Technical Reports

# Multi-epitope chimeras as a syphilis IVD working pair (RAG0046/RAG0046BIOT) for IgG+IgM antibody detection by a double-antigen sandwich (DAS) immunoassay format.

**THE AIM OF THIS STUDY IS THE ANALYSIS OF THE ANTIGENIC CAPACITY OF THE MULTI-EPITOPE BIOMARKERS RAG0046 AND RAG0046BIOT AS A WORKING PAIR IN A DOUBLE-ANTIGEN SANDWICH IMMUNOASSAY, TO DETECT SIMULTANEOUSLY IgG + IgM ANTIBODIES IN A SYPHILIS LAST GENERATION IVD TEST**

Syphilis is a multistage progressive disease caused by the spirochete *Treponema pallidum* subsp. *pallidum* and is characterized by localized, disseminated, and chronic stages. *T. pallidum* research, including the identification of antigens, has been hindered by the inability to culture the bacterium continuously *in vitro*, requiring the propagation of organisms by experimental rabbit infection.

The serological tests used most often are the nontreponemal tests (the Venereal Disease Research Laboratory test and the rapid plasma reagin test, RPR) and the treponemal tests (microhemagglutination assay for *Treponema pallidum,* MHATP, and fluorescent treponemal antibody absorption test, FTAAbs). The older methods, such as the RPR test, use phospholipid (nontreponemal) antigens. These are generally used to screen large numbers of samples. However, they are nonspecific and react with lipoid antigens resultant from cellular destruction or from other treponemal species, and as a consequence, false-positive reactions may occur. Newer serologic tests use specific *T. pallidum* antigens. When the sensitivities of the ELISA, RPR test, and MHA-TP were compared by using the FTA-Abs test as the standard, the ELISA technique had a higher sensitivity and specificity.

Moreover, it has been described innumerable times in bibliography that the use of selected antigens such as recombinant proteins may prevent many false-reactive results due to other, less specific polypeptides. Thus, recombinant antigens can favourably replace whole *T. pallidum* extracts in human syphilis diagnosis and thus avoid the use of live animals in the production of treponemal antigens.

*T. pallidum* possesses several abundant integral membrane lipoproteins. Among them, lipoprotein Tpp17 has been described as highly immunogenic. In order to take advantage of the antigenic determinants of this antigen, Rekom Biotech has performed the design and production of a new recombinant chimera formed by fusion of several epitopes from this and other syphilis lipoproteins biomarkers. In the design, it has been considered as an essential part of the chimeric multi-epitope biomarker, the independent folding of all the domains, formed each by the antigenic determinants of every selected protein (figure 1).

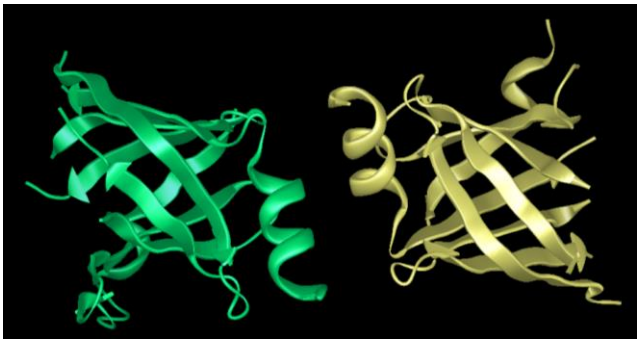

**Figure 1.** Tpp17 3D structure.

Different design strategies were used in order to get the recombinant chimera, our aims were (i) to obtain a multi-epitope syphilis biomarker, with highly antigenic characteristics and (ii) also, and not less important, a recombinant protein with good physical-chemical characteristics in order to get a soluble and versatile molecule, easy to manipulate and to conjugate. After examination 3D structures and physical-chemical characteristics of each selected domain separately, two strategies were carried out, obtaining two different recombinant chimeras (Chim1 and Chim2). In the production of recombinant multi-epitope antigens, the selected domains are very important but also the order of each domain in the whole protein and the linkers between every pair of domains, their composition



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and length. Each domain should be separately enough from the rest, to perform a good folding in order to maintain all the conformational epitopes. Also, each antigenic determinant should be able to interact with the antibodies with its proper orientation and without steric hindrance problems.

Several expressions and solubilization studies were performed with the recombinant chimeras in order to obtain a high production yield and a good solubility profile (figure 2-A). The recombinant chimeras were purified to homogeneity (figure 2-B) and afterwards several ELISA analyses were performed.

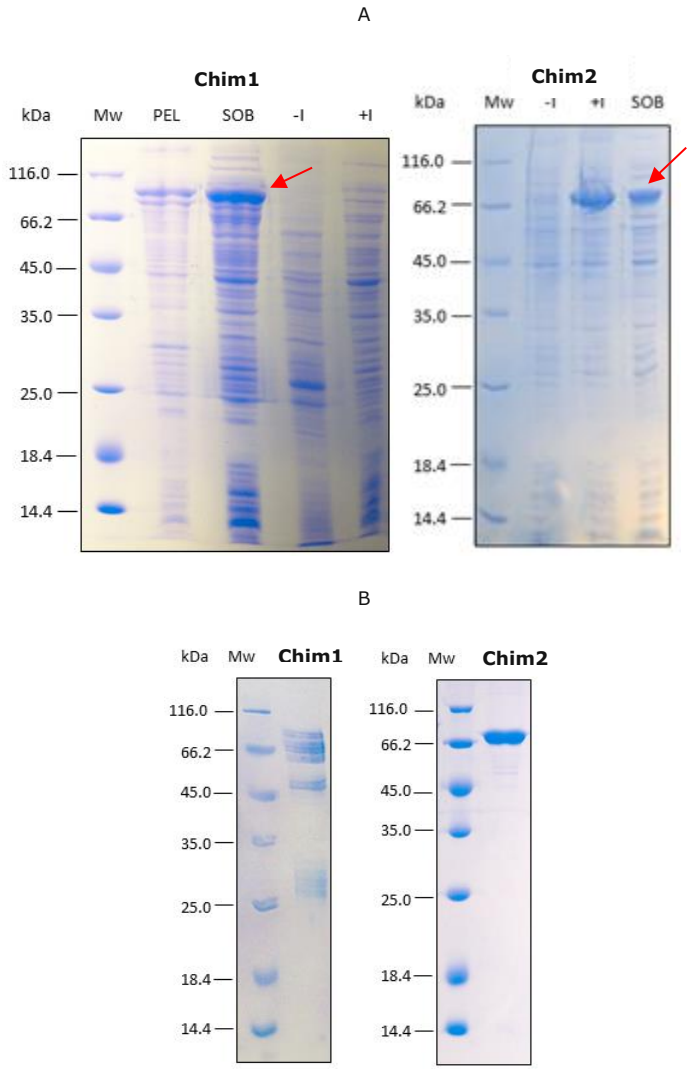


**Figure 2.** SDS-PAGE analysis of both chimeras produced from two different design strategies: A) expression of the recombinant chimeras for syphilis: (-I) no induction of the heterologous gene in the culture; (+I) induction of the target gene expression in the culture; (SOB) presence of the recombinant chimera in the soluble fraction of the culture, the clarified extract; (PEL) presence in the pellet fraction. B) both proteins purified to homogeneity.

Chim2 seemed to be more stable to degradation, as it can be seen once the protein has been purified. The protein produced by the strategy 1 appeared to be unstable, sensible to proteases and a bit insoluble (as it can be seen in the pellet fraction).

Also, as we were interested in their performance in the discrimination between positive and negative syphilis specimen sera, both proteins were analysed by an in-house ELISA assay, in order to compare both.

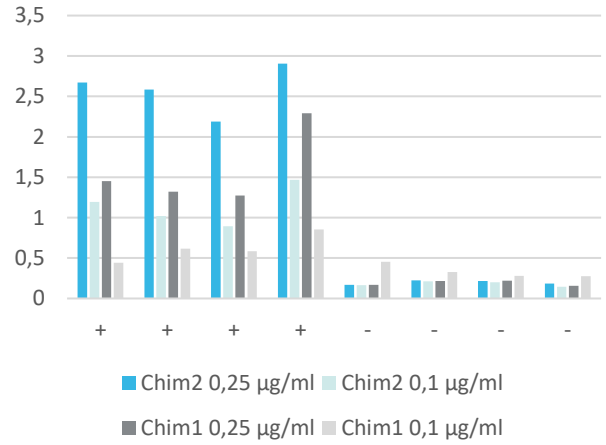


**Figure 3**. IgG indirect ELISA assay of Chim1 vs Chim2. Two different concentrations were used from Chim1 and Chim2 in order to discriminate between positive and negative syphilis specimen sera pre-validate by using Architeck ELISA commercial assay.

Chim2 showed more reactivity with positive sera and lower background with negative specimen sera. Maybe this is an effect of the initial degradation of Chim1 as it is more unstable than Chim2 or maybe the domains in Chim2 are positioned in a better way, with the suited orientation to interact with the specific antibodies.

Based on these results, the following ELISA studies were performed only with the Chim2 antigen (RAG0046), as this protein proved to be more stable and reactive than Chim1 (figure 4).



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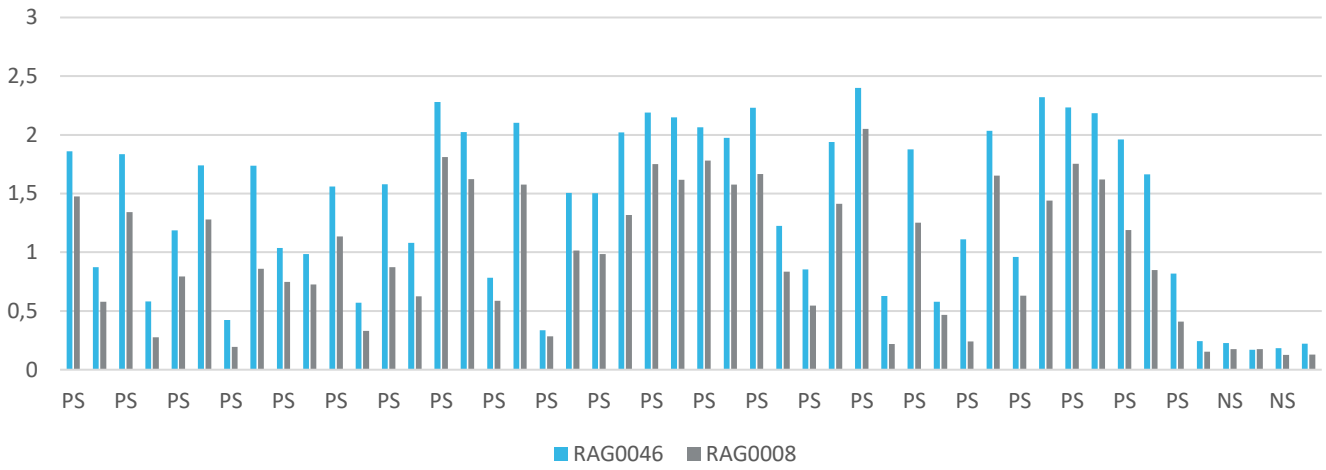


**Figure 4**. Indirect IgG ELISA assay. In order to coat the antigens to the plate in equimolecular proportions, Tpp17 was coated at 0.3 μg/ml and the chimera was coated at 0.4 μg/ml. The sera were used at 1/20 dilution and the anti-IgG conjugated was used at 1/100,000 titer.

In the IgG indirect ELISA assay performed, Tpp17 (RAG0008) was also tested as reference antigen for our new recombinant chimera (RAG0046).

To carry out a third generation ELISA by performing a double-antigen sandwich ELISA assay (DAS-ELISA), this recombinant chimera was prepared also as a conjugated protein with biotin. This syphilis biomarker (RAG0046BIOT) was also purified to homogeneity and a western blot with streptavidin was performed to control the presence of biotin in the protein (figure 5).

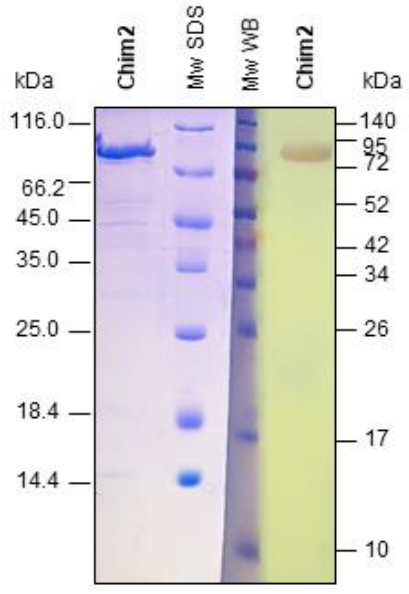


**Figure 5.** Western blot analysis of RAG0046BIOT to detect streptavidin/biotin reaction. The incubation was performed with HRP conjugated streptavidin (1/2500).

The working pair formed by the two recombinant multi-epitope syphilis biomarkers (RAG0046/RAG0046BIOT) was used to carry out a DAS-ELISA assay which is outlined in figure 6.

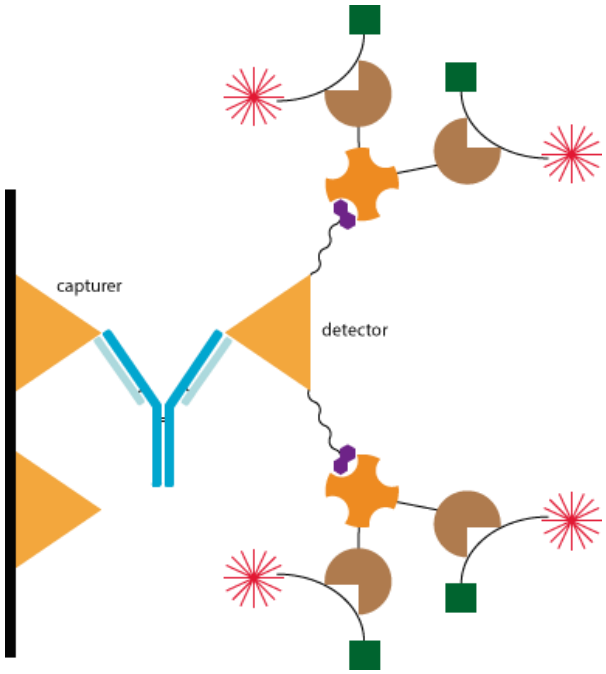


**Figure 6.** Scheme of a double-antigen sandwich ELISA (DAS).

This kind of format allows the detection at the same time of IgGs and IgMs, being highly sensitive. Moreover, as the antigen is used at the same time as a capturer of the antibody, and as detector (conjugated antigen), the antibody should bind an identical epitope in both antigen molecules at the



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same time, being this method highly specific, although it can have at the same time some steric hindrance problems.

Plates were coated by the non-biotinylated antigen at a concentration of 0.5 μg/ml and different concentrations of the biotinylated antigen were used as a detector with streptavidin. Again, several pre-validated sera by Architeck commercial ELISA test, were used in these analyses at 1/20 dilution (figure 7). The detection complex is the one formed by the biotinylated antigen plus HRP-streptavidin.

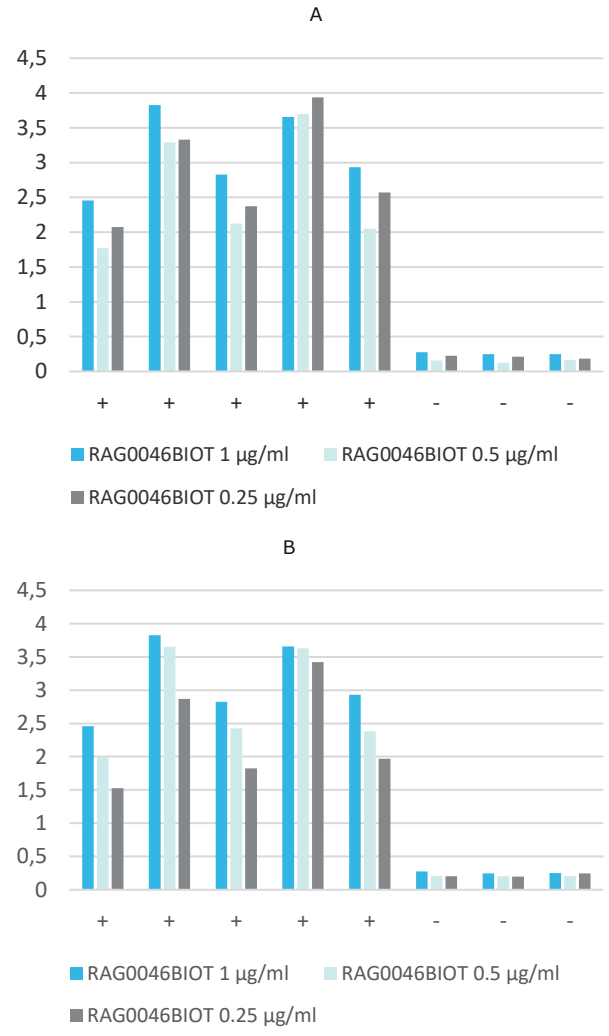


**Figure 7.** DAS-ELISA assay with different concentrations of RAG0046BIOT. RAG0046 was coating the plate at 0.5 μg/ml and different concentrations of RAG0046BIOT were used as detector. HRP-streptavidin was used at 1/10,000 dilution. A) RAG0046BIOT and HRP-strep were preincubated at RT for 1 h and the detector complex was used once formed; B) no pre-formed complex was used, first RAG0046BIOT was incubated on the plates and afterwards, the HRP-strep was added.

The comparison between the pre-formed detection complex and no pre-formed complex is shown in figure 8.

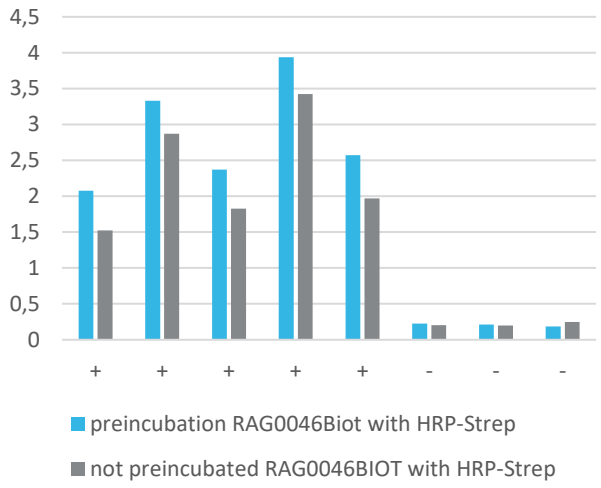


**Figure 8.** DAS-ELISA assay of pre-incubated detector complex vs non detector complex. RAG0046 was coating the plate at 0.5 μg/ml and RAG0046BIOT was used as detector at the concentration of 0.25 μg/ml. HRP-streptavidin was used at 1/10,000 dilution and sera at 1/20 dilution. The graphic shows the reactivity with positive and negative specimen sera of the pre-formed RAG0046BIOT-HRP-strep vs the non-formed detection complex.

## CONCLUSION

Our construction Chim2 proved to be better than Chim1 regarding stability, solubility and reactivity in ELISA assays discriminating syphilis positive and negative specimen sera. Also, comparative studies by an indirect IgG ELISA of the Chim2 vs Tpp17 showed a high sensitivity of our multi-epitope biomarker. At the same time, with the same multi-epitope antigen but biotinylated, we developed an in-house DAS-ELISA assay to detection at the same time of IgG+IgM.

Our biomarkers RAG0046 and RAG0046BIOT form a perfect working pair to develop a third generation ELISA based in a DAS-ELISA format, which is improving sensitivity not only by detecting at the same time IgG+IgM, but also due to the wide spectrum of epitopes located at these biomarkers´ structure. No steric hindrance problems seem to be arisen during the assay development.



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Moreover, we have analysed if there is any advantage by pre-incubating the detector RAG0046BIOT with the HRP-streptavidin previously at the assay development and we have found that positive/negative sera discrimination is nearly not modified (figure 7). There are slightly higher signals in the case of the preincubation and the procedure is quicker (45 min shorter), as the detector complex can be formed previously (figure 8).

Finally, these two proteins are very soluble and show very good physico-chemical characteristics therefore we can already foresee that they will be very stable in plastic surfaces. Antigenicity and stability of the biomarkers are properties very appreciated by IVD test manufacturers and strengthen the antigen versatility.

Also, by several performed in-house DAS-ELISA assays, we have demonstrated that both proteins have a very good reactivity with no steric hindrance in their interaction with antibodies at the same time, been a perfect working pair for third generation ELISA or other immunoassays with a double-antigen sandwich format.



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