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1 # A real-time colorimetric reverse transcription loop-mediated isothermal
2 # amplification (RT-LAMP) assay for the rapid detection of highly pathogenic H5
3 # clade 2.3.4.4b avian influenza viruses

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9 **Keywords:** RT-LAMP, highly pathogenic avian influenza virus, clade 2.3.4.4b molecular detection,
10 clinical diagnostic, field detection.

11

12 **Abstract**

13 Highly pathogenic avian influenza viruses (HPAIV) are a major threat to the global poultry industry
14 and public health due to their zoonotic potential. Since 2016, Europe and France have faced major
15 epizootics caused by clade 2.3.4.4b H5 HPAIV. To reduce sample-to-result times, point-of-care testing
16 is urgently needed to help prevent further outbreaks and the propagation of the virus. This study
17 presents the design of a novel real-time colorimetric reverse transcription loop-mediated isothermal
18 amplification (RT-LAMP) assay for the detection of clade 2.3.4.4b H5 HPAIV. A clinical validation
19 of this RT-LAMP assay was performed on 198 pools of clinical swabs sampled in 52 poultry flocks
20 during the H5 HPAI 2020-2022 epizootics in France. This RT-LAMP assay allowed the specific
21 detection of HPAIV H5Nx clade 2.3.4.4b within 30 minutes with a sensitivity of 86.11%. This rapid,
22 easy-to-perform, inexpensive, molecular detection assay could be included in the HPAIV surveillance
23 toolbox.

24

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25 **Introduction**

26 Avian influenza viruses (AIV) are enveloped negative-sense segmented single-stranded RNA viruses
27 belonging to the *Orthomyxoviridae* family. AIV are commonly classified into subtypes based on the
28 combination of their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) (Wahlgren,
29 2011). Importantly, AIV also can be differentiated by their pathogenicity: low pathogenic avian
30 influenza viruses (LPAIV) and highly pathogenic avian influenza viruses (HPAIV). LPAIV are the
31 most predominant worldwide and cause no to mild symptoms in infected individuals (Germeraad *et*
32 *al*., 2019; Jourdain *et al.*, 2010). Most of the time, LPAIV are undetected in flocks. However, due to
33 genetic changes, H5 and H7 HA sometimes can shift from low pathogenic to highly pathogenic forms
34 (Abdelwhab *et al*., 2013; Dupré *et al.*, 2021; Wahlgren, 2011). Unlike LPAIV, HPAIV induce severe
35 disease associated with strong typical clinical signs and high mortality rates (Lean *et al*., 2022).

36 HPAIV display a tremendous evolutionary potential, driven by mutations, indels and reassortments (D.
37 Lee *et al*., 2017; D. H. Lee *et al*., 2021; L. Shi *et al.*, 2019). This property has led to the emergence of
38 the A/goose/Guangdong/1996(Gs/GD) lineage. All subsequent viruses derived from this lineage have
39 been classified into clades and subclades. Over the years, clade 2.3.4 has become dominant, and
40 subclade 2.3.4.4b has been widely persistent in Eurasia since 2016 (W. Shi & Gao, 2021). Viruses
41 belonging to clade 2.3.4.4b have led to major epizootics, mostly due to H5N8 subtypes from 2016/2017
42 to 2020/2021, and then an H5N1 subtype in 2021/2022. HPAIV epizootics have caused the death of
43 millions of wild and domestic birds, threatened public health due to zoonotic risks, and generated major
44 economic losses to the poultry industry (Adlhoch *et al*., 2022). In addition, HPAIV epizootics seem to
45 be spreading more widely over time, with new territories infected worldwide.

46 HPAIV infections are characterized by a massive viral shedding in the early stages of the infection,
47 especially in ducks (Gaide *et al*., 2021; Vergne *et al.*, 2021), and a rapid spread of the disease. These
48 findings raise numerous challenges for the control and the surveillance of the infection, especially

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49 during epizootics. Despite the reinforcement of biosecurity measures since 2016 (Delpont *et al*., 2021)
50 and the application of new control measures by public authorities each year, the virus has acquired the
51 ability to spread rapidly and uncontrollably (Guinat *et al*., 2020; Lewis *et al*., 2021; Vergne *et al*.,
52 2021). New strategies for the early detection of HPAIV, therefore are needed to better control viral
53 spread.

54 Currently, European and French official guidelines for HPAIV detection and surveillance require an
55 rRT-PCR analysis on tracheal swabs (Nielsen *et al*., 2021). Positive results for HPAIV systematically
56 lead to the culling of entire poultry flocks. However, although rRT-PCR is considered as the gold
57 standard for HPAIV detection based on its analytical sensitivity, this method is still rather expensive,
58 time-consuming (~80 min), and requires sophisticated equipment that is difficult to transport and must
59 be operated by trained staff. This ultimately reduces its in-field diagnosis potential and can delay the
60 rapid response needed during an epizootic.

61 Easy-to-perform, fast, low-cost, low-tech, sensitive, and specific methods are needed to improve the
62 rapidity of HPAIV detection and develop new strategies for HPAIV surveillance in the field. The loop-
63 mediated isothermal amplification (LAMP) assay is a molecular biology technology that has been
64 developed since 2000 (Notomi *et al*., 2000). The LAMP assay is an end-point nucleic acid
65 amplification method based on a DNA polymerase. The technology requires a set of 2 or 3 pairs of
66 primers targeting 6 to 8 binding sites, making it highly specific. The fast and isothermal amplification
67 (15-40 min) can be performed by standard transportable equipment such as a heat block or water bath.
68 This, combined with user-friendly read-outs such as fluorescence, turbidity and even colorimetric
69 changes, enables its utilization in a field point-of-care strategy. Overall, LAMP assays are inexpensive
70 (~1.5 euros/reaction, for indication of magnitude only), rapid, easy-to-perform, and robust against the
71 well-known PCR reaction inhibitors. For all of these reasons, LAMP assays have been largely
72 developed for the detection of viruses (Golabi *et al*., 2021; Padzil *et al*., 2022; Vanhomwegen *et al*.,

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73 2021). More importantly, the low-technology required and the possible lyophilization of reagents allow
74 its utilization in remote locations where resources are scarce or non-existent (Howson *et al*., 2017;
75 Kumar *et al*., 2021; Vanhomwegen *et al.*, 2021).

76 Our research focused on developing a real-time colorimetric reverse transcription LAMP (RT-LAMP)
77 assay for the detection of H5 HPAIV clade 2.3.4.4b. We designed a primer set for the detection of H5
78 HPAIV clade 2.3.4.4b, and assessed its sensitivity and specificity on eight different AIV subtypes and
79 pathotypes, including viruses from diverse clades. Finally, we assessed the performance of this real-
80 time colorimetric RT-LAMP assay on tracheal and cloacal swabs sampled in France during the
81 2020/2021 and 2021/2022 H5 HPAIV epizootics.

82 **Materials and Methods**

83 **Primer design**. A set of 626 HA sequences from H5 HPAIV clade 2.3.4.4b HA available on GISAID
84 up to 7 February 2022, *via* https://gisaid.org or from our laboratory, were aligned using Geneious Prime
85 2021.2.2 (https://www.geneious.com). From this alignment, a consensus sequence with a 95% base
86 identity threshold was extracted to target a 200 to 350 base-long region with low base diversity. Finally,
87 a 308 base region was selected and passed through the PrimerExplorer V5 online tool for LAMP primer
88 design. A set of primers was selected (Table 1) and their specificity was checked through a BLAST
89 alignment with eight selected AIV subtypes (Table 2).

90 **Inclusivity and exclusivity.** The analytical inclusivity and exclusivity of the designed primers were
91 tested *in silico*, by rRT-PCR and by our RT-LAMP assay, on eight different avian influenza subtype
92 viruses available in our laboratory. The selection included three H5 HPAIV clade 2.3.4.4b, one H5
93 HPAIV from the European lineage (not part of clade 2.3.4.4b) (Briand *et al*., 2017) and four LPAIV
94 (Table 2).

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95 **RNA extraction.** Viral RNA was extracted using the magnetic bead-based ID Gene Mag Fast
96 Extraction Kit (IDvet, Grabels, France) combined with the IDEAL 32 extraction robot (IDvet),
97 following the manufacturer's instructions. The extracted RNA was stored at -20 °C before use.

98 **Colorimetric Real-time RT-LAMP.** RT-LAMP reactions were done using the WarmStart
99 Colorimetric LAMP 2X Master Mix kit (M1800, NEB, Hitchin, UK) following the manufacturer's
100 instructions. Briefly, a 25 µL reaction mix was prepared by mixing 12.5 µL of the WarmStart
101 Colorimetric LAMP 2X Master Mix, 5 µL of the sample viral RNA, 5 µL nuclease free water and 2.5
102 µL of a 10x primer solution mix prepared beforehand. The 10x primer solution mix was prepared with
103 16 µM of each FIP/BIP, 2 µM of each B3/F3, and 4 µM of each LF/LB. The reaction mix was incubated
104 at 65 °C for 30 min and the colour switch, from purple to yellow in case of positive reaction, was
105 assessed with the naked eye.

106 **Real-time RT-qPCR.** Two quantitative RT-PCR were used in this study. A real-time quantitative RT-
107 PCR (rRT-PCR) with the officially-approved IDvet M gene and H5/H7 one-step rRT-PCR kit (Idvet,
108 https://www.id-vet.com) was used for clinical validation as the 'gold standard' molecular-based
109 HPAIV detection method. All reactions were realized following the manufacturer's instructions. A
110 second RT-qPCR was used for the limit of detection assay. Viral RNA absolute quantification was
111 performed using the iTaq Universal SYBR green one-step kit (#1725150, Bio-rad). The H5 gene was
112 targeted with HA $H5N8_{HP}$ primers (5'-GACCTCTGTTACCCAGGGAGCCT-3', 5'-
113 GGACAAGCTGCGCTTACCCCT-3') (Bessière *et al.*, 2021). The absolute quantification was
114 performed using a standard curve based on 10-fold serial dilution of a plasmid containing the H5 HA
115 gene. RT-qPCR reaction and results analysis were performed on a LightCycler 96 instrument (Roche).

116 **Limit of detection assay.** To investigate the detection limit of this RT-LAMP assay, a viral RNA
117 absolute quantification was performed. Two serial dilutions of extracted viral RNA from embryonated

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118 eggs HPAIV H5N8 2020/2021 and HPAIV H5N1 2021/2022 amplification were realized. Each
119 dilution was systematically analyzed by our RT-LAMP assay and the iTaq RT-qPCR.

120 **Clinical validation.** The designed real-time colorimetric RT-LAMP was tested for clinical use on
121 swabs in an epizootic context. A total of 198 swabs pools, corresponding to 52 poultry flocks (32 duck,
122 19 chicken, 1 quail flocks), was included in this validation (Supplementary Table 1). The pools
123 consisted of a mix of five tracheal or cloacal swabs sampled in infected or suspected farms during the
124 HPAIV H5N8 2020/2021 and HPAIV H5N1 2021/2022 epizootics in France. All samples were tested
125 in accordance with the European guidelines for HPAIV diagnosis. First, the total RNA was extracted
126 using the magnetic bead-based ID Gene Mag Fast Extraction Kit IDvet (https://www.id-vet.com)
127 combined with the IDEAL 32 extraction robot (IDvet), following the manufacturer's instructions.
128 Then, HPAIV H5Nx viral RNA detection was simultaneously performed by rRT-PCR with the IDgene
129 H5/H7 one-step rRT-PCR kit and the real-time colorimetric RT-LAMP assay. To validate the
130 feasibility of the protocol under field conditions, this clinical validation was performed by a non-trained
131 member of staff.

132 **Results**

133 The designed RT-LAMP primers were analyzed *in silico* to confirm their complementarity to binding
134 regions. Multiple alignments with publicly available HA sequences from different AIV subtypes,
135 including HPAIV from different clades and LPAIV, were selected. The alignment revealed the high
136 binding complementary to the targeted regions of the clade 2.3.4.4b H5 HPAIVs only. The overall
137 binding complementary reaches a 97.64%, 100%, and 99.4% ratio for the HPAIV H5N8 2016/2017,
138 2020/2021 and the HPAIV H5N1 2021/2022, respectively. However, for the non-clade 2.3.4.4b
139 viruses, the overall complementary rates do not exceed 84.12% (Supplementary Table 2). This low
140 binding affinity is theoretically not sufficient to allow amplification (26,27) (Figure 1). To investigate
141 the analytical inclusivity and exclusivity of the different AIV subtypes, eight AIV from distinct

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142 subtypes and pathotypes, available in our laboratory, were used (Table 2, Figures 1 and 2). All samples
143 were analyzed using the RT-LAMP assay in parallel with the gold standard rRT-PCR targeting both
144 the M, H5 and H7 genes as controls for the detection of viral RNA. The results showed a 100% (3/3)
145 analytical reactivity for the detection of clade 2.3.4.4b H5 HPAIV. Additionally, the exclusivity test
146 showed a 100% primer specificity as none of the non-H5 clade 2.3.4.4b viruses tested positive (Figure
147 2). These results, associated with the *in silico* analysis, tend to confirm the specificity of the RT-LAMP
148 assay for HPAIV H5Ny from clade 2.3.4.4b.

149 Furthermore, the RT-LAMP assay detection limit was investigated by absolute quantification of the
150 viral RNA by the iTaq RT-PCR. Two separate 2-fold serial dilutions of HPAIV H5N8 2020/2021 and
151 H5N1 2021/2022 viral RNA samples were analyzed by RT-LAMP assay, rRT-PCR and RT-qPCR
152 (Supplementary Table 3). The results were globally similar for both viruses. Indeed, the lowest RNA
153 concentrations testing positive with the RT-LAMP assay were 9.66 and 18.44 copy /µL for the H5N8
154 HP 2020/2021 and the H5N1 HP 2021/2022, respectively (Supplementary Table 3). These findings are
155 in agreement with the expected detection limit of a RT-LAMP assay, usually ranging from 100 to 1000
156 copies/reaction (Zhang *et al*., 2020). Moreover, the rRT-PCR results indicate a shared limit of detection
157 with a cycle threshold (C_t) of around 30 for both viruses (Supplementary Table 3).

158 Finally, a clinical validation of this RT-LAMP assay was performed on clinical swabs sampled during
159 the H5N8 HP 2020/2021 and H5N1 HP 2021/2022 epizootics in France. Following the European
160 guidelines for HPAIV surveillance and detection, a total of 198 pools of five tracheal or cloacal swabs
161 each, corresponding to 52 poultry flocks (32 duck, 19 chicken, 1 quail flocks) (Supplementary Table
162 4), were analyzed simultaneously by rRT-PCR, considered the gold standard method, and by the RT-
163 LAMP assay developed.

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164 Firstly, the swabs were grouped, regardless of the flocks, based on their C_t values obtained by rRT-
165 PCR to investigate the sensitivity and specificity of the RT-LAMP assay depending on the viral RNA
166 loads. Therefore, samples were divided for into four categories: C_t <25, 25< C_t <30, C_t >30 and 0<C_t
167 (Supplementary Table 4). The results of the rRT-PCR and RT-LAMP showed a very good agreement
168 for samples with C_ts below 30. Indeed, the RT-LAMP assay showed a 100% and 86.27% sensitivity
169 for samples with C_t<25 and C_ts between 25 and 30, respectively. For C_t values above 30, the sensitivity
170 decreased drastically to 18.18%. When all data were considered altogether without differentiation
171 based on C_ts, the overall sensitivity and specificity reached 86.11% and 100%, respectively
172 (Supplementary Table 4).

173 Secondly, the data were analyzed grouped by flocks. Our data regrouped a total of 52 flocks with
174 between 1 and 9 swab pools. According to the HPAIV detection guidelines (Nielsen *et al.*, 2021), only
175 one pool must be detected as positive to consider the whole flock positive. Based on this guidance,
176 only five flocks showed result discrepancies between RT-LAMP and rRT-PCR assays (RT-LAMP
177 assay negative while rRT-PCR positive) (Supplementary Table 1). In this context, data analysis showed
178 a 90% sensitivity and 100% specificity for HPAIV detection in flocks. Interestingly, the five flocks
179 with divergent results correspond to samples with RT-PCR C_t>30.

180 **Discussion**

181 To improve the molecular detection of HPAIV circulating worldwide, this study aimed to develop a
182 real-time colorimetric RT-LAMP assay. Taken together, our findings suggest that this new real-time
183 colorimetric RT-LAMP assay may offer, in specific contexts and purposes, an alternative to the gold
184 standard rRT-PCR for the detection of HPAIV from clade 2.3.4.4b provided the primers are regularly
185 updated. The three couples of designed primers have shown to be highly specific to the clade 2.3.4.4b
186 H5 HPAIV in both *in silico* and *in vitro*. However due to the multiplicity of primers binding to a total
187 of 8 binding sites, a high primer-to-binding site complementarity is required. Even though a relative

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188 difference of detection limit and sensitivity can be noted between distinct viruses (Table 2,
189 Supplementary Table 3 and 4), this assay has proven to be very sensitive, with a detection threshold
190 determined below 20 copy/ μL. Previous knowledge of the circulating strains (i.e., obtained by
191 sequencing in a context of diagnosis or surveillance) is highly recommended to avoid false negatives
192 due to a lack of specificity. Following the HPAIV detection guidelines, RT-LAMP detection of the M
193 gene could be performed simultaneously (Golabi *et al.*, 2021).

194 A clinical validation performed on 198 pools of clinical swabs sampled in 52 poultry flocks have shown
195 an overall sensitivity of 86.11% with up to 100% for samples with C_t below 25. Moreover, even though
196 both detection limit investigations done by RT-qPCR and the clinical assay showed suboptimal results
197 for samples with $C_t>30$, this does not seem to be a major limitation in the context of an HPAIV
198 epizootic. Indeed, most HPAIV infections induce high viral shedding, even in the earliest stages of the
199 infection, leading to high loads of viral RNA (Criado *et al.*, 2021; Filaire *et al.*, 2022; Germeraad *et*
200 *al.*, 2019) especially in the respiratory tract (Gaide *et al.*, 2021). Therefore, low viral RNA loads,
201 associated with $C_t>30$, which corresponds to approximately less than 20 copies / µl (Table 2 and
202 Supplementary Table 4), are infrequent in HPAIV-infected birds.

203 This 30 min colorimetric RT-LAMP reaction could be performed in the field for point-of-care
204 application. Additionally, this strategy could be included in a workflow comprising fast lysis and
205 extraction methods and environmental sampling methods for the rapid detection of new outbreaks
206 directly on-farm, especially in a context of clinical suspicions, where viral RNA loads are the highest.
207 Further validation by proficiency tests in reference laboratories would be required before
208 implementation in official surveillance but in the principle, this rapid, easy-to-perform (even for non-
209 trained staff), inexpensive, low-tech molecular detection assay could be included in the HPAIV
210 surveillance toolbox and improve the response capacity during epizootics.

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220 **Disclosure statement**

221 The authors declare no conflict of interest.

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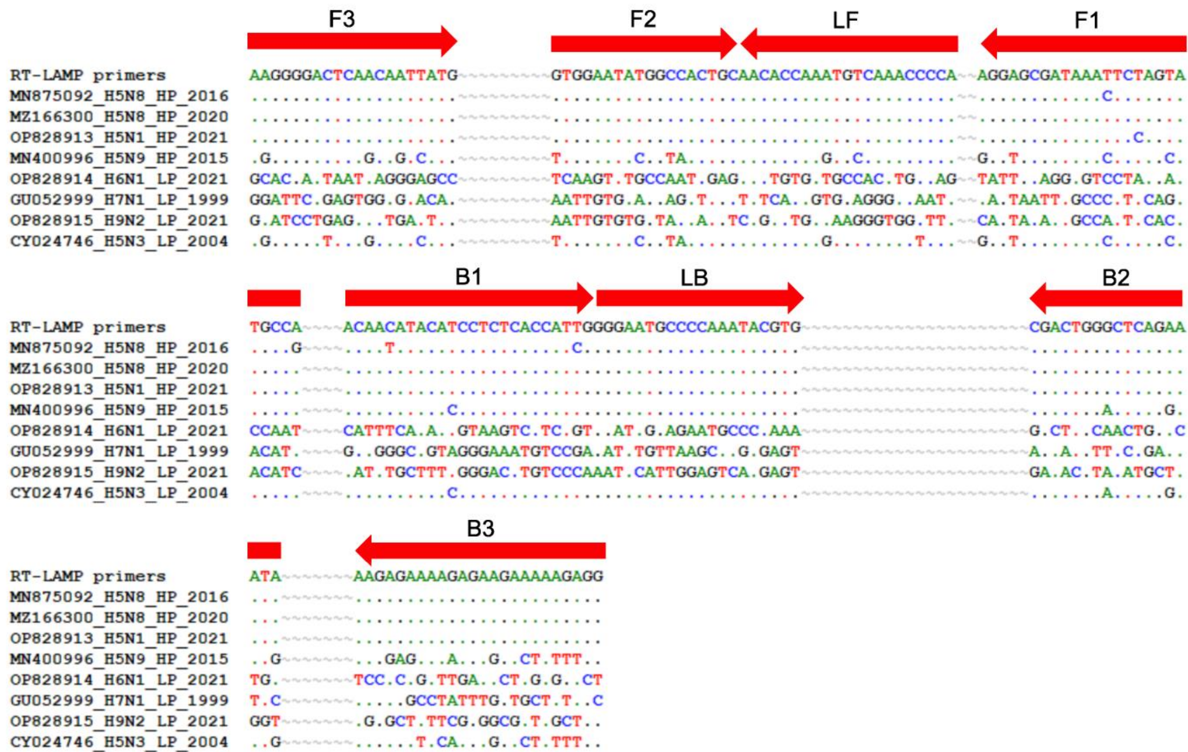

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337 **Figure 1.** LAMP primers alignment to the sequence of the 8 AIV viruses included in the study.

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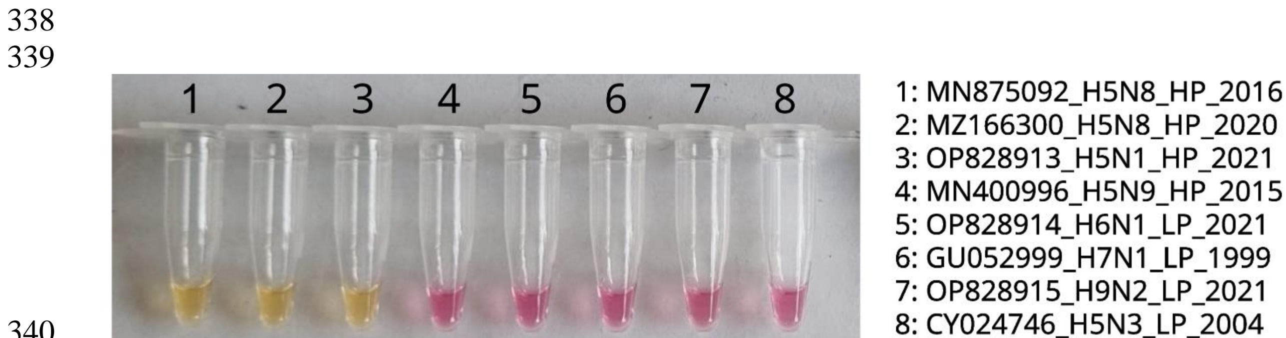

340

341 **Figure 2.** Real-time colorimetric LAMP results on different AIV viruses. Positive amplification

342 induces a colorimetric change from purple to yellow. 1, 2016/2017 H5N8 HP clade 2.3.4.4b virus. 2,

343 2020/2021 H5N8 HP clade 2.3.4.4b virus. 3, 2021/2022 H5N1 clade 2.3.4.4b virus. 4, H5N9 HP

344 none clade 2.3.4.4b virus. 5, H6N1 LP virus. 6, H7N1 LP virus. 7, H9N2 LP virus. 8, H5N3 LP

345 virus.

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348 **Tables**

349 Table 1: List and sequences of the designed RT-LAMP primers. The nucleotide position corresponds

350 to the HA sequence of the HPAIV H5N8 2020/2021 (accession number MZ166300).

Gene	Primer	Length (bp)	Sequence (5' to 3')	Location on H5 gene
HA	F3	20	AAGGGGACTCAACAATTATG	824-843
HA	B3	24	CCTCTTTTTCTTCTCTTTTCTCTT	1014-1037
HA	FIP	43	TGGCATACTAGAATTTATCGCTCCT-GTGGAATATGGCCACTGC	F1 (894-918), F2 (853-870)
HA	BIP	42	ACAACATACATCCTCTCACCATTG-TATTTCTGAGCCCAGTCG	B1(923-946), B2(989-1006)
HA	LPF	21	TGGGGTTTGACATTTGGTGTT	871-891
HA	LPB	20	GGGAATGCCCCAAATACGTG	947-966

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352 Table 2: Viruses selected to test the designed primer set

Subtype	Pathogenic form	Clade 2.3.4.4b	Sampling location	Sampling time	Host	Amplification	Virus reference	Accession number
H5N8	HP	Yes	France	2016	Duck	Embryonated eggs	A/mallard duck/France/171201g/2017 (H5N8)	MN875092
H5N8	HP	Yes	France	2020	Duck	Embryonated eggs	A/Mule_duck/France/20353/2020(H5N8)	MZ166300
H5N1	HP	Yes	France	2021	Duck	Embryonated eggs	A/Mule_duck/France/21348/2021 (H5N1)	OP828913
H5N9	HP	No	France	2015	Guinea Fowl	Embryonated eggs	A/Guinea Fowl/France/129/2015(H5N9)	MN400996
H9N2	LP	No	Tunisia	2021	Chicken	None	A/Gallus gallus/Tunisia/20057/2020(H9N2)	OP828915
H6N1	LP	No	France	2021	Duck	Embryonated eggs	A/Pekin_duck/France/21114/2021(H6N1)	OP828914
H7N1	LP	No	Italy	1999	Turkey	Cells (MDCK)	A/Turkey/Italy/977/1999/H7N1	GU052999
H5N3	LP	No	Italy	2004	Duck	Embryonated eggs	A/duck/Italy/775/2004(H5N3)	CY024746

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354

Supplementary Material

355

356 **Supplementary Table 1:** Summary of clinical sample information

357

Flock ID	Sample ID	Animal species	Sampling year	Viral strain	Swab	Ct	LAMP	qPCR flock result	LAMP flock result
#1	#1.1	Chicken	2020	H5N8	Cloacal	28.34	Pos	Pos	Pos
	#1.2	Chicken	2020	H5N8	Cloacal	29.78	Pos		
	#1.3	Chicken	2020	H5N8	Tracheal	20.38	Pos		
	#1.4	Chicken	2020	H5N8	Tracheal	20.50	Pos		
#2	#2.1	Duck	2021	H5N8	Tracheal	21.26	Pos	Pos	Pos
	#2.2	Duck	2021	H5N8	Tracheal	24.10	Pos		
	#2.3	Duck	2021	H5N8	Tracheal	22.85	Pos		
	#2.4	Duck	2021	H5N8	Tracheal	24.70	Pos		
#3	#3.1	Chicken	2021	H5N8	Tracheal	21.94	Pos	Pos	Pos
	#3.2	Chicken	2021	H5N8	Tracheal	22.64	Pos		
	#3.3	Chicken	2021	H5N8	Tracheal	21.78	Pos		
	#3.4	Chicken	2021	H5N8	Tracheal	23.21	Pos		
#4	#4.1	Duck	2021	H5N8	Tracheal	15.72	Pos	Pos	Pos
	#4.2	Duck	2021	H5N8	Tracheal	19.59	Pos		
	#4.3	Duck	2021	H5N8	Tracheal	16.93	Pos		

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	#4.4	Duck	2021	H5N8	Tracheal	20.27	Pos		
#5	#5.1	Duck	2021	H5N8	Tracheal	18.73	Pos	Pos	Pos
	#5.2	Duck	2021	H5N8	Tracheal	23.31	Pos		
	#5.3	Duck	2021	H5N8	Tracheal	23.43	Pos		
	#5.4	Duck	2021	H5N8	Tracheal	24.32	Pos		
#6	#6.1	Chicken	2021	H5N8	Tracheal	-	Neg	Pos	Neg
	#6.2	Chicken	2021	H5N8	Tracheal	30.32	Neg		
#7	#7.1	Duck	2021	H5N8	Tracheal	25.98	Pos	Pos	Pos
	#7.2	Duck	2021	H5N8	Tracheal	24.02	Pos		
	#7.3	Duck	2021	H5N8	Tracheal	21.50	Pos		
	#7.4	Duck	2021	H5N8	Tracheal	35.32	Neg		
#8	#8.1	Duck	2021	H5N8	Tracheal	21.78	Pos	Pos	Pos
	#8.2	Duck	2021	H5N8	Tracheal	23.05	Pos		
	#8.3	Duck	2021	H5N8	Tracheal	22.83	Pos		
	#8.4	Duck	2021	H5N8	Tracheal	20.67	Pos		
#9	#9.1	Duck	2021	H5N8	Tracheal	18.66	Pos	Pos	Pos
	#9.2	Duck	2021	H5N8	Tracheal	20.01	Pos		
	#9.3	Duck	2021	H5N8	Tracheal	20.85	Pos		
	#9.4	Duck	2021	H5N8	Tracheal	21.10	Pos		
	#9.5	Duck	2021	H5N8	Tracheal	19.00	Pos		
	#9.6	Duck	2021	H5N8	Tracheal	19.34	Pos		

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	#9.7	Duck	2021	H5N8	Tracheal	20.88	Pos		
#10	#10.1	Duck	2021	H5N8	Tracheal	34.93	Neg	Pos	Neg
	#10.2	Duck	2021	H5N8	Tracheal	34.82	Neg		
	#10.3	Duck	2021	H5N8	Tracheal	34.26	Neg		
	#10.4	Duck	2021	H5N8	Tracheal	-	Neg		
#11	#11.1	Duck	2021	H5N8	Tracheal	20.52	Pos	Pos	Pos
	#11.2	Duck	2021	H5N8	Tracheal	20.91	Pos		
	#11.3	Duck	2021	H5N8	Tracheal	18.57	Pos		
	#11.4	Duck	2021	H5N8	Tracheal	20.64	Pos		
#12	#12.1	Duck	2021	H5N8	Tracheal	23.91	Pos	Pos	Pos
	#12.2	Duck	2021	H5N8	Tracheal	24.11	Pos		
	#12.3	Duck	2021	H5N8	Tracheal	28.44	Pos		
	#12.4	Duck	2021	H5N8	Tracheal	23.53	Pos		
#13	#13.1	Duck	2021	H5N8	Cloacal	25.27	Pos	Pos	Pos
	#13.2	Duck	2021	H5N8	Cloacal	23.38	Pos		
	#13.3	Duck	2021	H5N8	Cloacal	28.51	Pos		
	#13.4	Duck	2021	H5N8	Cloacal	24.82	Pos		
#14	#14.1	Duck	2021	H5N8	Cloacal	28.46	Pos	Pos	Pos
	#14.2	Duck	2021	H5N8	Cloacal	35.54	Pos		
	#14.3	Duck	2021	H5N8	Cloacal	22.07	Pos		
	#14.4	Duck	2021	H5N8	Cloacal	35.20	Neg		

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	#14.5	Duck	2021	H5N8	Tracheal	26.48	Pos		
	#14.6	Duck	2021	H5N8	Tracheal	27.28	Pos		
	#14.7	Duck	2021	H5N8	Tracheal	23.46	Pos		
	#14.8	Duck	2021	H5N8	Tracheal	28.86	Neg		
#15	#15.1	Duck	2021	H5N8	Tracheal	25.06	Pos	Pos	Pos
	#15.2	Duck	2021	H5N8	Tracheal	19.84	Pos		
	#15.3	Duck	2021	H5N8	Tracheal	22.40	Pos		
	#15.4	Duck	2021	H5N8	Tracheal	23.62	Pos		
#16	#16.1	Duck	2021	H5N8	Tracheal	20.92	Pos	Pos	Pos
	#16.2	Duck	2021	H5N8	Tracheal	19.08	Pos		
	#16.3	Duck	2021	H5N8	Tracheal	22.70	Pos		
	#16.4	Duck	2021	H5N8	Tracheal	19.80	Pos		
#17	#17.1	Duck	2021	H5N8	Cloacal	25.12	Pos	Pos	Pos
	#17.2	Duck	2021	H5N8	Cloacal	24.60	Pos		
	#17.3	Duck	2021	H5N8	Cloacal	26.17	Pos		
	#17.4	Duck	2021	H5N8	Cloacal	24.17	Pos		
	#17.5	Duck	2021	H5N8	Tracheal	19.26	Pos		
	#17.6	Duck	2021	H5N8	Tracheal	18.31	Pos		
	#17.7	Duck	2021	H5N8	Tracheal	21.01	Pos		
#18	#18.1	Duck	2021	H5N8	Tracheal	20.46	Pos	Pos	Pos
	#18.2	Duck	2021	H5N8	Tracheal	20.89	Pos		

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	#18.3	Duck	2021	H5N8	Tracheal	19.18	Pos		
	#18.4	Duck	2021	H5N8	Tracheal	22.59	Pos		
	#18.5	Duck	2021	H5N8	Tracheal	22.03	Pos		
#19	#19.1	Duck	2022	H5N1	Tracheal	22.66	Pos	Pos	Pos
	#19.2	Duck	2022	H5N1	Tracheal	29.36	Neg		
	#19.3	Duck	2022	H5N1	Tracheal	27.85	Pos		
	#19.4	Duck	2022	H5N1	Tracheal	29.39	Pos		
	#19.5	Duck	2022	H5N1	Tracheal	31.02	Neg		
#20	#20.1	Duck	2022	H5N1	Tracheal	23.58	Pos	Pos	Pos
	#20.2	Duck	2022	H5N1	Tracheal	26.97	Pos		
	#20.3	Duck	2022	H5N1	Tracheal	27.36	Pos		
	#20.4	Duck	2022	H5N1	Tracheal	26.19	Pos		
	#20.5	Duck	2022	H5N1	Tracheal	-	Neg		
	#20.6	Duck	2022	H5N1	Tracheal	35.28	Neg		
	#20.7	Duck	2022	H5N1	Tracheal	-	Neg		
#21	#21.1	Duck	2022	H5N1	Tracheal	27.51	Pos	Pos	Pos
	#21.2	Duck	2022	H5N1	Tracheal	21.13	Pos		
	#21.3	Duck	2022	H5N1	Tracheal	25.46	Pos		
	#21.4	Duck	2022	H5N1	Tracheal	23.97	Pos		
#22	#22	Duck	2022	H5N1	Tracheal	28.65	Pos	Pos	Pos
#23	#23	Duck	2022	H5N1	Tracheal	32.64	Neg	Pos	Neg

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#24	#24.1	Chicken	2022	H5N1	Tracheal	-	Neg	Neg	Neg
	#24.2	Chicken	2022	H5N1	Tracheal	-	Neg		
#25	#25.1	Chicken	2022	H5N1	Tracheal	35.11	Neg	Pos	Pos
	#25.2	Chicken	2022	H5N1	Tracheal	29.31	Pos		
	#25.3	Chicken	2022	H5N1	Tracheal	28.87	Neg		
#26	#26.1	Chicken	2022	H5N1	Tracheal	21.59	Pos	Pos	Pos
	#26.2	Chicken	2023	H5N2	Tracheal	22.51	Pos		
#27	#27.1	Chicken	2022	H5N1	Tracheal	29.69	Pos	Pos	Pos
	#27.2	Chicken	2022	H5N1	Tracheal	26.14	Pos		
	#27.3	Chicken	2022	H5N1	Tracheal	-	Neg		
	#27.4	Chicken	2022	H5N1	Tracheal	28.38	Pos		
	#27.5	Chicken	2022	H5N1	Tracheal	-	Neg		
#28	#28.1	Chicken	2022	H5N1	Tracheal	-	Neg	Pos	Pos
	#28.2	Duck	2022	H5N1	Tracheal	22.95	Pos		
	#28.3	Duck	2022	H5N1	Tracheal	20.19	Pos		
	#28.4	Duck	2022	H5N1	Tracheal	18.70	Pos		
	#28.5	Duck	2022	H5N1	Tracheal	21.09	Pos		
#29	#29	Chicken	2022	H5N1	Tracheal	18.07	Pos	Pos	Pos
#30	#30	Chicken	2022	H5N1	Tracheal	15.14	Pos	Pos	Pos
#31	#31	Chicken	2022	H5N1	Tracheal	31.95	Neg	Pos	Neg
#32	#32	Chicken	2022	H5N1	Tracheal	36.03	Pos	Pos	Pos

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#33	#33	Chicken	2022	H5N1	Tracheal	-	Neg	Neg	Neg
#34	#34	Chicken	2022	H5N1	Tracheal	33.79	Neg	Pos	Neg
#35	#35.1	Chicken	2022	H5N1	Tracheal	22.14	Pos	Pos	Pos
	#35.2	Chicken	2022	H5N1	Tracheal	21.39	Pos		
#36	#36.1	Chicken	2022	H5N1	Tracheal	19.90	Pos	Pos	Pos
	#36.2	Chicken	2022	H5N1	Tracheal	23.67	Pos		
#37	#37.1	Chicken	2022	H5N1	Tracheal	20.95	Pos	Pos	Pos
	#37.2	Chicken	2022	H5N1	Tracheal	25.22	Pos		
#38	#38	Chicken	2022	H5N1	Tracheal	18.88	Pos	Pos	Pos
#39	#39	Chicken	2022	H5N1	Tracheal	24.32	Pos	Pos	Pos
#40	#40.1	Duck	2022	H5N1	Cloacal	-	Neg	Pos	Pos
	#40.2	Duck	2022	H5N1	Cloacal	26.61	Pos		
	#40.3	Duck	2022	H5N1	Tracheal	33.98	Pos		
	#40.4	Duck	2022	H5N1	Tracheal	-	Neg		
#41	#41	Duck	2022	H5N1	Tracheal	24.94	Pos	Pos	Pos
#42	#42.1	Duck	2022	H5N1	Tracheal	25.35	Pos	Pos	Pos
	#42.2	Duck	2022	H5N1	Tracheal	17.65	Pos		
	#42.3	Duck	2022	H5N1	Tracheal	23.35	Pos		
	#42.4	Duck	2022	H5N1	Tracheal	22.30	Pos		
#43	#43.1	Duck	2022	H5N1	Tracheal	25.84	Pos	Pos	Pos
	#43.2	Duck	2022	H5N1	Tracheal	24.67	Pos		

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	#43.3	Duck	2022	H5N1	Tracheal	29.49	Neg		
#44	#44.1	Duck	2022	H5N1	Cloacal	22.53	Pos	Pos	Pos
	#44.2	Duck	2022	H5N1	Cloacal	29.65	Neg		
	#44.3	Duck	2022	H5N1	Cloacal	29.46	Pos		
	#44.4	Duck	2022	H5N1	Cloacal	28.78	Neg		
	#44.5	Duck	2022	H5N1	Tracheal	33.48	Neg		
	#44.6	Duck	2022	H5N1	Tracheal	-	Neg		
	#44.7	Duck	2022	H5N1	Tracheal	28.60	Neg		
#45	#45.1	Chicken	2022	H5N1	Cloacal	24.46	Pos	Pos	Pos
	#45.2	Chicken	2022	H5N1	Cloacal	25.19	Pos		
	#45.3	Chicken	2022	H5N1	Cloacal	26.33	Pos		
	#45.4	Chicken	2022	H5N1	Cloacal	27.86	Pos		
	#45.5	Chicken	2022	H5N1	Tracheal	23.00	Pos		
	#45.6	Chicken	2022	H5N1	Tracheal	24.98	Pos		
	#45.7	Chicken	2022	H5N1	Tracheal	23.59	Pos		
	#45.8	Chicken	2022	H5N1	Tracheal	27.28	Pos		
#46	#46.1	Duck	2022	H5N1	Cloacal	23.57	Pos	Pos	Pos
	#46.2	Duck	2022	H5N1	Cloacal	23.22	Pos		
	#46.3	Duck	2022	H5N1	Cloacal	25.74	Pos		
	#46.4	Duck	2022	H5N1	Cloacal	21.88	Pos		
	#46.5	Duck	2022	H5N1	Cloacal	33.68	Neg		

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	#46.6	Duck	2022	H5N1	Tracheal	17.03	Pos		
	#46.7	Duck	2022	H5N1	Tracheal	16.01	Pos		
	#46.8	Duck	2022	H5N1	Tracheal	15.63	Pos		
	#46.9	Duck	2022	H5N1	Tracheal	17.03	Pos		
#47	#47.1	Duck	2022	H5N1	Tracheal	33.28	Neg	Pos	Pos
	#47.2	Duck	2022	H5N1	Tracheal	31.12	Neg		
	#47.3	Duck	2022	H5N1	Tracheal	28.39	Pos		
	#47.4	Duck	2022	H5N1	Tracheal	28.29	Pos		
	#47.5	Duck	2022	H5N1	Tracheal	26.04	Pos		
	#47.6	Duck	2022	H5N1	Tracheal	26.22	Pos		
#48	#48.1	Duck	2022	H5N1	Tracheal	23.71	Pos	Pos	Pos
	#48.2	Duck	2022	H5N1	Tracheal	22.55	Pos		
	#48.3	Duck	2022	H5N1	Tracheal	21.79	Pos		
	#48.4	Duck	2022	H5N1	Tracheal	23.85	Pos		
#49	#49.1	Duck	2022	H5N1	Cloacal	23.08	Pos	Pos	Pos
	#49.2	Duck	2022	H5N1	Cloacal	25.39	Pos		
	#49.3	Duck	2022	H5N1	Cloacal	27.47	Pos		
	#49.4	Duck	2022	H5N1	Cloacal	23.48	Pos		
	#49.5	Duck	2022	H5N1	Tracheal	22.42	Pos		
	#49.6	Duck	2022	H5N1	Tracheal	22.74	Pos		
	#49.7	Duck	2022	H5N1	Tracheal	20.88	Pos		

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	#49.8	Duck	2022	H5N1	Tracheal	21.31	Pos		
#50	#50.1	Duck	2022	H5N1	Tracheal	23.39	Pos	Pos	Pos
	#50.2	Duck	2022	H5N1	Tracheal	22.61	Pos		
#51	#51.1	Duck	2022	H5N1	Cloacal	26.55	Pos	Pos	Pos
	#51.2	Duck	2022	H5N1	Cloacal	27.61	Pos		
	#51.3	Duck	2022	H5N1	Cloacal	26.16	Pos		
	#51.4	Duck	2022	H5N1	Cloacal	31.97	Neg		
#52	#52.1	Duck	2022	H5N1	Cloacal	-	Neg	Pos	Pos
	#52.2	Quail	2022	H5N1	Cloacal	-	Neg		
	#52.3	Quail	2022	H5N1	Cloacal	-	Neg		
	#52.4	Quail	2022	H5N1	Cloacal	-	Neg		
	#52.5	Quail	2022	H5N1	Cloacal	21.58	Pos		
	#52.6	Quail	2022	H5N1	Tracheal	-	Neg		
	#52.7	Quail	2022	H5N1	Tracheal	34.01	Pos		
	#52.8	Quail	2022	H5N1	Tracheal	34.35	Neg		

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361 **Supplementary Table 2:** Summary of the mismatches base number between the LAMP primers and
362 viral RNA.

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Primer	F3	B3	FIP		BIP		LF	LB	Overall nucleotide identity %
			F1	F2	B1	B2			
Size	20	24	25	18	24	18	21	20	
H5N8 HP 2015/2016	0	0	2	0	2	0	0	0	97,65
H5N8 HP 2020/2021	0	0	0	0	0	0	0	0	100
H5N8 HP 2021/2022	0	0	1	0	0	0	0	0	99,41
H5N9 HP	4	10	4	4	1	3	2	0	83,53
H5N3	4	9	4	4	1	3	2	0	84,12
H6N1	17	15	19	16	19	12	14	15	25,29
H7N1	16	15	18	11	19	9	14	15	31,18
H9N2	13	16	18	13	20	4	14	18	31,76

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Supplementary Table 3: HPAIV H5N8 2020/2021 and H5N1 2021/2022 RT-LAMP detection sensitivity assay.

Dilution number		1	2	3	4	5	6	7	8	9	10	11
H5N8 2020/2021	rRT-PCR C_t	20.81	22.44	24.53	26.58	28.38	30.76	31.54	33.21	34.46	36.01	-
	RT-qPCR quantification copy/µL	33270	11760	3061	874.4	214.3	86.56	18.44	0.94	-	-	-
	Colorimetric detection	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg
H5N1 2021/2022	rRT-PCR C_t	21.64	23.01	25.40	27.36	29.27	31.62	33	35.30	36.91		
	RT-qPCR quantification copy/µL	25320	7477	2424	665.30	230.60	84.85	14.70	9.66	-	-	-
	Colorimetric detection	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Pos	Neg	Neg	Neg

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381 **Supplementary Table 4:** Overview of the LAMP results in comparison to the rRT-PCR considered
382 as the gold standard.

Virus subtypes	Ct range	Number of samples	True positive	True negative	False positive	False negative	Sensitivity	Specificity
H5N8 HP 2020/2021	Ct<25	59	59	-	-	-	100%	-
	25<Ct<30	13	12	-	-	1	92.31%	-
	Ct>30	9	1	2	-	6	14.29%	100%
	All values	81	72	2	-	7	91.14%	100%
H5N1 HP 2021/2022	Ct<25	48	48	-	-	-	100%	-
	25<Ct<30	38	32	-	-	6	84.21%	-
	Ct>30	31	3	16	-	12	20%	100%
	All values	117	83	16	-	18	82.18%	100%
H5N8 & H5N1 HP	Ct<25	107	107	-	-	-	100%	-
	25<Ct<30	51	44	-	-	7	86.27%	-
	Ct>30	40	4	18	-	18	18.18%	100%
	All values	198	155	18	-	25	86.11%	100%

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