TITLE: DETERMINATION OF INFLUENZA VIRUS SUSCEPTIBILITY TO NEURAMINIDASE INHIBITORS USING A FLUORESCENT SUBSTRATE

WRITTEN BY: ANGIE LACKENBY

SUMMARY

This SOP describes the method used to determine influenza virus neuraminidase (NA) activity and sensitivity to neuraminidase inhibitor (NI) drugs using an enzyme assay with a fluorescent substrate. The NA enzyme cleaves sialic acid from adjacent sugar residues. Inhibitors block this activity by competitively binding the enzyme active site. NA activity and sensitivity to inhibitors can be determined using the fluorogenic substrate, MUNANA (2' 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate). This substrate is cleaved by NA to yield free 4-Methyumbelliferone which fluoresces at high pH. The increase in fluorescence is quantified to give a measure of NA activity. Performing this assay in the presence of inhibitors allows the concentration of drug required for inhibition of enzyme activity by 50% (IC_{50}) to be determined.

SAFETY

Good Laboratory Practice supplemented with local COSHH and risk assessments

This assay is suitable for tissue culture and egg grown influenza A and influenza B viruses.

1.0 CROSS REFERENCE PROTOCOLS

1.1 Influenza virus inoculation of original cell culture fluids

1.2 HA of Influenza from tissue culture

2.0 MATERIALS

2.1 *EQUIPMENT*

2.1.1 Fluorescence plate reader (355nm and 460nm filters) **See useful note 8.1**

2.1.2 96 well plate shaker

2.1.3 Single channel pipettes suitable for 10µl to 900µl volumes

2.1.4 8-well and 12-well multi-channel pipettes suitable for 10µl to 150µl volumes

2.1.5 Filtered tips suitable for 10µl-900µl volumes

2.1.6 Multi-well reservoirs (Thermo Electron Cat. No. RTP/08200-10)

2.1.7 Pipette boy

2.1.8 10 and 25 ml disposable pipettes

2.1.9 Warm Room (+37^{o}C)

2.1.10 Fridge (+4^{o}C)

2.1.11 Freezer (- 20^{o}C and - 80^{o}C)

2.1.12 Black 96 well flat bottom plates (Corning 3915 or similar)

2.1.13 Adhesive plate sealers (or plastic lids are also suitable)

2.2 *REAGENTS*

2.2.1 Influenza virus isolates derived from passage in tissue culture or egg fluids with an HA titre of 20 Units or higher **(see useful notes 8.2 and 8.3)**

2.2.2 Subtype matched reference viruses (**see section 6 for details**)

2.2.3 2-Morpholinoethanesulfonic acid (MES) (Sigma-Aldrich M3671 or similar)

2.2.4 Calcium chloride (VWR 5701 or similar)

2.2.5 Oseltamivir Carboxylate (Roche. Product no. GS4071 or Ro64-0802)

2.2.6 Zanamivir (Glaxo-Smithkline Product no. GR121167X or GG167)

2.2.7 MUNANA (2' 2'-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate) (Sigma-Aldrich M8639 or similar)

2.2.8 4-Methylumbelliferone sodium salt (Sigma-Aldrich M1508)

2.2.9 Glycine (VWR 1517 or similar)

2.2.10 Absolute Ethanol (VWR 101077Y or similar)

2.2.11 Sodium Hydroxide (VWR 101182 or similar)

2.2.12 Distilled water

3.0 PREPARATION OF BUFFERS AND SOLUTIONS

All solutions and buffers should be stored at room temperature unless otherwise stated. Working solutions for use in the assay are prepared from master stock solutions where stated, for accuracy. The working solution of MUNANA should be made freshly before each assay.

3.1 *MASTER STOCK SOLUTIONS AND BUFFERS*

325mM MES: 31.72g MES in 500ml ddH_2O,
pH to 6.5 with concentrated NaOH

100mM CaCl2: 5.55g $CaCl_2$ in 500ml ddH_2O,

1M Glycine: 37.5g in 500ml ddH_2O,
pH to 10.7 with concentrated NaOH

10mM Oseltamivir Carboxylate: 250mg GS4071 in 87.92ml ddH_2O, store at -80°C or
250mg Ro64-0802 in 64.7ml ddH_2O, store at -80°C

10mM Zanamivir: 200mg in 60.18ml ddH_2O, store at -80°C

1mM MUNANA: 25mg in 51ml MES assay buffer, store at -20C

3.2 *WORKING SOLUTIONS AND BUFFERS*

100µM Oseltamivir Carboxylate: 500µl of 10mM Oseltamivir carboxylate stock solution
49.5ml H_2O Store at -20°C

100µM Zanamivir: 500µl 10mM Zanamivir stock solution
49.5ml H_2O Store at -20°C

MES Assay Buffer:

32.5mM MES: 50ml of 325mM MES stock solution

4mM $CaCl_2$: 20ml of 100mM $CaCl_2$ stock solution

ddH_20: 430ml

pH to 6.5 with concentrated NaOH

100µM MUNANA: 300µl of 1mM stock solution
2.7ml MES assay buffer

N.B. Prepare freshly, do not store. This volume is sufficient for one 96 well plate.

Stop Solution (500ml):

0.1M Glycine: 50ml (1M stock)

25% Ethanol: 125ml absolute ethanol

ddH_2O: 325ml

pH to 10.7 with concentrated NaOH

4.0 NA Activity Determination (MUNANA Assay)

This section describes how to measure the neuraminidase (NA) activity of influenza virus.
The optimal virus sample dilution to standardise virus dose when measuring virus IC_{50} to neuraminidase inhibitors (NIs) can be determined using this method.
Each assay should include subtype matched validated reference viruses. **Section 6** gives details of suitable references and validation criteria for the assay.

4.1 Add 20µl MES assay buffer to each well of a black 96 well flat bottomed plate.

4.2 Make duplicate two-fold dilutions of virus material, with a starting dilution of 1/2, by adding 20µl of the first virus to wells A1 and B1, 20µl of the second virus to wells C1 and D1 and so on until row 1 is filled (see table 4.1). Mix buffer and virus by pipetting up and down several times.

4.3 Serial dilute the viruses down the plate by carrying over 20µl from row 1 to row 2 and so on, **stopping at row 11**. Discard 20µl from row 11. Row 12 contains buffer only as a blank control.

Table 4.1 Plate layout for virus addition

		H	G	F	E	D	C	B	A
1/2	**1**	Virus 4	Virus 4	Virus 3	Virus 3	Virus 2	Virus 2	Virus 1	Virus 1
1/4	**2**								
1/8	**3**								
1/16	**4**								
1/32	**5**								
1/64	**6**								
1/128	**7**								
1/256	**8**								
1/512	**9**								
1/1024	**10**								
1/2048	**11**								
Buffer Only	**12**								

4.4 Prepare 3ml of MUNANA substrate working stock (100µM) **per plate** and add 30µl of substrate to each well including the blank row 12, ensuring virus titration and substrate mix.

4.5 Seal plate with a plastic seal and incubate at 37^{o}C for 60 minutes with shaking, in the dark.

4.6 Terminate the reaction by adding 150µl of stop solution to all wells including the blank row 12.

4.7 Read the plate within 20 minutes of adding stop solution detecting fluorescence using an excitation wavelength of 355nm and an emission wavelength of 460nm.

4.8 A mean value for the blank buffer only wells is calculated and taken from each data point. The data are then plotted as relative fluorescence units against virus dilution. This plot should yield a sigmoid dose-response curve (**see section 6 and useful note 8.4**). Each replicate is plotted independently, and the virus dose calculation carried out on each replicate separately. Virus dose dilutions should not differ for replicates by more than 1 dilution factor.

4.9 **Standard virus dose calculation**: Define the virus dilution in which enzyme activity yields the equivalent level of fluorescence in one hour as 10μM of 4-methyllumelliferone sodium salt (**see useful note 8.1 and appendix 1**). This dilution is then used in the IC_{50} assay in order to ensure equivalent activities for each virus are compared. This cut off should be within the linear range of the enzyme activity curve. Examples of expected curves are given in appendix 2.

Appendix 1 gives details of how to generate the standard curve of 4 methylumbelliferone sodium salt.

5.0 Neuraminidase Inhibition Assay

This section describes how to determine the IC_{50} of a virus to a neuraminidase inhibitor (NI). The NA activity of each virus should be measured (**MUNANA assay: section 4.0**) before performing the NI assay. For best results both assays should be performed on the same day (**See useful note 8.5**).

Each assay should include the subtype matched validated reference viruses (**see section 6**).

5.1 Dilute each virus in MES assay buffer according to the factor determined in the MUNANA assay.

5.2 Add 10µl of diluted virus to 2 columns (wells 1-11) of a black flat bottomed 96 well plate (i.e. column A +B wells 1-11 virus 1, column C+D wells 1-11 virus 2 etc. See table 5.1).

Table 5.1 Plate layout for virus addition

	H	G	F	E	D	C	B	A
1	Virus 4	Virus 4	Virus 3	Virus 3	Virus 2	Virus 2	Virus 1	Virus 1
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								

5.3 Prepare four-fold dilutions of drug beginning at 20,000nM (see table 5.2)

5.4 Add 10µl of each drug dilution to a full row of a 96 well plate (i.e. Row 1 A-H: 20,000nM, row 2 A-H: 2000nM, row 3 A-H: 1250nM etc). Ensure that the virus and drug are mixed.

Table 5.2 Method of preparing drug dilutions

Step	Dilution series	Drug Concentration (nM)	'In Assay' Concentration (nM)
1	200µl of 100µM working stock+800µl MES	20000	4000
2	300µl of step 1 +900 MES	5000	1000
3	300µl of step 2 +900 MES	1250	250
4	300µl of step 3 +900 MES	312.5	62.5
5	300µl of step 4 +900 MES	78.13	15.63
6	300µl of step 5 +900 MES	19.53	3.91
7	300µl of step 6 +900 MES	4.88	0.98
8	300µl of step 7 +900 MES	1.22	0.24
9	300µl of step 8 +900 MES	0.31	0.061
10	300µl of step 9 +900 MES	0.076	0.015
11	Buffer only	Virus/Substrate control	0
12	Buffer only	Substrate/Buffer control	0

5.5 Seal plate with a plastic seal and incubate for 30 minutes at 37^{o}C with shaking.

5.6 Prepare 3ml of MUNANA working stock (100µM) **per plate** and add 30µl of substrate to each well including the blank row 12, ensuring virus/drug and substrate mix.

5.7 Seal plate with a plastic seal and incubate at 37^{o}C for 60 minutes, with shaking.

5.8 Terminate the reaction by adding 150µl stop solution to all wells including the blank row 12.

5.9 Read the plate within 20 minutes of adding stop solution detecting fluorescence using an excitation wavelength of 355nm and an emission wavelength of 460nm.

5.10 The data are plotted as relative fluorescence units against NA inhibitor concentration which should yield a sigmoid does-response curve (**see useful note 8.4**). An example is given in **appendix 3**. Refer to section 6 for validation criteria.

5.11 IC_{50} values are calculated for the replicates independently, and the mean IC_{50} taken as the final value. IC_{50} values are calculated by first subtracting the mean blank value from all data points. Next, the number of relative fluorescence units given by 50% of the virus control value is calculated, and the drug dilution corresponding to this level of fluorescence is the IC_{50} value.

5.12 Section 7 gives brief details of data handling and analysis.

6.0 Neuraminidase Inhibition Assay Validation and Reference Criteria

6.1 Reference viruses are included in all NA activity and IC_{50} assays and should be subtype matched to the samples undergoing testing. If the sample subtype is unknown, reference strains of all subtypes should be used. Suggestions of reference suitable strains are given in the table below (**See useful note 8.6**).

Subtype	Reference Virus	Mutation
H1N1	A/Texas/36/91	Wild Type (274H)
		274Y
H3N2	A/Sydney/5/97	Wild Type (292R)
		292K
	A/Wuhan/359/95	Wild Type (119E)
		119V
Flu B	B/Malaysia/256/04	Wild Type
	B/Florida/7/05	Wild Type
	B/Memphis/20/96	Wild Type (152R)
	B/Memphis/20/96	152K

6.2 All curves in both the NA activity and IC_{50} assays should be manually checked for points which do not fit the sigmoidal shape and to ensure replicate curves match.

6.3 In the NA activity assay, the dilution factor calculated in the replicate testing for a given virus giving equal fluorescence units to that generated by 10nmol/ml should be no more than one dilution apart.

6.4 IC_{50} calculations are carried out on replicates independently and the final IC_{50} is a mean of the two values.

6.5 Any sample for which replicate titrations show greater than 20% difference in values should be repeated.

6.6 Validation limits for each reference virus should be determined. Limits are defined as 3 standard deviations above and below the median IC_{50}. The median is calculated from a minimum of ten independent assays of the reference virus.

6.7 If a reference virus IC_{50} value fails to meet validation criteria for a given drug, the test using that drug is invalidated and all samples repeated. Trends in reference IC_{50} performance should be monitored. A batch which fails to meet validation criteria in three consecutive assays should be discarded.

7.0 Data Analysis

7.1 An algorithm for samples testing is given in appendix 4A. Samples with a valid IC_{50} value greater than 1.65SD above the median for the subtype and season are retested twice and the mean IC_{50} value calculated. If this mean IC_{50} value is greater than 1.65SD above the median the sample is classified as a minor outlier and subjected to further characterisation and genotyping as detailed in appendix 4B. Any sample with a mean IC_{50} value greater than 3SD above the median for the season and subtype is classified as a major outlier and subjected to further characterisation and genotyping according to appendix 4B.

7.2 As cut off criteria are based on the median IC_{50} value of isolates per season and subtype, they cannot be applied until a significant number of isolates have been tested. The cut off criteria should therefore be calculated only after at least 20 isolates have been assayed and applied in a retrospective fashion to those isolates. The cut off criteria can be re-evaluated at the end of the season to ensure they are set at an appropriate level.

7.3 If insufficient viruses from a given subtype or season are available to determine robust cut off criteria, data should be used from isolate results for the previous season, or from other laboratories where values are given on the EISS website.

8.0 Useful Notes

8.1 **Fluorescence is measured in relative fluorescence units. Because of this, different fluorimeters have different ranges of values. Raw data values measured in RFU cannot be compared from one machine to another, and can only be compared from one assay to the next if settings are not changed.**

8.2 Virus isolates which have been stored frozen can be analysed for IC50. After thawing, virus should be stored at +4oC until the IC50 determination is complete upon which virus can be stored frozen once again. Refer to useful note 8.4.

8.3 Samples with low titre may not have sufficient NA activity for inhibition testing and may give inaccurate IC_{50} values. Only samples with HA titres of 20 and over and/or peak NA activity equivalent to 10μM 4-methylumbelliferone sodium salt can be reliably tested. Samples not meeting these criteria should be passaged to yield a higher titre.

8.4 Plotted data should yield sigmoid curves. Strains with flat curves or low neuraminidase activity (despite reasonable HA titre) may be exhibiting reduced sensitivity to neuraminidase inhibitors, even if their IC_{50} value is within the normal range. These samples should be subject to further characterisation.

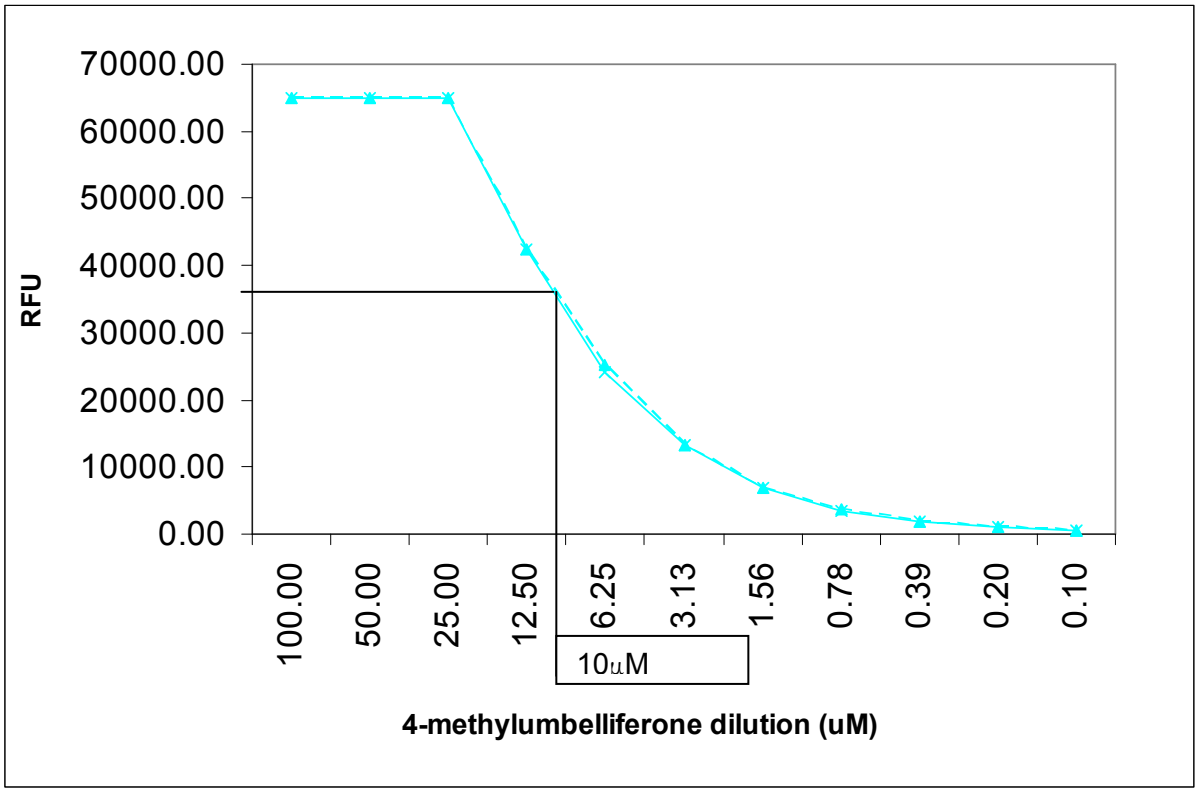
8.5 Between performing the MUNANA assay and IC50 determination assay, viruses should be stored at +4^{o}C. No more than 24 hours should elapse between the 2 tests; otherwise virus dilutions should be determined by MUANA assay again. This is because the NA activity of viruses, particularly those with mutations in the NA gene causing resistance can be unstable.

8.6 As described in section 6, subtype matched NI sensitive and resistant viruses should be included as reference standards in all assays. If resistant viruses are not available then subtype matched sensitive strains can be used as reference standards provided the performance of such viruses in IC_{50} assays is well characterised. The performance of selected viruses can be evaluated by measuring the IC_{50} value against NI drugs 10-20 times in independent assays. This will allow a median value for the IC_{50} of neuraminidase inhibitor susceptibility for that particular reference virus to be determined. Assay performance can then be validated according to the criteria described in section 6. Whilst this approach to standardisation will not absolutely guarantee the ability of the test to determine neuraminidase resistance it will provide a means to ensure day to day variation is minimised.

9.0 Appendices

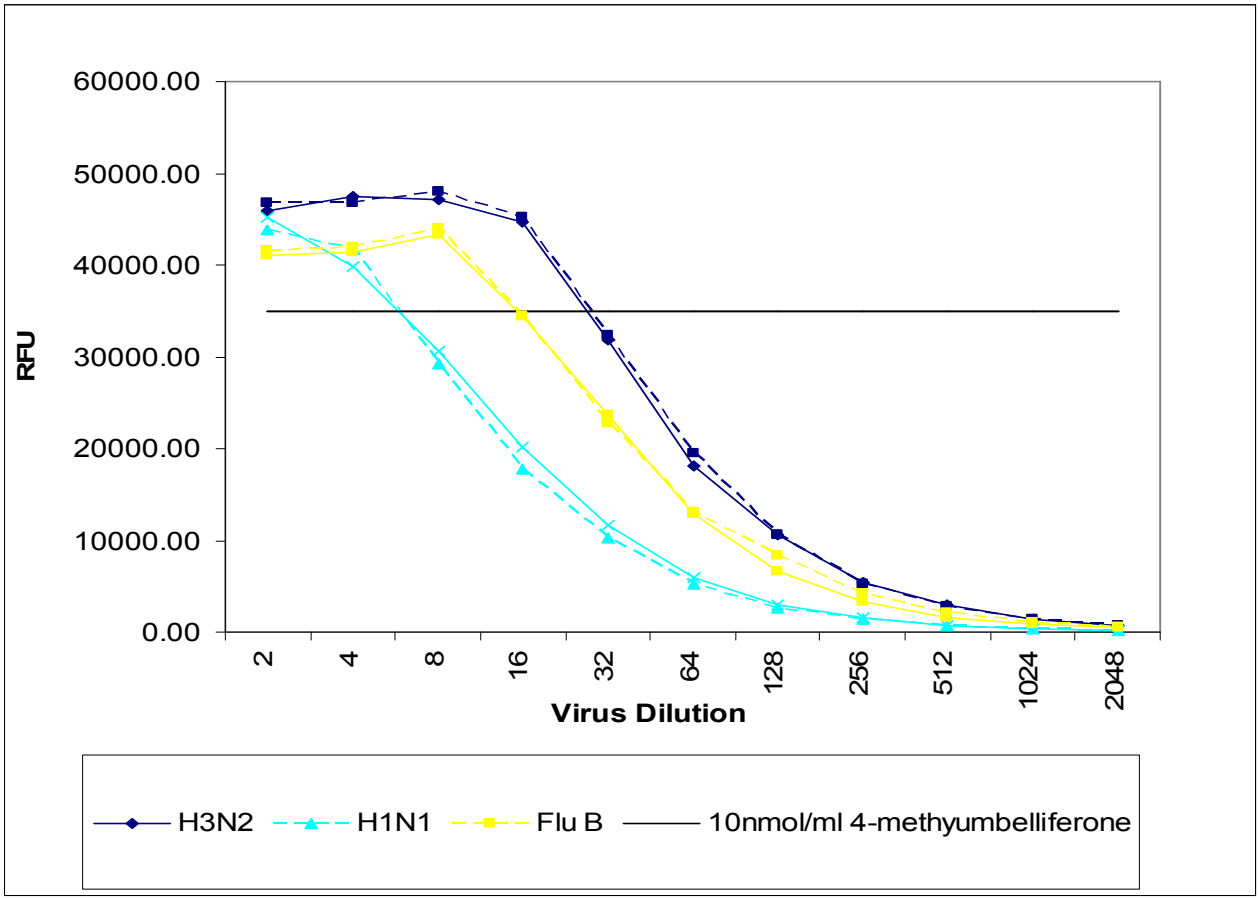
APPENDIX 1: 4-METHYLUMBELLIFERONE SODIUM SALT STANDARD CURVE

1. Dilute 4-methylumbelliferone sodium salt (4-MUSS) in water to 100μM concentration.
2. Serial dilute the 4-MUSS in 1/2 steps, in stop solution (used for NA activity and IC50 tests). The 4-MUSS must be titrated in stop solution to ensure that the fluorophore is fluorescing (requires high pH).
3. Pipette 200μl of each dilution of 4-MUSS onto the same plates which are used in IC50 testing (black, flat bottomed).
4. Measure the fluorescence activity of the 4-MUSS titration series. The volume of 200μl must be measured as this is equal to the final volume which is measured in the NA activity and IC50 assays.
5. An example curve for the 4-MUSS titration is given below.
6. Determine the relative fluorescence units generated by 10μM 4-MUSS.
7. The number of RFU given by 10μM 4-MUSS can then be applied to curves generated by viral titrations to determine standard dose for IC50 testing.
8. For example, based on the curve below, a cut off of 37500 RFU would be applied to all virus titrations. The total number of RFU will be different on different fluorimeters (see useful note 7.1)



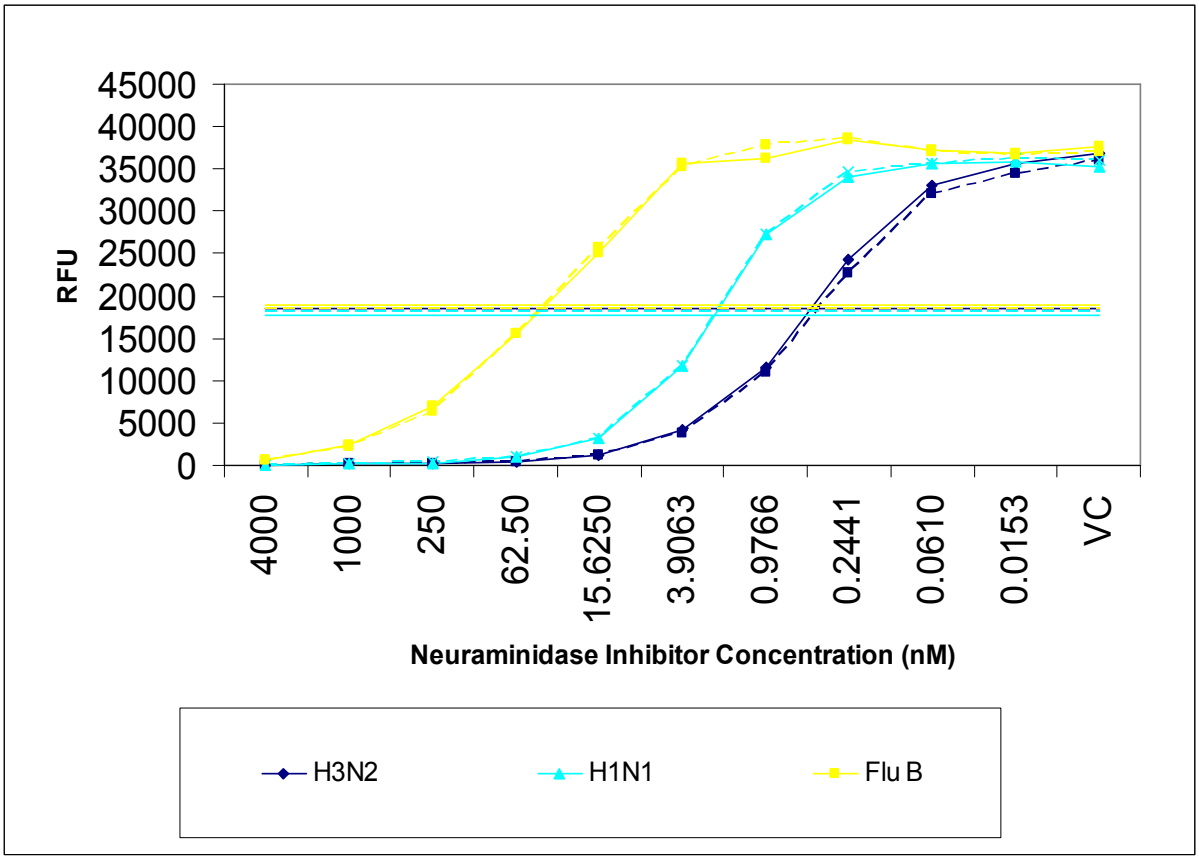
APPENDIX 2: ANALYSES OF MUNANA RESULTS

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1		Titration													
2	Virus	2	4	8	16	32	64	128	256	512	1024	2048	blank		
3	H3N2	46345	47947	47473	45120	32176	18463	11005	5856	3325	1869	1138	418		
4		47251	47133	48491	45649	32787	19892	11059	5605	3186	1742	1125	468		
5	H1N1	44181	42140	29676	18238	10763	5613	3101	1812	1058	768	617	393		
6		45646	40263	31110	20586	12149	6305	3447	1931	1174	797	626	426		
7	Flu B	42002	42304	44404	34911	23155	13420	8788	4550	2513	1427	985	417		
8		41511	41839	43760	34927	24147	13301	6991	3711	2060	1323	891	405		
9											Average Blank		421.17		
10															
11	Minus Blank	0.30103000	0.60205999	0.90308999	1.20411998	1.50514998	1.80617997	2.10720997	2.40823997	2.70926996	3.01029996	3.31132995	CUT OFF		
12	H3N2	45923.83	47525.83	47051.83	44698.83	31754.83	18041.83	10583.83	5434.83	2903.83	1447.83	716.83	35000		
13		46829.83	46711.83	48069.83	45227.83	32365.83	19470.83	10637.83	5183.83	2764.83	1320.83	703.83	35000		
14	H1N1	43759.83	41718.83	29254.83	17816.83	10341.83	5191.83	2679.83	1390.83	636.83	346.83	195.83	35000		
15		45224.83	39841.83	30688.83	20164.83	11727.83	5883.83	3025.83	1509.83	752.83	375.83	204.83	35000		
16	Flu B	41580.83	41882.83	43982.83	34489.83	22733.83	12998.83	8366.83	4128.83	2091.83	1005.83	563.83	35000		
17		41089.83	41417.83	43338.83	34505.83	23725.83	12879.83	6569.83	3289.83	1638.83	901.83	469.83	35000		
18	H3N2	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00			
19		35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00			
20	H1N1	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00			
21		35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00			
22	Flu B	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00			
23		35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00	35000.00			
24															
25	Find Conc	0.30103000	0.60205999	0.90308999	1.20411998	1.50514998	1.80617997	2.10720997	2.40823997	2.70926996	3.01029996	3.31132995	Conc	Dilution	Dilution for IC50
26	H3N2				1.429679296								26.89547981	13	14
27					1.443498355								27.76504329	14	
28	H1N1		0.764332967										5.812098516	3	3
29			0.76130145										5.771669436	3	
30	Flu B			1.187942223									15.41495365	8	8
31				1.187278707									15.39142062	8	

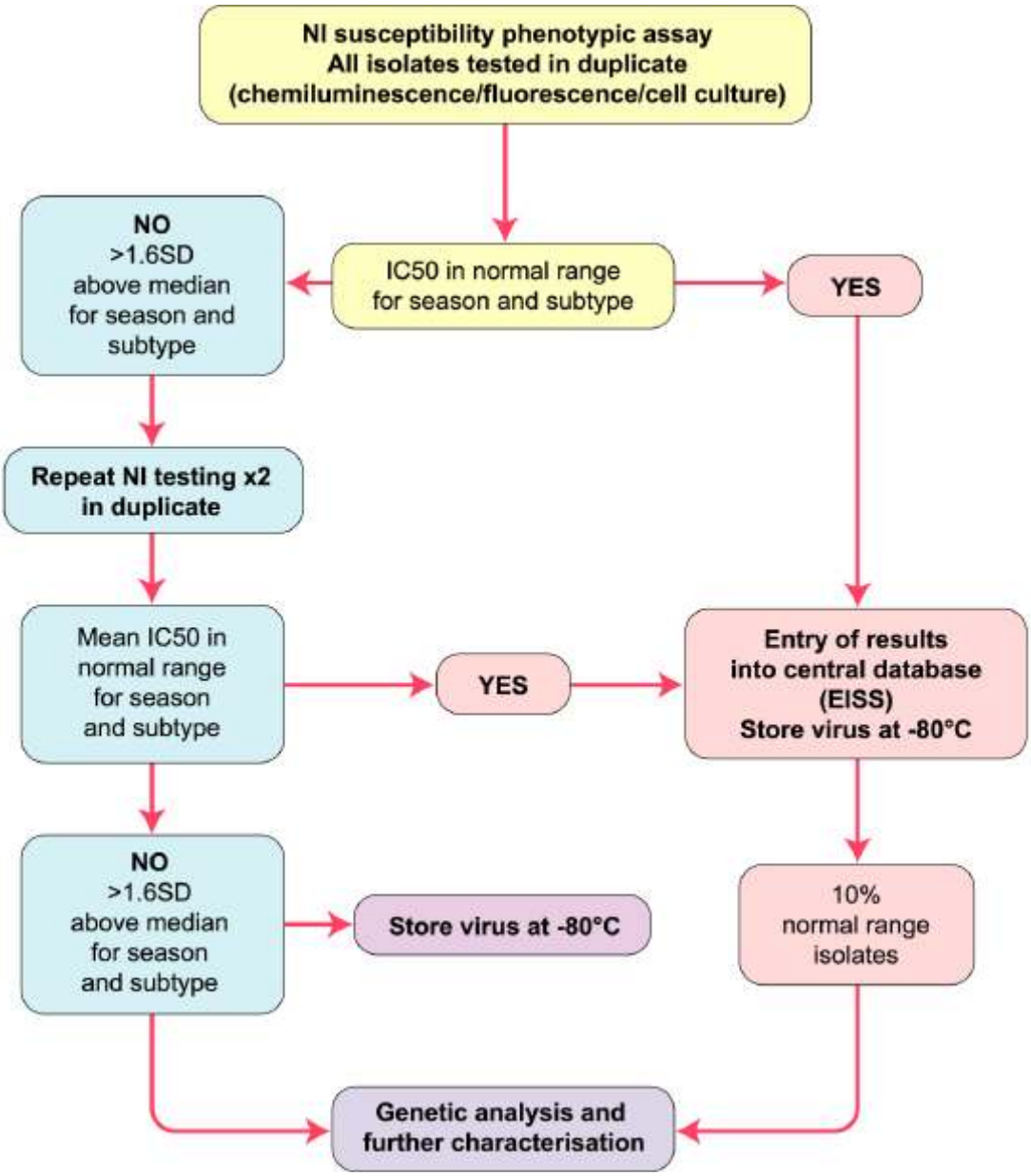


APPENDIX 3: ANALYSES OF NA INHIBITION ASSAY RESULTS

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1		Neuraminidase Inhibitor Concentration												
2	Strain Name	4000	1000	250	62.50	15.6250	3.9063	0.9766	0.2441	0.0610	0.0153	VC	neg	
3	H3N2	482	504	553	772	1663	4590	11854	24588	33455	36000	37282	398	
4		473	501	542	745	1601	4209	11300	23014	32402	34929	36436	398	
5	H1N1	484	504	679	1301	3577	12225	27581	34470	36110	36156	35728	395	
6		468	512	698	1305	3653	12197	27573	35037	36121	36664	36525	397	
7	Flu B	1068	2688	7348	15971	25511	35980	36675	38762	37647	37247	38029	390	
8		990	2621	6725	15872	26068	35666	38123	39018	37376	37103	37328	350	
9												NEG MEAN	388.00	
10														
11	Log(Conc)	3.60206	3	2.39794	1.79588	1.19382	0.59176	-0.0103	-0.61236	-1.21442	-1.81648	VC	50%CUT	
12	H3N2	94	116	165	384	1275	4202	11466	24200	33067	35612	36894	18447	
13		85.00	113.00	154.00	357.00	1213.00	3821.00	10912.00	22626.00	32014.00	34541.00	36048.00	18024	
14	H1N1	96.00	116.00	291.00	913.00	3189.00	11837.00	27193.00	34082.00	35722.00	35768.00	35340.00	17670	
15		80.00	124.00	310.00	917.00	3265.00	11809.00	27185.00	34649.00	35733.00	36276.00	36137.00	18068.5	
16	Flu B	680.00	2300.00	6960.00	15583.00	25123.00	35592.00	36287.00	38374.00	37259.00	36859.00	37641.00	18820.5	
17		602.00	2233.00	6337.00	15484.00	25680.00	35278.00	37735.00	38630.00	36988.00	36715.00	36940.00	18470	
18	H3N2	18447.00	18447.00	18447.00	18447.00	18447.00	18447.00	18447.00	18447.00	18447.00	18447.00	18447.00		
19		18024.00	18024.00	18024.00	18024.00	18024.00	18024.00	18024.00	18024.00	18024.00	18024.00	18024.00		
20	H1N1	17670.00	17670.00	17670.00	17670.00	17670.00	17670.00	17670.00	17670.00	17670.00	17670.00	17670.00		
21		18068.50	18068.50	18068.50	18068.50	18068.50	18068.50	18068.50	18068.50	18068.50	18068.50	18068.50		
22	Flu B	18820.50	18820.50	18820.50	18820.50	18820.50	18820.50	18820.50	18820.50	18820.50	18820.50	18820.50		
23		18470.00	18470.00	18470.00	18470.00	18470.00	18470.00	18470.00	18470.00	18470.00	18470.00	18470.00		
24														
25	FINDIC50	3.60206	3	2.39794	1.79588	1.19382	0.59176	-0.0103	-0.61236	-1.21442	-1.81648	Res	IC50	Mean IC50
26	H3N2							-0.34036				-0.34036	0.45671	0.44
27								-0.375833				-0.375833	0.420889	
28	H1N1						0.363067					0.363067	2.307101	2.26
29							0.346664					0.346664	2.221591	
30	Flu B				1.591565							1.591565	39.04492	40.34
31					1.619561							1.619561	41.6448	



APPENDIX 4A: ALGORITHM FOR SAMPLE TESTING



APPENDIX 4B: ALGORITHM FOR SAMPLE CHARACTERISATION