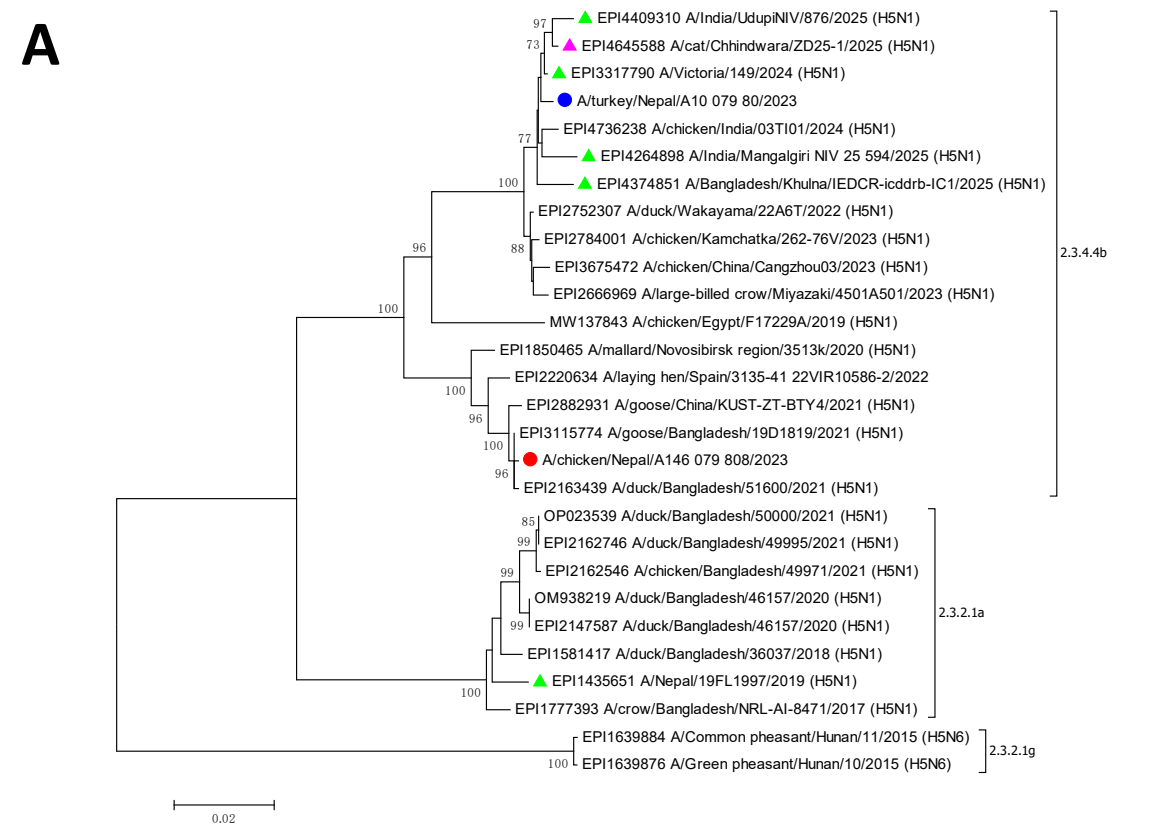
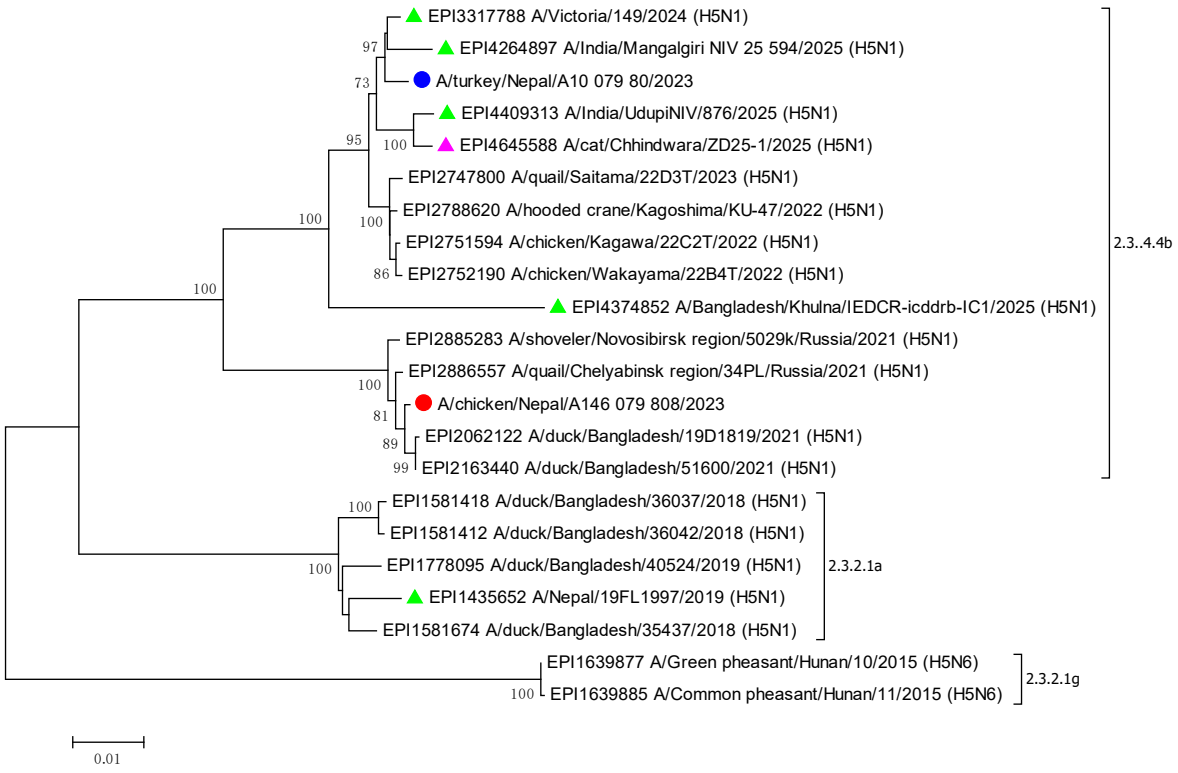
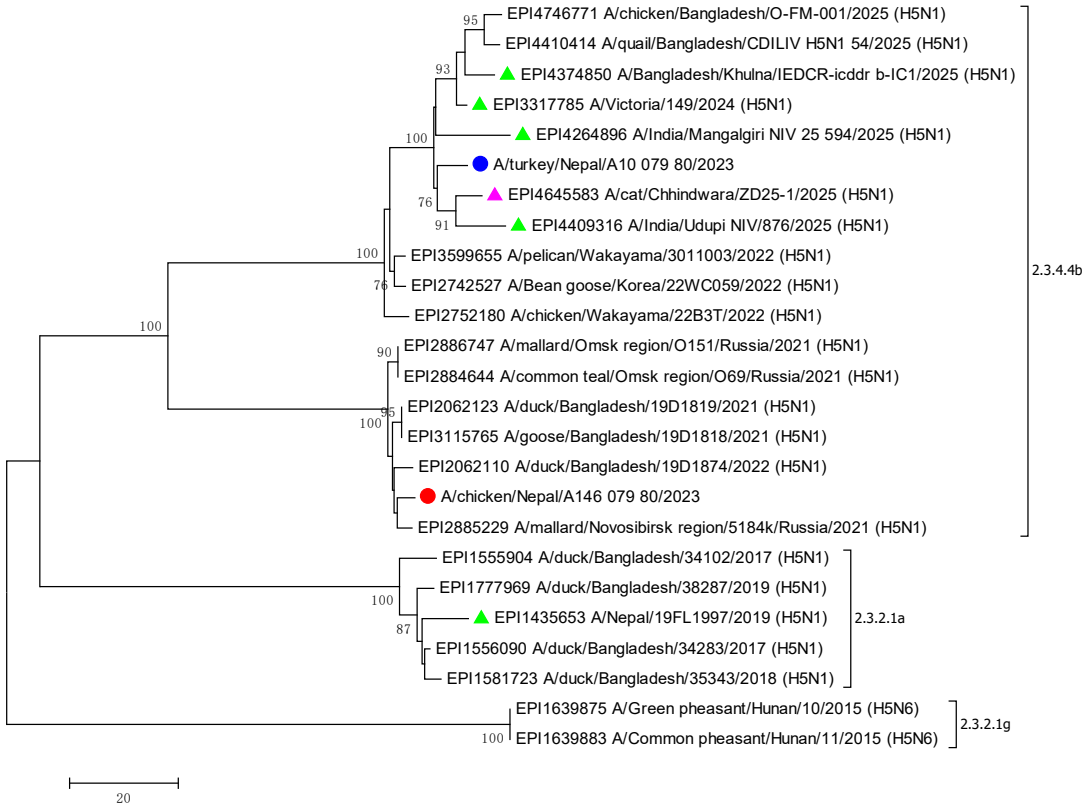
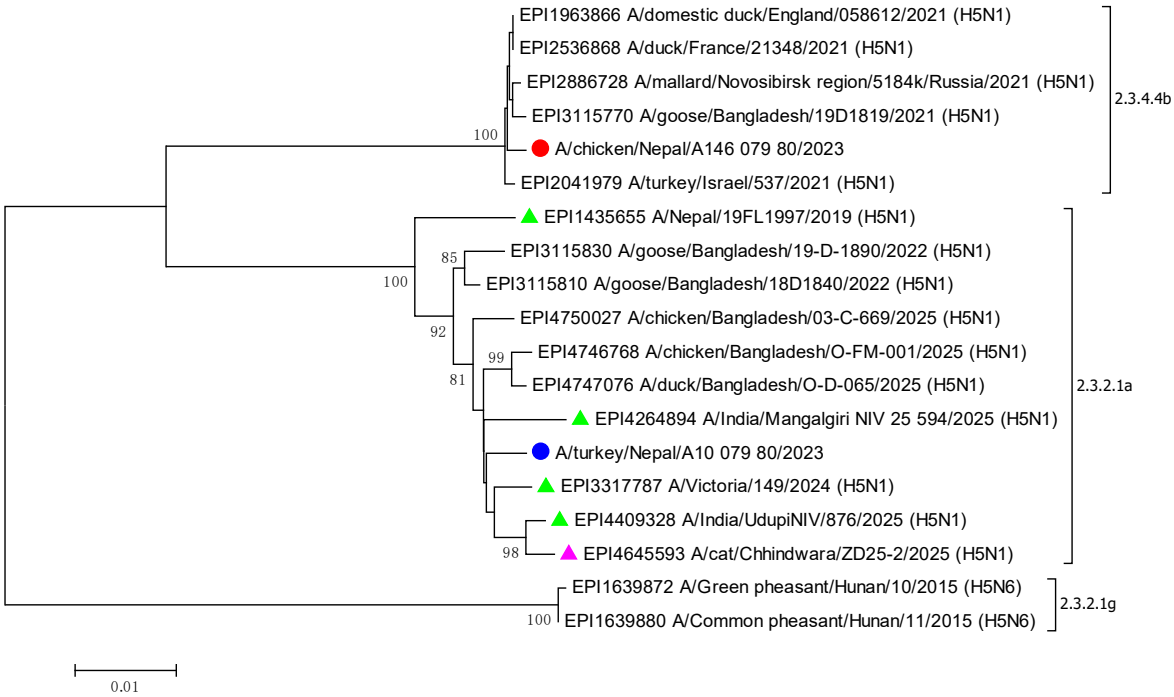

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2.3.2.1a
2.3.2.1g
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2.3.2.1a
2.3.2.1g
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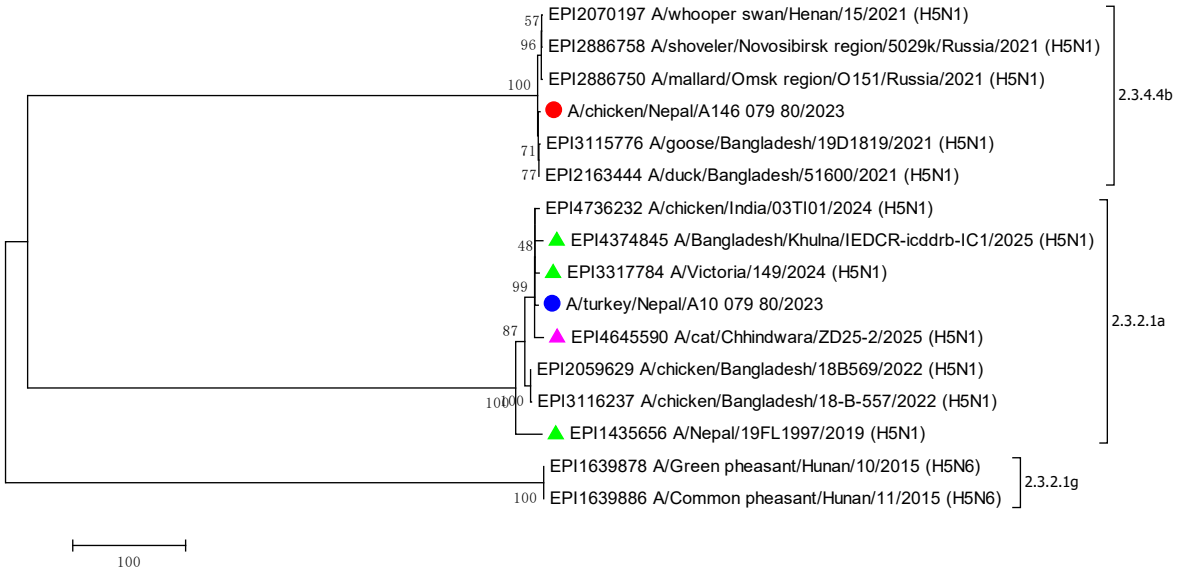
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EPI1581723 A/duck/Bangladesh/35343/2018 (H5N1)
EPI1639875 A/Green pheasant/Hunan/10/2015 (H5N6)
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2.3.2.1a
2.3.2.1g
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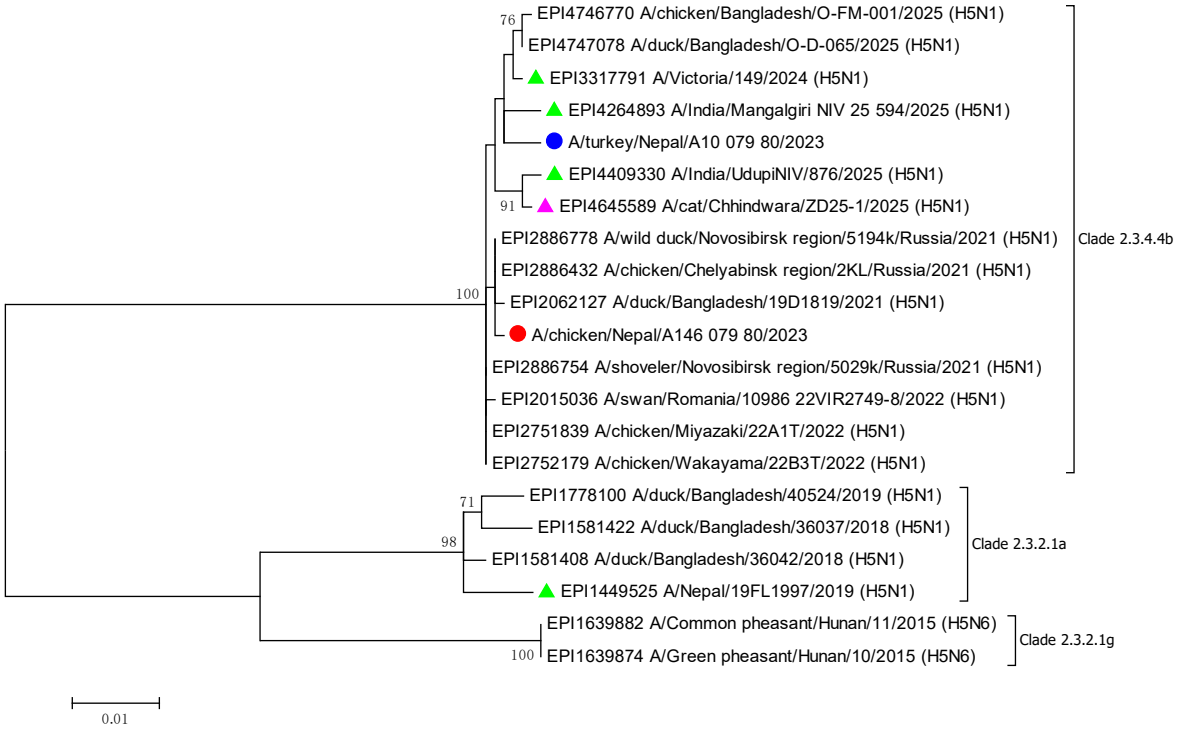
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EPI3317787 A/Victoria/149/2024 (H5N1)
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2.3.2.1a
2.3.2.1g
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E


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EPI3317784 A/Victoria/149/2024 (H5N1)
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EPI4645590 A/cat/Chhindwara/ZD25-2/2025 (H5N1)
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EPI3116237 A/chicken/Bangladesh/18-B-557/2022 (H5N1)
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2.3.2.1g
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96
100
71
77
48
99
87
100
100
100
100

F


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EPI4264893 A/India/Mangalgiri NIV 25 594/2025 (H5N1)
A/turkey/Nepal/A10 079 80/2023
EPI4409330 A/India/UdupiNIV/876/2025 (H5N1)
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EPI2886432 A/chicken/Chelyabinsk region/2KL/Russia/2021 (H5N1)
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A/chicken/Nepal/A146 079 80/2023
EPI2886754 A/shoveler/Novosibirsk region/5029k/Russia/2021 (H5N1)
EPI2015036 A/swan/Romania/10986 22VIR2749-8/2022 (H5N1)
EPI2751839 A/chicken/Miyazaki/22A1T/2022 (H5N1)
EPI2752179 A/chicken/Wakayama/22B3T/2022 (H5N1)
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EPI1581408 A/duck/Bangladesh/36042/2018 (H5N1)
EPI1449525 A/Nepal/19FL1997/2019 (H5N1)
Clade 2.3.2.1a
EPI1639882 A/Common pheasant/Hunan/11/2015 (H5N6)
EPI1639874 A/Green pheasant/Hunan/10/2015 (H5N6)
Clade 2.3.2.1g
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91
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71
98
100
0.01

G

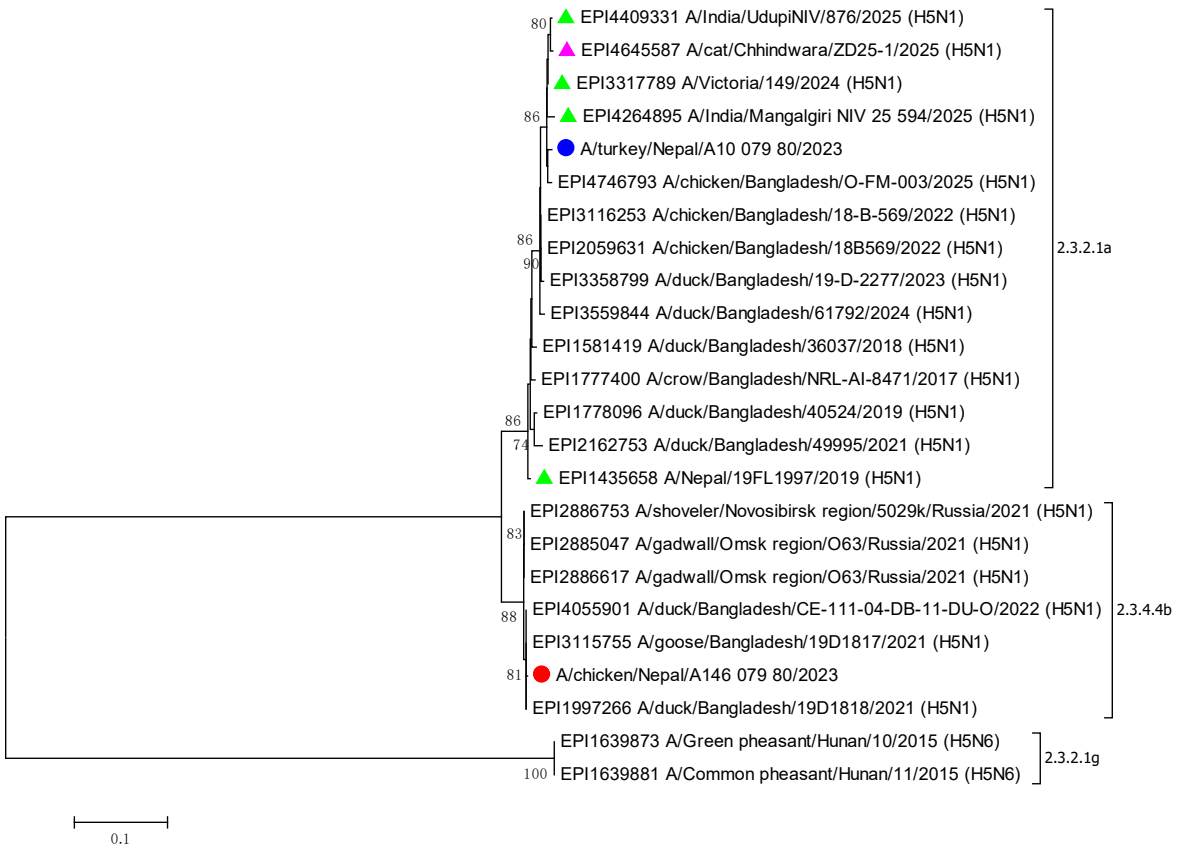


Figure S1 Maximum-likelihood phylogenetic tree of a (**A**) PB2 (**B**) PB1, (**C**) PA, (**D**) NP, (**E**) NA, (**F**) M and (**G**) NS segments. The two samples from this study are highlighted with a red and blue dot. Human and feline samples are shown with green and pink triangles respectively. Clades are identified and bootstrap values >70 are shown.

Supplementary File S1: Avian influenza virus subtype N1 protocol

Avian Influenza Virus Subtype N1 Quick Reference Sheet

General Information			
Taxonomy	Genus *influenza A*, family *Orthomyxoviridae*		
Genome	Segmented negative sense single stranded RNA		
Host species	Avian		
Reference	Wang et al unpublished (In-house AAHL AI N1 assay)		
CAUTION!!	**Zoonosis. Potential Human pathogen**		
Suitable sample	Tracheal and cloacal swabs or faeces, tissue homogenates, pools of organs, allantoic fluid or others as deemed appropriate.		
Real-Time TaqMan PCR Assays			
Gene target	NA gene of avian influenza virus. This assay also detects pandemic H1N1 (2009) influenza A virus.		
Recommendations for use	Is not intended as a primary detection assay. Can be used as a follow-up assay after confirmation as positive in Matrix and H5 PCR assay systems. Positive, uncharacteristic and late cycle curve amplification needs to be followed up with nucleotide sequence.		
AI N1 Assay	**AI N1 PP Mix** Assay is provided as 80uL liquid. **Add 720uL RNase free water, Use 1.5uL per reaction**		
Primer and probe details	**Name**	**Sequence**	**FINAL Concentration**
	AI N1 1316Fm Fwd:	5'- GYGGGAGCAGCMTWTCYTT-3'	800nM
	AI N1 1379R Rev:	5'-CCGTCTGGCCAAGACCAA-3'	800nM
	AI N1 1336Pm Probe:	5'- FAM-TGTGGTGTAAAYAGTGAMAC-MGB-3'	200nM
Thermal cycler program	1 x 45°C 10 min, 95°C 10 min 45 x 95°C 15 sec, 60°C 45 sec	**These are standard AgPath amplification conditions. If other PCR reagents are used these conditions will need to be modified according to the manufacturers guidelines.**	
Positive controls	H5N1 strong and weak positive extraction controls		
Result Analysis and Interpretation			
Threshold setting	0.1 (ABI 7500)		
Interpretation	Positive: Ct less than 40; Having a characteristic amplification curve Negative: Ct greater than 45; Ct undetectable; No characteristic amplification curve Indeterminate: Ct between 40 and 45		

Influenza A N1 RT-PCR Master Mix Sheet (AI N1)

Samples:_____ Lab Code:_____

Extraction Operator: _____ Extraction Date: _____

Extraction Kit used:_____ Extraction Kit Batch:_____

PCR Operator: _____ PCR date:_____

REACTION COMPONENT	VOLUME PER REACTION (µL)	VOLUME FOR ..... REACTIONS (µL)
Nuclease Free water	0.4	
2X AgPath RT-PCR Buffer (Ambion P/N AM1005) Lot #:	7.5	
25X RT PCR Enzyme Mix	0.6	
AI N1 PP Mix Batch #:	1.5	
Total volume	**10.0**	
Template RNA	5.0	

AgPath Cycling Parameters			
1X	45°C 10 min, 95°C 10 min	Standard AgPath amplification conditions. Note cycling parameters may differ when using different kits	Reporter Dye: FAM Quencher: MGB Set Quencher as **MGB**
45X	95°C 15 sec, 60°C 45 sec		

Quality Control				
Control Description: Strong/Weak/NTC/ Extraction Pos or Neg	Batch #	Expected Result (e.g. Ct =29-32, Negative)	Actual result	QC pass/fail

Table S1 Mutations identified in the genomic segments of A/chicken/Nepal/A146_079_808/2023 (**A**), A/turkey/Nepal/A10_079_80/2023 (**B**), and A/Victoria/149/2024 (**C**) according to the FluServer tool available in GISAID. Mutations discussed in the main text are highlighted in red while differences between A/turkey/Nepal/A10_079_80/2023 and A/Victoria/149/2024 are highlighted in yellow.

Virus	Segment	List of mutations
A	PB2	N102T, V292I, V356I, M444V
B	PB2	I67V, N82S, K116R, V139I, V255L, I292V, V338D, R355G, K389R, L464M, M676A, A717T
C	PB2	I67V, N82S, K116R, V139I, V255L, I292V, V338D, K389R, L464M, M676A, A717T
A	PB1	N16D, R430K, D619G, A652T, S694I, G733R
B	PB1	E75G, M317T, Y324F, E581G, M655I, L695I
C	PB1	E75G, E97G, K121R, E581G, M655I, L695I
A	PB1-F2	NONE
B	PB1-F2	NONE
C	PB1-F2	NONE
A	PA	I61M, T85A, T97N, D160E, Y176F, I201T, K262R, I322L, I354F, K391R, I505V
B	PA	V63M, D216N, K262R, S296T, Y305H, S388G, F520Y, Y535H, I543L, K615R
C	PA	V63M, D216N, S296T, Y305H, E327K, S388G, D479E, F520Y, Y535H, I543L, K615R
A	PA-X	T97N, D160E, N245S
B	PA-X	V63M, K195R, K252R
C	PA-X	V63M, Q210P
A	HA	V14A, N170D
B	HA	V5M, L6F, T10A, I11T, I67V, D104G, Q131R, N136D, S137Y, E142D, K156N, S157P, D170N, A172S, G179S, V190I, R205N, T211I, V226I, K228R, S336N, R339K, I390M, R420K, P505Q, I528V
C	HA	V5M, L6F, T10A, I11T, I67V, D104G, Q131R, N136D, E142D, K156N, S157P, D170N, A172S, G179S, V190I, R205N, T211I, V226I, K228R, R339K, I528V
A	NP	D51E, M105V
B	NP	I41V, K48Q, V67A, A85P, V183I, I347L
C	NP	I41V, K48Q, A85P, V183I, I475V
A	NA	K6R, T8I, I10T, Y44H, S70N, E77K, D259E, D287E, M289I, Q308H, V394I, S405T
B	NA	V8I, I13V, M29I, H36Q, I38F, G41R, N42F, T59A, S70P, L120M, I143V, K197N, R200G, E239K, I269M, K312T, K332R, H346N, T361N, G362N, E366D, D431E
C	NA	V8I, I13V, M29I, H36Q, I38F, G41R, N42L, T59A, S70P, L120M, I143V, K197N, R200G, V221I, E239K, I269M, K312T, K332R, H346N, T361N, G362N, E366D, D431E
A	M1	NONE
B	M1	V115I
C	M1	NONE
A	M2	NONE
B	M2	W41C
C	M2	NONE
A	NS1	S48I, S83P, L147I, D171N, G210E, V226I

B	NS1	S3P, G47S, I64V, S73P, A82T, A86T, R88H, R100K, R118K, N127T, F138L, D139N, L166F, I194V, R200K, S206C, N207D, D209G, P212T, P215S
C	NS1	G47S, S73P, A82T, A86T, R88H, R100K, R118K, D125N, F138L, D139N, L166F, I194V, S206C, N207D, D209G, P212T, P215S
A	NEP	G53R, G63E
B	NEP	S3P, K39R, A49V, M52V, D54E, E75D, N92S
C	NEP	K39R, M52V, D54E, E75D