Addressing avian influenza A(H7N9)

Laboratory protocols and algorithms
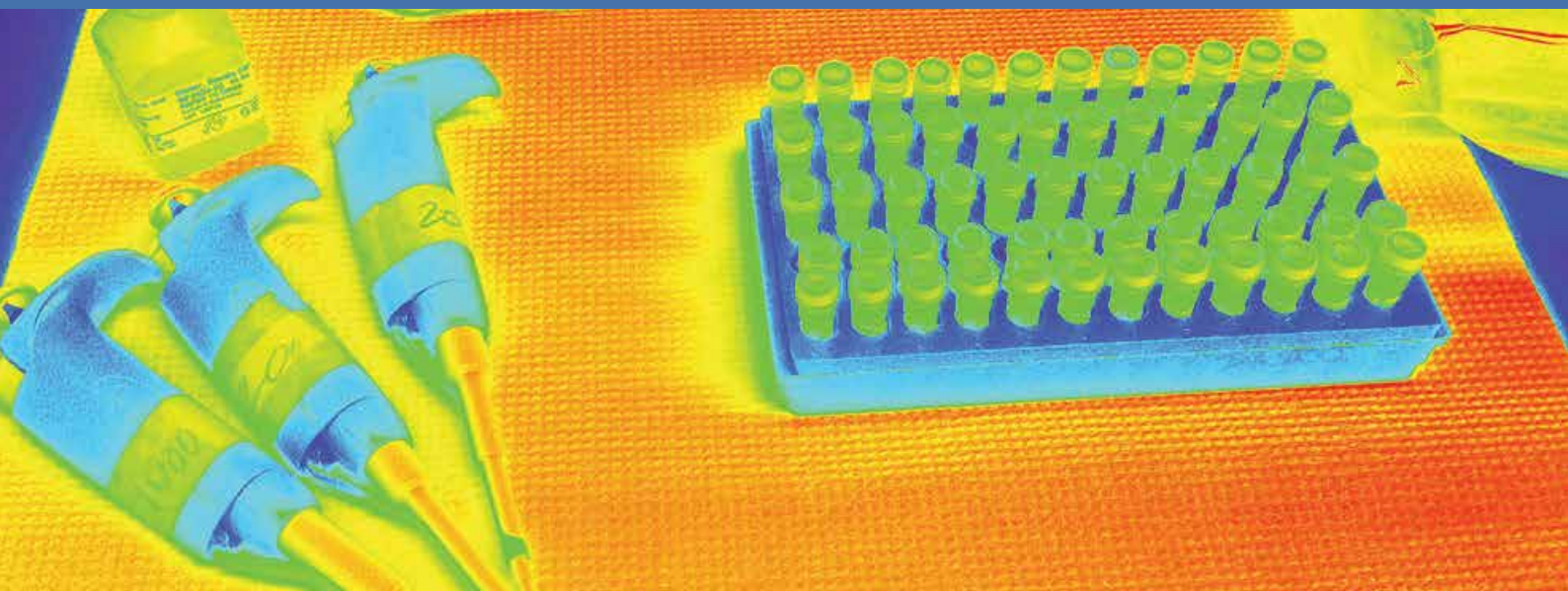
CONTENTS

INTRODUCTION

In response to the emergence of the novel avian influenza A(H7N9) virus in China in March 2013, public and veterinary health sectors have collaborated to make diagnostic protocols available to governments and research institutions. Chinese authorities have been sharing H7N9 virus isolates and their sequences and other phenotypic and genotypic data. This has enabled international laboratories to evaluate the biological properties of the virus and improve their own diagnostic protocols and procedures.

The Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (OIE), the World Health Organization (WHO) and other partners have been working with the Government of the People's Republic of China to help respond to the situation. The OIE/FAO network of expertise on animal influenza (OFFLU) has compiled laboratory algorithms, protocols and validation data for the detection and characterization of H7N9. This guidance has been validated by OFFLU reference centres and WHO collaborating centres.

FAO recommends member countries apply OFFLU guidance for H7N9 diagnosis. All protocols are fit for purpose, since they are highly sensitive and specific in detecting H7 viruses in the field, including H7N9.

Early during the H7N9 event, FAO in collaboration with its reference centres chose to proceed with the Friedrich Löffler Institut (FLI) protocols as preferred procedures for the detection of H7N9. FAO made this choice on the predicted reaction of these primers and probes with the sequences that became available. There were no mismatches in sequences when compared to A/Anhui/1/2013. Information on this and other validated protocols for the detection of H7N9 are publicly available on the OFFLU website: www.offlu.net.

The Australian Animal Health Laboratories (AAHL) in Geelong developed the previous diagnostic algorithms for the detection of avian influenza viruses (AIVs) in the Southeast Asia region. AAHL developed these algorithms in consultation with the Association of Southeast Asian Nations (ASEAN) regional animal health laboratory network. FAO updated the algorithms to accommodate H7N9 screening in addition to H5N1 highly pathogenic avian influenza which continues to circulate in the region.

This document describes the protocols from FLI and the updated laboratory algorithms from AAHL and ASEAN. It also provides links to other reference documents. These include the WHO N9 protocol, alternative H7 conventional and reverse-transcriptase (RT) polymerase chain reaction (PCR) protocols and validation data provided by OFFLU contributing laboratories.

FAO has provided three categories of guidance for the laboratory testing of animal samples for the presence of avian influenza A(H7N9) virus:

1. **Overview of primers and probes**
2. **Validated RT-PCR protocols**
3. **Surveillance laboratory flow chart**

1. OVERVIEW OF PRIMERS AND PROBES

1.1 Primers and probes for the detection of H7N9 in real-time RT-PCR assays (*see table below*)

H7 ASSAYS	Prediction for H7N9 detection	Primer forward	Primer reverse	Probe	Dye	Quencher
CNIC H7(N9) rRT-PCR	VALIDATED	AGAAA TGAAATGGCT CCTGTCAA	GGTTTTTTCTT GTATTTTTATA TGACTTAG	AGATAATGCT GCATTCCCGC AGATG	FAM	BHQ1
rRT_PCR_CODA	VALIDATED	GYAGYGGYTACAAA GATGTG	GAAGACAAG GCCCATTGC AA	TGGTTTAGCTT CGGGGC	not specified	
rRT-PCR_IZSVe	VALIDATED	TTTGGTTTAGCTTCGGG	GAAGAMAAG GCYCATTG	CATCATGTTTC RTACTTCT	VIC	MGB
Eurasian H7 HA2 rRT-PCR-AHVLA	VALIDATED	GGCCAGTA TTAGAAACAAC ACCTATGA	GCCCCGAAGC TAAACCAAAG TAT	CCGCTGCTTA GTTTGACTGG GTCAATCT	FAM	BHQ-1
rRT-PCR H7.2-FLI	VALIDATED	AYA GAA TAC AGA TWG ACC CAGT	TAG TGC ACY GCA TGT TTC CA	TGG TTT AGC TTC GGG GCA TCA TG	FAM	BHQ-1
Eurasian H7 rRT-PCR assay 2-HKU	PREDICTED to work	ATAGATAGCAG GGCAGTTGG	GATCWATTGC HGAYTGRGTG	CCYTCYCCYTG TGCRTTYTG	FAM	3BHQ1
Eurasian H7 rRT-PCR assay 1-HKU	PREDICTED to work	ATMAATAGCAG RGCARTRGG	GATCWATTGC HGAYTGRGTG	CCYTCYCCYTG TGCRTTYTG	FAM	3BHQ1
H7 CS rRT-PCR-AHVLA	PREDICTED to work	CGTGCAAGTTTT CTGAGAGG	GACCTTCCCAT CCATTTTCA	AAC CCG CTA TGG CAC CAA ATA GGC CTC	FAM	BHQ-1
N9 assays	**Prediction for H7N9 detection**	**Primer forward**	**Primer reverse**	**Probe**	**Dye**	**Quencher**
CNIC (H7)N9 rRT-PCR	VALIDATED Detection of N9	TGGCAATGACA CACACTAGTCA GT	ATTACCTGGA TAAGGGTCGT TACACT	AGACAATCCC CGACCGAATG ACCC	FAM	BHQ1
Eurasian N9 rRT-PCR-SEPRL	VALIDATED Detection of N9	GCA TTG ACA GAT GAT AGA TCA AA	AGC CCA ATA GTC CAT GAA AGA	CTG ACT GGA / ZEN/GTG GTT ACA GTG G	FAM	IABkFQ
M assays	**Prediction for H7N9 detection**	**Primer forward**	**Primer reverse**	**Probe**	**Dye**	**Quencher**
InfA rRT-PCR (by CDC/WHO)	VALIDATED Detection of H7N9 as FluA positive	GACCRATCCTGT CACCTCTGA C	AGGGCATTYT GGACAAAKCG TCTA	TGC AGT CCT CGC TCA CTG GGC ACG	FAM	BHQ1

1.2 Additional information

RT-PCR assays:
http://www.offlu.net/fileadmin/home/en/guidance/pdf/OFFLU_real-time_diagnostics_of_H7N9_Dv07.pdf
Conventional PCR assays:
http://www.offlu.net/fileadmin/home/en/guidance/pdf/OFFLU_conventional_diagnostics_of_H7N9_Dv05.pdf

2. VALIDATED RT-PCR PROTOCOLS

The below mentioned protocols describe recommended real-time RT-PCR procedure [1] (rRT-PCR) for the detection of influenza A virus of subtype H7

2.1 Purpose

- This assay can be used to detect viral ribonucleic acid (RNA) of AIV subtype H7 strains including the novel avian H7N9 (A/Anhui/1/2013-like).
- RNA isolated from various sample types (e.g. blood, tissues and swabs) can be used.
- The AIV-H7.2 method was developed by FLI and validated for Eurasian and some North American AIV H7 strains as well as novel avian H7N9.
- The assay will detect a broad variety of H7 strains. A positive result does not prove H7N9 presence in the sample. Further testing is required through sequencing or N9 RT-PCR.
- Weak cross-reaction with H10 strains is possible.

2.2 RNA extraction

- This assay is to be used with sample RNA that has already been tested positive for AIV (by M-gene RT-PCR).

---

1 Based upon 'FLI Method AIV-H7.2; Code: NRLAI_PCR_4V1', courtesy of Timm Harder, Friedrich Löeffler Institut, Insel Riems, Germany

2.3   Real-time RT-PCR procedure

2.3.1 Kits and controls

- Validation was based on SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (#12574-026; Invitrogen). Different One-Step RT-PCR kits are expected to work as well, but procedures likely need to be adjusted to accommodate for different settings required by other kits.
- Run controls: a 'no template' control (NTC), positive control (PC).
- In order to prevent/minimize cross contamination, the concentration of the positive control should be low (with a Cq-threshold of approximately 33 in the M-gene assay).
- After preparation of the mastermix (see table below), 20 μl will be used for each reaction and 5 μl template RNA are to be added for 25 μl total volume. The volume can be adjusted to accommodate other detection equipment.

2.3.2 Preparation of primer/probe mix

AIV-H7.2-Mix-FAM (10 pmol Primer/μl + 1,25 pmol probe/μl)

Primer/probe name	Sequence	Volume
AIV-HA7_1593-F (100 pmol/μl)	5´-AYA GAA TAC AGA TWG ACC CAG T -3´	20 μl
AIV-HA7_1740-R	5´-TAG TGC ACY GCA TGT TTC CA -3´	20 μl
AIV-HA7_1649-**FAM** (100 pmol/μl)	5´-**FAM**- TGG TTT AGC TTC GGG GCA TCA TG-**BHQ2**-3´	2,5 μl
TE buffer (pH 8,0)	-----	157,5 μl
Total volume	**200 μl AIV-H7-2-Mix-FAM**	

2.3.3 Preparation of the mastermix

Mastermix components	Volume
RNase free water	4,5 μl
2x reaction mix	12,5 μl
Enzyme mix	1,0 μl
Primer-probe mix: AIV-H7.2-mix-FAM	2,0 μl
Total volume mastermix	**20 μl/ reaction tube or well**

Addition of template RNA	Volume
Samples	5 μl
NTC	
PC	
Total volume of reaction mixture	**25 μl**

2.3.4 Programming the PCR cycler

- This can be adjusted to accommodate other One-Step kits.

Setting	Temperature	Time	Cycles
Reverse transcription	50°C	30 min	
Inactivation RT /activation Taq	94°C	2 min	
Denaturing	94°C	30 sec	
Annealing *	56°C	30 sec	42-45 cycles
Elongation	68°C	30 sec	

* Fluorescence is recorded in the FAM-channel during annealing

2.3.5 Analysis

- Analyse results as for M-gene or H5 rRT-PCR
- Failure to detect the positive control means the run is invalid.
- If negative controls are "positive" reagents may be RNA-contaminated. The run is invalid.

2.4   Example laboratory chart

Real-time RT-PCR run #: _____   Date: _____

Processed by: _____   Time: _____

Remarks: _____

When added, tick box	Mastermix	AIV-H7.2	
		1 x	**x**
	RNase free water	4,5 µl	
	2x Reaction mix	12,5 µl	
	Enzyme mix	1,0 µl	
	AIV-H7.2-Mix-FAM	2 µl	
	Total volume mastermix per reaction	**20 µl**	
	Addition of template -RNA Field samples PC, NTC	5 µL each	
	Total volume per reaction	**25 µl**	

96-well-plate: SAMPLE-IDs


```
1 cycle:
RT              30min   50°C
ActivationTaq   2min    94°C
42 cycles:
Denat.          30sec   94°C
Annealing 30sec 56°C
Elongation 30sec 68°C
```

Name: _____

Date: _____

Valid □ Yes   □ No

Results:

_____

_____

_____

_____

2.5 Algorithm for the detection of AIV through PCR and/or virus isolation
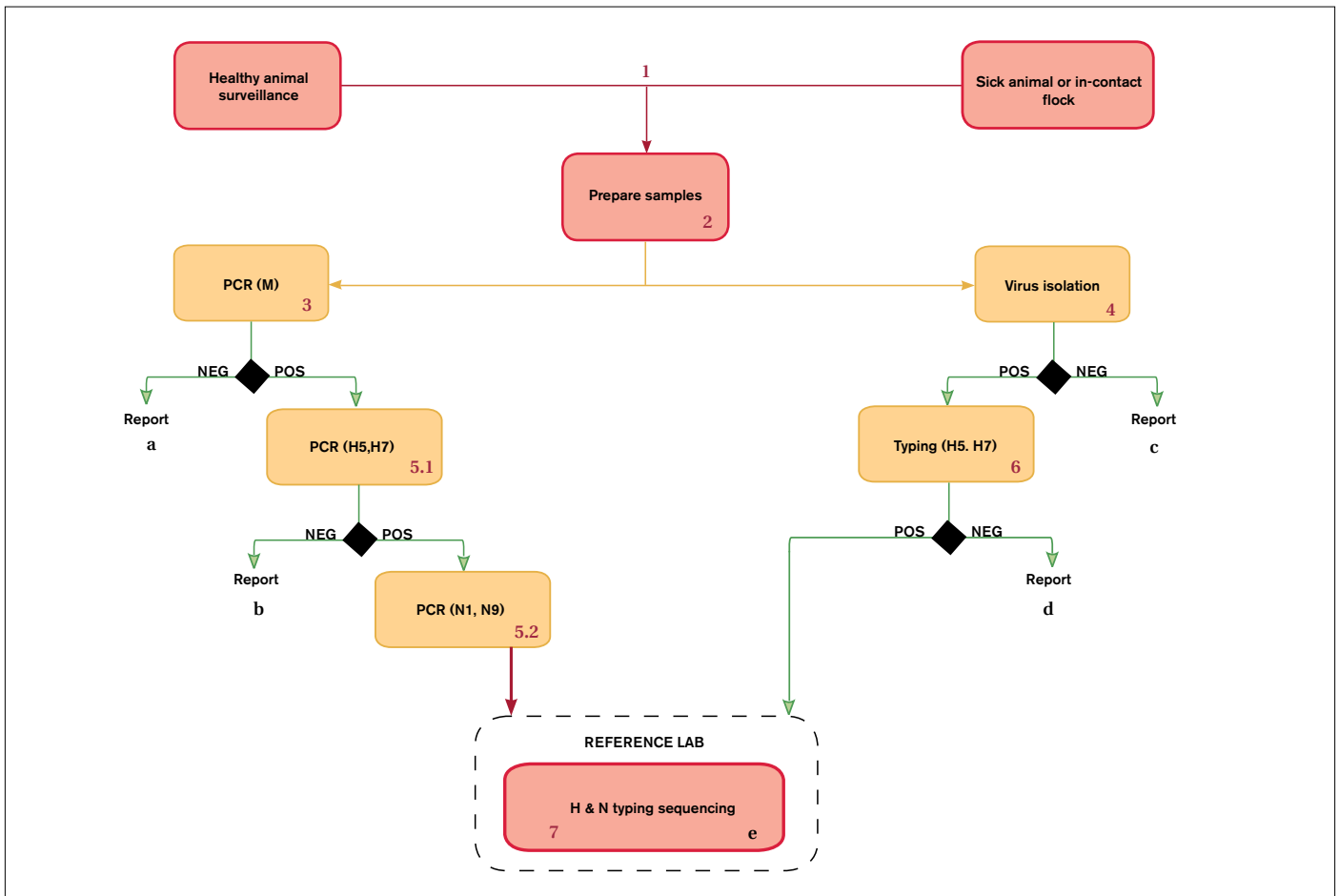
Notes

1	Diagnosis: Use PCR or virus isolation (preferably PCR); Surveillance: Start with PCR, then isolate virus from positive sample.
2	Avoid pooling samples in the field whenever possible. When required for testing purposes, conduct pooling at the laboratory by combining a maximum of five similar samples per pool from the same sample species, epidemiological unit and type (i.e. either oropharyngeal/tracheal or cloacal, but not mixed cloacal and oropharyngeal).
3	Screening flocks for all influenza viruses using PCR (M) is recommended where possible. Specific virus PCR can be used first (e.g. H5 or H7) where a diagnosis is required for a specific virus in an emergency.
4	For isolation use up to three passages in eggs and hemagglutinin assay (HA).
5.1 & 5.2	Use recommended regional PCR primers and probes.
6	Use hemagglutinin inhibition (HI) assays for subtyping of isolates and/or PCR and/or sequencing. HI antigens and antisera must match the circulating H5 or H7 clade.
7	Confirmation of H and N type requires sequencing of the isolate.
a	Not influenza A. Differential diagnosis (Newcastle disease, infectious bursal disease, duck viral enteritis; optional).
b	Report as influenza A, not H5 and not H7. Optional: further subtyping to determine H and N.
c	Not influenza A by virus isolation.
d	Report as influenza A, not H5, not H7. Optional: further subtyping to determine H and N.
e	Whole genome sequencing (optional).

2.6   Additional information

OFFLU protocols: available at http://www.offlu.net/index.php?id=267
WHO protocols (H7 and N9) available at http://www.who.int/influenza/gisrs_laboratory/cnic_realtime_rt_pcr_protocol_a_h7n9.pdf

3.   SURVEILLANCE LABORATORY FLOW CHART

3.1   Serological considerations for the detection of poultry exposure to avian influenza A(H7N9)

In vivo infectivity studies in poultry with H7N9 virus have shown that the virus is shed for a limited period of time following infection. This significantly reduces the opportunity to detect the virus in poultry populations during targeted surveillance. Therefore serological surveillance may be an appropriate method of choice for detection of H7N9 infection when conducting an active surveillance programme.

Laboratory experiments with H7N9 in various animal species (e.g. chickens, quails, turkeys, pigeons, and ducks) have shown that HI testing can be used to detect exposure of animals to H7N9 infections. Data show that poultry become seropositive at 5 to 7 days after infection; the longevity of the antibody response is currently being investigated, but it will wane over time.

AAHL and the Animal Health and Veterinary Laboratories Agency (AHVLA) have found high levels of cross-reactivity in HI assay results using various antisera against H7N9 as an antigen. The AAHL and AHVLA results have been posted by OFFLU. [2]

Therefore, standard HI assays using the Chinese H7N9 or Eurasian H7 antigens can be used for the serosurveillance for H7N9. These methods have been described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012. [3]

However, when performing serosurveillance for H7N9, it is essential to consider that:

• Antibodies to the avian influenza A(H7N9) virus and antibodies to other Eurasian-lineage H7Nx influenza viruses are highly cross-reactive.

• Anti-H7 antibodies do not unequivocally proof infection with H7N9 as the hemagglutinin inhibition assay cannot distinguish between antibodies to H7N9 and antibodies to other Eurasian-lineage H7Nx influenza viruses.

• In infected areas where H7N9 virus has been detected, the presence of anti-H7 antibodies in sera should be considered highly indicative of previous H7N9 infection.

• Neuraminidase inhibitor (NAI) assays using HxN9 antigens should be utilized to determine presence of anti-N9 antibodies, however, other HxN9 viruses will also cross-react with novel H7N9.

---

2. OFFLU results:
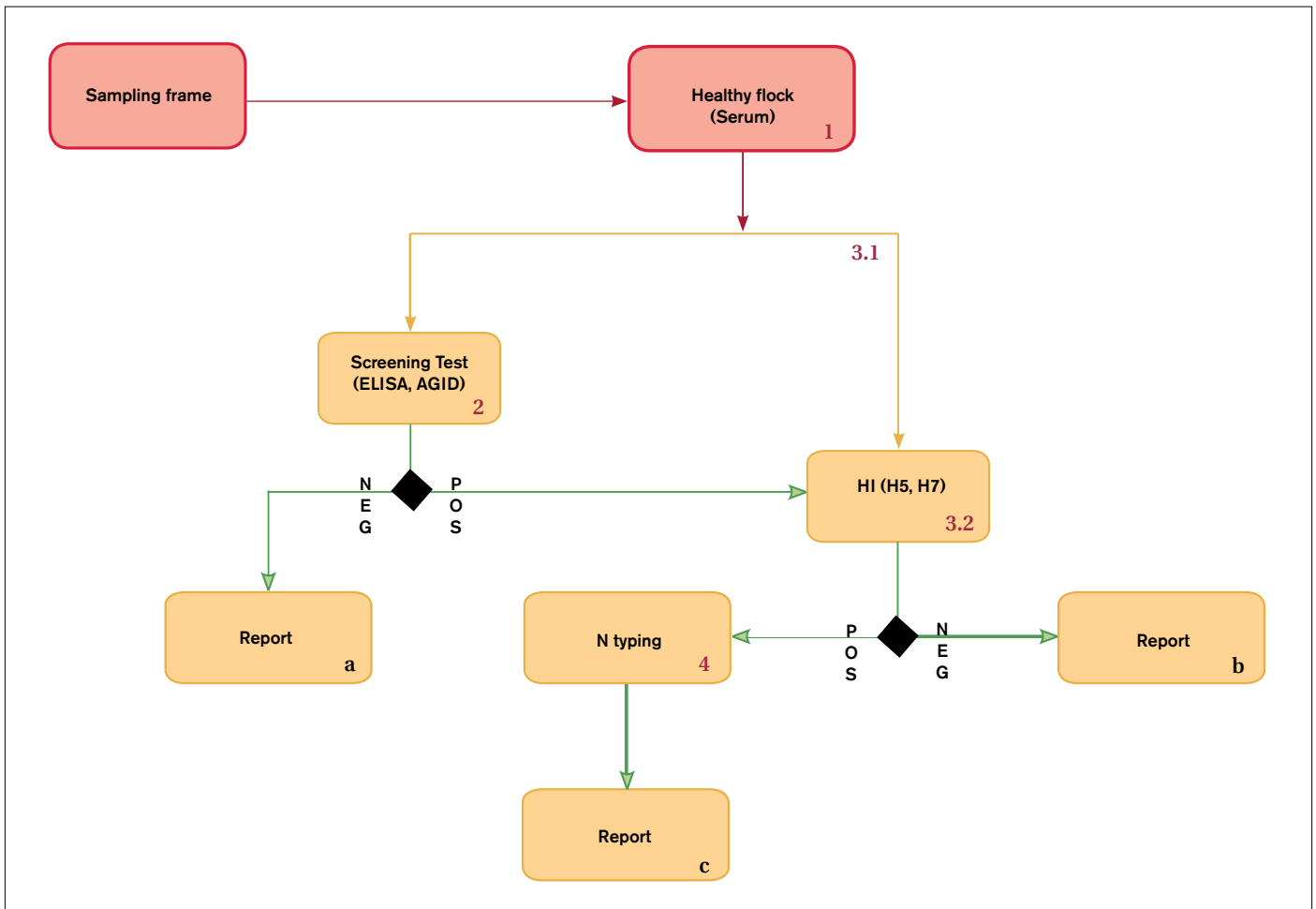   i) http://www.offlu.net/fileadmin/home/en/guidance/pdf/HI_and_NI_results_AHVLA_7May13.pdf; and
    ii) http://www.offlu.net/fileadmin/home/en/resource-centre/pdf/AAHL_H7N9_Experiment_17May.pdf
3.   ttp://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.04_AI.pdf
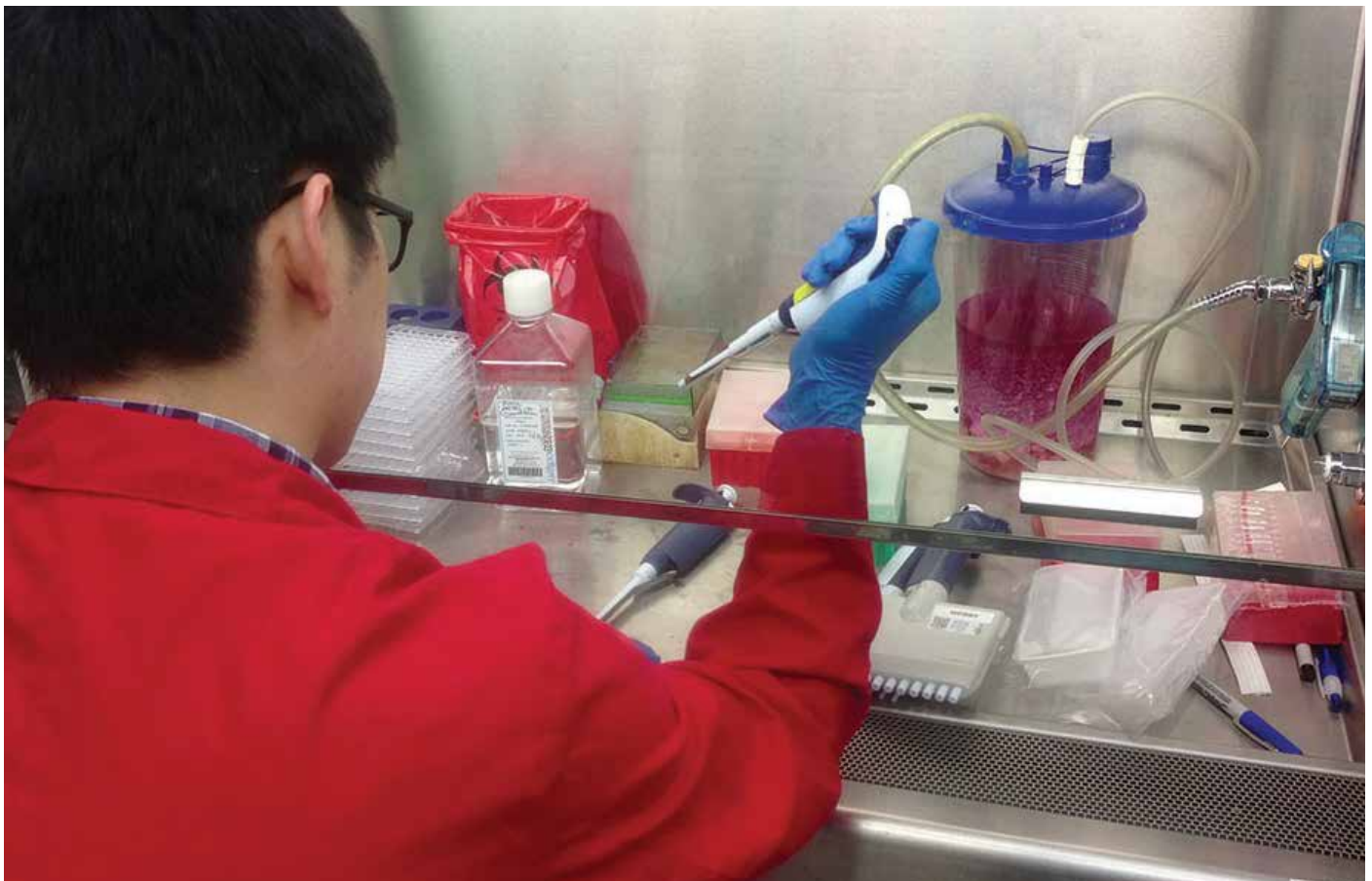
© FAO/Stephanie Sonnberg

3.2 Laboratory diagnostic algorithm for the serosurveillance of AIV

Notes

1   Investigate vaccination history of flock

2   Avoid pooling of serum samples

3   If flock has been previously vaccinated against H5N1 or H7Nx:
- 3.1 Use homologous antigen to determine vaccination coverage;
- 3.2 Screen for H5 or H7 antibodies using appropriate antigens for these subtypes

4   To determine the NA type: use NAI assays

a   No influenza A antibodies present. Therefore; there is no evidence of previous infection, though infection with H5 or H7 viruses can fail to cause seroconversion. In chickens seroconversion requires approximately 5–7 days after infection and antibodies wane over time.

b   Report as influenza A antibodies present, but not anti-H5, not anti-H7; optional: further subtyping of HA-specific antibodies using full HI panel (H1-H16).

c   Report either as H5- or H7-specific antibodies; optional: also include NA subtype.

I3596E