

NATIONAL STANDARD METHOD

REAL-TIME QUADRIplex PCR FOR THE DETECTION OF INFLUENZA

VSOP 25

Issued by Standards Unit, Evaluations and Standards Laboratory
Centre for Infections

REAL-TIME QUADRIplex PCR FOR THE DETECTION OF INFLUENZA

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VSOP 25i1

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STATUS OF NATIONAL STANDARD METHODS

National Standard Methods, which include standard operating procedures (SOPs), algorithms and guidance notes, promote high quality practices and help to assure the comparability of diagnostic information obtained in different laboratories. This in turn facilitates standardisation of surveillance underpinned by research, development and audit and promotes public health and patient confidence in their healthcare services. The methods are well referenced and represent a good minimum standard for clinical and public health microbiology. However, in using National Standard Methods, laboratories should take account of local requirements and may need to undertake additional investigations. The methods also provide a reference point for method development.

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The HPA aims to be a fully Caldicott compliant organisation. It seeks to take every possible precaution to prevent unauthorised disclosure of patient details and to ensure that patient-related records are kept under secure conditions¹.

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APPENDIX 3: VALIDATION DATA FOR THE QUADRIPLEX INFLUENZA A/B H5 MS2 RNA ON ROTOR-GENE. 18

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AMENDMENT PROCEDURE

Controlled document reference	VSOP 25
Controlled document title	Real-Time Quadriplex PCR for the detection of Influenza

Each National Standard Method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from standards@hpa.org.uk.

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment

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REAL-TIME PCR FOR THE DETECTION OF QUADRIplex INFLUENZA

The assay described below was developed as part of an ongoing collaboration project between HPA East of England, Cambridge and the Respiratory Virus Reference Laboratory at the Centre for Infections, London. The information presented within should be treated as confidential. These protocols should not be distributed and results obtained with these assays should not be published or presented in a public forum without the explicit consent of the Respiratory Virus Unit, Centre for Infections.

SCOPE OF DOCUMENT

This document describes a new REAL-TIME assay that can detect all generic influenza A subtypes (H1-H15), specifically identify the presence of H5 and simultaneously detect Influenza B and an internal control in clinical specimens (Respiratory secretions).

INTRODUCTION

The advantage of this approach is monitoring of efficiency of extraction and reverse transcription/amplification. Generic PCR primers/Taqman® probes for influenza A/B were designed from multiple alignments of the matrix and nucleoprotein gene sequences. The H5 specific primers/probe were those currently used in the Cfl H5 assay (VSOP 41). MS2 was chosen as an internal control because of its ease of propagation and because it represents encapsidated RNA, with specific primers/Taqman® probe designed near the 5' end of the genome.

The primers and probe detailed in this SOP have been extensively optimised and tested against a range of Influenza A H5 viruses, including recent Influenza A H5 virus strains isolated in Vietnam in 2004 AND Turkey in 2005. The specificity of this assay has been evaluated against a panel of influenza A virus (subtype H1-H15), Influenza B strains and a respiratory panel of pathogens to ensure specificity of the assay for Flu A, H5 and Flu B. See validation data near the end of this document.

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1 SAFETY CONSIDERATIONS²⁻¹¹

1.1 SPECIMEN COLLECTION

Appropriate hazard labelling according to local policy should be used.

1.2 SPECIMEN TRANSPORT AND STORAGE

Compliance with current postal and transport regulations is essential.

A suitable virus transport system must be used (where appropriate) and the specimen placed in a sealed plastic bag or pouch, separately from the request form.

Appropriate hazard labelling according to local policy should be used.

1.3 SPECIMEN PROCESSING

All handling of clinical material from suspected avian Influenza patients must be carried out in a containment level 3 (CL3) facility within a class I/III bio-safety cabinet. Once clinical material has been added to the lysis buffer employed in the extraction procedure, subsequent extraction of viral nucleic acid can be carried out at CL2. * See – Useful notes on choice of extraction method.

Good laboratory practice.

The above guidance should be supplemented with local COSHH and risk assessments.

PPE to be worn at all times.

2 SPECIMEN COLLECTION

2.1 OPTIMAL TIME OF SPECIMEN COLLECTION

Specimens should be processed as soon as possible. Ideally, transportation systems should ensure that specimens arrive in the laboratory within 24 hours.

2.2 CORRECT SPECIMEN TYPE AND METHOD OF COLLECTION

Respiratory secretions: Nasopharyngeal Aspirates (NPA), Bronchoalveolar Lavage (BAL), Endotracheal Aspirates (ETA), Nose and Throat Swabs (in virus transport medium) and Sputum.

Note: Based on recent avian H5 data from Vietnam and recent publications, analysis of blood and faeces specimens during the acute presentation part of the illness is clinically useful/relevant. While the protocol described herein has been validated using respiratory secretions, analysis of plasma/serum shouldn't pose any difficulties with the nucleic extraction protocols described herein. Faeces, however, would be problematic and would need a modified extraction protocol that is fully validated.

[A suggested starting point would be the pre-treatment protocol optimised for Norovirus PCR testing using Roche STAR (stool transport and recovery) buffer (03335 208 001) for stabilisation, followed by chloroform treatment (as detailed in the Roche STAR buffer insert) prior to nucleic acid extraction. A local risk assessment should be performed before carrying out this procedure in your CL3 laboratory.]

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2.3 ADEQUATE QUANTITY AND APPROPRIATE NUMBER OF SPECIMENS

It is imperative that duplicate samples should be taken (in the event that the sample is found to be H5 positive). The second sample can be sent to Cfl. for additional confirmation. Duplicates may also be required for the exclusion of other microbial pathogens.

A minimum of 300 μ L of sample is required for extraction of Influenza A/B/H5.

3 SPECIMEN TRANSPORT AND STORAGE

3.1 TIME BETWEEN SPECIMEN COLLECTION AND PROCESSING

Specimens should be transported to the laboratory and processed as soon as possible. Specimens that may be delayed should be refrigerated prior to transportation to the laboratory.

3.2 SPECIAL CONSIDERATIONS TO MINIMISE DETERIORATION

Specimens should be refrigerated at 4°C if there is likely to be a delay in processing. If the delay is likely to exceed 24 h, the sample should be stored at -70°C and thawed prior to processing. Repeated freezing and thawing should be avoided.

4 EQUIPMENT AND REAGENTS

4.1 EQUIPMENT

Rotor-Gene™ 3000 Real-time thermal cycler (Corbett Research Limited)

Microcentrifuge

Vortex Genie® 2

0.2 mL or 0.1 mL tubes for the Rotor-Gene

Pipettes with disposable filter tips

4.2 REAGENTS

SuperScript™ III Platinum® one-step qRT-PCR system (Invitrogen cat no: 11732-088)

RNase-free water (Invitrogen Gibco : cat no: 10977035)

QIAamp® Viral RNA mini kit (250) (Qiagen cat no: 52906)

Control virus = Influenza A H5N3 A/Duck/Singapore/3/97 virus is available from ERNVL

(<http://hpa.org.uk/cfi/esl/gcru/products.htm>)

Forward and reverse primers (as detailed in section 5.3) were obtained from Metabion (<http://www.metabion.com>).

Probes, PCR-7 and AM, containing taqman minor groove binders (MGB) were purchased from Applied Biosystems while the other two probes, BNP and MS2 taq, with Cy5 and ROX labels were purchased from Metabion.

RNasin 40U/ μ L (Promega cat no: N211A)

L2 buffer (cat no:20-8200), L6 buffer (cat no:20-8600) and Silica (cat no: 20-8000) were purchased from Severn Biotech Ltd.

Bacteriophage MS2 (ATCC 15597-B1) and its *E. coli* Host (ATCC 15597) was purchased from the American Type Culture Collection and propagated as detailed in the product information sheets provided^{12,13}.

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5 PROCEDURE

5.1 BOOM METHOD FOR NUCLEIC ACID EXTRACTION

1. To a clean labelled 1.5 mL microcentrifuge tube, add the following in order: (perform extraction in duplicates if sufficient sample volume)

Perform this in the extraction room

20 µL of size fractionate silica -

840 µL of L6 Buffer

20 µL of MS2 internal control (10 - 4) i.e. 4600 pfu per extraction

Take these tubes to CL-3 room and add 150 µL of specimen and controls to their respective tubes in the class-III cabinet. Close the tubes and vortex for 5 seconds.

2. In the extraction room, place the tubes on a rotary mixer (Genie 2) at RT for 10 min on a setting of 5.

3. Centrifuge for 15 sec at 13 000 rpm, remove the supernatant using an extended fine tip pastette without disturbing the pellet. Collect waste into a universal bottle marked G (Guanidinium waste).

4. Wash with 1 mL of L2 buffer (x2) and vortex on Genie 2 for 10 sec (make sure the silica is completely re-suspended). Proceed as at step (3).

5. Repeat step (4) with 1 mL of 70% ethanol (x2). Pour off the ethanol gently into a universal bottle marked S (solvent waste).

6. Repeat step (5) with 1 mL of acetone (x1).

7. After removing the acetone (perform carefully as pellet may become dislodged) place the tubes with lid open at 56°C in a dry heating block for 10 min.

8. Elute the nucleic acid from the silica by adding 30 µL RNase- free water containing 1 µL of RNasin (see table below) and vortex for 5 sec. Incubate at 56°C for 10 min.

Note: Prepare RNasin in RNase-free water (n + 2 [1µL Rnasin + 29µL H₂O] reactions and add 30 µL to each tube)

9. Vortex and centrifuge at 13 000 rpm for 3 min.

10. Carefully collect eluate into a clean labelled 0.2 mL avoiding any silica (any silica will inhibit RT step).

11. Proceed immediately to RT-PCR step and store the remaining elute at -20°C.

5.2 QIAAMP NUCLEIC ACID EXTRACTION PROCEDURE

QIAamp Viral RNA Kit: Cat no: 52906 (250 preps)

1. To a clean labelled 1.5 mL micro-centrifuge tube, add the following in order (perform extraction in duplicates if sufficient sample volume):

Perform this in the extraction room

560 µL of AVL Buffer

20 µL of MS2 internal control (10-4) i.e. ~ 4600 pfu per extraction

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Take these tubes to CL-3 room and add 140 µL of specimen and controls to their respective tubes in the class-III cabinet. Close the tubes and vortex for 5 sec.

2. In the extraction room, place the tubes on a rotary mixer (Genie 2) at RT for 10 min on a setting of 5.

During incubation stage remove individual QIAamp columns from their packaging and place in a rack and label the tops with the corresponding sample number.

3. Pulse spin and add 560 µL of ethanol (97-100%) to the sample, mix thoroughly by vortexing and pulse spin.

4. Carefully apply half of the mixture from step (3) to the QIAamp spin column without wetting the rim, close the cap and centrifuge at 8 000 rpm for 1 min. Discard the waste from the collection tube into a universal bottle marked G (guanidinium waste) and discard the used collection tube. Place QIAamp spin in a clean 2 mL collecting tube.

5. Repeat step (4).

6. Carefully add 500 µL of buffer AW-1 to the column (without wetting the rim) and centrifuge at 8 000 rpm for 1 min. Discard the waste from the collection tube into a universal bottle marked S (solvent waste) and discard the used collection tube. Place QIAamp spin column in a clean 2 mL collecting tube.

7. Repeat step (6) with wash buffer AW-2 follow by a further centrifugation at 13 000rpm for 1 min to remove any residual buffer.

8. Place the QIAamp spin column in a labelled (MD number) clean 1.5 mL flip-top tube (cut the tops out with scissors and keep for later use).

9. Add 60 µL AVE buffer / RNase-free water drop wise at the centre of the column and close the lid. Incubate for 1 min.

10. Elute the RNA by centrifugation at 8 000 rpm for 1 min.

11. Discard the spin columns and recap the tubes.

12. Discard the waste labelled G into a waste container marked Guanidinium isothiocyanate and discard the waste labelled S into a solvent waste bottle.

5.3 TAQMAN FLU A, B, H5 & IC AMPLIFICATION ON THE ROTOR GENE™

1. In the clean room, prepare the following Quadriplex Flu Real-time master mix (MMX). The PCR MMX should be N + 2 (N = No of tests including controls). Vortex and pulse spin.

Reagent for x1 reaction	Volume (µL)
RNase free water	1.38
2 x Buffer	12.5
PCR-7F (New-10 pmol/µl) 5'-GCC GAA TGA TGC MAT MAA YT -3'	1.0
PCR-7R (10 pmol/µl) 5'-CGC ACC CAT TGG AGT TTG AC -3'	1.0
PCR-7 Probe (New-5 pmol/µl) 5'-FAM- CAT TGC TCC AGA AWA T -MGBNFQ-3'	0.5
AM-F (20 pmol/µl) 5'-GAG TCT TCT AAC MGA GGT CGA AAC GTA -3'	0.5
AM-R (20 pmol/µl) 5'-GGG CAC GGT GAG CGT RAA -3'	1.0

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AM Probe (10 pmol/μl) 5' – VIC – TCC TGT CAC CTC TGA C - MGBNFQ-3'	0.4
BNP-F (20 pmol/μl) 5' – GCA GCT CTG ATG TCC ATC AAG CT - 3'	0.16
BNP-R (20 pmol/μl) 5'-CAG CTT GCT TGC TTA RAG CAA TAG GTC T -3'	0.16
BNP-Probe (3.3 pmol/μl) 5' – Cy5 – CCA GAT CTG GTC ATT GGR GCC CAR AAC TG – BHQ3-3'	0.2
MS2 F1 (20 pmol/μl) 5' TGG CAC TAC CCC TCT CCG TAT TCA CG- 3'	0.1
MS2 R1 (20 pmol/μl) 5' GTA CGG GCG ACC CCA CGA TGA C- 3'	0.1
MS2 Taq (10 pmol/μl) 5' Rox- CAC ATC GAT AGA TCA AGG TGC CTA CAA GC– BHQ2 3'	0.2
Superscript™ III Platinum one-step enzyme	0.8
Total Volume	20.0

- In the extraction room, load 0.2 mL flat top clear tubes on an appropriate cooled metal block beginning at position 1.
- Pipette 20 μL of reaction mix into the 0.2 mL tubes.
- Add 5μL of RNA extract to respective tubes, close the tube after each addition.
- In the amplification room, transfer the tubes into a 36 well carousel at their corresponding locations and load it into the Rotor-Gene™. Apply the locking ring (for 36 well Rotor only).
- Open a previous Quadriplex Flu folder. Click 'NEW' from the menu and 'NEW' again from the tool box.
- Select the Rotor type as 36, tick the 'No domed' and click 'Next'
- Type in 'operator Name' in the operator box – set reaction volume at 25 μL and click 'Next'.
- A 'Quadriplex Flu-Taqman' run profile should be displayed as outline below:

'Quadriplex Flu-' Programme on the Rotor – Gene™

Programme	Temp (°C)	Hold Time (min)	Acquisition mode	No. of Cycles
RT	50	30	Not Acquiring	x 1
Denature	95	2	Not Acquiring	x 1
Cycling	95	15 (sec)	Not Acquiring	x 45
	60	1	Acquiring on Fam, Joe, Cy5 & Rox	

- Select 'calibration', this will display Auto gain calibration set-up menu, ensure FAM channel is displayed. Set temperature to 60°C and Click 'Start' – this will adjust the gains for the FAM channel selected, when completed click on 'close' followed by 'close' on the calibration menu.
- Click 'Next', followed by 'Start Run'. Save the current run as 'Flu ddmmy' in the Flu-H5 runs folder.

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12. The run will start and after few seconds will display the run worklist to edit.

13. Enter sample ID from the worklist including positive controls and leave type as unknown. For water negative control select type as NTC.

No	Colour	Name	Type	Group	Given Conc	Selected
1		MD NO	Unknown			Yes
2		Duck H5	Unknown			Yes
3		Water	NTC			Yes

14. Click on 'Finish' and the Rotor-Gene™ will display the run screen.

5.4 DATA ANALYSIS

15. Following completion of run select 'Analysis' in the view menu. An analysis tool box will appear, click on the following sequence: 'Quantification' – 'Cycling Channel Fam' – 'show'. Close the calculation tool box and click linear scale which will change to log scale (this will display amplification of Flu-H5 in the samples).

16. In the menu box; Click on 'Dynamic Tube', 'Slope correction' and set the threshold to 10% with efficiency left blank in the 'More setting' tool box.

17. At the 'Eliminate cycle box' enter a value of 10 and on the 'Threshold' box, enter a value between 0.01-0.05 until the threshold value line just clears the base line.

18. Click on 'Report' select standard report format and print hard copy for record.

19. Repeat step 15. For 'Cycling Channel's - 'Joe for Flu A' - 'Cy5 for Flu-B' and 'Rox for internal MS2 control'.

5.5 RUN VALIDATION

Ensure that positive controls - Duck-H5, Flu A & Flu B and negative controls together with the MS2 IC have worked before accepting results.

* USEFUL NOTES:

For H5 suspect cases perform both test samples and control reactions in duplicate or triplicate, depending upon material available.

Each laboratory should perform a risk assessment of the likely risk of the samples to be tested containing Influenza A H5 virus, prior to aliquotting of the sample(s) into lysis buffer for extraction. The ratio of lysis buffer to sample is less when a MagNApure extraction is performed than for manual Boom extraction. A low ratio of lysis buffer to sample may not be sufficient to inactivate high titred Influenza A H5 virus.

Qiagen AVL buffer has been shown to render clinical samples non-infectious at the volumes used herein¹⁴.

Optimisation experiments indicated 20 µL of a 10⁻⁴ dilution (PBS) of our stock (2.3 X 10⁹ pfu/mL) was the most reliable input per extraction for both reproducible MS2 detection and non-interference with other routine amplification targets. This equates to 4600pfu per extraction and therefore in the case of a 60µL elution, followed by addition of 5µL of extract, 383 pfu per PCR reaction.

Purified MS2 RNA is available from Roche (cat no: 165 948) at a concentration of 0.8µg/µL. When used as standards in a Quantitative MS2 assay we determined the genome copy number to be 9.18 per pfu of our stock. For those who would prefer to add purified RNA to the

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master-mix, I would suggest 0.5 µL of a 10⁻⁶ dilution of the MS2 RNA (RNase free water containing 0.1 ng/µL of PolyA carrier RNA) per PCR reaction as a starting point.

6 QUALITY ASSURANCE

6.1 ASSESSMENT OF PREPARATION

All new oligonucleotide reagents (P/P) are batch tested prior to aliquotting, batch numbering and freezing. Each assay run has positive and negative controls for each target at the appropriate levels and their Ct values monitored for each run over time to assess any assay drift.

6.2 INTERNAL AND EXTERNAL QUALITY ASSURANCE

This assay is included in the IQA specimen resubmission programme (1% of all samples submitted).

Influenza A H5 : proficiency panel for molecular detection run by Cfl Colindale.

QCMD panels for Flu A, B and Flu A Haemagglutinin sub-typing.

Control material used :

The positive H5 control virus (H5N3 A/Duck/Singapore/3/97) available from ERNVL is employed for each run. This control material has been prepared as aliquots of 600µL virus in 200µL lysis buffer. For routine use this is further diluted 1:1000 in L2 buffer and 150uL of this dilution extracted for each run.

Positive controls for Flu A and Flu B are prepared from culture positive material; again diluted appropriately in L2 buffer and 150µL extracted for each run.

The negative control for each run is molecular grade water.

7 LIMITATIONS

Successful isolation of organisms depends on correct specimen collection, transport, storage and processing; and the provision of adequate/suitable clinical information.

The procedure(s) in these documents aim to describe good microbiological standard methods for the specimen types specified. Other procedures may be required and professional interpretation by qualified staff is essential. Please note that knowledge of infectious diseases changes constantly and although this NSM is under regular review it may not include emerging pathogens.

The above guidance should be supplemented with local assessments of limitations.

8 REPORTING PROCEDURE

Negative specimens should be reported as eg "virus not isolated". Ensure satisfactory detection of the internal control prior to reporting a negative result.

Positive specimens should be reported as eg "Influenza A or B isolated".

In the case of A H5 positive, if a confirmatory test has not been performed in parallel, then a H5 confirmatory test should be carried out immediately.

If further tests are to be carried out a report should be issued stating further results are pending.

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9 REFERENCE LABORATORY

Send Influenza A H5 positive samples via a notified courier (see below) to the Respiratory Virus Unit, Cfl, Colindale for immediate confirmation.

Influenza A/B PCR positives should ideally be cultured from the original material and then sent to the Respiratory Virus Unit, Cfl, Colindale for confirmation/analysis. A specific arrangement with the reference laboratory should be established prior to sending material of this nature.

Main Contractor for H5 Courier service: **TNT Same Day**

24/7 contact telephone number – 0208 804 8467

Quoting Account Number – 0330212524

Service Contact – Stuart Bradford 07768 792783

Back up Contractor **City Sprint**

24/7 contact telephone number – 0207 880 1121 quoting Account number – M7100

Service Contacts	Andrew Turner	07989 857816
	David Potter	07989 855424
	Nick Brennan	07738 144591

10 REPORTING TO THE HPA (LOCAL AND REGIONAL SERVICES AND CDSC CENTRE FOR INFECTIONS)

Any positive result indicating infection with Influenza A H5 must be reported promptly to ensure that the appropriate public health actions are undertaken.

Guidance on the current reporting procedures for this infection is available on the HPA website www.hpa.org.uk. Any query regarding the reporting procedure. Or any other aspect of the public health management, should be discussed with the local Health Protection Unit (HPU) in the first instance.

Influenza A and B should also be reported promptly (ensuring appropriate actions are undertaken) and clinical and/or risk factor data desirable as part of report.

11 ACKNOWLEDGEMENTS AND CONTACTS

This National Standard Method has been developed, reviewed and revised by the Standard Methods Working Group for Virology (http://www.hpa-standardmethods.org.uk/wg_virology.asp). The contributions of many individuals in clinical virology laboratories and specialist organisations who have provided information and comment during the development of this document, and final editing by the Medical Editor are acknowledged.

The National Standard Methods are issued by Standards Unit, Evaluations and Standards Laboratory, Centre for Infections, Health Protection Agency London.

For further information please contact us at:

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APPENDIX 1: INFLUENZA A H5 RNA TARGET SEQUENCE IN THE HAEMAGGLUTININ GENE

H5 Taqman Assay (151bp)

```
GCCGAATGATGCMATMAAYTtgagagtaatggaatttCATTGCTCCAGAATWAT  
aaaattgtaaaaaggaggctcagcaatcatgaaaagtgattggaatgtaactgcaaccaagt  
GTCAAACCTCCAATGGGTGCG
```

MS2 (Internal Control) Target Sequence (99bp)

```
TGGCACTACCCCTCTCCGTATTCACGggggcgtaagtgtCACATCGATAGATCAAGGTGCCTACAA  
GCgaagtggGTCATCGTGGGGTCGCCGTAC
```

Influenza A matrix gene target sequence (205bp)

```
GAGTCTTCTAACCGAGGTCGAAACGTAcgttctctatcatcccgtcaggccccct  
caaagccgagatcgcacagagactgaagatgtctttgcaggaagaacaccgatct  
tgaggctctatggaatggctaaagacaagaccaaTCCTGTACCTCTGACTaaggg  
gattttaggattgtgTTCACGCTCACCGTGCCC
```

Influenza B NP gene target sequence (148bp)

```
GCAGCTCTGATGTCCATCAAGCTcCAGTTTTGGGCTCCAATGACCAGATCTGGgggg  
aacgaagtaggtggagacggagggtctggccaaataagctgcagcccagtgtttgca  
gtggaaAGACCTATTGCTCTAAGCAAGCAAGCTG
```

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APPENDIX 2: PREPARATION OF INFLUENZA A, B, H5 & IC PRIMER / PROBE MIX

Stock conc. (pmol/ μ L)	Volume of stock / reaction (μ L)	For 100 reactions (μ L)
Taqman quadriplex Assay		
PCR7for(new) 10	1.0	100
PCR7rev 10	1.0	100
PCR7 probe (H5) 5	0.5	50
AM-F 20	0.5	50
AM-R 20	1.0	100
Probe-A 10	0.4	40
BNP-F 20	0.16	16
BNP-R 20	0.16	16
Probe-B 3.3	0.2	20
MS2-F 20	0.1	10
MS2-R 20	0.1	10
Probe- MS2-Taq 10	0.2	20
Total volume	4.92	492

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APPENDIX 3: VALIDATION DATA FOR THE QUADRIplex INFLUENZA A/B H5 MS2 RNA ON ROTOR-GENE.

- **SENSITIVITY**

Determined using titrated stocks provided by Cfl.

A/Taiwan/1/86 (1.6×10^8 pfu/ml) detected down to -7 which equates to 0.2 pfu/reaction

A/Moscow/10/99 (2.6×10^8 pfu/ml) detected down to -6 which equates to 3.25 pfu/reaction

B/Panama/45/90 (8×10^7 pfu/ml) detected down to -8 which equates to 0.01 pfu/reaction

The Cfl H5 component was as sensitive as the normal Cfl taqman assay when compared in a head-to-head.

- **H5 Isolates**

The full range of Influenza A H5 viruses held in Cfl, including recent Influenza A H5 virus strains isolated in Vietnam, Indonesia and Turkey were detected by the Quadriplex and no noticeable interference in sensitivity or specificity was observed.

- **Flu A subtypes**

All 15 subtypes (H1 – H15) are detected by the Flu A matrix component. Sequencing analysis of the amplified sequences for each of 15 subtypes confirmed the consensus nature of the designed taqman probe. The assay also successfully identified the index H7N3 case in the poultry farm in Norfolk.

- **Blind Respiratory Panel**

When a respiratory panel (N=24) was processed by the assay the Flu A (H1, H3 and H5) and Flu B samples in the panel were correctly detected/identified and all other pathogens (Adenovirus, Flu C, RSV, etc) were negative, assuring the specificity of the assay.

- **Primer/probe compatibility**

The Flu A and B components of the assay were optimised separately and brought together (making the necessary adjustments to primers/probe concentrations) in a manner that maintained the sensitivity for each target. The H5 Cfl assay was then introduced, again making adjustments but maintaining sensitivity. Finally the MS2 primers and probes were introduced in a similar manner, ensuring MS2 was always detected.

- **NPA analysis**

When applied retrospectively to examine 118 consecutive clinical samples found negative by IF for Flu A/B in Cambridge, nine in total were found to be positive, 5 for Flu A and 4 for Flu B. All 9 positives were sequenced and confirmed as circulating Flu A matrix and Flu B NP sequences. MS2 was amplified in all samples, within 1 or 2 Ct's of the mode, and H5 was not detected.

- **RCGP validation.**

201 clinical samples from the RCGP study, containing Flu A positives (N=53; H1 & H3), Flu B positives (N=57) and Flu A/B Negatives (N=91) were extracted on the Magnapure in Cfl, with and without MS2 phage (4600 pfu per extraction). The extracts were then subjected to the quadriplex assay in

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Cambridge and the two available real-time Flu A/B assays (Generic Flu A/B and multiplex H1/H3/B) in RVU at Cfl.

The inclusion of MS2 did not affect the quadriplex results with the Ct values broadly identical for all the A/B positives found with and without MS2. No H5 positives were found and MS2 was always detected.

		Cfl Flu A		
		+	-	Total
Quadriplex Flu A	+	52	0	52
	-	1	91	92
	Total	53	91	144

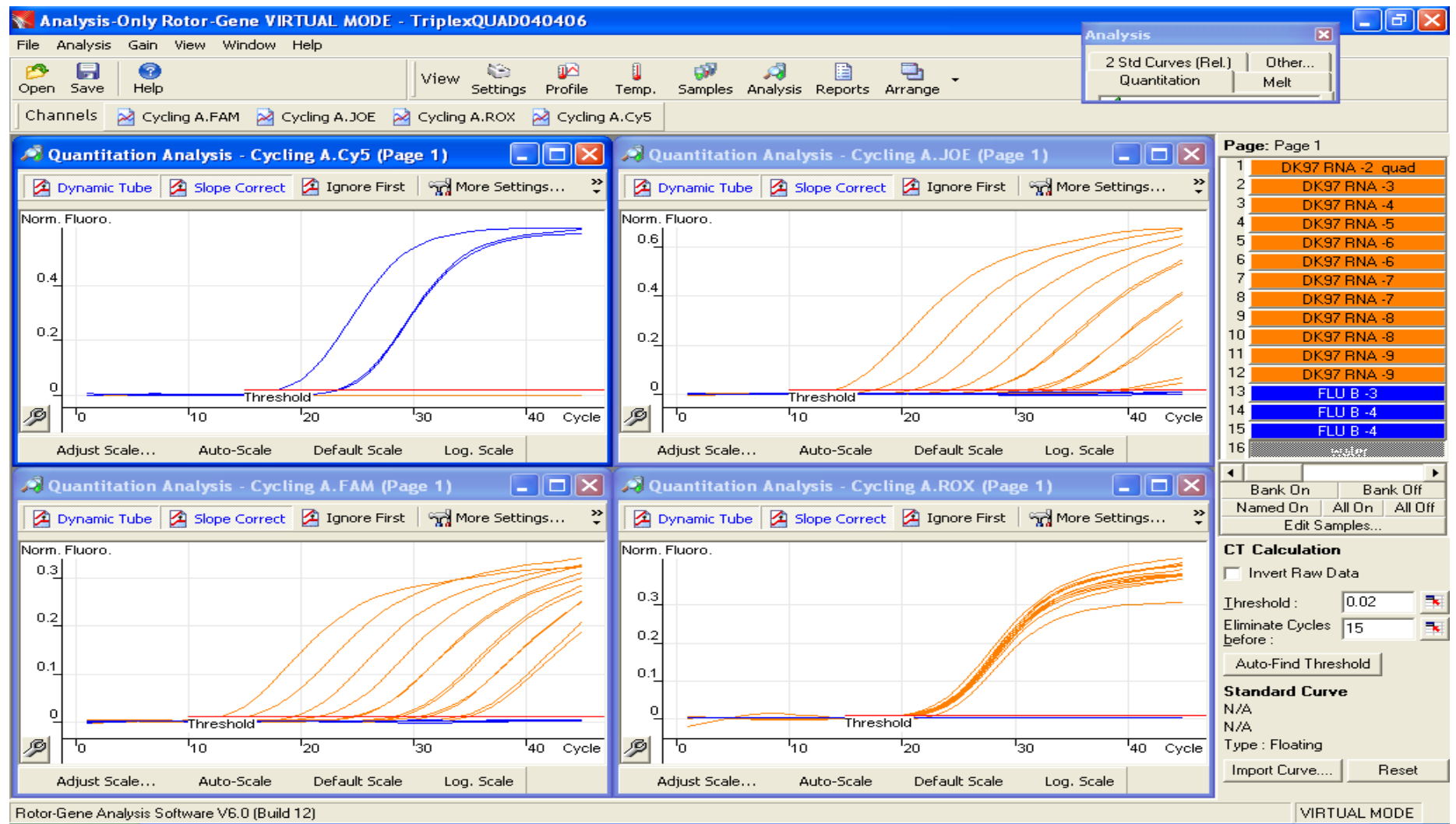
Sensitivity 98.1%, Specificity 100%, PPV 100% and NPV 98.9%

		Cfl Flu B		
		+	-	Total
Quadriplex Flu B	+	57	0	57
	-	0	91	91
	Total	57	91	148

Sensitivity 100%, Specificity 100%, PPV 100% and NPV 100%

- See figure on the next page for a representative figure of the data obtained with the quadriplex assay for a dilution series of DK97 Singapore H5N3 control material.

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