

www.pei.de

Alternative Approaches for Modification of the classical (WHO-manual) Microneutralisation Assay

Ralf Wagner
Paul-Ehrlich-Institut
Section Viral Vaccines



Use of Colon Carcinoma (CaCO2) cells

*Continuous line of human intestinal epithelial differentiated cells
(colorectal adenocarcinoma)*

Rationale to use CaCo2 cells:

Contain proteases for the activation of low-pathogenic Influenza viruses
(containing HA with a monobasic cleavage site)

- TMPRSS2
- TMPRSS4

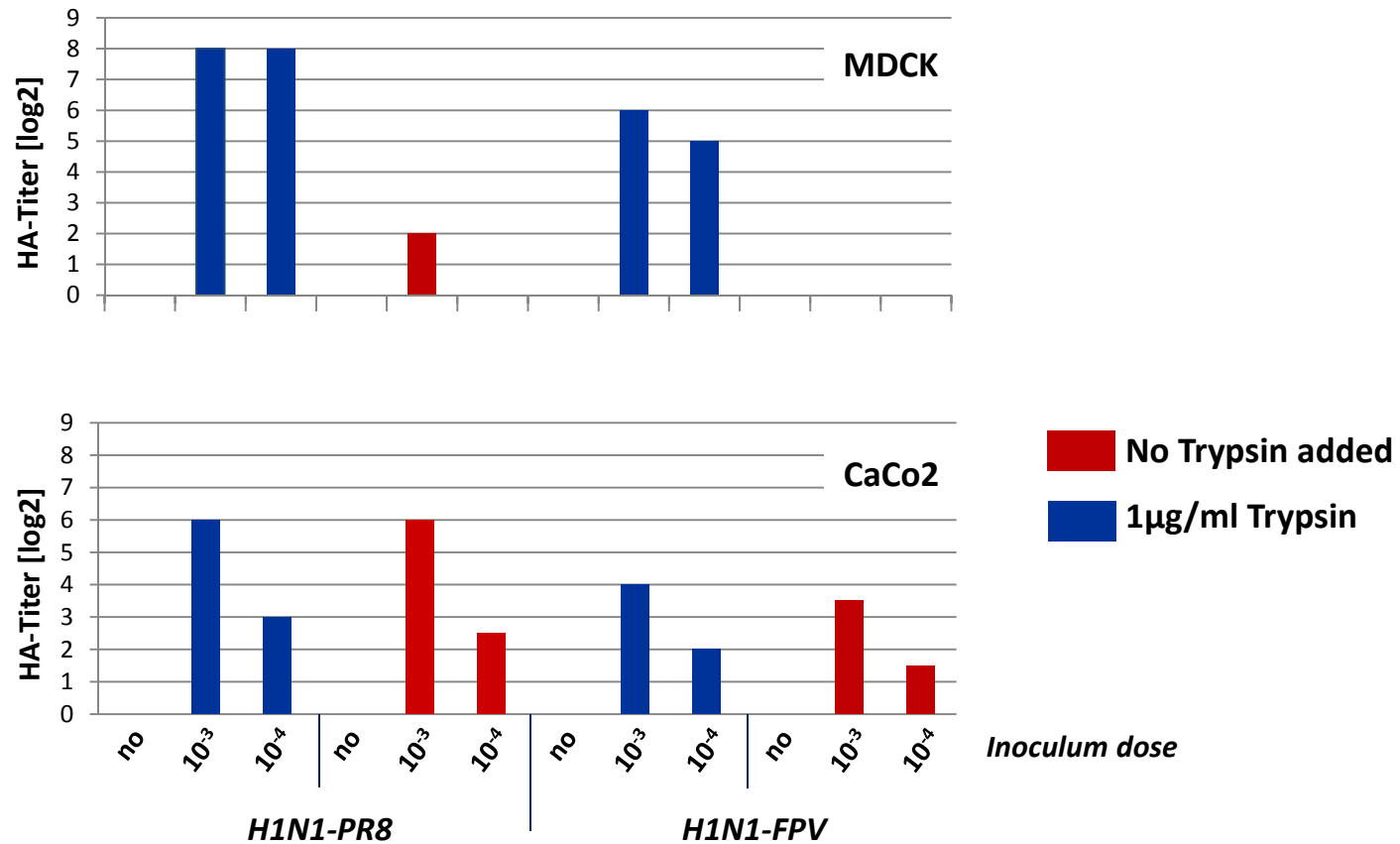
(Pöhlmann et al., 2010; Zhirnov and Klenk, 2003)

⇒ **No exogenous addition of trypsin required**
Trypsin could lead to degradation of antibodies to be assayed
(especially during longer incubation periods)

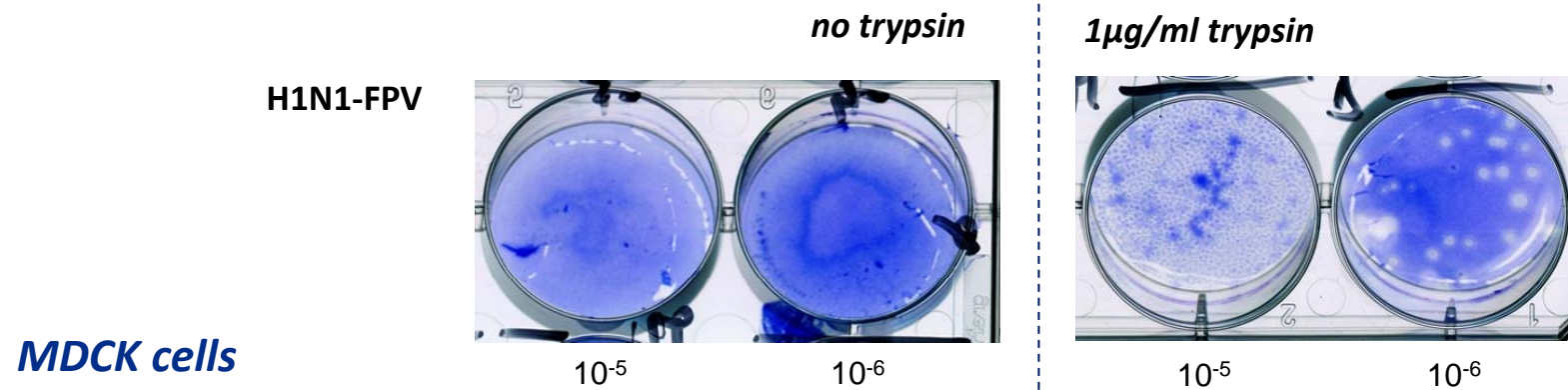
Multicycle growth of H1N1-Reassortants in CaCo2 cells is Trypsin-independent



HA-titers in culture supernatants 72 h.p.i.
MDCK cells as a control

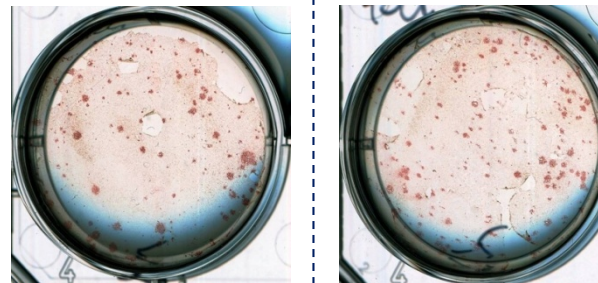


Trypsin-independent cell-to-cell spread in CaCo2 cells

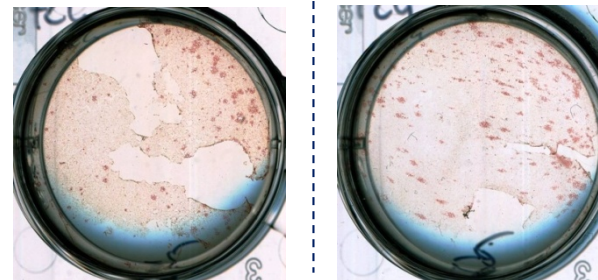


CaCo2 cells

A/Victoria/361/2011 (H3N2)



**A/Hamburg/05/2009 (H1N1)
in FPVmut background
(H1N1-FPV)**



→ MNT-assays done with (MDCK cells) or without (CaCo2 cells) trypsin



MNT results for H3N2 seasonal virus

Alternative approaches addressed:

- **MDCK versus CaCo2 cells**
- **Longer Incubation period (1d, 3d, 6d)**
- **Read-out-methods**
“Classical” anti-NP stain (WHO) versus HA, CPE, WST-reagent)
- **Viral isolate versus reassortant**

➤ Mostly, data presented are GMTs of three independent assay repeats

CPE examination compared to classical anti-NP readout



Reassortant IVR-165

| | | anti-NP readout (WHO) | | | | CPE readout (Visual inspection) | | |
|--------------------|---------------------------|-----------------------|--------|----------|----------|---------------------------------|----------|------|
| | | at day 1 | | at day 3 | | at day d3 | | |
| Inoculum dilution | | 1:1500 | 1:4000 | 1:30.000 | 1:30.000 | 1:30.000 | 1:30.000 | |
| Cell type | | CaCo2 | MDCK | CaCo2 | MDCK | CaCo2 | MDCK | |
| Human adult | Adult, 52 yoa | 95 | 127 | 28 | 202 | na | 320 | |
| | Adult, 58 yoa | 202 | 453 | 160 | 403 | na | 368 | |
| | Adult, 48 yoa | 202 | 508 | 320 | 422 | na | 422 | |
| | Adult, 47 yoa | 127 | 453 | 80 | 508 | na | 570 | |
| | Adult, 53 yoa | 403 | 453 | 359 | 453 | na | 508 | |
| | Adult, 28 yoa | 320 | 538 | 160 | 453 | na | 453 | |
| | Adult, 47 yoa | 806 | 718 | 806 | 640 | na | 557 | |
| | Adult, 50 yoa | 2560 | 2560 | 1280 | 2560 | na | 3620 | |
| | Adult, 24 yoa | 2560 | 3225 | 2560 | 2281 | na | 2560 | |
| | Adult, 32 yoa | 1810 | 2281 | 2032 | 1140 | na | 1613 | |
| | Ferrets | Ferret 315 - pre | 1600 | 2941 | 4525 | 970 | na | 3378 |
| | | Ferret 319 - pre | 3200 | 5120 | 5382 | 1470 | na | 2560 |
| Ferret 320 - pre | | 3200 | 6756 | 7611 | 2560 | na | 3880 | |
| Ferret 315 - post | | 3200 | 5881 | 2540 | 1810 | na | 2874 | |
| Ferret 319 - post | | 2016 | 3620 | 2540 | 1280 | na | 2281 | |
| Ferret 320 - post | | 3200 | 3620 | 2540 | 1140 | na | 2281 | |
| Children | Child, 4 yoa | 3620 | 10240 | 5120 | 7241 | na | 7241 | |
| | Child, 6 yoa | 28 | 90 | 36 | 127 | na | 143 | |
| | Child, 4 yoa | 5120 | 5120 | 5120 | 3620 | na | 5120 | |
| | Child, 5 yoa | 640 | 718 | 905 | 508 | na | 508 | |
| Controls | Positive Control (12/116) | ≥20480 | 102400 | ≥20480 | 51200 | na | 51200 | |
| | Negative Control | 718 | 805 | 570 | 402 | na | 479 | |

Observations:

- ❑ No marked impact of different incubation periods (1 and 3 days) with classical anti-NP readout (for ferret sera: titers lower at day3 for MDCK cells, no consistent trend in CaCo2 cells)
- ❑ Quite good correlation with CPE – but virus strain-specific – no clear CPE detection in CaCo cells

Alternative: CPE-Detection by use of WST-1 reagent



Assay principle:

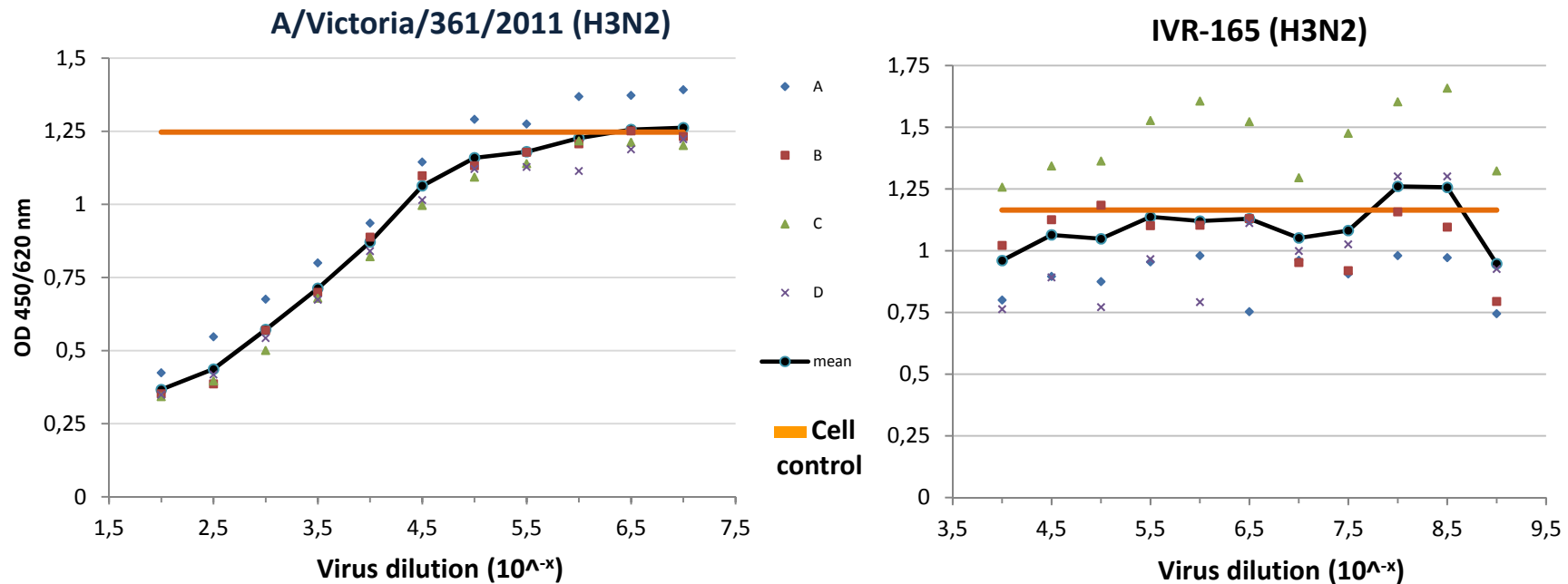


→ Amount of formazan directly correlates with number of metabolically active (viable) cells

⇒ Applicable to quantify CPE in infected cells (*Lehtoranta et al., 2009*)

⇒ To allow for a more standardised quantifiable determination of cell infection

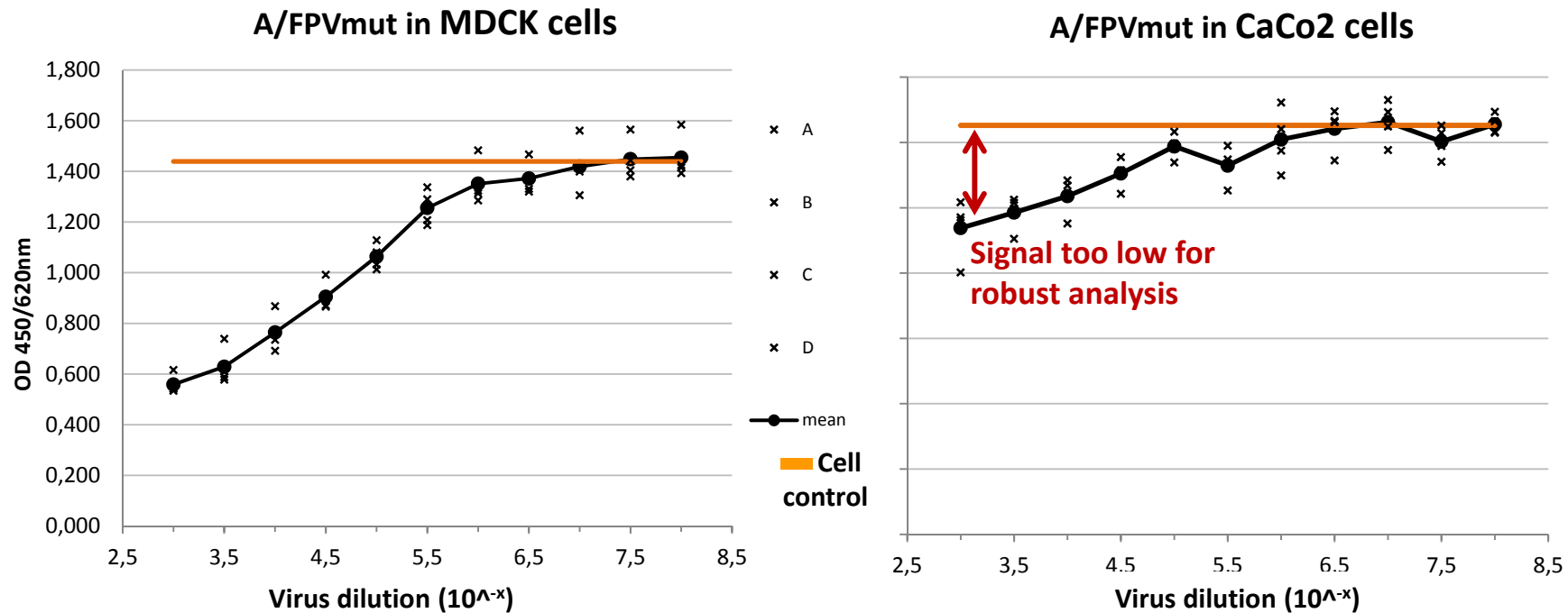
However: Results are highly dependent on the virus used!



Alternative: CPE-Detection by use of WST-1 reagent



...Results also highly dependent on cell type...!



Hence, at present not qualified as a reliable and robust indicator of CPE!!

HA readout compared to classical anti-NP readout



Reassortant IVR-165

| Inoculum dilution | Readout: HA with turkey RBC | | | | HA with guinea pig RBC | | | | anti-NP | |
|---------------------------|-----------------------------|---------|----------|----------|------------------------|---------|----------|----------|----------|----------|
| | at day 1 | | at day 3 | | at day 1 | | at day 3 | | at day 3 | |
| | 1:1.500 | 1:4.000 | 1:30.000 | 1:30.000 | 1:1.500 | 1:4.000 | 1:30.000 | 1:30.000 | 1:30.000 | 1:30.000 |
| Cell type | CaCo2 | MDCK | CaCo2 | MDCK | CaCo2 | MDCK | CaCo2 | MDCK | CaCo2 | MDCK |
| Adult, 52 yoa | na | 453 | 80 | 403 | na | 254 | 40 | 254 | 28 | 202 |
| Adult, 58 yoa | na | 2153 | 202 | 368 | na | 844 | 180 | 403 | 160 | 403 |
| Adult, 48 yoa | na | 1076 | 320 | 381 | na | 640 | 359 | 422 | 320 | 422 |
| Adult, 47 yoa | na | 1280 | 101 | 640 | na | 640 | 71 | 570 | 80 | 508 |
| Adult, 53 yoa | na | 1810 | 320 | 570 | na | 508 | 359 | 453 | 359 | 453 |
| Adult, 28 yoa | na | 3620 | 2560 | 453 | na | 761 | 2560 | 538 | 160 | 453 |
| Adult, 47 yoa | na | 2153 | 1437 | 844 | na | 970 | 905 | 640 | 806 | 640 |
| Adult, 50 yoa | na | 7241 | 20480? | 5120 | na | 1613 | 1280 | 3620 | 1280 | 2560 |
| Adult, 24 yoa | na | 5120 | 5747 | 4064 | na | 3225 | 3620 | 2281 | 2560 | 2281 |
| Adult, 32 yoa | na | 5120 | 4064 | 2560 | na | 3620 | 2560 | 1437 | 2032 | 1140 |
| Ferret 315 - pre | na | 7241 | 3805 | 5881 | na | 4457 | 3805 | 2560 | 4525 | 970 |
| Ferret 319 - pre | na | 10240 | 5382 | 5120 | na | 6756 | 4525 | 2941 | 5382 | 1470 |
| Ferret 320 - pre | na | 10240 | 7611 | 7241 | na | 6756 | 7611 | 3378 | 7611 | 2560 |
| Ferret 315 - post | na | 10240 | 5702 | 7241 | na | 7241 | 4032 | 3620 | 2540 | 1810 |
| Ferret 319 - post | na | 10240 | 2851 | 6451 | na | 5747 | 2851 | 2874 | 2540 | 1280 |
| Ferret 320 - post | na | 10240 | 5080 | 5120 | na | 6451 | 4032 | 2560 | 2540 | 1140 |
| Child, 4 yoa | na | ≥20480 | 10240 | 10240 | na | 10240 | 10240 | 7241 | 5120 | 7241 |
| Child, 6 yoa | na | 905 | 45 | 226 | na | 254 | 28 | 143 | 36 | 127 |
| Child, 4 yoa | na | 10240 | 10240 | 5120 | na | 10240 | 10240 | 5120 | 5120 | 3620 |
| Child, 5 yoa | na | 3620 | 718 | 806 | na | 1437 | 718 | 570 | 905 | 508 |
| Positive Control (12/116) | ≥20480 | ≥20480 | ≥20480 | 102400 | ≥20480 | ≥20480 | ≥20480 | 51200 | ≥20480 | 51200 |
| Negative Control | 806 | 569 | 806 | 569 | 640 | 689 | 640 | 479 | 570 | 402 |

Observations:

- In MDCK cells no significant differences between D1 and D3 – and to anti-NP detection
Overall, titers tend to be somewhat lower after 3 days
- Better pronounced gradual titer responses in CaCO₂ as compared to MDCK

Comparison: Virus Isolate versus Reassortant (H3N2)



Viruses:

A/Victoria/361/2011

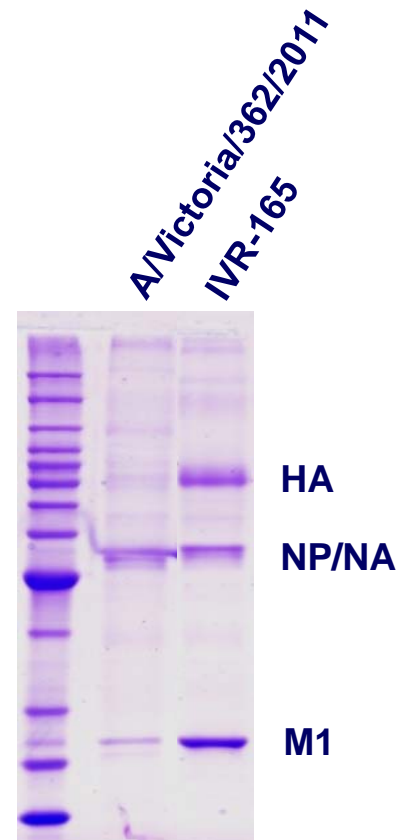
IVR-165

| | Anti-NP readout | | | | | HA: Turkey RBC | | HA: GP RBC | | |
|---------------------------|-----------------|--------|---------|-------|--------|----------------|--------|------------|-------|--------|
| | 1 day | | 3 days | | 6 days | 3 days | | 3 days | | 6 days |
| | A/Vict | IVR | A/Vict | IVR | IVR | A/Vict | IVR | A/Vict | IVR | IVR |
| Adult, 52 yoa | <5 | 127 | 160 | 202 | 320 | 320 | 403 | 320 | 254 | 320 |
| Adult, 58 yoa | 28 | 453 | 320 | 403 | | 320 | 368 | 320 | 403 | |
| Adult, 48 yoa | 40 | 508 | 640 | 422 | | 640 | 381 | 640 | 422 | |
| Adult, 47 yoa | 40 | 453 | 453 | 508 | 640 | 640 | 640 | 640 | 570 | 640 |
| Adult, 53 yoa | 57 | 453 | 160 | 453 | | 320 | 570 | 320 | 453 | |
| Adult, 28 yoa | 20 | 538 | 640 | 453 | | 640 | 453 | 640 | 538 | |
| Adult, 47 yoa | 80 | 718 | 905 | 640 | 1280 | ≥640 | 844 | ≥640 | 640 | 640 |
| Adult, 50 yoa | 226 | 2560 | 453 | 2560 | | 640 | 5120 | 453 | 3620 | |
| Adult, 24 yoa | 320 | 3225 | 640 | 2281 | 2560 | ≥1280 | 4064 | ≥1280 | 2281 | 2560 |
| Adult, 32 yoa | 160 | 2281 | 320 | 1140 | 1280 | 640 | 2560 | 453 | 1437 | 1280 |
| Ferret 315 - pre | 800 | 2941 | 800 | 970 | 3200 | 320 | 5881 | 113 | 2560 | 400 |
| Ferret 319 - pre | 1600 | 5120 | 800 | 1470 | 1600 | 453 | 5120 | 226 | 2941 | 1600 |
| Ferret 320 - pre | 1600 | 6756 | 1600 | 2560 | 1600 | 320 | 7241 | 226 | 3378 | 1600 |
| Ferret 315 - post | 1131 | 5881 | 800 | 1810 | 1600 | 160 | 7241 | 160 | 3620 | 800 |
| Ferret 319 - post | 800 | 3620 | 566 | 1280 | 1600 | 113 | 6451 | 113 | 2874 | 1600 |
| Ferret 320 - post | 800 | 3620 | 800 | 1140 | 1600 | 226 | 5120 | 160 | 2560 | 800 |
| Child, 4 yoa | | 10240 | | 7241 | | | 10240 | | 7241 | |
| Child, 6 yoa | <5 | 90 | 160 | 127 | | 320 | 226 | 320 | 143 | |
| Child, 4 yoa | | 5120 | | 3620 | | | 5120 | | 5120 | |
| Child, 5 yoa | 40 | 718 | 320 | 508 | | 320 | 806 | 320 | 570 | |
| Positive Control (12/116) | ≥12.800 | 102400 | ≥12.800 | 51200 | 102400 | ≥1280 | 102400 | ≥1280 | 51200 | 51200 |
| Negative Control | 640 | 805 | 640 | 402 | 640 | ≥1280 | 569 | ≥1280 | 479 | 640 |

Comparison: Virus Isolate versus Reassortant (H3N2) – Protein composition



Different protein pattern – in particular regarding HA and M1 band intensities
Could clearly affect results obtained in MNT





That's All

Thank you