Humanization and Expression of IgG and IgM Antibodies in Plants as Potential Diagnostic Reagents for Valley Fever

Collin Jugler[1,2], Francisca J. Grill[2], Douglas F. Lake[1,2], Qiang Chen[1,2*]

[1] The Biodesign Institute, Arizona State University, Tempe, Arizona, USA

[2] School of Life Sciences, Arizona State University, Tempe, Arizona, USA

*Corresponding author

Qiang Chen, Ph.D. Arizona State University, Mail Zone 5401, 1001 S. McAllister Avenue, Tempe, AZ 85287. T: (480) 239-7802, F: (480) 727-7615.

Email: qiang.chen.4@asu.edu

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Abstract

Monoclonal antibodies (mAbs) are important proteins used in many life science applications, from diagnostics to therapeutics. High demand for mAbs for different applications urges the development of rapid and reliable recombinant production platforms. Plants provide a quick and inexpensive system for producing recombinant mAbs. Moreover, when paired with an established platform for mAb discovery, plants can easily be tailored to produce mAbs of different isotypes against the same target. Here, we demonstrate that a hybridoma-generated mouse mAb against chitinase 1 (CTS1), an antigen from *Coccidioides* spp., can be biologically engineered for use with serologic diagnostic test kits for coccidioidomycosis (Valley Fever) using plant expression. The original mouse IgG was modified and recombinantly produced in plants as IgG and IgM isotypes with human kappa, gamma, and mu constant regions. The two mAb isotypes produced in plants were shown to maintain target antigen recognition to CTS1 using similar reagents as the Food and Drug Administration (FDA)-approved Valley Fever diagnostic kits. As none of the currently approved kits provide antibody dilution controls, humanization of antibodies that bind to CTS1, a major component of the diagnostic antigen preparation, may provide a solution to the lack of consistently reactive antibody controls for Valley Fever diagnosis. Furthermore, our work provides a foundation for reproducible and consistent production of recombinant mAbs engineered to have a specific isotype for use in diagnostic assays.

32 ## Introduction

33 Antibodies are a leading class of biological molecules utilized in numerous research, diagnostic,
34 and treatment settings. Monoclonal antibodies (mAbs) have a strong research and development interest
35 for therapeutic purposes due to a highly profitable market, predicted to be upwards of $300 billion by
36 2025[1]. The market for antibodies as research reagents or diagnostic tools is estimated to reach levels of
37 $6.3 billion by 2027 and $42.56 billion by 2029, respectively[2,3]. Many antibodies used for these latter
38 purposes are polyclonal in nature, allowing for more economical production. However, polyclonal
39 antibodies may introduce a large degree of batch-to-batch variation, causing reproducibility issues[4,5].
40 These issues can be overcome by identifying sequences of mAbs for targets of interest, thereby allowing
41 recombinant production processes to replace mammalian serum collection as an antibody source.
42 Strong market projections, in parallel with the reproducibility crisis, urges innovation in both identifying
43 new mAbs, as well as improving production methods for sustainable, reliable sources for reagents.

44 Generating mAbs to targets of interest can be accomplished in several ways. Isolation of human
45 B cells, followed by antigen-specific sorting and single-cell variable region sequencing to identify high
46 affinity and specific antibodies is a high throughput method employed when convalescent individuals
47 are available[6]. However, this method may not be useful for discovering antibodies against antigens that
48 individuals may not been exposed to and thus, do not have B cells that have undergone proper affinity
49 maturation. Also, this method is not cost-effective, requiring cutting-edge technology for successful mAb
50 generation, limiting its accessibility. Phage display is another technology that has generated useful mAbs
51 for therapeutic purposes, although it also has limitations, including libraries not representing all
52 antibodies and potential mispairing of variable heavy and light regions, compared to *in vivo* generation
53 of antibodies[7,8]. The traditional and widely used method of hybridoma generation following mouse
54 immunization can generate highly pure, full length mAbs that can be continuously cultured for

55 production[9]. However, even hybridoma technology can suffer from instability of the fused cells and
56 there is constant risk of contamination, which can limit sustainable production of a consistent mAb.
57 Furthermore, traditional hybridoma-derived mAbs are of mouse origin, making them unsuitable for
58 human applications. This limitation also pertains to certain *in vitro* diagnostic applications. For example,
59 hybridoma-produced murine mAbs cannot be used as positive controls in commercial assays for
60 diagnosis of antibody responses to pathogens in human patients, as the secondary antibodies (usually
61 anti-human IgG or IgM) cannot recognize the control mAbs of mouse origin.

62 Recombinant protein expression in plants is an alternative to other expression systems that has
63 gained momentum in recent decades. MAbs against a diverse array of targets have been generated and
64 characterized, with some being produced under current Good Manufacturing Practices (cGMP) as proof-
65 of-principle that plants can be a viable alternative to mammalian-made biopharmaceuticals[10,11]. There
66 are several advantages of using plants as mAb production hosts. Plants do not require sterile growth
67 conditions or expensive growth media, making them a more cost-effective production method
68 compared to Chinese hamster ovary (CHO) or hybridoma-based methods, while also greatly reducing
69 the risk of contamination of mammalian viruses or other pathogens[12,13]. Once the variable gene
70 sequences of a mAb of interest are defined, simple cloning procedures followed by agroinfiltration[14,15]
71 allows the rapid generation of high levels of mAbs in plants. The flexibility of plant-based expression
72 systems has allowed the production of multiple mAbs and mAb variants for a diversity of applications,
73 including engineering the same Fab region to different antibody isotypes and subtypes, and mAbs for
74 diagnostic purposes[16–21].

75 Serological diagnostic tests are used to help identify many different diseases in patients. The
76 specificity of antibodies generated from the immune response against pathogens can be detected by
77 enzyme immunoassays (EIA) to inform the infection status. Coccidioidomycosis (Valley Fever) is a fungal
78 respiratory disease caused by *Coccidioides* spp[22]. The primary clinical diagnostic test for Valley Fever

79 detects the antibody response in patient serum to the fungi[23]. Specifically, patient IgG and IgM against
80 Valley Fever are detected in an EIA by reactivity to *Coccidioides* spp. antigen coated in individual wells on
81 a plate[24]. Clinical laboratories performing serology tests using Food and Drug Administration (FDA)-
82 approved kits for diagnosis of Valley Fever are required to run dilution controls when testing patient
83 sera, per Clinical Laboratory Improvement Amendments (CLIA) regulations[25], ensuring that patient
84 samples and controls are diluted in the same manner while performing the assay. Since human IgG and
85 IgM dilution controls are not provided in the diagnostic serology kits, human sera that demonstrated
86 previous IgG and/or IgM reactivity to *Coccidioides* spp. antigen must be identified, stored and re-used
87 with the reagents in the diagnostic kit every time the test is run to comply with federal requirements.
88 The supply of positive human sera is uncertain, and the inherent variability of human sera may lead to
89 inconsistent diagnostic results. The lack of a consistent control presents a major challenge to all clinical
90 laboratories that perform serologic testing for Valley Fever and provides an opportunity to generate
91 human IgG and IgM reagents that can be used as dilution controls with the serologic test kits.

92 Here, we present the development of a *Coccidioides* spp. antigen-specific mAb produced by
93 murine hybridomas and its conversion to humanized chimeric IgG and IgM isotypes by using a plant-
94 based expression system. The two mAb human isotypes are produced efficiently in plants. Furthermore,
95 they effectively recognize their target, chitinase 1 (CTS1), from *Coccidioides* spp. using similar reagents
96 to those used clinically in Valley Fever diagnostic kits. This study may provide a solution to a particular
97 "pain-point" in clinical laboratories that run serologic tests for the diagnosis of coccidioidomycosis. This
98 may also set a foundation for using plants to provide various human antibody isotypes as human
99 diagnostic reagents, inexpensively and quickly with consistent quality.

100 Methods & Materials

101 Antigen preparation and mouse immunization

102 Antigen was prepared as a culture filtrate of the mycelial phase of *Coccidioides* spp. as
103 previously detailed[26]. Mycelial culture filtrate (MCF) was concentrated using an Amicon centrifugal filter
104 unit (Millipore) and the total protein concentration was measured by bicinchoninic acid (Thermo
105 Scientific). MCF was mixed with Magic Mouse adjuvant (Creative Diagnostics) at 1:1 volume and used to
106 immunize one BALB/c mouse under an Institutional Animal Care and Use Committee–approved protocol
107 at Mayo Clinic (50 µg per dose). Subcutaneous (SQ) immunization was repeated with half the dose of
108 MCF at 3-week intervals. Antibody titers to MCF were monitored by indirect ELISA and when an
109 acceptable level was reached (>1:32,000), a final SQ boost was given, and the mouse was sacrificed
110 three days later for hybridoma generation.

111 **Generation and purification of mAbs**

112 Hybridomas were generated using a standard technique[9]. Briefly, the spleen was excised and
113 processed into a single-cell suspension, followed by lysis of erythrocytes with 1X red blood cell lysis
114 buffer (Invitrogen). The remaining 4 x 10^7 splenocytes were fused with 8 x 10^7 murine myeloma cells
115 (P3X63Ag8.653) using 1 mL of 50% polyethylene glycol solution (Sigma-Aldrich). Fused cells were
116 resuspended in 20% fetal bovine serum complete Dulbecco's modified Eagle medium with
117 hypoxanthine-aminopterin-thymidine selection supplement (Sigma-Aldrich) and plated in 96-well plates
118 at 5 x 10^4 splenocytes per well. Plates were incubated for ten days at 37˚C in a 5% CO_2 incubator. Culture
119 supernatant was tested for antibodies to MCF by indirect ELISA. Cells positive by ELISA were subcloned
120 by limiting dilution and screened using the same method after ten days. Positive subclones were
121 expanded and supernatant was purified by protein A/G chromatography (Thermo Scientific). Purified
122 mAbs were evaluated for specificity by Western blotting. One mAb, 4H2, bound to a known antigen in
123 MCF, CTS1, and was pursued for gene rescue.

124 **ELISA for Hybridoma Screening**

125 Purified MCF was coated on an ELISA plate at 20 µg/mL. Then, the plate was washed with 1X
126 phosphate-buffered saline + 0.05% Tween-20 (PBST) three times and blocked in 1% bovine serum
127 albumin in phosphate-buffered saline (BSA-PBS). For monitoring mouse antibody titers, mouse serum
128 was diluted in 1% BSA-PBS to various dilutions and allowed to incubate on the ELISA plate for 1 hour. For
129 testing hybridoma cultures, neat supernatant was added directly to the blocked ELISA plate and
130 incubated for 1 hour. After primary antibody incubation, plates were washed three times with PBST
131 then incubated with horseradish peroxidase (HRP)-conjugated goat-anti mouse IgG antibody (1:5000
132 dilution, Jackson ImmunoResearch, Cat. No. 115-035-071) for 1 hour. Plates were washed four times
133 with PBST and TMB substrate was added. After color development, the reaction was stopped with
134 0.16M sulfuric acid and absorbance was read at 450nm.

135 **Mouse immunoglobulin gene rescue**

136 Cell lysate of mAb 4H2 was prepared using the Cells-to-cDNA™ II Kit (Invitrogen). The cell lysate
137 was subsequently used to synthesize cDNA using a reverse transcription reaction with reagents included
138 in the kit. The resultant cDNA was subject to polymerase chain reaction (PCR) amplification of unknown
139 mouse immunoglobulin light chain (LC) and heavy chain (HC) variable genes using previously published
140 degenerate primers[27]. Each PCR reaction contained 2 µL cDNA, 0.5 µM 5' and 3' primers, 25 µL Phusion
141 Flash High-Fidelity PCR Master Mix (Thermo Scientific), and ultra-pure water to bring the reaction
142 volume to 50 µL. Cycling conditions were as follows: initial denaturation at 98˚C for 10 seconds followed
143 by 30 cycles of a three-step program (98˚C, 1 second; 55˚C, 5 seconds; 72˚C, 6 seconds), and a final
144 extension at 72˚C for 1 minute. Amplification was confirmed by running 8 µL of the PCR reaction on a 1%
145 agarose gel. The remaining 42 µL of the PCR product were subject to DNA cleanup using the QIAquick®
146 PCR Purification Kit (Qiagen) and the approximate concentration of DNA was determined using a
147 NanoDrop spectrophotometer (Thermo Scientific). Cleaned DNA from each PCR reaction was cloned into
148 pcDNA™3.1V5/HisA vector (Thermo Scientific) using the Cold Fusion Cloning Kit (System Biosciences).

149 DNA from resultant clones was extracted using the QIAprep®Spin Miniprep Kit (Qiagen) and sent for
150 Sanger sequencing.

151 **Human IgG and IgM Chimera Construction**

152 Primers were designed to add a plant-specific secretion signal peptide to murine variable
153 regions of the LC and HC clones. The resulting PCR products were then digested and ligated onto a
154 human kappa chain backbone, along with a human IgG_1[28] and a human mu chain backbone[17],
155 respectively, before being cloned into a plant expression vector as described[29]. Positive clones
156 containing the human chimeric IgG and IgM constructs were transformed in *Agrobacterium tumefaciens*
157 and verified by PCR.

158 **Agroinfiltration and Temporal Expression in *Nicotiana benthamiana***

159 Transgenes were introduced into *N. benthamiana* leaves by agroinfiltration as previously
160 described[14,30] and leaves expressing the recombinant IgG or IgM were collected for temporal expression
161 analysis. Leaves collected in the range of 2 – 9 days after transgene introduction were homogenized in
162 extraction buffer (1X PBS containing 1mM ethylenediaminetetraacetic acid [EDTA], 2mM
163 phenylmethylsulfonyl fluoride [PMSF], and 10 mg/mL sodium L-ascorbate) at a 1:1.5 ratio of fresh leaf
164 weight (FLW) to buffer volume. An ELISA plate that had been coated overnight at 4°C with a goat anti-
165 human kappa chain antibody (Southern Biotech, Cat. No. 2060-01) at 2 µg/mL, was washed four times
166 with PBST, followed by blocking with 5% dry milk dissolved in PBST (DM-PBST). Dilutions in DM-PBST of
167 homogenized leaf samples and an isotype control of known concentration were added to the plate and
168 incubated for 1 hour at 37°C. After washing four times, a goat anti-human IgG-HRP antibody (1:4000
169 dilution, Southern Biotech, Cat. No. 2040-05) or a goat anti-human IgM mu chain-HRP (1:5000 dilution,
170 Abcam, Cat. No. ab97205) was diluted in 5% DM-PBST and added to the plate for 1 hour at 37°C. After

171 an additional four washes, TMB substrate was added to the plate and a 1M sulfuric acid stop solution
172 was used prior to reading absorbance at 450nm.

173 **Plant-Made mAb Purification and SDS-PAGE Analysis**

174 *N. benthamiana* leaves expressing either the humanized 4H2 IgG or IgM were harvested and
175 homogenized in the same extraction buffer described above for temporal analysis. The protein extract
176 containing IgG or IgM was processed as described[29,31], followed by Protein A (Cytiva) or human anti-mu
177 chain (Sigma-Aldrich, Cat. No. A9935) affinity chromatography. Eluted mAbs were further concentrated
178 and buffer exchanged into 1X PBS, pH 7.4 by ultrafiltration in an Amicon centrifugal filter unit
179 (Millipore). Purified mAbs were then subjected to SDS-PAGE and Coomassie Blue R-250 staining under
180 both reducing and non-reducing conditions on a Mini-Protean TGX 4-20% gel (Bio-Rad).

181 **Western Blotting**

182 Western blots were performed as described previously[29]. In brief, to validate the plant-made
183 mAbs identity, plant-made 4H2 IgG (P-4H2 IgG) or plant-made 4H2 IgM (P-4H2 IgM), were subjected to
184 SDS-PAGE under reducing and non-reducing conditions and transferred onto polyvinylidene difluoride
185 (PVDF, Bio-Rad) membranes. Membranes were blocked with 5% DM-PBST for 1 hour, followed by
186 washing with PBST. Goat anti-human kappa-HRP (1:5000 dilution, Southern Biotech, Cat. No. 2060-05),
187 goat anti-human IgG-HRP (1:10,000, Southern Biotech, Cat. No. 2040-05) or goat anti-human IgM mu
188 chain-HRP (1:5000 dilution, Abcam, Cat. No. ab97205) were diluted in 5% DM-PBST and incubated for 1
189 hour. After washing with PBST, membranes were developed using with SuperSignal® West Pico
190 Chemiluminescent Substrate (Thermo Scientific) according to manufacturer's instructions and images
191 were taken with an ImageQuant instrument.

192 For validation of the plant-made mAbs' target recognition, recombinant CTS1 and *Coccidioides*
193 MCF were separated under reducing conditions on 12% polyacrylamide gels and transferred to PVDF

194 membrane. Following blocking in 1% BSA-PBST, either the parental murine 4H2 IgG (M-4H2 IgG), P-4H2
195 IgG, or P-4H2 IgM were diluted to 500 ng/mL in 1% BSA-PBS and incubated for 1 hour. The membranes
196 were then probed with the appropriate secondary antibody [1:10,000 dilution, goat-anti-mouse IgG Fc-
197 specific-HRP, Jackson ImmunoResearch, Cat. No. 115-035-071; 1:10,000 dilution, goat-anti-human IgG
198 Fc-specific-HRP, Sigma-Aldrich, Cat. No. A0170; 1:10,000 dilution, goat-anti-human IgM Mu-HRP, Caltag
199 Cat. No. H15007], followed by a 1-hour incubation. After washing with PBST, a KPL TrueBlue Peroxidase
200 substrate (SeraCare) was added, followed by image capture.

201 **Antigen Binding ELISA by Chimeric IgG and IgM**

202 To evaluate P-4H2 IgG and P-4H2 IgM antigen binding, recombinant CTS1 was coated on an
203 ELISA plate at 2 µg/mL overnight at 4˚C. Then, the plate was washed with 1X PBST three times and
204 blocked in 1% BSA-PBS. MAb isotypes were tested at 1 µg/mL and diluted two-fold in 1% BSA-PBS. After
205 1 hour of incubation, plates were washed three times with PBST then incubated with HRP-conjugated
206 anti-human secondary antibody [1:20,000 dilution, goat-anti-human IgG Fc-specific-HRP, Sigma-Aldrich,
207 Cat. No. A0170; 1:5,000 dilution, goat-anti-human IgM Mu-HRP, Caltag Cat. No. H15007]. Hybridoma-
208 produced parent mAb was used as a positive control, with an HRP-conjugated anti-mouse secondary
209 antibody [1:20,000 dilution, goat-anti-mouse IgG Fc-specific-HRP, Jackson ImmunoResearch, Cat. No.
210 115-035-071]. Plates were washed four times with PBST and TMB substrate was added. The reaction
211 was stopped with 0.16M sulfuric acid and absorbance was read at 450nm.

212 ## Results

213 ***Engineering and Expression of Coccidioides-Specific Human of IgG and IgM Chimeras in***
214 ***Nicotiana benthamiana***

215 A mAb of interest, 4H2, was identified by immunizing mice with MCF from *Coccidioides* spp.,
216 followed by hybridoma generation and gene rescue. The resulting murine variable heavy region was
217 genetically fused onto both human IgG_1 and IgM heavy constant chains, in parallel with the variable light
218 region being grafted onto a human kappa constant chain. Gene constructs for both humanized IgG and
219 IgM were introduced into leaves of *N. benthamiana* and recombinant expression of the 4H2 IgG and IgM
220 chimeras was monitored over time by an anti-human IgG or IgM ELISA. Peak expression of P-4H2 IgG
221 was observed at day 7 after agroinfiltration, reaching 39.95 ± 2.5 µg/g FLW (**Figure 1A**). P-4H2 IgM
222 reached peak expression of 33.3 ± 1.19 µg/g FLW at day 3 after introduction of the transgenes (**Figure**
223 **1B**).

224 ***Recombinant Chimeric IgG and IgM Purification and Characterization***

225 P-4H2 IgG was purified to a high degree of homogeneity by Protein A affinity chromatography,
226 comparable to a control IgG mAb (**Figure 2A**). The recombinant P-4H2 IgM was purified by affinity
227 chromatography with a goat anti-human mu chain agarose resin (**Figure 2B**) and more bands were
228 observed than the IgG isotype. To verify the identity of the observed bands from affinity purified P-4H2
229 IgG and P-4H2 IgM, western blotting was performed. The bands observed with SDS-PAGE analysis of the
230 IgG isotype indeed correspond to both human kappa chain at ~25 kDa (**Figure 3A and 3C**), as well as
231 human gamma chain at ~50 kDa (**Figure 3B**). Likewise, the bands observed for the purified P-4H2 IgM
232 represent the same human kappa chain (**Figure 4A and 4C**), as well as the human mu chain (**Figure 4B**),
233 with an expected size of ~60-65 kDa. Western blot analysis also revealed that the major protein bands
234 observed in the purified, P-4H2 IgM falling between 50 and 37 kDa represent either proteolysis of intact
235 human mu chain or a truncated mu chain, in addition to the expected band at ~60-65 kDa (**Figure 2B**
236 **and 4B**). As expected, both P-4H2 IgG and P-4H2 IgM under non-reducing conditions are composed of

237 two heavy and two light chains, for monomeric sizes of ~150 kDa (**Figure 3C**) and ~170 kDa (**Figure 4C**),
238 respectively.

239 To ensure the human IgG and IgM chimeras produced in plants maintained antigen binding
240 specificity of M-4H2 IgG, P-4H2 IgG and P-4H2 IgM were also tested for binding to recombinant CTS1
241 and MCF. As determined by indirect ELISA, both recombinant isotypes of the chimeric mAb bound to
242 recombinant CTS1 in a dose-dependent manner (**Figure 5**), with nearly identical activity to M-4H2 IgG.
243 Similarly, western blot analysis also indicated specific interaction between the plant-made chimeric IgG
244 and IgM mAbs and both recombinant CTS1 and MCF (**Figure 6**), further validating the binding activity of
245 the P-4H2 IgG and P-4H2 IgM to the antigen of interest.

246 Discussion

247 In this study, we utilized plants as an alternative recombinant protein expression platform to
248 express two isotypes of a hybridoma-produced mAb against a fungal antigen, CTS1, from *Coccidioides*
249 spp. To our knowledge, this is only the second report of generating an IgM molecule in plants, but a first
250 for our intentional monomeric IgM design (pairing of two heavy chains), as opposed to a pentameric
251 design[17,32]. Both the humanized P-4H2 IgG and P-4H2 IgM were efficiently expressed within a week of
252 transgene delivery, with levels of 39.95 ± 2.5 and 33.3 ± 1.19 µg/g FLW, respectively. These expression
253 levels fall within the range of previously reported plant-made mAbs, but recombinant IgG levels vary
254 widely, with observations ranging from 4 – 1500 µg/g FLW[33,34], while a previous expression of a
255 pentameric IgM reached levels of approximately 84 µg/g FLW[17]. The moderate expression levels of the
256 IgG and IgM observed in this study may be attributed to several factors, including the use of non-codon-
257 optimized plant protein expression, specifically in the variable regions of the mAbs, and the use of
258 different expression vectors than those reported in the literature. Increasing expression levels of these

259 mAbs is possible by addressing these points and efforts to increase protein expression levels are
260 currently ongoing.

261 The sandwich ELISA used to monitor expression level was designed to only recognize
262 recombinant mAbs that contained both a human kappa light chain and either a human gamma (for IgG)
263 or human mu (for IgM) heavy chain. The proper assembly of both the IgG and IgM monomers was
264 further validated by reducing and non-reducing western blot. Indeed, the IgG kappa chain was detected
265 at the expected size of ~150 kDa. Furthermore, the kappa and mu chain components of the IgM
266 observed in SDS-PAGE were identified and pairing of two mu heavy chains and two kappa light chains to
267 form a monomeric IgM was shown to occur, indicated by the ~170 kDa band under non-reducing
268 conditions both on western blot and SDS-PAGE. We expected no more than a monomeric form of the
269 IgM, due to the intentional absence of the joining (J)-chain gene, yet a similar band pattern can be
270 observed when comparing the plant-made monomeric IgM with the pentameric human IgM control,
271 indicating possible formation of other IgM oligomers without the J-chain, which has been observed in
272 other studies[35–39]. The smeared banding pattern of the plant-made IgM can be attributed to the
273 presence of heterologous populations of IgM in the purified product, likely due to proteolysis.
274 Optimization of purification of IgM can be performed in the presence of protease inhibitors to limit
275 potential proteolysis. If proteolysis cannot be avoided, the target IgM population could be further
276 purified by other additional chromatography steps.

277 Both the chimeric P-4H2 IgG and P-4H2 IgM were purified by simple affinity chromatography
278 methods, specific for their respective heavy constant regions. The IgG reached comparable levels of
279 purity to a mammalian cell-produced IgG subjected to the same purification scheme. In contrast, the
280 homogeneity of P-4H2 IgM was lower than the pentameric IgM control made in mammalian cells.
281 Indeed, the major smaller-than-expected bands between 37 and 50 kDa appeared as a smear of protein
282 fragments, with a minor band appearing just above the 20 kDa marker. We can conclude that the bands

283 of ~45 kDa in size are degradation products from human mu chain, due to the use of an anti-human mu
284 chain agarose as the affinity resin in purifying the mAb isotype. It is also reasonable to infer that the
285 band near 20 kDa is a mu chain degradation product, given that the summation of the ~45 kDa and the
286 ~20 kDa bands would give an expected size of the non-degraded human mu chain we observe of ~60-65
287 kDa. Thus, all the major non-target bands found in the purified, recombinant IgM are accounted for and
288 can be linked back to the constant human mu chain.

289 For ensuring a consistent and reliable IgM product, several steps can be taken to reduce or
290 eliminate the unwanted bands we observed here. Our monomeric design may just be inherently
291 unstable. Including a J chain gene can be implemented to preferentially produce a pentameric IgM
292 product, as has been performed previously in plants[17], which would likely increase the stability of the
293 protein, help protein folding, and may make it less prone to proteolytic degradation during protein
294 extraction and purification. We can also adjust the purification method to include a second affinity
295 chromatography step specific for human kappa constant chain, potentially only purifying active species
296 of IgM (those that have both mu and kappa chains). This would provide a consistent product for the
297 intended use as an antigen-specific human dilution control.

298 Valley Fever is a fungal disease that requires an accurate and timely diagnosis. However, current
299 FDA-approved diagnostic kits lack a dilution control. Currently, clinical laboratories who run serologic
300 tests to detect antibodies against *Coccidioides spp*. are forced to identify, collect, store and re-use
301 previously IgG and IgM-positive sera for use in subsequent test runs. This leads to inconsistency in
302 diagnoses due to inherent variability of storing and re-using human sera. In response to this unmet need
303 for a consistent diagnostic control, we developed a mouse mAb specific to a *Coccidioides* spp. antigen
304 used in the clinical diagnostic kits and engineered it into human IgG and IgM chimeras. We confirmed
305 that the human chimeras retain original antigen recognition by indirect ELISA with recombinant CTS1.
306 This binding was validated in parallel by Western blotting using recombinant CTS1, alongside the

307 immunogen, MCF. Taken together, these data provide evidence that the plant-made chimeric IgG and
308 IgM specifically recognize the antigen of interest found in the immunogen, in a manner consistent with
309 the parental murine mAb. Furthermore, the humanization allows these chimeric mAb isotypes to be
310 recognized by anti-human IgG and IgM secondary antibodies in an ELISA, like those used in Valley Fever
311 diagnostic kits. Therefore, these human chimeric mAbs may be useful as positive controls to replace
312 positive human sera currently used to comply with federal law. This may lead to more consistent
313 diagnostic results and a sustainable supply. The next phase of research will involve investigating the
314 utility of these mAbs in the commercial kits with clinical samples and gaining regulatory approval.
315 Overall, our study highlights the opportunity of plant-based, recombinant production of both IgG and
316 IgM isotypes against CTS1, as consistent controls for Valley Fever diagnostic kits. This platform may
317 provide a consistent and reproducible source of low-cost mAbs as diagnostic reagents for other diseases
318 as well.

319 **Author Contributions**

320 Q.C. and D. L. conceptualized research; C.J. and F.G. designed experiments, performed experiments, and
321 analyzed data. C.J., F.G. and Q.C. wrote the paper with revision by D.L. All authors have reviewed and
322 agreed to the submitted version of the manuscript.

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326 **Conflicts of Interest**

327 The authors declare no conflict of interest.

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435

436 **Figure Legends**

437 **Figure 1: Expression of 4H2 Isotypes in *Nicotiana benthamiana* leaves.**

438 Temporal expression of P-4H2 IgG (**A**) and P-4H2 IgM (**B**) human chimeras was analyzed by sandwich
439 ELISA that detects IgG or IgM containing both human kappa light chain and either human gamma or
440 human mu heavy chain from total soluble plant protein extracts. Mean ± SEM from two independent
441 experiments is plotted.

442 **Figure 2: Purification of P-4H2 IgG and P-4GH2 IgM from *N. benthamiana* leaves.**

443 Affinity-purified, humanized P-4H2 IgG (**A**) and P-4H2 IgM (**B**) were separated by SDS-PAGE on 4-20%
444 polyacrylamide gels under reducing and non-reducing conditions. One representative of multiple
445 experiments is shown. Lanes 1 and 3, plant-made mAb isotypes. Lanes 2 and 4, control human mAb
446 isotypes. M, molecular weight marker.

447 **Figure 3: Assembly of 4H2 IgG purified from *N. benthamiana* leaves.**

448 Purified recombinant P-4H2 IgG was separated under reducing (**A** and **B**) and non-reducing (**C**)
449 conditions by SDS-PAGE and proteins was transferred to PVDF membranes. Proteins were then detected
450 with antibodies specific for human kappa chain (**A** and **C**) or human gamma chain (**B**) to verify protein
451 identity and assess IgG assembly. Lane 1, Humanized, P-4H2 IgG, Lane 2, Human kappa IgG control.

452 **Figure 4: Assembly of 4H2 IgM purified from *N. benthamiana* leaves.**

453 Purified recombinant P-4H2 IgM was separated under reducing (**A** and **B**) and non-reducing (**C**)
454 conditions by SDS-PAGE and proteins was transferred to PVDF membranes. Proteins were then detected
455 with antibodies against human kappa chain (**A** and **C**) or human mu chain (**B**) to verify protein identity
456 and assess IgM assembly. Lane 1, Humanized, P-4H2 IgM, Lane 2, Human kappa IgM control.

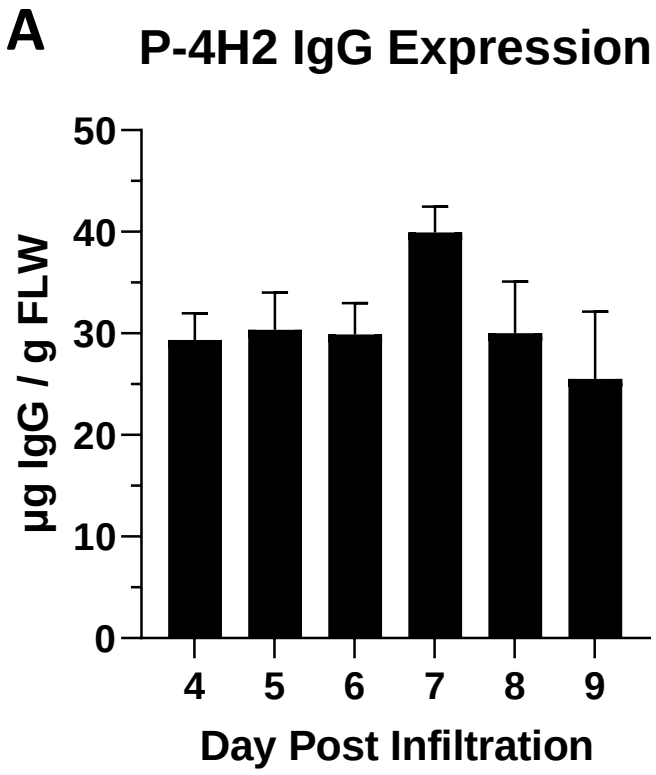
457 **Figure 5: Plant-Made 4H2 Isotypes Specifically Bind to Target Antigen.**

458 Serial dilutions of P-4H2 IgG and P-4H2 IgM isotypes were incubated with CTS1 immobilized on ELISA
459 plates. An HRP-conjugated anti-human IgG or anti-human IgM secondary antibody was used to detect
460 the specific interaction between the plant-made IgG or IgM isotype (P-4H2 IgG, P-4H2 IgM) with CTS1,
461 respectively. The parental mouse IgG (M-4H2 IgG) was used as a positive control, which was detected
462 with an anti-mouse IgG secondary antibody conjugated to HRP. Mean ± SEM is plotted from three
463 independent experiments.

464 **Supplemental Figure 1: Plant-Made 4H2 Isotypes Bind to Target Antigen on Western Blot.**

465 Recombinant CTS1 and *Coccidioides posadasii* mycelial culture supernatant were separated by SDS-PAGE
466 and either stained with Coomassie Blue (**A**) or transferred to PVDF membrane. Membranes were then
467 probed with the parental M-4H2 IgG (**B**), the humanized, P-4H2 IgG (**C**), or the humanized, P-4H2 IgM
468 (**D**), followed by the appropriate secondary HRP-conjugated antibody and development with a
469 colorimetric substrate. Lane 1, Recombinant CTS1. Lane 2, *Coccidioides posadasii* mycelial culture
470 supernatant. M, molecular weight marker.

Figure 1


A
P-4H2 IgG Expression
µg IgG / g FLW
50
40
30
20
10
0
4
5
6
7
8
9
Day Post Infiltration

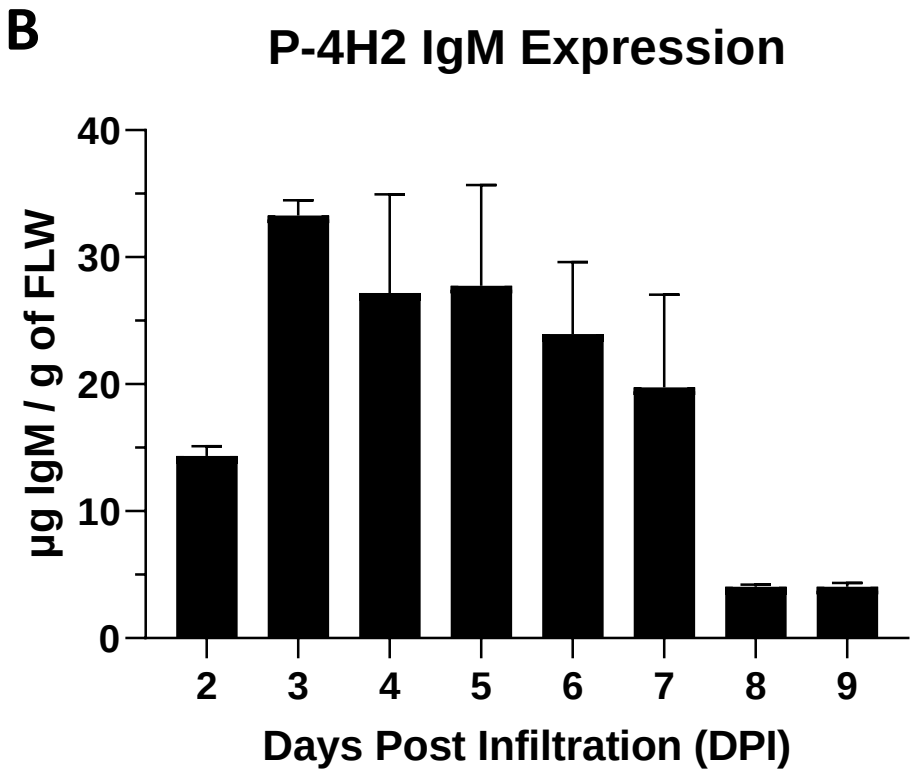

B
P-4H2 IgM Expression
µg IgM / g of FLW
40
30
20
10
0
2
3
4
5
6
7
8
9
Days Post Infiltration (DPI)

Figure 2

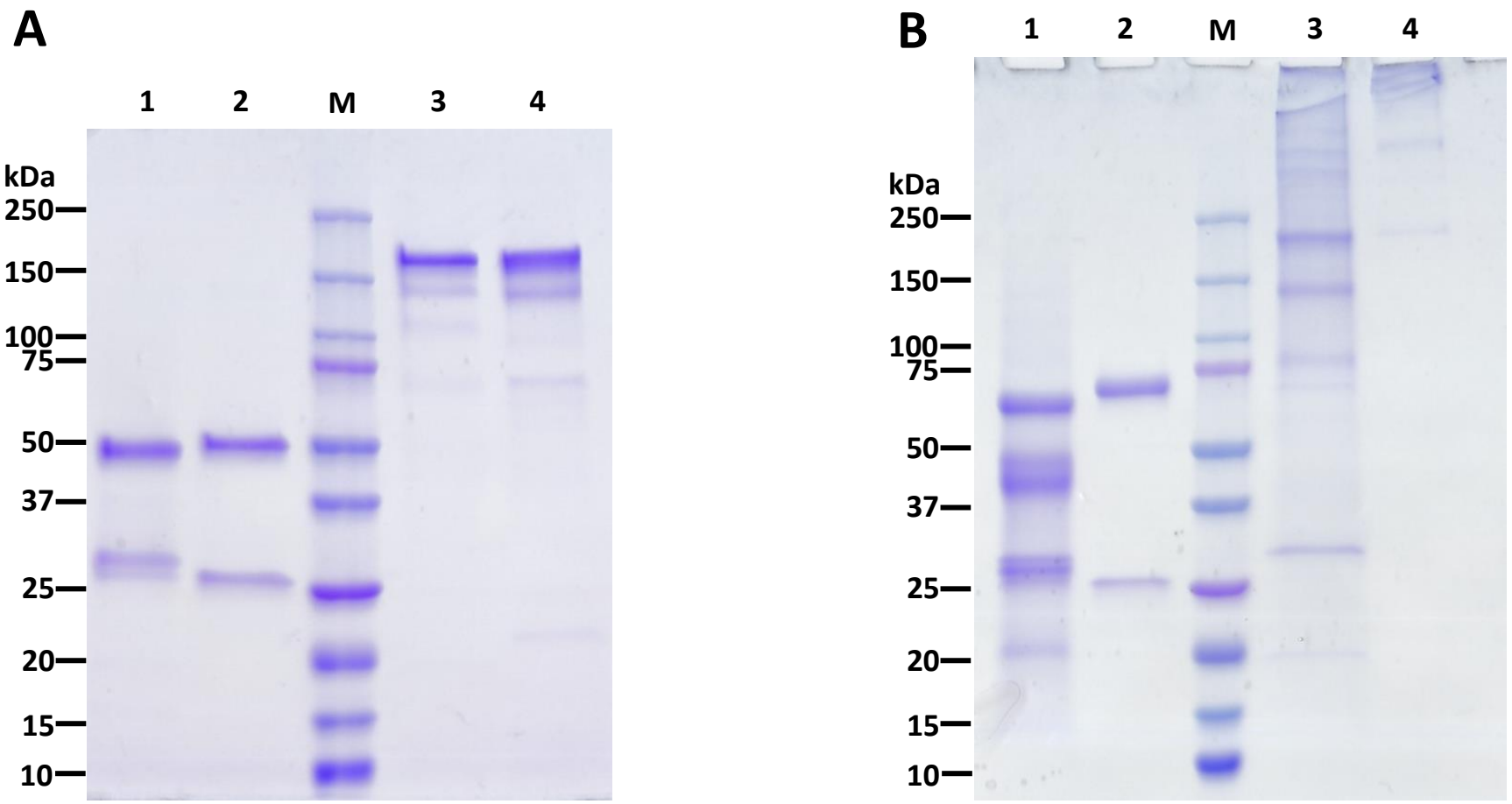
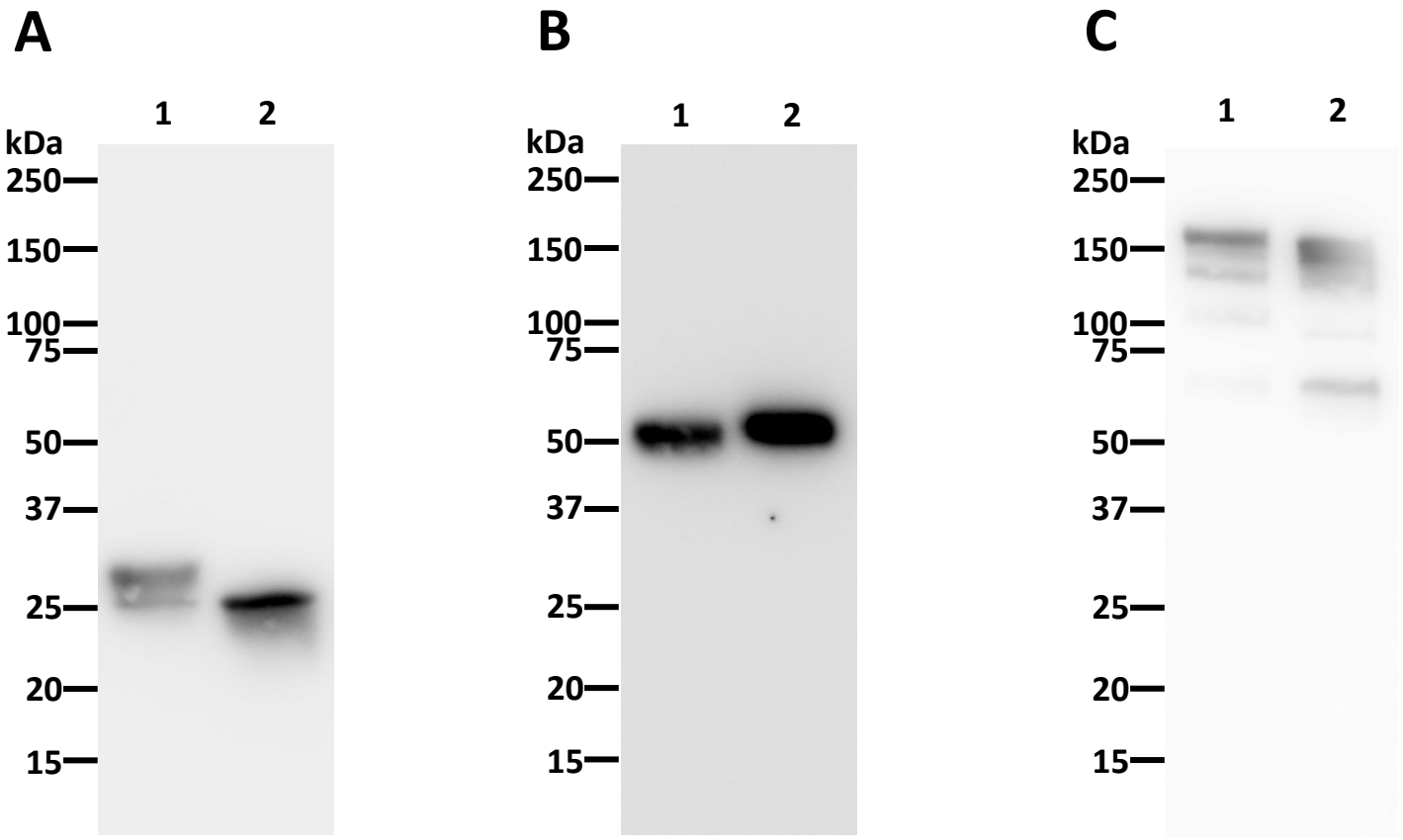

A
1
2
M
3
4
kDa
250
150
100
75
50
37
25
20
15
10
B
1
2
M
3
4
kDa
250
150
100
75
50
37
25
20
15
10

Figure 3


A
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37
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20
15
C
1
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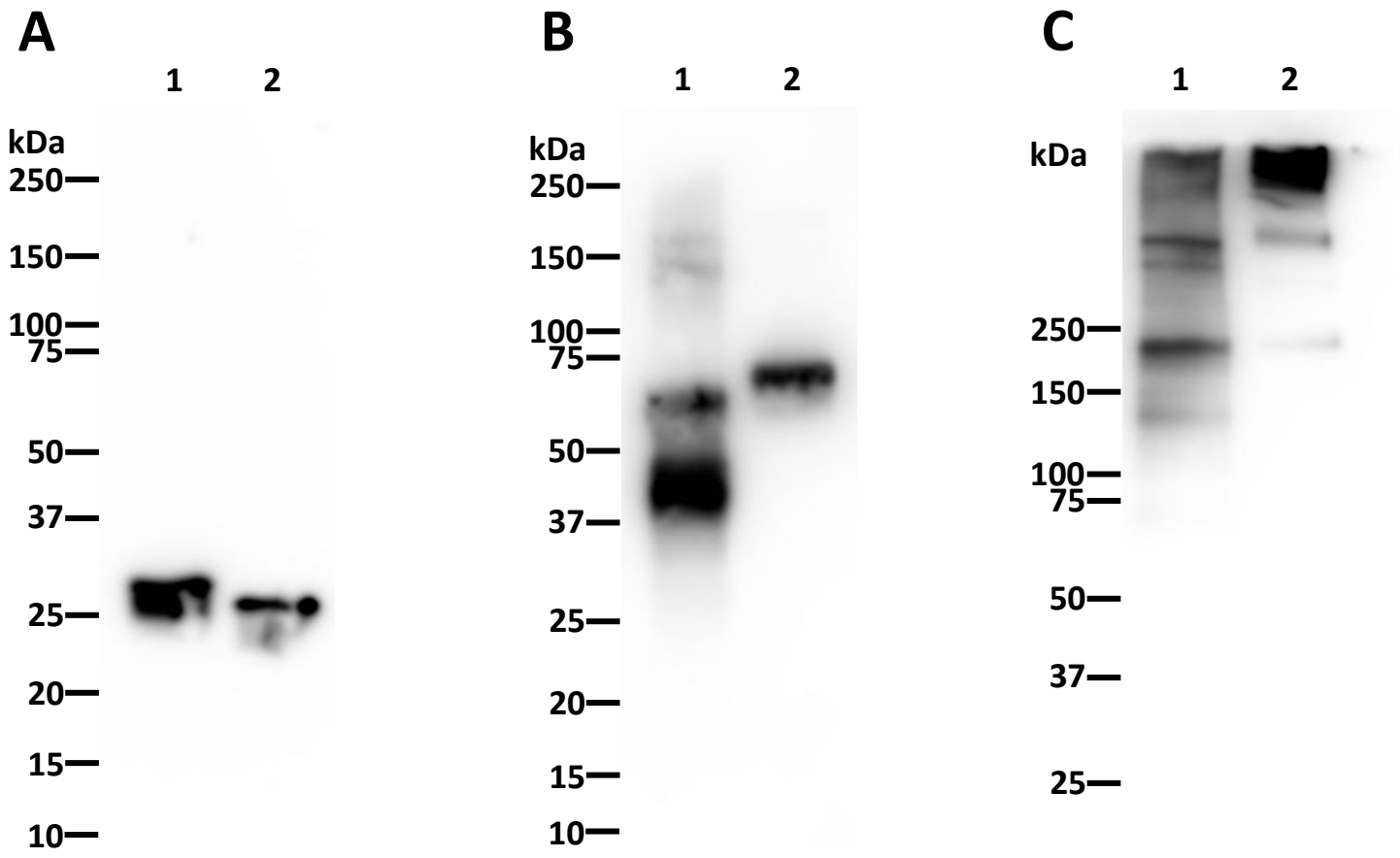
25
20
15

Figure 4


A
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75
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37
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20
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75
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37
25
20
15
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C
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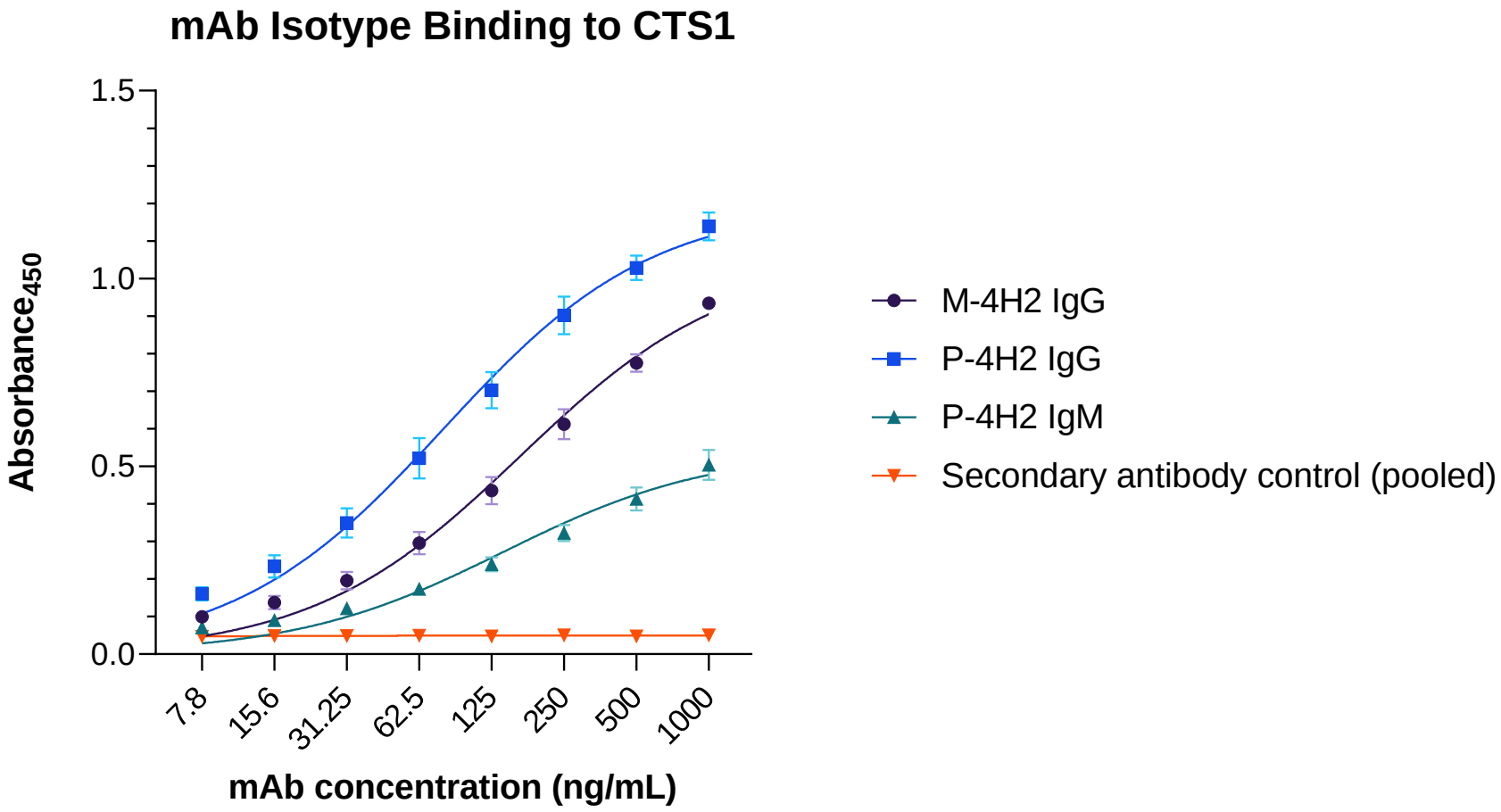
2
kDa
250
150
100
75
50
37
25

Figure 5


mAb Isotype Binding to CTS1
Absorbance450
1.5
1.0
0.5
0.0
7.8
15.6
31.25
62.5
125
250
500
1000
mAb concentration (ng/mL)
M-4H2 IgG
P-4H2 IgG
P-4H2 IgM
Secondary antibody control (pooled)

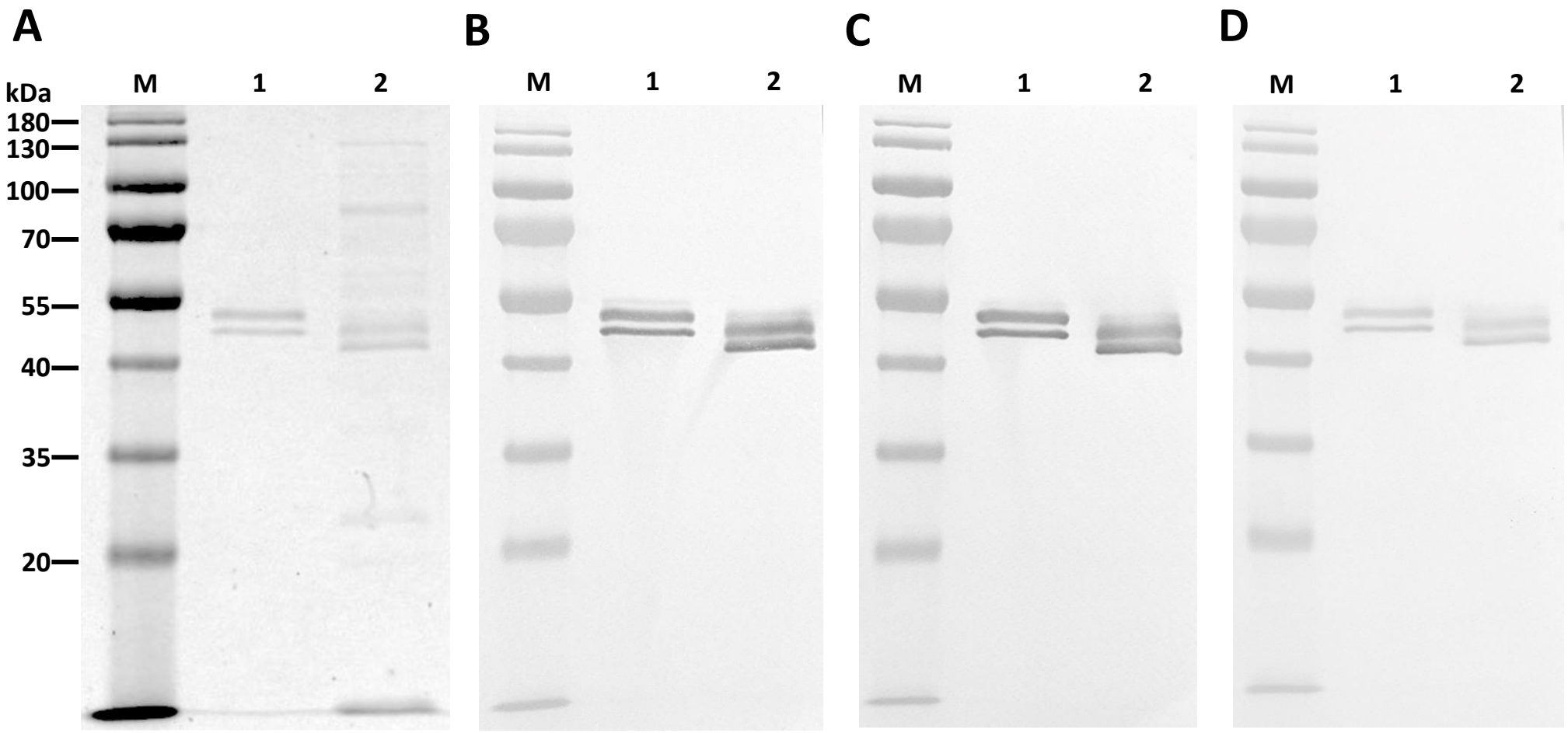



Figure 6