

1 Article

2 Antibody screening by microarray technology – 3 direct identification of selective high-affinity clones

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9

10 **Abstract:** The primary screening of hybridoma cells is a time-critical and laborious step during the
11 development of monoclonal antibodies. Often critical errors occur in this phase, which support the
12 notion that the generation of monoclonal antibodies with hybridoma technology is difficult to
13 control and hence a risky venture. We think that it is crucial to improve the screening process to
14 eliminate most of the immanent deficits of the conventional approach. With this new microarray-
15 based procedure, several advances could be achieved: Selectivity for excellent binders, high
16 throughput, reproducible signals, avoidance of misleading avidity (multivalency) effects, and
17 simultaneous performance of competition experiments. The latter can directly be used to select
18 clones of desired cross-reactivity properties. In this paper, a model system with two excellent clones
19 against carbamazepine, two weak clones and blank supernatant has been designed to examine the
20 effectiveness of the new system. The excellent clones could be detected largely independent of the
21 IgG concentration, which is unknown during the clone screening, since the determination and
22 subsequent adjustment of the antibody concentration is not possible in most cases. Furthermore, in
23 this approach, the enrichment, isolation and purification of IgG for characterization is not necessary.
24 Raw cell culture supernatant can be used directly, even when fetal calf serum (FCS) or other complex
25 media had been used. In addition, an improved method for the oriented antibody-immobilization
26 on epoxy-silanized slides is presented. Based on the results of this model system, we conclude that
27 this approach should be preferable to most other protocols leading to many of false positives,
28 causing expensive and lengthy confirmation steps to weed out the poor clones.

29 **Keywords:** Monoclonal antibodies, Mabs, fusion, false positives, hapten immunoassays,
30 competitive immunoassays, ELISA, antibody validation, antibody quality, microarray, hybridoma
31 technology, linker recognition, high-throughput screening, HTS, heterology concept.

32

33 1. Introduction

34 During antibody development, the screening of hybridoma cells is a crucial step. Several obstacles
35 may lead to a complete failure of the process. First, the assay needs to be selective (“specific”)
36 enough. Otherwise the researcher is flooded with seemingly positive clones, which in a later stage
37 turn out to be of poor quality or completely negative. Good clones might be irreversibly lost in this
38 phase, because in most cases it is not feasible for all clones to undergo an in-depth examination. The
39 second requirement is speed, since some irrelevant hybridoma cells might grow very fast and
40 overgrow some positive clones, if the final clonal state is not reached, yet. The third point is
41 parallelization, since the probability that an excellent clone is found, increases with the number of
42 clones tested. Due to technical and financial limitations, often too few clones are examined. The
43 fourth issue is mainly encountered with haptens. Quite often, antibodies, which bind to an
44 immobilized hapten-protein conjugate and hence are identified as positives, are found to be weak
45 or non-binders of the free analyte. This effect is known as spacer recognition, linker recognition or

46 bridging phenomenon [1-13]. Sometimes this effect can be reduced by application of linker or site
47 heterology using different linkers or conjugation reagents for the preparation of immunogens and
48 coating antigens or enzyme conjugates [14,15]. The fifth point refers to the affinity of the respective
49 clones. In most cases, an affinity ranking is required to identify the strongest binder, which often
50 shows the best performance in analytical applications [16]. The sixth risk factor is the screening
51 date. Since the antibody screening is a cost and time intense step, the procedure is usually
52 performed only once or twice. However, due to the varying growth rate of individual hybridoma
53 clones, the “best” time for a screening can hardly be determined. Consequentially, several rounds
54 are necessary to catch both, fast and slow cells. The fast cells need to be ranked immediately, since
55 they might be lost on a later date.

56 Most popular screening procedures show one or several of these drawbacks and therefore increase
57 the risk of an unsatisfactory antibody development. This might be the reason, why such projects
58 still are high-risk endeavors, which is particularly unpleasant, when project partners or customers
59 are critically dependent on a timely antibody deployment.

60 Here we present a novel screening format, which should be highly favorable for most projects
61 based on hybridoma technology and conveniently feasible for most laboratories. This protocol
62 overcomes the hurdles mentioned above and is based on microarrays performed on a standard-
63 slide format. The first important advancement is the use of an antibody-immobilized format, which
64 in this context has been proposed in [17], in contrast to antigen-immobilized formats, which are
65 recommended in most textbooks and articles. The second improvement is the miniaturization of the
66 assay, which is achieved by the use of a microarray format, which has been used favorably in many
67 applications, e.g. [18-22]. This enables the fast and easy performance of a screening, sometimes with
68 only a single chip. However, the third feature might be the most innovative in this context. The
69 microarray-based test can be performed in a true competitive format, which leads to both, the
70 identification of true positives and the affinity ranking of the clones. Even some basic cross-
71 reactivity tests might be possible. We have performed a model screening with known clones, which
72 had been identified and characterized previously [23,24].

73 In the context of hybridoma technology, microarray-based screening formats have been presented
74 in several publications [19,25-34]. Nearly all used antigen-immobilized formats with all of their
75 limitations mentioned above. Due to their fundamentally different approach, we do not discuss
76 them in more detail, here. Most of these protocols have found only very limited application until
77 today and as a consequence, a practical microarray approach for antibody screening is still lacking.

78 Carbamazepine (CBZ) is an important antiepileptic drug, which is prescribed in frequently and at a
79 relatively high dose. Due to its poor degradability, it is found in many surface and ground waters
80 and therefore can be used as an anthropogenic contamination marker. Several immunoassays have
81 been developed for the detection of CBZ, which require the availability of suitable antibodies,
82 which are nearly always a limiting resource in immunochemical applications. Recently, new
83 antibodies against CBZ have been developed in our department [23,24]. We used some of these
84 clones as model antibodies for the setup and optimization of a novel chip-based hybridoma
85 screening procedure, which is presented here as a feasibility study. The application of this approach
86 to complete projects for antibody development is planned to be performed and published in future.

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90 **2. Materials and Methods**

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92 2.1. Reagents, buffers, materials and equipment

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94 Transparent, flat-bottom non-binding 96 MTP plates were acquired from Greiner Bio-One
95 (Frickenhäusen, Germany), PD SpinTrap™ G-25 Desalting Columns were obtained from GE
96 Healthcare (Uppsala, Sweden), clear microscope slides were bought from Sigma-Aldrich (Darmstadt,
97 Germany). Recombinant Protein G (PRO-402) and Cys-Protein-G (PRO-1238) were purchased from
98 Prospec (Ness-Ziona, Israel), monoclonal anti-CBZ antibody BAM-mab 01 (CBZ) was obtained from
99 BAM (Federal Institute for Materials Research and Testing (BAM), Berlin), anti-CBZ antibody
100 B3212M (Meridian Life Science Inc, Memphis, USA) was kindly supplied by S. Flemig (BAM), the
101 clones 3B3 and 6C5 were supplied by M. Dippong [24] (BAM), fetal calf serum (Biochrom S0115), L-
102 glutamine, RPMI1640 and 2-mercaptoethanol were acquired from Biochrom (Berlin, Germany). The
103 fluorescence dyes Dy654-NHS and Dy554-NHS were purchased from Dyomics (Jena, Germany).
104 According to the manufacturer, the following properties of the dye Dy654 are given: Absorption /
105 emission max.: 653 / 677 nm (in ethanol), molar absorbance: 220.000 M⁻¹cm⁻¹, soluble in water,
106 methanol and DMF. The mono-protected PEG3 linker 1-(9-fluorenylmethoxycarbonyl-amino)-
107 4,7,10-trioxa-13-tridecanamine hydrochloride (Fmoc-TOTA·HCl) was bought from Iris-Biotech
108 (Marktredwitz, Germany). Carbamazepine (CBZ), Dibenz[b,f]azepin-5-carbonyl chloride (CBZ-Cl,
109 Sigma, 90 %), bovine serum albumin (BSA, Sigma, >98%) and (3-glycidyloxypropyl)trimethoxysilane
110 (Glymo, Sigma, >98%), DMSO (AppliChem, >99.5 %), glycerol (Sigma, Aldrich G2025), Tween 20
111 (Sigma, P7949), hydrochloric acid (Fluka, 84415), sodium hydroxide (Sigma, 30620), Mucosal (Sigma,
112 Z637203) tetrahydrofuran (THF, Chemsolute, >99.9 %) and toluene (Roth, >99.5 %) were obtained
113 from Sigma-Aldrich. Ultrapure water (MilliQ) was supplied by a Milli-Q Synthesis A10 (Merck,
114 Germany). Cyano-4-hydroxycinnamic acid was bought from Bruker (201344).

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117 The washing buffer was made of 10 mM phosphate, 150 mM sodium chloride and 0.05 vol. % of
118 Tween 20 (PBST005, adjusted to pH 7.4). The spotting buffer PBSGT (10 x PBS, adjusted to pH= 8.0)
119 contained 100 mM of sodium hydrogen phosphate, 1500 mM of sodium chloride, 2.5 vol. % of
120 glycerol and 0.00625 vol % of Tween 20. The cell culture medium (CCM) was prepared from 270 ml
121 of RPMI 1640 (Biochrom F1215), 30 ml of fetal bovine serum (S0115 Superior, 0439X), 3 ml of 200 mM
122 L-glutamine (Biochrom L0282) and 300 µl of 2-mercaptoethanol (Biochrome M3148). Cell culture
123 medium with glycerol and Tween 20 for spotting (CCMGT) was prepared by supplying CCM 24:1
124 with 50 vol.% glycerol containing 0.125 vol. % Tween 20.

125

126 The spotting was carried out with a BioOdyssey Calligrapher Miniarrayer (BioRad Laboratories,
127 München, Germany) equipped with MCP310S solid pins (spot diameter about 400 µm, about 1 nl,
128 BioRad Laboratories). The glass slides were scanned with a DITABIS Microarray Scanner MArS
129 (Pforzheim, Germany) using the red/green filter set in the 10 µm fast scanning mode. MALDI-TOF
130 MS was performed with a Bruker Autoflex II Smartbeam mass spectrometer.

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132 2.1. Preparation of Epoxy Slides

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134 Transparent glass slides (25 x 75 x 1 mm) were sonicated for 15 minutes at room temperature
135 (RT) in a 2 vol.% solution of Mucosal universal detergent, rinsed with pure water and etched for one
136 hour in sodium hydroxide solution (10%) and rinsed with pure water. The etched slides were treated
137 in 37% hydrochloric acid for 2 hours, washed with pure water and dried by placing the slides in a
138 gentle air-stream. 1 vol% of water was added to toluene and stirred for 5 minutes. Subsequently, 1
139 vol% of Glymo was added and stirred for another five minutes. The slides were incubated in this
140 solution for 18 hours at RT. Subsequently the slides were washed with isopropanol and pure water.
141 After the silanization, the slides were highly hydrophobic. The epoxy slides can be stored for a longer
142 time in a dry containment at RT.

143

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In a first step, the epoxy slides were spotted by Cys-Protein-G. The printing solution consisted of Cys-Protein-G (1 g/L) diluted 1:5 in PBSGT (pH 8.0). The spotting procedure was performed at 65% humidity and 15°C using MCP360S pins with 400 μm diameter, transferring approx. 1 nL of the solution per spot resulting in a 12x8 spot array with 1000 μm spot to spot distance. The printed slide was incubated for three days in an airtight 50 ml falcon tube over PBS with 1 % glycerol in the dark. After the incubation, the slide was washed with PBST, purged with 0.1 vol.% of glycerol, dried with nitrogen and directly used for the screening experiments. No further blocking steps were applied to the chip. These protein G chips might be stored cool or frozen in the dark for future projects.

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153 2.2. Sample printing and incubation

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In the next step, the immobilization of the antibodies from cell culture supernatants was examined. As a model system, we used a typical cell culture medium supplied with 10% of fetal calf serum, spiked with the respective antibodies at different concentration levels. The transfer from the 96-well source plate (non-binding, Greiner Bio-One, 655901, F-Bottom) to the microarray was performed with a Calligrapher™ MiniArrayer (BioRad). For the printing step, MCP360S solid ceramic pins were used, which have a tip diameter of 400 μm and transfer approximately 1 nL of sample. Thus, only extremely small amounts of supernatant are consumed, and a nearly unlimited number of replicates can be performed, if required. The samples were reprinted in a 12x8 subgrid at the very same coordinates on which the Cys-Protein-G was previously immobilized. For some assays (e.g. inhibition, see below) replicates are performed on the same chip. After 18 hours of incubation at 4°C, the chip was washed thoroughly again. It should be considered that no extended washing steps of the ceramic pin have been performed in the spotting procedure, due to time considerations. This and some other washing issues may lead to some carryover in rare cases. Nevertheless, these effects can be easily identified and corrected during data evaluation [see Fig. 8].

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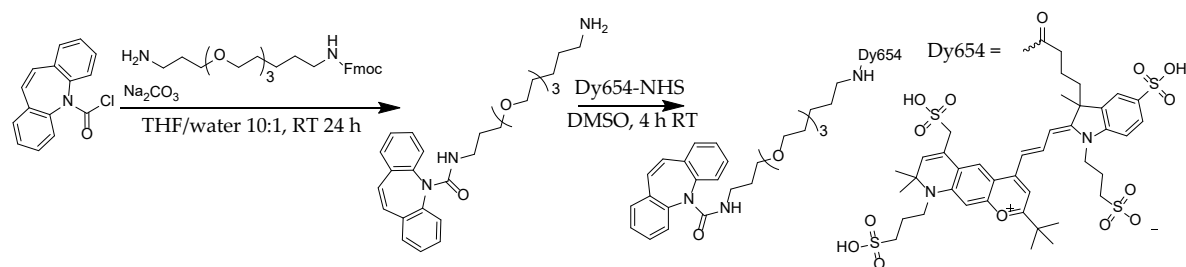
170 2.3. Design and synthesis of hapten-fluorophore conjugates

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The screening procedure relies on fluorophore-labeled antigens or haptens. The proper choice of a dye is of considerable relevance. Today, highly advanced fluorescence labels are available, which display many desirable properties like high quantum yield, high photostability, excellent water solubility, reduced aggregation and low non-specific binding. Based on the available laser excitation source of 635 nm, the dye Dy654 [Fig. 1] was chosen.

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180 Fig. 1: Synthesis of the CBZ-TOTA-Dy654 tracer [24].

181

182 First, CBZ-TOTA-amine was synthesized by a nucleophilic substitution reaction of
 183 dibenz[b,f]azepine-5-carbonyl chloride with a semi-protected Fmoc-TOTA spacer. The Fmoc group
 184 was subsequently cleaved under mildly basic conditions and the unprotected terminal amino group
 185 was reacted with an equimolar amount of NHS-activated Dy654 in DMSO as described in [24]. The
 186 conjugate was used without further purification.

187

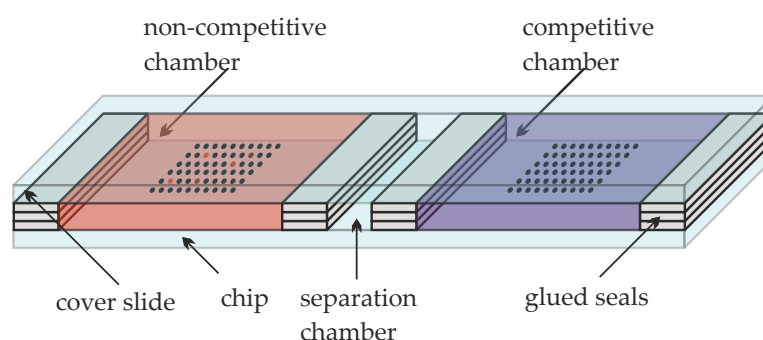
188

189 2.5. Competition experiments

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191 The glass slides were epoxy-functionalized, coated with Cys-Protein-G sub grids (12x8 spots)
192 and reprinted with the sample solutions as described above. For the competition experiments the slide
193 was divided in three different areas with glued seals. Four seals manufactured of three vertical stacks
194 of laboratory adhesive tape (*Toughtags*) were glued onto the slide and a blank slide was placed on top
195 of them. In this way, three separated chambers, the “non-competitive” and the “competitive” one,
196 with a separation chamber in between, were created, see Fig. 2. Each of the main chambers had
197 dimensions of approx. 25 mm x 20 mm x 0.3 mm. The first cavity, the non-competitive cavity of the
198 slide, was filled with approx. 150 μ l of diluted CBZ-TOTA-Dy654 tracer in PBST (1:10.000), the
199 separation cavity was kept empty, while the third cavity, the competitive chamber, was incubated
200 with approx. 150 μ l of CBZ-TOTA-Dy654 tracer in PBST (1:10.000) with addition of 26 mg L⁻¹ of CBZ.
201 The incubation was performed simultaneously in both cavities for one hour in the dark at RT.
202 Subsequently, the cover slide was removed, and the microarray was rinsed with PBST and 0.1 vol.%
203 of glycerol and dried quickly with nitrogen. The final washing steps after the tracer incubation
204 required approx. 1 minute in total. The slide was scanned with the microarray scanner at 100 % PMT
205 (photomultiplier) intensity in the 10 μ m fast scanning mode. For 635 nm excitation, the red filter was
206 used.

207



208

209

210 Fig. 2: Competition experiment on a microarray slide.

211

212 The scan of the whole slide consisting of a 16-bit TIF file, was imported in Fiji-ImageJ software
213 [35,36], corrected for angular misalignment and cropped into two separate files: the non-competitive
214 and the competitive array. Each array is saved individually as a 16-bit TIF file, without any additional
215 preprocessing applied on the raw data. For each crop file, the center X- and Y-coordinates of the
216 upper left spot are determined for further semi-automated data evaluation.

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218

219 2.6. Identification and ranking of hybridoma clones

220

221 As a model system, we used 3 positive clones of a previous hybridoma project for the
222 development of improved CBZ antibodies and one commercial antibody. The monoclonal antibodies
223 possessed quite different relative affinity constants against CBZ; they cover about four orders of
224 magnitude regarding the IC₅₀ value (see Table 1). These clones have been described in previous
225 publications [23,24,37]. As negative controls replicated spots of a typical cell culture medium (CCM)
226 were used. One of the most critical points of such hybridoma screenings is the influence of the
227 unknown IgG concentration. In theory, it might be possible to determine the IgG concentration for
228 each clone independently and dilute the supernatants accordingly. However, this approach seems to
229 be not feasible. Therefore, we tested three different antibody concentrations of 0.1, 1 and 10 mg/L. It
230 is obvious that this approach cannot cope with all situations, which might occur. First, heavy
231 contaminations of irrelevant immunoglobulins, e.g. caused by contaminated cell lines, might reduce

232 the signal considerably. And second, at a very early stage of development, a clone with a low
233 antibody production rate might be left undetected due to the very low IgG concentration in the
234 supernatant. Therefore, the first screening should be followed by a later screening after an additional
235 growth time of one or two weeks, if the results of the first screening were unsatisfactory.

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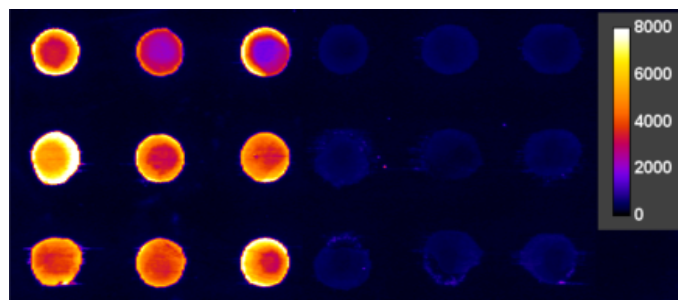
237 3. Results

238 3.1. The coating of microarray slides

239 Surface chemistry is a crucial point for microarrays. Epoxy-silanized glass slides were chosen here,
240 because they have been proven to show excellent performance in antibody applications [38,39]. In a
241 next step, they were coated with protein G [40], which enables a very efficient and oriented
242 immobilization [41] of most immunoglobulin classes. Finally, a protein G coating is expected to enrich
243 antibodies from the complex cell-culture supernatants due to the selective interaction between
244 protein G and immunoglobulins. For the repeated use of the same antibody-coated chip, a novel
245 preactivation crosslinking may be used [42]. However, this most advanced protocol was not applied
246 in this work, yet.

247 It could be shown in preliminary tests that a cysteine-modified protein-G, Cys-Protein-G [Fig. 3]
248 consistently showed higher immobilization efficiency for IgG, which supports the notion that the
249 additional cysteine leads to an improved immobilization on epoxy slides at a pH 7 and 8. Previously,
250 Cys-Protein-G was mainly used on gold surfaces [43,44], on which the strong thiol-gold interaction
251 leads to an oriented and efficient immobilization. Since gold-coated slides are quite expensive, we
252 prefer epoxy-silanized surfaces on conventional glass slides for our screening approach. In
253 experiments with epoxy-functionalized glass substrates, spotted Cys-Protein-G showed significantly
254 higher fluorescence signals for fluorescently labelled goat IgG [Fig. 3]. Therefore, in further
255 experiments, Cys-Protein-G was used exclusively. The selective pre-spotting of the chip with Cys-
256 Protein-G instead of the pre-coating of the whole chip with this reagent has the advantage of well-
257 defined spot shapes and a significantly reduced consumption of Cys-Protein-G.

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259

260

261 Fig. 3: Comparison of different surface coatings for antibody immobilization. In this experiment,
262 fluorophore-labeled goat IgG was incubated on spots of immobilized Cys-Protein-G (left) and protein
263 G (right). The spot to spot distance is 1 mm in x- and y-dimension.

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266 3.2. Antibody printing

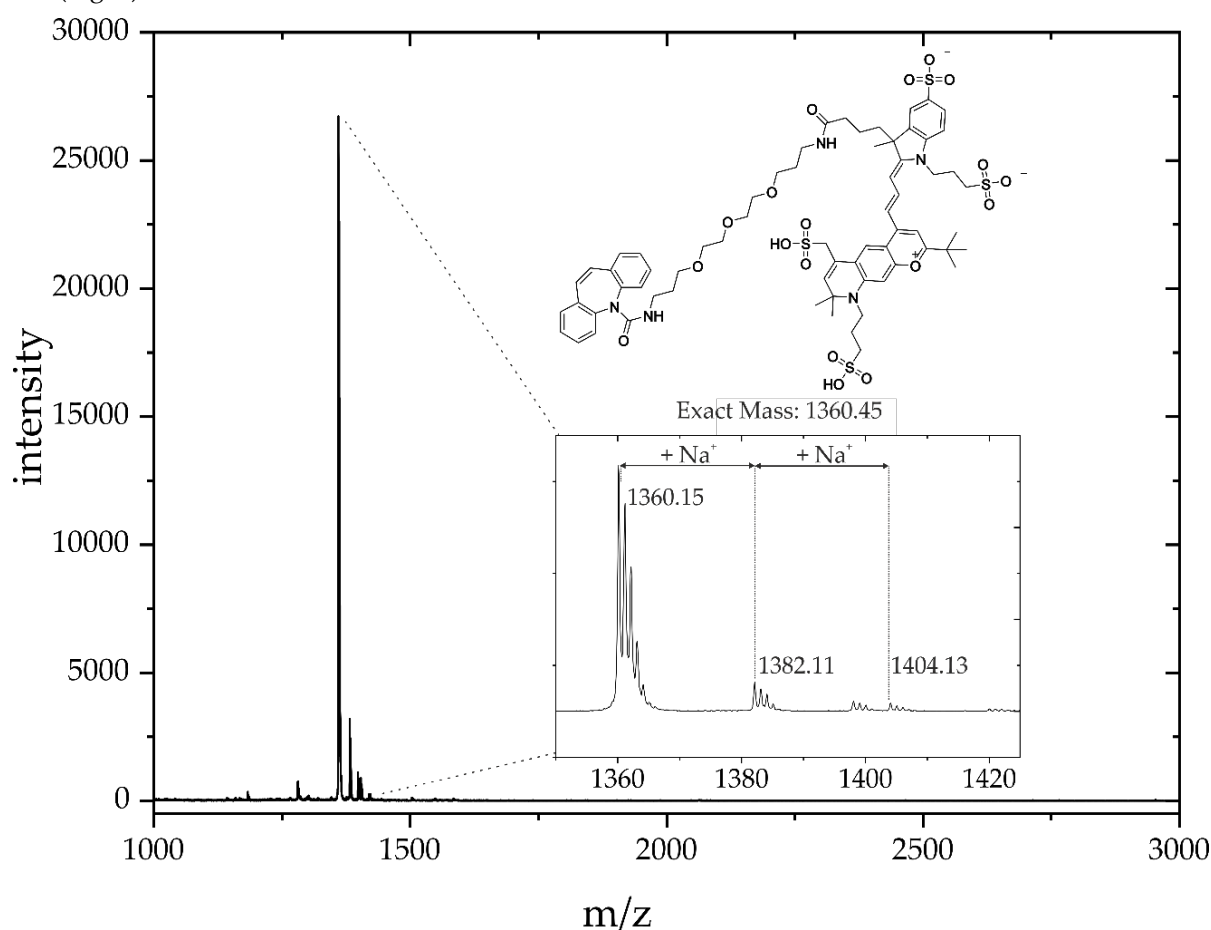
267 In a next step, the immobilization of the antibodies from protein-rich cell culture supernatants was
268 examined. As a model system, we used a typical cell culture medium supplied with 10% of fetal calf
269 serum with glycerol and Tween 20 to improve the spot shape, spiked with the respective antibodies
270 at different concentration levels. The transfer from the 96-well source plate to the microarray was
271 performed with a Calligrapher™ MiniArrayer (BioRad). For the printing, MCP360S solid ceramic
272 pins were used, which have a diameter of 400 μm , which transfer approximately 1 nL of sample
273 volume. Hence, only extremely small amounts of the supernatant are consumed, and a nearly

274 unlimited number of replicates can be performed, if required. The spots were reprinted in a 12x8
275 subgrid on the same coordinates on which the Cys-Protein-G had been immobilized previously.

276

277 3.3. Design and synthesis of hapten-fluorophore conjugates

278 The screening procedure relies on the use of monovalent, fluorophore-labeled antigens. The
279 proper choice of the dye is a relevant point. Today, highly improved fluorescence labels are available,
280 which display many desirable properties, e.g. high quantum yield, high photostability, excellent
281 water solubility and low non-specific binding. Based on the available laser excitation source of 635
282 nm, the dye Dy654 [Fig. 1] was chosen. To avoid steric hindrance and unwanted interaction between
283 hapten (immunoreactive group) and fluorescent dye, a short polyethylene glycol linker (TOTA)
284 was used. The CBZ-TOTA-Dy654 tracer was synthesized in two steps [24] and examined by MALDI-TOF-
285 MS (Fig. 4).



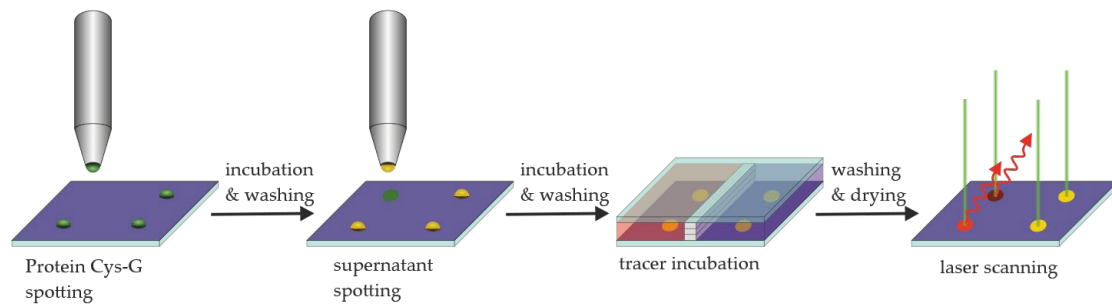
286 Fig. 4: MALDI-TOF-MS analysis of the carbamazepine-Dy654 conjugate. The expected molecular
287 mass of the compound in the negative mode is 1360,45. Due to the various sulfonic acids, the molecule
288 is prone to exhibit sodium adduct peaks.

289

290

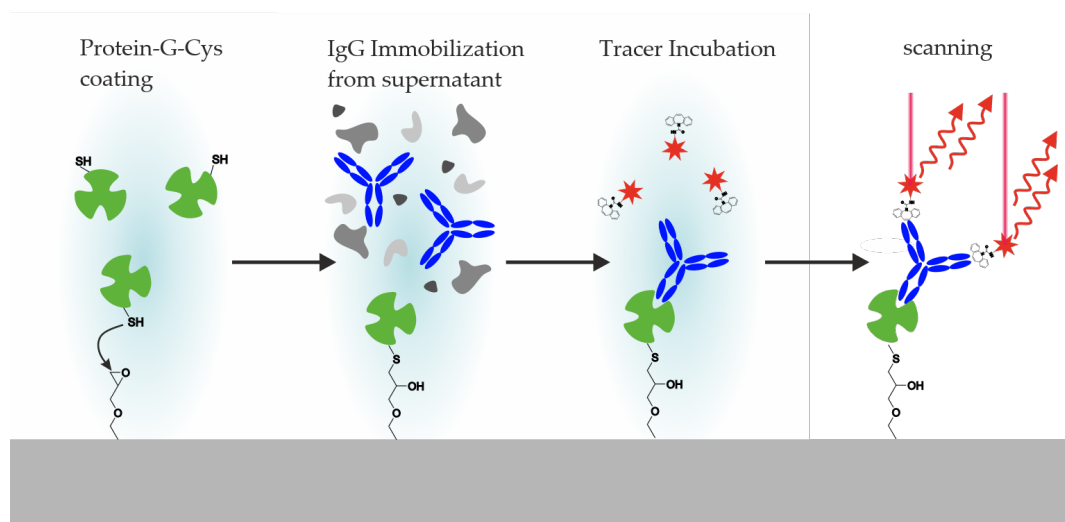
291 3.4. Incubation steps of reagents and hybridoma supernatants

292 All immunochemical steps were performed on epoxy-silanized glass slides (Fig. 5). In a first
 293 layer, Cys-Protein-G was printed on the slide with 400 μm pins in a 1 mm grid. After incubation for
 294 three days in an airtight 50 ml falcon tube over PBS with 1 % glycerol in the dark, the slides are
 295 washed thoroughly and dried. These protein G chips might be stored cool or frozen in the dark for
 296 future projects.



297 Fig. 5: General approach for the chip-based screening: A: Printing of Cys-Protein-G on an epoxy-
 298 silanized glass slide (may be prepared in advance), B: Printing of MAb supernatants, C: Incubation
 299 of labeled antigen/hapten. In separate chamber(s) but on the same slide, competition experiments can
 300 be performed D: Laser scan to quantify fluorescence signals.

301
 302 For the simulated screening process, raw hybridoma supernatants (here: model solutions of
 303 known clones), were stored in a 96-well microwell plate. The simulated hybridoma supernatants
 304 were transferred from this source plate to the Cys-Protein-G chip by the same 400 μm pins onto the
 305 same locations. For some assays (e.g. inhibition, see below) replicates are prepared on the same chip.
 306 After the incubation, the chip was washed again and subsequently incubated with the fluorescence
 307 tracer CBZ-TOTA-Dy654. After a short washing step, the chip was dried and examined by a
 308 conventional fluorescence scanner (Fig. 6).
 309



310
 311 Fig. 6: Incubation steps of the hybridoma screening process: 1. Printing and incubation of an
 312 epoxy-silanized glass chip with a Cys-Protein-G solution. Washing step. 2. Printing and incubation
 313 of hybridoma supernatant. Washing step. 3. Incubation of fluorescence tracer (labeled antigen or
 314 hapten). Washing step. Drying. 4. Fluorescence Scan (Exc. 635 nm, Em. 650-670 nm). (5. Regeneration
 315 is optional).
 316
 317

318 The assay type described above was rarely applied in the literature. It shows some distinct
 319 advantages:

320 a) The dye conjugate is monovalent, which avoids confusing avidity (multivalency) effects, which
 321 often are misinterpreted.

322 b) This monovalent binding restricts the signals to high-affinity antibodies. With medium or weakly
 323 binding antibodies, the tracer (labeled antigen) is washed away. The washing duration might
 324 modulate the affinity cutoff of the detected clones.

325 c) The tracer binding is highly reversible, which makes it possible to reuse the chip without strong
 326 regeneration steps.

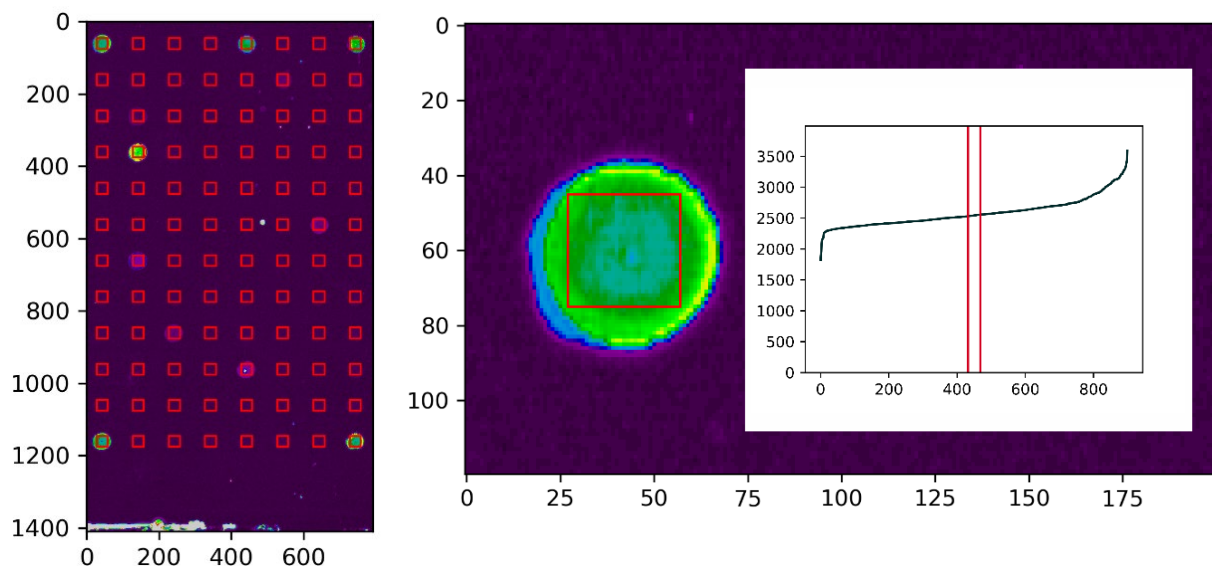
327 Multivalency also is a frequent problem in surface-plasmon resonance (SPR) measurements, which
 328 are often used for antibody characterization. Unperceived multivalency leads to misleadingly high
 329 affinity constants, overestimating the quality of an antibody [45-48].

330

331 3.5. Data Evaluation

332 In the semi-automated data evaluation with Python (Anaconda Spyder 3.3.2), the previously
 333 saved non-competitive and the competitive crop-files are imported and as a manual input, the x-y
 334 start coordinates along with basic grid parameters are entered. In the first step of the data evaluation
 335 by the script on the center of every spot in the array, a square region of interest (ROI) of 30 x 30 pixels
 336 was defined, see Fig. 7.

337



338

339 Fig. 7: Scan of the microarray (left) with generated square regions of interest (ROI) over all spots of
 340 the array for the semi-automated data evaluation with the Python script. ROI of the first spot (right)
 341 with included pixels were sorted by their intensity and the 2% of the central pixels used for the
 342 evaluation.

343

344 For every individual ROI, all included pixels were sorted according to their intensities. The
 345 central 2% of the pixels were used to calculate the truncated mean of the spot intensity, the remaining
 346 pixels in the ROI are trimmed (truncated) in order to achieve a highly robust estimate of the mean.
 347 This accounts for even severe spot inhomogeneities and significantly increases the robustness of the
 348 spot evaluation. The procedure was performed simultaneously for the non-competitive and the
 349 competitive array. Subsequently, the quotient of the corresponding non-competitive and competitive
 350 spot was calculated and stored in a table. This quotient was used to assess the quality of the clones.
 351 A high quotient translates to a clone with a high affinity for the fluorescent tracer, as the tracer was
 352 strongly bound by the captured IgG from the supernatant. Simultaneously a high quotient shows a

353 successful competition with the target analyte, as the analyte inhibited the binding of the tracer.
 354 Finally, the spot intensities, along with the quotient were exported as .txt file by the script.
 355

356 3.7. Identification and ranking of hybridoma clones

357 As a model system, we used three positive clones of a previous hybridoma project for the
 358 development of improved carbamazepine (CBZ) antibodies and one commercial clone of proven
 359 quality. The monoclonal antibodies possessed quite different affinities against their target; they cover
 360 about four decades (Table 1). These clones have been described or used in previous publications
 361 [23,24,37,49]. Dots of a cell culture medium were used as negative controls. One of the most critical
 362 points of such hybridoma screenings is the influence of the unknown IgG concentration. In theory, it
 363 might be possible to determine the IgG concentration independently and to dilute the supernatants
 364 accordingly. However, this approach seems to be quite impractical and hence not useful. Therefore,
 365 we tried to get along with varying antibody concentrations and tested three different levels of 0.1, 1
 366 and 10 mg/L.
 367

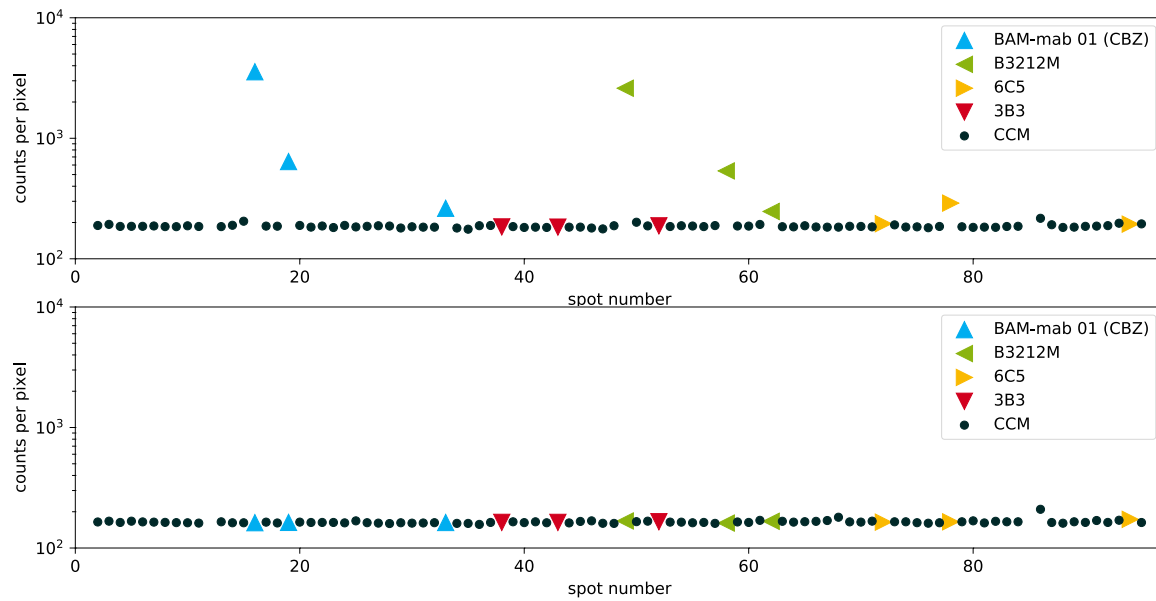
368 Table 1. Monoclonal antibodies used for the simulated screening

Antibody	IC ₅₀ [µg/L]	Isotype	Rating	Test result (1 mg/L)	Test result (10 mg/L)	References
BAM-mab 01 (CBZ)*	0.32	IgG1	good	++	++	[23,49]
B3212M	0.15	IgG1	good	++	++	[23,37,50-55]
6C5	23.0	IgG1	poor	--	--	[24]
3B3	1700	IgG1	very poor	--	--	[24]

369 * In the first publication, this clone is designated "clone 1"[23].
 370

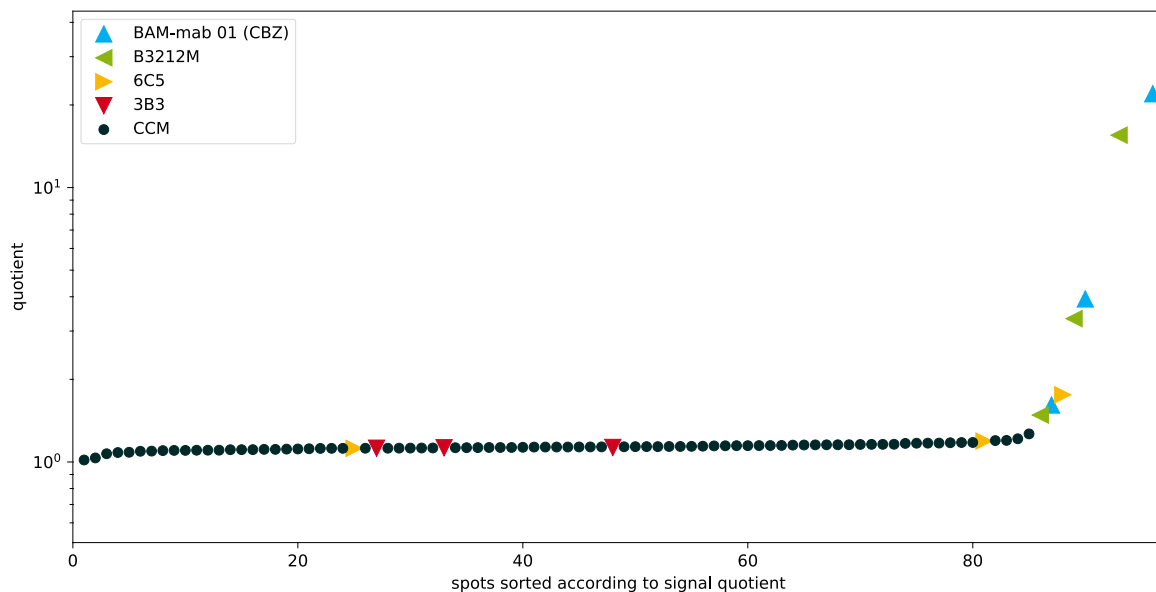
371 In Fig. 8, the normalized fluorescence signals of 92 samples are shown ordered by spot number.
 372 In typical screenings, the vast majority of all tested clones show no affinity for the hapten and
 373 therefore signals on the background level are available in abundance.
 374

375 Although, a small drift of the signal was found, the signal quotient is quite stable. Most
 376 important however, is the unambiguous identification of the high-affinity clones B3212M and BAM-
 377 mab 01 (CBZ). The quotients at all concentrations (0.1, 1 and 10 mg/L) were significantly above the
 378 negative controls. These excellent clones would have been identified under any circumstance. This is
 379 the most important finding of this work. However, also false positives need to be minimized. In
 380 theory, many other screening protocols can identify good clones, but they generate such a flood of
 381 false positives leading to the unfortunate situation that some or all of the good clones may be
 382 overlooked. Fig. 9 also shows that this method is not distracted by poor clones. Very weak antibodies
 383 go either completely undetected as the clone 3B3 (red triangles), or they are only slightly positive at
 384 very high concentrations (yellow triangles), but still remain below the cut-off value of 2. Nevertheless,
 385 from our point of view, the most practical way to choose positive clones is to avoid any cut-off values,
 386 but simply to start with the highest quotient, collect more clones in the direction from the highest to
 387 the lowest quotient and stop when you have a sufficient number of clones or there are no more clones
 388 significantly different from the background.
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Fig. 8 Signal for the spots on the Cys-Protein-G glass slide. The upper figure shows the non-competitive binding of the fluorescently labeled hapten (analyte) to the immobilized antibodies. The lower figure shows the same experiment under competitive conditions with an excess of haptens (27 mg l⁻¹ analyte CBZ). Known monoclonal antibodies diluted in cell culture medium are color coded, accordingly. CCM: Cell Culture Medium (negative controls). Blank measurements with slightly increased signals are caused by carryover effects, which can be easily identified and eliminated.



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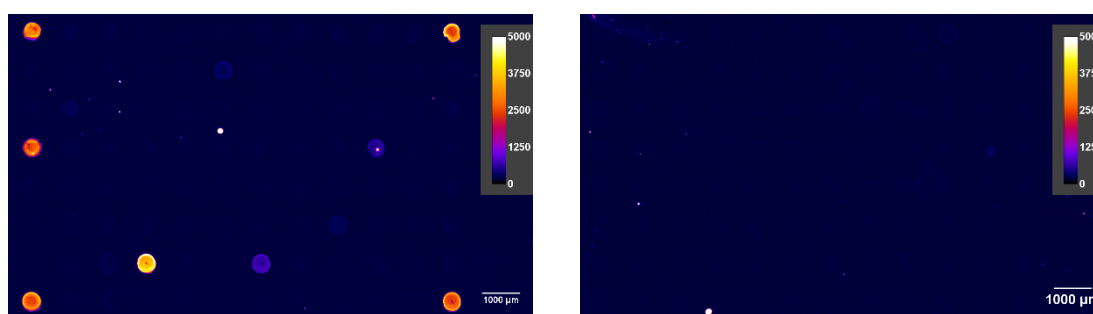
Fig. 9 Signal quotients of the non-competitive and the competitive spots for the spots on the Cys-Protein-G glass slide. Known monoclonal antibodies diluted in cell culture medium are color coded, accordingly. CCM: Cell Culture Medium (negative controls). The good antibodies BAM-mab 01 (CBZ) and B3212M are easily identified. With a cut-off value of 2 for the quotient, the weak antibody 6C5 is always below this value, even at high concentrations, and the poorest antibody 3B3 is not different from the blank values. It is noteworthy that 3B3 could not be distinguished from high-affinity clones in an original publication (Fig. 3 in [24]).

3.6. Competition experiments

Essentially all screening protocols published before did not use competitive assays for the primary examination of hybridoma clones. In contrast, the direct (antibody-immobilized) format used in this

411 work avoids avidity effects and efficiently suppresses other unwanted false positives, sometimes
412 loosely termed "linker recognition". Competitive formats provide additional evidence for the
413 performance of the respective clone in competitive assays. This approach directly rejects all clones,
414 which cannot be inhibited by the target analyte at a user-defined concentration (Fig. 10). However,
415 this format is even more powerful. It enables to examine cross-reactivity properties at a very early
416 stage of clone screening and thus to pick the best clones for a respective application. Considering the
417 perspective of regeneration of the slides, the cross-reactivity experiments may be repeated to check
418 all cross-reactants of interest. It is not necessary to postpone this characterization and selection to the
419 time after clone expansion. Due to the small spots and hence the density of the arrays, several
420 replicates of the clones can be printed on one slide. Using incubation chambers with not only two,
421 but several separated wells, parallel incubations without and with different competitors may be
422 performed in one run. The strict focus on the best clones and the rejection of non-binding and non-
423 inhibited clones saves a lot of time and money, since the recloning, expansion, antibody isolation and
424 purification are by far the most expensive and time-consuming steps in the development of
425 monoclonal antibodies, which have to be performed with each seemingly positive clone.

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431 Fig. 10 Non-competitive mAb incubation (left) and the competitive incubation with 27 mg
432 L⁻¹ carbamazepine (CBZ) (right). The positive spots are strongly inhibited by the haptens (analyte),
433 which means that the respective antibodies bind selectively to the target compound CBZ.

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436 4. Discussion

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438 The development of monoclonal antibodies is still a risky and expensive endeavor. Inefficient and
439 error-prone screening procedures cause unnecessary costs and project delays. We are convinced that
440 poor antibody clones should be eliminated in the development process as early as possible.
441 Unfortunately, nearly all text-book protocols rely on screening steps of limited selectivity. More
442 powerful validation steps are often shifted to later stages of the project, after recloning and expansion
443 of many seemingly positive clones have been performed. From this retrospective point of view, it is
444 often recognized that the selected clones are of disappointing quality or even negative. Our approach
445 uses several measures to improve this situation: First of all, the miniaturization of the process enables
446 to test a very high number of clones, which avoids that clones are lost due to arbitrary pre-selection
447 criteria or other limitations. The next improvement is based on the use of an antibody-immobilized
448 format, which is not yet routinely used. This leads to the suppression of unwanted avidity effects,
449 which usually causes the overestimation of the affinity of poor antibody clones. After efficient
450 washing steps, only strong binders are detected as true positives. Finally, the parallel performance of
451 inhibition assays confirms the selectivity and the focus on the right target. Otherwise, many
452 antibodies bind strongly to the immunogen conjugates, but not or only weakly to the intended target.
453 In addition, even a more complex inhibition screening might be performed, if very special cross-
454 reactivity restrictions must be met.

455 5. Conclusions

456 With the aid of known monoclonal antibodies, it could be shown that the presented approach is
457 extremely efficient to identify high-affinity clones, with essentially no false positives. We plan to
458 apply this protocol to our upcoming antibody projects to reduce the costs and the time efforts for the
459 development of high-quality antibodies, which are desperately needed in many bioanalytical
460 ventures [16,56].
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462 **Supplementary Materials:** The following are available online, Description of the semi-automated data
463 evaluation.

464 **Author Contributions:** Conceptualization, M.G.W.; methodology, M.P. and M.G.W.; software, M.P.; validation,
465 M.P. and M.G.W.; resources, M.G.W. data curation, M.P.; writing—original draft preparation, M.G.W.; writing—
466 review and editing, M.P. and M.G.W.; visualization, M.P.; project administration, M.G.W.; funding acquisition,
467 M.G.W.

468 **Funding:** This research received no external funding.

469 **Acknowledgments:** We thank Sabine Flemig (BAM) and Kristin Hoffmann (BAM) for the preparation of the cell
470 culture medium and the donation of the antibodies BAM-mab 01 (CBZ) and B3212M, Martin Dippong (BAM)
471 for the supply of the clones 6C5 and 3B3, Timm Schwaar (BAM) for MALDI measurements, Peter Carl (BAM)
472 for fruitful discussions and assistance with the contact spotter.

473 **Conflicts of Interest:** The authors declare no conflict of interest.
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