微滴式数字PCR培训及应用分享

Bio-Rad FAS　罗艳

2019-03-11

- 微滴式数字PCR技术
  - ddPCR原理
  - ddPCR技术特点
- QX200 ddPCR系统
  - QX200 ddPCR系统
  - ddPCR实验操作流程
- ddPCR相关应用及实验设计

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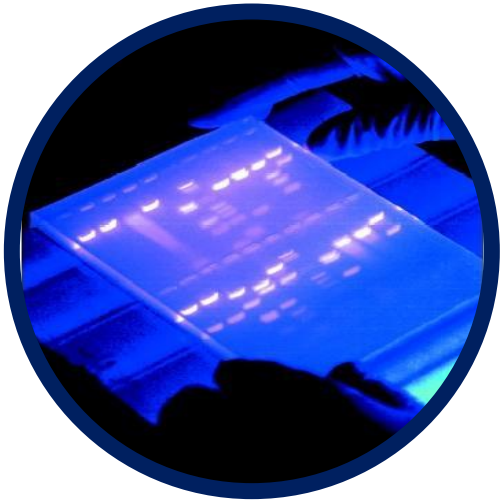
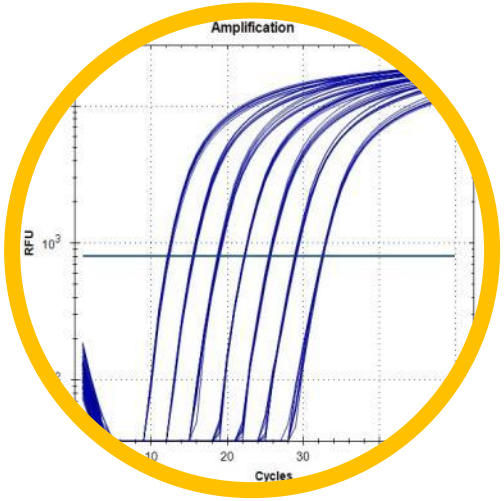
PCR技术的发展历史

1st



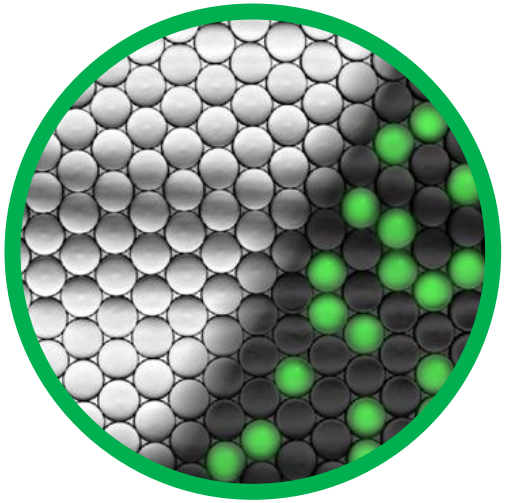
Routine PCR
定性检测

2nd



Real-time PCR
相对定量

3rd



Digital PCR
绝对定量

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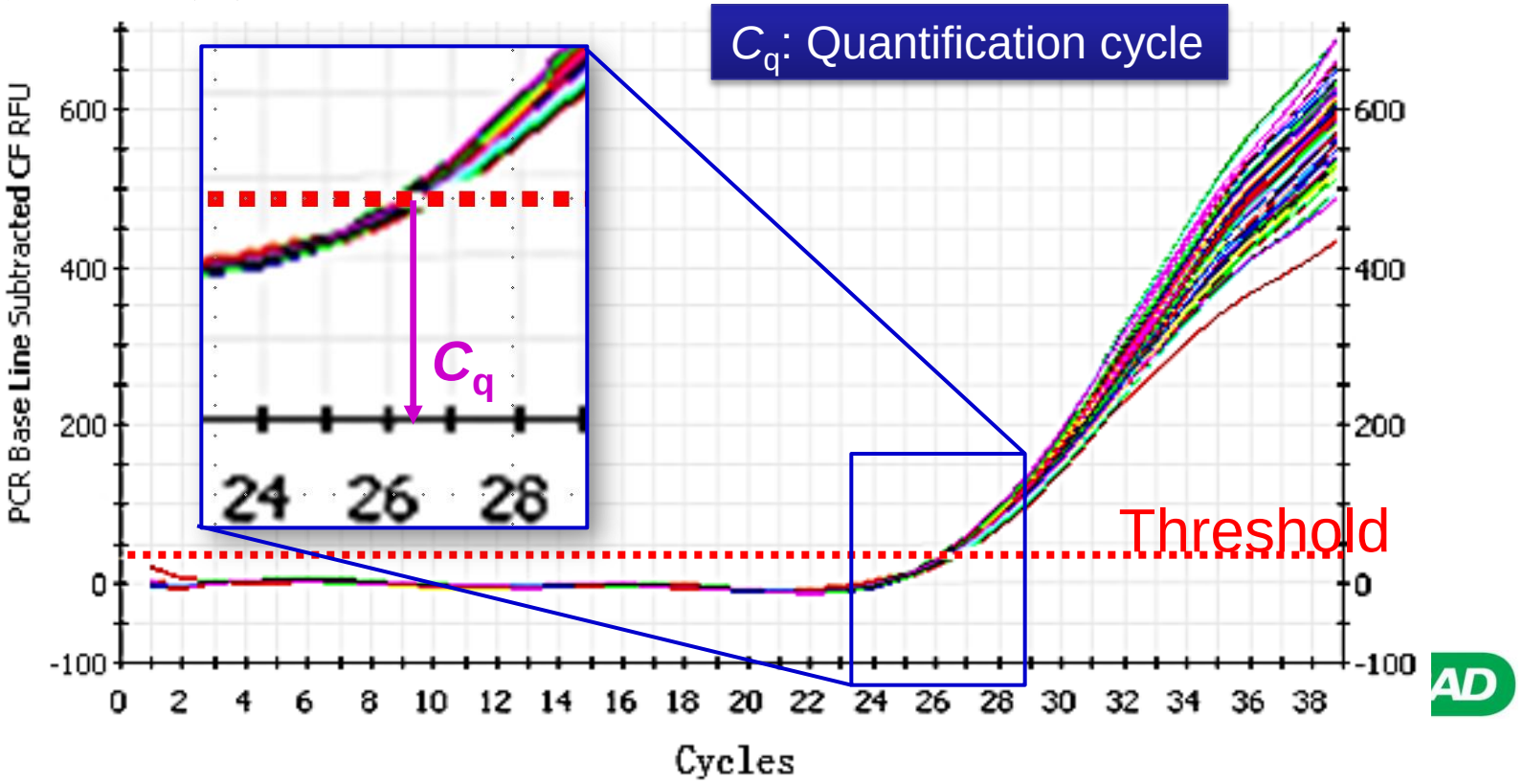
Real-Time PCR的基本原理

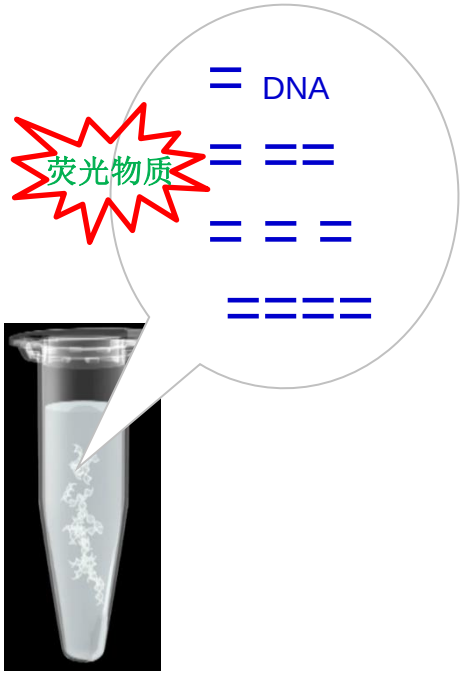
实时检测：在普通PCR反应体系中加入荧光物质（染料或探针），然后实时记录PCR过程中与目标分子量相关的荧光信号强度

定量原理：利用指数期C_q值与起始模板量对数值间的线性相关性，对DNA进行定量

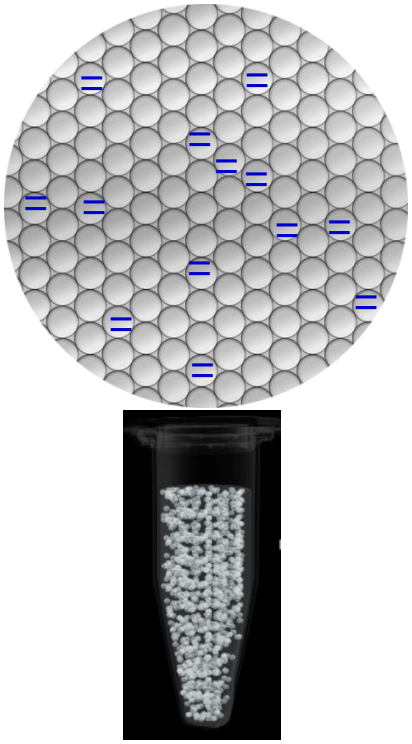


Droplet digital PCR技术原理

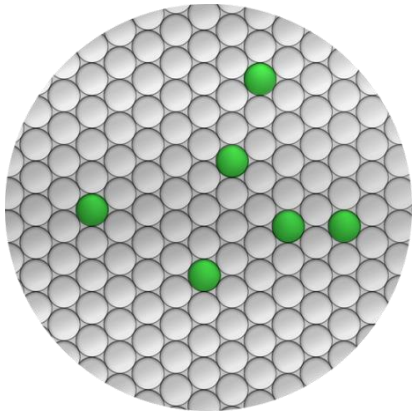
PCR反应体系配制



微滴化处理



微滴的荧光信号检测



Positive reactions

Negative reactions

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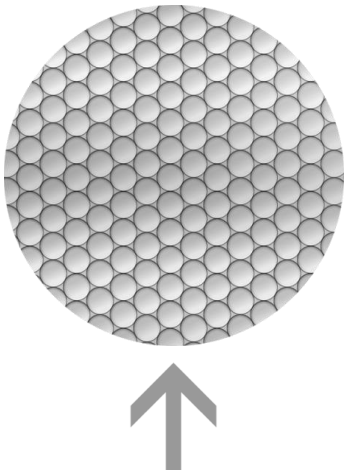
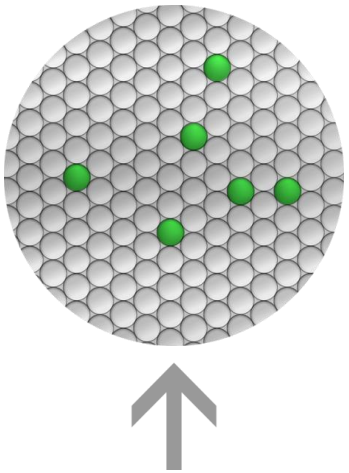
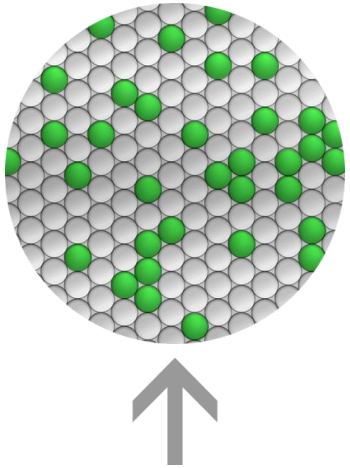
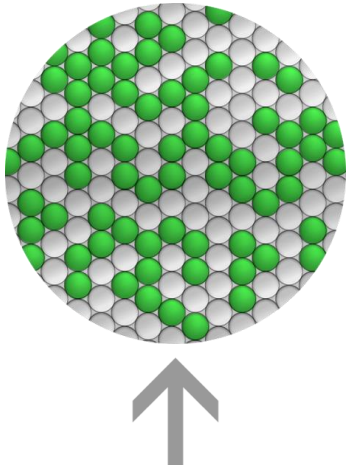
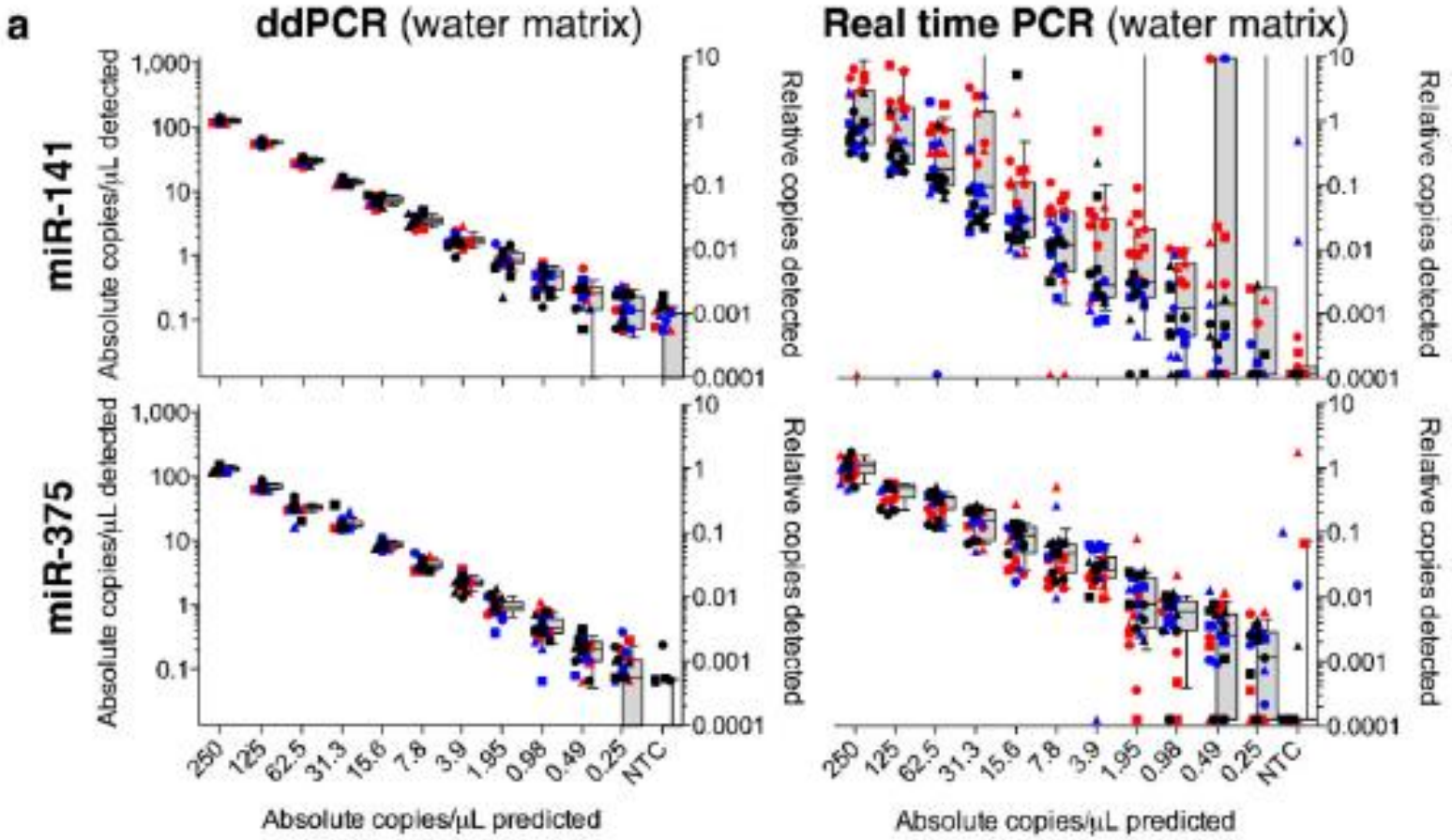
ddPCR绝对定量原理

Sample 1	Sample 2	Sample 3	Sample 4
			
<u>NO</u> targets	Low concentration	Medium concentration	High concentration
p= 0 *positive* /143 *total*	*p*= 6/143	*p*= 34/143	*p*= 70/143
	Poisson corrected **6.2**/143	Poisson corrected **38**/143	Poisson corrected **96**/143

ddPCR实现精准定量和高度重复



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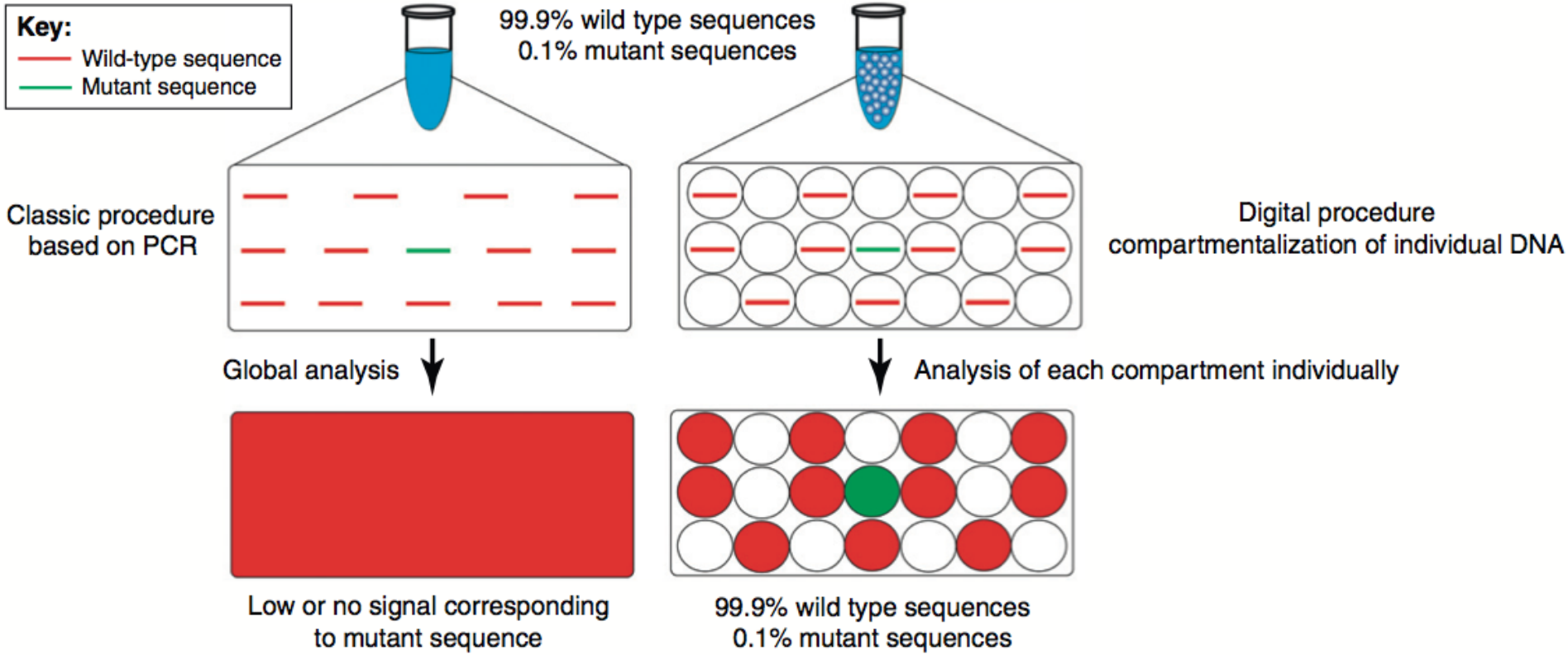
Nat Methods. 2013

ddPCR实现痕量样品的检测



TRENDS in Molecular Medicine

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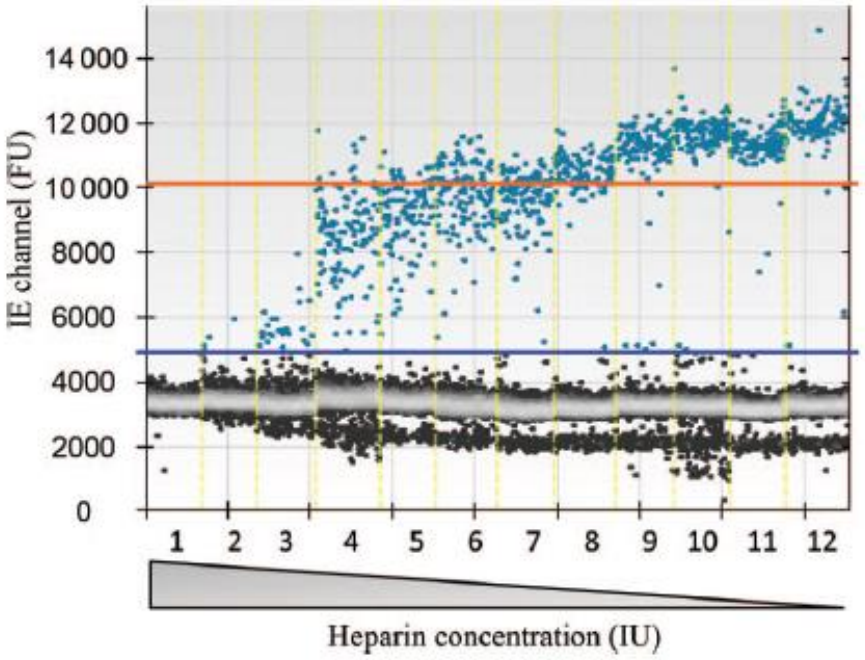
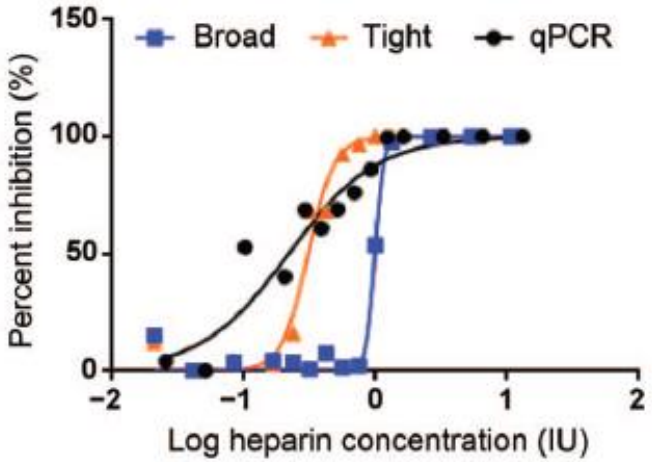
ddPCR不易受扩增效率的影响





ddPCR offers an advantage over qPCR when dealing with inhibition-prone samples

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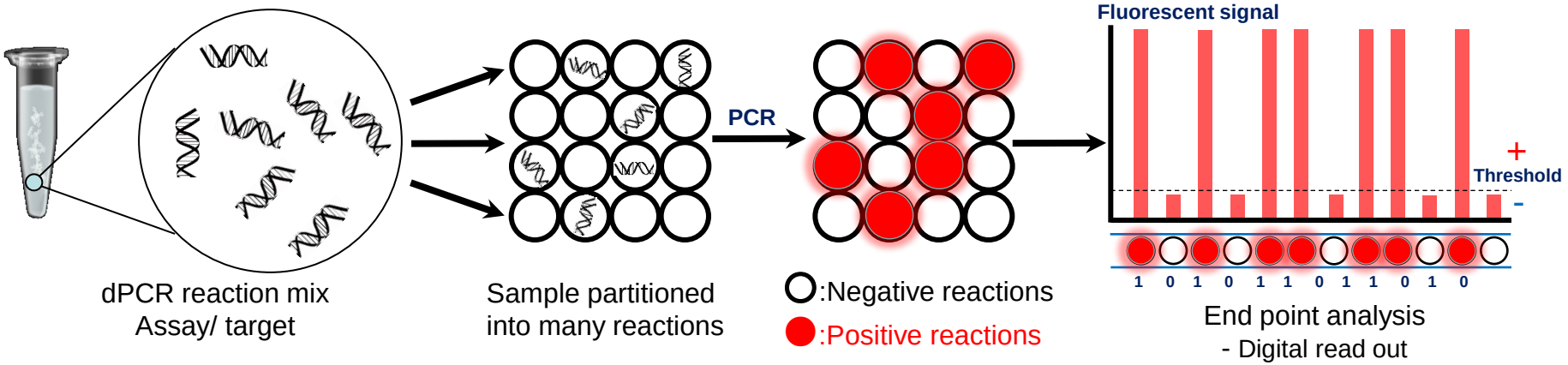
Clin Chem, 2013

微滴式数字PCR的特点和优势



Key Benefits of ddPCR:

01 无需标准曲线的绝对定量（DNA/mRNA/miRNA/LncRNA）

02 更高的检测灵敏度（提升高丰度野生型背景下的<0.1%突变检出率）

03 更高的数据精密度（能识别1.1倍以内的浓度变化）

04 更好的数据重复性（对靶标核酸含量的连续监测、横向比较）

05 提高对抑制物的耐受度（克服抑制物对定量结果的干扰）

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QX200™微滴式数字PCR系统

微滴分析仪

微滴发生器

Reagents

Assays

Consumables

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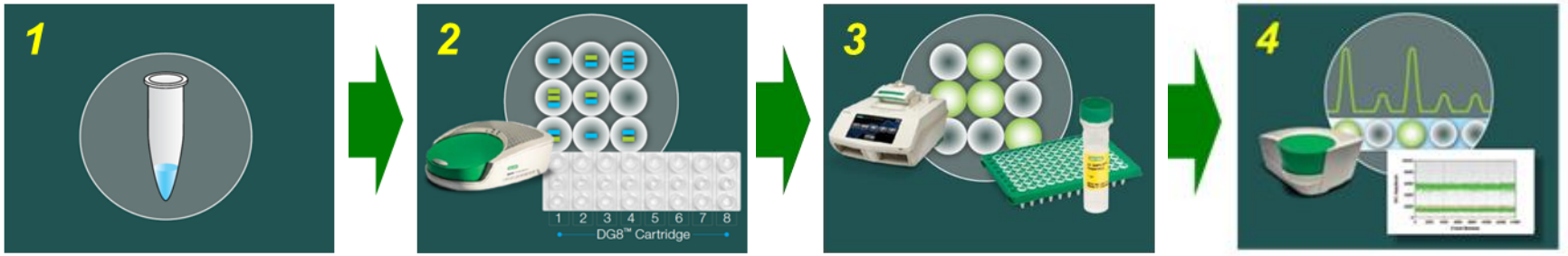
微滴式数字PCR的操作流程



ddPCR反应体系配制

- 将DNA/RNA样品、引物和探针/EvaGreen染料与ddPCR预混液混合（20 µl）

微滴制备

- 将ddPCR反应体系添加至微滴发生器芯片的小孔中
- 微滴发生器每次运行最多可以产生8×20,000个油包水的微滴
- 目标核酸和背景核酸随机分布于微滴中

微滴PCR

- 将微滴转移至96孔PCR反应板中，并密封
- 进行微滴PCR

微滴分析

- PCR反应后，将96孔PCR反应板转移至微滴分析仪上
- 分析每个样品的微滴的荧光信号
- 软件根据阴性微滴的比例，结合泊松分布原理，定量DNA/RNA浓度（copies/µl）

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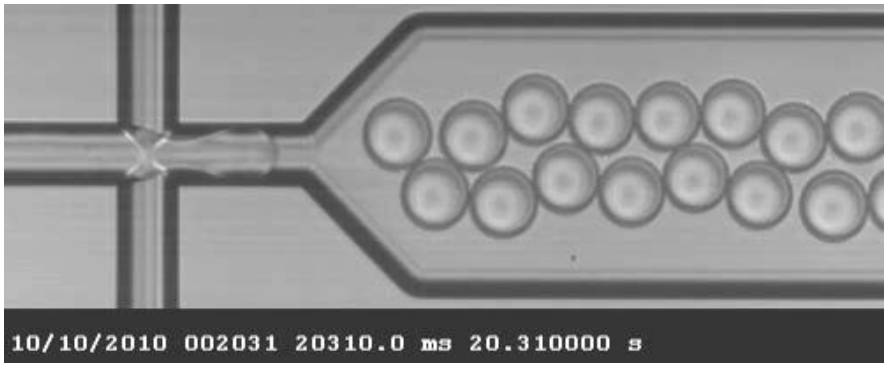
微滴制备

将含样品的预混液和油加入微滴发生卡的指定位置，放入微滴发生器内。每次对8个样品进行微滴化处理，2 min完成，每个样品可生成20,000个微滴。



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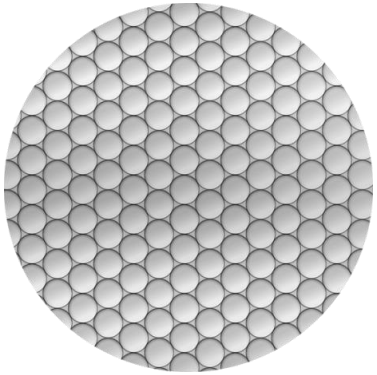
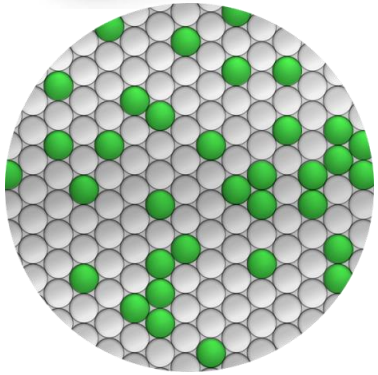
微滴扩增

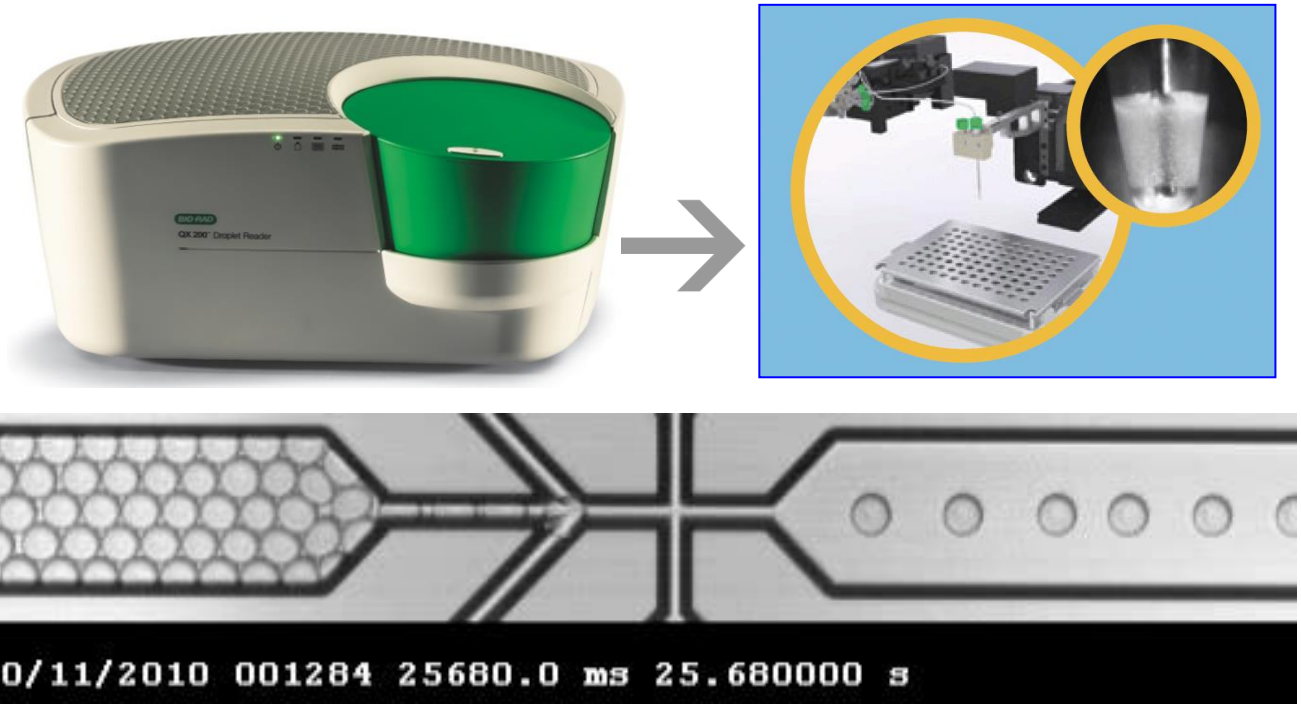
普通PCR仪即可，无需实时检测荧光。





微滴检测

将PCR反应板直接放入微滴分析仪，自动对样品进行逐个分析，每个微滴逐个通过检测器，每小时可完成32个样品的检测。



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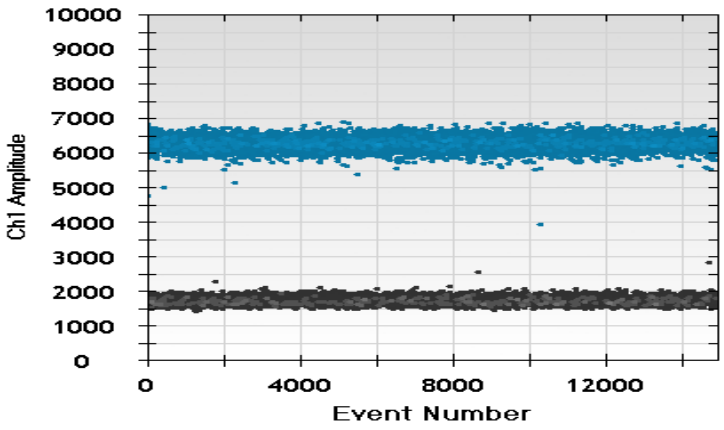
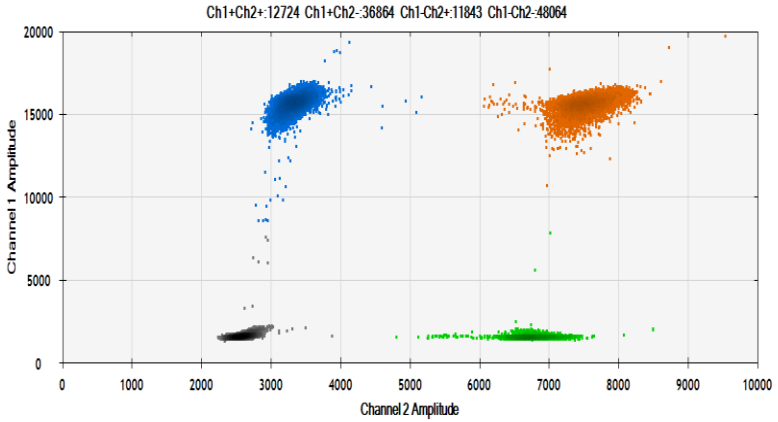
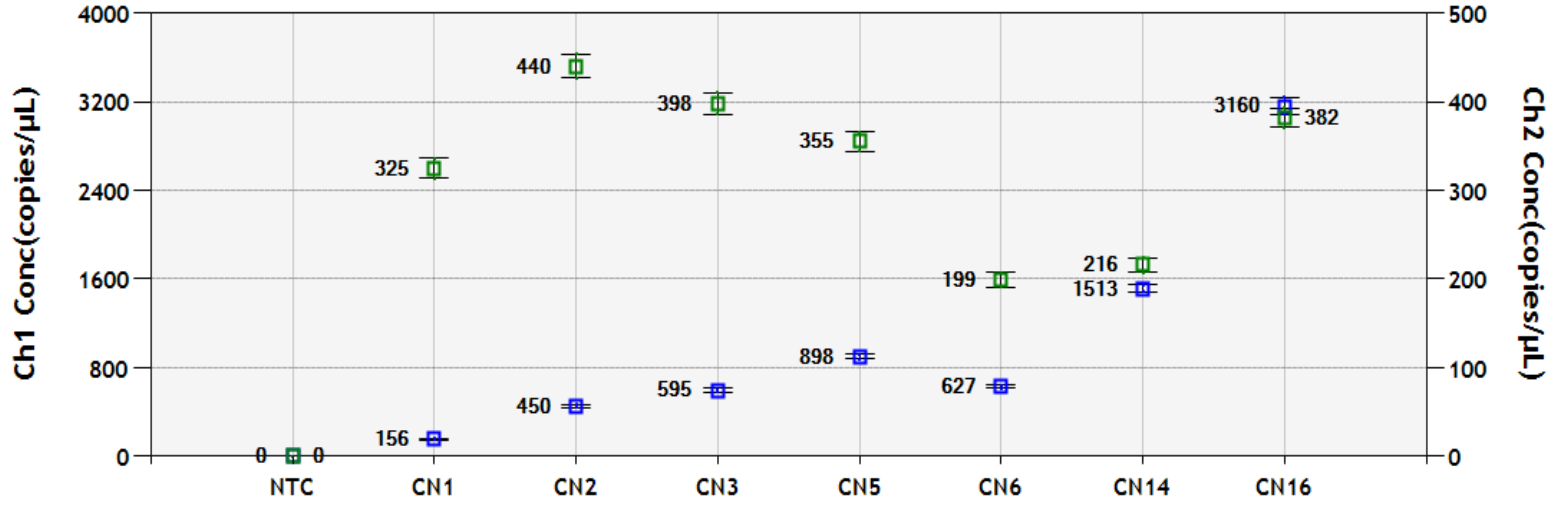
软件自动分析结果，无需计算







简易操作流程 & 软件使用指南

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ddPCR相关配套试剂耗材

ddPCR预混液

微滴发生油

微滴发生卡及密封垫

引物/探针/Assay

96孔反应板及封膜

微滴读取油

货号	名称
1864002	QX200 droplet generator
1864003	QX200 droplet reader
1814000	PX1 PCR Plate sealer
	微滴发生专用油
186-3005	Droplet Generator Oil for Probes, 10 x 7 ml (1000 reactions)
186-4005	Droplet Generator Oil for EvaGreen, 2 x 7 ml (200 reactions)
1863004	微滴检测专用油
1864007	微滴发生卡及密封垫
	ddPCR预混液
186-3023	ddPCR Supermix for Probes (no dUTP), 2 ml (2 x 1 ml), 200 reactions
186-3024	ddPCR Supermix for Probes (no dUTP), 5 ml (5 x 1 ml), 500reactions
186-4033	QX200 ddPCR EvaGreen Supermix, 2 ml (2 x 1 ml), 200 x 20 µl reactio
186-4034	QX200 ddPCR EvaGreen Supermix, 5 ml (5 x 1 ml), 500 x 20 µl reactio
12001925	96孔板（Bio-Rad）
1814040	Pierceable Foil Heat Seal

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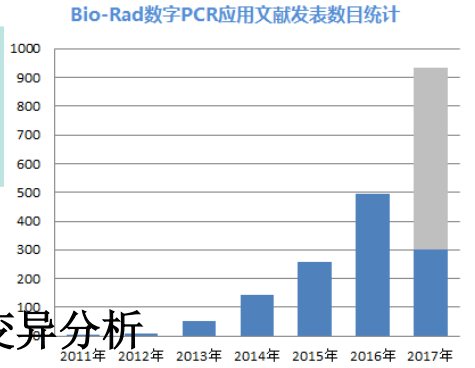
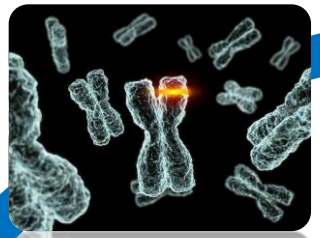
ddPCR应用广泛



稀有突变检测



微生物检测

拷贝数变异分析

DNA残留检测
/CRMCs质控标定

微小差异基因表达分析

环境监测

无创产前检查

NGS测序文库质控

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ddPCR的主要应用

- **绝对定量：**标准物质的标定，没有标准品但需要定量检测的样品……
- **高灵敏度的检测：**痕量样品，珍稀样品，突变检测，单细胞研究……
- **高精密度的检测：**微小表达差异分析，拷贝数变异分析（CNV），动态监测……
- **复杂样品的检测：**环境样品，土壤，水样……
- NGS结果的验证，基因编辑检测……

标准物质的标定

Anal Bioanal Chem (2014) 406:1701–1712
DOI 10.1007/s00216-013-7546-1

中国计量科学研究院

RESEARCH PAPER

Evaluation of droplet digital PCR for characterizing plasmid reference material used for quantifying ammonia oxidizers and denitrifiers

Lianhua Dong · Ying Meng · Jing Wang · Yingying Liu

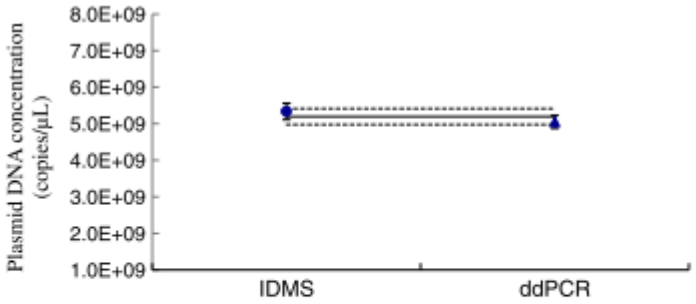
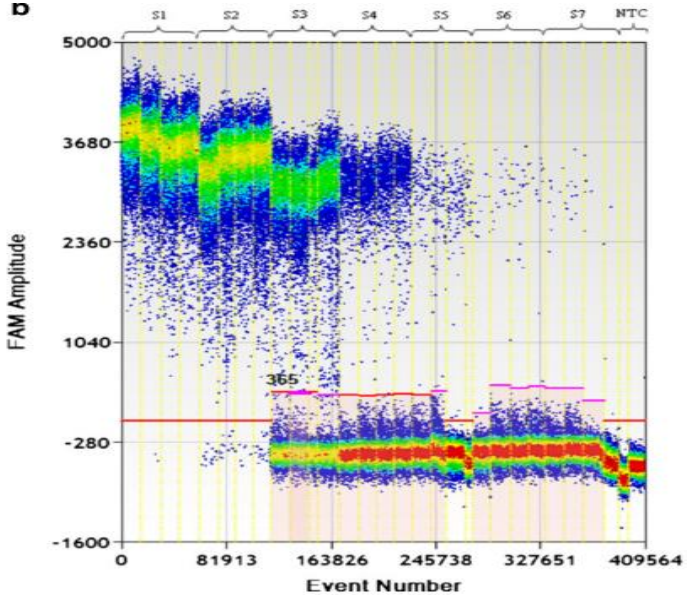


Fig. 7 Measured plasmid pNIM-003 DNA concentrations, with the expanded uncertainty (k=2) (copies μL^{-1}), obtained by droplet digital PCR (ddPCR) and isotope dilution mass spectrometry (IDMS). The attributed concentration for the plasmid DNA stock (*continuous line*) with the expanded uncertainty (*dashed lines*) was calculated by averaging the two measurement results



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Anal Bio Chem, 2014

ddPCR用于HIV DNA的高灵敏检测

The NEW ENGLAND JOURNAL of MEDICINE

BRIEF REPORT

Absence of Detectable HIV-1 Viremia after Treatment Cessation in an Infant

Deborah Persaud, M.D., Hannah Gay, M.D., Carrie Ziemniak, M.S., Ya Hui Chen, B.A., Michael Piatak, Jr., Ph.D., Tae-Wook Chun, Ph.D., Matthew Strain, M.D., Ph.D., Douglas Richman, M.D., and Katherine Luzuriaga, M.D.

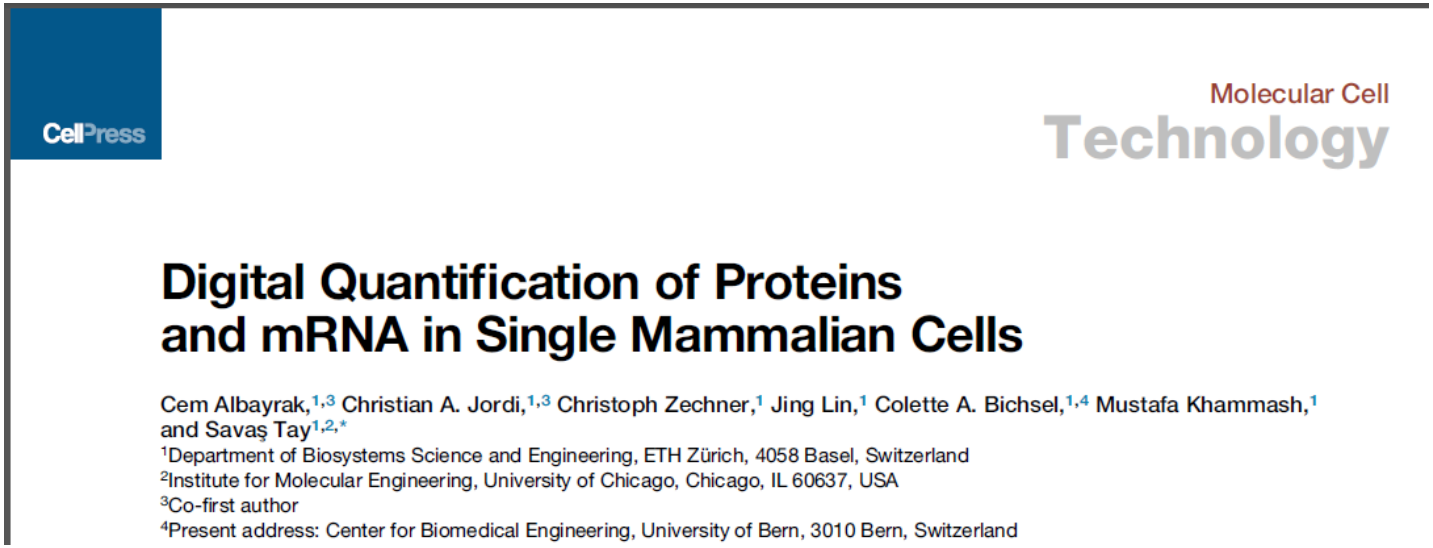
- Background
- 出生时为HIV 阳性，进行抗病毒治疗，29天后即无法通过常规检测手段检测到HIV
- Solution
- 24,26月龄时，采用ddPCR技术对该名婴儿HIV DNA进行检测

Age	HIV DNA detected by ddPCR	Replication-competent virus detected
24 months	**37 copies/ million PBMC**	**No**
26 months	**4 copies/ million PBMC**	**No**

灵敏度高达0.0004%

NEJM, 2013

ddPCR用于单细胞mRNA和Protein检测



CellPress

Molecular Cell
Technology

Digital Quantification of Proteins and mRNA in Single Mammalian Cells

Cem Albayrak,[1,3] Christian A. Jordi,[1,3] Christoph Zechner,[1] Jing Lin,[1] Colette A. Bichsel,[1,4] Mustafa Khammash,[1] and Savaş Tay[1,2,*]
[1]Department of Biosystems Science and Engineering, ETH Zürich, 4058 Basel, Switzerland
[2]Institute for Molecular Engineering, University of Chicago, Chicago, IL 60637, USA
[3]Co-first author
[4]Present address: Center for Biomedical Engineering, University of Bern, 3010 Bern, Switzerland

Highlights

- Digital PLA protocol allows ultrasensitive protein quantification from single cells
- Combination with digital PCR allows joint single-cell protein and mRNA measurements
- Absolute mRNA and protein abundances measured jointly from single mammalian cells
- Joint mRNA-protein data was used to build a two-step model of gene expression

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ddPCR用于微小基因表达差异分析研究

A male-determining factor in the mosquito *Aedes aegypti*

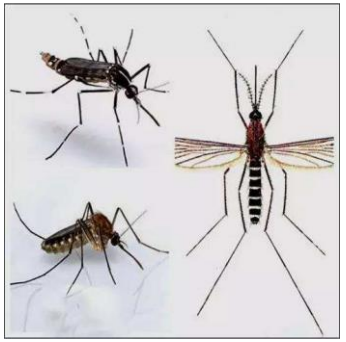
Andrew Brantley Hall,[1,2,3*] Sanjay Basu,[3,4*] Xiaofang Jiang,[1,2,3] Yumin Qi,[2,3] Vladimir A. Timoshevskiy,[3,4] James K. Biedler,[2,3] Maria V. Sharakhova,[3,4] Rubayet Elahi,[2] Michelle A. E. Anderson,[3,4] Xiao-Guang Chen,[5] Igor V. Sharakhov,[1,3,4] Zach N. Adelman,[1,3,4†] Zhijian Tu[1,2,3†]

[1]Interdisciplinary PhD Program in Genetics, Bioinformatics, and Computational Biology, Virginia Tech, Blacksburg, VA, USA. [2]Department of Biochemistry, Virginia Tech, Blacksburg, VA, USA. [3]Fralin Life Science Institute, Virginia Tech, Blacksburg, VA, USA. [4]Department of Entomology, Virginia Tech, Blacksburg, VA, USA. [5]School of Public Health and Tropical Medicine, Southern Medical University, Guangdong, People's Republic of China..

*These authors contributed equally to this work.

†Corresponding author. E-mail: jaketu@vt.edu (Z.T.); zachadel@vt.edu (Z.N.A.)

Sex determination in the mosquito *Aedes aegypti* is governed by a dominant male-determining factor (M factor) located within a Y chromosome–like region called the M locus. Here, we show that an M-locus gene, *Nix*, functions as an M factor in *A. aegypti*. *Nix* exhibits persistent M linkage and early embryonic expression, two characteristics required of an M factor. *Nix* knockout with CRISPR/Cas9 resulted in largely feminized genetic males and the production of female isoforms of two key regulators of sexual differentiation: *doublesex* and *fruitless*. Ectopic expression of *Nix* resulted in genetic females with nearly complete male genitalia. Thus, *Nix* is both required and sufficient to initiate male development. This study provides a foundation for mosquito control strategies that convert female mosquitoes into harmless males.

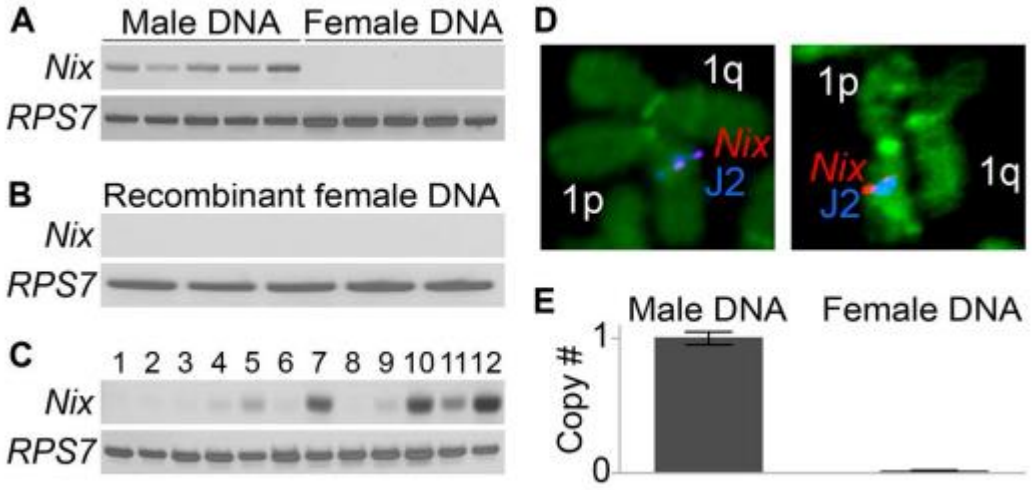


研究内容：发现埃及伊蚊的性别决定因子*Nix*基因，采用ddPCR确定基因的拷贝数及对其他基因表达的调控

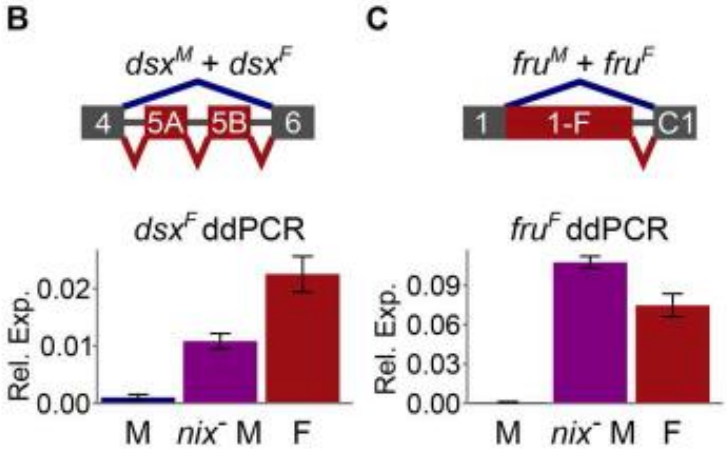
BIO-RAD

Science, 2015

ddPCR用于微小基因表达差异分析研究



采用了PCR，FISH和ddPCR的方法对Nix基因在雌雄性中的拷贝数进行检测分析



采用ddPCR方法，检测*Nix*基因敲除后雌性分化相关因子*dsx*和*fru*的表达水平

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Science, 2015

ddPCR用于凡纳滨对虾免疫基因表达分析

Aquaculture 434 (2014) 403–410

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Mannooligosaccharides from copra meal improves survival of the Pacific white shrimp (*Litopenaeus vannamei*) after exposure to *Vibrio harveyi*

Wanilada Rungrassamee [a,*], Yutthana Kingcha [b], Yanee Srimarut [b], Sawarot Maibunkaew [a], Nitsara Karoonuthaisiri [a], Wonnop Visessanguan [b]

[a] Microarray Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), Khlong Luang, Pathum Thani, Thailand
[b] Food Biotechnology Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), Khlong Luang, Pathum Thani, Thailand

近年来，人们对抗生素的过度使用越来越关注，在这样的背景下，人们希望能有替代措施来避免或者减少抗生素的使用。本文评价了来源于椰子的低聚甘露糖(MOS)作为添加剂，在凡纳滨对虾养殖过程中对对虾的生长、抗Vibrio harveyi弧菌能力等方面的作用。通过微滴式数字PCR对免疫基因表达水平的分析，证明MOS可以通过调节凡纳滨对虾自身免疫力相关基因的表达，从而提高抗Vibrio harveyi弧菌的能力。

ddPCR用于凡纳滨对虾免疫基因表达分析

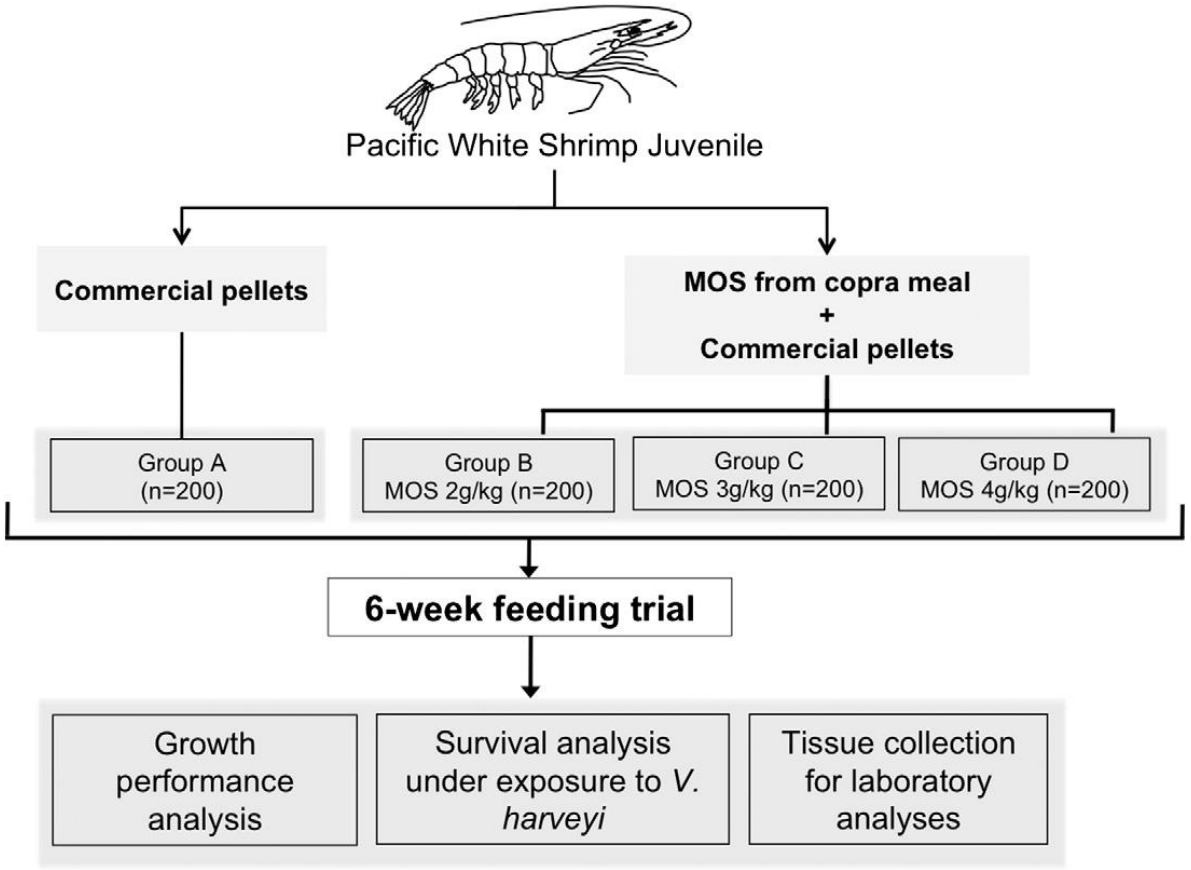

Fig. Overview of experimental plan to determine effects of copra meal mannooligosaccharide (MOS) supplementation on shrimp growth and immune performance.

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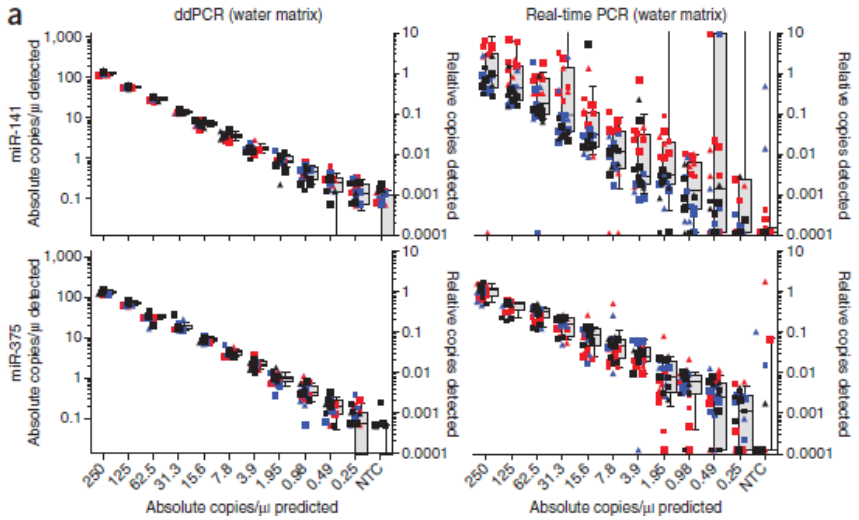
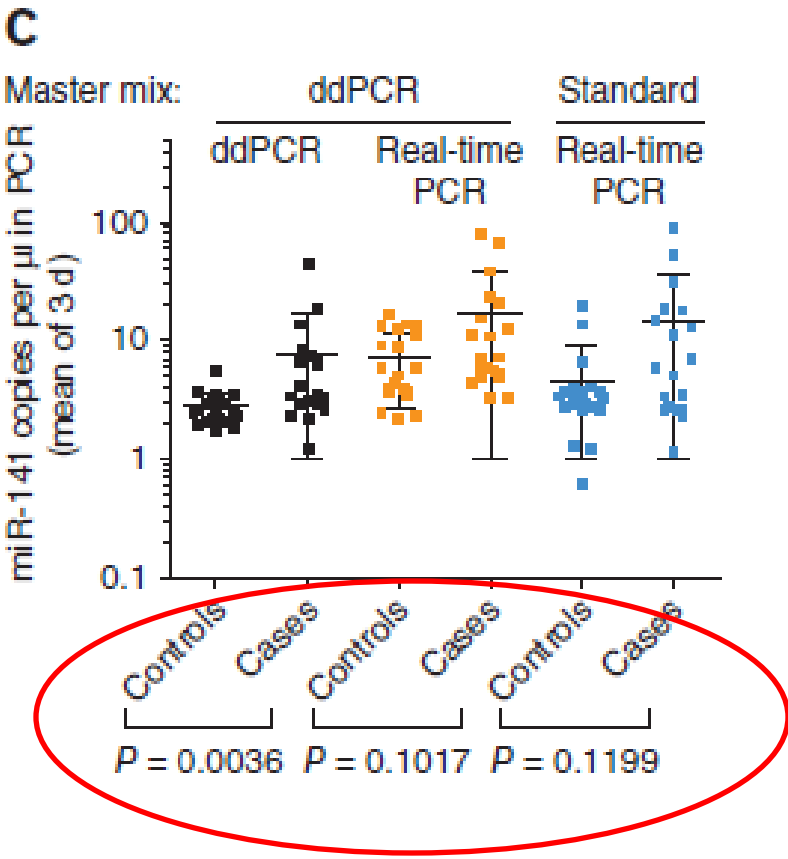
ddPCR检测miRNA更灵敏、准确

Absolute quantification by droplet digital PCR versus analog real-time PCR

Christopher M Hindson[1,6,7], John R Chevillet[2,7], Hilary A Briggs[2], Emily N Gallichotte[2], Ingrid K Ruf[2], Benjamin J Hindson[1,6], Robert L Vessella[3] & Muneesh Tewari[2,4,5]





BIO-RAD

Nat Methods. 2013

双重ddPCR检测2种蓝藻

AEM Journals.ASM.org

Comparison of Quantitative PCR and Droplet Digital PCR Multiplex Assays for Two Genera of Bloom-Forming Cyanobacteria, *Cylindrospermopsis* and *Microcystis*

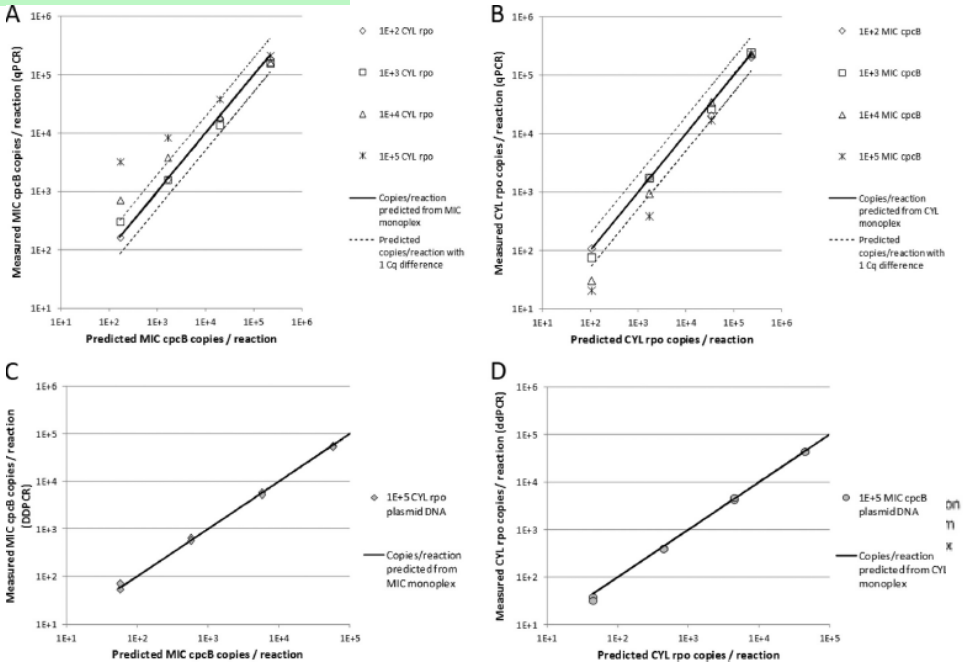
Shu Harn Te, Enid Yingru Chen, Karina Yew-Hoong Gin

NUS Environmental Research Institute, Department of Civil and Environmental Engineering, National University of Singapore, Singapore

qPCR与ddPCR相关性较好；

qPCR线性范围更宽，耗时更短，成本更低，适合初筛；

ddPCR重复性更好，更耐受PCR抑制物以及多重反应的竞争抑制，检测水华样本的准确度和精确度更高。



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AEM, 2017

ddPCR检测eDNA预测种群状态

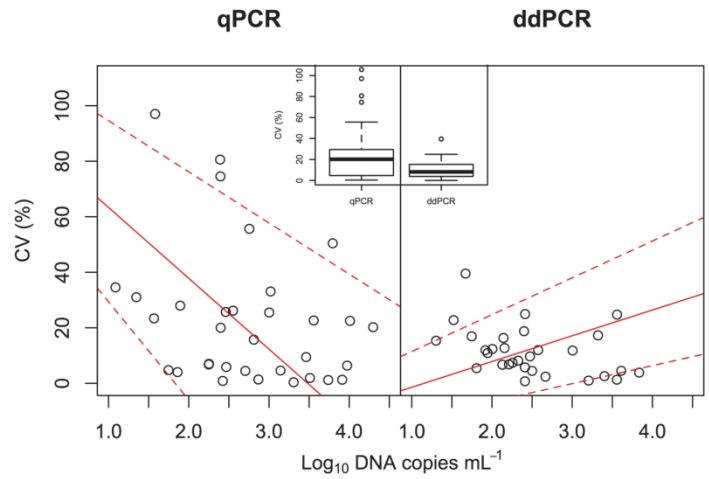
PLOS ONE

RESEARCH ARTICLE

Use of Droplet Digital PCR for Estimation of Fish Abundance and Biomass in Environmental DNA Surveys

Hideyuki Doi[1]*, Kimiko Uchii[1,2], Teruhiko Takahara[3], Saeko Matsuhashi[1], Hiroki Yamanaka[4], Toshifumi Minamoto[5]

1 Institute for Sustainable Sciences and Development, Hiroshima University, Higashi-Hiroshima, Japan, **2** Faculty of Pharmacy, Osaka Ohtani University, Tondabayashi, Japan, **3** Graduate School of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima, Japan, **4** Department of Environmental Solution Technology, Faculty of Science and Technology, Ryukoku University, Otsu, Japan, **5** Graduate School of Human Development and Environment, Kobe University, Kobe, Japan



ddPCR检测的重复性及准确度更高，CV值更小

Environmental Science & Technology

Article

Droplet digital PCR outperforms real-time PCR in the detection of environmental DNA from an invasive fish species

Hideyuki Doi, Teruhiko Takahara, Toshifumi Minamoto, Saeko Matsuhashi, Kimiko Uchii, and Hiroki Yamanaka

Environ. Sci. Technol., **Just Accepted Manuscript** • DOI: 10.1021/acs.est.5b00253 • Publication Date (Web): 07 Apr 2015

PCR reagents, especially at low DNA concentrations. Limits of DNA detection, which were tested by spiking the bluegill DNA to DNA extracts from the ponds containing natural inhibitors, found that ddPCR had higher detection rate than real-time PCR. Our results suggest that ddPCR is more resistant to the presence of PCR inhibitors in field samples than real-time PCR. Thus, ddPCR outperforms real-time PCR methods for detecting eDNA to document species distributions in natural habitats, especially in habitats with high concentrations of PCR inhibitors.

ddPCR对环境因子的耐受程度更高，在检测真实样本时性能优于qPCR

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ddPCR在基因编辑事件中的应用

Strategy for mutant detection

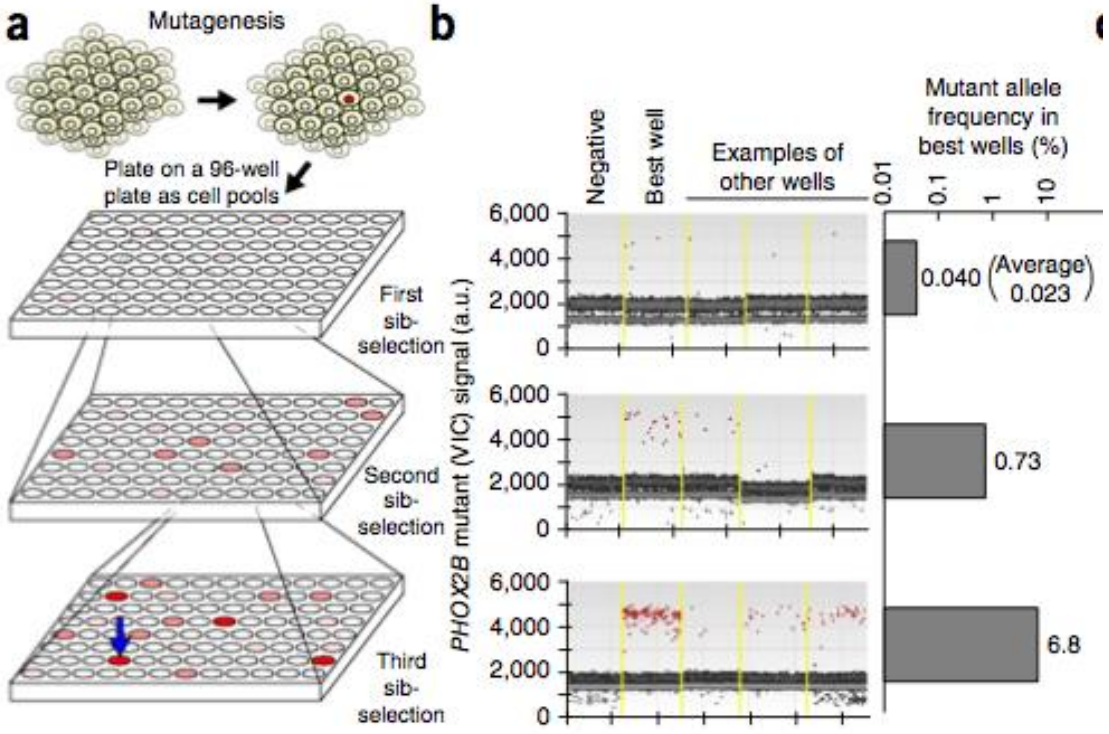

Figure 2 | Point mutagenesis in human iPS cells. (**a**) Overview of the approach to is

ddPCR detects and quantifies mutant allele freq of 0.02%

- ddPCR detected gene editing events as low as 0.02% frequency
- 10-fold decrease in active work time over existing methods (screen 11 clones instead of 2,000!)
- Over 20 independent iPSC clones isolated to date using ddPCR

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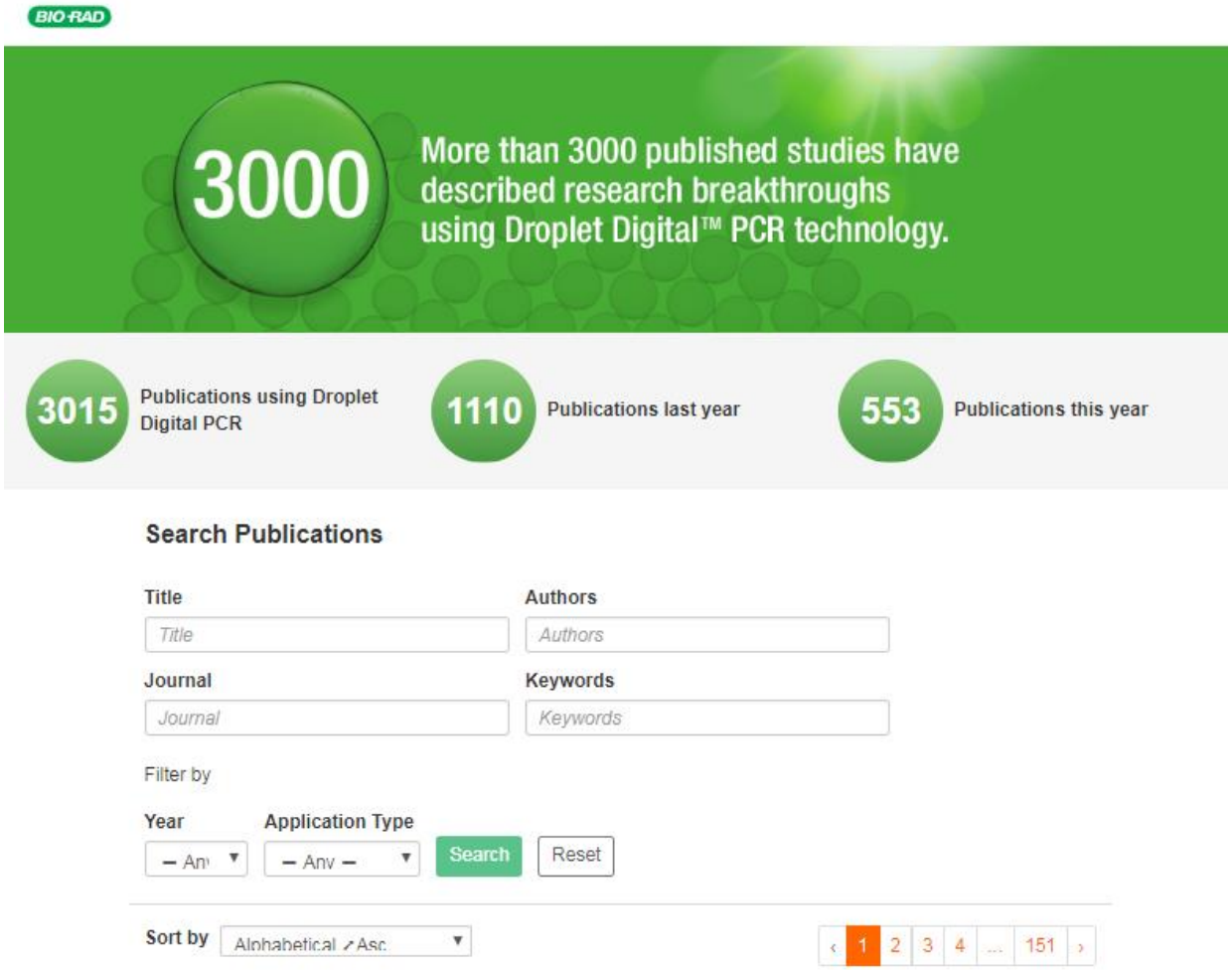
ddPCR文献搜索工具



https://www.bio-rad.com/ddPCR/publications/?WT.mc_id=180514024366

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ddPCR实验设计——金黄色葡萄球菌的检测

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Accurate Detection of Methicillin-Resistant *Staphylococcus aureus* in Mixtures by Use of Single-Bacterium Duplex Droplet Digital PCR

Jun Luo,[a,b] Junhua Li,[a] Hang Yang,[a] Junping Yu,[a] Hongping Wei[a]

Key Laboratory of Special Pathogens and Biosafety, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China[a]; University of Chinese Academy of Sciences, Beijing, China[b]

Initial testing of 104 nasal swabs showed that the ddPCR method had 100% agreement with the standard culture method, while the normal duplex qPCR method had only about 87.5% agreement.

The single-bacterium duplex ddPCR assay is rapid and powerful for more accurate detection of MRSA directly from clinical specimens.

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J Clin Micro, 2017

ddPCR实验设计

Accurate Detection of Methicillin-Resistant *Staphylococcus aureus* in Mixtures by Use of Single-Bacterium Duplex Droplet Digital PCR

Assay validation

Primers/probes design

annealing temperature optimization

Evaluation of the duplex ddPCR performance

Performance of duplex ddPCR for spiking experiment

Optimizing conditions for bacteria lysis

Sample test

(nasal swab specimens)

duplex ddPCR and qPCR comparison

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ddPCR实验设计

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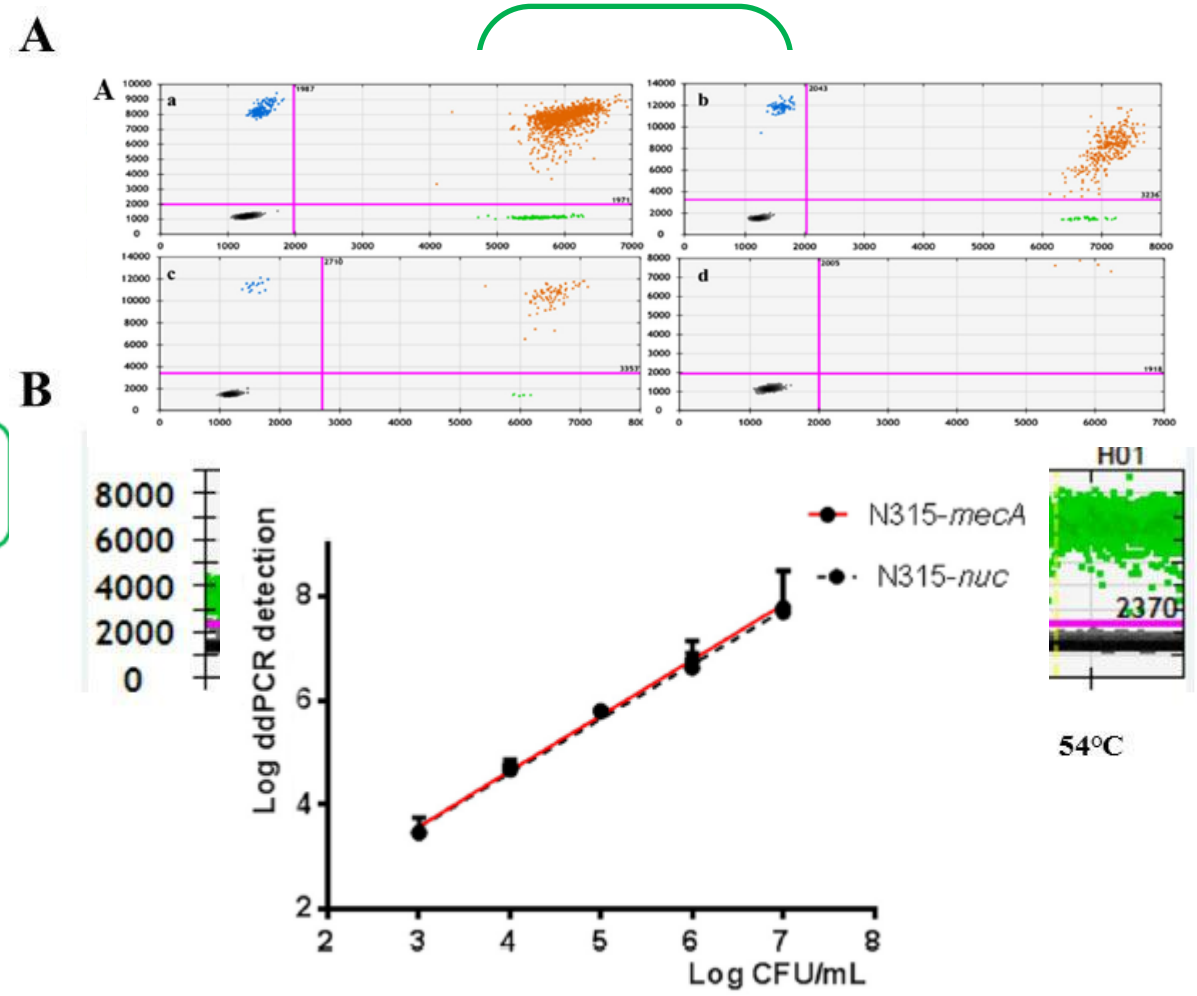

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J Clin Micro, 2017

ddPCR实验设计

Accurate Detection of Methicillin-Resistant *Staphylococcus aureus* in Mixtures by Use of Single-Bacterium Duplex Droplet Digital PCR

nasal swab specimens detection

duplex ddPCR and qPCR comparison

TABLE 1 Detection results of 18 simulative nasal swab samples based on the MRSA index ratios of duplex ddPCR

Sample no.[a]	Sample contents	Average M[b]	Average N	Average MN	MIR	MIR_{cutoff}	Detection result
1	1×10^5 CFU/ml *S. aureus* 91118	0	295	0	0	0	MSSA
2	1×10^6 CFU/ml *E. coli*	0	0	0	0	0	No MRSA
3	2.7×10^5 CFU/ml MR-CoNS	512	0	0	0	0	MR-CoNS
4	3×10^3 CFU/ml MRSA ZX3	4	4	4	100	0	MRSA
5	4×10^5 CFU/ml MRSA ZX108	826	768	690	89.84	8.65	MRSA
6	4×10^4 CFU/ml MRSA FY16	91	84	77	91.67	0	MRSA
7	7×10^3 CFU/ml MRSA WH70	12	13	11	91.67	0	MRSA
8	3×10^3 CFU/ml MRSA N315	4	4	4	100	0	MRSA
9	4.5×10^5 CFU/ml MRSA YN22	833	805	691	85.84	8.65	MRSA
10	3×10^4 CFU/ml MRSA KQ6	63	52	43	82.69	0	MRSA
11	2.5×10^4 CFU/ml MRSA FY17	48	49	39	81.25	0	MRSA
12	2×10^5 CFU/ml MRSA ZX54	433	422	386	91.47	8.65	MRSA
13	5×10^3 CFU/ml MRSA N315 + 1×10^5 CFU/ml MR-CoNS + 1.7×10^6 CFU/ml MSSA	207	3,427	51	24.63	23.96	MRSA with mixture of MSSA (more) and MR-CoNS (less)
14	1×10^5 CFU/ml MR-CoNS + 1.7×10^6 CFU/ml MSSA	193	3,419	37	19.17	23.96	Mixture of MSSA and MR-CoNS
15	2×10^6 CFU/ml MRSA N315 + 1×10^6 CFU/ml MR-CoNS + 1.5×10^6 CFU/ml MSSA	6,037	7,204	4,768	78.79	37.62	MRSA with nearly equal mixture of MSSA and MR-CoNS
16	4×10^3 CFU/ml MR-CoNS + 4×10^3 CFU/ml MSSA	8	8	0	0	0	Mixture of MSSA and MR-CoNS
17	1.5×10^5 CFU/ml MRSA N315 + 1×10^6 CFU/ml MR-CoNS + 3×10^6 CFU/ml MSSA	2,305	6,261	951	41.25	37.62	MRSA with nearly equal mixture of MSSA and MR-CoNS
18	1×10^6 CFU/ml MR-CoNS + 3×10^6 CFU/ml MSSA	2,051	5,996	681	33.2	37.62	Mixture of MSSA and MR-CoNS

TABLE 2 Sensitivity and specificity of the qPCR and ddPCR methods for detection of MRSA in 104 nasal specimens

Method and result	Culture result: Positive	Culture result: Negative	Sensitivity (% [95% CI])	Specificity (% [95% CI])	Agreement (% [95% CI])
qPCR			38.89 (18.26–63.86)	97.67 (91.06–99.6)	87.5 (79.22–92.91)
Positive	7	2			
Negative	11	84			
ddPCR			100 (78.12–100)	100 (94.67–100)	100 (95.56–100)
Positive	18	0			
Negative	0	86			

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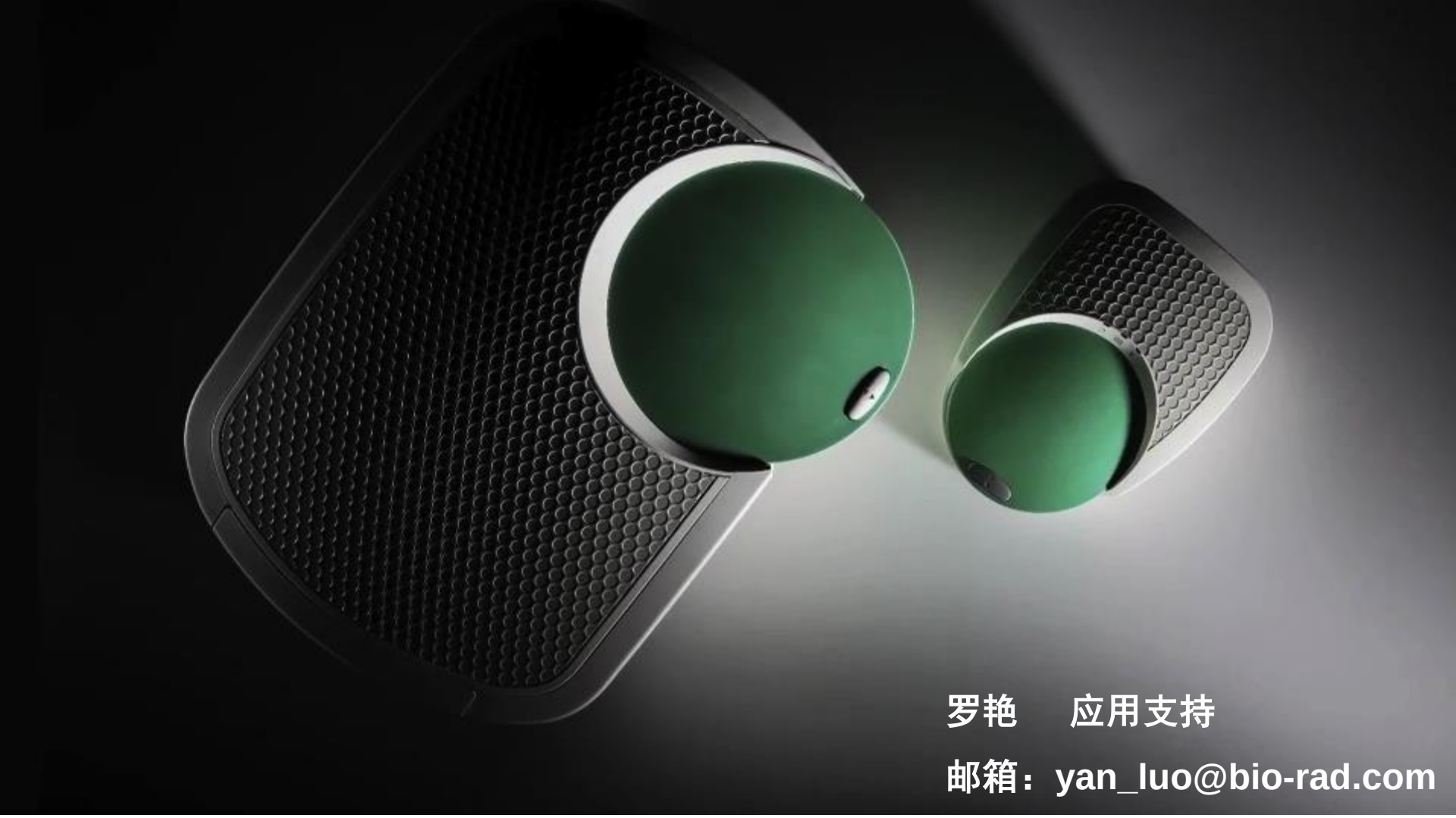

罗艳　应用支持

邮箱：yan_luo@bio-rad.com

Thank you !