UNIVERSITEIT GENT

FACULTY OF MEDICINE AND HEALTH SCIENCES

Droplet Digital PCR, the new tool in HIV reservoir quantification?

Ward De Spiegelaere

Droplet Digital PCR,
the new tool in HIV reservoir quantification?

Content:

- **Digital PCR**
- Applications
  - Total HIV DNA
  - 2LTR
  - CA HIV RNA
- Conclusions

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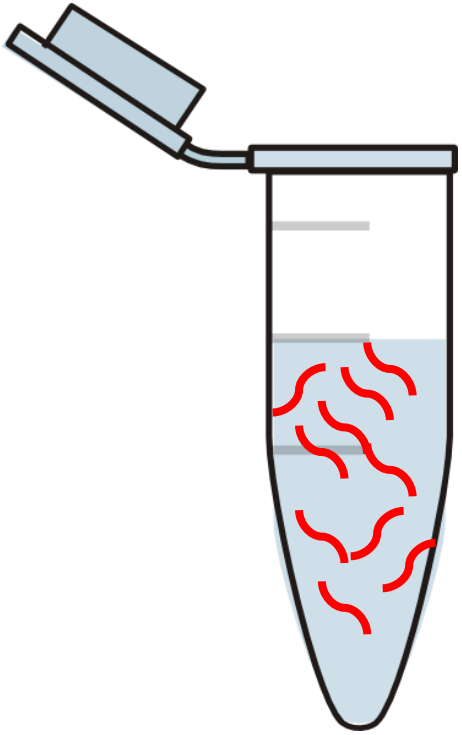
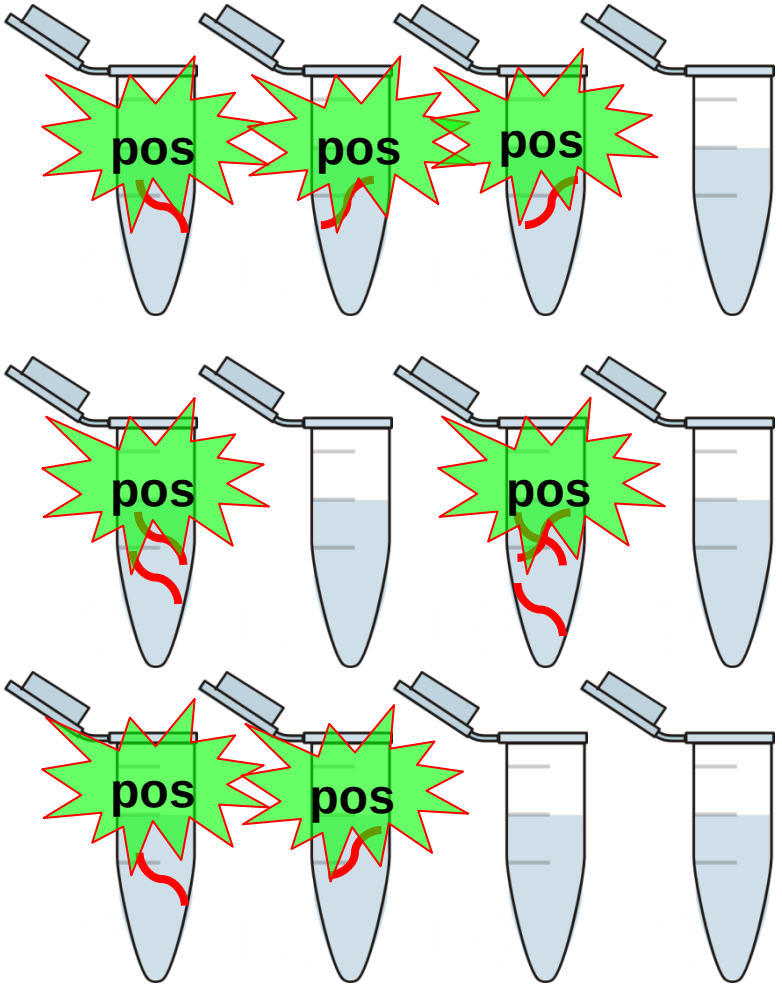
What is digital PCR (dPCR)?

qPCR

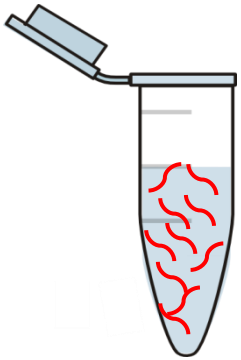


10 amplicons

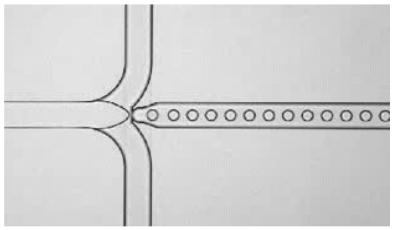
dPCR

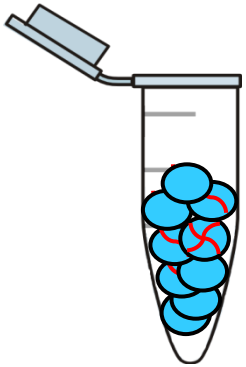


Droplet digital PCR (ddPCR)



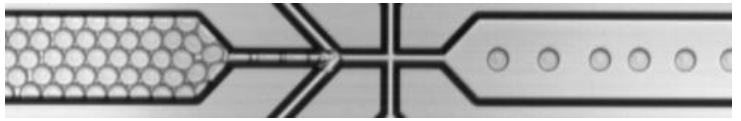
20 µl



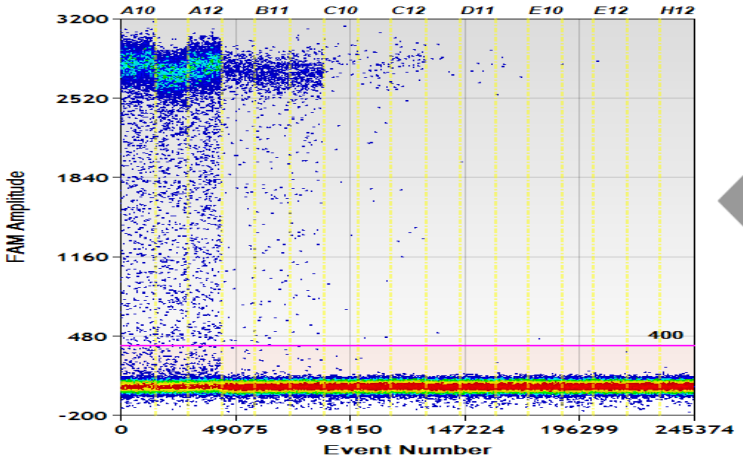


20,000 reactions

PCR



fluorescent read-out



Advantages of digital PCR

Direct absolute quantification:

- **No standard curve**
- **LOD = LOQ**

End point PCR:

- **Less susceptible to inhibition**
  - input ↑
  - mismatches
- **Higher flexibility in assay design**

Low level detection

- **Higher accuracy vs qPCR**
- **Not higher sensitivity**

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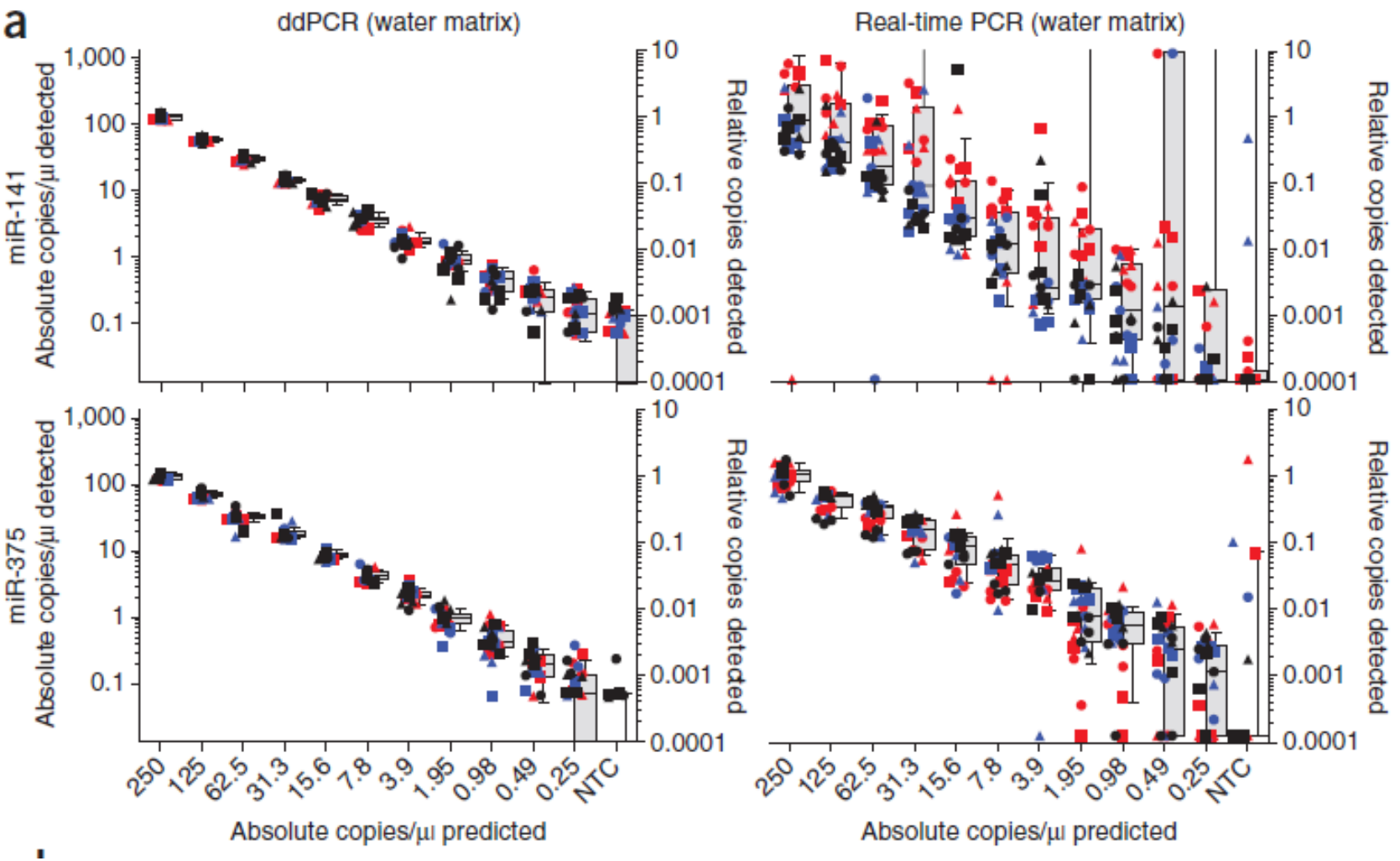
Accuracy of ddPCR for DNA quantification

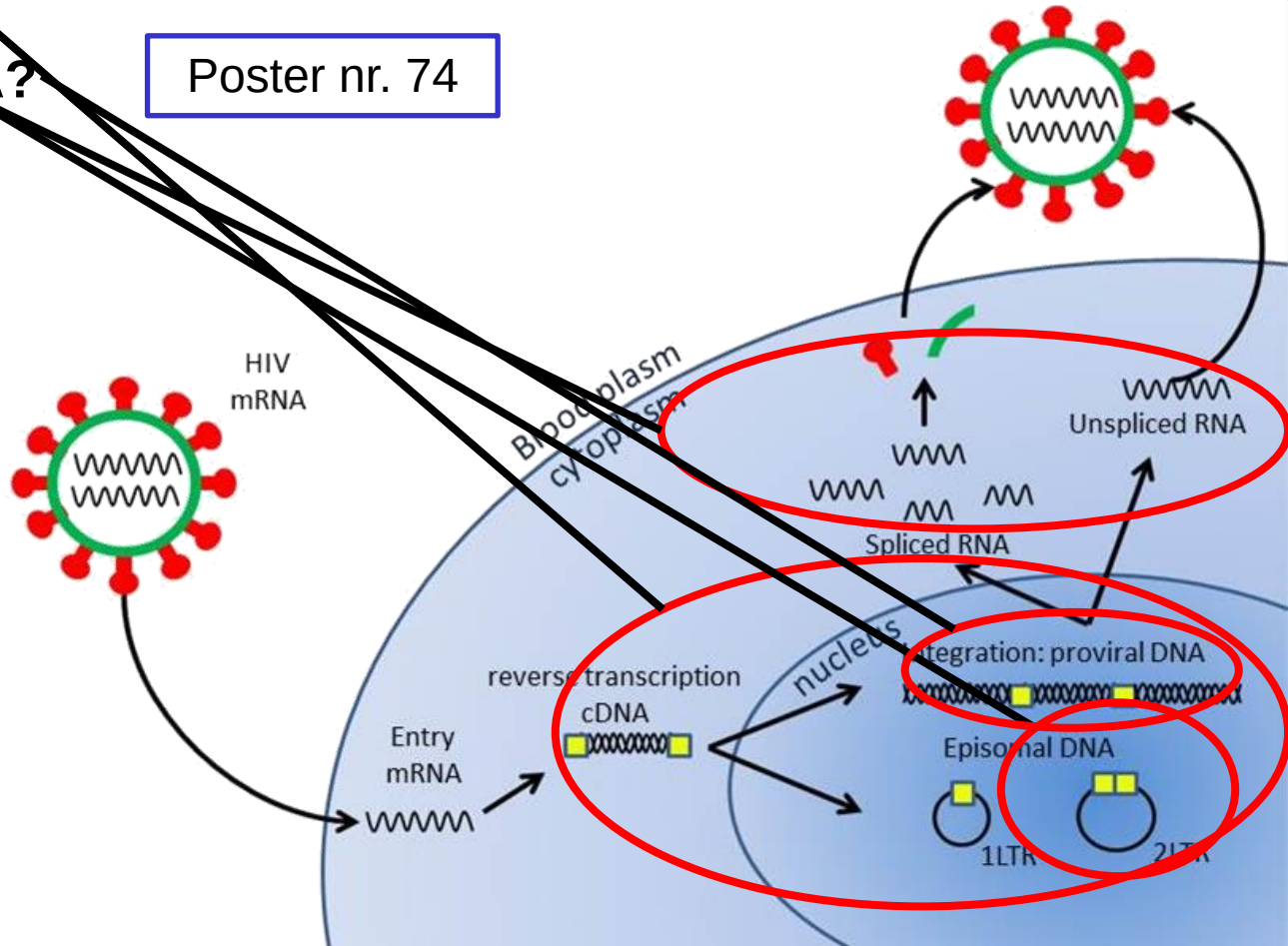


Hindson CM, Chevillet JR, Briggs HA et al. Nat Methods (2013):

Implementing PCR-based HIV reservoir diagnostics on the ddPCR platform

- **Total HIV**
- **2LTR**
- **HIV RNA**
- **Integrated HIV DNA?**

Poster nr. 74



Pitfalls

Maximal amount of total DNA input

False positive droplets

Treshold setting

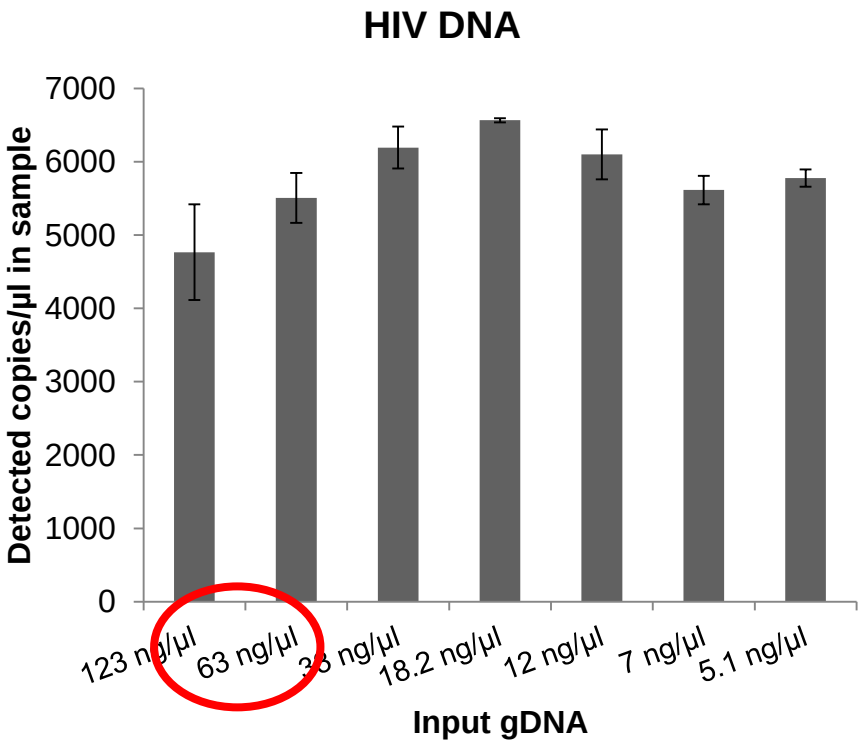
Qualitative assessment of droplets

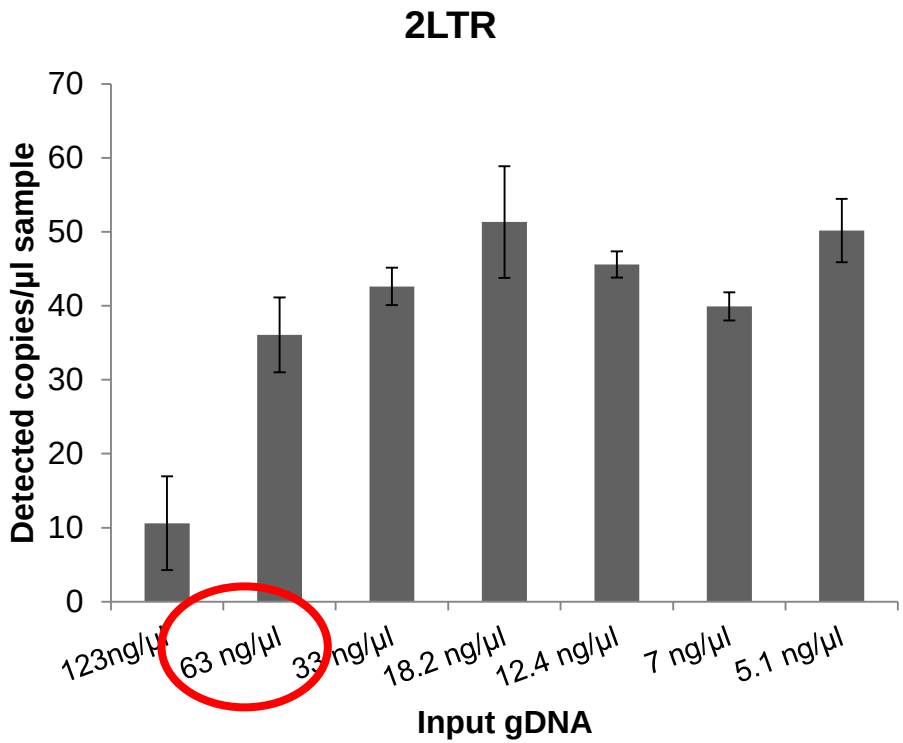
- **raw ddPCR data needed**
- **droplet loss**

Molecular dropout: failed amplification in some droplets

Maximal input gDNA

Different total gDNA amounts

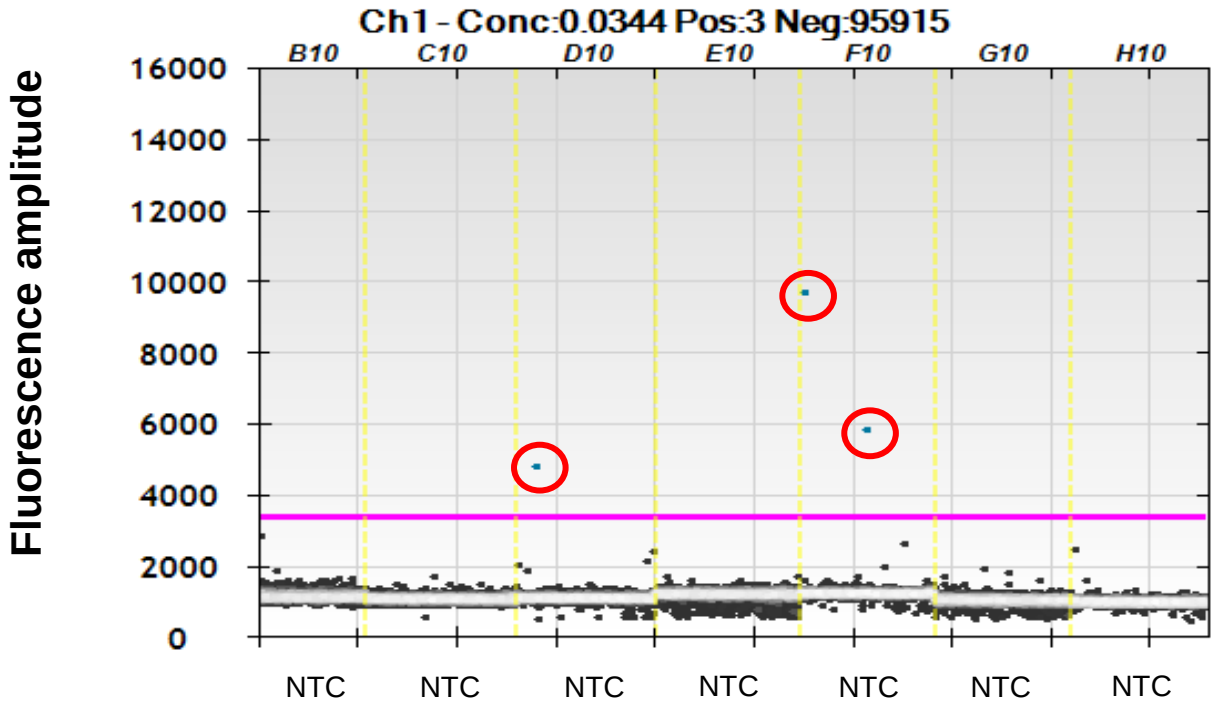




Limited to < 200,000 cell equivalents/20µl reactions

Solution? Pool droplets from separate wells

False positives



Freqeuncy of occurence: 1/3 or <1/10 wells

Maximally 3 droplets/well

LOD ≠ LOQ

Pooling data from multiple wells is not possible

Droplet Digital PCR,
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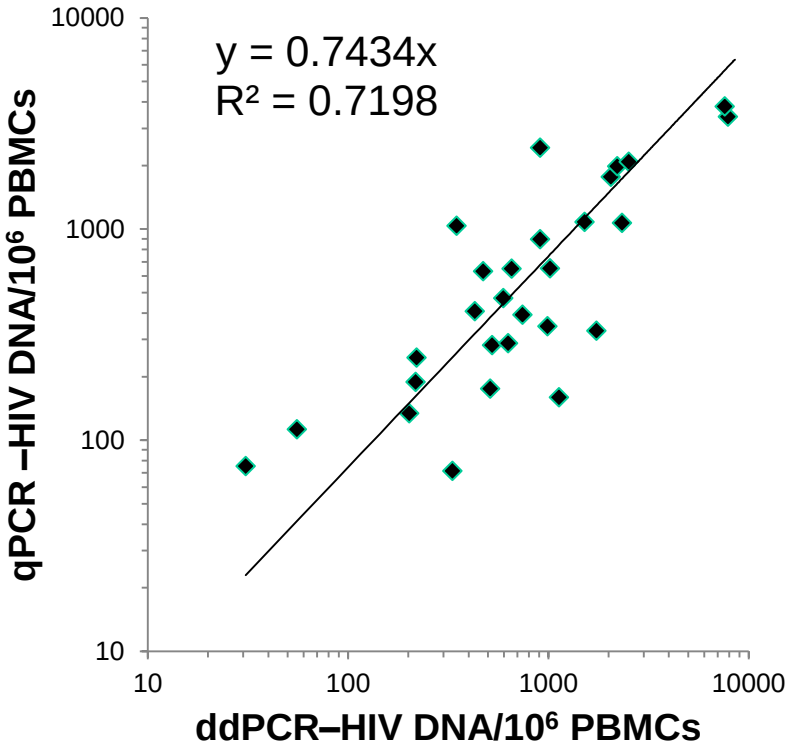
HIV DNA

Good correlation

Slight overestimation with ddPCR compared to qPCR

Possibly due to mismatches in patient samples

Patient samples



n=28

In collaboration with Una O'Doherty, UPENN

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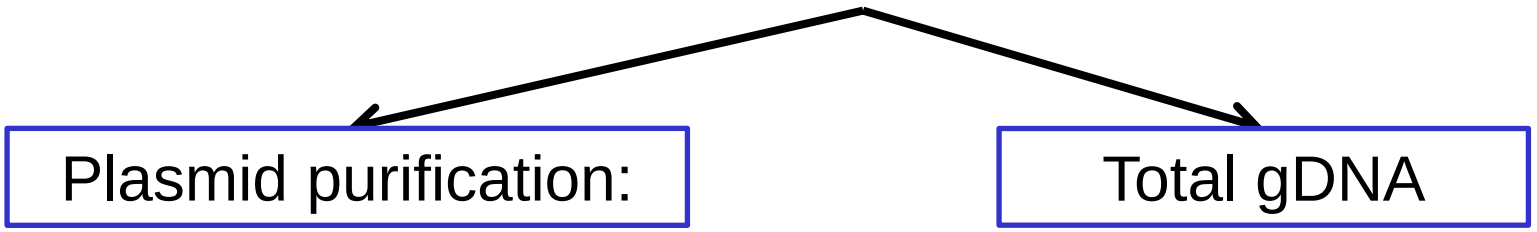
2LTR

Low abundance of 2LTR!



Pro:

- High cellular input

Contra:

- Loss of low abundant episomal DNA?
- Normalization strategy?

Pro:

- Internal reference gene

Contra:

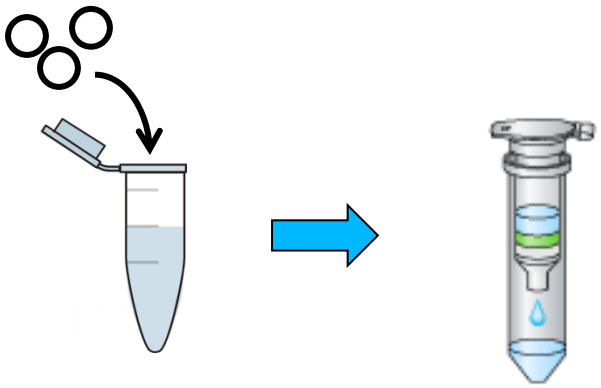
- High DNA content: inhibition?

Plasmid pur vs gDNA

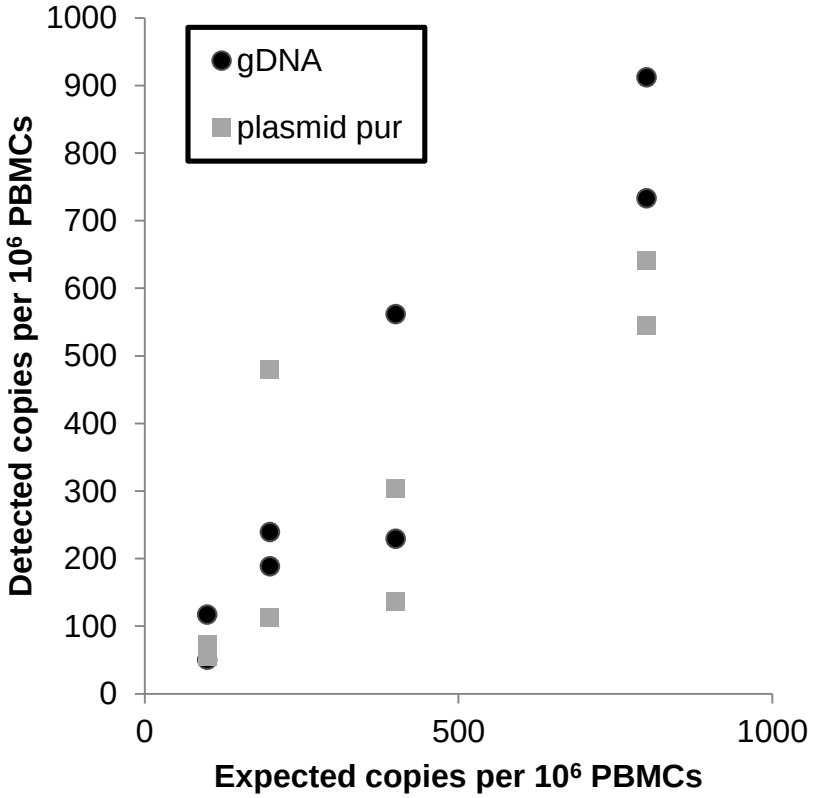
Modified plasmid purification

Addition of a non HIV reference plasmid before plasmid pur

pSIF (6.3 kb)
FIV-based



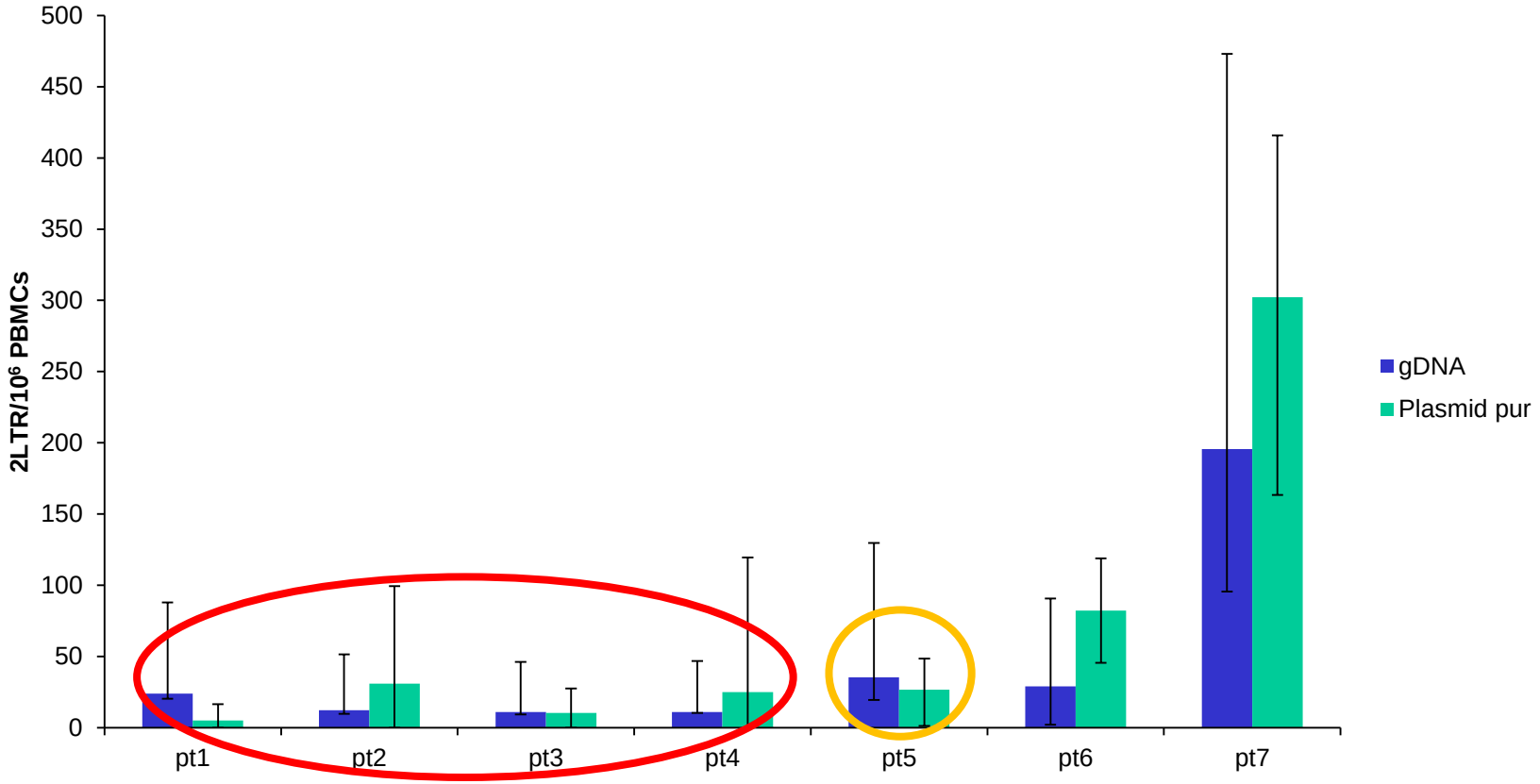
Serial dilution of infected cells in 10^7 PBMCs



Patient derived PBMCs

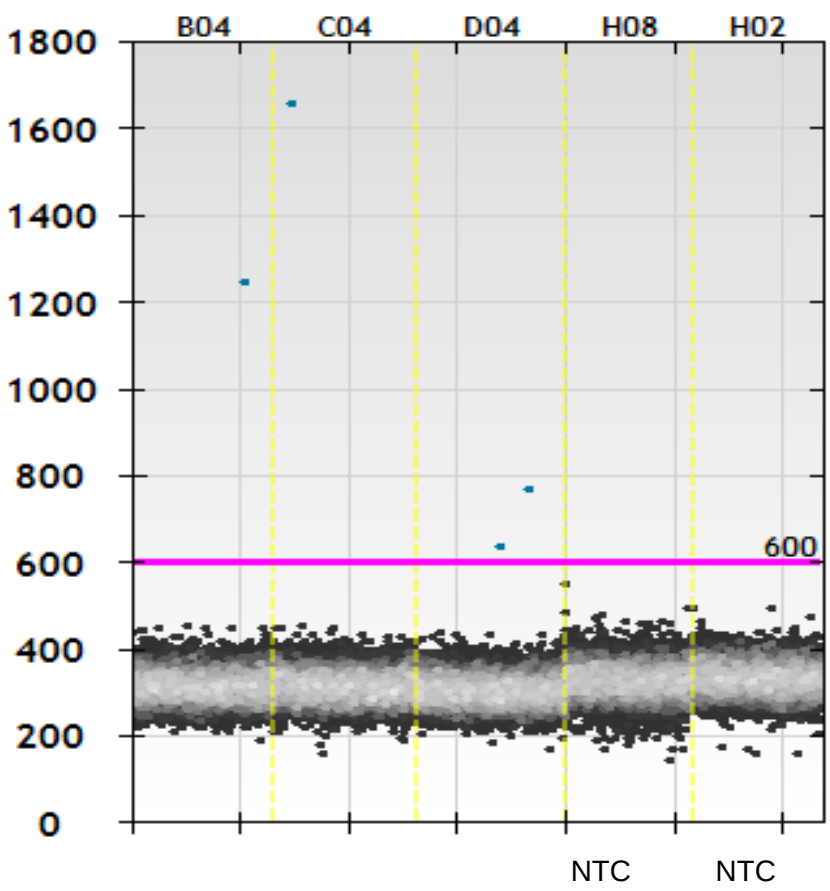
gDNA: < 2x10^5 cells/reaction

Plasmid pur: 2x10^5 - 10^7 cells/reaction

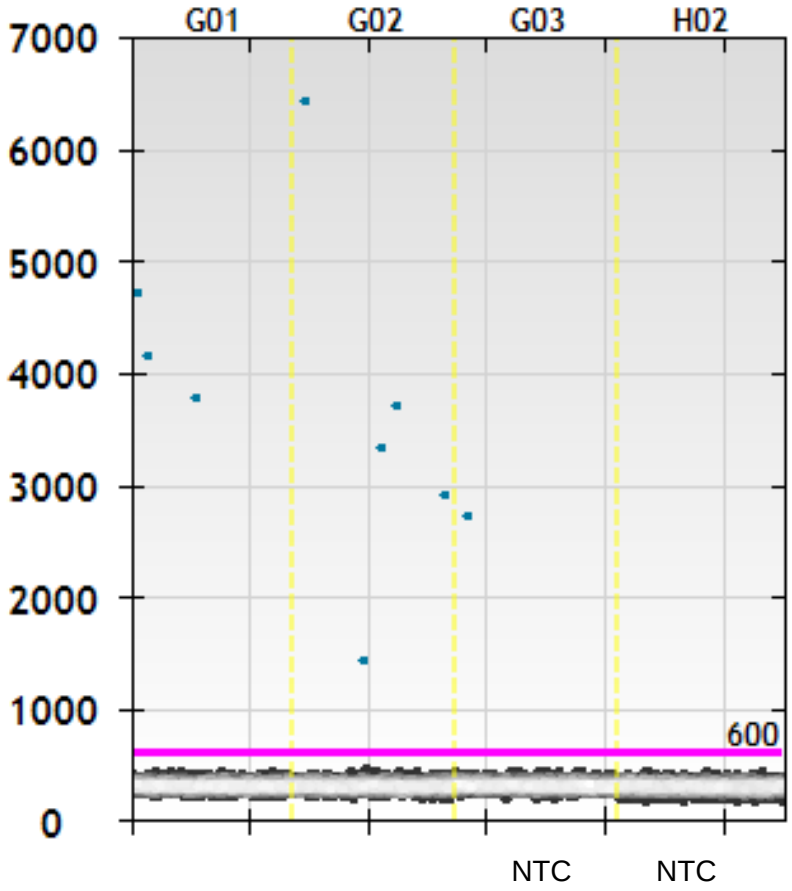


Raw data pt5

gDNA

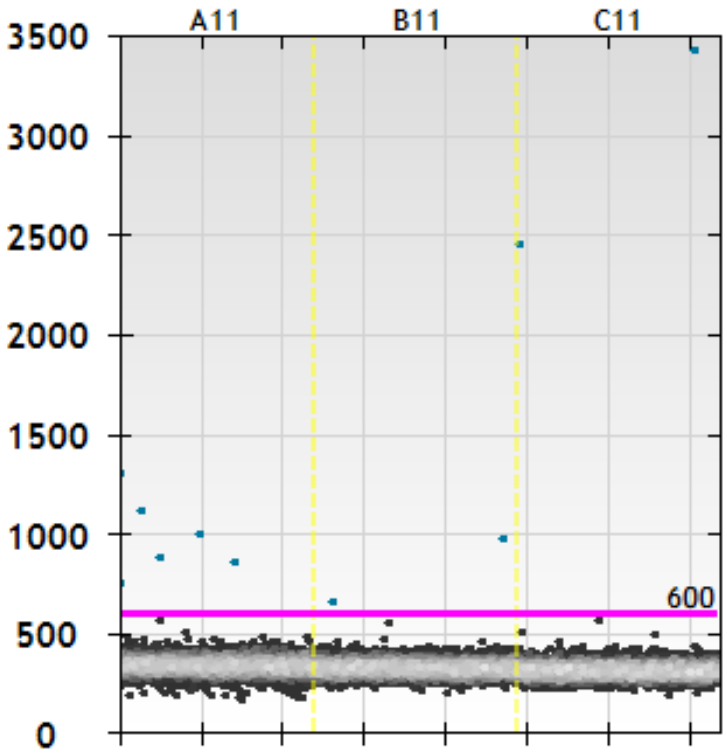


Plasmid purified DNA

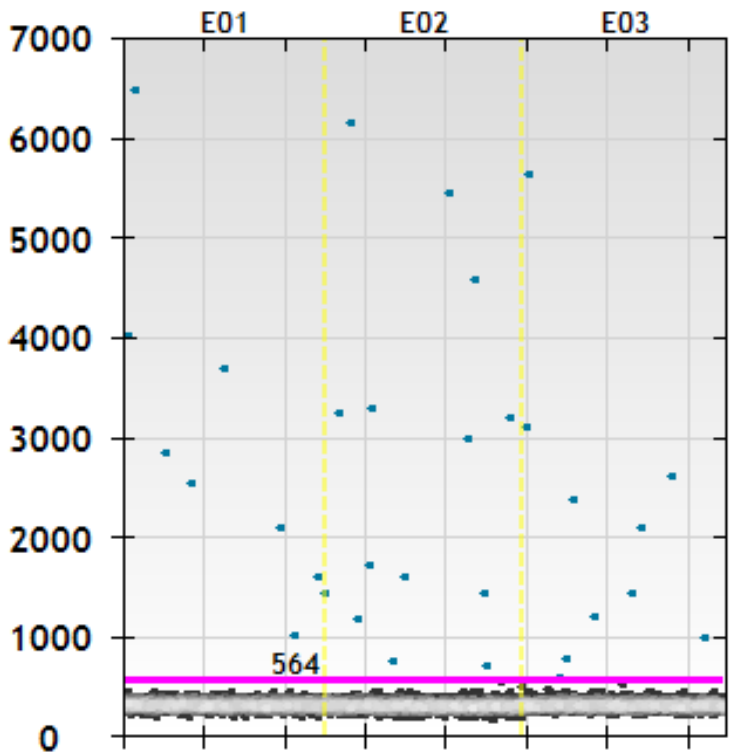


Raw data: pt6

gDNA



Plasmid purified DNA



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CA HIV RNA

DdPCR vs seminested PCR

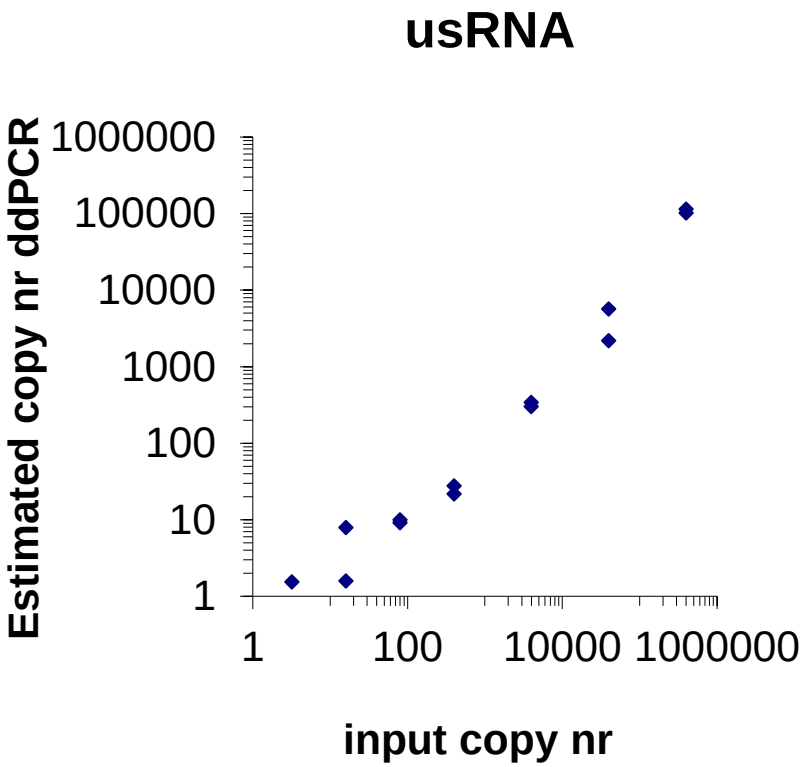
RNA-cDNA conversion

slope: 0.1015

10-fold underestimation

Need for conversion factor!

Standard Dilution series



Kiselinova , Pasternak, De Spiegelaere, et al., Plos One (in revision)

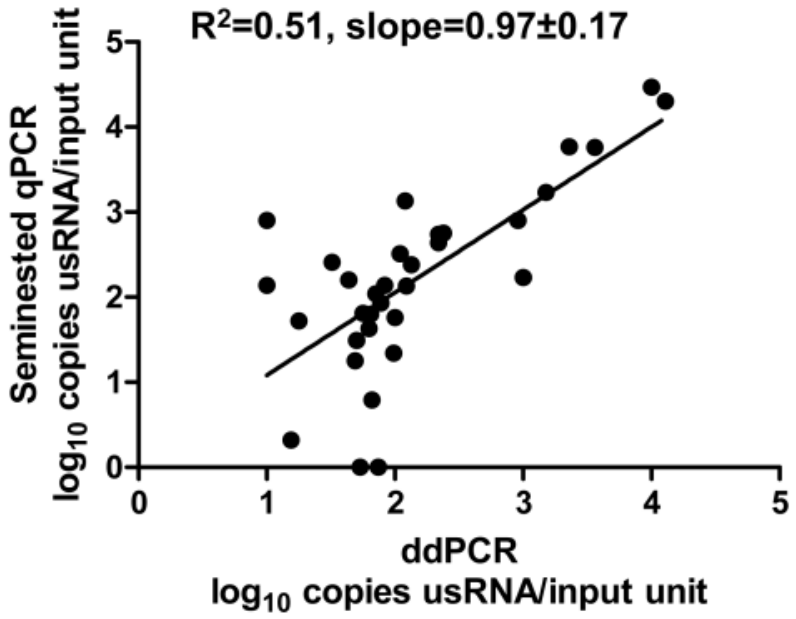
CA HIV RNA (ddPCR vs seminested PCR)

Patient samples

Good correlation in higher ranges

Sample error in low abundant samples

Unspliced HIV RNA



n=34

Kiselinova , Pasternak, De Spiegelaere, et al., Plos One (in revision)

CA HIV RNA (ddPCR vs seminested PCR)

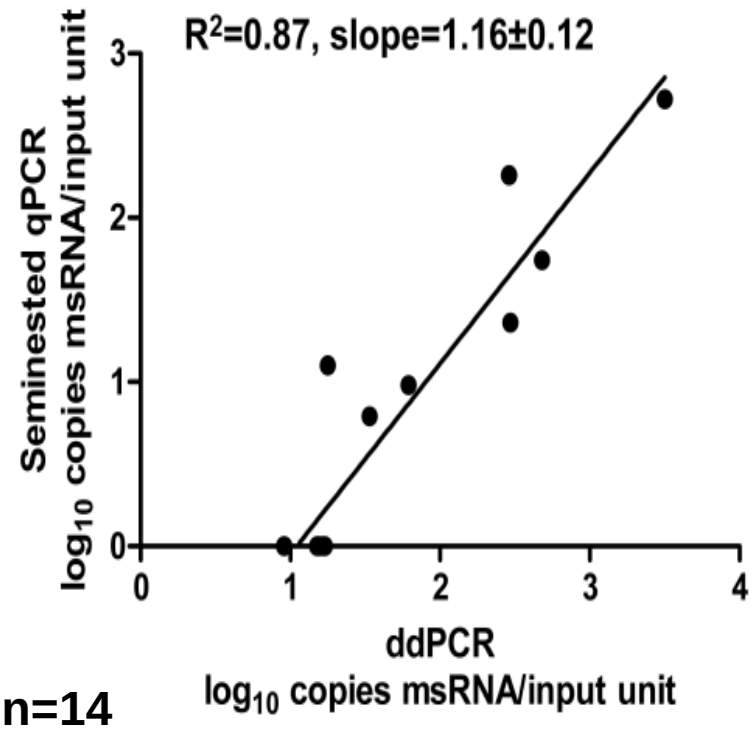
Patient samples

Good correlation in higher ranges

Higher estimation with ddPCR

→ mismatches

Multiple spliced HIV RNA



n=14

Summary

	Total HIV	2LTR	RNA
Comparison with qPCR	+	+	+
Pre PCR processing?			
Effect of false positives			
Sequence variation			
Standard required			

Conclusion

advantages over qPCR:

- **Accuracy**
- **Mismatches**
- **Inhibition**

But suffers drawbacks

- **False positives**
- **Maximal input**

Guidelines for reporting ddPCR methods are required!

Guidelines

To improve interpretation and reproducibility

- Number of replicates
- Template input/sample
- Normalization strategy

- **Raw data**
  - Number of droplets assessed/sample
  - Number of droplets found positive
  - Level of False positives

Guidelines should be set-up and adopted **by the community!**

Acknowledgements

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UPENN

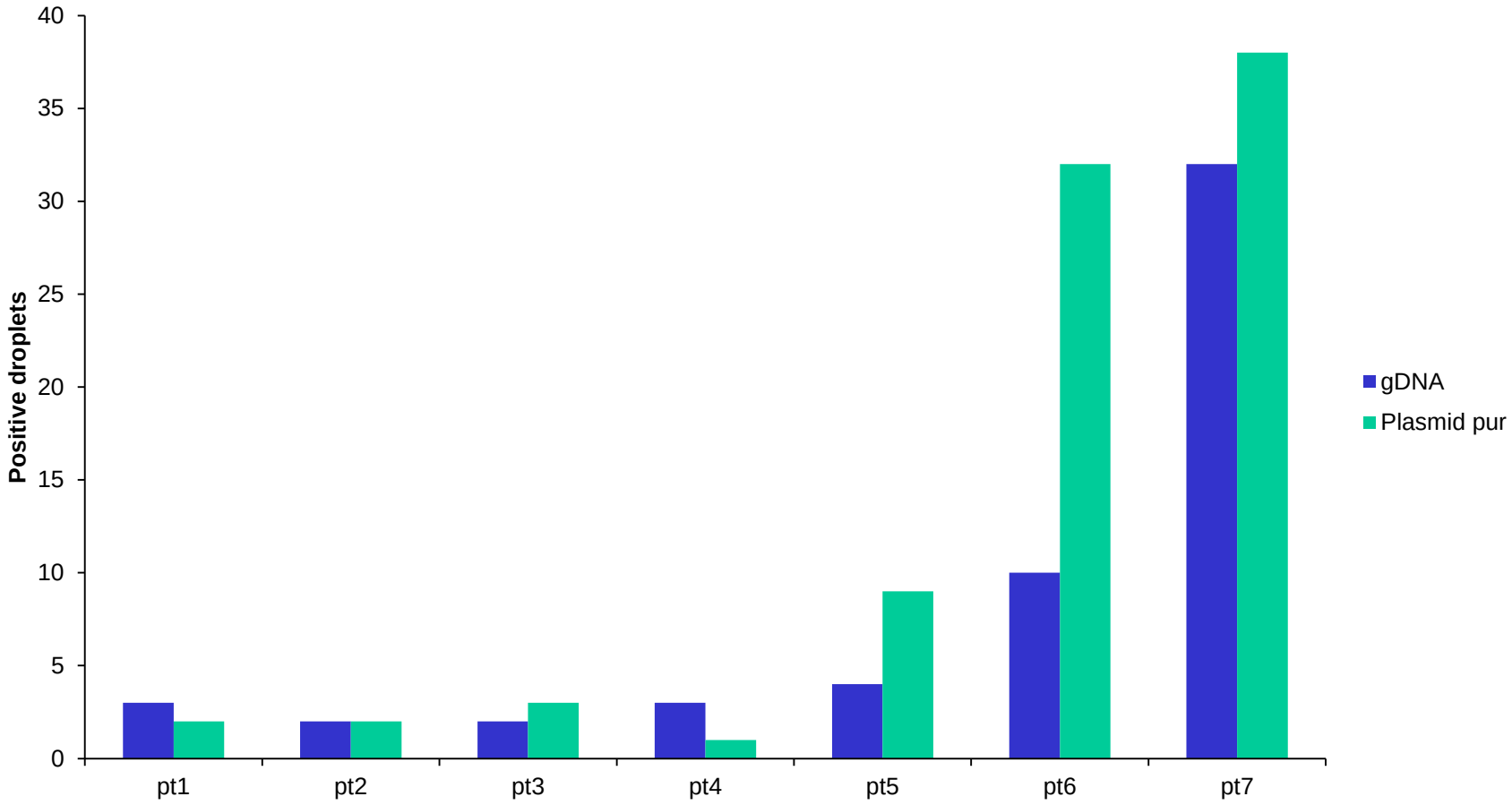
Una O'Doherty

Lindsay Lynch

University of Amsterdam

Alexander Pasternak

2LTR droplet count



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