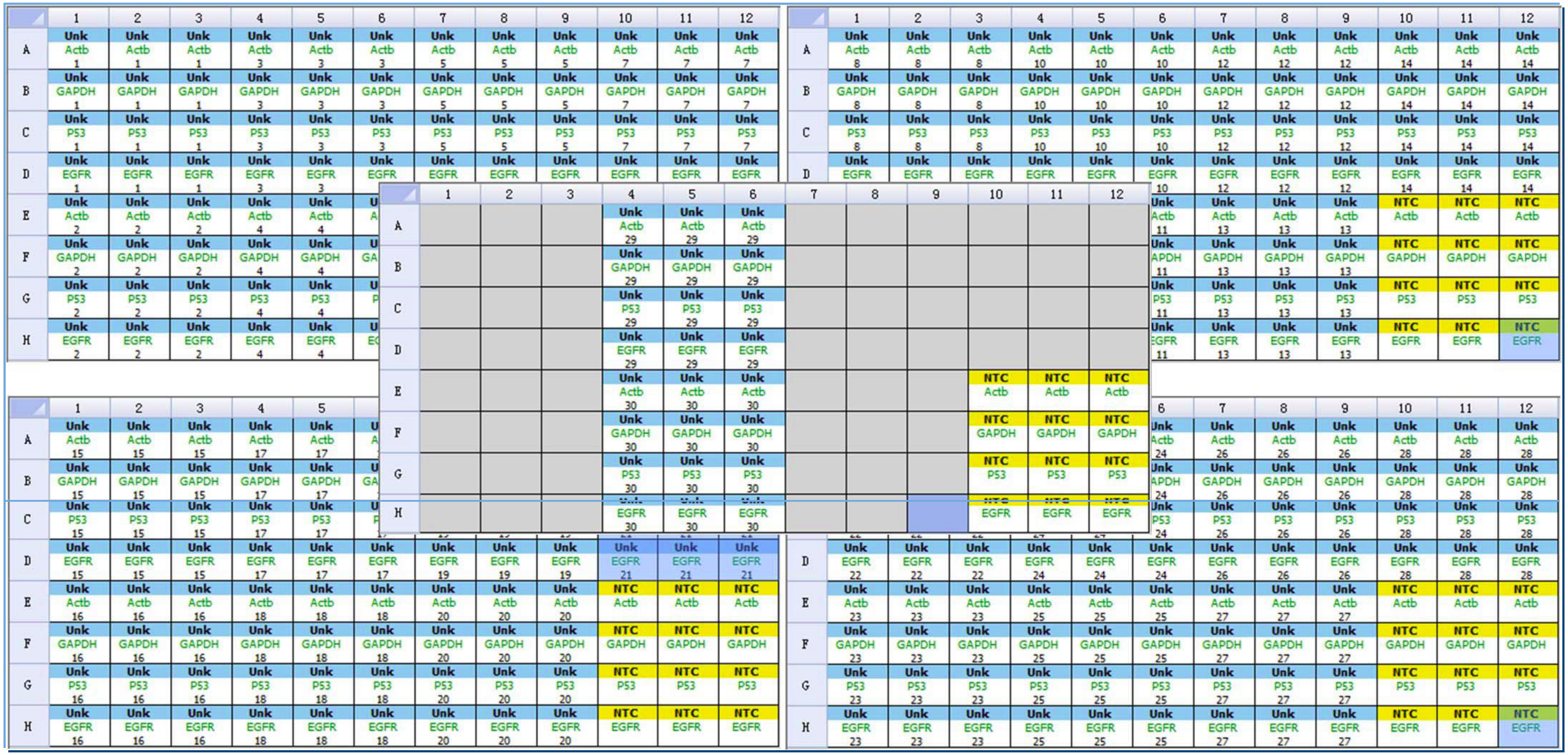
B	Unk-2 P53	Unk-2 P53	Unk-2 P53	Unk-10 P53	Unk-10 P53	Unk-10 P53	Unk-18 P53	Unk-18 P53	Unk-18 P53	Unk-26 P53	Unk-26 P53	Unk-26 P53
C	Unk-3 P53	Unk-3 P53	Unk-3 P53	Unk-11 P53	Unk-11 P53	Unk-11 P53	Unk-19 P53	Unk-19 P53	Unk-19 P53	Unk-27 P53	Unk-27 P53	Unk-27 P53
D	Unk-4 P53	Unk-4 P53	Unk-4 P53	Unk-12 P53	Unk-12 P53	Unk-12 P53	Unk-20 P53	Unk-20 P53	Unk-20 P53	Unk-28 P53	Unk-28 P53	Unk-28 P53
E	Unk-5 P53	Unk-5 P53	Unk-5 P53	Unk-13 P53	Unk-13 P53	Unk-13 P53	Unk-21 P53	Unk-21 P53	Unk-21 P53	Unk-29 P53	Unk-29 P53	Unk-29 P53
F	Unk-6 P53	Unk-6 P53	Unk-6 P53	Unk-14 P53	Unk-14 P53	Unk-14 P53	Unk-22 P53	Unk-22 P53	Unk-22 P53	Unk-30 P53	Unk-30 P53	Unk-30 P53
G	Unk-7 P53	Unk-7 P53	Unk-7 P53	Unk-15 P53	Unk-15 P53	Unk-15 P53	Unk-23 P53	Unk-23 P53	Unk-23 P53	NTC P53	NTC P53	NTC P53
H	Unk-8 P53	Unk-8 P53	Unk-8 P53	Unk-16 P53	Unk-16 P53	Unk-16 P53	Unk-24 P53	Unk-24 P53	Unk-24 P53			



	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk-1 EGFR	Unk-1 EGFR	Unk-1 EGFR	Unk-9 EGFR	Unk-9 EGFR	Unk-9 EGFR	Unk-17 EGFR	Unk-17 EGFR	Unk-17 EGFR	Unk-25 EGFR	Unk-25 EGFR	Unk-25 EGFR
B	Unk-2 EGFR	Unk-2 EGFR	Unk-2 EGFR	Unk-10 EGFR	Unk-10 EGFR	Unk-10 EGFR	Unk-18 EGFR	Unk-18 EGFR	Unk-18 EGFR	Unk-26 EGFR	Unk-26 EGFR	Unk-26 EGFR
C	Unk-3 EGFR	Unk-3 EGFR	Unk-3 EGFR	Unk-11 EGFR	Unk-11 EGFR	Unk-11 EGFR	Unk-19 EGFR	Unk-19 EGFR	Unk-19 EGFR	Unk-27 EGFR	Unk-27 EGFR	Unk-27 EGFR
D	Unk-4 EGFR	Unk-4 EGFR	Unk-4 EGFR	Unk-12 EGFR	Unk-12 EGFR	Unk-12 EGFR	Unk-20 EGFR	Unk-20 EGFR	Unk-20 EGFR	Unk-28 EGFR	Unk-28 EGFR	Unk-28 EGFR
E	Unk-5 EGFR	Unk-5 EGFR	Unk-5 EGFR	Unk-13 EGFR	Unk-13 EGFR	Unk-13 EGFR	Unk-21 EGFR	Unk-21 EGFR	Unk-21 EGFR	Unk-29 EGFR	Unk-29 EGFR	Unk-29 EGFR
F	Unk-6 EGFR	Unk-6 EGFR	Unk-6 EGFR	Unk-14 EGFR	Unk-14 EGFR	Unk-14 EGFR	Unk-22 EGFR	Unk-22 EGFR	Unk-22 EGFR	Unk-30 EGFR	Unk-30 EGFR	Unk-30 EGFR
G	Unk-7 EGFR	Unk-7 EGFR	Unk-7 EGFR	Unk-15 EGFR	Unk-15 EGFR	Unk-15 EGFR	Unk-23 EGFR	Unk-23 EGFR	Unk-23 EGFR	NTC EGFR	NTC EGFR	NTC EGFR
H	Unk-8 EGFR	Unk-8 EGFR	Unk-8 EGFR	Unk-16 EGFR	Unk-16 EGFR	Unk-16 EGFR	Unk-24 EGFR	Unk-24 EGFR	Unk-24 EGFR			

关于IRC（inter-run calibration）

gene maximization method



	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk Actb 1	Unk Actb 1	Unk Actb 1	Unk Actb 3	Unk Actb 3	Unk Actb 3	Unk Actb 5	Unk Actb 5	Unk Actb 5	Unk Actb 7	Unk Actb 7	Unk Actb 7
B	Unk GAPDH 1	Unk GAPDH 1	Unk GAPDH 1	Unk GAPDH 3	Unk GAPDH 3	Unk GAPDH 3	Unk GAPDH 5	Unk GAPDH 5	Unk GAPDH 5	Unk GAPDH 7	Unk GAPDH 7	Unk GAPDH 7
C	Unk P53 1	Unk P53 1	Unk P53 1	Unk P53 3	Unk P53 3	Unk P53 3	Unk P53 5	Unk P53 5	Unk P53 5	Unk P53 7	Unk P53 7	Unk P53 7
D	Unk EGFR 1	Unk EGFR 1	Unk EGFR 1	Unk EGFR 3	Unk EGFR 3	Unk EGFR	Unk EGFR	Unk EGFR	Unk EGFR	Unk EGFR	Unk EGFR	Unk EGFR
E	Unk Actb 2	Unk Actb 2	Unk Actb 2	Unk Actb 4	Unk Actb 4	U A						
F	Unk GAPDH 2	Unk GAPDH 2	Unk GAPDH 2	Unk GAPDH 4	Unk GAPDH 4	U GA						
G	Unk P53 2	Unk P53 2	Unk P53 2	Unk P53 4	Unk P53 4	U P						
H	Unk EGFR 2	Unk EGFR 2	Unk EGFR 2	Unk EGFR 4	Unk EGFR 4	U E						

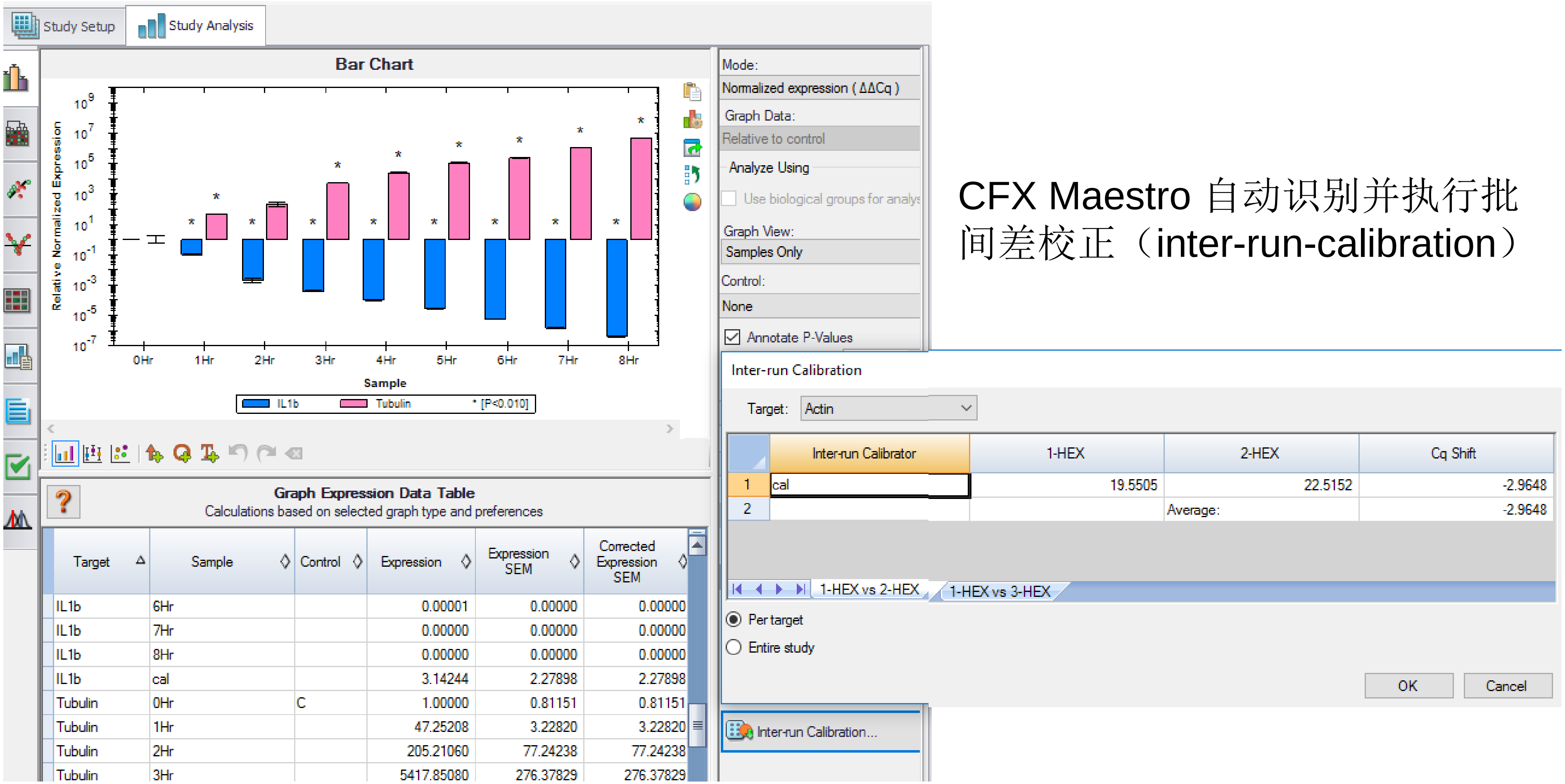
	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk Actb 8	Unk Actb 8	Unk Actb 8	Unk Actb 10	Unk Actb 10	Unk Actb 10	Unk Actb 12	Unk Actb 12	Unk Actb 12	Unk Actb 14	Unk Actb 14	Unk Actb 14
B	Unk GAPDH 8	Unk GAPDH 8	Unk GAPDH 8	Unk GAPDH 10	Unk GAPDH 10	Unk GAPDH 10	Unk GAPDH 12	Unk GAPDH 12	Unk GAPDH 12	Unk GAPDH 14	Unk GAPDH 14	Unk GAPDH 14
C	Unk P53 8	Unk P53 8	Unk P53 8	Unk P53 10	Unk P53 10	Unk P53 10	Unk P53 12	Unk P53 12	Unk P53 12	Unk P53 14	Unk P53 14	Unk P53 14
D	Unk EGFR	Unk EGFR	Unk EGFR	Unk EGFR	Unk EGFR	Unk EGFR 10	Unk EGFR 12	Unk EGFR 12	Unk EGFR 12	Unk EGFR 14	Unk EGFR 14	Unk EGFR 14
E						Unk Actb 11	Unk Actb 13	Unk Actb 13	Unk Actb 13	NTC Actb	NTC Actb	NTC Actb
F						Unk APDH 11	Unk GAPDH 13	Unk GAPDH 13	Unk GAPDH 13	NTC GAPDH	NTC GAPDH	NTC GAPDH
G						Unk P53 11	Unk P53 13	Unk P53 13	Unk P53 13	NTC P53	NTC P53	NTC P53
H						Unk GFR 11	Unk EGFR 13	Unk EGFR 13	Unk EGFR 13	NTC EGFR	NTC EGFR	NTC EGFR

	1	2	3	4	5	6	7	8	9	10	11	12
A				Unk Actb 29	Unk Actb 29	Unk Actb 29						
B				Unk GAPDH 29	Unk GAPDH 29	Unk GAPDH 29						
C				Unk P53 29	Unk P53 29	Unk P53 29						
D				Unk EGFR 29	Unk EGFR 29	Unk EGFR 29						
E				Unk Actb 30	Unk Actb 30	Unk Actb 30				NTC Actb	NTC Actb	NTC Actb
F				Unk GAPDH 30	Unk GAPDH 30	Unk GAPDH 30				NTC GAPDH	NTC GAPDH	NTC GAPDH
G				Unk P53 30	Unk P53 30	Unk P53 30				NTC P53	NTC P53	NTC P53
H				EGFR 30	EGFR 30	EGFR 30				EGFR	EGFR	EGFR

	1	2	3	4	5							
A	Unk Actb 15	Unk Actb 15	Unk Actb 15	Unk Actb 17	Unk Actb 17	U A						
B	Unk GAPDH 15	Unk GAPDH 15	Unk GAPDH 15	Unk GAPDH 17	Unk GAPDH 17	U GA						
C	Unk P53 15	Unk P53 15	Unk P53 15	Unk P53 17	Unk P53 17	U P						
D	Unk EGFR 15	Unk EGFR 15	Unk EGFR 15	Unk EGFR 17	Unk EGFR 17	Unk EGFR 17	Unk EGFR 19	Unk EGFR 19	Unk EGFR 19	Unk EGFR 21	Unk EGFR 21	Unk EGFR 21
E	Unk Actb 16	Unk Actb 16	Unk Actb 16	Unk Actb 18	Unk Actb 18	Unk Actb 18	Unk Actb 20	Unk Actb 20	Unk Actb 20	NTC Actb	NTC Actb	NTC Actb
F	Unk GAPDH 16	Unk GAPDH 16	Unk GAPDH 16	Unk GAPDH 18	Unk GAPDH 18	Unk GAPDH 18	Unk GAPDH 20	Unk GAPDH 20	Unk GAPDH 20	NTC GAPDH	NTC GAPDH	NTC GAPDH
G	Unk P53 16	Unk P53 16	Unk P53 16	Unk P53 18	Unk P53 18	Unk P53 18	Unk P53 20	Unk P53 20	Unk P53 20	NTC P53	NTC P53	NTC P53
H	Unk EGFR 16	Unk EGFR 16	Unk EGFR 16	Unk EGFR 18	Unk EGFR 18	Unk EGFR 18	Unk EGFR 20	Unk EGFR 20	Unk EGFR 20	NTC EGFR	NTC EGFR	NTC EGFR

						6	7	8	9	10	11	12
						Unk Actb 24	Unk Actb 26	Unk Actb 26	Unk Actb 26	Unk Actb 28	Unk Actb 28	Unk Actb 28
						Unk APDH 24	Unk GAPDH 26	Unk GAPDH 26	Unk GAPDH 26	Unk GAPDH 28	Unk GAPDH 28	Unk GAPDH 28
						Unk P53 24	Unk P53 26	Unk P53 26	Unk P53 26	Unk P53 28	Unk P53 28	Unk P53 28
D	Unk EGFR 22	Unk EGFR 22	Unk EGFR 22	Unk EGFR 24	Unk EGFR 24	Unk EGFR 24	Unk EGFR 26	Unk EGFR 26	Unk EGFR 26	Unk EGFR 28	Unk EGFR 28	Unk EGFR 28
E	Unk Actb 23	Unk Actb 23	Unk Actb 23	Unk Actb 25	Unk Actb 25	Unk Actb 25	Unk Actb 27	Unk Actb 27	Unk Actb 27	NTC Actb	NTC Actb	NTC Actb
F	Unk GAPDH 23	Unk GAPDH 23	Unk GAPDH 23	Unk GAPDH 25	Unk GAPDH 25	Unk GAPDH 25	Unk GAPDH 27	Unk GAPDH 27	Unk GAPDH 27	NTC GAPDH	NTC GAPDH	NTC GAPDH
G	Unk P53 23	Unk P53 23	Unk P53 23	Unk P53 25	Unk P53 25	Unk P53 25	Unk P53 27	Unk P53 27	Unk P53 27	NTC P53	NTC P53	NTC P53
H	Unk EGFR 23	Unk EGFR 23	Unk EGFR 23	Unk EGFR 25	Unk EGFR 25	Unk EGFR 25	Unk EGFR 27	Unk EGFR 27	Unk EGFR 27	NTC EGFR	NTC EGFR	NTC EGFR

多板合并分析与批间差校正（Gene study）



Mode: Normalized expression (ΔΔCq)

Graph Data: Relative to control

Analyze Using

☐ Use biological groups for analys

Graph View: Samples Only

Control: None

☑ Annotate P-Values

Graph Expression Data Table

Calculations based on selected graph type and preferences

Target	Sample	Control	Expression	Expression SEM	Corrected Expression SEM
IL1b	6Hr		0.00001	0.00000	0.00000
IL1b	7Hr		0.00000	0.00000	0.00000
IL1b	8Hr		0.00000	0.00000	0.00000
IL1b	cal		3.14244	2.27898	2.27898
Tubulin	0Hr	C	1.00000	0.81151	0.81151
Tubulin	1Hr		47.25208	3.22820	3.22820
Tubulin	2Hr		205.21060	77.24238	77.24238
Tubulin	3Hr		5417.85080	276.37829	276.37829

Inter-run Calibration

Target: Actin

	Inter-run Calibrator	1-HEX	2-HEX	Cq Shift
1	cal	19.5505	22.5152	-2.9648
2			Average:	-2.9648

● Per target

○ Entire study

CFX Maestro 自动识别并执行批间差校正（inter-run-calibration）

统计学检验

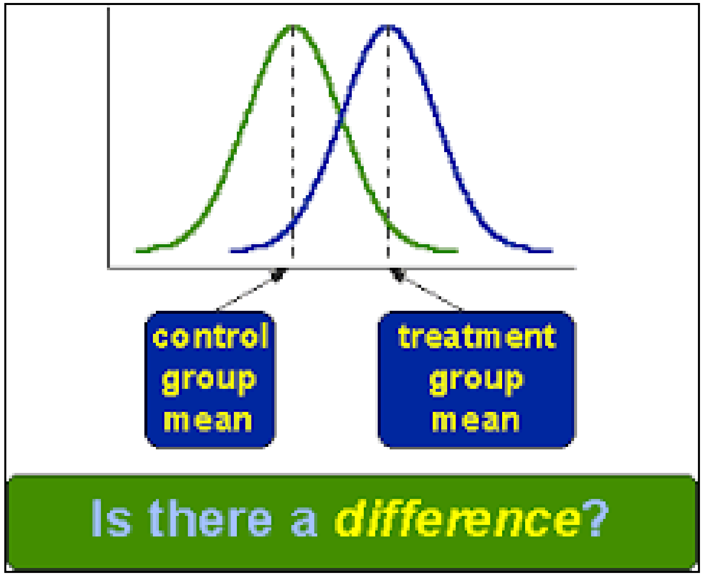
- 生物学数据的一个最重要特征是其不确定性.
  - 细胞经过处理后某基因相对于对照表达量升高

问题：是生物体本身的不确定性引起的，还是处理因素作用的结果？

- 如何对数据进行评价？
  - 显著性检验：利用统计学的方法，检验被处理的问题 是否存在 统计上的显著性差异。
  - CFX Maestro软件集成t-test和ANOVA

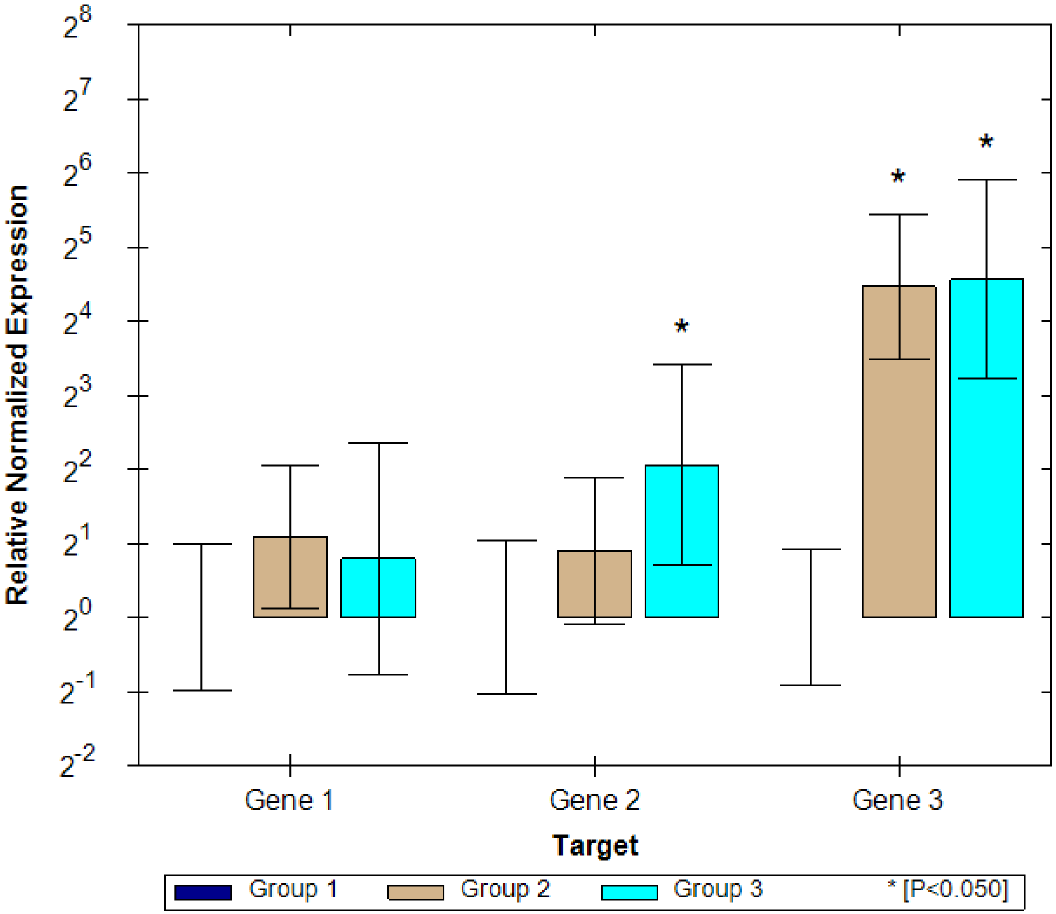
统计学检验 T-test

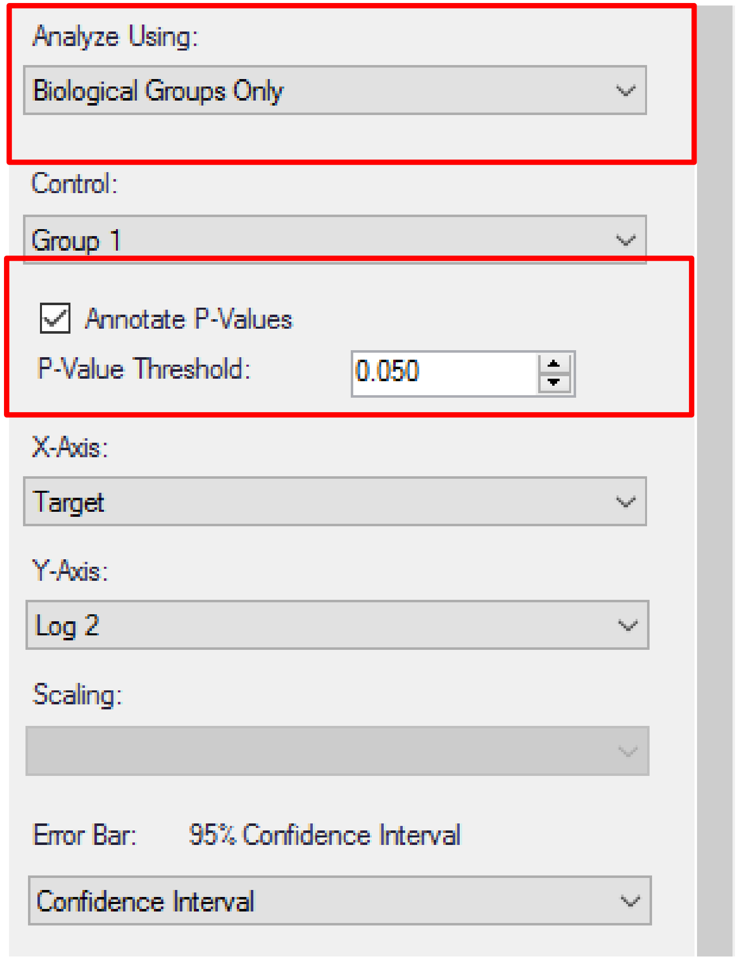
- T检验是用于小样本（样本容量小于30）的两个正态总体平均值差异程度的检验方法。它是用T分布理论来推断差异发生的概率，从而判定两个平均数的差异是否显著。
- T-tests 得出 p-values,对于检验水平α=0.05或0.01
  - P>α，不拒绝H0，无统计学差异
  - P<α，接受H1，有统计学差异



$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{s_1^2}{N_1} + \frac{s_2^2}{N_2}}}$$

CFX Maestro 中使用T-test





Analyze Using: Biological Groups Only

Control: Group 1

☑ Annotate P-Values

P-Value Threshold: 0.050

X-Axis: Target

Y-Axis: Log 2

Scaling:

Error Bar: 95% Confidence Interval

Confidence Interval

假设检验— 方差分析ANOVA

方差分析ANOVA： 又称为F-检验，用于两个及两个以上样本均数差别的显著性检验。

- CFX Maestro 1.1 提供**单因素方差分析（one-way ANOVA）**，用于同一个自变量的多个总体均数（多个数据集）间的比较（即观察一个因素）
- 方差分析首先是比较各组间总的差异，如总差异有显著性，再进行组间的两两比较（多重比较检验）

零假设H_0：$\mu_1 = \mu_2 = \mu_3$

备选假设H_1：$\overline{X_1} \neq \overline{X_2} \neq \overline{X_3}$

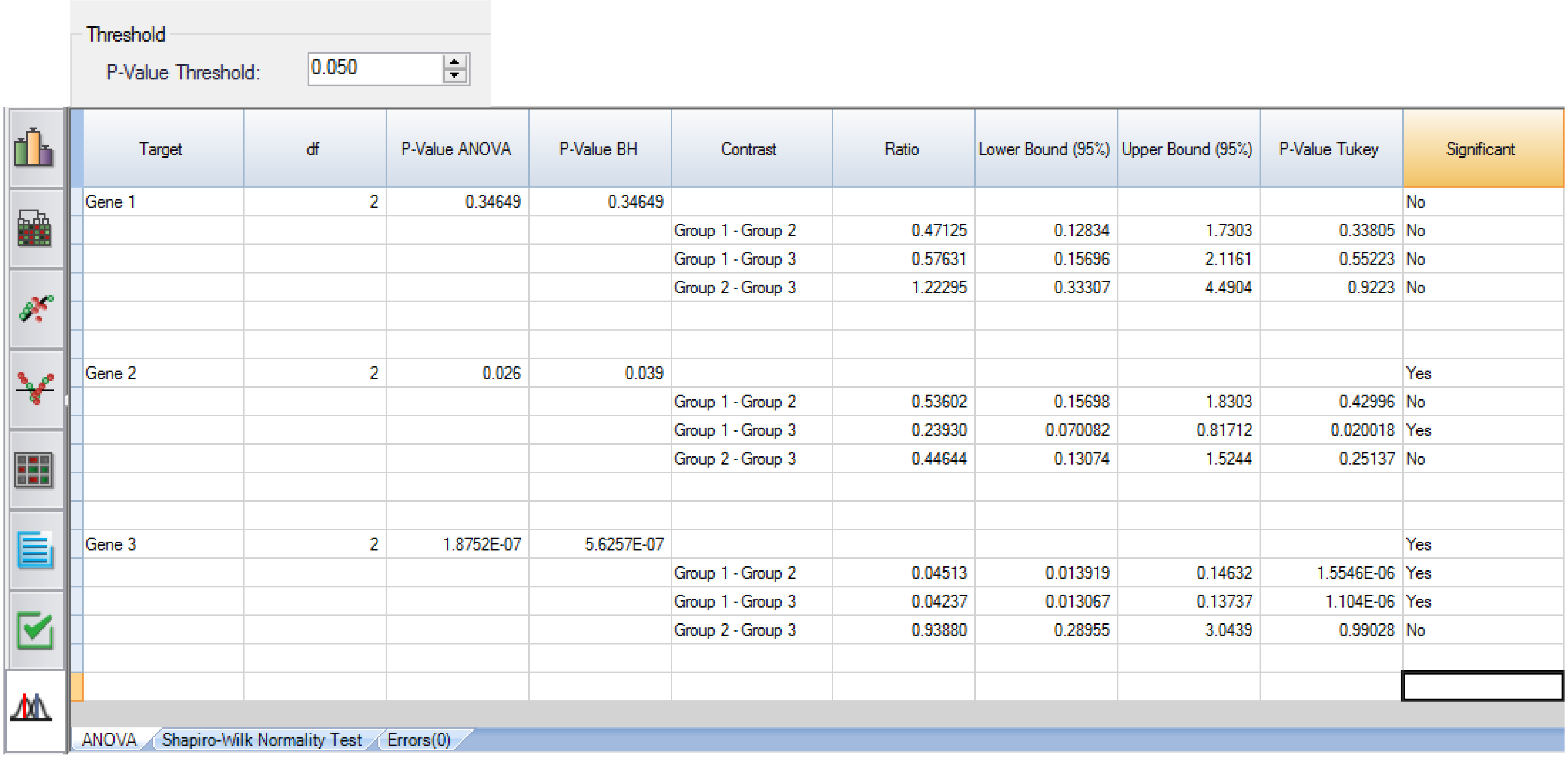
$$F=\frac{S_1{}^2/\sigma_1{}^2}{S_2{}^2/\sigma_2{}^2} \sim F（n_1-1, n_2-1）$$

应用ANOVA的条件：

1. 随机样本
2. 来自正态分布
3. 两均数比较时，要求总体方差相当

例如：用5中不同的药物处理小鼠（5个处理组），同时设置未处理组（对照组），观察Gene X在这6组小鼠中的表达量是否有差异。这里有一个自变量，即处理方式。可使用单因素ANOVA

CFX Maestro 中使用ANOVA



Threshold

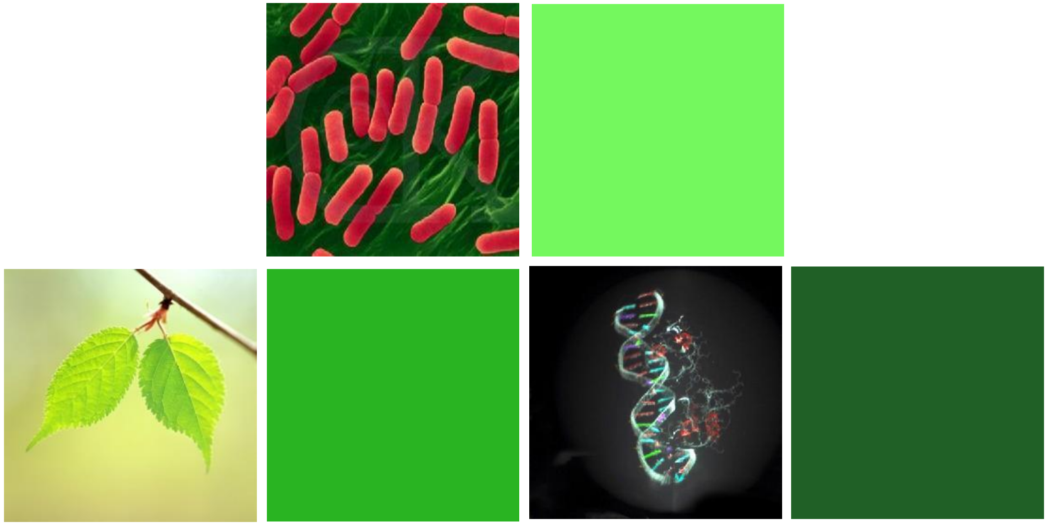
P-Value Threshold: 0.050

Target	df	P-Value ANOVA	P-Value BH	Contrast	Ratio	Lower Bound (95%)	Upper Bound (95%)	P-Value Tukey	Significant
Gene 1	2	0.34649	0.34649						No
				Group 1 - Group 2	0.47125	0.12834	1.7303	0.33805	No
				Group 1 - Group 3	0.57631	0.15696	2.1161	0.55223	No
				Group 2 - Group 3	1.22295	0.33307	4.4904	0.9223	No
Gene 2	2	0.026	0.039						Yes
				Group 1 - Group 2	0.53602	0.15698	1.8303	0.42996	No
				Group 1 - Group 3	0.23930	0.070082	0.81712	0.020018	Yes
				Group 2 - Group 3	0.44644	0.13074	1.5244	0.25137	No
Gene 3	2	1.8752E-07	5.6257E-07						Yes
				Group 1 - Group 2	0.04513	0.013919	0.14632	1.5546E-06	Yes
				Group 1 - Group 3	0.04237	0.013067	0.13737	1.104E-06	Yes
				Group 2 - Group 3	0.93880	0.28955	3.0439	0.99028	No

产品介绍-软件系统

软件提供从Cq值到统计学检验结论的一键式分析工具：

- 完备的数据计算方法，符合MIQE规范
- Gene Study 功能，无限合并多板分析功能
- 可带入实际扩增效率、可以进行多内参相对定量分析
- 自动计算内参稳定系数M值，方便内参筛选
- 多种实验结果可视化显示功能，柱形图、箱线图、点状图、聚类图、散点图、火山图、热图，图形输出功能强大，图形导出可直接用于发表
- 中文版本、安全版本和Mac版本
- 整合统计学分析工具（t-test, 单因素方差分析）



谢谢大家！