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], Thomas E. Grys[3], 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

[3]Laboratory Medicine and Pathology, Mayo Clinic, Phoenix, 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

Keywords: Coccidioidomycosis, Valley Fever (VF), Diagnostics, Plant-made Diagnostics, IgG, IgM, Humanization

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.

33 ## Introduction

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

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

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

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

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

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

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

101 Methods & Materials

102 Antigen preparation and mouse immunization

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

112 **Generation and purification of mAbs**

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

125 **ELISA for Hybridoma Screening**

126 Purified MCF was coated on an ELISA plate at 20 µg/mL. Then, the plate was washed with 1X
127 phosphate-buffered saline + 0.05% Tween-20 (PBST) three times and blocked in 1% bovine serum
128 albumin in phosphate-buffered saline (BSA-PBS). For monitoring mouse antibody titers, mouse serum
129 was diluted in 1% BSA-PBS to various dilutions and allowed to incubate on the ELISA plate for 1 hour. For
130 testing hybridoma cultures, neat supernatant was added directly to the blocked ELISA plate and
131 incubated for 1 hour. After primary antibody incubation, plates were washed three times with PBST
132 then incubated with horseradish peroxidase (HRP)-conjugated goat-anti mouse IgG antibody (1:5000
133 dilution, Jackson ImmunoResearch, Cat. No. 115-035-071) for 1 hour. Plates were washed four times
134 with PBST and TMB substrate was added. After color development, the reaction was stopped with
135 0.16M sulfuric acid and absorbance was read at 450nm.

136 **Mouse immunoglobulin gene rescue**

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

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

152 **Human IgG and IgM Chimera Construction**

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

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

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

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

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

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

182 **Western Blotting**

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

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

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

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

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

213 ## Results

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

216 A mAb of interest, 4H2, was identified by immunizing mice with MCF from *Coccidioides* spp.,
217 followed by hybridoma generation and gene rescue. The resulting murine variable heavy region was
218 genetically fused onto both human IgG_1 and IgM heavy constant chains, in parallel with the variable light
219 region being grafted onto a human kappa constant chain. Gene constructs for both humanized IgG and
220 IgM were introduced into leaves of *N. benthamiana* and recombinant expression of the 4H2 IgG and IgM
221 chimeras was monitored over time by an anti-human IgG or IgM ELISA. Peak expression of P-4H2 IgG
222 was observed at day 7 after agroinfiltration, reaching 39.95 ± 2.5 µg/g FLW (**Figure 1A**). P-4H2 IgM
223 reached peak expression of 33.3 ± 1.19 µg/g FLW at day 3 after introduction of the transgenes (**Figure**
224 **1B**).

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

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

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

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

247 Discussion

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

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

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

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

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

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

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

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

320 **Author Contributions**

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

324 **Acknowledgements**

325 The authors wish to thank Dr. Herta Steinkellner for the sequence of mu constant region. We also thank
326 Katherine Nguyen, Joshua Lesio and Akhil Mahant for helping with *N. benthamiana* maintenance.

327 **Conflicts of Interest**

328 The authors declare no conflict of interest.

329 **References**

330 1. Lu, R. M. *et al.* Development of therapeutic antibodies for the treatment of diseases.
331 *Journal of Biomedical Science* **27**, 1–30 (2020).

332 2. Meticulous Research. Research Antibodies and Reagents Market worth $6.32 billion by
333 2027 | Meticulous Market Research Pvt. Ltd.
334 https://www.meticulousresearch.com/pressrelease/273/research-antibodies-reagents-
335 market-
336 2027?utm_source=Globnewswire&utm_medium=Paid&utm_campaign=Product&utm_co
337 ntent=18-01-2022 (2020).

338 3. Meticulous Research. Diagnostic Specialty Antibodies Market Worth $42.56 Billion by
339 2029 | Meticulous Market Research Pvt. Ltd.
340 https://www.meticulousresearch.com/pressrelease/462/diagnostic-specialty-antibodies-
341 market-
342 2029?utm_source=Globnewswire&utm_medium=Paid&utm_campaign=Product&utm_co
343 ntent=01-02-2022 (2022).

344 4. Gray, A. *et al.* Animal-free alternatives and the antibody iceberg. *Nature Biotechnology* **38**,
345 1234–1239 (2020).

346 5. Bradbury, A. & Plückthun, A. Reproducibility: Standardize antibodies used in research.
347 *Nature* **518**, 27–29 (2015).

348 6. Gilchuk, P. *et al.* Integrated pipeline for the accelerated discovery of antiviral antibody
349 therapeutics. *Nature Biomedical Engineering* **4**, 1030–1043 (2020).

350 7. Alfaleh, M. A. *et al.* Phage Display Derived Monoclonal Antibodies: From Bench to
351 Bedside. *Frontiers in Immunology* **11**, 1986 (2020).

352 8. Hammers, C. M. & Stanley, J. R. Antibody Phage Display: Technique and Applications. *The*
353 *Journal of Investigative Dermatology* **134**, e17 (2014).

354 9. Köhler, G. & Milstein, C. Continuous cultures of fused cells secreting antibody of
355 predefined specificity. *Nature* **256**, 495–497 (1975).

356 10. Qiu, X. *et al.* Reversion of advanced Ebola virus disease in nonhuman primates with
357 ZMapp. *Nature* **514**, 47–53 (2014).

358 11. Lai, H. *et al.* Monoclonal antibody produced in plants efficiently treats West Nile virus
359 infection in mice. *Proceedings of the National Academy of Sciences* **107**, 2419–2424
360 (2010).

361 12. Chen, Q., Santi, L. & Zhang, C. Plant-made biologics. *BioMed Research International* **2014**,
362 (2014).

363 13. Chen, Q. & Davis, K. R. The potential of plants as a system for the development and
364 production of human biologics [version 1; referees: 3 approved]. *F1000Research* **5**, (2016).

365 14. Leuzinger, K. *et al.* Efficient agroinfiltration of plants for high-level transient expression of
366 recombinant proteins. *Journal of Visualized Experiments* e50521 (2013)
367 doi:10.3791/50521.

368 15. Chen, Q. *et al.* Agroinfiltration as an Effective and Scalable Strategy of Gene Delivery for
369 Production of Pharmaceutical Proteins. *Advanced Techniques in Biology & Medicine* **01**,
370 (2013).

371 16. Sun, L. *et al.* Increased in vitro neutralizing activity of SARS-CoV-2 IgA1 dimers compared
372 to monomers and IgG. *Proceedings of the National Academy of Sciences* **118**, 2107148118
373 (2021).

374 17. Loos, A. *et al.* Expression and glycoengineering of functionally active heteromultimeric
375 IgM in plants. *Proceedings of the National Academy of Sciences* **111**, 6263–6268 (2014).

376 18. Ma, J. K. C. *et al.* Generation and Assembly of Secretory Antibodies in Plants. *Science* **268**,
377 716–719 (1995).

378 19. Kallolimath, S. *et al.* Highly active engineered IgG3 antibodies against SARS-CoV-2.
379 *Proceedings of the National Academy of Sciences* **118**, (2021).

380 20. He, J., Lai, H., Brock, C. & Chen, Q. A novel system for rapid and cost-effective production
381 of detection and diagnostic reagents of West Nile virus in plants. *Journal of Biomedicine*
382 *and Biotechnology* **2012**, (2012).

383 21. Sun, L. *et al.* Increased in vitro neutralizing activity of SARS-CoV-2 IgA1 dimers compared
384 to monomers and IgG. *Proceedings of the National Academy of Sciences* **118**,
385 e2107148118 (2021).

386 22. van Dyke, M. C. C., Thompson, G. R., Galgiani, J. N. & Barker, B. M. The Rise of
387 Coccidioides: Forces Against the Dust Devil Unleashed. *Frontiers in Immunology* **10**, 2188
388 (2019).

389 23. Khan, S. *et al.* Interlaboratory agreement of coccidioidomycosis enzyme immunoassay
390 from two different manufacturers. *Medical Mycology* **57**, 441–446 (2019).

391 24. Malo, J. *et al.* Comparison of three anti-coccidioides antibody enzyme immunoassay kits
392 for the diagnosis of coccidioidomycosis. *Medical Mycology* **58**, 774–778 (2020).

393 25. Centers for Medicare & Medicaid Services & Department of Health and Human Services.
394 493.1256 Standard: Control procedures. *CFR-42-493-1256* vol. 5 (2011).

395 26. Mead, H. L., van Dyke, M. C. C. & Barker, B. M. Proper Care and Feeding of Coccidioides: A
396 Laboratorian's Guide to Cultivating the Dimorphic Stages of C. immitis and C. posadasii.
397 *Current Protocols in Microbiology* **58**, e113 (2020).

398 27. Wang, Z. *et al.* Universal PCR amplification of mouse immunoglobulin gene variable
399 regions: the design of degenerate primers and an assessment of the effect of DNA
400 polymerase 3' to 5' exonuclease activity. *Journal of Immunological Methods* **233**, 167–177
401 (2000).

402 28. Hurtado, J. *et al.* In vitro and in vivo efficacy of anti-chikungunya virus monoclonal
403 antibodies produced in wild-type and glycoengineered Nicotiana benthamiana plants.
404 *Plant Biotechnology Journal* **18**, 266–273 (2020).

405 29. Jugler, C., Sun, H. & Chen, Q. SARS-CoV-2 Spike Protein-Induced Interleukin 6 Signaling Is
406 Blocked by a Plant-Produced Anti-Interleukin 6 Receptor Monoclonal Antibody. *Vaccines*
407 **9**, 1365 (2021).

408 30. Esqueda, A., Jugler, C. & Chen, Q. Design and expression of a bispecific antibody against
409 dengue and chikungunya virus in plants. *Methods in Enzymology* (2021)
410 doi:10.1016/BS.MIE.2021.05.003.

411 31. Jugler, C., Joensuu, J. & Chen, Q. Hydrophobin-Protein A Fusion Protein Produced in Plants
412 Efficiently Purified an Anti-West Nile Virus Monoclonal Antibody from Plant Extracts via
413 Aqueous Two-Phase Separation. *International Journal of Molecular Sciences* **21**, 2140
414 (2020).

415 32. Vasilev, N., Smales, C. M., Schillberg, S., Fischer, R. & Schiermeyer, A. Developments in the
416 production of mucosal antibodies in plants. *Biotechnology Advances* **34**, 77–87 (2016).

417 33. Shanmugaraj, B., Rattanapisit, K., Manopwisedjaroen, S., Thitithanyanont, A. &
418 Phoolcharoen, W. Monoclonal Antibodies B38 and H4 Produced in Nicotiana benthamiana
419 Neutralize SARS-CoV-2 in vitro. *Frontiers in Plant Science* **11**, (2020).

420 34. Diamos, A. G. *et al.* High Level Production of Monoclonal Antibodies Using an Optimized
421 Plant Expression System. *Frontiers in Bioengineering and Biotechnology* **7**, (2020).

422 35. Randall, T. D., Parkhouse, R. M. E. & Corley, R. B. J chain synthesis and secretion of
423 hexameric IgM is differentially regulated by lipopolysaccharide and interleukin 5.
424 *Proceedings of the National Academy of Sciences* **89**, 962–966 (1992).

425 36. Randall, T. D., Brewer, J. W. & Corley, R. B. Direct evidence that J chain regulates the
426 polymeric structure of IgM in antibody-secreting B cells. *Journal of Biological Chemistry*
427 **267**, 18002–18007 (1992).

428 37. Niles, M. J., Matsuuchi, L. & Koshland, M. E. Polymer IgM assembly and secretion in
429 lymphoid and nonlymphoid cell lines: Evidence that J chain is required for pentamer IgM
430 synthesis. *Proceedings of the National Academy of Sciences* **92**, 2884–2888 (1995).

431 38. Cattaneo, A. & Neuberger, M. S. Polymeric immunoglobulin M is secreted by transfectants
432 of non-lymphoid cells in the absence of immunoglobulin J chain. *The EMBO Journal* **6**,
433 2753–2758 (1987).

434 39. Hiramoto, E. *et al.* The IgM pentamer is an asymmetric pentagon with an open groove
435 that binds the AIM protein. *Science Advances* **4**, (2018).

436

437 **Figure Legends**

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

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

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

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

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

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

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

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

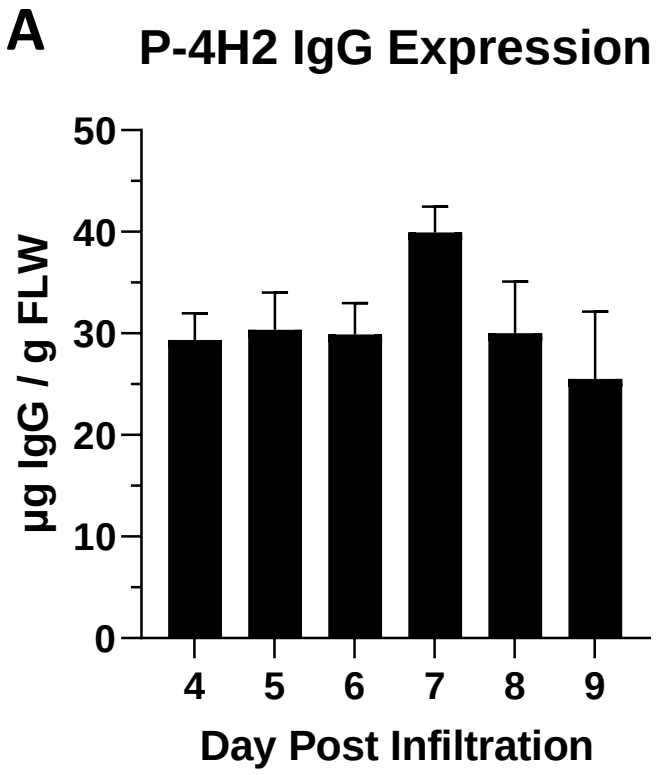
458 **Figure 5: Plant-Made 4H2 Isotypes Specifically Bind to Target Antigen.**

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

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

466 Recombinant CTS1 and *Coccidioides posadasii* mycelial culture supernatant were separated by SDS-PAGE
467 and either stained with Coomassie Blue (**A**) or transferred to PVDF membrane. Membranes were then
468 probed with the parental M-4H2 IgG (**B**), the humanized, P-4H2 IgG (**C**), or the humanized, P-4H2 IgM
469 (**D**), followed by the appropriate secondary HRP-conjugated antibody and development with a
470 colorimetric substrate. Lane 1, Recombinant CTS1. Lane 2, *Coccidioides posadasii* mycelial culture
471 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

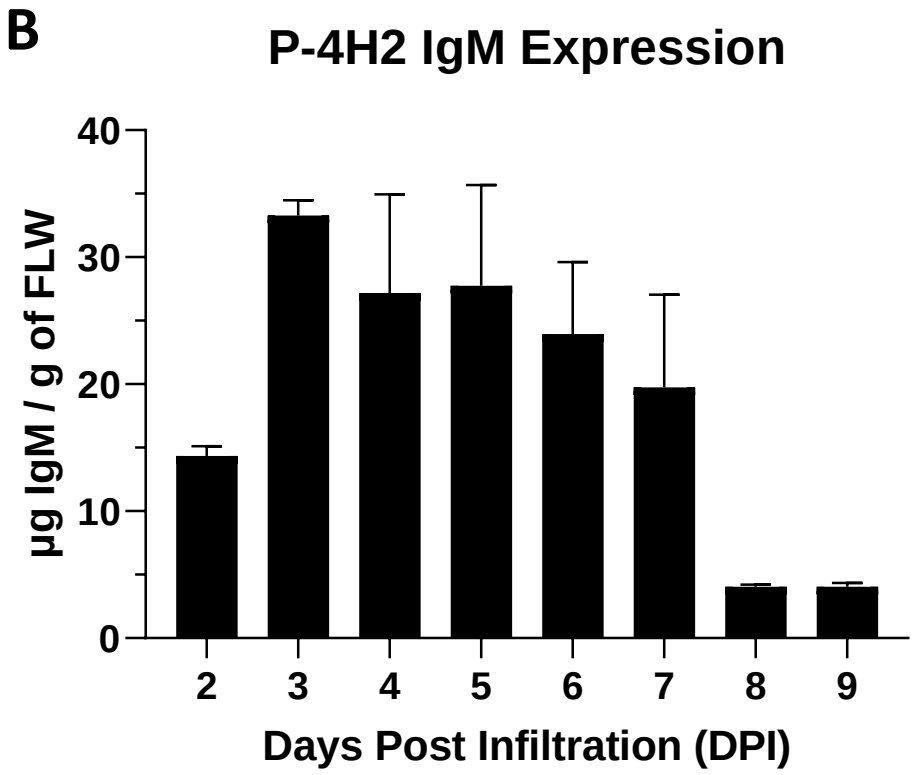

B
P-4H2 IgM Expression
µg IgM / g of FLW
40
30
20
10
0
2
3
4
5
6
7
8
9
Days Post Infiltration (DPI)

Figure 2

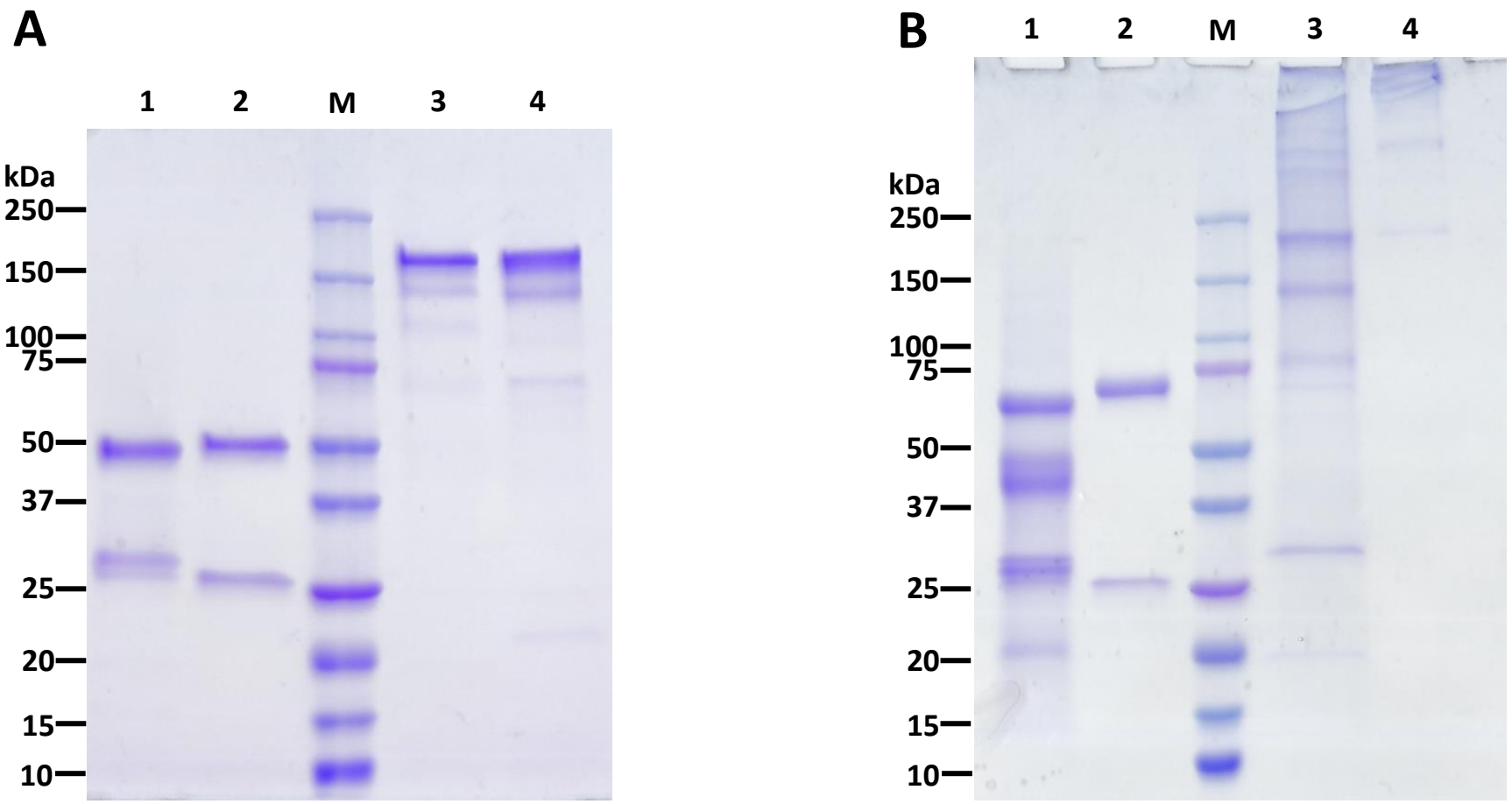

A
1
2
M
3
4
kDa
250
150
100
75
50
37
25
20
15
10
B
1
2
M
3
4
kDa
250
150
100
75
50
37
25
20
15
10

Figure 3

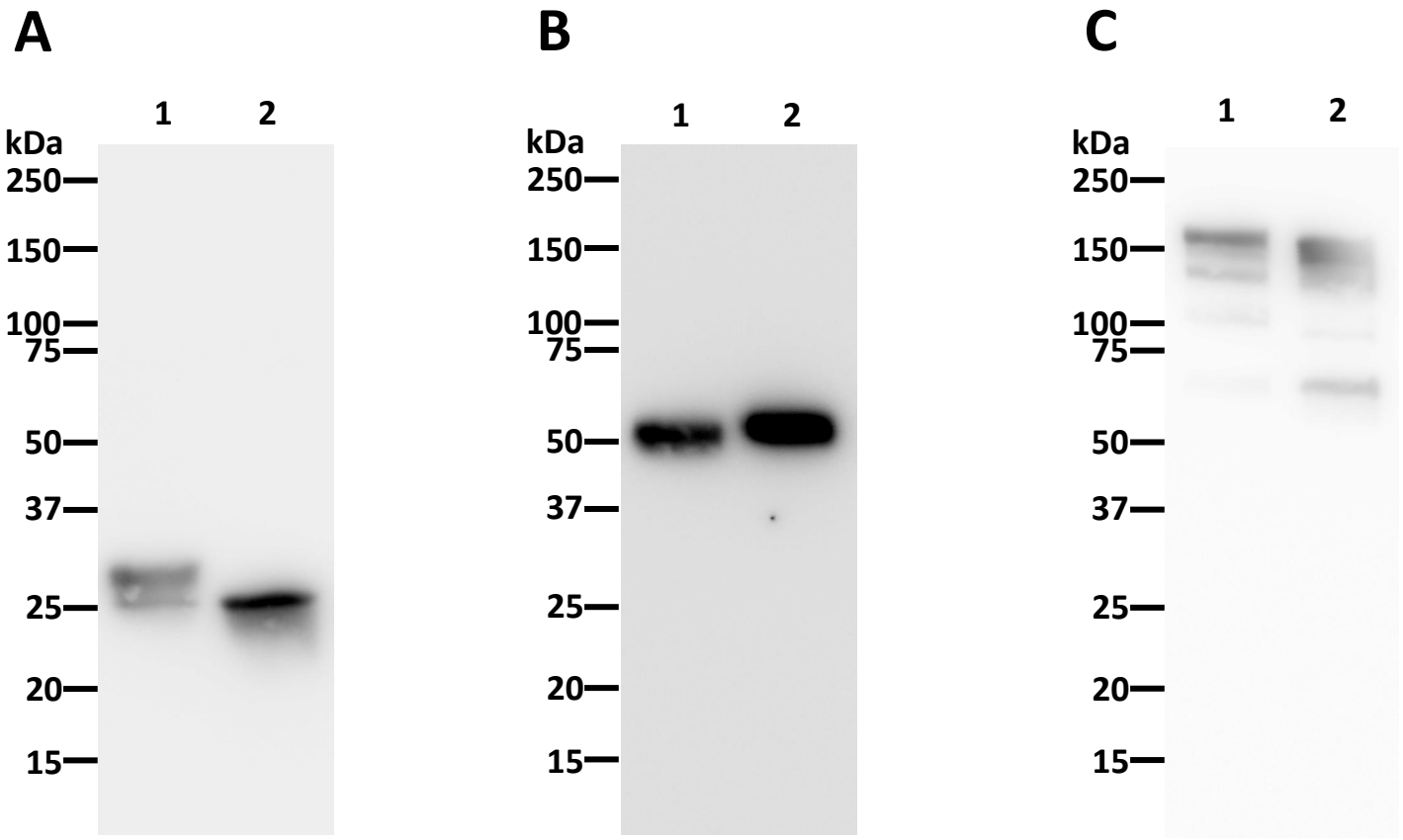

A
1
2
kDa
250
150
100
75
50
37
25
20
15
B
1
2
kDa
250
150
100
75
50
37
25
20
15
C
1
2
kDa
250
150
100
75
50
37
25
20
15

Figure 4

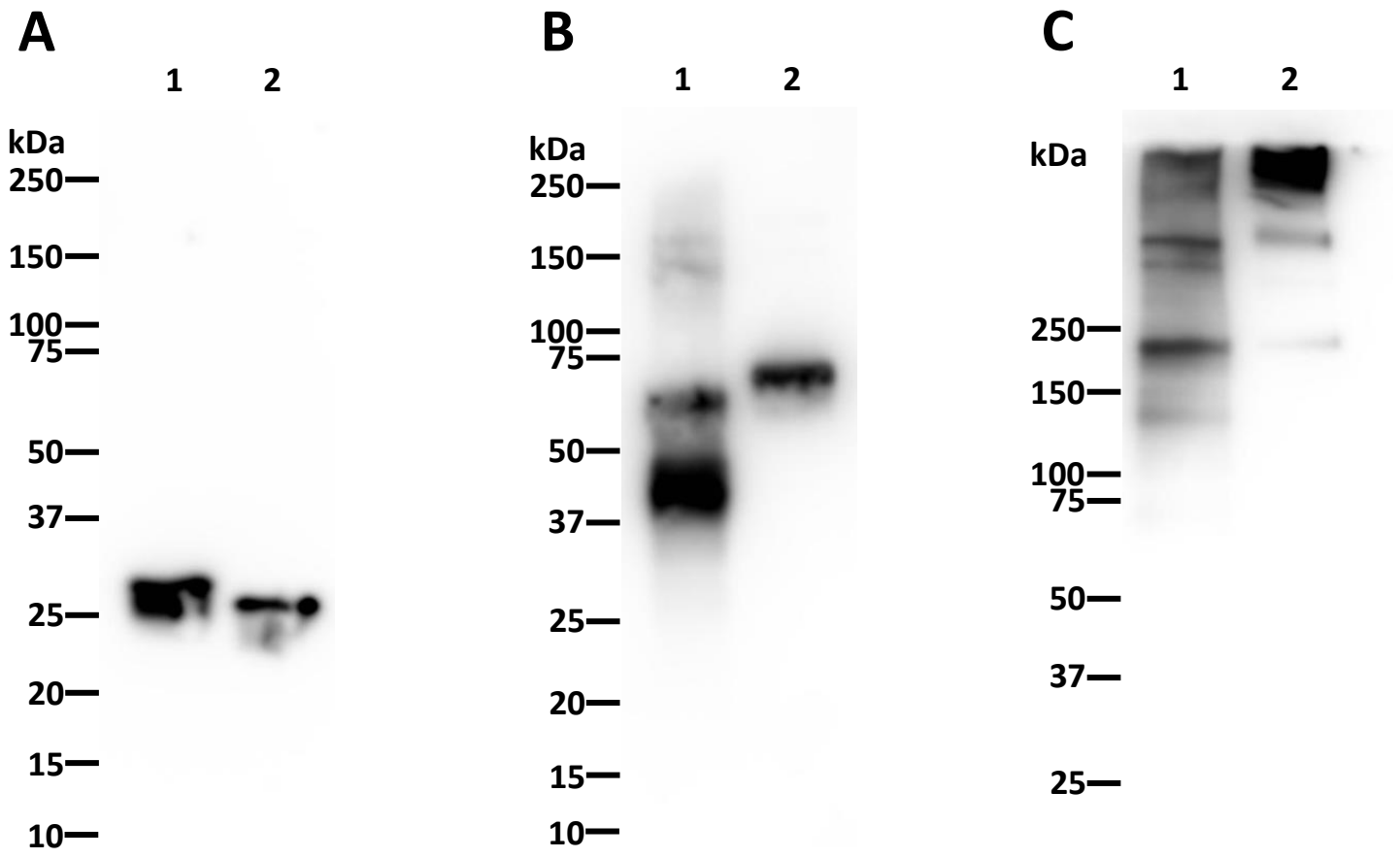
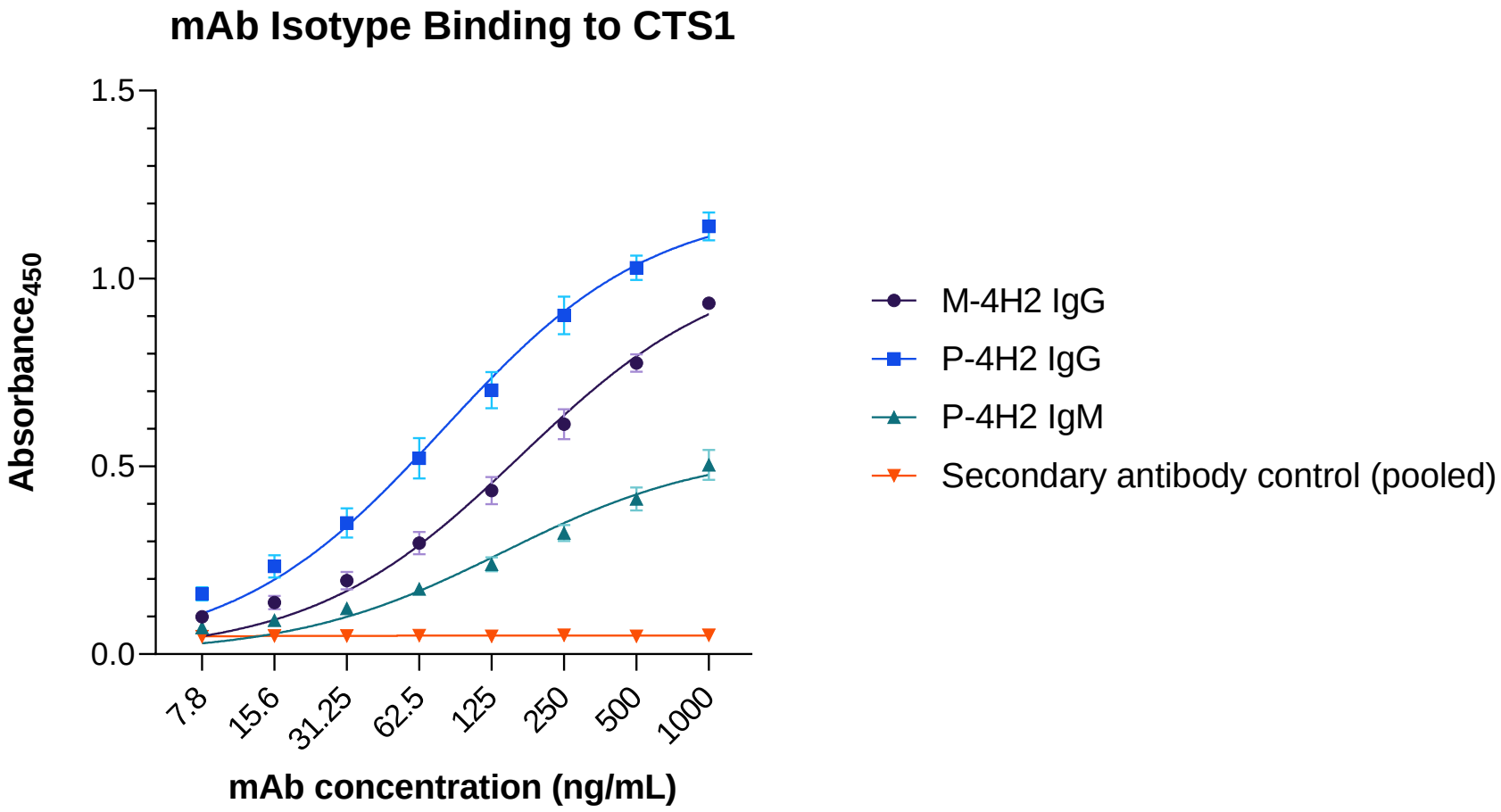

A
1
2
kDa
250
150
100
75
50
37
25
20
15
10
B
1
2
kDa
250
150
100
75
50
37
25
20
15
10
C
1
2
kDa
250
150
100
75
50
37
25

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)

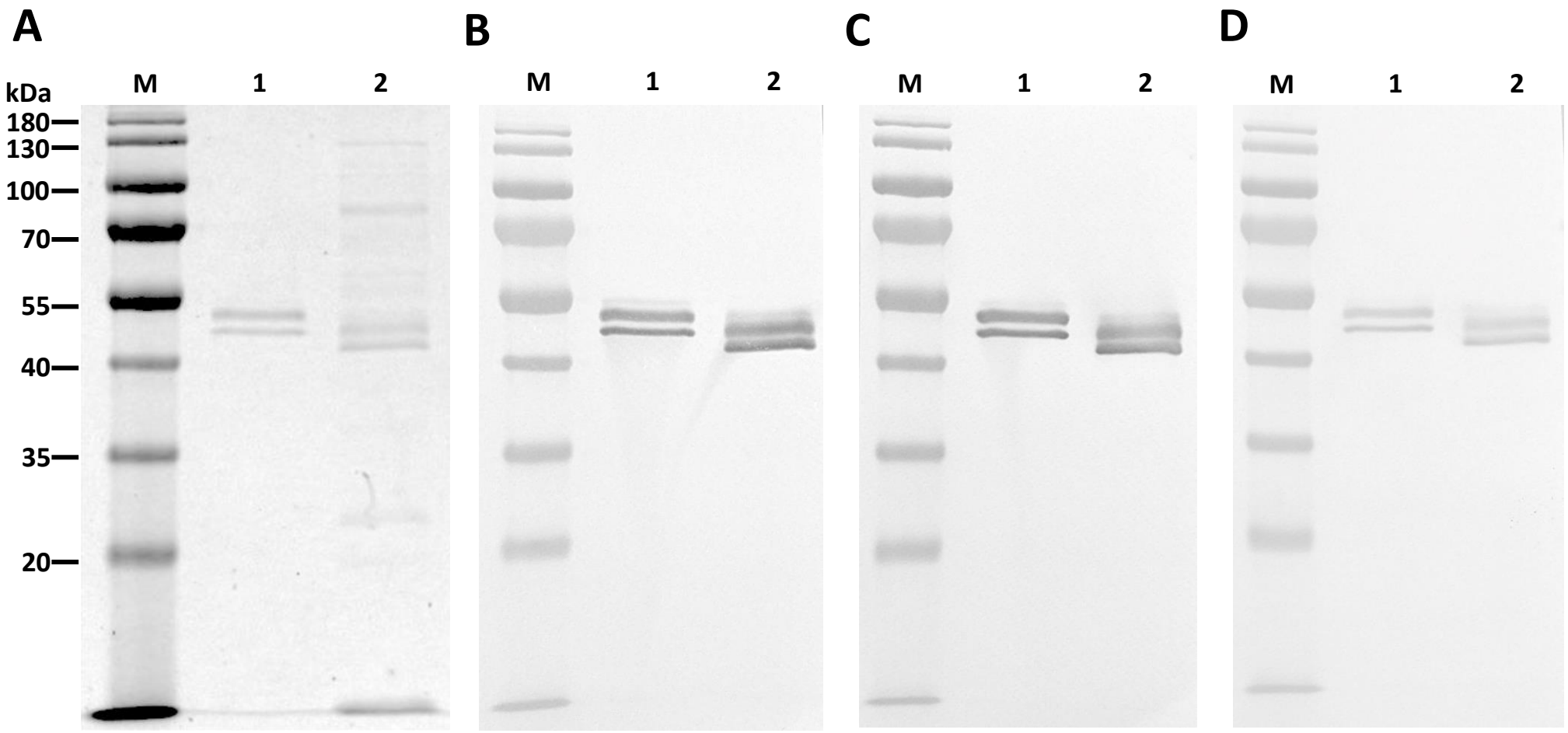



Figure 6