HEALTH PROTECTION AGENCY

MICROBIOLOGY SERVICES COLINDALE

VIRUS REFERENCE DEPARTMENT

STANDARD OPERATING PROCEDURE

TITLE: INFLUENZA MUNANA NEURAMINIDASE ACTIVITY AND INHIBITION ASSAY (FLUORESCENT IC_{50} ASSAY)

SOP NO. **V-6815/01-10**

AUTHOR: **ANGIE LACKENBY**

SUMMARY

This SOP describes the method to determine influenza virus neuraminidase (NA) activity and sensitivity to neuraminidase inhibitors (NI) using an enzyme assay with a fluorescent substrate. NA activity and NI sensitivity 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, and the quantitative increase in fluorescence gives a measure of NA activity. The concentration of drug needed for inhibition of enzyme activity by 50% (IC_{50}) is determined by assay in the presence of NIs.

SAFETY

This assay is suitable for tissue culture and egg grown influenza A and influenza B viruses
Good Laboratory Practice and refer to VRD Safety Manual (V6764)
COSHH Risk Assessment No.s:
VB37 Influenza Virus Neuraminidase Inhibition
VB551 Influenza Virus Neuraminidase Inhibition with strains to be handled at CL3
VC893 Influenza virus Neuraminidase Inhibition with Fluorescent substrate
VB545 Control measures for work with pandemic influenza H1N1 viruses

1.0 CROSS REFERENCE

1.1 VW0771 IC50 Data Management Workflow
1.2 VW0770 IC50 Dilution Ranges and Layouts
1.3 VW0772 IC50 Reference Virus Validation Limits
1.4 VW0574 Munana Neuraminidase Activity Assay Worksheet
1.5 VW0575 IC50 Results Record Sheet: Neuraminidase Inhibitor Susceptibility
1.6 SOP V6816 IC50 Outlier Identification and Trend Monitoring
1.7 SOP V5402 Haemagglutination (HA) Test for Influenza Virus

2.0 PERSONNEL

2.1 All medical microbiologists, clinical scientists, biomedical scientists, and healthcare scientists with suitable training.

3.0 EQUIPMENT

3.1 Fluorescence plate reader (355nm and 460nm filters) (useful note 9.1)
3.2 Plate shaker
3.3 Single channel pipettes suitable for 10 to 900µl volumes and filtered tips
3.4 8 and/or 12-well multi-channel pipettes suitable for 10 to 150µl volumes and filtered tips
3.5 Multi-well reservoirs (Thermo Electron Cat. No. RTP/08200-10)
3.6 10 and 25 ml disposable pipettes and pipette boy
3.7 Warm Room (+37^{o}C), Fridge (+4^{o}C), Freezer (- 20^{o}C and - 80^{o}C)
3.8 Black 96 well flat bottom plates (Corning 3915 or equivalent)
3.9 Adhesive plate sealers (useful note 9.2)

4.0 REAGENTS

4.1 Influenza virus isolates (tissue culture/egg fluids) HA ≥16 Units (useful note 9.3)
4.2 Subtype matched reference viruses (see section 8 for details)
4.3 2-Morpholinoethanesulfonic acid (MES) (Sigma-Aldrich M3671 or equivalent)
4.4 Calcium chloride (VWR 5701 or equivalent)
4.5 Oseltamivir Carboxylate (Roche. Product no. GS4071 or Ro64-0802)
4.6 Zanamivir (Glaxo-Smithkline Product no. GR121167X or GG167)
4.7 MUNANA (2’ 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate) (Sigma-Aldrich M8639)
4.8 4-Methylumbelliferone sodium salt (Sigma-Aldrich M1508)
4.9 Glycine (VWR 1517 or equivalent)
4.10 Absolute Ethanol (VWR 101077Y or equivalent)
4.11 Sodium Hydroxide (VWR 101182 or equivalent)
4.12 Distilled water

5.0 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 must be made freshly for each assay.

5.1 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

5.2 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 (per plate): 300µl of 1mM stock solution
2.7ml MES assay buffer

Stop Solution (500ml): 0.1M Glycine: 50ml (1M stock)
25% Ethanol: 125ml absolute ethanol
ddH_2O: 325ml
pH to 10.7 with concentrated NaOH

6.0 NA Activity Determination (MUNANA Assay)

Use work instruction VW0574 for sample worksheet and results. Follow VW0771 for data management workflow.
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 8** gives details of suitable references and validation criteria for the assay.

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

6.2 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 A2, 20µl of the second virus to wells A3 and A4 and so on until row A is filled (see table 4.1). Mix buffer and virus by pipetting up and down several times, taking care not to create aerosols.

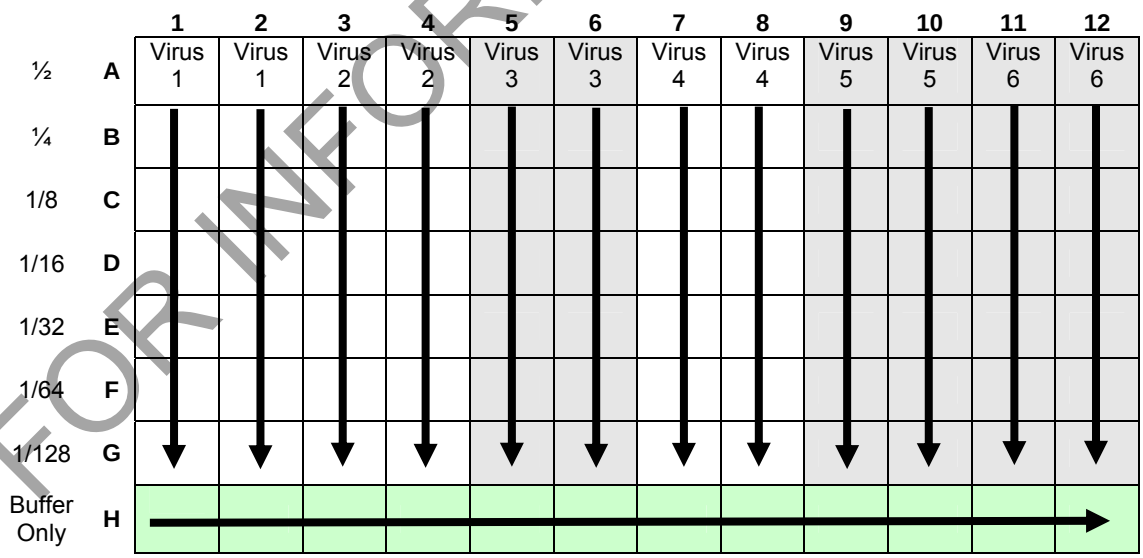
6.3 Serial dilute the viruses down the plate by carrying over 20µl from row A to row B and so on, stopping at row G. Discard 20µl from row G. The final row of the plate contains buffer only as a blank control.

6.4 Prepare 3ml of MUNANA substrate working stock (100µM) per plate and add 30µl to each well including the blank row H.

6.5 Seal the plate(s) and incubate at 37°C for 60 minutes with shaking, in the dark.

6.6 Terminate the reaction by adding 150µl of stop solution to all wells.

Table 4.1 Plate layout for virus addition



6.7 Read the plate within 30 minutes of adding stop solution using the MUNANA test protocol on the Flurostar optima plate reader (appendix 2).

6.8 The data are plotted as relative fluorescence units (RFU) against virus dilution, with the mean blank (buffer only) value subtracted. This plot should yield a sigmoid dose-response curve (see section 8 and useful note 9.4).

6.9 To calculate standard virus dose for IC_{50} testing, transfer the 96 well plate data to the MUNANA Results excel template (VW0574).

6.10 The standard virus dose is calculated by defining the virus dilution in which enzyme activity for a given isolate yields the equivalent level of fluorescence in one hour as 10μM of 4-methyllumelliferone sodium salt (useful note 9.1 and appendix 1). This cut off should be within the linear range of the enzyme activity curve. Examples of expected curves are given in appendix 3.

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

7.0 Neuraminidase Inhibition Assay

Use work instruction VW0575 for sample worksheet and results. Follow VW0771 for data management workflow.

This section describes how to determine the IC_{50} of a virus to a neuraminidase inhibitor (NI).

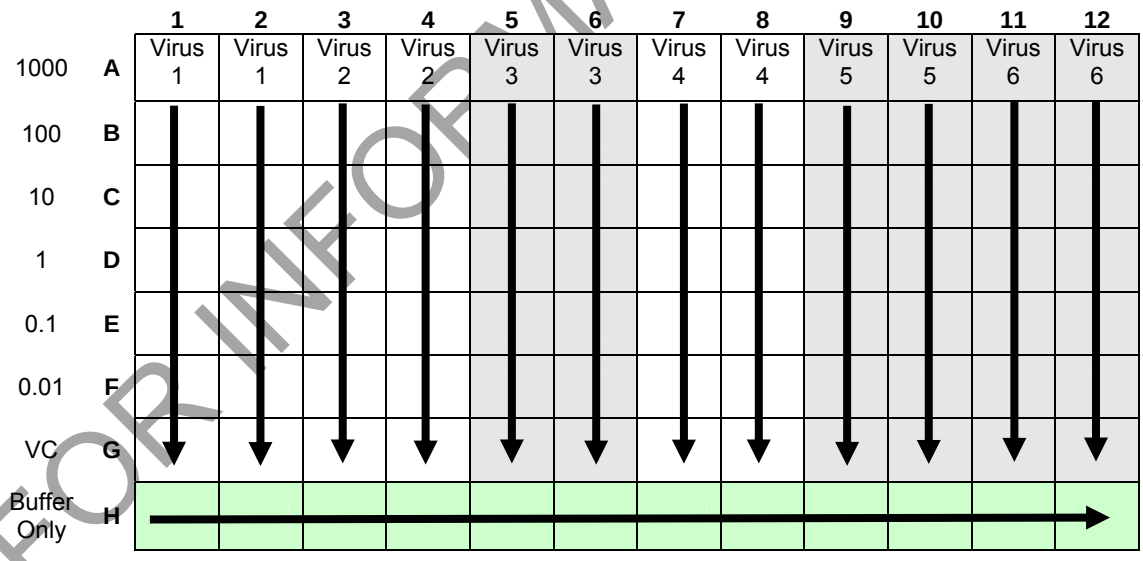
For best results calculate the standard virus dose by measuring the NA activity of each virus (**NA Activity assay: section 6.0**) performing both assays on the same day (useful note 9.5).

Each assay should include the subtype matched validated reference viruses (**see section 8**).

7.1 Dilute each virus appropriately in MES assay buffer.

7.2 Add 10µl of diluted virus to 2 columns (wells A-G) of a black flat bottomed 96 well plate (i.e. column 1 +2 wells A-G virus 1, column 3+4 wells A-G virus 2 etc. See table 5.1).

Table 5.1 Plate layout for virus addition



7.3 In an 8 well reservoir, prepare ten-fold dilutions of drug (see table 5.2)

7.4 Add 10µl of each drug dilution to a full row of a 96 well plate (i.e. Row A 1-12: 1,000nM, row B 1-12: 100nM, row C 1-12: 10nM etc). Ensure that the virus and drug are mixed.

7.5 Seal the plate(s) and incubate for 30 minutes at 37^{o}C with shaking.

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

7.7 Seal the plate(s) and incubate at 37^{o}C for 60 minutes with shaking, in the dark.

7.8 Terminate the reaction by adding 150µl of stop solution to all wells.

Table 5.2 Method of preparing drug dilutions*

Step	Dilution series	Drug Concentration (nM)	'In Assay' Concentration (nM)
1	100µl of 100µM working stock+1900µl MES	5000	1000
2	150µl of step 1 +1350 MES	500	100
3	150µl of step 2 +1350 MES	50	10
4	150µl of step 3 +1350 MES	5	1
5	150µl of step 4 +1350 MES	0.5	0.1
6	150µl of step 5 +1350 MES	0.05	0.01
7	Buffer only	Virus/Substrate control	0
8	Buffer only	Substrate/Buffer control	0

*NB: The drug dilution range can be changed to suit the known or suspected IC_{50} values of the test isolates. A narrow range for more precise calculation of low IC_{50} values (250nM-0.08nM) and an extended range for highly resistant isolates (10,000nM-0.1nM) have been calculated in work instruction VW0770.

7.9 The data are plotted as RFU against NA inhibitor concentration, with the mean blank (buffer only) value subtracted. This plot should yield a sigmoid dose-response curve (see section 8 and useful note 9.4).

7.10 To calculate the IC_{50} values, transfer the plate data to the NAI Results excel template (VW0575).

7.11 IC_{50} values are calculated for the duplicates independently, and the mean IC_{50} taken as the final value. The RFU 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. An example is given in appendix 4.

7.12 Trends in IC_{50} values for given subtypes and seasons are monitored, refer to SOP V6816 for details.

8.0 Neuraminidase Inhibition Assay Validation and Reference Criteria

8.1 Current reference strains and batches in use and the IC50 and MUNANA validation limits are given in VW0772.

8.2 Reference viruses are included in all NA activity and IC_{50} assays and should be subtype matched to the samples undergoing testing, wherever possible. 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 (Useful note 9.6).

8.3 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.

8.4 In the NA activity assay, the virus dose calculated from the replicates should be no more than one dilution factor apart.

8.5 The final IC_{50} is a mean of independently calculated values from the replicates.

8.6 Validation limits for each reference virus should be determined. Maximum 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.

8.7 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.

9.0 Useful Notes

9.1 Fluorescence is measured in relative fluorescence units (RFU). 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.

9.2 Where biosafety levels dictate, plates can be read whilst sealed with an optical clear plate seal. This will reduce the overall fluorescence units slightly, but will not impact on the shape of the curves, nor the virus dilution/IC_{50} value. In this instance, stop solution should be added to the plates, and then left to equilibrate to room temperature for 10 minutes prior to sealing the plate to minimise condensation.

9.3 Samples with low titre may have insufficient NA activity for inhibition testing and may give inaccurate IC_{50} values. Only samples with HA titres of ≥ 16 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.

9.4 Plotted data should yield sigmoid curves. Strains with low neuraminidase activity (despite reasonable HA titre) or flattened curves in presence of drug 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.

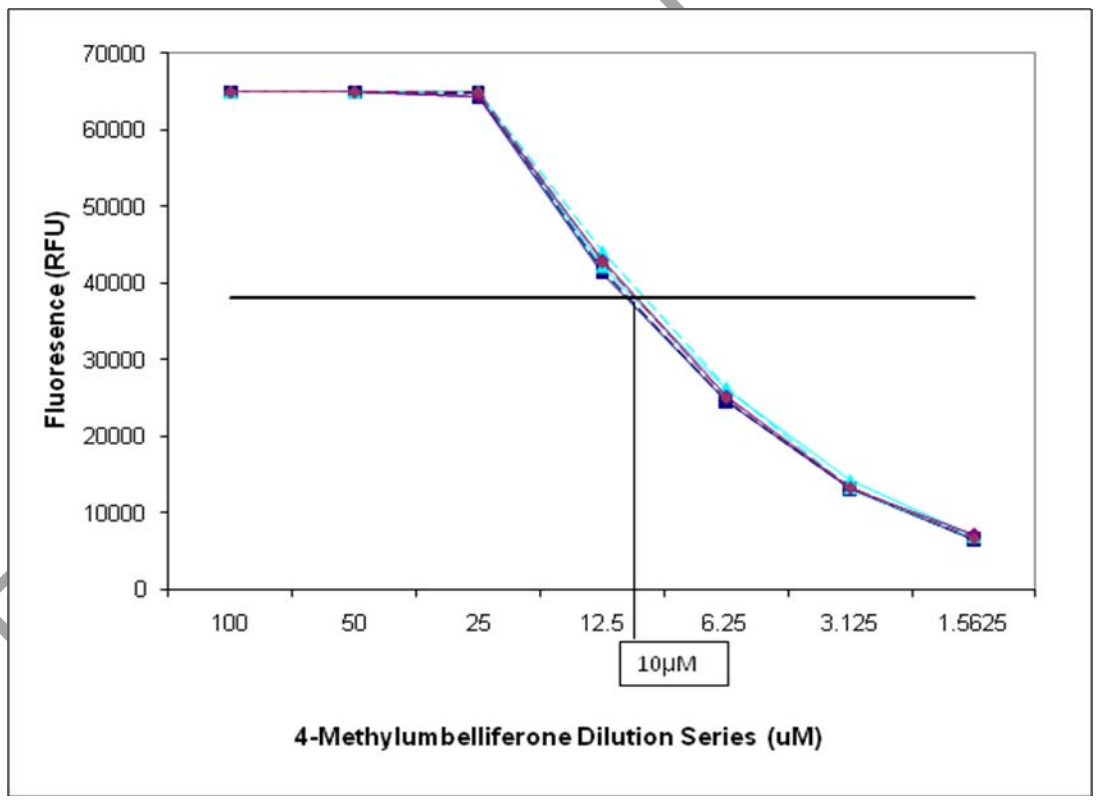
9.5 The NA activity of influenza viruses, particularly those with mutations in the NA gene causing resistance can be unstable. Isolates should be stored at -80^{o}C and kept at +4oC for minimal times. Virus dilutions calculated by MUNANA assay are only valid up to 24 hours after testing if isolates are stored at +4^{o}C and should assayed again if isolates are frozen/left longer.

9.6 As described in section 8, subtype matched NI sensitive and resistant viruses should be included as references in all assays. If resistant viruses are not available, subtype matched sensitive strains can be used as references provided the performance of such viruses in IC_{50} assays is well characterised, (e.g. evaluated in 10-20 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 8. 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.

10.0 Appendices

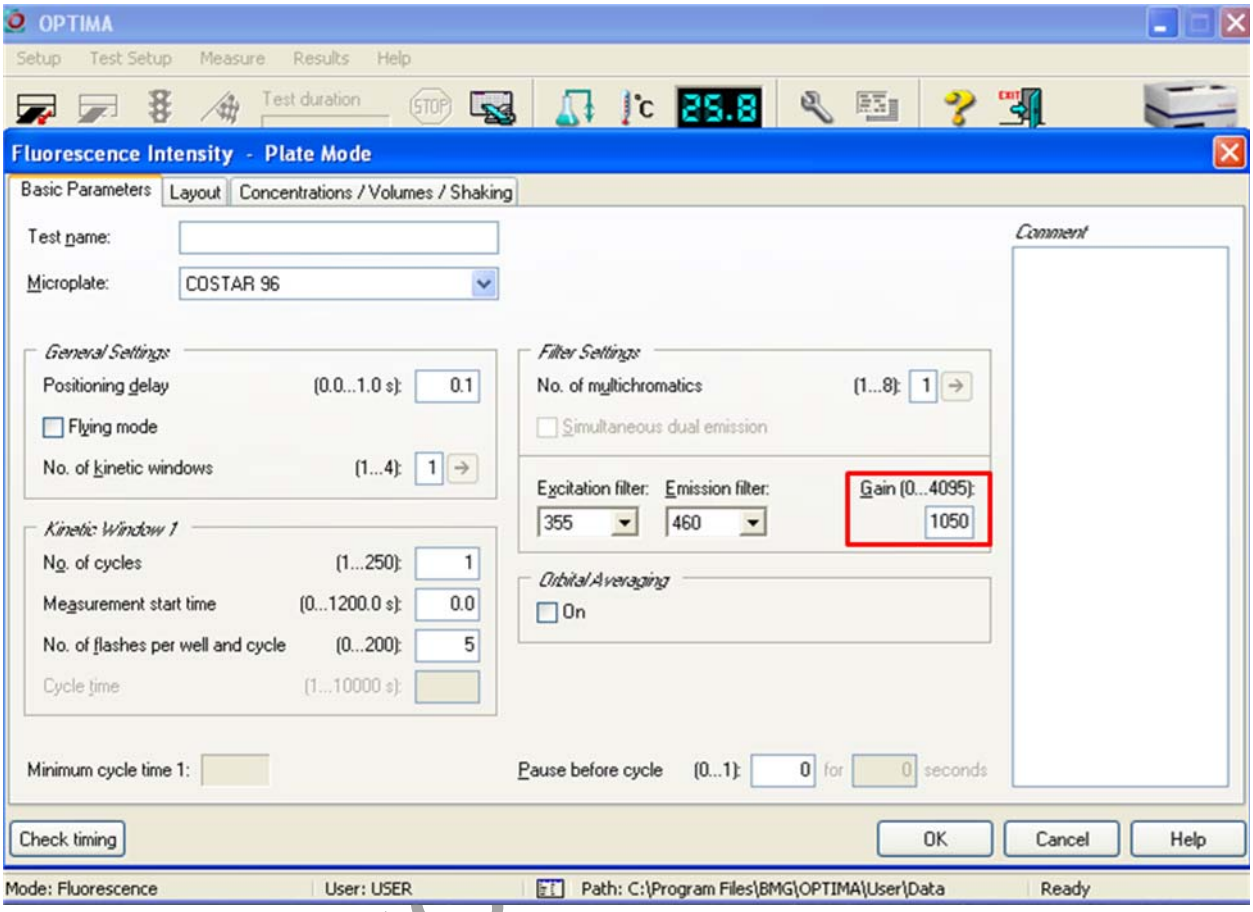
APPENDIX 1: 4-METHYLUMBELLIFERONE SODIUM SALT STANDARD CURVE

1. Dilute 4-methylumbelliferone sodium salt (4-MUSS) in water to 1mM concentration.
2. Serial dilute the 4-MUSS in 1/2 steps, in stop solution (used for NA activity and IC_{50} 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 IC_{50} 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 IC_{50} assays.
5. An example curve for the 4-MUSS titration is given below.
6. Determine the RFU 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 IC_{50} testing.
8. For example, based on the curve below, a cut off of 38000 RFU would be applied to all virus titrations. The total number of RFU will be different on different fluorimeters (useful note 9.1)



APPENDIX 2: PLATE READER TEST PROTOCOL

Note: Gain settings are specific to the machine

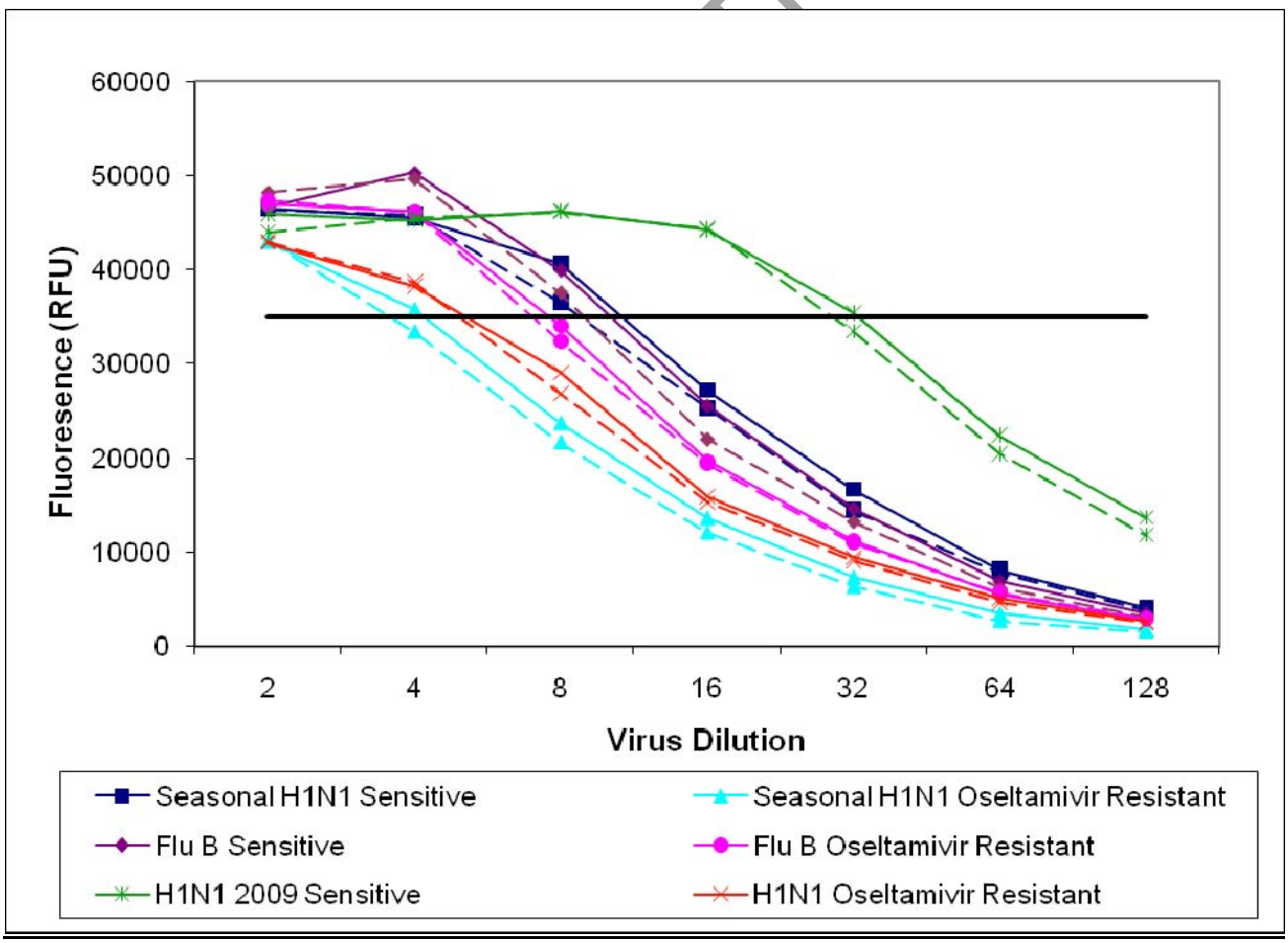


APPENDIX 3: ANALYSES OF MUNANA RESULTS

Virus	Seasonal H1N1 Sensitive		Seasonal H1N1 Oseltamivir Resistant		Flu B Sensitive		Flu B Oseltamivir Resistant		H1N1 2009 Sensitive		H1N1 Oseltamivir Resistant	
2	47044	46833	43411	43619	47026	48649	47335	47848	46387	44472	43293	43427
4	45984	46279	36239	33912	50775	50145	46586	46583	45778	45969	38722	39179
8	41221	36954	24199	22160	40454	38032	34489	32862	46731	46563	29529	27309
16	27688	25737	14115	12554	26047	22445	20149	19835	44679	44788	16343	15715
32	17135	14903	7792	6798	15133	13690	11638	11452	35847	33868	9894	9517
64	8642	8234	3936	3155	7413	6593	6067	6128	22881	20878	5553	5051
128	4599	4310	2248	2025	3977	3507	3309	3445	14136	12257	3066	2948
Blank	458	446	464	454	467	444	446	460	455	444	461	457

Average-Blank	Seasonal H1N1 Sensitive		Seasonal H1N1 Oseltamivir Resistant		Flu B Sensitive		Flu B Oseltamivir Resistant		H1N1 2009 Sensitive		H1N1 Oseltamivir Resistant	
0.301029996	46589.33	46378.33	42956.33	43164.33	46571.33	48194.33	46880.33	47393.33	45932.33	44017.33	42838.33	42972.33
0.602059991	45529.33	45824.33	35784.33	33457.33	50320.33	49690.33	46131.33	46128.33	45323.33	45514.33	38267.33	38724.33
0.903089987	40766.33	36499.33	23744.33	21705.33	39999.33	37577.33	34034.33	32407.33	46276.33	46108.33	29074.33	26854.33
1.204119983	27233.33	25282.33	13660.33	12099.33	25592.33	21990.33	19694.33	19380.33	44224.33	44333.33	15888.33	15260.33
1.505149978	16680.33	14448.33	7337.33	6343.33	14678.33	13235.33	11183.33	10997.33	35392.33	33413.33	9439.33	9062.33
1.806179974	8187.33	7779.33	3481.33	2700.33	6958.33	6138.33	5612.33	5673.33	22426.33	20423.33	5098.33	4596.33
2.10720997	4144.33	3855.33	1793.33	1570.33	3522.33	3052.33	2854.33	2990.33	13681.33	11802.33	2611.33	2493.33
0.301029996	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000
0.602059991	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000
0.903089987	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000
1.204119983	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000
1.505149978	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000
1.806179974	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000
2.10720997	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000	35000

Find Conc	Seasonal H1N1 Sensitive		Seasonal H1N1 Oseltamivir Resistant		Flu B Sensitive		Flu B Oseltamivir Resistant		H1N1 2009 Sensitive		H1N1 Oseltamivir Resistant	
0.301029996				0.554219367								
0.602059991			0.621670279				0.879059679	0.846208533			0.709050673	0.696511217
0.903089987	1.031357134	0.943327511			1.007549576	0.952865739						
1.204119983										1.461410577		
1.505149978									1.514258732			
1.806179974												
2.10720997												
Concentration	10.7487295	8.776624362	4.18475733	3.582773613	10.17535513	8.97151399	7.569369036	7.01792193	32.67824557	28.93413992	5.117415418	4.971772146
Dilution	5.4	4.4	2.1	1.8	5.1	4.5	3.8	3.5	16.3	14.5	2.6	2.5
Final Dilution	**5**		**2**		**5**		**4**		**15**		**3**	

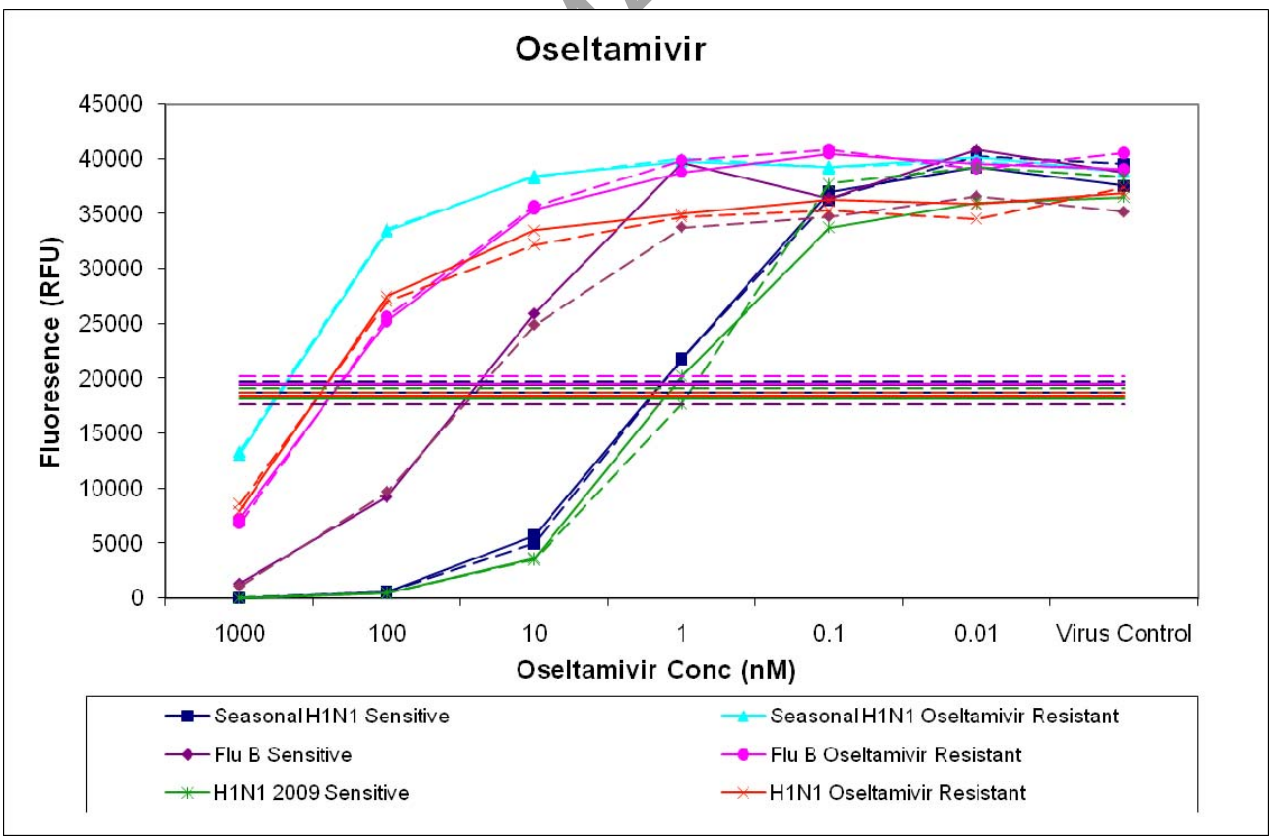


APPENDIX 4: ANALYSES OF NA INHIBITION ASSAY RESULTS

Strain Name	Seasonal H1N1 Sensitive		Seasonal H1N1 Oseltamivir Resistant		Flu B Sensitive		Flu B Oseltamivir Resistant		H1N1 2009 Sensitive		H1N1 Oseltamivir Resistant	
1000	515	547	13598	13856	1827	1589	7760	7315	508	528	8463	9095
100	1069	1075	33951	34144	9724	10196	25728	26179	962	959	27950	27532
10	6195	5454	38990	38796	26434	25404	35927	36181	4129	4010	34008	32705
1	22318	22259	40284	40521	40162	34296	39326	40378	20767	18191	35511	35257
0.1	37496	36824	39830	39691	36946	35288	40963	41339	34293	38282	36742	35774
0.01	39719	40702	40671	40216	41378	37116	40032	39582	36520	39726	36377	35019
Virus Control	38052	39977	39393	39357	39347	35693	39559	41017	37024	38823	37357	37883
Blank	483	497	487	482	492	428	473	492	470	472	672	555

Average-Blank	Seasonal H1N1 Sensitive		Seasonal H1N1 Oseltamivir Resistant		Flu B Sensitive		Flu B Oseltamivir Resistant		H1N1 2009 Sensitive		H1N1 Oseltamivir Resistant	
3	14.75	46.75	13097.75	13355.75	1326.75	1088.75	7259.75	6814.75	7.75	27.75	7962.75	8594.75
2	568.75	574.75	33450.75	33643.75	9223.75	9695.75	25227.75	25678.75	461.75	458.75	27449.75	27031.75
1	5694.75	4953.75	38489.75	38295.75	25933.75	24903.75	35426.75	35680.75	3628.75	3509.75	33507.75	32204.75
0	21817.75	21758.75	39783.75	40020.75	39661.75	33795.75	38825.75	39877.75	20266.75	17690.75	35010.75	34756.75
-1	36995.75	36323.75	39329.75	39190.75	36445.75	34787.75	40462.75	40838.75	33792.75	37781.75	36241.75	35273.75
-2	39218.75	40201.75	40170.75	39715.75	40877.75	36615.75	39531.75	39081.75	36019.75	39225.75	35876.75	34518.75
Virus Control	37551.75	39476.75	38892.75	38856.75	38846.75	35192.75	39058.75	40516.75	36523.75	38322.75	36856.75	37382.75
50% Cut	**18775.88**	**19738.38**	**19446.38**	**19428.38**	**19423.38**	**17596.38**	**19529.38**	**20258.38**	**18261.88**	**19161.38**	**18428.38**	**18691.38**
3	18775.88	19738.38	19446.38	19428.38	19423.38	17596.38	19529.38	20258.38	18261.88	19161.38	18428.38	18691.38
2	18775.88	19738.38	19446.38	19428.38	19423.38	17596.38	19529.38	20258.38	18261.88	19161.38	18428.38	18691.38
1	18775.88	19738.38	19446.38	19428.38	19423.38	17596.38	19529.38	20258.38	18261.88	19161.38	18428.38	18691.38
0	18775.88	19738.38	19446.38	19428.38	19423.38	17596.38	19529.38	20258.38	18261.88	19161.38	18428.38	18691.38
-1	18775.88	19738.38	19446.38	19428.38	19423.38	17596.38	19529.38	20258.38	18261.88	19161.38	18428.38	18691.38
-2	18775.88	19738.38	19446.38	19428.38	19423.38	17596.38	19529.38	20258.38	18261.88	19161.38	18428.38	18691.38
Virus Control	18775.88	19738.38	19446.38	19428.38	19423.38	17596.38	19529.38	20258.38	18261.88	19161.38	18428.38	18691.38

FINDIC50	Seasonal H1N1 Sensitive		Seasonal H1N1 Oseltamivir Resistant		Flu B Sensitive		Flu B Oseltamivir Resistant		H1N1 2009 Sensitive		H1N1 Oseltamivir Resistant	
3			2.68807424	2.700678973			2.317140194	2.287339642			2.462943244	2.45237159
2					1.389609515	1.480495463						
1	0.188666811	0.120224636							0.12049976			
0										-0.073198198		
-1												
-2												
RES	0.188666811	0.120224636	2.68807424	2.700678973	1.389609515	1.480495463	2.317140194	2.287339642	0.12049976	-0.073198198	2.462943244	2.45237159
IC50	1.544069382	1.318938773	487.6118369	501.9713989	24.52502819	30.23398985	207.5583424	193.7936942	1.319774581	0.844893175	290.3643167	283.381562
Mean IC50	1.4		494.8		27.4		200.7		1.1		286.9	



11.0 SUMMARY OF REVISIONS

RETRAINING REQUIRED YES ☐ NO ☒
N/A. First Issue.