µFlot: New Microfluidic Dot-Blot-Based Device for Antibody Quality Evaluation

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ABSTRACT: The key factor in the development of antibody-based assays is to find an antibody that has an appropriate affinity, high specificity, and low cross-reactivity. Here, we report on a new microfluidic dot-blot-based device (µFlot) that simultaneously screens the quality of antibodies destined for research applications. The µFlot design combines the advantages of traditional dot-blot and microfluidic techniques for antibody selection including affinity, cross-reactivity, and batch-to-batch comparisons. The µFlot eliminated several labor- and time-consuming steps associated with traditional ELISA and western blotting. The µFlot device can be prepared using micro-milling or 3D printing, compares up to five antibodies per run, and enables to select the most appropriate antibody for each specific application. The only peripheral equipment required for assays with µFlot are a vacuum pump, a camera, and densitometry software. The µFlot prototype was validated with several Ag-Ab complexes and the results were confirmed using ELISA and conventional dot-blot analysis. The µFlot was then used to test commercial antibodies with vendor-declared specificity/affinity for the target antigens apolipoprotein E and EpCAM, as well as whole bacteria including Salmonella, Listeria, and Escherichia. The µFlot affinities and cross-reactivities were evaluated and the antibodies best suited for each application were identified.

Antibody quality and validation are often problematic in research. Studies from the past ten years have reported low quality for a substantial proportion of the antibodies on the market [1-8]. The antibodies may fail to recognize the target molecule correctly [4]. They might also lack specificity, bind a nontarget molecule, or not bind at all [3]. Vendors may inappropriately develop and validate the antibodies. Moreover, researchers may incorrectly use or store them [6, 9]. Further, there could be wide batch-to-batch variability [10]. Hence, researchers may find it difficult if not impossible to replicate published results based on experiments involving antibodies [3]. This problem applies both to polyclonal and monoclonal antibodies [11]. However, complaints about the former are comparatively more frequent [1, 4]. Certain authors proposed the exclusive use of recombinant antibodies or protein affinity reagents rather than monoclonal or polyclonal antibodies [2, 12]. The International Working Group for Antibody Validation (IWGAV) issued a "proposal for (the) validation of antibodies" [13] comprising a thorough verification process. They recommended performing at least one of five “pillars of validation” to be able to claim that a particular antibody correctly recognizes its target molecule. The first pillar proposes genetic strategies (use of control cells in which the target gene or epitope has been knocked out or knocked down), the second names orthogonal strategies (antibody-independent method for target quantification to correlate with antibody-based target quantification), the third are independent antibody strategies (antibodies that recognize the same target but bind to different regions of the protein), the fourth is expression of tagged proteins (antibodies target a protein containing an affinity tag), and the fifth is immunoprecipitation followed by mass spectrometry[12]. The pros and cons of each proposed antibody validation pillar have been discussed by Edfors et al. (2018) [14]. Western blotting was suggested by Sikorski et al. (2018) for antibody validation [15]. Certain authors recommended biologically rigorous antibody evaluation using surface plasmon resonance, isothermal titration calorimetry, and/or immunoprecipitation/matrix-assisted laser desorption-ionization/mass spectrometry (IP/MALDI-MS) [16, 17]. Slaastad et al. developed microsphere affinity proteomics (MAP) wherein peptides bind antibodies conjugated with fluorescent dyes [18]. A comprehensive investigation into antibody quality and validation issues was published by Taussig et al. [19].

Antibody quality and validation deficiencies waste valuable materials, time, effort, and money. Bradbury and Plückthun [2] reported that > $350 million per year are misspent in biomedical research in the United States alone. The authors mentioned several examples of research projects that failed this way. They believe that these losses are incurred mainly because of poorly characterized and ill-defined antibodies. Moreover, some of the research, involving these antibodies, is irreproducible and unreliable [1, 3, 20]. There have been enormous international initiatives on the part of the research community and numerous antibody producers and vendors to ameliorate antibody production and validation. Nevertheless, commercial antibodies

are not yet systematically generated, validated, or unambiguously identified [13, 21-23].

The antibody market comprises > 300 registered commercial research suppliers [1]. Hundreds of antibody products targeting specific molecules are usually available. These include polyclonals vs. monoclonals, purified antibodies vs. non-purified antibodies in the form of lyophilized powders vs. liquid stocks with various buffer compositions, and coming from different host animals. Affinity reagents other than antibodies such as DARPins [24], affibodies, affimers, aptamers [25], and protein affinity reagents are also available [7, 26, 27]. There are substantial differences in the prices of the antibody products. Several hundreds of antibody products were found for apolipoprotein E. The 2018 price for 100 µg anti-ApoE antibody was in the range of 250–980 €. Further, the target structure is seldom specified by the supplier. Therefore, the interaction with the target molecule in the intended application such as recombinant vs. native molecule, cell suspension vs. fixed/stained tissue, and soluble vs. conjugated vs. solid-phase-bound target molecules may differ [13]. Target molecule conformation may change depending upon the pH, buffer, reagent composition, or molarity because of chaotropic and steric effects [6]. Vendors cannot forecast every intended application or experimental condition. For this reason, final antibody selection and validation are the responsibilities of the end user. Hence, selection of the appropriate antibody for the intended experiment may be very challenging. Researchers seeking new antibodies can consult various databases [28], published papers, and selection guides [21, 23, 29] that cite previously performed applications involving these agents. While this information increases the probability that the antibody will be suitable for a particular application, it can never predict all potential interferences arising from variability in the antigens used and the application conditions. Thus, antibodies that are fit for one application may be unsuitable for another.

Here, we describe the novel µFlot device that tests and compares the quality of preselected antibodies. This tool implements principle of affinity dot-blot that employs a chaotropic reagent step. It rapidly and semi-quantitatively determines whether the selected antibody interacts with a particular target with sufficient affinity under specific conditions. It also helps to assess whether the antibody has acceptably low cross-reactivity with nontarget structures. Moreover, µFlot can perform classic dot-blot, evaluate the condition of long-stored antibodies, and identify batch-to-batch variability.

The µFlot features a special lid with a system of reservoirs on the upper side and a system of microfluidic drainage channels on the bottom side. The construction of the lid enables that the membrane remains inside the µFlot device during the whole experiment. No membrane replacement or cutting, or individual dot treatment is required. Compared to enzyme-linked immunosorbent assay (ELISA) and western blot [30], µFlot is considerably less time-consuming and labor-intensive. However, it is not appropriate for antigen mixtures or non-purified antigens since they are directly placed on the membrane and further separation is impossible. Only isolated soluble protein/peptides or bacterial cells were tested on µFlot. We demonstrated on several examples of various antibodies how the device works and what kind of information we can gain from it.

Experimental

Materials

Bovine serum albumin (BSA) was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The detection solution for horseradish peroxidase (HRP)-labeled secondary antibody was Opti-4CN™ substrate and a diluent concentrate from a commercial kit (Bio-Rad Laboratories, Hercules, CA, USA). Other chemicals used in buffer preparation were purchased from Penta (Chrudim, Czech Republic). The washing buffer was PBS-T (PBS with 0.05% Tween 20). The equilibration buffer was 10 mM phosphate buffer (pH 7.3). The blocking buffer was 5% BSA in PBS-T. The primary antibody buffer was 0.25% BSA in PBS-T. The antigens used for testing were bovine α-chymotrypsin (Cat. No. C4129; Sigma-Aldrich Corp., St. Louis, MO, USA), apolipoprotein E3 (ApoE3, human; Cat. No. ABIN573259; BioVision, Milpitas, CA, USA), and epithelial cell adhesion molecule (EpCAM, human, recombinant; Cat. No. 10694-H08H; Sino Biological, Beijing, China). The bacterial species used in antibody testing were *Salmonella typhimurium, Listeria monocytogenes, Escherichia coli,* and *Bacillus cereus* (American Type Culture Collection (ATCC), Manassas, VA, USA). The primary antibodies were pig and rat polyclonal anti-chymotrypsin IgG molecules, pig and sheep serum, mouse monoclonal anti-ApoE3 IgG, and rabbit polyclonal anti-ApoE3 IgG (Moravian Biotechnology, Brno, Czech Republic), mouse monoclonal anti-EpCAM IgG1: HEA 125 (Cat. No. BK61004-N; Progen, Heidelberg, Germany), C10 and 323/A3 (Cat. No. A0510; Santa Cruz Biotechnology, Dallas, TX, USA), anti-*Salmonella*: rabbit polyclonal (O and H Ag; Cat. No. 0701) and mouse monoclonal (flagellar protein; Cat. No. 6321; ViroStat; Portland, ME, USA); goat polyclonal CSA-1 (common structural Ag; Cat. No. 01-91-99; KPL, Gaithersburg, MD, USA), and mouse monoclonal (*Salmonella*; Cat. No. MBS531315) and rabbit polyclonal with HRP (O and H Ag; Cat. No. MBS 536004; MyBioSource, San Diego, CA, USA). Secondary HRP-labeled antibodies were anti-pig-IgG (rabbit; Cat. No. A5670), anti-sheep-IgG (rabbit; Cat. No. A0510), anti-mouse-IgG (goat; Cat. No. A5278), anti-rabbit-IgG (goat; Cat. No. A8275), anti-goat IgG (rabbit; Cat. No. A5420; Sigma-Aldrich Corp., St. Louis, MO, USA).

µFlot Device

The µFlot prototype was fabricated from transparent polycarbonate (PC) sheets (Palsun 10 mm; Gutta, Kladno, Czech Republic) using CNC micro-milling. It consisted of a vacuum manifold (bottom plate), a support plate (middle plate), and a sample template (lid; Fig. 1Aa-c). The top view shows that the lid is square and has 25 drilled holes arranged in a 5 × 5 array. Each five-hole row had a reservoir to hold 1 mL reagent. The bottom surface of the lid had a microfluidic drainage system (Fig. 1B) that emptied the liquid in the holes into larger collector channels and thence the output (Fig. 1B-2). The system comprised five collector channels and 25 short microchannels each 200 µm wide and 50 µm deep. A short and sharp expansion was included midway through the microchannels to increase their width tenfold. The expansion prevented spontaneous drainage caused by the capillary effect. The middle plate had the same square hole pattern as the lid and supported the blotting membrane (Fig. 1Af). The bottom plate

had a retention chamber for the drained liquid. The lid and the bottom plate had outputs (Fig. 1B-2,3) for a vacuum pump connected by plastic fittings to plastic tubing. The three aforementioned components were fastened with four bolts and wingnuts (Fig. 1Ai). The parts were sealed with 2 mm thickness of microporous silicone (SP/16; Gumex, Straznice, Czech Republic). The membrane was inserted between two polyethylene (PE) foils (Fig. 1Ae) with the same square hole pattern as the lid and mounted between the lid and the middle plate. In this manner, fluids were prevented from leaking out of the drainage channels and permeating the membrane outside the target areas.

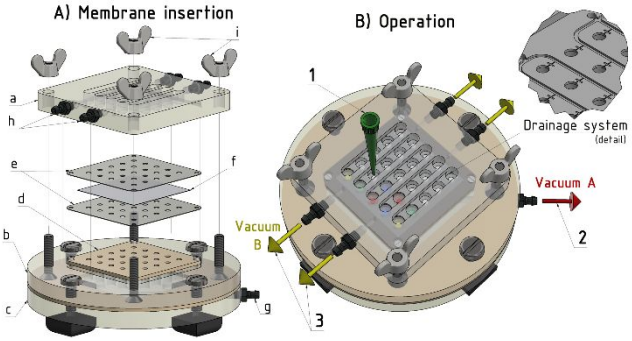

Fig. 1. A. µFlot construction and compartments. a-sample template/lid; b-support plate; c-vacuum manifold; d-membrane gasket; e-PE foils; f-membrane; g-vacuum chamber outlet; h-drainage system outlets; i-wingnuts; B. Top view of µFlot device: 1-solution application; 2-vacuum outlet to deposit target onto membrane; 3-vacuum outlets to drain reagents and buffers.

Affinity/avidity determination by dot-blot

This technique was previously described in Svobodova et al. [31]. Briefly, a commercially available DHM-96 dot-blot manifold (Scie-Plas; Cambridge, UK) with 96 dots each 3 mm in diameter was utilized. It consisted of three layers. The top had an 8 x 12 well array while the bottom served as a fluid retention chamber with a vacuum output. The membrane was set on top of the middle layer. The target molecule was applied to the blotting membrane with the dot-blot manifold. The membrane was then removed from the device, allowed to dry, placed in a Petri dish, incubated with a blocking buffer (BB) for 1 h, and cut into 5 x 5 mm squares with scissors (one dot per square). The membrane pieces were transferred to a segmented dish with 4 x 5 wells (one dot per well). Each membrane was separately treated with the reagents and rinsed with washing buffer (WBu). The liquids were removed and separately reapplied to each well. The dots were incubated with primary antibodies for 1 h and each row was incubated with one of the tested antibodies. The membrane dots were washed thrice with WBu by alternately applying and removing 1 mL liquid each time. Chaotropic reagent solutions of increasing molarity (1, 1.0, 1.5, 2.0M NH_4SCN) were prepared and applied to the wells in ascending order of concentration. Rapid pipetting was required to ensure the same incubation time for each chaotropic reagent concentration. After 5 min incubation, the chaotropic reagent was discarded and washing was repeated. The appropriate HRP-labeled secondary antibody was applied and incubated for 1 h. Another washing step was then performed. Incubation Opti-4CN substrate kit and densitometry were then conducted (Fig. 2A).

Affinity/avidity determination by avidity ELISA

In avidity ELISA, an enzyme-linked immunosorbent assay is enhanced by incubation with a mild chaotropic reagent in order to evaluate immunocomplex stability [32, 33]. Each well of a type P microtiter plate was filled with 150 µL of 2.5% (v/v) glutaraldehyde in carbonate buffer (pH 9.49). The plate was incubated at laboratory temperature for 2 h. Each well was drained and its contents were replaced with 100 µL antigen solution (40 µg/mL). Incubation was conducted at 4 °C for 20 h. The plate was washed thrice with distilled water and 150 µL blocking solution per well was added. After 1 h incubation at 37 °C, 100 µL diluted pig or sheep serum (1:2,000) was dispensed into each well. Other 1h incubation and washing steps followed. Then 100 µL NH_4SCN chaotropic reagent solutions in 0.1 M sodium potassium phosphate buffer (pH 6.2) in an optimized concentration range (0.5 M, 1 M, 1.25 M, 1.5 M, 1.75 M, and 2 M) were successively dispensed into the wells using a multichannel pipette.

After incubation for 15 min at 37 °C, the well contents were drained and the plate was washed five times with distilled water. Then 100 µL of the selected and 1:8,000-diluted HRP-labeled secondary antibody was pipetted into each well and the plate was incubated for 1 h at 37 °C. The plate was washed five times and each well was filled with 100 µL substrate solution (5 mg *o*-phenylenediamine and 5 µL of 30% (v/v) H_2O_2 in 10 mL of 0.1 M phosphate buffer; pH 6.2). Incubation was conducted in darkness and at 37 °C for 15 min. The reaction was stopped by adding 50 µL of 1 M H_2SO_4 to each well. The absorbances of the well contents were measured on a microplate reader (LabSystems Multiskan RC; Thermo Fisher Scientific, Waltham, MA, USA) at 492 nm. The measured data were evaluated and graphically processed.

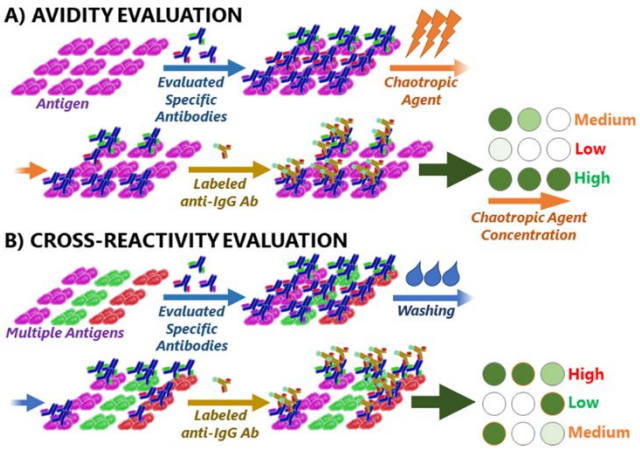

Fig. 2. Principle of avidity/affinity evaluation (A) and cross-reactivity (B) of primary Abs on µFlot device.

Affinity/avidity determination by µFlot device

A polyvinylidene difluoride (PVDF) blotting membrane was cut into a strip 45 x 50 mm long which was then treated with methanol for 1–3 min and placed inside the device (Fig. 1Af). The membrane was equilibrated with 10 mM phosphate equilibration buffer (pH 7.3) (EB). The target molecule and the positive and negative controls were dissolved in EB and applied by pipette to their appropriate positions (100 µL per dot). Vacuum A was applied (Fig. 1B), the reagents were affixed to the membrane surface, and the liquid passed into the retention chamber in the bottom plate (Fig. 1Ac). This procedure was heretofore identical to that reported by Svobodova et al. [31]. For

the blocking step, the reservoirs (Fig. 1B-1) were filled with blocking buffer (BB, 5% BSA in PBS-T). After 1 h incubation at RT, the BB was drained (Fig. 1B; vacuum B) and each reservoir was filled thrice with 1 mL washing buffer (WBu; PBS with 0.05% Tween 20). The vacuum pump was continually running and the liquid was constantly drained through the microchannels. Then 1 mL of each primary antibody dissolved in primary antibody buffer (PAB; 0.25% (v/v) BSA in PBS-T) was added to each reservoir and incubated for 1 h at RT. The primary antibodies were drained and the lid (Fig. 1Aa) was released and rotated by 90°. Each row in each reservoir was filled with 1 mL of each chaotropic reagent dilution in increasing order of concentration (range: 0.5–2 M). After 5 min incubation, the reagent was drained, and the lid was rotated 90° back to its initial position. Each reservoir was washed six times with 1 mL WBu and continual vacuum draining was applied. The horseradish peroxidase (HRP)-labeled secondary antibodies were then added to the reservoirs. After 1 h incubation at laboratory temperature, the membrane was washed six times with WBu, removed from the device, and dried on an absorbent tissue. Spots on the membrane were developed in the Opti-4CN substrate solution. After 5–15 min incubation, the membrane was washed in distilled water and photographed with a ChemiDoc™ XRS+ system (Bio-Rad Laboratories, Hercules, CA, USA). The image was processed with Image Lab™ software (Bio-Rad Laboratories, Hercules, CA, USA). Classic dot-blot can be performed on the µFlot device by omitting the chaotropic reagent and lid rotation steps.

Results and Discussion

There were three phases to the experiments conducted in this study. First, we developed the µFlot prototype and verified its accuracy and functionality. Next, we compared the measurements made by the µFlot against those obtained by conventional techniques such as dot-blot and ELISA. Then, we used the µFlot to test specific antibodies varying in quality and origin and furnished by different vendors. Our aim was to determine how effectively the µFlot could detect the most appropriate antibodies. We performed affinity evaluation on the soluble protein antigens ApoE and EpCAM and whole bacteria. We also assessed cross-reactivity with other bacterial species.

Testing µFlot device functionality

In the first test, we checked for any possible leakage between device components and/or spontaneous entry of the liquid from the wells to the drainage channels in the lid. To this end, we added methyl green or methyl orange to the lid reservoirs and wells. The solution did not enter the drainage channels until the vacuum pump was powered on. Thence, the colored solution either passed to the retention chamber (Fig. 1–B2, vacuum B) or drained off (Fig. 1–B3, vacuum B). We observed no leakage or spontaneous draining into the channels. Thus, the µFlot was ready for use in the subsequent experiments.

Antibody evaluation on µFlot device

The µFlot device can test antibody affinity/avidity. We used the µFlot to establish whether two interacting molecules can resist the effects of increasing the concentration of a chaotropic reagent (Fig. 2A). Antibodies with strong specific bonds presented with horizontal or slightly declining trend lines while those with weak bonds had steeply declining trend lines. Certain applications such as protein isolation and cell sorting require high affinity antibodies with strong, specific binding. In contrast, medium/low affinity antibodies are suitable for affinity purifications such as isolated target molecules that must be released into the buffer, and competitive enzyme immunoassays (EIA). Antibodies that did not interact with antigens had straight trend lines approaching zero and were unsuitable for any application. The results of the new batch could also be compared against those for the preceding one. We could also use the µFlot to determine the impact of long-term storage on antibody function. The ability of an antibody to recognize its target may change over time.

The µFlot can also evaluate cross-reactivity. We applied various antigens to a single line on the membrane and tested each antibody. We incubated the primary and secondary antibodies and conducted colorimetry and densitometry. We compared the intensities of the positive control dots with those for the nontarget molecules and expressed the differences in terms of percentage of positive control (Fig. 2B). We deemed antibodies with the most appropriate affinity and the lowest cross-reactivity for nontarget molecules as the most suitable for a particular application.

We then tested µFlot on a model system consisting of chymotrypsin and two polyclonal anti-chymotrypsin antibodies also subjected to conventional dot-blot. We applied a chymotrypsin concentration series of 0.005 µg/100 µL, 0.01 µg/100 µL, 0.025 µg/100 µL, 0.05 µg/100 µL, and 0.1 µg/100 µL on the membrane in quadruplicate. The concentration increased from left to right in rows 1 and 4 and decreased from left to right in rows 2 and 5 (Fig. S1). The wells in row 3 were filled with washing buffer and served as blanks. We used hyperimmune pig serum diluted to 1:2,000 as the anti-chymotrypsin IgG source. We diluted the corresponding HRP-labeled secondary antibodies to 1:8,000 with WBu and performed a classic dot-blot procedure on the µFlot. However, we omitted the chaotropic reagent step. We averaged the intensities of the four spots of the corresponding concentrations. The relative signal standard deviation (SD) was < 5% except for 0.025 µg/100 µL in which case it was 6%. We determined the working range with a model system at concentrations in the range of 1–50 ng chymotrypsin/100 µL. The plotted signal trend was a plateau starting at ~50 ng chymotrypsin/100 µL. Thus, 20 ng chymotrypsin/100 µL was determined to be optimal and was selected for use in the subsequent experiments.

Avidity evaluation by µFlot, commercial dot-blot, and avidity ELISA

We compared the performance of µFlot with those of conventional affinity dot-blot and avidity ELISA. We conducted the experiment using two polyclonal anti-chymotrypsin antibodies acquired from sheep and pig booster vaccinations. We compared the aforementioned antibodies for their abilities to recognize chymotrypsin deposited on the membrane and resist increasing concentrations of chaotropic reagent. We established that 0.02 µg chymotrypsin/100 µL sufficed for the µFlot and avidity dot-blot. For the ELISA, however, 4 µg chymotrypsin/100 µL was necessary as this method is less sensitive than the other two. We diluted the primary antibodies to 1:2,000 and the appropriate secondary antibodies (anti-sheep- and anti-pig antibody conjugated with HRP) to 1:8,000. We used 0.5–2 M ammonium thiocyanate as the chaotropic agent. The results of the avidity dot-blot and ELISA were consistent with those obtained from the µFlot device (Fig. 3A–3C). Pearson´s correlation coefficient was close to unity. The correlations between µFlot and dot-blot were 0.949 for pig antibody and 0.987 for sheep antibody. The

correlations between µFlot and ELISA were 0.982 for pig antibody and 0.950 for sheep antibody. Hence, both pairs of methods were positively linearly correlated.

The preceding experiments demonstrated that the µFlot device had several important advantages over the two conventional methods. Antigen application in µFlot was faster and easier than it was in ELISA. The performed ELISA test took 25h due to the necessary overnight incubation with the antigen (20h). This could be minimized to 2h if another type of ELISA plate is used for antigen coating. Still, µFlot does not require this step. In addition, compare to ELISA has higher sensitivity. Western blot requires electrophoresis of an antigen for approx. 1h and subsequent protein transfer can take 2h or more. Furthermore, western blot does not allow the chaotropic reagent step which is crucial in antibody comparison and affinity/avidity evaluation. On the other hand, western blot enables to load protein mixture or unpurified protein. Thus, time-demanding protein loading is in µFlot replaced by rapid application of the antigen directly on the membrane using vacuum albeit µFlot can process purified or recombinant proteins or peptides and whole bacteria, not a mixture of proteins. Further, the µFlot is far less labor-intensive and time-consuming than avidity dot-blot as the former does not require blotting membrane cutting, incubation of individual membrane pieces, or frequent manual buffer replacement. The microfluidic channels and reservoirs in the lid of the µFlot enabled simultaneous application, performance, removal of each reagent solution, and accelerated washing. The applied fluid can be quickly and reproducibly drained off by a vacuum pump. Moreover, for the final membrane image capture, no imaging system with original software is necessary. In fact, an ordinary camera and free software such as ImageJ suffice for dot-blot evaluation. Thus, the technique and requirements of this method are readily accessible and affordable.

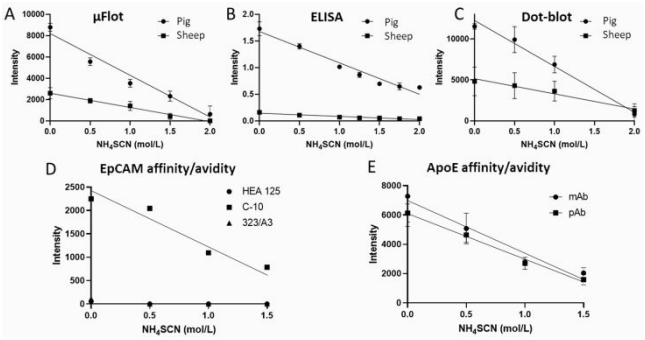

Fig. 3. Affinity/avidity evaluation of two anti-chymotrypsin antibodies by A) µFlot, B) ELISA, and C) dot-blot. Results for experiments repeated in quintuplicate. Affinity/avidity evaluation. D) anti-EpCAM antibodies. E) anti-ApoE antibodies. Results for experiments repeated in quadruplicate.

Testing of various antibodies and critical evaluation of results

To confirm the ability and robustness of the µFlot device, we used it to test various monoclonal or polyclonal antibodies varying in specificity and purchased from different vendors.

We endeavored to prove the stability of three different anti-EpCAM monoclonal antibodies stored long-term in a freezer at -20 °C. We subjected them to the µFlot procedure and compared them with corresponding freshly prepared aliquots. We monitored the differences in their ability to interact with the antigen. We analyzed the C10, 323/A3, and HEA 125 antibody clones using the standard µFlot protocol. The concentration of the recombinant EpCAM antigen was 10 µg per dot. The primary anti-EpCAM antibodies were diluted to 1:2,000 with WBu Ammonium thiocyanate solutions of 0.5 M, 1.0 M, and 1.5 M served as the chaotropic reagents. Densitometry revealed that only the C10 antibody spots produced specific signals in both experiments. Despite the medium-high initial affinities of the HEA 125 and 323/A3 antibodies, their aliquots under long-term storage lost all affinity for the EpCAM antigen (Fig. 3D).

To compare the performances of commercial polyclonal and monoclonal antibodies, we evaluated affinity/avidity for anti-ApoE antibodies. We applied 10 ng/100 µL ApoE antigen per dot on the membrane. We diluted primary mouse monoclonal and rabbit polyclonal anti-ApoE to 1:3,000 and incubated them with antigen exposed to 0.5 M, 1 M, and 1.5 M ammonium thiocyanate. The monoclonal and polyclonal antibodies presented with nearly identical affinity/avidity for the antigen, albeit the polyclonal antibodies had slightly higher avidity than the monoclonal antibodies (Fig. 3E). However, the former also demonstrated twice as much nonspecific blank adsorption as the latter (data not shown). Overall, then, monoclonal antibodies would be relatively more effective for this type of application.

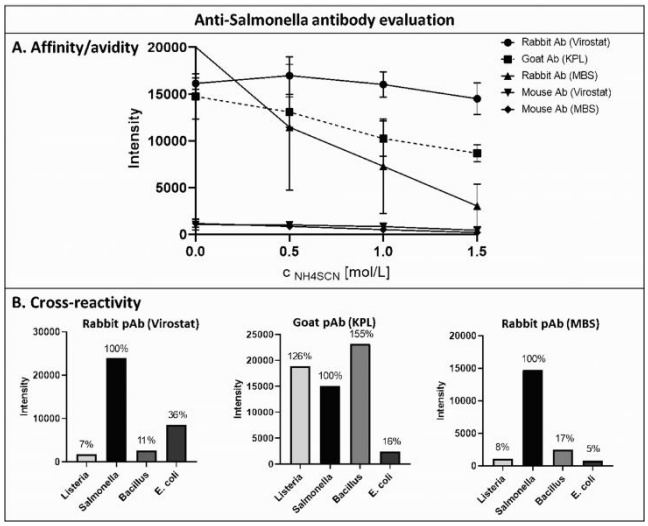

Fig. 4. Evaluation of anti-*Salmonella* antibodies. A) affinity/avidity (repeated thrice). B) cross-reactivity performed on µFlot.

µFlot testing of antibacterial antibodies and critical evaluation of the results

The preceding experiments showed that the µFlot device can test antibody reactivity regardless of antigen origin or structure. We compared the efficacies of antibacterial antibodies against whole bacteria deposited on the membrane (Fig. 2B). We conducted affinity/avidity and cross-reactivity evaluations on 24-h cell suspensions diluted with PBS to match McFarland turbidity standard No. 1.5 (~4.5 x 10^8 CFU/mL). We then immediately diluted this stock cell suspension to 1:10, 1:5, 1:1, and 2:1 with PBS and obtained a concentration series. The bacteria selected for these experiments were all pathogens known to occur in cow milk, namely, *Salmonella typhimurium* (five antibodies), *Escherichia coli* (two antibodies), and *Listeria monocytogenes* (three antibodies). *Bacillus cereus* served as the negative control. The primary antibodies were diluted 1:1,000 and the HRP-labeled secondary antibodies were diluted 1:5,000. The antibody evaluation procedure used was identical to that described in the previous subsection.

We tested the affinity/avidity of the five anti-*Salmonella* antibodies using *S. typhimurium* cell stock suspension diluted to 1:5. We exposed the immune complexes to 0 M, 0.5 M, 1 M, and 1.5 M ammonium thiocyanate. The results are shown in Fig. 4A. The avidity evaluation data for the polyclonal antibodies showed typical trend lines for both high- and low-affinity antibodies. In contrast, neither mouse monoclonal antibody recognized the targets on *Salmonella.* Hence, both were excluded from the subsequent experiments.

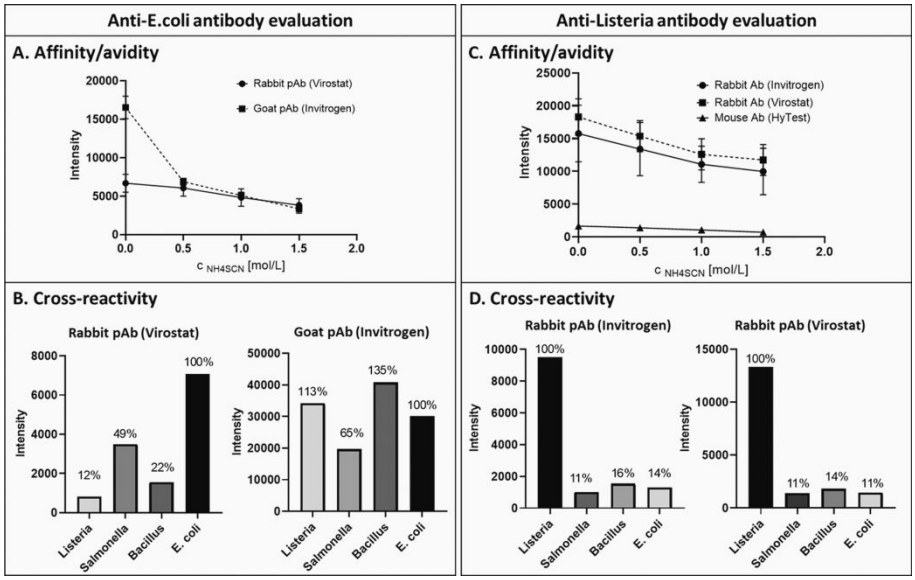

Fig. 5. Evaluation of affinity/avidity and cross-reactivity of two anti-E. coli antibodies (left) and three anti-Listeria antibodies (right) on µFlot.

We used the µFlot device to test the three different polyclonal antibodies for cross-reactivity with *S. typhimurium* as a positive control, and *L. monocytogenes*, *E. coli*, and *B. cereus* as nontarget antigens. All of them were derived from stock suspensions diluted to 1:5. Both rabbit polyclonal antibodies showed low to moderate cross-reactivity with *E. coli* (36%) (Fig. 4B). However, the goat antibody presented with severe cross-reactivity. The anti-*Salmonella* antibody more strongly interacted with the *Listeria* and *Bacillus* antigens than it did with the *Salmonella* antigen itself. We based the final evaluation on the intended application, namely, the selective isolation of whole bacteria. The results of the avidity comparison and cross-reactivity evaluation indicate that the polyclonal antibody with slightly worse cross-reactivity but higher avidity is the best choice. Strong avidity is required for this application. Any potential cross-reactivity with *E. coli* would be eliminated by a selective polymerase chain reaction (PCR). In contrast, applications such as immunoassays require antibodies with medium affinity, high specificity, and low cross-reactivity.

In the µFlot, we tested two polyclonal anti-*E. coli* antibodies produced in goat and rabbit for avidity and cross-reactivity. We diluted the bacterial culture stock suspensions to 1:1. The avidity comparison revealed that the goat polyclonal antibody had higher initial signal intensity. However, the signal substantially dropped with increasing chaotropic reagent concentration (Fig. 5A). On the other hand, the rabbit polyclonal antibodies had lower initial avidity but it did not markedly change with increasing chaotropic reagent concentration.

The cross-reactivity test (Fig. 5B) disclosed that the goat antibodies had higher cross-reactivity with all nontarget bacteria species than they did with the target *E. coli*. The rabbit antibody had dramatically lower nonspecific interaction with nontarget bacteria than the goat antibody. Hence, the rabbit polyclonal antibody is the preferred selection in this case. Nevertheless, other antibodies might be considered and tested as well.

We also tested two polyclonal and one monoclonal anti-*Listeria monocytogenes* antibodies for avidity and cross-reactivity on the µFlot. The monoclonal antibody did not interact with whole-cell antigen and was excluded from the subsequent experiments. Both polyclonal antibodies only weakly responded to changes in chaotropic reagent concentration. Thus, they had high avidity for whole-cell antigen (Fig. 5C). The cross-reactivity test on the µFlot revealed that both polyclonal antibodies had very low nonspecific adsorption (Fig. 5D). Thus, both polyclonal antibodies are suitable for applications requiring high avidity. Competitive pricing might be the selection criterion in this case.

Conclusions

The µFlot is a palm-sized device that researchers, laboratories, and institutions can use to evaluate the quality and assess the validity of antibodies and other affinity binding reagents. Our aim was to provide an easy and affordable tool for simultaneous antibody screening. The principle of the device is the predetermination of the ability of antibody candidates to recognize targets to be used in the intended application. The proviso is that the test conditions resemble those of the target application. In this manner, the optimal antibody for the application may be identified. The target can be a peptide, protein, or whole bacterial cell. Only a vacuum pump, camera, and densitometry software are required as peripheral equipment for the µFlot. The technique presented here is not intended to replace conventional methods such as ELISA, immunohistochemistry, or western blotting. However, the µFlot could semiquantitatively, rapidly, and economically

evaluate and compare antibody properties and its output would be simple to interpret. The most promising antibodies could then be thoroughly validated by more demanding and precise techniques such as surface plasmon resonance, isothermal colorimetric titration, fluorescence resonance energy transfer (FRET), IP-MALDI and other techniques mentioned in the introduction.

The μFlot device depicted here was prepared using micro-milling. This method is suitable for the preparation of the prototype but not for serial production. Therefore, the 3D printing is under our investigation to implement its easy and cost-effective development and distribution. We have machined the whole device using the CAD data on both the 3D printer (using ABS and PLA strings) and the CNC router. Both techniques offer good results with the CNC router allowing a wider selection of the device materials.

At present, much research is being directed towards elucidating the characteristics of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19). Scientists are attempting to establish the mode of transmission and mechanism by which the virus attaches to various organs, identify its molecular targets, and develop novel drugs with efficacy against it. In such research, techniques employing antibodies are utilized. However, Antibodies available on the market are not yet thoroughly validated and there are few published references at this time. A device such as the μFlot could help researchers rapidly select the most suitable antibody of the highest quality for use in this vital work. Therefore, our future intent will include the evaluation of several anti-SARS-CoV-2 antibodies for research use. Another goal would include exploring other possibilities of the device to be applied for nucleic acids and other bioreagents. We assume that its construction provides various opportunities for improvements according to the planned application.

The researchers definitely should validate and unequivocally specify the antibodies that are used in their research. Therefore, a practical tool that will help them to refine suitable antibody for their application.

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