Digital PCR in HIV Reservoir quantification

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Outline

1) What is digital PCR

2) Comparison of digital PCR with qPCR

3) Applications of digital PCR in Virology

4) Future perspectives
What is digital PCR

Sven Sachsalber
Quantification (Using the Poisson distribution)

**Unknown:** concentration of target molecules ($\lambda$)

**Known:**
The ratio of positive partitions ($P$)

$$\lambda = -\ln(1 - \hat{p})$$
What is digital PCR

Sample & Assay  Divide  PCR Amplification  Count
dPCR is not a novel technology!

Limiting dilution of samples for single molecule PCR

Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase

RANDALL K. SAIKI, DAVID H. GELFAND, SUSANNE STOFFEL, STEPHEN J. SCHARF, RUSSELL HIGUCHI, GLENN T. HORN, KARY B. MULLIS,* HENRY A. ERLICH

A thermostable DNA polymerase was used in an in vitro DNA amplification procedure, the polymerase chain reaction. The enzyme, isolated from *Thermus aquaticus*, greatly simplifies the procedure and, by enabling the amplification reaction to be performed at higher temperatures, significantly improves the specificity, yield, sensitivity, and length of products that can be amplified. Single-copy genomic sequences were amplified by a factor of more than 10 million with very high specificity, and DNA segments up to 2000 base pairs were readily amplified. In addition, the method was used to amplify and detect a target DNA molecule present only once in a sample of 10⁵ cells.

Fig. 3. Poisson distribution of single target sequences in samples of 10⁵ cells. (A) Electrophoretic analysis of PCR products in a set of 15 samples (lanes 1 to 15). The arrow indicates the position of the 150-bp amplification product that is visible in some samples (lanes 3, 4, and 6).

9 October 1987; accepted 17 December 1987
Human Immunodeficiency Virus-Infected Individuals Contain Provirus in Small Numbers of Peripheral Mononuclear Cells and at Low Copy Numbers

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double PCR method with a limit dilution approach, both the proportion of infected cells and the number of molecules of HIV provirus per cell can be accurately estimated. Results obtained with PBMCs from 12 HIV-positive hemophiliacs
Why did people stop using digital PCR?

100 µl reactions! Assay costs!

Automatization? Operating costs and troubles:
New technologies make dPCR possible

Arrays:
Multiple partitions on a plate
New technologies make dPCR possible

**picodroplets:**
Droplets in oil
A typical dPCR read-out
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Theoretical benefits of digital PCR

• Direct quantification of DNA molecules
  • No standard curve
  • No variability due to lower efficiency

➢ Higher accuracy
➢ Higher precision
➢ Higher sensitivity
➢ Higher reproducibility
dPCR is more precise compared to qPCR

Precision:
- ddPCR is superior when compared to qPCR
- CV 4 to 20-fold lower in ddPCR compared to qPCR
dPCR is more precise compared to qPCR

Comparison of ddPCR versus seminested qPCR for HIV DNA quantification

Coefficient of Variation (CV) between triplicates

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>replicates</th>
<th>CV ddPCR</th>
<th>CV qPCR</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard curve</td>
<td>7</td>
<td>3</td>
<td>14%</td>
<td>43%</td>
<td>3</td>
</tr>
<tr>
<td>Patient samples</td>
<td>17</td>
<td>3</td>
<td>8%</td>
<td>29%</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Bosman et al., Scientific Reports 2015 5:13811
Comparison of HIV DNA between ddPCR and seminested qPCR

No significant bias compared to qPCR on HIV DNA measurement

Bland Altman plot

Bosman et al., Scientific Reports 2015 5:13811
dPCR Performance of current technology

- Better accuracy and precision ✓
- Less refractory to inhibition
- Better reproducibility
- More sensitive?
Inhibition

- dPCR outperforms qPCR comparing inhibitory substances (SDS, EDTA and Heparin)

*Dingle et al., Clin Chem (2013)59:1670-2*
HIV template in background of HIV negative DNA

33 ng/µl DNA = 660ng/20µl reaction = 100,000 cell equivalents per reaction

dPCR and inhibition: priming mismatches

Mismatches in the probe region

Lower fluorescence would harm qPCR but less harm to ddPCR

Trypsteen et al., Anal Bioanal Chem (2015) 407:5827-34 (suppl data)
dPCR Performance of current technology

- Better accuracy and precision ✓
- Less refractory to inhibition ✓
- Better reproducibility
- More sensitive?
Replicate experiments on 3 consecutive days

Factor of 7 more reproducible compared to qPCR

*Hindson et al., Nature Methods (2013) 10:1003-5*
dPCR is highly reproducible

DNA isolation
HIV ddPCR (triplicate)
RPP30 ddPCR (duplicate)

Median CV 6%

Unpublished data
dPCR Performance of current technology

- Better accuracy and precision
- Less refractory to inhibition
- Better reproducibility
- More sensitive?
Sensitivity

Some reports indicate higher sensitivity of ddPCR

At the limit of detection, more samples are positive with ddPCR

But false positives in ddPCR…

Kiselinova et al., Plos One 2014 9:e85999; Bosman et al., Scientific Reports 2015 5:13811
False positives in ddPCR

Kiselinova et al., Plos One (2014) 9: e85999
dPCR Performance

- Better accuracy and precision
- Less refractory to inhibition
- Better reproducibility
- More sensitive?

When to use dPCR or qPCR depends on the question:

Template present? → go for standard PCR or nested

How much? → go for digital PCR
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Applications of digital PCR in virology

- HIV reservoir analysis in Cure research
- Difficult samples
- Mutation detection
- Relative quantification
HIV reservoirs

Healthy subject  HIV infection  cART  cART stop

- T cells
- HIV reservoir
HIV reservoir in early ART

Applications of digital PCR in virology

- HIV reservoir analysis in Cure research
- Difficult samples
- Mutation detection
- Relative quantification
Difficult samples

Low sample volumes
- Direct quantification after lysis

Pavsic et al., Digital PCR for direct quantification of viruses without DNA extraction. Analytical and Bioanalytical Chemistry (2016) Vol 408:67-75
Direct quantification of RNA viruses in wastewater

Detection of Rotavirus in wastewater

No RNA isolation step

Applications of digital PCR in virology

- HIV reservoir analysis in Cure research
- Difficult samples
- Mutation detection
- Relative quantification
Oseltamivir Resistant mutants in influenza A H1N1

Higher sensitivity of dPCR to detect resistant mutations

A

\[ y = 0.5429x + 1.700 \]

\[ R^2 = 0.9223 \]

B

% SNP abundance (+/- 95% CI)

Applications of digital PCR in virology

- HIV reservoir analysis in Cure research
- Difficult samples
- Mutation detection
- Relative quantification
Discriminating chromosomal integrated (ci)HHV-6 from genuine viral replication.

Relative quantification

Merkel cell polyomavirus in cancer

Ota et al., Quantitative analysis of viral load per haploid genome revealed the different biological features of Merkel Cell Polyomavirus infection in skin tumor
Perspectives of dPCR in virology

• Several applications possible:
  • Low level quantification
  • Mutation/resistance detection
  • Relative quantification

• Current drawbacks:
  • False positives
    • New technologies?
  • Need for objective data analysis
    • Has received little analysis, but new technique, new issues.
What is positive, what is negative?

Future perspectives: devices and software

Automatic threshold setting (extreme value theory)


Trypsteen et al., Anal Bioanal Chem (2015) 407:5827-34 (suppl data)
General Conclusion

Current state of the ART

- Specific applications
- Do we need the sensitivity?

Future perspectives

- May partially/totally replace real time PCR
- Need for high throughput and low cost
- Ease of analysis, ease of interlaboratory standardization
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