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Technical Note

Reducing the risk of misdiagnosis of indirect ELISA by normalizing serum-specific background noise: The example of detecting anti-FGFR3 autoantibodies

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ABSTRACT

Indirect enzyme-linked immunosorbent assay (ELISA) is an important diagnostic method as it enables the quantification of the presence of autoantibodies in human blood sera. However, unspecific binding of antibodies to the solid phase causes considerable serum-specific background noise (SSBN), involving the risk of false positive diagnosis. Therefore, we present a simple and concise, yet obvious proof-of-principle of a recently suggested normalization method. The method is based on subtracting SSBN by using non-coated ELISA wells as a control for each serum-of-interest. We performed ELISA to quantify anti-fibroblast growth factor receptor 3 (FGFR3) antibody levels in three positive controls (two anti-FGFR3-positive patients and a rabbit antiserum against FGFR3) and 58 negative controls (healthy blood donors). In all subjects, we found considerable unspecific reactivity which strongly varied among subjects. The conventional normalization method was not able to balance this strong SSBN, as demonstrated by 2/58 false positive healthy controls and one FGFR3-positive patient that was hidden in the noise (false negative). SSBN normalization reduced the frequency of false-positives to 0/58. Further, all three anti-FGFR3-positive sera were successfully detected and even doubled their z-score used to determine positivity. Albeit occupying more space on the ELISA plate, we strongly recommend considering this normalization method when working with blood sera. To better put the idea across to the community, we depict the SSBN issue and its solution in a graphic scheme. We conclude that SSBN normalization increases the sensitivity and specificity of indirect ELISA and thereby reduces the risk of false positive and false negative diagnosis.

1. Introduction

Indirect enzyme-linked immunosorbent assay (ELISA) enables the quantification of the presence of certain autoantibodies in human blood sera and is therefore an important diagnostic method. However, unspecific IgG binding to the solid phase (e.g., the polystyrene), causes considerable variation among different human sera, referred to as serum-specific background noise (SSBN). SSBN sometimes even exceeds the true antibody-antigen reactions and hence leads to the risk of false positive diagnosis (Güven et al., 2014; Terato et al., 2016; Elshafie et al., 2016; Kenna et al., 1985). SSBN can be caused by heterophile antibodies, anti-animal antibodies, or polystyrene-affine antibodies, combined with the inadequate blocking of polystyrene or blocking with IgG-reactive proteins (summarized in (Güven et al., 2014)). In numbers, depending on the assay conditions and serum properties, 4–32% of the sera showed non-specific binding (Güven et al., 2014). This can cause erroneous interpretations of diagnostical data. Some authors even discuss “numerous uncertain conclusions” and “misuse” in this context

Abbreviations: ELISA, enzyme-linked immunosorbent assay; SSBN, serum-specific background noise; FGFR3, fibroblast growth factor receptor 3; BSA, bovine serum albumin; PBS, phosphate-buffered saline; OD, optical density

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(Terato et al., 2016; Waritani et al., 2017).

Although unspecific background noise can be reduced through the use of detergents such as Tween-20 (Ravindranath et al., 1994), none of the conventional ELISA buffers are capable of blocking unspecific antibody binding (Terato et al., 2014). Hence, there is a need for improved buffers (Terato et al., 2014) or study designs in order to avoid those issues. In this study, we address the latter aspect: the study design.

Notably, an improved study design has been suggested predominantly in articles published in the Journal of Immunological Methods (Elshafie et al., 2016; Terato et al., 2014). In detail, these articles recommend quantifying SSBN by using non-coated, but blocked wells incubated with each sera-of-interest. The exact loading pattern was illustrated by Waritani et al. (Waritani et al., 2017). As correctly stated by these authors, appropriate data normalization based on SSBN is unfortunately often skipped, however (Waritani et al., 2017). Thus, we infer that the ELISA community has either not yet been reached or inadequately convinced.

In a recent article, Haberland et al. (2018) nicely summarized the ongoing discussion and speculate whether calculating the difference between coated and non-coated wells (as already used in some studies (Trier et al., 2016, 2018a,b)) would resolve or increase the issue. They kindly invited the research community to contribute with ideas and wet lab experiments. We gladly accept this invitation. Therefore, the aim of this study is to present a simple and concise, yet obvious proof-of-principle of subtractive normalization using the SSBN. We fulfill this aim by showing the power of background subtraction for indirect ELISA with two sensory neuropathy patients known to be positive for anti-fibroblast growth factor receptor 3 (FGFR3) antibodies (Antoine et al., 2015) and 58 healthy controls. Further, we illustrate the issue and its solution by a summary diagram.

2. Material and methods

2.1. Patients and samples

The sera of patients were obtained from a multicenter prospective study led by neurologists at the University Hospital of Saint-Etienne on the clinical and electrophysiological characterization of patients with sensory neuropathy associated with anti-FGFR3 antibodies. FGFR3 antibodies have recently been described as a useful biomarker of a subset of inflammatory neuropathies affecting the dorsal root ganglia (Antoine et al., 2015). The study was approved by the ethical committee at the University Hospital of Saint-Etienne (reference number CPP 2014–27) and all participants gave written consent for the study and for the use of their serum sample as well as clinical and electrophysiological data. To be included, patients had to be ≥18 years old and have sensory neuronopathy, small fiber neuropathy, pure sensory chronic inflammatory demyelinating polyneuropathy or length-dependent neuropathy. Blood sampling was performed by trained clinical staff in each center. Sera were obtained by coagulation and centrifugation of blood samples for 15 min at 1000*g* at room temperature and then aliquoted, frozen and sent to our laboratory at −20 °C. On arrival, the sera were frozen at −80 °C before analysis.

All control sera were collected from healthy donors as part of the blood donation organized by the French Blood Establishment. These sera were prepared and stored in the same way as the patient sera.

2.2. Indirect ELISA

Detection of FGFR3 antibodies was performed by a standard indirect ELISA test. Maxisorb 96-well plates (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were coated overnight at 4 °C with 100 µL of 5 µg/mL of the human recombinant intracellular domain of FGFR3 (Invitrogen, Carlsbad, California, USA) in coating buffer of carbonate/bicarbonate 0.05 M, pH 9.6 (Sigma-Aldrich, Saint-Louis, Missouri, USA). To quantify the SSBN, a non-coated well was prepared for each sera-of-interest by incubating 100 µL of coating buffer without the FGFR3 protein. After one wash, all wells were blocked with 300 µL of 0.06% Tween-20, 0.1% fish gelatin and 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (Sigma-Aldrich) for 2 h at room temperature. The plates were washed four times (0.1% Tween-20 in PBS) and incubated overnight at 4 °C with 100 µL of sera samples (1:30 in blocking buffer). For each serum, three wells were incubated: two wells coated with the human intracellular domain of FGFR3 and one without the coating of the protein (non-coated well). A serum-free blank control was also used in each plate by incubating blocked wells only with the secondary antibody. As a non-human positive control, we used 1:3000 (i.e., 0.33 µg/L) rabbit-anti-FGFR3 antibody (polyclonal IgG, GeneTex, Irvine, USA). The plates were washed seven times and incubated for 2 h at 4 °C with 100 µL of the appropriate peroxidase-conjugated secondary antibody solutions: 1:3000 (i.e., 0.43 µg/L) of rabbit-anti-human IgG (Dako) in the blocking buffer for human sera samples and 1:3000 (i.e., 0.43 µg/L) of swine-anti-rabbit Ig (Dako) in the blocking buffer for the rabbit-anti-FGFR3 control antibody. The plates were then washed ten times and were incubated for 30 min with 100 µL of 0.4 mg/mL of *O*-phenylenediamine dihydrochloride in phosphate/citrate buffer (0.05 mM, pH 5, Sigma-Aldrich). Optical density (OD) was measured at 450 nm with Multiscan EX Elisa Reader (Thermo Fisher Scientific).

To ensure the reliability of the results, the OD inter-assay (or inter-plates) variability was evaluated using three samples in each plate (two sera from healthy donors and the positive control with anti-FGFR3 antibody) and acceptability limits were defined as the OD mean ± 2 standard deviations. Thus, each ELISA plate was interpreted only if ODs of controls were within acceptable limits. In all experiments, the inter-assay coefficient of variation of OD was < 10% for all three controls (8.1%, 9.3% for healthy donor sera and 7% for anti-FGFR3 antibodies). Data normalization based on SSBN was realized for each serum: the difference between the OD of the FGFR3-coated well and that of of the non-coated well was calculated and the test was considered positive when the difference was 3 SDs above the average healthy control ($n = 58$) signal (z-score > 3, corresponding difference on OD scale > 0.200). To test the impact of data normalization, using the same criteria, we also defined thresholds for the OD of unnormalized coated wells (z > 3, = OD > 0.658) and for conventionally normalized wells, i.e., the difference between the OD of the FGFR3-coated well and the mean of the blank controls of the same plate (z > 3, = OD > 0.584).

3. Results

For indirect ELISA, we coated FGFR3 onto ELISA plates and incubated 1:30 dilutions of human sera. We recorded the reactivity of 58 negative controls (healthy blood controls) and three positive controls (two anti-FGFR3-positive patients (Antoine et al., 2015) and the rabbit antiserum against FGFR3). Without normalization (Fig. 1A) and with the conventional normalization method (subtracting a serum-free blank; Fig. 1B), we identified 2/58 (5.2%) false positive healthy controls, while one anti-FGFR3-positive patient was false negative. The rabbit antiserum against FGFR3 and the other patients were positive.

The non-coated control wells (Fig. 1C) nicely illustrate the source of the problem: even without antigen coating, sera bound the wells in a strongly inter-individually varying way. As the antigen was absent, reactivity must have resulted from unspecific antibody binding. The surprisingly similar signal intensities and patterns of antigen-coated versus non-coated wells suggest that most of the signal in antigen-coated wells arose from unspecific binding. This finding shows that a normalization of the SSBN is necessary.

As we quantified the SSBN for each of our samples via non-coated wells (Fig. 1C), we were able to subtract it from the corresponding values of the coated wells (Fig. 1D). The resulting difference represents the antigen-specific reactivity of each serum, which showed 0/58 false

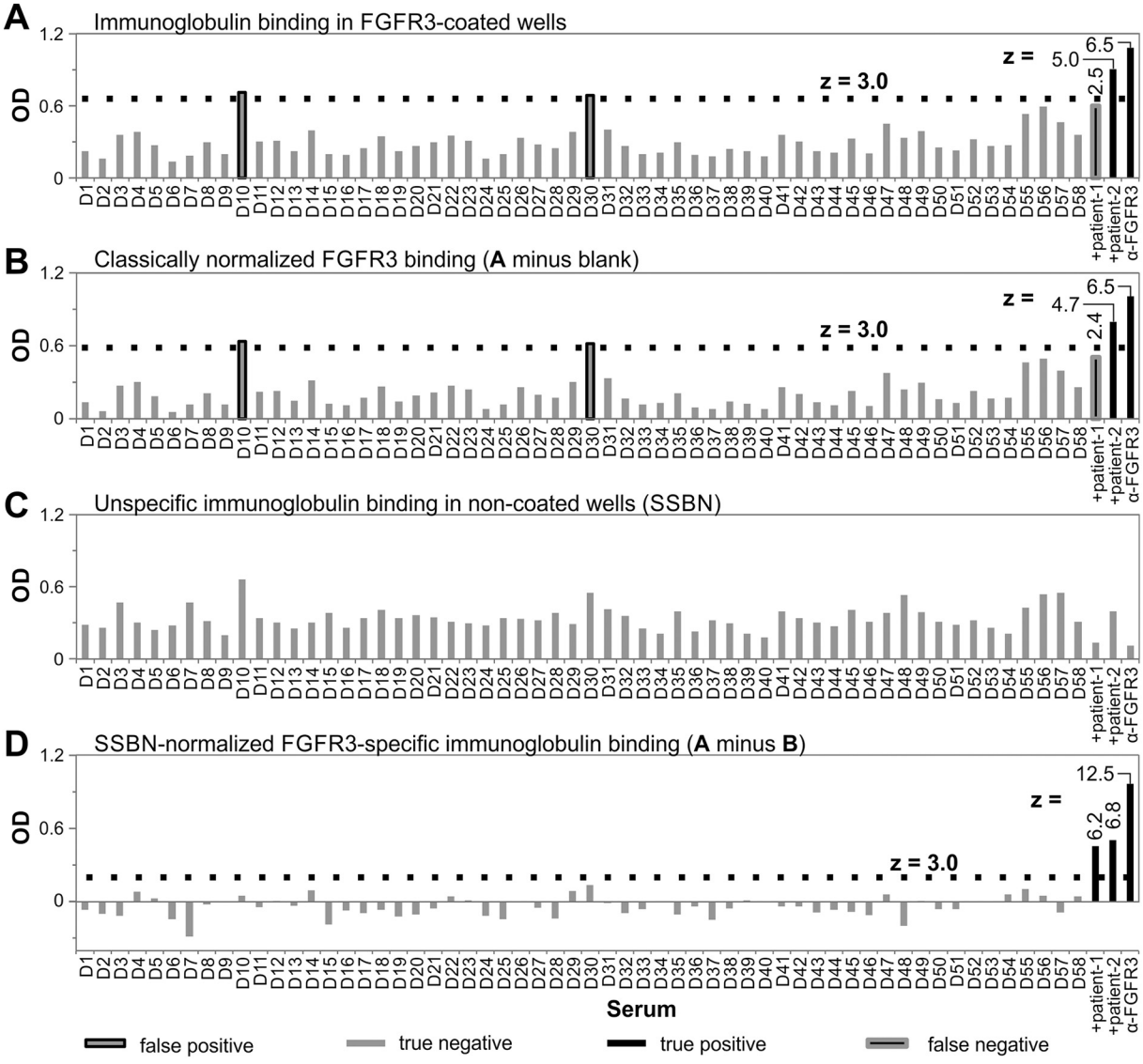

Fig. 1. The serum-specific background noise and its handling. A: Optical densities of healthy controls (D1 - D58), two anti-FGFR3-positive patients (+patient-1 and -2), and rabbit anti-FGFR3 serum (α-FGFR3) in FGFR3-coated ELISA wells. B: Difference between values in A and a corresponding serum-free blank. C: Optical densities of the same sera used in A, but in non-coated, blocked ELISA wells. D: Difference between corresponding values in A and C, representing FGFR3-specific reactivity. A z-score (z) ≥3 above the healthy donors' mean value defines positivity (dotted line). The legend allocates result categories (true/false positive/negative) to panels A, B, and D.

positives, while both FGFR3-positive patients and the rabbit antiserum were true positives. The SSBN-normalized reactivity of the two positive sera, indicated by the z-score, was 1.4–2.6 times higher than that of the conventional normalization approach (6.2 versus 2.4 for patient-1, 6.8 versus 4.7 for patient-2, and 12.5 versus 6.5 for rabbit-anti-FGFR3; Fig. 1D).

By having estimates for both unspecific and specific binding of human serum antigens (Fig. 1C versus D), we were able to compare them directly. Interestingly, all healthy controls (58/58) exhibited stronger unspecific binding (Fig. 1C) than specific binding (Fig. 1D). Only the anti-FGFR3-positive patients had a stronger specific than unspecific reactivity.

We repeated the experiment, with serum dilutions of 1:50, and obtained the same number of false positives and false negatives as well as the same level of improvement when using SSBN normalization (data not shown).

4. Discussion

Recently, Haberland et al. posed the question as to whether it might be helpful to calculate the difference between the ODs obtained by coated wells (i.e., the sum of specific and unspecific reactivity) and the OD obtained by non-coated wells (i.e., the unspecific reactivity) (Haberland et al., 2018). Our research shows – at least within the conditions of our experiments – that calculating this difference strongly improved our outcomes.

Our simple approach of applying 3 positive controls and 58 negative controls shows the power of quantifying and subtracting serum-specific background reactivity in ELISA approaches. While the conventional normalization approach resulted in 2/58 false positive diagnosis and one missed positive patient, the serum-specific data normalization detected all three positive patients and found 0/58 false positives. By using SSBN normalization, the ELISA approach became (1) more sensitive by detecting more true positives and (2) more specific by detecting less false positives.

Fig. 1C intriguingly demonstrates strong variation among healthy

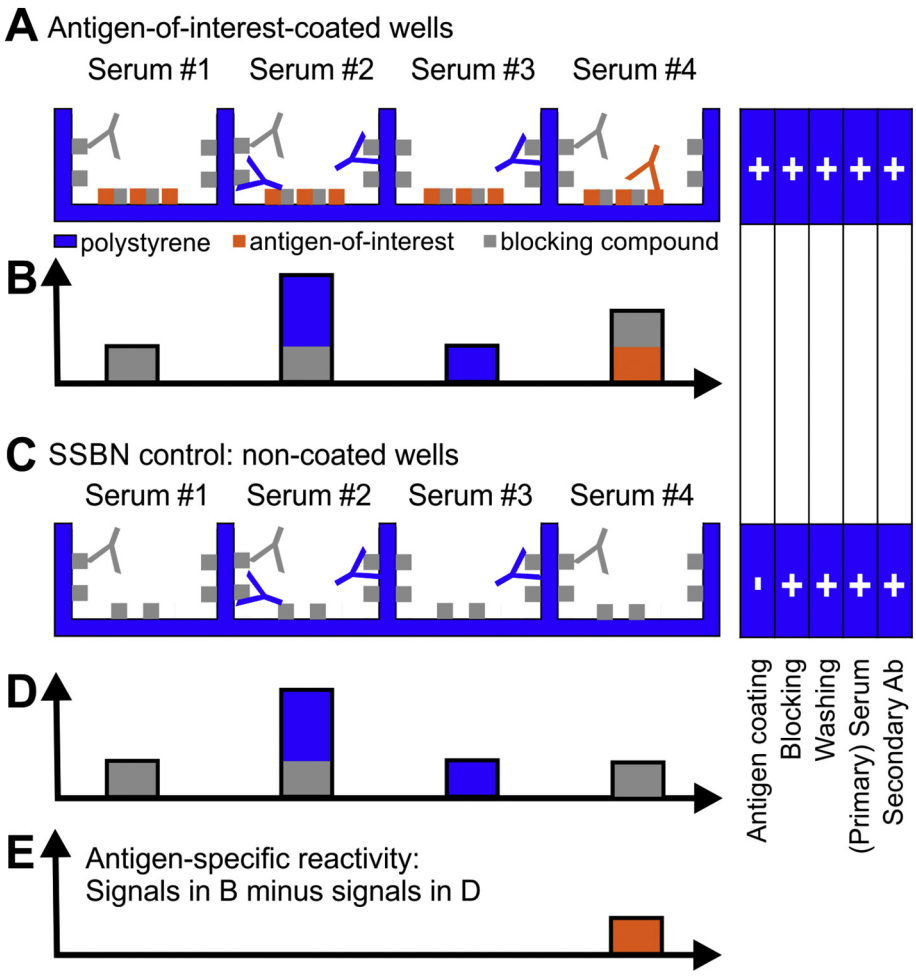

Fig. 2. Schematic demonstration of the benefit of SSBN normalization. A: Four different sera are tested in four antigen-coated and blocked wells. Antibodies (Y, color-coded) are binding their targets. B: Signal quantification based on bound antibodies in A. C: SSBN control: the same four sera as those in A are tested in non-coated wells, but ones which otherwise are treated the same (cf. table on the right). D: Signal quantification based on bound antibodies in C. E: Antigen-specific reactivity.

controls resulting in exceptionally dominant unspecific reactivity that would have been misinterpreted as positivity while using inappropriate normalization methods. The situation is schematically shown in Fig. 2. The false-positive reactivity results from the unspecific binding of immunoglobulins to components of the blocking solution or the plastic wells (Fig. 2C). By quantifying the unspecific reactivity of each well using non-coated control wells (e.g. Figs. 1C and 2C, D) and by subtracting this from the total reactivity (Figs. 1A and 2A, B), the *specific* reactivity against the protein of interest (here: FGFR3) can be arithmetically extracted (Figs. 1D and 2E).

Surprisingly, 100% of the tested healthy control sera showed a significant unspecific reactivity, which strongly varied among subjects, with two cases (5.2%) which would have been diagnosed as positive. It has to be noted that we chose BSA as one of the blocking compounds, which can generate false positive reactivity (Terato et al., 2014). Notably, our proof-of-principle shows that SSBN normalization is able to improve ELISA outcomes even though we have not used optimal blocking conditions.

For a false positive estimation, we used healthy controls instead of anti-FGFR3-negative neuropathy patients in order to avoid potential bias resulting from the misdiagnosis of patients, which can never be completely excluded. By using healthy controls instead of disease controls, it is possible that we may have obtained only a conservative estimate for the false positive rate, as the frequency of patients with high unspecific binding may be lower in healthy controls compared to patients (Waritani et al., 2017), albeit this has not been demonstrated for neuropathies. To highlight this last point, we estimated the false positives and false negatives in a parallel study using a large cohort of neuropathy patients (Tholance et al., in preparation). Here, 13 out of 366 patients (3.5%) patients would have been classified as positive using the conventional approach, but negative using the SSBN normalization (false positive). In contrast, only 40 out of 65 anti-FGFR3-positive patients would have been positive with conventional normalization (25/65, 38.5% of false negatives). This supports our view that SSBN normalization improves specificity and sensitivity of the test.

Besides SSBN normalization, several other strategies have been suggested in order to handle the unspecific binding of sera IgG, such as blocking with casein (Kenna et al., 1985), blocking with commercialized compounds (Waritani et al., 2017), washing with adapted concentrations of Tween-20 (Ravindranath et al., 1994), and changing the BSA preparation (Xiao and Isaacs, 2012). Nevertheless, as those strategies are probably never 100% efficient, we recommend using SSBN normalization in addition to adapting blocking or washing buffers.

For other methods, such as Western blotting, normalization methods are improving as well (Moritz, 2017). As is common in method development, the pros and cons of new applications must be balanced. In the case of SSBN normalization, it must be noted that there is an inevitable, inherent disadvantage to the method, as can be seen in Fig. 2. In contrast to the conventional ELISA normalization, where a duplicate or triplicate of serum-free control wells is sufficient, SSBN normalization by definition requires a control well for each tested serum. Despite occupying more space of the ELISA plate by these

means, we are convinced that reducing the risk of misdiagnosis (both false negatives and false positives) outbalances these disadvantages.

5. Conclusion

Here we present a simple and concise, yet obvious proof-of-principle of data normalization based on SSBN in indirect ELISA. Although employing this approach means occupying more space on the ELISA plate, we strongly recommend considering this normalization method when working with blood sera, as it increases the specificity and sensitivity of indirect ELISA and thereby reduces the risk of false positive and false negative misdiagnosis.

Declaration of interest

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