Technical Reports

Titration experiments

Technical Report: Titration experiments

The aim of the research presented in this technical report is to identify good practices for antigen titration for discrimination between positive and negative sera for a given disease. These good practices will be applied to the products of Rekom Biotech S. L. The document is organized in two parts. Firstly, a Montecarlo simulation is driven to identify these good practices. For this, a model of the chemical processes that take place in the Enzyme-Linked ImmunoSorbent Assay (ELISA) is identified. Secondly, the results are validated with a number of experiments. The complete investigation is performed for the 15-kDa lipoprotein of Treponema pallidum (Tpp15 or TpN15) for syphilis detection.

The sections of the document are as follows: In Section 1 the process model is identified. In Section 2, real data from Tpp15 is employed to identify the model parameters. In Section 3, the Montecarlo simulation is established. In Section 4, different titration strategies are evaluated. Section 5 presents an experimental part devoted to briefly validate and illustrate the titration protocol.

1 ELISA model

Let α be the number of antigen molecules linked to the surface of the polystyrene well in a microtiter plate in the correct position for linking the antibody. When a given volume of sera with β molecules of antibody is introduced in the well, the following process takes place:

$$s.Ag + Ab \text{ € } \quad s.Ag.Ab \tag{0.1}$$

where s.Ag represents one antigen molecule liked to the polystyrene surface, Ab is one antibody molecule in the sera dilution and s.Ag.Ab the complex antigen-antibody liked to the surface. The reaction is fast and reversible, and therefore it can be considered to reach equilibrium:

$$r = k_a(\alpha - x)(\beta - x) - k_d x = 0 \qquad \frac{x}{(\alpha - x)(\beta - x)} = \frac{k_a}{k_d} = K \tag{0.2}$$

being x the number of molecules of the complex antigen-antibody linked to the polystyrene surface. Generally speaking, there is a high affinity between antibody and antigen so that the equilibrium constant is high. Equation (1.2) can be put into the following form:

$$x^2 - \left(\alpha + \beta + \frac{1}{K}\right)x + \alpha\beta = 0 \tag{0.3}$$

with solution:

$$x = \frac{1}{2}\left(\left(\alpha + \beta + \frac{1}{K}\right) - \sqrt{\left(\alpha + \beta + \frac{1}{K}\right)^2 - 4\alpha\beta}\right) \tag{0.4}$$

where the second summand is only subtracted due to x needs to be lower than the lowest of the initial values α and β. Equation (1.4) can be used to simulate the response of an ELISA assay to multiple dilution of sera:

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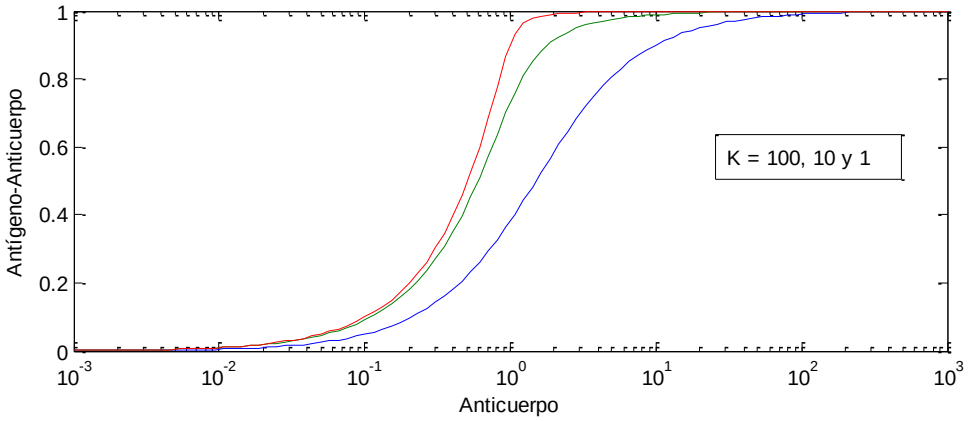

Figure 1. Simulated curves with equation (1.4) for different values of K.

After washing and any other necessary treatment, a direct measurement is applied of the well (e.g. spectrometry): Y, which can be thought of being related to x according to:

$$Y = ax + b \qquad Y = \frac{a}{2}\left(\left(\alpha + \beta + \frac{1}{K}\right) - \sqrt{\left(\alpha + \beta + \frac{1}{K}\right)^2 - 4\alpha\beta}\right) + b \qquad (0.5)$$

This model can also be represented by the more used "4-Parameter Logistic" model:

$$y = D + \frac{A - D}{1 + (\beta / C)^B} \qquad (0.6)$$

where β is the independent variable, the antibody quantity, *y* is the dependent variable, the measurement quantity, and A, B, C and D are the model parameters. A and D are in the same units as *y*, C is in the same units as β, and B is dimensionless. Although the 4PL model was not derived from first principles, its use is simpler than that of (1.5) and the results are fairly similar:

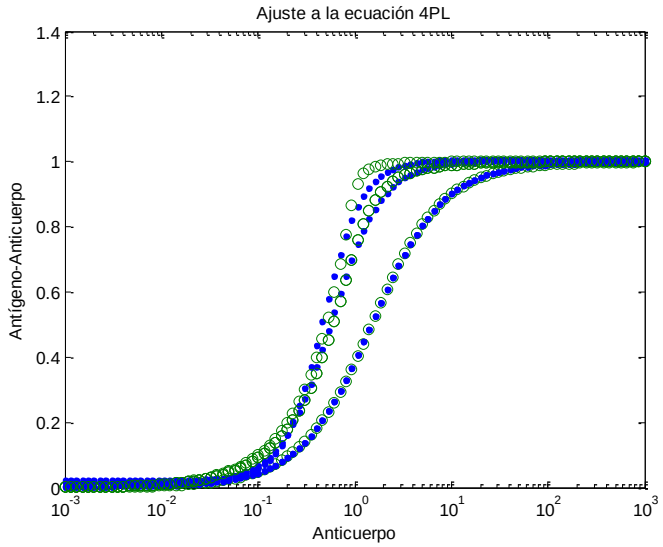

Figure 2.Comparison between the physico-chemical model (green circles) and the 4PL model (blue dots)

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2 Model calibration

In the Appendix, three titration experiments for Tpp15 with 4 positive and 4 negative sera for syphilis are presented. Three replicates are used in each experiment and four antigen dilutions are considered. These experiments will be employed to calibrate the parameters of the 4PL model.

Firstly, the first experiment, with dilutions 1/1000, 1/2000, 1/3000 and 1/4000, will be analyzed. Figure 3 shows the tendency of the mean value of the replicates in each titration. In the figure, the optic density (OD) of the positive sera decreases with decreasing dilution of the antigen. Negative sera, nevertheless, remain almost steady. Regarding the variability among the replicates (not shown) in general higher values are found for higher ODs. Similar results are found for the second experiment in the Appendix. In the third experiment, the OD values for dilutions 1/500, 1/750, 1/1000 and 1/1500 seem to remain steady, reflecting a platoon due to excess of antigen.

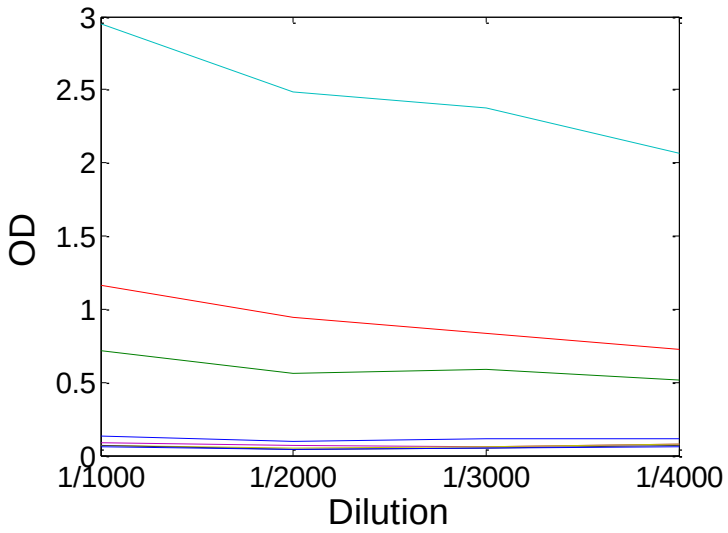

Figure 3: Average optic density (OD) for the three replicates corresponding to each serum and dilution in the first experiment in the Appendix.

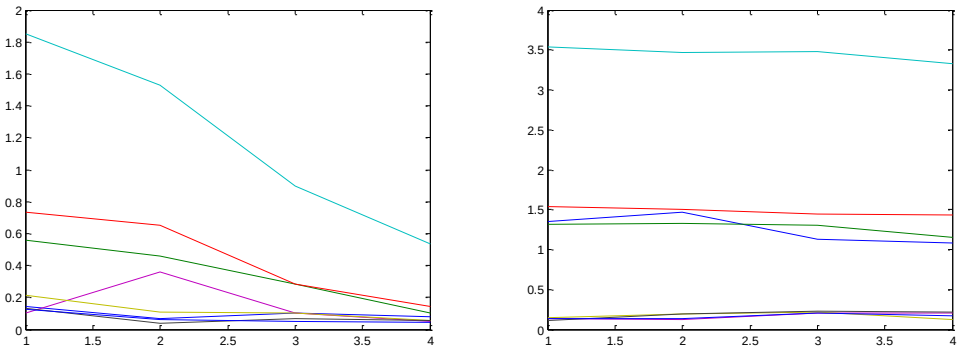

Figure 4: Average optic density (OD) for the three replicates corresponding to each serum and dilution in the second (right) and third (second) experiment in the Appendix.

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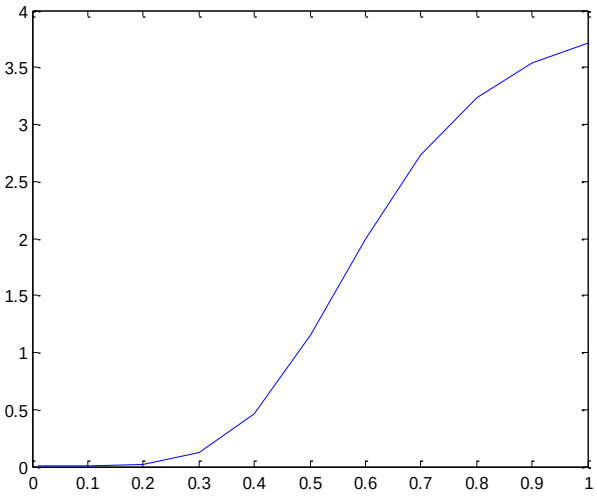
To simulate the observed behavior in the data, the 4PL model with parameters A=0, D=4 for minimum and maximum asymptotes, C=0.6 for inflexion point and B=-5 will be used. A serial dilution of a serum with antibody concentration of 1 is shown in the following figure:

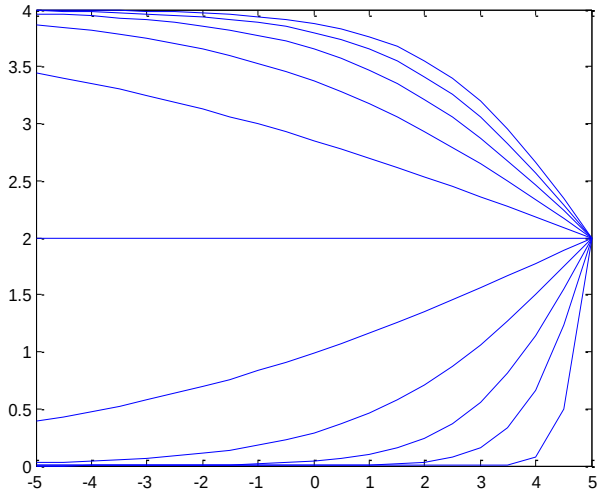



```
% matlab

vi=[0.01 0.1:0.1:1];
a=[];
for i=1:length(vi),
a(i)=PL4(vi(i),0, -5,0.6,4);
end
plot(vi,a)
```

Figure 5: Simulation with the 4PL model of a serial dilution of a serum with concentration 1 (left) and Matlab code used (right).

Now it is necessary to determine an adequate procedure to simulate the variation of actual data with respect to the dilution. Several considerations are in due. Firstly, the minimum asymptote (A) will be left to 0. Respecting the rest of the parameters, in the following figures the effect of varying each of them while maintaining the others in the reference value is shown.




```
% matlab

vi=[0.01 0.1:0.1:1];
vj=[-5:0.5:5];
for i=1:length(vi),
a=[];
for j=1:length(vj),
a(j)=PL4(vi(i),0,-
+vj(j),0.5,4);
end
plot(vj,a)
hold on
end
```

Figure 6: Simulation with the 4PL model for fixed A=0, C=0.5, D=4 and varying values of B (left) and Matlab code used (right).

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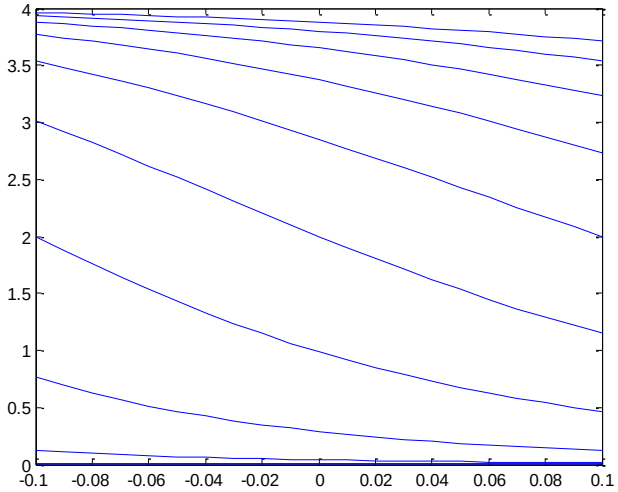
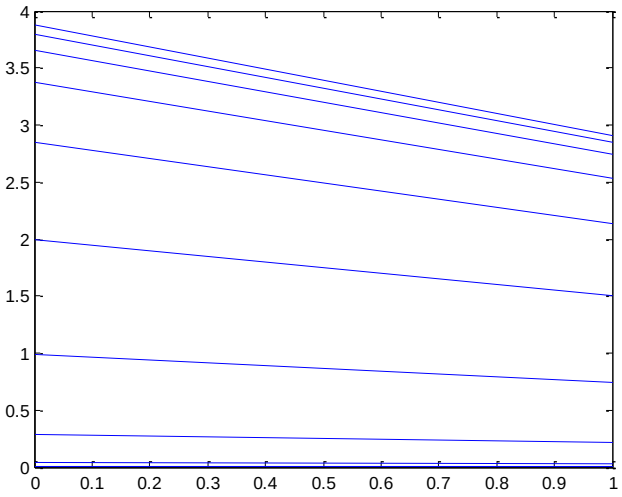


```
% matlab

vi=[0.01 0.1:0.1:1];
 vj=[-0.1:0.01:0.1];
 for i=1:length(vi),
a=[];
for j=1:length(vj),
a(j)=PL4(vi(i),0,-
5,0.5+vj(j),4);
end
plot(vj,a)
hold on
end
```

Figure 7: Simulation with the 4PL model for fixed A=0, B=-5, D=4 and varying values of C (left) and Matlab code used (right).



```
% matlab

vi=[0.01 0.1:0.1:1];
vj=0:1;
for i=1:length(vi),
a=[];
for j=1:length(vj),
a(j)=PL4(vi(i),0,-
5,0.5,4-vj(j));
end
plot(vj,a)
hold on
end
```

Figure 8: Simulation with the 4PL model for fixed A=0, B=-5, C=0.5 and varying values of D (left) and Matlab code used (right).

The effect of varying B (Figure 6) is not, in principle, useful for our purposes. Therefore, B will be left to -5. Augmenting the value of C (Figure 7) implies separating mainly higher concentrations. Nevertheless, the most realistic effect is the one in Figure 8 compared to that in Figure 3, for instance. D can be modified to simulate different dilutions, so that the maximum asymptote decreases for decreasing dilutions. This holds up to a point where D does not grow any more (third experiment in the Appendix) since the antigen is in excess.

3 Parameters in the Montecarlo Simulation

For the Montecarlo simulation, variations in parameters C and D in the 4PL model are used as discussed in the previous section. A grid of 2 x 3 possibilities for D=4, D=3 and D=2, and C=0.6 y C=0.7 are used to simulate 3 dilutions of antigen plus 2 different dilutions of conjugate, inspired by the first two experiments in the Appendix. The simulated OD are

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corrupted with Gaussian nose of mean 0 and standard deviation equal to 0.05xOD + 0.1, simulating heteroscedastic variability.

In order to simulate the concentration for positive and negative sera, two distributions are employed. Negative sera are simulated with mean 0 and standard deviation 0.1. Only positive values are accepted. Positive sera are simulated with mean 0.5 and standard deviation 0.1. Maximum and minimum concentration values are normalized to 1 and 0.01, respectively.

To identify the optimum dilutions for the simulation, which should be found in a successful titration experiment, the ROC curves are computed using a wide sample of simulated sera: 5,000 positive and 5,000 negative individuals:

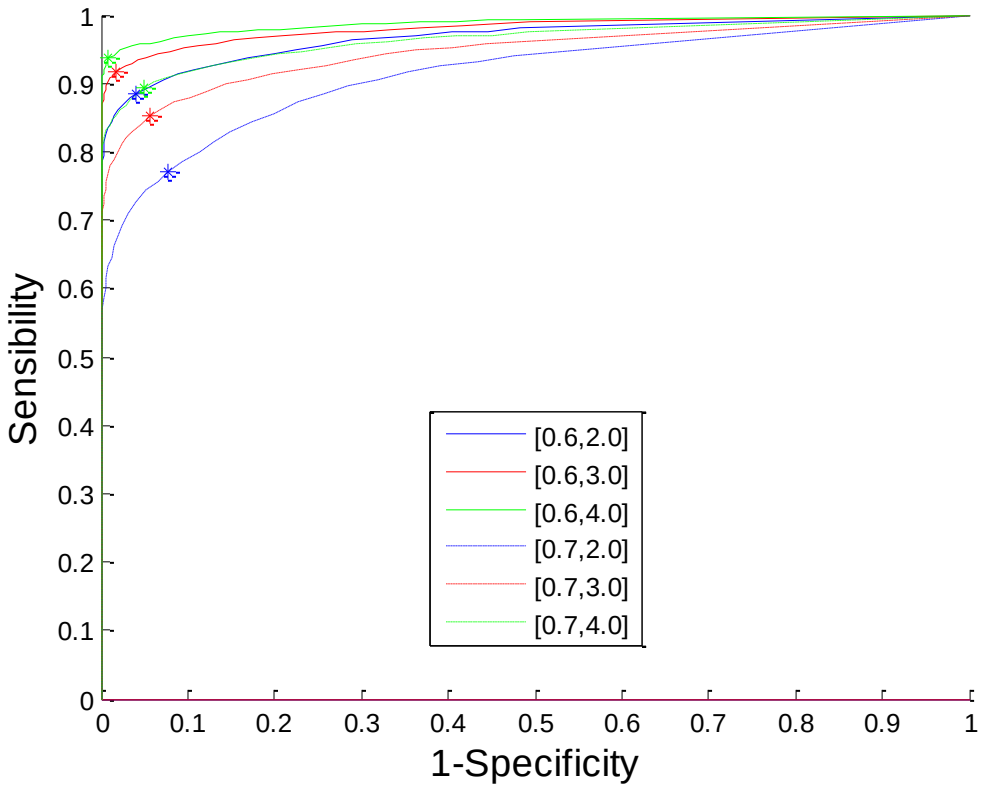

Figure 9: ROC curve from 10,000 simulated sera for the different dilutions of antigen and conjugate.

Thus, according to Figure 9, a successful titration experiment should reveal that the best titration choice is C=0.6 and D=4, with little difference comparing to C=0.6 and D=3, which goes next. It is noteworthy that the fact that the maximum asymptote (D) is higher does not mean better discrimination power between positive and negative sera. For instance, C=0.6 and D=2 has similar discrimination power than C=0.7 and D=4, but lower maximum asymptote.

4 Montecarlo Simulation

Several are the possibilities in a titration experiment. Here, the following decisions in the titration are investigated:

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- How to select the sera for the titration experiment.
- How many sera are necessary.
- How many replicates.
- How to assess the discrimination capability between positive and negative sera at a specific titration.

From the simulation procedure defined in the previous section, a 100 sera sample is obtained: 50 positive sera and 50 negative sera. This is a realistic (or maybe slightly optimistic) number for the sera at the disposal of Rekom Biotech S.L. everytime a titration experiment is started. Since less than 10% of these sera will be used in the titration, one of the objectives of this section is to identify an adequate strategy to select a sub-sample of sera for titration from the complete sample at our disposal. Thus, in the simulation, from the entire sample (50+ vs 50-) a sub-sample or 4+ vs 4- sera is chosen according to the following strategies:

- Centered: choose the 4 sera of each class (positive or negative) which are closest to the class mean.
- Clearest: choose the 4 highest positive and the 4 lowest negative.
- Least clear: choose the 4 lowest positive and the 4 highest negative.
- Homogeneus: choose homogeneusly from the quartiles of each class.
- Centered Homogeneus: the same as iv. restricted to the interval between 1 standard deviation around the mean value.

Another point under research is how to assess the discrimination capability at a titration. For this, there are several possibilities:

a. The ratio between the positive mean and the negative mean. The highest this value is, the more discrimination power. There are two drawbacks in this index: there is no standard procedure to identify when the titration is adequate (in other words, the notion of a "high" and "low" index is not clearly defined) and the variability within each class is not considered.

$$R = \frac{\bar{X}_+}{\bar{X}_-}$$

where $\bar{X}_+$ is the sample mean OD of the positive sera (at a given antigen concentration) and $\bar{X}_-$ the corresponding mean for negative sera.

b. A statistical test of significance for the comparison of means. This overcomes both drawbacks of the preceding index. The Welch's test may be the most approapriate one. It is an extension of the Student's test for possibly unequal variances in the two clases compared. The Welch's test is performed on the following statistic:

$$t = \frac{\bar{X}_+ - \bar{X}_-}{\sqrt{\frac{s_+^2}{N_+} + \frac{s_-^2}{N_-}}}$$

where s_+^2 and s_-^2 are the sample variances for positive and negative sera, respectively, and N_+ and N_- are the number of sera in each class. To perform the test, the number of degrees of freedom are estimated following the Welch-Satterthwaite equation:

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$$v = \frac{\left(\frac{s_+^2}{N_+} + \frac{s_-^2}{N_-}\right)^2}{\frac{s_+^4}{N_+^2 \cdot (N_+ - 1)} + \frac{s_-^4}{N_-^2 \cdot (N_- - 1)}}$$

The test is computed comparing the statistic to a Student's distribution with *v* degrees of freedom. If the resulting p-value is below 0.05, the null hypothesis is rejected which means that the two classes (positive and negative sera) can be discriminated at that titration.

c. A permutation test. The advantages of this test are the same as those of the statistical test based on the t-Student's distribution, and the adtional advantage that normallity is not assumed. In a permutation test, a statistic is computed from the positive and negative sera. Then, the pertenence of the sera to positive and negative groups is randomly permuted and the statistic is recomputed. This is repeated many times to provide a background distribution for the statistic. This is useful to determine the values this statistic can take just because of chance. Also, a p-value can be computed with this test. The statistic used in this work is.

$$P = \bar{X}_+ - \bar{X}_-$$

In the Montecarlo simulation, 10,000 simulations will be repeated for each sera selection strategy and discrimination metric. These simulations will be analyzed using a univariate Analysis of Variance (ANOVA) with the following factor, which is coherent with the 6 dilutions ordering considered in the ROC curves in Figure 9:

	C = 0.6	C = 0.7
D = 2	X1 = 1	X1 = 4
D = 3	X1 = 2	X1 = 5
D = 4	X1 = 3	X1 = 6

The objective (for a succesful titration) is to find the following ordering of the different dilutions: X1 = {3,2,6,1,5,4} or X1 = {3,2,1,6,5,4}, according to Figure 9.

4.a Ratio R

The LSD intervals plot corresponding to the ANOVA for the 5 selection estrategies are shown in the following figure. The correct ordering, X1 = {3,2,6,1,5,4} or X1 = {3,2,1,6,5,4}, should be found in the figures from right to left, so that X1=3 should be the one more to the right and X1=4 the one more to the left. Also, the percentage out of the 10,000 titration simulations that the 6 strategies are identified in order and the percentage that the best strategy (X1=3) is correcctly identified are especified below each figure. According to Figure 11, the Ratio index has similar results with the 5 selection strategies. Approximately, in 1/3 of the titration simulations, X1=3 is recognized as the best titration.

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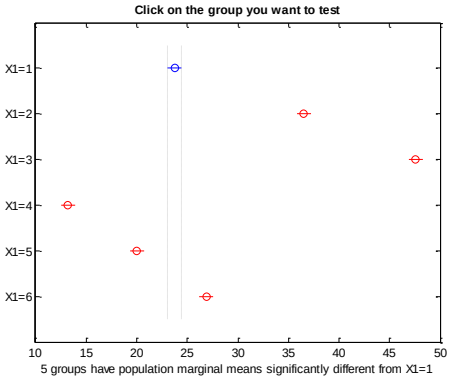
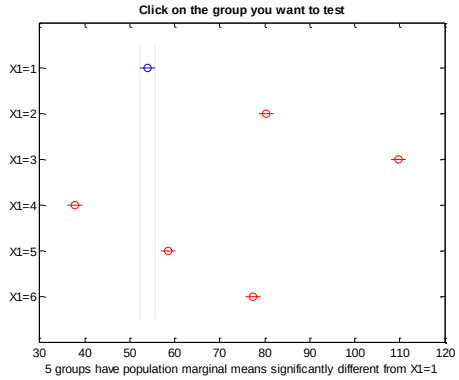
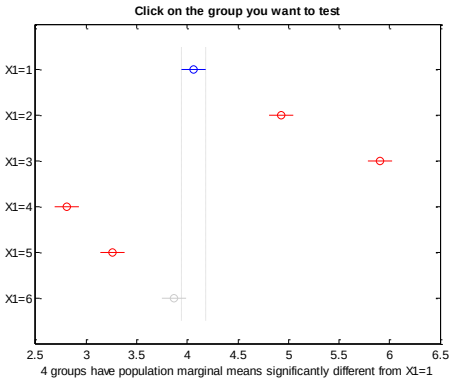


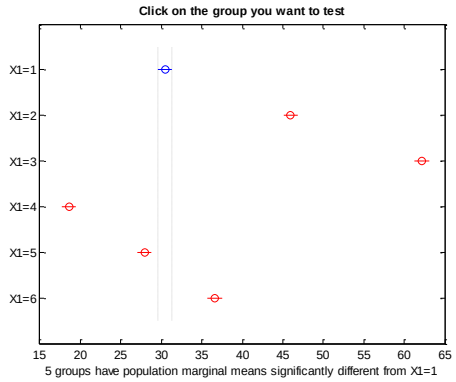
Centered (2.6/34.7 %)



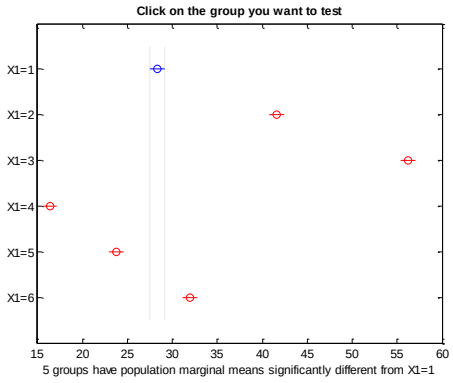
Clearest (1.8/31.1 %)



Least clear (0.9/30.9 %)



Homogeneus(2.1/35.1 %)



Homogeneus Centered (2.5/35.1 %)

Figure 11: LSD intervals plot for the 5 selection strategies and the Ratio as discrimination index.

4.b Welch's test

In this case, the true ordering X1 = {3,2,6,1,5,4} or X1 = {3,2,1,6,5,4} according to the ROC curves should be seen from left to right. According to Figure 12, the Welch's test should be used with the Centered selection strategy. In 40% of the titration simulations, X1=3 is recognized as the best titration. The LSD intervals tend to be more distorted with the Welch's test. Thus, selection strategies such as "Clearest", "Homogeneus" and "Homogeneus

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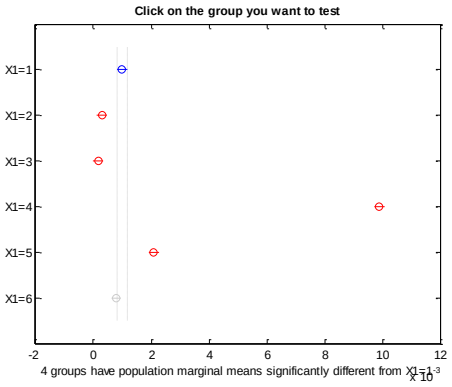
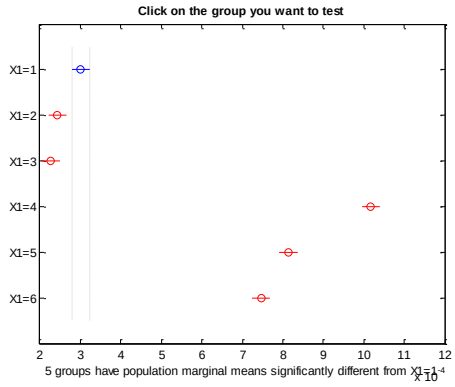
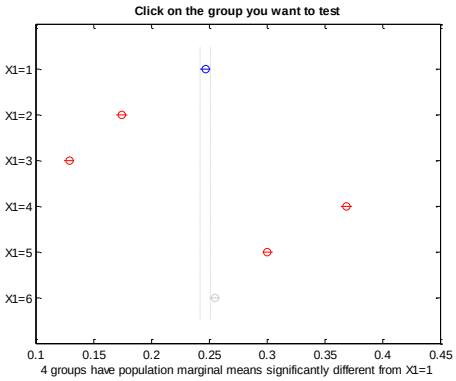
Centered" tend to penalize more changes in parameter C in the 4PL model than in parameter D. Also, there is a clear loss of performance in these strategies compared to Centered.

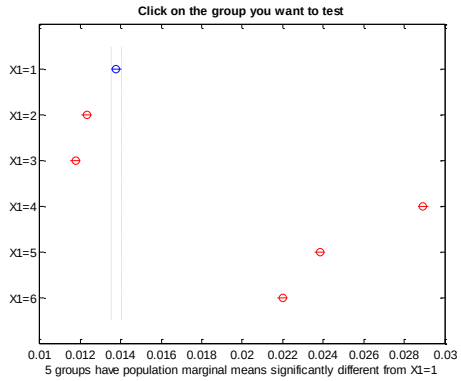




Centered (5.0/40.1 %)

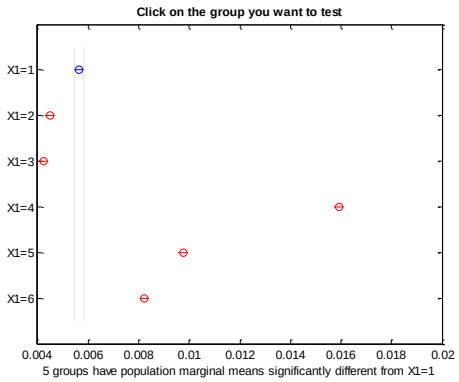
Clearest (1.2/22.7 %)





Least clear (2.0/35.9 %)

Homogeneus(3.0/26.7 %)



Homogeneus Centered (2.0/20.2 %)

Figure 12: LSD intervals plot for the 5 selection strategies and the Welch's test as discrimination index.

4.c Permutation test

Again, the true ordering X1 = {3,2,6,1,5,4} or X1 = {3,2,1,6,5,4} according to the ROC curves should be seen from left to right. In each test, 100 permutations are computed.

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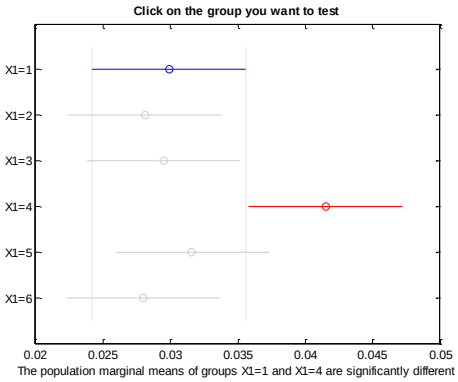
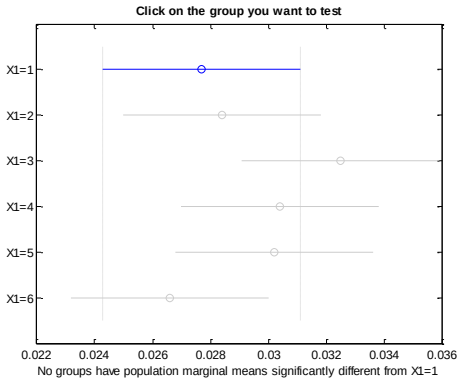
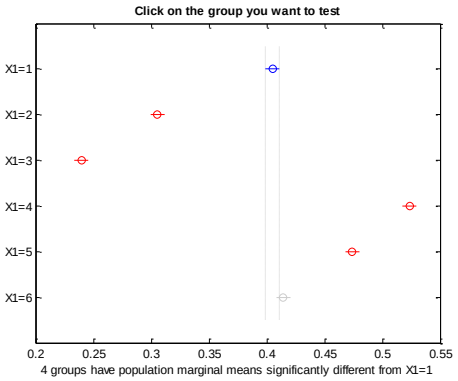
Because the permutation test is much more time consuming than the other two approaches, only 100 simulations are performed (instead of 10,000) except for the best selection strategy: Least Clear, for which 10,000 simulations are performed. According to the results, the permutation test should be used with the selection strategy least clear. Still, the Welch's test gives better results.



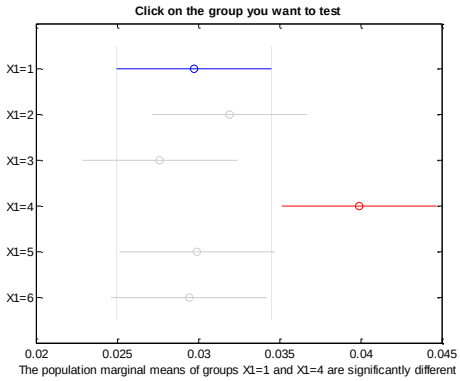
Centered (0/16 %)



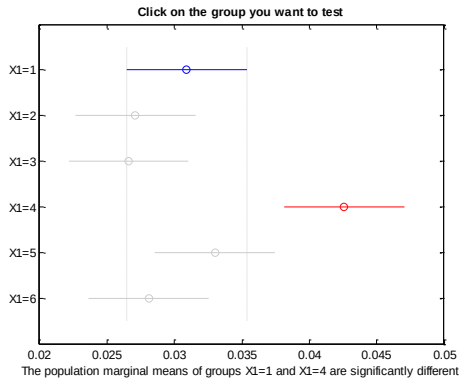
Clearest (0/12 %)



Least clear (0.9/30.1 %)



Homogeneus(0/21 %)



Homogeneus Centered (0/14 %)

Figure 13: LSD intervals plot for the 5 selection strategies and the permutation test as discrimination index.

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4.d Some additional questions

The results show that there are statistical significant differences among the selection strategies and also among the discrimination indices. This justifies the convenience of the present simulation. The sera should be selected according to the Centered strategy and the Welch's test is the most appropriate way to measure discrimination capability. Nevertheless, the convenient size of the sub-sample of sera for titration has not been studied yet, since all the experiments were performed for 4 positive against 4 negative sera (4:4). In this section, we study if the results of the test can be improved by using more sera and/or replicate measures. For this, a number of questions will be posed and investigated through Montecarlo simulations of 1,000 simulations. The numerical results are presented in the Tables below.

4.d.i Do we obtain better results with larger sub-samples?

Ratio: All strategies remain uniform and a slight improvement is found.

Sub-sample	Centered	Clearest	Least clear	Homogeneus	Hom. Cent.
3:3	2.0/33.1	1.1/27.4	1.1/29.0	3.1/34.1	1.9/32.8
4:4	3.4/36.1	1.2/32.7	1.1/26.5	2.3/34.2	3.1/35.1
5:5	3.8/39.6	2.8/35.6	1.5/31.6	1.9/35.0	2.5/39.5
6:6	4.8/41.4	2.7/36.7	2.4/33.3	3.7/40.0	2.7/39.4
7.7	4.8/41.7	2.5/38.8	2.8/39.0	4.4/42.4	4.8/41.7
8:8	5.9/49.2	4.7/41.2	3.4/40.4	4.2/46.2	5.6/47.1

Welch: There is a slight improvement.

Muestra	Centered	Clearest	Least clear	Homogeneus	Hom. Cent.
3:3	2.5/38.2	1.0/22.7	1.2/34.7	2.2/23.8	2.2/18.4
4:4	5.4/40.2	1.1/22.5	2.4/36.4	2.7/28.0	2.0/20.3
5:5	4.7/38.7	1.0/22.9	3.3/43.3	4.6/30.4	2.3/23.8
6:6	6.3/37.2	1.7/23.3	3.1/48.0	3.7/32.3	3.0/25.5
7.7	7.6/43.0	2.3/26.1	4.5/50.6	5.6/34.9	4.3/29.2
8:8	5.5/38.2	2.7/28.7	5.6/50.0	5.4/40.3	4.3/32.2

Permutation: There is no clear improvement but a worsening. Only the selection strategy Least Clear works correctly. This is the only strategy considered from this point onwards for the permutation test.

Muestra	Centered	Clearest	Least clear	Homogeneus	Hom. Cent.
4:4	0.2/16.8	0.3/17.9	1.2/31.3	0/17.0	0.2/16.5
5:5	0/16.1	0/13.5	1.3/35.5	0/12.7	0.2/12.7
6:6	0/3.6	0/3.5	1.6/37.5	0/2.8	0/3.1
7.7	0/0	0/0.4	1.5/34.9	0/0.4	0/0.4
8:8	0/0	0/0.1	2.1/25.8	0/0	0/0

4.d.ii Do we obtain better results with a skew sub-sample, with more positive sera?

Ratio: No clear improvemet.

Muestra	Centered	Clearest	Least clear	Homogeneus	Rep. Cent.
4:4	3.8/38.2	2.1/29.0	0.9/23.0	2.3/33.2	2.4/34.6
5:3	2.0/36.9	0.9/29.5	0.9/21.5	1.9/33.3	1.7/33.1
6:2	1.3/33.4	1.0/28.4	0.4/20.7	1.1/31.9	1.3/33.0
7.1	2.5/37.0	1.1/34.7	0.8/22.4	1.0/36.0	1.0/39.4

Welch: No clear improvement.

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Muestra	Centered	Clearest	Least clear	Homogeneus	Rep. Cent.
4:4	4.3/41.8	1.6/21.6	2.0/35.9	2.9/26.8	1.6/20.3
5:3	7.0/48.2	3.4/36.8	1.7/28.8	4.4/37.4	3.4/37.0
6:2	3.1/40.0	2.5/33.2	0.6/23.6	2.9/34.0	1.8/33.1
7.1	2.2/38.2	0.9/31.4	0.3/11.3	1.7/31.2	1.7/32.3

Permutación: There is a clear worsening.

	4:4	5:3	6:2	7:1
Menos	1.2/31.4	0.8/25.2	0.5/17.6	0.1/9.6

4.d.ii Do we obtain better results introducing replicates?

Ratio: The replicates improve the performance. 2 replicates are similar to a 8:8 experiment.

Replicates	Centered	Clearest	Least clear	Homogeneus	Rep. Cent.
1	2.0/36.7 %	1.3/32.8 %	0.7/23.7 %	2.2/32.7 %	2.5/33.1 %
2	6.5/47.1 %	2.2/39.7 %	2.2/33.4 %	4.4/46.8 %	4.7/45.7 %
3	9.9/57.4 %	3.4/47.7 %	3.8/39.8 %	8.2/53.5 %	9.1/57.5 %
4	10.5/62.4 %	5.7/54.2 %	4.2/44.2 %	9.6/61.1 %	10.2/61.9 %

Welch: The replicates improve the performance.

Replicates	Centered	Clearest	Least clear	Homogeneus	Rep. Cent.
1	5.2/38.6 %	1.0/24.1 %	2.9/37.2 %	2.9/26.6 %	1.6/22.1 %
2	9.9/47.7 %	1.7/24.9 %	4.1/45.5 %	5.6/34.7 %	5.1/29.7 %
3	14.5/52.7 %	2.8/28.3 %	5.5/55.0 %	6.2/37.4 %	8.0/33.6 %
4	20.2/58.4 %	2.8/28.5 %	8.8/60.8 %	8.4/40.2 %	8.8/36.7 %

Permutación: There is no clear improvement.

	1	2	3	4
Menos	1.6/30.5 %	0.7/34.5 %	1.0/31.4 %	2.1/26.5 %

4.e Some final experiments

To confirm the previous results, a last experiment with 10,000 simulations and the following strategies will be performed:

1. Ratio – Centered, 4:4
2. Ratio – Centered, 8:8
3. Ratio – Centered, 2 Replicates
4. Welch – Centered, 4:4
5. Welch – Centered, 8:8
6. Welch – Centered, 2 Replicates

In this case, a different comparison strategy will be used. To compare the 6 approaches, the number of coincident elements with the ordering in the ROC curves (X1 = {3,2,6,1,5,4} or X1 = {3,2,1,6,5,4}) is computed from 0 (if the best titration identified is not X1=3) to 6 (if the correct order is completely identified). This value is the one compared in the ANOVA. The result is shown in the following figure (notice that the factor X1 in the figure corresponds to the six approaches listed above). The best approach is to use a Welch's test with replicates and 4:4 sera.

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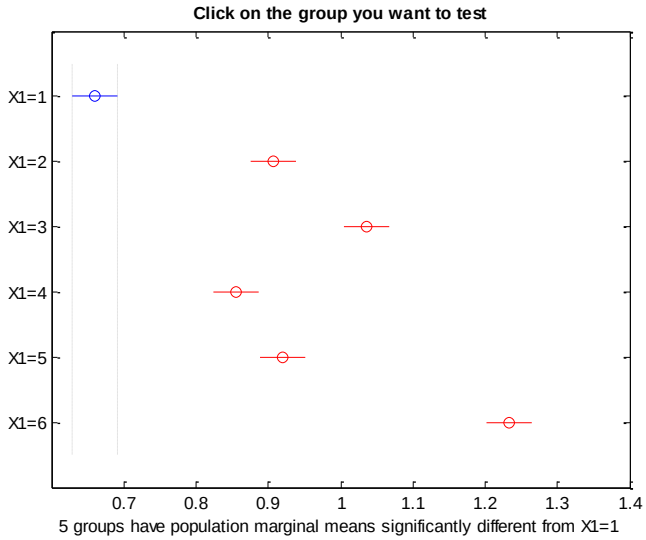

Figure 14: LSD intervals plot for the comparison among the 6 strategies: X1 from 1 to 3 employ the Ratio index for titration experiments 4:4, 8:8 and 4:4 with two replicates, respectively, and X1 from 4 to 6 the same with the Welch's test. There are statistically significant differences between the Welch's test 4:4 with 2 replicates (X1=6 and the best approach) and the rest.

4.f Discusion and Conclusion

According to the results in the Montecarlo simulation, the conclusions are:

- The best procedure to compare positive and negative sera in a titration experiment is the Welch's test.
- If a previous indication of the concentration of antibody in the sera is available, the sub-sample for titration should be selected so that positive and negative sera are the closest to the class mean.
- The selection of 4:4 sera is appropriate, but the introduction of replicates is very convenient.

There are several comments on these conclusions. Firstly, these results have been also found for other Montecarlo simulations, with different ROC curves associated. Nevertheless, it is not clear which of these are dependent on the specific conditions considered or the way to design the Montecarlo experiment. Thus, the conclusions should be understood as indications rather than strong and definitive statements. Secondly, the objetive in the Montecarlo experiment was to obtain a procedure to identify the best antigen concentration among a number of possible ones. Nevertheless, in real situations to find one or several antigen concentrations where positive and negative sera can be dishtinguished is more than enough. For this, the Welch's test is also appropriate. Furthermore, it should be noted that with a 4:4 titration, 2 replicates and the Welch's test less that 50% of the times the best concentration is identified. Therefore, with such a titration experiment it cannot be guarantized that the lowest p-value is achieved by the best concentration. Still, those concentration where positive and negative sera are discriminated can be sucessfully found. Finally, the fact that the Centered selection criteria presented nice results is quite convenient. This is because this approach affects very little the confidence in the result of the Welch test. Contrarily, the selection strategy "Clearest" may yield too optimistic results in the Welch's test, rejecting the null hypothesis even in cases when it should not be rejected.

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5 Experimental validation and illustration of the titration protocol

The experimental validation of the portocol has been performed with Tpp15. More than a detailed validation, this section should be seen as an ilustration of the titration protocol followed in Rekom Biotech S. L.

Tpp15 recombinant antigen was over-produced in the heterologous expression system *Escherichia coli* and it was then purified to homogeneity. Sera used to perform the experiments were pre-validated as syphilis positive sera by ELISA (Abbott: Architeck), TPHA (Spin React) and RPR (BectonDickinson) assays.

ELISA measurement. Microtiter plates were coated with different antigen concentration solutions ranged between 1.55 μg/ml and 0.20 μg/ml and prepared in phosphate-buffered saline (PBS). Plates were incubated overnight at room temperature, blocked with newborn calf serum, washed, and then incubated with sera from syphilis patients or healthy controls in PBS containing newborn calf serum for 45 min at 37°C. After a wash, a 1:100,000 or 1:50,000 dilution of peroxidase conjugated goat anti-human immunoglobulin G (IgG) was added and incubated at 37°C for 30 min. Finally, the peroxidase reaction was visualized by using a tetramethylbenzidine-hydrogen peroxide solution as a substrate (Neogen Corporation).

First of all, to decide a working point for the titration, we need to estimate a convenient concentration of sera and also a convenient titration area for the antigen. For this, a checkerboard titration experiment with few measurements is used. In this experiment, the OD of a single positive and negative sera are obtained for different dilutions of sera and antigen. The result for Tpp15 are the following:

Serum ++	**DO 450/620**						
		1/10	**1/20**	**1/40**	**1/80**	**1/160**	**PBS 1x**
20 ng/μl	**A**	2.513	2.253	1.923	1.449	0.99	0.022
10 ng/μl	**B**	2.281	2.009	1.761	1.274	0.848	0.016
1 ng/μl	**C**	2.008	1.811	1.449	0.971	0.557	0.016
0.75 ng/μl	**D**	2.179	1.846	1.413	0.952	0.566	0.025
Dil sueros	**E**	0.116	0.084	0.069	0.04	0.038	0.028

Serum --	**DO 450/620**						
		1/10	**1/20**	**1/40**	**1/80**	**1/160**	**PBS 1x**
20 ng/μl	**A**	0.859	0.543	0.291	0.185	0.112	0.026
10 ng/μl	**B**	0.831	0.534	0.44	0.332	0,197	0.021
1 ng/μl	**C**	0.243	0.158	0,096	0.08	0,089	0.021
0.75 ng/μl	**D**	0.187	0.136	0,103	0.067	0,049	0.028
Dil sueros	**E**	0.121	0.097	0.063	0.061	0,051	0.029

Computing the ratio of the measurements of the positive serum with the measurements of the negative serum the following values are obtained:

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		1/10	1/20	1/40	1/80	1/160	PBS 1x
20 ng/µl	A	2.925494761	4.14917127	6.60824742	7.83243243	8.83928571	0.84615385
10 ng/µl	B	2.74488568	3.76217228	4.00227273	3.8373494	4.30456853	0.76190476
1 ng/µl	C	8.263374486	11.4620253	15.09375	12.1375	6.25842697	0.76190476
0.75 ng/µl	D	11.65240642	13.5735294	13.7184466	14.2089552	11.5510204	0.89285714
Dil sueros	E	0.958677686	0.86597938	1.0952381	0.6557377	0.74509804	0.96551724

The highest ratio (maximum discrimination power) is obtained for antigen concentration 0,75 ng/ul. Observing also the row for 1 ng/ul, a good dilution for the sera is 1/40.

Now the working area is set, the same experiment as in the Montecarlo simulation will be repeated. Firstly, the ROC curves, using as ground-truth, are computed from 20 positive sera and 20 negative sera. The concentrations of antigen considered are 0,5 ng/ul, 0,75 ng/ul y 1 ng/ul, being the sera dilution 1/40. These ROC curves are computed for validating the protocol, but they are not customary computed for titration.

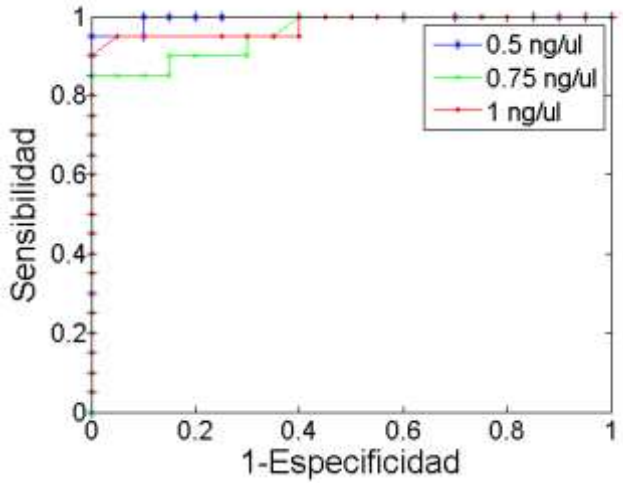

Figure 15: ROC curves for the Tpp15 experiment.

Figure 15 shows that the performance of the antigen is excelent for the three concentrations. As a matter of fact, differences are only found due to a single positive serum out of the total 20 which presents lower OD value than several negative sera. Because of this, we can consider that the three dilutions attain an adequate performance.

According to the Montecarlo simulation, the titration will be evaluated with the Welch's test and using 4:4 sera with 2 replicates. The following figure presents the result:

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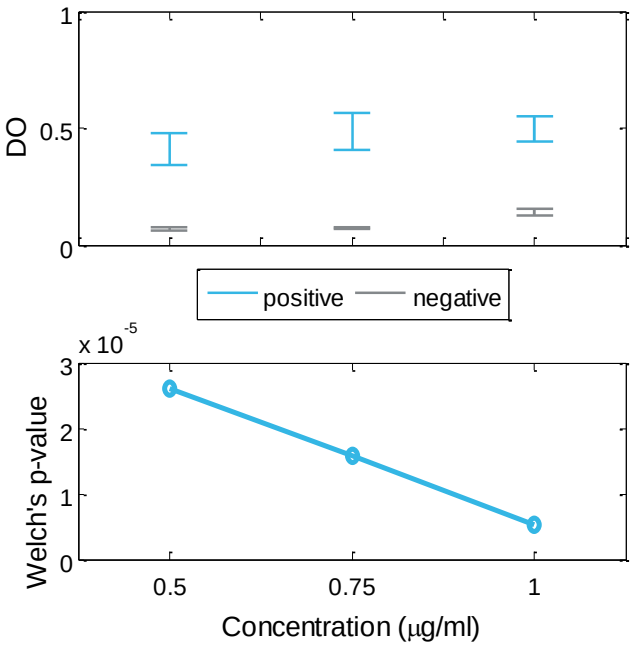

Figure 16: At the top, 95% confidence intervals for discrimination using the Welch's test. At the bottom, the p-value of the test.

At the bottom of Figure 16 the p-values of the Welch's test for the three antigen concentrations are shown. The corresponding intervals are shown at the top. If for a given concentration the p-value is below 0.05, or equivalently the intervals do not overlap, it means that the concentration is adequate to distinguish between positive and negative sera. As it can be seen, the result is coherent with the ROC curves, validating the protocol. In a normal titration experiment in Rekom Biotech, only the checkerboard titration and the experiment in the last figure (4:4 with two replicates) is performed.

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ANNEXED: Tpp15, lot: 11RAG0009001

Assay 1

Date: 10/05/07 Cod: **10052011#1** Conjugate dil: 1:100,000 Assay: IgG

Description: Initial assay for the recombinant antigen for syphilis detection Tpp15 lot 11RAG0009001. It was tested with four syphilis positive sera and four negative sera. Four dilutions of the recombinant antigen around 0.25 μg/ml, were tested. Positive sera were pre-validated as syphilis positive sera by ELISA (Abbott: Architeck), TPHA (Spin React) and RPR(BectonDickinson) assays.

Sera Nº	1	2	3	4	5	6	7	8	9	10	11	12
A	24	24	24	24	24	24	24	24	24	24	24	24
B	47	47	47	47	47	47	47	47	47	47	47	47
C	73	73	73	73	73	73	73	73	73	73	73	73
D	275	275	275	275	275	275	275	275	275	275	275	275
E	13	13	13	13	13	13	13	13	13	13	13	13
F	25	25	25	25	25	25	25	25	25	25	25	25
G	41	41	41	41	41	41	41	41	41	41	41	41
H	53	53	53	53	53	53	53	53	53	53	53	53
	1/1000			1/2000			1/3000			1/4000		

OD 450/620	1	2	3	4	5	6	7	8	9	10	11	12
A	0.203	0.136	0.087	0.071	0.077	0.06	0.073	0.135	0.096	0.074	0.066	0.104
B	0.612	0.486	0.572	0.435	0.51	0.43	0.33	0.364	0.162	0.117	0.111	0.076
C	0.718	0.615	0.875	0.628	0.656	0.676	0.347	0.335	0.173	0.151	0.148	0.135
D	1.725	1.734	2.09	1.628	1.622	1.332	0.973	0.897	0.83	0.586	0.447	0.564
E	0.122	0.094	0.085	0.07	0.121	0.887	0.109	0.119	0.073	0.053	0.052	0.044
F	0.401	0.064	0.168	0.108	0.118	0.102	0.139	0.095	0.074	0.054	0.052	0.058
G	0.237	0.096	0.068	0.001	0.051	0.056	0.065	0.067	0.071	0.063	0.052	0.053
H	0.204	0.153	0.026	0.087	0.057	0.033	0.047	0.057	0.046	0.044	0.039	0.049
	1/1000			1/2000			1/3000			1/4000		

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Tpp15, lot: 11RAG0009001

Assay 2

Date: 10/05/07 Cod: **10052011#1** Conjugate dil: 1:50,000 Assay: IgG

Description: The assay is exactly the same as the last one but the conjugated was risen to a 1:50,000-dilution in order to increase the signal.

Sera Nº	1	2	3	4	5	6	7	8	9	10	11	12
A	24	24	24	24	24	24	24	24	24	24	24	24
B	47	47	47	47	47	47	47	47	47	47	47	47
C	73	73	73	73	73	73	73	73	73	73	73	73
D	275	275	275	275	275	275	275	275	275	275	275	275
E	13	13	13	13	13	13	13	13	13	13	13	13
F	25	25	25	25	25	25	25	25	25	25	25	25
G	41	41	41	41	41	41	41	41	41	41	41	41
H	53	53	53	53	53	53	53	53	53	53	53	53
	1/1000			1/2000			1/3000			1/4000		

OD 450/620	1	2	3	4	5	6	7	8	9	10	11	12
A	0.18	0.098	0.13	0.078	0.108	0.1	0.079	0.089	0.173	0.094	0.097	0.143
B	0.756	0.679	0.709	0.557	0.593	0.537	0.588	0.612	0.568	0.477	0.551	0.529
C	1.73	1.176	1.25	0.919	0.869	1.036	0.825	0.902	0.767	0.737	0.715	0.728
D	2.964	2.985	2.901	2.485	2.442	2.524	2.396	2.29	2.453	1.975	2.146	2.088
E	0.094	0.075	0.082	0.054	0.059	0.08	0.062	0.074	0.051	0.065	0.074	0.094
F	0.081	0.059	0.058	0.052	0.042	0.054	0.068	0.059	0.039	0.089	0.073	0.06
G	0.091	0.056	0.067	0.041	0.045	0.047	0.044	0.066	0.037	0.087	0.068	0.061
H	0.087	0.043	0.055	0.041	0.034	0.035	0.05	0.046	0.041	0.07	0.052	0.043
	1/1000			1/2000			1/3000			1/4000		

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Tpp15, lot: 11RAG0009001

Assay 3

Date: 25/05/11 Cod: **25052011#1** Conjugate dil: 1:50,000 Assay: IgG

Description: Plates were coated with a higher concentration of the recombinant antigen (around 1 μg/ml), maintaining the conjugated dilution in 1:50,000. One of the positive sera seemed not be positive for the recombinant antigen Tpp15, therefore, it was replaced by sera Nº 19, previously validated by ELISA(Abbott: Architeck), TPHA(Spin React) and RPR(BectonDickinson) as a positive serum for syphilis.

Sera Nº	1	2	3	4	5	6	7	8	9	10	11	12
A	19	19	19	19	19	19	19	19	19	19	19	19
B	47	47	47	47	47	47	47	47	47	47	47	47
C	73	73	73	73	73	73	73	73	73	73	73	73
D	275	275	275	275	275	275	275	275	275	275	275	275
E	13	13	13	13	13	13	13	13	13	13	13	13
F	25	25	25	25	25	25	25	25	25	25	25	25
G	41	41	41	41	41	41	41	41	41	41	41	41
H	53	53	53	53	53	53	53	53	53	53	53	53
	1/500			1/750			1/1000			1/1500		

OD 450/620	1	2	3	4	5	6	7	8	9	10	11	12
A	1.252	1.342	1.469	1.485	1.46	1.464	1.193	1.043	1.157	0.978	1.174	1.106
B	1.425	1.37	1.144	1.301	1.196	1.47	1.362	1.304	1.233	1.079	1.191	1.203
C	1.479	1.678	1.455	1.609	1.484	1.418	1.333	1.529	1.474	1.325	1.587	1.395
D	3.513	3.555	3.53	3.52	3.46	3.415	3.371	3.468	3.606	3.549	2.914	3.523
E	0.121	0.114	0.153	0.172	0.103	0.103	0.105	0.286	0.24	0.177	0.157	0.274
F	0.163	0.122	0.153	0.214	0.149	0.23	0.215	0.125	0.3	0.196	0.167	0.017
G	0.109	0.124	0.108	0.181	0.276	0.129	0.172	0.216	0.309	0.256	0.226	0.183
H	0.175	0.084	0.134	0.126	0.087	0.188	0.137	0.285	0.206	0.159	0.217	0.133
	1/500			1/750			1/1000			1/1500		

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