digital PCR

Maido Remm

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

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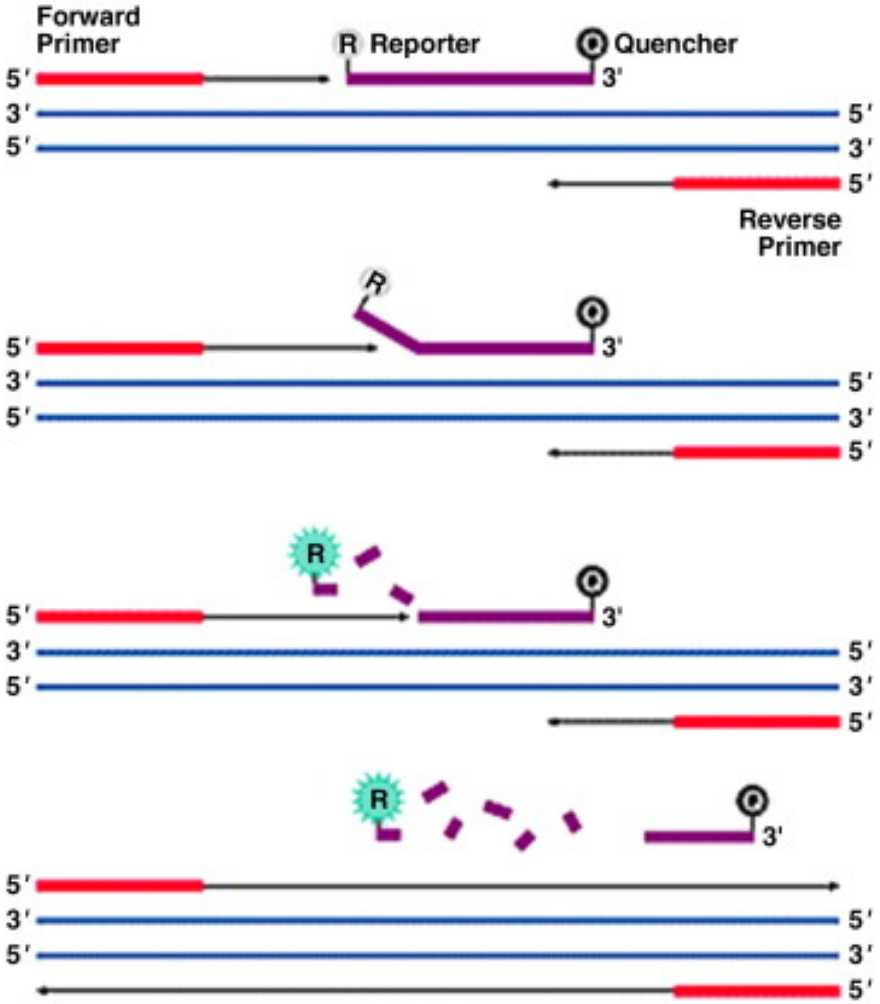
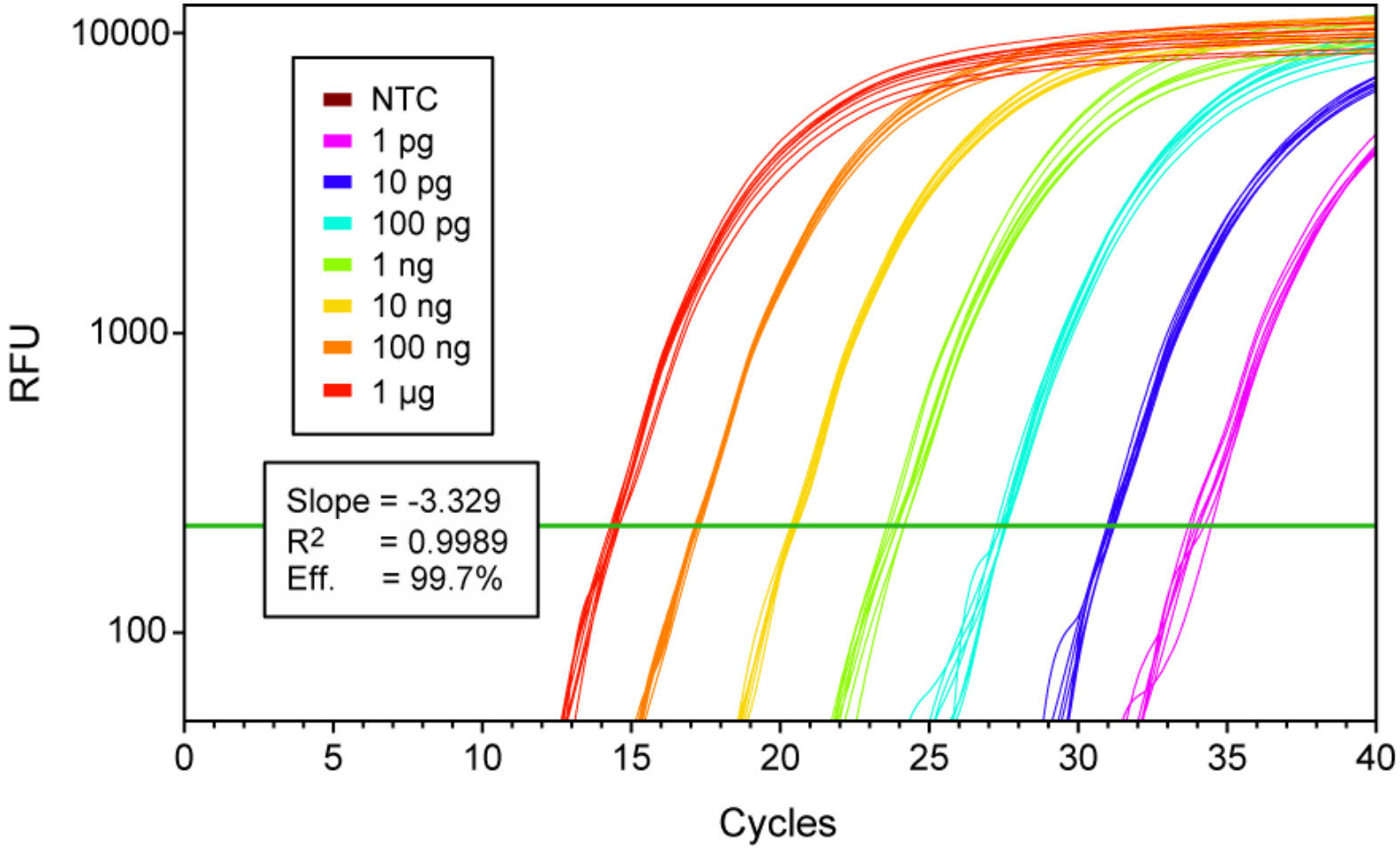
real-time PCR (qPCR) = analog PCR



http://origin-ars.els-cdn.com/content/image/1-s2.0-S073497501000090X-gr3.jpg

real-time PCR (qPCR)

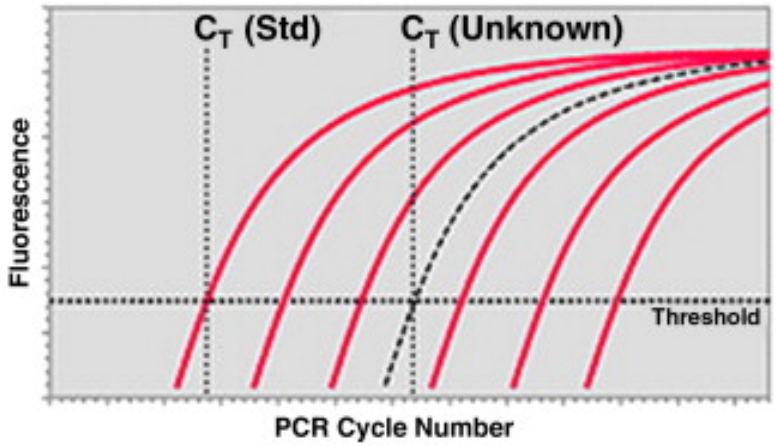
increase in fluorescence is dependent on amount of template DNA

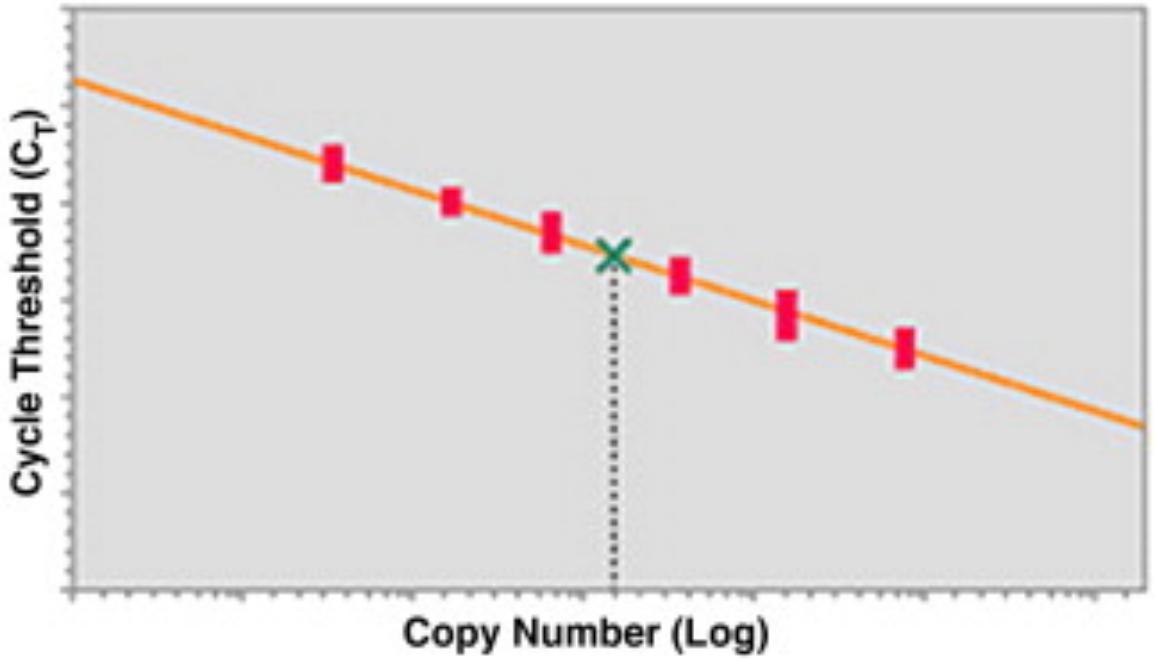


http://www.5prime.com/media/437867/ampl%20of%20actb.png

real-time PCR (qPCR)

estimates quantity of DNA through comparison with calibration curves





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digital PCR

Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 9236–9241, August 1999
Genetics

Digital PCR

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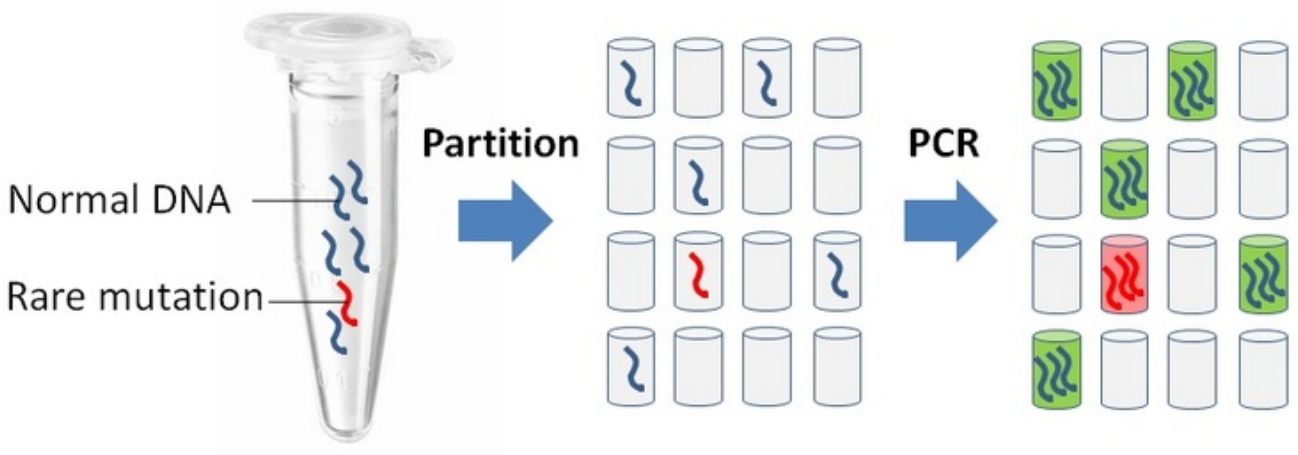


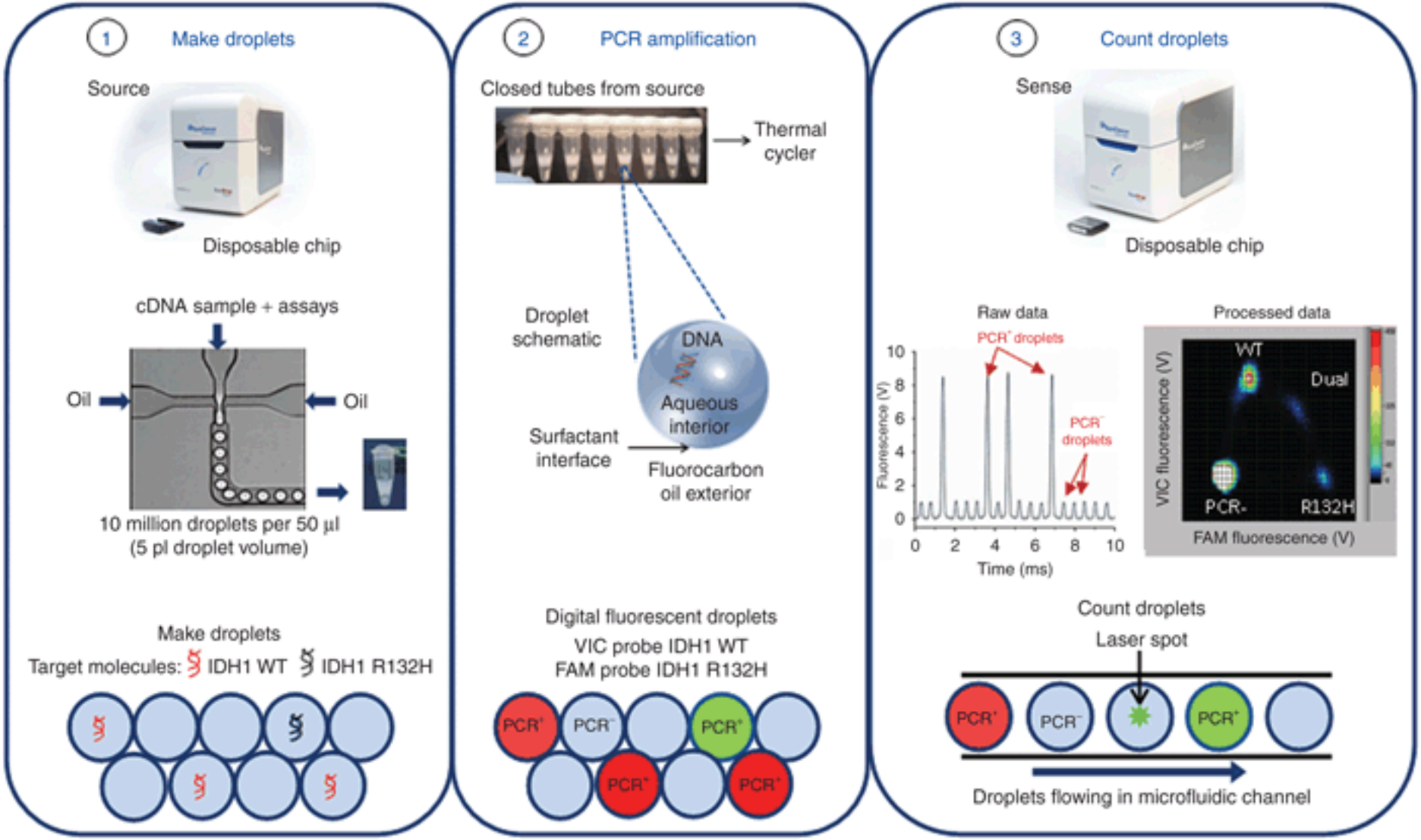
-PCR+ or PCR+ detection (1 or 0)

- No need for precise measure-ments of fluorescence levels

- Quantification through counting

http://www.jnmedsys.com/digital-pcr.html

droplet digital PCR



Citation: *Molecular Therapy Nucleic Acids* (2013) **2**, e109; doi:10.1038/mtna.2013.28
Published online 23 July 2013

2 main droplet dPCR technologies

BIO-RAD

QX200™ droplet-digital™ PCR system (ddPCR™)

RainDance Technologies

RainDrop™ Digital PCR System

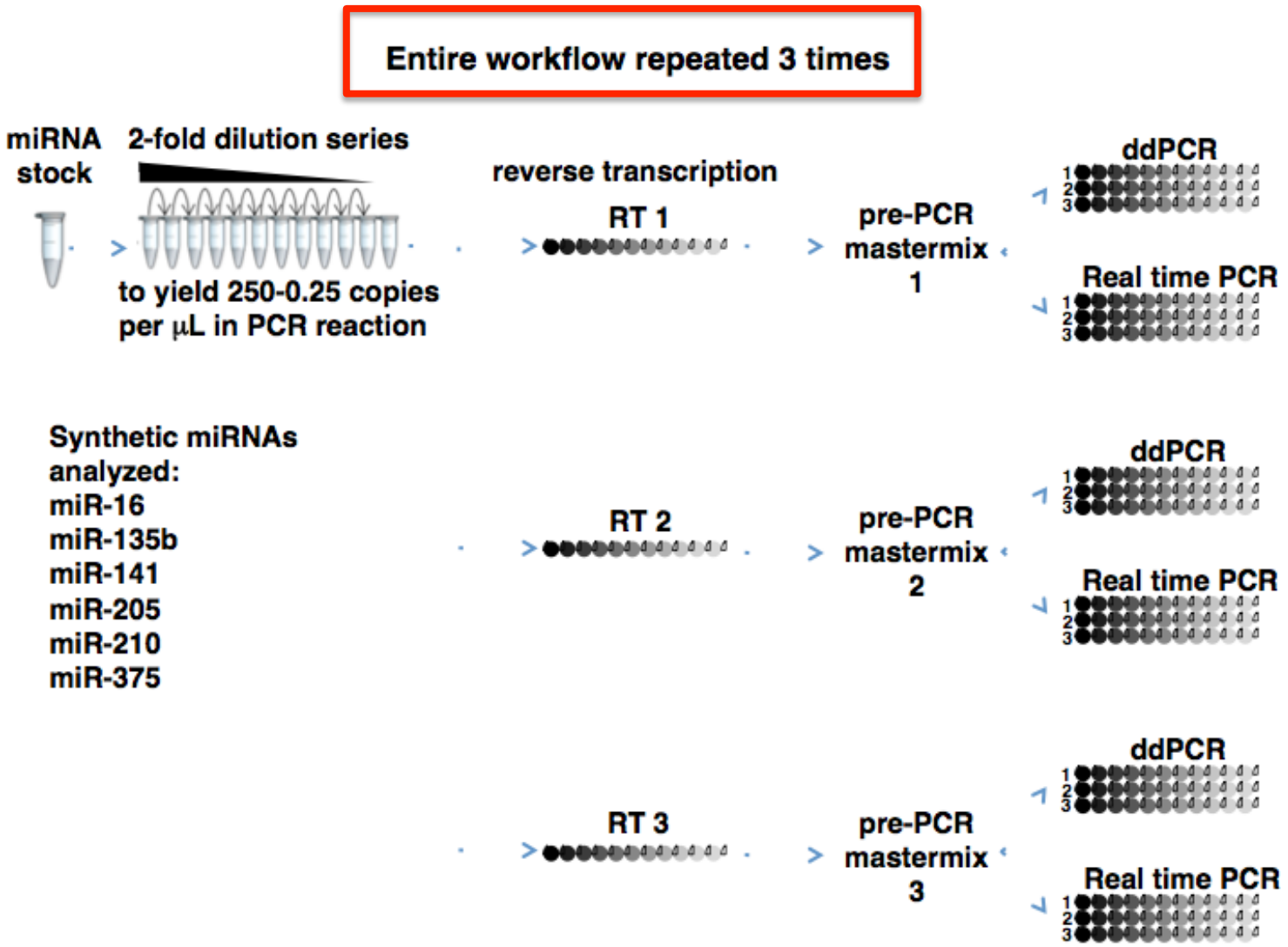
2 main droplet dPCR technologies

Table 1 | Commercial digital PCR offerings

Vendor	Instruments and list price	Consumables and list price	Number and volume of partitions	Volumes required	qPCR capacity	Multiplexing
Bio-Rad Laboratories	QX100 ddPCR System (machines to generate and read droplets): $89,000	8 samples per chip (14,000–16,000 droplets per sample): $3 per sample	Up to 96 samples per run (assumes manual pipetting into PCR plate); 1,344,000 partitions per run (assuming 12 chips at once); 1 nl per partition	Up to 9 µl per sample (20,000 droplets made); an average of 70% read	No	Uses 2 colors to detect 2 targets
RainDance[c]	RainDrop Digital PCR (machines to generate, collect and read droplets): $100,000	8 samples per chip (up to 10,000,000 droplets per sample): $10–$30 per sample	8 samples per run; up to 80,000,000 partitions per run; 5 pl per partition	5–50 µl per sample	No	Uses 2 colors, but can use varying concentrations of probes to detect up to 10 targets

Digital PCR hits its stride
Monya Baker
Nature Methods **9**, 541–544 (2012)

Testing sensitivity and precision



Sensitivity

water

Supplementary Table 4	Limit of Quantification (copies per μL PCR)	
miRNA	ddPCR	Real time PCR
miR-141	0.5	0.5
miR-375	0.25	0.5
miR-210	1	1
miR-135b	1	8
miR-205	0.5	0.5
miR-16	0.25	4

plasma

Supplementary Table 5	Limit of Quantification (copies/μL PCR)	
miRNA	ddPCR	Real time PCR
miR-141	0.25	1
miR-375	2	8
miR-210	0.5	8
miR-135b	1	2
miR-205	1	1
miR-16	NA*	NA*

Supplementary Table 4| Operating characteristics based on ddPCR and real time PCR analysis of synthetic miRNA oligonucleotides (water matrix). Limit of quantification (LOQ) was defined as the lowest concentration tested that remained above or equal to the limit of detection (LOD)* and above or equal to the lower limit of linear range of the assay (LLLR)**. * LOD was defined as = <*x*>bi + *k*sbi, were <*x*>bi = mean of the no- template controls, *s*bi = standard deviation of no-template controls, *k* = 2.479 (99% confidence interval). LOD determinations here are likely to be biased in favor of real time PCR, as undetermined Cts are set uniformly to 40, artificially lowering *s*bi estimates for real time PCR. ** LLLR was determined by runs-testing, removing successive lowest dilution points until the *P*-value was >0.05, indicating no significant deviation from linearity.

Precision

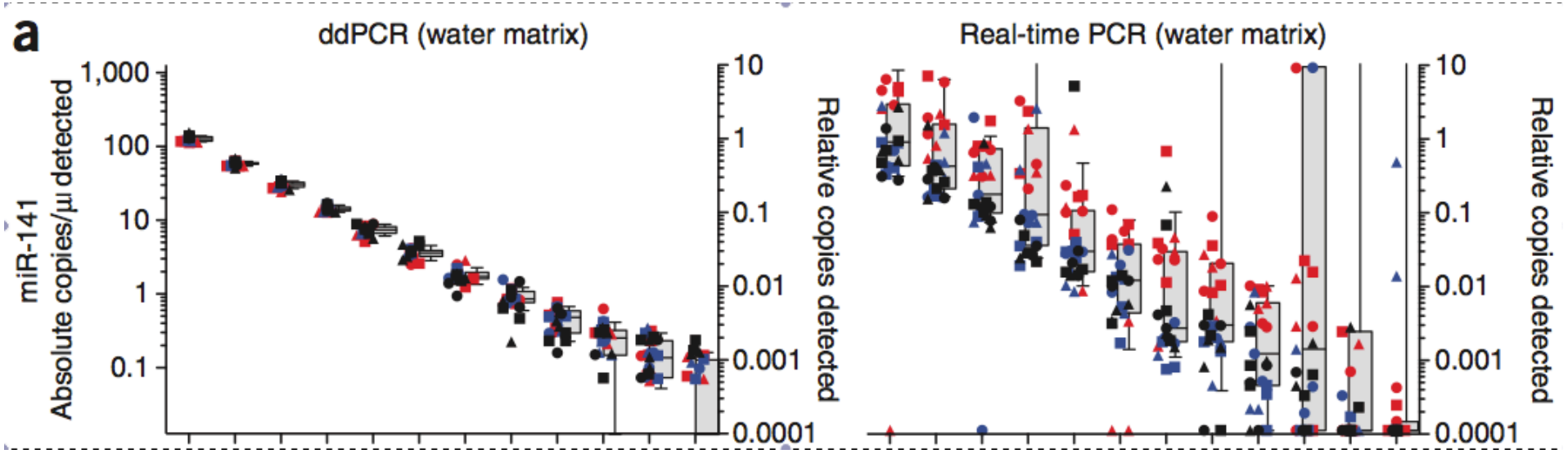
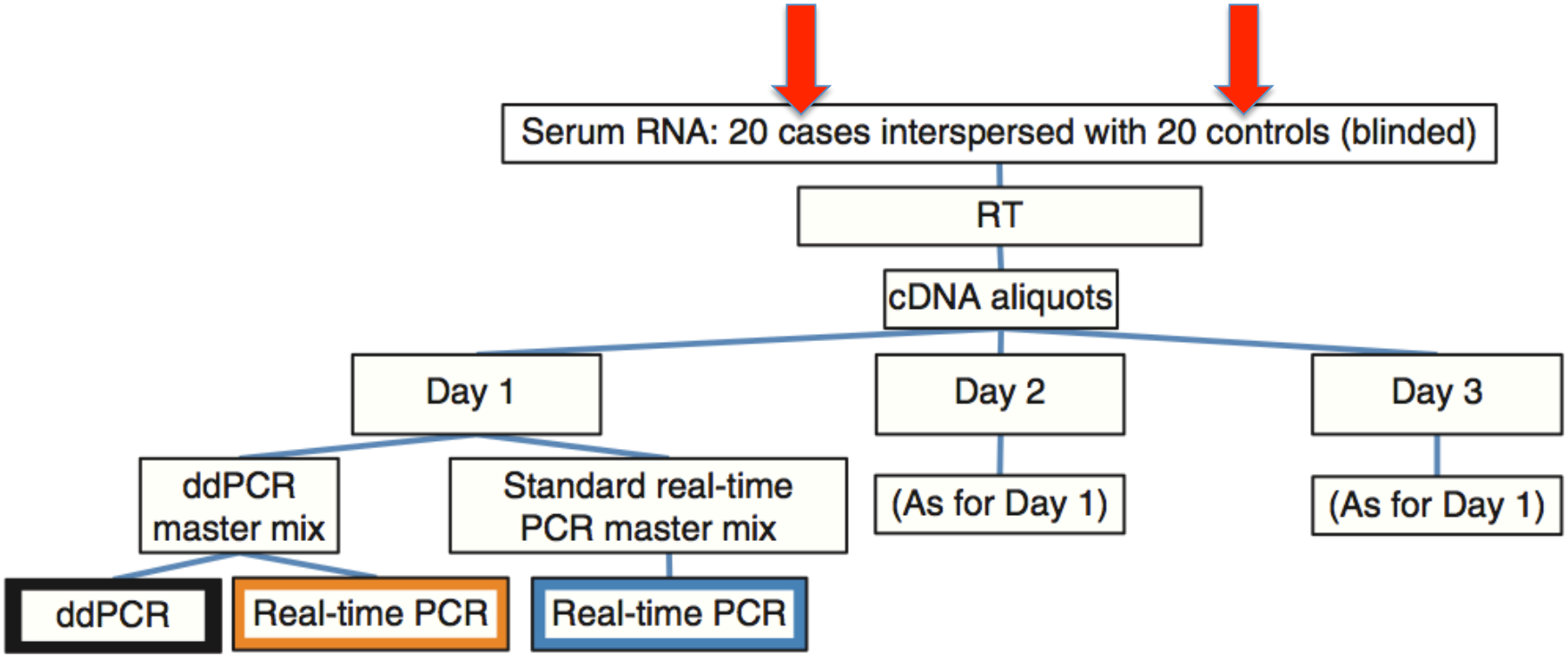


Figure 1 | Quantification of synthetic miRNA oligonucleotides by ddPCR and real-time PCR. (**a**) Comparative analysis of dilution series of indicated miRNAs in water. Each color represents one preparative (independent preparation of a dilution series) replicate and each shape represents individual RT reactions (RT 1, circle; RT 2, square; and RT 3, triangle). Box and whisker plots (gray) show median (center line), 25th and 75th percentiles (box), and 10th and 90th percentiles (whiskers). NTC, no-template control.

Test with real prostate cancer samples

We focused on the quantification of cDNAs corresponding to microRNAs (miRNAs), which are small regulatory RNA molecules with diverse cellular functions[7]. miRNAs also exist in highly stable extracellular forms in the vascular circulation[8,9] with potential hormonal function[10] and can be useful as blood-based biomarkers for cancer[8] and other diseases[11].



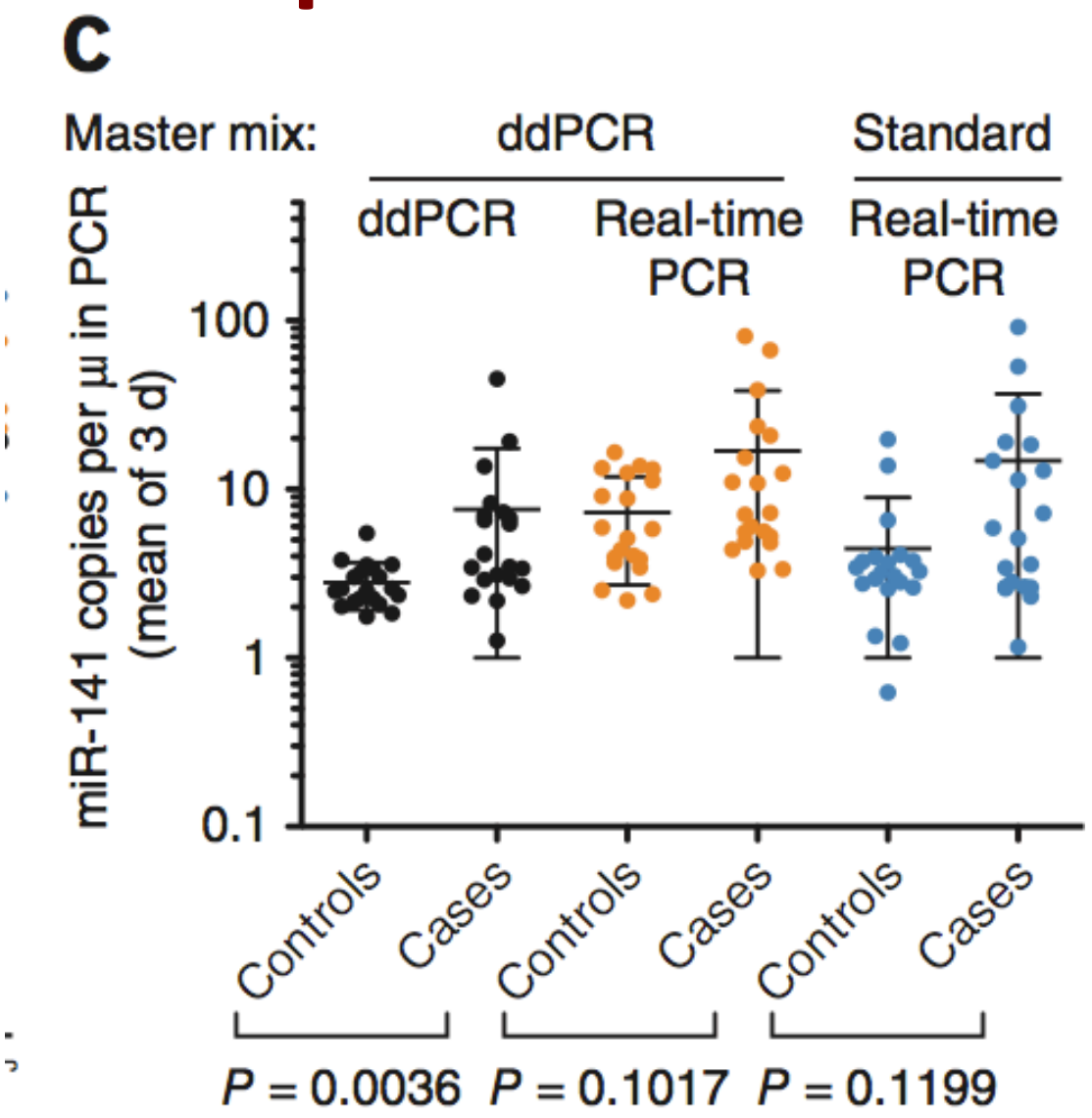
Day-to-day reproducibility

Supplementary Table 9| Day-to-day variability of miR-141 quantification from clinical samples (ddPCR vs. standard real time PCR). Results are presented as copies of miR-141 per µL in PCR reaction.
s.d.: standard deviation. %CV: Coefficient of variation calculated from replicates over 3 days (percentage). **Fold-change of %CV was calculated as the ratio of %CV for real time PCR results to %CV for ddPCR results.**

Supplementary Table 9

Sample Number	ID	Status	ddPCR (copies per µL in PCR)						Standard real-time PCR (copies per µL in PCR)						CV Fold Difference
			Day 1	Day 2	Day 3	Mean	SD	%CV	Day 1	Day 2	Day 3	Mean	SD	%CV	
1	A	Control	2.20	2.21	3.03	2.48	0.48	19	7.78	2.14	0.39	3.44	3.86	112	6
2	B	Case	3.59	2.40	2.89	2.96	0.60	20	1.54	5.62	0.76	2.64	2.61	99	5
3	C	Control	6.70	4.32	5.47	5.50	1.19	22	7.01	3.77	0.52	3.76	3.25	86	4
4	D	Case	2.52	1.63	2.41	2.19	0.49	22	4.21	3.19	0.50	2.63	1.92	73	3
5	E	Control	4.01	3.09	3.60	3.57	0.46	13	8.91	1.49	0.77	3.72	4.50	121	9
6	F	Case	2.95	2.06	3.01	2.67	0.53	20	3.48	2.02	2.25	2.58	0.78	30	2
7	G	Control	2.70	1.85	2.21	2.26	0.43	19	6.66	2.50	1.81	3.66	2.63	72	4
8	H	Case	6.94	6.23	7.08	6.75	0.46	7	21.76	9.17	13.19	14.71	6.43	44	6
9	I	Control	3.99	3.47	3.91	3.79	0.28	7	9.87	3.23	27.77	13.62	12.70	93	13
10	J	Case	44.45	44.64	46.44	45.18	1.10	2	202.81	46.22	25.77	91.60	96.85	106	43
11	K	Control	2.31	2.38	2.82	2.51	0.28	11	9.62	3.60	46.53	19.92	23.25	117	11
12	L	Case	4.22	3.68	4.49	4.13	0.41	10	0.53	1.18	159.11	53.61	91.37	170	17
13	M	Control	4.04	3.10	2.47	3.20	0.79	25	4.26	2.56	1.03	2.62	1.62	62	3
14	N	Case	8.30	6.88	5.84	7.01	1.23	18	22.36	9.54	2.19	11.36	10.21	90	5
15	O	Control	3.67	3.03	2.35	3.01	0.66	22	3.36	4.23	0.71	2.77	1.84	66	3
16	P	Case	8.70	8.63	7.47	8.27	0.69	8	39.37	12.83	2.95	18.38	18.83	102	12
17	Q	Control	3.59	2.78	2.68	3.02	0.50	16	7.37	4.05	0.50	3.97	3.43	86	5
18	R	Case	21.52	19.05	17.07	19.21	2.23	12	57.76	26.66	8.71	31.04	24.82	80	7

How well miR-141 levels predict cancer?



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NATURE METHODS | VOL.10 NO.10 | OCTOBER 2013 | 1003

Competing financial interests

C.M.H.and B.J.H. were formerly employees of Quantalife, Inc. and Bio-Rad, Inc., including during periods that the work was done. M.T.'s laboratory received some consumable supplies from Quantalife, Inc. and Bio-Rad, Inc. during the course of the studies. M.T. is an inventor on patent application US 12/993,828 pertaining to extracellular microRNA biomarkers.

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

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CONCLUSIONS:

Our comparison of microRNA quantification by ddPCR and real-time PCR revealed **greater precision** (coefficients of variation decreased 37–86%) and **improved day-to-day reproducibility** of ddPCR but with **comparable sensitivity**.

When we applied ddPCR to serum microRNA biomarker analysis, this translated to **superior diagnostic performance** for identifying individuals with cancer.

Differences between droplet PCR and conventional PCR

The relative numbers of reactants are still large; in a 5 picoliter-sized drop, there are approximately 2,500,000 molecules of each primer (900 nM) and 750,000 probe molecules (250 nM).

In ddPCR, **the absolute concentration of a single target molecule, relative to the PCR reagents, is substantially higher** in the nanoliter volume than in conventional microliter-scale PCR. The likelihood of favorable primer-template interactions, and thus, the efficiency, specificity, and sensitivity of ddPCR, is increased in comparison to that of conventional PCR tests.

Similarly, **the fluorescent product is confined to the droplet volume, and so, small changes in fluorescence intensity are more readily detected** by photonics equipment than a similar absolute amount of fluorescence would be by conventional qPCR platforms.

ddPCR is robust against many of the factors that can negatively influence conventional PCR because **the DNA template is sequestered from cross-reacting DNA templates and inhibitory moieties**.

Roberts et al. JCM 51: 2195

PCR primers and probes?

Plan to amplify a **75–200 bp product**. Short PCR products are typically amplified with higher efficiency than longer ones, but a PCR product should be at least 75 bp long to allow room for placement of the probe.

Design primers that have a GC content of 50–60%.
Strive for a **Tm between 50 and 65°C.**
Calculate Tm values using the nearest-neighbor method, with values of **50 mM for salt concentration and 300 nM for oligonucleotide concentration**.
Adjust primer locations so they are outside the target sequence secondary structure. Avoid repeats of Gs or Cs longer than 3 bases.
Check the forward and reverse primer sequences to ensure that there is no 3' complementarity (to avoid primer-dimer formation).

When designing **probes**, follow these guidelines:
The Tm of each hydrolysis probe should be 5–10°C higher than that of the corresponding primers. In most cases, the probe should have <30 nucleotides between the fluorophore and the quencher to avoid affecting baseline signal intensity. It must not have a G at its 5' end, because this may quench the fluorescence signal even after hydrolysis. Choose a sequence within the target that has a GC content of 30–80% and design the probe to anneal to the strand that has more Gs than Cs (so the probe contains more Cs than Gs). The QX100 system is compatible with FAM dye and either HEX or VIC as a secondary dye.
The QX100 system is not compatible with SYBR® Green or EvaGreen double-stranded DNA-binding dyes.

Applications

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Journals.ASM.org

Development and Evaluation of a Next-Generation Digital PCR Diagnostic Assay for Ocular *Chlamydia trachomatis* Infections

Using ddPCR to measure *C. trachomatis* infectious load in clinical samples (conjuctival swab samples) in presence of inhibitory agents and competing DNA

OPEN ACCESS Freely available online

PLOS ONE

Quantitative Analysis of Food and Feed Samples with Droplet Digital PCR

Detection of GMO maize strains in concentrations of < 1%

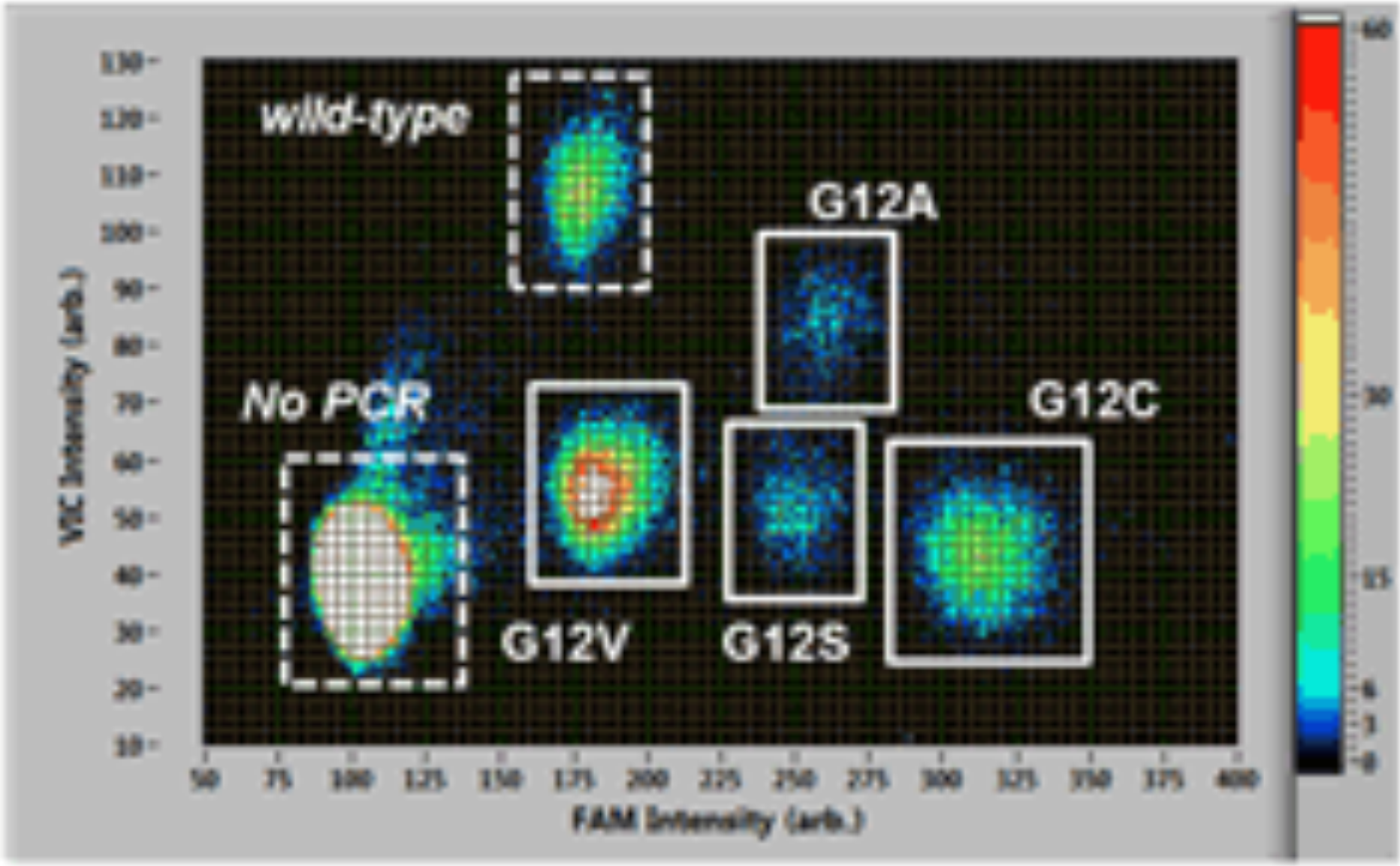
RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

A Sensitive and Specific Diagnostic Test for Hearing Loss Using a Microdroplet PCR-Based Approach and Next Generation Sequencing

Amplification of known candidate genes before sequencing. A deafness RDT primer library was then created that consisted of 1,209 amplicons and associated primer pairs.

multiplex dPCR



The method does not need extremely low dilutions