Heliyon 6 (2020) e03270

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Autoantibody testing by enzyme-linked immunosorbent assay-a case in which the solid phase decides on success and failure

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ARTICLE INFO

Keywords:
Biochemistry
Coatings
Surface chemistry
Immunology
Proteins
Laboratory medicine
Clinical research
Enzyme-linked immunosorbent assay
Biomarker discovery
Reproducibility
Solid-phase
Autoantibody

ABSTRACT

Background: The enzyme-linked immunosorbent assay (ELISA) is an indispensable tool for clinical diagnostics to identify or differentiate diseases such as autoimmune illnesses, but also to monitor their progression or control the efficacy of drugs. One use case of ELISA is to differentiate between different states (e.g. healthy vs. diseased). Another goal is to quantitatively assess the biomarker in question, like autoantibodies. Thus, the ELISA technology is used for the discovery and verification of new autoantibodies, too. Of key interest, however, is the development of immunoassays for the sensitive and specific detection of such biomarkers at early disease stages. Therefore, users have to deal with many parameters, such as buffer systems or antigen-autoantibody interactions, to successfully establish an ELISA. Often, fine-tuning like testing of several blocking substances is performed to yield high signal-to-noise ratios.

Methods: We developed an ELISA to detect IgA and IgG autoantibodies against chitinase-3-like protein 1 (CHI3L1), a newly identified autoantigen in inflammatory bowel disease (IBD), in the serum of control and disease groups ($n = 23$, respectively). Microwell plates with different surface modifications (PolySorp and MaxiSorp coating) were tested to detect reproducibility problems.

Results: We found a significant impact of the surface properties of the microwell plates. IgA antibody reactivity was significantly lower, since it was in the range of background noise, when measured on MaxiSorp coated plates ($p < 0.0001$). The IgG antibody reactivity did not differ on the diverse plates, but the plate surface had a significant influence on the test result ($p = 0.0005$).

Conclusion: With this report, we want to draw readers' attention to the properties of solid phases and their effects on the detection of autoantibodies by ELISA. We want to sensitize the reader to the fact that the choice of the wrong plate can lead to a false negative test result, which in turn has serious consequences for the discovery of autoantibodies.

1. Introduction

Immunoassays were first reported in the 1960. This so-called radioimmunoassay (RIA) was used to determine the insulin concentration in human plasma [1]. As the RIA technique relies on the radioactive labelling of antigens or antibodies with iodine-131 and later iodine-125, and could therefore only be used in laboratories with special safety equipment, alternative detection methods were sought. In 1971, two groups developed independently and simultaneously immunoassays, which instead of a radioactive reporter label used an enzyme, marking the beginning of enzyme immunoassays (EIA) and enzyme-linked immunosorbent assays (ELISA) era. The first published ELISA employed alkaline phosphatase as reporter label for the quantitative measurement of IgG in rabbit serum [2]. Since then, the ELISA has been further developed and widely used in medical and research laboratories for the assessment of autoantibodies as well as for commercial applications. Furthermore, the ELISA technology was employed for the verification of candidate biomarkers to improve the diagnostic and control of various diseases as well as the monitoring of drug response [3, 4].

Our team is concerned with the identification of new serological markers for the diagnosis and differentiation of chronic inflammatory bowel diseases (IBD). In this context, we identified chitinase-3-like

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https://doi.org/10.1016/j.heliyon.2020.e03270
Received 10 October 2019; Received in revised form 12 December 2019; Accepted 16 January 2020

protein 1 (CHI3L1) as an autoantigenic target in patients with Crohn's disease (CD) [5]. Hence, ELISA was used to detect immunoglobulin G and A against CHI3L1 in serum of healthy controls and patients.

Although there is a large number of commercially available ELISA kits that can be used for the detection and quantification of antigens or antibodies, it is necessary to develop new ELISAs especially in the context of biomarker discovery and verification. This also applies to our case, because the only ELISAs available focus on the detection of CHI3L1 and not on the detection of antibodies against CHI3L1. Although the principle of the ELISA seems relatively simple, there are many possible sources of error described extensively in a variety of troubleshooting guides. However, these usually deal with the causes of unexpected test results. These include, for example, excessive or missing signals, high background signals, lack of interassay reproducibility, inconsistencies in plate adsorption or the plate edge effect (unexpectedly low or high signals in peripheral wells) [6]. Often, assay components such as autoantibody or antigen concentration, antigen-autoantibody interactions, buffer compositions, incubation times and temperatures or washing steps were discussed. However, these troubleshooting instructions rarely contain information on the characteristics of the solid phase, mostly 96-well plates, which can also have a significant influence on the success or failure of an ELISA development. Many manufacturers of ELISA test plates do point out that the plate surface properties have a significant impact. At this point our attention was aroused by an article published already in 1983 reporting the adsorption of proteins on plastic surfaces. The authors showed that bovine albumin adsorbed differently well to a variety of polystyrene microplates, whereas this effect could not be demonstrated for human immunoglobulin G [7].

With this report we would like to underline the importance of the solid phase for the proper immobilization of a novel autoantigenic target regarding the interaction with the corresponding autoantibody as biomarker in question. In this context, the appropriate selection of plates during assay development determined the signal-to-noise (S/N) ratios and thus the assay confidence. In general, low S/N ratios correlate negatively with the confidence in an assay [8]. Choosing the right microwell plate, keeping track of batch numbers of the plates and keeping “backup plates” should be mandatory for the development of ELISA tests. However, anecdotes of peers and our own experience gives a different scenario.

2. Material and methods

2.1. Antigen, microwell plates and serum samples

Based on results from two-dimensional electrophoresis (2DE), Western Blot and subsequent identification of proteins by Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry (MALDI-TOF-MS), candidate autoantigenic target for IBD, Chitinase-3-like protein 1 (CHI3L1) was identified, following recombinant expression and His-Tag affinity purification as described previously [5]. Purity of the antigen was validated by means of SDS PAGE and Western Blot using antibodies directed against either human CHI3L1 or 6 x His-Tag. 96 well plates were purchased from Thermo Scientific and display either a hydrophobic surface (Nunc LockWell breakable strips, C8 flat bottom, PolySorp Cat. Nr. 446442) or a hydrophilic surface (Nunc LockWell breakable strips, C8 flat bottom, MaxiSorp, Cat. Nr. 446469). Sera from 23 healthy controls (in.vent Diagnostica, Henningsdorf, Germany) and 23 CD patients (kindly provided by Dr. Maria Papp, University of Debrecen, Hungary) were used.

2.2. Enzyme-linked immunosorbent assay

For the detection of CHI3L1-specific antibodies in human serum, the ELISA was designed as follows: 96 well plates were coated overnight at 4 °C with 2 μg/mL CHI3L1 diluted in bicarbonate buffer (pH 9.5). After three washing steps (1.5 M NaCl, 25 mM M KCl, 3 mM M Tris Base, 47 mM Tris-HCl, 1 % Tween 20, pH 7.3, supplemented with formaldehyde-free biocidal preservatives) the plates were incubated for one hour with 2% bovine serum albumin (BSA) in phosphate-buffered saline solution (PBS, pH 7.4) to reduce the non-specific binding. The plates were then washed and incubated with serum samples (diluted 1:100 in 8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl, pH 7.6, supplemented with BSA and formaldehyde-free biocidal preservatives) for one hour. The plates were washed again and then incubated with horseradish peroxidase (HRP)- conjugated anti-human IgA or IgG (Dianova GmbH, Hamburg, Germany) diluted in 120 mM NaCl (supplemented with stabilizers) for one hour. After the last wash, the plates were incubated with a ready-to-use TMB substrate (Seramun Diagnostica GmbH, Heidesee, Germany). The color development was stopped after 10 min with 0.25 M sulfuric acid and the optical density (OD) was read at 450 nm with 620 nm background compensation in a microplate reader (Sunrise, Tecan Trading AG). All incubation steps were performed at room temperature without shaking the plates.

2.3. Statistics

Measured optical densities (OD) were background corrected and the Kruskal-Wallis Test was used to determine statistical difference of multiple groups whereas two-tailed, nonparametric Mann-Whitney U test was used to analyze significant differences between two groups. Bonferroni correction was used on the basis of a significance level of 0.05 to account for multiple comparison problems.

For bioinformatic analyses, the ProteinAnalysis() function of Biopython (v. 1.75) under Python 3.7 was used to calculate characteristics of the protein.

3. Results

3.1. Assay development

Since there is no ELISA available for the detection of antibodies against CHI3L1, we have developed a corresponding assay. The IUPAC average molecular mass weight of the protein sequence sp|P36222|CH3L1_HUMAN is 42.6 kDa, (389 amino acids) with an aromaticity according to Lobry, 1994 of 0.118. It has an theoretical isoelectric point of 8.69 and a charge of 4.56 at pH 7.3 and -10.3 at a pH of 9.5. The molar extinction coefficient assuming cysteines (reduced) is 67840 and 68215 with cysteines residues (Cys-Cys-bond). We followed common guidelines and investigated different components of the ELISA [9]. These included the blocking solution, the antigen concentration and the concentration of the HRP conjugated secondary antibody. Regarding the blocking solutions, the commercial solution Roti®-Block (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) appeared to be unsuitable for our assay. Compared to a solution of 2 % bovine serum albumin (BSA) or 5 % skimmed milk powder (SMP), Roti®-Block showed significantly higher OD values for healthy control and serum samples (Roti®-Block vs. BSA or SMP; $p < 0.0001$, respectively) (Figure 1 A). The differences between the healthy control and the serum samples, with the exception of healthy control and serum 1 with Roti®-Block ($p = 0.0011$), did not change due to the different blocking solutions (Table 1). Since very high OD values were measured with Roti®-Block, which indicates that it is not suitable as a blocking solution for our assay, we continued to work with 2 % BSA as the standard blocking solution.

Regarding the antigen concentration, decreasing OD values were observed with decreasing antigen concentration (Figure 1 B). Significant differences between healthy control and the two serum samples remained unaffected with the exception of healthy control vs. serum 1 at 0.5 μg/mL antigen concentration. When comparing the antigen concentration with the OD values of the individual sera, serum 2 showed significantly lower OD values at 1, 0.75 and 0.5 μg/mL compared to 2 μg/mL ($p = 0.0008$; $p = 0.0127$ and $p < 0.0001$, respectively) (Table 1). Therefore, we continued to work with higher antigen concentrations.

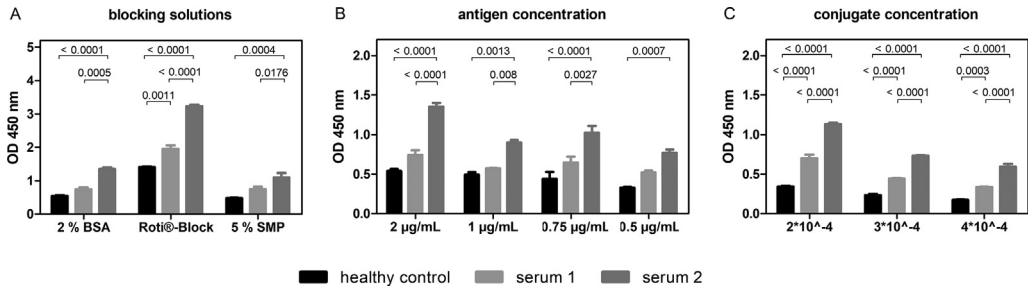

Figure 1. Relevant data for determination of ELISA conditions. One healthy control (serum) and two sera from Crohn's disease patients were measured in duplicate. A) Three different blocking solutions were used with the specified concentrations (Roti® -Block, according to manufacturer's specifications). B) ELISA plates were coated with the specified concentrations of antigen. C) 2 µg/mL antigen coated plates were used to evaluate different dilutions of the secondary antibody. Significant differences are given in the individual graphs. OD, optical density; BSA, bovine serum albumin; SMP, skimmed milk powder.

Table 1. Significance values and 95 % confidence interval of differences of varying ELISA conditions. The data show differences in OD values obtained from individual sera under different blocking solutions, antigen concentrations and secondary antibody concentrations (conjugate). BSA, bovine serum albumin; SMP, skimmed milk powder.

	healthy control		serum 1		serum 2	
	p	95 % CI of difference	p	95 % CI of difference	p	95 % CI of difference
Blocking						
2 % BSA vs. Roti® -Block	<0.0001	-1.157 to -0.5838	<0.0001	-1.495 to -0.9213	<0.0001	-2.169 to -1.596
2 % vs. SMP	>0.9999	-0.219 to 0.3543	>0.9999	-0.294 to 0.2793	0.0898	-0.035 to 0.5383
Roti® -Block vs. SMP	<0.0001	0.6515 to 1.225	<0.0001	0.914 to 1.487	<0.0001	1.847 to 2.421
Concentration						
2 g/mL vs. 1 g/mL	>0.9999	-0.2247 to 0.32	0.6537	-0.1012 to 0.4435	0.0008	0.1778 to 0.7225
2 g/mL vs. 0.75 g/mL	>0.9999	-0.1707 to 0.374	>0.9999	-0.1767 to 0.368	0.0127	0.05582 to 0.6005
2 g/mL vs. 0.5 g/mL	0.2233	-0.05885 to 0.4859	0.1819	-0.05085 to 0.4939	<0.0001	0.3117 to 0.8564
1 g/mL vs. 0.75 g/mL	>0.9999	-0.2183 to 0.3263	>0.9999	-0.3478 to 0.1968	>0.9999	-0.3943 to 0.1503
1 g/mL vs. 0.5 g/mL	0.7468	-0.1065 to 0.4382	>0.9999	-0.222 to 0.3227	>0.9999	-0.1385 to 0.4062
0.75 g/mL vs. 0.5 g/mL	>0.9999	-0.1605 to 0.3842	>0.9999	-0.1465 to 0.3982	0.0758	-0.01652 to 0.5282
Coating						
1:10000 vs. 1:20000	0.0137	0.01737 to 0.2043	<0.0001	0.1644 to 0.3513	<0.0001	0.305 to 0.492
1:10000 vs. 1:40000	0.0002	0.07721 to 0.2641	<0.0001	0.2692 to 0.4561	<0.0001	0.4425 to 0.6295
1:20000 vs. 1:40000	0.531	-0.03363 to 0.1533	0.0214	0.01137 to 0.1983	0.0019	0.04404 to 0.231

With decreasing concentration of the secondary antibody (conjugate), OD values also decreased (Figure 1 C). Significant differences between the healthy control and the two sera did not change. If we look at the sera individually and compare the different conjugate concentrations, we obtained significantly lower OD values for all sera (Table 1). We continued to work with the higher concentration.

3.2. Troubleshooting

After the assay was developed by our group (group A), it should be validated by our cooperation partner (group B) under the same conditions and the data should be reproduced. Although it appeared that the same assay components (BSA, buffer, TMB substrate, sample and conjugate dilutions) and conditions (number of wash steps, incubation time and temperature) were used, the assay was not reproducible by group B. Since the signals from group B were significantly lower, we first suspected the reaction components (TMB substrate, secondary antibodies). Next, we even checked whether it was a problem of the handling of the performers. This did not lead to restoration but rather to great skepticism about the entire ELISA concept. After a great deal of time, even the batch numbers of the used plates were compared. In the end, the plates of the same manufacturer were used, but they had a different surface coating.

Both groups used Nunc LockWell breakable strips, C8 flat bottom plates (Thermo Scientific), however, group A developed the assay on plates with a so-called PolySorp coating (hydrophobic) and group B repeated the assay on plates with a MaxiSorp coating (hydrophilic). Group A then directly compared the plate types with each other.

With the ELISA we aimed to detect different antibody subclasses, IgG and IgA, against a specific autoantigen in human serum. The results showed significant differences in reactivity, which appeared to be related to the surface coating of the 96 well plates used (Figure 2, Table 2). Regarding the IgA reactivity of the healthy controls and disease group, a significant difference of the measured OD values could be observed (Figure 2 B). When the assay was performed on Nunc Lockwell MaxiSorp plates, very low OD values were obtained, which are rather in the range of background noise. On the contrary, the same sera measured on Nunc Lockwell PolySorp plates showed significantly higher OD values. However, this difference does not affect the general assay assumption namely that IgA reactivity in patients is significantly higher than in healthy controls. However, there was a difference regarding OD values of the IgG reactivity (Figure 2 A). Although no significant differences could be shown between the plates, the type of surface coating influenced the assay result. While the sera were measured on Nunc Lockwell MaxiSorp plates, no difference in IgG reactivity between the control and disease groups could be demonstrated ($p = 0.0358$) by applying Bonferroni correction. Measurement of the same sera on Nunc Lockwell PolySorp plates revealed a significant difference between the control and disease groups. To find an explanation for this effect, which could possibly be due to the interaction of serum antibodies with the plate surface [10], the assay was repeated on Nunc Lockwell PolySorp plates with half of the sera showing the highest OD values. Part of the plate was coated with antigen, while the other part was incubated with carbonate buffer only. OD values measured on the uncoated part were subtracted from the values measured in coated wells (Figure 3, Table 1). Considering the

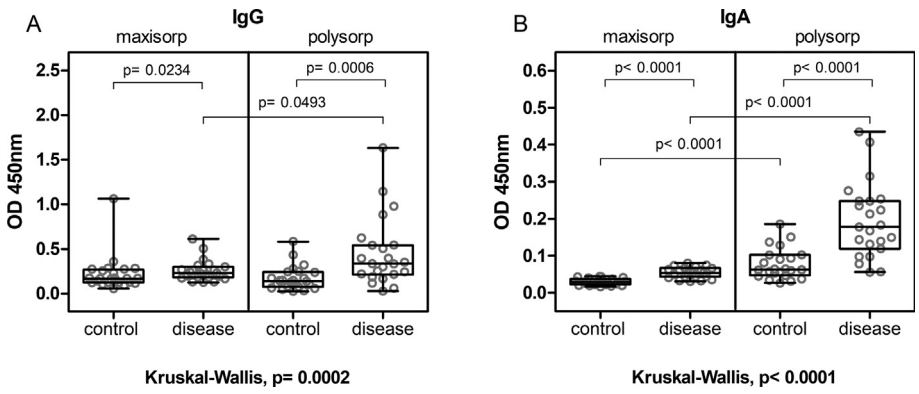

Figure 2. OD values of IgG and IgA reactivity from control (n = 23) and disease group (n = 23) obtained on different microwell plates. A) IgG reactivity to the coated antigen. Significant differences between four groups was calculated by Kruskal- Wallis Test. Mann-Whitney U test revealed significant difference of control and disease group, when measured on Nunc Lockwell PolySorp plates. B) IgA reactivity to the coated antigen. Kruskal-Wallis Test revealed significant differences between all groups. Mann-Whitney U test showed that there is a significant difference between control and disease groups on both, Nunc Lockwell MaxiSorp and PolySorp plates. Significance was also observed when comparing the control or disease groups on different plate types. Box plot represents median OD values and whiskers the minimum and maximum values. OD, optical density; IgG and IgA, immunoglobulin G and A.

control group, there was no significant difference (p = 0.0754). The same result was observed for the disease group (p = 0.321). Furthermore, the significant difference between the control and disease groups was maintained with and without this background correction.

4. Discussion

In summary, our data show that the development of new ELISAs for biomarker research should take into account the type of plates used. It may seem obvious that the plates are an important part of the ELISA, but sometimes it is a small detail, such as the surface coating, that can lead to large delays in assay development.

In general, antibodies or antigens can bind to the solid phase by passive adsorption and by hydrophobic interactions [9]. A bioinformatical analysis via Kyte-Doolitte hydropathy plot (Expasy Protscale, UniProtKB accession number P36222) (Figure 4) [11] suggested that CHI3L1 is more hydrophilic, but better ELISA results were obtained with the hydrophobic surface modification. However, different studies have shown that surface hydrophobicity or hydrophilicity has an influence on protein adsorption. It was shown that hydrophilic Pluronic-coated surfaces attenuate the adsorption of plasma proteins such as globulin, fibrinogen or albumin [12], which was confirmed by a second study [13] and that better adsorption of fibrinogen on hydrophobic polymers does not imply better interaction of fibrinogen with its binding partner (in this case platelet adhesion). Another study demonstrated that albumin and fibrinogen adhere similarly to hydrophobic and hydrophilic surfaces, although they have a less organized secondary structure when adsorbed to hydrophobic surfaces [14]. Therefore, we concluded that the nature of the surface has an influence on the conformation of the protein. In the case of CHI3L1, this may mean that the antigen adsorbs similarly to both used surfaces, resulting in conformational structural changes. This could make CHI3L1, which adsorbs to the hydrophobic surface, more accessible to antibodies, and in our case IgA in particular. In addition, CHI3L1 has two globular structures and it was shown that globular proteins have better adsorption kinetics and higher amounts of irreversibly bound protein on hydrophobic surfaces [15, 16]. However, it was not our goal to investigate the binding properties of CHI3L1, but to draw attention to the possible problems of the appropriate plate.

We are convinced that our case is representative at least for autoantibody test development since this is a common working scheme in many laboratories. In particular IBD-specific autoantibodies seem to interact mainly with conformational epitopes requiring special attention for their presentation by the corresponding absorbed targets [17]. In theory, once established in a laboratory, the assays should be ready to be applied in other laboratories as in our case. Science is facing a reproducibility crisis, and a plethora of survey studies (2/3) uncovered that the results in their respective field were irreproducible [18, 19, 20]. Seemingly simple but overseen information might render a whole assay non-functional as in our case. Unlike others (e.g. [21]), we were able to eliminate the source of error. Our intention was to illustrate which effect the selection of the plate surface may have on the performance of an autoantibody ELISA. In this context, we emphasize that the consequences of such an effect for the discovery of new biomarkers and reproducibility can be enormous. For example, the choice of the wrong plate could lead to potential new biomarkers being evaluated as negative because the measured signals or OD values are too low and thus in the range of background noise. However,

Table 2. Median OD obtain from 11 healthy control and 11 disease group sera before and after background correction from uncoated plates. OD, optical density; CI, Confidence Interval; IgG and IgA, immunoglobulin G and A.

		Nunc Lockwell MaxiSorp		Nunc Lockwell PolySorp	
	n	Median	95 % CI	Median	95 % CI
control IgA	23	0.0295	0.0225 to 0.0325	0.0625	0.0535 to 0.0935
disease IgA	23	0.0530	0.0440 to 0.0630	0.1780	0.1315 to 0.2475
control IgG	23	0.1680	0.1265 to 0.2545	0.1420	0.0790 to 0.2355
disease IgG	23	0.2300	0.1803 to 0.2655	0.3350	0.2195 to 0.5405
measured OD control	11			0.3345	0.2839 to 0.4275
measured OD disease	11			0.8915	0.6110 to 1.006
corrected OD control	11			0.2680	0.2218 to 0.3452
corrected OD disease	11			0.7400	0.5174 to 0.9060

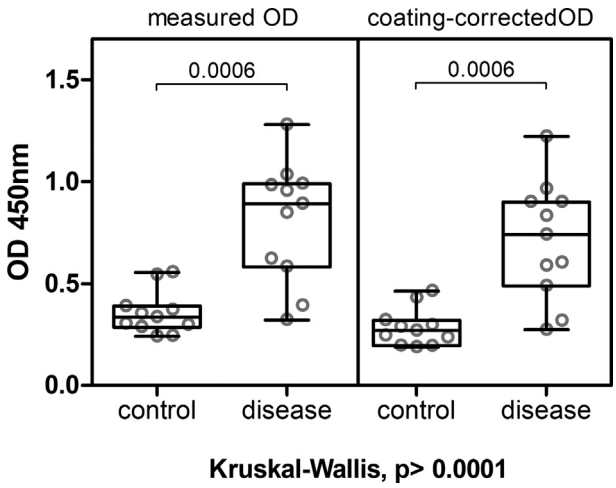

Figure 3. Comparison of measured OD values in control (n = 13) and disease (n = 13) group, with and without background correction. Data marked with "measured OD" represent OD values measured on coated plates. "Corrected OD" represents data of the same sera obtained after subtraction of OD values measured on uncoated plates. OD, optical density.

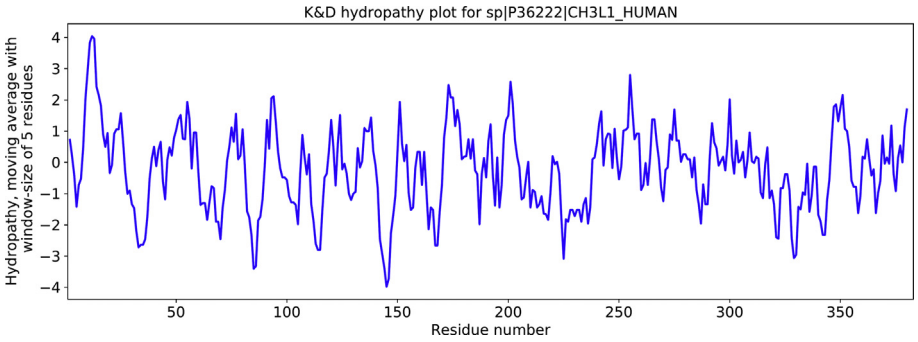

Figure 4. Kyte-Doolittle-Hydropathy Plot for Human CHI3L1 (UniProtKB accession number P36222). The plot was created based on the parameters, described by Kyte and Doolittle [11], with a customized script for Python 3.7. A moving average (window size 5) was used to smooth the residue data by averaging each residue value for residue with its 4 nearest neighbours.

similar reports show that intensive washing or insufficient antigen coating of the plate can also lead to such false negative results [22, 23]. Except for validation guidelines, we found no international standard for the development of ELISA techniques in biomarker discovery. Checklists, quality assurance (QA) and comprehensive description of the research work might help to improve the reproducibility [21, 24, 25]. Perhaps this report can serve to stimulate a discussion on whether such a guideline should be established, similar to the MIAPE (Minimum Information About a Proteomics Experiment) guidelines [26]. For other array technologies, such as microarrays, there are plenty of guidelines for the evaluation of the results. These include records of the probe preparation, array preparation and information about the signal intensities [27, 28, 29, 30].

5. Conclusion

In conclusion, it can be stated that the selection of the right solid phase material can have an enormous influence on the development of ELISA assays. Especially in the context of autoantibody testing, it is important that as much information as possible is available regarding the nature of the antigen, assay construction and solid phase material to avoid a time delay in assay development. Possibly internationally standardized guidelines could help to improve reproducibility.

Declarations

Author contribution statement

C. Deutschmann: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

D. Roggenbuck and S. Rödiger: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

P. Schierack: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was supported by the German Federal Ministry of Education and Research (BMBF- Wachstumskern-PRAEMED.BIO, grant number: 03WKDB2C).

Competing interest statement

The authors declare the following conflict of interests: D. Roggenbuck has a management role and is a shareholder of GA Generic Assays GmbH.

Additional information

TBC No additional information is available for this paper.

Acknowledgements

We would like to thank Dr. Maria Papp (Department of Internal Medicine, Division of Gastroenterology, Faculty of Medicine, University of Debrecen) for providing the patient sera.

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