Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59933R2
Full Title:	Activated Cross-Linked Agarose for the Rapid Development of Affinity Chromatography Resins - Antibody Capture as a Case Study
Keywords:	Protein A replacements; chromatography resin; downstream processing (DSP); design of experiments (DoE); plant-made pharmaceuticals (PMPs); primary recovery
Corresponding Author:	Johannes Felix Buyel, Dr. Fraunhofer-Gesellschaft zur Förderung der angewandten Forschung e. V. Aachen, NRW GERMANY
Corresponding Author's Institution:	Fraunhofer-Gesellschaft zur Förderung der angewandten Forschung e. V.
Corresponding Author E-Mail:	johannes.buyel@rwth-aachen.de
Order of Authors:	Matthias Knödler
	Clemens Rühl
	Patrick Opdensteinen
	Johannes Felix Buyel
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Aachen, Germany

1 **TITLE:**
2 **Activated Cross-Linked Agarose for the Rapid Development of Affinity Chromatography**
3 **Resins - Antibody Capture as a Case Study**
4
5 **AUTHORS:**
6 Knödler, Matthias[1]
7 matthias.knoedler@ime.fraunhofer.de
8
9 Rühl, Clemens[2]
10 clemens.ruehl@gmail.com
11
12 Opdensteinen, Patrick[1]
13 patrick.opdensteinen@ime.fraunhofer.de
14
15 Buyel, Johannes F.[1,3]
16 johannes.buyel@ime.fraunhofer.de
17
18 1.   Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Fraunhofer-
19 Gesellschaft zur Förderung der angewandten Forschung e. V., Aachen, Germany
20 2.   Sanofi Deutschland GmbH, Industriepark Hoechst, Frankfurt am Main, Germany
21 3.   Institute for Molecular Biotechnology, RWTH Aachen University, Aachen, Germany
22
23 **CORRESPONDING AUTHOR:**
24 Johannes F. Buyel
25

30 **SUMMARY:**
31 In this procedure, a DsRed-based epitope ligand is immobilized to produce a highly selective
32 affinity resin for the capture of monoclonal antibodies from crude plant extracts or cell culture
33 supernatants, as an alternative to Protein A.
34

35 **ABSTRACT:**
36 The purification of monoclonal antibodies (mAbs) is commonly achieved by Protein A affinity
37 chromatography, which can account for up to 25% of the overall process costs. Alternative, cost-
38 effective capture steps are therefore valuable for industrial-scale manufacturing, where large
39 quantities of a single mAb are produced. Here we present a method for the immobilization of a
40 DsRed-based epitope ligand to a cross-linked agarose resin allowing the selective capture of the
41 HIV-neutralizing antibody 2F5 from crude plant extracts without using Protein A. The linear
42 epitope ELDKWA was first genetically fused to the fluorescent protein DsRed and the fusion
43 protein was expressed in transgenic tobacco (*Nicotiana tabacum*) plants before purification by
44 immobilized metal-ion affinity chromatography. Furthermore, a method based on activated

45 cross-linked agarose was optimized for high ligand density, efficient coupling and low costs. The
46 pH and buffer composition and the soluble ligand concentration were the most important
47 parameters during the coupling procedure, which was improved using a design-of-experiments
48 approach. The resulting affinity resin was tested for its ability to selectively bind the target mAb
49 in a crude plant extract and the elution buffer was optimized for high mAb recovery, product
50 activity and affinity resin stability. The method can easily be adapted to other antibodies with
51 linear epitopes. The new resins allow gentler elution conditions than Protein A and could also
52 reduce the costs of an initial capture step for mAb production.
53
54 **INTRODUCTION:**
55 Biopharmaceutical products are important for the treatment of a broad spectrum of diseases in
56 nearly every branch of medicine[1]. Monoclonal antibodies (mAbs) dominate the
57 biopharmaceutical market, with worldwide sales expected to reach almost €110 billion in 2020[2].
58 The favored expression platform for mAbs are Chinese hamster ovary cell lines, which typically
59 produce high mAb titers of up to 10 g·L^{-1} in the culture supernatant[3,4]. However, the production
60 of mAbs in mammalian cell cultures is expensive due to the high cost of the medium and the
61 need for sterile fermentation[5]. Alternative expression platforms such as plants potentially offer
62 a faster, simpler, less expensive and more scalable approach for industrial manufacturing[6,7].
63
64 In addition to the costs associated with mammalian cell cultures, the widespread use of Protein
65 A affinity chromatography for product capture is a major cost driver for the industrial production
66 of mAbs. Protein A is naturally found on the surface of *Staphylococcus aureus* cells and it binds
67 to the fragment crystallizable (Fc) region of certain murine and human antibodies, thereby acting
68 as a defense mechanism to evade the humoral immune system[8]. Protein A has become the
69 industry gold standard for the capture of mAbs from cell culture supernatants and is also widely
70 used by the research community because it is highly selective, typically achieving mAb purities of
71 ~95% in a single step[8]. Unsurprisingly, sales of Protein A over the last two decades have closely
72 mirrored the sales of mAbs[8]. Depending on the production scale, the costs of Protein A can
73 correspond to more than 25% of the total process costs and thereby affect the market price of
74 therapeutic mAbs, which can be up to €2000 g^{-1} [5,9]. Therefore, alternative chromatography resins
75 with a similar purification performance have the potential to substantially reduce production
76 costs, making antibody-based therapies accessible for a larger number of patients[10-12]. Such
77 alternatives may also circumvent the disadvantages of Protein A chromatography, including the
78 harsh elution conditions at low pH (typically <3.5) that can potentially cause mAbs to undergo
79 conformational changes that promote aggregation[13]. Importantly, Protein A is selective only for
80 the Fc region of certain IgG subclasses, so non-functional molecules with truncated binding
81 domains may co-purify with the intact product[5], whereas mAb derivaties such as single-chain
82 variable fragments do not bind to Protein A at all.
83
84 Here, we describe an alternative affinity chromatography resin for the capture of the HIV-
85 neutralizing mAb 2F5 using its linear epitope ELDKWA (one letter amino acid code)[5,14]. We
86 genetically fused the 2F5 epitope to the C-terminus of the fluorescent protein DsRed, which
87 functioned as a carrier and reporter molecule, and produced the resulting protein **D**sRed-2**F**5-
88 **E**pitope (DFE) in transgenic tobacco (*Nicotiana tabacum*) plants. DFE was purified by single-step

89   immobilized metal-ion affinity chromatography (IMAC). The immobilization of the purified DFE
90   affinity ligand onto a cross-linked agarose resin was achieved by chemical coupling using N-
91   hydroxysuccinimide (NHS)-activated cross-linked agarose columns. Statistical experimental
92   designs were then used to optimize the immobilization procedure and coupling efficiency[15]. The
93   purification strategy for mAb 2F5 was evaluated in terms of antibody purity, yield and ligand
94   stability. In contrast to Protein A, which binds the Fc region, DFE bound to the complementarity-
95   determining region of 2F5, ensuring the purification of molecules with an intact paratope. Our
96   concept can easily be adapted to any mAb with a linear epitope or to other peptide-based affinity
97   ligands which can be easily identified by microarray studies[16].
98
99   [Place Figure 1 here]
100
101   **PROTOCOL:**
102
103   **1.   Cultivate the transgenic tobacco plants**
104
105   NOTE: The design of the DFE fusion protein and the generation of transgenic plants are described
106   elsewhere[5,17].
107
108   1.1)   Germinate the transgenic tobacco seedlings in soil. Irrigate the plants with a 1.0 g L^{-1}
109   fertilizer solution. Transfer the plants to single, 100 mm x 100 mm x 60 mm (length, width, height)
110   pots when they grow to a diameter of ~0.04 m.
111
112   1.2)   Cultivate the transgenic tobacco plants in a greenhouse with a 16-h photoperiod (25/22
113   °C light/dark temperature regime), with 70% relative humidity and automated fertilization with
114   a 1.0 g L^{-1} fertilizer solution for 15 min every hour.
115
116   1.3)   After 7 weeks, harvest all leaves except the four cotyledon leaves at the base of the plant
117   stem. Immediately process the harvested leaves as described below.
118
119   **2.   Heat precipitation of host cell proteins**
120
121   2.1)   Set up a water bath with a 20 L working volume in a stainless-steel heated vessel (0.3 m x
122   0.3 m x 0.3 m). At the start, place a magnetic pump in the water bath to agitate the liquid
123   permanently with a flow of 5 L min^{-1}. Mount an adjustable thermostat on the water bath for
124   temperature control (steps 2.4 and 2.8).
125
126   CAUTION: All the following steps up to 2.10 involve the handling of hot liquid. Wear appropriate
127   personal protective equipment including thermally insulated gloves and goggles.
128
129   2.2)   Add 15 L of deionized water to the water bath and heat the liquid to 70 °C.
130
131   2.3)   Place a 10 L bucket filled with 5 L of deionized water on a magnetic stirrer. Add sodium
132   phosphate to a final concentration of 120 mM. Adjust the pH to 8.14 using 10 M hydrochloric

133 acid. When all the components have dissolved, add the resulting concentrated blanching buffer
134 (5 L) to the water bath from step 2.2.

136 2.4)   Agitate the water bath until the temperature reaches 70 °C. Use 10 M hydrochloric acid
137 to adjust the pH to 8.14 if necessary. Continue agitating for at least 15 min after the required
138 temperature and pH are reached to ensure that the entire assembly is in thermal equilibrium.

140 2.5)   Prepare a 20 L bucket (0.3 m x 0.3 m x 0.3 m) filled with deionized water at a temperature
141 of ~17 °C (required for step 2.9).

143 2.6)   Prepare 400 g aliquots of the harvested tobacco leaves from step 1.3. Place one aliquot
144 in a perforated basket (0.2 m x 0.2 m x 0.2 m; pore width at least 0.02 m × 0.02 m). Avoid
145 overfilling the basket with plant material or packing the leaves too tightly to avoid damaging the
146 tissue.

148 2.7)   Fully submerge the basket in the hot blanching buffer from step 2.4 and make sure all
149 leaves remain under the liquid surface. Use a thermostable silicone spoon to hold the leaves
150 under the surface if necessary.

152 2.8)   Incubate the tobacco leaves for 1.5 min in the blanching fluid while the pump is still
153 agitating the liquid. Monitor the liquid temperature during the entire incubation period. Avoid
154 blocking the pump inlet with tobacco leaves.

156 2.9)   Remove the basket of tobacco leaves from the blanching buffer and drain residual buffer
157 from the leaves for 30 s. Transfer the basket to the bucket filled with cold water (from step 2.5)
158 and immerse the leaves for 30 s. Remove the basket and drain residual water from the leaves for
159 30 s before homogenization (step 3).

161 2.10)   Repeat steps 2.6 to 2.9 with fresh aliquots from 2.6 until the entire harvested biomass is
162 processed. Constantly monitor and adjust the pH and the temperature of the blanching buffer in
163 the water bath.

165 NOTE: Blanched plant material can be stored on ice for up to 30 min when several blanching
166 cycles are required to process the entire biomass. Blanched leaves can also be stored in vacuum-
167 sealed bags at −80 °C for at least 3 weeks. However, immediate processing is recommended
168 because prolonged storage can reduce the DFE yield.

170 **3.   Protein extraction and clarification**

172 CAUTION: The following steps involve a blender with rotating blades. Do not work in the blender
173 vessel when powered or mounted on the motor unit.

175 3.1)   Place 150 g (wet mass) of blanched tobacco leaves (step 2.9) in the blender vessel and
176 add 450 mL of extraction buffer (50 mM sodium phosphate, 500 mM sodium chloride, 10 mM

177 sodium disulfite, pH 7.5). Close the blender cap tightly to prevent the spilling of plant material or
178 buffer.

180 3.2)   Homogenize the leaves for 3 × 30 s with 30 s breaks between each pulse. Ensure that all
181 leaves are homogenized and that none stick to the top of the blender vessel. If necessary, open
182 the blender during the breaks and push down leaves that stick at the upper part of the vessel,
183 using a clean silicone spoon.

185 3.3)   After homogenization, take a 1.0 mL sample of the homogenate for subsequent analysis
186 (step 7).

188 3.4)   Collect the homogenate in a vessel of adequate size (e.g. if working with a total biomass
189 of 1.0 kg, use a vessel with a capacity of at least a 5 L). Repeat steps 3.1 to 3.3 until all of the
190 blanched biomass is homogenized.

192 3.5)   Mount a bag filter into a filter mount and place another adequately sized vessel (see 3.4)
193 beneath the assembly. Apply the homogenate to the bag filter at a rate of ~0.15 L min^{-1}. Take
194 samples of the bag filtrate after each liter for subsequent analysis (step 7) and measure the
195 turbidity of the bag filtrate pool as a 1:10 dilution in extraction buffer using a turbidimeter or
196 similar device.

198 3.6)   Further clarify the bag filtrate pool using 0.02 m^2 of a K700 (top) and KS50 (bottom) depth
199 filter layer combination per liter of bag filtrate pool. Apply a flow rate of 3.0 L min^{-1} m^{-2} up to a
200 maximum pressure of 0.2 MPa. Then remove residual particles by passing the clarified filtrate
201 through a 0.2-μm sterile filter as previously described[5].

203 **4.   Purification of the DFE affinity ligand**

205 4.1)   Prepare a fast protein liquid chromatography system by flushing with elution buffer
206 (10 mM sodium phosphate, 300 mM imidazole, pH 7.4), wash buffer (10 mM sodium phosphate,
207 pH 7.4) and equilibration buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 7.4).
208 Mount a column containing ~50 mL of chelating cross-linked agarose resin per kilogram of leaf
209 biomass (~2.5 L depth filtrate).

211 4.2)   Charge the column with nickel ions by applying 5 column volumes (CV) of 200 mM nickel
212 sulfate solution and wash it with 5 CV of elution buffer. Use a flow rate of 50 cm h^{-1} and monitor
213 the UV signals at 260, 280 and 558 nm for all subsequent chromatography steps.

215 4.3)   Equilibrate the column with 10 CV of equilibration buffer. Then load 50 CV of the clarified
216 plant extract (from step 3.6) onto the conditioned column.

218 4.4)   Wash the column with 10 CV of wash buffer. Elute the DFE fusion protein with 5 CV
219 elution buffer and collect the product-containing fraction once the UV signals at 280 nm and 558
220 nm have increased to more than 5 mAU above the baseline.

221

4.5)   Take a 0.2 mL sample from the elution fraction in order to measure the total soluble protein (TSP) concentration (step 7.1), DFE concentration (steps 7.2 and 7.3), and DFE purity (step 7.4).

4.6)   Mount a column containing ~50 mL of cross-linked dextran resin on a chromatography system. Equilibrate the column with 5 CV of coupling buffer (200 mM HEPES, 500 mM sodium chloride, pH 8.5). Inject 10 mL of the DFE elution fraction (step 4.4) for buffer exchange and monitor the UV absorbance at 280 and 558 nm.

4.7)   Collect the DFE-containing fraction once the UV signals at 280 nm and 558 nm have increased to more than 5 mAU above the baseline. Take a 0.2 mL sample and determine the TSP concentration, DFE concentration, and DFE purity (step 7).

4.8)   Concentrate the purified DFE sample (from step 4.7) to 15 g L^{-1} using a centrifugal concentrator tube at 3,000 × g at 4 °C for 30 min in a centrifuge. Continue with the coupling reaction (step 5).

NOTE: Store the DFE solution at 4 °C if the concentration or coupling steps cannot be carried out immediately.

5.   **Coupling DFE to the activated cross-linked agarose resin**

NOTE: Do not replace the isopropanol used for the storage of NHS-activated columns until all equipment and solutions for coupling are ready. Never let the columns run dry.

5.1)   Setup a design of experiments (DoE) model to optimize the coupling of DFE to the activated resin, with pH, buffer composition and DFE concentration as factors. The details of the DoE method are described elsewhere[15].

5.2)   Prepare the affinity ligand solution (from step 4.8) in a concentration range from 0.5–15 g L^{-1} or as defined in the DoE and store it on ice until the coupling reaction is ready (step 5.5). Fill at least ten 2 mL syringes with the DFE solution and prepare an adapter to mount the syringes on NHS-activated cross-linked agarose columns with a bed volume of 1.0 mL.

5.3)   For every 10 columns used for coupling, prepare 30 mL of deactivation solution (0.5 M ethanolamine, 0.5 M sodium chloride, pH 8.3), 30 mL of low-pH solution (0.1 M sodium acetate, 0.5 M sodium chloride, pH 4.0) and 10 mL of storage solution (0.05 M disodium phosphate, 0.1% (m/v) sodium azide, pH 7.0).

5.4)   Prepare 20 mL of 1 mM hydrochloric acid in a tube and incubate it on ice for at least 20 min. Prepare a precision scale to monitor the flow-through fractions for all steps during the coupling reaction (step 5.7).

265   5.5)   Open a sealed NHS-activated cross-linked agarose column and mount the syringe adapter
266   at the column inlet. Prevent any air entering the column by applying a drop of buffer onto the
267   adapter inlet before connecting it to the syringe.
268
269   5.6)   Wash the column with 6 mL of ice-cold 1 mM hydrochloric acid (from step 5.4) at a flow
270   rate of <1 mL min^{-1} and immediately proceed to step 5.7.
271
272   5.7)   Inject 1.5 mL of DFE solution (step 5.2) using a 2 mL syringe at a flow rate of <1 mL min^{-1}
273   and collect the flow-through fraction on a precision scale (step 5.4) for subsequent analysis (step
274   7). Seal the column at both ends and incubate for 15–45 min at 22 °C, depending on the DoE
275   setup.
276
277   5.8)   Inject 6 mL of deactivation solution followed by 6 mL of low-pH solution at a flow rate of
278   <1 mL min^{-1} to remove non-covalently bound ligands from the resin. Then inject 6 mL of
279   deactivation solution and incubate the column for 15 min.
280
281   5.9)   Inject 6 mL of low-pH solution into the column, followed by 6 mL of deactivation solution.
282   Then inject another 6 mL of low-pH solution into the column.
283
284   5.10)   Inject 2 mL of storage solution into the column and store at 4 °C.
285
286   **6.   Testing the purification of mAbs from clarified plants extracts**
287
288   6.1)   Prepare 100 mL of clarified plant extract containing 2F5[5] or the supernatant from the
289   preferred cell-based expression system, also containing 2F5.
290
291   6.2)   Prepare equilibration buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH
292   7.4), low-pH elution buffer (0.05 M citrate, 0.05 M sodium chloride, pH 4.0–3.25), and high-ionic-
293   strength elution buffer (1.0–4.0 M magnesium chloride, 0.1 M HEPES, pH 8.0)
294
295   6.3)   Flush the chromatography system with the buffers. Mount a DFE affinity column (from
296   step 5.10) on the chromatography system and equilibrate with 5 CV of equilibration buffer at a
297   flow rate of 1.0 mL min^{-1}. Monitor the UV absorbance at 280 nm.
298
299   NOTE: Loading plant extract or cell culture supernatant onto the column can cause an increase
300   in backpressure. Set a high-pressure alert at 0.2 MPa to avoid damage to the chromatography
301   system or DFE column.
302
303   6.4)   Load 80 mL of the clarified plant extract or supernatant (step 6.1) onto the column at a
304   flow rate of 0.5 mL min^{-1} to guarantee a contact time of 2 min. Collect the flow-through samples
305   in 2 mL fractions for breakthrough curve reconstruction (step 7.3). Store the flow-through
306   samples at 4 °C if immediate sample analysis is not possible.
307
308   6.5)   Wash the column with 6 CV of equilibration buffer. Collect a sample of the wash at the

309   <mark>beginning, middle and end of this step.</mark>

310

311   <mark>6.6)   Elute mAb 2F5 with 5 CV of low-pH elution buffer or high-ionic-strength elution buffer</mark>
312   <mark>(0.1 M HEPES, 1.25 M magnesium chloride, pH 8.0). Collect the DFE fraction when the UV 280 nm</mark>
313   <mark>signal has increased to 5 mAU above the baseline.</mark>

314

315   <mark>6.6.1) Optimize the elution buffer for each epitope–antibody pair. For 2F5, 1.25 M magnesium</mark>
316   <mark>chloride achieved an optimal balance between product recovery and ligand stability.</mark>

317

318   NOTE: <mark>The magnesium chloride solution is prone to precipitation. Therefore, dissolve the</mark>
319   <mark>magnesium chloride in ~700 mL of water. Separately dissolve the HEPES in 100 mL of water and</mark>
320   <mark>adjust the pH to 8.0. Add the dissolved magnesium chloride solution to the HEPES solution and</mark>
321   <mark>add water to a final volume of 1.0 L. Do not adjust the pH after dissolving the magnesium chloride</mark>
322   <mark>because this will cause precipitation.</mark>

323

324   6.7)   Analyze all samples taken during steps 6.4–6.6 using the Bradford method, lithium
325   dodecylsulfate polyacrylamide gel electrophoresis (LDS-PAGE) and surface plasmon resonance
326   (SPR) spectroscopy (step 7).

327

328   **7.   Sample analysis**

329

330   7.1.   Measure the TSP concentration using the Bradford method[18,19].

331

332   7.1.1. In triplicate, pipette 2.5 µL of each sample into a single well of a 96-well plate. Use eight
333   bovine serum albumin (BSA) standards in triplicate covering the range 0–2,000 mg L^{-1}.

334

335   7.1.2. Add 200 µL of Bradford reagent to each well and mix by gently pipetting up and down.
336   Keep the pipette level to avoid the formation of bubbles that distort the subsequent readout.

337

338   7.1.3. Incubate the plate for 10 min at 22 °C and measure the absorbance at 595 nm in a
339   spectrophotometer. Calculate the TSP concentration in the samples based on a standard curve
340   through the BSA reference points.

341

342   7.2.   Quantify DFE by fluorimetry

343

344   7.2.1. In triplicate, pipette 50 µL of each sample into single wells of a black 96-well half-area
345   plate. Include six DsRed standards covering the range 0–225 mg L^{-1}.

346

347   7.2.2. Measure the fluorescence twice using a 530 ± 30 nm excitation filter and a 590 ± 35 nm
348   emission filter in a spectrophotometer. Calculate the DFE concentration in the samples based on
349   a standard curve through the DsRed reference points.

350

351   7.3.   Measure the 2F5 concentration by SPR spectroscopy[20].

352

353 7.3.1. Prepare the HBS-EP running buffer (10 mM 4-(2-hydroxyethyl)-1-
354 piperazineethanesulfonic acid (HEPES), 3 mM ethylenediaminetetraacetic acid (EDTA), 150 mM
355 sodium chloride, 0.005% v/v Tween-20, pH 7.4) and filter sterilize it by passing it through a 0.2
356 µm vacuum bottle-top filter. Degas the buffer for 15 min.

358 7.3.2. Connect the HBS-EP running buffer to the SPR instrument before docking a
359 carboxymethylated dextran surface chip and equilibrate the system using the prime function.
360 Start a manual run with a flow rate of 30 µL min^{-1} and condition the chip surface by alternately
361 injecting 30 mM hydrochloric acid and 25 mM sodium hydroxide (two injections each) over flow
362 cells 1 and 2 at a flow rate of 30 µL min^{-1} for 1 min.

364 7.3.3. Prepare 300 µL of a 500 mg L^{-1} Protein A solution in 10 mM sodium acetate (pH 4.0). Thaw
365 vials containing 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and
366 0.1 M NHS and centrifuge at 16,000 × g for 1 min before mixing 70 µL of EDC and 70 µL of NHS.

368 7.3.4. Transfer the EDC/NHS mixture and the Protein A solution to 7-mm plastic sample vials
369 and place in the sample rack. Activate the carboxymethylated dextran chip surface by injecting
370 the EDC/NHS mixture over flow cells 1 and 2 at a flow rate of 10 µL min^{-1} for 10 min.

372 7.3.5. Couple Protein A to the activated carboxymethylated dextran surface by injecting the
373 Protein A solution over flow cell 2 at a flow rate of 15 µL min^{-1} for 15 min.

375 7.3.6. While coupling the Protein A, thaw a vial of 1.0 M ethanolamine hydrochloride (pH 8.5)
376 and centrifuge at 16,000 × g for 1 min. Transfer 150 µL to a 7-mm plastic vial and place into the
377 instrument. When step 7.3.5 is complete, deactivate the chip surface by injecting the
378 ethanolamine solution over flow cells 1 and 2 at a flow rate of 10 µL min^{-1} for 7 min.

380 7.3.7. Condition the chip surface by alternately injecting 30 mM hydrochloric acid and 25 mM
381 sodium hydroxide (two injections each) over flow cells 1 and 2 at a flow rate of 30 µL min^{-1} for
382 0.5 min.

384 7.3.1) Prepare standards of antibody 2F5 in HBS-EP running buffer at a concentration of 500 µg
385 L^{-1} and dilute samples containing 2F5 in HBS-EP to a final concentration in the range 20–1,000 µg
386 L^{-1}. Inject samples and standards over flow cells 1 and 2 at a flow rate of 30 µL min^{-1} for 3 min.
387 Inject a 2F5 standard after every 15 samples.

389 7.3.2) Subtract the relative units (RU) signal of flow cell 1 (reference cell) from the signal of flow
390 cell 2 (measurement cell) for each sample and calculate the antibody concentration based on the
391 RU signal of 2F5 standard injections.

393 7.3.3) After every sample or standard injection, regenerate the chip surface by injecting 30 mM
394 hydrochloric acid at a flow rate of 30 µL min^{-1} for 0.5 min over flow cells 1 and 2.

396 7.3.4) Plot the 2F5 concentration for each flow-through sample from step 6.4 against the flow

397 through volume to obtain the 2F5 breakthrough curve. Calculate the amount of injected 2F5
398 when 10% of the loading 2F5 concentration was reached to obtain the 10% dynamic binding
399 capacity (DBC).
400
401 7.4.   Analyze protein samples by LDS-PAGE.
402
403 7.4.1) Open a ready-to-use 4–12% BisTris LDS polyacrylamide gel and place it in an
404 electrophoresis module. Transfer 800 mL of running buffer (50 mM MES, 50 mM Tris base, 0.1%
405 (m/v) SDS, 1 mM EDTA, pH 7.3) into the module and add 0.5 mL of antioxidant solution.
406
407 7.4.1. In a 1.5-mL reaction tube, mix 10 µL of loading buffer with 4 µL of reducing agent and 26
408 µL of the protein sample. Incubate the tube in a heat block for 10 min at 80 °C. Centrifuge the
409 tube at 500 × g for 30 s.
410
411 7.4.2. Load the LDS polyacrylamide gel (10 µL of sample per lane) and load 5 µL of pre-stained
412 protein standard (10–180 kDa) in a separate lane.
413
414 7.4.3. Close the electrophoresis chamber and connect the power supply. The electrophoresis
415 should run for 40 min and at constant 200 V.
416
417 7.4.4. Remove the gel from the electrophoresis chamber. Open the gel sheathing and wash the
418 gel for 15 min in water on a shaker at 19 rpm. Stain the gel for 1 h in staining solution on a shaker
419 at 19 rpm.
420
421 7.4.5. Destain the gel for 1 h in water on a shaker at 19 rpm. Scan the gel using a film scanner.
422
423 **REPRESENTATIVE RESULTS:**
424 *Expression and purification of the affinity ligand*
425 The fusion protein DFE was expressed in transgenic tobacco plants grown in a greenhouse. The
426 yield was ~120 mg kg^{-1} leaf mass with an average biomass of ~130 g per plant. The DFE purity
427 was <5% of TSP in crude plant extracts before blanching but increased to ~40% after heat
428 treatment at 70 °C for 1.5 min, which precipitated >97% of the host cell proteins. The blanching
429 step was easily integrated into the harvesting and extraction routine (**Figure 1**) and took less than
430 2 h of extra time, including setting up the water bath. The overall recovery of DFE was
431 23.5 mg kg^{-1} with a purity of >90%. The steps responsible for product loss were blanching, depth
432 filtration and IMAC, with specific losses of 40%, 27% and 45%, respectively. The depth filter
433 capacity was on average 135 ± 36 L m^{-2} (±SD, n=3) and thus in the upper range of values reported
434 in the literature[21]. The DFE yield increased with plant age (**Figure 2**).
435
436 *Immobilization of the affinity ligand on NHS-activated chromatography columns*
437 During initial coupling tests, we found that HEPES buffer (pH 8.3) increased the coupling
438 efficiency to 89 ± 6% (±SD, n=3) compared to 78 ± 9% (±SD, n=3) for the bicarbonate buffer
439 recommended by the manufacturer. Therefore, HEPES was used for all subsequent coupling
440 experiments. A DoE approach was selected to optimize the coupling efficiency of DFE to NHS-

441 activated cross-linked agarose resin. The absolute amount of DFE immobilized on the resin
442 increased with the mass of DFE injected into the column and plateaued at ~15 g L^{-1} whereas the
443 coupling yield declined continuously as more DFE was injected (**Figure 2**). The coupling yield was
444 also >50% lower in an acidic buffer, indicating the need to screen for suitable coupling conditions
445 for each ligand on a case-by-case basis. Ideal conditions in terms of coupling yield, absolute
446 quantity of immobilized DFE and column costs were identified using the numerical optimization
447 tool of the DoE software. The most desirable conditions (pH 9.0 and 7.0 mg of DFE per 1 mL of
448 cross-linked agarose resin) were located on a large plateau and were therefore robust. The DFE
449 molecules retained their red fluorescence even after coupling, and the color intensity
450 corresponded to the total amount of immobilized DFE (**Figure 2**). Therefore, column color can be
451 used as a simple quality control parameter to estimate the coupling efficiency and column
452 quality. The fluorescence also confirmed that DFE fusion protein assembled in the tetrameric
453 state of native DsRed.
454
455 [Place Figure 2 here]
456
457 *Testing 2F5 isolation using the DFE affinity resin*
458 The recombinant 2F5 antibody was transiently produced in *Nicotiana benthamiana* plants grown
459 in a phytotron[5]. The capture of 2F5 from the crude plant extract was tested using affinity columns
460 coupled with ~7.0 mg purified DFE (step 6). Elution from Protein A resins usually involves an acidic
461 buffer (pH ~3.3)[13]. Therefore, we initially evaluated different low-pH elution buffers (pH 6.0–
462 3.25) for the DFE columns. The elution of 2F5 was successful at pH values below 4.5 with the
463 highest recovery of ~35% at pH 3.25. However, low-pH elution inactivated both the antibody (as
464 confirmed by SPR spectrometry) and the DFE ligand (as indicated by the loss of color and the
465 lower DBC, Figure 3). The latter was anticipated given that native DsRed denatures at pH <4.0[22,23].
466 To avoid product and ligand denaturation, we tested magnesium chloride as an alternative
467 elution agent because it has previously been used to elute mAbs from other affinity resins[24]. A
468 magnesium chloride concentration of 1.25 M was sufficient to elute 2F5 from the DFE affinity
469 resin with a recovery of 105 ± 11% (±SD, n=3) and a purity of 97 ± 3% (±SD, n=3). This
470 performance was comparable to Protein A resins[25,26]. The equilibrium dissociation constant (K$_D$)
471 of DFE-purified 2F5 antibody and the synthetic ligand Fuzeon was 791 pM whereas that of a
472 Protein A-purified counterpart was 763 pM[5]. Furthermore, no substantial color loss was observed
473 in the resin over a total of 25 bind-and-elute cycles. The DBC of the DFE affinity resin at 10% 2F5
474 breakthrough declined linearly over the course of 25 cycles to ~15% of the initial value (**Figure 3**).
475
476 [Place Figure 3 here]
477
478 **FIGURE LEGENDS:**
479
480 **Figure 1: Process flow scheme for the preparation of epitope affinity resins that can be used**
481 **for the capture of mAbs from crude plant extracts or cell culture supernatants.** (**A**) The affinity
482 ligand DFE was expressed in transgenic tobacco plants. A heat precipitation step (**B**) was included
483 before harvested leaves were homogenized (**C**). (**D**) The crude plant extract was clarified by bag
484 filtration, depth filtration and 0.2-μm sterile filtration. (**E**) DFE was then purified by IMAC. (**F, G**)

485 The purified DFE affinity ligand was immobilized on EDC/NHS-activated cross-linked agarose
486 columns. (**H**) Bacterial cultures carrying T-DNA encoding antibody 2F5 were used for transient
487 expression in *N. benthamiana* plants (**I**) grown in a phytotron. (**J**) *N. benthamiana* leaves were
488 harvested and processed as described in D. (**K**) mAb 2F5 was purified from the clarified extract
489 using the DFE affinity columns (**L**).
490
491 **Figure 2: Optimization of DFE immobilization to NHS-activated cross-linked agarose resin.**
492 (**A**) LDS-PAA gel with western blot overlay of homogenate and elution samples from unblanched
493 and blanched transgenic DFE plant extracts. Harvest of plants was performed 38, 45 or 52 days
494 after seeding. Western blots were performed using an anti-His$_6$-antibodies[5]. (**B**) Total amount of
495 coupled DFE affinity ligand in dependence if coupling pH and overall mass of purified DFE injected
496 onto NHS-activated cross-linked agarose columns. Red dots indicate the actual experiments
497 performed to build the response surface model. (**C**) DFE affinity columns after the coupling
498 procedure. The numbers correspond to the coupling conditions highlighted in panel B. dps – days
499 post seeding.
500
501 **Figure 3: Testing the isolation of 2F5 from clarified plant extracts using the DFE affinity resins.**
502 (**A**) DFE affinity resins after one elution cycle using buffers with different pH values in the range
503 4.0–3.0 and the same resins after a neutralization step at pH 6.0. (**B**) DFE affinity resins after one
504 and six cycles of 2F5 purification using 1.25 M or 4.00 M magnesium chloride as eluent. (**C**)
505 Chromatograms of frontal loading experiments (break-through curves) to determine the cycle-
506 dependent dynamic binding capacity of DFE resin using magnesium chloride as eluent. The break-
507 through curves were measured for 4.0 M and 1.25 M magnesium chloride elution buffers and
508 various cycle numbers. (**D**) Cycle-dependent dynamic binding capacity of DFE using 1.25 M
509 magnesium chloride as eluent.
510
511 **Table 1: Trouble-shooting**
512
513 **DISCUSSION:**
514 *Applications of the novel affinity resin*
515 We have shown that custom affinity chromatography resins for the capture of mAbs can be
516 manufactured by immobilizing a ligand containing a mAb-specific epitope to NHS-activated cross-
517 linked agarose. To design such a resin, it was necessary to know the epitope sequence and to use
518 a linear epitope. The resulting resins are advantageous for the capture of mAbs because they
519 could potentially replace expensive Protein A affinity chromatography steps. The interaction
520 between 2F5 and DFE in our case study was mediated by epitope–paratope binding, so our ligand
521 should be more selective than Protein A, which binds to the Fc region of most murine and human
522 IgGs. Because individual ligands are needed for each mAb, our method may initially seem suitable
523 mainly for antibodies that are produced on a very large scale. However, by combining our
524 approach with rapid plant-based transient protein expression, new affinity ligands can be
525 prepared in less than 2 weeks[27] with minimal effort[28]. Hence, the method is also suitable for
526 small-scale mAb purification.
527
528 *Production and potential improvements of the affinity ligand*

529 Plants offer a fast and safe production platform for affinity ligands[5,29,30], such as the DFE fusion
530 protein featured in our case study. Blanching the plant material greatly reduced the quantity of
531 host cell proteins in a single step and was easily integrated into a standard clarification routine.
532 However, the recovery of the ligand was low in the current setup, probably due to its moderate
533 thermal stability and some non-specific binding to the filter layers, as reported for other
534 products[31-33]. Engineering the carrier to increase its thermal stability may therefore help to
535 improve the ligand yield in the future, as described for the malaria vaccine candidate CCT, the
536 antitumor enzyme PpADI or a mesophilic β-glucosidase[34-36]. The same holds true for the depth
537 filtration step, where protein engineering may help to reduce non-specific binding to the filter
538 material[37]. The production costs for DFE and similar ligands could also be reduced by improving
539 the overall efficiency of clarification using flocculants or filter additives[38,39].
540
541 When DsRed is used as a carrier, it forms a tetrameric complex. This is advantageous because it
542 increases the number of epitopes per ligand, but it may also render the ligand more susceptible
543 to disassembly or denaturation during affinity chromatography. A monomeric carrier protein
544 such as mCherry may therefore be preferable, because it is stable at low pH[40], and the inclusion
545 of tandem repeats of the epitope would increase the avidity of the ligand and thus increase resin
546 capacity[5,26,41]. For simple carrier-epitope proteins (i.e., those with no disulfide bonds or post-
547 translational modifications) microbial production systems may reduce the manufacturing costs
548 and make the ligands more competitive with Protein A. For example, green fluorescent protein
549 has been expressed in bacterial cells with a yield of ~1 g kg^{-1} biomass, which would significantly
550 reduce ligand production costs[42].
551
552 Regardless of the expression host, a purified affinity ligand was required during coupling to
553 minimize the immobilization of host cell proteins or media components that can otherwise
554 reduce resin selectivity and capacity. The inclusion of a poly-histidine tag for IMAC purification
555 increased the purity to ~90% in a single step, facilitating rapid and inexpensive ligand
556 production[5,43,44]. However, the position of the fusion tag is important because it has the potential
557 to sterically hinder epitope binding or to induce the cleavage of either the tag or the epitope from
558 the carrier[45,46].
559
560 *Immobilization of the affinity ligand on NHS-activated chromatography columns*
561 Immobilization was carried out manually or using a chromatography system. The small buffer
562 volumes per column seemed to favor manual handling (e.g., due to the minimal waste volumes).
563 However, if multiple/larger columns are needed, the chromatography system makes the coupling
564 conditions easier to control (e.g., regulated flow rates) and is therefore more likely to achieve
565 reproducible results in terms of DBC. Our data suggest that the coupling buffer and pH have an
566 important effect on the coupling efficiency and overall column costs. Screening factors that
567 influence the coupling reaction and adjusting them for each carrier protein (or even for each
568 carrier–ligand fusion) could therefore improve coupling efficiency and resin performance, and
569 we recommend this approach.
570
571 *Testing 2F5 isolation using the DFE affinity resin*
572 Product yield and purity are important aspects of resin performance, and in the case of DFE we

573 achieved a yield of 105 ± 11% (±SD, n=3) and a purity of 97 ± 3% (±SD, n=3), which is comparable
574 with the performance of benchmark Protein A resins[25,26]. Another key performance indicator for
575 resins in general (and particularly for those based on affinity ligands) is the DBC at 10% product
576 breakthrough, because this parameter affects the amount of resin required for a specific process
577 and thus the costs. For the DFE ligand, the initial DBC was ~4 g L^{-1} resin, which is ~13% of the
578 corresponding value for Protein A under similar conditions (only 2 min contact time)[25,47] but
579 about 15-fold higher compared to other custom affinity resins such as the anti-FSH-
580 immunoaffinity ligand using the same residence time of 2 minutes[48]. The DBC of DFE declined to
581 15% of the starting value after 25 bind-and-elute cycles, whereas more than 50 cycles are
582 required for the same loss of DBC in commercial Protein A resins[49]. However, it is important to
583 note that our carrier has not yet been optimized to the same extent as Protein A, which has been
584 comprehensively investigated and improved over the last four decades[8].

586 Thus far we have improved the resin stability and product recovery by switching from a low-pH
587 elution buffer to a high concentration of magnesium chloride (**Figure 3**), as recommended in
588 earlier studies[13]. The characteristic red color of the affinity ligand did not fade substantially during
589 the 25 bind-and-elute cycles, so we speculate that endogenous plant proteases in the clarified
590 plant extracts[31] may have truncated and thus inactivated the epitope of the ligand. Therefore,
591 designing protease-resistant linkers to connect the carrier and epitope may help to maintain the
592 initial DBC over an extended number of cycles. Given the rapid and simple expression and
593 purification of the DFE ligand, its straightforward coupling to commercial chromatography resins,
594 and its excellent product yield and purity, we believe that our method offers a suitable alternative
595 to Protein A for the purification of mAbs and antibody derivatives which do not bind to Protein
596 A, especially if improvements to the carrier and linker can improve the DBC and ligand stability.
597 This assumption was supported by the small difference in the dissociation constant of DFE-
598 purified and Protein A-purified 2F5 antibody[5], indicating that our new affinity ligand allows the
599 recovery of high-quality mAbs.

601 *Benefits and current limitations of the method*
602 Producing the affinity ligand as a genetic fusion with a carrier protein increases solubility in
603 aqueous buffers and thus compatibility with typical ligand coupling conditions. In contrast, blank
604 peptides derived from solid phase peptide synthesis may have limited solubility under these
605 conditions due to their sequence[50], which cannot be changed because it is dictated by the amino
606 acid sequence of epitope recognized by the mAb to be purified. Others have therefore used an
607 on-resin synthesis of peptide ligands[51]. The static binding capacity of the resulting resin was high
608 (~ 80 g L^{-1}), but the process of resin preparation is lengthy, a dynamic binding capacity was not
609 reported and the obtained purity and recovery were lower than in our approach. An additional
610 advantage of a fusion protein ligand in laboratory scale is that the ligand and variants thereof can
611 be rapidly produced, purified and tested with minimal effort in an easy-to-use high-through
612 expression system[52].

614 The two current limitations of the method presented here are the low dynamic binding capacity
615 of 3 g L^{-1} and its 90% reduction over the course of 25 bind-and-elute cycles[5]. These limitations
616 can be addressed in the future by applying less stringent loading conditions and replacing the

current DsRed carrier with an engineered, more stable variant respectively. For example, doubling the current contact time from 2 to 4 minutes has the potential to double the dynamic binding capacity as was shown for some Protein A resins[26].

Troubleshooting
The following table highlights potential problems that can be encountered during this protocol and provides hints on how to solve them (**Table 1**).

[place table 1 here]

DISCLOSURES:
The authors have no conflicts of interest to disclose.

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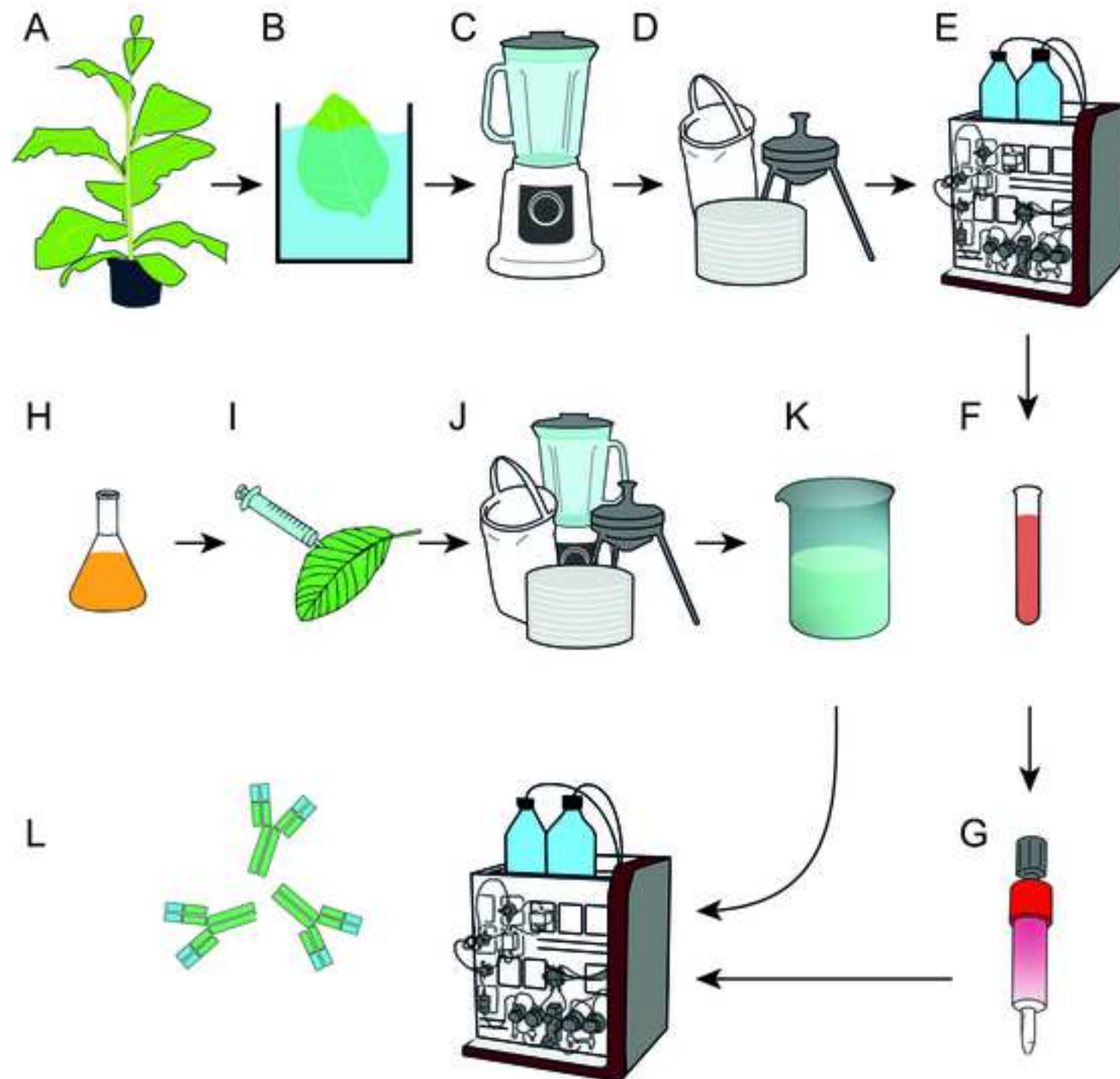787 (2019).

788
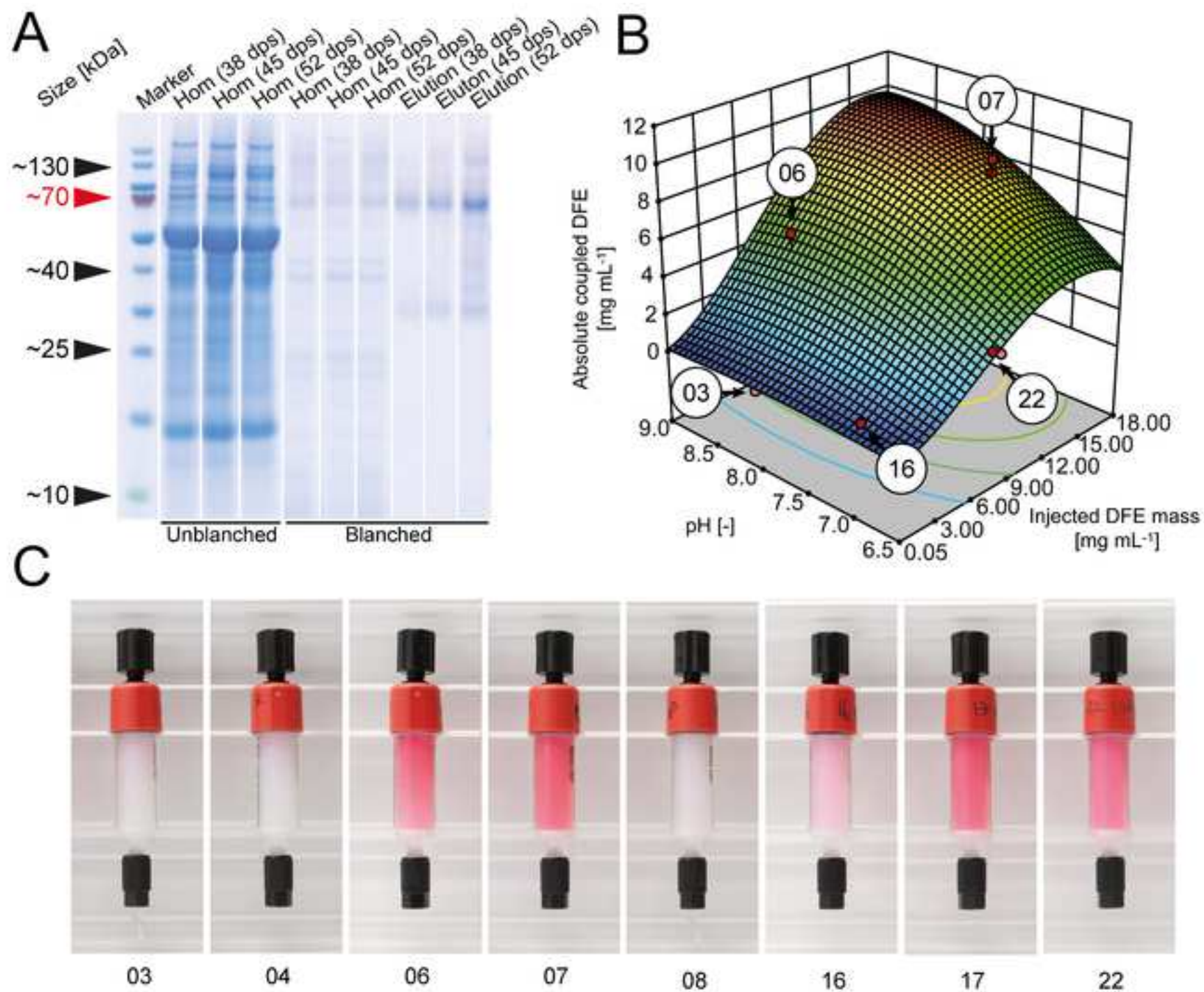
Figure 1

Figure 2
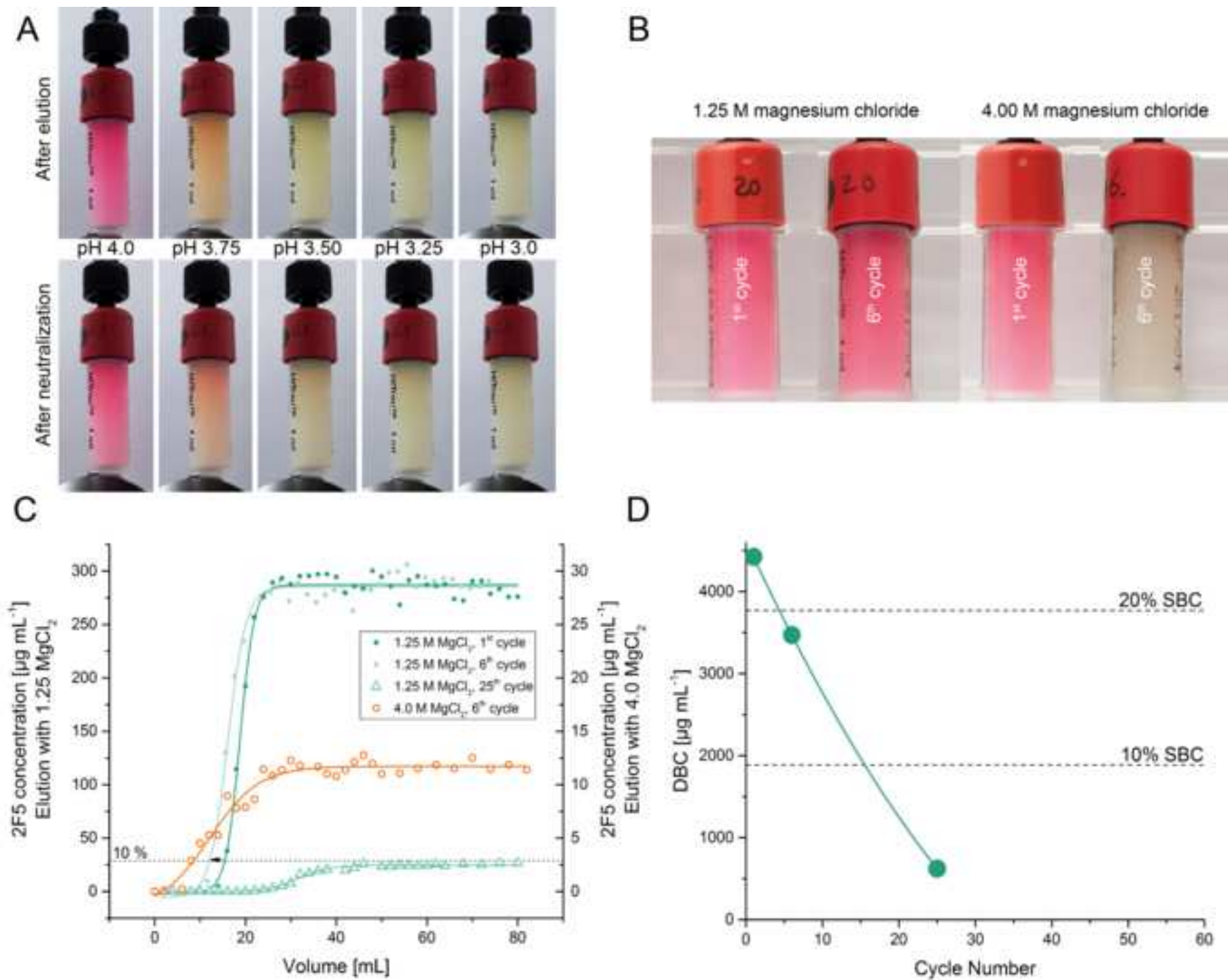
Table_1

Table 1: Potential problems that can be encountered and possible fixes.

Protocol step	Problem
1	Plants do not grow
2 and 3	Large quantities of host cell proteins are present
2 and 3	No product found in the plant extract
3	Large stem or leaf parts remain after extraction
3	Rapid pressure increase during depth filtration
4	Little fusion protein during elution / a lot of fusion
4	Fusion protein lost during concentration
5	Low coupling yield
5 and 6	Low mAb yield
7	Very low/high protein concentrations in Bradford
7	Low mAb concentration during SPR measurement

Couse	Fix
Compromised growth conditions	Check the pH and conductivity of the fertilizer
	Check the temperature and light conditions
Incomplete precipitation	Check the temperature during blanching
	Check the agitation in the blanching bath
Blanching temperature too high	Check temperature and pH during blanching
pH in blanching buffer too low	
Incomplete mixing in blender	Make sure the plant material does not form a plug
Incorrect filter selection and/or orientation	Check the filter type and orientation
IMAC resin was not charged with metal ions	Check if the IMAC resin was correctly charged wit
Fusion protein lost the affinity tag	Avoid intense sunlight and high temperatures dur
Fusion protein bound to the membrane	Check the membrane type
	Make sure the concentration factor was not too hi
Incorrect sequence of coupling reagent addition	Check the reagents labels and sequence of addit
Incorrect preparation of the columns before coupl	Check the conditions of column preparaiton
Low mAb expression in the plant biomass	Test mAb expression in biomass
Low ligand density	Check the purity of the fusion protein preparation
Bubble formation during pipetting	Check for bubbles in the 96-well palte
Compromised Protein A chip	Compare with results of standard mAb with know
Incorrect sample dilution	Check the dilution rate and buffer

ɟ in the blender

th ions
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n concentration

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10L/20L Bucket	n/a	n/a	Blanching equipment
2100P Portable Turbidimeter	Hach	4650000	Turbidimeter
ÄKTApure	GE Helthcare	29018226	Chromatography system
Allegra 25R	Beckman Coulter	369434	Centrifuge
Amine Coupling Kit	GE Healthcare	BR100050	SPR chip coupling kit
Amine Coupling Kit	GE Healthcare	BR100050	SPR chip coupling kit
Antibody 2G12	Fraunhofer IME	n/a	Standard for SPR quantification
Blender	Waring	800EG	Blender
BP-410	Fuhr	2632410001	Bag filter
CanoScan 5600F	Canon	2925B009	Scanner
Centrifuge tube 50 mL self-standing	Labomedic	1110504	Reaction tube
Chelating Sepharose FF	GE Helthcare	17-0575-01	Chromatography resin
Cond 3320	WTW	EKA 3338	Conductometer
Design-Expert(R) 8	Stat-Ease, Inc.	n/a	DoE software
Discovery Comfort	Gilson	F81029	Multichannel pipette
Disodium phosphate	Carl Roth GmbH	4984.3	Media component
Diverse bottles	Schott Duran	n/a	Glas bottles
Dri Block DB8	Techne	Z381373	Heat block
DsRed	Fraunhofe IME	n/a	Standart
EDTA	Carl Roth GmbH	8043.2	Buffer component
EnSpire	Perkin Elmer	2390-0000	Plate reader
ETHG-912	Oregon Scientific	086L001499-230	Thermometer
F9-C	GE Helthcare	29027743	Fraction collector
Ferty 2 Mega	Kammlott	5.220072	Fertilizer
Forma -86C ULT freezer	ThermoFisher	88400	Freezer
HEPES	Carl Roth GmbH	9105.3	Buffer component
Hettich Centrifuge Mikro 200	Hettich	2400	Centrifuge
HiPrep 26/10	GE Helthcare	GE17-5087-01	Chromtography column
HiTrap NHS-activated Sepharose HP, 1 mL	GE Helthcare	17-0716-01	Chromatography columns
Hydrochloric acid	Carl Roth GmbH	4625.1	Buffer component
Imidazole	Carl Roth GmbH	3899.2	Buffer component
K700	Pall	5302305	Depth filter layer
KM02 basic	IKA	n/a	Magnetic stirrer
KS50P 60D	Pall	B12486	Depth filter layer
L/S 24	Masterflex	SN-06508-24	Tubing
Lauda E300	Lauda Dr Wobser GmbH	Z90010	Immersion circulator
Magnesium chloride	Carl Roth GmbH	KK36.2	Buffer component
Masterflex L/S	Masterflex	HV-77921-75	Peristaltic pump
Minisart 0.2 µm	Sartorius	16534K	Filter unit
Nalgene Rapid-Flow PES bottle top filter	Thermo Fischer Scientific	595-4520	Vacuum filtration of SPR buffers
Nickel sulphate	Carl Roth GmbH	T111.1	Buffer component
Novex NuPAGE 4-12% BisTris LDS gels	Invitrogen	NP0336BOX	LDS-PAA gels
Novex X-cell Mini Cell	Invitrogen	EI0001	PAGE chamber
NuPAGE 20x running buffer	Invitrogen	NP0002	Buffer concentrate
NuPAGE antioxidant	Invitrogen	NP0005	Antioxidant
PageRuler protein ladder (10-180 kDa)	Invitrogen	26616	Protein standart
Perforated bucked	n/a	n/a	Blanching
PH 3110	WTW	2AA110	PH meter
PowerPac HC	Biorad	1645052	Electrophoresis module
Protein A from Staphylococcus aureus	Sigma-Aldrich	P7837-5MG	Coating of SPR chips
Sephadex G-25 fine, cross linked dextran	GE Helthcare	17003301	Chromatography resin
Silicone spoon	n/a	n/a	Spoon
Simply Blue SafeStain	Invitrogen	LC6060	Gel staining solution
Sodium acetate	Carl Roth GmbH	6773.1	Buffer component
Sodium acetate	Carl Roth GmbH	X891.1	Media component
Sodium azide	Sigma Aldrich	S2002-100G	Media component
Sodium chloride	Carl Roth GmbH	P029.2	Buffer component
Sodium citrate	Carl Roth GmbH	HN13.2	Buffer component
Sodium bisulfite	Carl Roth GmbH	243973-100G	Media component
Sodium phosophate	Carl Roth GmbH	T877.2	Media component
SPR Affinity Sensor - High Capacity Amine	Sierra Sensors GmbH/Bruker Daltonics	SPR-AS-HCA	SPR chip
SPR-2/4 Surface Plasmon Resonance Analyzer	Sierra Sensors GmbH/Bruker Daltonics	n/a	SPR device
SSM3	Stuart	10034264	Mini Gyro-rocker
Heated vessel, 20 L	Clatronic	n/a	Blanching chamber
Sterile syringes, 2 mL	B. Braun	4606027V	Syringes
Syringe adpter (Union Luer F)	GE Helthcare	181112-51	Syringe adapter
TE6101	Sartorius	TE6101	Precision scale
Tween-20 (Polysorbate)	Merck	8170721000	Buffer component
Unicorn 6.4	GE Helthcare	29056102	Chromatography software
Vacuum bags	Ikea	203.392.84	Plant storge
VelaPad 60	Pall	VP60G03KNH4	Filter housing
Vortex-Genie 2	Scientific industries	SI-0236	Vortex
XK-26/20 column housing	GE Helthcare	28-9889-48	Chromtography column

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Author(s):

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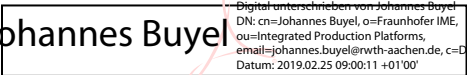
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CORRESPONDING AUTHOR

Name: Johannes Buyel

Department: Integrated Production Platforms

Institution: Fraunhofer IME

Title: Head of Department

Signature: Johannes Buyel
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DN: cn=Johannes Buyel, o=Fraunhofer IME,
ou=Integrated Production Platforms,
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Response to review comments
Review Manuscript Number: 59933_R0_030119
Title: Activated Sepharose for the rapid development of affinity resins - antibody capture as a case study

Editorial comments:
Editor comment 1: 4.2: How do you charge the column with nickel ions?
Author response 1: We have added the information of how to charge the column with nickel ions.

Editor comment 2: Figure 2A: Please define 'dps' in the legend.
Author response 2: We added an definition of the abbreviation to the figure legend.

Editor comment 3: Figure 2C: Most of the numbers here are not in Figure 2B.
Author response 3: The figure legends states that the numbers in panel C correspond to those in panel B. In our understanding, this means: #3 in panel C = #3 in panel B. It does not imply completeness (i.e. for every number in C there is a number in B). We will gladly change the legend text if the Editor can suggest a better way to describe what we had in mind. Additionally, even though we did not claim this, most of the numbers (i.e. >50%) from panel C can be found in panel B (i.e. 5/8 =62.5%).

Reviewers' comments:
Reviewer #1:
Reviewer comment 1.1: The concept is good. However, the paper is written like a internal lab work protocol instead of publication
Author response 1.1: The paper is written as a lab work protocol indeed, because this is the requirement of the journal.

Major Concerns:
Reviewer comment 1.2: There are no results for product quality or comparison to product quality with historical methods of protein purification. It is difficult to assess efficiency or efficacy of product without any product quality impact. One of the reasons why other resins have failed to displace ProA is the efficiency of ProA resin to remove HCP. The author does not show any data.
Author response 1.2: We agree that product quality is an important criterion when assessing a new purification method. Even though the manuscript at hand is meant to focus on the method of preparing the affinity ligand, rather than analyzing it, we mentioned in the results section "Testing 2F5 isolation using the DFE affinity resin" of the text that the recovery and purity (the latter being an indicator for the HCP removal mentioned by the reviewer) were similar to those achieved with Protein A. We have now expanded this information with data from our initial publication (Rühl et al. 2018 in J. Chrom. A) reporting that the equilibrium dissociation constant (K_D) for DFE-purified and Protein A-purified 2F5 antibody were 791 pM and 763 pM respectively and thus very similar. We therefore believe that the 2F5 quality of both preparations was comparable.

Reviewer #2:
Reviewer comment 2.1: My only point that presented a slight pause in my assessment of this paper was that the authors do not describe the reason that they choose to display their epitope on a protein, rather than using perhaps chemical synthesis to produce structurally similar compounds or even immobilize peptides on Sepharose. It is not an easy process to cultivate tobacco plants, produce and isolate the required protein and then immobilize it. IS it really significantly more advantageous than using Protein A,

because it does seem equally laborious and costly. Perhaps the authors should spend a paragraph commenting on this issue. However, I do believe that this paper is worthy of publication. A lot of work has been done and it will certainly prove useful.

Author response 2.1: The reviewer raises a valid point and we have added a paragraph in the discussion section to explain our perspective on the matter.

Reviewer #3:

Reviewer comment 3.1: Please add a Table to present the main faced problems during experiments and their possible solutions, for instance, a failure mode and effects analysis (FMEA) of the protocol proposed. This Table will contribute to troubleshooting when other people try to reproduce the experiment by following the protocol.

Author response 3.1: This is a good idea, we have added an according table at the end of the protocol section.

Reviewer comment 3.2: Please add some lines about the limitations and/or disadvantages and areas of improvement of the proposed method.

Author response 3.2: We have added a section highlighting the current limitations and challenges with the method at the end of the discussion.