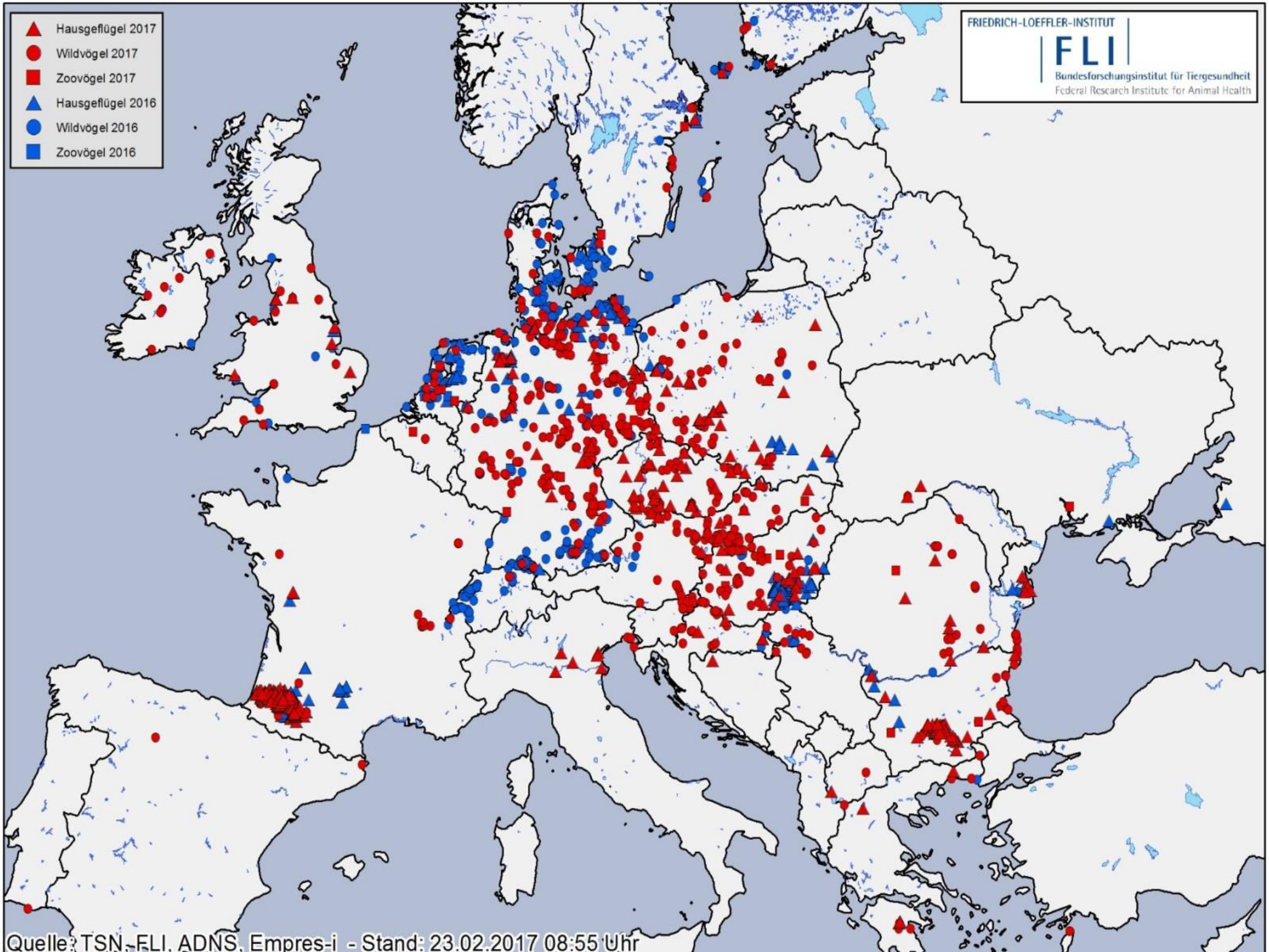
OFFLU avian influenza virus characterisation
29 – 30 March 2017
FAO Headquarters, Rome, Italy

Timm Harder

Friedrich-Loeffler-Institute,
Isle of Riems, Germany

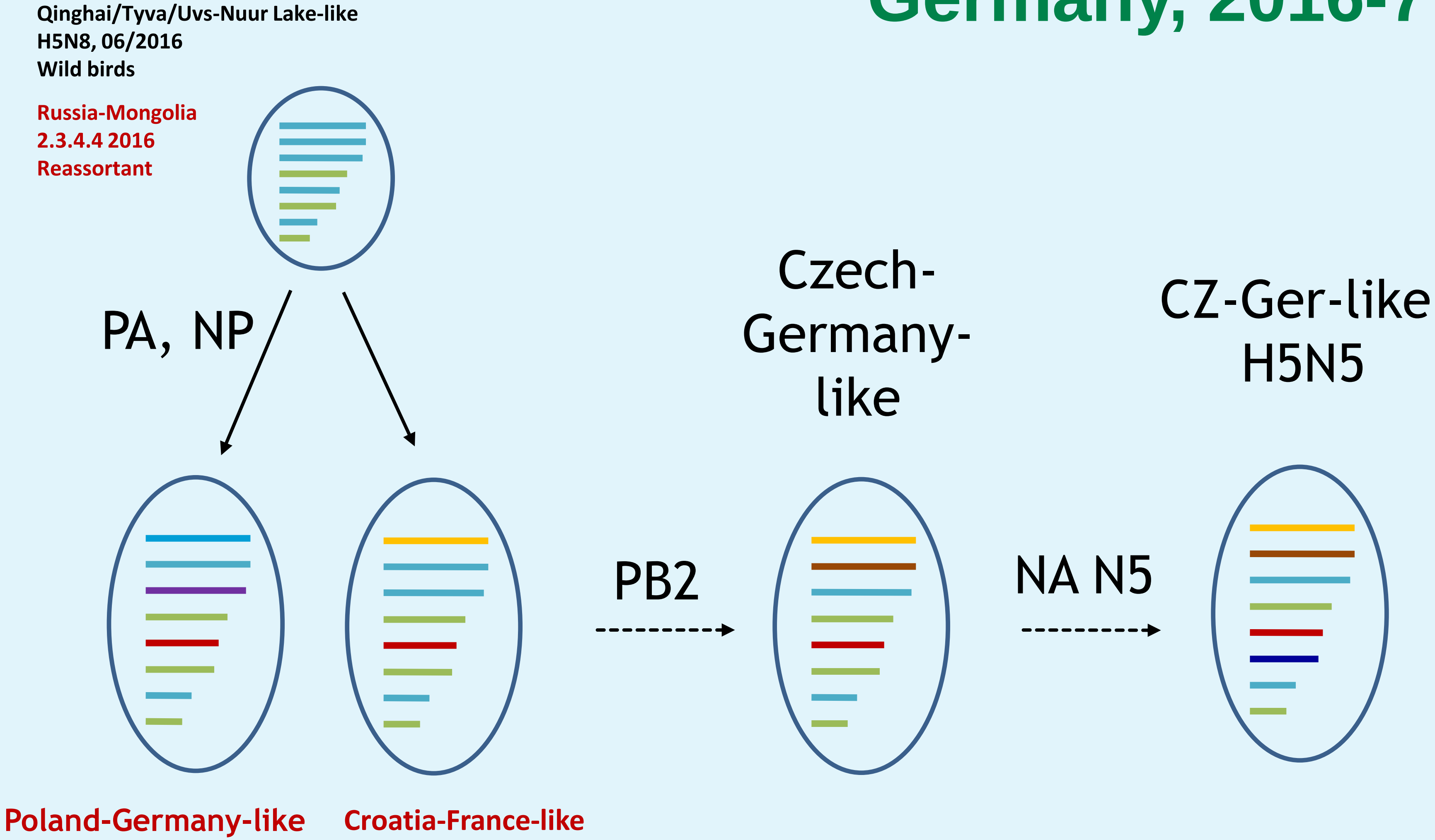
Who did the work?

- PCR development: Mahmoud Naguib, Annika Graaf, Donata and Bernd Hoffmann
- NGS sequencing and phylogenies: Anne Pohlmann, Dirk Hoeper, Patrick Zitzow
- Sample analysis, magical lab management: Diana Wessler, Aline Maksimov
- Epidemiologic analyses: A lot of people from the FLI Epi institute
- R software and related mysteries: Aline and Pavlo Maksimov
- Christian Grund, Martin Beer, Franz Conraths



Quelle: TSN, FLI, ADNS, Empres-i - Stand: 23.02.2017 08:55 Uhr

Genotypes of HP AIV H5N8, Germany, 2016-7



Qinghai/Tyva/Uvs-Nuur Lake-like
H5N8, 06/2016
Wild birds

Russia-Mongolia
2.3.4.4 2016
Reassortant

PA, NP

PB2

Czech-Germany-like

NA N5

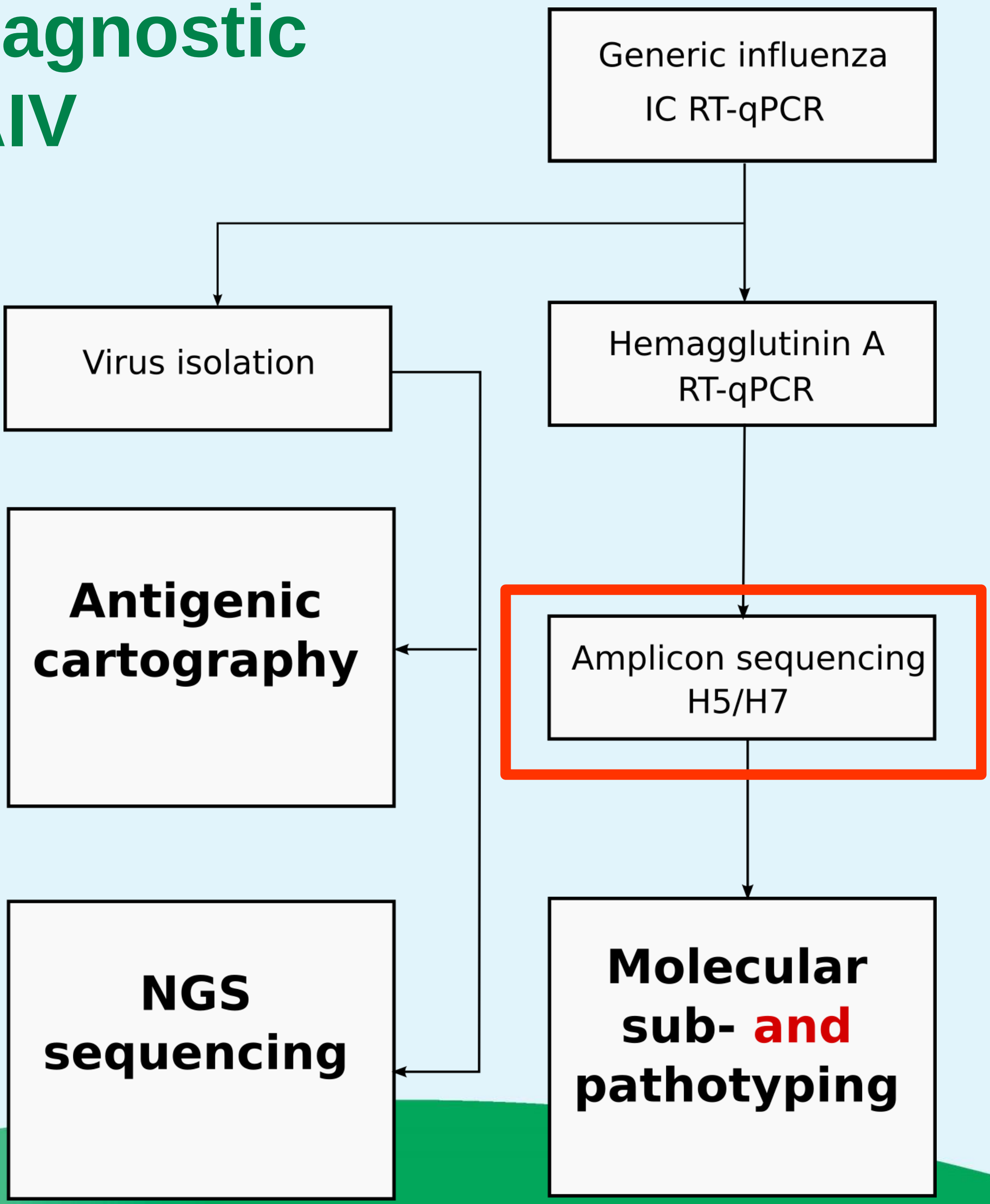
CZ-Ger-like H5N5

Poland-Germany-like

Croatia-France-like

offlu
OIE FAO
network of expertise on animal influenza

General diagnostic scheme, AIV



Generic influenza IC RT-qPCR

Virus isolation

Hemagglutinin A RT-qPCR

Antigenic cartography

Amplicon sequencing H5/H7

NGS sequencing

Molecular sub- and pathotyping

Sequencing-independent pathotyping - H5

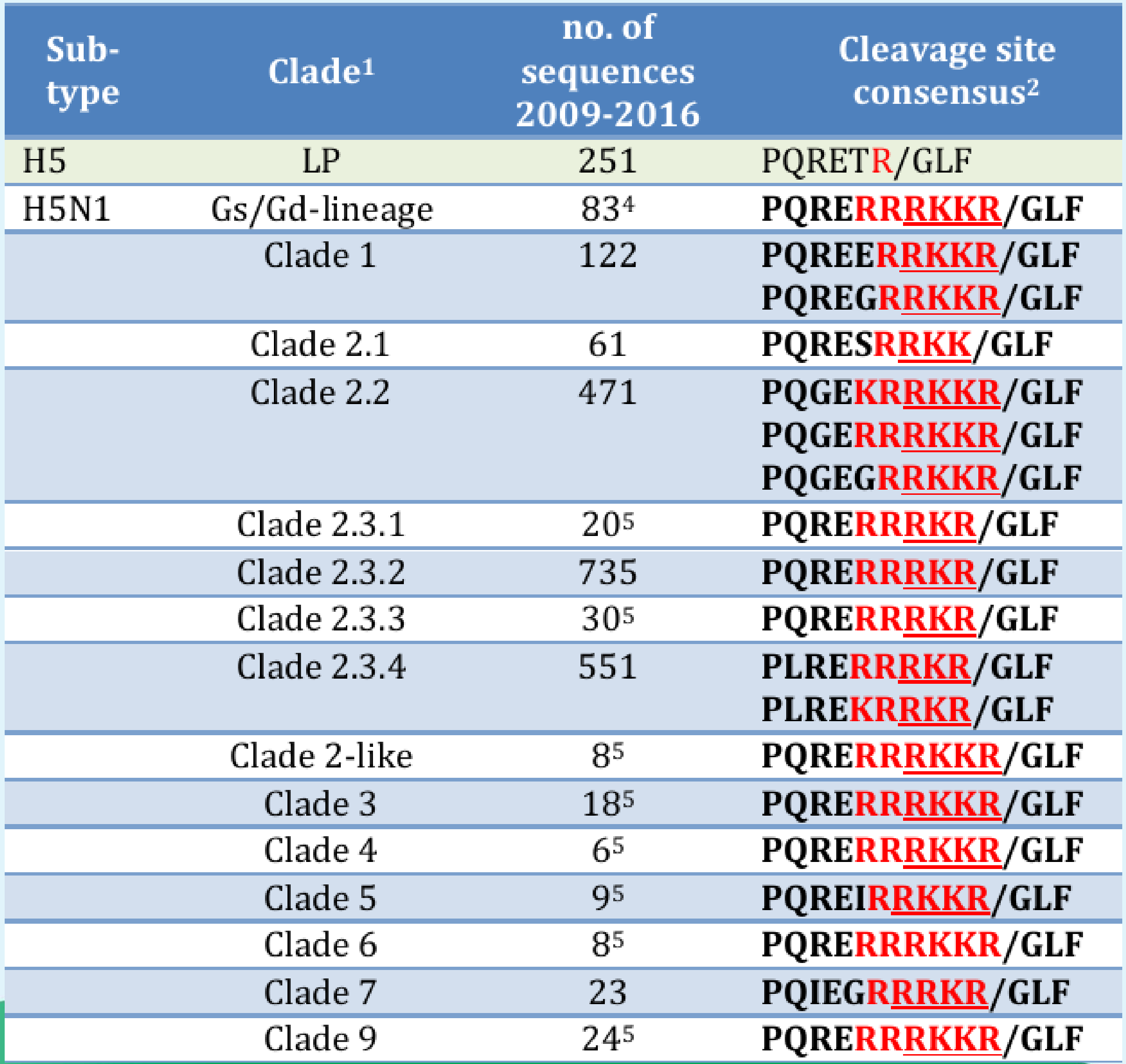
LPAI: Monobasic cleavage site

```
GGAATGTCCCTCAAAGAGAGACAA------------GAGGACTATTTGGGGCTATAGCAGGCTTTATAGAAGGAGGGTGGC
GGAATGTTCCTCAAAGAGAAACAA------------GAGGACTGTTTGGGGCTATAGCAGGCTTTATAGAAGGAGGGTGGC
GGAATGTTCCTCAAAGAGAAACAA------------GAGGACTATTTGGGGCTATAGCAGGCTTTATAGAAGGAGGGTGGC
GGAATGTCCCTCAAAGAGARACAA------------GAGGACTATTTGGGGCTATAGCAGGCTTTATAGAAGGAGGGTGGC
GGAATGTCCCTCAAAGAGAGACAA------------GAGGACTATTTGGGGCTATAGCAGGCTTTATAGAAGGAGGGTGGC
GGAATGTCCCTCAGAGAGAGACGA------------GAGGACTATTTGGGGCTATAGCAGGCTTTATAGAAGGAGGGTGGC
GGAATGTTCCTCAAAGAGAGACAA------------GAGGTCTATTTGGAGCAATAGCAGGCTTTATAGAGGGAGGATGGC
GGAATGTTCCTCAAAGAGAGACAA------------GAGGTCTATTTGGAGCAATAGCAGGCTTTATAGAGGGAGGATGGC
GGAATGTTCCTCAAAGAGAGACAA------------GAGGTCTATTTGGAGCAATAGCAGGCTTTATAGAGGGAGGATGGC
GGAACGTTCCTCAAAGAGAAACAA------------GAGGTCTATTTGGAGCTATAGCAGGCTTTATAGAGGGAGGATGGC
GGAACGTTCCTCAAAGAGAAACAA------------GAGGTCTATTTGGAGCTATAGCAGGCTTTATAGAGGGAGGATGGC
GAAATAGCCCTCAAGGAGAGAGAAGAAGAAAAAAGAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGC
GAAATAGCCCTCAAGGAGAGAGAAGAAGAAAAAAGAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGC
GAAATAGCCCTCAAGGAGAGAGAAGAAGAAAAAAGAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGC
GAAATAGCCCTCAAGGAGAGAGAAGAAGAAAAAAGAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGC
GAAATAGCCCTCAAGGAGAGAGAAGAAGAAAAAAGAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGC
GAAATAGCCCTCAAGGAGAGAGAAGAAGAAAAAAGAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGC
GAAATAGCCCTCAAGGAGAGAGAAGAAGAAAAAAGAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGC
GAAATAGCCCTCAAGGAGAGAGAAGAAGAAAAAAGAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGC
GAAATAGCCCTCAAGGAGAGAGAAGAAGAAAAAAGAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGC
GAAATAGCCCTCAAGGAGAGAGAAGAAGAAAAAAGAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGC
```

HPAI: Insertion of multiple basic AA

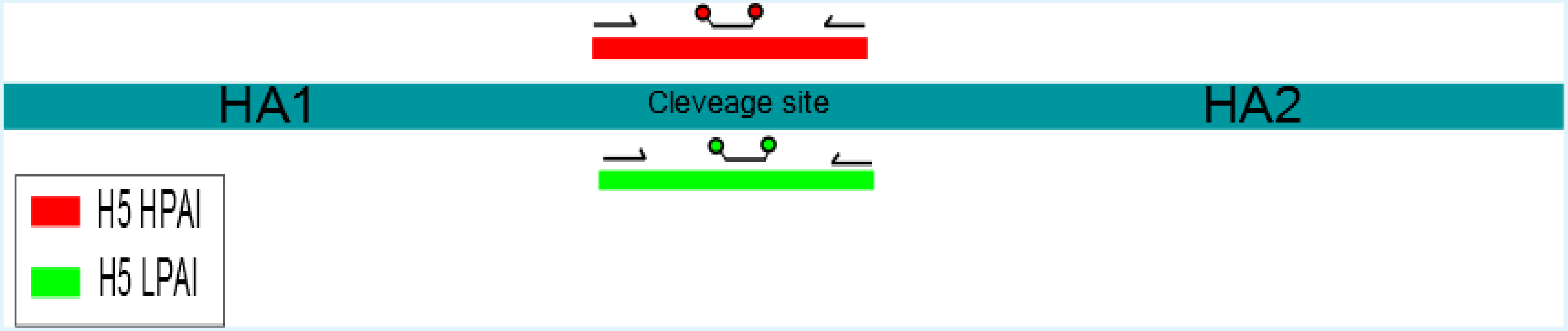
gs/GD-like H5 HP cleavage sites are fairly conserved

Data from OFFLU webpage



Sub-type	Clade[1]	no. of sequences 2009-2016	Cleavage site consensus[2]
H5	LP	251	PQRETR/GLF
H5N1	Gs/Gd-lineage	83[4]	**PQRERRRKKR/GLF**
	Clade 1	122	**PQREERRKKR/GLF** **PQREGRRKKR/GLF**
	Clade 2.1	61	**PQRESRRKK/GLF**
	Clade 2.2	471	**PQGEKRRKKR/GLF** **PQGERRRKKR/GLF** **PQGEGRRKKR/GLF**
	Clade 2.3.1	20[5]	**PQRERRRKR/GLF**
	Clade 2.3.2	735	**PQRERRRKR/GLF**
	Clade 2.3.3	30[5]	**PQRERRRKR/GLF**
	Clade 2.3.4	551	**PLRERRRKR/GLF** **PLREKRRKR/GLF**
	Clade 2-like	8[5]	**PQRERRRKKR/GLF**
	Clade 3	18[5]	**PQRERRRKKR/GLF**
	Clade 4	6[5]	**PQRERRRKKR/GLF**
	Clade 5	9[5]	**PQREIRRKKR/GLF**
	Clade 6	8[5]	**PQRERRRKKR/GLF**
	Clade 7	23	**PQIEGRRRKR/GLF**
	Clade 9	24[5]	**PQRERRRKKR/GLF**

Design of pathotype-specific probes

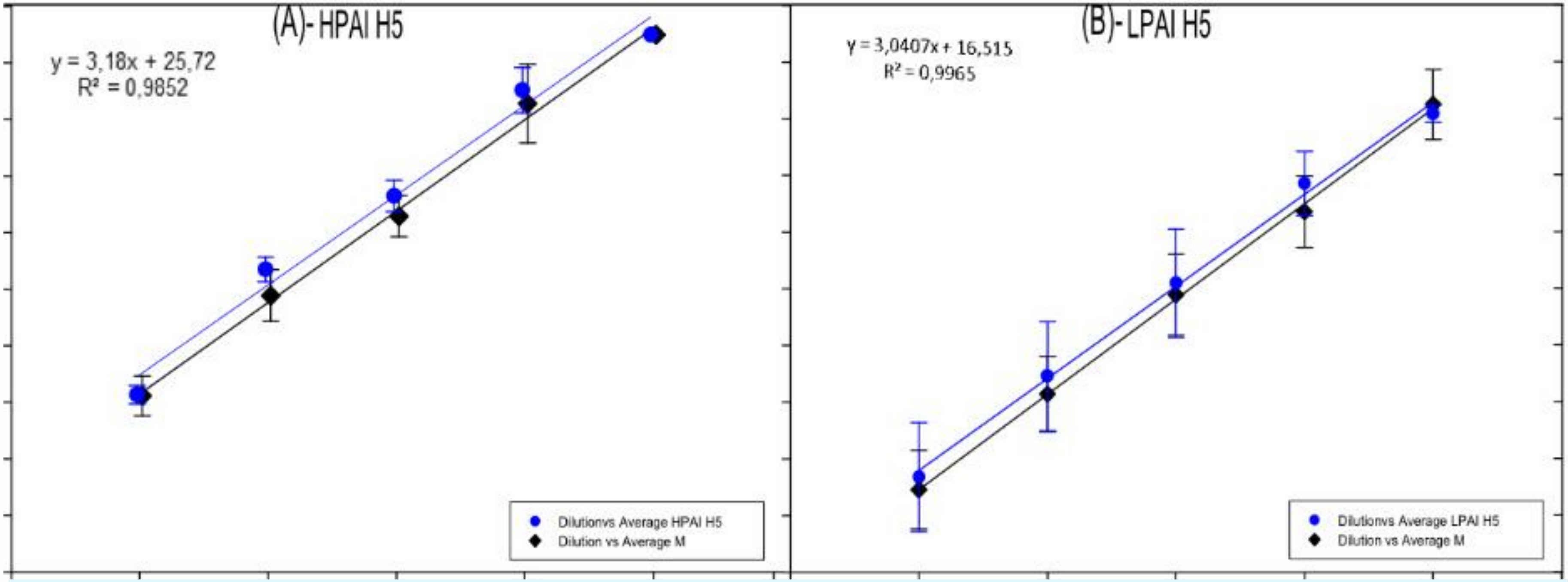


Probes span cleavage site and are anchored in the fusion peptide encoding sequence stretch

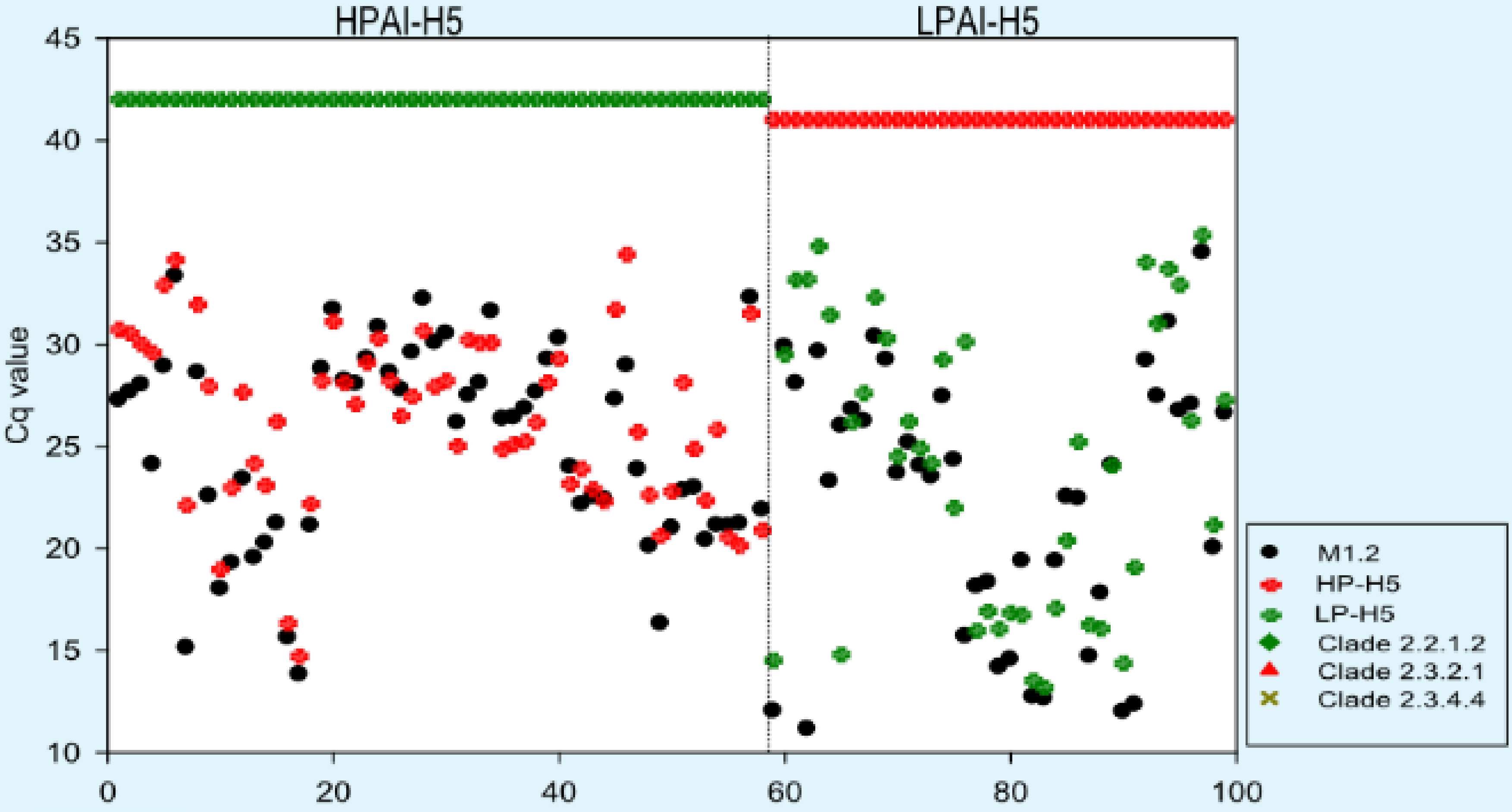
Naguib et al., 2016

Virus	**Patho- and phylotype**	**PCR method**	
		H5-HPAI	**H5-LPAI**
A/turkey/Turkey/1/2005 (H5N1)	HP Clade 2.2	+	-
A/chicken/Egypt/0879-NLQP/R737/2008 (H5N1)	HP Clade 2.2.1.1	+	-
A/chicken/Egypt/NLQP7FL-AR747/ 2013 (H5N1)	HP Clade 2.2.1.2	+	-
A/duck/Egypt/AR236-A3NLQP/2015 (H5N1)	HP Clade 2.2.1.2	+	-
A/turkey/Egypt/AR238-SD177NLQP/2014 (H5N1)	HP Clade 2.2.1.2	+	-
A/Falco peregrinus/Dubai/AR3430/2014 (H5N1)	HP Clade 2.3.2.1c	+	-
A/quail/Dubai/AR3445-2504.3/2014 (H5N1)	HP Clade 2.3.2.1c	+	-
A/duck/Bangladesh/D3-AR2111/2013 (H5N1)	HP Clade 2.3.2.1a	+	-
A/turkey/Germany/AR2485-86/2014 (H5N8)	HP Clade 2.3.4.4a	+	-
A/turkey/Germany-MV/AR2472/2014 (H5N8)	HP Clade 2.3.4.4a	+	-
A/Tufted-duck/Germany/AR8444/2016 (H5N8)	HP Clade 2.3.4.4b	+	-
A/chicken/Indonesia/R132/2004 (H5N1)	HP Clade 2.1.1	+	-
A/chicken/Indonesia/R134/2003 (H5N1)	HP Clade 2.1.1	+	-
A/chicken/Indonesia/R60/2005 (H5N1)	HP Clade 2.1.1	+	-
A/Vietnam/1194/2004 (H5N1)	HP Clade 1.1	+	-
A/chicken/GXLA/1204/2004 (H5N1)	HP Clade 2.4	+	-
A/chicken/Vietnam/P41/2005 (H5N1)	HP Clade 1.1	+	-
A/chicken/Vietnam/P78/2005 (H5N1)	HP Clade 1.1	+	-
A/common teal/Germany/Wv1378-79/2003(H5N2)	LP	-	+
A/duck/Germany/R1789/2008 (H5N3)	LP	-	+
A/turkey/Germany/AR915/2015 (H7N7)	-	-	-
A/chicken/Egypt/AR754-14/2013 (H9N2)	-	-	-
A/chicken/Sudan/AR251-15/2014 (IBV)	-	-	-
A/chicken/Egypt/AR254-15/2014 (NDV)	-	-	-

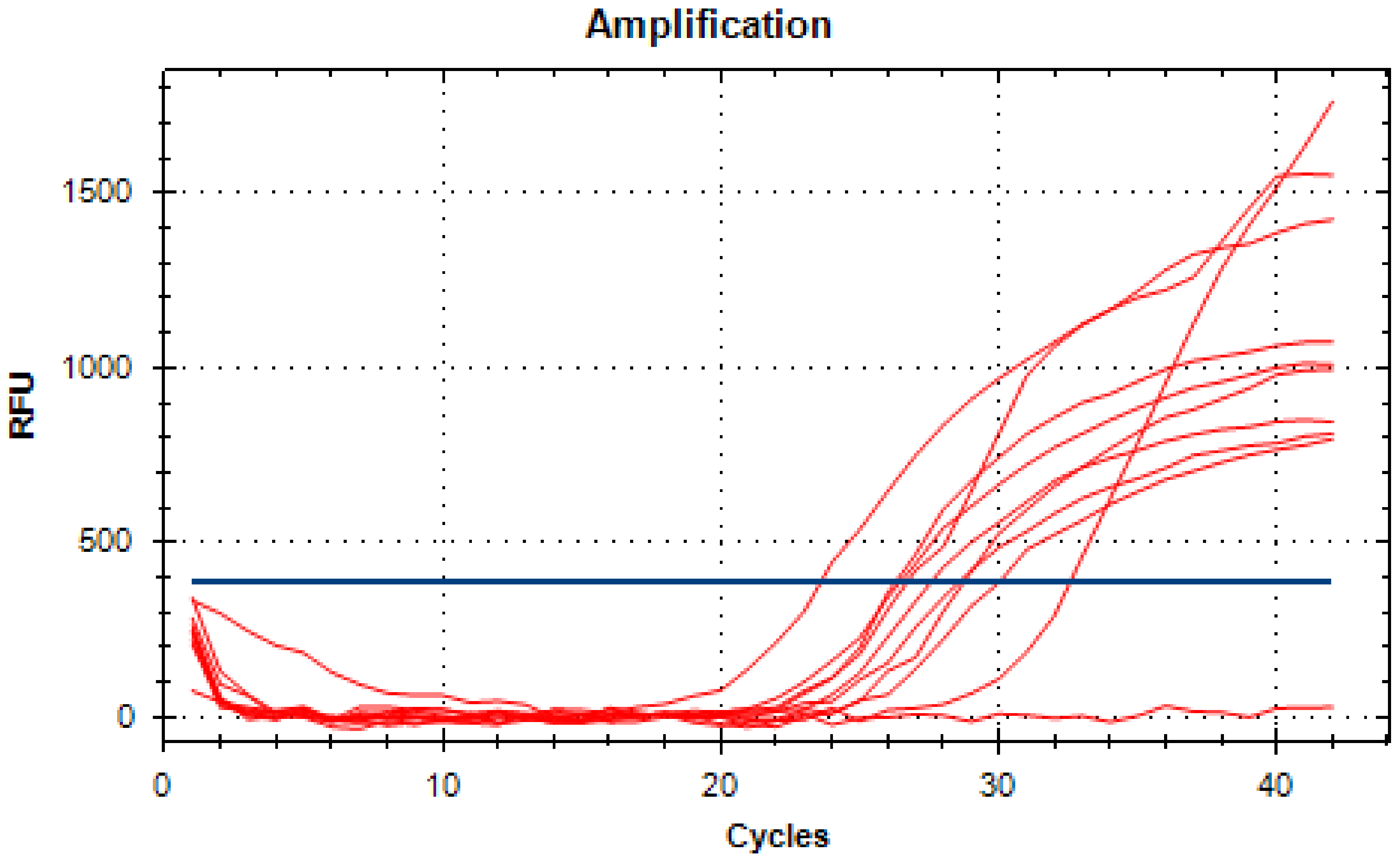
Similar sensitivity of H5 pathotyping and M1.2 RT-qPCRs



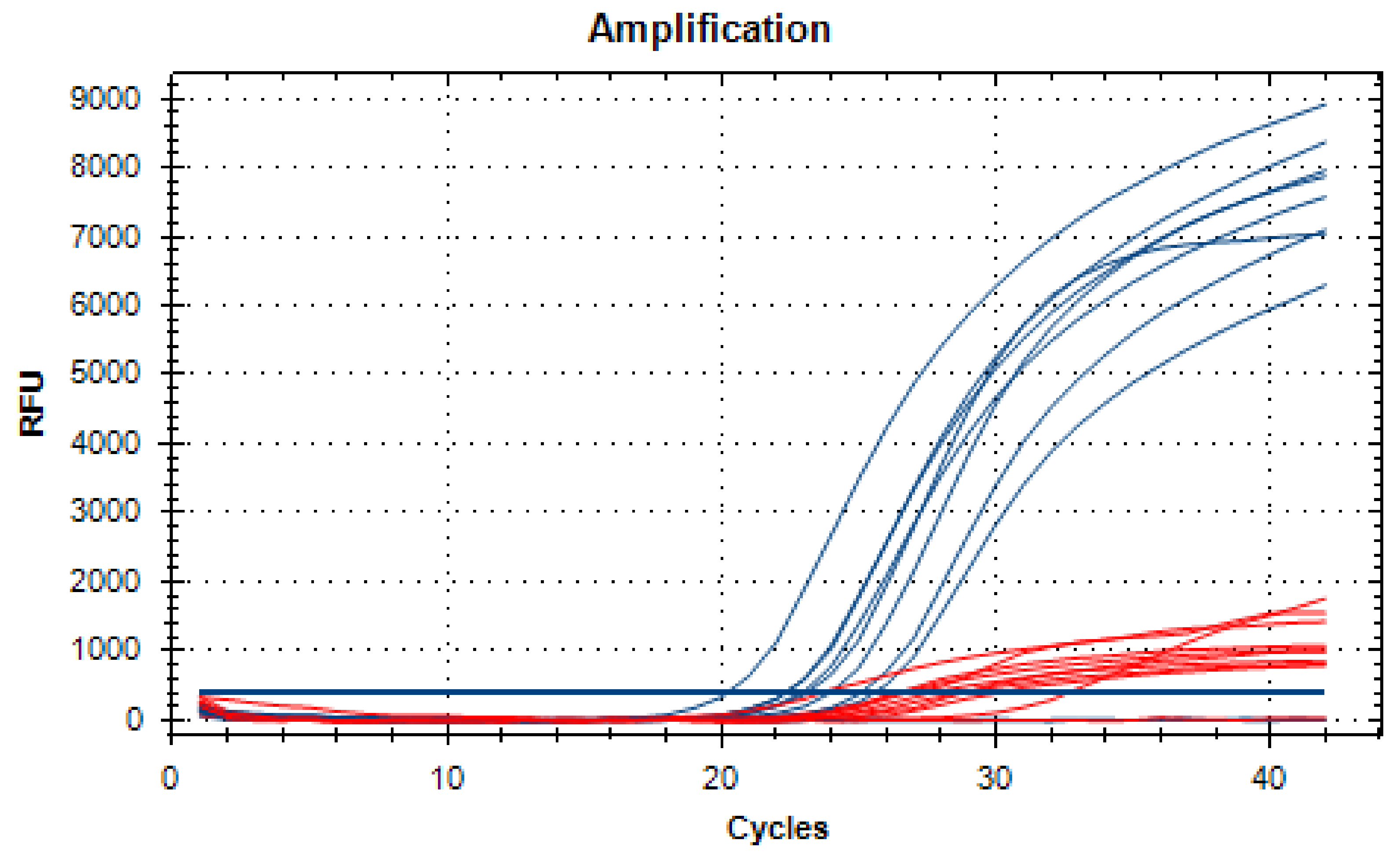
H5 pathotyping RT-qPCRs, field samples



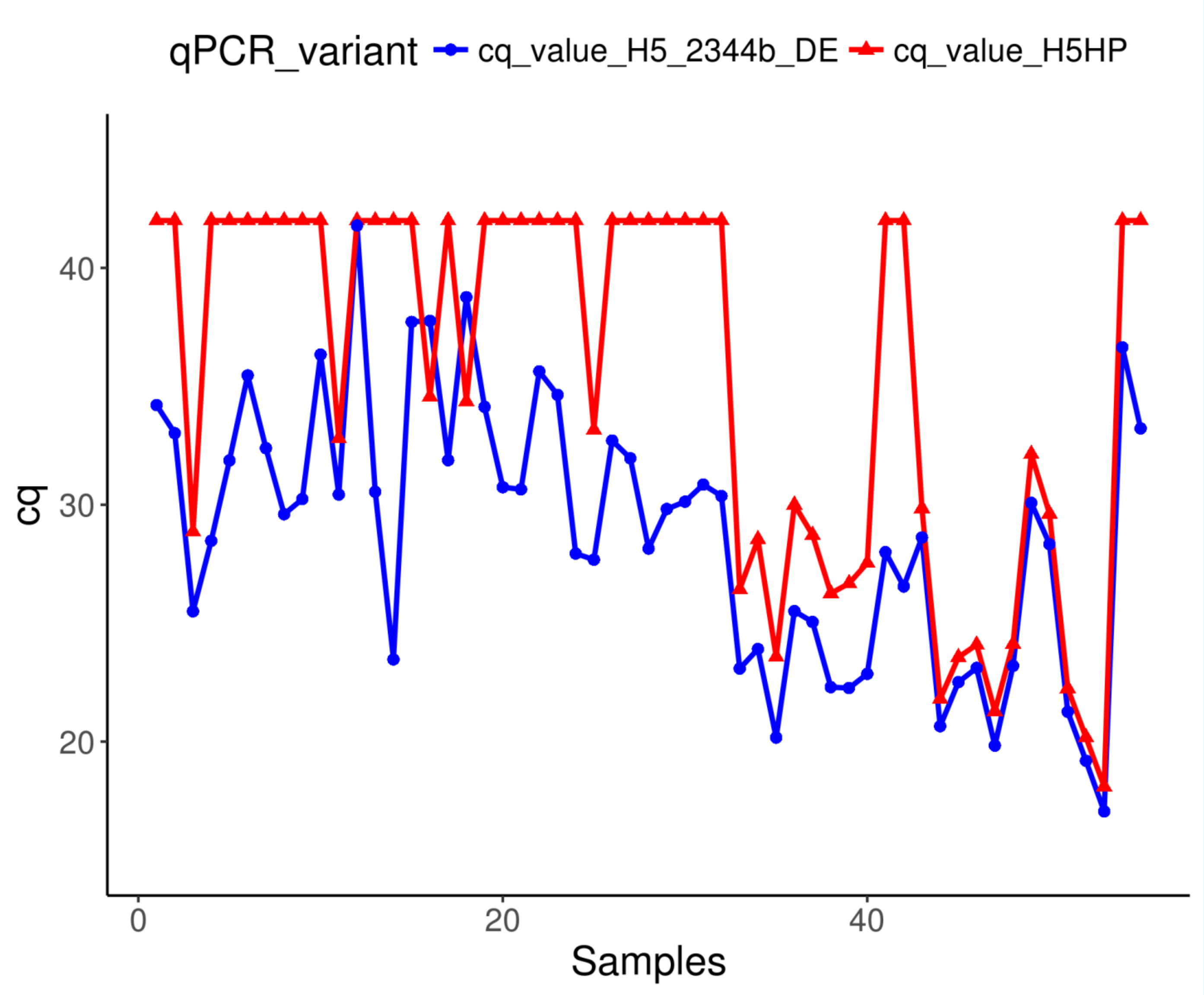
Subtyping RT-qPCR H5HP - Naguib



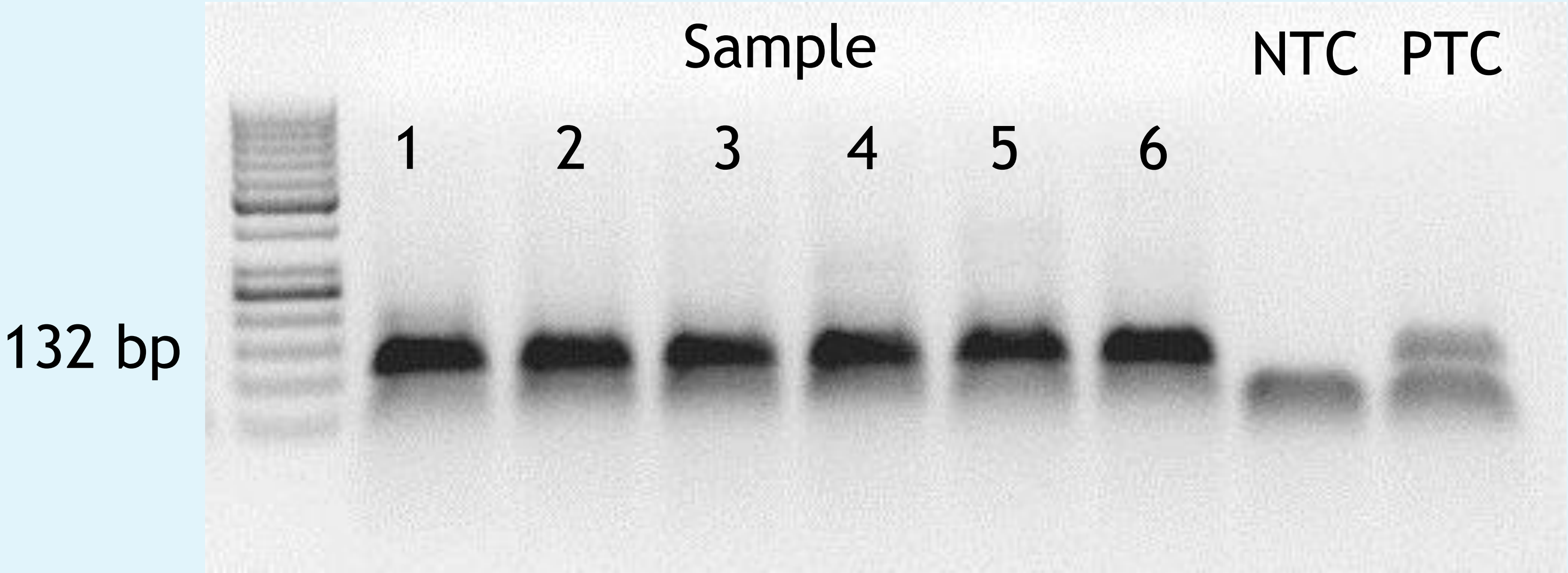
Subtyping RT-qPCR H5HP - De



Subtyping RT-qPCR H5HP - comp

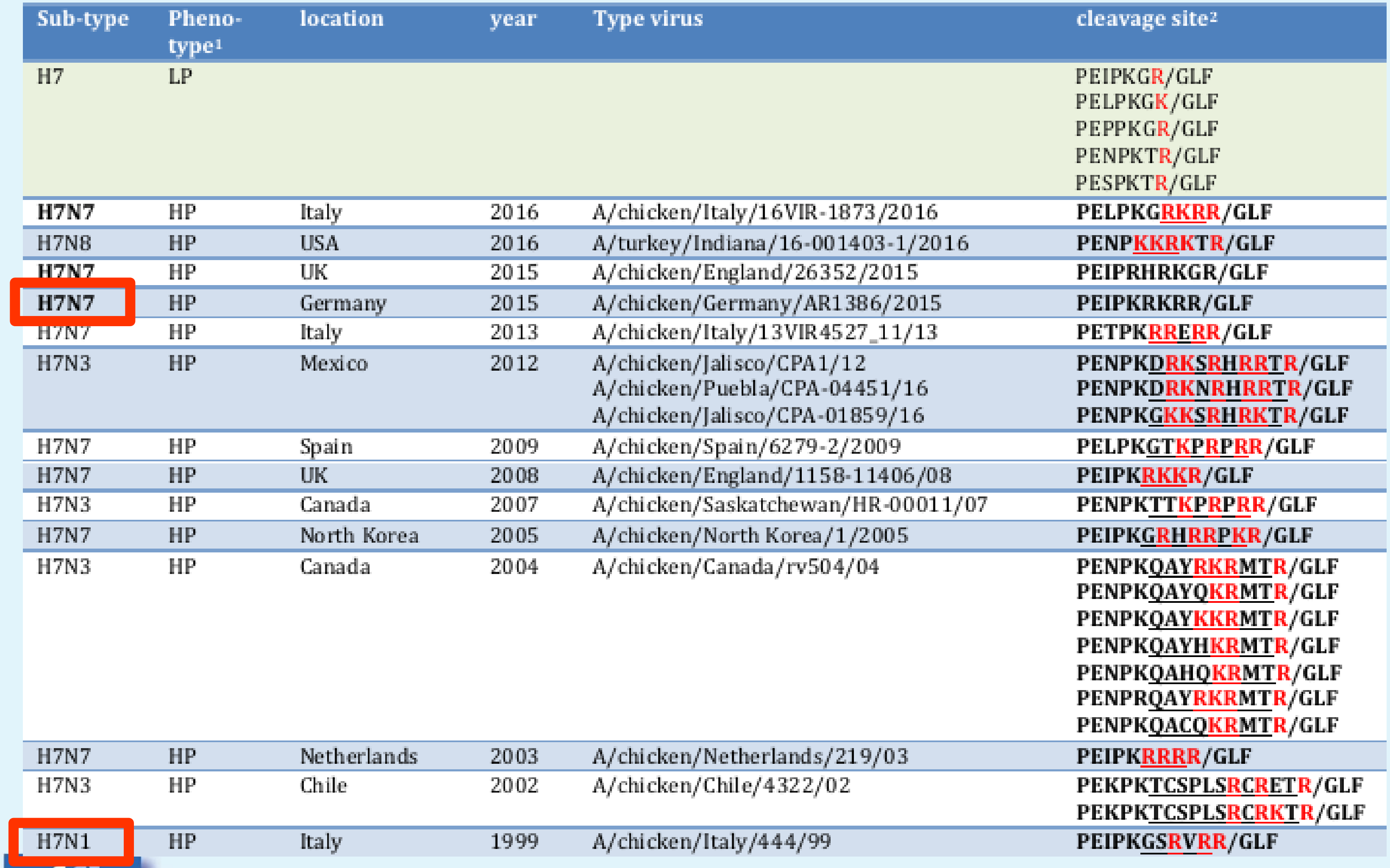


Sequencing from RT-qPCR amplificates



- HP H5 RT-qPCR 2.3.4.4b
- Expected product size 124-138 bp
- Tubes need to be opened (cave contamination)

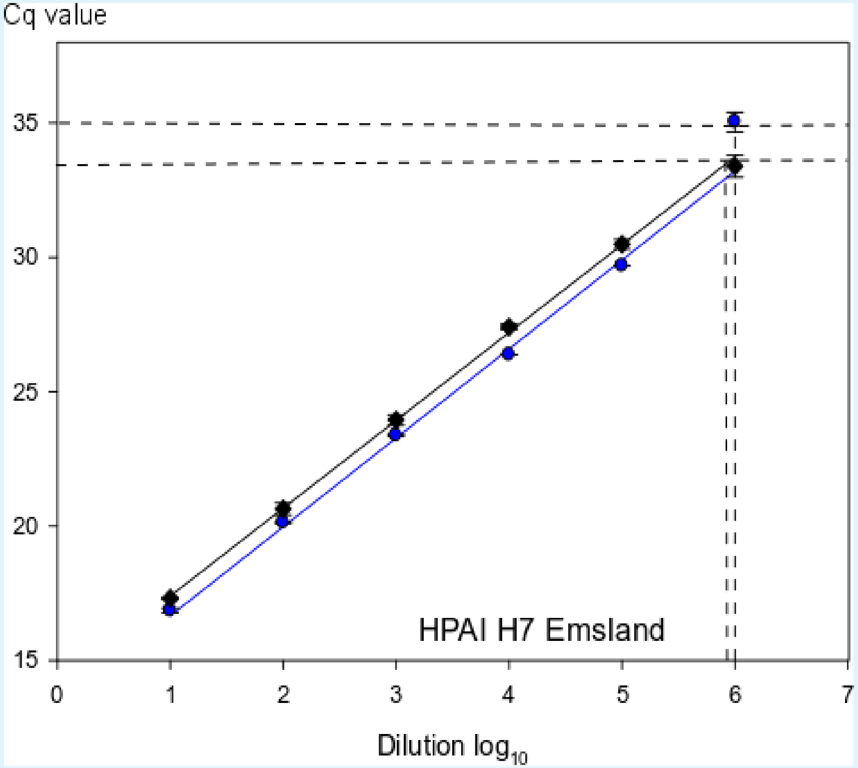
H7 HP cleavage sites are not at all conserved

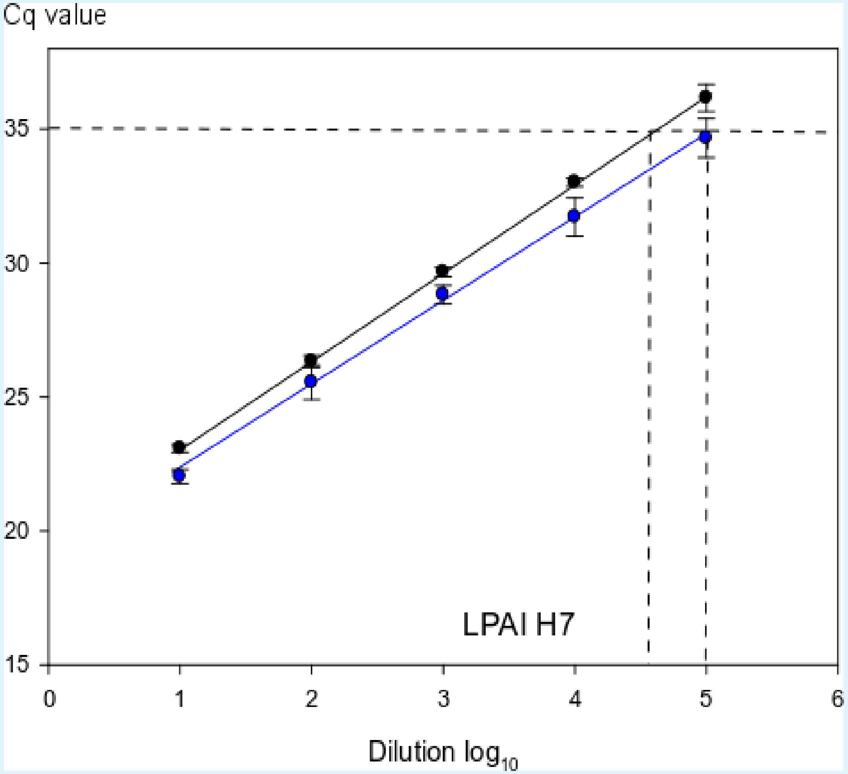


Sub-type	Pheno-type[1]	location	year	Type virus	cleavage site[2]
H7	LP				PEIPKGR/GLF PELPKGK/GLF PEPPKGR/GLF PENPKTR/GLF PESPKTR/GLF
H7N7	HP	Italy	2016	A/chicken/Italy/16VIR-1873/2016	**PELPKGRKRR/GLF**
H7N8	HP	USA	2016	A/turkey/Indiana/16-001403-1/2016	**PENPKKRKTR/GLF**
H7N7	HP	UK	2015	A/chicken/England/26352/2015	**PEIPRHRKGR/GLF**
H7N7	HP	Germany	2015	A/chicken/Germany/AR1386/2015	**PEIPKRKRR/GLF**
H7N7	HP	Italy	2013	A/chicken/Italy/13VIR4527_11/13	**PETPKRRERR/GLF**
H7N3	HP	Mexico	2012	A/chicken/Jalisco/CPA1/12 A/chicken/Puebla/CPA-04451/16 A/chicken/Jalisco/CPA-01859/16	**PENPKDRKSRHRRTR/GLF** **PENPKDRKNRHRRTR/GLF** **PENPKGKKSRHRKTR/GLF**
H7N7	HP	Spain	2009	A/chicken/Spain/6279-2/2009	**PELPKGTKPRPRR/GLF**
H7N7	HP	UK	2008	A/chicken/England/1158-11406/08	**PEIPKRKKR/GLF**
H7N3	HP	Canada	2007	A/chicken/Saskatchewan/HR-00011/07	**PENPKTTKPRPRR/GLF**
H7N7	HP	North Korea	2005	A/chicken/North Korea/1/2005	**PEIPKGRHRRPKR/GLF**
H7N3	HP	Canada	2004	A/chicken/Canada/rv504/04	**PENPKQAYRKRMTR/GLF** **PENPKQAYQKRMTR/GLF** **PENPKQAYKKRMTR/GLF** **PENPKQAYHKRMTR/GLF** **PENPKQAHQKRMTR/GLF** **PENPRQAYRKRMTR/GLF** **PENPKQACQKRMTR/GLF**
H7N7	HP	Netherlands	2003	A/chicken/Netherlands/219/03	**PEIPKRRRR/GLF**
H7N3	HP	Chile	2002	A/chicken/Chile/4322/02	**PEKPKTCSPLSRCRETR/GLF** **PEKPKTCSPLSRCRKTR/GLF**
H7N1	HP	Italy	1999	A/chicken/Italy/444/99	**PEIPKGSRVRR/GLF**

- Data from OFFLU

Similar sensitivity of H7 pathotyping and M1.2 RT-qPCRs





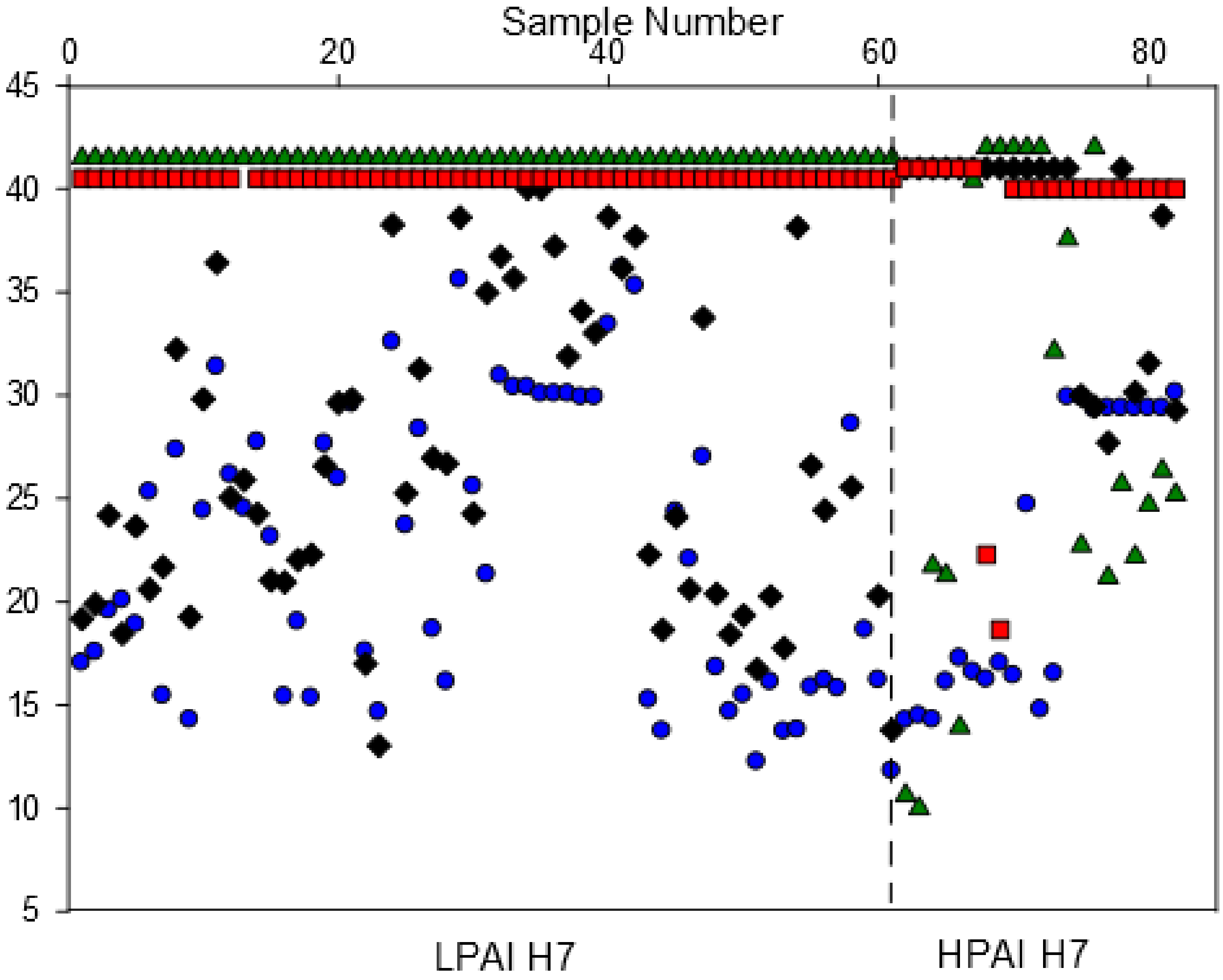
Sequencing-independent pathotyping H7 LP and HP RT-qPCRs, field samples

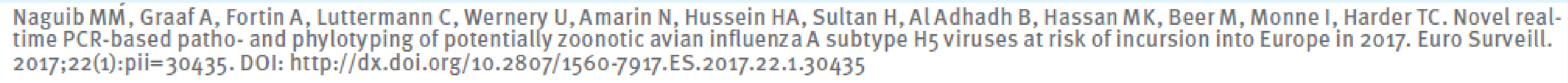
● M 1.3

◆ H7 LP

🟥 H7 HP-It

▲ H7 HP-De

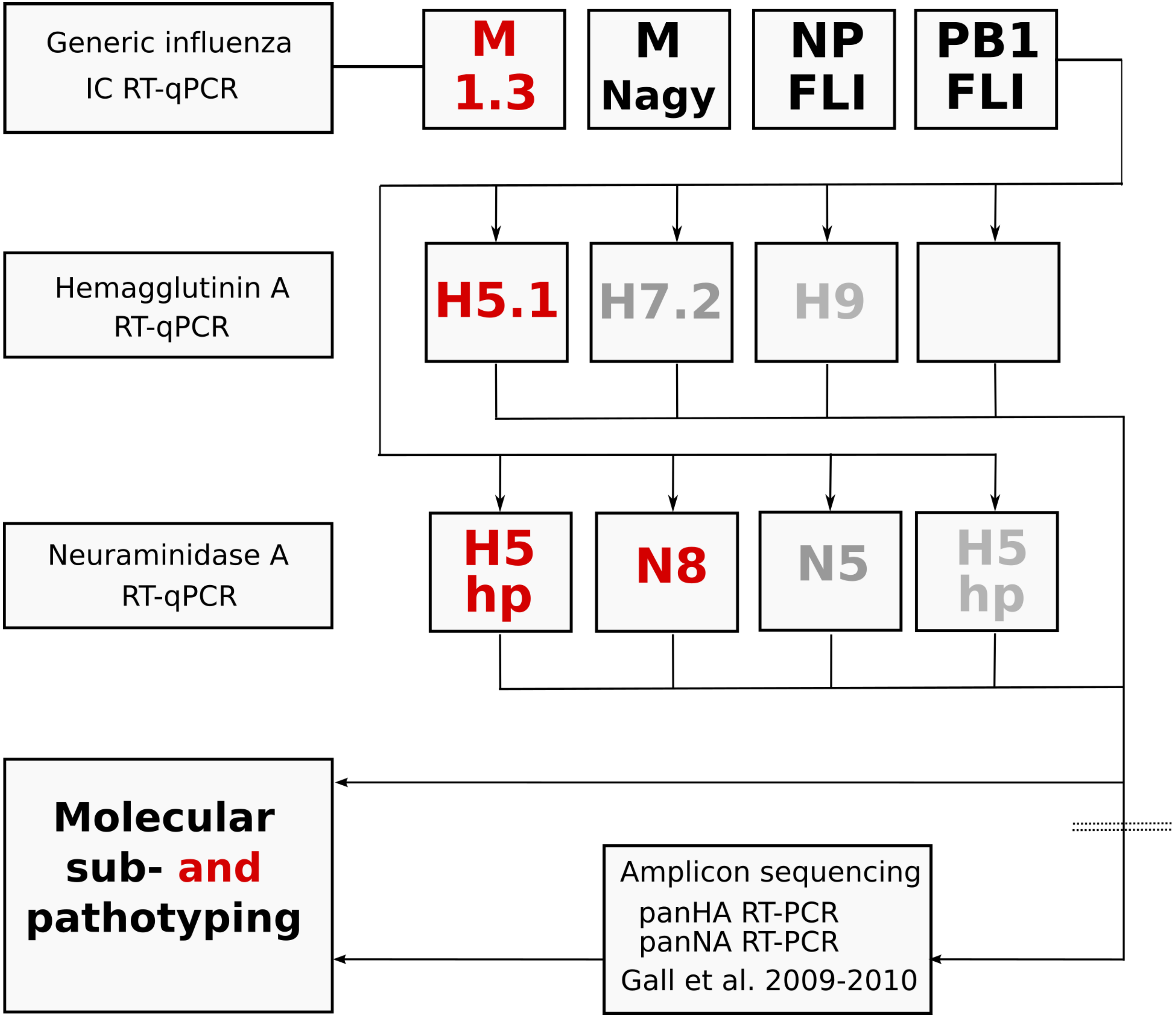




Naguib MM, Graaf A, Fortin A, Luttermann C, Wernery U, Amarin N, Hussein HA, Sultan H, Al Adhadh B, Hassan MK, Beer M, Monne I, Harder TC. Novel real-time PCR-based patho- and phylotyping of potentially zoonotic avian influenza A subtype H5 viruses at risk of incursion into Europe in 2017. Euro Surveill. 2017;22(1):pii=30435. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2017.22.1.30435

SISPA

New proposed diagnostic algorithm for sequencing-independent sub- and pathotyping of avian influenza viruses



Generic influenza
IC RT-qPCR

M 1.3

M Nagy

NP FLI

PB1 FLI

Hemagglutinin A
RT-qPCR

H5.1

H7.2

H9

Neuraminidase A
RT-qPCR

H5 hp

N8

N5

H5 hp

Molecular sub- and pathotyping

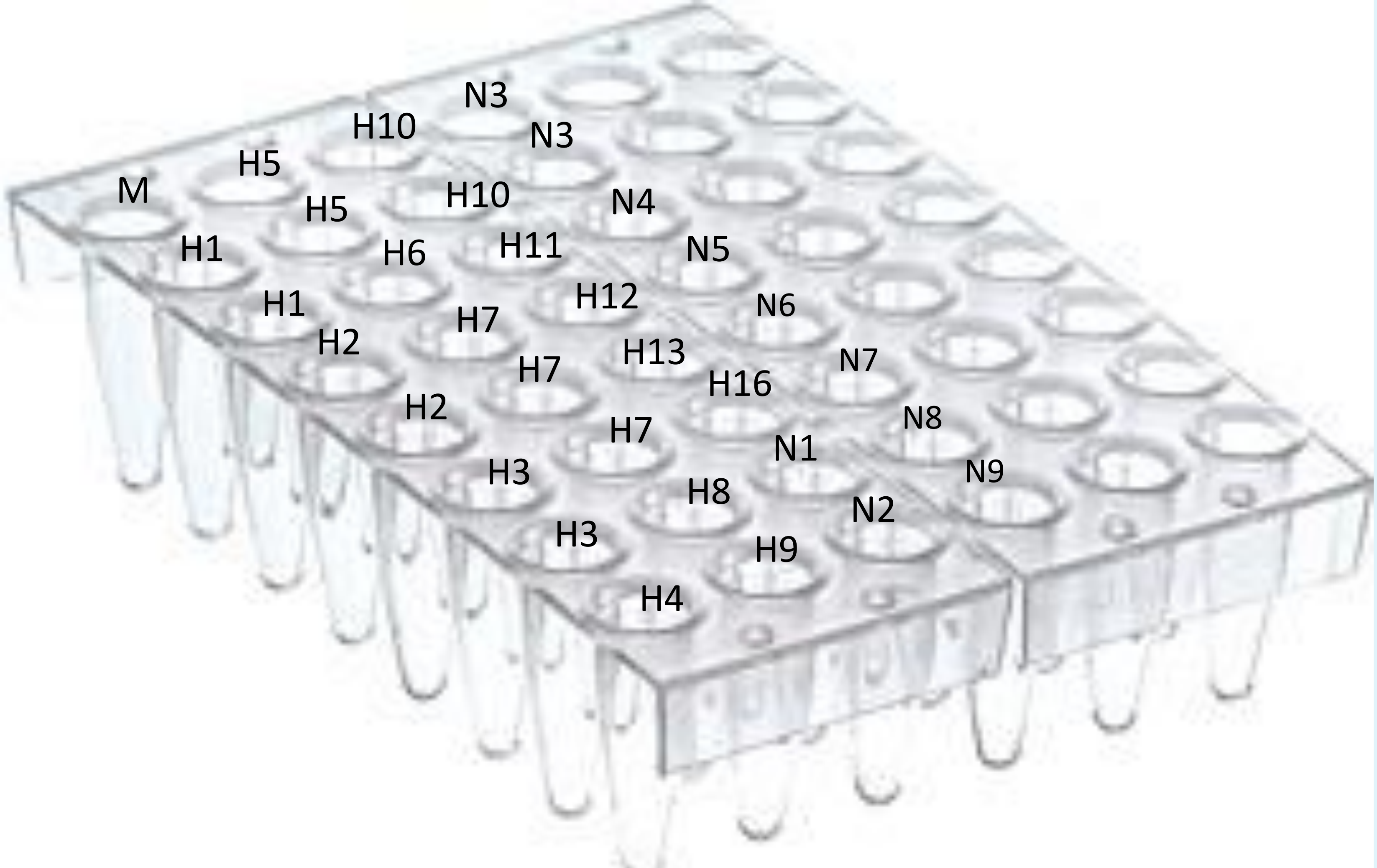
Amplicon sequencing
panHA RT-PCR
panNA RT-PCR
Gall et al. 2009-2010

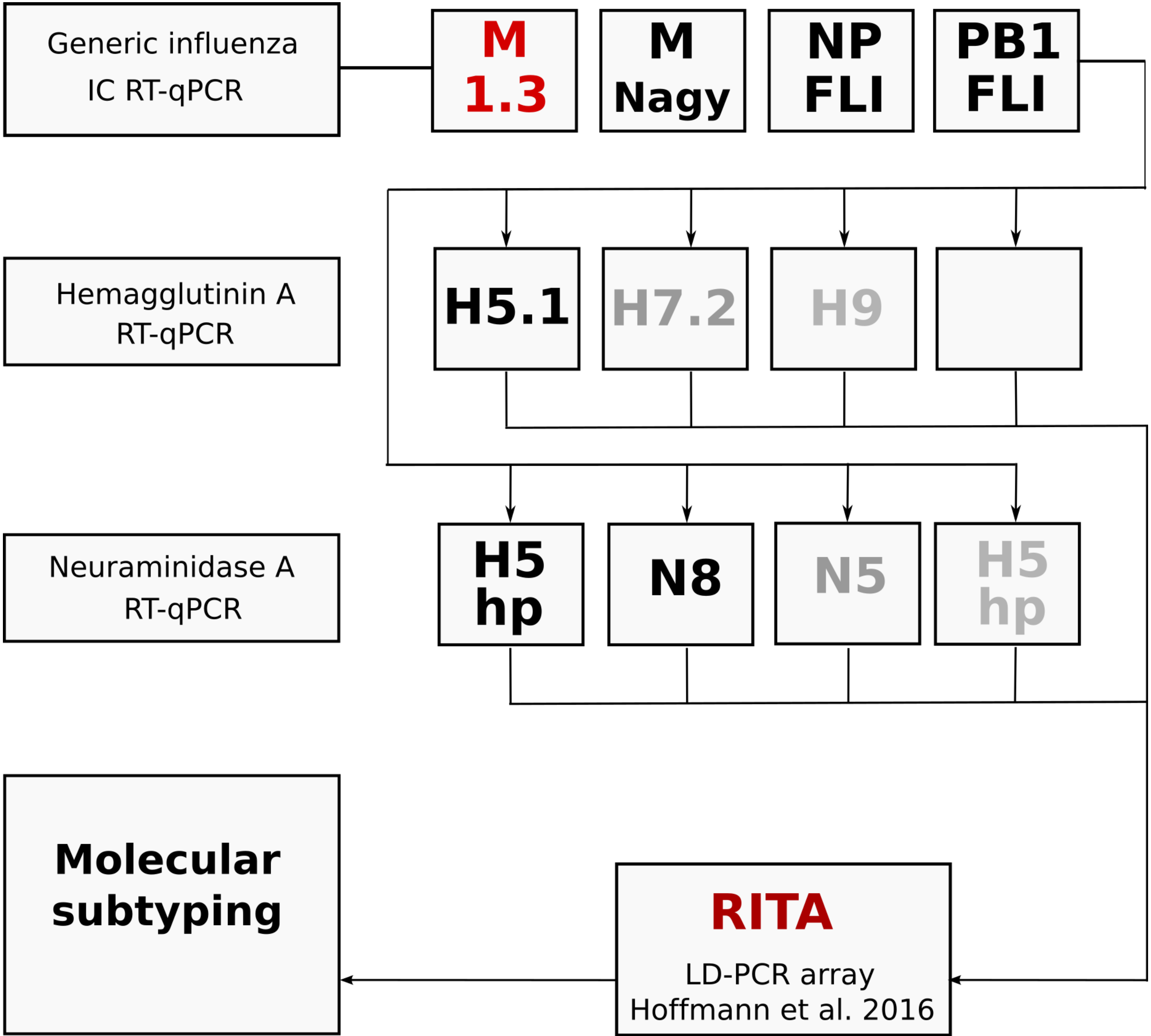
SCIENTIFIC REPORTS | 6:27211 | DOI: 10.1038/srep27211

RITA

Riems influenza typing assay: Extended sets of RT-qPCR to detect 16 HA and 9 NA subtypes of avian influenza viruses

RITA – a low density PCR array





Generic influenza
IC RT-qPCR

M 1.3

M Nagy

NP FLI

PB1 FLI

Hemagglutinin A
RT-qPCR

H5.1

H7.2

H9

Neuraminidase A
RT-qPCR

H5 hp

N8

N5

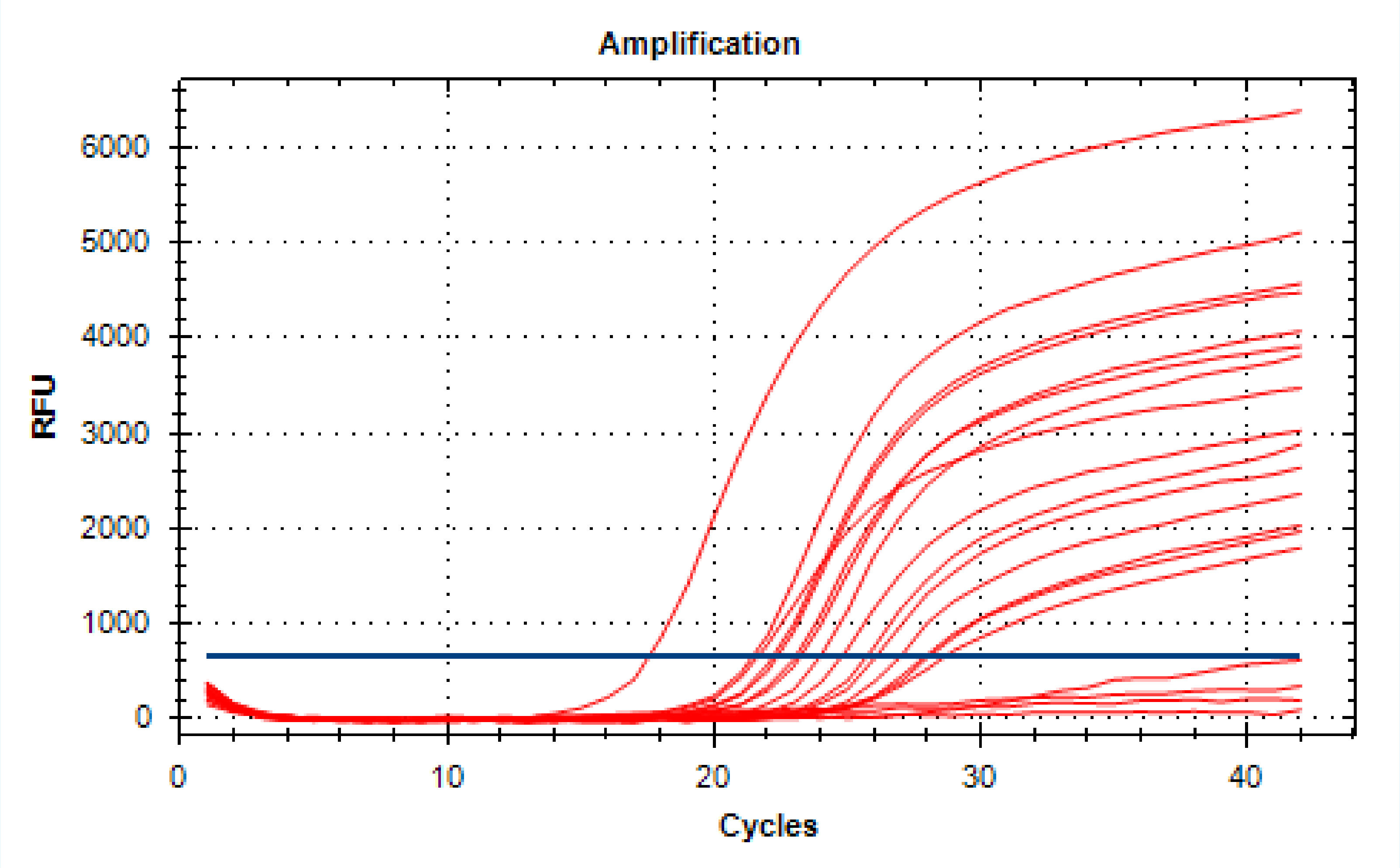
H5 hp

Molecular subtyping

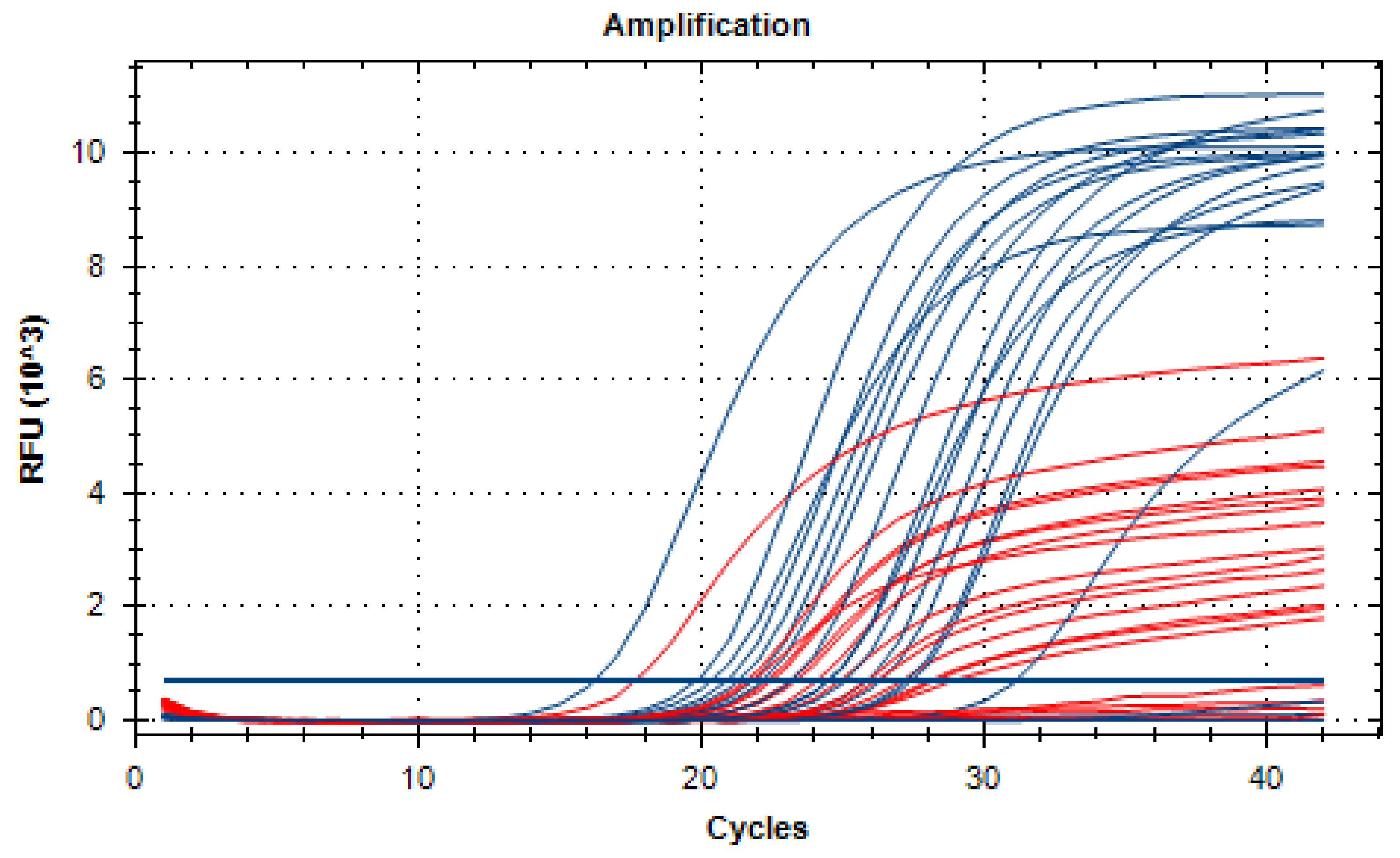
RITA

LD-PCR array
Hoffmann et al. 2016

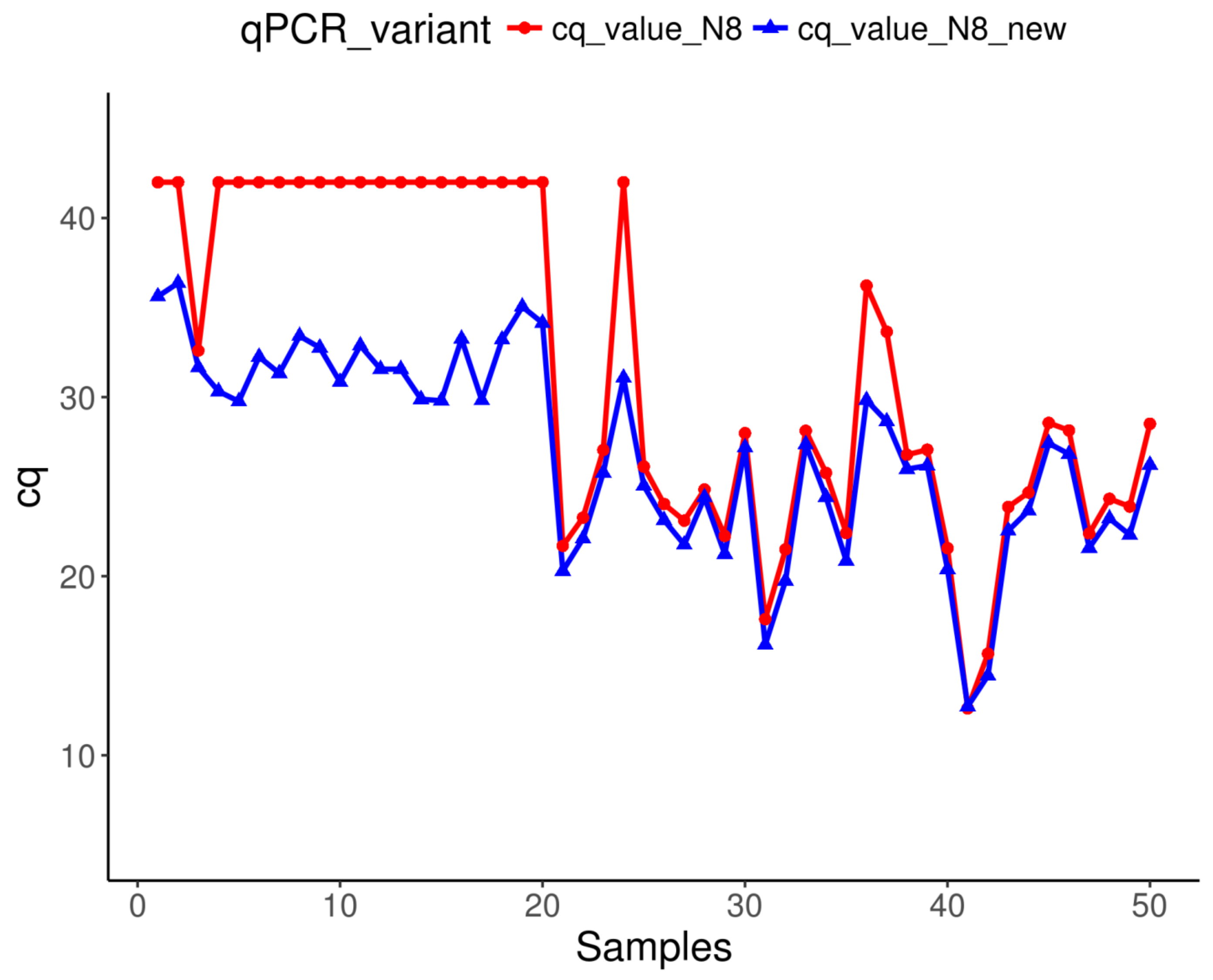
Subtyping RT-qPCR N8 - RITA



Subtyping RT-qPCR N8 - De

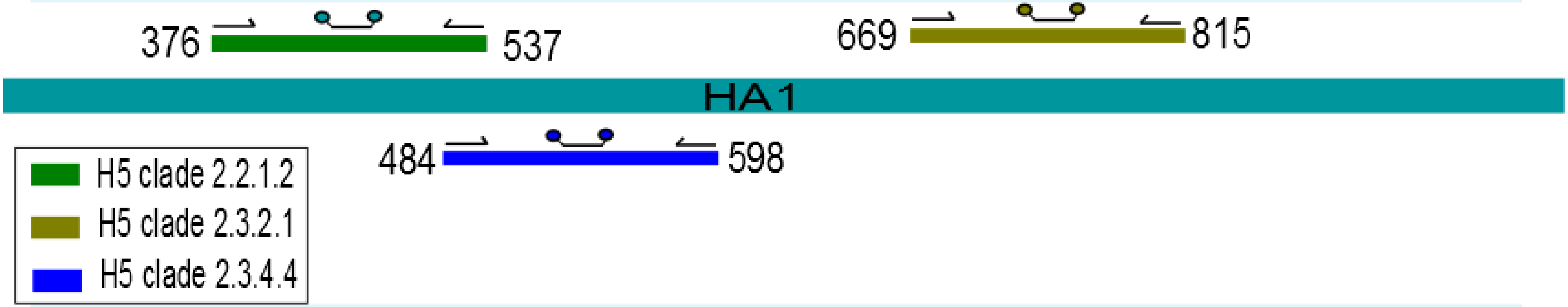


Subtyping RT-qPCR N8 - comp



Distinguishing gs/GD clades und lineages by RT-qPCR

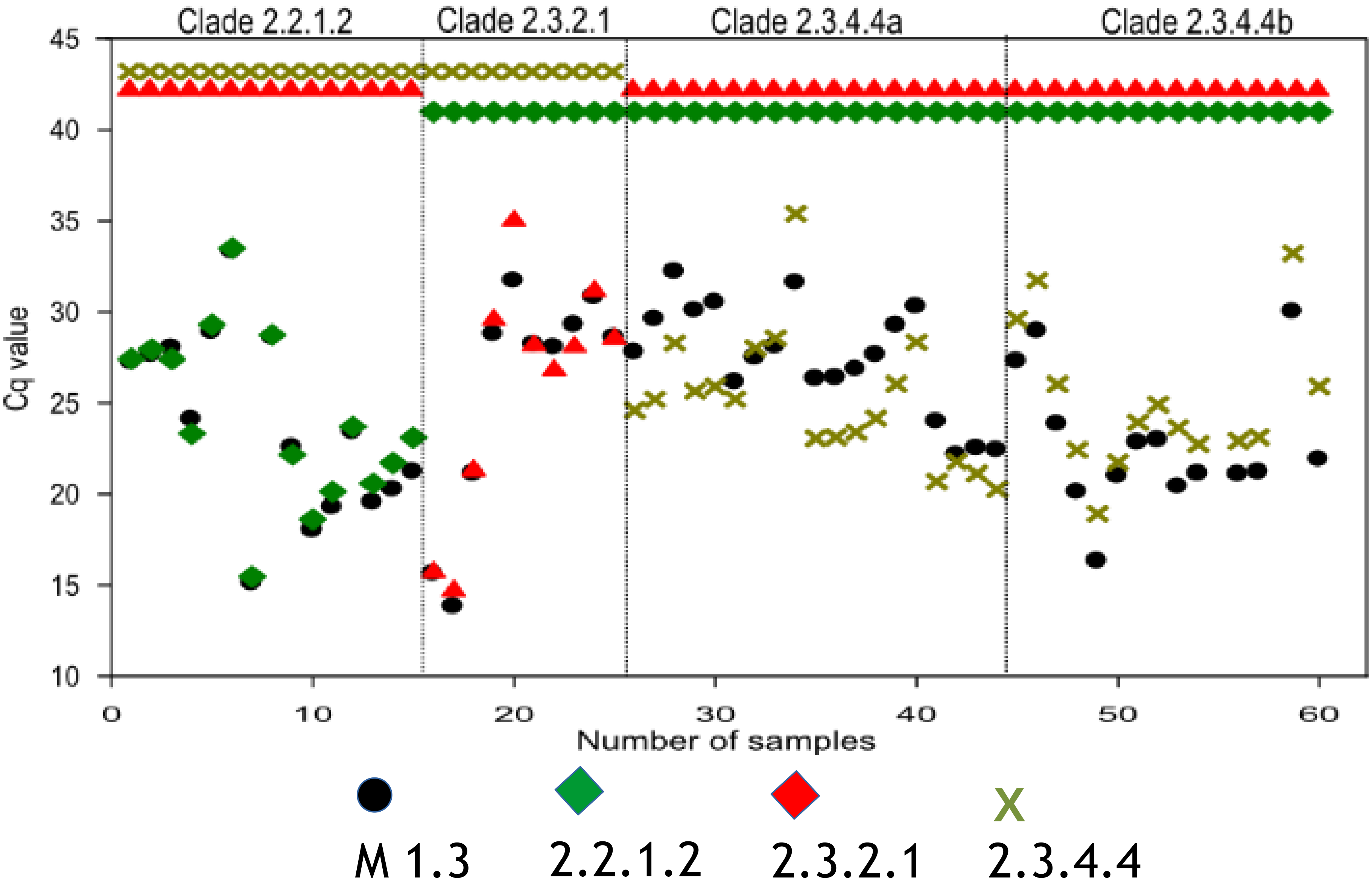
- Antigenic variation affecting vaccine efficacy between and within gs/GD clades
- Co-circulation of different clades/lineages/escape mutants reported from Vietnam, China, **Egypt**



Naguib et al., 2016

Virus	Patho- and phylotype	Clade 2.2.1.2	Clade 2.3.2.1	Clade 2.3.4.4
A/turkey/Turkey/1/2005 (H5N1)	HP Clade 2.2	+	-	-
A/chicken/Egypt/0879-NLQP/R737/2008 (H5N1)	HP Clade 2.2.1.1	-	-	-
A/chicken/Egypt/NLQP7FL-AR747/ 2013 (H5N1)	HP Clade 2.2.1.2	+	-	-
A/duck/Egypt/AR236-A3NLQP/2015 (H5N1)	HP Clade 2.2.1.2	+	-	-
A/turkey/Egypt/AR238-SD177NLQP/2014 (H5N1)	HP Clade 2.2.1.2	+	-	-
A/Falco peregrinus/Dubai/AR3430/2014 (H5N1)	HP Clade 2.3.2.1c	-	+	-
A/quail/Dubai/AR3445-2504.3/2014 (H5N1)	HP Clade 2.3.2.1c	-	+	-
A/duck/Bangladesh/D3-AR2111/2013 (H5N1)	HP Clade 2.3.2.1a	-	+	-
A/turkey/Germany/AR2485-86/2014 (H5N8)	HP Clade 2.3.4.4a	-	-	+
A/turkey/Germany-MV/AR2472/2014 (H5N8)	HP Clade 2.3.4.4a	-	-	+
A/Tufted-duck/Germany/AR8444/2016 (H5N8)	HP Clade 2.3.4.4b	-	-	+
A/chicken/Indonesia/R132/2004 (H5N1)	HP Clade 2.1.1	-	-	-
A/chicken/Indonesia/R134/2003 (H5N1)	HP Clade 2.1.1	-	-	-
A/chicken/Indonesia/R60/2005 (H5N1)	HP Clade 2.1.1	-	-	-
A/Vietnam/1194/2004 (H5N1)	HP Clade 1.1	-	-	-
A/chicken/GXLA/1204/2004 (H5N1)	HP Clade 2.4	-	-	-
A/chicken/Vietnam/P41/2005 (H5N1)	HP Clade 1.1	-	-	-
A/chicken/Vietnam/P78/2005 (H5N1)	HP Clade 1.1	-	-	-
A/common teal/Germany/Wv1378-79/2003(H5N2)	LP	-	-	-
A/duck/Germany/R1789/2008 (H5N3)	LP	-	-	-

Sequencing-independent „clading“ of gs/GD HPAIV H5, field samples



Conclusions

- Generic detection of influenza viruses by RT-qPCRs is an established routine measure.
- Subtyping by RT-qPCR can be applied to all sub-types and various lineages of AIV.
- Sequencing-independent pathotyping by RT-qPCRs can be applied to routine diagnostics.
- Established RT-qPCRs and proposed algorithms may require adaptation to and fine tuning for improved performance in circumscript outbreaks.
- All PCRs published and/or available from FLI .