

Optimizing Reagents and Substrates for Standardizing RPPA

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INTRODUCTION

Reverse phase protein arrays (RPPA) offer great promise for the identification and analysis of critical biomarkers for disease states in patient samples and for high-throughput drug screening using cultured cell systems. Porous nitrocellulose (NC) film slides continue to be the preferred substrate for RPPA due to their high protein binding capacity and many functional advantages. Additionally, the compatibility of NC with mass spectrometry allows for the extension of RPPA technology with emerging downstream protein characterization methodologies.

NC is well suited for colorimetric detection, but has seen limited use for fluorescence detection primarily due to high intrinsic fluorescence. However, fluorescence detection offers significant advantages over colorimetric methods – namely enhanced sensitivity and the ability to perform multiplexed assays. Grace Bio-Labs has developed a nitrocellulose chemistry that combines the advantages of high protein binding and low auto-fluorescence, allowing for either color or fluorescence detection. Furthermore, we have developed and optimized a blocking chemistry for this substrate which improves fluorescence-based results obtained with our NC.

The desire to take RPPAs to the clinic will require consistency in methodologies, materials, and reagents utilized for RPPA to allow for cross-study comparisons, obtain consensus results and ultimately, derive consistent and reliable patient diagnosis. Data presented here highlight the variation which may be introduced into RPPA experiments from choice of substrate, blocking reagents, and lysis reagents. These results underscore the need for standardized reagents for RPPA and offer effective solutions for both colorimetric- and fluorescence-based RPPA assays.

METHODS

Microarray Printing and Detection: Protein microarrays were spotted using a SciFLEXARRAYER S3 printer (Sciencion). Fluorescence intensities were obtained at 532 nm or 635 nm using a GenePix 4000B Scanner (Molecular Devices). All comparative data shown were obtained using the same scanning parameters. Color intensities were obtained using a UMAX PowerLook 2100XL scanner.

Assays: NC binding capacities were determined after spotting Cy3-labeled goat IgG and measuring post-wash fluorescence (standard wash: 3x PBST 5 min. ea.; 3x PBS, 5 min. ea.; 1x H₂O, 1min.). Immunoassay data are normalized and background-subtracted, collected after detection of IgG from goat serum with a 1 hr. incubation with anti-goat IgG-Cy3 (fluorescence) or anti-goat IgG followed by HRP/AEC staining (colorimetric). Blocking efficiency was determined after 1 hr. blocking followed by fluorescent detection of purified IgG, IL-1 α , IL-1 β , IL6, TNF α , TNF β , or INF γ with the following incubations: 1hr. goat α -antigen detection antibody (plus wash (3x PBST, 5 min. ea.)), 1hr. α -goat-IgG-Alexa635 or-TRITC reporter antibody, and standard wash (as above).

RPPA lysis buffers were prepared following published recipes (Pawelz *et al.*, 2001; Chan *et al.*, 2004; Tibes *et al.*, 2006; Spurrier *et al.*, 2008) and supplemented with inhibitor tablets from Roche. Equal amounts of mouse liver tissue (approx. 40 mg) were homogenized in respective buffers. Protein extraction efficiency, spotting/binding efficiency with NC, and functional assessment with fluorescent and colorimetric endpoints were assessed for two cytosolic proteins (Actin, GAPDH) and two nuclear proteins (Histone H2A, PARP). Total protein was determined by Sypro Ruby (Life Technologies) staining with BSA as a standard.

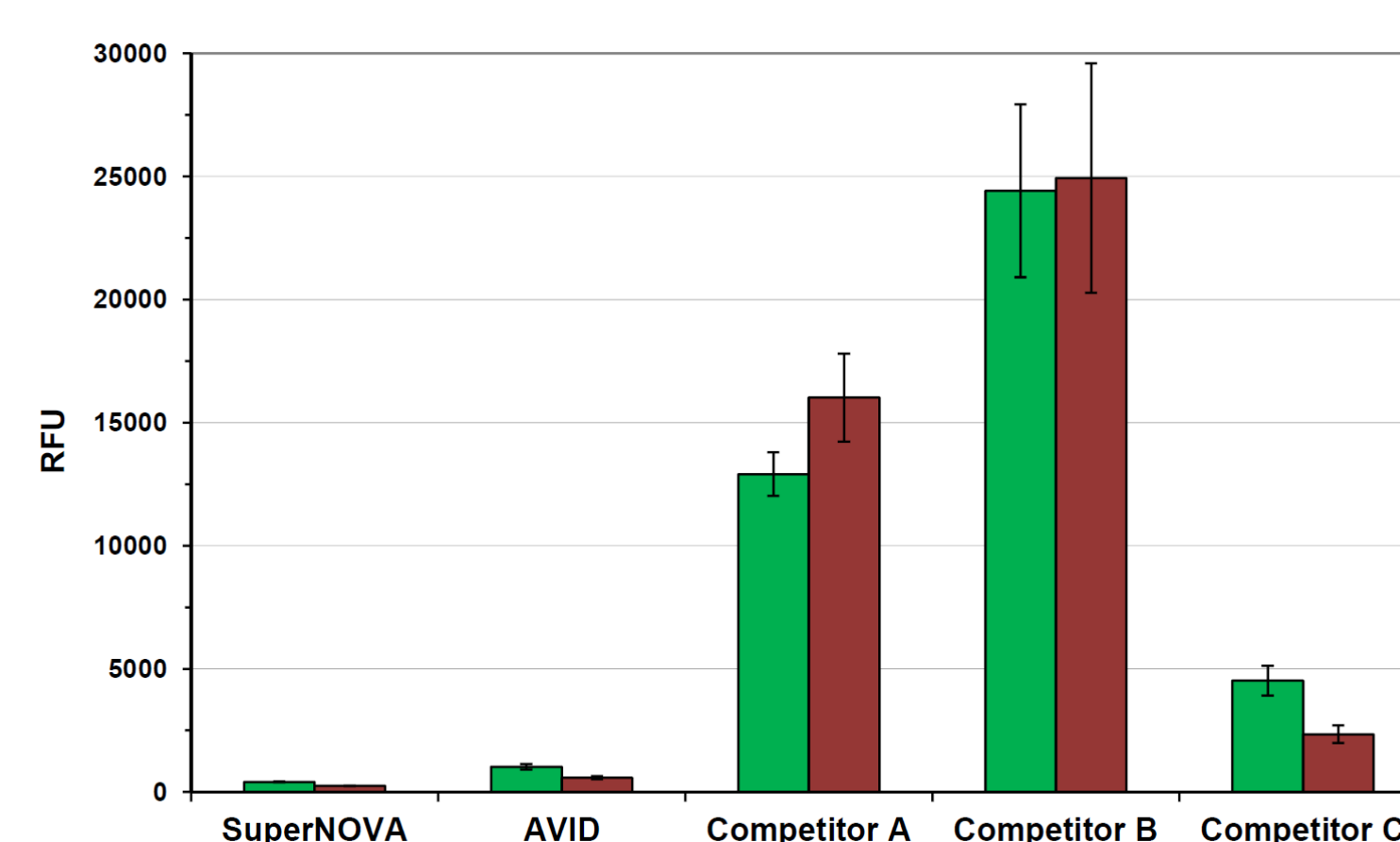


Figure 1. SuperNOVA porous nitrocellulose slides have 10- to 60-fold lower backgrounds in both the 532 nm and 635 nm fluorescence channels – a further improvement over our AVID formulation. Shown are the average backgrounds obtained for 532 nm (green, 500 PMT/33% LP) and 635nm (red, 650 PMT/100% LP).

RESULTS

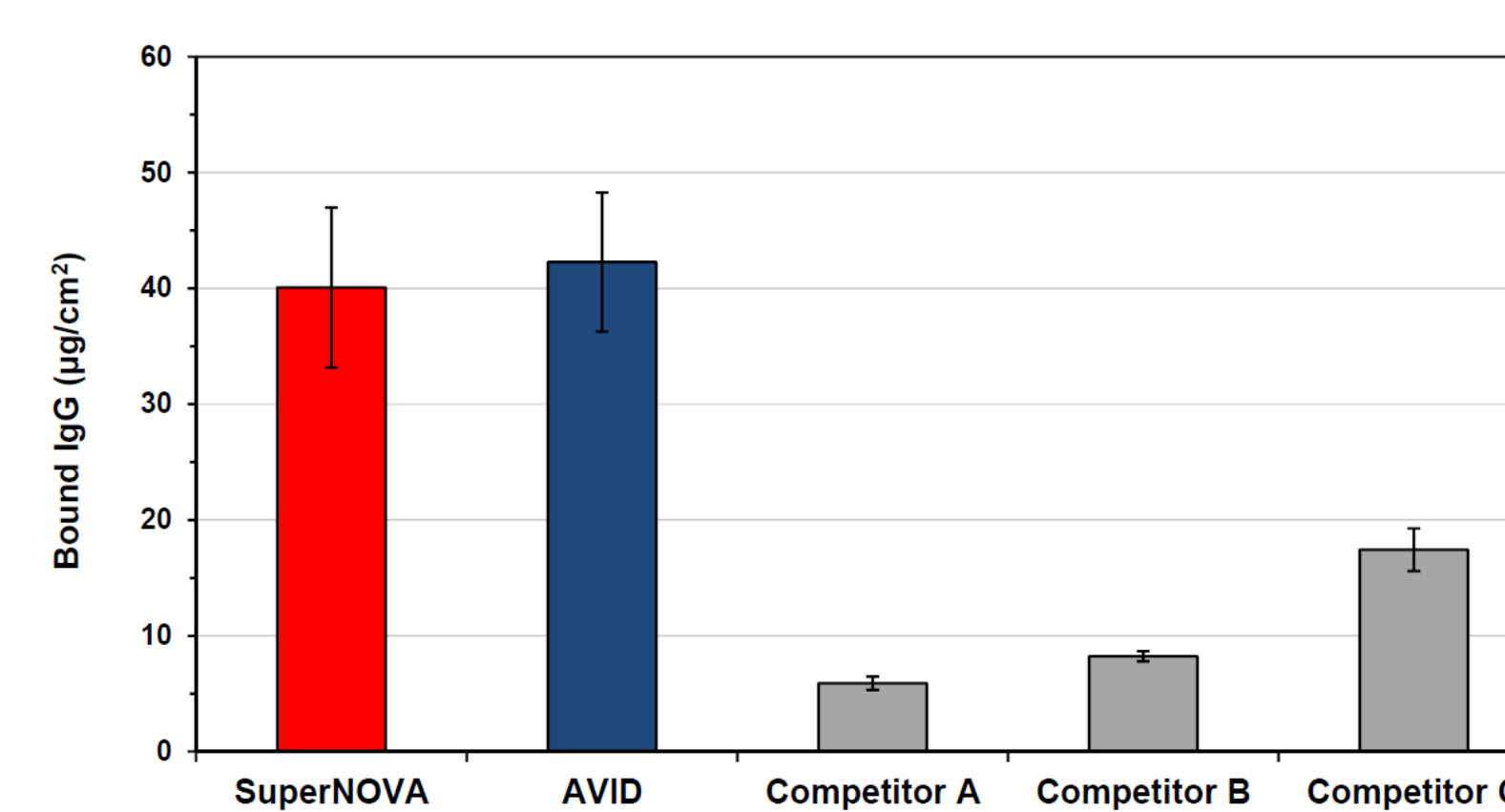


Figure 2. SuperNOVA slides have 2- to 7-fold higher binding capacity than competing film slides and maintain the same protein binding capacity compared to AVID. Shown is the average binding of IgG on different porous nitrocellulose slides.

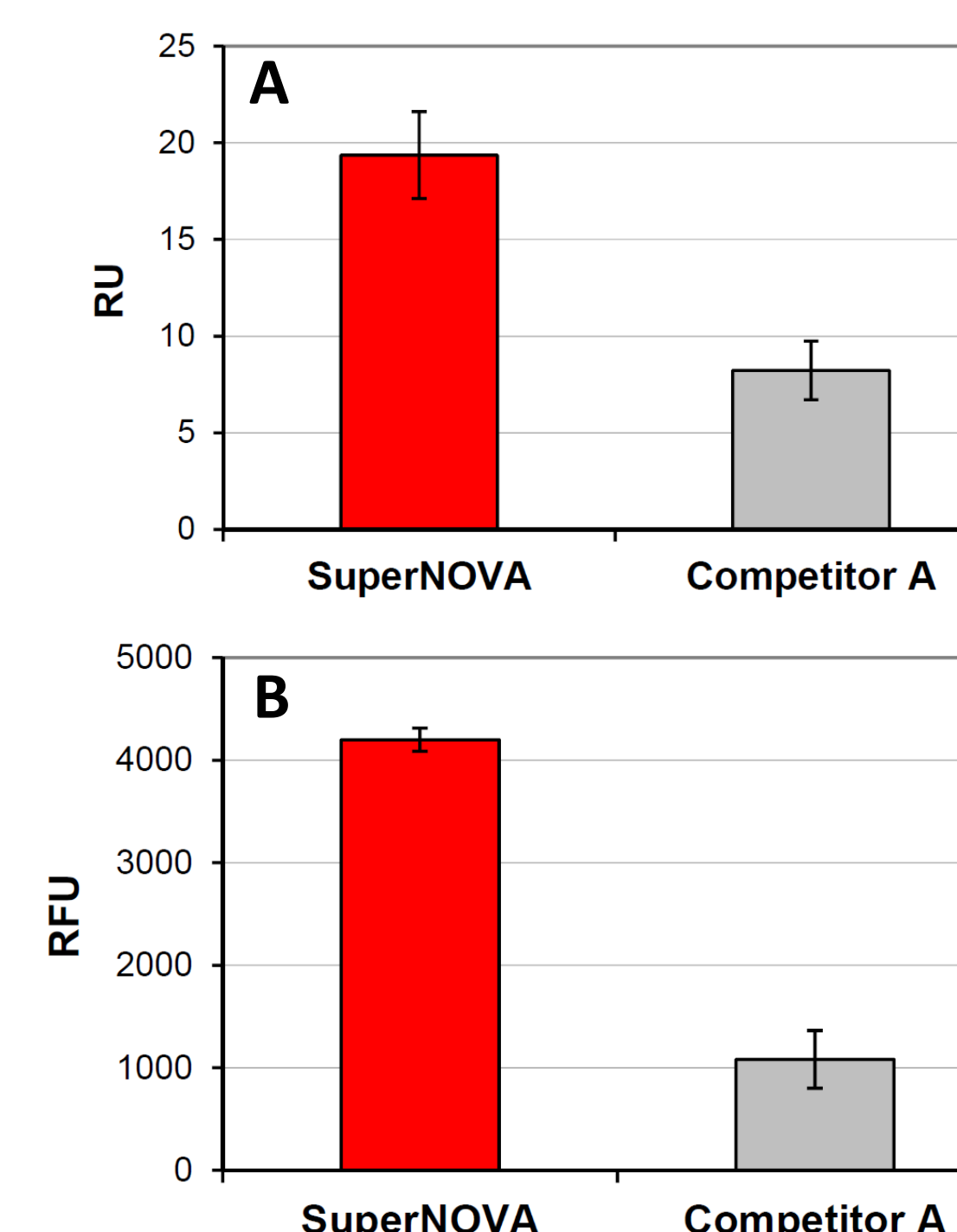


Figure 3. Assay results comparing SuperNOVA to a representative competing NC film slide. High protein binding capacity translates to significantly better signal after functional (A) colorimetric- or (B) fluorescence-based immunoassays.

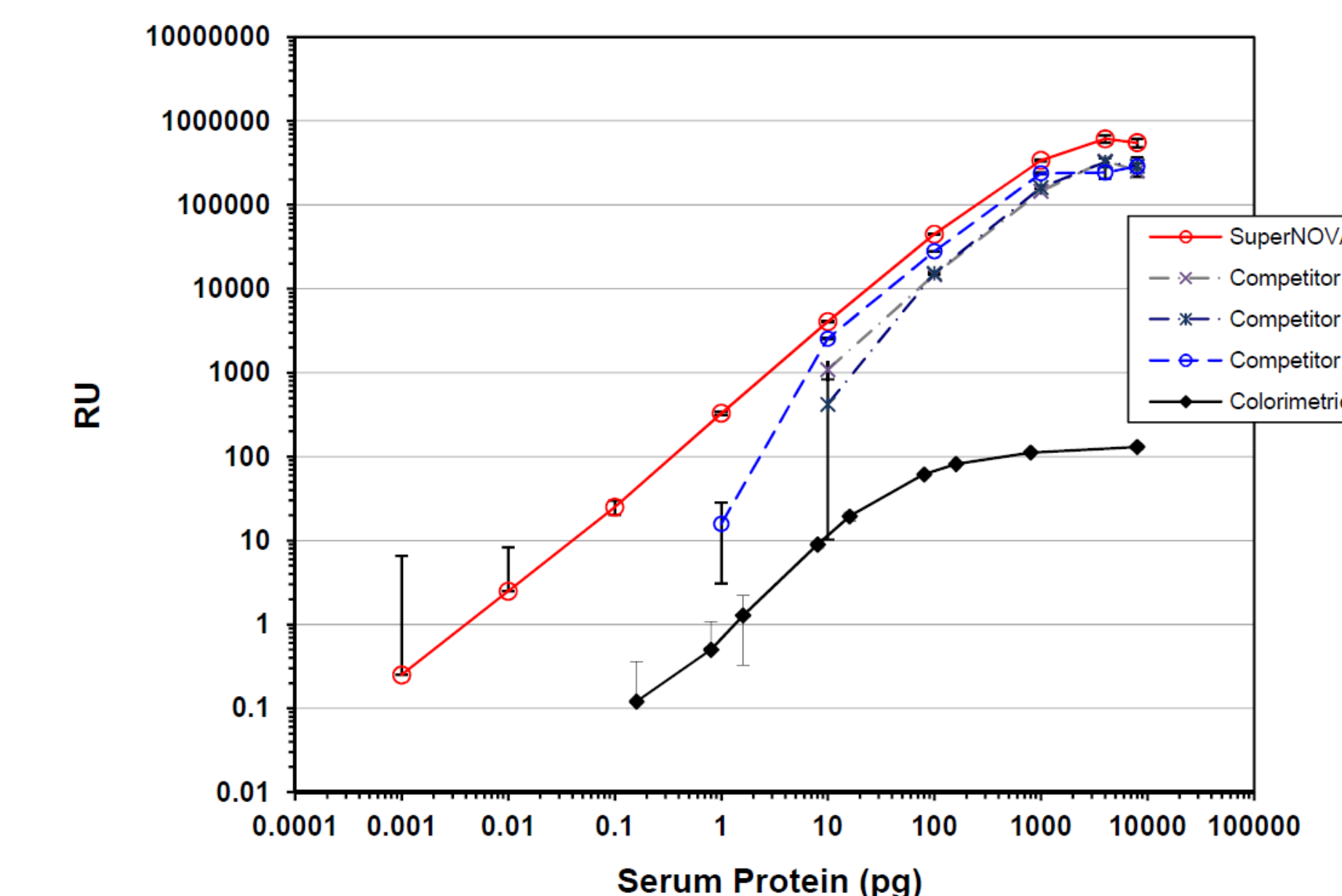


Figure 4. The dynamic range of a typical fluorescent immunoassay on SuperNOVA is linear over 6 orders of magnitude compared to approx. 2 for competing film slides. Shown are fluorescence measurements at 532 nm for IgG detected from a serial dilution of goat serum. Similar results were obtained in the 635 nm channel (data not shown). Additionally, data from a colorimetric assay for detection of the same antigen show approx. 2 orders of magnitude linearity and comparatively lower sensitivity to fluorescence when assessing non-amplified signal.

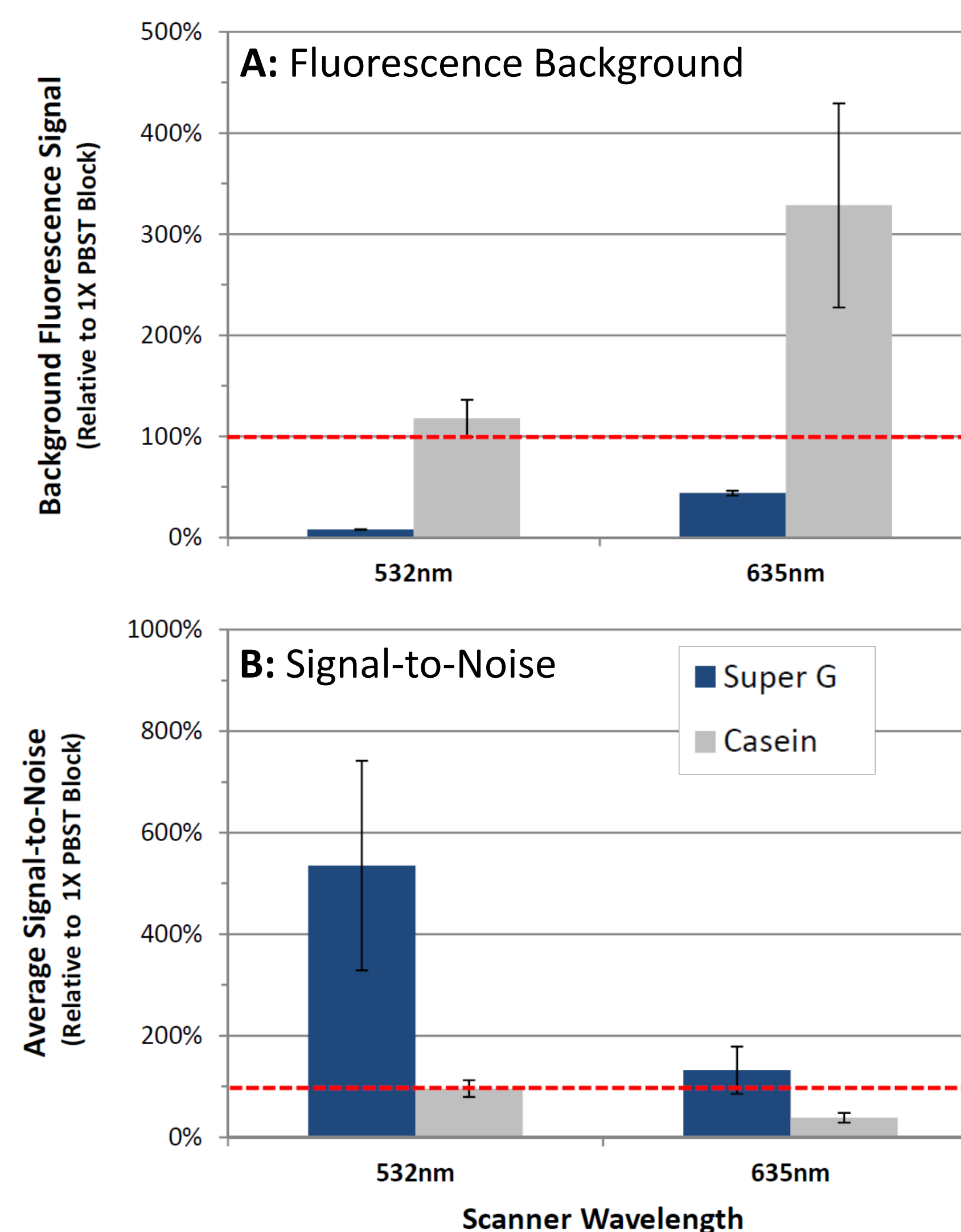


Figure 5. Background fluorescence and signal-to-noise from SuperNOVA film slides blocked with Super G Blocking reagent or casein relative to blocking with a common blocker (PBS with 0.1% Tween-20). (A) Background fluorescence using Super G are 10-fold lower at 532 nm and 5-fold lower at 635 nm. (B) Conversely, fluorescence Signal-to-Noise is 5.5-fold higher at 532 nm and 3.5-fold higher at 635 nm after blocking with Super G. Data presented are the average signal-to-noise from the detection of 7 purified antigens.

Table 1

Lysis Buffer	Components
A	Tris/SDS/Glycerol/T-PER
B	Tris/SDS/Glycerol
C	Tris/SDS/Glycerol/Triton X-100
D	Urea/CHAPS/Pharmalyte
E	Grace Bio-Labs

CONCLUSIONS

In addition to consistent methodologies for sample handling and processing for RPPA analysis, there is a clear need for optimized and standardized substrates and reagents for RPPA.

- Choice of porous nitrocellulose film has a significant impact on both colorimetric- and fluorescence-based RPPA assay sensitivity. To obtain the highest assay signal and greatest signal to noise for RPPA assays, nitrocellulose films with the highest binding capacity obtainable are shown to perform best.
- Choice of blocking reagent has a substantial impact on results obtained from fluorescent assays. Although casein is a common blocking reagent for colorimetric RPPA assays, it is not recommended for fluorescence-based assays.
- Choice of tissue/cell lysis buffer contributes to RPPA assay signal variation. Characteristics contributing to differences between these reagents are protein yield, spot size, extent of protein binding to NC, and potential effects on antibody binding during the RPPA assay.

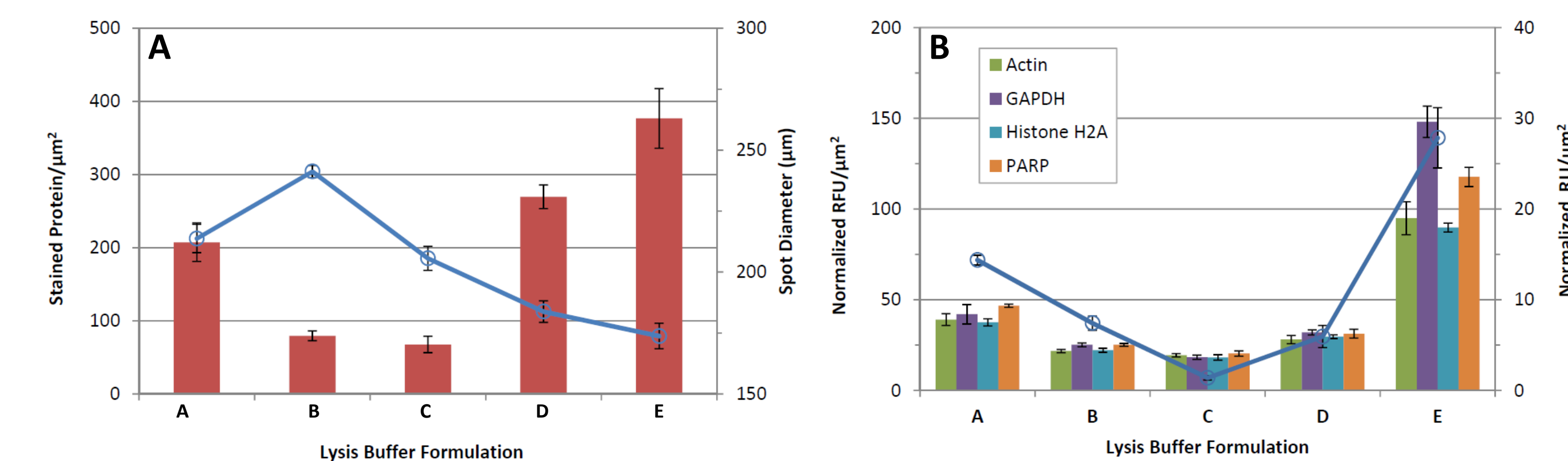


Figure 6. RPPA lysis buffers (from Table 1) were compared in their ability to extract protein, their binding characteristics to NC, and their performance in RPPA assays. Protein extraction efficiency from mouse liver tissue (data not shown) were similar for Buffers B, D, and E (80 µg protein / mg tissue), slightly lower for Buffer A (50 µg/mg) and higher for Buffer C (120 µg/mg). (A) Data show total protein bound (bar, left axis) to SuperNOVA slides and corresponding spot diameters (line, right axis). (B) RPPA assays for Actin, GAPDH, Histone H2A, and PARP proteins show varying results in both fluorescent (bar, left axis) and colorimetric (line, right axis) assays depending on buffer content.

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