DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service
Centers for Disease Control
and Prevention (CDC)
Atlanta, GA 30329

LP-492: Baloxavir Susceptibility Assessment using Influenza Replication Inhibition Neuraminidase-based Assay (IRINA)

Virology, Surveillance and Diagnosis Branch, Influenza Division

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1.0 Purpose

This protocol describes procedures for assessing influenza susceptibility to baloxavir using the Influenza Replication Inhibition Neuraminidase-based Assay (IRINA). The inhibition of virus replication is determined by measuring the enzymatic activity of neuraminidase protein expressed on the surface of virus-infected cells. The neuraminidase cleaves MUNANA and a fluorescent product is released. This method can also be used to test other antivirals and biologicals (e.g., antiserum).

2.0 Scope

The procedure was developed by Molecular Epidemiology Team (MET) of the Virology, Surveillance and Diagnosis Branch (VSDB), Influenza Division.

3.0 Responsibility

Laboratory Personnel must adhere to all biosafety guidelines and are responsible for following this procedure as written.

4.0 Definitions

4.1 BSA: Bovine Serum Albumin
4.2 BSC: Class II Biosafety Cabinet
4.3 BXA: Baloxavir Acid
4.4 diH_2O: Deionized distilled water
4.5 DMSO: Dimethyl Sulfoxide
4.6 FBS: Fetal Bovine Serum
4.7 MDCK-SIAT1 cells: Madin-Darby canine kidney cells overexpressing human α-2,6-sialyltransferase
4.8 MES: 2-(N-morpholino) ethanesulfonic acid
4.9 MUNANA: 4-(methylumbelliferyl)-N-acetylneuraminic acid
4.10 4-MU: 4-methylumbelliferone
4.11 MW: Molecular Weight
4.12 NA: Neuraminidase
4.13 PA: polymerase acidic (protein)
4.14 PBS: Phosphate Buffered Saline
4.15 RFU: Relative Fluorescence Unit
4.16 RT: Room Temperature
4.17 VGM: Virus Growth Media

5.0 Equipment

5.1 Microplate reader equipped with excitation (λ=360nm) and emission (λ=460nm) filters for fluorescence signal measurement: e.g., BioTek Cytation or Synergy and accompanying Gen5 software

5.2 Class II BSC

5.3 37ºC CO_2 incubator with thermometer

5.4 Bio-Rad TC20™ Automated Cell Counter, or equivalent

5.5 BioTek BioStack 3 Microplate Stacker (BioTek Instruments, Inc.) integrated with BioTek Cytation Multi-Mode Reader

5.6 Centrifuge with swing-bucket rotor for 50 mL tubes

5.7 Vortex Mixer

6.0 Materials

6.1 MDCK-SIAT1 cells (e.g., MilliporeSigma #05071502-1VL; IRR #FR-1380, w/ MTA)

6.1.1 Prepare MDCK-SIAT1 cell suspension in cell culture media to achieve 5-7×10^5 cells/mL (follow **9.1.3.1 – 9.1.3.4** and use the viable cell count to make the necessary dilution). Transfer 15 mL of cell suspension to 75 cm^2 flask (~10×10^6 cells) or 30 mL to 162 cm^2 flask (~20×10^6 cells).

This should be done 16-24h prior to test. 3-4×10^6 cells are needed to test one 96-well plate.

Note: If MDCK-SIAT1 cells are maintained in medium containing geneticin, exclude geneticin from medium when seeding cells 16-24h prior to titration or inhibition with BXA.

6.2 Cell Culture Media with 10% FBS

DMEM	1000 mL (e.g., Gibco #11965-084)
FBS, heat-inactivated	100 mL (e.g., Cytiva HyClone #SH30071.03HI)
Sodium Bicarbonate, 7.5%	16 mL (e.g., Gibco #25080-094)
200 mM L-Glutamine	10 mL (e.g., Gibco #25030-081)
100 mM Sodium Pyruvate	10 mL (e.g., Gibco #11360-070)
100X Nonessential amino acid	10 mL (e.g., Gibco #11140-050)
1 M HEPES	10 mL (e.g., Gibco #15630-080)
100X Pen Strep	10 mL (e.g., Gibco #15140-122)

6.3 Trypsin-EDTA solution (0.05%) (e.g., Gibco #25300-054)

6.4 VGM with 0.2% BSA (**Note:** TPCK-trypsin is omitted to achieve single-cycle virus replication)

DMEM	500 mL (e.g., Gibco #11965-092)
7.5% BSA	14 mL (e.g., Gibco #15260-037)
100X Pen Strep	5 mL (e.g., Gibco #15140-122)
1 M HEPES	12.5 mL (e.g., Gibco #15630-080)

6.5 1X PBS, no Ca^{+2}, no Mg^{+2} (e.g., Gibco #14190-144)

6.6 Trypan Blue solution, 0.4% (e.g., Gibco #15250-061)

6.7 Hemacytometer (e.g., Fisher Sci. #02-671-54) or cell counting slides (e.g., for TC20™ Bio-Rad #1450011)

6.8 DMSO (e.g., Sigma #D2438)

6.9 Antiviral drug – PA inhibitor BXA (e.g., MedChemExpress #HY-109025A: powder or 10 mM solution in DMSO; MW 483.49)

6.9.1 BXA powder should be dissolved in DMSO to make a 10 mM concentrated stock.

	10 mM stock		
BXA	1 mg	5 mg	10 mg
DMSO	0.207 mL	1.034 mL	2.068 mL

6.9.2 A stock of 1 mM BXA in DMSO should be prepared for later use. For example, dilute 10 mM BXA 1/10 using DMSO to get 1 mM (e.g., 100 µL of 10 mM BXA added to 900 µL DMSO). Make 200 µL stock aliquots of the 1 mM BXA and store at -70°C or lower. Stock is good for up to 4 years.

6.10 Reference viruses

6.10.1 CDC Baloxavir Susceptibility Reference Virus Panel, version 1.1, IRR #FR-1678

6.10.1.1 A/Louisiana/50/2017(PA-I38; wild-type) and A/Louisiana/49/2017 (PA-I38M)

6.11 NA-Fluor™ Influenza Neuraminidase Assay Kit (Applied Biosystems, #4457091) containing:

Note: Store contents of the kit as suggested by manufacturer.

6.11.1 NA-Fluor™ MUNANA substrate.

6.11.1.1 To prepare a 2.5 mM stock of NA-Fluor™ substrate, add 5 mL of diH_2O into the substrate vial and mix gently by inverting vial 3 times.

Note: Excess 2.5 mM substrate stock solution can be stored in 500 µL aliquots in opaque centrifuge tubes at -20°C. Freeze/thaw only once.

6.11.1.2 To prepare a 200 µM working solution of NA-Fluor™ substrate, make a 1/12.5 dilution of the 2.5 mM stock solution in 1X NA-Fluor™ assay buffer (see **6.11.2**). Working solution of substrate must be protected from exposure to light (e.g., cover container with aluminum foil) and stored at 4°C or on ice until use (step **9.1.5**).

Notes:

The 200 µM working solution of NA-Fluor™ substrate should be prepared fresh for each test.

To prepare 6 mL of 200 µM working solution (enough for one 96-well plate), add 0.48 mL of 2.5 mM stock solution to 5.52 mL of 1X NA-Fluor™ assay buffer. The extra volume allows for potential loss due to pipetting.

To use the entire content (5 mL) of the 2.5 mM stock solution, add it to 57.5 mL of 1X NA-Fluor™ assay buffer. This yields 62.5 mL of 200 µM working solution, which is sufficient for 10 96-well plates.

6.11.2 NA-Fluor™ 2X assay buffer (66.6 mM MES, 8 mM $CaCl_2$, pH 6.5).

6.11.2.1 To prepare 1X NA-Fluor™ assay buffer, mix equal volumes of 2X NA-Fluor™ assay buffer and diH_2O (1:1 dilution).

Note: 1X NA-Fluor™ assay buffer can be stored at 4°C for 1 month.

6.11.3 NA-Fluor™ Stop solution (0.2 M Na_2CO_3). Use as provided.

6.12 diH_2O (e.g., Corning Cellgro #25-055-CM)

6.13 4-MU (e.g., MilliporeSigma #M1381; MW 176.17)

6.13.1 4-MU powder should be stored at RT. Make a 40 mM stock by dissolving 70 mg of 4-MU in 5 mL of absolute ethanol and add 0.9% NaCl to bring the volume to 10 mL. Prepare 0.5 mL aliquots in opaque tubes to protect from light and can be stored for up to 1 year at -20°C.

6.13.2 Prepare a 4 mM 4-MU solution from 40 mM stock by diluting 1/10 (e.g., transfer 100 µL of the 40 mM stock solution to 900 µL of 1:1 mixture of absolute ethanol and 0.9% NaCl). Prepare 0.5 mL aliquots in opaque tubes to protect from light and can be stored for up to 6 months at -20°C.

6.13.3 On the day of NA activity measurement (step **9.1.5**), dilute the 4 mM 4-MU by 1/20 to a final concentration of 200 µM in 1X NA-Fluor™ assay buffer (equilibrated to RT) (e.g., add 50 µL of 4 mM 4-MU to 950 µL of 1X NA-Fluor™ assay buffer). Remaining un-used 200 µM of 4-MU should be discarded.

Note: Before use, equilibrate tube of 4 mM 4-MU at RT, vortex for 5-10 seconds. Also, mix 200 µM 4-MU by vortexing for 5-10 seconds.

6.14 Ethanol, absolute (e.g., MilliporeSigma #E7023)

6.15 0.9% sodium chloride (NaCl) solution (e.g., MilliporeSigma #S8776)

6.16 Tissue culture-treated 96-well black plates with clear bottom (e.g., Agilent #204626-100, Corning #3603)

6.17 Clear adhesive seal sheets/film for plates (e.g., Thermo Sci. #AB1170)

6.18 Assorted pipettes and filtered tips

6.19 Sterile tubes/bottles and troughs for making dilutions (e.g., 8 or 12-channel reservoirs, 50 mL conical tubes, 250 mL bottles)

7.0 Controls

The fluorescent metabolite of MUNANA is 4-MU; it is used as a control to normalize virus inoculum. Fluorescent signal (RFU) produced by 1500 pmol/well of 4-MU is the target enzyme activity for type A viruses, while for type B viruses it equals 50% of this value. This control (4-MU) is used in both, titration and inhibition, portions of this assay.

8.0 Safety Precautions

8.1 Institutional safety guidelines must be adhered to at all times.

8.2 Manipulations with live infectious material must be done within a BSC (it is advisable to use a designated BSC, when testing clinical specimens).

8.3 Refer to Safety Data Sheets provided by reagent manufacturer before storing, handling, or working with or disposing of chemicals or hazardous materials.

9.0 Procedure

Note: See **Figure 1** for a schematic of the full procedure.

9.1 Part 1: Determination of working virus dilution

Note: See **Figure 2** for a diagram of a typical IRINA titration plate set-up.

9.1.1 Thaw virus aliquots in tap water (~10 minutes) and transfer immediately to a metal tube holder on ice.

Note: It is advisable to use the same virus aliquot for both determination of working virus dilution (step **9.1**) and inhibition (step **9.2**). Re-freeze virus after setup for determining working virus dilution.

9.1.2 In 96-well black clear-bottom plate, make seven 10-fold serial dilutions of viruses in VGM (row A-G):

9.1.2.1 Add 100 µL of VGM to all wells.

9.1.2.2 Add 11 µL of virus to row A of the respective column.

Note: One column per virus allows for 12 viruses to be titrated per plate.

9.1.2.3 Using a 12-channel P20, mix by pipetting suspension in row A and transfer 11 µL of virus dilution from row A to row B. Change tips and repeat procedure until row G; discard final 11 µL from row G. Row H is the no virus control.

9.1.3 Prepare cell suspension.

9.1.3.1 Must use flask(s) containing confluent cell monolayer of MDCK-SIAT1 cells prepared 16-24h before the test. One 162-cm^2 flask typically contains enough cells for 6-12 plates (20-40x10^6 cells), depending on cell confluency.

Decant cell growth media from flask(s) and wash cells with PBS, no Ca^{+2}, no Mg^{+2} (using 15 mL or 25 mL, depending on flask size). To dissociate cell monolayer, add 5 mL of trypsin-EDTA to a 162 cm^2 flask(s) or 3 mL to a 75 cm^2 flask(s). **Do not agitate contents of the flask(s)** and place flask(s) into CO_2 incubator at 37°C for 35-45 minutes.

9.1.3.2 Gently shake flask side to side, without tapping, so cells can detach. Slightly tilt flask while shaking to ensure cells are detached from the entire area.

9.1.3.3 Add 10 mL cell culture media (warmed at 37°C) to a 162-cm^2 flask (6 mL for a 75 cm^2 flask) and re-suspend cells by pipetting at least five times to break up clumps, while avoiding bubbling. Transfer cell suspension into a 50 mL conical tube.

Note: Clumped cells can be identified in the cell counting step **9.1.3.4**. Clumping appears as aggregates of 3 or more cells. This can cause the automated cell counting to be inaccurate and may also affect morphology (consistency) of cell monolayer. If there is substantial clumping, continue mixing the cell suspension until clumping is minimal.

9.1.3.4 Count cells using a Bio-Rad TC20™ Automated Cell Counter or manually with a hemacytometer. Cell viability should be >95% by trypan blue staining.

9.1.3.4.1 Trypan blue exclusion procedure for cell counting:

9.1.3.4.1.1 If using Automated Cell Counter, add 100 µL cell suspension to 100 µL of 0.4% trypan blue and mix. If using phase contrast hemacytometer, add 50 µL cell suspension to 450 µL of 0.4% trypan blue and mix.

9.1.3.4.1.2 Load cell counting slide or hemacytometer with 10 µL mixture of cell suspension and trypan blue.

9.1.3.4.1.3 Place slide in Bio-Rad TC20™ Automated Cell Counter. Automated Cell Counter would provide number of cells/mL and % of viability. If counting manually, place hemacytometer under a light microscope to count unstained (viable) and stained (nonviable) cells manually then calculate cell viability and viable cell count (https://www.hemacytometer.org/).

9.1.3.5 Transfer necessary quantity of cells to a 50 mL conical tube (3-4×10^6 cells are needed for one 96-well plate). The maximum volume of cell suspension added to the tube should not exceed 10 mL. If needed, use an additional 50 mL conical tube(s). Add PBS to bring the volume to 50 mL then close tube cap tightly and gently mix by inversion.

Note: Prepare enough cells to accommodate the number of test plates, plus 1-2 extra.

9.1.3.6 Pellet cells by centrifugation at ~150×g (800 rpm) for 8 minutes at 4°C.

9.1.3.7 Remove supernatant and re-suspended cells in VGM to achieve ~0.6-0.8×10^6 cells/mL.

9.1.3.8 Pour cell suspension into a reservoir and transfer 50 µL into each well of the 96-well plate containing viruses using a multi-channel pipet by hovering the tips over the wells while dispensing. Be careful not to touch the contents of the wells.

Notes:

Cells will settle to the bottom of a container (tube or reservoir), keep cell suspension homogenous by mixing every 5-10 minutes.

Cells should be used immediately (within 1h) after preparation.

If using an automatic repeat pipettor, after collecting cells into the tips, dispense the first aliquot back into the cell reservoir while hovering. Continue hovering and dispense into wells of a 96-well plate.

9.1.4 Incubate plate(s) 22-24h at 37°C with 5% CO_2.

9.1.5 Determine NA activity.

9.1.5.1 Prepare NA-Fluor™ Influenza Neuraminidase Assay Kit reagents (step **6.11**).

Alternatively, in-house prepared reagents can be used (see **10.3 - Appendix C**).

9.1.5.2 Prepare 200 µM 4-MU in 1X NA-Fluor™ assay buffer (step **6.13.3**).

9.1.5.3 Move plate(s) into BSC and aspirate supernatant from all wells using either a 12-channel manual pipette or by aspiration with vacuum.

Note: Be sure that almost all the supernatant is removed, or the results may be influenced by free NA in leftover supernatant.

9.1.5.4 Add 7.5 µL of 200 µM 4-MU to three wells containing only cells (e.g., H10-H12) (see **Figure 2**).

Note: Before use, mix tube of 200 µM 4-MU by vortexing for 5-10 seconds.

9.1.5.5 Add 50 µL of 200 µM NA-Fluor™ substrate solution to all wells and cover plate(s) with lid(s).

Note: Ensure the tips touch the bottom corner of the wells when dispensing. In addition, covered plate(s) can be quickly mixed at high pulse on plate shaker to make sure entire content reach to the bottom to uniformly cover the cell monolayers.

9.1.5.6 Incubate plate(s) at 37°C for 1h.

Note: Do not stack more than 5 plates in the incubator for uniform heat transfer.

9.1.5.7 Bring plate(s) back into the BSC and add 50 µL of NA-Fluor™ Stop solution to each well.

9.1.5.8 Seal the plate(s) with film.

Note: If the reader can only read from the top of the plate, before sealing, allow plate(s) to cool after adding Stop solution (~15 minutes) to prevent condensation on the seal.

9.1.6 Read signal

9.1.6.1 Open the Gen5 software and select/create a protocol with the following parameters:

9.1.6.1.1 Excitation λ=360nm ± 20nm; Emission λ=460nm ± 20nm

9.1.6.1.2 During calibration of a reader, make sure RFU signal for 1500 pmol/well of 4-MU falls in the linear range, i.e., signal should not be near background or at the saturation (see **10.1 – Appendix A**).

9.1.6.2 Read plate(s) and export the data.

9.1.7 Open “CDC_Dilution_calculations_IRINA_12viruses_v3.xlsx” and use generated RFU data to determine the working virus dilution for each virus. Directions to use the virus dilution calculator are provided within the tool.

9.2 **Part 2: Inhibition – testing susceptibility of viruses to BXA**

Note: See **Figure 3** for a diagram of a typical IRINA BXA inhibition plate set-up.

9.2.1 Prepare drug dilutions.

Notes:

If a new preparation of BXA is being used, it must be first verified by comparing results with a previously verified stock (see **10.2 – Appendix B**).

The 3-fold serially diluted 3X working BXA dilutions (333 – 0.018 nM) should be prepared fresh on the day of the assay. Remaining un-used diluted BXA should be discarded.

9.2.1.1 Let VGM equilibrate to RT or warm it to 37°C before preparing the drug dilutions. Otherwise, BXA may precipitate out of solution.

9.2.1.2 Prepare a 3 µM solution of BXA from 1 mM BXA working stock by diluting 1/333 (e.g., add 6 µL of 1 mM BXA to 1.994 mL VGM).

Note: Pipetting very small quantities (volumes < 5 µL) is not recommended.

9.2.1.3 Dilute the 3 µM BXA to 1 µM by diluting 1/3 (e.g., transfer 250 µL of 3 µM solution to 500 µL of VGM).

9.2.1.4 Prepare 3-fold serial dilutions of BXA using a 12-channel reservoir, starting with 333 nM in channel 1.

To test 8 viruses (one plate), 500 µL is needed per dilution of BXA: fill all 12 channels with 500 µL of VGM, then transfer 250 µL of 1 µM BXA to channel 1 and mix, then transfer 250 µL from channel 1 to channel 2 and mix; repeat until channel 10. Channels 11 (virus only) and 12 (cell only) are controls.

Adjust volume for more plates as needed.

Note: Each dilution will be 3X concentrated. After mixing with virus and cell suspension the final drug concentration will be 1X. Final concentrations range from 111 to 0.006 nM.

9.2.1.5 Set aside the reservoir in BSC at RT for later (step **9.2.3**).

9.2.2 Prepare working virus dilution

9.2.2.1 Prepare working virus dilutions for viruses to be tested in a reservoir with 8 channels using calculations made in step **9.1.7**. Each channel corresponds to one row of a 96-well plate. 800 µL of diluted virus is sufficient for one replicate (one row).

Notes:

It may be necessary to initially dilute the virus 1/10 – 1/1000 prior to making the working dilution in the reservoir.

A 5 mL 6x8 reservoir or 2 mL 96 deep-well plate may be used for preparing multiple plates; each column in the reservoir or deep-well plate can be used for one test plate. One 96 deep-well plate can be used to dilute viruses for up to 12 test plates.

For quality control purposes, include a reference virus pair (wild-type virus and PA mutant displaying reduced susceptibility to BXA) in each plate, if possible (see **6.10**).

9.2.3 Transfer 50 µL/well of drug dilutions and control VGM (see **9.2.1.4**) to rows of a 96-well plate(s) using a manual or automatic 12-channel pipette; channel 1 corresponds to column 1, etc.

Note: Ensure the tips touch the bottom corner of the wells when dispensing.

9.2.4 Add working virus dilutions to plate(s) containing drug:

9.2.4.1 Mix virus dilutions by pipetting, before transferring.

9.2.4.2 Use an 8-channel automatic P1000 pipette with setting for 12 dispensations of 50 µL. Aspirate and release the first dispensation back into the chamber, because volume of the first dispensation might be inaccurate.

9.2.4.3 Dispense 50 µL of diluted viruses to respective rows of the 96-well plate, starting with column 11 and ending with column 1 (pipetting from the lowest drug concentration to the highest); eleven total dispensations.

Notes:

To ensure accurate dispensation, allow the tips to touch the bottom of the well.

After tips have touched the bottom of the well, neither tips nor the content should be put back in the reservoir. This will avoid contamination with drug.

Column 12 serves as a negative control (cells only) and does not require an additional 50 µL to supplement volume of virus; 50 µL VGM (step **9.2.3**) plus 50 µL of cells is sufficient.

9.2.5 Prepare cell suspension (see step **9.1.3**).

Note: Trypsinization of cells can be started right before step **9.2.3** or **9.2.4**, depending on the number of plate(s).

9.2.6 Add 50 µL of the cell suspension to each well without touching well contents (see **9.1.3.8**).

9.2.7 Incubate plates for 22-24h at 37°C with 5% CO_2.

9.2.8 Determine NA activity (see **9.1.5**).

Note: 4-MU should be placed in wells G12 and H12 (see **Figure 3**), instead of H10 – H12, as mentioned in **9.1.5.4**.

9.2.9 Read signal (see **9.1.6**).

9.2.10 Transfer RFU data to JASPR v5.0 (a CDC in-house developed tool) to determine effective concentration of BXA yielding 50% reduction in NA activity (EC_{50}) for each virus.

Alternatively, any program for curve-fitting analysis (e.g., GraphPad Prism) can be used to determine EC_{50}.

Notes:

For type A viruses, the RFU of control well with no BXA (column 11) should be between 3 times lower or higher the value of 1500 pmol of 4-MU. For example, if RFU of 1500 pmol 4-MU is 18,000, the acceptable range for type A viruses would be 6,000 – 54,000.

For type B viruses, the target RFU is 50% that of type A (i.e., 50% RFU of 1500 pmol 4-MU). Using the example above, acceptable range for type B would be 3,000 – 27,000.

The RFU value in column 11 should be higher than or close to the RFU in the lowest drug concentration well (column 10) of the respective row.

Column 12 of rows A-F serves as a no drug, no virus control and should have background RFU signal. See **12.3** (Figure 3).

The inhibition curve should be sigmoidal. A curve that is not sigmoidal signifies high BXA resistance and will affect EC_{50} values. In this case, the virus should be retested with increased drug concentrations until a sigmoidal curve is achieved.

Results are expressed as fold increase in EC_{50} of test virus compared to (sub)type specific median EC_{50} values of viruses tested during previous influenza season(s). Reduced susceptibility to BXA is defined by an arbitrary threshold of ≥ 3-fold increase in EC_{50} compared to either a (sub)type-specific median EC_{50} values or sequenced-matched control virus.

10.0 Appendices

10.1 **Appendix A:** Use of 4-MU for signal calibration

10.1.1 **Introduction**

Before performing IRINA on a given plate reader for the first time, a 4-MU-based calibration must be performed to determine the optimal settings. This procedure needs to be repeated only when the reader undergoes a major repair or recalibration.

The enzymatic cleavage of the fluorogenic substrate MUNANA results in the product 4-MU, a fluorophore. Commercially available 4-MU is used to determine a plate reader's optimal settings. The RFUs generated by the specified serial dilutions of 4-MU must be within the instrument's linear range of detection. If not, please consult the manufacturer or use a different fluorimeter.

10.1.2 **Preparing and measuring 4-MU serial dilutions**

10.1.2.1 Prepare 200 µM 4-MU in 1X NA-Fluor™ assay buffer (see step **6.13.3**).

10.1.2.2 In a 96-well black plate with clear bottom, add 47.5, 45.0, 42.5, 35.0, and 20.0 µL of 1X NA-Fluor™ assay buffer in triplicate into wells 1 through 5, respectively, of rows A, B, and C.

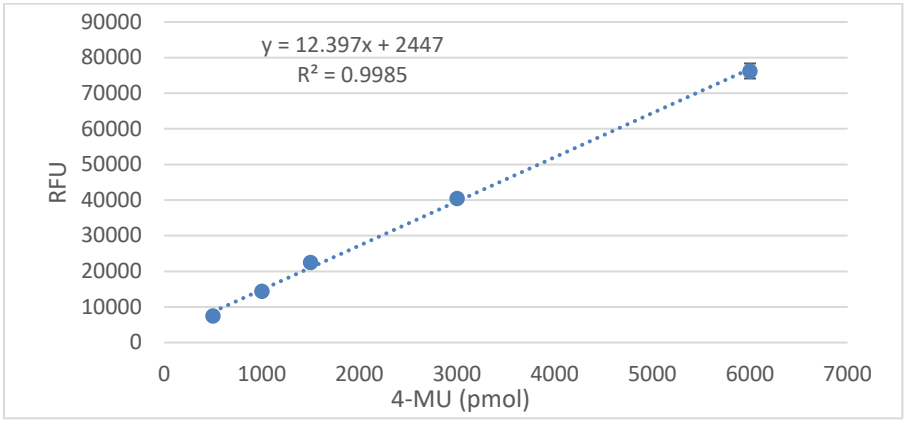
10.1.2.3 Add 2.5, 5.0, 7.5, 15, and 30 µL of 200 µM 4-MU working solution to each respective well to bring the total volume in each well to 50 µL. The final concentrations of 4-MU will be 10, 20, 30, 60, and 120 µM (total amount of 4-MU per well will be 500, 1000, 1500, 3000, and 6000 pmol, respectively). Add 50 µL of 1X NA-Fluor™ assay buffer to remaining wells in rows A, B, and C to serve as the background.

10.1.2.4 Add 50 µL of stop solution to each well in rows A, B, and C.

10.1.2.5 Initiate software for the plate reader and run fluorescence reading of the plate with Excitation λ=360nm ± 20nm; Emission λ=460nm ± 20nm.

10.1.2.6 Achieve linear correspondence of signal (RFU) and 4-MU amount (**Figure A**) by adjusting sensor distance from plate, gain, and/or time of signal acquisition in the reader's setting. The signal for the increasing concentrations of 4-MU should rise in a linear fashion (signal saturation should not be reached).

Figure A. Linear correspondence of fluorescent signal (RFU) to amount of 4-MU



10.2 **Appendix B:** Verification of new BXA stocks

10.2.1 Before using a new preparation of BXA, it must be first verified by testing reference viruses in parallel with the previously used (verified) 1 mM working stock.

10.2.2 Prepare the same dilution series as described in **9.2.1** for the new and current BXA stocks.

Note: Prepare enough for 4 virus tests per stock of BXA (300 µL per dilution is sufficient).

10.2.3 Transfer 50 µL of each dilution and VGM controls to a 96-well black clear-bottom plate. Place the current BXA stock in rows A through D, and the new BXA stock to rows E through H.

Note: See **Figure B** for a diagram of a typical BXA stock verification plate.

10.2.4 Prepare 3 mL of working virus dilution for a pair of reference viruses from the IRR Baloxavir Panel: (e.g., A/Louisiana/50/2017 and A/Louisiana/49/2017) (see **6.10**).

10.2.5 Transfer 50 µL of each working virus dilution to columns 1 through 11 of the test plate such that rows A, B, E, and F are occupied by the wild-type virus and C, D, G, and H are occupied by the mutant.

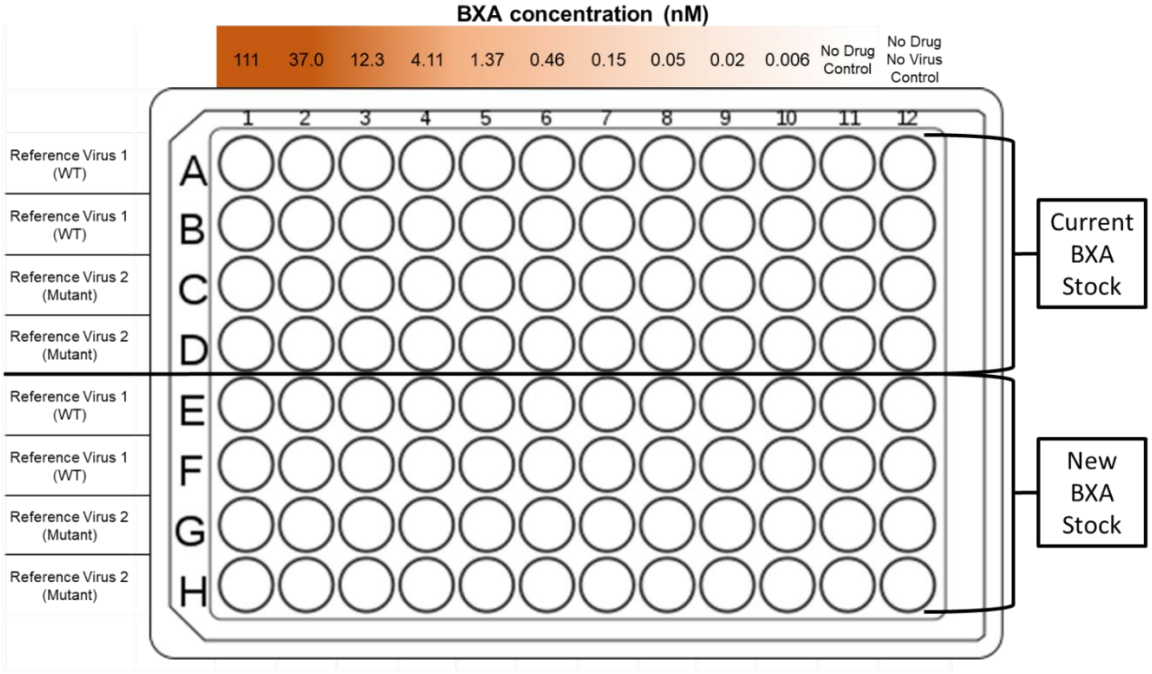
10.2.6 Prepare and add cell suspension according to step **9.1.3** (3-4×10^6 cells per plate is sufficient).

10.2.7 Incubate plate for 22-24h at 37°C with 5% CO_2, then follow steps **9.2.8** – **9.2.10** to determine EC_{50}s.

10.2.8 Ensure that EC_{50} results from the new BXA stock are in close agreement (< 30% difference) with the results from the current BXA stock. Average the duplicates to make comparisons.

Note: Because EC_{50} may vary from test to test, conducting this comparison in parallel (same test and plate) is essential.

Figure B. Baloxavir stock verification plate set-up diagram



10.3 **Appendix C:** Preparing in-house reagents to measure NA activity; an alternative to NA-Fluor™ kit.

10.3.1 MUNANA (e.g., MilliporeSigma #M8639; Biosynth Carbosynth #EM05195; MW 489.41)

10.3.1.1 MUNANA powder should be stored at -20°C. Make a 100 mM stock by dissolving the powder in diH_2O. Swirl and/or invert to mix; do not vortex. Prepare 0.5 mL aliquots in black tubes to protect from light; stored for up to 1 year at -20°C.

	100 mM stock			
MUNANA	5 mg	25 mg	100 mg	1 g
diH_2O	0.102 mL	0.511 mL	2.043 mL	20.430 mL

10.3.1.2 For measuring NA activity (step **9.1.5**), dilute 100 mM MUNANA substrate to 200 µM in 1X assay buffer (see recipe in step **10.3.2**). For example, add 50 µL of 100 mM MUNANA substrate to 25 mL of 1X assay buffer.

10.3.2 1X assay buffer (33.3 mM MES, 4 mM $CaCl_2$, pH 6.5): add 33.3 mL 1.0 M MES, pH 6.5 (e.g., Alfa Aesar #J61587) and 4.0 mL 1.0 M $CaCl_2$ (e.g., MilliporeSigma #C5080) into 963.7 mL of diH_2O.

10.3.3 Stop solution (0.2 M Na_2CO_3, pH 11.5): weigh 21.2 g of Na_2CO_3 (anhydrous, e.g., MilliporeSigma #222321) and dissolve in 1 L of diH_2O, adjust pH to 11.5 using 1 N NaOH (e.g., Fisher #SS266-1).

10.4 **Appendix D:** Influenza hemagglutinin antigenic testing

Note: Typically, ferret antiserum is treated with Receptor Destroying Enzyme (RDE) prior to testing, which dilutes antiserum 10-fold.

10.4.1 Antiserum dilutions:

Make a 1/40 working dilution; dilute RDE-treated antiserum 1/4. Make nine 2-fold serial dilutions, mix each dilution by pipetting, resulting in ten dilutions in total. Adjust volume for the number of plates as needed; 500 µL per dilution is needed for one plate, which includes extra volume for pipetting.

For example: add 250 µL of RDE-treated antiserum to 750 µL VGM, then transfer 500 µL into 500 µL VGM, repeat.

If using a 12-channel trough, add appropriate volume of VGM to channels 11 and 12 for controls.

Note: A 1/40 working dilution will become a final dilution of 1/80 (starting) in the assay after a 1:1 mixture of antiserum with virus.

10.4.2 Transfer 50 µL/well of antiserum dilutions and control VGM to rows 96-well plate(s) using a manual or automatic 12-channel pipette; channel 1 corresponds to column 1, etc.

10.4.3 Add 50 µL/well of virus to plates (step **9.2.4.3**) and incubate plates at RT for 1-1.5h.

10.4.4 Add 50 µL/well of cells (step **9.1.3**) and place plate(s) in incubator at 37°C with 5% CO_2 for 3-4h.

10.4.5 After the 3-4h incubation, carefully remove supernatant from all wells (can use a low-pressure vacuum) and supplement the monolayer with 100 µL of VGM (equilibrated to RT).

Note: Removing the supernatant after 3-4h post infection will eliminate the potential interference of anti-neuraminidase antibodies in the antiserum during the neuraminidase activity determination step.

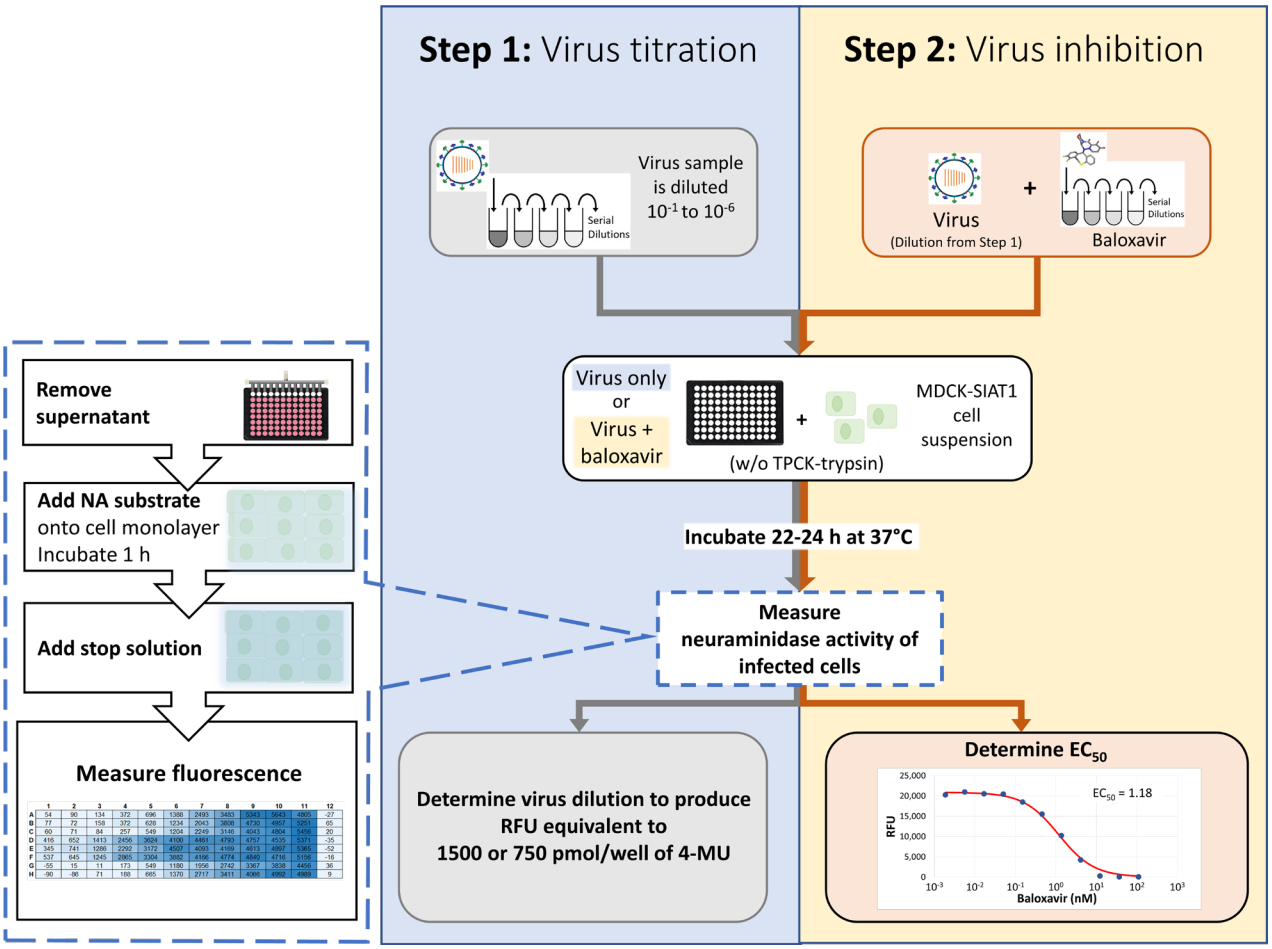
10.4.6 Continue incubation at 37°C with 5% CO_2 for an additional 19-21h (total incubation 22-24h post infection).

10.4.7 Proceed to determination of NA activity (step **9.1.5**).

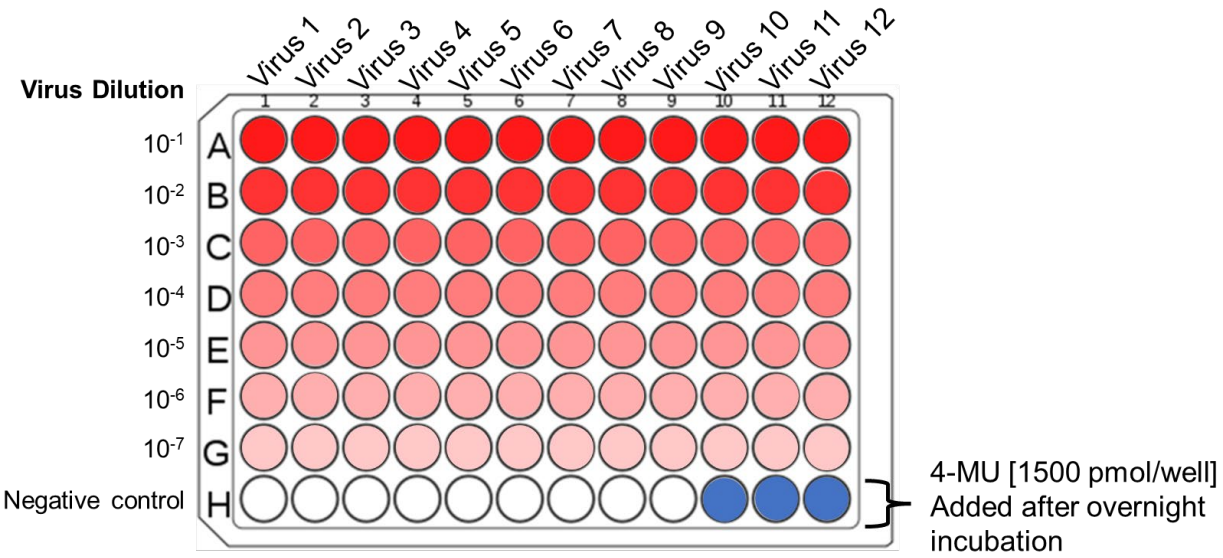
10.4.8 Analyze data using IRINA_AFLUT for antigenic analysis.

11.0 Figures

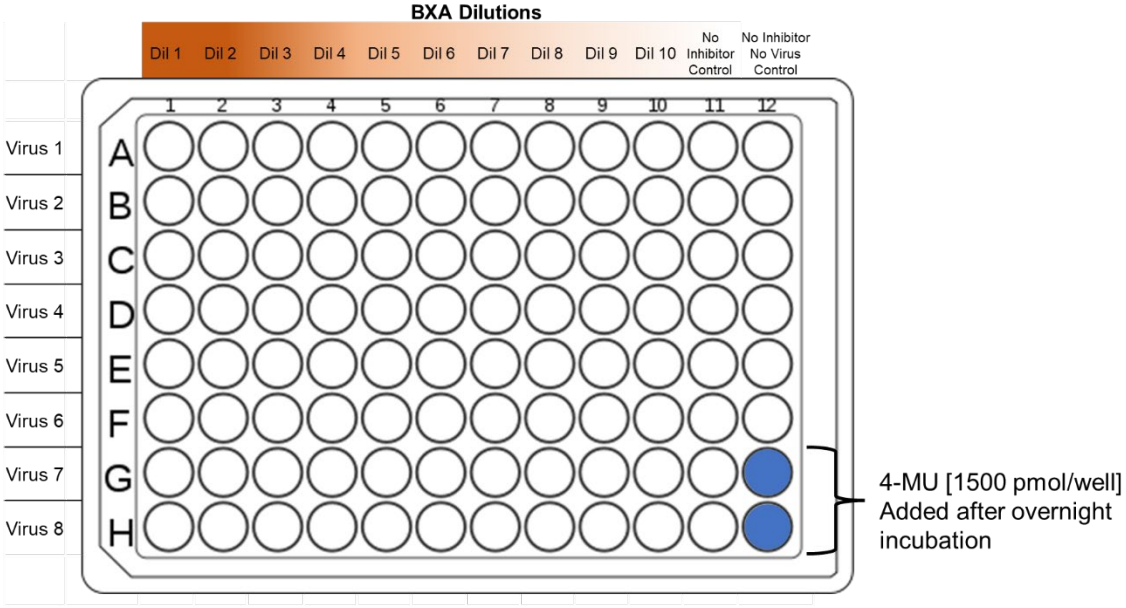
11.1 Figure 1. Baloxavir susceptibility testing using IRINA: Workflow Diagram



11.2 **Figure 2. IRINA titration plate set-up**



11.3 **Figure 3. IRINA BXA inhibition plate set-up**



Note: Reference viruses should be included at the bottom of each plate (Rows G and H) for quality control. References should include one virus with a mutation conferring reduced susceptibility to the agent being tested (BXA) and one matching wild-type virus.