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Step-by-step full factorial design to optimize a quantitative sandwich ELISA

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ABSTRACT

In this work, a quantitative sandwich ELISA was optimized, through a full factorial design of experiments (DOE) in successive steps of a preliminary protocol obtained by the method of one factor at a time (OFAT). The specificity of the optimized ELISA, the lower limit of quantification, the quantification range and the analytical sensitivity of the antigen quantification curve were evaluated, in comparison with the curve obtained from the preliminary protocol. The full factorial DOE was linked to a simple statistical processing, which facilitates the interpretation of the results in those laboratories where there is no trained statistician. The step-by-step optimization of the ELISA and the successive incorporation into the protocol of the best combination of factors and levels, allowed obtaining a specific immunoassay, with an analytical sensitivity 20 times greater and with a lower limit of antigen quantification that decreased from 156.25 at 9.766 ng/mL. As far as we know, there are no reports of optimization of an ELISA following the step-by-step scheme used in this work. The optimized ELISA will be used for the quantification of the TT-P0 protein, the active principle of a vaccine candidate against sea lice.

1. Introduction

Although enzyme-linked immunosorbent assay (ELISA) is a potentially robust and sensitive ligand binding assay, its development and optimization can be challenging. Since the method involves the assembly of a large immune complex with several components, the assay signal can be affected by many factors. Several parameters must be considered during the optimization process, such as the initial immobilization of the biomolecule on the solid surface, the buffers used in the assay, the incubation time and temperature, depending on the detection system used [1]. Optimization is intended to increase the sensitivity and the robustness of the ELISA [2,3].

Identification of key trial variables has long been done following the one factor at a time (OFAT) method. In this model, the optimization of many variables or factors that condition the assay is done by studying each factor independently, keeping all the others constant, which often leads to incomplete results because it does not explore or determine important interactions between the factors. In addition, it implies a high consumption of time and resources [4].

As an alternative to OFAT, design of experiments (DOE) is a systematic and statistically based approach to evaluate factor combinations and adjust parameters to optimize trial results [5]. In this method, multiple experimental factors are varied simultaneously and, unlike OFAT, individual and interactive effects can be accurately interpreted, performing fewer experiments in less time than the OFAT approach would require [6].

The DOEs are based on factorial designs that can be fractional or complete. Fractional factorial designs are valid considering that some interactions are not as significant as the main effects [7]. Instead, a full factorial design consists of all possible factor combinations in an assay, which means that it is a more powerful design than the fractional one. This design is very useful, but requires a large number of test points as the levels of a factor or the number of factors increase [8].

In [9]. affirmed that, despite its advantages and reliable results, the full factorial DOE had been infrequently used to optimize immunoassays. It is a deficiency that persists to this day. Since ELISA is a multistep procedure, it would be convenient to apply full factorial designs that successively include each of the factors and levels that intervene in these steps, which has not been described in scientific papers. Due to experimental limitations, the number of factors under study is typically up to six [10]. If these designs are restricted to six factors or fewer per stage, they could be performed and processed by researchers without implying

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high experimental or statistical complexity, which may be the cause of the limited application of this type of DOE.

The objective of this work was to optimize a quantitative sandwich ELISA, through the application of full factorial experiments in successive steps of the assay. This ELISA will be used for the quantification of the TT-P0 protein, active principle of a vaccine candidate against sea lice (*Lepeophtheirus salmonis*) produced by the Center for Genetic Engineering and Biotechnology of Camagüey, Cuba. In addition, it could be applicable to pharmacokinetic studies of this molecule.

2. Materials and methods

2.1. Biological reagents

The recombinant TT-P0 protein (1.0 mg/mL), with molecular weight of 8.3 kDa and purified by affinity chromatography to metal chelates (Center for Genetic Engineering and Biotechnology, CIGB, Havana, Cuba), was the antigen for the generation of the monoclonal antibodies CBSSP0-Ls.3 and CBSSTT.6 (CIGB Sancti Spiritus, Cuba), which specifically recognized P0 (peptide derived from ribosomal protein P0) and TT (tetanus toxoid derived P2 peptide TT830-844), respectively. The monoclonal antibody CBSSTT.6 was conjugated to horseradish peroxidase (CBSSTT.6-HRP) by the method proposed by Ref. [11]. The TT-P0 protein was also used as antigen in the sandwich ELISA protocols.

2.2. Initial sandwich ELISA protocol

The initial homemade sandwich ELISA was obtained by the OFAT method. This procedure is described below and was used as the starting point for the optimization of the ELISA through a sequence of full factorial experiments in the assay steps. The buffers were prepared according to the recommendations of [12] and the plates were not shaken during the incubation times.

a) Costar® 3590 high binding plates were coated with 100 μL/well of 10 μg/mL of CBSSP0-Ls.3 diluted in 100 mM carbonate-bicarbonate buffer (pH 9.6) and incubated 2 h at 37 °C.
b) Apply three washes of 10 s duration with 380 μL/well of Phosphate Buffered Saline with 0.05% Tween 20 (PBST)
c) The wells were blocked with 380 μL of a solution of 3% (w/v) nonfat-dried milk in PBS for 1 h at 37 °C.
d) After one washing for 10 s, 100 μL/well of TT-P0 were added, diluted in PBS with 2% (w/v) nonfat-dried milk, in two-fold serial dilutions, from 40000 ng/mL to 156.2 ng/mL. The plate was incubated 1 h at 37 °C.
e) As step b)
f) 100 μL/well of CBSSTT.6-HRP, diluted 1:1600 in PBS and 2% nonfat-dried milk, was incubated 1 h at 37 °C.
g) As step b)
h) Plates were incubated in the dark with 100 μL/well ortho-phenylenediamine (OPD, 0.5 mg/mL) dissolved in 75.7 mM citrate/phosphate buffer and 0.015% (w/v) hydrogen peroxide, for 20 min at room temperature (RT).
i) The colorimetric reaction was terminated with 100 μL/well 2 M sulfuric acid and the Absorbance at 492 nm (A492nm) was measured by the ELISA plate reader (PR-621, Tecnosuma Internacional, Havana, Cuba). Nonfat dried-milk in PBS solution at 2% (w/v) was used as assay blank.

2.3. Optimization of the initial sandwich ELISA protocol

For the optimization of each step of this ELISA by means of full factorial analysis, two concentrations of TT-P0 antigen were used: high concentration (20 μg/mL) and low concentration (0.3 μg/mL). Factors and levels optimized in a step of the ELISA were incorporated into the protocol to optimize the corresponding next step.

2.3.1. Plate coating

The effects of coating parameters were analyzed using a 3x2x2x2x2 full factorial design.

- CBSSP0-Ls.3 concentration: 3.0, 5.0 and 10 μg/mL
- Coating buffer: Carbonate-bicarbonate buffer (50 mM; pH 9.6) and Phosphate Buffered Saline (PBS, pH 7.4)
- Incubation temperature: RT and 37 °C
- Incubation time: 2 h and 3 h
- Plate type: Costar® 3590 high binding and Greiner Bio-One high binding

Four plates of each type were coated. Half of each one was coated with PBS and the other half with carbonate-bicarbonate buffer (pH 9.6). Two columns per antibody concentration were placed in each half. The distribution of the temperature-time combinations by plate type was organized as follows: RT-2h; RT-3h; 37'C-2h; 37'C-3h. Three replicates of each antigen concentration were prepared and plated in duplicate for each treatment tested.

2.3.2. Antigen binding to CBSSP0-Ls.3

For this evaluation, the plates were coated with the optimal conditions obtained in 2.3.1.

A 3 × 2 × 3 full factorial design was used to optimize this stage.

- Nonfat-dried milk concentration in the antigen dilution buffer: 1, 2 and 3%
- Incubation temperature: RT and 37 °C
- Incubation time: 30 min, 1 h and 2 h

Six plates were used, one for each temperature-time combination. Three sections were distributed per plate, one for each nonfat-dried milk concentration. Six replicates of each antigen concentration were prepared and plated in duplicate per treatment evaluated.

2.3.3. Binding of TT-P0 to CBSSTT.6-HRP enzyme conjugate

For the optimization of this step, the best conditions obtained in two previous steps were used. The same combination of factors, levels and replicates described in 2.3.2 was followed. The 1:1600 dilution of the CBSSTT.6-HRP enzyme conjugate was applied, according to the initial ELISA.

After defining the best conditions for this step, an experiment was performed to verify the optimal dilution of the conjugate. Six dilutions of the conjugate were tested: 1:1000, 1:1300, 1:1600, 1:2000, 1:4000 and 1:8000. Three replicates per dilution were prepared and placed in triplicate in the plate with the corresponding blank. For this experiment, the antigen concentration used was 20 μg/mL.

2.3.4. Horseradish peroxidase-OPD reaction

The optimal conditions achieved in the three previous stages of the ELISA were applied to optimize this step. A 2 × 2 full factorial design was used. The factors evaluated were the type of substrate buffer (75.7 mM citrate/phosphate buffer, pH 5.0 and 100 mM acetate buffer, pH 5.0) and the OPD concentration (0.5 and 0.6 mg/mL).

Half of the plate was used for each buffer, and the two OPD concentrations were evaluated in each one. Three replicates of each antigen concentration were prepared and plated in triplicate per treatment assayed.

2.3.5. Washing steps

After optimizing the four previous stages, new washing conditions were tested considering two parameters: number of washes (2, 3 and 4) and washing time (10 s and 30 s). A 3 × 2 full factorial design was applied. Six plates were used, one for each combination of number of washes and time. The same combination of time and number of washes was applied per plate after each ELISA step, except after blocking, which

was maintained with 1 wash during 10 s as in the initial ELISA protocol. Six replicates of each antigen concentration were prepared and plated in duplicate per plate.

The MW-2001 PROWASH model plate washer (Tecnosuma Internacional, Havana, Cuba) was used with a dispensing pressure of 3.5 psi. This plate washer has, at the suction head, a vacuum pressure ≥10.06 psi and a vacuum flow ≥ 5L/min.

2.4. Specificity

This parameter was evaluated using the optimized ELISA with an additional step. This step consisted of pre-incubating the mixture of the CBSSP0-Ls.3 antibody with the TT-P0 antigen in solution before adding it to the plate.

Fifteen concentrations of CBSSP0-Ls.3 antibody, from 1.57 nM to 0.0956 pM, were prepared by two-fold serial dilutions using PBS with 1% (w/v) nonfat-dried milk. Each antibody concentration was mixed with 5 μg/mL of the TT-P0 antigen in eppendorf® tubes. The incubation was maintained for 1 h at RT. Thereafter, three replicates of this reaction mixture for each dilution point were added to the plate previously coated and blocked. A solution of 5 μg/mL of the TT-P0 antigen with no added antibody was used as control. From here, the assay continued with the subsequent steps of the optimized ELISA.

The specificity of the assay was expressed by the percentage of inhibition (%I), calculated as:

$$\%I = 100 - \frac{IR}{Control} \times 100$$

Where: IR is the A492nm of each antigen-antibody reaction mixture.

2.5. Antigen concentration curve to evaluate the effect of the ELISA optimization

The new antigen concentration curve was prepared with two-fold serial dilutions from 2500 ng/mL to 9.766 ng/mL. This curve was compared with that described in the ELISA initial protocol, in terms of lower limit of quantification, range of quantification, range relative to the lower limit of quantification and analytical sensitivity. Curves were fitted by five-parameter logistic regression using the GraphPad Prism software.

Both curves were repeated in triplicated during six days by two analysts, three days each one. For the evaluation of the intra-curve repeatability, the accuracy of 75% of the points of the curve, assessed for percent of relative error (%RE), should be less than 20%, except at the lower point of the curve, which should be less than 25%. The precision and accuracy inter-curves were assessed by the coefficient of variation expressed as a percentage (%CV) and %RE, respectively. Both should be less than 15%, except at the lower point of the curve, which should be less than 20% [13].

%RE and %CV were determined by the formulas:

$$\%RE = \frac{Mean\ of\ back\ calculated\ concentration - Nominal\ concentration}{Nominal\ concentration} \times 100$$

$$\%CV = \frac{Standard\ deviation}{Mean\ of\ back\ calculated\ concentration} \times 100$$

The range of quantification in each curve was defined by the maximum (Upper limit of quantification, ULOQ) and minimum (Lower limit of quantification, LLOQ) points that complied with the accuracy and precision acceptance criteria. The range relative to the lower limit of quantification was calculated by the formula: (ULOQ - LLOQ)/LLOQ.

The analytical sensitivity represents the variation of the A492nm signal per unit variation of the antigen concentration. The lower half of the curves was fitted to a second degree polynomial and the first derivative of that function was evaluated in the LLOQ to get the analytical sensitivity value.

2.6. Statistical analysis

Data processing was performed using the Statistical Package for Social Sciences 21.0 (SPSS).

The dependent variable used to follow the optimization of the ELISA, with the high and low concentrations of the antigen, was the signal to noise ratio (S/N), which is the ratio of the A492nm signal corrected with the assay blank and the signal from the blank. The solution of PBS with the corresponding concentration (%) of nonfat-dried milk powder was used as assay blank. The best combination of factors for each step of the ELISA was defined as the one with the highest value of signal to noise ratio. The means of these values were obtained from the output of the SPSS program, following the menu options: Analyze/General linear model/Univariate/Descriptive statistics.

In each step, the individual and interactive effects of the factors were determined by means of a complete factorial ANOVA, where the statistical significance ($\alpha = 0.05$) of the main effects of each factor and the interaction between factors were evaluated. Effect sizes were assessed using the Eta-square partial statistic.

3. Results

3.1. Optimization of the ELISA steps

3.1.1. Plate coating

In this stage, five factors and their 26 possible interactions were evaluated. The factors CBSSP0-Ls.3 concentration, buffer, incubation temperature and plate type were significant ($p \geq 0.000$) for both antigen levels, but incubation time was not significant for high ($p = 0.209$) or low ($p = 0.064$) antigen concentration. Fig. 1 shows the interactions that were significant for high and low antigen concentrations. The first-order interactions buffer-CBSSP0-Ls.3 concentration, CBSSP0-Ls.3 concentration-plate type, CBSSP0-Ls.3 concentration-temperature, and buffer-temperature had higher partial eta-square values. The effect sizes of these interactions were in the range 0.50–0.87 and 0.61–0.92 for low and high antigen concentrations, respectively. The buffer-plate type interaction had the smallest effect size (Eta-square = 0.04) when a high concentration of antigen was used (Fig. 1B); however, this interaction was the seventh most effective at low antigen concentration (Fig. 1A). The smallest effect size (Eta-square = 0.04) found with this antigen concentration was in the second-order interaction between buffer-temperature-type of plate, which with the highest concentration of antigen reached a partial eta-square value of 0.12 (Fig. 1B). With both concentrations of antigen, significant third-order interactions were found and the interaction between the five factors was also significant.

Non-matching significant interactions were found between the two antigen concentrations tested. The interactions temperature-plate type, first order, and buffer-temperature-time-plate type, third order, were only significant at low antigen concentration (Fig. 1A). Third-order interactions buffer-CBSSP0-Ls.3 concentration-temperature-plate type and buffer- CBSSP0-Ls.3 concentration-temperature-time were significant only at high antigen concentration (Fig. 1B).

The combination between the levels of the factors that produced the highest signal-to-noise ratio, both at low (S/N = 7.35) and high (S/N = 48.05) antigen concentration at this stage of the ELISA was.

- CBSSP0-Ls.3 concentration: 10 μg/mL
- Coating buffer: PBS
- Incubation temperature: RT
- Incubation time: 3 h
- Plate type: Costar® 3590 high binding

When 3 μg/mL of capture antibody was dissolved in carbonate-

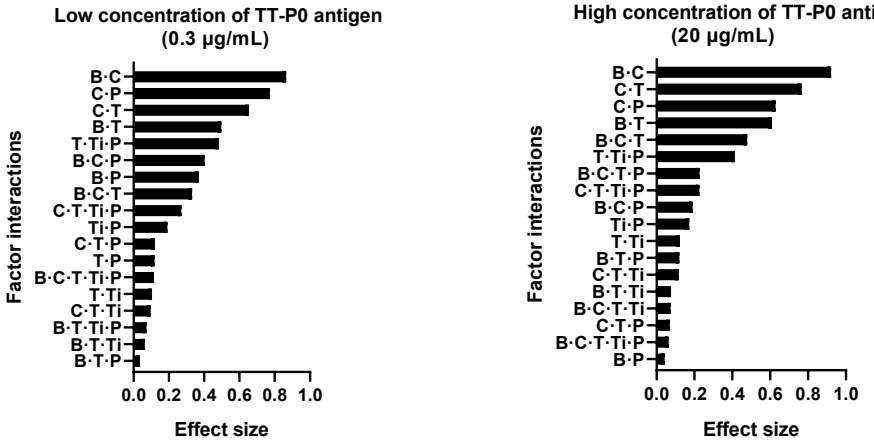


Fig. 1. Significant interactions between factors for coating of the ELISA plate, obtained from 3x2x2x2x2 full factorial ANOVA ($p \leq 0.05$). B: buffer; C: concentration of the monoclonal antibody CBSSP0-Ls.3; P: plate type; T: incubation temperature; Ti: incubation time. The dependent variable was the signal to noise ratio, which is the quotient of the A492nm signal corrected with the assay blank and the blank signal. The size of the effect of the factor interactions was measured according to the partial Eta-square.

bicarbonate buffer and incubating for 2 h at 37 °C the minimum signal to noise ratio was obtained for high (S/N = 5.18) and low (S/N = 1.25) antigen level.

3.1.2. Antigen binding to CBSSP0-Ls.3

Three factors and four interactions were assayed for the optimization of the antigen binding to the antibody that coated the plate. The factors time and concentration of nonfat-dried milk in the dilution buffer caused the greatest effect at the two antigen concentrations tested (Fig. 2). The most important interaction was detected between these two factors at both antigen concentrations, but it was slightly higher with 20 μg/mL TT-P0 (Eta-square = 0.66). Temperature was the factor that, in both cases, produced a smaller effect size.

The non-significant interactions contrasted at 0.3 μg/mL and 20 μg/mL of antigen: in the first case, the second-order interaction of non-fat milk concentration-time-temperature resulted (Fig. 2A), and in the second, the first-order interaction of non-fat milk concentration-temperature (Fig. 2B).

The maximum signal to noise ratio, 14.25 and 58.42 for low and high antigen concentration, respectively, was produced with the follow combination between the levels of the factors.

- Nonfat-dried milk concentration in the antigen dilution buffer: 1%
- Incubation temperature: 37 °C
- Incubation time: 2 h

The worst signal to noise ratio involved totally different levels than those that gave the maximum signal at both antigen concentrations: 3% of nonfat-dried milk in the antigen dilution buffer and incubation at RT during 30 min. With 0.3 μg/mL of antigen the signal to noise ratio was 1.73 and with 20 μg/mL it was 7.19.

3.1.3. Binding of TT-P0 to CBSSTT.6-HRP enzyme conjugate

The factor with the largest effect size was the concentration of nonfat-dried milk in the enzyme conjugate dilution buffer. The most important interaction was temperature-time, with partial Eta-square values of 0.46 and 0.62 for low and high antigen concentrations, respectively (Fig. 3).

Temperature was a non-significant factor at a high concentration of TT-P0, as were the first-order nonfat-dried milk concentration-time interaction and the only second-order one: nonfat dried milk concentration-temperature-time (Fig. 3B). However, these last two interactions were significant at low levels of TT-P0 (Fig. 3A).

The results of the evaluation of different dilutions for the enzyme conjugate are showed in Table 1. The optimal dilution, according to the highest value of the signal to noise ratio, was 1:1300.

The values in the second and third columns represent the mean of the absorbance values of three replicates at each conjugate dilution. The coefficient of variation was in the range 4.03%–8.82%.

The optimal conditions for the incubation of the CBSSTT.6-HRP enzyme conjugate in the ELISA plate, supported by the highest signal to noise values (S/N = 10.10 and S/N = 55.00 for 0.3 μg/mL and 20 μg/mL of antigen, respectively) were the following.

- Nonfat-dried milk concentration in the antigen dilution buffer: 1%
- Incubation temperature: RT
- Incubation time: 1 h
- Dilution for the CBSSTT.6-HRP enzyme conjugate: 1:1300

The signal to noise ratio decreased almost 2-fold for both antigen

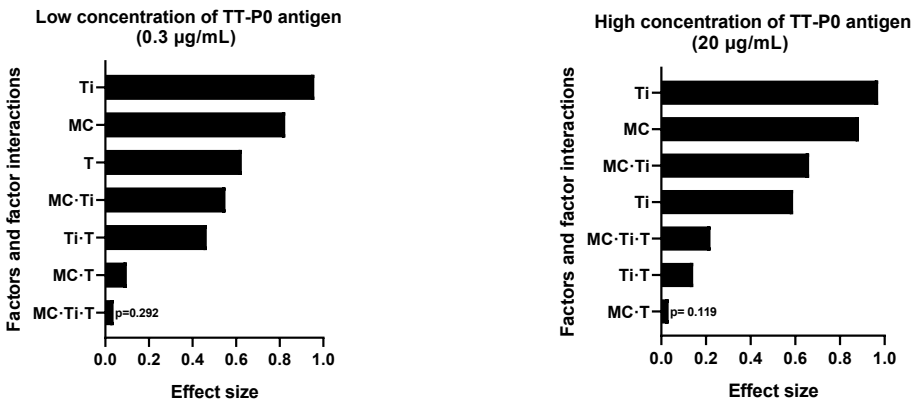


Fig. 2. Factors and factor interactions for the binding of TT-P0 antigen to the CBSSP0-Ls.3 monoclonal antibody that coated the ELISA plate. MC: nonfat-dried milk concentration; T: incubation temperature; Ti: incubation time. The dependent variable was the signal to noise ratio, which is the quotient of the A492nm signal corrected with the assay blank and the blank signal. The size of the effect of the factor interactions was measured according to the partial Eta-square from a 3 × 2 × 3 full factorial ANOVA. The statistical significance was set at 0.05.

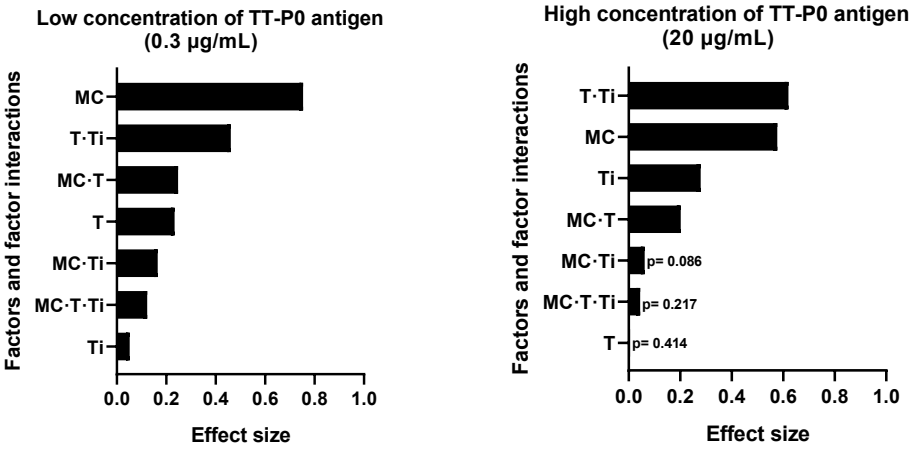


Fig. 3. Factors and factor interactions for the binding of CBSSTT.6-HRP enzyme conjugate to the TT-P0 antigen. MC: nonfat-dried milk concentration; T: incubation temperature; Ti: incubation time. The dependent variable was the signal to noise ratio, which is the quotient of the A492nm signal corrected with the assay blank and the blank signal. The size of the effect of the factor interactions was measured according to the partial Eta-square from a 3 × 2 × 3 full factorial ANOVA. The statistical significance was set at 0.05.

Table 1
Evaluation of dilution factors for the CBSSTT.6-HRP enzyme conjugate.

Dilution factor	A492nm Assay blank[a]	A492nm Signal	Signal to noise ratio
1000	0.046	1.827	39.7
1300	0.045	1.831	40.7
1600	0.042	1.563	37.2
2000	0.043	1.393	32.4
4000	0.041	0.934	22.8
8000	0.045	0.575	12.8

[a] assay noise.

concentrations when the concentration of milk was increased from 1% to 3% in the dilution buffer, but the levels of the other two factors were the same.

3.1.4. Horseradish peroxidase-OPD reaction

In optimizing this ELISA step, the type of buffer was the only significant factor for the two antigen concentrations included in the study. The OPD concentration and its interaction with the type of buffer were not significant at any of the antigen levels (Fig. 4).

In this ELISA step, the combination that produced the maximum signal to noise ratio (S/N = 6.25 and S/N = 33.34 for 0.3 µg/mL and 20 µg/mL of antigen, respectively) was.

- Type of substrate buffer: 100 mM acetate buffer, pH 5.0
- OPD concentration: 0.5 mg/mL

The use of citrate/phosphate buffer and 0.6 mg/mL of OPD produced the lowest signal to noise ratios: 4.64 and 27.49 for low and high concentration of antigen, respectively.

3.1.5. Washing steps

The number of washes was the factor that had a significant impact on the size of the effect, both in the low and in the high concentration of antigen (Fig. 5). The washing time and the interaction between both factors did not significantly affect the partial Eta-squared value. The best combination for washing was.

- number of washes: 2
- washing time: 10 s

The worst condition for this step consisted of applying 4 washes for 30 s, which resulted in the minimum signal to noise ratio, 7.38 and 25.31 for the two levels of antigen, in that order. The signal to noise ratio found with the best condition was 1.5 times higher in both cases.

3.2. Specificity

The evaluation of specificity was done by an inhibition assay, which started with the reaction in solution of the TT-P0 antigen with the CBSSP0-Ls.3 monoclonal antibody. Fig. 6 shows that this antigen-

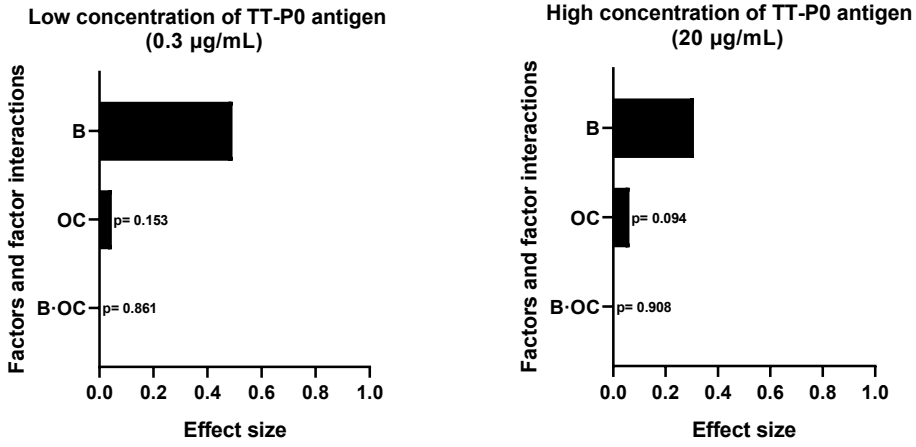


Fig. 4. Factors and factor interactions for the Horseradish peroxidase-OPD reaction. B: type of substrate buffer; OC: ortho-phenylenediamine concentration. The dependent variable was the signal to noise ratio, which is the quotient of the A492nm signal corrected with the assay blank and the blank signal. The size of the effect of the factor interactions was measured according to the partial Eta-square from a 2 × 2 full factorial ANOVA. The statistical significance was set at 0.05.

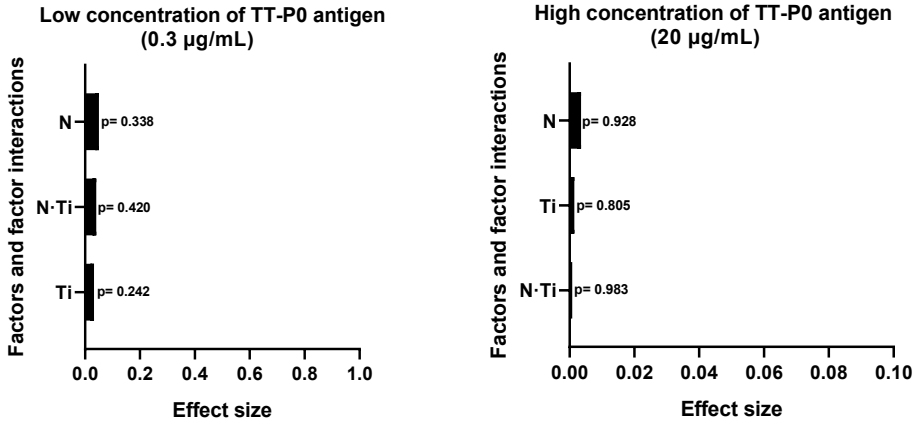


Fig. 5. Factors and factor interactions for the washing steps. N: number of washes; Ti: washing time. The dependent variable was the signal to noise ratio, which is the quotient of the A492nm signal corrected with the assay blank and the blank signal. The size of the effect of the factor interactions was measured according to the partial Eta-square from a 3 × 2 full factorial ANOVA. The statistical significance was set at 0.05.

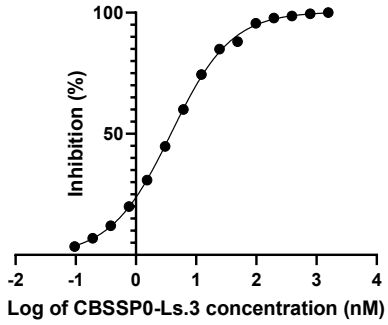


Fig. 6. Estimation of the specificity of the ELISA. An inhibition assay was performed in solution with 5 µg/mL of the TT-P0 antigen and the CBSSP0-Ls.3 monoclonal antibody at different concentrations, from 1.57 nM to 0.0956 pM, obtained by two-fold serial dilutions. Three replicates of the reaction mixture were used for each dilution point. A control with no added antibody was used. The percentage of inhibition was calculated by the formula: 100-(A492nm of each antigen-antibody reaction mixture/A492nm of Control) x100. Values represent the mean of percent of inhibition per dilution point. The coefficient of variation of these data was in the interval 0.29%–11.28%.

antibody reaction was almost 100% inhibited at the highest concentration of CBSSP0-Ls.3 (1.57 nM), which confirmed the specificity of the ELISA. This percentage gradually declined as the antibody concentration decreased, until the reaction was practically not inhibited (3.45%) at the last dilution point.

3.3. Antigen concentration curve to evaluate the effect of the ELISA optimization

Two antigen concentration curves were prepared for this assay, using 1:2 serial dilutions. The first curve, with a concentration range between 40000 ng/mL and 156.25 ng/mL, was evaluated using the initial ELISA protocol (Fig. 7A). With the optimized ELISA, a second curve was prepared in the range from 2500 ng/mL to 9.766 ng/mL (Fig. 7B). These curves were fitted using a five-parameter logistic regression. Since the standard deviation augmented with increasing antigen concentration, it was necessary to determine a weighting factor as described in the GraphPad Prism software. The calculated weighting factor was 1.5.

Both curves met the preset accuracy and precision acceptance criteria. The curve corresponding to the initial protocol showed an accuracy range of $0.12 \leq \%RE \leq 2.41$ and a precision of $4.46 \leq \%CV \leq 9.95$ (table attached to Fig. 7A), while in the optimized ELISA the ranges obtained were $0.27 \leq \%RE \leq 4.84$ and $4.36 \leq \%CV \leq 9.94$, for accuracy and precision, respectively (table attached to Fig. 7B).

The results of the comparison between the antigen concentrations curves obtained with the two ELISAs are summarized in Table 2. The optimized ELISA showed a LLOQ 16 times lower than the initial ELISA, without affecting the range relative to the LLOQ. The analytical sensitivity was 20 times higher in the optimized ELISA.

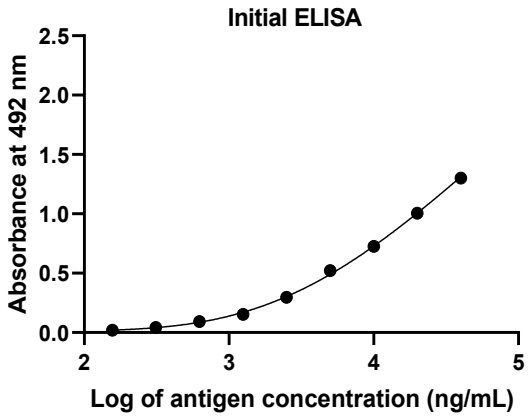
The optimal conditions for each step of the ELISA are presented in Table 3, as a compilation of all the results of the full factorial design applied in this study.

4. Discussion

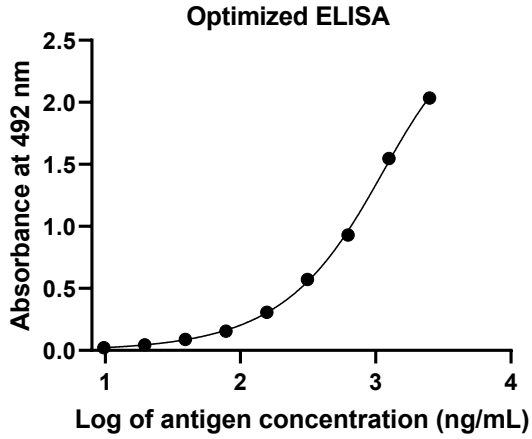
The application of full factorial design of experiments in the successive steps of the ELISA was effective to optimize the assay. A key benefit of this type of DOE is the ability to clearly identify important interactions between assay parameters that would have been missed with the traditional OFAT approach. This was certainly the case in our study and previous studies have also made this observation [4]. It is an iterative model that provides the ability to obtain valuable information about a ligand binding assay with a minimal number of experiments [14]. It would be very expensive, experimentally intensive, and time consuming to test so many factors and levels in our immunoassay using OFAT. Although OFAT was useful for studying some main effects of the ELISA initial protocol, it is an approach that involves evaluating each factor separately and provides little information about how the factors interact. It would be very difficult to optimize a robust and sensitive ELISA using the OFAT method. In this work, a classical and uncomplicated statistical analysis of the data, generated from full factorial experiments, clearly establishes the relationship between the variable of interest (signal to noise ratio) and the factors studied, and allows identifying the main effects of the factors or the interactions between them in each step of the ELISA.

Coating is the first step in any ELISA and is dependent on plate type, time, temperature, coating buffer, and coating agent concentration. These five factors were evaluated in this work. In fact, this was the stage of the ELISA where the greatest number of factors and levels intervened. Its optimization allowed increasing 9 times the signal to noise ratio at high concentration of antigen, and almost 6 times at low concentration. All factors evaluated at this stage are applicable to antigen coating in another type of ELISA.

The interaction between the incubation time and the nonfat-dried milk concentration was the most important in the capture antibody-antigen binding step, although these factors separately showed the largest effect sizes. In the binding of the CBSSTT.6-HRP enzyme conjugate to TT-P0, the temperature and incubation time had the key interaction, unlike the previous step. However, nonfat-dried milk



Nominal Concentration (ng/mL)	Mean A492nm	Back-calculated concentration (ng/mL)	RE (%)	CV (%)
40000	1.301	39854	-0.37	6.16
20000	1.006	20072	0.36	4.54
10000	0.725	10034	0.34	5.12
5000	0.522	5006	0.12	9.95
2500	0.296	2471	-1.14	7.93
1250	0.153	1248	-0.18	9.01
625	0.092	640	2.41	6.92
312.5	0.043	305,9	-2.12	4.46
156.25	0.020	157.11	0.55	8.51



Nominal Concentration (ng/mL)	Mean A492nm	Back-calculated concentration (ng/mL)	RE (%)	CV (%)
2500	2.034	2470	-1.20	8.32
1250	1.547	1288	3.02	4.44
625.0	0.931	595.6	-4.70	5.01
312.5	0.572	324.8	3.94	4.36
156.3	0.307	158.2	1.23	7.76
78.13	0.156	74.34	-4.84	8.82
39.06	0.089	40.39	3.39	6.78
19.53	0.044	19.29	-1.22	9.94
9.766	0.022	9.792	0.27	6.03

Fig. 7. Antigen TT-P0 concentration curves with initial and optimized ELISA. Curves were fitted by five-parameter logistic regression using the GraphPad Prism software. The adjacent tables show the respective coefficients of variation (CV) and relative error (RE) of the six ELISAs performed with each protocol, as a measure of the precision and accuracy of the back-calculated concentration at each point.

Table 2
Comparison between the antigen concentration curves obtained with the initial ELISA and with the optimized ELISA.

Parameters	Initial ELISA (IE)	Optimized ELISA (OE)	Ratio
Lower limit of quantification (ng/mL)	156.2	9.766	16 (IE/OE)
Range of quantification (ng/mL)	156.2–40000	9.766–2500	–
Range relative to the lower limit of quantification[a]	255	255	1
Analytical sensitivity[b](mL/ng)	0.0001	0.0020	20 (OE/IE)

[a] The range relative to the lower limit of quantification was calculated by the formula: (ULOQ - LLOQ)/LLOQ, where ULOQ is the upper limit of quantification and LLOQ is the lower limit of quantification.

[b] Represents the variation of the A492nm signal per unit variation of the antigen concentration. The lower half of the curves was fitted to a second degree polynomial and the first derivative of that function was evaluated in the LLOQ to get the analytical sensitivity value.

concentration was again an important factor. This factorial design makes it possible to properly evaluate, in addition to the interactions between factors, the main effect of a factor on the dependent variable [8]. The nonfat-dried milk concentration at 1% in the antigen dilution buffer, with the incubation 1 h at RT, was identified as the best combination of levels per factor in this step. However, when the percentage of nonfat-dried milk is triplicated, keeping the incubation time and temperature the same, the signal to noise ratio decreases drastically in the two antigen concentrations. This confirms that the nonfat-dried milk concentration is the determining factor in the optimization of the binding of the CBSSTT.6-HRP enzyme conjugate to TT-P0. The calculation of the partial eta squared also supports this result, because it evaluates the nonfat-dried milk concentration as the factor with the largest effect size.

In these two previous steps of antigen-antibody binding, the optimal temperature was different: the antigen binding to the monoclonal antibody CBSSP0-Ls.3 was at 37 °C, while the binding of the CBSSTT.6-HRP enzyme conjugate to TT-P0 occurred at RT. This could mean that the interaction epitope-paratope is distinct in both bonding.

No significant interactions were found between the factors analyzed in the horseradish peroxidase-OPD reaction and in the washing steps. Nevertheless, it was possible to determine the optimal conditions for these steps, according to the combination of factors that yielded the maximum signal to noise ratio. The fact that there are no significant interactions does not invalidate the application of full factor analysis in these steps in other ELISAs. In addition, other factors could be suggested to make the study broader, for example: temperature and incubation time of the enzyme-substrate reaction, or changes in the composition of the washing buffer.

The main disadvantage of full factorial experiments is that they should not evaluate more than six factors at a time [10]. In this study, this was not a problem because a maximum of five factors were analyzed

Table 3
Optimized ELISA protocol.

Number	ELISA Steps	Procedures
1	Plate coating	Costar® 3590 High Binding Plate coated with 100 μL/well of 10 μg/mL of the monoclonal antibody CBSSP0-Ls.3, diluted in Phosphate Buffered Saline (PBS), is incubated 3 h at room temperature.
2	Washing	Apply two washes of 10 s duration with 380 μL/well of PBS with 0.05% Tween 20.
3	Blocking	Block the plate wells with 380 μL of a solution of 3% (w/v) nonfat-dried milk in PBS for 1 h at 37 °C.
4	Washing	Apply a wash of 10 s duration with 380 μL/well of PBS with 0.05% Tween 20.
5	TT-P0 antigen binding to CBSSP0-Ls.3	Apply 100 μL/well of the TT-P0 antigen diluted in a solution of 1% (w/v) nonfat-dried milk in PBS. Incubate 2 h at 37 °C.
6	Washing	As in step 2
7	Binding of CBSSTT.6-HRP enzyme conjugate to TT-P0	Apply 100 μL/well of the CBSSTT.6-HRP enzyme conjugate diluted 1:1300 in a solution of 1% (w/v) nonfat-dried milk in PBS. Incubate 1 h at room temperature.
8	Washing	As in step 2
9	Horseradish peroxidase-OPD reaction	Plate is incubated in the dark with 100 μL/well OPD (0.5 mg/mL) dissolved in 100 mM acetate buffer and 0.015% (w/v) hydrogen peroxide, for 20 min at room temperature.
10	Reaction stop	100 μL/well 2 M sulfuric acid
11	Absorbance measurement	Absorbance at 492 nm is measured in an ELISA plate reader

simultaneously and it was only in the coating, which is the ELISA step where it is mandatory to consider the largest number of factors at the same time. In the rest of the steps, the number of factors and interactions were reduced, but key parameters, such as buffer preparation, temperature and incubation time, were taken into account. As far as we know, there are no reports of ELISA optimization following the step-by-step scheme that was used in this work. The analysis carried out was comprehensive and with a simple statistical processing. Our results have been obtained without resorting to DOEs that use the response surface method and desirability functions, which have been applied by other authors [15,16]. We consider that these methods make complex the data processing for the researchers who not have access to the service of a trained statistician. This could be one of the causes of the non-generalization of the step-by-step full factorial design for the optimization of ELISA.

The specificity of the optimized ELISA was verified. Moreover, the validity of the optimization was confirmed by the results of the comparison between the antigen concentration curves obtained with the initial ELISA and with the optimized ELISA. In the ligand binding assays designed for pharmacokinetic studies and clinical diagnostics, regulatory agencies require the development and optimization of assays with LLOQ that are capable of detecting analytes at clinically relevant concentrations [17]; FDA, 2019). In this work, it is demonstrated that the optimization of the ELISA by means of full factorial experiments was able to significantly reduce the LLOQ of the TT-P0 with respect to the assay improved by an OFAT method. This is an important result for the application of the optimized ELISA to pharmacokinetic studies related to the vaccine candidate against sea lice. The analytical sensitivity evaluated in the LLOQ, as it is the zone of highest experimental error, increased 20 times after the application of the step-by-step full factorial design. Therefore, with the optimized ELISA it will be possible to detect smaller changes in the concentration of TT-P0, which would not be detected by the initial ELISA. For example, a 0.001 increase in A492nm, at a point near the LLOQ of the initial curve, can be detected with a 10 ng/mL increase in TT-P0 concentration; but, with the optimized curve, the change can be perceived with a 0.5 ng/mL increase in this concentration. The LLOQ and the analytical sensitivity were improved without reducing the range relative to the LLOQ. Contracting this range would have been an undesirable result due to the format of many immunoassays, whose quantification range can be narrow, sometimes <1 order of magnitude [13].

5. Conclusions

Step-by-step optimization of a quantitative sandwich ELISA by a full factorial design of experiments, and the successive incorporation into the protocol of the best combination of factors and levels, is a key strategy to obtaining a specific and more sensitivity immunoassay, with reduced LLOQ as a better antigen detectability criterion. Since DOE are performed on the basis of statistical theory, the experimental design proposed in this work has been linked to a simple statistical processing, which facilitates the interpretation of the results in those laboratories where a trained statistician is not available.

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CRediT authorship contribution statement

Carlos A. Hernández: Conceptualization, Investigation, Formal analysis, Writing – review & editing. **Maylin Pérez-Bernal:** Conceptualization, Investigation, Visualization, Writing – review & editing. **Daymí Abreu:** Investigation, Data curation. **Onel Valdivia:** Investigation, Data curation. **Magali Delgado:** Investigation. **Dayamí Dorta:** Investigation. **Andy G. Domínguez:** Investigation. **Enrique R. Pérez:** Project administration, Supervision. **José M. Sánchez-Ríos:** Project administration, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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