



MRC PPU  
**Reagents  
and Services**

## **ANTIBODY TESTING RESULTS** **Standard Reporting Template**

**Antibody Name:** Anti-IAV-PA-X

**Full Antigen Name:** PA-X (accessory protein) protein of influenza A virus (IAV) strain A/Puerto Rico/8/1934 (H1N1) ('PR8')

**Antigen Species:** Virus

**Antigen:** GST-IAV PA-X (192 – 252) DU73913

**Sheep Number:** DA224

Bleeds Tested: 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup>, 5<sup>th</sup>

Recommended Bleed: see results.

## **Immunoblotting:**

### **Method**

*MDCK cells were infected with IAV strains PR8 or A/WSN/33 ('WSN') at an MOI of 3 PFU/cell, or mock infected.*

*293T cells were transfected with PR8 pDUAL:PA (a bidirectional reverse genetics plasmid that encodes both the PA and PA-X proteins) or mock transfected, using Lipofectamine 2000 (Thermo Fisher) using cell density and plasmid mass as recommended in the manufacturer's protocol.*

*Cells were lysed in Laemmli buffer at 24 h post-infection or 48 h post-transfection, separated by electrophoresis on 12 % polyacrylamide gels and transferred to nitrocellulose membranes.*

*Membranes were blocked with 5 % milk in PBS-T (phosphate buffered saline with 0.1 % Tween 20) overnight at 4 °C, rinsed in PBS-T then probed with primary antibodies at 1 µg/ml in PBS-T for 1 h at room temperature. Membranes were washed ×3 in PBS-T followed by further incubation with anti-sheep IR680 or IR800 (Thermo Fisher) at 1 in 10,000 in PBS-T, for 1 h at room temperature.*

*Membranes were scanned with a Licor Odyssey CLx Infrared imaging system.*

### **Results**

IAV PA-X = 29.43 kDa. Ladder is the Page Ruler Prestained NIR Protein Ladder (Thermo Fisher).

No visible bands observed using any of 1<sup>st</sup> to 5<sup>th</sup> bleeds anti-PR8-PA X when used at 1 µg/ml or at 5 µg/ml to probe blots.

This was true for both infected and transfected cell lysates.

Negative results (data not shown).

### **Recommendation**

Antisera not suitable for use in immunoblotting under conditions described above.

## **ELISA:**

### **Method**

*96 well ELISA plates were coated overnight at room temperature with lysates of PR8 infected or uninfected MDCK cells, with loading amounts normalised by tubulin concentration.*

*Plates were blocked for 2 h with 5 % milk in PBS-T.*

*All antibody dilutions were carried out using PBS-T.*

*Dilution series of the test antisera were added to the plates, prepared in duplicate from an initial concentration of 50 µg/ml using three-fold serial dilutions. Plates were incubated for 2 h at room temperature and washed 3 times in PBS-T.*

*Donkey Anti-Sheep IgG (H+L) Horseradish Peroxidase (HRP) Conjugate (Life Technologies/Novex) was added to wells at 1 in 20,000 dilution and incubated for 1 h at room temperature.*

*OPD substrate (Merck) was then added to wells and left to develop for 15 minutes in the dark and at room temperature, before stopping the reaction with 10 % H<sub>2</sub>SO<sub>4</sub>.*

*Absorbance (A490) was measured immediately using a Varioskan Lux (Thermo).*

### **Results:**

Results were plotted as average absorbance at 490nm at each antibody concentration with results obtained using uninfected lysate coated plates subtracted from results obtained using infected lysate coated plates to correct for any non-specific binding.

No signal above background was detected using any of 1<sup>st</sup> to 5<sup>th</sup> bleeds anti-PR8-PA X using this method.

Negative results (data not shown).

### **Recommendation**

Antisera not suitable for use in ELISA using this method

## Immunofluorescence:

### Method

A549 and 293T cells were seeded at  $2 \times 10^5$  cells/well in 24 well plates containing 13 mm coverslips.

For 293T cells, coverslips were first coated with poly-D-Lysine to promote adhesion.

The day after seeding, A549 cells were infected with PR8 at an MOI of 2 PFU/cell and 293T cells were transfected with as above using Lipofectamine 2000, following the manufacturer's protocol.

Cells were fixed at 24 h post-infection or post-transfection in 4 % formaldehyde in PBS, then permeabilised using 0.2 % Triton-X100 in PBS/2 % fetal bovine serum (FBS) before blocking for up to 1 h in PBS/2 % FBS.

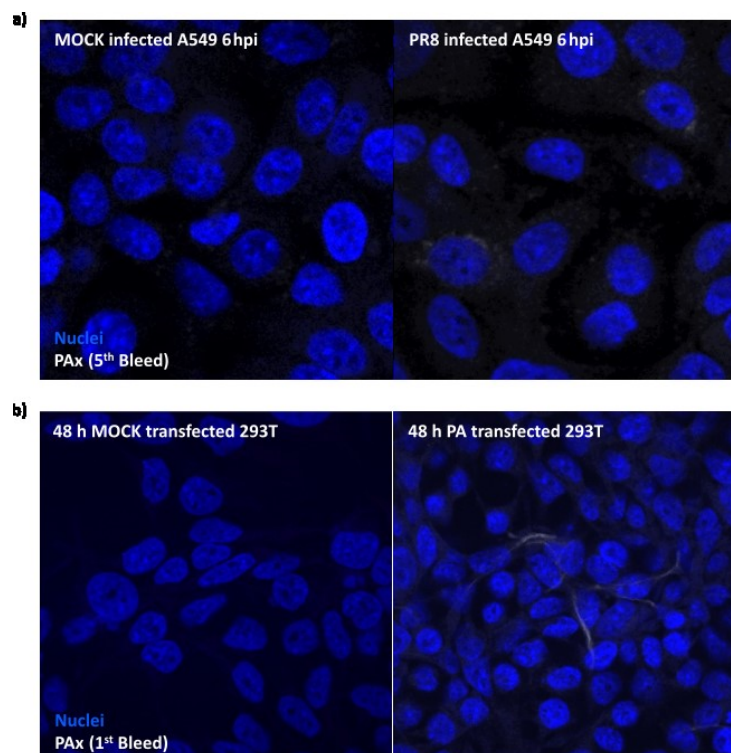
All antibody dilutions were carried out in PBS/2 % FBS. Cells were incubated with primary antibodies at 1:50 for 1 h at room temperature, washed 3 times in PBS/2 % FBS then probed with donkey anti-sheep IgG (H+L) Cross-Adsorbed Secondary antibody, AlexaFluor 647 (Invitrogen) at 1:500 and DAPI at 1:1000 for 45 minutes at room temperature.

Cells were washed 3 times in PBS/2 % FBS and once in PBS, excess moisture was removed before mounting coverslips on slides using ProLong Gold Antifade mounting agent (Thermo Fisher).

Mounting agent was allowed to harden overnight before observation using a Zeiss 710 confocal microscope.

### Results

A weak but specific signal was detected for all bleeds in both infection and transfection. Images show 5<sup>th</sup> bleed antibody at 6 h post-infection and 1<sup>st</sup> bleed antibody at 48 h post-transfection; similar results were observed for the other bleeds.



- a) Weak signal detected using all bleeds at 1 in 50 dilution in PR8 infected cells.
- b) Stronger signal detected using all bleeds at 1 in 50 dilution in PA transfected cells.

### Recommendation

All 5 bleeds, but particularly 1<sup>st</sup> bleed are suitable for use in IF for detection of PA-UFO protein in infected cells at a 1 in 50 dilution with the consideration that this is a non-abundant protein and therefore that the signal will always be faint and dispersed.

**PUBLICATIONS:** None to date

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