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## A Rapid, Multiplex Dual Reporter IgG and IgM SARS-CoV-2 Neutralization Assay for a Multiplexed Bead-based Flow Analysis System

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1 **TITLE:**
2 A Rapid, Multiplex Dual Reporter IgG and IgM SARS-CoV-2 Neutralization Assay for a Multiplexed
3 Bead-based Flow Analysis System
4
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23 **KEYWORDS:**
24 multiplex, microsphere, bead, SARS-CoV-2, serological assay, neutralization assay
25
26 **SUMMARY:**
27 A flow analysis system for bead-based multiplexed assays which provides a two-reporter readout
28 was used for the development of multiplex serological and antibody neutralization assays that
29 can simultaneously measure neutralizing IgG and IgM antibodies for SARS-CoV-2.
30
31 **ABSTRACT:**
32 The COVID-19 pandemic has underscored the need for rapid high-throughput methods for
33 sensitive and specific serological detection of infection with novel pathogens, such as SARS-CoV-
34 2. Multiplex serological testing can be particularly useful because it can simultaneously analyze
35 antibodies to multiple antigens that optimizes pathogen coverage, and controls for variability in
36 the organism and the individual host response. Here we describe a SARS-CoV-2 IgG 3-plex
37 fluorescent microsphere-based assay that can detect both IgM and IgG antibodies to three major
38 SARS-CoV-2 antigens—the spike (S) protein, spike angiotensin-converting enzyme-2 (ACE2)
39 receptor-binding domain (RBD), and nucleocapsid (Nc). The assay was shown to have comparable
40 performance to a SARS-CoV-2 reference assay for IgG in serum obtained at ≥21 days from
41 symptom onset but had higher sensitivity with samples collected at ≤5 days from symptom onset.
42 Further, using soluble ACE2 in a neutralization assay format, inhibition of antibody binding was
43 demonstrated for S and RBD.
44

**INTRODUCTION:**

The COVID-19 pandemic has emphasized the importance of being able to rapidly develop and implement fast, high-throughput, high-performing serological tests that can be used for diagnosis and surveillance of novel pathogens such as SARS-CoV-2. Multiplex serological testing can be extremely useful in a pandemic, as it can simultaneously analyze antibodies to multiple antigens, which provides for broad pathogen coverage and controls for variability in the organism and the host response to infection. The development of a multiplex fluorescent bead-based assay, the SARS-CoV-2 IgG 3Flex (3Flex), that can detect antibodies to the spike (S) protein, the spike angiotensin-converting enzyme-2 (ACE2) receptor-binding domain (RBD), and nucleocapsid (Nc)1 of SARS-CoV-2 was recently reported[1]. 3Flex was shown to have comparable performance to a reference SARS-CoV-2 IgG assay for serum obtained at ≥21 days form symptom onset but had higher sensitivity with samples collected at ≤5 days from symptom onset. Additionally, inhibition of antibody binding was demonstrated for S and RBD antigens using soluble ACE2 in a neutralization assay format. Multiplex bead-based technology has been widely adopted as a proven technology for multiplex serological testing and has distinct advantages for large-scale screening, including short time-to-results, small sample requirements, and reduced labor[2-4].

Recently, a new flow analysis instrument has become available that provides the same high-plex, high-throughput capability of the system demonstrated in previous work[1], but with the added benefit of two reporter channels. The ability to measure two fluorescent reporter signals in a single reaction allows the assessment of two results per analyte for each sample and is ideal for isotyping applications, which typically must be performed in separate reaction wells[5]. The dual reporter capability provides twice the data for each sample in half as many reaction wells with half the sample volume requirements, and thus has a clear advantage over single reporter systems. This study details the standard serological assay protocol and describes the adaptation to a neutralization assay. Additionally, this report describes the conversion of the single reporter assay to a dual reporter serological assay, and subsequently, a dual reporter neutralization assay, to simultaneously measure neutralizing antibodies of both IgG and IgM isotypes against the SARS-CoV-2 S, RBD, and Nc antigens. A visual overview of the assay protocol is provided in **Figure 1**.

**PROTOCOL:**

Residual human serum samples previously tested for SARS-CoV-2 diagnostic analyses were used in this study which was approved by the University of Rochester Institutional Review Board.

**1.      Reagents and equipment**

1.1      Obtain coronavirus antigens and human IgG and IgM from commercial sources.

1.2      Obtain assay control (AC) bead sets (45 and 220) from the instrument vendor. The AC bead set 45 monitors Median Fluorescence Intensity (MFI) collection on single reporter channel (RP1) instruments. The AC bead set 220 monitors MFI collection on the second reporter channel (RP2).

89   1.3    Obtain detection antibodies and antigen-specific rabbit sera and antibodies used for
90   coupling confirmation.
91
92   1.4    Perform the detection with biotin-labeled antibodies with either a streptavidin,
93   R-phycoerythrin conjugate (SAPE) or a streptavidin conjugate of fluorophore Super Bright 436
94   (SASB; excitation at 405 nanometers (nm) and emission at 436 nm).
95
96   1.5    Obtain Human ACE2 protein (i.e., the SARS Receptor). Prior to use, rehydrate lyophilized
97   ACE2 protein by dissolving to a 1 mg/mL concentration in nuclease-free water (Not DEPC-treated,
98   autoclaved and 0.2-μm sterile filtered) and store at 4 °C.
99
100  1.6    Perform all reactions in 96-well nonbinding surface (NBS) black flat-bottom plates. Seal
101  the plates with 96 well microplate aluminum sealing tape for assay incubation steps. Use a
102  magnetic plate separator for the assay wash steps.
103
104  **2.     Antigen-coupled and antibody-coupled control beads preparation**
105
106  2.1    Covalently couple magnetic, polystyrene, and color-coded microsphere sets (6.5-μm
107  diameter) with S, RBD, and Nc antigens as described in Cameron et al.[1]. Coronavirus antigens are
108  coupled at 10 pmol/$10^6$ beads (S) and 100 pmol/$10^6$ beads (RBD and Nc). Control beads coupled
109  with human IgG or IgM are generated as previously described[1]. IgG and IgM are coupled at 5
110  μg/$10^6$ beads.
111
112  2.2    After coupling, determine the bead concentrations and dilute each stock to 1 x $10^6$
113  beads/mL using described procedures[6].
114
115  2.3    Perform confirmation of antigen coupling with antigen-specific antibodies from rabbit or
116  with positive human sera as described in Cameron et al.[1].
117
118  2.3.1  Confirm human IgG coupling with the assay's biotinylated anti-human IgG/SAPE detection
119  reagents, and IgM coupling with a phycoerythrin (PE)-labeled goat anti-human IgM.
120
121  NOTE: Coupling confirmation is performed using a titration of protein-specific serum or antibody,
122  because the optimal concentration to use for these reagents is not known. For serum, serial fold
123  dilutions are used whereas for antibodies supplied as mg/mL stocks, serial μg/mL dilution series
124  are used.
125
126  2.4    Dilute assay control (AC) beads that monitor MFI collection in the two reporter channels
127  to a concentration of 1 x $10^6$ beads/mL prior to using in multiplex bead mixes.
128
129  **3.     Fresh multiplex bead mix preparation**
130
131  3.1    Prepare multiplex beads mixes freshly each day from the individual coupled bead and
132  control bead stocks at 1 x $10^6$ beads/mL.

133

134 NOTE: Multiplex bead mixes are prepared to deliver 2,500 beads for each bead region in a 50- μL
135 volume. Calculations for how to prepare multiplex bead mixes for any number of reactions are
136 described in the blog published by Angeloni (2020)[6].

137

138 **4.     Sample dilution**

139

140 4.1     Prepare a 1:100 serum sample dilution by mixing 10 μL of serum into 990 μL of phosphate-
141 buffered saline (PBS) containing 0.05% Tween 20, 0.02% sodium azide, and 1% of bovine serum
142 albumin (BSA) (PBS-TBN buffer).

143

144 4.2     Dilute samples again 10-fold by adding 20 μL of the 1:100 dilution to 180 μL of PBS-TBN.

145

146 NOTE: Serum samples consist of negative sera, from specimens collected in 2019 prior to the
147 COVID-19 pandemic, and positive sera from SARS-CoV-2 PCR-positive patients, representing low
148 and high antibody titers. Positive samples were collected between ≈3 to >60 days from symptom
149 onset (DFSO). All serum samples and IgG-stripped negative control sera are diluted to 1:1000 as
150 described below. For neutralization assays, sera are diluted 1:500 as described in the
151 neutralization protocols below (sections 6 and 8).

152

153 **5.     Single reporter serological assay protocol**

154

155 5.1     Add 50 μL of multiplex bead mix to each assigned well of a 96-well non-binding microtiter
156 plate.

157

158 5.2     Pipette 50 μL of 1:1000 serum or PBS-TBN into the appropriate wells; the final dilution of
159 serum in the well is 1:2000.

160

161 5.3     Cover the plate with a microplate foil seal and incubate on a heated plate shaker at 37 °C
162 for 15 min with shaking at 600 rpm.

163

164 5.4     Remove the plate from the plate shaker and place onto the magnetic plate separator for
165 2 min to separate the beads from the reaction mixture.

166

167 5.5     While retaining the plate on the magnet, remove the foil seal, carefully invert the plate
168 over a waste container, and gently flick the supernatant out of the wells.

169

170 5.6     Blot the plate on absorbent paper while still holding the plate on the magnet.

171

172 5.7     Wash the reaction wells as described below (first post-sample incubation wash).

173

174 5.7.1   Remove the plate from the plate magnet and add 150 μL of PBS-TBN to each well.

175

176 5.7.2   Cover the plate with a fresh foil seal and shake at 37 °C for 2 min at 600 rpm.

177

178     5.7.3    Remove the plate from the plate shaker and place onto the plate magnet for 2 min to
179     separate the beads from the reaction mixture.

180

181     5.7.4    While retaining the plate on the magnet, remove the foil seal, carefully invert the plate
182     over a waste container, and gently flick the supernatant out of the wells.

183

184     5.7.5    Blot the plate on absorbent paper while still holding the plate on the magnet.

185

186     5.8       Wash the reaction wells as described below (second post-sample incubation wash).

187

188     5.8.1    Remove the plate from the plate magnet and add 150 µL of PBS-TBN to each well.

189

190     5.8.2    Cover the plate with a fresh foil seal and shake at 37 °C for 2 min at 600 rpm.

191

192     5.8.3    Remove the plate from the plate shaker and place onto the plate magnet for 2 min to
193     separate the beads from the reaction mixture.

194

195     5.8.4    While retaining the plate on the magnet, remove the foil seal, carefully invert the plate
196     over a waste container, and gently flick the supernatant out of the wells.

197

198     5.8.5    Remove the plate from the magnet.

199

200     5.9       Make a fresh mix of biotin-anti-human IgG with SAPE by diluting the antibody to 0.62
201     µg/mL and the SAPE to 1 µg/mL. Add 100 µL to each well.

202

203     5.10    Cover the plate with a microplate foil seal and incubate on a heated plate shaker at 37 °C
204     for 15 min with shaking at 600 rpm.

205

206     5.11    Remove the plate from the plate shaker and place onto the magnetic plate separator for
207     2 min to separate the beads from the reaction mixture.

208

209     5.12    While retaining the plate on the magnet, remove the foil seal, carefully invert the plate
210     over a waste container, and gently flick the supernatant out of the wells.

211

212     5.13    Blot the plate on absorbent paper while still holding the plate on the magnet.

213

214     5.14    Wash the reaction wells as described below (first post detection antibody incubation
215     wash).

216

217     5.14.1  Remove the plate from the plate magnet and add 150 µL of PBS-TBN to each well.

218

219     5.14.2  Cover the plate with a fresh foil seal and shake at 37 °C for 2 min at 600 rpm.

220

221 5.14.3  Remove the plate from the plate shaker and place onto the plate magnet for 2 min to
222 separate the beads from the reaction mixture.
223
224 5.14.4  While retaining the plate on the magnet, remove the foil seal, carefully invert the plate
225 over a waste container, and gently flick the supernatant out of the wells.
226
227 5.14.5  Blot the plate on absorbent paper while still holding the plate on the magnet.
228
229 5.15    Wash the reaction wells as described below (second post-detection antibody incubation
230 wash).
231
232 5.15.1  Remove the plate from the plate magnet and add 150 µL of PBS-TBN to each well.
233
234 5.15.2  Cover the plate with a fresh foil seal and shake at 37 °C for 2 min at 600 rpm.
235
236 5.15.3  Remove the plate from the plate shaker and place onto the magnetic plate separator for
237 2 minutes to separate the beads from the reaction mixture.
238
239 5.15.4  While retaining the plate on the magnet, remove the foil seal, carefully invert the plate
240 over a waste container, and gently flick the supernatant out of the wells.
241
242 5.15.5  Remove the plate from the magnet.
243
244 5.16    Add 100 µL of PBS-TBN to each well.
245
246 5.17    Cover with foil seal and shake for 2 min at 37 °C.
247
248 5.18    Remove the plate from the plate shaker. Then remove the foil seal and read 50 µL of each
249 well on the flow analyzer according to the User Manual. A brief description is provided below.
250
251 5.18.1  Select the drop-down menu in the upper left corner of the screen and navigate to **Plate
252 Configuration**.
253
254 5.18.2  Select **Run Plate**.
255
256 5.18.3  Select the **Eject** icon to eject the plate carrier.
257
258 5.18.4  Load the plate onto the plate carrier and select the **Retract** icon to retract the plate
259 carrier.
260
261 5.18.5  Select the **Run** icon to run the plate.
262
263 **6.      Single reporter neutralization assay protocol**
264

265    6.1    Prepare a fresh multiplex bead mix as described in section 3 above.
266
267    6.2    Dilute patient and control sera 1:500 as follows.
268
269    6.2.1  Dilute serum 1:100 by mixing 10 µL of serum into 990 µL of PBS-TBN.
270
271    6.2.2  Dilute serum a further 5-fold by adding 40 µL of 1:100 serum into 160 µL of PBS-TBN.
272
273    6.3    Add 50 µL of multiplex bead mix to each assigned well of a 96-well non-binding microtiter
274    plate.
275
276    6.4    Add 25 µL of 2 µg/mL ACE2 dilution to each well.
277
278    6.5    Cover the plate with a microplate foil seal and incubate on a heated plate shaker at 37 °C
279    for 2 min with shaking at 600 rpm.
280
281    6.6    Remove the plate from the plate shaker and remove the foil seal.
282
283    6.7    Add 25 µL of 1:500 serum or PBS-TBN to the assigned wells.
284
285    6.8    Cover the plate with a microplate foil seal and incubate on a heated plate shaker at 37 °C
286    for 15 min with shaking at 600 rpm.
287
288    6.9    For the remainder of the assay procedure, follow steps 5.4 to 5.18.5 as described above.
289
290    **7.     Conversion to a dual reporter IgG and IgM serological assay**
291
292    7.1    Prepare antigen-coupled beads as described in Section 2 above.
293
294    NOTE: An additional bead set, coupled with human IgM, is included to monitor for the addition
295    of anti-IgM detection reagents, as well as an additional AC bead set (220) to monitor collection
296    of MFI for RP2. All bead stocks are at 1 x $10^6$ beads/mL for inclusion in multiplex bead mixes as
297    described below.
298
299    7.2    Prepare fresh multiplex bead mixes as described in Section 3 above.
300
301    7.3    Dilute serum samples and IgG-stripped negative control sera are diluted 1:1000 in Section
302    4 above.
303
304    7.4    Add 50 µL of multiplex bead mix to each assigned well of a 96-well non-binding microtiter
305    plate.
306
307    7.5    Pipette 50 µL of 1:1000 serum or PBS-TBN to the appropriate wells. The final dilution of
308    serum in the well is 1:2000.

309

7.6     Cover the plate with a microplate foil seal and incubate on a heated plate shaker at 37 °C for 15 min with shaking at 600 rpm.

7.7     Remove the plate from the plate shaker and place onto the magnetic plate separator for 2 min to separate the beads from the reaction mixture.

7.8     While retaining the plate on the magnet, remove the foil seal, carefully invert the plate over a waste container, and gently flick the supernatant out of the wells.

7.9     Blot the plate on absorbent paper while still holding the plate on the magnet.

7.10    Wash the reaction wells as described below (first post-sample incubation wash).

7.10.1  Remove the plate from the plate magnet and add 150 μL of PBS-TBN to each well.

7.10.2  Cover the plate with a fresh foil seal and shake at 37 °C for 2 min at 600 rpm.

7.10.3  Remove the plate from the plate shaker and place onto the plate magnet for 2 min to separate the beads from the reaction mixture.

7.10.4  While retaining the plate on the magnet, remove the foil seal, carefully invert the plate over a waste container, and gently flick the supernatant out of the wells.

7.10.5  Blot the plate on absorbent paper while still holding the plate on the magnet.

7.11    Wash the reaction wells again with PBS-TBN (second post-sample incubation wash), exactly as described above in steps 7.10.1–7.10.5.

7.12    Remove the plate from the magnet.

7.13    Make a fresh Detection Reagent mix with the required combination of reagents and add 50 μL or 100 μL to each well.

NOTE: Detection Reagent mixes containing the anti-Human IgG conjugated to the Brilliant Violet 421 reporter dye (BV; excitation at 405 nm and emission at 421 nm) were used at 50 μL/well due to limiting amounts of the stock reagents available. All other Detection Reagent mixes are used at 100 μL/well.

7.14    Cover the plate with a microplate foil seal and incubate on a heated plate shaker at 37 °C for 15 min with shaking at 600 rpm.

7.15    Remove the plate from the plate shaker and place onto the magnetic plate separator for 2 min to separate the beads from the reaction mixture.

353

354 7.16    While retaining the plate on the magnet, remove the foil seal, carefully invert the plate
355 over a waste container, and gently flick the supernatant out of the wells.

356

357 7.17    Blot the plate on absorbent paper while still holding the plate on the magnet.

358

359 7.18    Wash the reaction wells as described below with PBS-TBN (first wash after detection
360 antibody incubation).

361

362 7.18.1  Remove the plate from the plate magnet and add 150 µL of PBS-TBN to each well.

363

364 7.18.2  Cover the plate with a fresh foil seal and shake at 37 °C for 2 min at 600 rpm.

365

366 7.18.3  Remove the plate from the plate shaker and place onto the magnetic plate separator for
367 2 min to separate the beads from the reaction mixture.

368

369 7.18.4  While retaining the plate on the magnet, remove the foil seal, carefully invert the plate
370 over a waste container, and gently flick the supernatant out of the wells.

371

372 7.18.5  Blot the plate on absorbent paper while still holding the plate on the magnet.

373

374 7.19    Wash the reaction wells again with PBS-TBN (second wash after detection antibody
375 incubation), exactly as described above in steps 7.17.1–7.17.5.

376

377 7.20    Remove the plate from the magnet.

378

379 7.21    Add 100 µL of PBS-TBN to each well.

380

381 7.22    Cover with foil seal and shake for 2 min at 37 °C.

382

383 7.23    Remove the plate from the plate shaker. Then remove the foil seal and read 50 µL of each
384 well on the flow analyzer as described above in steps 5.18.1–5.18-5.

385

386 **8.      Conversion to dual reporter neutralization assay**

387

388 8.1     Use the coupled beads as described in step 7.1 above.

389

390 8.2     Prepare a fresh multiplex bead mix as described in step 7.2.

391

392 8.3     Serum samples and IgG-stripped negative control sera are diluted 1:500 as follows.

393

394 8.3.1   Prepare a 1:100 dilution by mixing 10 µL of serum into 990 µL of PBS-TBN.

395

396 8.3.2   Dilute the 1:100 samples a further 5-fold by adding 40 µL of the 1:100 dilutions to 160 µL

397     of PBS-TBN.

398

399     8.4      Add 50 µL of multiplex bead mix to each assigned well of a 96-well non-binding microtiter
400     plate.

401

402     8.5      Add 25 µL of 2 µg/mL ACE2 dilution to each well.

403

404     8.6      Cover the plate with a microplate foil seal and incubate on a heated plate shaker at 37 °C
405     for 2 min with shaking at 600 rpm.

406

407     8.7      Remove the plate from the plate shaker and remove the foil seal.

408

409     8.8      Add 25 µL of 1:500 serum or PBS-TBN to the assigned wells.

410

411     8.9      Cover the plate with a foil seal and incubate on a heated plate shaker at 37 °C for 15 min
412     with shaking at 600 rpm.

413

414     8.10    Remove the plate from the plate shaker and place onto the magnetic plate separator for
415     2 min to separate the beads from the reaction mixture.

416

417     8.11    With the plate retained on the magnet, remove the foil seal, carefully invert the plate
418     over a waste container, and gently flick the supernatant out of the wells.

419

420     8.12    Blot the plate on absorbent paper while still holding the plate on the magnet.

421

422     8.13    Wash the reaction wells as described below (first post sample incubation wash).

423

424     8.13.1  Remove the plate from the plate magnet and add 150 µL of PBS-TBN to each well.

425

426     8.13.2  Cover the plate with a fresh foil seal and shake at 37 °C for 2 min at 600 rpm.

427

428     8.13.3  Remove the plate from the plate shaker and place onto the magnetic plate separator for
429     2 min to separate the beads from the reaction mixture.

430

431     8.13.4  With the plate retained on the magnet, remove the foil seal, carefully invert the plate
432     over a waste container, and gently flick the supernatant out of the wells.

433

434     8.13.5  Blot the plate on absorbent paper while still holding the plate on the magnet.

435

436     8.14    Wash the reaction wells again with PBS-TBN (second post-sample incubation wash),
437     exactly as described above in steps 8.13.1–8.13.5.

438

439     8.15    Remove the plate from the magnet.

440

441    8.16    Make a fresh Detection Reagent mix with the required combination of reagents and add
442    50 µL or 100 µL to each well.

443

444    NOTE: Detection Reagent mixes containing the anti-Human IgG conjugated to the BV reporter
445    dye were used at 50 µL/well due to limiting amounts of the stock reagents available (see **Table**
446    **of Materials**). All other Detection Reagent mixes are used at 100 µL/well.

447

448    8.17    Cover the plate with a microplate foil seal and incubate on a heated plate shaker at 37 °C
449    for 15 min with shaking at 600 rpm.

450

451    8.18    Remove the plate from the plate shaker and place onto the magnetic plate separator for
452    2 min to separate the beads from the reaction mixture.

453

454    8.19    With the plate retained on the magnet, remove the foil seal, carefully invert the plate
455    over a waste container, and gently flick the supernatant out of the wells.

456

457    8.20    Blot the plate on absorbent paper while still holding the plate on the magnet.

458

459    8.21    Wash the reaction wells as described below (first was after detection antibody
460    incubation).

461

462    8.21.1  Remove the plate from the plate magnet and add 150 µL of PBS-TBN to each well.

463

464    8.21.2  Cover the plate with a fresh foil seal and shake at 37 °C for 2 min at 600 rpm.

465

466    8.21.3  Remove the plate from the plate shaker and place onto the magnetic plate separator for
467    2 min to separate the beads from the reaction mixture.

468

469    8.21.4  With the plate retained on the magnet, remove the foil seal, carefully invert the plate
470    over a waste container, and gently flick the supernatant out of the wells.

471

472    8.21.5  Blot the plate on absorbent paper while still holding the plate on the magnet.

473

474    8.22    Wash the reaction wells again with PBS-TBN (second wash after detection antibody
475    incubation), exactly as detailed above in steps 8.21.1–8.21.5.

476

477    8.22.1  Remove the plate from the plate magnet and add 150 µL of PBS-TBN to each well.

478

479    8.22.2  Cover the plate with a fresh foil seal and shake at 37 °C for 2 min at 600 rpm.

480

481    8.22.3  Remove the plate from the plate shaker and place onto the magnetic plate separator for
482    2 minutes to separate the beads from the reaction mixture.

483

484    8.22.4  With the plate retained on the magnet, remove the foil seal, carefully invert the plate

485    over a waste container, and gently flick the supernatant out of the wells.

486

487    8.22.5  Remove the plate from the magnet.

488

489    8.23    Add 100 μL of PBS-TBN to each well.

490

491    8.24    Cover with foil seal and shake for 2 min at 37 °C.

492

493    8.25    Remove the plate from the plate shaker. Then remove the foil seal and read 50 μL of each
494    well on the flow analyzer as described above in steps 5.18.1–5.18-5.

495

496    **REPRESENTATIVE RESULTS:**

497

498    **Optimization of the Single Reporter Serological Assay.**
499    The coupling strategy for all SARS-CoV-2 antigens is described in Cameron, et al., 2020[1] and in
500    step 2.1 above. The strategy for coupling confirmation is described in step 2.3 above. For an
501    efficient coupling, the median fluorescence intensity (MFI) values should be significantly higher
502    than the no serum/no antibody negative control reaction(s) and should demonstrate a dose
503    response of decreasing MFI signal with decreasing amounts of detection reagent. MFIs of
504    approximately 1,000 MFI units or greater over background MFI generally indicate efficient
505    protein coupling and are dependent on the concentration and specificity of the detection reagent
506    used. Using this method, confirmation of S, RBD and Nc antigen coupling were tested for two lots
507    of beads (**Figure 2**). The MFI signals for all antigens were significantly higher than the background
508    MFI and showed a dose response with titration of the detection reagents. The data also showed
509    good reproducibility of MFI levels between the two different lots of coupled beads.

510

511    Optimization of the single reporter assay was done as described in Cameron, et al., 2020[1]. Each
512    antigen was tested at different coupling concentrations with different dilutions of several serum
513    samples representing high, medium, low, and negative samples. In addition, because the assay
514    measures IgG titers, an IgG-stripped serum was used as an additional negative sample. Results of
515    a representative sample dilution series with different antigen coupling levels are shown in **Figure
516    3**. This initial dilution series was tested with a fixed concentration of detection reagents to get a
517    potential range of representative antigen-specific and background MFI levels. From these data,
518    additional sample dilution ranges and antigen coupling levels were determined and tested
519    further with additional samples (data not shown).

520

521    Next, the concentration of the detection reagent was optimized for use with the different serum
522    dilutions and antigen coupling levels. Representative data using 6 positive and 2 negative samples
523    are shown in **Figure 4**. Following additional testing with several hundred negative pre-COVID-19
524    serum samples, the final optimal antigen coupling levels and detection regent concentrations
525    were selected for the final assay protocol, as described in section 5.

526

527    **Conversion to a single reporter neutralization assay**
528    Conversion of the single reporter serological assay to a neutralization assay was achieved by

529  adding an incubation step using ACE2 (as a competing reagent) with the beads prior to adding
530  the serum sample. By titrating the amount of ACE2 added, inhibition of IgG binding to the S and
531  RBD antigens was observed, as shown in **Figure 5**. While the 11 patient sera had different titers
532  of IgG, each showed a significant decrease in MFI signal with increasing ACE2 concentration. As
533  expected, ACE2 only impacts the MFI signals of S and RBD, but not the Nc antigen.
534
535  The percent residual MFI signal relative to 0 µg/mL ACE2 for all samples is shown in **Figure 6**. For
536  most samples, a signal loss >30% was observed for S and RBD with increasing ACE2 concentration.
537  As expected, there was no effect of ACE2 on the Nc signal.
538
539  **Conversion to a dual reporter IgG and IgM serological assay**
540  For the dual reporter IgG and IgM assay, the standard single reporter assay conditions were used
541  to test different antibody and reporter combinations for the two reporter channels. The RP1
542  channel is similar to previous flow analysis instruments with detection of "orange" fluorescence
543  for reporter dyes such as PE. The second channel, RP2, employs a violet excitation laser that can
544  be used for detecting the "blue" fluorescence of dyes excited at 402 nm with emission spectra
545  peaks in the 421–441 nm range.
546
547  Several different anti-human IgG and IgM antibody combinations were tested at various
548  concentrations with the standard 2,000-fold sample dilution and incubation conditions detailed
549  in section 6. An initial test of anti-IgM conjugated to the DyLight 405 reporter dye (DL 405;
550  excitation at 400 nm and emission at 420 nm) for the RP2 channel was performed using a set of
551  11 PCR-positive samples with DFSO ranging from 5 to >60 days, and an IgG-stripped serum
552  sample. This detection reagent did not produce a high signal in the RP2 channel as shown in
553  **Figure 7A,B,C**. For samples with the high IgM RP2 MFI, the highest signals were seen for RBD and
554  Nc (**Figure 7B,C**). While IgM titers should be elevated at this time in some samples, the observed
555  MFI levels did not exceed 140 MFI units with the IgM detection reagent. Furthermore, the control
556  bead for IgM lacked a significant dynamic range for MFI when using DL 405 conjugated to anti-
557  IgM compared to a PE-labeled anti-IgM RP1 reporter used at the same concentrations (**Figure
558  7D**).
559
560  Because a suitable RP2 reporter-labeled detection reagent for IgM was not available, the original
561  biotin anti-IgG with SASB was tested for use in the RP2 channel. The signal for IgG in the RP2
562  channel with SASB did not have the same dynamic range as with SAPE, but it still had a suitable
563  range for measuring IgG titers for S, RBD, and Nc (**Figure 8**). These data led to testing different
564  RP1 IgM and RP2 IgG detection reagent combinations, as described below.
565
566  **Conversion to dual reporter neutralization assay**
567  The dual reporter serological assay was converted to a neutralization assay by adding an
568  incubation step with ACE2 and beads, prior to adding the serum sample. A 2-fold dilution series
569  of ACE2 starting at 16 µg/ml was used, and then tested with sets of 11 samples and stripped sera
570  as described above. In addition, 3 different combinations of RP1 IgM and RP2 IgG detection
571  antibody mixes were tested on a set of 11 samples and stripped serum controls. The final
572  combinations and reagent concentrations determined to be optimal are detailed in **Table 1**.

573
574  For IgG detection, the biotin anti-human IgG/SASB used in the original single reporter assay was
575  tested along with another anti-IgG conjugated to the BV reporter dye. While the BV conjugate
576  had high RP2 MFI signals, the MFI signal intensity for IgG titer varied across the ACE2 titrations
577  (**Figure 9A,C,E**). The biotin anti-human IgG/SASB combination had more consistent MFI signal
578  decrease across the ACE2 titration compared to the BV conjugate, demonstrating a dose
579  response, although the MFI levels were lower. The signal fluctuation by the BV conjugate across
580  ACE2 concentrations also interfered with determining the percent inhibition by ACE2 across the
581  range of IgG titers compared to the biotin anti-human IgG/SASB combination (**Figure 9B,D,F**).
582  Samples with low MFI signals, such as the DFSO 3 sample, do not have high enough titers to
583  evaluate the performance of these reagents or measure IgG neutralizing capacity.
584
585  For determining IgM titers in the RP1 channel, a PE-conjugated anti-IgM was compared to an
586  anti-human IgM conjugated to the DyLight 549 reporter dye (DL 549; excitation at 562 nm and
587  emission at 576 nm) at the concentrations listed in **Table 1**. Of these two reagents, the PE anti-
588  IgM displayed higher MFIs than those generated by DL 549 anti-human IgM for the samples
589  tested (**Figure 9A,C,E**). The IgM control bead measuring the addition of these reagents had
590  different average MFIs of ≈17,000 for the PE anti-IgM and 2,723 for the DL 549 conjugate. Even
591  with these differences, the impact of the various ACE2 concentrations on MFI was measurable
592  with the DL 549 conjugate. For determining ACE2 percent inhibition of IgM binding, there was a
593  slight but insignificant difference between the two IgM detection reagents (**Figure 9B,D,F**).
594
595  **FIGURE AND TABLE LEGENDS:**
596
597  **Table 1: List of RP1 and RP2 reporter dye combinations tested**. Several different RP1 and RP2
598  reporter dyes were evaluated for measuring IgG and IgM titers. The three combinations shown
599  above were tested at different RP1 detection modes and for optimization of the neutralization
600  assay. For RP2 combinations using SASB, concentrations for the biotinylated detection antibody
601  and SASB are shown. *Final Concentration represents the final concentration in the reaction well.
602
603  **Figure 1: Flowchart highlighting main assay protocol steps.**
604
605  **Figure 2: Confirmation of antigen coupling.** Antigens were coupled at 100, 10 and 1 pmol/$10^6$
606  beads. For S and RBD antigens, rabbit anti-S sera was used and detected with PE anti-rabbit IgG.
607  For Nc, a Protein G-purified rabbit polyclonal anti-Nc was used. The titration curves for beads
608  coupled with 10 pmol S (**A**), 100 pmol RBD (**B**) and 100 pmol Nc (**C**) are shown.
609
610  **Figure 3: Optimization of serum dilution with different antigen coupling levels.** Serum samples
611  representing high, medium, low, and negative samples were tested with the different pmol
612  antigen/$10^6$ bead couplings. A 4-fold serum titration was tested with a multiplex mix of
613  antigen-coupled beads using the assay protocol described in section 5. The MFI values of the
614  titration curves for S (**A**), RBD (**B**), and Nc (**C**) are shown.
615
616  **Figure 4: Determination of optimal serum dilution and detection reagent concentration.** Eight

617  serum samples, including negative patients (2 samples) and low- to high-positive (6 samples),
618  were tested at additional serum dilutions with three different detection reagent concentrations.
619  The MFI results are shown for S (**A**), RBD (**B**), and Nc (**C**). Based on these and other data (not
620  shown), a sample dilution of 1:2,000 and a biotin detection antibody concentration of 0.62 µg/mL
621  was selected.

623  **Figure 5: Optimization of single reporter neutralization assay.** Modification of the single
624  reporter serological assay to a neutralizing antibody detection assay was performed by adding an
625  incubation step with ACE2 as a competitor, as described in Section 6. A set of 11 samples, ranging
626  from low- to high-positive titer sera, and an IgG-striped serum sample were tested with a two-
627  fold dilution series of ACE2 starting at 4 µg/mL, using standard assay conditions. Across this range
628  of ACE2 concentrations, a decrease in MFI can be seen with increased ACE2 for S (**A**) and RBD (**B**),
629  but not for Nc (**C**).

631  **Figure 6: Percent inhibition of signal with ACE2.** The percent residual signals for the set of 11
632  samples (from **Figure 4**) are shown. For each sample, regardless of its IgG titer, a maximum
633  decrease of ≥30% MFI signal was achieved for S (**A**) and RBD (**B**) at the highest ACE2
634  concentration. For the Nc antigen (**C**) there was no significant inhibition of signal with any amount
635  of ACE2 tested.

637  **Figure 7: Test of DyLight 405 anti-IgM as RP2 IgM detection reagent.** A set of 11 samples and
638  IgG-stripped sera were used to test DL 405-conjugated anti-IgM as an RP2 detection reagent. The
639  RP2 IgM MFI signals for S (**A**), RBD (**B**), and Nc (**C**) are shown. The highest MFI signal for any
640  antigen with any sample was approximately 140 MFI units for RBD with sample H (**B**). Even the
641  signal on the IgM control bead set was less than 1,000 MFI in the RP2 channel across the whole
642  antibody titer range and did not show the increased dynamic range observed with the PE anti-
643  IgM detection antibody in the RP1 channel (**D**).

645  **Figure 8: Optimization of SASB as RP2 reporter with Biotin anti-IgG.** A set of 11 samples and IgG-
646  stripped sera were used to test a dilution series of SASB with the standard 0.62 µg/mL biotin anti-
647  IgG. The resulting IgG MFIs in the RP2 channel for S (**A**), RBD (**B**), and Nc (**C**) are shown.

649  **Figure 9: Comparison of three different combinations of RP1 IgM and RP1 IgG detection mixes**.
650  Three different detection antibody mixes were tested on 11 samples and IgG stripped sera. The
651  combinations and final concentrations used are described in **Table 1**. All samples were tested
652  with a 2-fold ACE2 dilutions, starting at 16 µg/mL. Data for samples at DFSO of 3, 12, and 30 days
653  are shown. The MFI signals for RP1 IgM (orange) and RP2 IgG (blue) are shown in **A** (DFSO 3), **C**
654  (DFSO 12), and **E** (DFSO 30). The ACE2 percent residual MFIs are shown in **B** (DFSO 3), **D** (DFSO
655  12), and **F** (DFSO 30). Signals representing a ≥30% decrease as compared to No ACE2 controls are
656  highlighted light green.

658  **DISCUSSION:**
659  This study expanded the functionality of a multiplex fluorescent microsphere assay, 3Flex, that
660  qualitatively assesses the IgG response to infection by SARS-CoV-2[1]. While many serological

assays for COVID-19 detect a single antigen, this assay simultaneously evaluates S, RBD, and Nc antigens, and includes an internal antibody isotype control[7]. In addition, ACE2 is used as an indicator to detect neutralizing antibody titers to SARS-CoV-2.

Multi-antigen assays may be particularly useful in the future for discriminating immune responses between infected and vaccinated persons. With SARS-CoV-2, if only S and RBD antibodies are evaluated, it would be impossible to discern whether this was due to a past infection or to a vaccination antibody response. None of the vaccines that are currently in use target the Nc antigen, so by evaluating S/RBD and Nc antigens, it is possible to distinguish past viral infection (Nc- and S/RBD-positive) from a vaccination response (S/RBD-positive only; Nc-negative).

In the current study, the 3Flex serological and neutralization assays (sections 5 and 6) were transferred onto a new dual reporter flow analysis instrument (sections 7 and 8). Typical immunoassay protocols for bead-based multiplex assays are similar to standard ELISA protocols, following comparable steps for sample incubation, detection antibody/reporter incubation, and analysis of results[8]. Previous studies have also demonstrated how standard ELISA assays can be transferred to a bead-based multiplexing platform[9].

The assay protocols could be seamlessly transferred from the predecessor single reporter instrument to the new dual reporter system, as shown by the results obtained for the test samples and controls (**Figure 4** and **Figure 5**). In the serological assay, all positive serum samples demonstrated a characteristic dose-responsive decrease in signal corresponding to both a higher sample dilution and a lower detection antibody concentration, whereas the signals for the negative samples remained at background levels. Similarly, for the neutralization assay, decreasing signals for S and RBD, but not Nc, corresponded to increasing concentrations of the ACE2 competitor, whereas the IgG stripped serum was much less affected by the addition of ACE2. Pre-incubating the beads with ACE2 for 2 min prior to addition of the serum sample (as described in sections 6 and 8), was also compared to combining the beads with ACE2 and serum at the same time and produced little difference in the results (data not shown). However, because the results obtained with the beads plus ACE2 pre-incubation step were more consistent, it was the final procedure adopted for the neutralization assay protocol (sections 6 and 8).

Because the dual reporter system incorporates a second fluorescent reporter channel, both assays were converted into isotyping assays to simultaneously measure both IgG and IgM responses. The dual reporter functionality of the flow analyzer allows collection of fluorescent signals from two reporter detection reagents for each analyte in the multiplex for each sample, effectively doubling the data generated per sample in an assay. For development and optimization of the dual reporter assay protocols, several commercially available reporter dyes and detection antibodies were evaluated for the new RP2 channel (**Figure 7** and **Figure 8**) to determine the best option(s) available. The anti-IgM antibody conjugated with the DL 405 reporter dye did not perform well in the RP2 channel (**Figure 7**), so biotin anti-IgG with SASB was subsequently evaluated for detection in the RP2 channel (**Figure 8**). Three combinations of RP1

705 and RP2 detection reagents were compared in the dual reporter neutralization assay (**Table 1**
706 and **Figure 9**) to determine the best performing combinations for simultaneous detection of IgG
707 and IgM neutralizing antibodies. While there were significant differences in the signal intensity
708 between the RP1 channel using PE-anti IgM and the RP2 channel using DL 549 anti-IgM, there
709 was no significant difference in the measurement of the percent binding inhibition by ACE2.

710
711 Several limitations to the current method and this study are acknowledged. First, the samples
712 tested and used in the method development and optimization were limited to sets of 8-11
713 samples. More samples will be tested in expanded studies to verify the method is fully optimized.
714 Second, a limited number of reporter fluorophores and reporter pairs were tested. Further
715 testing of all available reporters in all possible combinations will determine which reagents can
716 be used successfully in this method. The anti-IgG antibody conjugated to biotin was different
717 than the anti-IgG antibody conjugated to the BV 421 reporter. So, although variability in the signal
718 intensity for the BV 421 anti-IgG existed, it could be due to the specificity of the antibody and not
719 related to the reporter dye. Testing other available BV 421-conjugated antibodies may identify
720 another antibody that would perform well in the RP2 channel. Future studies will also evaluate
721 the combination of PE anti-IgM with BV 421 anti-human IgG for detection in RP1 and RP2,
722 respectively. Last, even with the dual reporter capability of the instrument, assays are limited to
723 two isotypes (two parameters) per reaction. If additional isotypes are to be measured or full
724 isotyping is desired, then additional reactions using the same two reporter channels would need
725 to be run in separate wells.

726
727 Bead-based multiplexing technology allows for rapid and flexible assay design with easy
728 implementation into routine laboratory workflows[3,4,10]. This study demonstrated the ease in
729 transferring the previously described 3Flex serological and neutralization assays for SARS-CoV-2
730 onto a flow analysis system for measuring IgG with a single reporter, or with slight modification,
731 simultaneously measuring both IgG and IgM responses using two reporters. The dual reporter
732 option has clear advantages over single reporter platforms because it can provide twice as much
733 data per sample, using half the sample volume, and in half the number of reaction wells. With
734 the appropriate reporter dyes and detection antibodies, isotyping assays can be easily developed
735 and performed on the dual reporter flow analysis instrument.

736

744
745 **REFERENCES:**
746
747 1.      Cameron, A. et al. A multiplex microsphere IgG assay for SARS-CoV-2 using ACE2-
748 mediated inhibition as a surrogate for neutralization. *Journal of Clinical Microbiology.* **59** (2),

749 e02489-20 (2020).

2. Waterboer, T. et al. Multiplex human papillomavirus serology based on in situ-purified glutathione s-transferase fusion proteins. *Clinical Chemistry.* **51** (10), 1845–1853 (2005).

3. Pickering, J. W. et al. A multiplexed fluorescent microsphere immunoassay for antibodies to pneumococcal capsular polysaccharides. *American Journal of Clinical Pathology.* **117** (4), 589–596 (2002).

4. Ayouba, A. et al. Development of a sensitive and specific serological assay based on Luminex technology for detection of antibodies to Zaire Ebola virus. *Journal of Clinical Microbiology.* **55** (1), 165–176 (2017).

5. Hornbeck, P., Fleisher, T.A., Papadopoulos, N.M. Isotype determination of antibodies. In: *Current Protocols in Immunology.* Vastl, J., ed. **116**, 2.2.1–2.2.7 (2017).

6. Angeloni, S. *Multiplex Bead Mixes Made Easy with an Excel-Based Bead Calculator*, <https://www.luminexcorp.com/blog/multiplex-bead-mixes-made-easy-with-an-excel-based-bead-calculator/> (2020).

7. FDA. *EUA Authorized Serology Test Performance*. <https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/eua-authorized-serology-test-performance> (2020).

8. Abcam. *Indirect ELISA protocol*. <https://www.abcam.com/ps/pdf/protocols/Indirect%20ELISA%20protocol.pdf> (2021).

9. Baker, H.N., Murphy, R., Lopez, E., Garcia, C. Conversion of a capture ELISA to a Luminex xMAP assay using a multiplex antibody screening method. *Journal of Visualized Experiments.* (65), e4084 (2012).

10. Pickering, J.W., Martins, T.B., Schroder, M. C., Hill, H. R. Comparison of a multiplex flow cytometric assay with enzyme-linked immunosorbent assay for auantitation of antibodies to tetanus, diphtheria, and Haemophilus influenzae Type b. *Clinical and Diagnostic Laboratory Immunology.* **9** (4), 872–876 (2002).
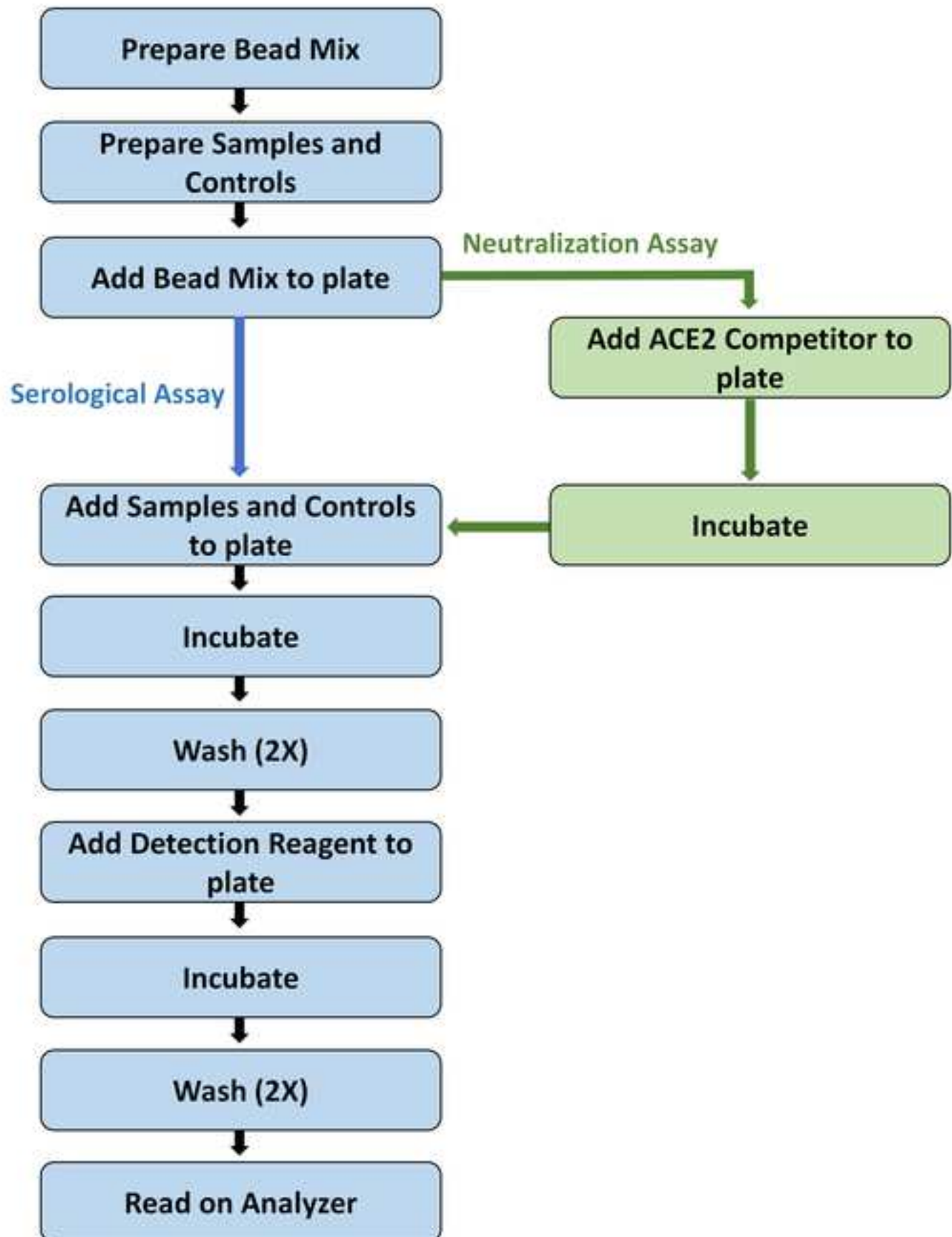
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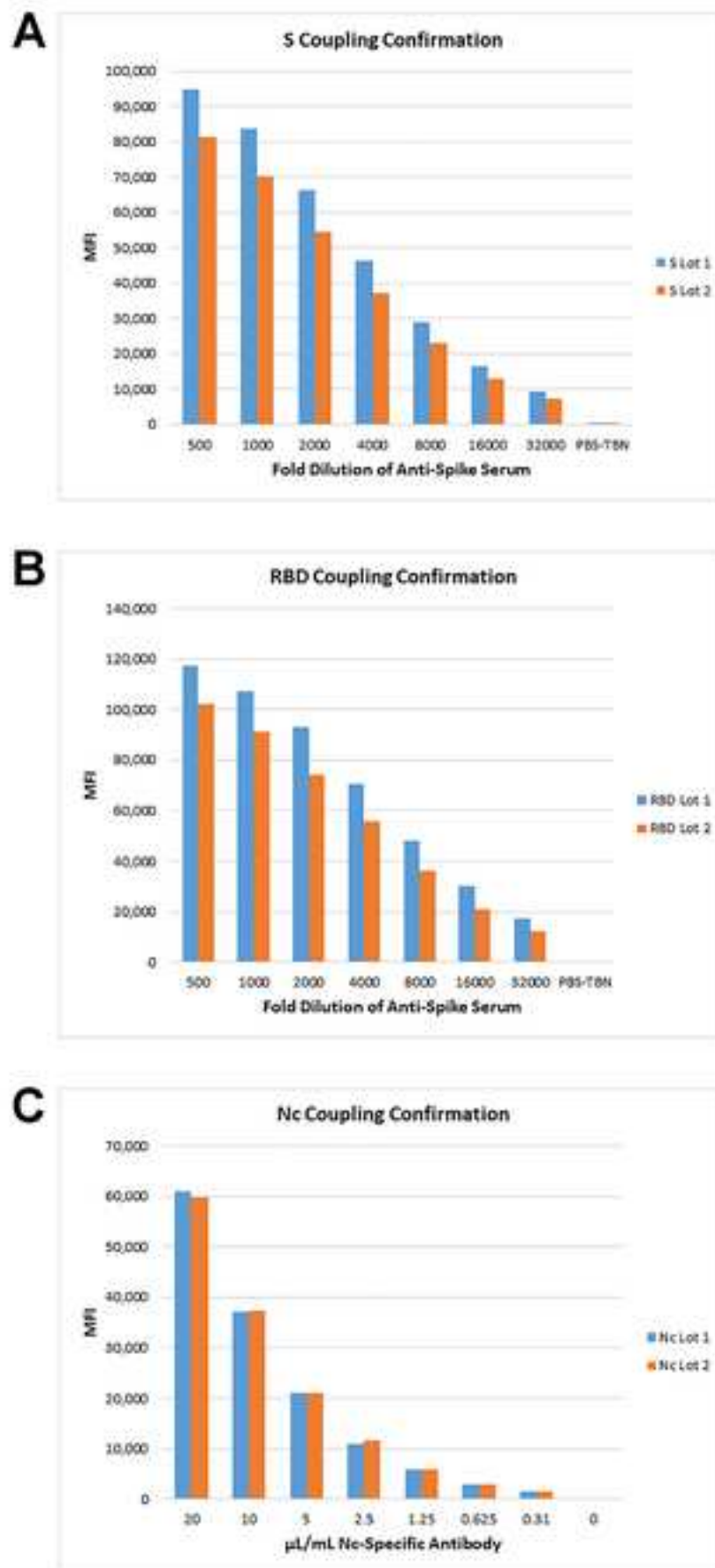
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Figure 2
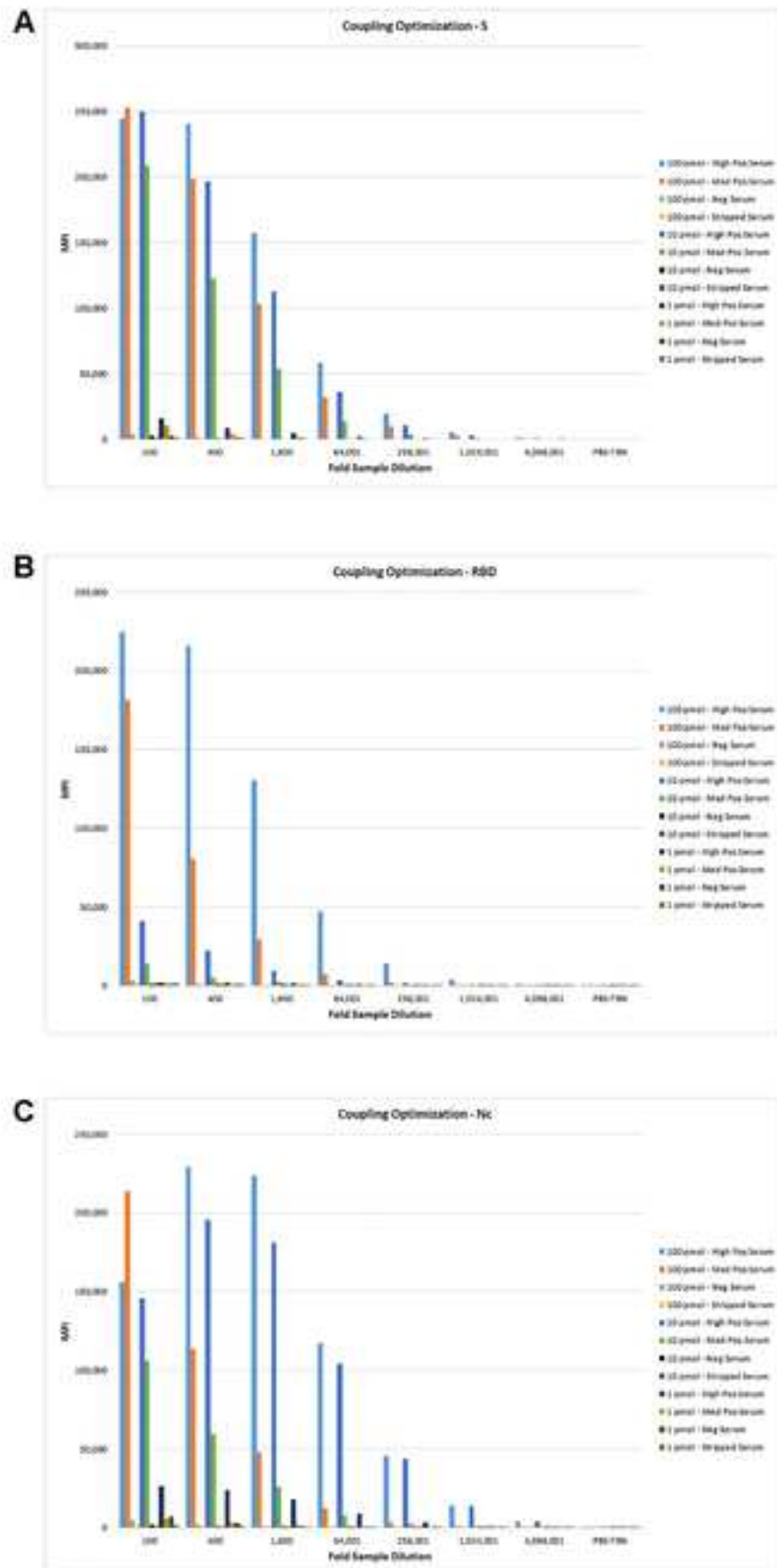
Click here to access/download;Figure;Angeloni Figure 2.jpg ⬇



A — S Coupling Confirmation
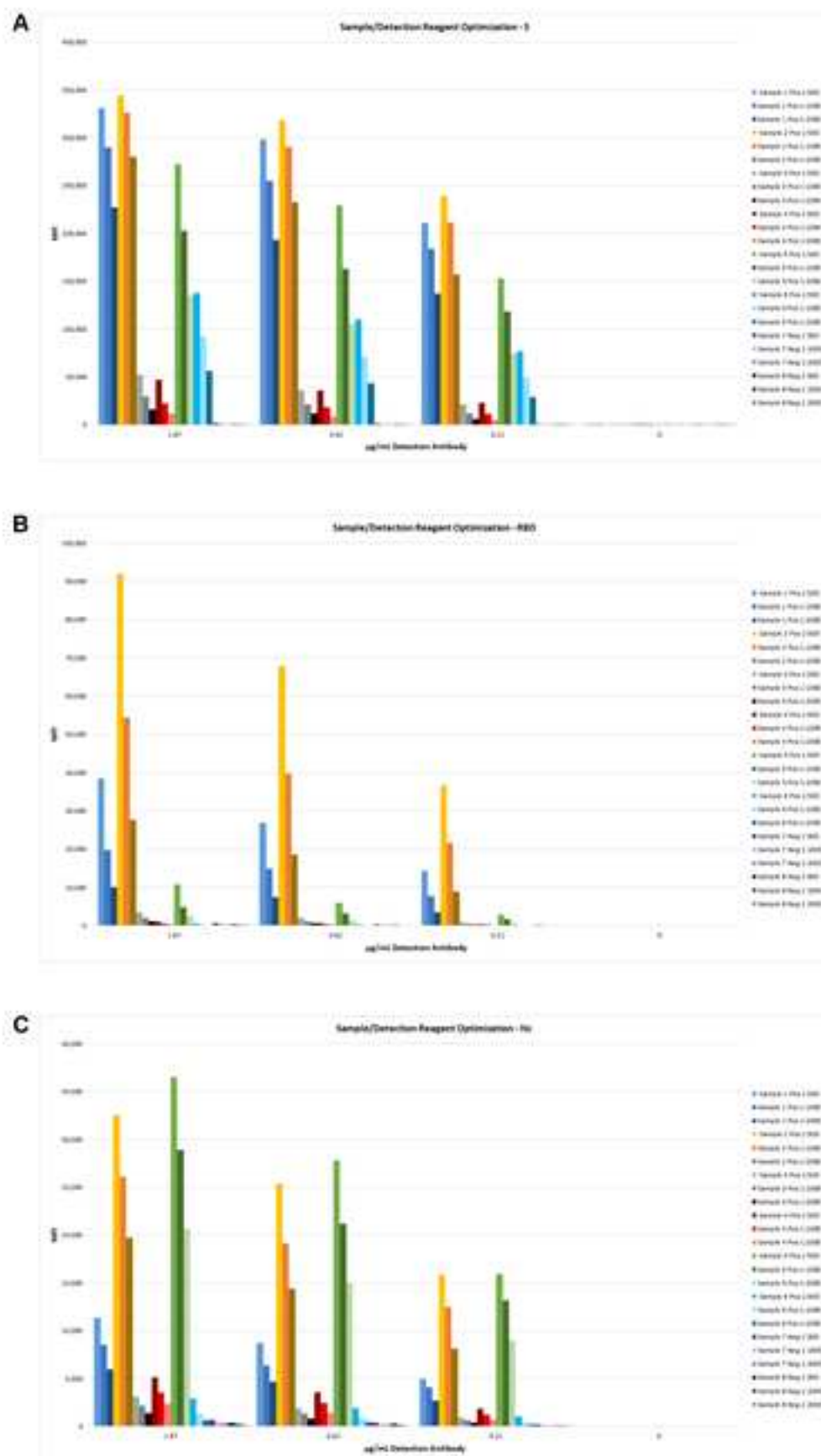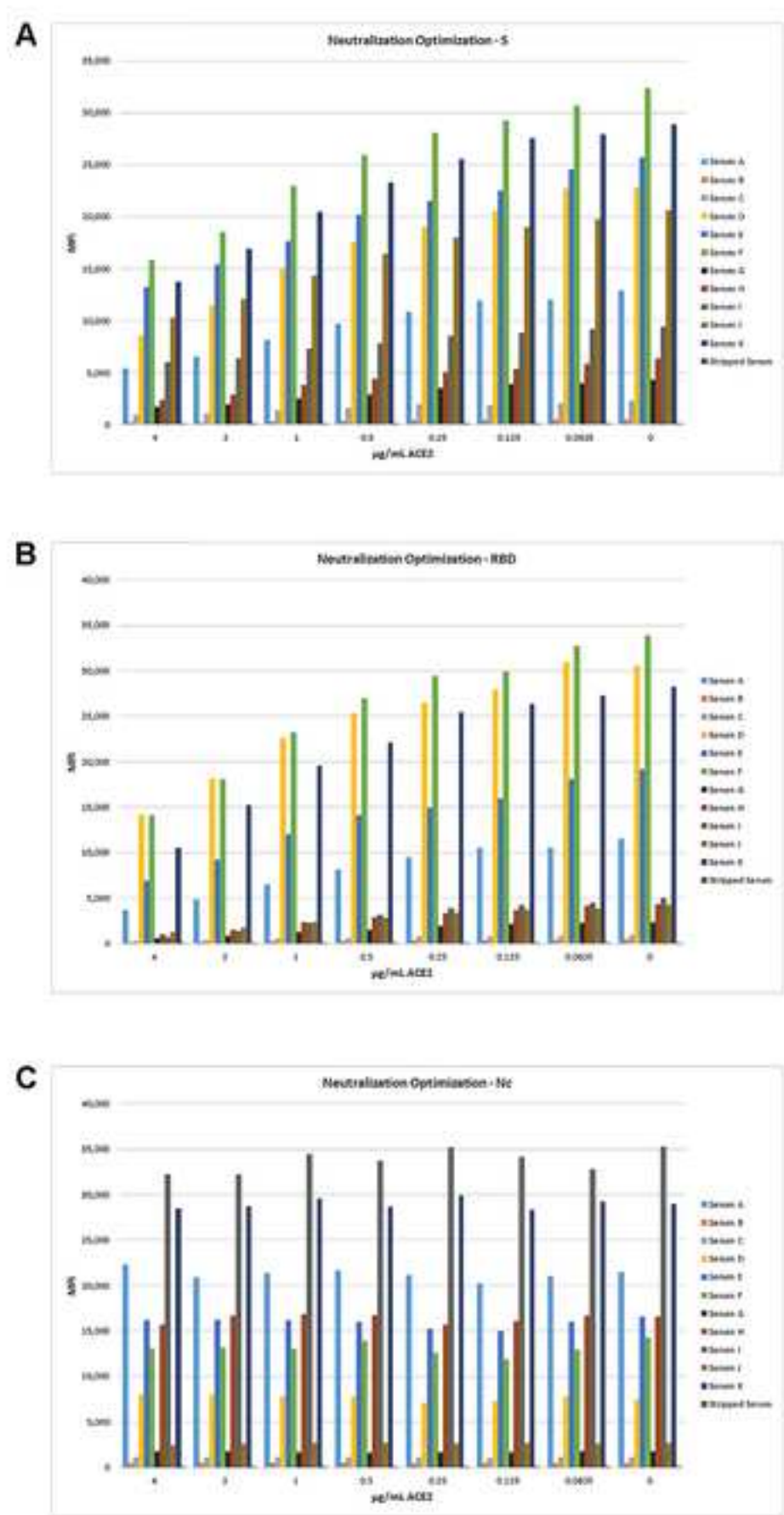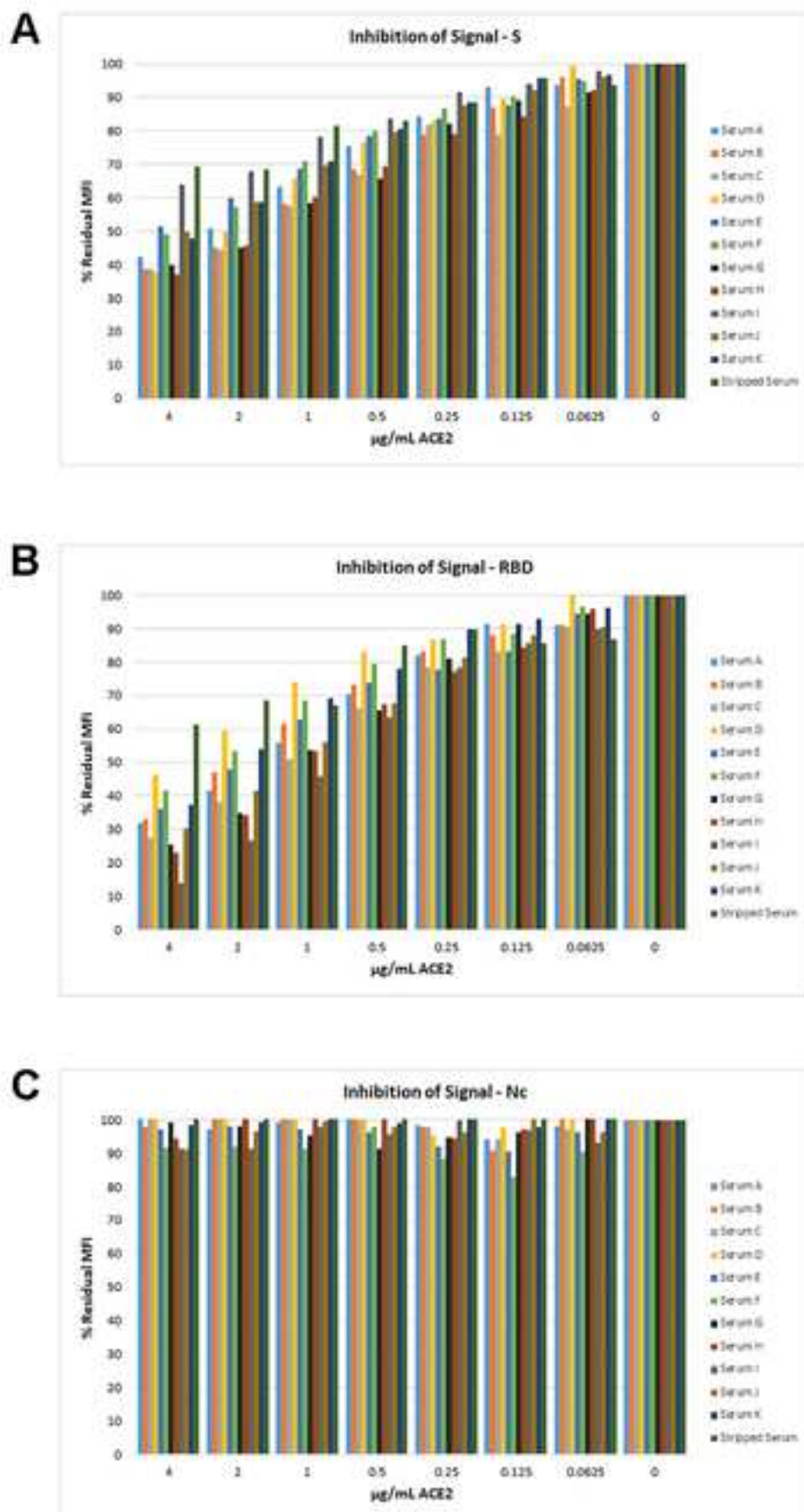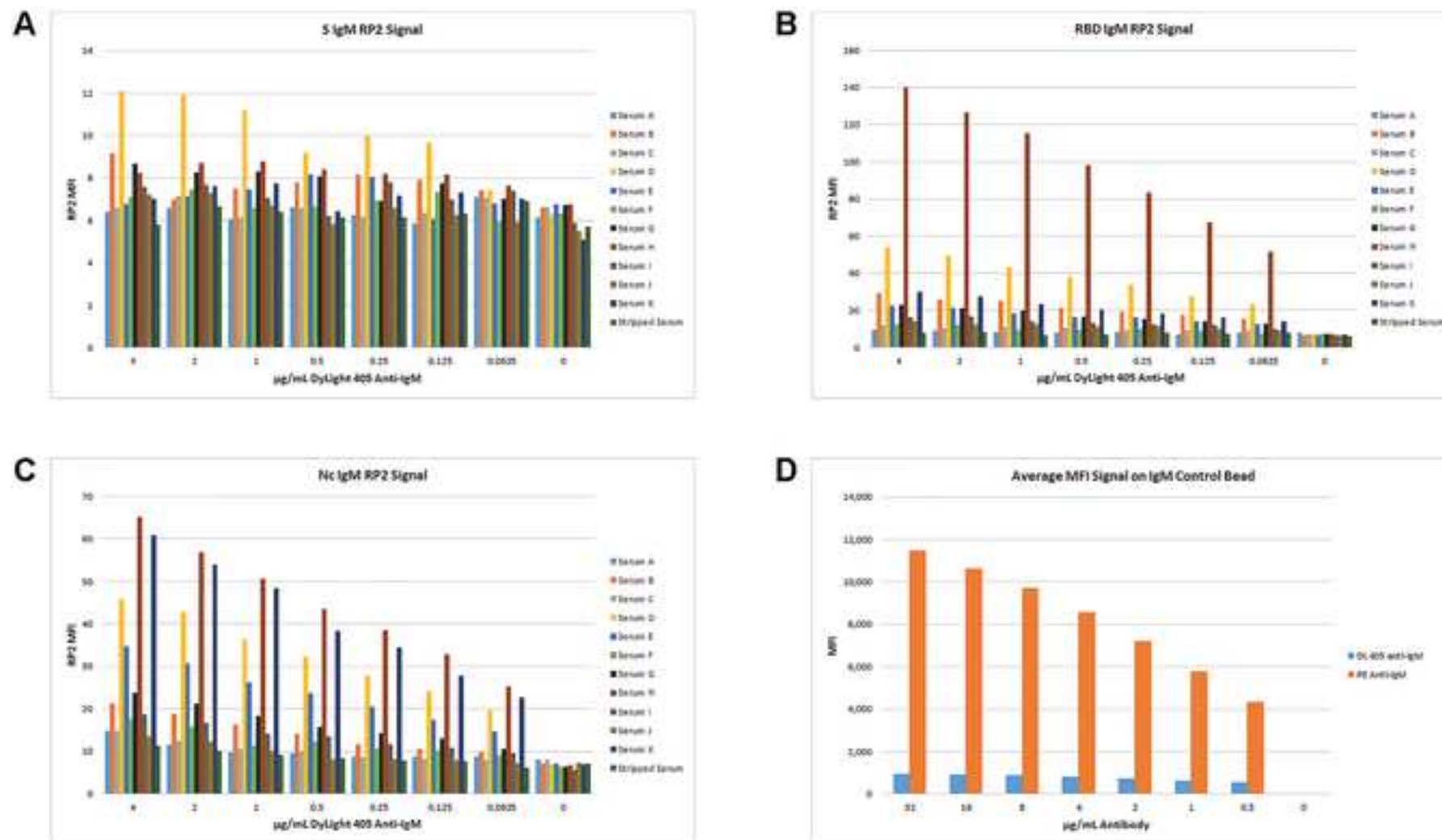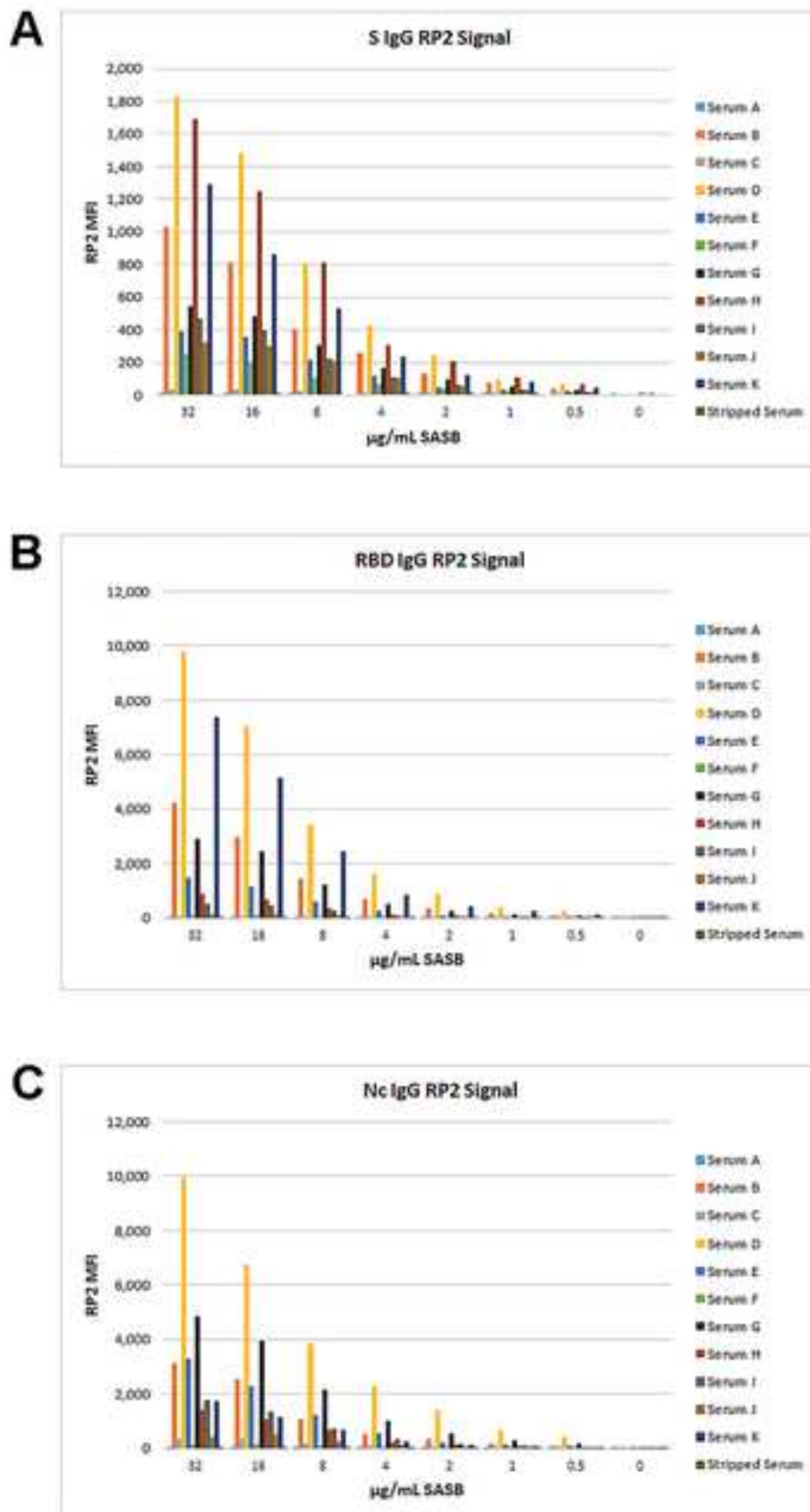
B — RBD Coupling Confirmation

C — Nc Coupling Confirmation

Figure 4
Click here to access/download;Figure;Angeloni Figure 4.jpg

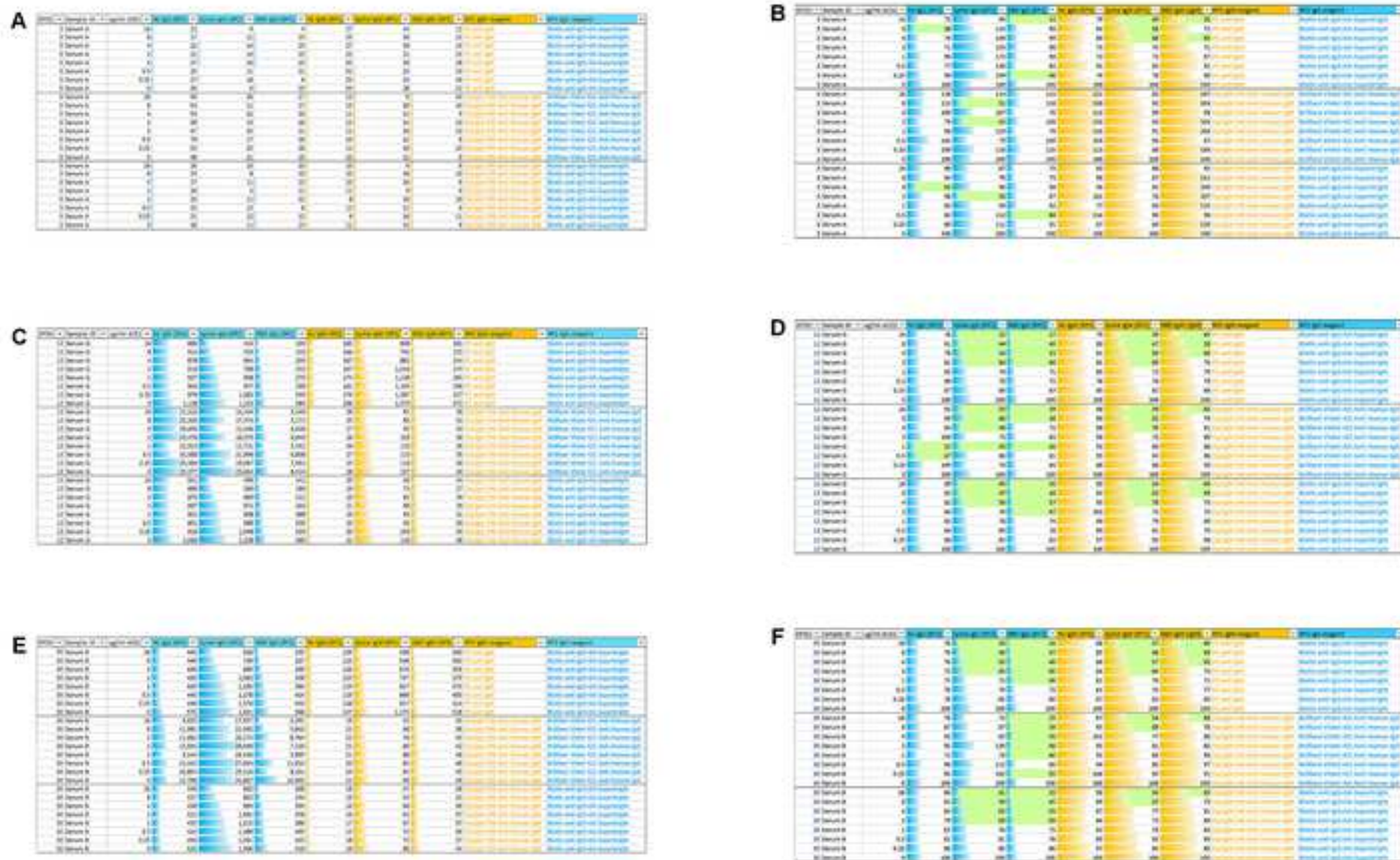Figure 5 Click here to access/download;Figure;Angeloni Figure 5.jpg ±

Figure 6

Figure 7

Figure 8 Click here to access/download;Figure;Angeloni Figure 8.jpg ⬇



A — S IgG RP2 Signal

B — RBD IgG RP2 Signal

C — Nc IgG RP2 Signal

Figure 9

Table 1

| Combination | Reporter |
|---|---|
| 1 | PE anti-IgM |
| | Biotin-Ig |
| | SASB |
| 2 | DyLight 549 Anti-Human IgM |
| | Brilliant Violet 421 Anti-Human IgG |
| 3 | DyLight 549 Anti-Human IgM |
| | Biotin-Ig |
| | SASB |

| Final Concentration In Well (µg/mL) | |
| --- | --- |
| RP1 | RP2 |
| 2 | - |
| - | 0.62 |
| - | 4 |
| 1 | - |
| - | 1 |
| 1 | - |
| - | 0.62 |
| - | 4 |

## Name of Material/ Equipment

ACE2 (human) (rec.)

Biotin-SP (long spacer) AffiniPure Goat Anti-Human IgG, Fcγ fragment specific

Bovine Serum Albumin

Branson 1800 Cleaner/water bath sonicator

Brilliant Violet 421 AffiniPure Donkey Anti-Human IgG (H+L)

Corning 96-well Black Flat Bottom Polystyrene NBS Microplate

Corning 96-well Microplate Aluminum Sealing Tape, Nonsterile

DyLight 405 AffiniPure Goat Anti-Human IgM, Fc5μ fragment specific

DyLight 549 AffiniPure F(ab')$_2$ Goat Anti-Human IgM Fc5μ specific

eBioscience Streptavidin Super Bright 436 Conjugate

Fisherbrand Incubating Microplate Shaker

Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, PE

Luminex Magnetic Plate Separator

MagPlex beads

PE AffiniPure F(ab')$_2$ Goat Anti-Human IgM Fc5μ specific

Phosphate Buffered Saline

SARS Nucleocapsid Protein Antibody

SARS Nucleocapsid Protein Antibody

SARS Spike Protein Antibody

SARS Spike Protein Antibody

SARS-CoV-2 (2019-nCoV) Spike S1 Antibody, Rabbit MAb
Sodium Azide
Streptavidin, R-Phycoerythrin Conjugate (SAPE)

Tube Revolver/ Rotator

TWEEN 20

xMAP Antibody Couplng Kit

xMAP INTELLIFLEX DR-SE

| Company | Catalog Number |
| --- | --- |
| AdipoGen Life Sciences | AG-40B-0192-3050 |
| Jackson ImmunoResearch Laboratories | 109-065-098 |
| MilliporeSigma | A7888 |
| Various | |
| Jackson ImmunoResearch Laboratories | 709-675-149 |
| Corning | 3650 |
| Corning | 6570 |
| Jackson ImmunoResearch Laboratories | 109-475-043 |
| Jackson ImmunoResearch Laboratories | 109-506-129 |
| ThermoFisher Scientific | 62-4317-82 |
| ThermoFisher Scientific | 02-217-757 |
| ThermoFisher Scientific | P-2771MP |
| Luminex Corporation | CN-0269-01 |
| Luminex Corporation | MC100XX-ID |
| Jackson ImmunoResearch Laboratories | 109-116-129 |
| MilliporeSigma | P3813 |
| Novos Biologicals | NB100-56683 |
| Novos Biologicals | NB100-56049 |

| | |
|---|---|
| Novos Biologicals | NB100-56047 |
| Novos Biologicals | NB100-56048 |
| SinoBiological | 40150-R007 |
| MilliporeSigma | S2002 |
| ThermoFisher Scientific | S21388 |
| ThermoFisher Scientific | 88881001 |
| MilliporeSigma | P9416 |
| Luminex Corporation | 40-50016 |
| Luminex Corporation | INTELLIFLEX-DRSE-RUO |

**Comments/Description**

Sigma-Aldrich

Discontinued but available from multiple vendors

Fisher Scientific

Sigma-Aldrich
Unconjugated, rabbit, polyclonal, Protein G purified
Unconjugated, rabbit, polyclonal, Protein A purified

Unconjugated, rabbit, polyclonal, Protein G purified
Unconjugated, rabbit, polyclonal, Protein G purified

Sigma-Aldrich
premium grade

Sigma-Aldrich

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March 16, 2021

Dear Dr. Bajaj,

We thank you, your editorial team, and the reviewers for your ongoing support in developing our manuscript, JoVE62487R1 "A Rapid, Multiplex Dual Reporter IgG and IgM SARS-CoV-2 Neutralization Assay for a Multiplexed Bead-based Flow Analysis System". We are confident that the changes resulting from your guidance have greatly enhanced the quality of our report.

We have strived to make all requested amendments to our paper, and outline all changes made below on a point-by-point basis, provided below. Our responses are shown in italic font immediately after each specific editorial comment. We have also provided two versions of our revised manuscript, one with changes showing/tracked and a second "Clean Copy" with changes accepted for easier reading.

Thank you again for your ongoing assistance.

Sincerely,

Sherry Dunbar, PhD

_____

**<span style="color:red">Editorial comments:</span>**
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

*1. We have thoroughly reviewed our manuscript and have repaired any spelling or grammatical inconsistencies that may have existed. We also have made certain that all abbreviations used in the report are defined at first mention.*


**2. Please make the title concise by deleting "Development of".**

*2. As requested, we have deleted "Development of" from the beginning of our paper's title.*

**3. Please provide an email address for each author.**

*3. Per editorial reminder and JoVE Author Instructions, we now include on the Title Page the email addresses for all coauthors.*

**4. Please revise the following lines to avoid overlap with previously published work: 32-34; 313-314; 343-344; 362-363; 378-379; 394-395; 712-714**

*4. We have reviewed and revised all editor-identified lines mentioned above to avoid overlap with previously published work to the best of our ability. The lines indicated at 313-395 are protocol steps but we have also condensed several sections of these protocol steps themselves, cited in the above pagination, to avoid unnecessary redundancy. This is discussed again in more detail in Editorial Comment #11, below.*

**5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript (text, figure legends, figures, tables) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.**
**For example: Streptavidin Super Bright 436 Conjugate (SASB); Tween®20; AC220; Brilliant Violet 421; DyLight 549 etc**

*5. We have deleted all trademark, registry and company names, and any commercial language from all sections of the manuscript. We have by necessity retained the commercial names for several of the fluorophores used, simply because there is no generic term available to adequately describe them without excessive verbiage—in these few instances, we were certain not to include company sources or any commercial language or symbols and we created abbreviations for these names for any subsequent mentions in the report.*

**6. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).**

*6. We have reworded all sentences in which personal pronouns had been used to eliminate their presence; these revisions are clearly shown in the tracked changes.*

**7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.**

*7. We have reviewed all manuscript text and have modified several sections and phrases to be more instructive and written in the imperative tense. We have occasional added explanatory*

*notes where descriptions are ancillary to protocol performance, but provide essential background information. We have not included any safety warnings because there are really no specific safety precautions needed beyond usual laboratory practices.*

**8. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.**

*8. We have rewritten the protocol steps so that they only contain "action items".*

**9. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.**

*9. We are confident that our protocol is now sufficiently detailed to allow easy and uncomplicated performance by any competent laboratory technician. There really are not many areas where settings and button clicks are used in the protocol, besides the final step of actually reading results on the flow cytometry unit. The User Manual is now referenced in these steps, and the key steps for loading and running the instrument are now briefly described. The start-up, calibration, programming, etc., for the instrument is conducted prior to running an assay and requires dozens of pages to describe in detail every step. Several chapters in the User Manual are devoted to these topics. The laboratory will also have their own preferred instrument settings, and procedures which may vary from lab-to-lab and run-to-run. Thus, we feel that detailed instructions for operating the instrument are beyond the scope of this report. Any technician performing this assay will already have received training and have experience with their instrument and will possess the equipment User Manual. We are certain that our protocol as provided is sufficient for assay reproduction that produces consistent and reliable results. We can also add the User Manual to the list of references if desired; however, it is not available for free download and is only provided with the instrument.*

**10. 2.3.1 (as an example): Please describe in detail how coupling was confirmed here or cite references that describe this so that readers know how to do this.**

*10. The procedures for coupling and coupling confirmation are described and cited in the first reference (Cameron, et al.). We have added a notation to coupling confirmation as a "Note" to this step, and have made certain that all other steps have sufficient explanatory detail. Coupling confirmation is a standard technique that is not unique to this protocol and is described in the cited prior work and its references.*

**11. Please make sure that you have highlighted only up to 3 pages of the protocol text including headings and spacings to indicate what is to be filmed.**

*11. We fully appreciate that our highlighted text was very close to the 3-page limit of what is to be filmed. We have condensed several steps throughout the protocol, including in the highlighted regions, to avoid redundancy. This has resulted in our highlighted steps for filming occupying less than the 3-page maximum allowance. Thank you for bringing this to our attention.*

**12. Please consider combining Tables 1 and 2 with the Table of Materials because they also contain information about materials used in the protocol.**

*12. As requested we have merged these sections, and appreciate that this recommended change greatly streamlines our materials presentation.*

**13. Please include an Acknowledgements section, containing any acknowledgments and all funding sources for this work**.

*13. We have now included an Acknowledgements section that provides funding information for this project, and also recognizes our enlistment of a professional medical writer to assist in making the editorial-requested changes to this report.*

**14. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (italics). Volume (bold) (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references. Please do not abbreviate journal names; use title case for all journal names.**

*14. We have revised our Bibliography to precisely conform with the formatting specifications provided by our Editor, and in line with published JoVE Author Instructions.*

*15. Miscellaneous modifications performed during revision.*

> *a. We have included a new Figure 1 Flowchart that highlights primary assay steps. This was a minor point suggested by Reviewer #2, which we were happy to accommodate.*

> *b. We have performed minor edits specified by JoVE Author Instructions such as italicizing all Latin abbreviations, abbreviating "minutes" to "min", etc.*

**Author Response to Reviewer Comments:**

*We thank the reviewers for their positive assessment of our report. Reviewer #1 had no modification request, neither minor nor major. Reviewer #2 made requested the inclusion of "summarized scheme of the whole protocol…", which we now do with the creation and addition of new Figure 1, a Flowchart that clearly delineates the major steps associated with our assay. We appreciate this critique and agree that it enhances reader/operator comprehension. We have also reviewed all other included Tables and Figures for easy readability, including seeking impartial outside assessments, and have identified no inordinate issues that require modification. All Figures are at 300 dpi and we have enlarged them to ensure that they are at 11 inches at their tallest or widest dimension.*

## Reviewers' Comments:
### Reviewer #1:
Manuscript Summary:
The manuscript "Development of a Rapid, Multiplex Dual Reporter IgG and IgM SARS-CoV-2 Neutralization Assay for a Multiplexed Bead-based Flow Analysis System" describe a flow analysis system for bead-based multiplexed assays which provides a two-reporter readout was used for the development of a multiplex antibody neutralization assay that can simultaneously measure neutralizing IgG and IgM antibodies for SARS-CoV-2. The author describe the limitations to the current method and the small number of samples.
The method described bead-based multiplexing technology can be useful for rapid and flexible assay design with easy implementation into routine laboratory workflows and can be useful tool to monitor the waves of infection.

Major Concerns:
none
Minor Concerns:
none

### Reviewer #2:
Manuscript Summary:
The authors have developed a multiplex bead-based neutralizing assay for SARS-CoV-2 antibodies against 3 antigens, using flow cytometry. Steps of optimization are described to show the relevance of this method.

Major Concerns:
There are not major concerns about this manuscrit, except the lack of comparison with an other neutralizing method, to confirm the results obtained. Thus, this manuscrit should be considered as a protocol development study and may be confirm latter in clinical situations.

Minor Concerns:
Figures are not very easy to read.
Authors should provide a summarized scheme of the whole protocole to improve the understanding of the method developed.