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# Robotic selection for the rapid development of stable CHO cell lines for HIV vaccine for production

**Short Title:** Rapid development of CHO cell lines for vaccine production**.**

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1 **Abstract**

2 The production of envelope glycoproteins (Envs) for use as HIV vaccines is challenging.
3 The yield of Envs expressed in stable Chinese Hamster Ovary (CHO) cell lines is
4 typically 10-100 fold lower than other glycoproteins of pharmaceutical interest.
5 Moreover, Envs produced in CHO cells are typically enriched for sialic acid containing
6 glycans compared to virus associated Envs that possess mainly high-mannose
7 carbohydrates. This difference alters the net charge and biophysical properties of Envs
8 and impacts their antigenic structure. Here we employ a novel gene-edited CHO cell
9 line (MGAT1$^-$ CHO) to address the problems of low expression, high sialic acid content,
10 and poor antigenic structure. We demonstrate that stable cell lines expressing high
11 levels of gp120, potentially suitable for biopharmaceutical production can be created
12 using the MGAT1$^-$ CHO cell line. We also show that the efficiency of this process can be
13 greatly improved with robotic selection. Finally, we describe a MGAT1$^-$ CHO cell line
14 expressing A244-rgp120 that exhibits improved binding of three major families of bN-
15 mAbs compared to Envs produced in normal CHO cells. The new strategy described
16 has the potential to eliminate the bottleneck in HIV vaccine development that has limited
17 the field for more than 25 years.

18

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19 **1 Introduction**

20 The development of a safe, effective, and affordable HIV vaccine is a global
21 public health priority. After more than 30 years of HIV research, a vaccine with these
22 properties has yet to be described. To date, the only clinical study to show that
23 vaccination can prevent HIV infection is the 16,000-person RV144 trial carried out in
24 Thailand between 2003 and 2009 (1). This study involved immunization with a
25 recombinant canarypox virus vector to induce cellular immunity (2-4) and a bivalent
26 recombinant gp120 vaccine designed to elicit protective antibody responses (5-7).
27 Although statistically significant, the protective efficacy of this vaccination regimen was
28 low (31.2%, P=0.04). Several correlates of protection studies suggested that the
29 protection observed was primarily due to antibodies to rgp120 (8-10). Thus, there is
30 considerable interest in finding ways to improve the level of protection that can be
31 achieved with rgp120 vaccine regimens. Improving an existing vaccine such as RV144,
32 with an established record of safety, would be faster and more cost-effective than
33 developing a new vaccine concept from scratch. A roadmap to improve the rgp120
34 vaccine used in the RV144 trial has been provided by the recent studies of broadly
35 neutralizing monoclonal antibodies (bN-mAbs) to gp120 as well as studies of the
36 carbohydrate content of virion associated Env proteins. Beginning in 2009, studies of
37 bN-mAbs isolated from HIV infected subjects revealed that many recognized unusual
38 glycan dependent epitopes requiring high-mannose glycans that are early intermediates
39 in the N-linked glycosylation pathway (11-20). Passive transfer studies reviewed by
40 Stephenson & Barouch (21) confirmed that these bN-mAbs could protect animals from
41 infection by SHIV viruses (22-27) and lower virus loads in HIV infected individuals (28,

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42 29). Multiple studies have now demonstrated that the carbohydrate present on virion
43 associated envelope glycoprotein, representing approximately 50% of its molecular
44 weight, is enriched for simple, high-mannose forms of N-linked carbohydrates rather
45 than the complex, sialic acid containing glycans found on most membrane bound and
46 secreted glycoproteins (20, 30-32). Since the rgp120 vaccine used in the RV144 study
47 and other clinical trials (33-35) was enriched for complex glycans (36), they lacked
48 multiple epitopes targeted by the high-mannose specific bN-mAbs. Thus the possibility
49 exists that rgp120s such as A244-rgp120 used in the RV144 trial, produced with the
50 glycans required to bind bN-mAbs, might be more effective in eliciting a protective
51 immune response than the previous rgp120 vaccines. To test this hypothesis in human
52 clinical trials, a practical way to produce large quantities of Env proteins possessing the
53 high-mannose glycans is required.

54 The production of recombinant HIV envelope proteins (rgp120 and rgp140) for
55 clinical research and commercial deployment has historically been challenging. Not only
56 is it labor intensive to isolate stable cell lines producing commercially acceptable yields
57 (e.g. >50 mg/mL) but it is also difficult to consistently manufacture a high quality, well
58 defined product with uniform glycosylation. Replacement of the native envelope signal
59 sequence (37, 38) and codon optimization (39) improved yields, but generating stable
60 CHO cell lines suitable for vaccine production remained difficult. Consequently, the
61 antigens used in the RV144 trials manufactured more than 20 years ago are still being
62 used in multiple clinical trials (40-44).

63 Recombinant gp120 typically possesses 25 or more potential N-linked
64 glycosylation sites making up more than 50% of the protein's mass (7, 45). Each

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65 glycosylation site can possess as many as 4 sialic acid residues, with up to as 79
66 different glycoforms (46) possible at a single site, resulting in enormous heterogeneity in
67 net charge and biophysical properties. This variability makes it hard to purify and define
68 the precise chemical structure of the recombinant protein. As pharmacokinetic and
69 pharmacodynamic properties of glycoproteins are in large part determined by the sialic
70 acid content, glycan heterogeneity represents a major source of product variability (47).
71

72 **2 Results**

73 Efforts to produce HIV Env proteins for clinical testing have been complicated by
74 problems of poor expression, heterogeneity in N-linked glycosylation and net charge,
75 and low yields from downstream purification (36, 46, 48-56). To address these problems
76 we combined a high efficiency electroporation device (MaxCyte STX, MaxCyte Inc.,
77 Gaithersburg, MD), a robotic cell selection system, ClonePix2 (Molecular Devices,
78 Sunnyvale, CA) and a novel cell line (MGAT1$^-$ CHO 3.4F10) that was recently
79 developed in our lab (57). The MGAT1$^-$ CHO cell line has a mutation in the Mannosyl
80 (Alpha-1,3-)-Glycoprotein Beta-1,2-N-Acetylglucosaminyl-transferase gene (MGAT1)
81 introduced by CRISPR/Cas9 gene editing. Recombinant gp120 produced by transient
82 transfection in MGAT1$^-$ CHO exhibited enhanced binding to three major families of
83 glycan dependent bN-mAbs (PG9, PGT128, and PGT121/10-1074) compared to
84 rgp120s produced in normal CHO or 293 cell lines. To explore the utility of MGAT1$^-$
85 CHO cells as a cellular substrate for biopharmaceutical manufacturing of HIV vaccines,
86 we attempted to create a stable MGAT1- CHO cell line expressing a variant of the
87 A244-rgp120 envelope protein that was used in the RV144 HIV-1 vaccine trial (1). This

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88 variant, A244_N332-rgp120, differed from the A244-rgp120 immunogen in that a single
89 N-linked glycosylation site was moved from N334 to N332 (58).

90 **2.1 Replacement of chemical transfection with electroporation.**

91 Estimating that the frequency of cells expressing high levels of rgp120 might be
92 in the range of one in $10^{-4}$ (59-61), we calculated that we needed to screen 10- 100
93 thousand transfectants. To optimize transfection efficiency, we replaced cationic lipid
94 transfection that we had previously used to transiently produce rgp120's (36, 57, 58, 62)
95 with electroporation. Use of the MaxCyte STX system resulted in reproducible
96 transfections with efficiencies typically greater than 80% in CHO-S or MGAT1$^{-}$ CHO
97 cells when GFP expression was quantified by flow cytometry, and viabilities greater
98 than 95% measured by trypan-blue exclusion (Fig. 1). Based on these results, MGAT1$^{-}$
99 CHO cells were electroporated with a plasmid designed for the expression of A244-
100 N332-rgp120, and the aminoglycoside 3'-phosphotransferase gene that confers
101 resistance to selectable marker, G418.

102 **2.2 Selection of stable MGAT1$^{-}$ CHO cell lines expressing A244_N332-rgp120**
103 **envelope proteins**

104 We used the ClonePix2 cell screening and selection robot to identify and select
105 the comparatively few transfectants secreting high levels of rgp120. In this system, high
106 producing cell colonies are visualized by the formation of a "halo" or immunoprecipitin
107 band formed by fluorescently labeled antibodies suspended in a semi-solid,
108 methylcellulose containing, cell culture matrix. After electroporation, cells were
109 suspended in a semisolid matrix containing the selectable marker G418 and antibodies
110 to rgp120 labeled with the Alexa 488 fluorophore. After six days, distinct colonies were

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111 visible. By sixteen days, 45,000 colonies dispersed among 8 six-well cell culture plates
112 had grown sufficiently for robotic screening and selection (Fig. 2A). When viewed under
113 fluorescent light (Fig. 2B), a small fraction of the cells exhibited halos resulting from
114 antibody-antigen precipitin bands that formed around the colonies secreting high levels
115 of rgp120. The top 0.1% (44) of colonies selected based upon morphology and halo
116 intensity were picked by the robot and expanded for further analysis (Fig. 2C). Selected
117 colonies were subsequently screened by ELISA for the ability of secreted rgp120 to bind
118 polyclonal antibodies, and the prototypic, glycan dependent bN-mAb PG9. PG9
119 recognizes an epitope in the V1/V2 domain and requires Man5 at position N160 for
120 binding (18). Based on the ELISA results, cells from the top 25 rgp120 producing
121 colonies were transferred to 24 well plates and screened by ELISA and immunoblot.
122 The best 15 lines were then expanded into 125 mL cultures for quantitative protein
123 expression assay under the same expression conditions used for transient expression
124 i.e. CD-CHO opt-CHO supplemented with glucose, glutamine, CHO- Feed A and
125 yeastolate. Six of these cultures were expanded for growth in 1.6 L shake flask cultures.
126 Immunoblot analysis (Fig. 3A) revealed that rgp120 made by the 6 MGAT1$^{-}$ CHO
127 colonies were smaller in size (85 kDa) compared to A244-rgp120 produced in normal
128 DG44 CHO cells (120 kDa). Comparison of reduced and non-reduced proteins detected
129 a trace amount of aggregated rgp120 protein and no proteolytic degradation (clipping).
130 Cultures were harvested when cell viability dropped to 50%. When culture supernatant
131 was assayed by ELISA (Fig. 3B) rgp120 titers of approximately 400 mg/L were
132 observed in in two cell lines (5D and 5F) with the 5C line exhibiting the lowest rgp120
133 titer (approximately 125 mg/L). Examining the kinetics of cell growth and rgp120

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134 accumulation in cell culture medium (Fig. 3C), we found that rgp120 production
135 increased after the addition of sodium butyrate at day six with the rate of accumulation
136 stabilizing between 10-14 days of cell culture. During this period, rgp120 became the
137 major protein secreted into the cell culture medium.

138 **2.3 Growth characteristics of MGAT1$^-$ CHO cells expressing A244_N332-rgp120**

139 Several experiments were performed to characterize the growth characteristics of
140 MGAT1$^-$ CHO cells expressing A244_N332-rgp120. The initial 125-500mg/L yield, was
141 obtained using culture conditions primarily designed for transient expression of
142 recombinant proteins following electroporation. Data is shown in Figure 4A-C from
143 triplicate cultures of 5F MGAT1$^-$ CHO (600 mL cultures grown in 1.6 L shake flasks) for
144 a 13-day culture period. Cells were grown at 37$^o$C until they reached late log growth
145 phase (six days) then sodium butyrate was added to enhance protein expression and
146 the temperature was shifted to 32$^o$C for the remainder of the experiment (Fig. 4A). The
147 cell viability ranged from 90-100% for the first 8 days of culture and then steadily
148 declined. In contrast the cumulative amount of rgp120 in the cell culture medium
149 continued to accumulate over the entire 13-day culture (Fig. 4C) reaching a maximum of
150 800 mg/L by harvest. Figure 4 panels D-F show a similar batch fed experiment to test
151 the effect of different feed additives on protein production by the 5F MGAT1$^-$ CHO line.
152 Five (duplicate) batch fed culture of the 5F MGAT1$^-$ CHO isolate were grown in shake
153 flasks in balanced CHO-Growth A (Irvine Scientific, Santa Ana, CA) media
154 supplemented with CHO Feed C, glucose, glutamine and one of each of a panel of
155 peptone hydrolysates; yeastolate, cottonseed, pea, wheat or CD-hydrolysate, which
156 support protein expression in CHO cells, reviewed in (63). The cells were again grown

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157 at 37$^{o}$C until they reached late log growth phase (six days) with a viable cell density
158 approaching 1x$10^{7}$ cells per ml, adding Sodium butyrate (1mM) and shifting the
159 temperature 32$^{o}$C for the remainder of the experiment (Fig. 4D). There were small
160 differences in cell growth and viability (Fig. 4D and 4F) and productivity with the different
161 peptone hydrolysate additives which might be further explored prior to large scale
162 production, however, all supported 1g/L production of rgp120 at harvest (Fig. 4F).
163 These studies demonstrate that the 5F MGAT1$^{-}$ CHO cell line expressing A244_N332-
164 rgp120 can be grown to high cell densities and is productive for up to 12-14 days in
165 culture. It is likely that media optimization and a regulated bioreactor system can
166 improve cell viability, cell densities, and rgp120 expression

167 **2.4 Sensitivity of A244-N322-rgp120 to digestion by Peptide-N-Glycosidase F and**
168 **Endoglycosidase H**

169 Recombinant gp120 produced in the 5F MGAT1$^{-}$ CHO cell line exhibited an
170 apparent molecular weight (MW) of (85 kDa). The same protein produced in CHO-S
171 cells, exhibited an apparent molecular weight of 120 kDa (Fig. 3A). This difference in
172 size would be expected if the glycans present in the protein produced in the MGAT1$^{-}$
173 CHO cell line were limited to Mannose -5 (Man5) or earlier intermediates in the N-linked
174 glycosylation pathway, and the glycans present in the protein produced in the CHO-S
175 cells consisted of the normal sialic acid containing complex carbohydrates. To test this
176 hypothesis, the proteins were digested with endoglycosidase H (EndoH) or Peptide-N-
177 Glycosidase F (PNGase F) (Fig. 5). EndoH selectively cleaves within the chitobiose
178 core of high-mannose and some hybrid oligosaccharides and thus cleaves the simple,
179 high mannose forms, of N-linked glycans but not the mature sialic acid containing N-

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180 linked glycans. In contrast, PNGase F cleaves between the innermost N-
181 acetylglucosamine and asparagine residues of high mannose, hybrid, and complex
182 oligosaccharides and is able to digest both simple and complex N-linked glycans. We
183 observed that PNGase F treatment converted the proteins produced in both the
184 MGAT1⁻ CHO and CHO-S cells to a common molecular weight of approximately 56
185 kDa. This result confirmed that the difference in molecular weight between the proteins
186 produced in the MGAT1⁻ CHO cell line and the CHO-S cell line could be attributed to
187 differences in the type of glycosylation and that approximately 50% of the mass of
188 rgp120 is carbohydrate. When the sensitivity to EndoH was measured, we found the
189 rgp120 produced in the MGAT1⁻ cells was mostly sensitive to digestion by EndoH,
190 whereas the rgp120 produced in the CHO-S cells was resistant to EndoH digestion.
191 This result confirmed that rgp120 produced in MGAT1⁻ CHO cells is derivatized
192 primarily with simple, high mannose glycans whereas the protein produced in CHO-S
193 cells is derivatized primarily with the complex, mature form of N-linked glycosylation.

194 **2.5 Binding of A244_N332-rgp120 by bN-mAbs**

195 The functional differences in the antigenic structures of A244_N332-rgp120 produced in
196 the MGAT1⁻ CHO cells and A244 rgp120 produced in normal CHO-S cells was
197 measured in a series of antibody binding experiments (Fig. 6). For these studies, the
198 binding of bN-mAbs to rgp120s expressed in a stable MGAT1⁻ CHO cell line (5F
199 MGAT1⁻ CHO) was compared to bN-mAb binding to the same protein expressed by
200 transient transfection in MGAT1⁻ CHO cells, and the A244 rgp120 protein transiently
201 expressed in CHO-S cells. In this regard, the protein expressed in CHO-S cells closely
202 resembled the A244-rgp120 protein used in the RV144 clinical trial. The panel of bN-

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203 mAbs included both glycan dependent bN-mAbs PG9, PGT121/101074, and PGT128
204 (17, 18) as well as the CD4 supersite site VRC01 antibody (64, 65).
205 For purposes of comparison, rgp120 binding to the virus entry inhibitor CD4-IgG
206 served as a positive control. We first examined rgp120 binding by the prototypic glycan
207 dependent bNAb PG9, that binds the core mannose residues of two glycans at position
208 N160 and either N156 or N173 within the V1V2 domain (18). The glycan at N160 was
209 initially identified as Man5 in the context of a V1V2 scaffold structure (66) but recent
210 reports suggest that PG9 is more tolerant of heterogeneity than initially thought (14, 67).
211 Consistent with the hypothesis that a complex glycan at position N160 might interfere
212 with PG9 binding, glycan mapping of CHO-S produced monomeric rgp120 revealed
213 complex glycans at positions N156 and N160 (68, 69). We observe a quantitative
214 difference (Fig. 6A) between the binding affinity of rgp120 A224-N332 produced in
215 MGAT1$^{-}$ CHO cells as measured by EC50 (1.33 nM) for PG9, when compared to the
216 RV144 antigen, rgp120 A224, produced in CHO S cells (no binding plateau). This result
217 is in concordance with data from previous transient transfection studies (57, 58).
218 Binding of bN-mAbs to protein produced in the 5F MGAT1$^{-}$ CHO cell line was
219 indistinguishable to protein produced by transient transfection in MGAT1$^{-}$ CHO cells.
220 We examined rgp120 binding to four bN-mAbs from two different families that are
221 known to recognize glycan dependent epitopes in the stem of the V3 domain. PGT126
222 and PGT128 bN-mAbs are both members of the PGT128 family, and were derived from
223 a common ancestral immunoglobulin VH gene (17). The bN-mAb 10-1074 is a member
224 of the PGT121 family, and both antibodies were derived from a common ancestral
225 immunoglobulin VH region gene distinct from the PGT128 family (13, 17). We observed

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226 significantly improved binding to members of the PGT128 family to MGAT1⁻ CHO-S
227 produced A244_N332 rgp120 compared to the CHO-S A244 protein; however the
228 magnitude of the difference was much greater for PGT126 compared to PGT128 (Fig.
229 6B and 6C). Improved PGT128 family binding is consistent with enrichment of N332
230 with Man5-9 glycans as PGT126/128 epitopes bind core residues of high mannose at
231 positions N301 and N332, or N295 and N334 ( 15, 17, 19, 20). Mass spectroscopy of
232 virion-derived gp120 has shown that asparagine at position 332 is occupied by Man5-9
233 glycans with Man8-9 dominating (32). However on CHO-S derived rgp120, high
234 mannose is dominant but not exclusive (68, 69). Our binding data suggests that the
235 PGT126 epitope is more sensitive to occlusion by proximal processed glycans than
236 PGT128.
237 Members of the PGT121 family (PGT121 and 10-10-74) differed considerably in
238 their ability to bind MGAT1⁻ A244_N332 and CHO-S A244 rgp120 (Fig. 5D and 5E).
239 PGT121 is different from all of the other glycan dependent bN-mAbs tested in that it
240 accommodates either a sialylated or high mannose glycan at positions N332 or N334,
241 and a sialyated glycan at position N137, whereas 10-1074 is N332 high-mannose
242 restricted (13, 17). We observed significantly improved binding of 10-1074 to
243 A244_N332 rgp120 produced in the MGAT1⁻ CHO cell line, and poor binding of
244 PGT121. PGT121 did not bind rgp120 made in CHO-S cells. In control experiments, we
245 found that all of the proteins tested bound to VRC01 bN-mAb. We noted a small but
246 consistent higher maximal binding of VRC01 to proteins produced in the MGAT1⁻ CHO
247 cell line compared to the CHO-S cell line (Fig. 6F). This difference was also previously
248 reported (57). VRC01 is an anti-CD4 binding site antibody with low affinity for glycan in

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249 glycan-array assay (65). Glycans N197, N276 and N262 or N263 overlap with the
250 binding site, and an N276 mannose core /VRC01 light chain contact was recently
251 identified by (70). All proteins tested bound comparably to CD4-IgG regardless of the
252 expression system (Fig. 6G), indicating that the CD4 binding site was properly folded
253 and that the concentrations of the individual proteins were identical. Similarly, all of the
254 Env proteins captured with the 34.1 Mab used in this experiment, bound comparably to
255 the PB94 polyclonal rabbit sera (Fig. 6H). Thus, there was no significant difference in
256 the concentration or coating efficiencies of the proteins used for the binding studies.

257 **2.6 Pathogen testing**

258 In order for the 5F MGAT1$^-$ CHO cell line to be considered as a substrate for
259 vaccine production by current Good Manufacturing Processes (cGMP), it needs to be
260 free of contamination by other cell lines and adventitious agents. To obtain data
261 supporting these criteria, cells from the 5F MGAT1$^-$ CHO cell line were screened for
262 contamination by a commercial testing laboratory (IDEXX, Inc., Columbia, MO). This
263 analysis used validated PCR based techniques to screen for contamination by cells from
264 multiple other species (human, mouse) and for contamination by a large number of
265 human and animal pathogens including mycoplasma and minute virus of mice (MVM).
266 No cellular, viral, or microbial contamination of the original research cell bank was
267 detected (Supplemental Tables 1 and 2).

268 **2.7 Outline of new process for the development of a stable MGAT1$^-$ CHO cell line**
269 **expressing HIV-1 rgp120**

270 Based on our results we were able to devise a new standardized cell line
271 production strategy for creating stable CHO cell lines expressing rgp120 and other Env

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272 proteins in a timeline of 8-10 weeks. An outline for this new cell line development
273 process including the experimental timeline for electroporation, colony selection, and
274 protein expression/ purification is shown in Figure 7. The use of high efficiency
275 electroporation, robotic screening, and the elimination of gene amplification strategies
276 all contribute to a major reduction in the time and cost of producing stable CHO cell
277 lines.
278

279 **3 Discussion**

280 In this study we describe the development of a new robotic cell line screening
281 and selection strategy for the rapid production of stable, high yielding CHO cell lines
282 suitable for the production of HIV vaccines. We also demonstrate that this strategy can
283 be successfully employed using the recently described MGAT1$^-$ CHO cell line (MGAT1$^-$
284 CHO 3.4F10) that limits glycosylation primarily to Man5 glycans and, to date, has only
285 been used for transient transfection experiments (57). Finally, we describe a MGAT1$^-$
286 CHO cell line (5F MGAT1$^-$ CHO) that produces high levels of a variant of gp120 from
287 the A244 isolate of HIV-1. A244-rgp120 was a key component in the AIDSVAX B/E
288 vaccine (5, 6) used in the RV144 clinical trial. The Env protein (A244_N332-rgp120)
289 produced by the 5F MGAT1- CHO cell line differs from the A244-rgp120 used in the
290 RV144 trial in the location of a single N-linked glycosylation site (N332 compared to
291 N334) and that it incorporates primarily Man5 glycans. These changes enable the
292 A244_N332 Env protein to bind bN-mAbs from three major families of bN-mAbs (PG9,
293 PGT128, and PGT121/10-1074) that did not bind to the original vaccine immunogen.
294 The addition of these glycan dependent epitopes represents a significant improvement

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295 in the antigenic structure of A244-rgp120 and might improve the level of efficacy that
296 can be obtained from an RV144-like immunization regimen.

297 Prior to the development of the MGAT1$^{-}$ CHO 3.4F10 cell line there was no
298 practical means by which recombinant Env proteins enriched for high mannose glycans
299 could be produced at large scale for HIV vaccine production. In these studies, we
300 demonstrated that CHO cell lines expressing up to 1g/L of rgp120 that are potential
301 candidates for vaccine antigen production, can be produced within 2-3 months. In
302 contrast, the CHO cell line used to produce A244-rgp120 used in the RV144 trial
303 produced only 20 mg/L and took more than 18 months to develop (Berman, P.W.,
304 personal communication).

305 Previous studies have shown that early intermediates in the N-linked
306 glycosylation pathway (Man5 or Man9) are essential components of many epitopes
307 recognized by bN-mAbs (11-16, 18, 19, 20). Additionally, we now know that HIV virion-
308 associated Env proteins are enriched for these early intermediates in the glycosylation
309 pathway (20, 31, 50, 71). The A244_N332-rgp120 protein produced in the 5F MGAT1$^{-}$
310 CHO cell line possesses multiple glycan dependent epitopes recognized by bN-mAbs
311 and appears to possess a glycan structure that more closely resembles authentic HIV
312 Env proteins compared to Env proteins produced in normal CHO cells. We hypothesize
313 that this “glycan optimized” immunogen produced in the 5F MGAT1$^{-}$ CHO cell line
314 might increase the potential level of protection documented in the RV144 trial from
315 31.2% (P=0.04) to the efficacy level of 50% or more thought to be required for
316 regulatory approval and clinical deployment (72).

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317 These studies demonstrate that the MGAT1⁻ CHO cell line is a suitable substrate
318 for stable cell line development (57). The 5F MGAT1⁻ CHO line can be grown at high
319 densities ($2x10^7$cells/mL) in serum free media for the length of time (8-10 days)
320 consistent with modern manufacturing methods intended for the production of HIV
321 vaccine immunogens. The success in cell line isolation was greatly enhanced by the
322 use of robotic selection. The MaxCyte STX electroporation system achieved
323 transfection efficiencies of greater than 80% with linearized plasmid. Another key step
324 in the robotic screening strategy was the need to develop an imaging reagent that
325 formed "halos" around rgp120 transfected cells. We found that the best "halos" resulting
326 from the formation of immune-precipitin bands in semi-solid methylcellulose media were
327 only obtained with fluorescently labeled polyclonal antibodies. Although previous
328 anecdotal reports suggested that mixtures of fluorescently labeled monoclonal
329 antibodies could form the immune-precipitin bands required for robotic selection, we
330 were unable to confirm these reports. Finally, we attempted to see if the robotic
331 selection could overcome the need for time-consuming gene amplification experiments
332 (via methotrexate or methionine sulphoximine), and we found that directly screening
333 approximately 40,000 clones in a single ClonePix2 experiment was adequate to isolate
334 a cell line that produced approximately 1g/L of rgp120 with no gene amplification.
335 Studies are in progress to determine whether the high expression levels found in this
336 study results from the selection of cell lines with high levels of amplified genes or from
337 integration of the HIV transgene into transcriptionally active regions of chromatin.
338 The new technology outlined here should allow for the rapid production and
339 testing of multiple new Env based vaccine concepts that have not previously been

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340 tested for lack of a fast and cost effective manufacturing process. Such concepts
341 include multivalent rgp120 vaccines (73), guided immunization vaccine strategies (73-
342 77), and Env proteins optimized for the binding of inferred ancestral forms of bN-mAbs
343 (78-80). In summary, by combining recent developments in transfection technology,
344 robotic selection, and gene editing, we have developed a novel method for the
345 production of recombinant Env proteins has the potential to improve the potency,
346 shorten the time, and lower the cost of HIV vaccine production. These improvements
347 provide the means to break the bottleneck in HIV vaccine manufacturing that has limited
348 the field for the last 20 years (81).
349

350 **4 Materials and methods**

351 **4.1 Cells and antibodies**

352 The suspension adapted, stable MGAT1$^-$ CHO cell line was created by targeted
353 inactivation of the gene encoding the enzyme, Mannosyl (Alpha-1,3-)-Glycoprotein
354 Beta-1,2-N-Acetylglucosaminyl-transferase in CHO cells using CRISPR/Cas9 gene
355 editing (57). Suspension adapted CHO-S cells were obtained from Thermo Fisher
356 (Thermo Fisher, Life Technologies, Carlsbad, CA). GnTI$^-$ 293 HEK cells were obtained
357 from the American Tissue Type Collection (ATCC). Broadly neutralizing monoclonal
358 antibodies (bN-mAbs), PG9, PGT121, PGT126, PGT128, VRC01, and 10-1074, were
359 obtained from the NIH AIDS Reagent Program (Germantown, MD) or produced from
360 published sequence data. The entry inhibitor CD4-IgG was identical to that described by
361 Capon et al. (82). The 34.1 murine monoclonal antibody was developed in our
362 laboratory (62) and is specific for a 27 amino acid sequence of Herpes Simplex Virus

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363 Type 1 glycoprotein D (gD) that has been used previously as a purification tag (7, 38).
364 Polyclonal antibodies were raised according to the guidelines of the Animal Welfare Act.
365 The immunization protocol was reviewed and approved by the Animal Care and Use
366 Committee of the Pocono Rabbit Farm and Laboratory (Pocono Laboratories and
367 Rabbit Farm, Canadensis, PA). Polyclonal rabbit-serum (PB94) was obtained from
368 rabbits immunized with a mixture of A244 and MN rgp120 as previously described (6).
369 Polyclonal goat-serum was raised from goats immunized with a cocktail of purified
370 rgp120s from three clades of HIV (CRF01_AE, B, and C) produced in GnTI$^-$ 293 HEK
371 cells. Rabbit and goat polyclonal anti-gp120 for use in immunoassays were purified by
372 affinity chromatography using a HiTrap Protein G column (GE Healthcare, Little
373 Chalfont, United Kingdom). Immunoaffinity purified anti-gp120 for use in the ClonePix2
374 robot (Molecular Devices, Sunnyvale, CA) was isolated from goat sera by
375 immunoaffinity chromatography using a column consisting of gp120 coupled to
376 Sepharose 4B (GE Healthcare, Little Chalfont, United Kingdom). Immunoaffinity
377 purification involved successive passage over rgp120 bound affinity columns which
378 were then washed with 10 column volumes of 50 mM Tris, 0.5 M NaCl, 0.1 M TMAC
379 (tetramethyl-ammonium chloride) buffer (pH 7.4). Bound antibody was eluted with 0.1 M
380 sodium acetate buffer, pH 3.0, and the eluent neutralized by the addition of 1.0 M Tris
381 (1:10 ratio). The purified antibody was adjusted to a final concentration of 1-2 mg/mL in
382 PBS buffer as determined by bicinchoninic acid (BCA) and conjugated to Alexa 488
383 (Thermo Fisher Scientific, Waltham, MA), before 0.1 μM filtration.

384 **4.2 Cell culture conditions**

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385 MGAT1$^-$ and CHO-S cells were maintained in CD-CHO medium supplemented
386 with 8 mM GlutaMAX, 0.1 mM Hypoxanthine, and 0.16 mM thymidine (HT) in shake
387 flasks using a Kuhner ISF1-X shaker incubator (Kuhner, Birsfelden, Switzerland) at
388 37°C, 8% $CO_2$, and 125 rpm. Static cultures were maintained in 6, 24, and 96 well cell
389 culture dishes (Greiner, Kremsmünster, Austria) and grown in a Sanyo incubator
390 (Sanyo, Moriguchi, Osaka, Japan) at 37°C and 8% $CO_2$. For protein production, CD-
391 OPTI-CHO or CHO Balanced Growth A medium (Irvine, Santa Ana, CA) was
392 supplemented with 2 mM GlutaMAX, HT and MaxCyte CHO A Feed which is comprised
393 of 0.5% Yeastolate, BD, Franklin Lakes, NJ; 2.5% CHO-CD Efficient Feed A, 2 g/L
394 Glucose (Sigma-Aldrich, St. Louis, MO) and 0.25 mM GlutaMAX). Cell culture media
395 and additives were obtained from Thermo Fisher Life Technologies (Carlsbad, CA)
396 unless otherwise stated. In preliminary batch fed cell culture experiments to optimize
397 protein yield, we tested out an additional four peptone hydrolysates replacing yeastolate
398 with; Proyield Cotton, Proyield Pea, Proyield Wheat (Friesland Campira, Delhi, NY) and
399 CD-Hydrolysate (SAFC, Carlsbad, CA) and CHO-CD Feed efficient A with CHO- CD
400 Efficient Feed C (Thermo Fisher Life Technologies, Carlsbad, CA).

401 **4.3 Molecular cloning and sequencing**

402 Standard genetic engineering techniques were used to construct a G418
403 selectable expression vector (UCSC1331) that encodes gp120 from the clade
404 CRF01_AE strain of HIV-1. The protein produced is identical in sequence to the A244-
405 rgp120 protein used in the RV144 clinical trial with the exception that a single N-linked
406 glycosylation site at N334 has been moved to position N332, described by Doran et al.
407 (58), GenBank ref MG189369. The plasmid was similar to the commercially available

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408 pCDNA3.1 vector with the exception that methylation targets at positions C41 and C179
409 in the CMV promoter were deleted as described by Moritz and Gopfert (83). All
410 sequencing was performed at the University of California Core Sequencing Facility
411 (Berkeley, CA). pCI_GFP, a gift from Dr. James Brady (MaxCyte), was transfected in
412 parallel with the gp120 expression plasmid to monitor transfection efficiency. Plasmid
413 DNA was prepared using the endotoxin free Qiagen Giga Prep purification kit (Qiagen,
414 Hilden, Germany) and linearized by digestion with Pvu1 (New England Biolabs, Ipswich,
415 MA) prior to electroporation.

416 **4.4 Selection of stable MGAT1$^{-}$ CHO cell lines expressing A244-rgp120**

417 Electroporation of the UCSC1331 plasmid into MGAT1$^{-}$ CHO cells was
418 performed using a MaxCyte scalable transfection system (STX, MaxCyte Inc.,
419 Gaithersburg, MD) according to the manufacturer's instructions. Twenty-four hours after
420 electroporation, cells were diluted to a concentration of 1000 or 5000 cells/mL in
421 methylcellulose CHO-Growth A with L-glutamine (Molecular Devices, Sunnyvale, CA)
422 containing 500 µg/mL of G418 and 10 µg/mL of Alexa 488 labeled immunoaffinity
423 purified polyclonal goat anti-gp120 antibody. The plates were incubated at 37$^{o}$C with 8%
424 $CO_2$ and 85% humidity for 16 days, then colony selection was performed using a
425 ClonePix2 robot (Molecular Devices, Sunnyvale, CA). Colonies were imaged under
426 white and fluorescent light (470 nm excitation and 535 nm emission wavelength filter
427 set). Both images were superimposed, and colonies selected according to mean
428 exterior fluorescent intensity (84). The top ranking 0.1% of colonies were aspirated with
429 micro-pins controlled by the ClonePix2 system and dispersed automatically in a 96-well
430 plate containing CHO Growth A medium (Genetix Molecular Devices, Sunnyvale, CA)

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431 supplemented with HT, 8 mM GlutaMAX, and 500 µg/ml G418, and cultured at 37$^0$C,
432 with 8% $C0_2$ and 85% humidity. After 9 days in culture, protein production was assayed
433 by ELISA, and positive colonies transferred to 2 mL wells and shake flasks when cell
434 mass permitted transfer. Six lines were cultured for protein production (Section 2.2) at a
435 volume of 600 mL in 1.6 L shake flasks (Thompson, Oceanside, CA)

436 **4.5 Protein quantification**

437 Protein concentration was determined by capture ELISA (62). Purified protein
438 and cell culture supernatant were analyzed on 4-12% Bis-Tris PAGE SDS gels in either
439 MES or MOPS gel running buffer (Thermo Scientific, Waltham, MA). For Immunoblot,
440 proteins were electrophoresed on a 4-12% NuPage PAGE SDS gel in MES running-
441 buffer, transferred to a PDVF membrane, then probed with a polyclonal rabbit anti-
442 rgp120 antibody (PB94) and an affinity purified secondary HRP conjugated goat anti-
443 rabbit H+L chain antibody (Jackson ImmunoResearch, West Grove, PA) and visualized
444 using an Innotech FluoChem2 system (Genetic Technologies Grover, MO).

445 **4.6 Affinity purification of A244_N332-rgp120**

446 Recombinant proteins were immunoaffinity purified from cell culture media using
447 the gD purification tag as previously described (36) and protein concentrations
448 determined using bicinchoninic assay (BCA).

449 **4.7 Binding of bN-mAbs**

450 The binding to bN-mAbs to purified rgp120s from the MGAT1$^-$ CHO and CHO-S lines
451 was assayed with a capture Fluorescence Immunoassay (FIA) as described previously
452 (58). Briefly, Fluotrac high binding 96 well plates (Griener Bio-One Kremsmünster,
453 Austria) were coated with 2ug/ml 34.1 Mab overnight in PBS, then blocked with

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454 1%BSA/PBS 0.05% tween for 2 hours. Purified rgp120 was captured at 6ug/ml
455 overnight at $4^0$C. Three-fold serial dilutions of antibody, entry inhibitor or isotype control
456 were added to each well followed by Alexa 488 labelled polyclonal anti-species antibody
457 (Jackson ImmunoResearch, West Grove PA). Incubations were performed for 90 min at
458 room temperature followed by a 4x wash in PBS 0.05% tween buffer unless otherwise
459 noted. Absorbance was read using an EnVision Multilabel Plate Reader (PerkinElmer,
460 Inc Waltham, MA) using a FITC 353 emission filter and FITC 485 excitation
461 filter. Assays were performed in triplicate. EC50 was calculated from a plot of log
462 (agonist) vs response –variable slope (four parameters) on Graph Pad Prism 6 for Mac.,
463 GraphPad Software, La Jolla, CA.
464 Binding assays were carried out in triplicate.

465 **4.8 Enzymatic digestion of carbohydrate**

466 Enzymatic digestion of rgp120 was performed as described by Yu et al (36). For
467 molecular mass analysis post digestion, 2 µg of each protein was electrophoresed on a
468 4-12% Bis-Tris PAGE SDS gel in MOPS running buffer, and stained with Coomassie
469 Simply Blue (Thermo Scientific, Waltham, MA).
470
471

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476 **References**

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**by expression of green fluorescent protein (GFP).** MGAT1$^{-}$ CHO and CHO-S cells were transfected by electroporation with a linearized green fluorescent protein expression plasmid or mock electroporated. Forty-eight hours after transfection, viability was determined by Trypan blue exclusion on a BioRad TC10 as >95% by for both lines**.** Flow cytometry on a LSRII (Becton Dickinson, San Jose, CA) was used to calculate the percentage of transfected cells expressing GFP.

**Figure 2. Primary identification of high producer MGAT1$^{-}$ CHO lines expressing A244_N332 rgp120 by immunofluorescent labeling**. **(A)** G418 selected colonies visible in a single 35mm well illuminated with white light at 6 days. **(B)** The same single 35mm well illuminated with 490 nm wavelength light. Colonies actively secreting rgp120 have a green "halo" visible at 525 nm. **(C)** Relative mean exterior fluorescence of halo for more than 10,000 colonies imaged by the ClonePix2 plotted by rank. The top ranking 0.1% of colonies (44) were robotically picked and cultured. The six clones expressing 0.2-0.4 g/L at day 56 are shown in red.

**Figure 3. Analysis of A244_N332-rgp120 secreted from stable MGAT1$^{-}$ CHO cell lines.** Six stable MGAT1$^{-}$ CHO cell lines identified with the ClonePix2 were selected as potential substrates for HIV vaccine production. **(A)** Immunoblot of affinity-purified rgp120 (50 ng per lane) produced by each of six A244_N332-rgp120 cell lines: 3E, 5C, 5D, 3F, 6B, and 5F. Purified A244_N332-rgp120 produced in normal CHO DG44 cells

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(692) was shown for purpose of comparison. **(B)** Comparison of A244_N332-rgp120 protein yields as determined by ELISA from the six MGAT1$^{-}$ CHO cell lines. (**C)** SDS PAGE of rgp120 produced by the 5F MGAT1$^{-}$ CHO cell line. Supernatant samples (10 µl per lane) collected over the time course of the culture were electrophoresed on a 4-12% NuPage PAGE SDS gel in MOPS buffer (Thermo Scientific, Waltham, MA). The gel was stained with Simply Blue (Thermo Scientific, Waltham, MA) and visualized using an Innotech FluoChem2 system (Genetic Technologies, Grover, MO).

**Figure 4. Growth and expression of the 5F MGAT1$^{-}$ CHO cell line expressing A244_N332-rgp120 in shake flask cultures.** Cells were cultured under standard conditions until day 6 when 1 mM Sodium butyrate was added, and the temperature shifted to 32$^{o}$C. Panels **A-C**: cells were fed with CHO Feed A and yeastolate as indicated, and harvested at day 13 (data from 3 shake flasks averaged). **(A)** Timecourse graph of viable cell densities (VCD) determined by trypan-blue exclusion on a BioRad T20 cell counter. **(B)** Timecourse of cell viabilities determined by trypan-blue exclusion. **(C)** Timecourse of A244_N332-rgp120 protein accumulation determined by ELISA. Panels **D-F** demonstrate optimization of protein expression (at >1g/L) by use of different feed additives. Five duplicate pairs of cultures were fed (as indicated) with CHO Feed C and either yeastolate (BD, Franklin Lakes NJ), cottonseed, wheat, pea hydrolsate (Friesland Camparia, Delhi, NY) or CD-hydrolysate (SAFC, Calsbad CA) at days 6, 8 and 10, and harvested at day 12 (data from each pair of shake flasks is averaged). **(D)** Timecourse graph of viable cell densities (VCD) determined by trypan-blue exclusion on a BioRad T20 cell counter. **(E)** Timecourse of cell viabilities determined by trypan-blue

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exclusion. **(F)** Timecourse of A244_N332-rgp120 protein accumulation determined by ELISA.

**Figure 5. SDS-PAGE analysis of A244_N332 rgp120 HIV produced in 5F MGAT1$^-$ CHO and CHO-S cells treated with PNGase or EndoH**. Enzymes and buffers were purchased from (New England Biolabs, Ipswich, MA). Purified protein was denatured and reduced then incubated overnight at 37$^o$C with or without glycosidase. Protein was resolved (2 µg/lane) on 4–12% SDS-PAGE gel and stained with Simply Blue. Plus (+) indicates enzyme treatment, minus indicates untreated.

**Figure 6. Binding of bN-mAbs to A244-rgp120 produced in normal and A244_N332-rgp120 produced in MGAT1$^-$ CHO cell lines.** A244_N332-rgp120 was purified from the stable clone 5F MGAT1$^-$ CHO cell line (closed circles) or from the MGAT1$^-$ CHO cells (open circles) transiently transfected with the UCSC 1331 plasmid. A244-rgp120 expressed and purified from transiently transfected CHO-S cells (open squares). Antibody binding was measured by a fluorescent immunoassay (FIA).

**Figure 7. The timeline for development of stable MGAT1$^-$ CHO cell lines expressing HIV-1 rgp120.** Leading clones expressing 0.2-0.4 g/L in shake flasks under standard laboratory conditions were selected in less than two months. Production was subsequently increased to levels of g/L rgp120 production with minimal feed optimization.

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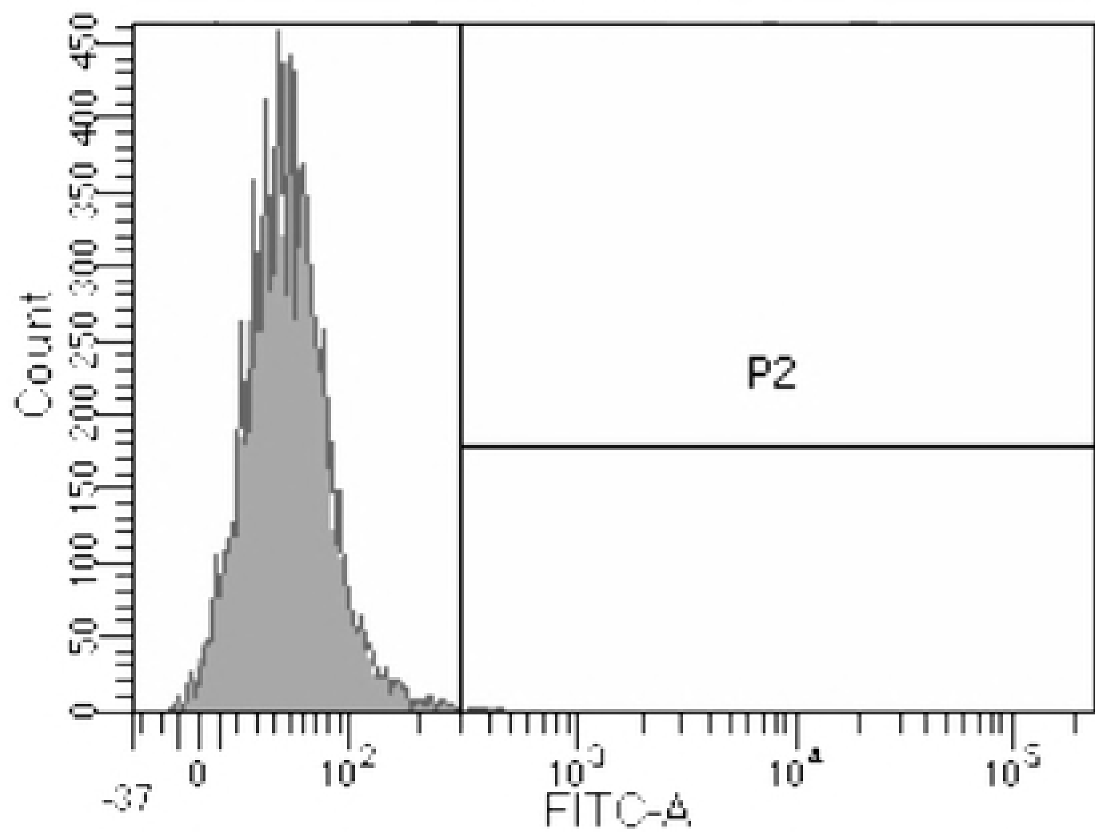
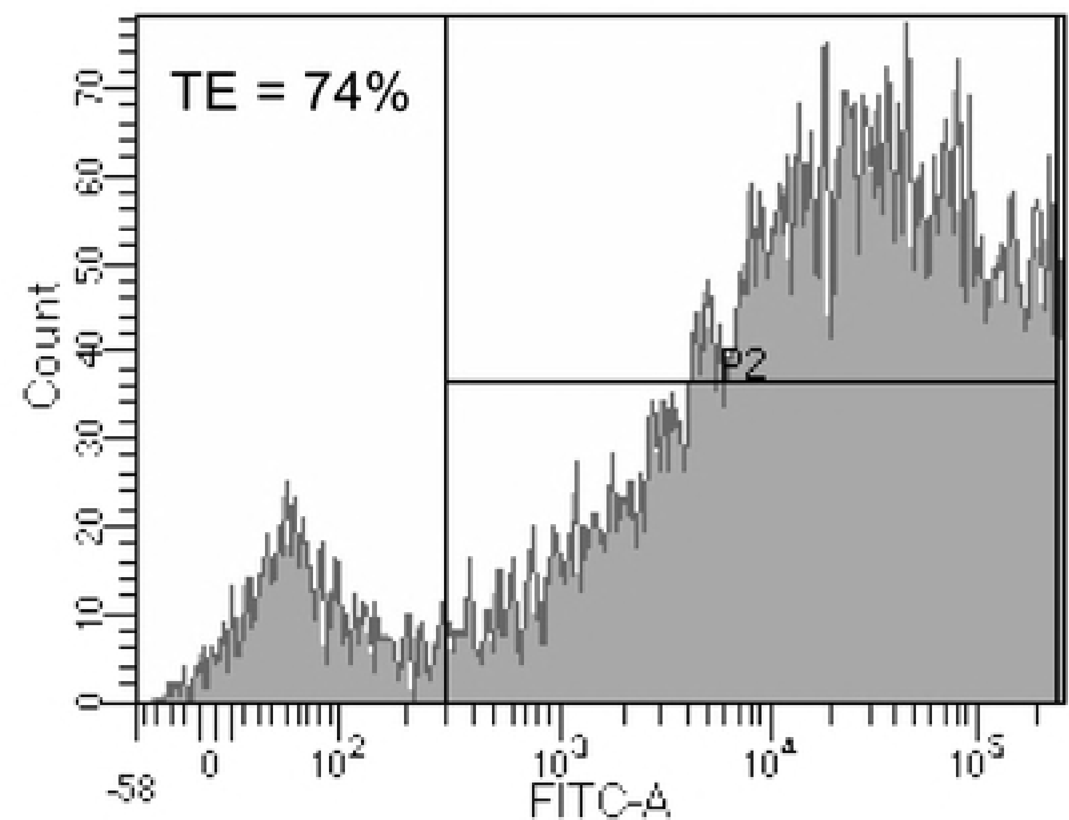
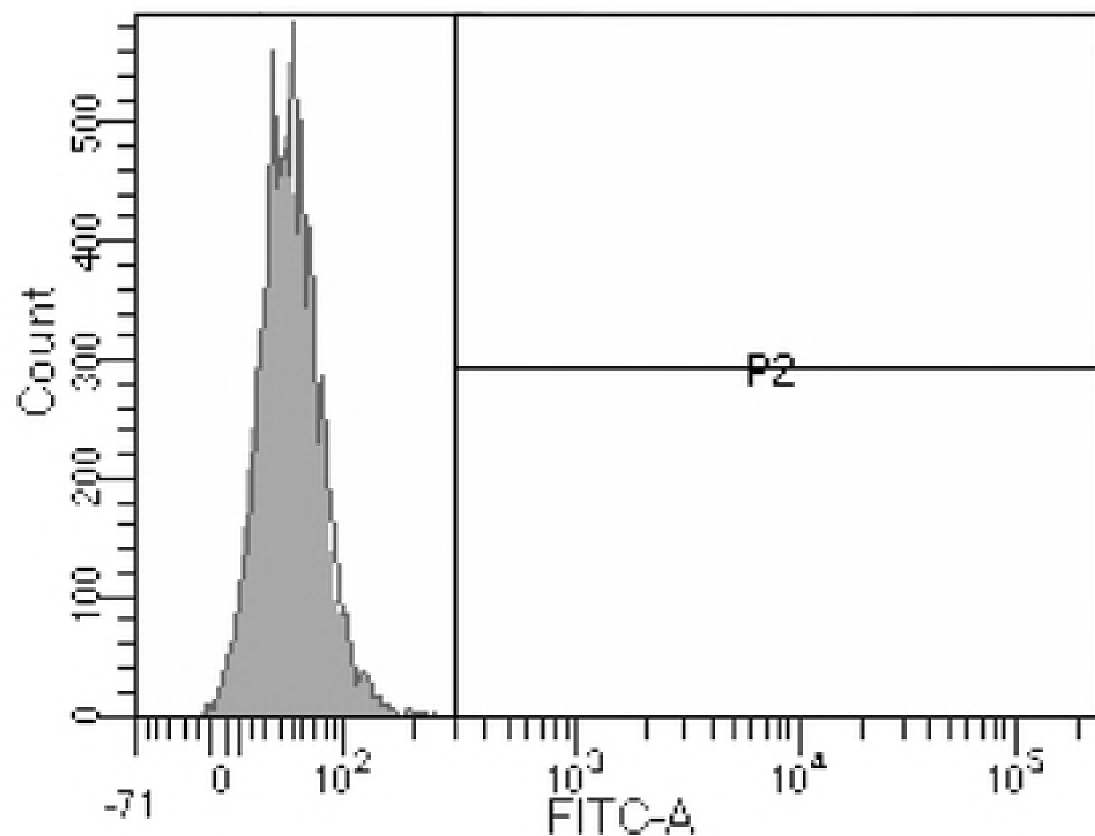
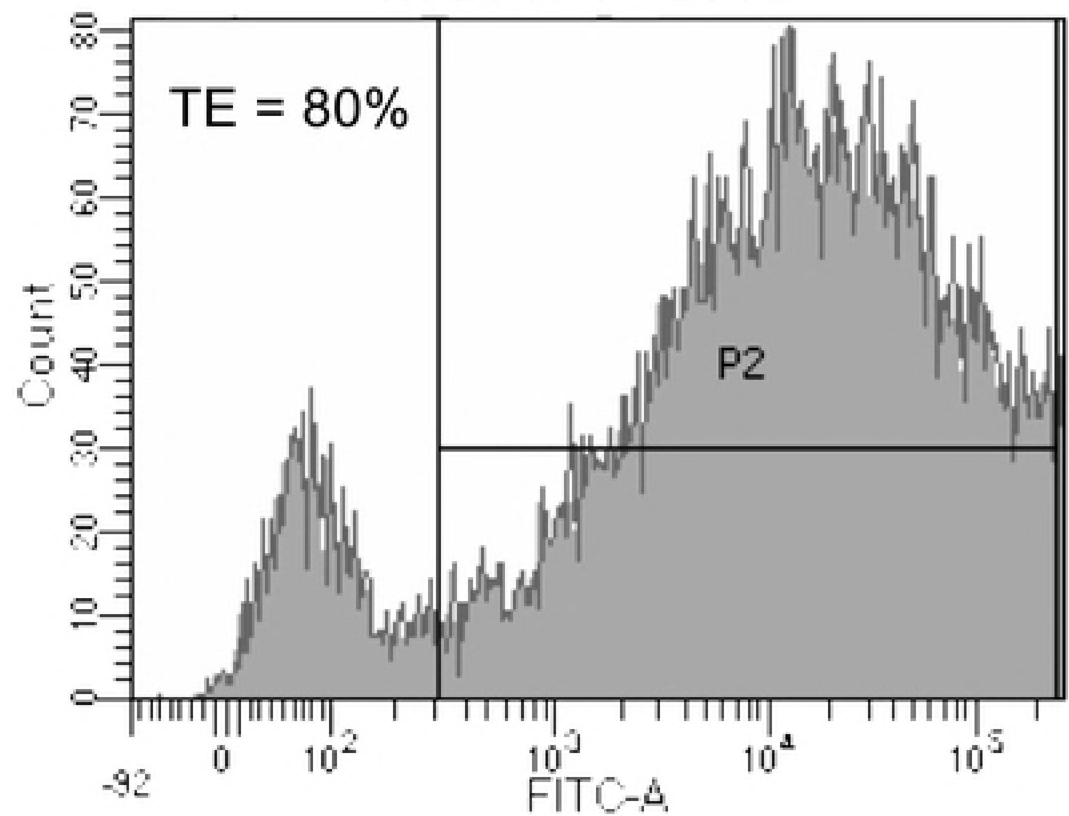
Mock Transfection

Transfection with GFP

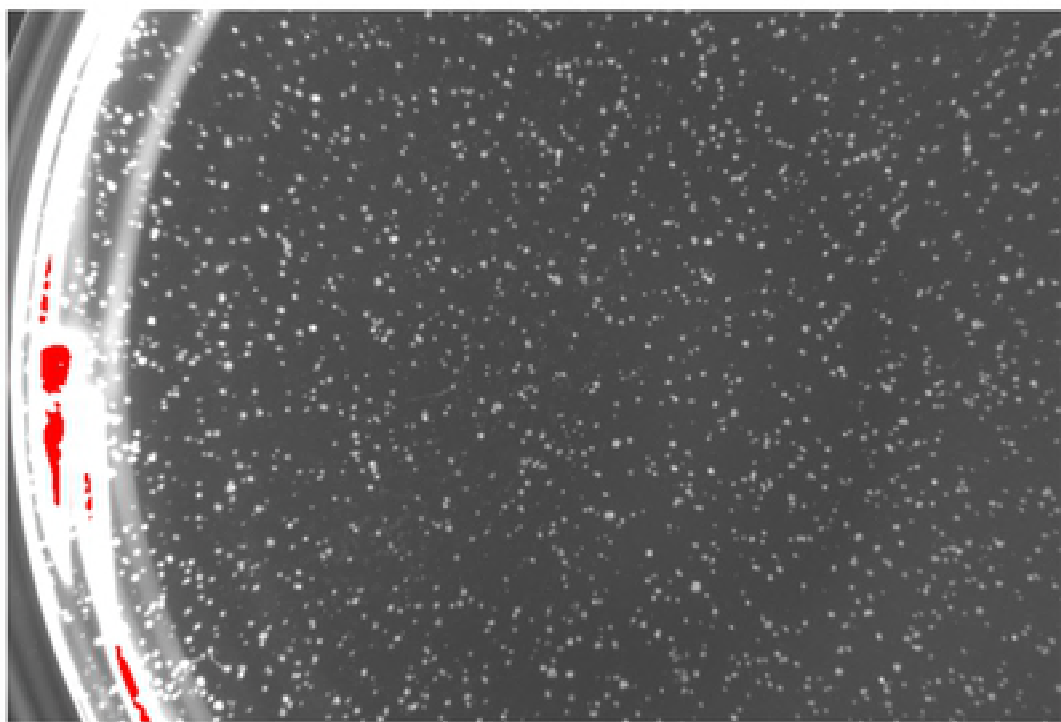


A



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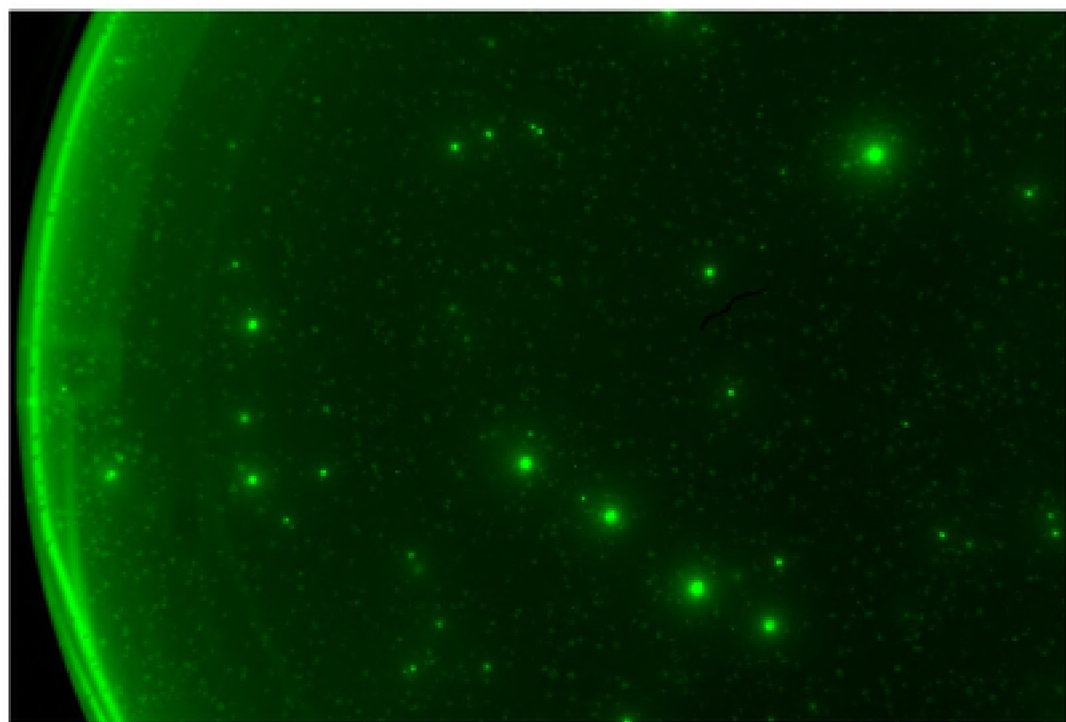


C

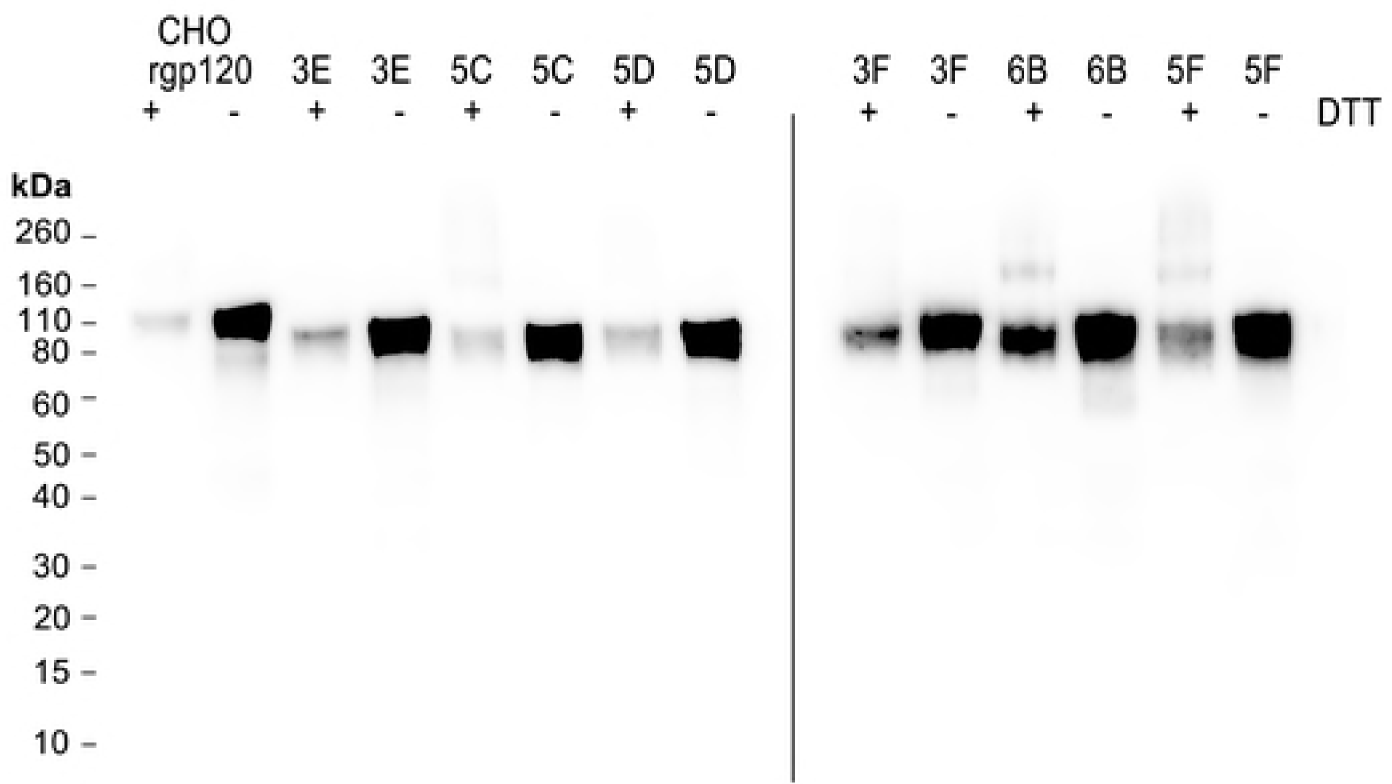
Mean Relative Exterior Intensity

10000
8000
6000
4000
2000
0

44 clones picked
top 6 clones at 56 days

0
15000
30000
45000

RANK

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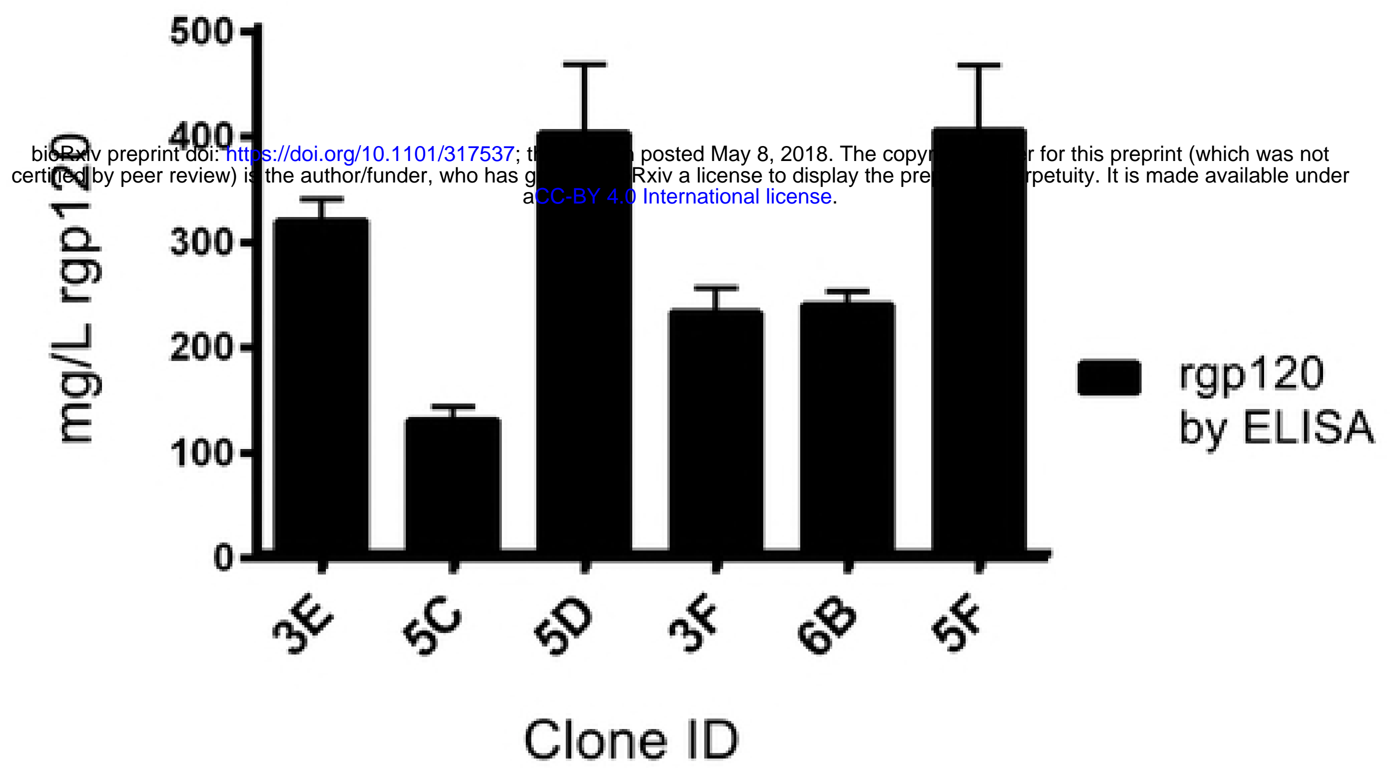


C

d0 d2 d4 d7 d9 d11 d12 d13 d14 rgp120 control

kDa
260
160
110
80
60
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40
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15
10

rgp120

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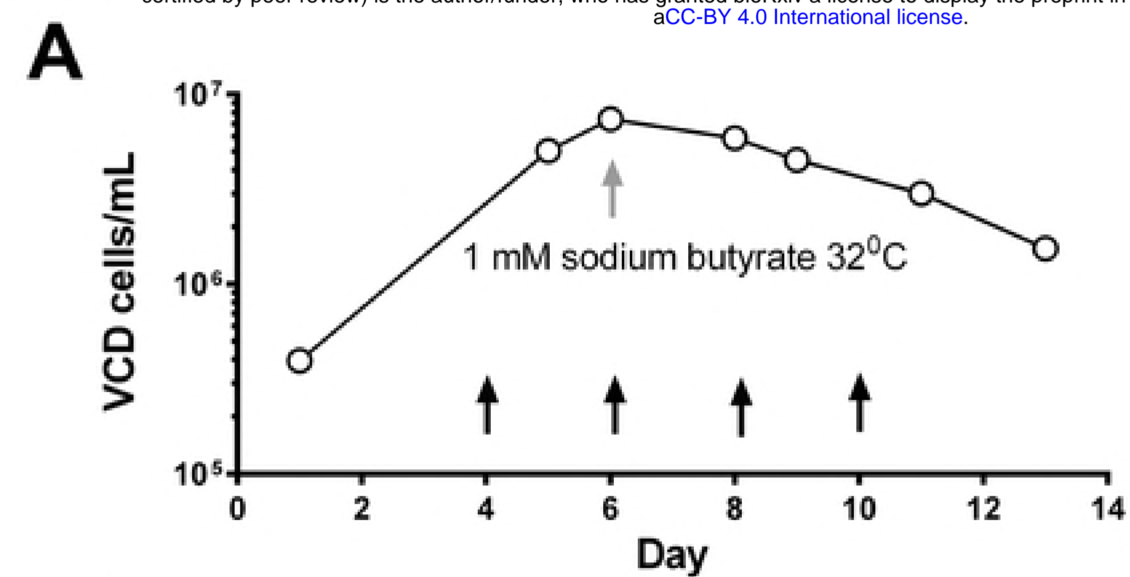
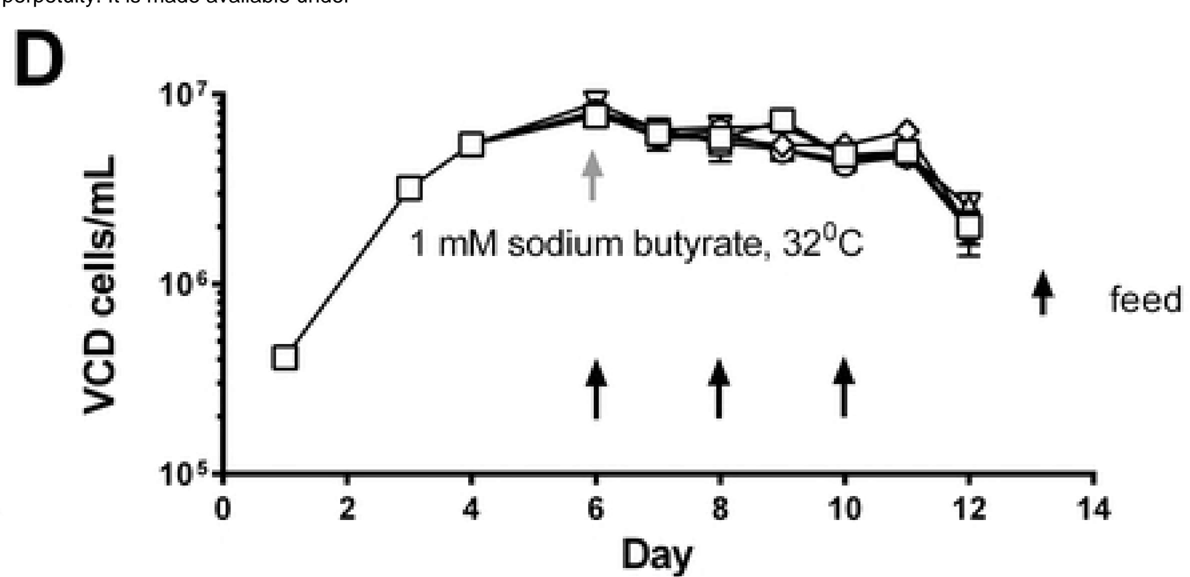
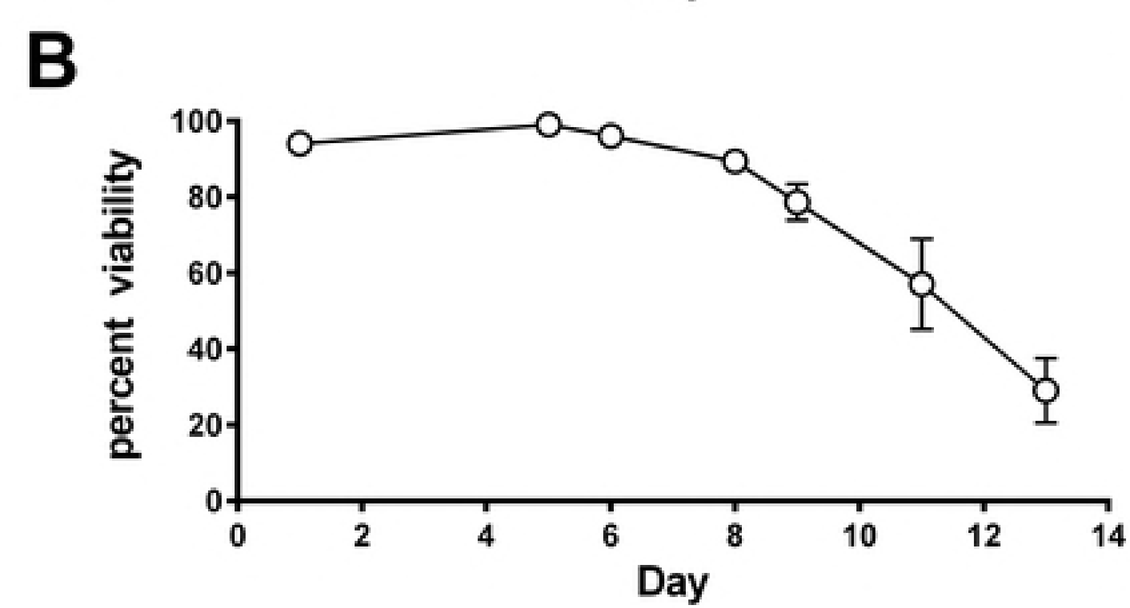
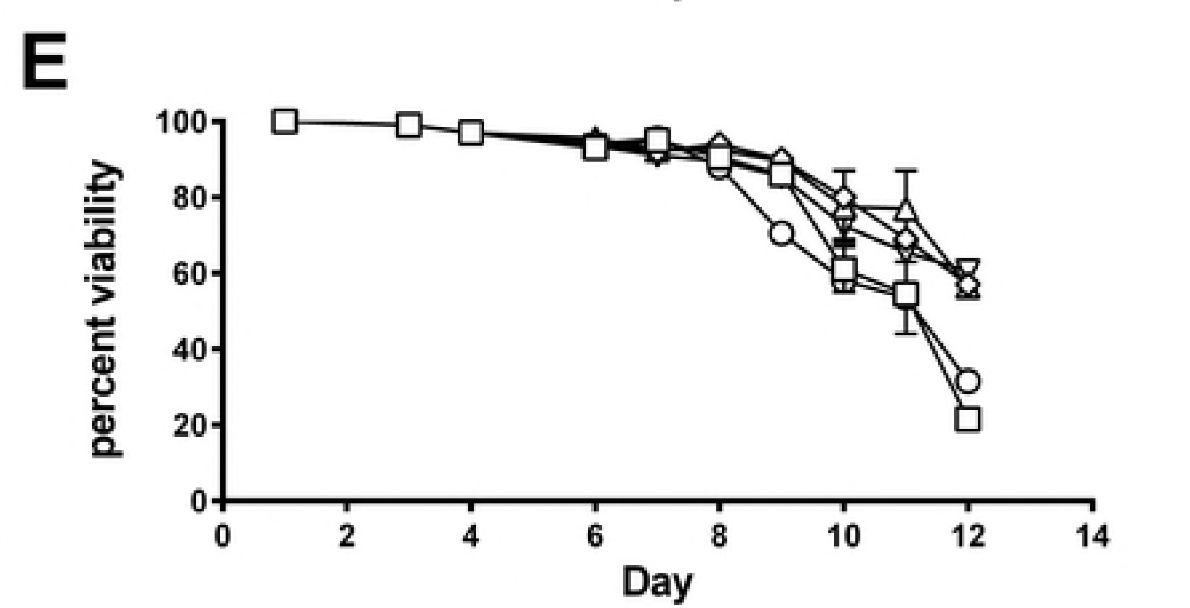
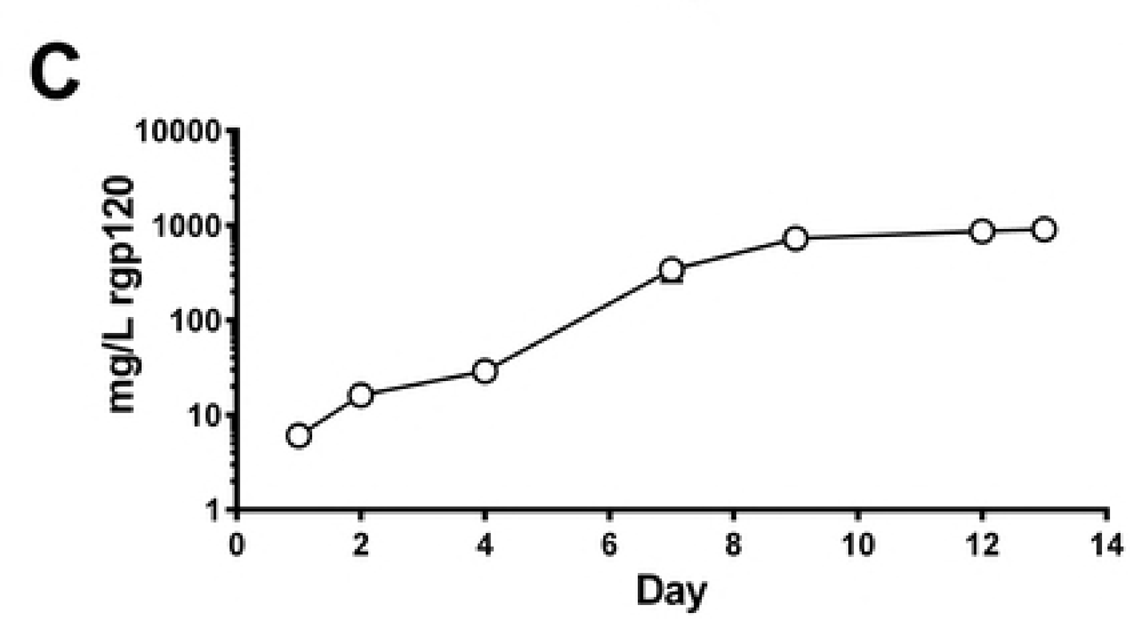
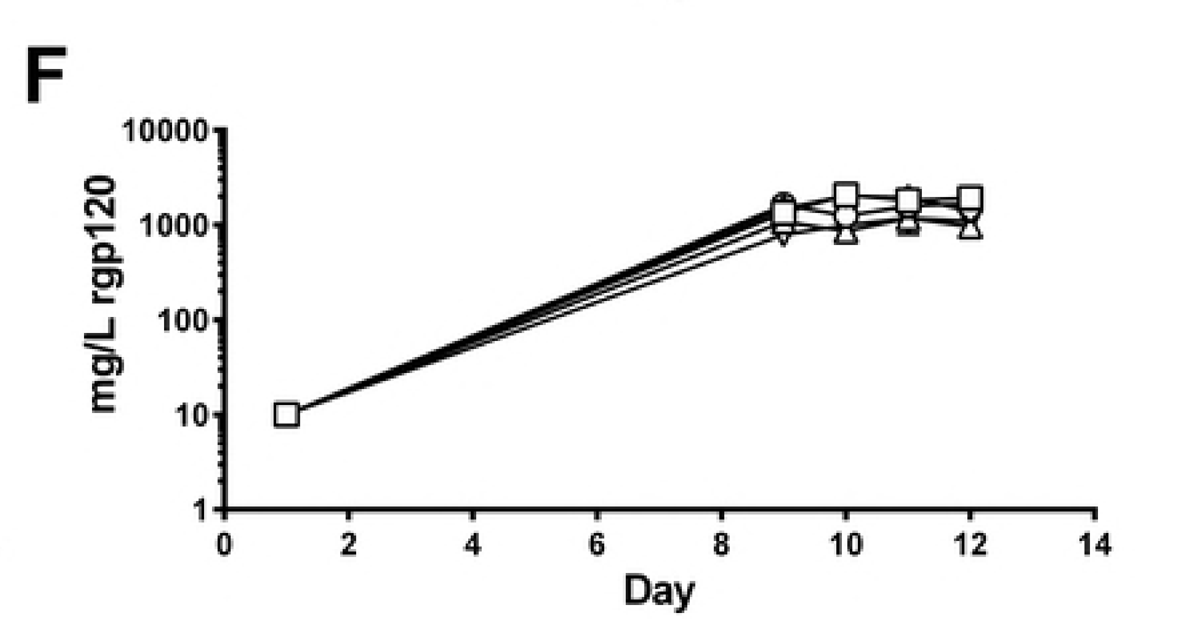


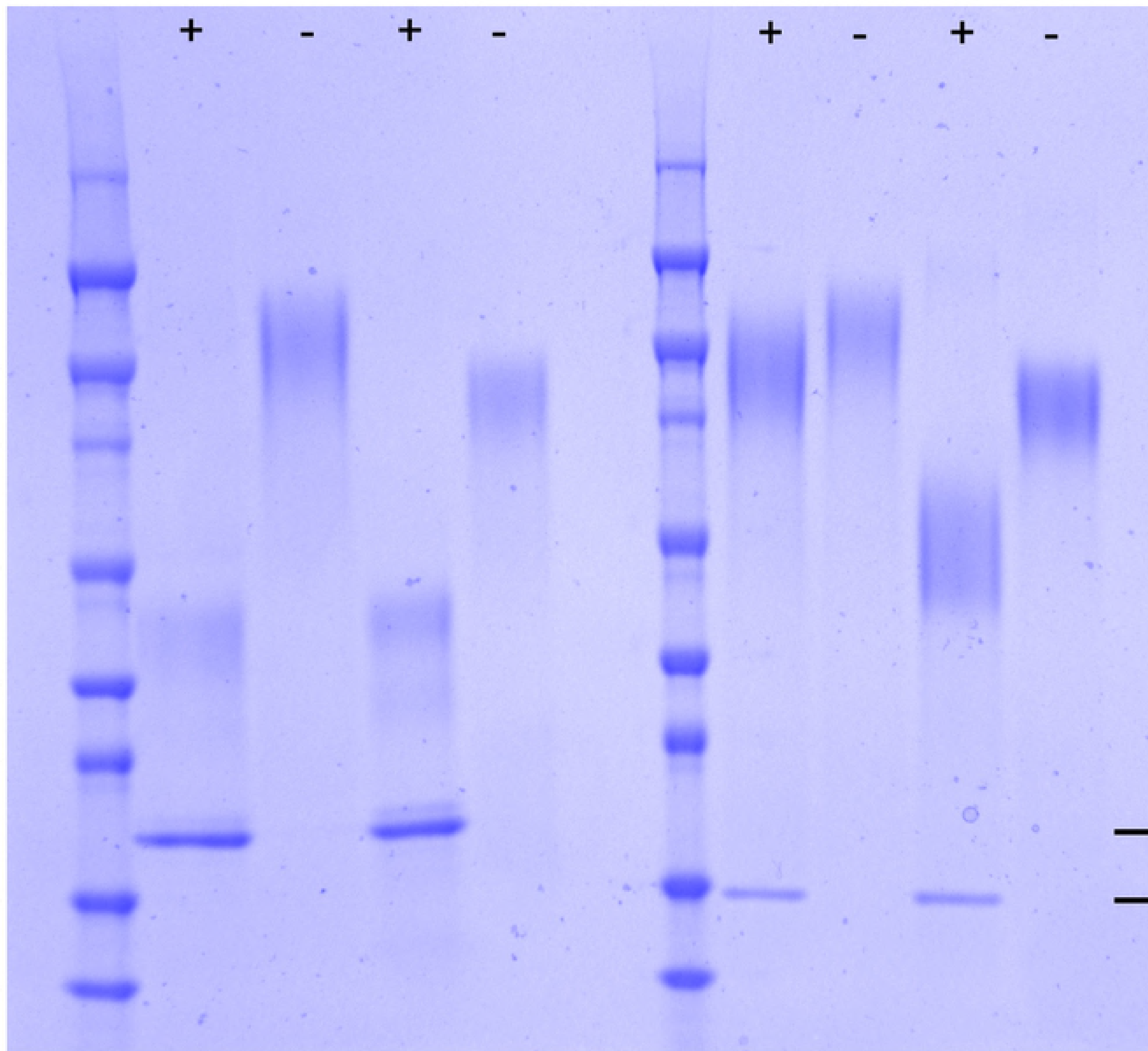




cottonseed  wheat  yeastolate
pea  CD-hydroysate


PNGase F Digestion
EndoH Digestion
CHO-S
MGAT1- (5F)
CHO-S
MGAT1- (5F)
A244_N332
A244_N332
A244_N332
A244_N332
+
-
+
-
+
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+
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kDa
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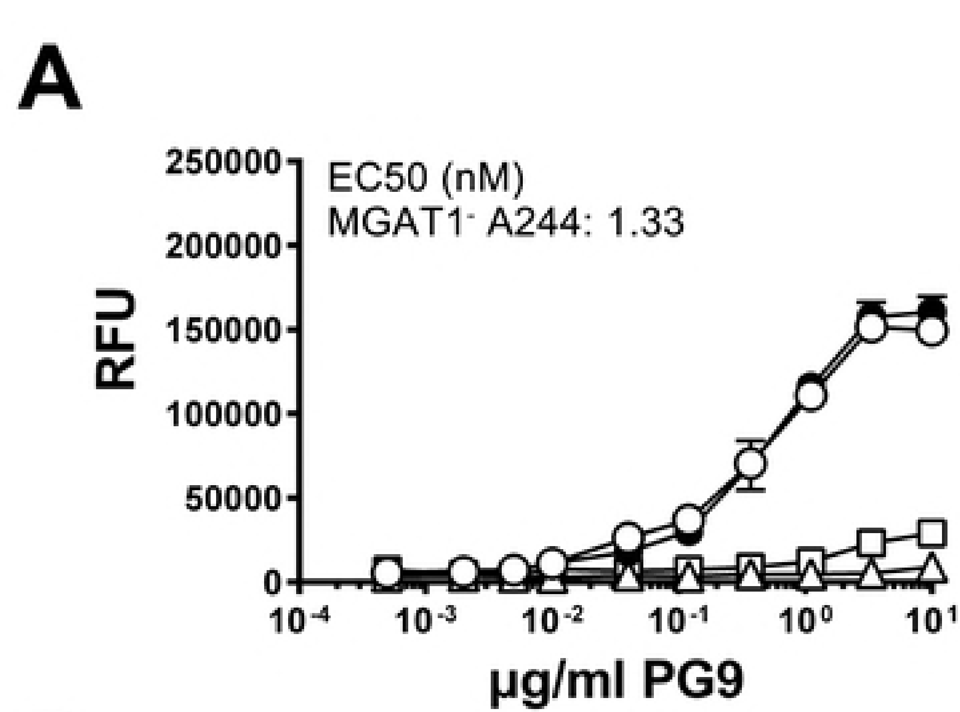
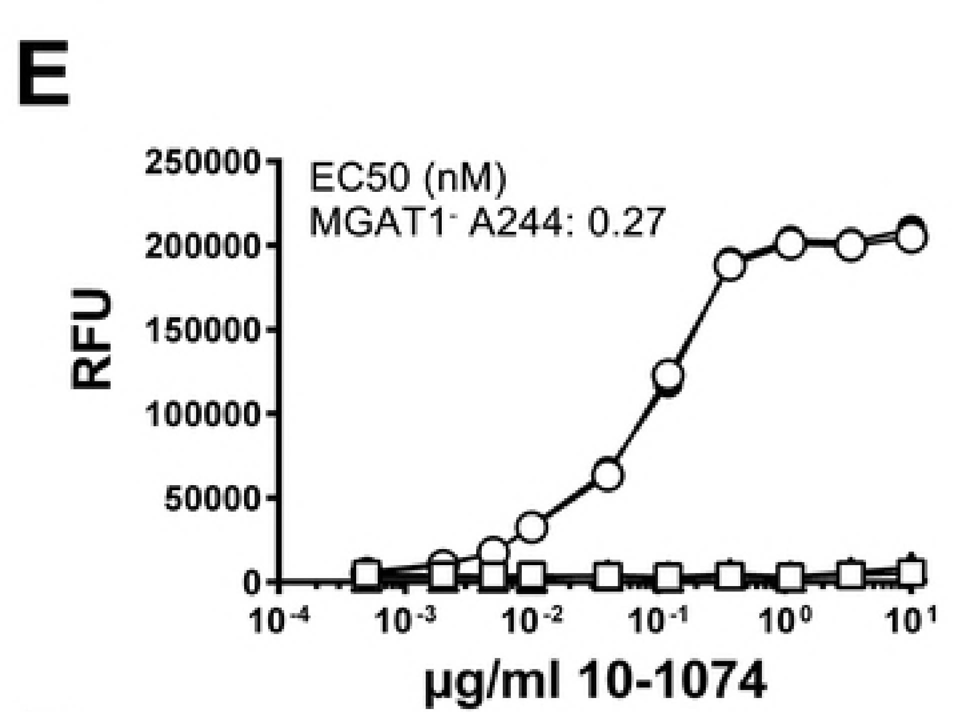
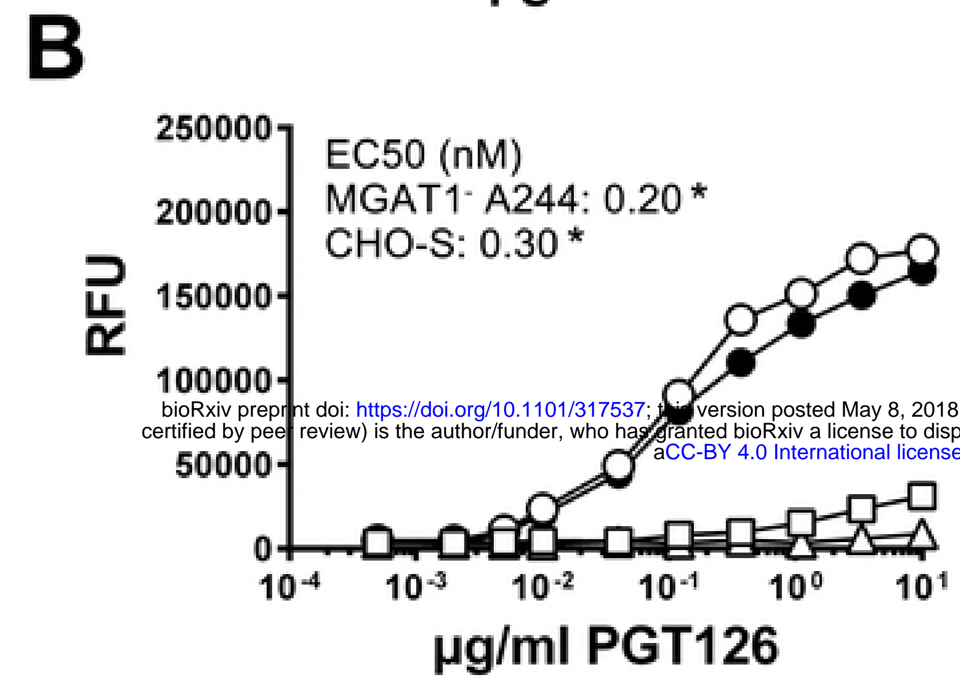
PNGase F
EndoH

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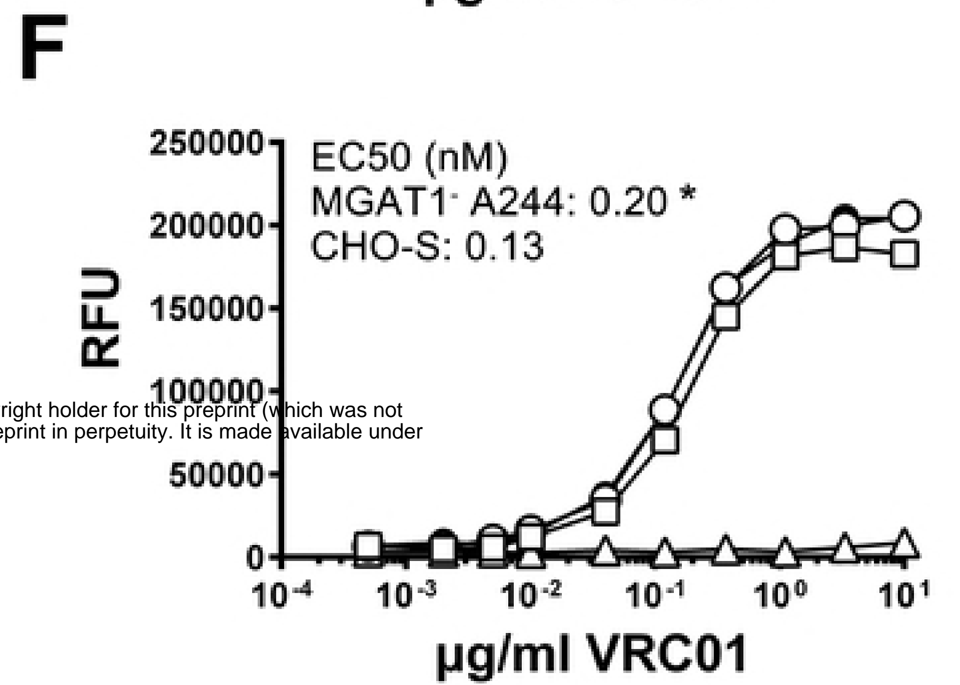
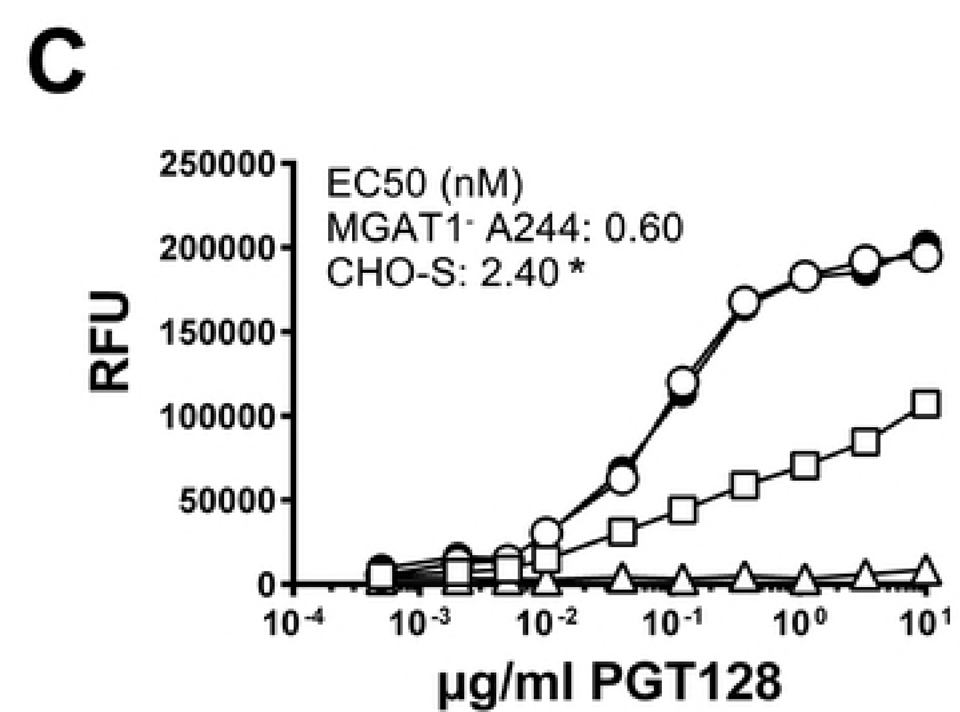
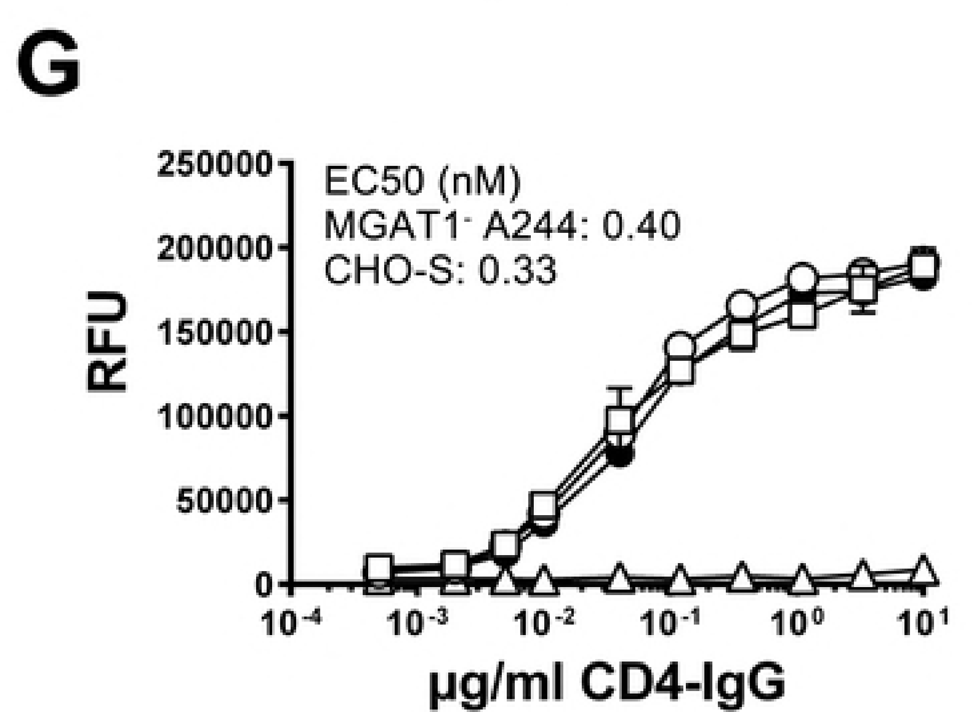
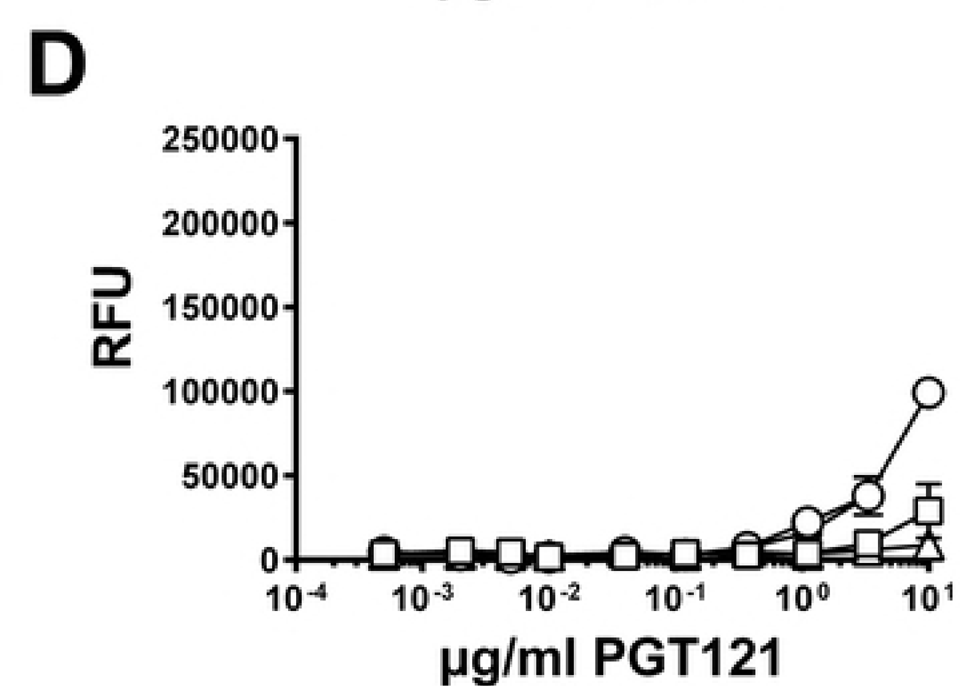
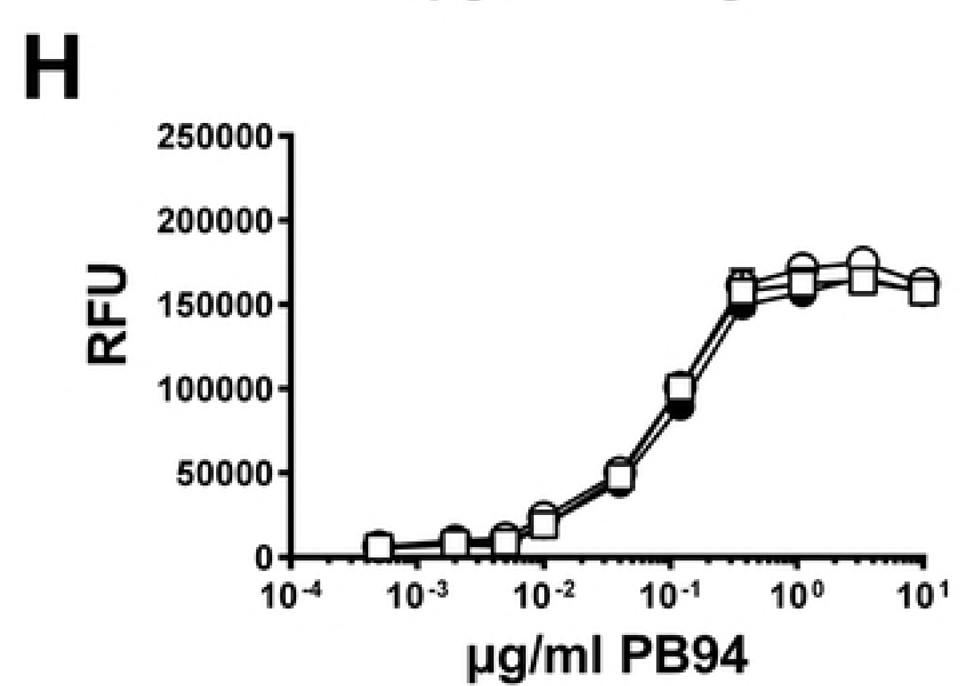


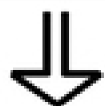



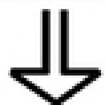




● A244_N332-rgp120 produced in MGAT1⁻ CHO cell line (clone 5F) stable
○ A244_N332-rgp120 produced in MGAT1⁻ CHO cell line by transient transfection
□ A244-gp120 produced in CHO-S cell line by transient transfection
△ isotype control mAb
* no plateau

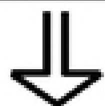
DAY 1: Electroporate MGAT1^{-} CHO cells with linearized plasmid



DAY 2: Plate cells with 500 µg/ml G418 and Alexa 488 polyclonal anti-gp120 (250,000 cells)

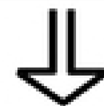


DAYS 14-16: Primary screening by ClonePix 2 (45,000 colonies), pick top 0.1% (44) into 96-well plate

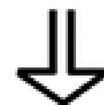


DAY 21: Secondary screening (43 colonies) ELISA

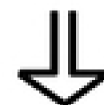
DAY 28: Continued screening and ELISA and Western Blot (15 colonies)



DAY 56: Scale up 6 clones and assay for protein production/characterization



Stability study, culture optimization, sterility testing, single cell re-clone



Master cell bank