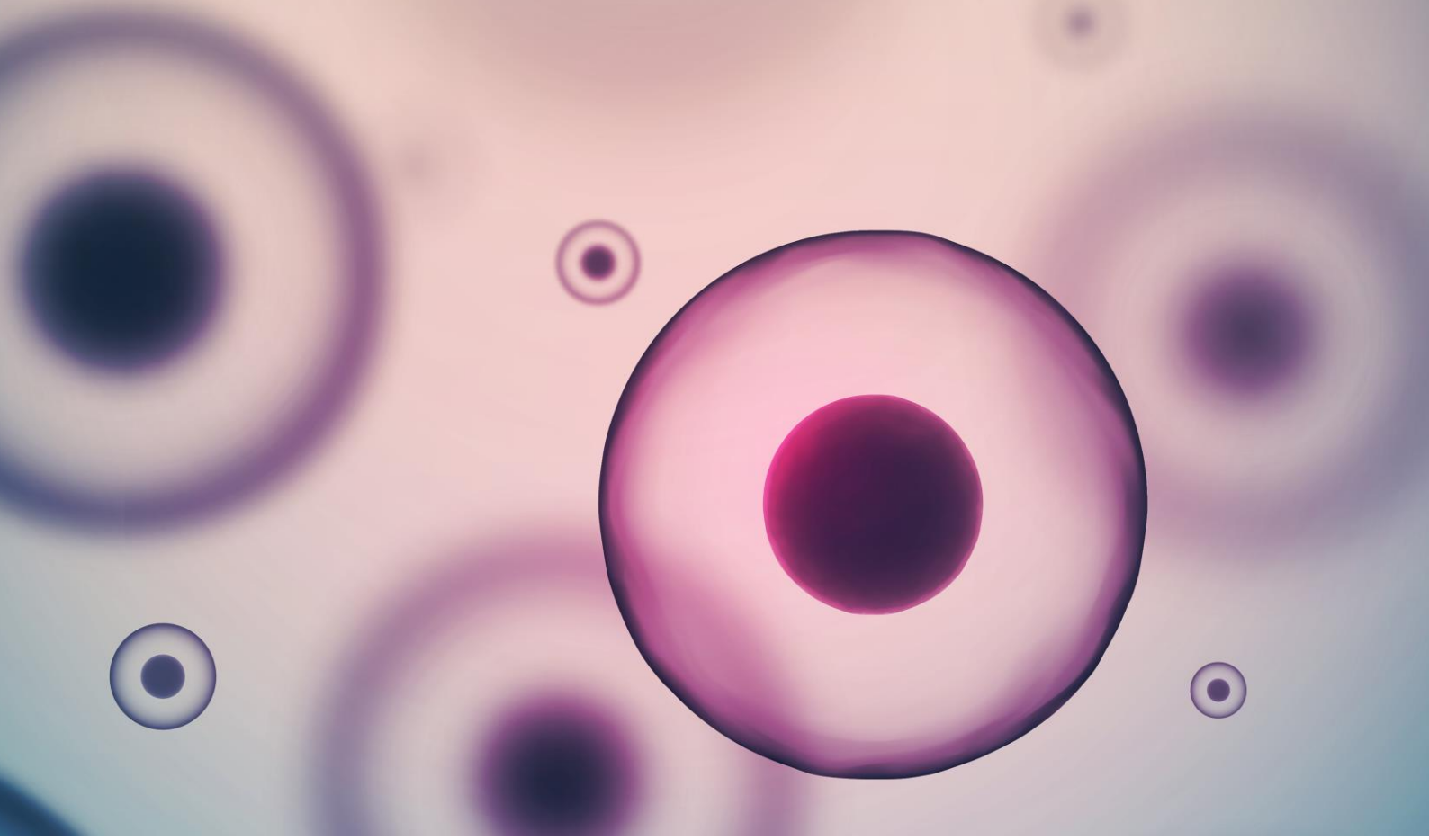

Technical Reports

Preparation of a detection complex RAG0109BIOT-Strep-HRP ready-to-use for CMV IgM immunocapture assay.

THE AIM OF THIS ASSAY IS THE OBTENTION OF A DETECTION COMPLEX FORMED BY RAG0109BIOT AND HRP-STREPTAVIDIN, READY-TO-USE, WHICH CAN BE POURED TO THE WELLS OF AN ELISA PLATE TO DEVELOP THE FINAL SIGNAL IN A IgM IMMUNOCAPTURE ASSAY.

Our preliminary experiments suggested that the recombinant poly-epitope chimeric antigen designed and produced by Rekom Biotech, RAG0109, recovers some pre-validated IgM positive sera which are not initially detected by two of the best antigens described in bibliography for CMV diagnosis: pp52 and pp150, showing the presence in merely one antigen of some important epitopes for this specific diagnostic (see https://www.rekombiotech.com/Content/Documents/support/technical-papers/recombinant-chimeric-antigen-chimcmv1.pdf)

Furthermore, this recombinant chimera was able to detect IgM Ab in an indirect and a capture ELISA formats. Besides, the IgM detection capacity of the protein was proven by using a commercial capture assay with very promising preliminary results.

In order to avoid the need of conjugating the protein with peroxidase, we have found a different alternative which is to conjugate the protein with biotin, which is a small, robust and stable molecule.

At Rekom Biotech, we have produced the CMV chimeric protein but conjugated with biotin (RAG0109BIOT) due to the market interest in CMV IgM detection. The extremely specific and high affinity binding of biotin by avidin and streptavidin (KD 10-15) results in specific detection systems of very high sensitivity. A clear advantage of this system is that with a common streptavidin-HRP system, we can obtain conjugated complex of all our references without the necessity of performing the peroxidation of each product.

Our aim is the obtention of a detection complex formed by a biotinylated antigen-Strep-HRP, which can be directly used in immunocapture and immunometric formats. For this purpose, several ELISA assays were performed in order to adjust the IgM detection in a capture format by using a conjugated complex produced by the biotinylated chimeric antigen plus HRP-streptavidin (figure 1).

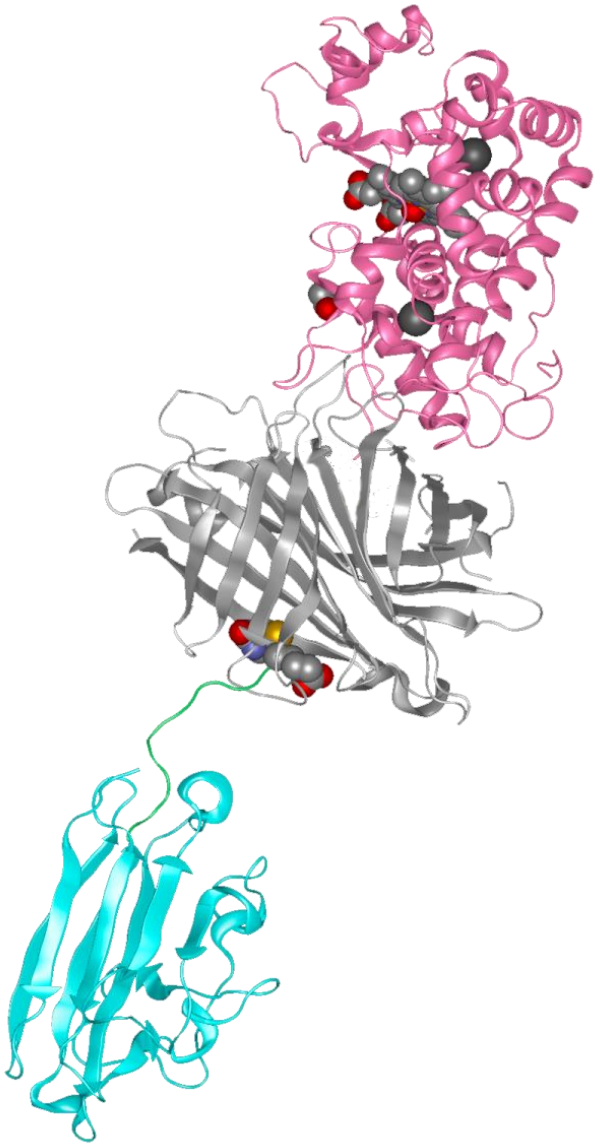

Figure 1. In blue is represented the RAG0109BIOT bonded to a biotin molecule which is specifically interacting with the HRP-streptavidin (pink and grey respectively).

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In order to develop the ELISA studies, the protein RAG0109BIOT was purified to homogeneity at Rekom Biotech (figure 2)

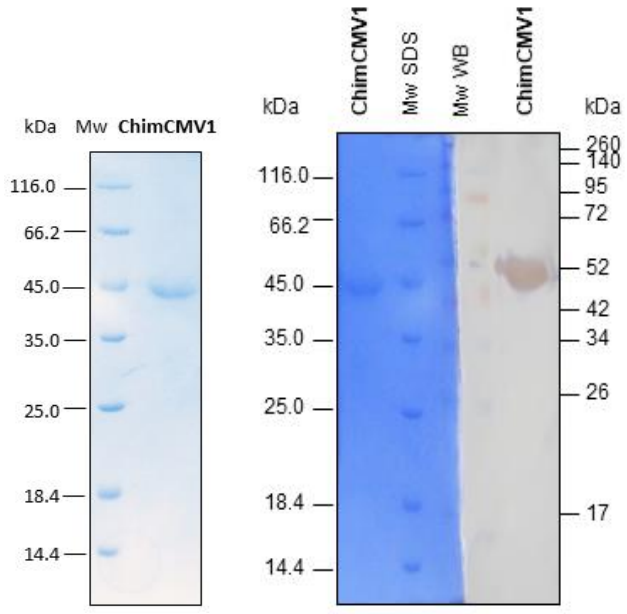

Figure 2. A) SDS-PAGE analysis of the biotinylated chimeric protein and B) SDS-PAGE and western blot analysis of the biotinylated ChimCMV1 protein. The western blot analysis was performed with conjugated streptavidin.

Initial assays were carried out in an indirect format, by coating the plate by the biotinylated antigen and developing the ELISA assay with HRP-Streptavidin in order to detect the specific interaction (figure 3).

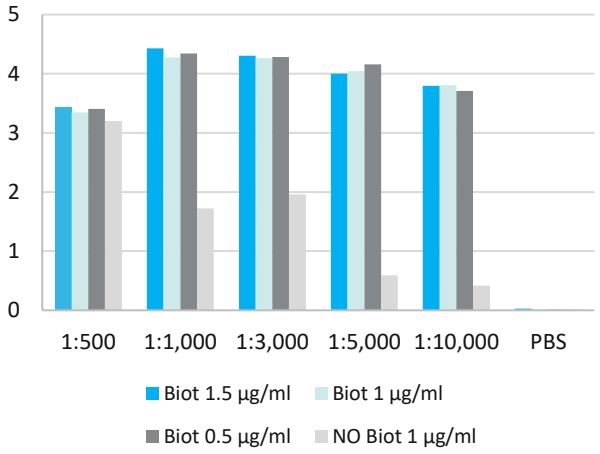

Figure 3. Different concentrations in plate of the biotinylated antigen RAG0109BIOT. The ELISA assay was developed with HRP-streptavidin. HRP-streptavidin titer is shown on the abscissa axis.

In order to avoid the high background with the negative control obtained with the non-biotinylated antigen, we have chosen a HRP-streptavidin titer of 1:5,000 and 1:10,000 to use in our analyses.

The IgM detection in CMV is normally performed by a capture format, thus, we analyzed our biotinylated antigen in this format, by using the commercial test CMV-IgM-eLA test PKS Medac and the positive and negative controls from this same test (figure 4).

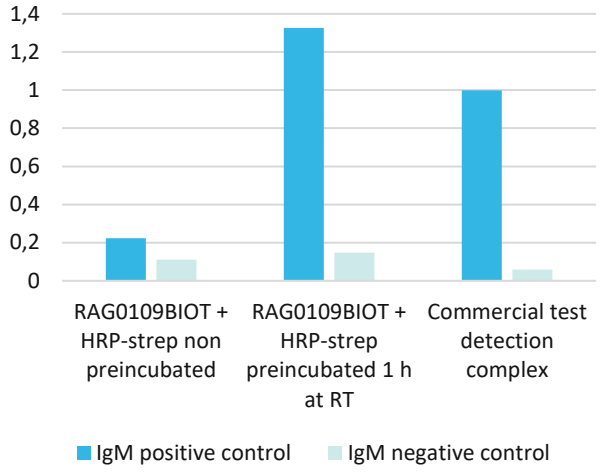

Figure 4. A capture ELISA was performed by using the commercial CMV-IgM test from Medac. Also, the positive and negative controls of the same test were used. HRP-streptavidin were used at 1:10,000 titer and RAG0109 at 1 µg/ml. Also, the detector complex from the commercial test was used as reference.

These analyses revealed that in this particular case, the pre-incubation of the biotinylated antigen and the streptavidin was an important prerequisite for the formation of the strong detection complex between the biotin and the streptavidin.

Also, an immunocapture format ELISA was performed to study the specifity of the interaction between HRP-streptavidin with the biotinylated antigen vs the non-biotinylated RAG0109 (figure 5) and to adjust the correct concentration of the biotinylated protein and the HRP-streptavidin in the detection complex for the capture ELISA (figure 6). For both objectives, the commercial test from Medac was used.

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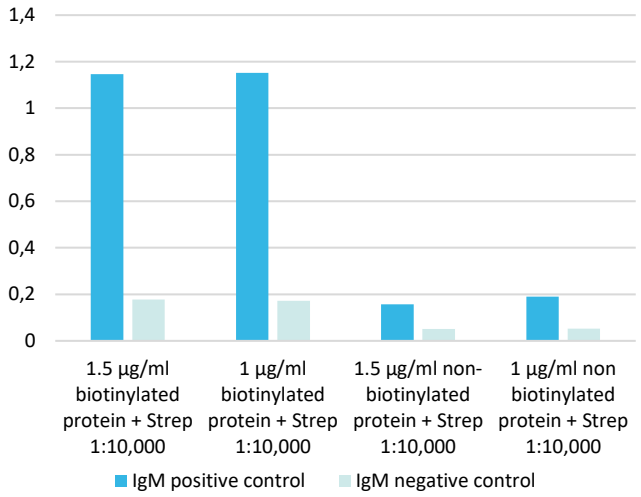

Figure 5. Biotinylated vs non-biotinylated antigen in a capture ELISA assay. A commercial immunocapture ELISA test (CMV-IgM-eLA test PKS Medac) was used with two different dilutions of the Rekom RAG0109BIOT and RAG0109, previously incubated with conjugated streptavidin. Positive and negative controls from the same test were used.

As it is shown in the figure, with these conditions, the assay is highly specific for biotinylated antigens.

Also, we can obtain different signals regarding antigen and streptavidin titer used in the ELISA assay (figure 6).

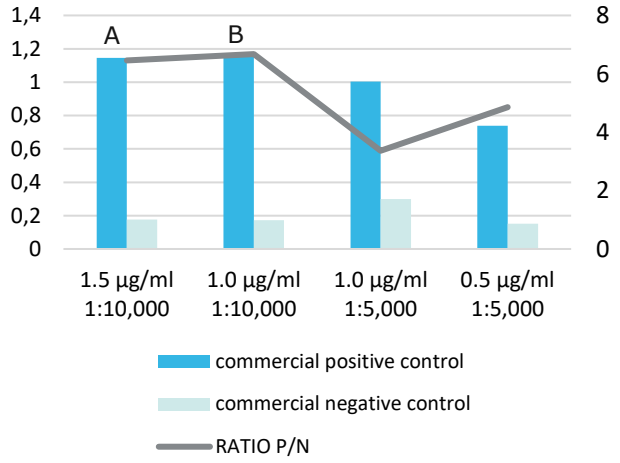

Figure 6. A capture ELISA assay was performed by using three different dilutions of the Rekom RAG0109BIOT (1.5, 1 and 0.5 µg/ml) in combination with two different HRP-streptavidin dilutions (1:10,000 and 1:5,000) as detector in the commercial test CMV-IgM-eLA test PKS medac. Both, positive and negative controls from the same commercial test were used for the assay. The complex for detection formed by the biotinylated protein and the conjugated streptavidin was previously incubated 1 hour at room temperature.

Conditions A and B were selected as the better P/N ratios for optimal discrimination between negative and positive sera.

Finally, the set-out conditions were used in an *in-house* ELISA developed in Rekom Biotech, with pre-validated real positive and negative IgM sera, to be used in a capture assay (figure 7).

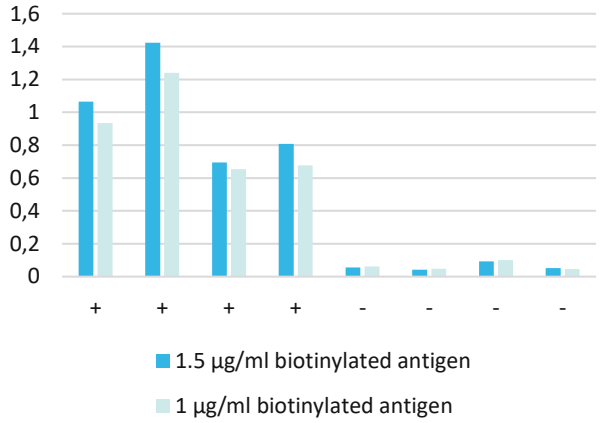

Figure 7. Immunocapture ELISA assay. 4 positive and 4 negative specimen sera, pre-validated with the IgM detection Vidas test, were used to study the signal difference between positive and negative sera. Anti-human IgM was used at a final concentration of 2.5 µg/ml to coat the plate.

CONCLUSION

In order to take advantage of the extremely specific and high affinity binding of biotin by streptavidin, at Rekom Biotech we have biotinylated some of our references, specifically the most interesting ones to develop capture and immunometric assays. In the particular case of the CMV chimera RAG0109, the biotinylated counterpart protein seemed to be highly effective in capture assays by interaction with HRP-streptavidin. We have analyzed that this interaction is highly specific of biotinylated proteins and the final signal is enhanced by pre-incubation 1 h at RT before use of the biotin-streptavidin complex. We have obtained the best conditions to obtain an optimal P/N ratio, promoting a good discrimination between positive and negative sera signal. Also, we have selected as optimun, a concentration of 1.5 ug/ml of the biotinylated antigen and a HRP-streptavidin titer of 1:10,000. This specific condition has worked with the capture plate and positive and negative IgM controls of a commercial IgM test, but also in a in-

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hose ELISA, by coating the plate with anti-IgM and using real specimen sera pre-validated with IgM Vidas test.

As in other cases, with this pre-incubation procedure, the assay is quicker (45 min shorter than a traditional ELISA), as the detector complex can be formed previously and simultaneously to other steps. Also, the reproducibility is possible as there is always only one biotin per protein molecule, and the protein is cheaper and more stable than the one conjugated with peroxidase, as biotin is a small, robust and stable molecule.

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