

荧光 PCR 原理及应用

周煜 ZHOU Yu

Application Specialist

cnmcbsupport@appliedbiosystems.com

Applied Biosystems



ABI公司及其荧光PCR仪器

Applied Biosystems

美国ABI 公司荧光定量PCR仪发展历史

1995－世界上第一台定量PCR仪7700型

1997－向医院用户推出5700型定量PCR仪

2000－推出7900型**384**孔荧光定量PCR仪

2001－　　7900型 96孔荧光定量PCR仪

} **公认最高端定量PCR仪**

2001－推出7000型荧光定量PCR仪取得巨大成功

2003－获得了荧光定量PCR仪专利－确立ABI在业界内的独特地位

2004－第3代荧光定量PCR仪7300和7500型

2007－第4代荧光定量PCR仪StepOne和StepOnePlus



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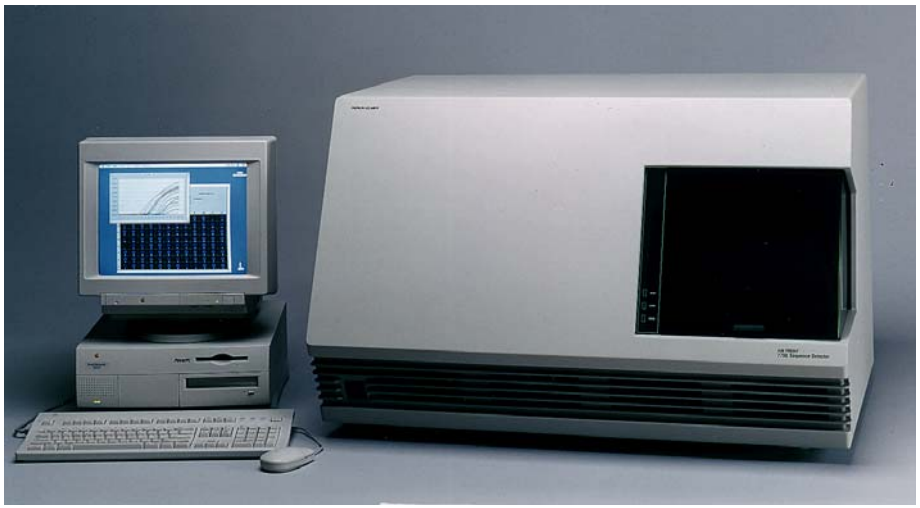
ABI 公司荧光PCR仪器



ABI 公司荧光PCR仪器

Applied Biosystems 7900HT Fast Real-Time PCR System

Applied Biosystems StepOne™ Real-Time PCR System

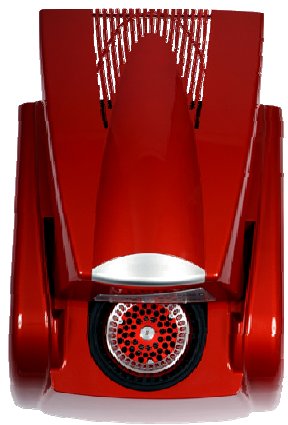
Applied Biosystems 7500 Fast Real-Time PCR System

Applied Biosystems 7500 Real-Time PCR System

Applied Biosystems 7300 Real-Time PCR System

Applied Biosystems

各种荧光PCR仪器





荧光PCR的化学

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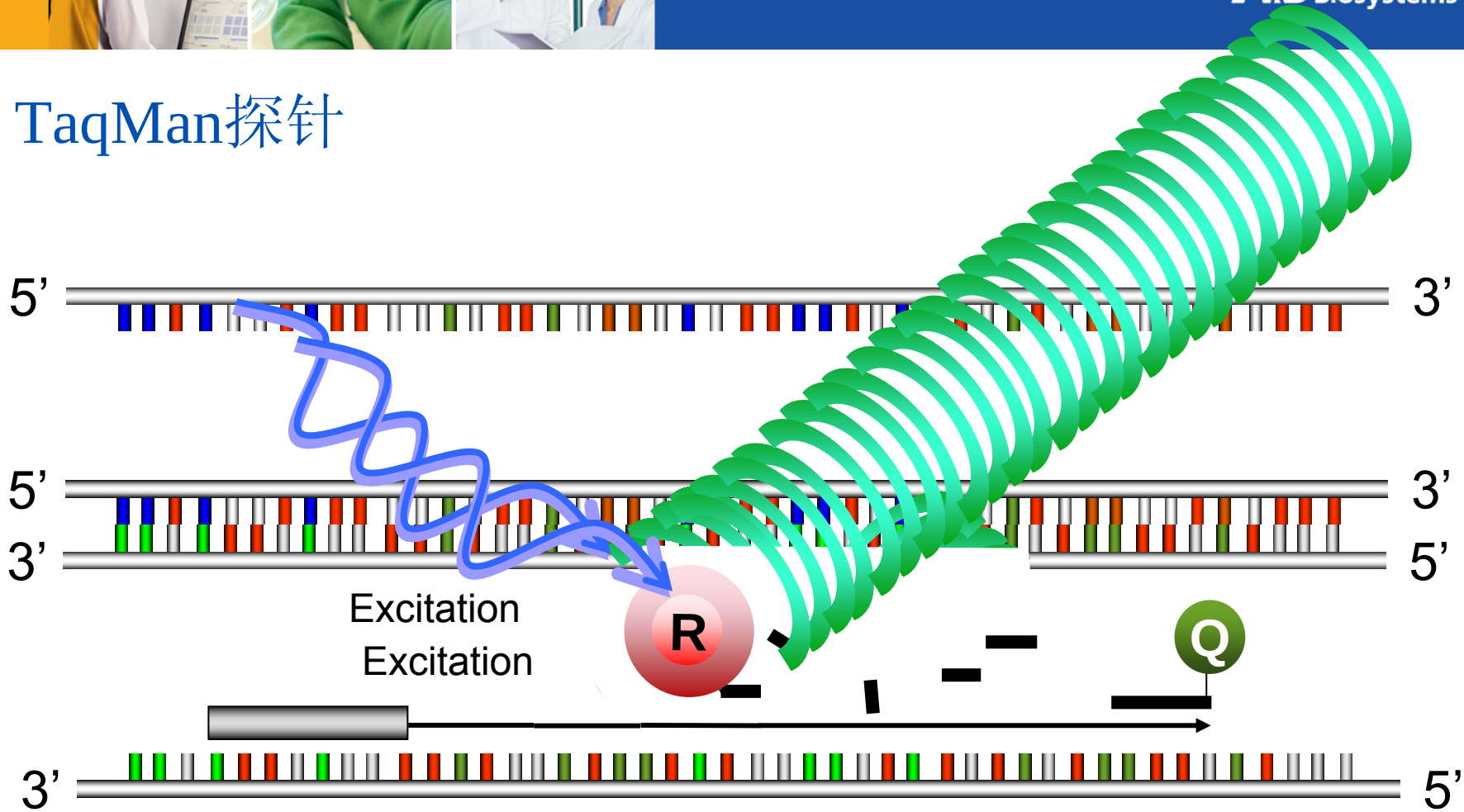
两种荧光化学原理

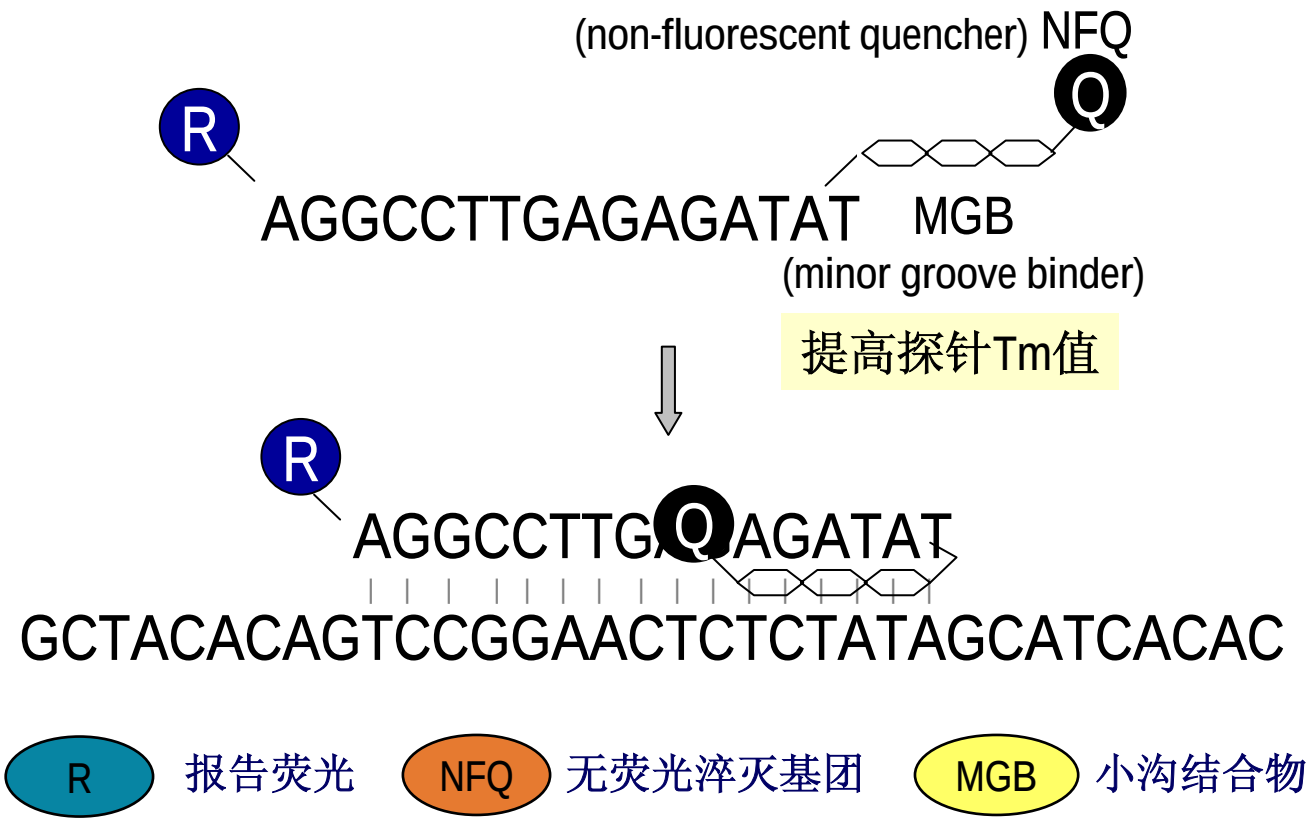
- Fluorogenic 5’ Nuclease Assay
  - An established and proven chemistry for high specificity nucleic acid real-time quantitation
  - The introduction of TaqMan® MGB (Minor Groove Binder) probes has produced a turn-key homogeneous chemistry for the detection of Single Nucleotide Polymorphisms (SNPs)

- SYBR® Green 1 Double Stranded DNA Binding Dye Assay
  - Ideal for target identification (screening) assays
  - A lower-cost alternative when high specificity is not required

TaqMan探针



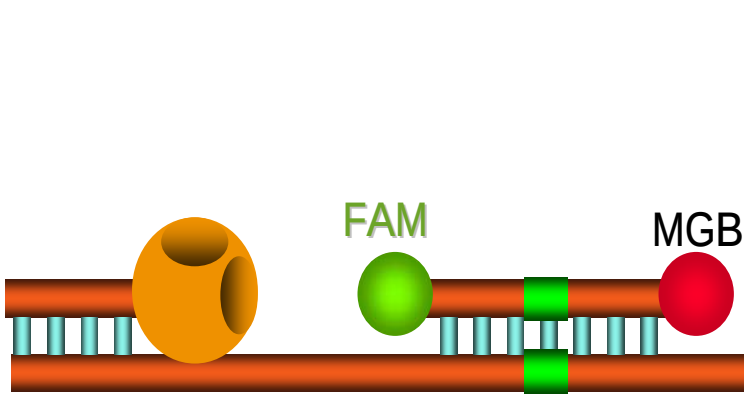
TaqMan MGB探针



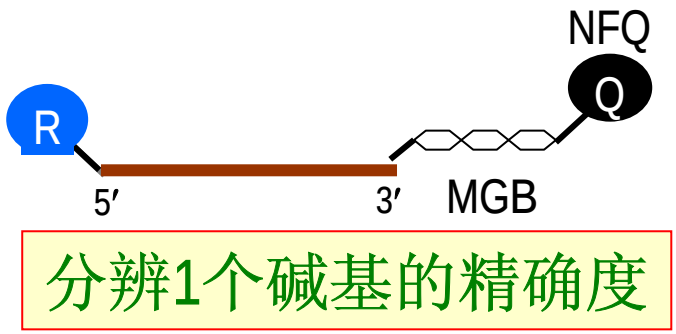
MGB探针的优点

- 提高淬灭效率
- 减少背景、提高信噪比
- 提高探针**Tm**值
  - **长15碱基，提高 18°C**
- 缩短探针长度
  - **最佳范围13-21 碱基**

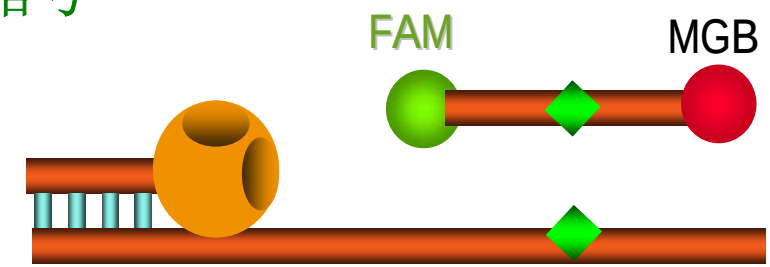
TaqMan MGB探针鉴别等位基因



完全配对，有信号



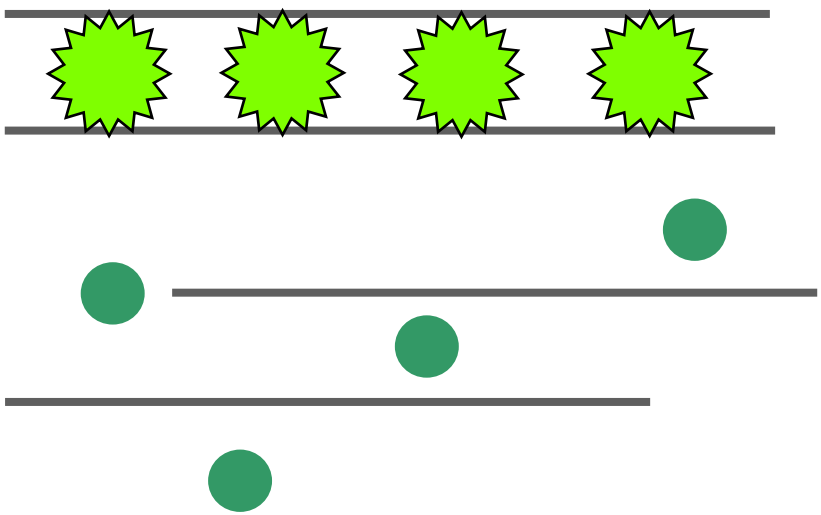
分辨1个碱基的精确度



一个碱基不配对，没有信号

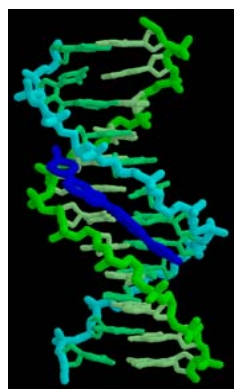
SYBR Green染料

SYBR Green I 与dsDNA 结合



与DNA结合时

游离时光

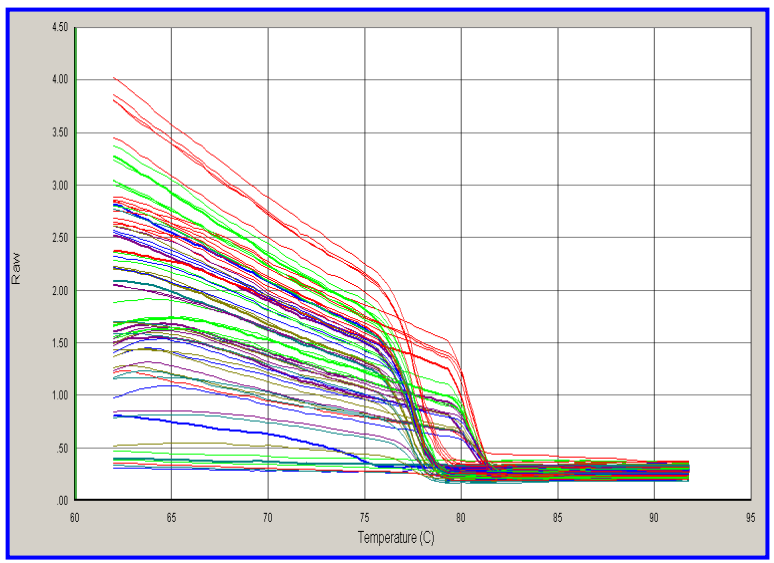


融解曲线(Dissociation curve)

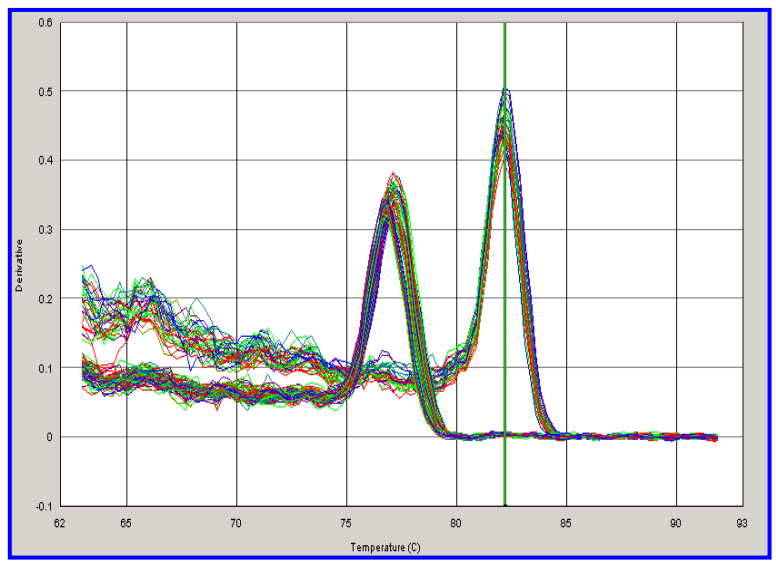
- Easily check for specificity of your SYBR assays immediately after PCR
- Thermal conditions (after last cycle of PCR):
  - Hold at 95° C for 20 sec
  - Hold at 60° C for 20 sec
  - Ramp and collect fluorescent data to 95° C over course of 20 minutes
- Software automatically generates melt curve profiles

融解曲线(Dissociation curve)

原始图谱



导数图谱

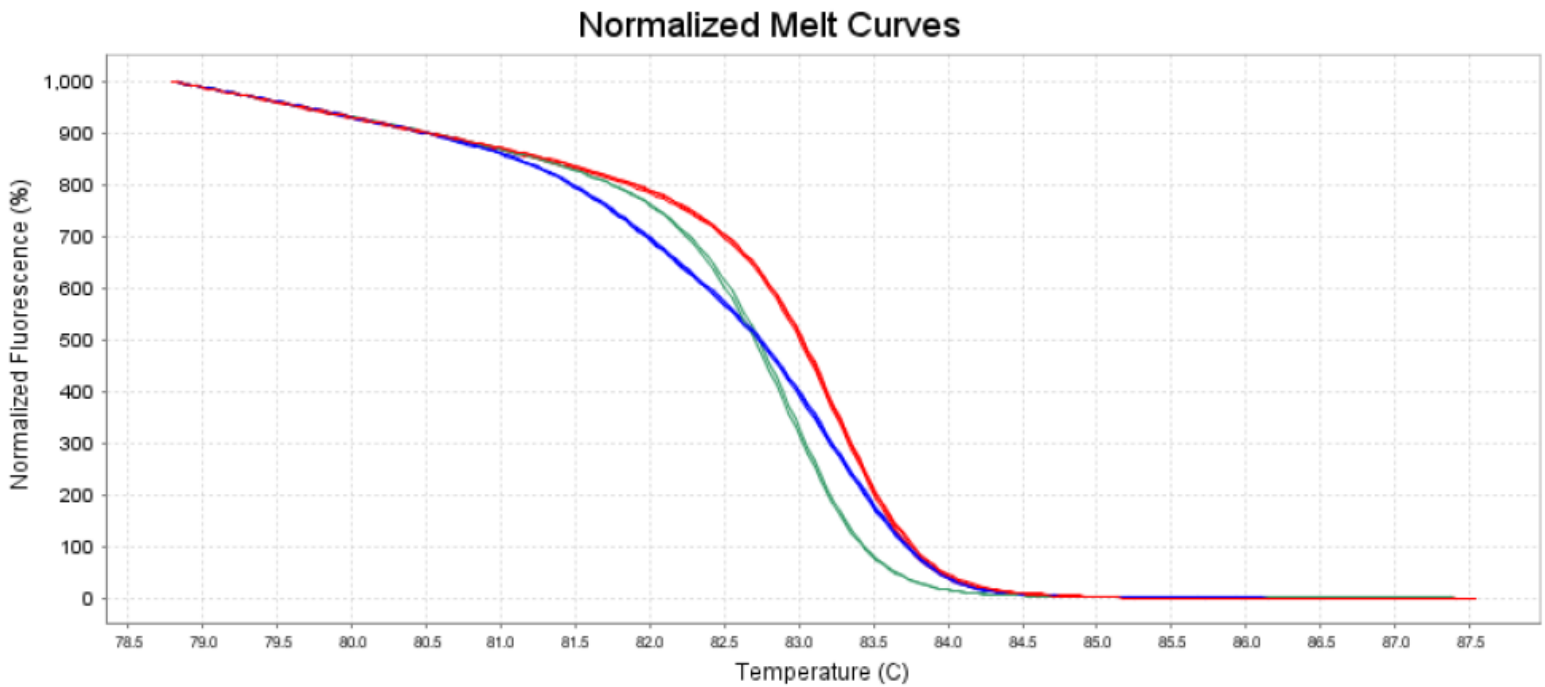


高分辨溶解曲线（HRM, High Resolution Melting ）

- Different from a regular SYBR® Green dye melt curve:
  - Chemistry: Saturating and brighter dsDNA binding dyes
    - LCGreen® EvaGreen™ SYTO®9
  - Instrument: More data points are collected
  - Software: New fluorescent normalization algorithms and plots

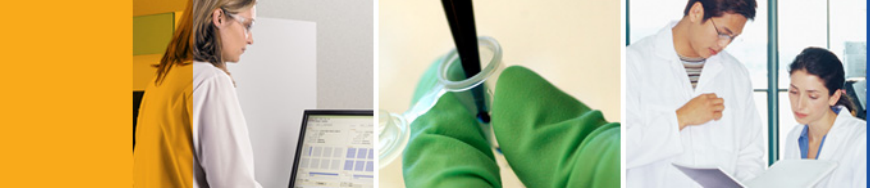
Now can be performed on the ABI 7500 Fast System

- The analysis is based on the difference in curve shape as well as Tm.
- HRM can identify mutations that cause Tm changes as minor as 0.1° C.

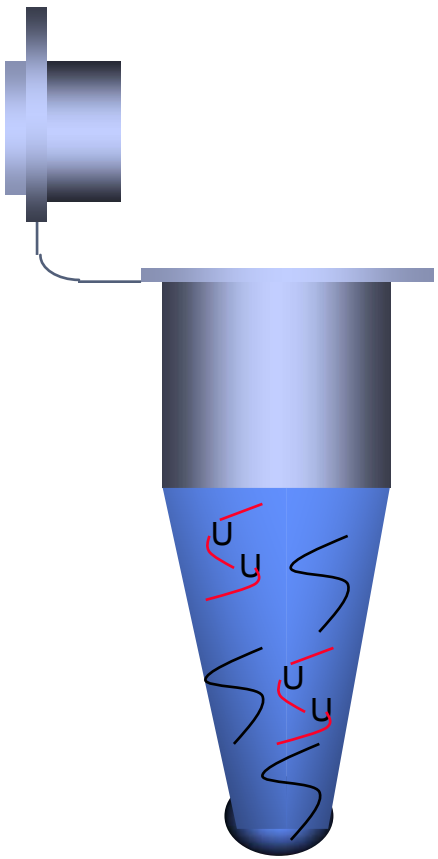


染料法的特点

- 成本低
- 兼容熔解曲线
- 特异性问题
- 不能进行多重扩增



荧光PCR的防污染技术

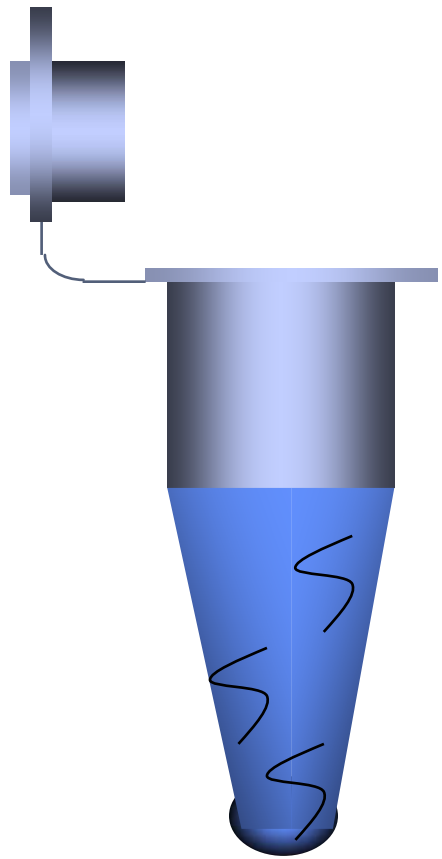


Uracil N-glycosylase

Chemical method to destroy contaminating amplicons.

All amplification chemistries use dUTP.

UNG does not function above 55°C



荧光参比— Passive Reference Dye: ROX

ROX以固定的浓度配在Master Mix中，不参与PCR扩增

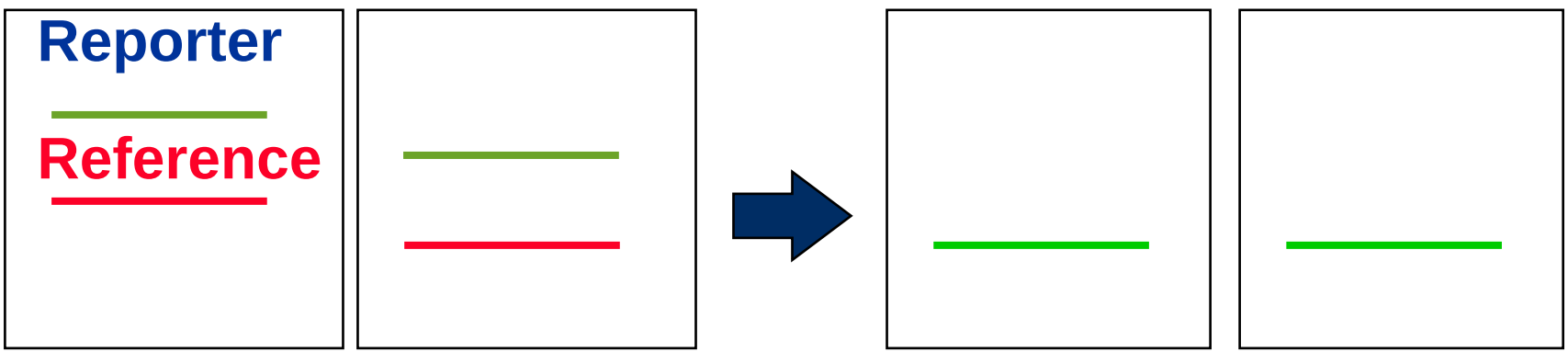
ROX的功能：

Improves precision.

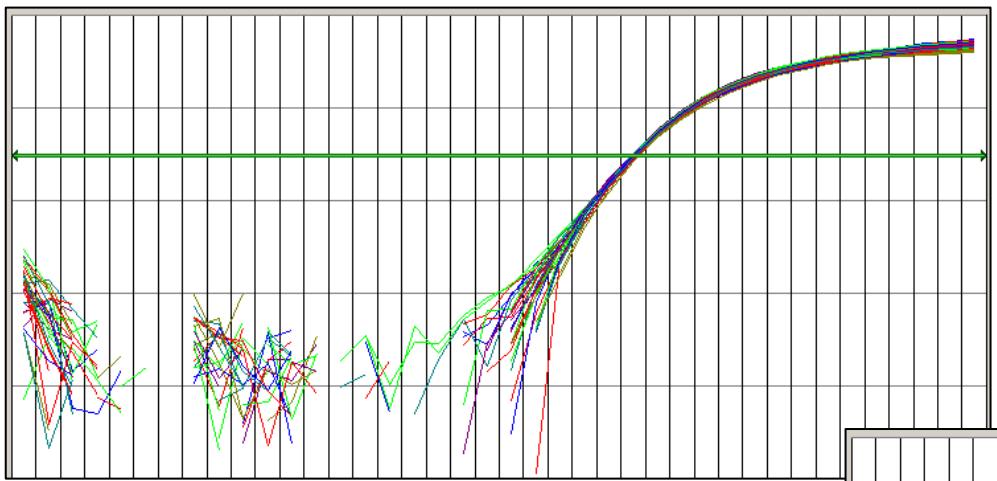
Compensates for small fluorescent fluctuations that can occur from well-to-well

Can be useful for diagnosing troubles

Rn = Normalization = Reporter / Reference

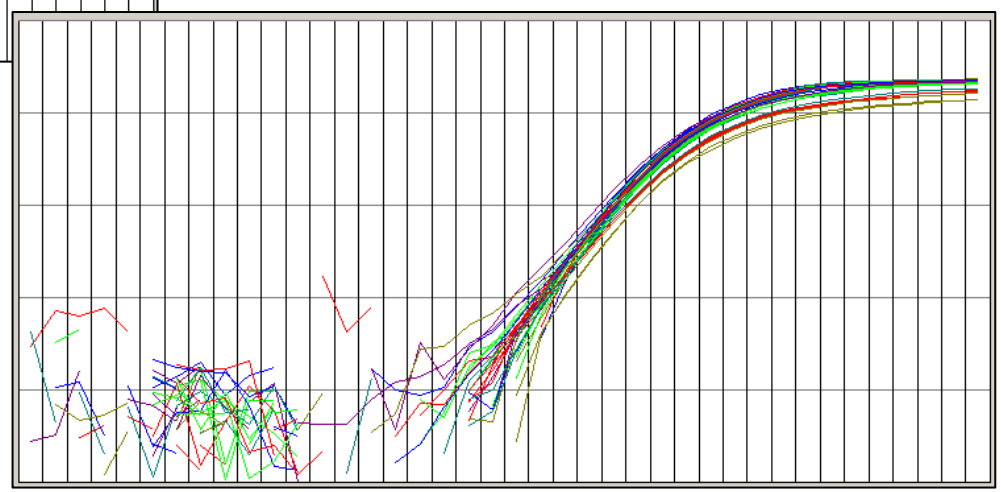


ROX Normalizer



36 Replicates analyzed with ROX passive reference dye

36 Replicates analyzed without ROX passive reference dye





定量PCR的数学原理

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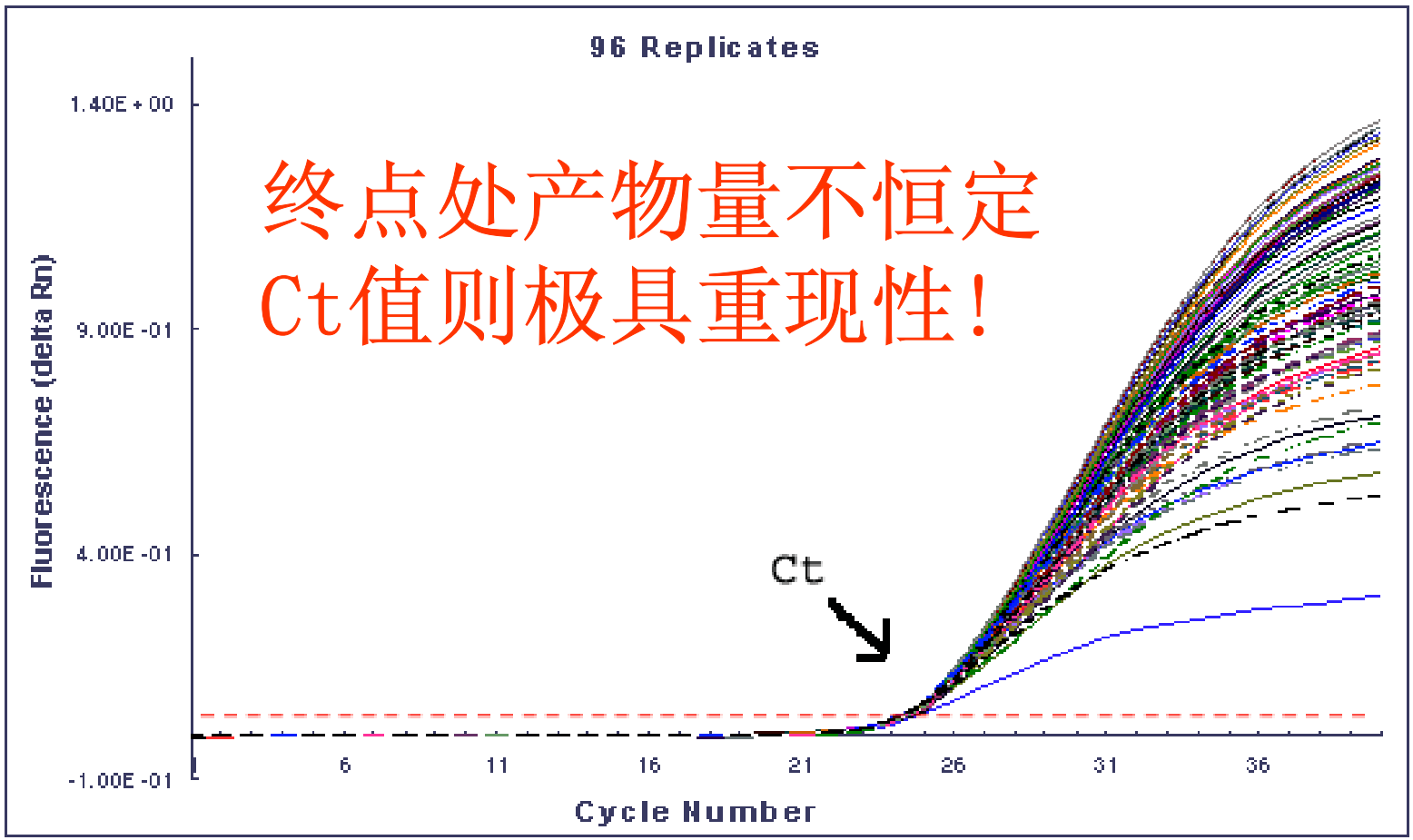
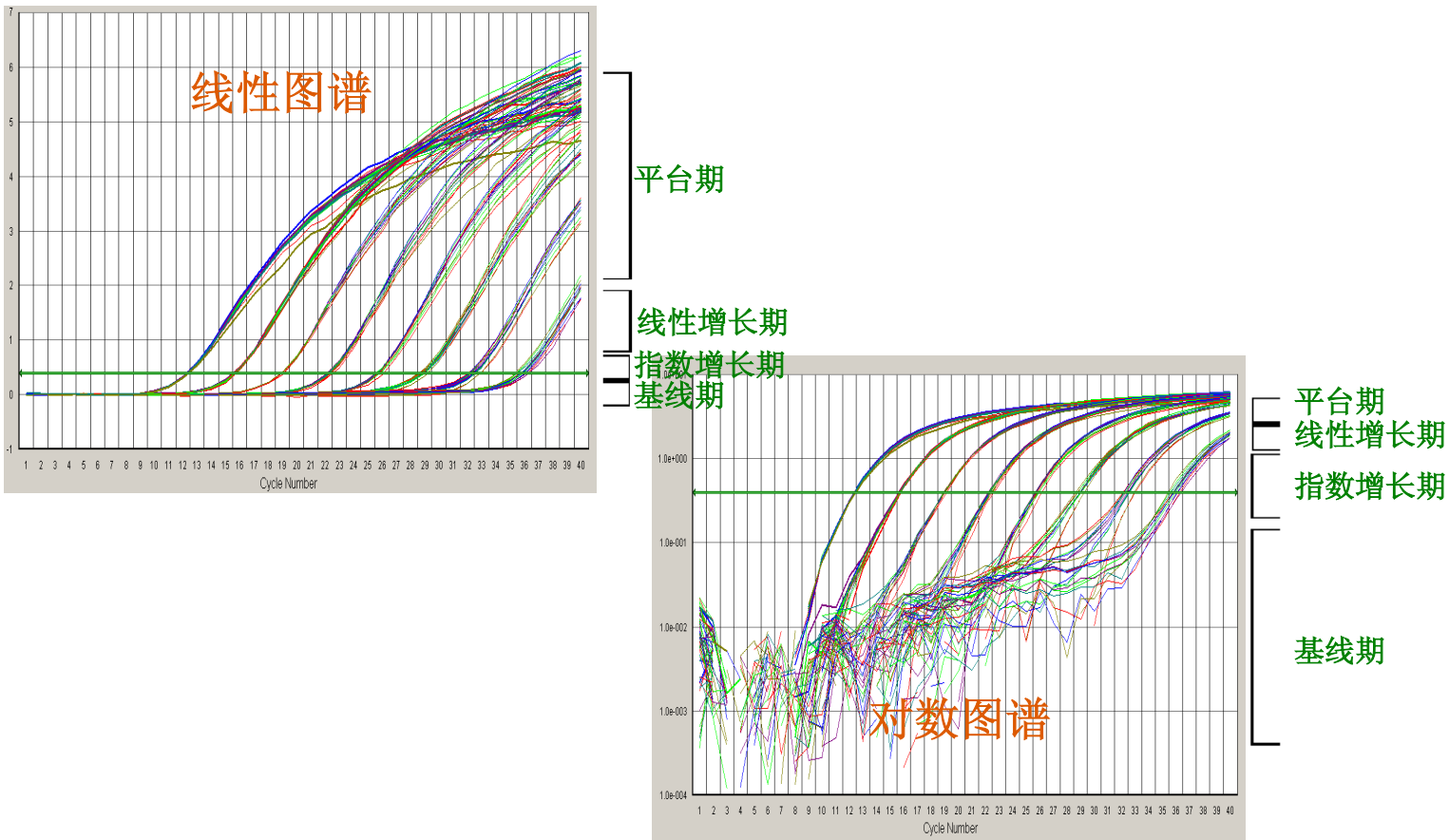
实时定量与终点定量


96 Replicates
1.40E+00
9.00E-01
4.00E-01
-1.00E-01
Fluorescence (delta Rn)
终点处产物量不恒定
Ct值则极具重现性!
Ct
1
6
11
16
21
26
31
36
Cycle Number

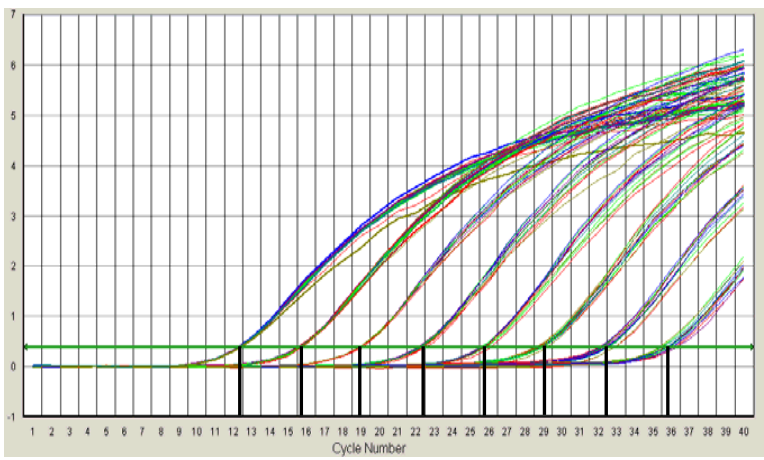
PCR动力学曲线和四个阶段



什么是C_T值？

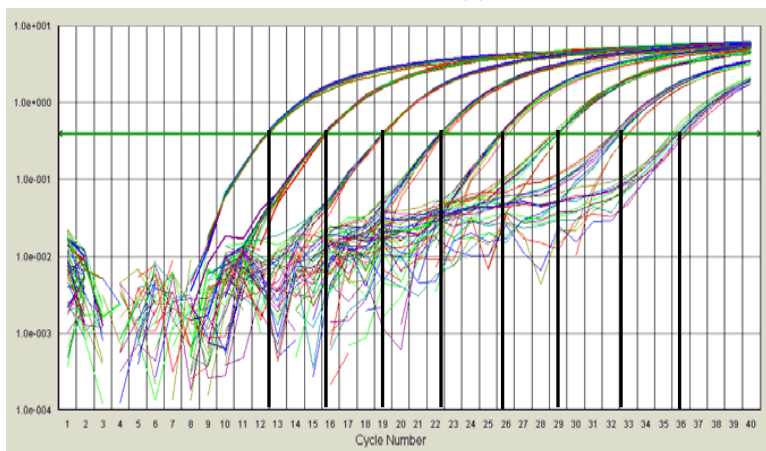
荧光信号 穿过阈值线时的循环次数－threshold cycle

线性图谱



C_T值

对数图谱



C_T值

PCR扩增的数学模型

- 扩增产物的总数量可以下式表示：

$$Y_n = X \cdot (1+E)^n$$

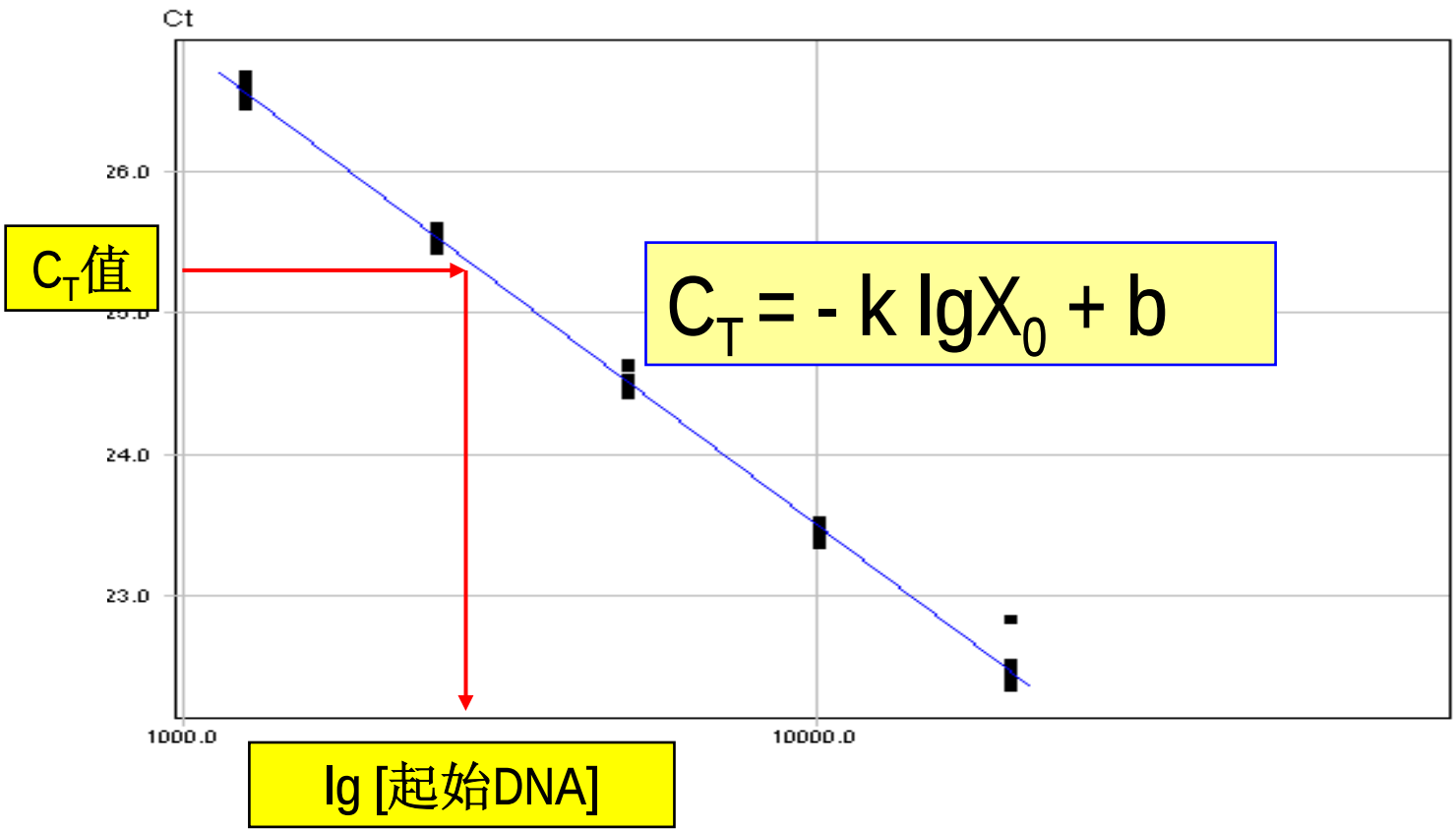
Y_n为PCR产物的分子数量
n为周期数
E为扩增效率（0<E<1）

- 理想条件下
  - 浓度增加 1倍－ C_T值减小1个单位
  - 浓度增加10倍－ C_T值减小3.3219280……

斜率与截距

- $C_T = - k \lg X_0 + b$
  - Slope=-1/log(1+E)
    - If E=1, Slope= -3.3219280……
    - Normally, slope is about -3.5~-4.2
  - Intercept
    - Normally, intercept is about 30~40

C_T值对[DNA]$_0$作图



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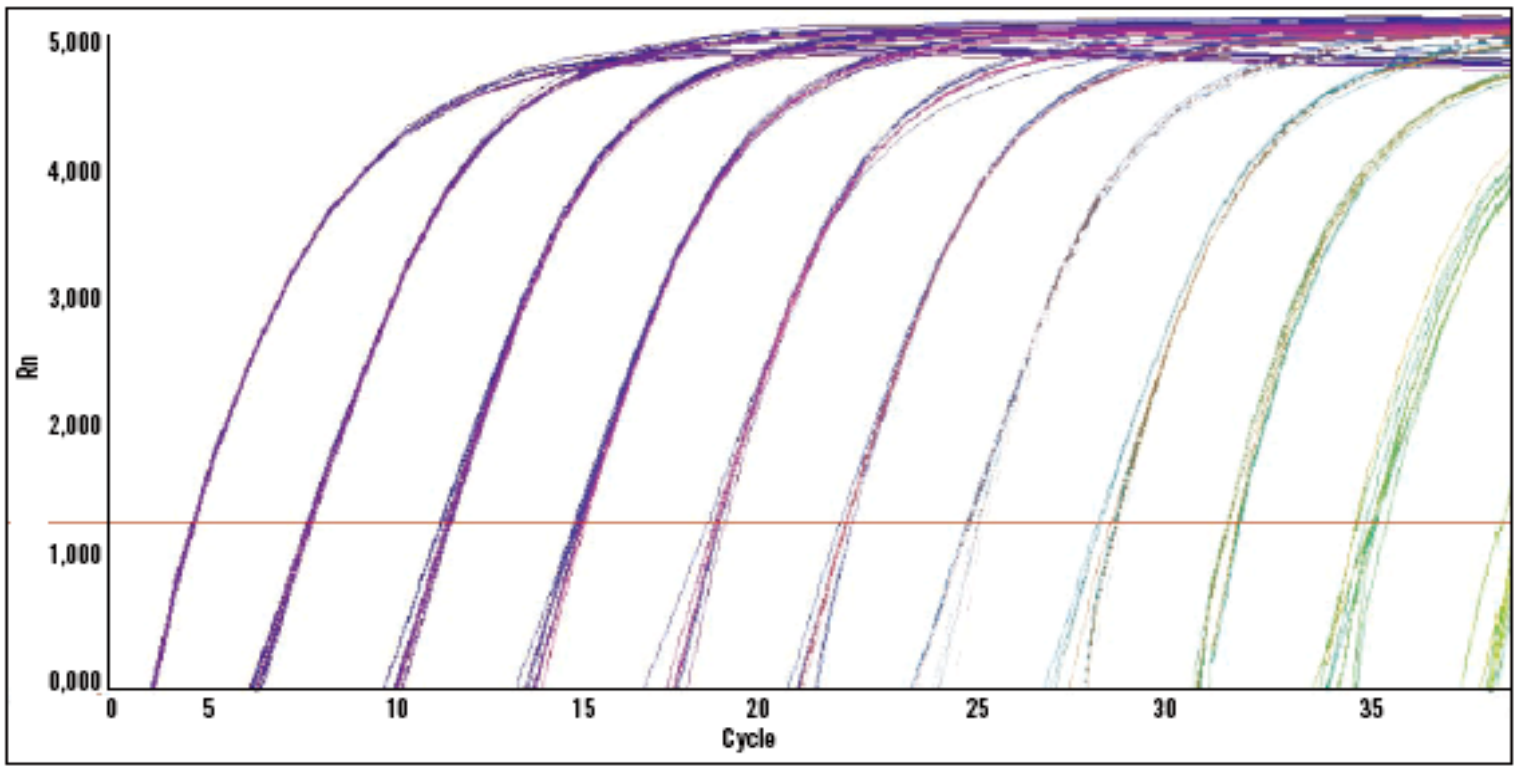
成功的定量PCR数据



实时荧光PCR技术的主要优点

- 可进行定量PCR检测，定量范围(Dynamic Range)宽；
- 操作方便－无复杂的PCR后分析步骤；
- 全封闭式检测－减少污染；
- 检测灵敏度及特异性高；
- 多通道分析；
- 熔点曲线分析　(Dissociation curve)……



荧光PCR技术的应用

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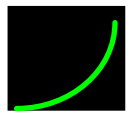
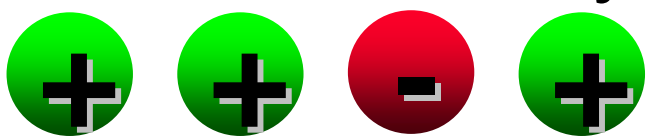
主要应用

Real time —— Quantitative PCR



End point

Plus/minus Assays



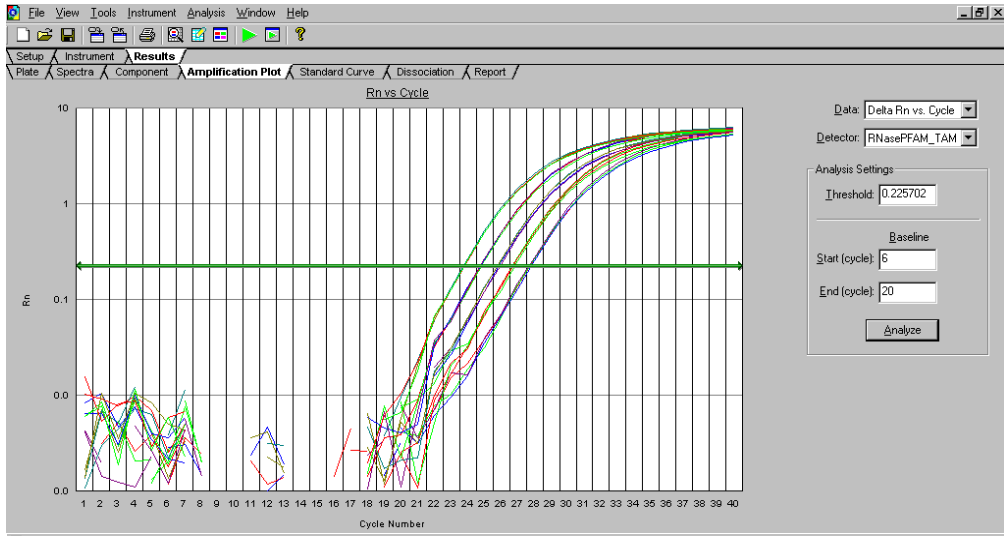
Allele Discrimination

定量PCR – Quantitative PCR

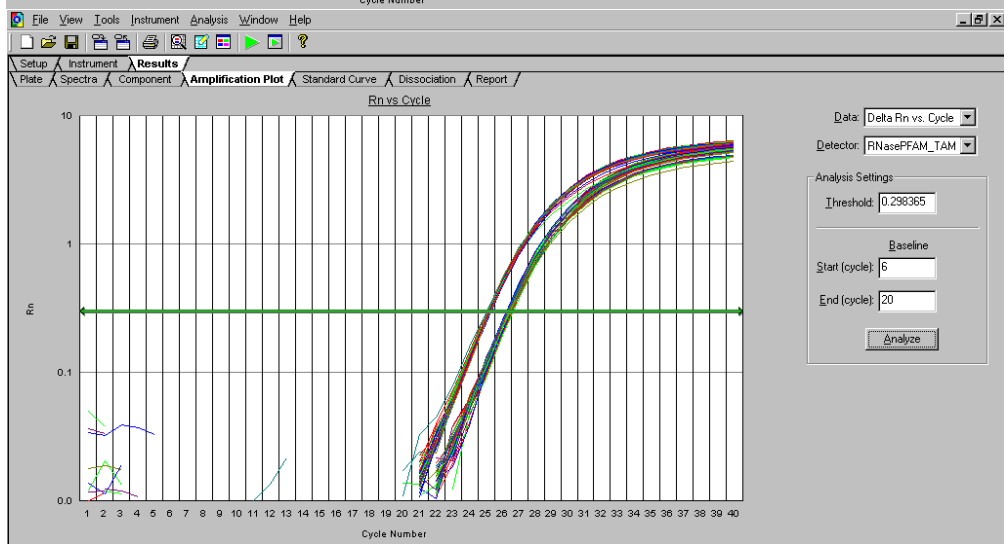
- How many?
  - 绝对定量 Absolute Quantitation
- How much?
  - 相对定量 Relative Quantitation

Results from RNase P Instrument Verification Plate

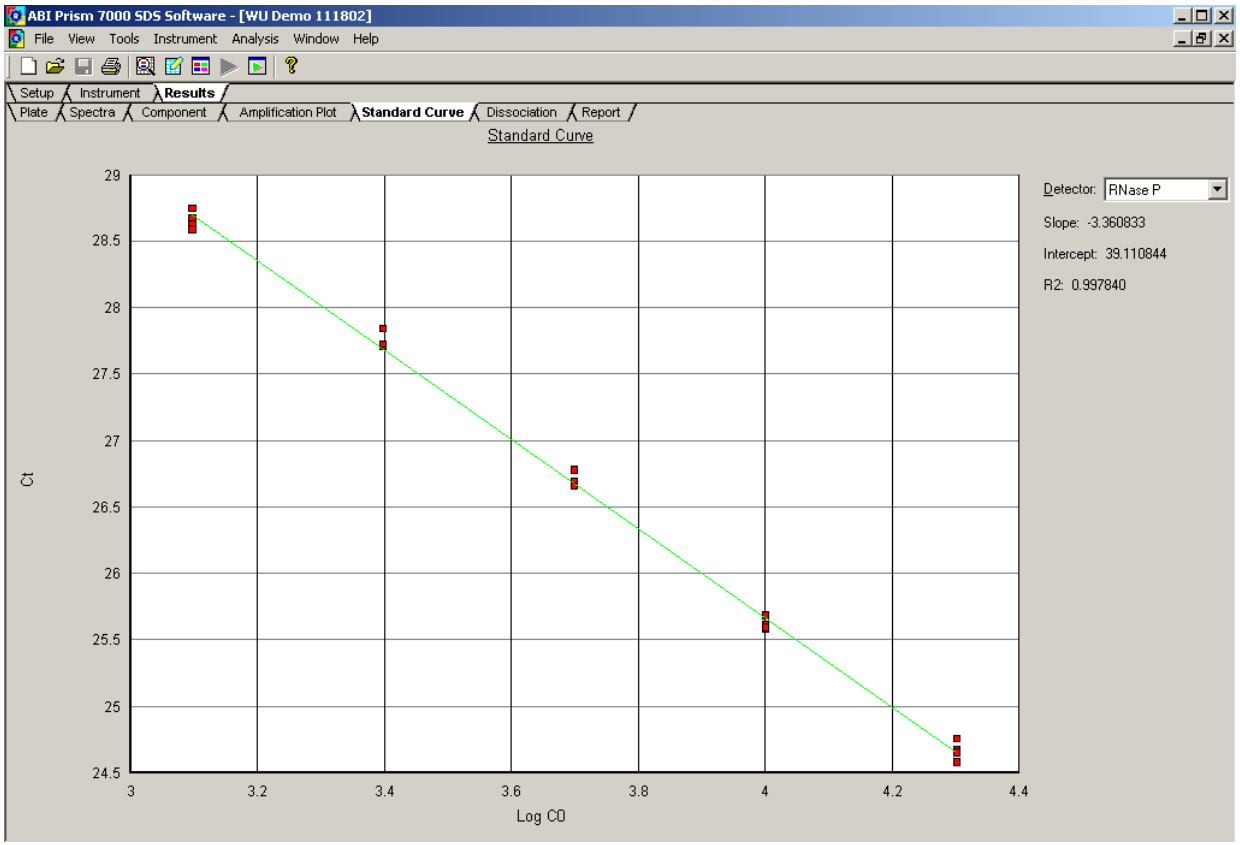
- Standard curve amplifications
- showing 20K, 10K, 5K, 2.5K
- and 1.25K copy populations
- in replicates of four



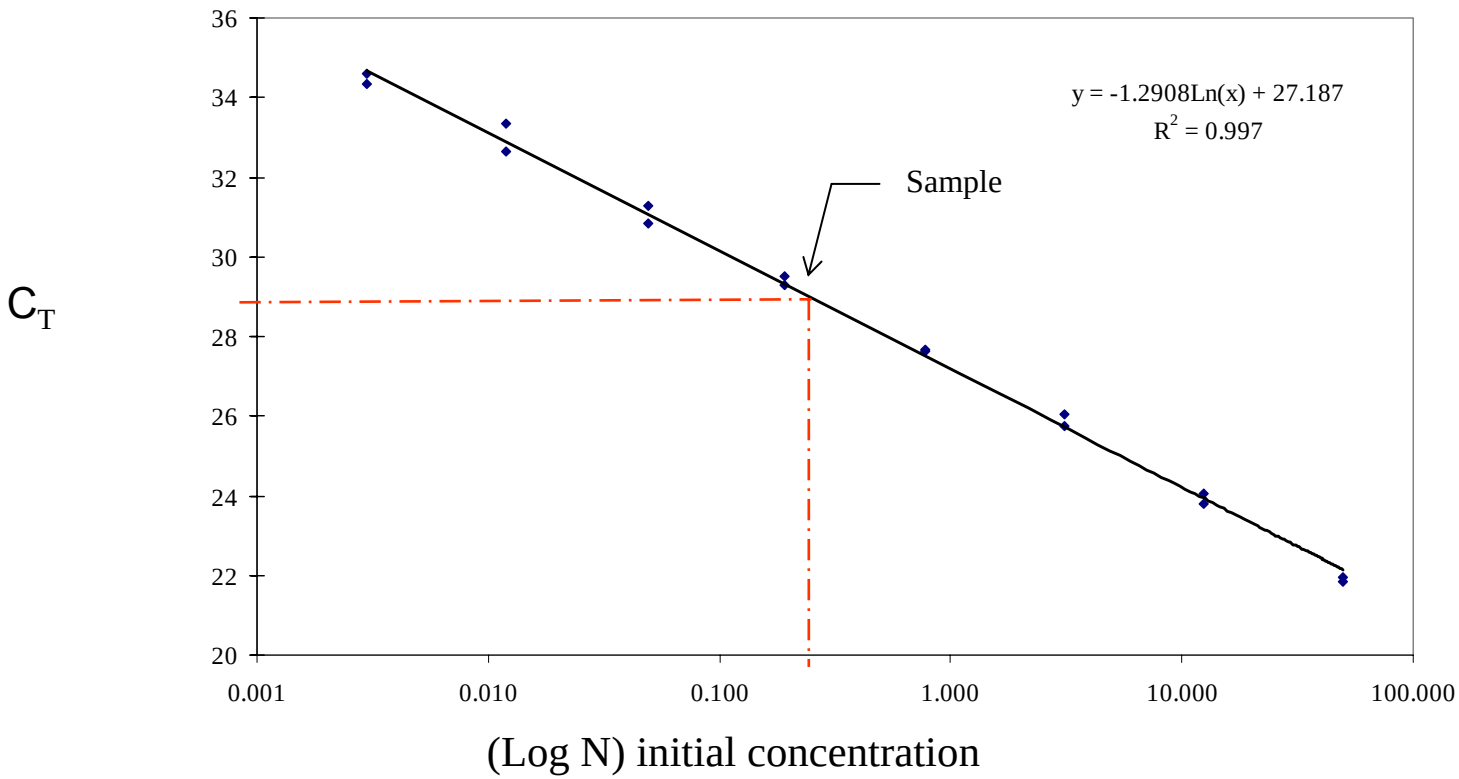
- "Unknown" amplifications
- showing 10K and 5K copy
- populations in replicates of 36



Results from RNase P Instrument Verification Plate



Calculating DNA Quantity of a Sample



Data Report Table

ABI Prism 7000 SDS Software - [DemoRNaseP.sds]

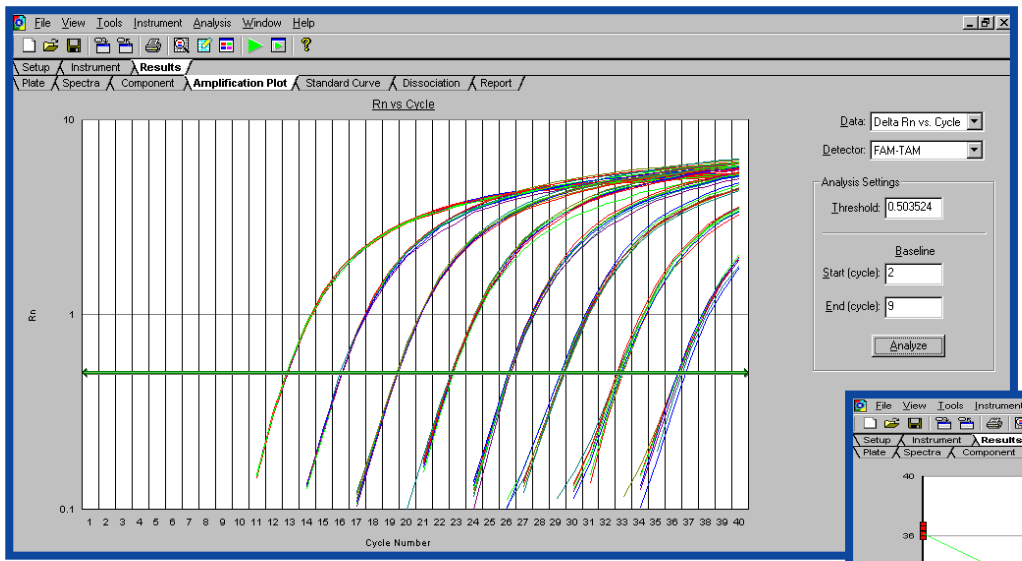
File View Tools Instrument Analysis Window Help

Setup | Instrument | Results

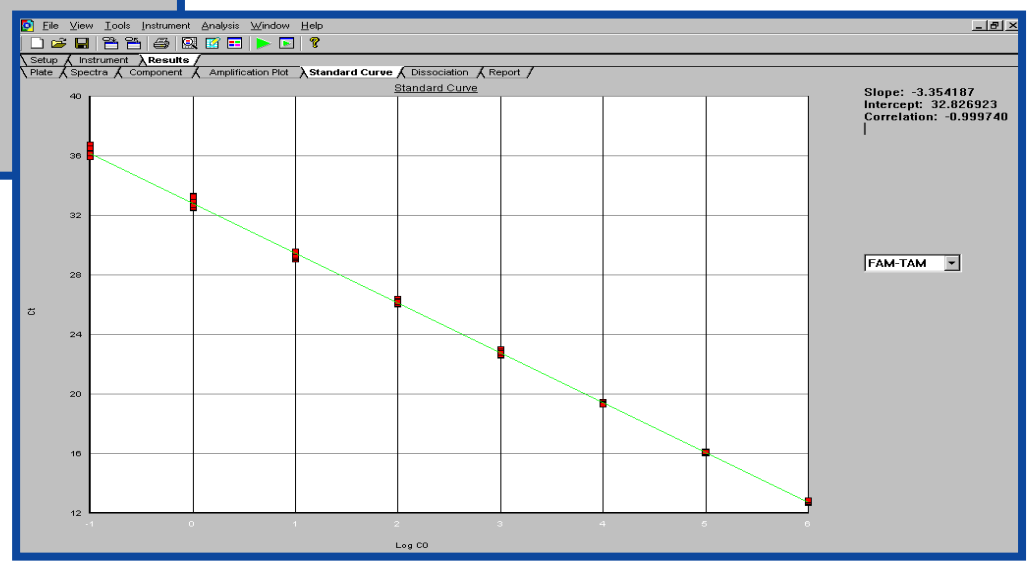
Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | Report

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty	Mean Qty	StdDev Qty
A1	5K	RNase P	Unknown	27.31	0.117	3980.24	4369.88	343.002
A2	5K	RNase P	Unknown	27.35	0.117	3869.92	4369.88	343.002
A3	5K	RNase P	Unknown	27.27	0.117	4066.20	4369.88	343.002
A4	5K	RNase P	Unknown	27.32	0.117	3941.69	4369.88	343.002
A5	5K	RNase P	Unknown	27.25	0.117	4133.08	4369.88	343.002
A6	5K	RNase P	Unknown	27.24	0.117	4162.23	4369.88	343.002
A7	5K	RNase P	Unknown	27.28	0.117	4050.88	4369.88	343.002
A8	5K	RNase P	Unknown	27.26	0.117	4118.60	4369.88	343.002
A9	5K	RNase P	Unknown	27.29	0.117	4028.91	4369.88	343.002
A10	5K	RNase P	Unknown	27.24	0.117	4152.21	4369.88	343.002
A11	5K	RNase P	Unknown	27.31	0.117	3956.82	4369.88	343.002
A12	5K	RNase P	Unknown	27.46	0.117	3583.97	4369.88	343.002
B1	5K	RNase P	Unknown	27.26	0.117	4103.47	4369.88	343.002
B2	5K	RNase P	Unknown	27.16	0.117	4390.18	4369.88	343.002
B3	5K	RNase P	Unknown	27.14	0.117	4469.72	4369.88	343.002
B4	5K	RNase P	Unknown	27.10	0.117	4589.70	4369.88	343.002
B5	5K	RNase P	Unknown	27.12	0.117	4516.37	4369.88	343.002
B6	5K	RNase P	Unknown	27.08	0.117	4636.35	4369.88	343.002
B7	5K	RNase P	Unknown	27.13	0.117	4498.48	4369.88	343.002
B8	5K	RNase P	Unknown	27.12	0.117	4529.29	4369.88	343.002
B9	5K	RNase P	Unknown	27.06	0.117	4697.71	4369.88	343.002
B10	5K	RNase P	Unknown	27.10	0.117	4565.45	4369.88	343.002
B11	5K	RNase P	Unknown	27.16	0.117	4394.29	4369.88	343.002
B12	5K	RNase P	Unknown	27.33	0.117	3922.80	4369.88	343.002
C1	5K	RNase P	Unknown	27.22	0.117	4221.09	4369.88	343.002
C2	5K	RNase P	Unknown	27.14	0.117	4466.49	4369.88	343.002
C3	5K	RNase P	Unknown	27.05	0.117	4753.63	4369.88	343.002

Dynamic Range Data



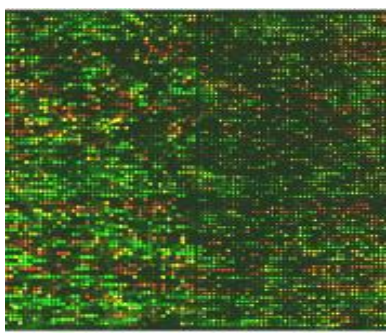
Amplification of serial dilutions of IL-10 target in replicates of 8

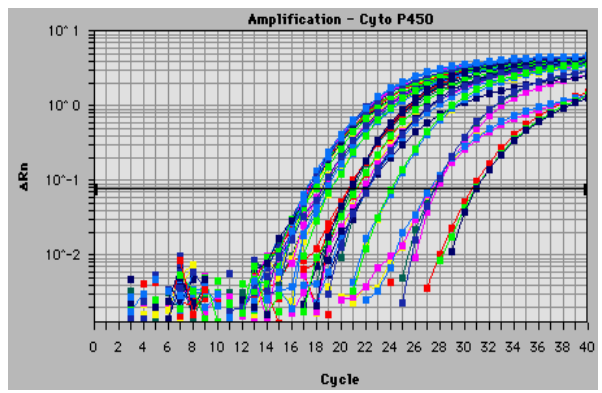


Standard curve showing 7 logs of linear dynamic range

相对定量

- Relative gene expression:
  - Compare treated samples vs. untreated samples (calibrator samples)
  - RNAi validation
  - Array validation



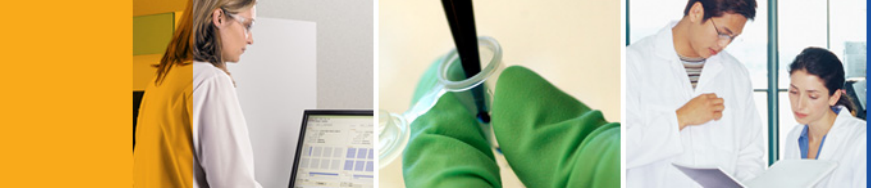


相对定量

- Δ Δ Ct
  - 相对量＝ $2^{-\Delta\Delta CT}$
  - 目标序列和内参序列的扩增效率相等（且接近100％）

- The Relative Standard Curve Method

$$R = \frac{(E_{target})^{\Delta Ct\ target\ (MEAN\ control - MEAN\ sample)}}{(E_{ref})^{\Delta Ct\ ref\ (MEAN\ control - MEAN\ sample)}}$$



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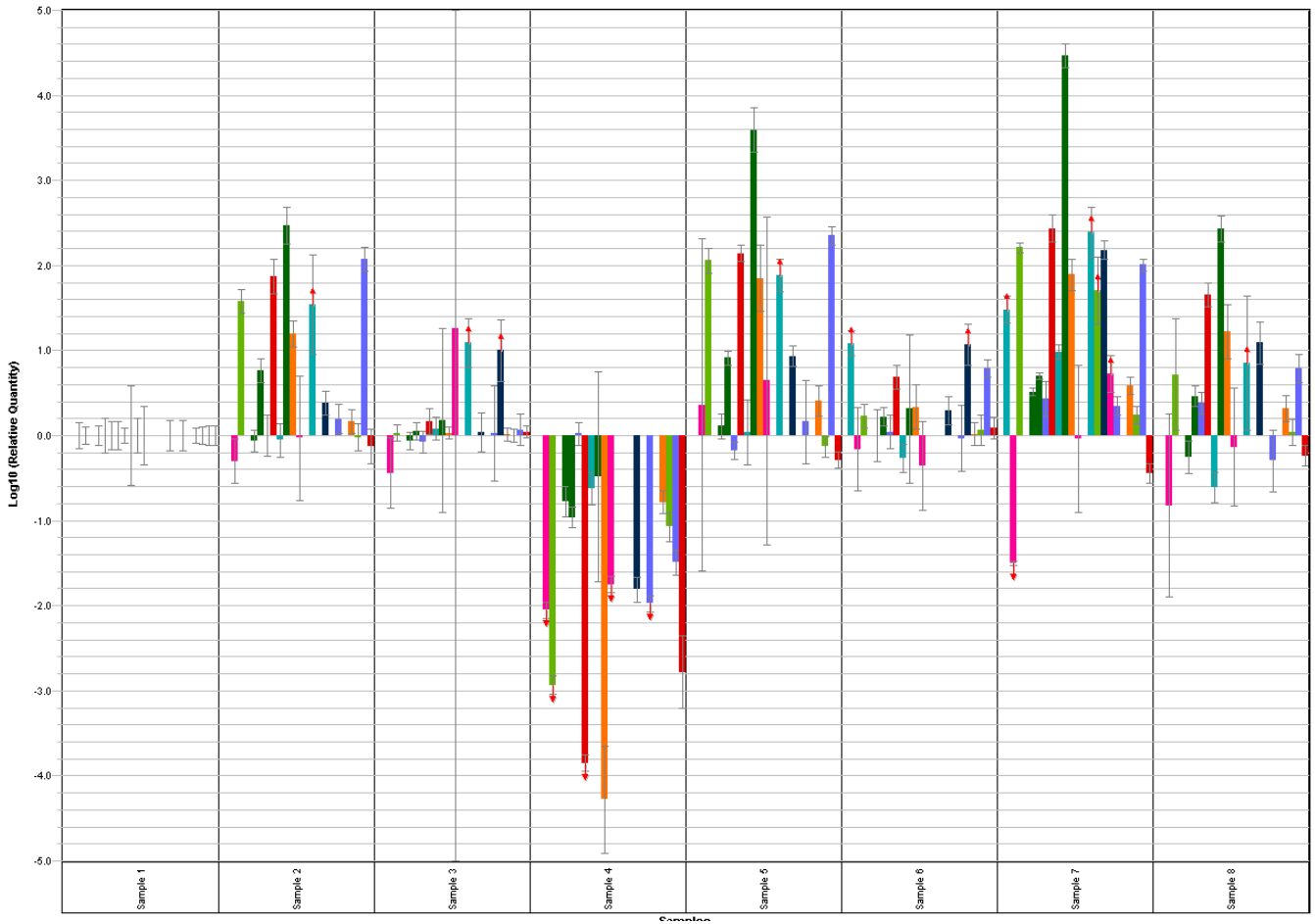
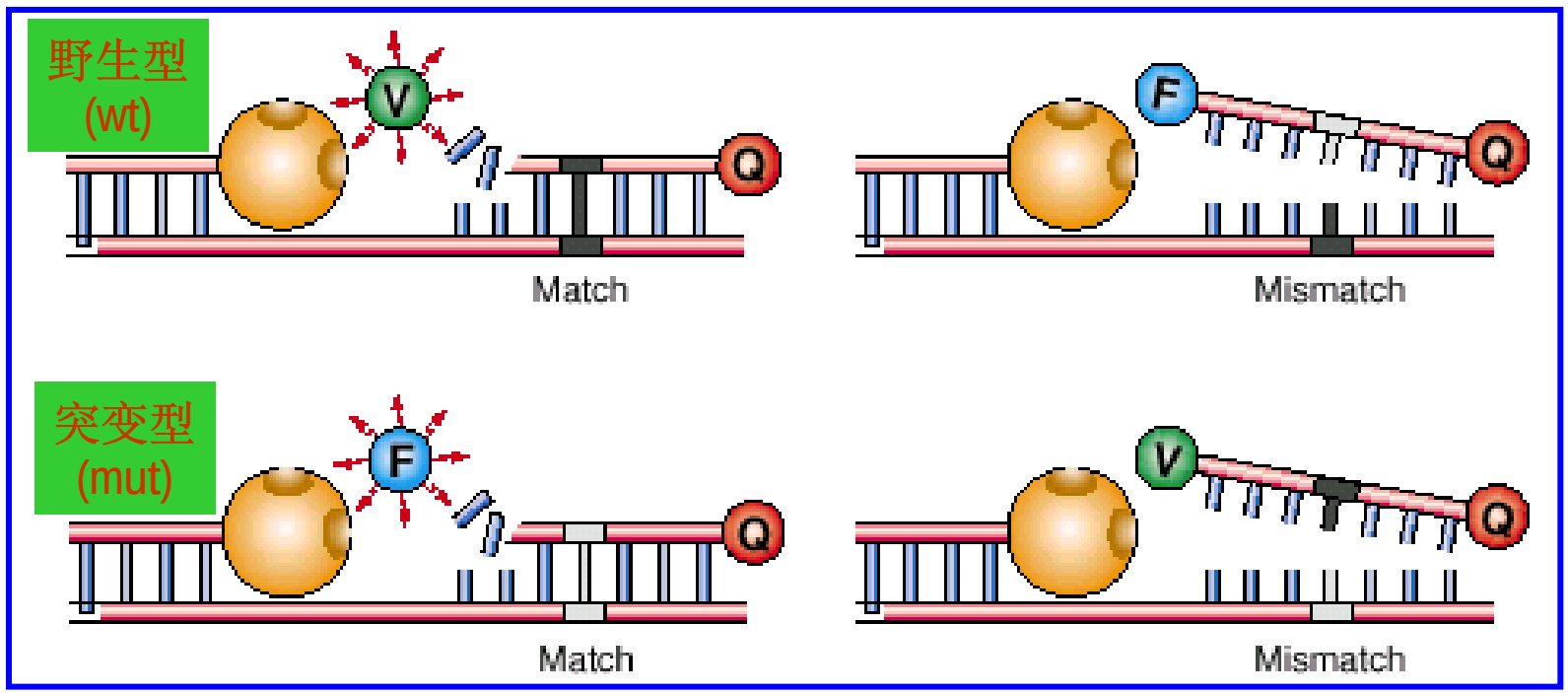
Relative Expression



等位基因分析

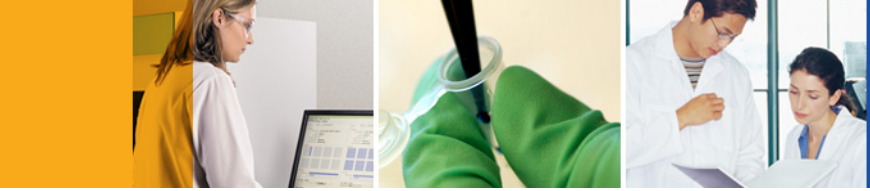
Allelic Discrimination (SNP Detection)

等位基因鉴定机理

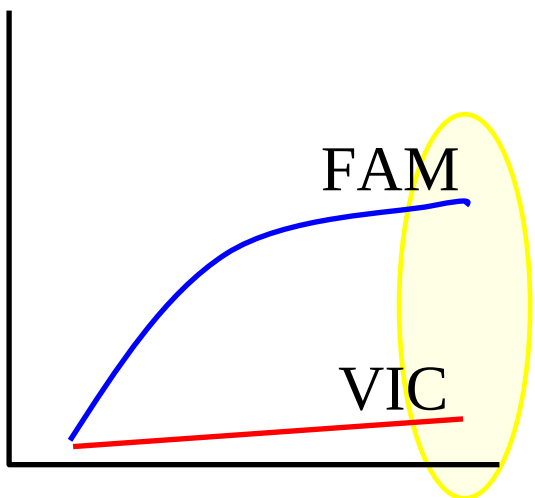


TaqMan® MGB 探针

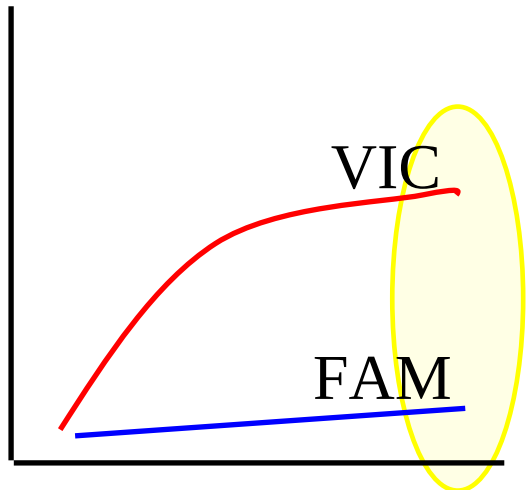
- Minor Groove Binder enhances the melting temperature (Tm) of the probe resulting in shorter probes
  - Shorter probes provide better discrimination
  - TaqMan® MGB probes provide excellent discrimination even with A/T rich sequences



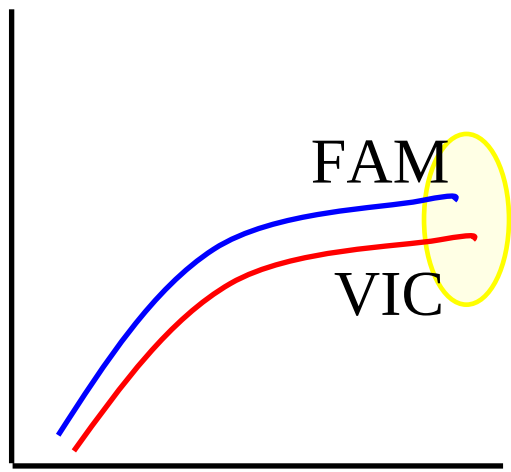
Allelic Discrimination Assay



Homozygote for FAM-specific allele



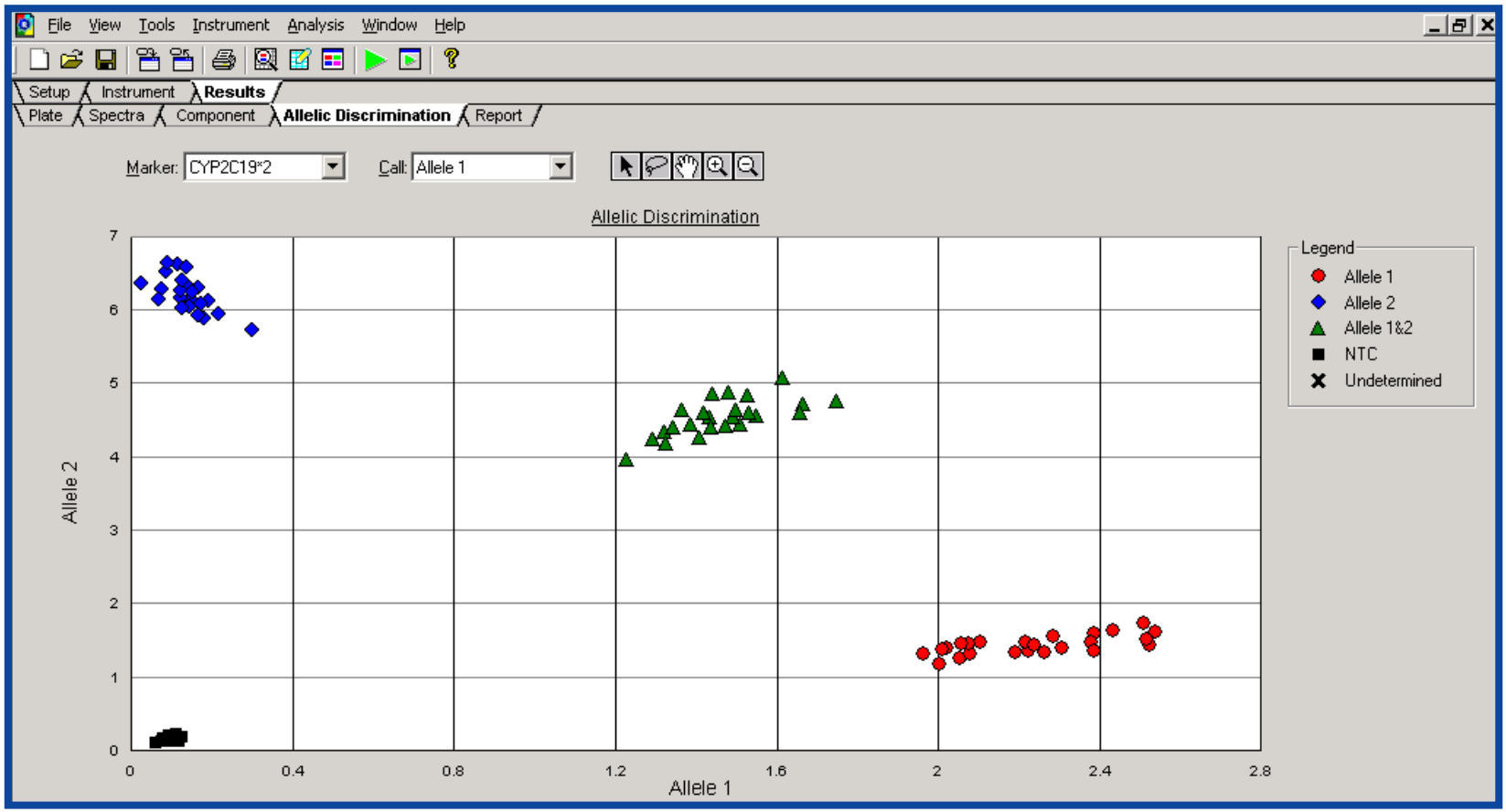
Homozygote for VIC-specific allele



Heterozygote for both alleles

Allelic Discrimination Dye Components Viewer

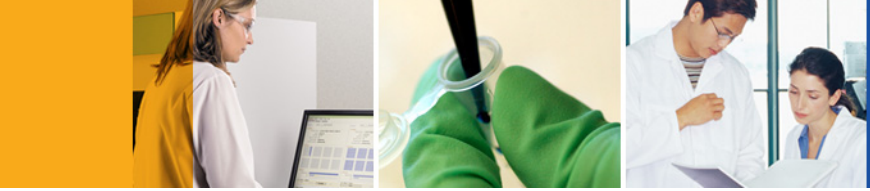
FAM



VIC

阴阳性鉴定

Plus/Minus Assay



Plus/Minus Calls = Automatic Results

Pathogen

Internal Positive Control

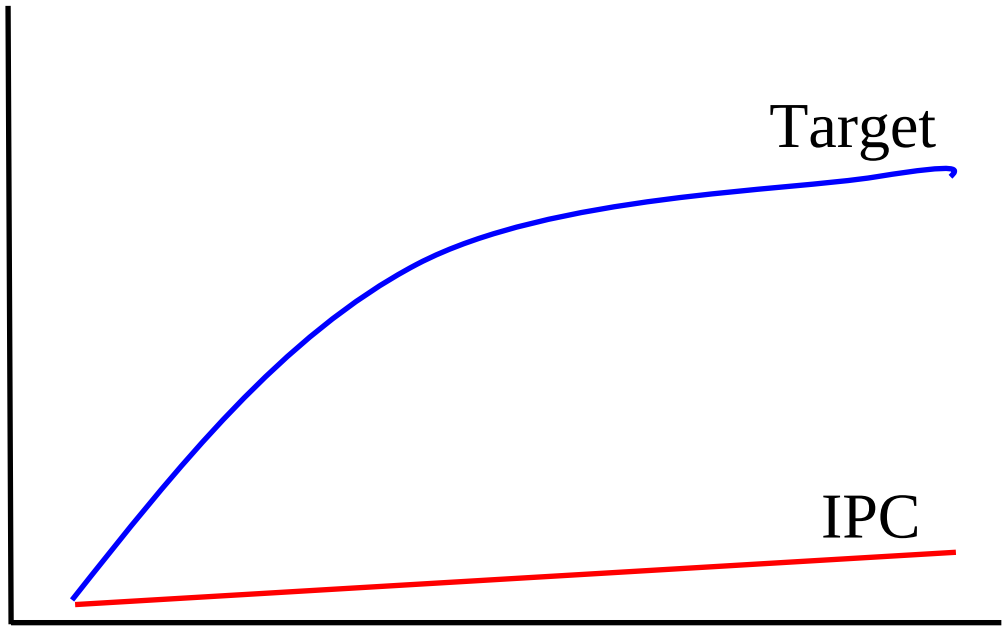
\+ − = Yes

\- + = No

\- − = No Amp

\+ + = Yes

Target vs. IPC Competition





荧光PCR光学原理

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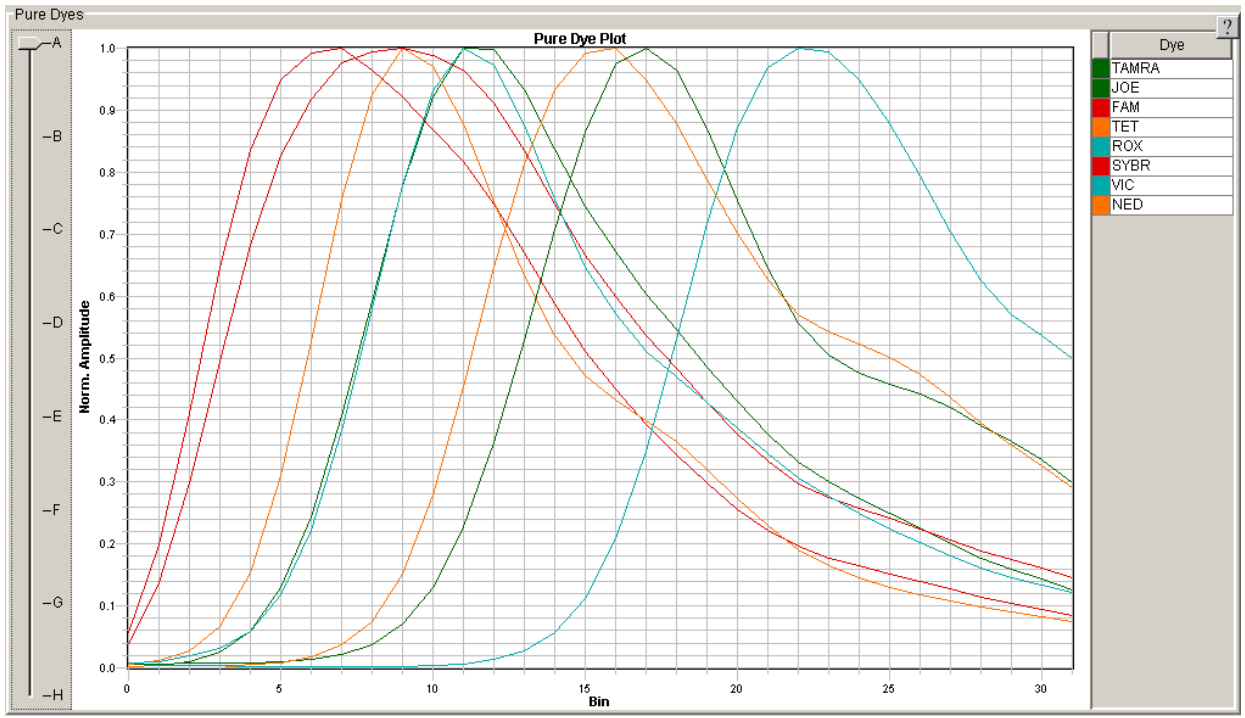
需要了解的问题

- 荧光染料
- 激发
  - 光源
  - 波长
- 检测器
  - 原件
  - 波长



- New Tungsten Halogen Excitation Lamp
  - Rated to 2,000 hours
  - Instrument has lamp life monitoring capability and notifies user when change is required or estimated lifetime is exceeded
  - User changeable

7500 System

Hardware Features - Optical

- 5 Excitation Filters
- 5 Emission Filters
- Filters are optimized to excite a broader range of dyes
  - FAM™, SYBR® Green I
  - VIC™, JOE™
  - NED™,TAMRA™, CY3 Dye™.
  - ROX™ (passive reference), Texas Red®
  - CY5 Dye™
- Multicomponenting algorithm means that we don’t require new filters to add new custom dyes

配套试剂

- TaqMan® Master Mix
- TaqMan® Gene Expression Master Mix
- TaqMan® SNP Genotyping Master Mix
- Fast SYBR® Green Master Mix
- TaqMan® Gene Expression Assays
- TaqMan® SNP Genotyping Assays
- Custom Primers & Probes



定量PCR的实验要素

Applied Biosystems



定量PCR实验要素

- 目标基因
- 标准曲线
- 监控系统故障
- 监控污染

样品
标准品
阳性对照
阴性对照

- 校准生物学误差
- 校准物理误差
- 降低其余误差

阳性内对照 (IPC)
参比荧光 (ROX)
重复实验

样　品

- DNA纯度：OD260/OD280=1.8，1.6 ~ 2.0之间
- DNA用量：0.05 ng – 100 ng

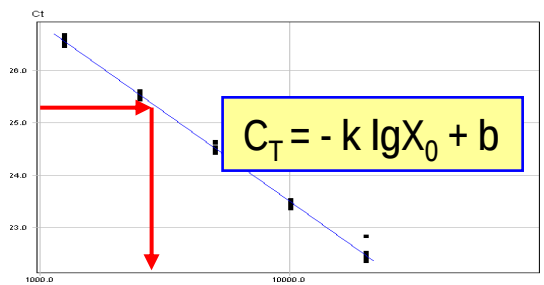
- RNA纯度：OD260/OD280=2.0
- cDNA用量：1-100 ng 总RNA反转录的cDNA取1 uL

标准品

- 目的：生成标准曲线，建立CT值与浓度之间的线性关系

- 要求：
  - 浓度已知
  - 5点以上
  - 标准品与待测样品的PCR效率一致，且接近100%
    - 仪器质量一致
    - 试剂质量一致：模板纯度、引物和探针的Tm值、酶活性、缓冲液成分
    - 反应条件一致：循环参数相同、同一次实验

- 不要求：
  - 不要求标准品与目标基因使用相同的DNA
  - 可以相同，也可以不同



标准品梯度稀释方法

- 选择目标→提取/PCR →纯化→测定浓度→调整浓度→梯度稀释
- CT值在18-30之间，覆盖全部样品浓度区间
- 10倍连续梯度稀释方法：
  - 1v原液(标准品i) +9v稀释缓冲液，得标准品ii
  - 1v标准品ii+9v稀释缓冲液，得标准品iii
  - 1v标准品iii +9v稀释缓冲液，得标准品iv
  - 1v 标准品iv +9v稀释缓冲液，得标准品v
  - 切不可由标准品i分别加不同体积的稀释缓冲液直接得到标准品ii、iii、iv、v

标准品单位

- 标准品的单位

- 根据最终目的选择标准品单位
  - 如果要求测定样品的基因拷贝数，则梯度稀释已知摩尔浓度的DNA片段
  - 如果要求测定样品的重量百分比[%(w/w)]，如转基因，标准品是将转基因与非转基因食品粉末按重量比混合，然后抽提混合DNA；切不可先抽好转基因DNA，再梯度稀释，也不可分别抽提转基因与非转基因DNA，再按重量比例混合（这样得出的是基因重量百分比，而不是样品重量百分比）

PCR效率对定量结果的影响

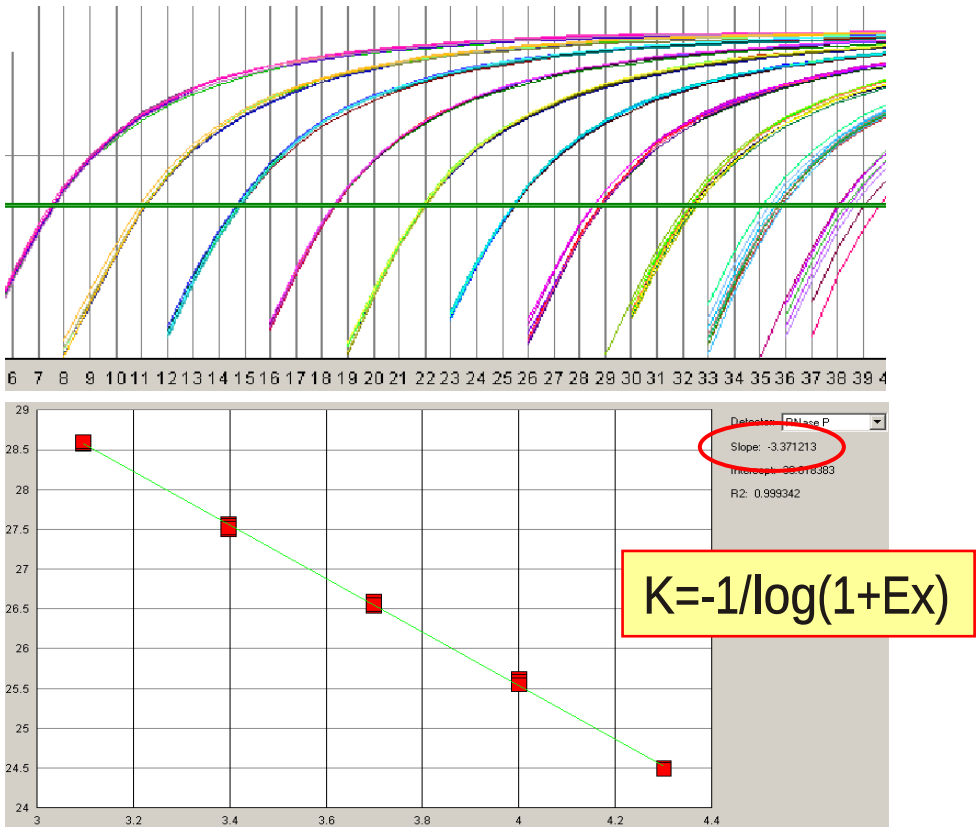
n = 30
1+Ex = 1.95 → $N = N_0\ 1.95^{30} = N_0 \times 5.0 \times 10^8$

相差2.2倍

n = 30
1+Ex = 1.90 → $N = N_0\ 1.90^{30} = N_0 \times 2.3 \times 10^8$

（Ex=扩增效率；n=循环圈数；No=起始模板分子数；N=扩增产物分子数）

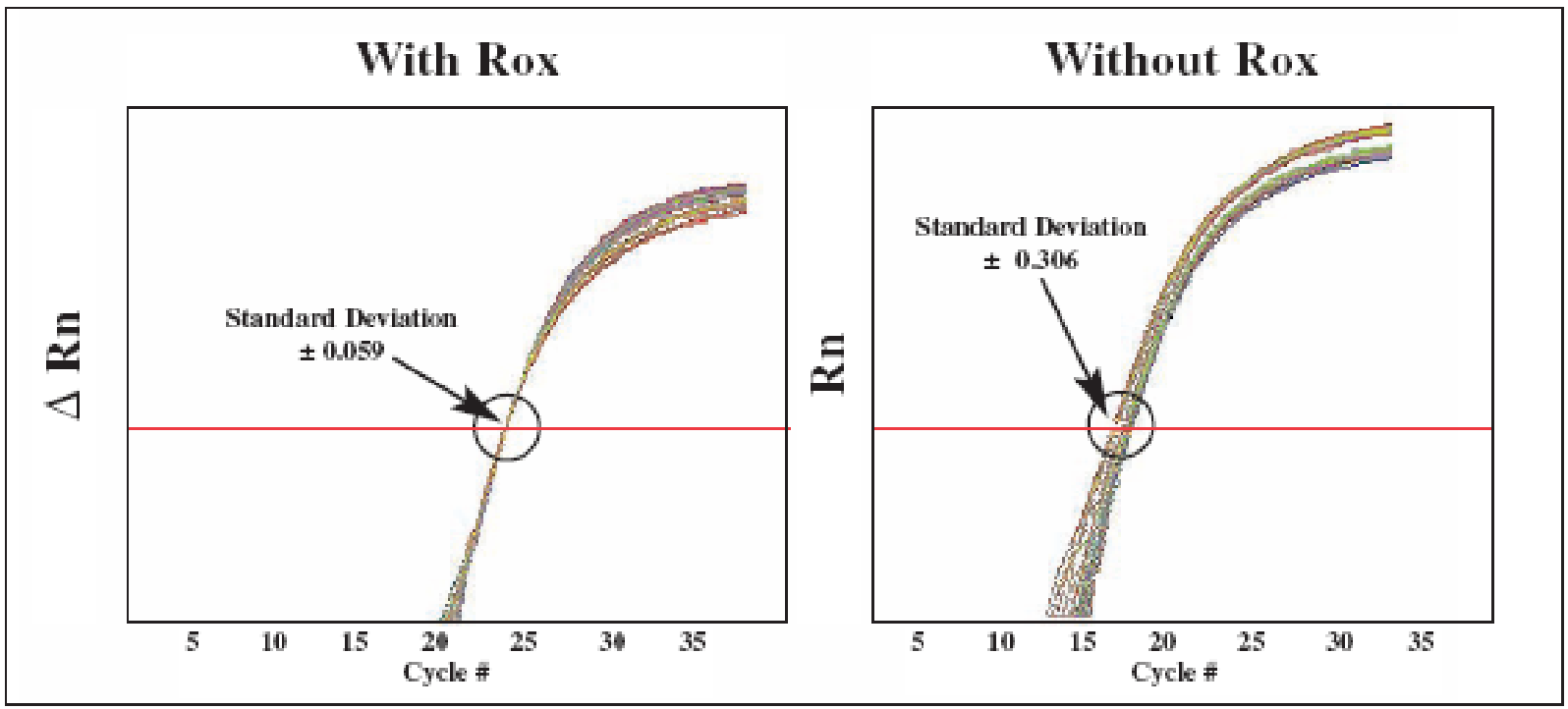
PCR效率测定


6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 4
29
28.5
28
27.5
27
26.5
26
25.5
25
24.5
24
3
3.2
3.4
3.6
3.8
4
4.2
4.4
Slope: -3.371213
R2: 0.999342
K=-1/log(1+Ex)

？ ？ ？

- 在使用TaqMan MGB探针的情况下，你认为管家基因与目标基因是在同一管内做多重定量好，还是分成两管分别定量好？哪一种检测的数据更精确？

ROX校准增加数据精度



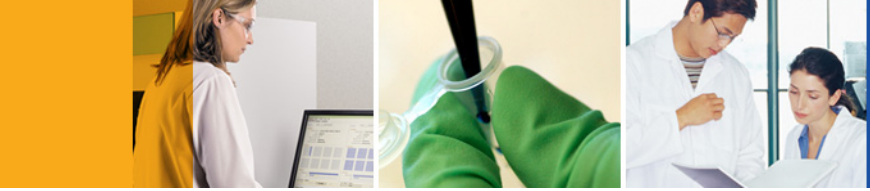


复管测试

- 样品和标准品都要重复
- 重复次数须遵循统计学要求

误差的校正

- 生物学方面的误差：IC校正
  - 细胞数量的差异、抽提效率、纯化损失、反转录效率等
- 物理方面的误差：ROX校正
  - 枪的误差（如试剂体积）、耗材质量（如管盖厚度、透光性能不一致）所导致的光能损失、仪器稳定性（如孔间、批间温度的波动）的误差等
- 其余的误差：统计校正
  - 重复实验，取平均值



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Q & A

术 → 道

生物学意义

↑

检测技术



谢谢！

8008203939

cnmcbsupport@appliedbiosystems.com

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