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Multiplex Dual-Target Reverse Transcription PCR for Subtyping Avian Influenza A(H5) Virus

Appendix

Methods

We performed reverse transcription quantitative PCR (RT-qPCR) by using the Invitrogen Superscript III Platinum One-Step qRT-PCR kit (Thermo Fisher Scientific, <https://www.thermofisher.com>) on the Rotor-Gene Q real-time PCR instrument (QIAGEN, <https://www.qiagen.com>). Each 25- μ L reaction contained 12.5 μ L of 2X buffer, 0.5 μ L of enzyme mix, 2 μ L of primer–probe mix, and 10 μ L of nucleic acids. Cycling conditions were as follows: hold at 52°C for 15 min, 94°C for 2 min, then 45 cycles of 94°C for 15 sec, 55°C for 40 sec, and 68°C for 20 sec. We analyzed the fluorescence signal in the green (H5), crimson (pan-influenza A), and yellow (RNase P) channels. We set thresholds at 0.1 normalized fluorescence units for all targets. We interpreted any exponential amplification curve crossing the green channel as subtype H5. The 95% lower limit of detection was determined in R (<https://www.r-project.org>) by using probit regression.

Appendix Table 1. Original Influenza A(H5) Primer and Probe Sequences

Name	Sequence (5'→3')	Source	Notes
H5.2344-1673F	TACCAAATAYTGTCAATTTATTCAAC	School of Public Health, Hong Kong University, Hong Kong SAR, China	A (H5) clade 2.3.4.4
H5.2344-1749R	GTAAYGACCCRTTRGARCACATCC		
H5.2344-1718Prb FAM	CTGGCAATCAT DRT GGCTGGTCT		
H5-1012F	TGGGTACCACCATAGCAATGAGCA	Centre for Health Protection, Hong Kong SAR, China	A (H5)
H5-1155R	AATCCCTTCCAACGGCCTCAAAC		
H5-1042P-P2_CFO560	TGGGTACGCTGCAGACAAAGAATCCA		

SAR, Special Administrative Region. Mixed bases are in bold

Appendix Table 2. Alignment of Primer/Probe Set 1 to North American Influenza A(H5) Hemagglutinin (HA) Sequences available from GISAID January 1, 2022 to May 29, 2024.

Reference	Forward Primer	Probe	Reverse Primer*	No.	%
Set 1	TACCAGATACTGTCAATTTATTCAAC	CTGGCAATCATG TR RGCTGGTCT	GGATGTGCTCCAATGGGTCGTTAC	5529	92.4
C.....	82	1.4
T.....	58	1.0
G.....G.....	44	0.7
T.....	39	0.7
A.....	36	0.6
T.....	25	0.4
A.....	24	0.4
A.....	21	0.4
T.....	13	0.2
A.....	11	0.2
T.....	8	0.1
A.....	8	0.1
C.....	8	0.1
G.....	7	0.1
G.....C.....	6	0.1
G.....	6	0.1
T.....	5	0.1
T.....	5	0.1
	4	0.1
T.....	4	0.1
A.....	4	0.1
T.....	4	0.1
	T.....	4	0.1
T.....T.....	4	0.1
	3	0.1

*The reverse primer sequence is presented as its reverse complement.

Sequences accessed May 29, 2024. Aligned sequences: 5987. GenBank: PP577943/GISAID: EPI3171488 (A/Texas/37/2024) is used as reference.

Primer and probe alignments are in-phase. Top 25 most abundant sequences are listed. Mixed bases are in bold.

Appendix Table 3. Alignment of Primer/Probe Set 2 to North American Influenza A(H5) Hemagglutinin (HA) Sequences available from GISAID January 1, 2022 to May 29, 2024.

Reference	Forward Primer	Probe	Reverse Primer*	No.	%
Set 2	TGGGTACCATCATAGCAATGAGCA	TGGGTACGCTGCGGACAAAGAATCCA	TTTGAGGCAGTTGGAAGGGAGTT	5085	84.9
	209	3.5
T.....A.....	93	1.6
	..A.....	74	1.2
T.....	48	0.8
C.....C.....	45	0.8
A.....	43	0.7
T.....	41	0.7
A.....	32	0.5
T.....	26	0.4
A.....	17	0.3
T.....G.....	15	0.3
	..T.....	15	0.3
T.....	14	0.2
A.....	13	0.2
C.....	12	0.2
T.....	12	0.2
A.....G.....	10	0.2
C.....	9	0.2
A.....	9	0.2
A..	9	0.2
T.....	8	0.1
G.....	8	0.1
A..	8	0.1
G.....	8	0.1

*The reverse primer sequence is presented as its reverse complement.
 Sequences accessed May 29, 2024. Aligned sequences: 5990. GenBank: PP577943/GISAID: EPI3171488 (A/Texas/37/2024) is used as reference.
 Primer and probe alignments are in-phase. Top 25 most abundant sequences are listed.

Appendix Table 4. Alignment of Primer/Probe Set 1 to Avian Influenza A(H5) Sequences from Human Cases and Dairy Cows.

Reference	Forward Primer	Probe	Reverse Primer*
Set 1	TACCAGATACTGTCAATTTATTCAAC	CTGGCAATCATGATGGCTGGTCT	GGATGTGCTCCAATGGGTCGTTAC
Human: A/Texas/37/2024
Human: A/Michigan/90/2024
Human: A/Michigan/91/2024
Human: A/Colorado/18/2022
A/dairy cow/Texas/24_009367-003/2024G.....
A/dairy cow/Texas/24_009367-005/2024G.....
A/dairy cow/Texas/24_009367-009/2024G.....
A/dairy cow/Texas/24_009367-010/2024G.....
A/dairy cow/Texas/24_009367-013/2024G.....
A/dairy cow/Texas/24_009367-014/2024G.....
Other 190 sequences from dairy cows

*The reverse primer sequence is presented as its reverse complement.

GenBank: PP577943/GISAID: EPI3171488 (A/Texas/37/2024) is used as reference. Mixed bases are in bold. Primer and probe alignments are in-phase.

Note: The 6 dairy cow sequences showing a mismatch with primer/probe set 1 do not contain any mismatches with primer/probe set 2.

Appendix Table 5. Alignment of Primer/Probe Set 2 to Avian Influenza A(H5) Sequences from Human Cases and Dairy Cows.

Reference	Forward Primer	Probe	Reverse Primer*
Set 2	TGGGTACCATCATAGCAATGAGCA	TGGGTACGCTGCCGACAAAGAATCCA	TTTGAGGCAGTTGGAAGGGAGTT
Human: A/Texas/37/2024
Human: A/Michigan/90/2024
Human: A/Michigan/91/2024
Human: A/Colorado/18/2022
A/dairy cow/Texas/24_009110-003/2024A.....
A/dairy cow/South Dakota/24_010354-001/2024A.....
A/dairy cow/Texas/24_009776-002/2024T.....
Other 193 sequences from dairy cows

*The reverse primer sequence is presented as its reverse complement.

GenBank: PP577943/GISAID: EPI3171488 (A/Texas/37/2024) is used as reference. Primer and probe alignments are in-phase.

Note: The 3 dairy cow sequences showing a mismatch with primer/probe set 2 do not contain any mismatches with primer/probe set 1.

Appendix Table 6. Single-Stranded DNA Sequences

Name	H5 Target Sequence (5'→3')	GenBank Reference	Location
ssD_FluA_H5_T1C1	GTATGACTACCCGCAGTATTCAGAAGAAGCAAGACTAAAAAGAGAGGAAATAAGTGGAGTAAAATTGGAATCAATAGGAACT <u>TACCAA</u> AATACTGTCAATTTATTCTACAGTGGCGAGTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCTTTATGGATG TGCTCCAATGGGTCGTTACAATGCAGAAAT	JQ966928	1534–1725
ssD_FluA_H5_T2C1	ATGGTTGGTATGGGTACCACCATAGCAATGAACAGGGGAGTGGGTACGCTGCAGACAAAGAATCCACTCAAAGGCAAT AGATGGAGTACCAATAAGGTCAACTCGATCATTGACAAAATGAACACTCAGTTT <u>GAGGCCGTTGGAAGGGA</u> TTTAATA ACTTA	JQ966928:	1122–1285
ssD_FluA_H5_T1C2	AGTAGGAACTTACCAGATACTGTCAATTTATTCAACAGCGGCAAGTCCCTAGCACTGGCAATCATGATGGCTGGTCTAT CTTTATGGATGTGCTCCAATGGGTCGTTACAGTGCAGAAAT	OP499866	1591–1710
ssD_FluA_H5_T2C2	ATGGTTGGTATGGGTACCATCATAGCAATGAGCAGGGGAGTGGGTACGCTGCGGACAAAGAATCCACCCAAAAGGCAAT AGATGGAGTTACCAATAAGGTCAACTCAATCATTGACAAAATGAACACTCAATTTGAGGCAGTTGGAAGGGAGTTAATAA CTTA	OP499866	1107–1270

Primer and probe targets are underlined. Mismatched bases are in bold. Single-stranded DNA was synthesized by Integrated DNA Technologies.

Appendix Table 7. Alignment of Primer/Probe Set 1 to Avian Influenza A (H5) American-non Goose GuangDong Sequences

Reference	Forward Primer	Probe	Reverse Primer*
Set 1	TACCAGATACTGTCAATTTATTCAAC	CTGGCAATCATGATGGCTGGTCT	GGATGTGCTCCAATGGGTCGTTAC
A/mallard/Minnesota/16–041335–3/2016 (H5N2)	. . T T . A C . . CG A T A . . G .
A/emperor goose/Alaska/17–004479–1/2016 (H5N2)	. . T A C . . C A A A . . G .
A/glaucous-winged gull/Alaska/16–041335–19/2016 (H5N2)	. . T A C A A A . . G .
A/northern pintail/Alaska/16–041335–5/2016 (H5N2)	. . T A C A A A . . G .

*The reverse primer sequence is presented as its reverse complement.

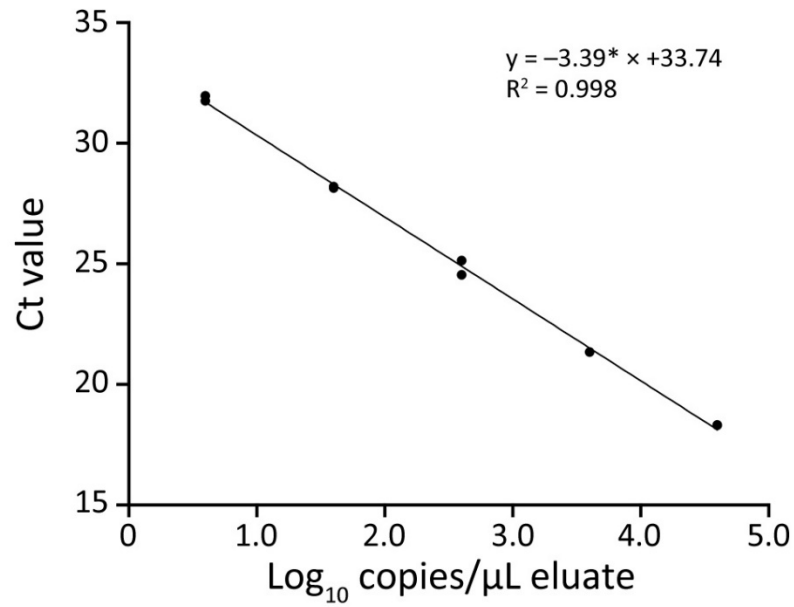
GenBank: PP577943/GISAID: EPI3171488 (A/Texas/37/2024) is used as reference. Mixed bases are in bold.

Appendix Table 8. Alignment of Primer/Probe Set 2 to Avian Influenza A(H5) American-non Goose GuangDong Sequences

Reference	Forward Primer	Probe	Reverse Primer*
Set 2	TGGGTACCATCATAGCAATGAGCA	TGGGTACGCTGCGGACAAAGAATCCA	TTTGAGGCAGTTGGAAGGGAGTT
A/mallard/Minnesota/16–041335–3/2016 (H5N2)	C A . . T A T .	. . C . . A . . T G . AA . . A . .
A/emperor goose/Alaska/17–004479–1/2016 (H5N2)	C . . A A . . T A T .	. . C . . A . . T AA . . A . .
A/glaucous-winged gull/Alaska/16–041335–19/2016 (H5N2)	C . . A A . . T A T .	. . C . . A . . C G . AA . . A . .
A/northern pintail/Alaska/16–041335–5/2016 (H5N2)	C . . A A . . T A T .	. . C . . A . . C G . AA . . A . .

*The reverse primer sequence is presented as its reverse complement.

GenBank: PP577943/GISAID: EPI3171488 (A/Texas/37/2024) is used as reference. Primer and probe alignments are in-phase.



Appendix Figure. Ten-fold serial dilutions of Kilbourne F181, A/duck/Singapore/645/1997 (H5N3) (BEI Resources Catalog # NR-9682) genomic RNA from 4.6 to 0.6 log₁₀ copies/mL were tested in duplicate using the H5 subtyping multiplex RT-qPCR. Filled circles, individual replicates. Solid line, simple linear regression (GraphPad Prism 10 for macOS, version 10.2.3).