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

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

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

1. Introduction

During antibody development, the screening of hybridoma cells is a crucial step. Several obstacles may lead to a complete failure of the process. First, the assay needs to be selective (“specific”) enough. Otherwise the researcher is flooded with seemingly positive clones, which in a later stage turn out to be of poor quality or completely negative. Good clones might be irreversibly lost in this phase, because in most cases it is not feasible for all clones to undergo an in-depth examination. The second requirement is speed, since some irrelevant hybridoma cells might grow very fast and overgrow some positive clones, if the final clonal state is not reached, yet. The third point is parallelization, since the probability that an excellent clone is found, increases with the number of clones tested. Due to technical and financial limitations, often too few clones are examined. The forth issue is mainly encountered with haptens. Quite often, antibodies, which bind to an immobilized hapten-protein conjugate and hence are identified as positives, are found to be weak or non-binders of the free analyte. This effect is known as spacer recognition, linker recognition or
bridging phenomenon [1-13]. Sometimes this effect can be reduced by application of linker or site
heterology using different linkers or conjugation reagents for the preparation of immunogens and
coating antigens or enzyme conjugates [14,15]. The fifth point refers to the affinity of the respective
clones. In most cases, an affinity ranking is required to identify the strongest binder, which often
shows the best performance in analytical applications [16]. The sixth risk factor is the screening
date. Since the antibody screening is a cost and time intense step, the procedure is usually
performed only once or twice. However, due to the varying growth rate of individual hybridoma
clones, the “best” time for a screening can hardly be determined. Consequentially, several rounds
are necessary to catch both, fast and slow cells. The fast cells need to be ranked immediately, since
they might be lost on a later date.

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

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

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

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

2. Materials and Methods
2.1. Reagents, buffers, materials and equipment

Transparent, flat-bottom non-binding 96 MTP plates were acquired from Greiner Bio-One (Frickenhausen, Germany). PD SpinTrap™ G-25 Desalting Columns were obtained from GE Healthcare (Uppsala, Sweden), clear microscope slides were bought from Sigma-Aldrich (Darmstadt, Germany). Recombinant Protein G (PRO-402) and Cys-Protein-G (PRO-1238) were purchased from Prospec (Ness-Ziona, Israel), monoclonal anti-CBZ antibody BAM-mab 01 (CBZ) was obtained from BAM (Federal Institute for Materials Research and Testing (BAM), Berlin), anti-CBZ antibody B3212M (Meridian Life Science Inc, Memphis, USA) was kindly supplied by S. Flemig (BAM), the clones 3B3 and 6C5 were supplied by M. Dippong [24] (BAM), fetal calf serum (Biochrom S0115), L-glutamine, RPMI1640 and 2-mercaptoethanol were acquired from Biochrom (Berlin, Germany). The fluorescence dyes Dy654-NHS and Dy554-NHS were purchased from Dyomics (Jena, Germany).

According to the manufacturer, the following properties of the dye Dy654 are given: Absorption / emission max.: 653 / 677 nm (in ethanol), molar absorbance: 220.000 M⁻¹cm⁻¹, soluble in water, methanol and DMF. The mono-protected PEG3 linker 1-(9-fluorenylmethyloxycarbonyl-amino)-4,7,10-trioxa-13-tridecanamine hydrochloride (Fmoc-TOTA·HCl) was bought from Iris-Biotech (Marktredwitz, Germany). Carbamazepine (CBZ), Dibenz[b,f]azepin-5-carbonyl chloride (CBZ-Cl, Sigma, 90 %), bovine serum albumin (BSA, Sigma, >98%) and (3-glycidyloxypropyl)trimethoxysilane (Glymo, Sigma, >98%), DMSO (AppliChem, >99.5 %), glycerol (Sigma, Aldrich G2025), Tween 20 (Sigma, P7949), hydrochloric acid (Fluka,84415), sodium hydroxide (Sigma, 30620), Mucasol (Sigma, Z637203) tetrahydrofuran (THF, Chemsolute, >99.9 %) and toluene (Roth, >99.5 %) were obtained from Sigma-Aldrich. Ultrapure water (MilliQ) was supplied by a Milli-Q Synthesis A10 (Merck, Germany). Cyano-4-hydroxycinnamic acid was bought from Bruker (201344).

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

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

2.1. Preparation of Epoxy Slides

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

2.2. Sample printing and incubation

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].

2.3. Design and synthesis of hapten-fluorophore conjugates

The screening procedure relies on fluorophore-labeled antigens or haptenes. 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.

![Synthesis of the CBZ-TOTA-Dy654 tracer](image)

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

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

Fig. 2: Competition experiment on a microarray slide.

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

2.6. Identification and ranking of hybridoma clones

As a model system, we used 3 positive clones of a previous hybridoma project for the development of improved CBZ antibodies and one commercial antibody. The monoclonal antibodies possessed quite different relative affinity constants against CBZ; they cover about four orders of magnitude regarding the IC50 value (see Table 1). These clones have been described in previous publications [23,24,37]. As negative controls replicated spots of a typical cell culture medium (CCM) were used. One of the most critical points of such hybridoma screenings is the influence of the unknown IgG concentration. In theory, it might be possible to determine the IgG concentration for each clone independently and dilute the supernatants accordingly. However, this approach seems to be not feasible. Therefore, we tested three different antibody concentrations of 0.1, 1 and 10 mg/L. It is obvious that this approach cannot cope with all situations, which might occur. First, heavy contaminations of irrelevant immunoglobulins, e.g. caused by contaminated cell lines, might reduce
the signal considerably. And second, at a very early stage of development, a clone with a low antibody production rate might be left undetected due to the very low IgG concentration in the supernatant. Therefore, the first screening should be followed by a later screening after an additional growth time of one or two weeks, if the results of the first screening were unsatisfactory.

3. Results

3.1. The coating of microarray slides

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

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

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

3.2. Antibody printing

In a next step, the immobilization of the antibodies from protein-rich cell culture supernatants was examined. As a model system, we used a typical cell culture medium supplied with 10% of fetal calf serum with glycerol and Tween 20 to improve the spot shape, spiked with the respective antibodies at different concentration levels. The transfer from the 96-well source plate to the microarray was performed with a Calligrapher™ MiniArrayer (BioRad). For the printing, MCP360S solid ceramic pins were used, which have a diameter of 400 µm, which transfer approximately 1 nL of sample volume. Hence, only extremely small amounts of the supernatant are consumed, and a nearly
unlimited number of replicates can be performed, if required. The spots were reprinted in a 12x8 subgrid on the same coordinates on which the Cys-Protein-G had been immobilized previously.

3.3. Design and synthesis of hapten-fluorophore conjugates

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

Fig. 4: MALDI-TOF-MS analysis of the carbamazepine-Dy654 conjugate. The expected molecular mass of the compound in the negative mode is 1360.45. Due to the various sulfonic acids, the molecule is prone to exhibit sodium adduct peaks.
3.4. Incubation steps of reagents and hybridoma supernatants

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

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

For the simulated screening process, raw hybridoma supernatants (here: model solutions of known clones), were stored in a 96-well microwell plate. The simulated hybridoma supernatants were transferred from this source plate to the Cys-Protein-G chip by the same 400 µm pins onto the same locations. For some assays (e.g. inhibition, see below) replicates are prepared on the same chip.

After the incubation, the chip was washed again and subsequently incubated with the fluorescence tracer CBZ-TOTA-Dy654. After a short washing step, the chip was dried and examined by a conventional fluorescence scanner (Fig. 6).

Fig. 6: Incubation steps of the hybridoma screening process: 1. Printing and incubation of an epoxy-silanized glass chip with a Cys-Protein-G solution. Washing step. 2. Printing and incubation of hybridoma supernatant. Washing step. 3. Incubation of fluorescence tracer (labeled antigen or hapten). Washing step. Drying. 4. Fluorescence Scan (Exc. 635 nm, Em. 650-670 nm). (5. Regeneration is optional).
The assay type described above was rarely applied in the literature. It shows some distinct advantages:

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

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

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

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

3.5. Data Evaluation

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

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

Finally, the spot intensities, along with the quotient were exported as .txt file by the script.

3.7. Identification and ranking of hybridoma clones

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

Table 1. Monoclonal antibodies used for the simulated screening

<table>
<thead>
<tr>
<th>Antibody</th>
<th>IC50 [µg/L]</th>
<th>Isotype</th>
<th>Rating</th>
<th>Test result (1 mg/L)</th>
<th>Test result (10 mg/L)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAM-mab 01 (CBZ)*</td>
<td>0.32</td>
<td>IgG1</td>
<td>good</td>
<td>+ +</td>
<td>+ +</td>
<td>[23,49]</td>
</tr>
<tr>
<td>B3212M</td>
<td>0.15</td>
<td>IgG1</td>
<td>good</td>
<td>+ +</td>
<td>+ +</td>
<td>[23,37,50-55]</td>
</tr>
<tr>
<td>6C5</td>
<td>23.0</td>
<td>IgG1</td>
<td>poor</td>
<td>- -</td>
<td>- -</td>
<td>[24]</td>
</tr>
<tr>
<td>3B3</td>
<td>1700</td>
<td>IgG1</td>
<td>very poor</td>
<td>- -</td>
<td>- -</td>
<td>[24]</td>
</tr>
</tbody>
</table>

* In the first publication, this clone is designated “clone 1”[23].

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

Although, a small drift of the signal was found, the signal quotient is quite stable. Most important however, is the unambiguous identification of the high-affinity clones B3212M and BAM-mab 01 (CBZ). The quotients at all concentrations (0.1, 1 and 10 mg/L) were significantly above the negative controls. These excellent clones would have been identified under any circumstance. This is the most important finding of this work. However, also false positives need to be minimized. In theory, many other screening protocols can identify good clones, but they generate such a flood of false positives leading to the unfortunate situation that some or all of the good clones may be overlooked. Fig. 9 also shows that this method is not distracted by poor clones. Very weak antibodies go either completely undetected as the clone 3B3 (red triangles), or they are only slightly positive at very high concentrations (yellow triangles), but still remain below the cut-off value of 2. Nevertheless, from our point of view, the most practical way to choose positive clones is to avoid any cut-off values, but simply to start with the highest quotient, collect more clones in the direction from the highest to the lowest quotient and stop when you have a sufficient number of clones or there are no more clones significantly different from the background.
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 hapten (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.

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

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

4. Discussion

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

With the aid of known monoclonal antibodies, it could be shown that the presented approach is extremely efficient to identify high-affinity clones, with essentially no false positives. We plan to apply this protocol to our upcoming antibody projects to reduce the costs and the time efforts for the development of high-quality antibodies, which are desperately needed in many bioanalytical ventures [16,56].

Supplementary Materials: The following are available online, Description of the semi-automated data evaluation.

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