# Application of Digital PCR for Sensitive Detection of Avian Influenza Viral RNA in Poultry Matrices with Low Viral Loads

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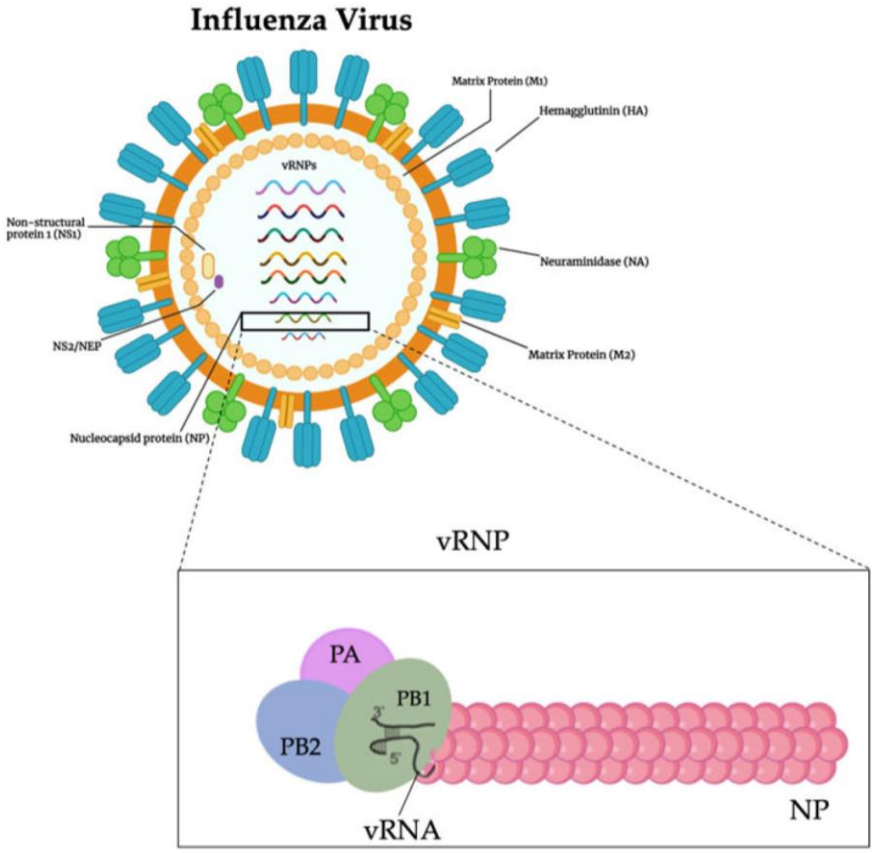
# Background




(Imperia et al., 2023)

**Avian influenza virus (AIV)** is a highly contagious disease of poultry that causes significant economic losses and poses serious risks to animal and public health worldwide.

Sensitive and accurate diagnosis is critical for early detection, effective outbreak control, and surveillance.

Although real-time RT-PCR is the current standard for AIV diagnosis, it may have limited sensitivity in low viral load and complex poultry samples.

**Digital PCR (dPCR) as a potential complementary/alternative method?**

# Objectives

1

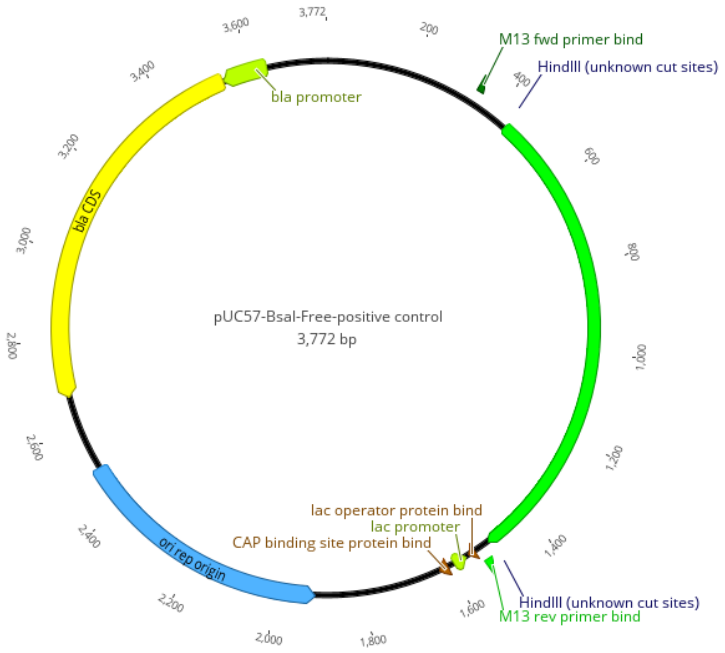
**To compare** the analytical performance of dPCR with real-time RT-PCR for the detection and quantification of AIV genomes.

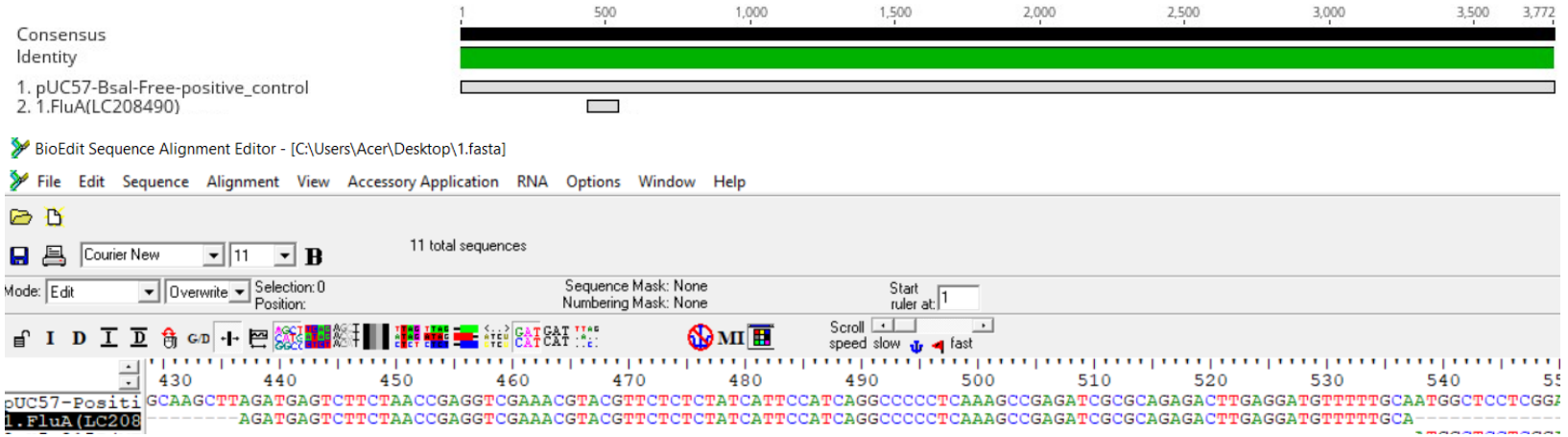
2

**To evaluate** the application of dPCR for sensitive and precise detection of AIV in diverse poultry sample matrices, particularly those with low viral loads.

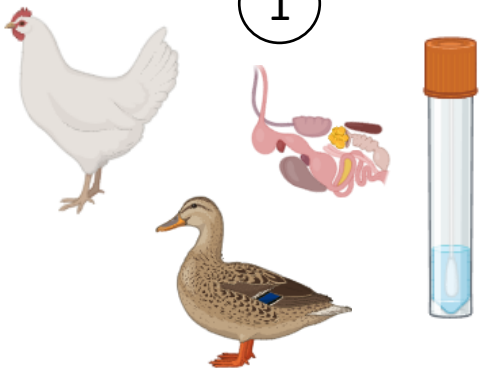
# Materials and methods

A synthetic plasmid containing the **matrix (M)** gene of AIV was used as the reference material for analytical evaluation.



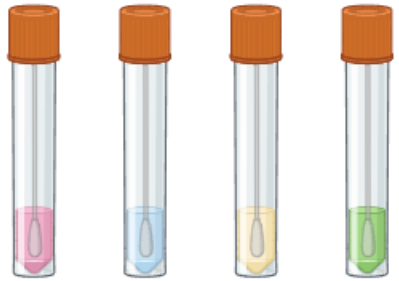



1



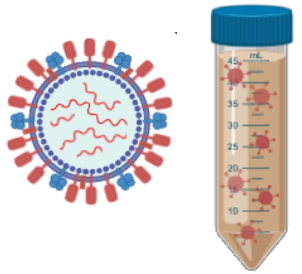
Sample collection

2



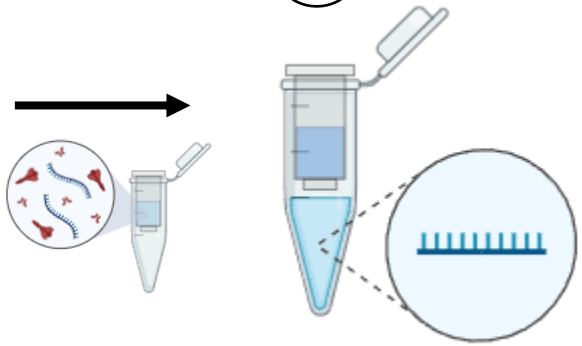
Four matrices

3



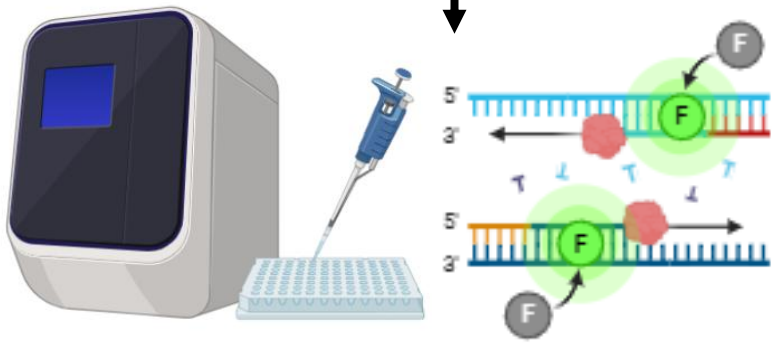
AIV reference viruses spike into matrices

4



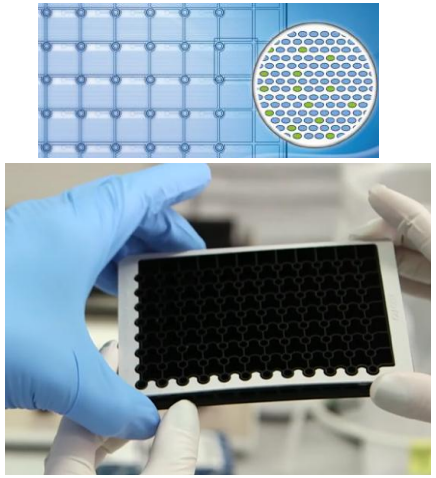
RNA extraction

5



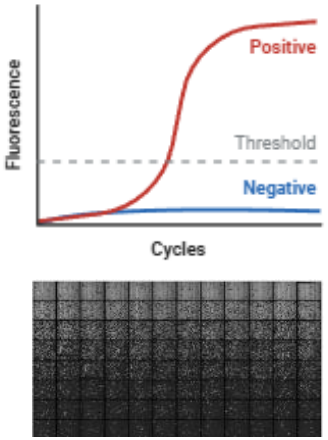

real-time RT-PCR

6



dPCR

7




Data analysis

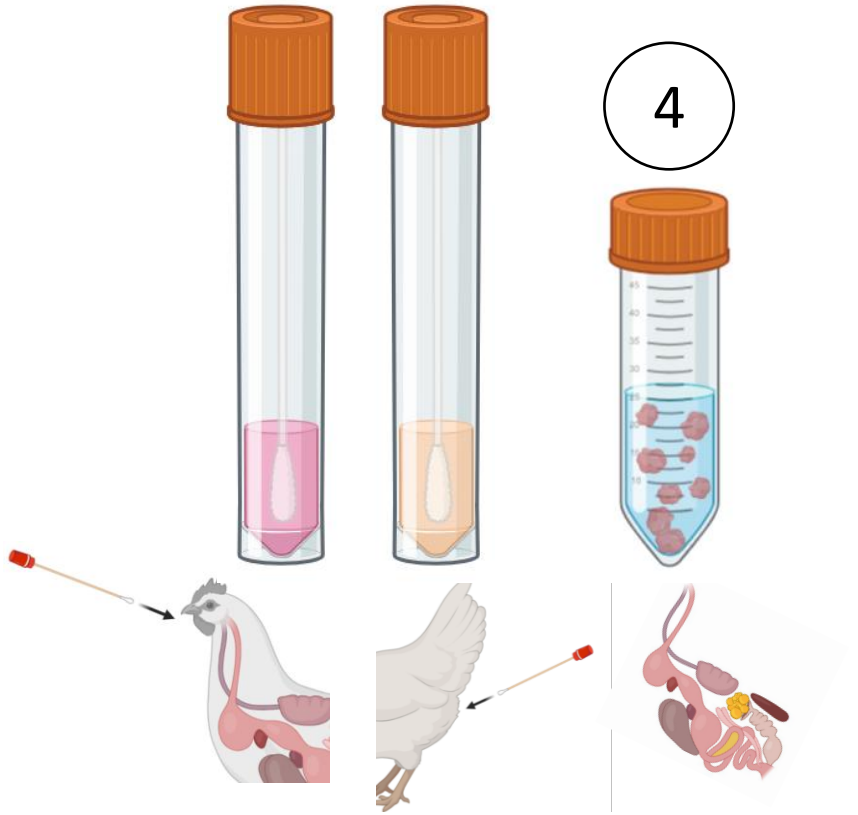
# Four matrices

1

Nuclease free $H_2O$

No inhibitor

2 3 4



Oropharyngeal swab | Cloacal swab | Tissue

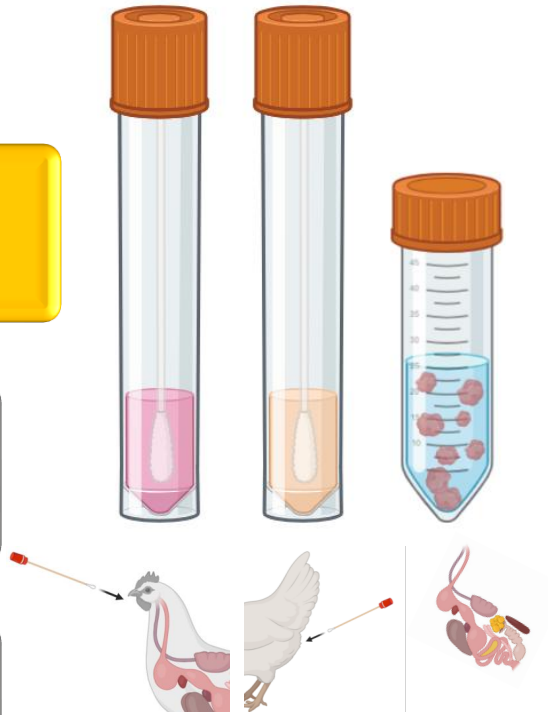
Variable inhibitors

Nuclease free $H_2O$

- Quantification range
- LOD
- Repeatability
- Specificity

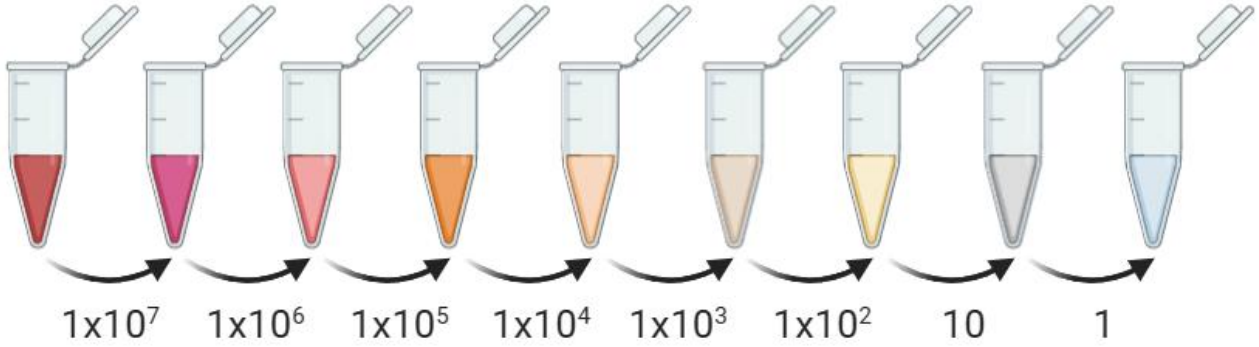
Three matrices

- Viral loads
- Correlation
- Bland-Altman

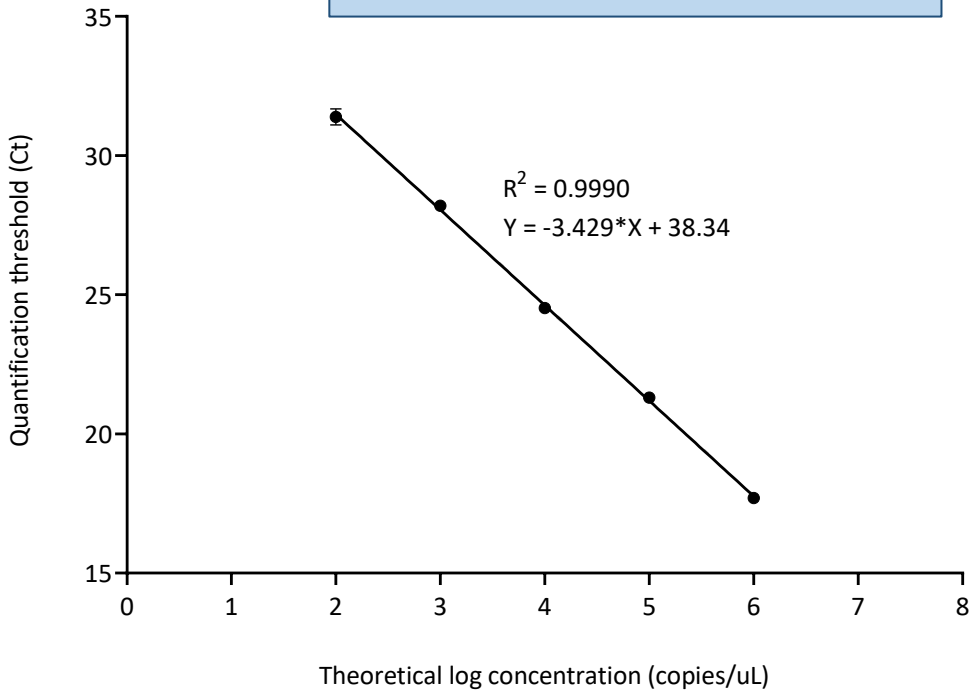


LOD : limit of detection

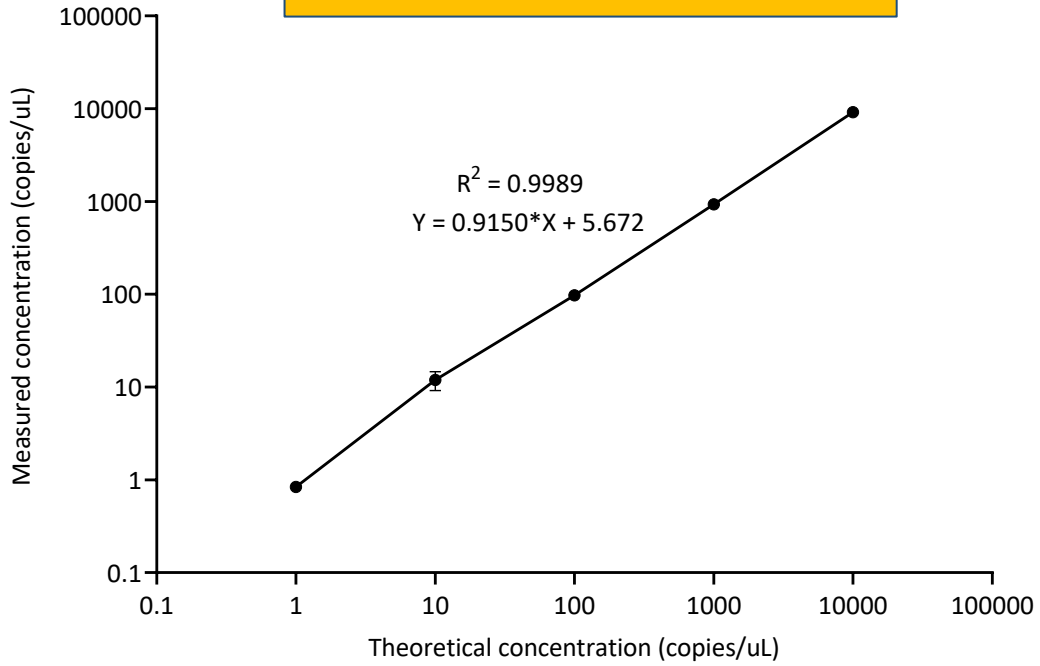
# Results




real-time RT-PCR




dPCR




Both methods showed <u>strong linearity</u> across 10-fold serial dilutions ($R^2 > 0.99$).

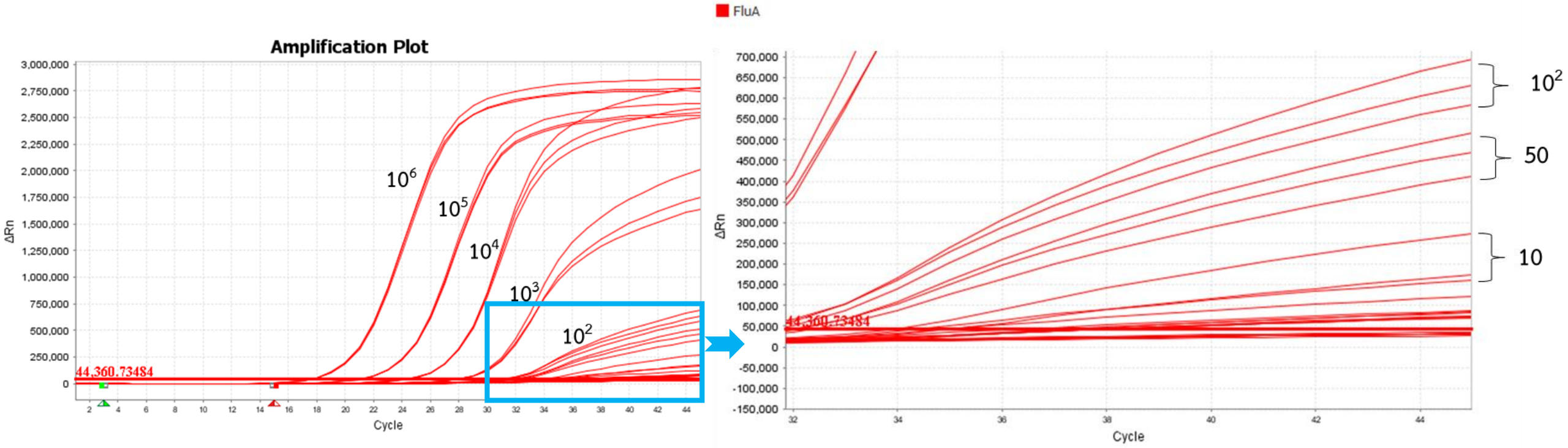
# Quantification range

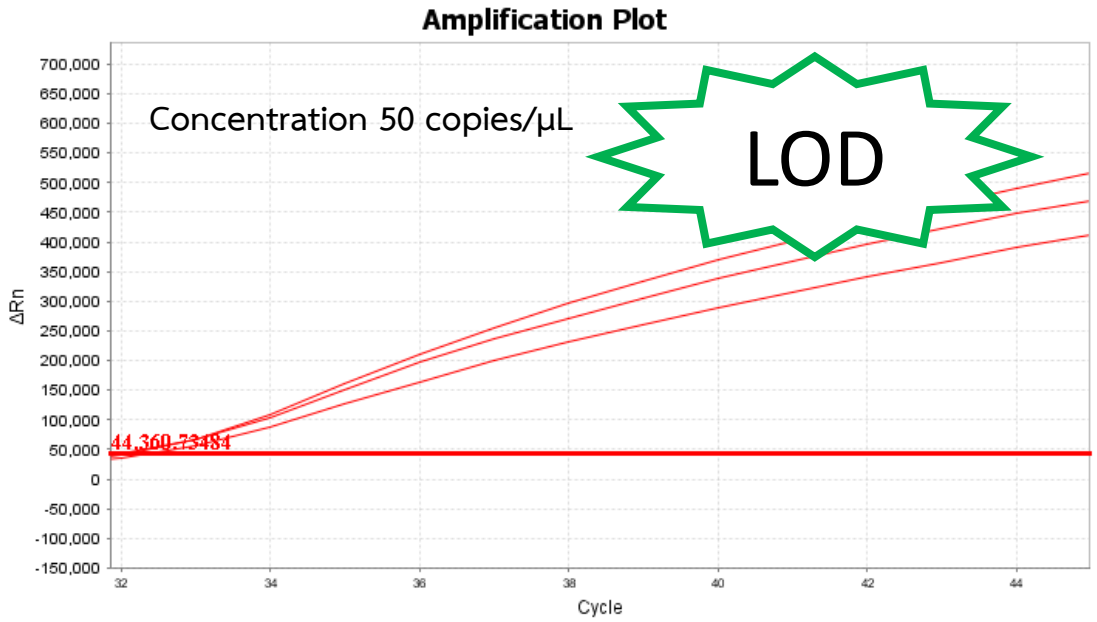
| Theoretical concentration (copies/µL) | real-time RT-PCR (ct) | | | | dPCR (copies/µL) | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Average | 1 | 2 | 3 | Average |
| $10^6$ | 17.74 | 17.67 | 17.67 | 17.69 | ADR | ADR | ADR | ADR |
| $10^5$ | 21.32 | 21.32 | 21.27 | 21.30 | ADR | ADR | ADR | ADR |
| $10^4$ | 24.53 | 24.44 | 24.59 | 24.52 | 8,776.8 | 9,366.2 | 9,322.4 | 9,155.14 |
| $10^3$ | 28.16 | 28.24 | 28.20 | 28.20 | 946.80 | 923.90 | 920.40 | 930.37 |
| $10^2$ | 31.11 | 31.68 | 31.38 | 31.39 | 95.80 | 101.90 | 93.78 | 97.16 |
| 50 | 31.98 | 32.41 | 32.15 | 32.18 | 50.39 | 46.67 | 52.91 | 49.99 |
| 10 | 38.20 | 34.52 | 35.19 | 35.97 | 11.92 | 14.62 | 9.15 | 11.89 |
| 5 | 37.56 | 37.37 | 36.57 | 37.17 | 4.21 | 3.64 | 7.40 | 5.08 |
| 2.5 | 38.41 | 37.96 | UND | 38.19 | 4.17 | 2.66 | 1.80 | 2.87 |
| 1 | UND | UND | UND | UND | 0.81 | 0.86 | 0.84 | 0.84 |

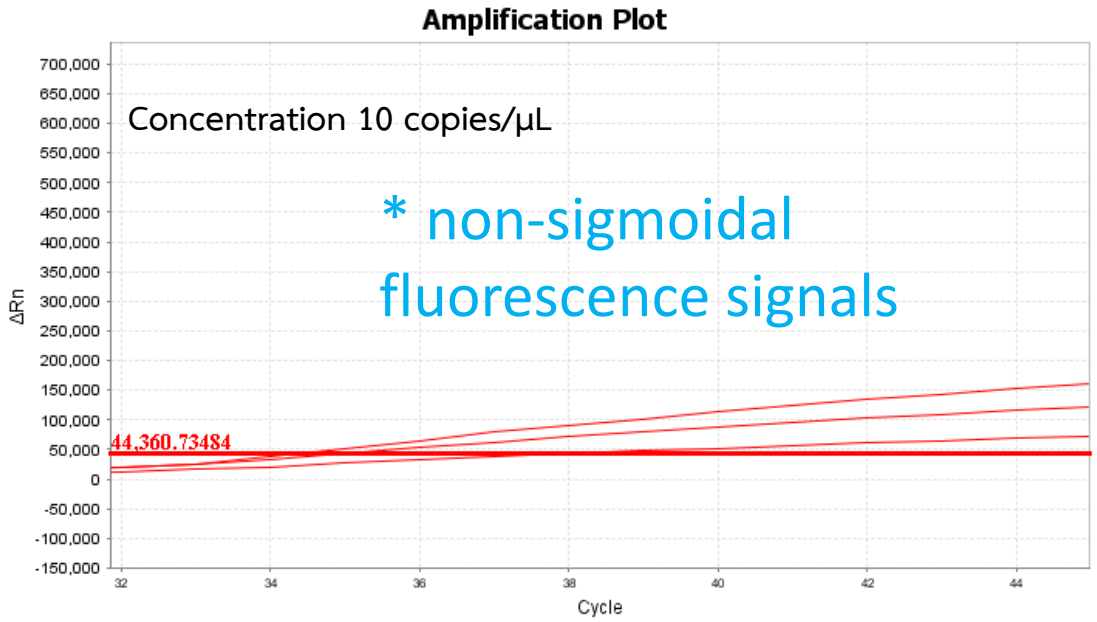
*UND = Under detected

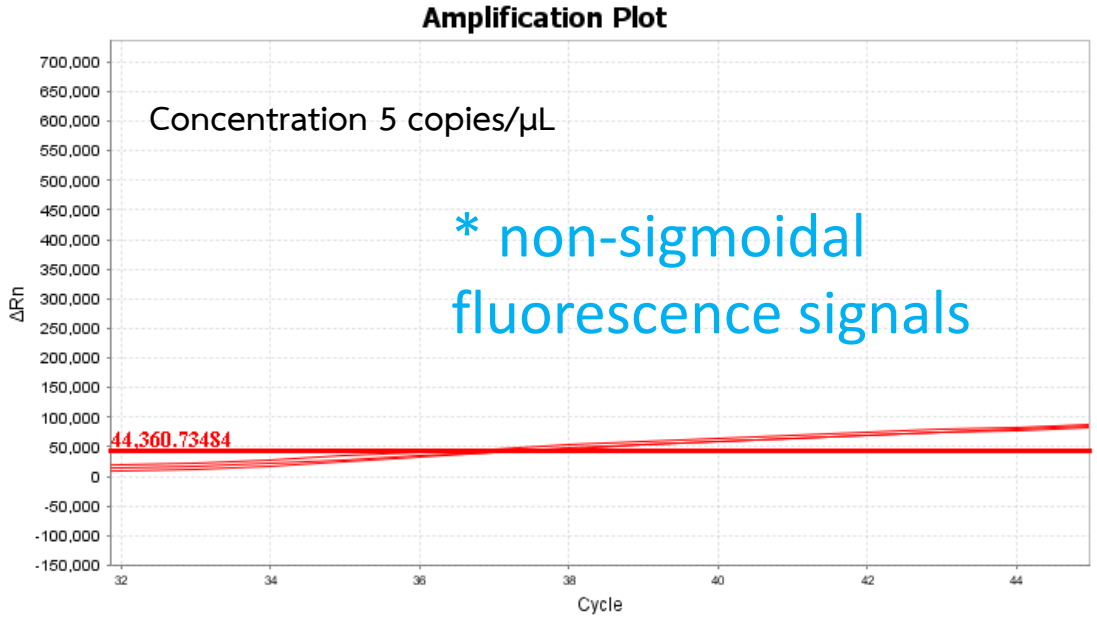
*ADR = Above dynamic range (saturated)

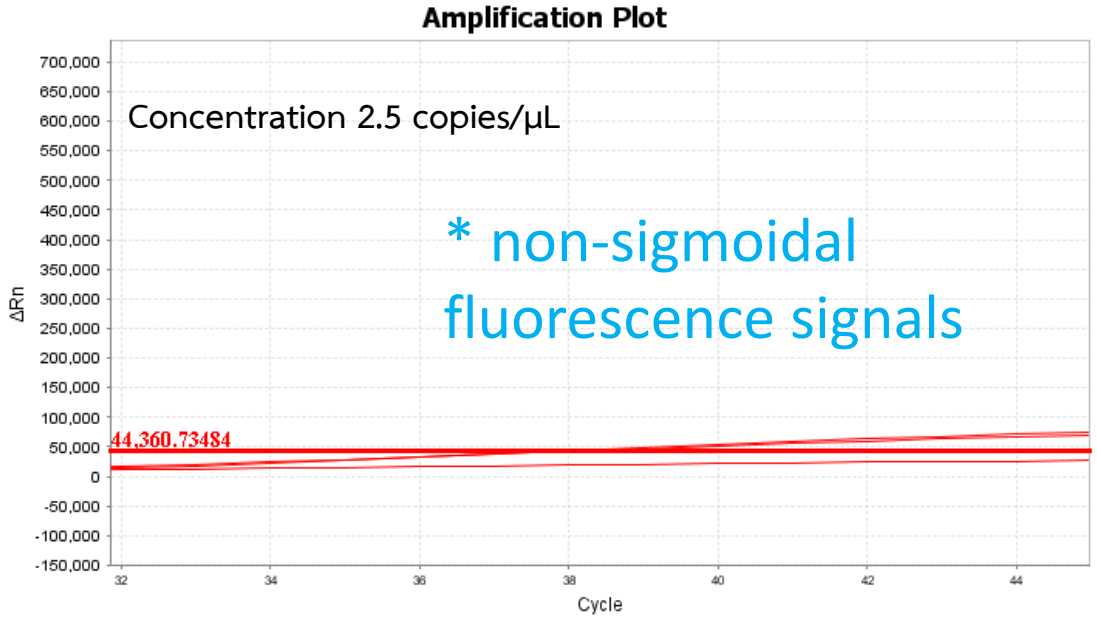
# real time RT-PCR




Amplification Plot
Concentration 50 copies/µL
LOD
ΔRn
Cycle
44,360.73484


Amplification Plot
Concentration 10 copies/µL
* non-sigmoidal
fluorescence signals
ΔRn
Cycle
44,360.73484


Amplification Plot
Concentration 5 copies/µL
* non-sigmoidal
fluorescence signals
ΔRn
Cycle
44,360.73484


Amplification Plot
Concentration 2.5 copies/µL
* non-sigmoidal
fluorescence signals
ΔRn
Cycle
44,360.73484

FluA

FluA

# Quantification range

| Theoretical concentration (copies/µL) | Real-time RT-PCR (ct) | | | | Digital PCR (copies/µL) | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Average | 1 | 2 | 3 | Average |
| $10^6$ | 17.74 | 17.67 | 17.67 | 17.69 | ADR | ADR | ADR | ADR |
| $10^5$ | 21.32 | 21.32 | 21.27 | 21.30 | ADR | ADR | ADR | ADR |
| $10^4$ | 24.53 | 24.44 | 24.59 | 24.52 | 8,776.8 | 9,366.2 | 9,322.4 | 9,155.14 |
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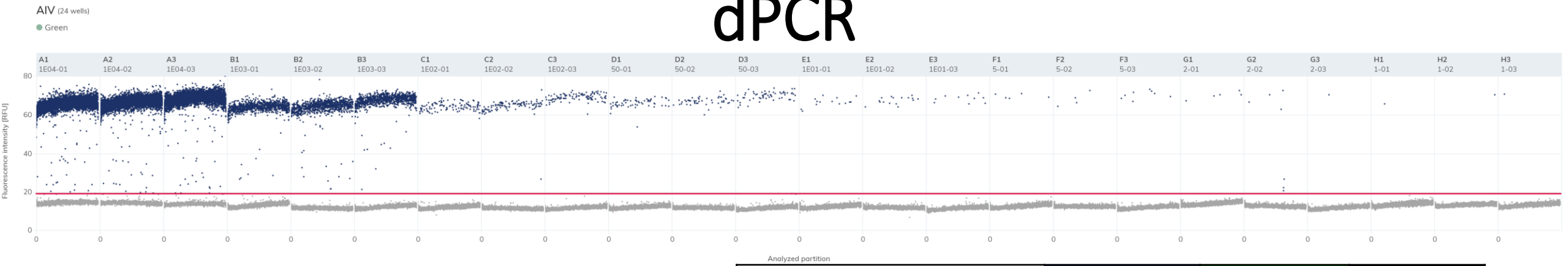
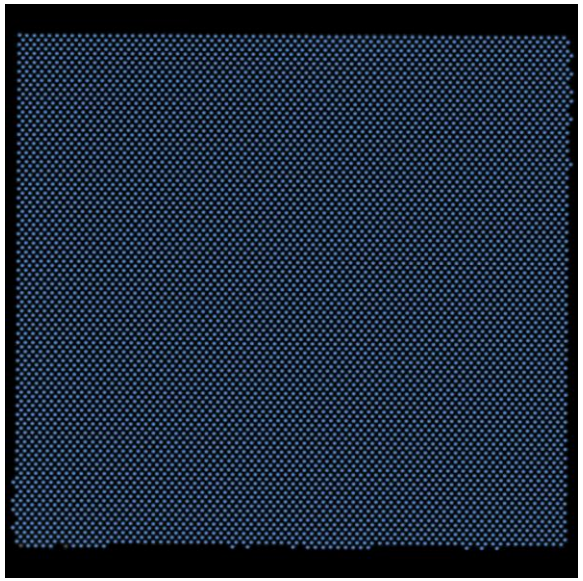
| 2.5 | 38.41 | 37.96 | UND | 38.19 | 4.17 | 2.66 | 1.80 | 2.87 |
| 1 | UND | UND | UND | UND | 0.81 | 0.86 | 0.84 | 0.84 |

*UND = Under detected

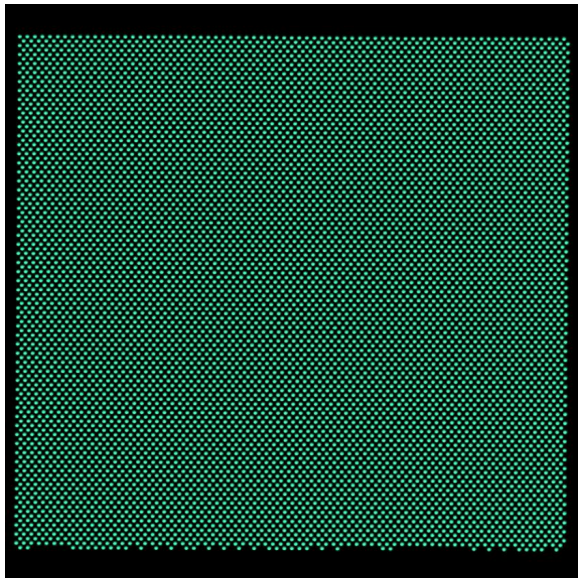
*ADR = Above dynamic range (saturated)

# dPCR



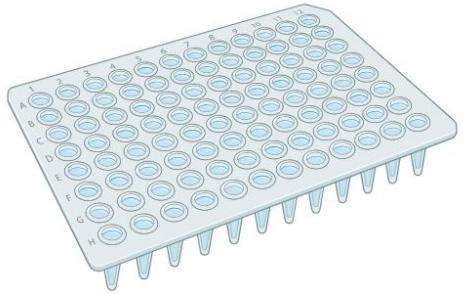
Valid partitions

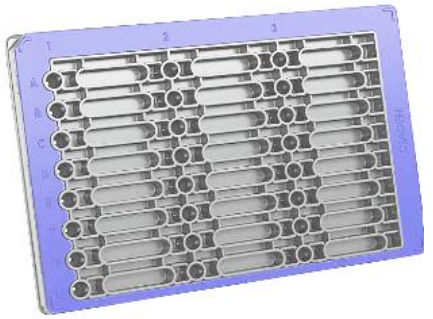


Positive partitions

| Theoretical concentration (copies/µL) | Valid | Positive | Negative |
|---|---|---|---|
| $10^7$ | 8,265 | 8,265 | 0 |
| $10^6$ | 8,276 | 8,276 | 0 |
| $10^5$ | 8,253 | 8,253 | 0 |
| $10^4$ | 8,277 | 6,102 | 2,175 |
| $10^3$ | 8,241 | 1,064 | 7,177 |
| $10^2$ | 8,279 | 114 | 8,165 |
| 50 | 8,253 | 53 | 8,202 |
| 10 | 8,282 | 14 | 8,268 |
| 5 | 8,276 | 5 | 8,271 |
| 2.5 | 8,264 | 3 | 8,261 |
| 1 | 8,256 | 1 | 8,255 |

*saturated





## real-time RT-PCR

Quantification range

50 - ≥ $10^6$ (copies/µL)

LOD

50 (copies/µL)

## dPCR

Quantification range

≤1 - $10^4$ (copies/µL)

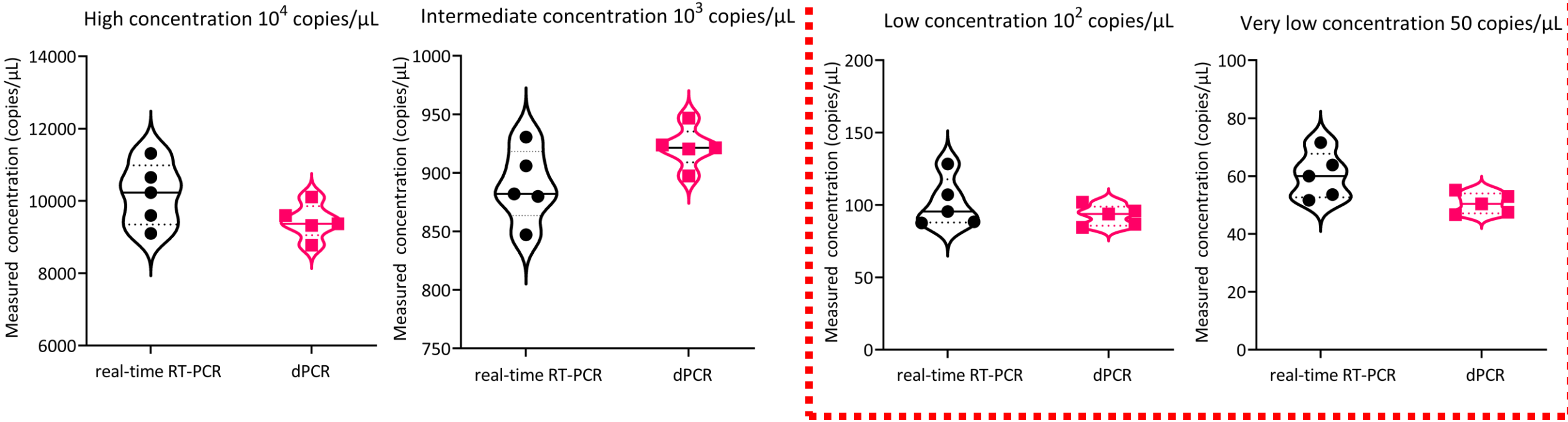
LOD

≤ 1 (copies/µL)

# Viral load categories (copies/µL)


| $10^4$ | $10^3$ | 100 | 50 |
|---|---|---|---|
| High | Intermediate | Low | Very low |

# Assay repeatability; intra-assay precision




Comparison of the repeatability is represented in a violin plot and it shows median values and the 25% and 75% interquartile ranges.

*dPCR demonstrated improved precision at low and very low viral loads*

# Assay specificity

| Agents | real-time RT-PCR | | | dPCR | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| HPAI H5N1 strain A/guinea fowl/Nigeria/KN-GF-14-16C_23VIR1455-1/2022 | Pos | Pos | Pos | Pos | Pos | Pos |
| HPAI H5N5 strain A/European herring gull/Norway/2023-07-2935_23VIR9749-12/2023 | Pos | Pos | Pos | Pos | Pos | Pos |
| HPAI H7N6 strain A/chicken/South Africa/SA2310_23VIR11588-2/2023 | Pos | Pos | Pos | Pos | Pos | Pos |
| LPAI H7N3 strain A/chicken/Netherlands/ 23025569-021_24VIR4103-4/2023 | Pos | Pos | Pos | Pos | Pos | Pos |
| LPAI H9N2 strain A/chicken/Niger/13-23_23VIR3551-49/2023 | Pos | Pos | Pos | Pos | Pos | Pos |
| AAvV-1/chicken/Macedonia/1400-1_20VIR1984-5/2020 virulent strain | Neg | Neg | Neg | Neg | Neg | Neg |
| AOAV-1/Gallus gallus/Belgium/8744/2022 avirulent strain | Neg | Neg | Neg | Neg | Neg | Neg |
| IBV | Neg | Neg | Neg | Neg | Neg | Neg |
| *E. coli* | Neg | Neg | Neg | Neg | Neg | Neg |
| negative extraction control (NEC) | Neg | Neg | Neg | Neg | Neg | Neg |
| no template control (NTC) | Neg | Neg | Neg | Neg | Neg | Neg |
| | | | | | Concordance | 100 |
| | | | | | Cohen's Kappa | 1.0 |

Inclusivity test

cross-reactivity panel

no contamination

*almost perfect agreement*

# Results



Nuclease free $H_2O$

Quantification range

LOD

Repeatability

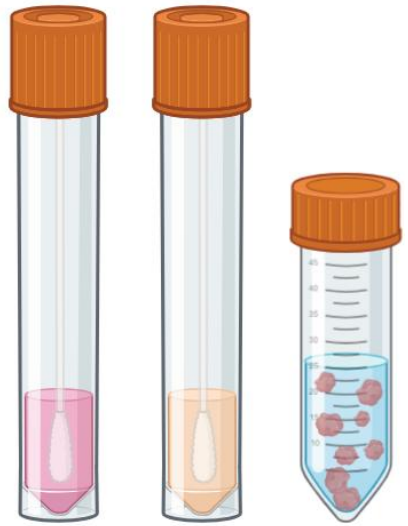
Specificity

**Three matrices**

Viral loads

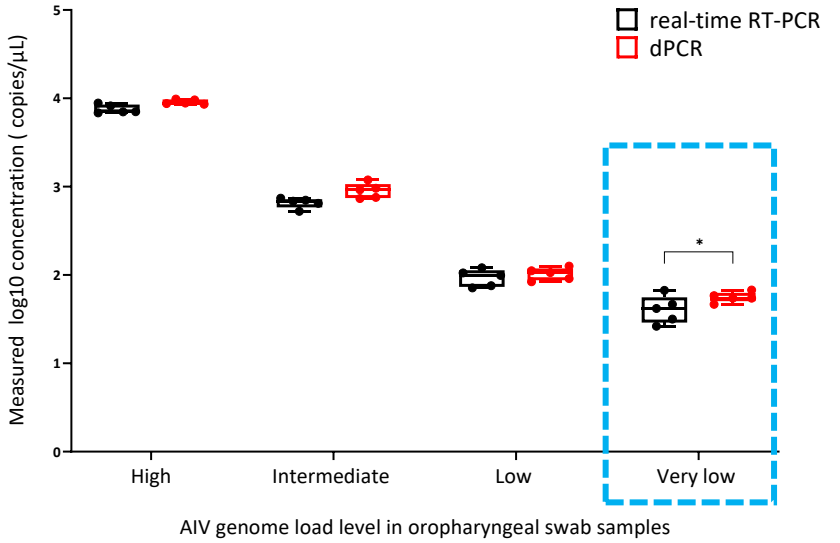
Correlation

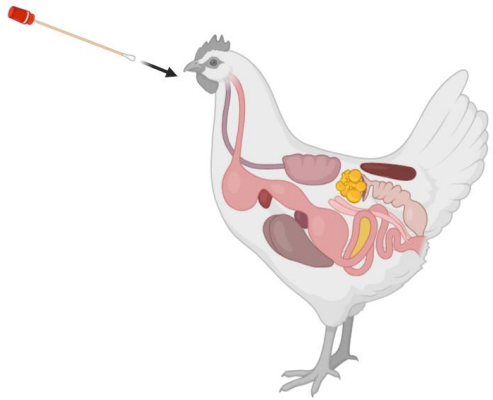
Bland-Altman



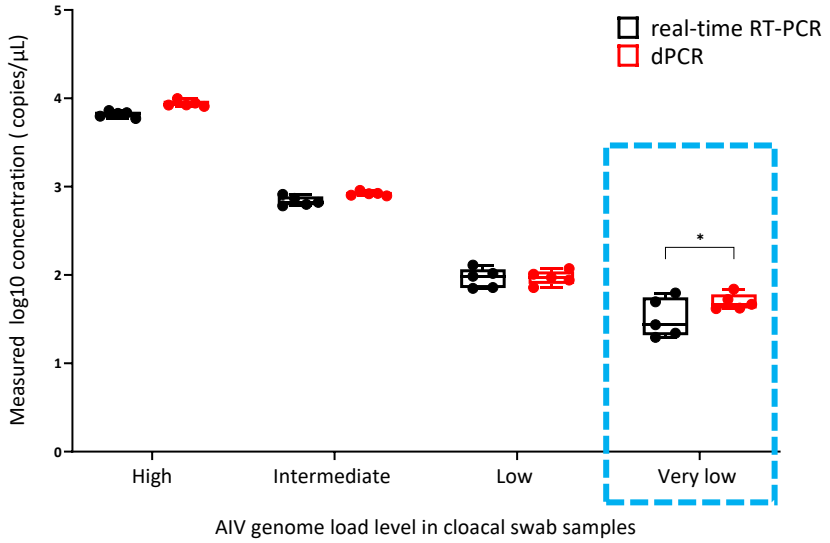
# Viral load in matrices

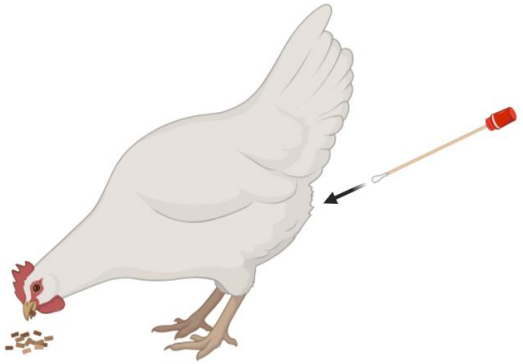
## Oropharyngeal swab



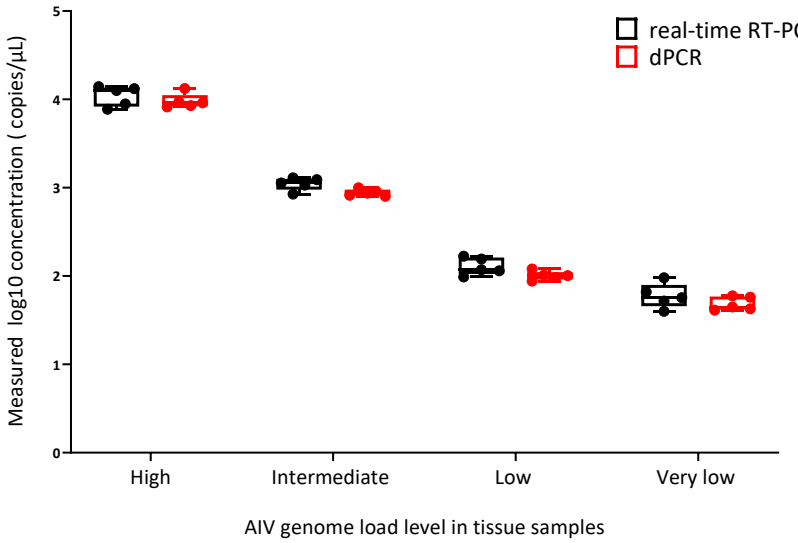



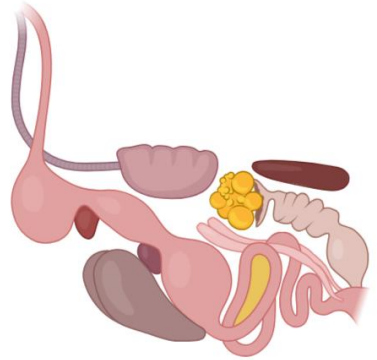
## Cloacal swab



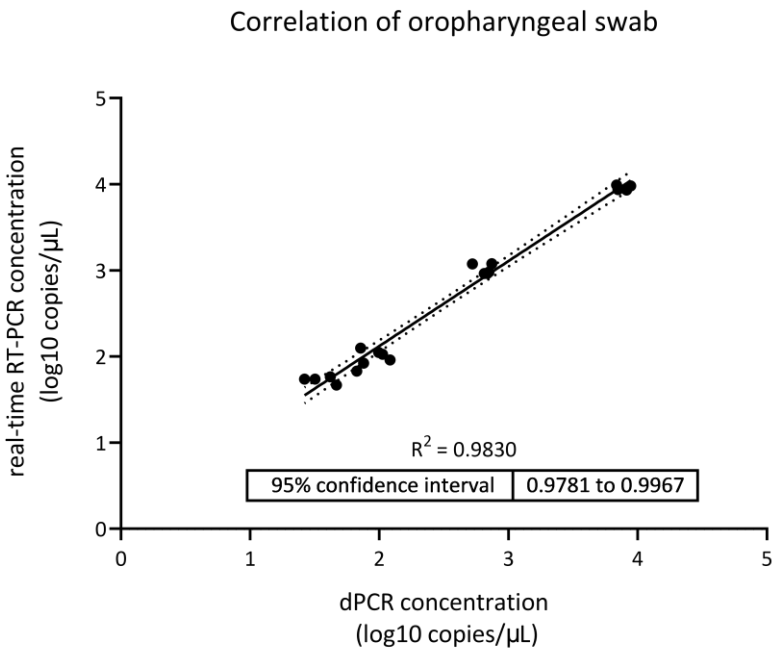
## Tissue

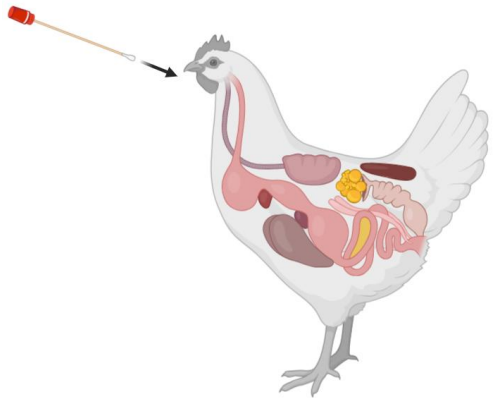


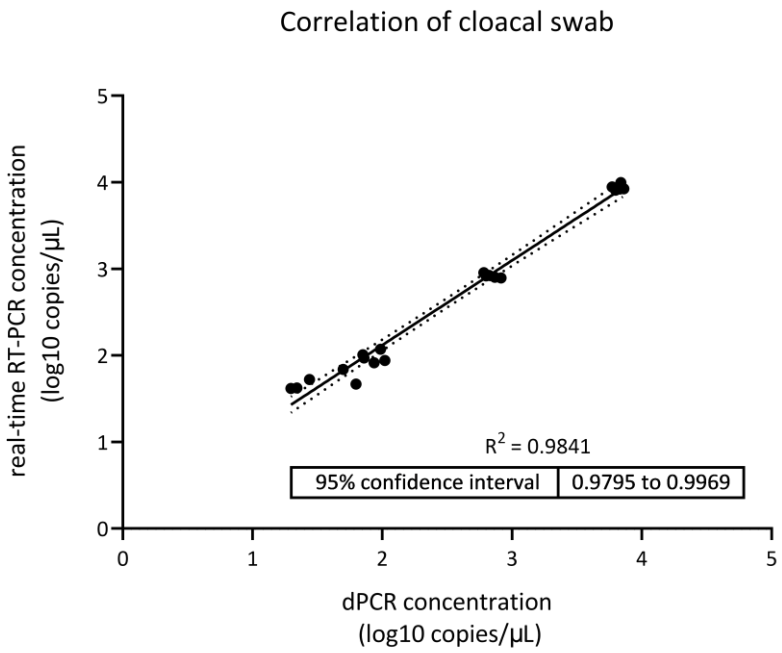
# Correlation analysis in matrices

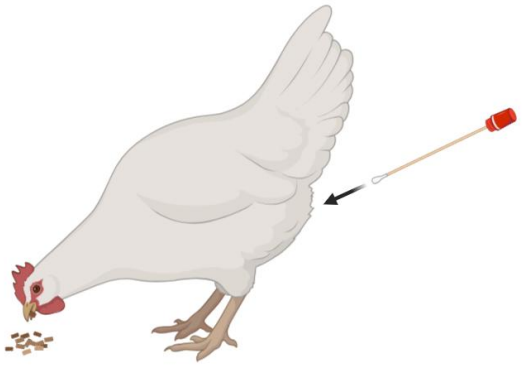
## Oropharyngeal swab



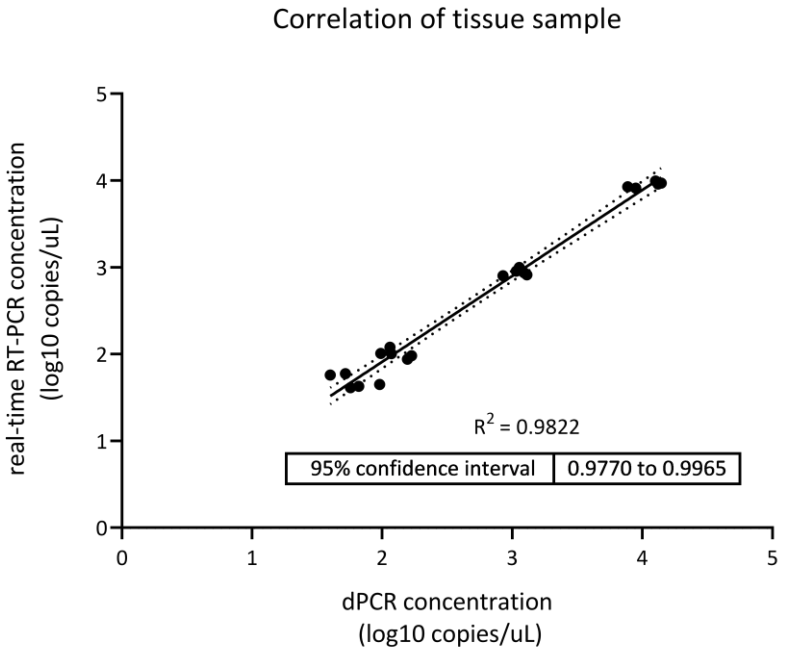



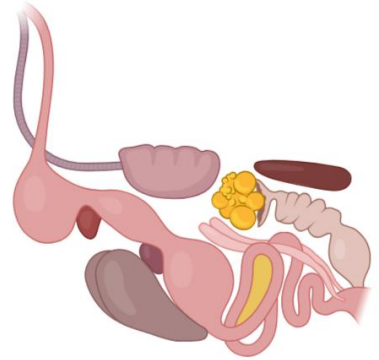
## Cloacal swab



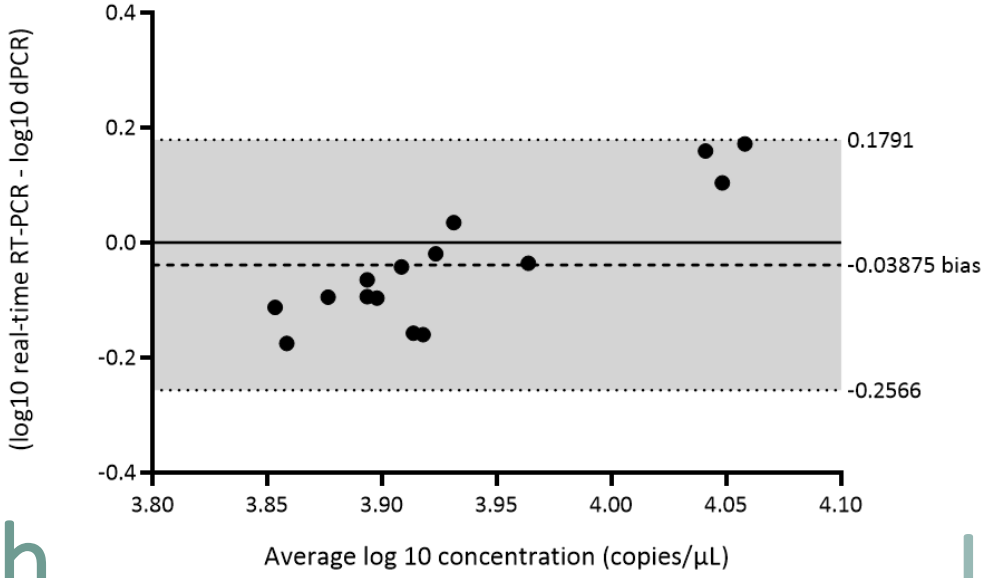
## Tissue



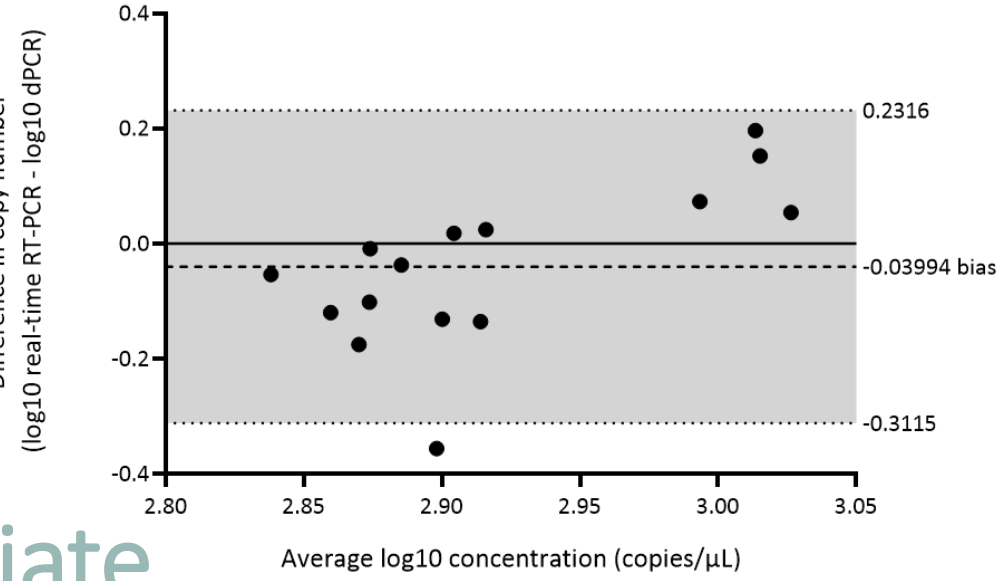
# Bland–Altman analysis


$10^4$



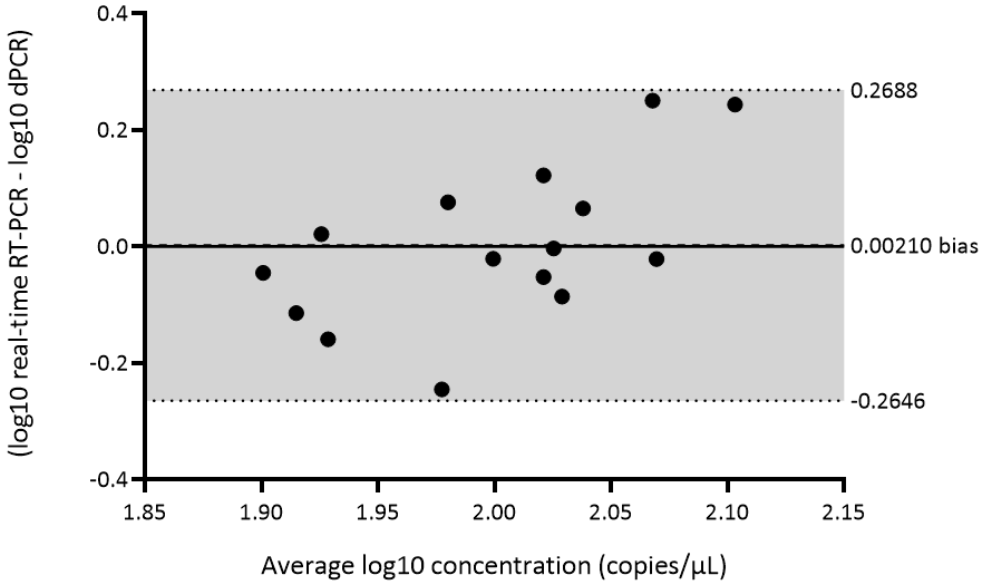

High


$10^3$



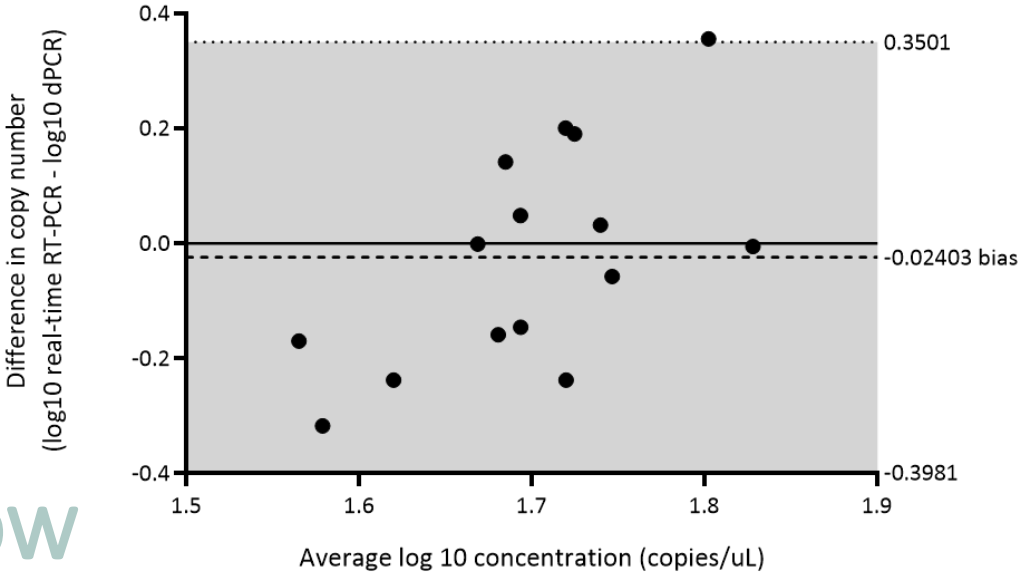

Intermediate


$10^2$




Low


50

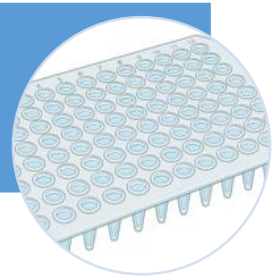



Very low

# Discussions and conclusion

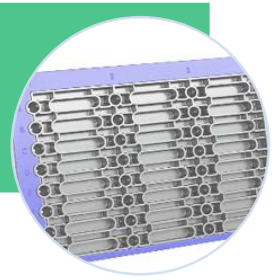
- real-time RT-PCR exhibited a **wider quantification** range, while dPCR was limited at high target concentrations because of partition saturation. dPCR showed **higher sensitivity**.

1



- dPCR performed better in swab samples, likely because it is more **tolerant** of PCR inhibitors, while both methods showed similar performance in tissue samples.

2



- Both methods showed excellent **agreement** and produced **comparable** results within the validated quantification range.

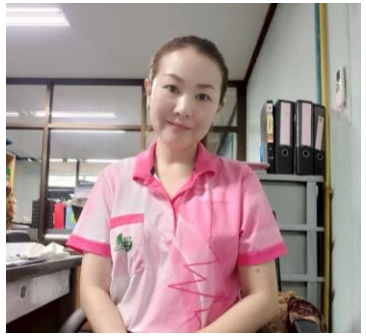
3

# Critiques and future directions

1. This study focused on influenza A genome detection only and **did not evaluate subtype** identification by dPCR.
2. Development of **subtype-specific** or multiplex dPCR assays for HA and NA genes (e.g., H5 and H7) is needed.
3. Future studies should validate these assays using **diverse field samples** to strengthen AIV surveillance and preparedness.

# Acknowledgements

I would like to express my sincere gratitude to my **Director** for continuous support and encouragement throughout this study.



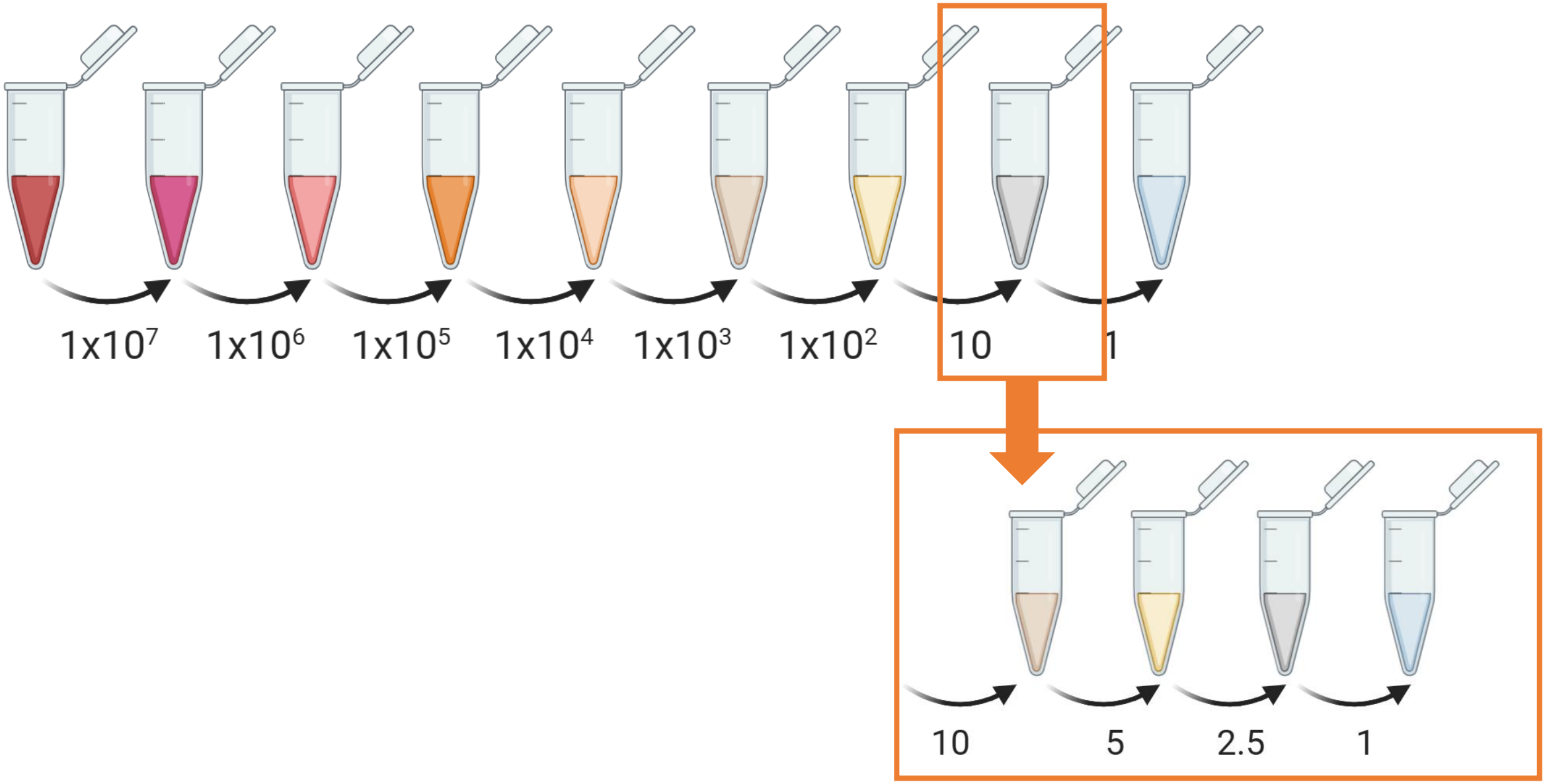
I would also like to thank my **co-authors** for their valuable guidance and contributions.

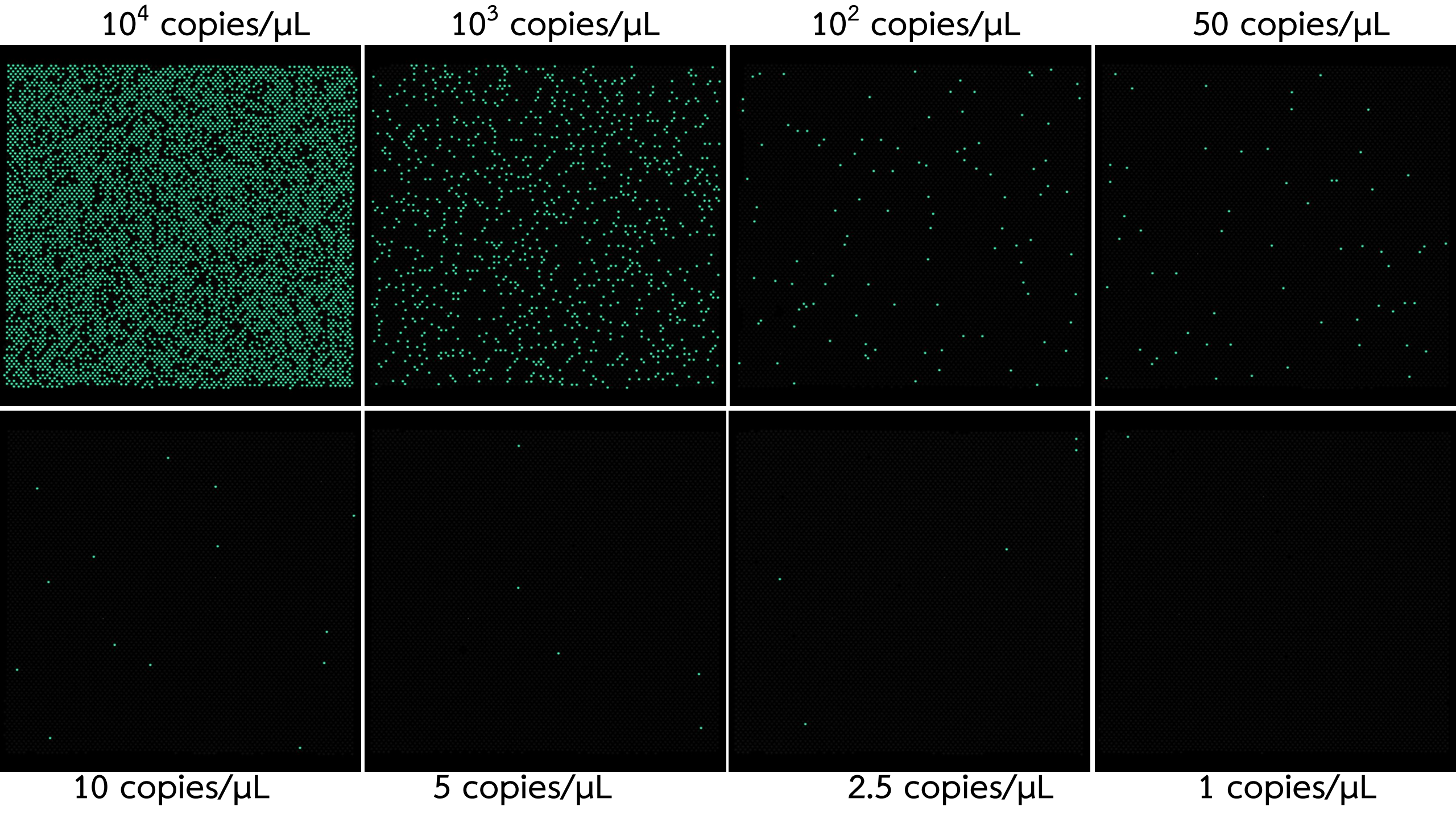
**The Virology Laboratory for their technical assistance, collaboration, and support.**

Thank you for your attention

# Additional data

# 10-fold serial dilutions & 2-fold serial dilutions




$10^4$ copies/µL
$10^3$ copies/µL
$10^2$ copies/µL
50 copies/µL
10 copies/µL
5 copies/µL
2.5 copies/µL
1 copies/µL

# Conclusion

- dPCR provides **highly sensitive** and **precise** detection with **absolute quantification** of AIV genomes.
- dPCR provides greater sensitivity and precision than real-time RT-PCR in **very low viral load** samples.
- dPCR can serve as a valuable **complementary tool** to real-time RT-PCR for **early** diagnosis and surveillance.