

The Digital PCR journey



Definition

Digital PCR concept was mentioned for the first time in *Nucleic Acids Res.* 1997 May 15;25(10):1999-2004 by Kalinina et al.

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Nucleic Acids Research, 1997, Vol. 25, No. 10 1999–2004

Nanoliter scale PCR with TaqMan detection

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ABSTRACT

We monitored PCR in volumes of the order of 10 nl in glass microcapillaries using a fluorescence energy transfer assay in which fluorescence increases if product is made due to template-dependent nucleolytic degradation of an internally quenched probe (TaqMan assay). This assay detected single starting template molecules in dilutions of genomic DNA. The results suggest that it may be feasible to determine the number of template molecules in a sample by counting the number of positive PCRs in a set of replicate reactions using terminally diluted sample. Since the assay system is closed and potentially automatable, it has promise for clinical applications.

INTRODUCTION

from these control reactions to that generated from an unknown sample (reviewed in 3). Later versions of this method used an 'internal control', i.e. a target nucleic acid added to the PCR that should amplify at the same rate as the unknown but which could be distinguished from it by virtue of a small sequence difference (e.g. a small insertion or deletion or a change that led to the gain or loss of a restriction site or reactivity with a special hybridization probe) (4,5). These methods have the disadvantage that slight differences in amplification efficiency between the control and experimental nucleic acids can lead to large differences in the amounts of their products after the million-fold amplification characteristic of PCR and it is difficult to determine relative amplification rates accurately. Newer quantitative PCR methods use the number of cycles needed to reach a threshold amount of PCR product as a measure of the initial concentration of target nucleic acid, with ethidium bromide (6) or TaqMan assays (7,8) used to follow the amount of PCR product accumulated in real

Definition

“Digital PCR” was published for the first time in PNAS Vol.96, August **1999** by **Bert Vogelstein** and **Kenneth Kinzler**:

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

Digital PCR

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Contributed by Bert Vogelstein, June 9, 1999

ABSTRACT The identification of predefined mutations expected to be present in a minor fraction of a cell population is important for a variety of basic research and clinical applications. Here, we describe an approach for transforming the exponential, analog nature of the PCR into a linear, digital signal suitable for this purpose. Single molecules are isolated by dilution and individually amplified by PCR; each product is then analyzed separately for the presence of mutations by using fluorescent probes. The feasibility of the approach is demonstrated through the detection of a mutant *ras* oncogene in the stool of patients with colorectal cancer. The process provides a reliable and quantitative measure of the proportion of variant sequences within a DNA sample.

We therefore sought to develop an approach to the problem that would overcome some of the aforementioned difficulties. The strategy described in this paper involves separately amplifying individual template molecules so that the resultant PCR products are completely mutant or completely WT. The homogeneity of these PCR products makes them easy to distinguish with existing techniques. Such separate amplifications are only useful in a practical sense, however, if a large number of them can be assessed simply and reliably. Techniques for such assessments were developed, with the output providing a digital readout of the fraction of mutant alleles in the analyzed population. A variety of applications for this technology are foreseeable.

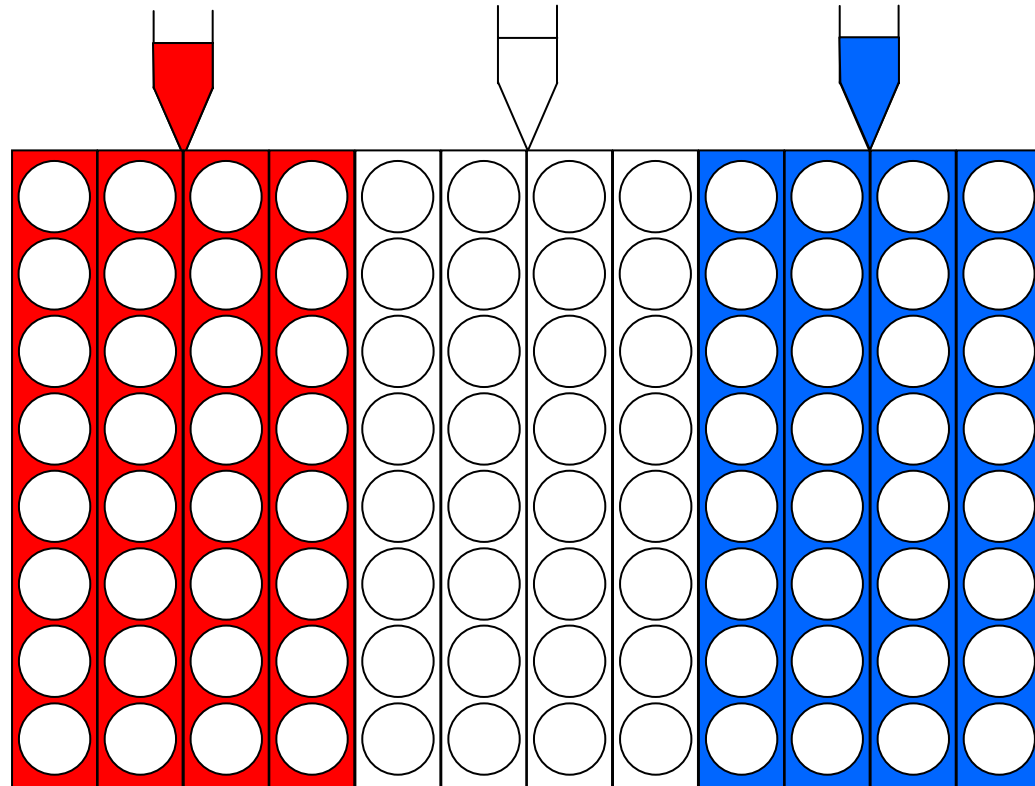
MATERIALS AND METHODS

Principle

Sample A

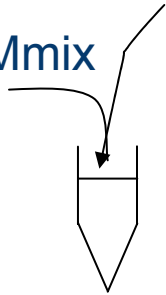
Sample B

Sample C



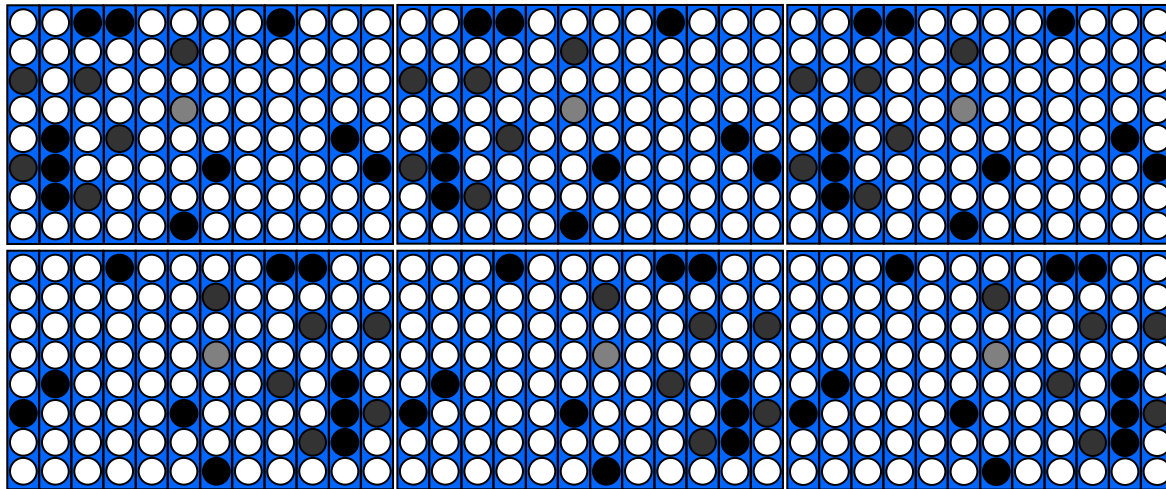
12.5 μ l Sample A

12.5 μ l Mmix



Limitations

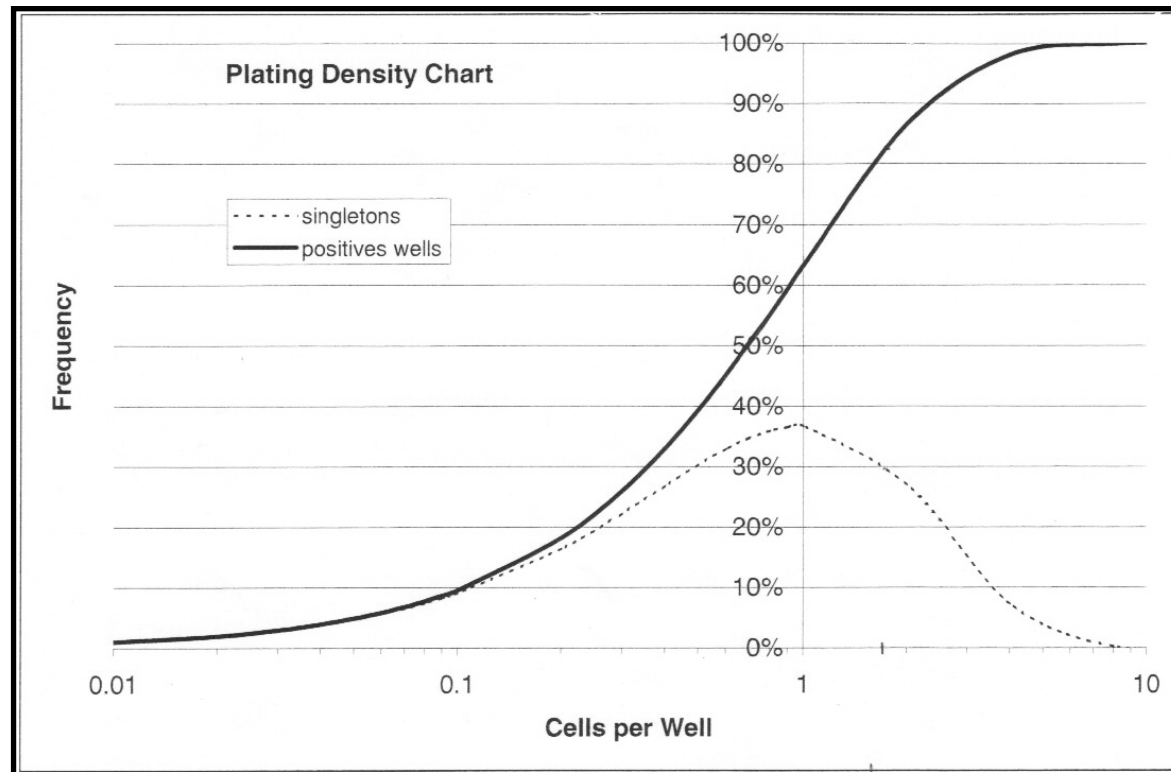
- The number of wells required



102 positives

- Cannot have more than 1 copy per well

Poisson distribution



Limitations

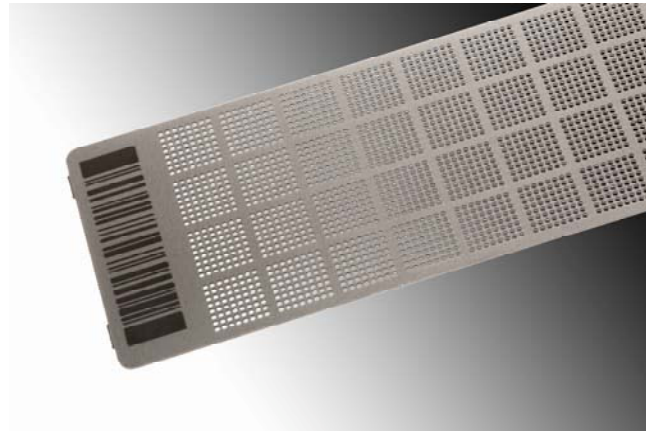
- Even if we use the Poisson distribution the experiment requires large numbers of wells
- This makes the experiment technically impractical to run
- The cost for each quantitation is elevated

Lead for new technology development

OpenArray™

By BioTrove

High-throughput PCR applications in nanoliter volumes



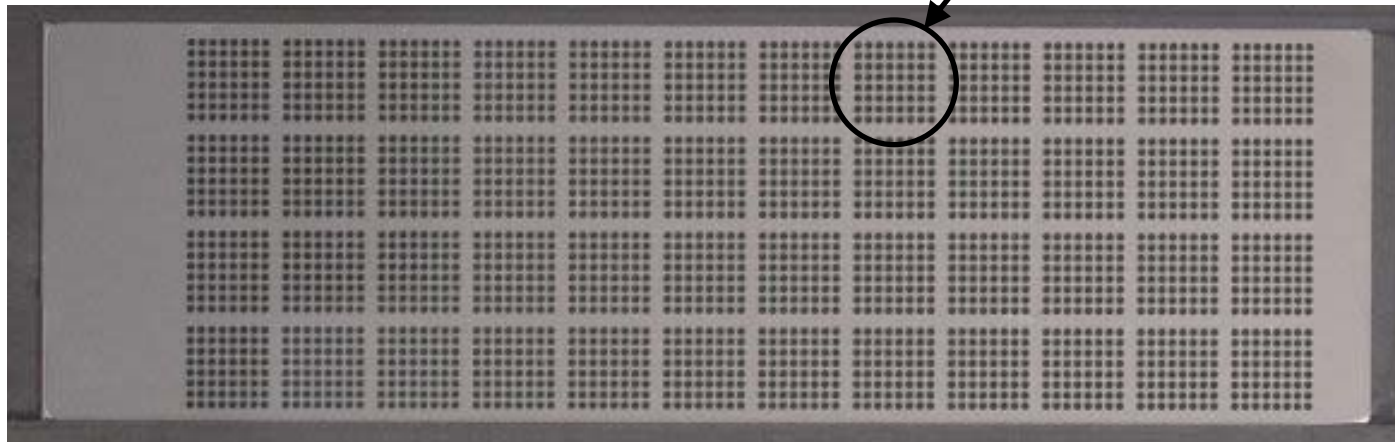
The OpenArray™ Plate

- A unique method to run ~3000 low-volume solution phase assays in parallel
- Equivalent to eight 384-well or thirty-two 96-well plates
- A platform for a wide variety of genomics applications based upon familiar qPCR formats

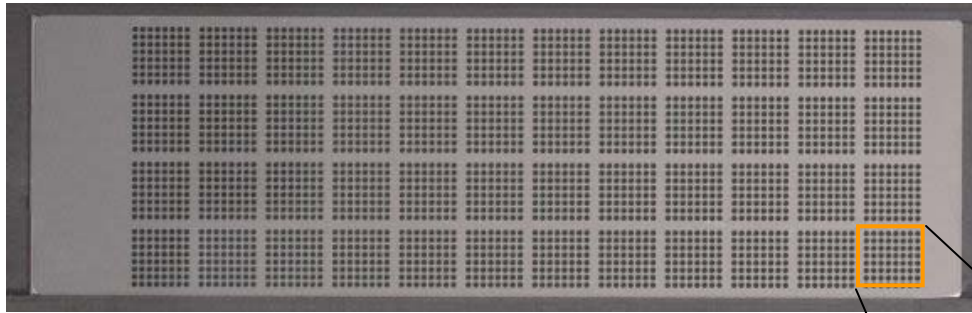


OpenArray™ Anatomy

- 3072 through-holes per OpenArray™ plate are arranged in 48 subarrays
- Each subarray consists of 64 through-holes in an 8x8 pattern.



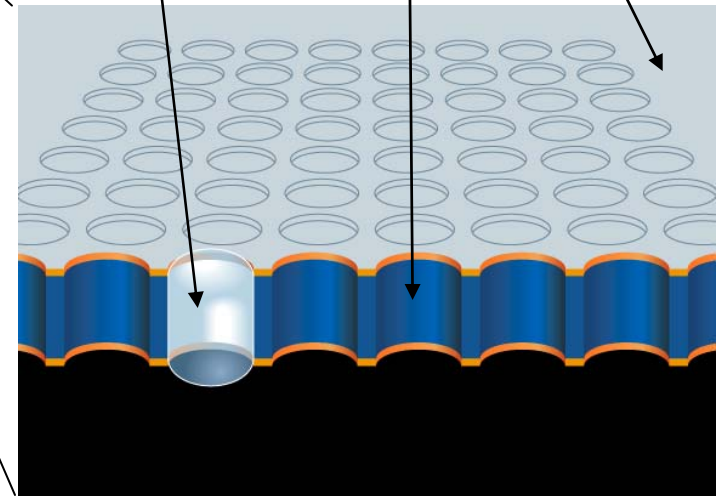
OpenArray™ Anatomy



33 nL volume

Hydrophobic

Hydrophilic



Hydrophilic and hydrophobic coatings enable reagents to load into and stay within the bottomless through-holes via passive capillary action.

OpenArray™ Loading Process



AutoLoader - Sample loading onto OpenArray™ plate

Can we use it for Digital PCR ?

Objectives: - Demonstrate Digital PCR on OpenArrays
- Determine the quantity of target in a background DNA

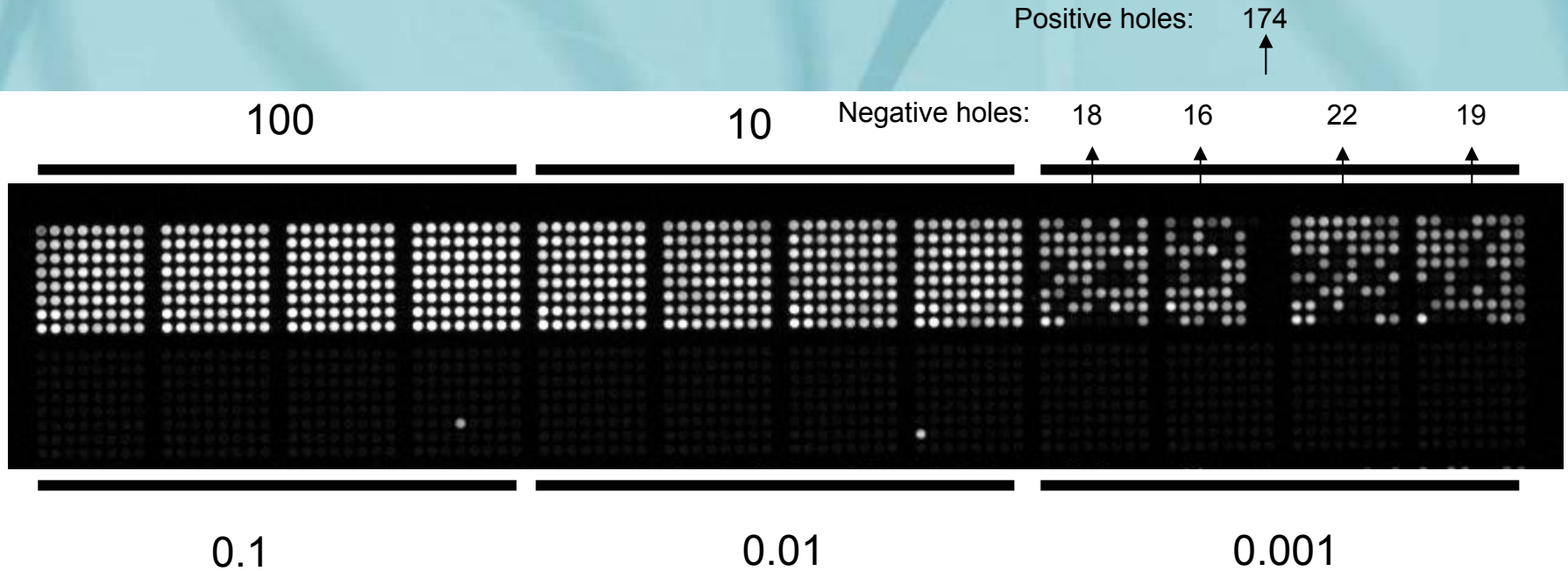
Material: - 5 different Standards corresponding to a 10 fold serial dilution
- Primers and Probes for the target to quantify
- Carrier DNA used to dilute Standards to be use as NTC

Method: - Assay are performed on empty OpenArrays using Taqman assay
- Measurements are done in real time PCR mode
- Computation are done using a Poisson distribution software

<http://www.uhnresearch.ca/labs/iscope/homebrew.html>

Poisson9

Std selection



Based on Poisson distribution, the standard providing 220/256 positives holes or less will be the more appropriate to use for a larger analysis.

Std 3 gave 174 positive.

Std3 and Std 4 to be run in larger number of holes

Protocol for RUN2

A	A	A	A	A	A	A	A	A	A	A	A
B	B	B	B	B	B	B	B	B	B	B	B
B	B	B	B	B	B	B	B	B	B	B	B
NT	NT C	NT C	NT C	NT C	NT C	NT C	NT C	NT C	NT C	NT C	NT C

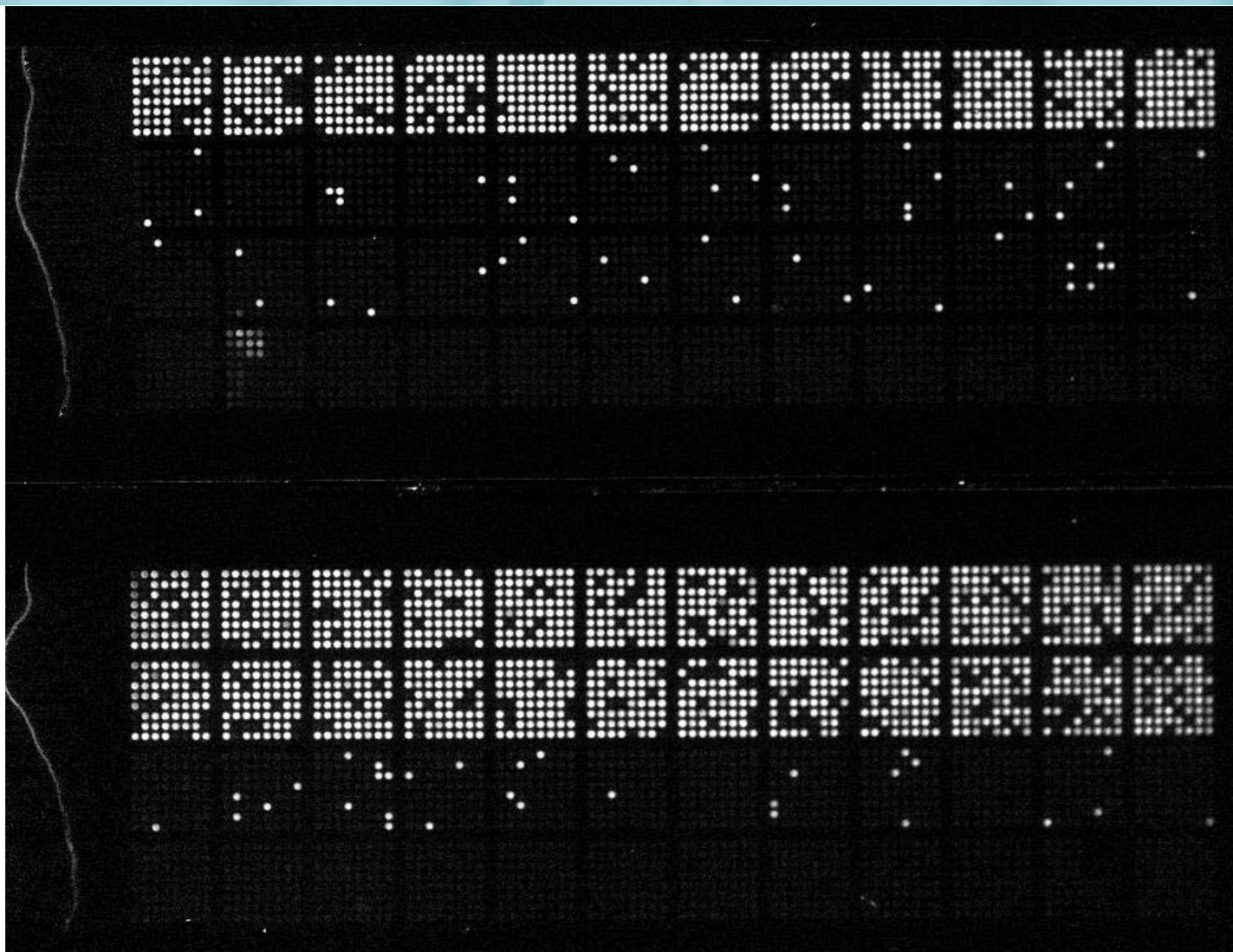
A= Std3

B= Std4

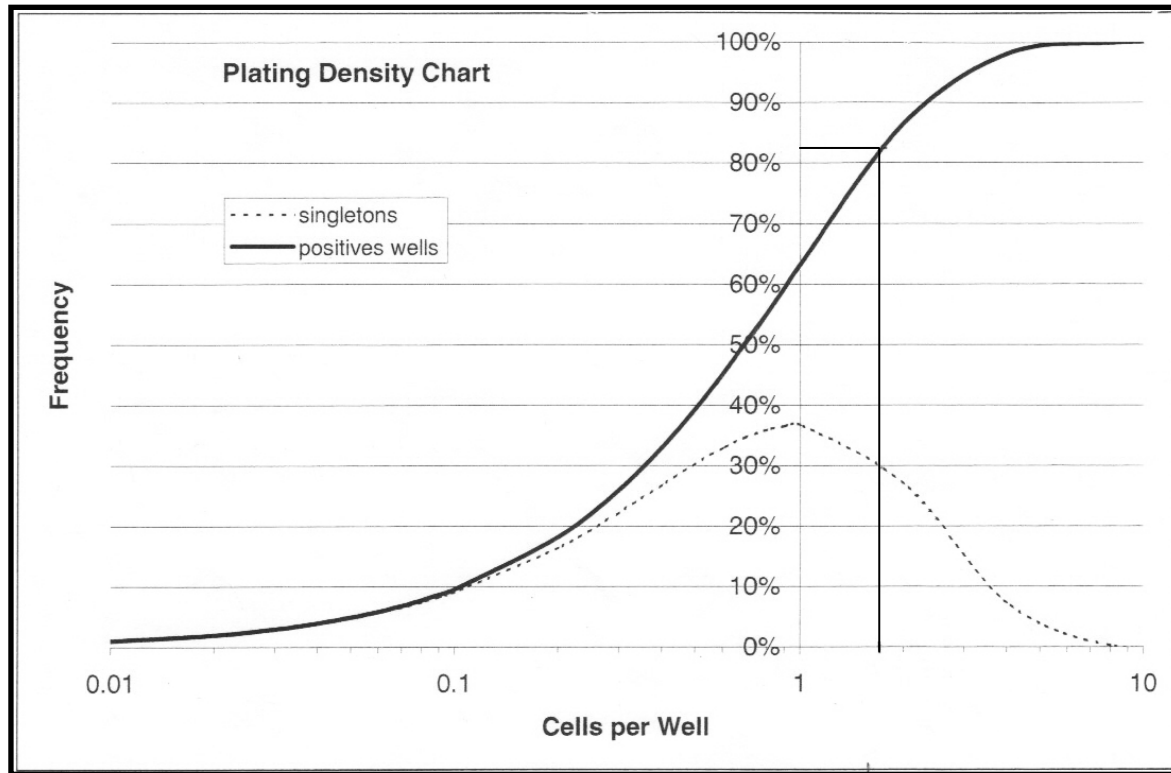
A	A	A	A	A	A	A	A	A	A	A	A
A	A	A	A	A	A	A	A	A	A	A	A
B	B	B	B	B	B	B	B	B	B	B	B
NT	NT	NT C	NT C	NT C	NT C	NT C	NT C	NT C	NT C	NT C	NT C

Run2 results

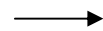
Std3 positives
1900/2304
82.5%



Calculations



Std3 positives
1900/2304
82.5%



Direct count not possible as
more than 1 copy per hole

Calculation using Poisson9

Std3 positives
190042304

1/Freq=0.574
Freq= 1.74 copies/33nl hole
Equivalent 52.7 copies/ μ l

Mmix μ l
DNA 3.5 \rightarrow 5 μ l per
1.5 SubArray

Std3 is 175.8 copies/ μ l

C:\DOCUME~1\Fabrice\LOCALS~1\Temp\TE40A3~1.ZIP\POISSON9.EXE

Press <Enter> to begin fresh data set, <ctrl-C> to exit?

```

-----
| Limiting Dilution Fit by Maximum Likelihood |
| Fazekas, J. Immunol. Meth. 49:R11-22 1982 |
| Adapted to permit single replica entries |
| N. Iscove Jan 1996 |
-----
    
```

Name of file for output: [<Enter> if none!]

```

Cells: ? 1
Reps : ? 2304
Negs : ? 404
p = .17534722
    
```

Cells: (enter '0' to terminate)?

```

k = 1
m = .57438678
    
```

```

cycle 1
y = 0
m = .57438678
Upper = .60498546
Lower = .5453357
X^2 [ 0 ] = 0
    
```

SET	CELLS	REPS	NEGS
1	1	2304	404

```

copies = 1
1/freq = .57438678
95% limits = .5453357 - .60498546
X^2 [ 0 ] = 0
    
```

Data not saved

Press <Enter> to begin fresh data set, <ctrl-C> to exit?

Experiment Conclusion

Std 3 shows a concentration of **175.8** copies/ μ l

The 95% confidence interval is **166.9 - 185.2** copies/ μ l

Level of Accuracy?

The sample used was a pure standard of **178** copies/ μ l

Digital PCR

- Small volume (33nl) reactions resolves the cost issue
- High throughput device increases the precision (3072 x 3 reactions)
- Automation makes it easy to perform
- Accurate volumes are essential for quantification

Applications

- Standard generation
 - Absolute count of the positive wells gives the real copy number
- Precise quantification for difficult samples or inhibited samples
 - Count all the positive wells for sample A and compare to sample B
- Low expressed target quantification
 - Not dependent on the number of PCR cycle, background, exponential phase...
- Rare mutation/SNP detection within a cell population
 - Detection of rare cells with tumorigenic markers or mutations within a population, even when they represent less than 1% as every target is amplified separately
- Small cell population detection (tumour, methylation...)

Conclusion

- The new generation of high throughput small volume real time PCR instrument will enable the conversion of analog PCR into Digital PCR in every lab
- Digital PCR will allow researchers to look for things that were not accessible before



Thank you!

