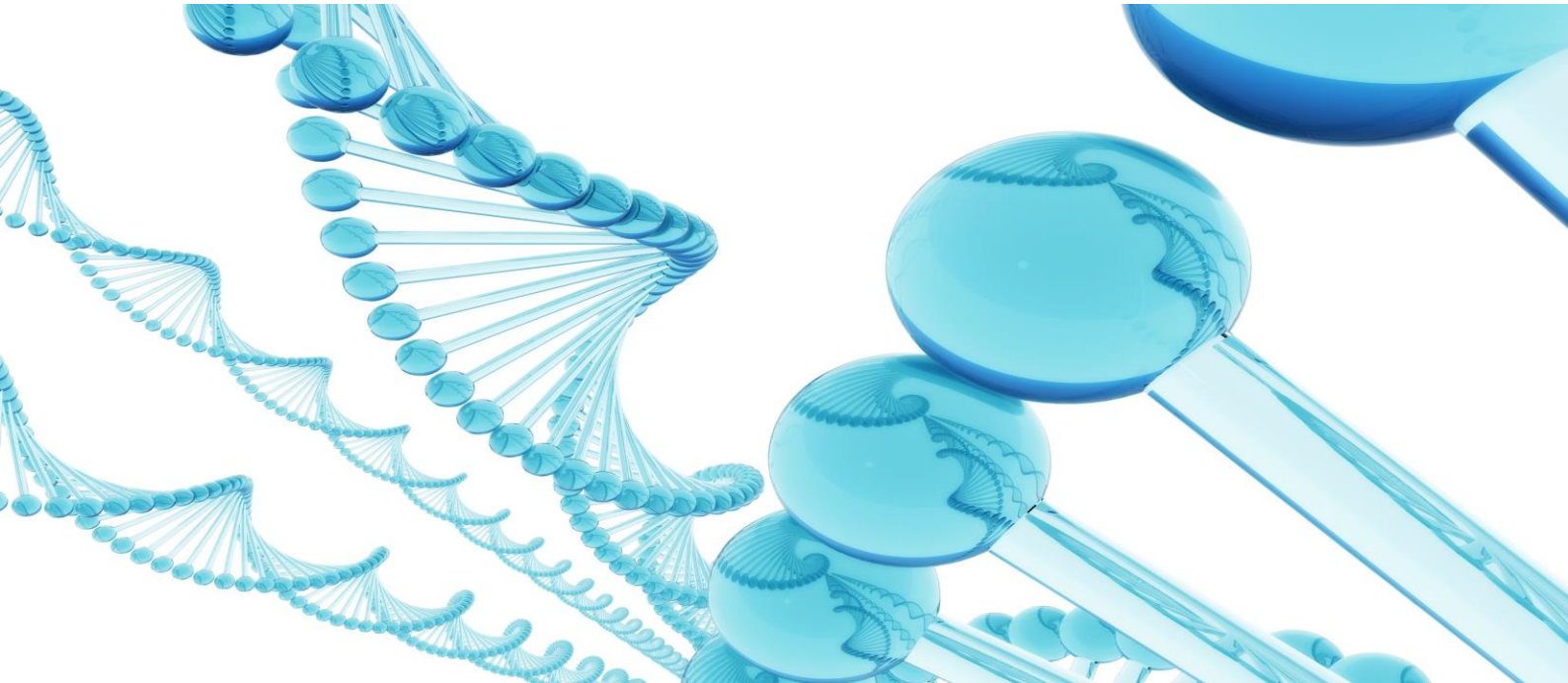
Technical Reports

OPTMIZATION OF THE RECOMBINANT EBV NUCLEAR ANTIGEN QUALITY BY IMPROVING ITS INTEGRITY IN ESCHERICHIA COLI



THE AIM OF THE RESEARCH PRESENTED IN THIS TECHNICAL REPORT IS TO SHOW THE IMPROVEMENT OBTAINED IN THE RECOMBINANT ANTIGEN EBNA1 FROM EPSTEIN-BARR VIRUS, AFTER A GENETIC ENGINEERING OF THE GENE BKRF1. THE IMPROVEMENTS ACHIEVED AFFECT ASPECTS RELATED TO THE FINAL DEVELOPMENT AND YIELD OBTAINED IN THE DSP (DOWNSTREAM PROCEDURE), STABILITY AND INTEGRITY OF THE PROTEIN, AND FINALLY CONCERNING DISCRIMINATION BETWEEN POSITIVE AND NEGATIVE SERA IN ELISA ASSAYS.

Epstein-Barr virus (EBV) is a member of the herpesvirus family and one of the most common human viruses. The virus occurs worldwide, and most people become infected with EBV sometime during their lives. Infants become susceptible to EBV as soon as maternal antibody protection disappears. When infection with EBV appears during adolescence or young adulthood, it causes infectious mononucleosis 35% to 50% of the time.

Furthermore, EBV is also a human carcinogen that has been implicated in the development of malignancies of lymphoid and epithelial origin, including Burkitt lymphoma, Hodgkin disease (HD), immunodeficiency-related B cell lymphoma, extranodal T/NK cell lymphomas, gastric carcinoma and nasopharyngeal carcinoma.

Currently, titers of IgA antibody against the EBV viral capsid antigen (VCA) (p18, p23) and the diffuse early antigens (EA-D) (p54 and p138) are regularly tested in many clinical centers. Moreover, many serological markers of EBV infection, including VCA, EA, nuclear antigen (EBNA1), Zta and DNAse, have also been developed in recent years. The protein EBNA1 (EBV Nuclear Antigen 1) plays a role in the maintenance of latent EBV infection and is expressed in all EBV infection and in all EBV-associated malignant tissues. The corresponding gene is BKRF1, which codifies for an antigen of 56,427.3 Da with a resolved crystal structure (figure 1). The amino-terminal half of EBNA1 contains a large domain of 239 amino acids with a repeated sequence of glycine-glycine-alanine.

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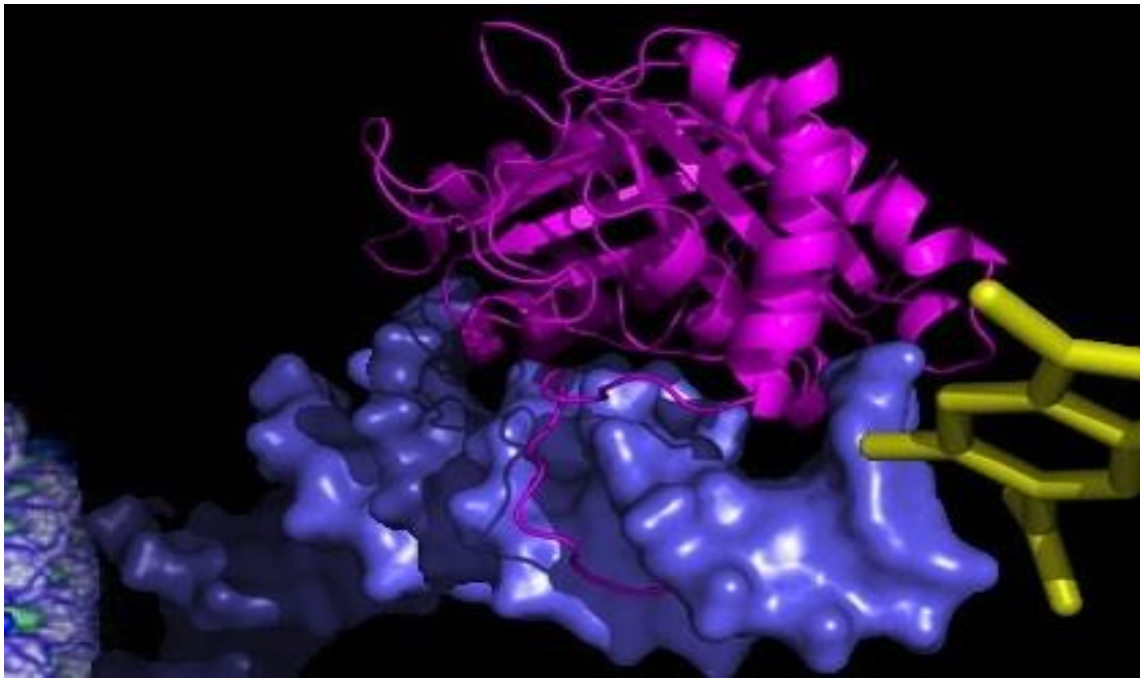

Figure 1. Crystal structure of the DNA-binding and dimerization domains of the Epstein-Barr virus nuclear antigen (EBNA1) (Bochkarev *et al.,* 1995).

In order to over-produce this protein in Escherichia coli, the gene BKRF1 was amplified and cloned into an expression vector. A recombinant antigen containing the complete ORF of the EBV BKRF1 gene plus a his-tag fused to the target protein, would have a theoretical molecular weight of 63 kDa. We added a fusion tag in order to improve its posterior purification. The initial expression of EBNA in *E. coli* as a heterologous system is showed in figure 2A. The recombinant antigen feature after a Ni affinity purification operation unit is showed in an analytical SDS-PAGE, a wide spread of the protein appears, with a massive degradation at the bottom of the gel. This profile was obtained from a standard expression culture, with 1 mM IPTG induction and 37ºC/shaking.

The main consequence of this fact is that it is not probable to develop a robust and accurate DSP in order to obtain a sole band which corresponds with the recombinant antigen. Also, the possibility of contamination with host cell proteins (HCP) is very high, being detrimental for the **specificity** of the recombinant antigen. On top of that, the degraded fragments observed which are producing this smear appearance in the gel and the vast degradation at the bottom, probably are truncated proteins which are losing some important epitopes probably essential for the **sensitivity** of the recombinant protein.

After an in-depth study of the EBNA1 gene sequence, the technical team of Rekom Biotech performed several changes in the expressed E. coli recombinant gene by genetic engineering, and different approaches were applied. The resulted expressed and purified protein, EBNA optimised (EBNAop) is showed in figure 2B. A unique, sharp band which maintained its integrity was obtained from a three-operation unit developed DSP. The achieved recombinant antigen was purified to homogeneity (approx. 95% pure), eluding the risk of contamination with HCP and the misplacing of important epitopes (figure 2).

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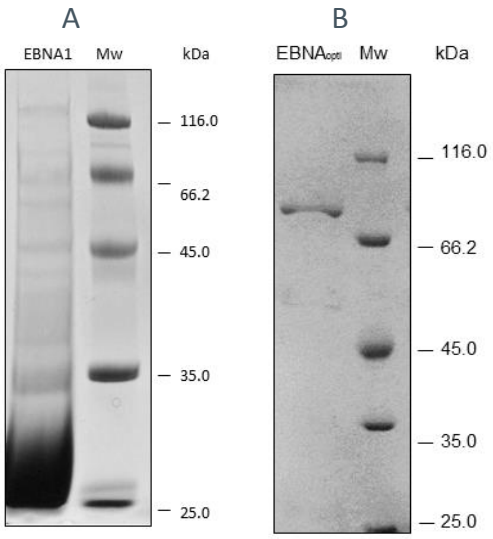

Figure 2. SDS-PAGE analysis of two proteins. A) a standard EBNA expression in E. coli by using 1 mM IPTG and shaker-37ºC incubation, after an affinity chromatography; B) EBNAop after a proprietary three-step DSP.

Other disadvantages are concluded from this situation. As we do not know which of these bands really correspond to our target protein, it is dangerous to make efforts to concentrate the recombinant antigen in order to obtain a higher titter for ELISA or lateral flow assay application. This is a risky option as we can enrich at the same time our protein preparation in HCP and therefore in unspecificity. The last applied operation unit favoured a good concentration of the protein, obtaining an IgG-ELISA titter up to 1:14,500, which meant 0.109 μg/ml of protein in plates (figure 3).

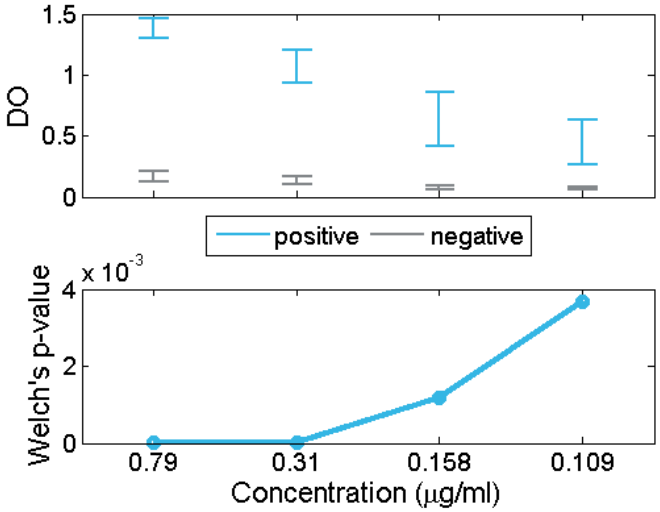

Figure 3. In this plot, the optical density at 450/620 nm for positive (blue) and negative (gray) sera are compared for each concentration of the recombinant antigen. An appropriate statistical test of significance for the comparison of means between both groups, the Welch's test, is employed. Eligible concentrations for the use of the antigen should present statistically significant differences between positive and negative sera. This happens when

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the intervals at the top do not overlap and, equivalently, when the p-value at the bottom is below 0.05. In the present figure, all p-values are below 0.05 and thus the intervals do not overlap. Therefore, any of the showed concentrations can be used to distinguish between positive and negative sera.

For the ELISA studies, sera from positive specimen were used. The status for EBV infection was previously confirmed by the VIDAS EBV VCA IgM assay (Biomérieux, France). Meanwhile EBNAop reached a concentration in plate as low as of 0.108 μg/ml with a very good discrimination between positive and negative sera, the titter obtained for the non-modified EBNA dropped at concentrations approximately of 1.7 ug/ml in plates, being therefore at least 16 times less sensitive than the optimized one (figure 4):

A

			EBNAopti							
Sera Nº	IgM VCA	IgG VCA	1,7 ug/ml		0,325 ug/ml		0,162 ug/ml		0,108 ug/ml	
1	nd	+	1,05	1,478	0,968	1,129	0,926	0,804	0,547	0,381
10	nd	+	1,848	1,908	1,623	1,677	1,59	1,543	1,435	1,441
506	nd	+	1,653	1,492	0,797	0,85	0,481	0,558	0,36	0,316
309	nd	+	1,765	1,962	1,624	1,581	1,333	1,399	1,042	1,21
121	+	-	0,196	0,093	0,089	0,087	0,103	0,107	0,077	0,095
300	+	-	0,309	0,348	0,151	0,143	0,16	0,182	0,082	0,105
632	+	-	0,345	0,378	0,133	0,146	0,115	0,114	0,104	0,088
652	+	-	0,081	0,113	0,073	0,063	0,075	0,075	0,068	0,066

B

			EBNA							
Sera Nº	IgM VCA	IgG VCA	1,7 ug/ml		0,325 ug/ml		0,162 ug/ml		0,108 ug/ml	
1	nd	+	0,585	0,547	0,181	0,177	0,15	0,123	0,12	0,088
10	nd	+	1,486	1,664	0,781	0,854	0,435	0,444	0,353	0,275
506	nd	+	0,123	0,183	0,08	0,086	0,095	0,098	0,091	0,116
309	nd	+	1,385	1,395	0,451	0,447	0,281	0,225	0,166	0,153
121	+	-	0,152	0,22	0,112	0,117	0,109	0,112	0,053	0,107
300	+	-	0,221	0,22	0,159	0,188	0,179	0,231	0,132	0,15
632	+	-	0,126	0,132	0,151	0,113	0,102	0,112	0,075	0,096
652	+	-	0,139	0,128	0,09	0,099	0,085	0,08	0,074	0,089

Figure 4: Data from an ELISA assay performed with EBNAop (A) and the non-modified EBNA (B). Four different concentrations in plates of the proteins were used: 1.7; 0.325; 0.162 and 0.108 μg/ml. Duplicates were performed with each concentration condition. Blue shade is used in positive non-detected sera. Sera Nº 506 is not detected even by the highest EBNA concentration in plates, displaying a defeat in sensitivity. The only concentration of EBNA which was able to discriminate positive and negative sera was 1.7 μg/ml. Conversely EBNAop carried out this discrimination even at 0.108 μg/ml.

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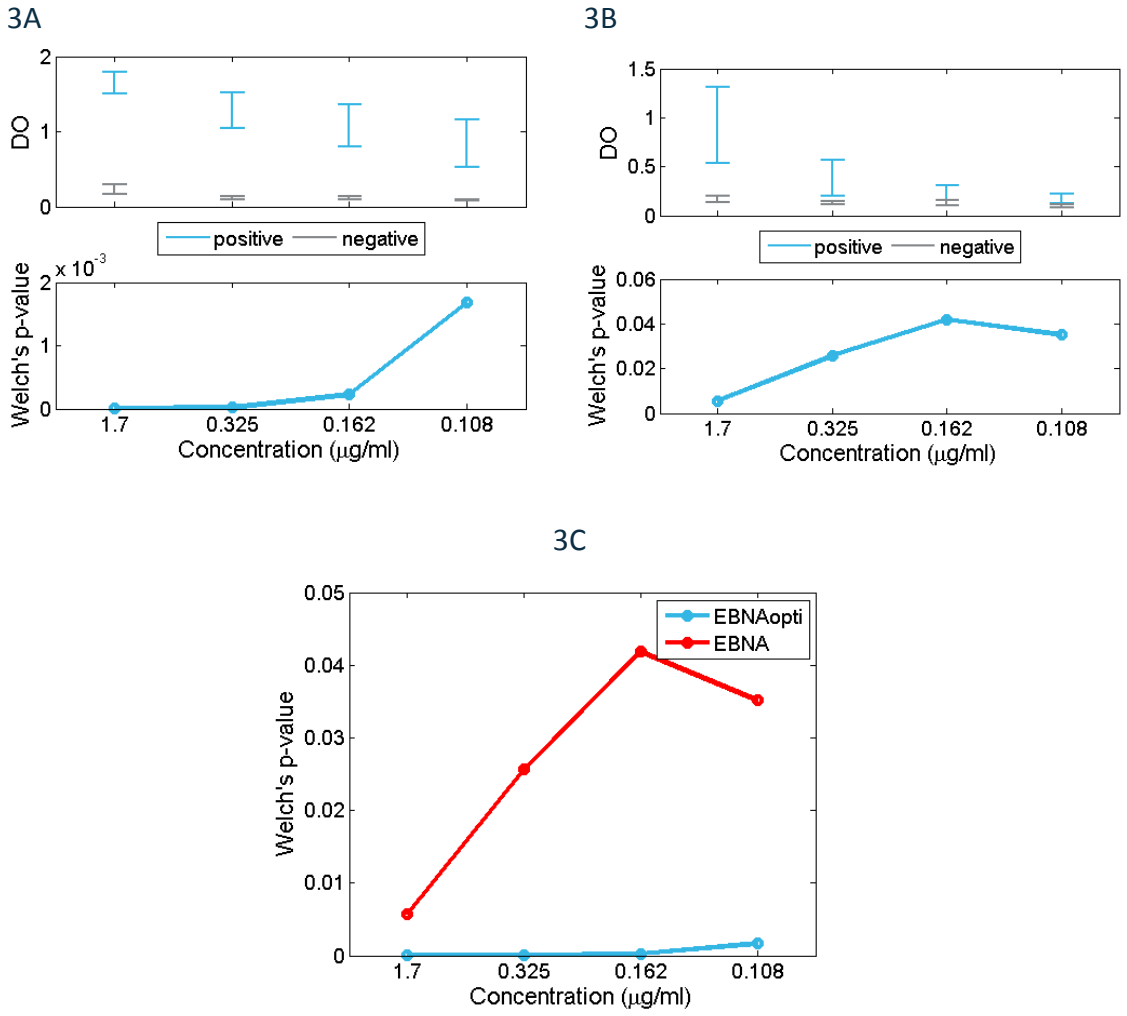

Figure 5. This figure shows the graphical representation of the above data. Comparison of the titer obtained with the EBNAop (3A) and with the non-modified one (3B). The EBNAop was concentrated until reaching a titer of 1:14,500 (0.108 µg/ml in plates), the non-modified was not able to discriminate at concentrations under 1.7 µg/ml in plates. 3C shows the comparison between the Welch´s p-value of both graphics; the lower the Welch´s p-value, the higher the discrimination between positive and negative sera.

CONCLUSION

The Rekom Biotech EBNA1 antigen corresponding to the complete EBNA1 EBV protein avoids the degradation observed in the directly *E. coli* expressed gene, allowing be purified to homogeneity and maintaining its integrity. This protein shows a high titter, permitting a really good discrimination between positive and negative sera. The protein allowed a 95% purity after a three-step DSP, guaranteeing also a high specificity.

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