HAL
open science

Fab is the most efficient format to express functional antibodies by yeast surface display

Coline Sivelle, Raphaël Sierocki, Kelly Ferreira-Pinto, Stéphanie Simon, Bernard Maillère, Hervé Nozach

To cite this version:

Coline Sivelle, Raphaël Sierocki, Kelly Ferreira-Pinto, Stéphanie Simon, Bernard Maillère, et al.. Fab is the most efficient format to express functional antibodies by yeast surface display. mAbs, 2018, 10 (5), pp.720-729. 10.1080/19420862.2018.1468952 . cea-02100007

HAL Id: cea-02100007

https://cea.hal.science/cea-02100007v1

Submitted on 15 Apr 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Fab is the most efficient format to express functional antibodies by yeast surface display

Coline Sivelle[1]*, Raphaël Sierocki[1]*, Kelly Ferreira-Pinto[1], Stéphanie Simon[2], Bernard Maillere[1], Hervé Nozach[1]

[1] Service d'Ingénierie Moléculaire des Protéines (SIMOPRO), CEA, Université Paris-Saclay, F-91191 Gif/Yvette, France.

[2] Service de Pharmacologie et Immunoanalyse (SPI), CEA, INRA, Laboratoire d'Etudes et de Recherches en Immunoanalyse, Université Paris-Saclay, F-91191 Gif/Yvette, France.

* These authors contributed equally to this work

Abstract

Multiple formats are available for engineering of monoclonal antibodies (mAbs) by yeast surface display, but they do not all lead to efficient expression of functional molecules. We therefore expressed four anti-tumor necrosis factor and two anti-IpaD mAbs as single-chain variable fragment (scFv), antigen-binding fragment (Fab) or single-chain Fabs and compared their expression levels and antigen-binding efficiency. Although the scFv and scFab formats are widely used in the literature, 2 of 6 antibodies were either not or weakly expressed. In contrast, all 6 antibodies expressed as Fab revealed strong binding and high affinity, comparable to that of the soluble form. We also demonstrated that the variations in expression did not affect Fab functionality and were due to variations in light chain display and not to misfolded dimers. Our results suggest that Fab is the most versatile format for the engineering of mAbs.

Keywords: yeast surface display, antibody engineering, scFv fragment, scFab fragment, Fab fragment, affinity maturation, monoclonal antibodies

Abbreviations:

BSA: bovine serum albumin; DSF: differential scanning fluorimetry; Fab: antigen-binding fragment; MFI: mean fluorescence intensity; PDI: protein disulfide isomerase; PEI: polyethylenimine; scFab: single-chain antigen-binding fragment; scFv: single-chain variable fragment; SEC: size exclusion chromatography; TNF: tumor necrosis factor; YSD: yeast surface display

Conflict-of-interest and financial disclosure

None

Introduction

Over the past 20 years, yeast surface display (YSD) has emerged as a very potent tool for protein engineering. It is a very proficient platform for *de novo* identification of new binders in naïve[1] or immune libraries.[2, 3] YSD is also used for the engineering of many antibodies, principally to identify mutants with improved affinity[4, 5] selectivity, stability and expression.[4-7] Affinity-matured antibodies have been developed against multiple antigens such as tumor necrosis factor (TNF),[8] fluorescein,[9] botulinum neurotoxin type A,[10] carcinoembryonic antigen,[11] epidermal growth factor receptor[12] and streptavidin.[13] Engineering of other properties of antibodies such as pH sensitivity or antigen specificity has also been described.[14, 15]

Engineering of pre-existing antibodies using YSD relies on the anchoring of antibody fragments on the surface of yeast cells via fusion with a cell wall protein. Various formats are described in the literature for the functional display of antibody fragments on yeast cells, such as single-chain variable fragment (scFv), antigen-binding fragment (Fab) or single-chain Fab (scFab), although they are known to lead to variable levels of antibody expression.[2, 4, 10, 16] Yeast display of IgG has also been reported with secretion and capture strategies,[17, 18] but, to our knowledge, not as a conventional fusion with a yeast surface anchor protein. In most cases, antibody fragments are presented using the Aga2p-based display system, which permits either N-terminal or C-terminal fusion,[4] but the lack of comparative studies does not facilitate the choice of the appropriate formats to be used.

51 ScFv molecules are the smallest functional entity (30 kDa) that includes the complete antigen-
52 binding site of an antibody. This entity comprises the two variables domains VH and VL connected by
53 a flexible linker that prevents their dissociation.

54 Fab are composed of the light chain (VL + CL) and a heavy chain (VH + CH1) connected by an
55 interchain disulfide bond. Several studies have reported the expression of Fab molecules on the
56 surface of yeast cells using either a bigenic plasmid[13] or two distinct plasmids[10, 14, 19] to allow separate
57 expression of the two chains. Expression levels of Fab molecules are generally considered as lower
58 than those of scFv molecules in *E. coli*,[20, 21] at the surface of phages[22, 23] and also on the surface of yeast
59 cells.[10]

60 The scFab derived from Fab is obtained through introduction of a polypeptide linker
61 connecting the light chain and the heavy chain. Thus, it combines some advantages of the scFv (single
62 chain and one expression plasmid, level of expression) with the stability and lower tendency to
63 aggregate of the Fab.[2, 23] The linker prevents the display of unpaired heavy chain antibodies (devoid of
64 light chain), which can sometimes interfere with the selection steps.[2] ScFab molecules carry a flexible
65 linker of variable length (32 to 80 amino acids) and an optional inter-chain disulfide bond.[2, 24] Deletion
66 of carboxy terminal cysteine residues (ΔC) of the CL and CH1 domains results in a scFabΔC format,
67 which has increased expression levels in *E. coli.* [23]

68 The scFv is the most popular format for antibody engineering,[4] probably because of its very
69 high level of expression, its small size and the presence of a single peptide chain, which is an advantage
70 for cloning or sequencing procedures. However, Fab and scFab formats have undeniable advantages,
71 especially when it comes to stability or for reformatting to IgG molecules after engineering.[22] To date,
72 there is no clear literature consensus regarding the choice of an antibody fragment format suitable for
73 engineering using yeast cell display. Also, no general study has yet explored the influence of antibody
74 fragment formats on a diversity of antibodies in this particular context.

75 Here, we report the comparison of 4 antibody fragment formats – scFv, scFab with and without
76 interchain disulfide bond and Fab - for the functional display of 6 antibodies. This study includes a first
77 set of well-known antibodies - adalimumab, infliximab, golimumab and certolizumab - already used in
78 clinical settings. This set of antibodies encompasses humanized, chimeric and fully human antibodies,
79 which share TNF as a common antigen.[25] The second set is composed of two chimeric antibodies
80 targeting the protein IpaD from the needle tip of type III secretion system of *Shigella* spp obtained
81 from mouse immunization. The objective of this study was to identify the most suitable format for the
82 functional expression of antibody fragments on the surface of yeast cells.

83

84

85 ## Results

86 **Conception of yeast surface display vectors allowing expression of scFv, scFab and Fab**
87 **antibody fragments**

88 To determine the influence of formats on the expression and functionality of antibody fragments, a
89 new subset of yeast display plasmids was designed. Four plasmids were constructed for respective
90 expression of scFv, scFab formats and Fab at the surface of *S. cerevisiae* cells. Those plasmids share a
91 common backbone from previously described pCT-L7.5.1,[26] including inducible promoter *Gal 1-10* and
92 Aga2p signal peptide [Fig. 1]. Antibody fragments were displayed with a free N-terminus and tethered
93 to the yeast cell wall by C-terminal fusion with Aga2p,[27] in order to avoid possible steric hindrance of
94 the fusion.

95 The plasmid pNT scFv encodes a typical scFv composed of VH/VL domains connected by a flexible linker
96 $(G4S)_3$. We also compared two previously described scFab formats, scFabΔC[2] and sc60Fab,[24] by
97 constructing the plasmids pNT scFabΔC and pNT sc60Fab. ScFabs are composed of a VL-C_K light chain
98 connected to the VH-CH1 heavy chain by a flexible linker. The difference between the two formats

99 resides in the nature and length of the linker (34 amino acids for scFabΔC versus 60 amino acids for
100 sc60Fab) and the presence of an inter-chain disulfide bond in sc60Fab connecting the two C-terminal
101 cysteine residues of C_K and CH1. Display of Fab molecules on yeast without any linker was achieved
102 using the bigenic plasmid pNT Fab where *Gal 1-10* promoter and terminator are duplicated to obtain
103 separate expression of the two chains.

104 Each plasmid includes a hemagglutinin (HA) tag to report expression of the complete antibody
105 fragment. For single chain formats (scFv and scFabs), the HA tag is located between the antibody
106 fragment and the Aga2p subunit. Full Fab expression can be controlled for pNT Fab through the
107 expression of the light chain with the HA tag or by direct labeling with anti-C_K antibody. Expression of
108 heavy chain can be revealed by the HIS tag located between the heavy chain and Aga2p.

109 The 6 selected antibodies are IgG1 molecules with C_K light chains. The sequences of TNF-specific
110 antibodies were obtained from the IMGT database.[28] The mAbs IpaD_301 and IpaD_318 were obtained
111 by mouse immunization [data not shown] and engineered to generate chimeric antibodies. These two
112 antibodies derive from the same parental genes *IGHV1-14*01*/ *IGKV1-117*01* from *Mus musculus* and
113 differ by only 7 and 8 mutations in the heavy chain and the light chain, respectively. Together, 24
114 plasmids corresponding to the sequences of 6 selected antibodies expressed in the 4 antibody formats
115 were successfully cloned and transformed into yeast. An exhaustive analysis of the 24 constructs was
116 performed in parallel to investigate the influence of the antibody fragment format on antigen binding.

117

118 **Fab format grants functional expression of the largest number of antibodies**

119 To assess the expression and functionality of the constructs, yeast cells were incubated with the
120 appropriate biotinylated antigen at a concentration far above the expected K_D to reach quantitative
121 binding of the antibody fragments to their antigen. When evaluated by fluorescence-activated cell
122 sorting (FACS), cells expressing scFvs of adalimumab, certolizumab, golimumab and IpaD_318

123 exhibited a high level of fluorescence, indicating quantitative binding of the antigen [Fig 2]. Weaker
124 fluorescence levels were observed for IpaD_301 scFv, whereas no signal was observed for infliximab
125 scFv, indicating antigen binding was weak or absent. Functionality of constructs displaying low or
126 moderate antigen binding was not improved by increased induction times [Supp Data Fig 3]. Antigen
127 binding was specific, as demonstrated by the lack of cross-reactivity with the non-relevant antigen:
128 anti-TNF antibodies showed no detectable IpaD binding and anti-IpaD antibodies showed no
129 detectable TNF binding [Supp Data Fig 1].

130 Yeast cells expressing scFab corresponding to adalimumab, golimumab and IpaD_318 displayed
131 quantitative binding to their antigen, both as scFabΔC and sc60Fab formats. Infliximab and IpaD_301
132 in scFab formats were not or weakly functional, respectively, as attested by the absence of
133 fluorescence for the corresponding cells. Interestingly, certolizumab scFabΔC showed significant
134 binding to TNF, while certolizumab sc60Fab with the 60-amino-acid linker and inter-chain disulfide
135 bond failed to bind TNF. All single-chain constructs were expressed at the surface of cells, as attested
136 by labeling with anti-HA antibodies, with the notable exception of certolizumab sc60Fab [Supp Data
137 Fig 2]. Except for this specific construct, the absence of binding of the antibody fragments to their
138 antigen could not be explained by lack of expression, but rather by a functional deficiency.

139 In contrast, all of the 6 tested antibodies bound to their antigen when expressed as Fab at the surface
140 of yeast cells. For each antibody, cells expressing functional Fabs form homogeneous populations with
141 a clear shift in fluorescence levels compared to non-expressing cells. The proportion of cells expressing
142 functional Fabs is relatively high (from 46% to 74%).

143 In our set-up, Fab is the only viable format for functional expression of infliximab and IpaD_301
144 antibody fragments. Besides, for 5 of 6 antibodies tested, the fluorescence of cells expressing
145 functional Fab was higher than the fluorescence of the equivalent cells expressing either scFvs or
146 scFabs. This suggested that Fab molecules were not only active, but also well expressed. For these
147 reasons, we focused on display of Fab molecules in the rest of the study.

148

149 **Variations of expression in Fab format among antibodies do not affect functionality**

150 We observed that maximal fluorescence levels of Fab-expressing cells vary among antibodies targeting
151 a common antigen. More specifically, the mean fluorescence intensity of cells displaying functional
152 infliximab Fabs was much lower than for adalimumab or golimumab, a phenomenon also observed for
153 IpaD_301 when compared to IpaD_318.

154 As variations of signal corresponding to antigen binding could be attributed either to differences of
155 expression levels or to functional differences, dissociation constants (K_D) were determined for each
156 displayed Fab [Fig. 3]. The measured K_D values for IpaD_301 and IpaD_318 are 3.2 nM and 1.7 nM,
157 respectively. This result is in accordance with the K_D evaluated by OctetRed with measured affinities
158 of 1.1 nM and 0.9 nM (Supplementary figure 4). Yeast expressing anti-TNF Fabs show subnanomolar
159 K_D, 161 pM for adalimumab, 102 pM for infliximab, 39 pM for golimumab and 11 pM for certolizumab.
160 These K_D values are consistent with the reported K_D determined for the corresponding IgG molecules
161 using SPR,[25] indicating that Fabs expressed on the surface of yeast cells have a very strong affinity for
162 their antigen.

163 Interestingly, higher affinity was not correlated with higher level of binding fluorescence. While having
164 a very good affinity for TNF, infliximab Fab displayed much weaker TNF binding signal compared to
165 adalimumab Fab, suggesting differences in their expression levels. Thus, additional labeling
166 experiments were performed to evaluate different expression reporters. In addition to antigen binding
167 labeling, light chain expression was evaluated with anti-HA or anti-C_K antibodies and heavy chain
168 expression was assessed with anti-HIS antibodies [Fig. 4].

169 Heavy chain expression showed slight variations between the 6 selected antibodies [Fig. 4a]. Labeling
170 with anti-HIS antibodies showed homogeneous expression of heavy chains, with IpaD_318 and
171 adalimumab being somewhat above average. This observation contrasted with light chain expression,

172 which showed strong differences across the Fab antibodies [Fig. 4b]. Light chain expression was
173 significantly higher for IpaD_318 and adalimumab Fabs than for IpaD_301 or infliximab, while
174 certolizumab and golimumab had intermediate light chain expression levels. Values observed for both
175 reporters anti-HA or anti-C_K were in good agreement, and the relative intensities of constructs were
176 very similar. Anti-C_K antibody yielded a very strong fluorescence signal, thus favoring easy
177 discrimination of C_K-expressing cells.

178 Antigen binding signals for IpaD_301 and IpaD_318 correlated well with their relative light chain
179 expression levels [Fig. 4c and Fig. 5]. Very similarly, the TNF binding signal was proportional to the
180 respective expression levels of adalimumab, infliximab, certolizumab and golimumab light chain of the
181 Fab [Fig 5]. Direct correlation between light chain expression and antigen binding was confirmed by
182 simultaneous labeling with anti-C_K antibody conjugated to allophycocyanin (APC) and streptavidin R-
183 phycoerythrin (streptavidin-PE) [Fig. 4d].

184 While having high sequence homology and similar affinities, IpaD_301 and IpaD_318 display significant
185 differences in expression and antigen binding in all formats tested. For this reason, we investigated a
186 possible correlation between the biophysical attributes of these two antibodies and their behavior on
187 the surface of yeast cells. Thus, recombinant expression of complete IgG1 IpaD_301 and IpaD_318 was
188 performed by transient transfection of HEK293 cells[29] followed by protein A purification. Production
189 yield was slightly higher for IpaD_318 than for IpaD_301 (20 and 14 mg per liter, respectively). Size-
190 exclusion chromatography revealed that both antibodies are predominantly produced as full-length
191 IgG with low amounts of aggregates for IpaD_318 and even lower amounts for IpaD_301 [Fig. 6]. In
192 contrast, differential scanning fluorimetry (DSF) analysis reveals that IpaD_301 has a lower melting
193 temperature (Tm) than IpaD_318, with Tm values of 67.6°C and 69.3°C, respectively [Fig. 6].

194

195 ## Discussion

196 We carried out a comparative study of expression formats using 6 different antibodies specific for two
197 different antigens, TNF and IpaD. In our hands, Fab appears to be the most versatile expression format
198 for display of functional antibodies on the surface of yeast cells.

199 ScFv is currently the most widespread format for antibody engineering. It has indisputable advantages,
200 such as its small size, which is particularly convenient for the cloning and sequencing of libraries. Also,
201 numerous studies have used scFvs successfully to engineer clones with improved properties,[4] including
202 clones derived from adalimumab with improved affinity towards TNF.[8] However, some antibodies
203 display reduced affinity and impaired biophysical properties when expressed as scFv as compared to
204 the corresponding Fab molecules.[23, 30] Indeed, CH1 and CL domains, which are not present in scFv
205 molecules, participate in the greater stability of Fab and scFab.[31, 32] The choice of an adapted format
206 has also proved to be an important parameter for other approaches such as phage display[33, 34] or
207 recombinant expression of antibody fragments.[35]

208 Here, we show that some antibodies do not seem to be active either in the scFv format or in the scFab
209 format when they are expressed at the surface of yeast cells. In our set-up, two antibodies, infliximab
210 and IpaD_301, appeared to lack functionality, as suggested by the total absence of antigen binding in
211 any single-chain format. The presence of C_K and CH1 domains, which could have a stabilizing role in
212 the scFab formats, had no effect on the functionality of the corresponding infliximab and IpaD_301
213 constructs. We cannot exclude that the presence of a linker between the light chain and the heavy
214 chain could also cause steric hindrance that impairs functionality.

215 We found that adalimumab, golimumab and IpaD_318 were well expressed and perfectly able to bind
216 their antigen as scFv constructs, but also in scFab or Fab formats. Certolizumab was also a very well
217 expressed and functional antibody in these formats, with the notable exception of the sc60Fab format,
218 which exhibited very poor expression and had no detectable antigen binding (Supp. Fig 2). These
219 particular results emphasize that the presence of the two C-terminal cysteine residues in scFab, as well

220 as the nature and length of the linker sequence, can dramatically alter expression of scFab molecules
221 for some antibodies. Moreover, the presence of the interchain disulfide bond can have a major
222 influence on the stability of the antibody fragment, as recently shown for the adalimumab Fab. [36] The
223 length of the linker also proved to be an important parameter for the proper packing of light and heavy
224 chains and possibly the accessibility of the paratope, thus ultimately affecting the function of the scFab
225 fragment.[23, 24]

226 In this study set-up, Fab was the only format that allowed functional display for each of the 6 tested
227 antibodies. FACS analysis revealed that a very high proportion of induced yeast cells (up to 70%)
228 expressed Fabs and produced a strong fluorescence signal corresponding to antigen binding. In the
229 context of protein engineering or affinity maturation, a strong signal would favor an easy
230 discrimination of high-affinity clones in libraries containing very high numbers of mutants.

231 Moreover, for anti-TNF molecules, all Fabs expressed on the surface of yeast cells exhibited a high
232 affinity for their antigen, with K_D values close to those reported in the literature, strongly suggesting
233 that the K_D of a protein-protein binding interaction measured on the surface of yeast cells was
234 essentially equal to that measured using soluble proteins. [37, 38]

235 Deeper analysis of expression reporters in the Fab format indicated a lower display level of light chains
236 for infliximab and IpaD_301 Fabs. Light chain display correlated perfectly with lower target binding of
237 these two constructs compared to the other anti-TNF Fabs or IpaD_318 Fab, respectively. Coexpression
238 of chaperones such as BiP, use of alternative promoters such as *Gal10* for the light chain expression,
239 or introduction of a new secretory leader sequence optimized for antibody secretion such as αMFpp8[39]
240 might be useful to promote the assembly of light and heavy chains of antibodies during Fab expression.
241 A recent study demonstrated that co-expression of protein disulfide isomerase (PDI) had a positive
242 effect on the functionality of some antibodies expressed as Fab on the surface of yeast cells.[40] In this
243 study, Georgiou and co-workers also showed that pairing of antibody chains could be promoted by
244 fusing the light and heavy chains with dimerization domains such as leucine-zipper proteins. Forced-

245 dimerization of antibody chains restored functionality for 3 of 13 tested anti-HA antibodies, although
246 with a lower proportion of cells displaying functional Fab.

247 Overall, infliximab and IpaD_301 exhibited relatively low expression in every tested format of antibody
248 fragment. We observed, however, marked differences in the expression profiles of IpaD_301 and
249 IpaD_318 on the surface of yeast cells in spite of their high sequence homology, with only 7 and 8
250 mutations in the heavy chain and the light chain, respectively. Production of these two antibodies in a
251 full-length IgG format reveal that IpaD_301 has lower production titers in HEK cells and also a lower
252 Tm (67.6°C vs 69.3°C). Interestingly, infliximab is also described as difficult to express in HEK cells[41] and
253 CHO cells,[42] with relatively low stability over time.[43] A comprehensive study comparing the biophysical
254 properties of clinical-stage antibodies indicated that infliximab has much lower production titers in
255 HEK cells than adalimumab, certolizumab and golimumab (6 mg/L for infliximab vs more than 100
256 mg/mL for the three other anti-TNF antibodies).[41] Wittrup and co-workers also reported that infliximab
257 had a lower Tm (64.5°C vs at least 70°C for the three other anti-TNF antibodies). These observations
258 are consistent with previously published studies indicating that protein secretion by yeast cells is
259 correlated with thermodynamic stability.[44, 45] Together, this suggests that antibodies with low
260 thermodynamic stability might have low expression on the surface of yeast cells.

261 Even as many alternative antibody formats are emerging, most of current therapeutic antibodies on
262 the market are antibodies in the IgG format.[21] Thus, it is essential that the conversion between the
263 screening format – the antibody fragment – and the IgG antibody does not impair the desired
264 properties, and in particular affinity for the antigen. From this perspective, use of Fab has strong
265 advantages. Several reports show that the Fab format tends to retain its binding affinity better than
266 scFv when converted into IgG.[19] Moreover, the presence of a peptide linker in the scFv and scFab
267 formats or introduction of additional domains such as leucine-zipper dimerization proteins might
268 introduce bias in the screening of improved clones because of subtle structural changes. Indeed, some

269 affinity-enhanced molecules engineered in scFv and scFabΔC formats lost affinity after conversion into
270 IgG.[22]

271 In conclusion, in spite of their practical convenience, single-chain antibody fragment formats such as
272 scFv and scFab do not always lead to a functional display of antibodies at the surface of yeast cells. In
273 contrast, we showed that all 6 tested antibodies were functional in the Fab format, with high affinities
274 for their antigen. Using our Fab expression plasmid, high proportions of yeast cells expressed Fab
275 molecules and exhibited strong antigen binding signals in FACS, two important properties when getting
276 started with the affinity maturation process by yeast display in good conditions. These advantages and
277 the lower risk of losing engineered properties upon conversion into IgG suggest that the Fab format is
278 the best alternative for the engineering of antibodies using YSD.

279

280

281

282 ## Material and methods

283 **Yeast display vectors construction**

284 Cloning were performed with the SLiCE recombination cloning method[46] or classic
285 restriction/ligature method using *E.coli* DH5α strain (Invitrogen). For each anti-TNF antibody,
286 optimized synthetic genes (VH and VL) were ordered from Eurofins Genomics based on the IMGT
287 sequences. All expression plasmids are derived from previously described pCT-L7.5.1[26] and share the
288 *CEN/ARS* replication origin, *TRP* auxotrophy marker, *colE1* replication origin, Ampicillin resistance gene
289 and galactose inducible promoter *GAL1*. Plasmid pCT-L7.5.1 was a gift from Prof. K. Dane Wittrup
290 (Addgene plasmid # 42900).

291 Plasmids pNT scFv were modified from parental pCT-L7.5.1 plasmid by modifying the aga2p
292 signal sequence with a dipeptide spacer[27] and a *NheI* restriction site. A stop codon was inserted at the
293 3' end of aga2p gene to obtain plasmids allowing the display of scFv with a free N-terminus end and a
294 C-terminal anchoring to the yeast cell wall. Synthetic genes corresponding to each scFv were then
295 cloned using *NheI* and *SpeI* restriction sites.

296 The pNT scFabΔC vector was generated by cloning the coding sequence of the scFabΔC of the
297 previously described pYDscFab[2] between the restriction sites *NheI* and *XhoI* of the pNT scFv plasmid.
298 Plasmid pYDscFab was a gift from Prof. Dennis Burton. Heavy chains and light chains of antibodies were
299 cloned between *NheI*/*Pfl23II* and *NcoI*/*SalI* restrictions sites, respectively.

300 The pNT sc60Fab vectors were generated from pNT scFab plasmids. The linker and the cysteine
301 residues allowing interchain disulfide bond formation were modified by PCR and homologous
302 recombination between *BssHII* and *NcoI* sites. Heavy chains and light chains of antibodies were cloned
303 between *NheI-Pfl23II* and *NcoI-SalI* restriction sites, respectively.

304 The bigenic pNT Fab vector was generated by ligation into pNT scFv between *SpeI* and *XhoI* of
305 a synthetic gene (Eurofins Genomics) containing a 6-HIS tag, the Aga2p domain, a *Gal1* promoter, an

306 aga2p signal sequence, an HA tag and a transcription terminator. The resulting plasmid has two distinct
307 expression cassettes corresponding to respective expression of light and heavy chains of the Fabs.
308 Heavy chains and light chains of antibodies were then cloned between *NheI*/*Pfl23II* and *NcoI*/*SalI*
309 restrictions sites, respectively.

310 **Yeast transformation**

311 Preparation of competent yeast cells EBY100 (ATCC® MYA-4941™; a *GAL1-AGA1* :: *URA3 ura3-*
312 *52 trp1 leu2 Δ1 his3 Δ*200 *pep4 :: HIS2 prb*1*Δ1.6R can1 GAL*) was performed according to Suga et al.[47]
313 Then, 100 µL of EBY100 electro-competent cells were mixed with 1-2 µg plasmid DNA, transferred to
314 a pre-chilled electroporation cuvette (Biorad, 165-2086) and pulsed at 2.5 kV, 25 µF (Biorad Gene
315 Pulser Xcell). Yeast cells were then diluted in 1 mL of sorbitol (1 M), and 200 µL were streaked on SD-
316 CAA agar plates [6.7 g/L yeast nitrogen base without casamino acids, 20 g/L dextrose, 5 g/L casamino
317 acids, 100 mM sodium phosphate pH 6.0].

318 **Growth and expression conditions**

319 Yeast cultures were performed in 24 deep-well round-bottom plates. Pre-cultures were
320 performed by inoculating 3 mL of SD-CAA medium with one colony from selective agar plate and
321 incubated overnight at 30°C, 200 rpm. The saturated pre-culture (typically OD_{600} of 8-10) was passaged
322 in order to obtain an initial culture OD_{600} of 0.25-0.50. The culture was grown at 30°C until its OD600
323 reached 0.5-1.0. Cells were centrifuged and re-suspended in 3 mL of SG-CAA galactose induction
324 medium [6.7 g/L yeast nitrogen base without casamino acids, 20 g/L galactose, 5 g/L casamino acids,
325 100 mM sodium phosphate, pH 6.0] and induced for 16-36 h at 20°C, 200 rpm.

326 **Yeast labelling and flow cytometry analysis**

327 10^6 cells were taken from the induced culture and washed with 1 mL PBSF (phosphate-buffered
328 saline (PBS), bovine serum albumin (BSA) 0.1%) buffer. Cells were resuspended in 50 µL of the target
329 protein at the desired concentration and incubated at 20°C, 1000 rpm until equilibrium is reached

330 (depending on target protein concentration). Cells were washed with 1 mL of ice-cold PBSF to avoid
331 dissociation and resuspended in ice-cold PBSF containing the appropriate fluorescent reporters:
332 Streptavidin-PE (Thermo Fisher scientific; catalog number S866; 1:100 dilution), Mouse anti-Human
333 Kappa Light Chain Antibody APC conjugate (Thermo Fisher scientific; catalog number MH10515; 1:100
334 dilution), 6x-His Epitope Tag antibody Dylight-650 conjugate (Thermo Scientific, catalog number MA1-
335 21315-D650; 1:100 dilution) or HA Tag Monoclonal Antibody APC conjugate (1:50 dilution). Cells were
336 incubated on ice in the dark for 15 minutes and analyzed with BD FACS Aria™ III cytometer.

337 **Cell-Binding assays for determining the affinity of antibodies for their antigen**

338 Cell binding assays were performed according to Hunter et al.[38] in order to avoid ligand
339 depletion. 10^4 to 10^5 cells were incubated in 50-500 µL of the target protein at the desired
340 concentrations and incubated at 20°C, 1000 rpm long enough to reach equilibrium. Cells were analyzed
341 by FACS for their binding fluorescence as described above. Auto-fluorescence was subtracted for each
342 measure and resulting values were normalized with maximum fluorescence. Data are then fitted with
343 a non-linear regression analysis to obtain the K_D.

344 **Production and purification of IgG IpaD_301 and IpaD_318**

345 IpaD_301 and IpaD_318 antibodies were produced via transient transfection of HEK293
346 Freestyle™ cells (HEK 293FS). HEK 293FS were transfected as previously described by Subedi *et al.*[48]
347 without the use of Valproic acid using the HEK transient transfection plasmids described by Smith *et*
348 *al.*[29]

349 The day before transfection, cells were sub-cultured in order to be at a density of 2-3.10^6
350 cells/mL the next day. The day of the transfection, cells were centrifuged 5 min at 100 g and
351 resuspended in fresh FreeStyle 293 Expression medium (Thermo-Fisher) to obtain a density of 2.5.10^6
352 cells/mL. For a transfection volume of 100 mL, 150 µg of each plasmid (coding for heavy chain and light
353 chain) was added to the cells at a concentration of 0.5 µg/µL. Cells were incubated 5 min at 37°C, 180

354 rpm, 8 % CO_2. 1.8 mL of polyethylenimine (PEI) was added at a concentration of 0.5 mg/mL to the cells
355 (PEI:DNA ratio = 3:1). Cells were subsequently incubated 24h at 37°C, 180 rpm, 8 % CO_2.

356 A day after transfection, 100 mL of fresh EX-CELL® Serum-Free Medium for HEK 293 (Sigma-
357 Aldrich), supplemented with 6 mM L-Glutamine, was added to the transfected culture. Cells were
358 finally incubated 4 more days at 37°C, 180 rpm, 8 % CO_2. Five days after transfection, the supernatant
359 was harvested by centrifuging the cells at 1000g for 15 min at 4°C and kept on ice until purification.
360 The supernatant was purified using a HiTrap™ Protein A HP column of 1 mL (GE Healthcare) on an
361 ÄKTA purifier (GE Healthcare) according to the manufacturer's description.

362 **Size-exclusion chromatography**

363 A Superdex-200 10/300 GL (GE Healthcare) column was used on an ÄKTA purifier (GE
364 Healthcare). Runs were monitored with Unicorn™ 5.31 software (GE Healthcare). 500 µL of each
365 sample was applied on the column using a flow rate of 0.7 mL/min and PBS as running buffer.

366 **Fab IpaD_301 and IpaD_318 affinity determination**

367 Fab were prepared from full-length IgG using a Pierce® Fab Micro Preparation Kit. The binding
368 affinity of Fab IpaD_301 and IpaD_318 was evaluated by biolayer interferometry using an Octet RED96
369 instrument (Pall ForteBio). Biotinylated IpaD was loaded onto streptavidin biosensors (ForteBio) at a
370 concentration of 5 µg/mL for 8 min in PBSF (1x PBS, pH 7.4, 0.1% BSA). Fab IpaD_301 and IpaD_318
371 were then associated with IpaD using 4 different concentrations (20, 10, 5 and 2.5 nM). Duration of
372 association and dissociation steps were 20 min and 120 min. All steps were performed at 20°C. Data
373 were collected and analyzed using Octet Software version 10.0.3 (Pall ForteBio). Binding kinetics were
374 fitted using a 1:1 Langmuir-binding model.

375

376 **Thermostability evaluation**

377 DSF was performed as previously described by Niesen *et al.*[49] using a StepOne Real-Time PCR
378 System from Applied Biosystems. IgG monomers of IpaD_301 and IpaD_318 purified via SEC were
379 mixed with the SYPRO Orange reagent. Final dye concentration was 5x and final IgG concentration was
380 150 µg/mL. The temperature gradient was run from 20 to 90 °C with 1 min equilibration at each degree
381 centigrade.

382

383

384 **Acknowledgements**

385

386 The authors would like to thank Dr Magali Aumont-Nicaise for her assistance with differential
387 scanning fluorimetry experiments and Dr Hervé Volland for anti-HA antibodies.

388

389 **Figure 1**. **Schematic representation of yeast surface display vectors used in this study.**
390 Antibody fragment constructs are tethered to the cell wall of *S. cerevisiae* cells by C-terminal fusion
391 with the Aga2p subunit of the a-agglutinin yeast cell surface anchor protein. Expression is controlled
392 by the tightly regulated galactose-inducible *Gal1* promoter. Aga2-ss: native signal sequence of Aga2p
393 for secretion of the constructs. Single-chain antibody fragments (scFv, scFabΔC and sc60Fab) include
394 flexible linkers connecting the light chain and the heavy chain. pNT Fab is a bigenic plasmid including
395 two identical GAL1 promoters for respective expression of light and heavy chains. Cysteine residues
396 responsible for the formation of the inter-chain disulfide bond connecting C_K and CH1 are indicated in
397 red. Expression of antibody fragments can be monitored using the HA tag for all constructs. A 6His-Tag
398 sequence can also be used for the monitoring of heavy chain expression when using pNT Fab.

399

400

401 **Figure 2**. **Antigen binding of the 6 selected antibodies expressed as different antibody**
402 **fragment formats on the surface of yeast cells.** Cells are labeled with biotinylated antigen then with
403 Streptavidin–PE (Target Binding) before FACS analysis. Selected antigen concentrations are far above
404 the expected K_D to reach a quantitative binding with the antibody fragments. Respective antigen
405 concentration were 20 nM for anti-TNF antibodies and 50 nM for anti-IpaD antibodies. For each
406 construct, a FACS histogram shows the fluorescence intensity corresponding to antigen binding (x-axis)
407 vs the number of detected events (y-axis). Interval gates (bars) are shown to differentiate cells with
408 detectable antigen binding (mean fluorescence intensity (MFI) score above 10^3, in green) from cells
409 with no detectable antigen binding (MFI score under 10^3, in grey).The 10^3 cutoff has been obtained by
410 measuring MFI of cells with a non-relevant antigen, cf. Supplementary figure1.

411

412 **Figure 3**. **Affinity of Fab antibody fragments expressed on the surface of yeast cells.** The mean
413 fluorescence intensity (MFI), determined by FACS analysis, is plotted against varying concentrations of
414 antigen for every Fab construct, and fit to a monovalent binding model. Measurements were done in
415 triplicate with independent cultures and inductions. (A) Equilibrium binding constants K_D of the four
416 anti-TNF Fabs for TNF at 20°C. Curve fitting results in a K_D of 114 ± 6 nM for adalimumab Fab, 102 ± 14
417 pM for infliximab Fab, 39 ± 4 pM for golimumab Fab and 11 ± 1 pM for certolizumab Fab. (B) Equilibrium
418 binding constants K_D of the two anti-IpaD Fabs for IpaD at 20°C. K_D IpaD_301 Fab = 3.2± 0.3 nM, K_D
419 IpaD_318 Fab = 1.7± 0.2 nM.

420

421 **Figure 4**. **Antigen binding and expression levels of heavy and light chains of the 6 selected**
422 **antibodies expressed as Fab on the surface of yeast cells.** Cells were labeled with the appropriate
423 expression reporter and the mean fluorescence intensity of positive cells was determined by FACS
424 analysis. For antigen binding, cells were double-labeled with biotinylated antigen and Streptavidin–PE
425 (target binding) before FACS analysis. (A) Heavy chain expression evaluated with anti-HIS antibody
426 reporter. (B) Light chain expression evaluated with anti-HA antibody reporter (C) Biotinylated antigen
427 binding evaluated with Streptavidin-PE (D) Light chain expression evaluated with anti-CK antibody
428 reporter. Measurements were made in triplicate using cells from independent cultures.

429

430 **Figure 5**. **Representative bivariate flow cytometric analysis of yeast cells expressing Fab**
431 **antibody fragments expressed on their surface.** Each single dot on the plot designates the APC (y-axis)
432 and PE (x-axis) fluorescence intensity values for a single yeast cell. Cells were double-labeled with
433 biotinylated antigen/Streptavidin–PE (target binding) and anti-CK APC labels (Fab expression). Antigen
434 concentrations in labeling experiments were 20 nM for anti-TNF antibodies and 50 nM for anti-IpaD
435 antibodies. Functional subpopulations were gated using an inverted gate of the double negative sub-
436 populations. The percentage number correspond to the mean percentage of cells expressing functional
437 Fabs from three separate inductions.

438

439 **Figure 6**. **Biophysical characterization of IpaD_301 IgG and IpaD_318 IgG expressed by HEK**
440 **cells.** (A)**:** Size-exclusion chromatograms of the protein A purified IgGs on a Superdex 200 10/300 GL
441 size-exclusion column for IpaD_301 IgG (black) and IpaD_318 IgG (grey). Monomeric IgG species elutes
442 at 15 mL and aggregates or multimeric species elutes between 10 to 12 mL. (B): Tm determination of
443 IgGs by differential scanning fluorimetry. The fluorescence intensities are normalized to peak values.
444 Error bars correspond to experiment triplicates.

445 **1. Chao G, Lau WL, Hackel BJ, Sazinsky SL, Lippow SM, Wittrup KD. Isolating and engineering**
446 **human antibodies using yeast surface display. Nat Protoc 2006; 1:755-68.**

447 **2. Walker LM, Bowley DR, Burton DR. Efficient recovery of high-affinity antibodies from a**
448 **single-chain Fab yeast display library. J Mol Biol 2009; 389:365-75.**

449 **3. Wang B, Lee CH, Johnson EL, Kluwe CA, Cunningham JC, Tanno H, et al. Discovery of high**
450 **affinity anti-ricin antibodies by B cell receptor sequencing and by yeast display of combinatorial**
451 **VH:VL libraries from immunized animals. mAbs 2016; 8:1035-44.**

452 **4. Boder ET, Raeeszadeh-Sarmazdeh M, Price JV. Engineering antibodies by yeast display.**
453 **Arch Biochem Biophys 2012; 526:99-106.**

454 **5. Pepper LR, Cho YK, Boder ET, Shusta EV. A decade of yeast surface display technology:**
455 **where are we now? Comb Chem High Throughput Screen 2008; 11:127-34.**

456 **6. Angelini A, Chen TF, de Picciotto S, Yang NJ, Tzeng A, Santos MS, et al. Protein Engineering**
457 **and Selection Using Yeast Surface Display. Methods Mol Biol 2015; 1319:3-36.**

458 **7. Gera N, Hussain M, Rao BM. Protein selection using yeast surface display. Methods 2013;**
459 **60:15-26.**

460 **8. Rajpal A, Beyaz N, Haber L, Cappuccilli G, Yee H, Bhatt RR, et al. A general method for**
461 **greatly improving the affinity of antibodies by using combinatorial libraries. Proc Natl Acad Sci U S**
462 **A 2005; 102:8466-71.**

463 **9. Boder ET, Midelfort KS, Wittrup KD. Directed evolution of antibody fragments with**
464 **monovalent femtomolar antigen-binding affinity. Proc Natl Acad Sci U S A 2000; 97:10701-5.**

465 **10. Lou J, Geren I, Garcia-Rodriguez C, Forsyth CM, Wen W, Knopp K, et al. Affinity maturation**
466 **of human botulinum neurotoxin antibodies by light chain shuffling via yeast mating. Protein Eng**
467 **Des Sel 2010; 23:311-9.**

468 **11. Graff CP, Chester K, Begent R, Wittrup KD. Directed evolution of an anti-carcinoembryonic**
469 **antigen scFv with a 4-day monovalent dissociation half-time at 37 degrees C. Protein Eng Des Sel**
470 **2004; 17:293-304.**

471 **12. Zhou Y, Drummond DC, Zou H, Hayes ME, Adams GP, Kirpotin DB, et al. Impact of single-**
472 **chain Fv antibody fragment affinity on nanoparticle targeting of epidermal growth factor receptor-**
473 **expressing tumor cells. J Mol Biol 2007; 371:934-47.**

474 **13. van den Beucken T, Pieters H, Steukers M, van der Vaart M, Ladner RC, Hoogenboom HR, et**
475 **al. Affinity maturation of Fab antibody fragments by fluorescent-activated cell sorting of yeast-**
476 **displayed libraries. FEBS Lett 2003; 546:288-94.**

477 **14. Schroter C, Gunther R, Rhiel L, Becker S, Toleikis L, Doerner A, et al. A generic approach to**
478 **engineer antibody pH-switches using combinatorial histidine scanning libraries and yeast display.**
479 **mAbs 2015; 7:138-51.**

480 **15. Garcia-Rodriguez C, Levy R, Arndt JW, Forsyth CM, Razai A, Lou J, et al. Molecular evolution**
481 **of antibody cross-reactivity for two subtypes of type A botulinum neurotoxin. Nat Biotechnol 2007;**
482 **25:107-16.**

483 **16. Rosowski S, Becker S, Toleikis L, Valldorf B, Grzeschik J, Demir D, et al. A novel one-step**
484 **approach for the construction of yeast surface display Fab antibody libraries. Microb Cell Fact**
485 **2018; 17:3.**

486 **17. Rhiel L, Krah S, Gunther R, Becker S, Kolmar H, Hock B. REAL-Select: full-length antibody**
487 **display and library screening by surface capture on yeast cells. PLoS One 2014; 9:e114887.**

488 **18. Rakestraw JA, Aird D, Aha PM, Baynes BM, Lipovsek D. Secretion-and-capture cell-surface**
489 **display for selection of target-binding proteins. Protein Eng Des Sel 2011; 24:525-30.**

490 **19. Weaver-Feldhaus JM, Lou JL, Coleman JR, Siegel RW, Marks JD, Feldhaus MJ. Yeast mating**
491 **for combinatorial Fab library generation and surface display. FEBS Lett 2004; 564:24-34.**

492 **20. Gaciarz A, Veijola J, Uchida Y, Saaranen MJ, Wang C, Horkko S, et al. Systematic screening**
493 **of soluble expression of antibody fragments in the cytoplasm of E. coli. Microb Cell Fact 2016;**
494 **15:22.**

495 **21. Holliger P, Hudson PJ. Engineered antibody fragments and the rise of single domains. Nat**
496 **Biotechnol 2005; 23:1126-36.**

497 **22. Steinwand M, Droste P, Frenzel A, Hust M, Dubel S, Schirrmann T. The influence of**
498 **antibody fragment format on phage display based affinity maturation of IgG. mAbs 2014; 6:204-18.**

499 **23. Hust M, Jostock T, Menzel C, Voedisch B, Mohr A, Brenneis M, et al. Single chain Fab**
500 **(scFab) fragment. BMC Biotechnol 2007; 7:14.**

501 **24. Koerber JT, Hornsby MJ, Wells JA. An improved single-chain Fab platform for efficient**
502 **display and recombinant expression. J Mol Biol 2015; 427:576-86.**

503 **25. van Schie KA, Ooijevaar-de Heer P, Dijk L, Kruithof S, Wolbink G, Rispens T. Therapeutic TNF**
504 **Inhibitors can Differentially Stabilize Trimeric TNF by Inhibiting Monomer Exchange. Scientific**
505 **reports 2016; 6:32747.**

506 **26. Hackel BJ, Kapila A, Wittrup KD. Picomolar affinity fibronectin domains engineered utilizing**
507 **loop length diversity, recursive mutagenesis, and loop shuffling. J Mol Biol 2008; 381:1238-52.**

508 **27. Wang Z, Mathias A, Stavrou S, Neville DM, Jr. A new yeast display vector permitting free**
509 **scFv amino termini can augment ligand binding affinities. Protein Eng Des Sel 2005; 18:337-43.**

510 **28. Lefranc MP, Giudicelli V, Duroux P, Jabado-Michaloud J, Folch G, Aouinti S, et al. IMGT(R),**
511 **the international ImMunoGeneTics information system(R) 25 years on. Nucleic Acids Res 2015;**
512 **43:D413-22.**

513 **29. Smith K, Garman L, Wrammert J, Zheng NY, Capra JD, Ahmed R, et al. Rapid generation of**
514 **fully human monoclonal antibodies specific to a vaccinating antigen. Nat Protoc 2009; 4:372-84.**

515 **30. Worn A, Pluckthun A. Stability engineering of antibody single-chain Fv fragments. J Mol**
516 **Biol 2001; 305:989-1010.**

517 **31. Rothlisberger D, Honegger A, Pluckthun A. Domain interactions in the Fab fragment: a**
518 **comparative evaluation of the single-chain Fv and Fab format engineered with variable domains of**
519 **different stability. J Mol Biol 2005; 347:773-89.**

520 **32. Quintero-Hernandez V, Juarez-Gonzalez VR, Ortiz-Leon M, Sanchez R, Possani LD, Becerril**
521 **B. The change of the scFv into the Fab format improves the stability and in vivo toxin neutralization**
522 **capacity of recombinant antibodies. Mol Immunol 2007; 44:1307-15.**

523 **33. Chan CE, Chan AH, Lim AP, Hanson BJ. Comparison of the efficiency of antibody selection**
524 **from semi-synthetic scFv and non-immune Fab phage display libraries against protein targets for**
525 **rapid development of diagnostic immunoassays. J Immunol Methods 2011; 373:79-88.**

526 **34. Hust M, Jostock T, Menzel C, Voedisch B, Mohr A, Brenneis M, et al. Single chain Fab**
527 **(scFab) fragment. BMC Biotechnol 2007; 7:14.**

528 **35. Rao MF, Li YJ, Dong JX, Wu WJ, Xu ZL, Sun YM, et al. Production and characterization of a**
529 **single-chain Fab fragment for the detection of O,O-diethyl organophosphorus pesticides. Anal**
530 **Methods-Uk 2016; 8:3140-7.**

531 **36. Nakamura H, Oda-Ueda N, Ueda T, Ohkuri T. A novel engineered interchain disulfide bond**
532 **in the constant region enhances the thermostability of adalimumab Fab. Biochem Biophys Res**
533 **Commun 2018; 495:7-11.**

534 **37. Cherf GM, Cochran JR. Applications of Yeast Surface Display for Protein Engineering.**
535 **Methods Mol Biol 2015; 1319:155-75.**

536 **38. Hunter SA, Cochran JR. Cell-Binding Assays for Determining the Affinity of Protein-Protein**
537 **Interactions: Technologies and Considerations. Methods Enzymol 2016; 580:21-44.**

538 **39. Rakestraw JA, Sazinsky SL, Piatesi A, Antipov E, Wittrup KD. Directed evolution of a**
539 **secretory leader for the improved expression of heterologous proteins and full-length antibodies in**
540 **Saccharomyces cerevisiae. Biotechnol Bioeng 2009; 103:1192-201.**

541 **40. Wang B, DeKosky BJ, Timm MR, Lee J, Normandin E, Misasi J, et al. Functional interrogation**
542 **and mining of natively paired human VH:VL antibody repertoires. Nat Biotechnol 2018.**

543 **41. Jain T, Sun T, Durand S, Hall A, Houston NR, Nett JH, et al. Biophysical properties of the**
544 **clinical-stage antibody landscape. Proc Natl Acad Sci U S A 2017; 114:944-9.**

545 **42. Le Fourn V, Girod PA, Buceta M, Regamey A, Mermod N. CHO cell engineering to prevent**
546 **polypeptide aggregation and improve therapeutic protein secretion. Metabolic engineering 2014;**
547 **21:91-102.**

548 **43. Lerch TF, Sharpe P, Mayclin SJ, Edwards TE, Lee E, Conlon HD, et al. Infliximab crystal**
549 **structures reveal insights into self-association. mAbs 2017; 9:874-83.**

550 **44. Kowalski JM, Parekh RN, Mao J, Wittrup KD. Protein Folding Stability Can Determine the**
551 **Efficiency of Escape from Endoplasmic Reticulum Quality Control. J Biol Chem 1998; 273:19453-8.**

552 **45. Kowalski JM, Parekh RN, Wittrup KD. Secretion efficiency in Saccharomyces cerevisiae of**
553 **bovine pancreatic trypsin inhibitor mutants lacking disulfide bonds is correlated with**
554 **thermodynamic stability. Biochemistry 1998; 37:1264-73.**

555 **46. Zhang Y, Werling U, Edelmann W. SLiCE: a novel bacterial cell extract-based DNA cloning**
556 **method. Nucleic Acids Res 2012; 40:e55.**

557 **47. Suga M, Isobe M, Hatakeyama T. Cryopreservation of competent intact yeast cells for**
558 **efficient electroporation. Yeast 2000; 16:889-96.**

559 **48. Subedi GP, Johnson RW, Moniz HA, Moremen KW, Barb A. High Yield Expression of**
560 **Recombinant Human Proteins with the Transient Transfection of HEK293 Cells in Suspension.**
561 **Journal of visualized experiments : JoVE 2015:e53568.**

562 **49. Niesen FH, Berglund H, Vedadi M. The use of differential scanning fluorimetry to detect**
563 **ligand interactions that promote protein stability. Nat Protoc 2007; 2:2212-21.**

564

565

566

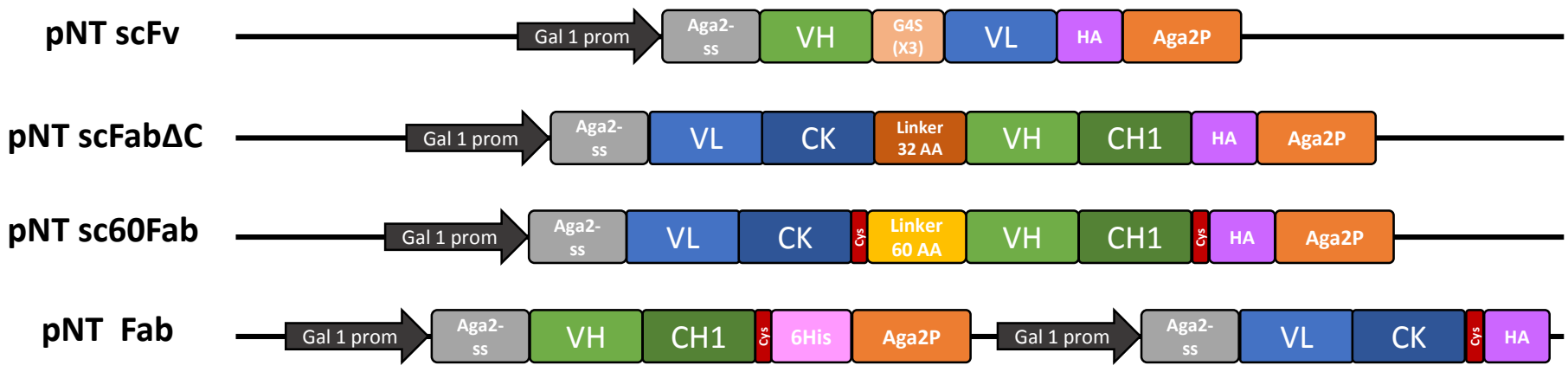

pNT scFv
Gal 1 prom
Aga2-ss
VH
G4S (X3)
VL
HA
Aga2P
pNT scFabΔC
Gal 1 prom
Aga2-ss
VL
CK
Linker 32 AA
VH
CH1
HA
Aga2P
pNT sc60Fab
Gal 1 prom
Aga2-ss
VL
CK
Cys
Linker 60 AA
VH
CH1
Cys
HA
Aga2P
pNT Fab
Gal 1 prom
Aga2-ss
VH
CH1
Cys
6His
Aga2P
Gal 1 prom
Aga2-ss
VL
CK
Cys
HA

Figure 1.

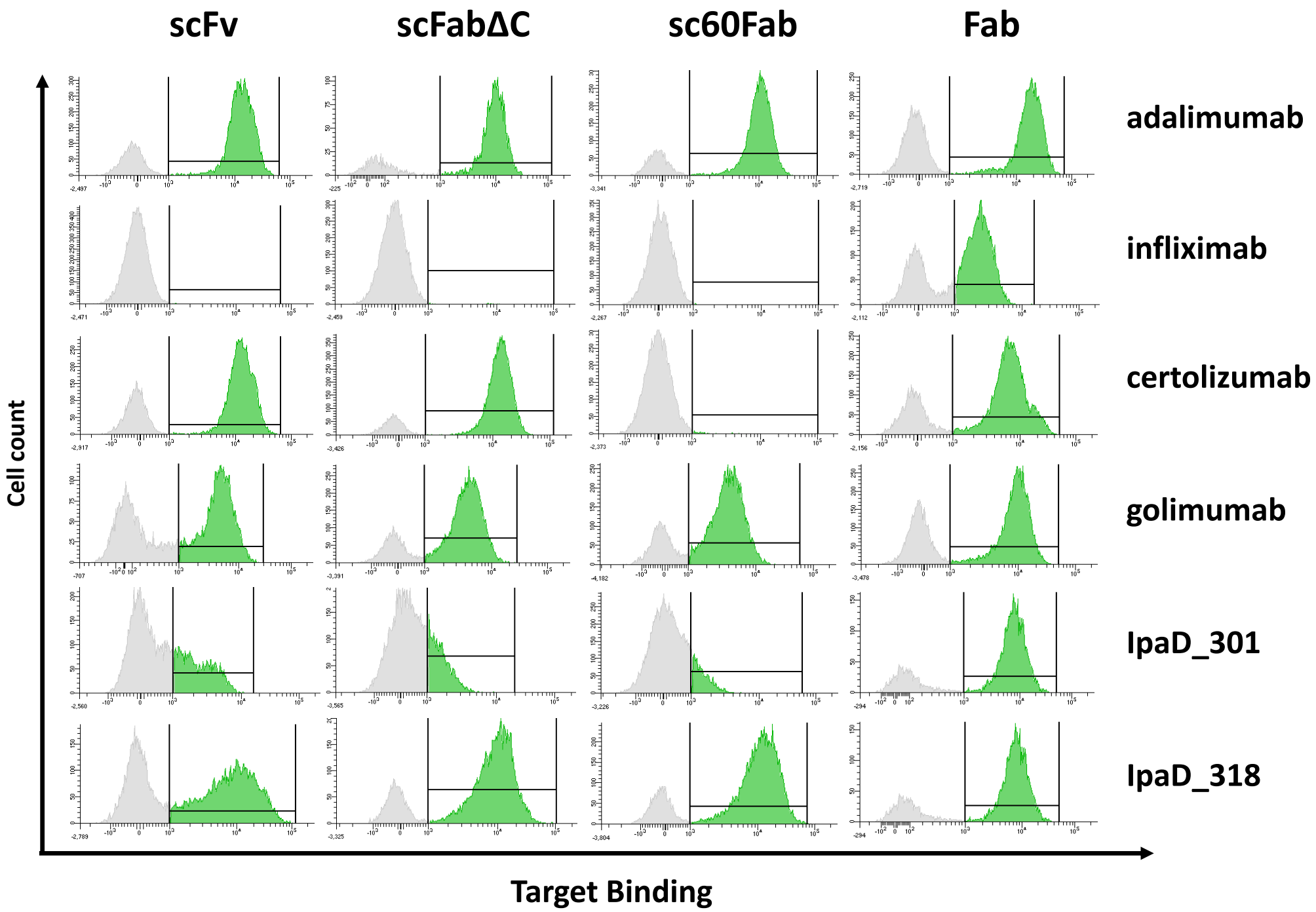

scFv
scFabΔC
sc60Fab
Fab
adalimumab
infliximab
certolizumab
golimumab
IpaD_301
IpaD_318
Cell count
Target Binding

Figure 2.

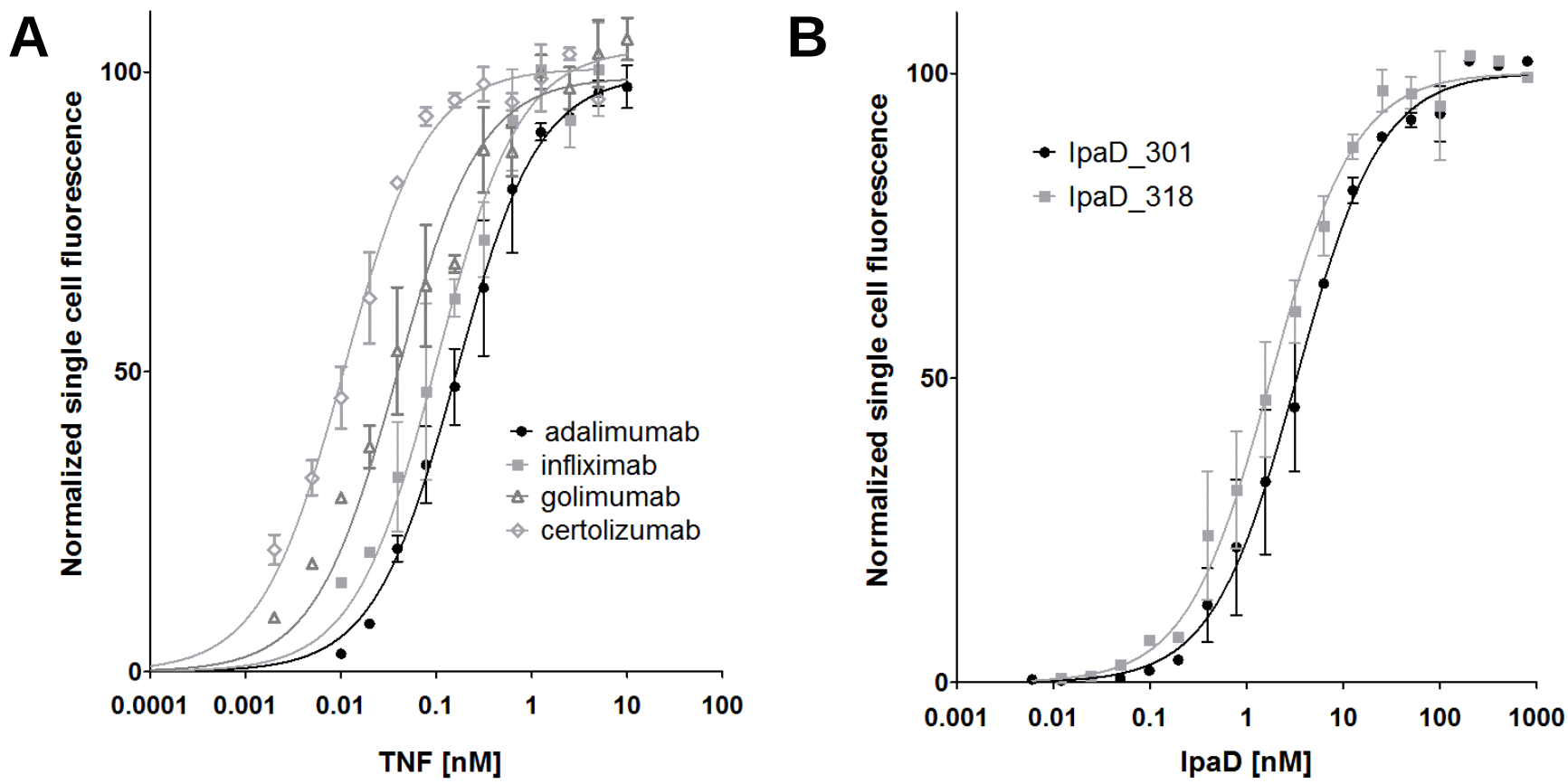

A
Normalized single cell fluorescence
100
50
0
0.0001
0.001
0.01
0.1
1
10
100
TNF [nM]
adalimumab
infliximab
golimumab
certolizumab
B
Normalized single cell fluorescence
100
50
0
0.001
0.01
0.1
1
10
100
1000
IpaD [nM]
IpaD_301
IpaD_318

Figure 3.

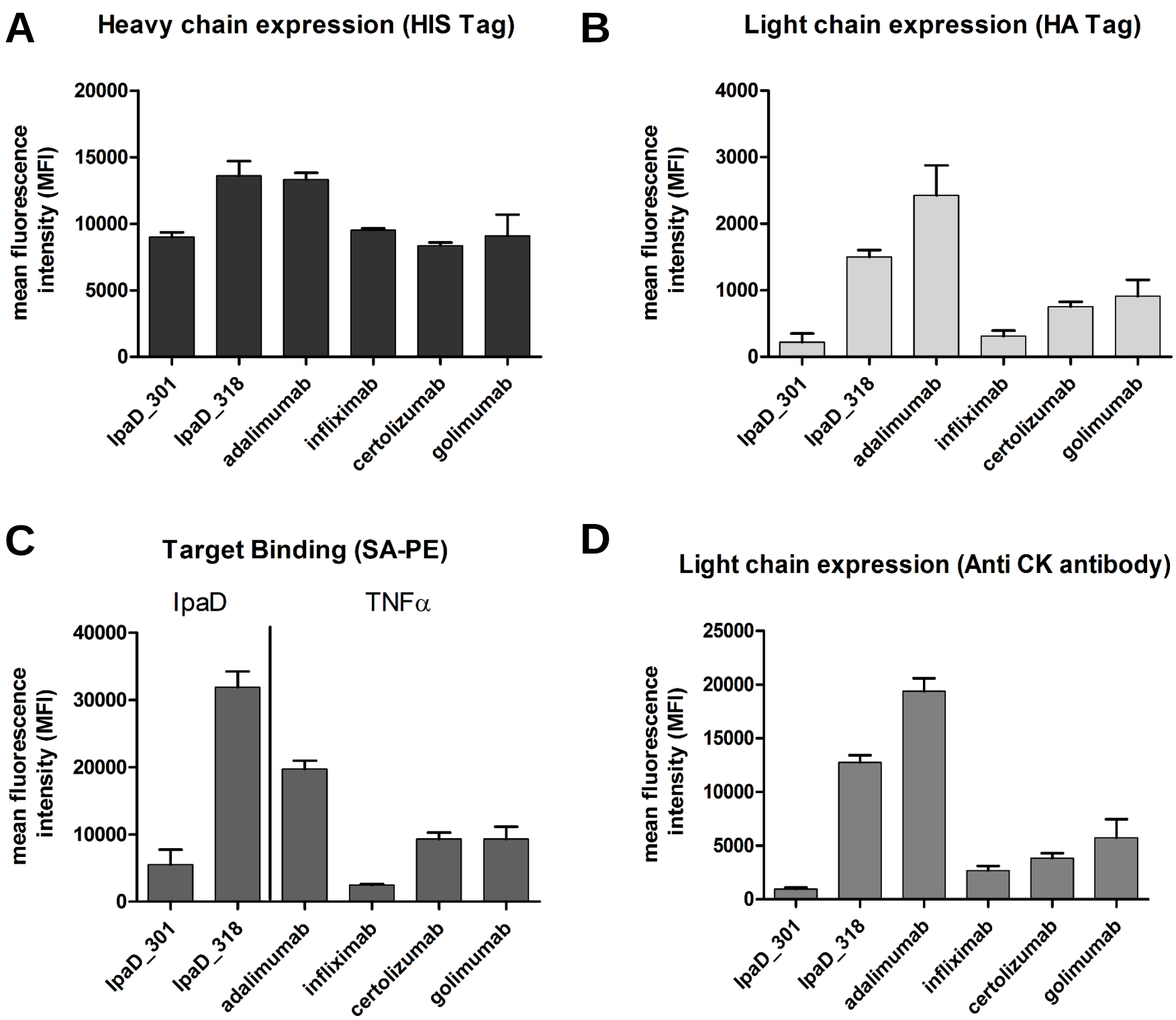

A
Heavy chain expression (HIS Tag)
mean fluorescence intensity (MFI)
20000
15000
10000
5000
0
IpaD_301
IpaD_318
adalimumab
infliximab
certolizumab
golimumab
B
Light chain expression (HA Tag)
mean fluorescence intensity (MFI)
4000
3000
2000
1000
0
IpaD_301
IpaD_318
adalimumab
infliximab
certolizumab
golimumab
C
Target Binding (SA-PE)
IpaD
TNFα
mean fluorescence intensity (MFI)
40000
30000
20000
10000
0
IpaD_301
IpaD_318
adalimumab
infliximab
certolizumab
golimumab
D
Light chain expression (Anti CK antibody)
mean fluorescence intensity (MFI)
25000
20000
15000
10000
5000
0
IpaD_301
IpaD_318
adalimumab
infliximab
certolizumab
golimumab

Figure 4.

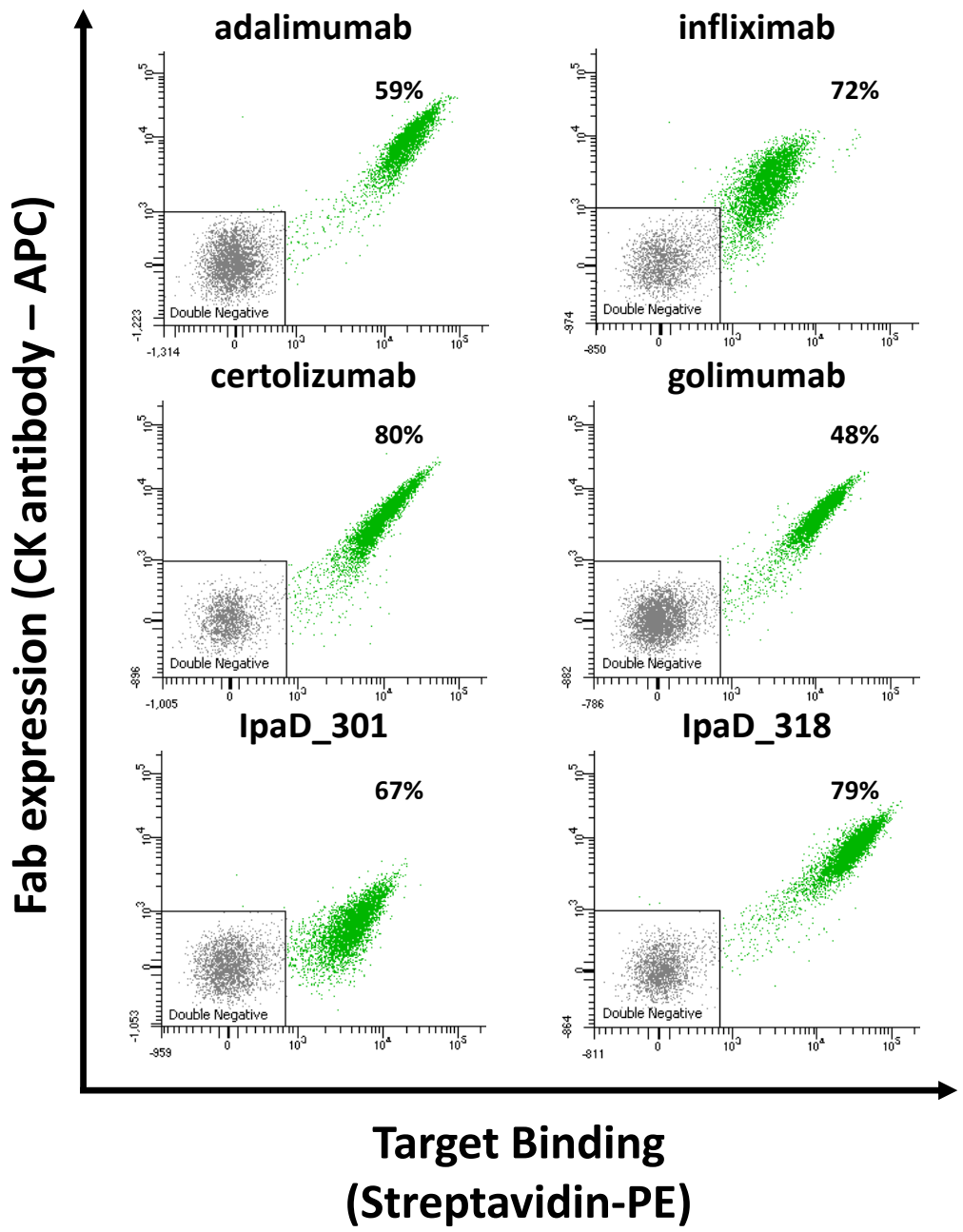

adalimumab
59%
infliximab
72%
certolizumab
80%
golimumab
48%
IpaD_301
67%
IpaD_318
79%
Double Negative
Fab expression (CK antibody – APC)
Target Binding
(Streptavidin-PE)

Figure 5.

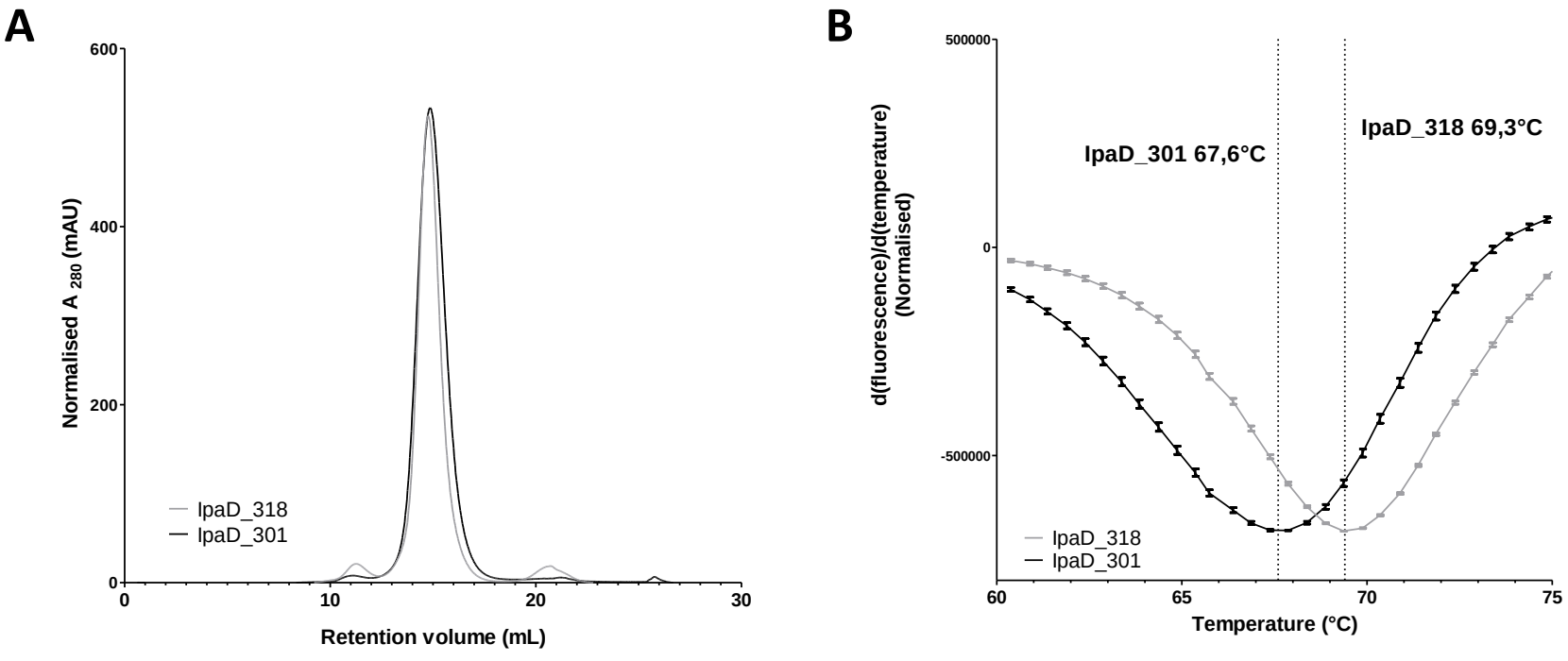
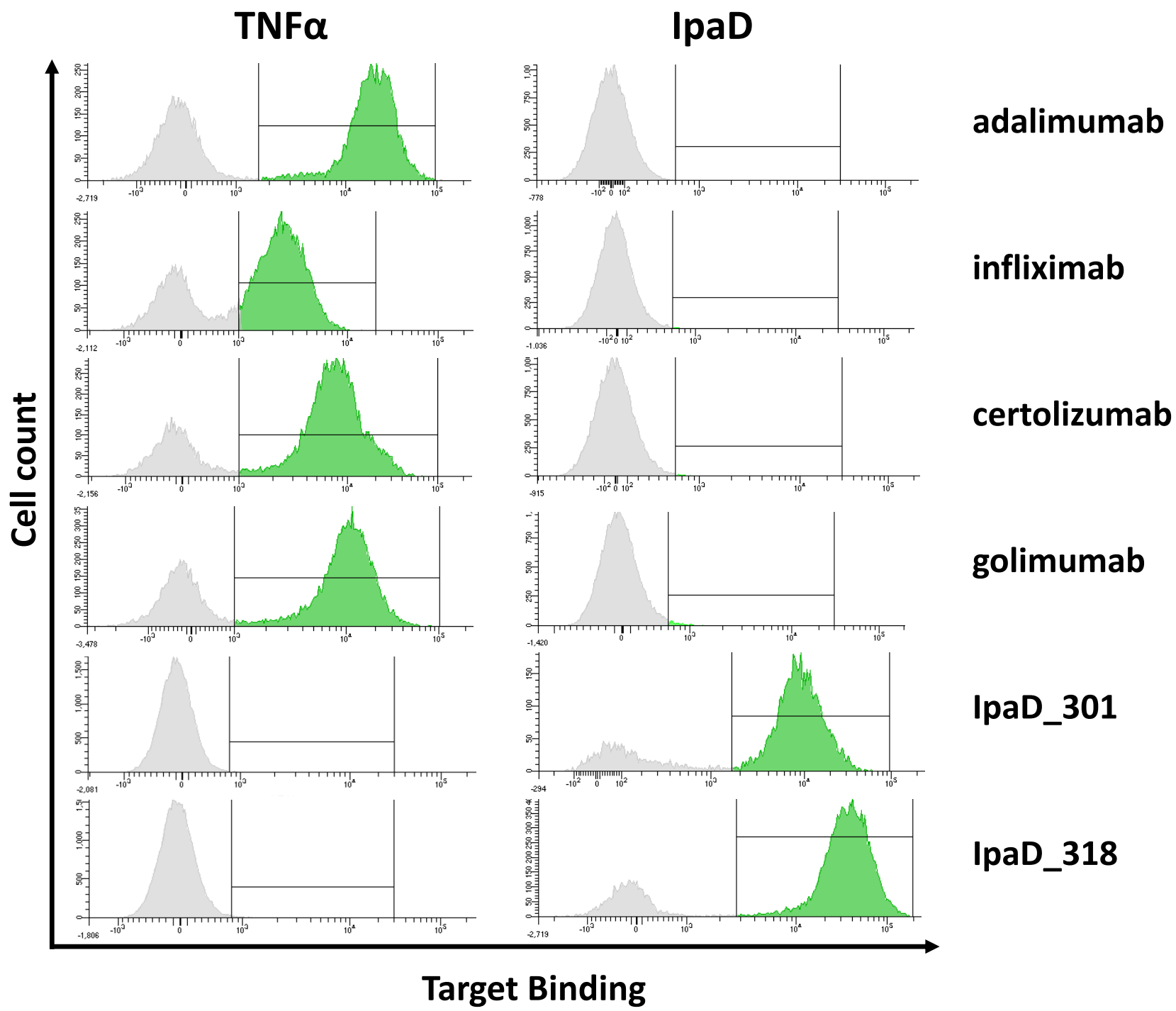
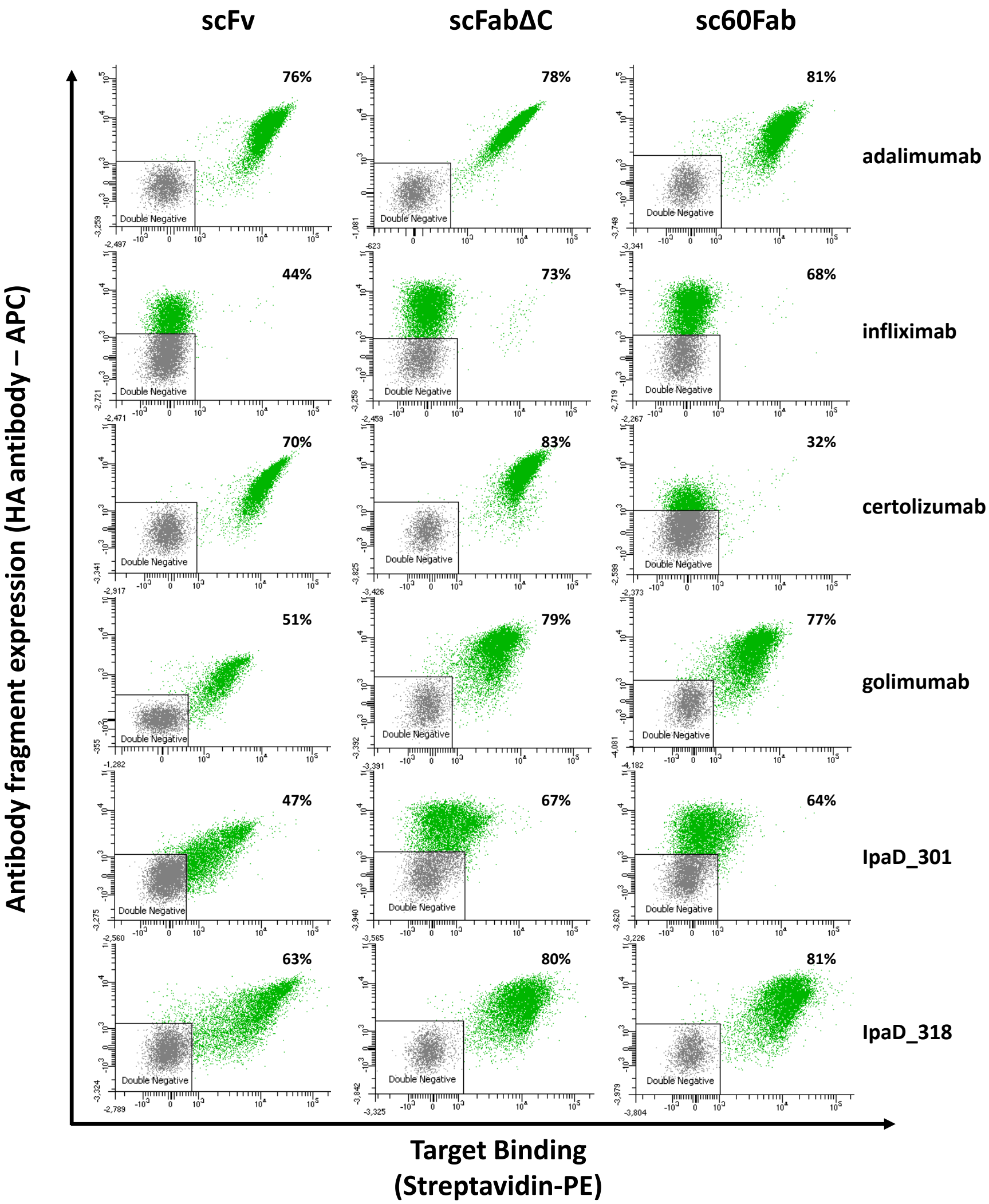

A
600
400
200
0
Normalised A 280 (mAU)
IpaD_318
IpaD_301
0
10
20
30
Retention volume (mL)
B
500000
0
-500000
d(fluorescence)/d(temperature)
(Normalised)
IpaD_301 67,6°C
IpaD_318 69,3°C
IpaD_318
IpaD_301
60
65
70
75
Temperature (°C)

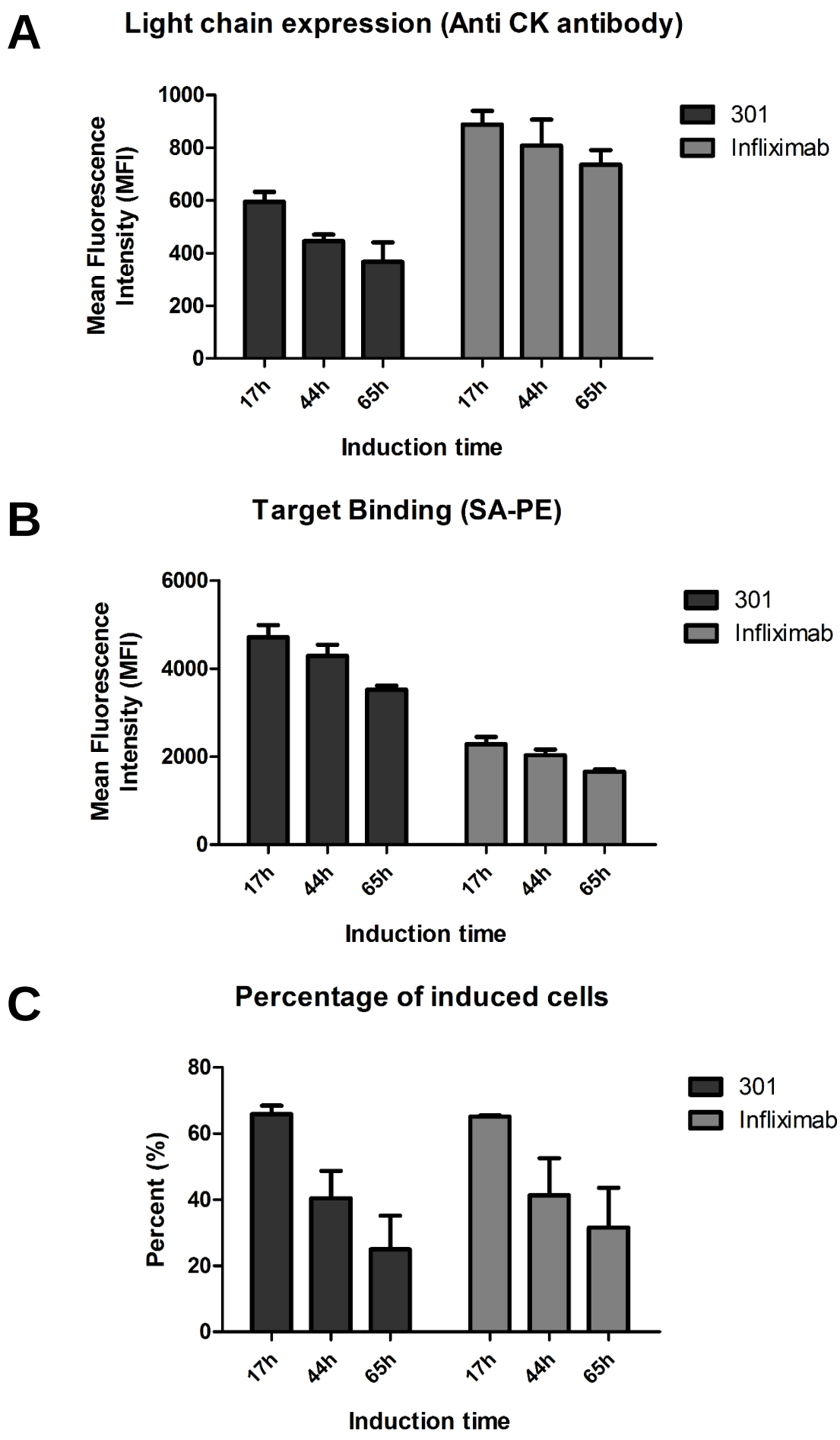
Figure 6.



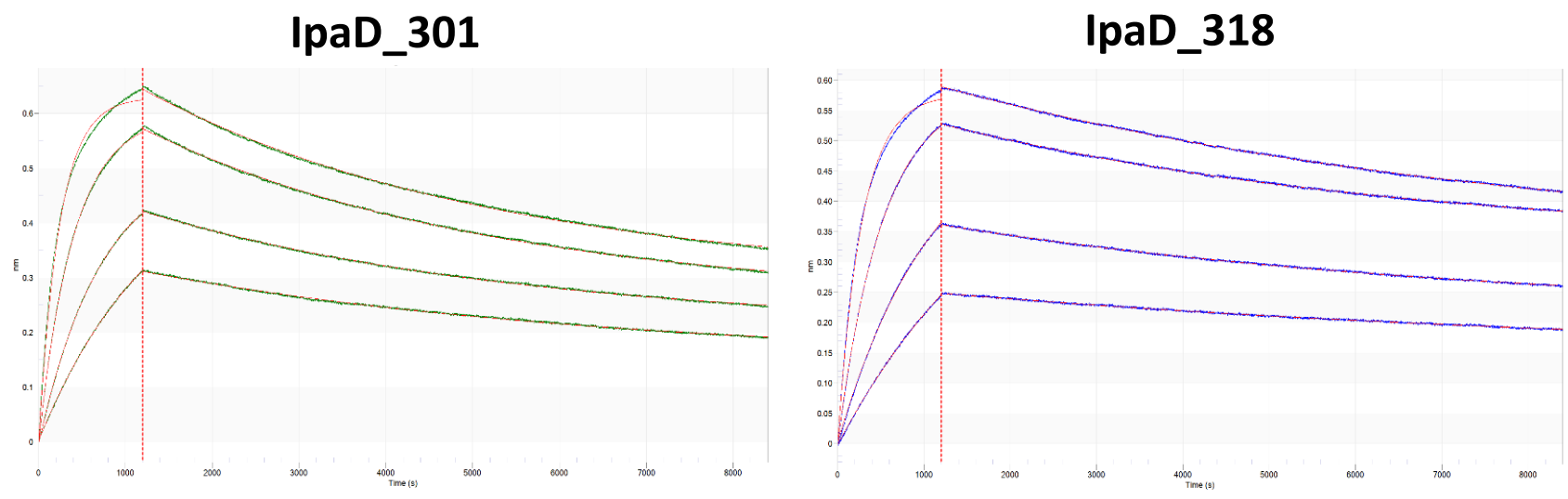
Supplementary figure 1. **TNFα and IpaD binding of the 6 selected antibodies expressed as Fab on the surface of yeast cells.** Cells are labeled with biotinylated antigen then with Streptavidin–PE (Target Binding) before FACS analysis. Selected antigen concentrations are far above the expected K_D to reach a quantitative binding with the antibody fragments. Respective antigen concentration were 20 nM for TNFα and 50 nM for IpaD. For each construct, a FACS histogram shows the fluorescence intensity corresponding to antigen binding (x-axis) vs the number of detected events (y-axis). Interval gates (bars) are shown to differentiate cells with detectable antigen binding (in green) from cells with no detectable antigen binding (in grey).



Supplementary figure 2. **Bivariate flow-cytometric analysis of yeast cells expressing various antibody fragments on their surface.** Each single dot on the plot designate the APC (y-axis) and PE (x-axis) fluorescence intensity values for a single yeast cell. Cells are double-labeled with biotinylated antigen/Streptavidin–PE (Target Binding) and anti-HA APC labels (Fab expression). Antigen concentration in labelling experiment were respectively 20 nM for anti-TNF antibodies and 50 nM for anti-IpaD antibodies. Functional subpopulations in green were gated using an inverted gate of the double negative subpopulations. The percentage is the subpopulation of cell expressing Fab.



Supplementary Figure 3. **Antigen binding and expression levels of antibodies IpaD_301 and Infliximab expressed as Fab antibody fragments on the surface of yeast cells.** Cells were labeled with the appropriate expression reporter and the mean fluorescence intensity of positive cells was determined by FACS analysis. For antigen binding, cells were double-labeled with biotinylated antigen and Streptavidin–PE (target binding) before FACS analysis. (A) Light chain expression evaluated with anti-CK antibody reporter (B) Biotinylated antigen binding evaluated with Streptavidin-PE (C) Percentage of cells with detectable antigen binding. Measurements were made in triplicate using cells from independent cultures.



	KD (M)	kon(1/Ms)	koff(1/s)
Fab IpaD_301	1.05E-09	2.16E+05	2.25E-04
Fab IpaD_318	9.25E-10	1.59E+05	1.47E-04

Supplementary figure 4. **Bio-layer interferometry (Octet Red) K_D determination of IpaD_301 Fab and IpaD_318 Fab.** Fab binding to recombinant IpaD was assayed by BLI. Biotinylated IpaD was loaded on Streptavidin biosensors and equilibrated in PBSF buffer (1X PBS pH 7.4, 0.01% (w/v) BSA) before being incubated with varying concentrations of each Fab for 20 min. Dissociation was performed in PBSF buffer to for 2 hours. For each Fab, real time binding profiles using multiple protein concentrations and fitted curves (1:1 Langmuir binding model) are displayed.