**Table S1: Infected cell population (ICP) and NA activity of influenza B viruses**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Virus name** | **Lineage** | **Parameter** | **Range** | **Median** | **Average ± SD** |
| B/North Dakota/07/2020 | Victoria | ICP | 709-1370 | 958 | 999 ± 264 |
| RFU | 9959-14307 | 11330 | 11731 ± 1813 |
| B/Maryland/01/2021 | Yamagata | ICP | 535-1022 | 768 | 773 ± 189 |
| RFU | 7628-9121 | 8152 | 8263 ± 606 |

RFU: relative fluorescence unit; SD: standard deviation

Target ICP was 1000. ICP and NA activity (expressed as RFU) were determined at 24 hpi. Results from two independent experiments.

**Table S2: Virus dilution factors determined using NA activity and ICP for surveillance viruses (n=96)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Type/subtype or lineage | No. of viruses | Range of dilutions factors (Min - Max) | | Ratio of dilution factors (IRINA/HINT) | |
| IRINA | HINT | Average ± SD | Range (Min - Max) |
| A(H1N1)pdm09 | 24 | 11 - 99213 | 9 - 93256 | 1.0 ± 0.3 | 0.3 - 1.9 |
| A(H3N2) | 23 | 17 - 25285 | 27 - 26050 | 0.8 ± 0.2 | 0.4 - 1.3 |
| A(H3N2) with NA-N419S | 1 | 1625 | 14481 | 0.1 | - |
| B/Victoria | 24 | 186 - 56434 | 116 - 61775 | 1.1 ± 0.3 | 0.5 - 1.8 |
| B/Yamagata | 24 | 1981 - 90463 | 1371 - 59856 | 1.2 ± 0.3 | 0.5 - 1.8 |

HINT: high content imaging-based neutralization test; IRINA: influenza replication inhibition neuraminidase-based assay; SD: standard deviation

The NA activity-based dilution factors were determined by extrapolating the virus dilution that would yield a fluorescence signal equivalent to ~1750 and ~900 pmol/well of 4-MU for influenza type A and type B, respectively. ICP-based dilution factors were calculated to yield ~1000 infected cells per well. When ratio of dilution factors (IRINA/HINT) is close to 1.0 that indicates that NA activity-based dilution factor is very similar to ICP-based dilution factor.

**Table S3: Virus dilutions used for IRINA and NI assays**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **(Sub)type or lineage** | **Virus name** | **NA AAa** | **Virus dilution factor** | |
| **IRINA** | **NI** |
| A(H1N1)pdm09 | A/Wyoming/02/2019 | I223 | 11350 | 18 |
| A/Argentina/264/2018 | I223K | 8600 | 21 |
| A/Hawaii/70/2019 | I223 | 6371 | 4 |
| A/New York/16/2020 | I223K | 2010 | 5 |
| A/Tennessee/17/2018 | I223 | 800 | 9 |
| A/Iowa/73/2018 | I223M | 22500 | 51 |
| A/California/18/2019 | I223 | 2600 | 21 |
| A/Santiago/108999/2018 | I223L | 5300 | 6 |
| A/Illinois/45/2019b | H275 | 2833 | 8 |
| A/Alabama/03/2020b | H275Y | 392 | 6 |
| A/Maine/01/2021 | N295 | 15600 | 22 |
| A/West Virginia/09/2020 | N295S | 20000 | 26 |
| A(H3N2) | A/Pennsylvania/46/2015b | E119 | 1070 | 121 |
| A/Washington/33/2014b | E119V | 410 | 10 |
| A/Washington/01/2007 | R292 | 3730 | 7 |
| A/Bethesda/956/2006 | R292K | 1363 | 2 |
| B/Victoria | B/Laos/0080/2016b | H134 | 67400 | 78 |
| B/Laos/0654/2016b | H134N | 44500 | 18 |
| B/Memphis/20/96 | R150 | 10000 | 13 |
| B/Memphis/20/96 | R150K | 2700 | 3 |
| B/North Carolina/25/2018b | D197 | 22400 | 13 |
| B/Missouri/12/2018b | D197E | 36200 | 138 |
| B/South Carolina/02/2020 | G243 | 48900 | 8 |
| B/New Jersey/02/2020 | G243S | 16500 | 14 |

AA: amino acid; IRINA: influenza replication inhibition neuraminidase-based assay; NI: neuraminidase inhibition assay

aAmino acid numbering is based on the NA (sub)type specific for type A viruses and the NA of type B viruses. NA sequences of control viruses are identical to mutant viruses, except for the indicated residue. For each pair, wildtype control virus is listed first followed by the respective mutant virus.

bViruses from the CDC neuraminidase inhibitor susceptibility reference virus panel, v3.0 (IRR cat. # FR-1755).

**Table S4: Antigenic analysis of influenza A(H3N2) viruses using IRINA and HINT: with and without removal of supernatant after infection**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Virus** | **NA amino acid changes compared to A/Darwin/6/2021a** | **Ferret antiserum raised against Cam/e0826360/20** | | | |
| **Titer (fold)** | | | |
| **Supernatant not removed**  **(same as Table 4)** | | **Supernatant removed**  **at 2-3 hpi** | |
| **IRINA** | **HINT** | **IRINA** | **HINT** |
| **Reference** |  |  |  |  |  |
| A/Cambodia/e0826360/2020 | I62M | *2207 (1.0)* | *2453 (1.0)* | *1908 (1.0)* | *1765 (1.0)* |
| A/Darwin/6/2021 |  | 178 (12.4) | <80 (>30.7) | <80 (>23.8) | <80 (>22.1) |
| **Test** |  |  |  |  |  |
| A/Montana/01/2021 | R150H | 176 (12.6) | <80 (>30.7) | <80 (>23.8) | <80 (>22.1) |
| A/Maryland/05/2021 | D346G | 339 (6.5) | 113 (21.8) | 88 (21.6) | 102 (17.3) |
| A/Arizona/02/2021 | S329N | 248 (8.9) | 103 (23.8) | <80 (>23.8) | 94 (18.8) |
| A/Michigan/05/2021 | E83G, V263I, R315S, D346G, N463D, S465N | 171 (12.9) | 162 (15.1) | 105 (18.2) | 135 (13.0) |
| A/Arizona/04/2022 | D346G/D | 273 (8.1) | 140 (17.5) | 108 (17.7) | 136 (13.0) |

HINT: high content imaging-based neutralization test; IRINA: influenza replication inhibition neuraminidase-based assay; NA: neuraminidase

Antigenic analysis of cell-propagated influenza A(H3N2) viruses, representing HA genetic clade 3C.2a1b.2a.2 was carried out by IRINA and HINT assays using post-infection ferret antiserum generated for the influenza A(H3N2) vaccine reference virus, A/Cambodia/e0826360/2020 (Cam/e0826360/20); 3C.2a1b.2a.1 virus selected for the 2021-2022 northern hemisphere season.

This experiment was carried out using two experimental conditions: (1) supernatant was not removed (same setup as done for Table 4), and (2) supernatant was removed following attachment of cells at 2-3 hpi and replenished with VGM without TPCK-trypsin. All plates were incubated for a total of 24 hpi. Remaining steps were as described in Materials and Methods. The neutralization titer is the reciprocal dilution of antiserum needed to reduce the RFU or ICP by 50%. Underlined italics indicate homologous titers. Number in parentheses indicate fold changes in titer compared to the respective homologous titers.

aConsensus amino acids in A/Darwin/6/2021 are: I62, E83, R150, V263, R315, S329, D346, N463, S465.