# Optimization of Fluorescent Detection of Rotavirus Protein NSP4 and a Cellular Receptor in two Cell Lines

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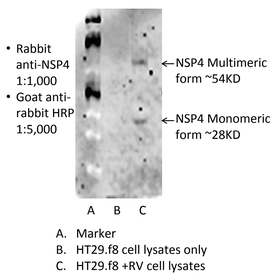
## Introduction

Rotavirus (RV) infections are the most common cause of severe diarrhea in infants and young children worldwide. The two licensed vaccines for RV protect children from common strains of RV, but they are less effective against new emerging RV strains. Therefore, new therapeutics to treat RV infections need to be developed. Recently, we have shown stilbenoids, *trans*-arachidin-1 (t-A1) and *trans*-arachidin-3 (t-A3), decrease progeny virus particles by one hundred fold. Likewise, western blot assays show a decrease in the amount of the viral protein NSP4 with the addition of the stilbenoids during a RV infection. This indicates an effect on viral replication. Immunoblot assays are a standard and cost effective means to analyze the effects of stilbenoids on RV infections.

## Problem

- Western blots have previously been performed with Pierce ECL Western blot substrate (Therrmo Scientific), visualized with x-ray film.
- The Typhoon 8600 laser scanner (GE Healthcare Life Sciences) is available to image our blots. But, experiments have shown that it is not optimized for ECL detection, and has produced poor quality images.
- Fluorescent imaging with the Typhoon 8600 would be more sensitive and cost effective.




A. Marker
B. HT29.f8 cell lysates only
C. HT29.f8 +RV cell lysates

## Experimental Design

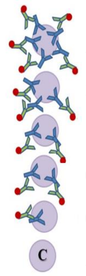
- To develop a more time and cost effective immunoblot assay, the enhanced chemiluminescence (ECL) assay previously used in our experiments was redesigned to an ECL plex fluorescent detection system (1,2).
- Both an African Green Monkey kidney cell line, MA104, and a human intestinal cell line, HT29.f8, were infected with RV. Control and RV-infected cell lysates were prepared and quantified using a micro-BCA protein assay.
- Two-fold dilutions of the cell lysates were added to nitrocellulose membranes in a slot blot apparatus. The concentration of both the antigen-specific primary antibodies and secondary antibodies were held constant at the dilutions that previously demonstrated a good signal in ECL assays.
- Alexa Fluor® 647 conjugated goat anti-rabbit antibodies (Life Technologies), were tested to determine the sensitivity and specificity for signal to noise ratios using the excitation/emission spectras of 633/670nm and 532/580nm.
- The images were collected with the Typhoon 8600 laser scanner (GE Healthcare Life Sciences).

## Material & Methods

Micro BCA Assays: Cells were infected with a human rotavirus (Wa strain) at an MOI of 0.2. At 24hours post infection, the cells were collect (1,2). Cell lysates were prepared and the total protein was quantified using the Pierce Micro BCA assay (Life Technologies) as described in the manual (3).

https://tools.lifetechnologies.com/content/sfs/prodImages/high/23225-BCA-Assay-Kit.jpg

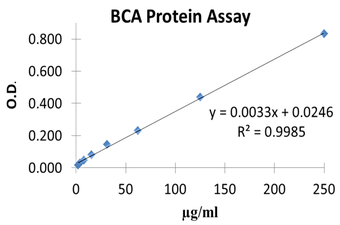
http://www.bio-rad.com/webroot/web/images/lsr/products/electrophoresis/product_detail/global/lsr_bio_dot_product.jpg




Immunoassays: Five microliter of cell lysates from RV-infected (5µg, 2.5µg, 1.25µg, and 0.625µg, respectively) and uninfected cell lysates (5µg) were loaded onto nitrocellulose membranes using a Bio-Dot SF apparatus microfiltration unit, probed with rabbit anti-NSP4-specific or cannabinoid receptor-1 antibodies. Goat anti-rabbit antibodies conjugated to Alexa Fluor® 647 were added and reactive bands were visualized using the Typhoon 8600 laser scanner.

## Results

### Micro BCA Protein Quantification




| O.D. | O.D. | O.D. | AVG O.D. | ug/ml |
|---|---|---|---|---|
| 0.818 | 0.811 | 0.868 | 0.832 | 250 |
| 0.415 | 0.422 | 0.480 | 0.439 | 125 |
| 0.257 | 0.266 | 0.261 | 0.261 | 62.5 |
| 0.149 | 0.144 | 0.147 | 0.146 | 31.3 |
| 0.082 | 0.080 | 0.081 | 0.081 | 15.6 |
| 0.051 | 0.043 | 0.044 | 0.046 | 7.8 |
| 0.027 | 0.021 | 0.035 | 0.028 | 3.9 |
| 0.013 | 0.012 | 0.016 | 0.014 | 1.95 |

| | O.D. | O.D. | O.D. | avg O.D. | 0.014 | mg/ml |
|---|---|---|---|---|---|---|
| MA104a | 0.1390 | 0.0947 | - | 0.12 | 27.97 | 1.40 |
| MA104b | 0.0927 | 0.0897 | 0.1030 | 0.10 | 21.39 | 1.07 |
| MA104c | 0.0608 | 0.0524 | 0.0872 | 0.07 | 12.80 | 0.64 |
| HT29.f8 a | 0.0324 | 0.0392 | 0.0223 | 0.03 | 2.04 | 0.10 |
| HT29.f8 b | 0.0755 | 0.0983 | - | 0.09 | 18.89 | 0.94 |
| HT29.f8 c | 0.0557 | 0.0622 | - | 0.06 | 10.42 | 0.52 |

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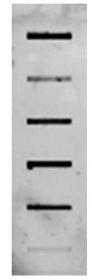
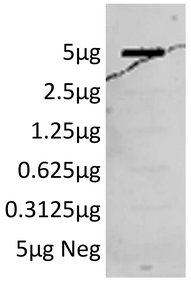
Poster presentation at Bright Ideas Conference April 29, 2015 Stephen F Austin State University

## Fluorescent Immunoassays using Alexa Fluor® 647 with HT29.f8 cell lysates



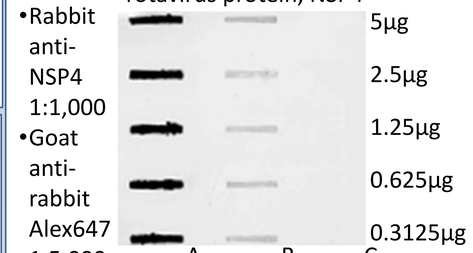

| Mode | Fluorescence |
|---|---|
| Sensitivity | Normal |
| PMT | 500 |
| Excitation | Red 633 |
| Emission | 670 |
| Primary Antibody | 1: 1000 Rabbit anti-NSP4 150-175 |
| Secondary Antibody | 1: 5000 Goat anti-Rabbit Alexa 647 |

| Mode | Fluorescence |
|---|---|
| Sensitivity | Normal |
| PMT | 500 |
| Excitation | Green 532 |
| Emission | 580 |
| Primary Antibody | 1: 1000 Rabbit anti-NSP4 150-175 |
| Secondary Antibody | 1: 5000 Goat anti-Rabbit Alexa 647 |



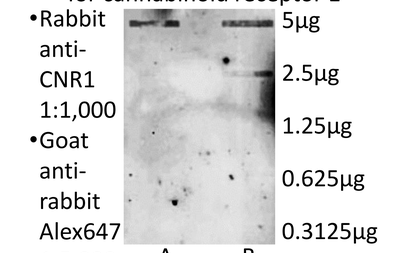

## Fluorescent Immunoassays using Alexa Fluor® 647 with MA104 and HT29.f8 cell lysates

Slot Blot analysis of MA104 and HT29.f8 cell lysates for rotavirus protein, NSP4




A.HT29.f8 +RV cell lysates
B.MA104 +RV cell lysates
C.MA104 cell lysates only

Slot Blot analysis of uninfected MA104 and HT29.f8 cell lysates for cannabinoid receptor 1




A. MA104 cell lysates
B. HT29.f8 cell lysates

## Discussion

Our data using the ECL plex fluorescent assays showed a strong signal with varying concentrations of cell lysates for both the rotavirus protein, NSP4, and a membrane-bound protein, the cannabinoid receptor 1. The Alexa Fluor® 647 secondary antibodies showed specificity at the excitation/emission 633/670nm, and produced a signal when excitation/emission 532/580nm was used. This suggests that the Alexa Fluor® 647 secondary antibody has a broader range for signal detection. These optimizations will enhance the sensitivity of antigen-specific signals for a variety of antigens, and will make future immunoassays more cost effective than detecting the signals with ECL.

## References

1. Smith, P.K., et al. (1985). Measurement of protein using bicinchoninic acid. Anal Biochem 150:76-85.
2. Gibbons, TF, Storey, SM, Williams, CV, McIntosh, A, Mitchel, DM, Parr, RD, Schroeder, ME, Schroeder, F and Ball, JM. 2011. *Virol. J.* 8:278-297.
3. Parr, RD, Storey, SM, Mitchell, DM, McIntosh, AL, Zhou, M, Mir, KD, and Ball, JM. 2006 *J.Virol.*80:2842-2854.