Development of a multiplex RT-PCR assay for the detection of influenza A(H5NX)

Mitchell Gell-Freeman, Jade Cogdale, Beatrix Kele, Katja Hoschler

Respiratory Virus Unit, UK Health Security Agency, Colindale, London, NW9 5EQ

INTRODUCTION

The Influenza A(H5) subtype is zoonotic and primarily affects birds but has been known to also infect mammals, including humans. There were two confirmed A(H5N1) human cases in the UK during the 2024-25 winter season. Circulating influenza A(H5NX) subtype viruses demonstrate vast genetic diversity and, the predominant subtype currently circulating worldwide is A(H5N1), clades 2.3.2.1 and 2.3.4.4 (figure 1) (1). In the USA, A(H5N1) clade 2.3.4.4b, B3.13 genotype is responsible for the current outbreak in dairy cattle.

Due to the genetic diversity of influenza A(H5), there is a need for multi-target testing strategy to be used for real-time PCR to ensure viral detection. Previously, two uniplex RT-PCR assays (European (2) and Eurasian (3,4)) were run in parallel as part of a suite of assays to confirm the presence of a A(H5) subtype virus in a clinical sample. The new multiplex assay combines the targets from the uniplex assays with the addition of internal control detection into a singular, independent assay. This streamlines the diagnostic workflow, ensuring the laboratory is better prepared for timely and accurate detection of any human H5 cases. This project has also validated the Roche MP96 extraction platform – a new, high throughput extraction platform, for the extraction of CL3 samples, further strengthening our capability to respond to an outbreak scenario.

Project aim: to develop a multiplex assay which has equivalent or improved diagnostic and analytical sensitivity and specificity to the existing uniplex versions, streamlining laboratory workflows and increasing testing capacity.

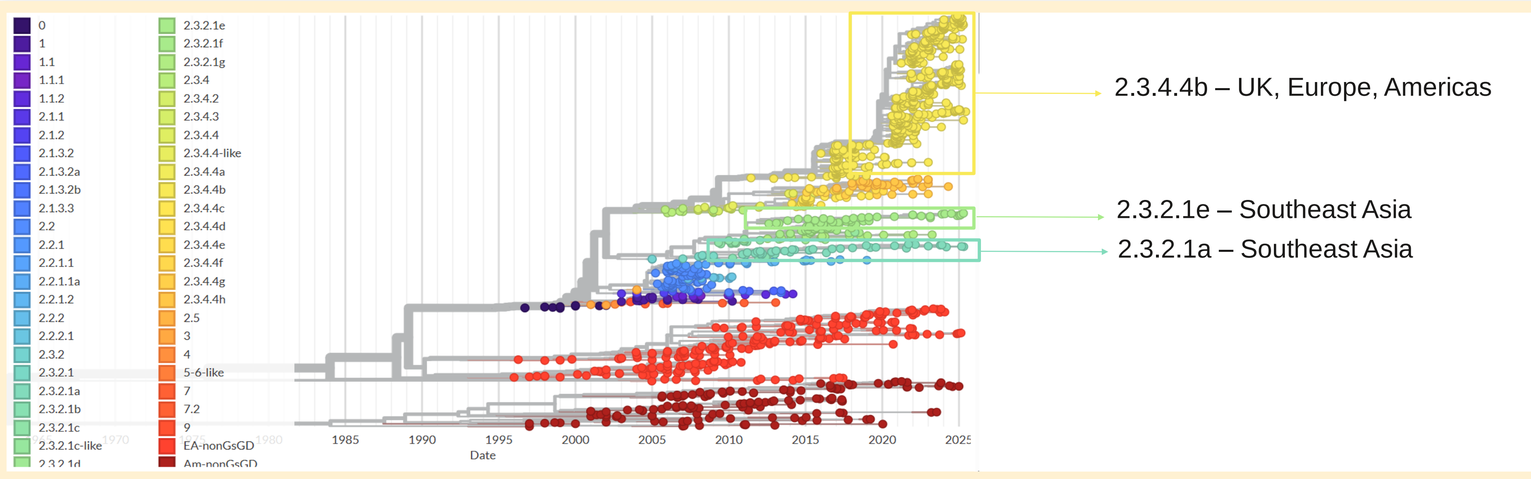

Figure 1. Phylogenetic tree showing genetic diversity of influenza A(H5Nx) by HA clade (https://nextstrain.org/avian-flu/h5nx/ha/all-time?c=h5_label_clade)

METHODS

The assay was optimised and validated using the following steps:

1. An *in-silico* review was conducted to determine the suitability of the existing primer and probe sequences in relation to current circulating strains. The dual target nature accounts for mismatches in one target.

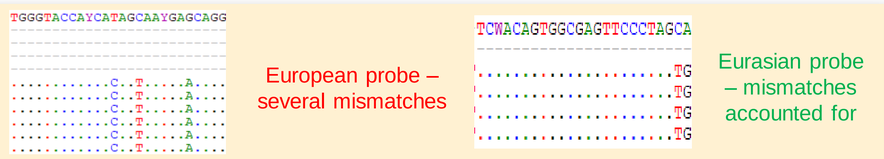

Figure 2. European and Eurasian probe sequence (top sequence) mismatches with circulating 2.3.2.1e clade genomes (shown in conservation plot).

2. Primer and probe concentrations were optimised to determine the best concentrations in the multiplex reaction. Concentrations have been optimised to reduce channel bleed and maximise sensitivity.

Table 1. Dye channels for each probe

x1m1	x2m2	x3m3	x4m4	x5m5	x6m6
FAM	VIC	ABY	ROX	MP	Cy5.5
European		Eurasian	IC	Passive ref	

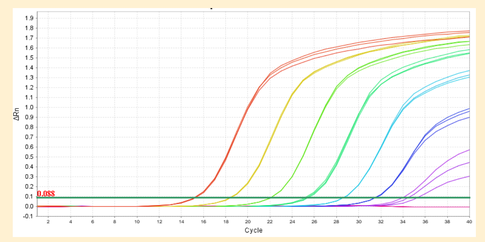

Figure 3. Amplification plot for SBCMV IC when there is H5 Eurasian positive amplification

3. Assay validation was performed using reference viruses, external quality assurance panels (EQAP) and patient samples held in the Respiratory Virus Unit to determine diagnostic and analytical specificity and sensitivity.

RESULTS

Analytical Sensitivity and Specificity

LoD95 was determined using Amplirun Molecular Control (Launch Diagnostics, MBC052) Influenza A(H5N1) RNA IVD control. This was diluted 2-fold and tested in 8 replicates. The data was examined by Probit analysis using RStudio and is summarised in table 3.

Table 1. LoD95 for Amplirun RNA control

H5N1 A/reassortant/NIBRG-14 (Viet Nam/1194/2004 x PR8) clade 1				
	Eurasian		European	
	Uniplex	Multiplex	Uniplex	Multiplex
	Copies/uL	Copies/uL	Copies/uL	Copies/uL
MD	0.83	1.61	6.44	0.23
LoD95	1.97	3.32	15.1	0.48
LCL 95% CI	1.40	2.44	10.8	0.35
UCL 95% CI	4.21	5.94	28.9	1.02

- The Eurasian multiplex target is slightly less sensitive than the uniplex targets, 3.32 vs 1.97 copies/µL
- The European multiplex target is more sensitive than the uniplex, 0.48 vs 15.1 copies/µL

Additional experiments were conducted to determine the LoD95 of the PCR. Two *in-vitro* transcripts (IVT) from the A(H5N1) 2.3.4.4b and 2.3.2.1e clades were diluted 2-fold and tested in 8 replicates, The data was examined by Probit analysis using Rstudio and is summarised in tables 4 and 5.

Table 2. LoD95 for H5N1 2.3.4.4b IVT

H5N1 2.3.4.4b A/chicken/Scotland/054477/2021				
	Eurasian		European	
	Uniplex	Multiplex	Uniplex	Multiplex
	Copies/uL	Copies/uL	Copies/uL	Copies/uL
MD	0.20	0.24	0.24	0.22
LoD95	0.43	0.45	0.65	0.45
LCL 95% CI	0.31	0.34	0.46	0.33
UCL 95% CI	0.78	0.78	1.31	0.81

- The European multiplex target is more sensitive than the uniplex, 0.45 vs 0.65 copies/µL
- The Eurasian multiplex target is comparable to the uniplex, 0.43 vs 0.45 copies/µL

Table 3. LoD95 for H5N1 2.3.2.1e IVT

H5N1 2.3.2.1e A/Cambodia/NPH230032/2023		
	Eurasian	
	Uniplex	Multiplex
	Copies/uL	Copies/uL
MD	0.92	3.63
LoD95	2.46	6.32
LCL 95% CI	1.71	4.84
UCL 95% CI	5.21	10.5

- The European target failed to detect this IVT, which was expected due to mismatches in the target region
- Initial data suggests that the multiplex assay is slightly less sensitive than the uniplex, 6.32 vs 2.46 copies/µl, respectively

Analytical specificity was determined by testing EQA panels and in-house reference viruses. The EQA panels included a range of respiratory viruses such as seasonal and non-seasonal influenza subtypes, RSV subtypes, and coronaviruses:

- PHE proficiency Panel 2021 (containing A/H5N1 clade 2.2.1.1)
- WHO EQAP 2023 (containing A/H5N1 clade 2.3.4.4b)
- WHO EQAP 2024 (containing A/H5N1 clades 2.3.4.4b and 2.3.2.1c)

All H5 samples were correctly subtyped. All negatives and non-H5 influenza viruses were correctly identified as negative in the assay under validation.

Diagnostic Sensitivity and Specificity

Since the first human case of human H5N1 in 2021, there have been 7 detections in England; however, due to the low number of H5N1 detections, diagnostic sensitivity and specificity could not be determined.

Despite this, one 2.3.4.4b clade clinical sample was tested and correctly identified as positive in the new multiplex assay (table 4).

Table 4. 2.3.4.4b clade clinical sample detection in uniplex and multiplex H5 assay

UNIPLEX		MULTIPLEX	
Current H5 Eurasian	Current H5 European	New H5 Eurasian	New H5 European
30.57	34.39	29.94	32.59

As mentioned, diagnostic specificity could not be determined, however, 149 respiratory samples tested were shown to be negative for influenza A(H5NX). The samples consisted of surveillance swabs which were negative or positive for various respiratory viruses, including seasonal influenza, coronaviruses, RSV subtypes, hMPV, and adenoviruses.

Multi-Platform Robustness

Further sensitivity experiments were performed by 10-fold serially diluting multiple subtypes and clades of influenza A(H5) reverse genetic viruses in viral transport medium to emulate a typical respiratory clinical sample matrix and extracted using the BioMerieux eMAG and Roche MagNA Pure 96 (MP96):

- RG8A (A/Astrakhan/3212/2020, H5N8, clade 2.3.4.4b)
- B3.13 (A/Dairy Cattle/Texas/24-008749-001/2024, H5N1, clade 2.3.4.4b, genotype B3.13).

Extracted RNA material was then tested in triplicate on the uniplex and new multiplex assays to determine relative LoD, multi-platform robustness, and PCR efficiency/linearity. The results are summarised in table 6, 7 and 8.

Table 6. Relative LoD for RG8A and B3.13 strains on extraction platforms under validation

Strain	Extract	Eurasian LoD PFU/mL		European LoD PFU/mL	
		Uniplex	Multiplex	Uniplex	Multiplex
RG8A	eMAG	2.90E+01	2.90E+01	2.90E+01	2.90E+01
	MP96	2.90E+01	2.90E+01	2.90E+01	2.90E+01
B3.13	eMAG	1.38E+02	1.38E+02	1.38E+02	1.38E+02
	MP96	1.38E+01	1.38E+02	1.38E+02	1.38E+02

- There is equivalent sensitivity of both targets in the new multiplex assay to validated workflows of the uniplex assays.

Table 7. Multiplatform robustness between extraction platforms under validation

Strain	Target	eMAG	MP96	Multi-platform CV% range
		CV% range	CV% range	
RG8A	Eurasian	0.1% - 1.1%	0.02% - 1.6%	0.5% - 3.3%
	European	0.1% - 1.9%	0.1% - 0.9%	1.1% - 3.5%
B3.13	Eurasian	0.3% - 5.0%	0.05% - 3.4%	1.1% - 5.0%
	European	0.2% - 1.0%	0.1% - 0.9%	1.8% - 2.7%

- All inter-extraction platforms CVs are in the acceptable range of <5%, demonstrating multi-platform robustness.

Table 8. PCR Linearity and Efficiency for eMAG and MP96 extraction platforms

Strain	Target	BioMérieux eMAG			Roche MP96 - LV Kit		
		Slope	R^2	% efficiency	Slope	R^2	% efficiency
RG8A	Eurasian	-3.435	0.999	95.5%	-3.227	0.999	104.1%
	European	-3.475	0.998	94.0%	-3.380	0.999	97.6%
B3.13	Eurasian	-3.228	0.995	104.1%	-3.317	0.994	100.2%
	European	-3.384	1	97.5%	-3.527	0.999	92.1%

- All PCR efficiencies lie within the acceptable range of 90-110% and R^2 values are all >0.99. This shows that the multiplex PCR has acceptable linearity and efficiency for both extraction methods

DISCUSSION

- LoD95 for the European and Eurasian targets show minor differences in the new multiplex assay compared to the uniplex assay; however, this is accounted for by the dual target approach. The multiplex LoD95 lies within the acceptable range.
- The European primer and probe set failed to detect clade 2.3.2.1e which was expected due to the multiple mismatches in the target sequence. Clade 2.3.2.1e strain was detected by the Eurasian primer and probe set, which reiterates the need for a multiple target testing strategy
- There is equivalent sensitivity, acceptable CV ranges, PCR efficiencies and R^2 values between extraction platforms, demonstrating multiplatform robustness.

CONCLUSIONS

- The multiplex assay has demonstrated equivalent sensitivity compared to previous uniplex assay workflow against contemporary strains
- This assay is fit for purpose, it can specifically detect target sequences with acceptable sensitivity, specificity, linearity, efficiency and precision.
- The MP96 has been validated for RNA/Total NA extraction of CL3 samples, increasing testing capacity.
- Currently, there is no 2.3.2.1a clinical material available in the UK, meaning the assay has not been validated against this clade circulating in India/Bangladesh. Further validation work will be performed when clinical material becomes available.

REFERENCES

1. Joint Food and Agriculture Organisation of the United Nations, World Health Organisation, World Organisation for Animal Health. Updated joint FAO/WHO/WOAH public health assessment of recent influenza A(H5) virus events in animals and people. https://www.woah.org/app/uploads/2025/04/2025-04-17-fao-woah-who-h5n1-assessment.pdf
2. Yves Thomas (National Centre for Influenza, Central Laboratory of Virology, University Hospital of Geneva, Switzerland), and the probe sequence amended subsequently by Terry Collins (NHS Greater Glasgow. North Glasgow University Hospitals Division, West of Scotland Specialist Virology Centre). Additional modifications to both primers and probe sequences were made by Jade Cogdale RVU, UKHSA in 2023
3. Slomka et al. AVIAN DISEASES 51:373-377, 2007.
4. Spackman et al. JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2002, p. 3256-3260. Vol 40, No. 9