

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**INHIBITION OF HBSAG WITH STABLE TRANSFECTION OF ANTI-HBSAG
RECOMBINANT ANTIBODY IN PLC/PRF/5 CELL LINE**

ELİF AKTAR
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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GEBZE

2019

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

ANTİ-HBSAG REKOMBİNANT ANTİKORUNUN
STABİL TRANSFEKSİYONU İLE PLC/PRF/5 HÜCRE
HATTINDA HBSAG'NİN İNHİBİSYONU

ELİF AKTAR
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

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SUMMARY

Prevalence of chronic HBV infection is approximately 3.5% worldwide, and affected people are at high risk for the development of liver cirrhosis and hepatocellular carcinoma. Despite the availability of effective treatments for HBV infection, there is still a need for curing chronic HBV disease. The main goal of this study is to determine the therapeutic capacity of the Lig7 single chain variable fragment (scFv), which is specific for Hepatitis B surface Antigen. We first assessed the binding capacity of anti-HBsAg Lig7 to HBsAg with western blot and ELISA by using PLC/PRF/5 cell line, which is a strong producer of HBsAg. Next, we transfected pIRES2 AcGFP1 vector containing scFv Lig7 sequence into PLC/PRF/5 cell line. Transfection efficiency was observed through expression of GFP1 protein under the fluorescence microscopy. Despite the apparent decrease in HBsAg levels, Lig7 scFV was barely detectable in Western blotting and ELISA assays. Alternatively, Lig7 scFV was incubated in HBsAg coated ELISA plates and then protein L-HRP was used as a detection system. This approach allowed us to successfully detect Lig7 scFv by ELISA. Then, in order to increase the stability of Lig7, we ligated the human IgG constant fragment (CH₂CH₃) to the scFv Lig7 fragment, and checked the ligation efficiency by PCR. The Lig7_Fc fragment was cloned into PBI-CMV1 vector, and the expected band size for fusion antibody was detected on an agarose gel. Then, pBI-CMV1 plasmid vector carrying Lig7_Fc fusion sequence was transfected into CHO-K1 cell line, and Lig7_Fc protein expression was determined by ELISA. Therefore, two ELISA systems were developed for the detection of scFv Lig7 and Lig7_Fc, respectively. In conclusion, our studies demonstrated that the recombinant scFv Lig7 antibody binds successfully to HBsAg, and Lig7_Fc and scFv Lig7 can be used as therapeutic agents in the future for the elimination of HBV.

Keywords: Hepatitis B, Hepatitis B surface antigen (HBsAg), Single chain variable fragment (scFv), inhibition of HBsAg.

ÖZET

Karaciğer sirozu ve hepatoselüler karsinom gelişme riski yüksek olan kronik HBV taşıyıcılarının dünya popülasyonunda görülme sıklığı yaklaşık % 3,5'tir. HBV enfektifine karşı geliştirilen etkili tedaviler olsa da, sorun devam etmektedir. Bu çalışmanın temel amacı, faj görüntüleme teknolojisine sahip Hepatit B yüzey antijenine (HBsAg) özgü Lig7 tek zincirli değişken fragmanının (scFv) terapötik kapasitesini belirlemektir. İlk önce anti-HBsAg Lig7'nin HBsAg'ye western blot ve ELISA ile bağlanma kapasitesini, HBsAg'nin güçlü bir üreticisi olan PLC/PRF/5 hücre hattını kullanarak değerlendirdik. Daha sonra scFv Lig7 sekansı içeren pIRES2 AcGFP1 vektörünü PLC/PRF/5 hücre hattına aktardık. Floresans mikroskopu altında GFP1 proteini ekspresyonu yoluyla transfeksiyon etkinliği gözlemlendi. HBsAg seviyelerinde belirgin bir düşüşe rağmen, Lig7 scFV Western blot ve ELISA deneylerinde zorlukla saptanabildi. Alternatif olarak, Lig7 scFV, HBsAg kaplı ELISA plakalarında inkübe edildi ve daha sonra bir tespit sistemi olarak protein L-HRP kullanıldı. Bu yaklaşım, ELISA ile Lig7 scFv'yi başarıyla tespit etmemizi sağlamıştır. Daha sonra, Lig7'nin stabilitesini arttırmak için, insan IgG sabit fragmanını (CH2CH3) scFv Lig7 fragmanına bağladık ve ligasyon verimliliğini PCR ile kontrol ettik. Lig7_Fc fragmanı pBI-CMV1 vektörüne klonlandı ve füzyon antikoru için beklenen bant boyutu bir agaroz jeli üzerinde tespit edildi. Daha sonra, Lig7_Fc füzyon dizisini taşıyan pBI-CMV1 plazmid vektörü CHO-K1 hücre hattına transfekte edildi ve Lig7_Fc protein ekspresyonu ELISA ile belirlendi. Bu nedenle, sırasıyla scFv Lig7 ve Lig7_Fc'nin tespiti için iki ELISA sistemi geliştirilmiştir. Sonuç olarak, çalışmalarımız rekombinant scFv Lig7 antikorunun HBsAg'ye başarılı bir şekilde bağlandığını ve Lig7_Fc ve scFv Lig7'nin gelecekte HBV'nin ortadan kaldırılmasında terapötik ajanlar olarak kullanılabileceğini göstermiştir.

Anahtar Sözcükler: Hepatit B, Hepatit B yüzey antijeni (HBsAg), Tek zincirli değişken fragmanı (scFv), HBsAg inhibisyonu.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations</u> <u>and Acronyms</u>	<u>Explanations</u>
µg	: Microgram
ng	: Nanogram
pg	: Picogram
pMol	: Picomole
mM	: Millimolar
M	: Molar
µl	: Microliter
bp	: Base pairs
kDa	: Kilodaltons
kb	: Kilobases
A	: Amper
V	: Volt
ABST	: 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ALT	: Alanine Aminotransferase
APS	: Ammonium persulfate
ATCC	: American Type Culture Collection
cccDNA	: Covalently Closed Circular DNA
CHB	: Chronic Hepatitis B
CHO-K1	: Chinese Hamster Ovary
EDTA	: Ethylenediaminetetraacetic Acid
ELISA	: Enzyme Linked Immunosorbent Assay
F	: Forward Primer
Fab	: The Antigen-Binding Fragment
FBS	: Fetal Bovine Serum
Fc	: Constant Fragment
G	: Gram

GFP	:	Green Fluorescent Protein
HBV	:	Hepatitis B virus
HBcAg	:	Hepatitis B core antigen
HBeAg	:	Hepatitis B e antigen
HBsAg	:	Hepatitis B surface antigen
HCC	:	Hepatocellular carcinoma
IRES	:	Internal Ribosome Entry Site
MAM	:	Marmara Research Center
mg	:	Milligram
min	:	Minute
ml	:	Milliliter
mAbs	:	Monoclonal Antibodies
NEB	:	New England Biolabs
PBS	:	Phosphate-Buffered Saline
PCR	:	Polymerase Chain Reaction
pH	:	Potential of Hydrogen
R	:	Reverse Primer
rcDNA	:	Relaxed Circular DNA
RNA	:	Ribonucleic Acid
Rpm	:	Revolutions per minute
scFv	:	Single chain variable fragment
SDS-PAGE	:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TAE	:	Tris Base, Acetic Acid, EDTA
TUBITAK	:	Scientific and Technological Research Council of Turkey
U	:	Enzyme Units
pNpp	:	p-Nitrophenyl phosphate disodium hexahydrate

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1. INTRODUCTION

Hepatitis B virus leads to chronic hepatitis B infection, which is among the most important risk factors for the development of liver fibrosis, hepatocellular carcinoma [Yuen et al., 2018], morbidities with fatal consequences [Balcioglu et al. 2014]. Every year, between 500000 and 1000000 people are getting infected with Hepatitis B Virus (HBV) and, more than 780000 people die due to hepatitis B. According to World Health Organization (WHO) reports, two billion of the global population were infected with hepatitis B [Başalp et al., 2017], and approximately 257 million people have been chronically suffering from hepatitis B [Yuen et al., 2018]. Transmission of HBV is mainly through blood and body fluids. Other transmission types are mainly due to sharing needle among the drug users, contaminated blood transfusion, tattooing, and body piercing. While mother to child transmission (MTCT) and sexual intercourse are common causes of HBV transmission in South-East Asian and Western Pacific regions [Lok et al., 2017], the transmission of HBV generally emerges in Western Europe, North America and Australia due to needle sharing between drug users [D'Souza & Foster, 2004].

HBV is a member of the Hepadnaviridae family. The infectious virion has a size of 3200 base pairs (bp), contains a partly double-stranded relaxed circular DNA (rcDNA). The rcDNA is transmitted to the nucleus and gets converted into totally double-stranded DNA [covalently closed circular DNA (cccDNA)], which is the persistent form of HBV in infected cells [W. Seto et al., 2018]. HBV genome includes four open reading frame, which encode polymerase, Pre-S/S, Pre-C/Core, and X regions. These genes are translated into the polymerase, hepatitis B e antigen, hepatitis B preS1, preS2 and surface antigen, hepatitis B core antigen and X proteins [Balcioglu, 2003].

According to WHO, the definition of HBV cure is the clearance of HBsAg and eradication of HBV cccDNA in the serum and acquisition of normal ALT levels. However, current therapies can rarely achieve complete treatment of patients with CHB [W. Seto et al., 2018]. Treatment of chronic HBV is achieved in two ways. The

first way is preventive and related to vaccination with recombinant HBsAg before the occurrence of an infection. According to the WHO report, vaccination has saved 37 million children who otherwise will suffer from chronic HBV between 1990 to 2014 in the western Pacific region. [W. Seto et al., 2018]. Antiviral therapy is applied to HBV infected people who used immunomodulatory and nucleos(t)ide analog drugs. While nucleoside analogs prevent HBV replication by inhibiting reverse transcriptase activity, immunomodulatory drugs such as IFN- α stimulate the host immune system to eradicate infected hepatocytes cells. However, HBV DNA and HBsAg seroclearance were failed in most patients with chronic hepatitis B [Dong, Ying, Qiu, Lu, & Zhang, 2017]. Furthermore, novel therapies are being developed to cure HBV. Several of the therapeutic drugs are in clinical phases and preclinical experimental stages. Drug design by using small molecules is based on the inhibition of HBV by targeting any protein in the HBV life cycle [Bloom, Maepa, Ely, & Arbuthnot, 2018]. Other ways of the treatment include the use of inhibitors for subviral particles such as HBsAg, HBcAg, HBeAg [Yu et al., 2012]. Another therapeutic modality is the stimulation of the host immune system with cytokines and interferons [Bloom et al., 2018].

Unfortunately, current therapies generally result in weak viral clearance, failure of the immune response, generation of virus strain with resistance against the drugs in long term therapy with current drugs. Furthermore, novel developed drugs continue to face the same problems [W. Tan et al., 2013]. In addition to these novel drugs and current therapies, the use of monoclonal antibodies (mAbs) has become an increasingly attractive area of research throughout the last three decades. Monoclonal antibodies that are isolated from the large antibody libraries by using several techniques have been included in the list of drugs that can be developed against many diseases due to the high affinity and negligible side effects of monoclonal antibodies [Hoogenboom, 2005]. While 18 monoclonal antibodies have been approved and are in use in pharmaceutical markets, the clinical trials assess more than 100 monoclonal antibodies against different diseases [Holliger & Hudson, 2005].

Furthermore, since the mAbs possess low toxicity, long half-life, and high specificity, they became favorite therapeutic agents against HBV [W. Tan et al., 2013].

Notably, smaller antibodies fragments such as Fab, single chain variable fragments (scFv) and variants generated with engineering (diabodies, triabodies, and single-domain antibodies) have emerged as safely and useable agents in clinical trials targeting many diverse disease. The small antibody fragments keep specification features against targeted proteins or antigens; also their production is more cost effective. However, it is not easy to detect the small antibody fragments in the cell or bacteria in some conditions, and different strategies were developed for the identification of the small antibody fragments [Holliger & Hudson, 2005]. One of the strategies is to form chimeric antibody fragments to observe how much the activity of a chimeric mouse-human antibody in the human cell line [Morrison et al., 1984]. Furthermore, scFv fragments are not as stable as the complete antibody structure (IgG) since the constant fragment (Fc) has crystallizable properties. Thus, the fusion of scFv and Fc provide more stable antibody structure [Nian et al., 2016].

In the present study, the therapeutic activity of anti-HBsAg scFv Lig7 fragment produced by phage display technology was assessed for its capacity to inhibit HBsAg through its transfection into PLC/PRF/5 cell line, which releases HBsAg into the culture medium. The binding of Lig7 to commercial HBsAg and HBsAg from the supernatant of PLC/PRF/5 cell line was tested in a Western blot experiment by using anti-HBsAg Lig7 as a primary antibody. Furthermore, existence of HBsAg was demonstrated in the culture medium of PLC/PRF/5 cell line by ELISA. Simultaneously, scFv Lig7 in pIRES2 AcGFP1 vector was transfected into the PLC/PRF/5 cell line. After its transfection, HBsAg inhibition was successfully demonstrated with ELISA assay. Furthermore, scFv Lig7 transfected into PLC/PRF/5 cells was determined with ELISA.

In order to determine the Lig7 easily, the constant human fragment (CH₂CH₃) was ligated to the scFv Lig7 fragment with PCR studies. Even though the ligation of Lig7_Fc into pBI-CMV1 expression vector was performed in all formed fusion Lig7_Fc antibodies, the whole sequence of the fusion antibodies was failed to be determined by using the Sanger sequencing method. The Lig7_Fc antibodies were transfected into CHO-K1 cell line due to their higher transfection efficiencies than PLC/PRF/5 cell line. Presence of Lig7_Fc insertion was successfully demonstrated with sandwich ELISA at 48 hours after the transfection. Consequently, HBsAg inhibition was defined

with a commercial ultra-ELISA HBsAg kit after the transfection of scFv Lig7 into PLC/PRF/5. Although, the complete sequence of the Lig7_Fc could not be determined with Sanger sequencing method, the expression of Lig7_Fc after the transfection was shown by ELISA.

2. LITERATURE REVIEW

2.1. Hepatitis B

Hepatitis B is a liver disease caused by hepatitis B virus with high morbidity and mortality rates [Yuen et al., 2018]. Hepatitis B is still a major health problem worldwide although the vaccination with HBsAg is compulsory in most countries [Maepa et al. 2015 and Yuen et al. 2018]. Chronic liver diseases such as cirrhosis, liver failure, fibrosis and hepatocellular carcinoma are common in patients with chronic hepatitis B infection [Fourati & Pawlotsky, 2016].

It is the complex interaction between the HBV and the host immune system. If the host infected to HBV in adult, defense of the host is stronger, as a result, most of the host overcome as acute HBV infection. In contrast, if the infected host term of perinatal or early childhood, most of the hosts become chronic HBV because of the insufficient immune response [Fourati & Pawlotsky, 2016].

According to the last reports, patients carrying HBV has classified as five subtitles in general [Vlachogiannakos and Papatheodoridis, 2018]. The non-inflammatory phase previously was known as immune tolerant phase. It was characterized with the presence of HBeAg, the high concentration of HBV DNA, normal ALT levels and absent or minimum levels of fibrosis and histological inflammation in the liver [Fourati and Pawlotsky, 2016]. The inflammatory phase was named formerly immune active or immune elimination phase. Distinctive features of the phase are the existence of HBeAg raised ALT concentrations, fluctuated HBV DNA levels in patients' serum. In additionally, liver necroinflammation and fibrosis generally emerges in the phase because of immune-mediated response [Terrault et al., 2018]. The inactive phase was identified as loss of HBeAg, emerging of anti-HBeAg, normal concentrations of ALT, low or undetectable levels HBV DNA in the serum [Cheng et al., 2018]. Immune escape phase is called HBeAg-negative inflammatory phase. In that phase, owing to genome mutations in HBV precore region, expression of HBeAg is failed in the patients having this phase.

Furthermore, liver disease emerges as actively accompany with high levels of ALT and permanent HBV DNA expression in the phase. Progression of liver diseases is faster than other phases [Fourati and Pawlotsky, 2016]. The last phase is called the occult phase. The patients in that phase have undetectable HBsAg levels, the existence of anti-hepatitis core antigen with the existence of anti-HBsAg or not. Also, normal ALT values and undetectable HBV DNA levels (generally) were identified in the phase [Vlachogiannakos and Papatheodoridis, 2018].

Hepatitis B surface antigen (HBsAg) can be detected in the serum between two and ten weeks of Hepatitis B infection. Following the appearance of HBsAg, the symptoms start, aminotransferase level raises. Most people recover between 2 to 6 months by being provided HBsAg seroclearance owing to express anti-HBsAg antibodies. If HBsAg still exists in the patient serum after six months from exposure to HBV, it is known chronic HBV infection [Seto et al., 2014].

2.1.1. Acute Hepatitis B

Almost 70 % of the patients with infected acute hepatitis B recover with mild symptoms or without symptomatic event. Symptoms are associated with HBV reveals that other patients are also having acute hepatitis B. These clinical symptoms are known as fever, fever, fatigue, anorexia, body aches. Fatigue and nausea, jaundice, and rarely liver failure. Besides, ALT levels increase, HBsAg, and HBV DNA reaches detectable levels in the patients' serum. The signs continue between 2 - 3 weeks and 1 - 6 months [Pawlotsky et al., 2008]. Acute liver failure has appeared on approximately 1 % of patients having acute hepatitis B [Liang, 2009].

2.1.2. Chronic Hepatitis B

In the early phase of chronic hepatitis B, HBsAg, HBeAg, HBV DNA progress with high titers in the patients' serum. Furthermore, aminotransferase concentrations appear at the normal range [Liang, 2009]. The evidence of chronic hepatitis B is defined as high ALT, fibrosis, and inflammation of the liver according to the result of the biopsy. Also, after six months from the infection, HBsAg is observed in the

patients' serum. Almost 40% of the patients having chronic hepatitis B develop the most important liver diseases such as hepatocellular carcinoma, cirrhosis, fibrosis, and liver failure because of long term infection and immune response [Jongh et al., 1992]. Chronic hepatitis B that causes related to liver diseases shows an alteration according to both viral conditions, such as HBV DNA levels, subviral particles (HBsAg, HBeAg), the genotype of HBV, mutation patterns and host-specific conditions such as gender, age, immune response, genetic susceptibility [Liang, 2009].

2.2. Hepatitis B Virus

Hepatitis B virus is known as a member of the Hepadnaviridae family. It leads to acute hepatitis B and chronic hepatitis B in humans [Mohebbi et al., 2018]. The virus is composed of minor virions [Mohebbi et al., 2018] and has partly double-stranded, relaxed circular DNA, which is the 3200 base pairs (bp) [W. K. Seto et al., 2014]. Covalently closed circular DNA (cccDNA) is essential for replication and surviving of the virus. HBV replication is performed with reverse transcription of pre-genomic RNA by using the reverse transcriptase enzyme, which has a high mutation rate. [W. K. Seto et al., 2014]. The genome has four main open reading frames. Pre-core and core reading frame (preC/C) contains two overlapping fragments to encode hepatitis B core antigen and hepatitis B e antigen, respectively [Dong et al., 2017]. The polymerase ORF (P) is used to express polymerase enzyme consist of reverse transcriptase (RT) [Ott et al., 2012]. Pre-S1, pre-S2, and surface (S) protein are transcribed by using Pre-S/S open reading frame [Fourati and Pawlotsky, 2016]. The last ORF encodes X proteins as a regulatory protein (Maepa et al., 2015), see figure 2.1 [W. S. Tan, 2017].

Genotypes of the HB virus was classified from A to J [Yuen et al., 2018]. The genotypes were determined according to geographically. For instance, genotype A is seen in North America, northern Europe as a common, genotypes B and C are frequent in Asia, genotype D emerge mostly the Middle East and India. [W. K. Seto et al., 2014].

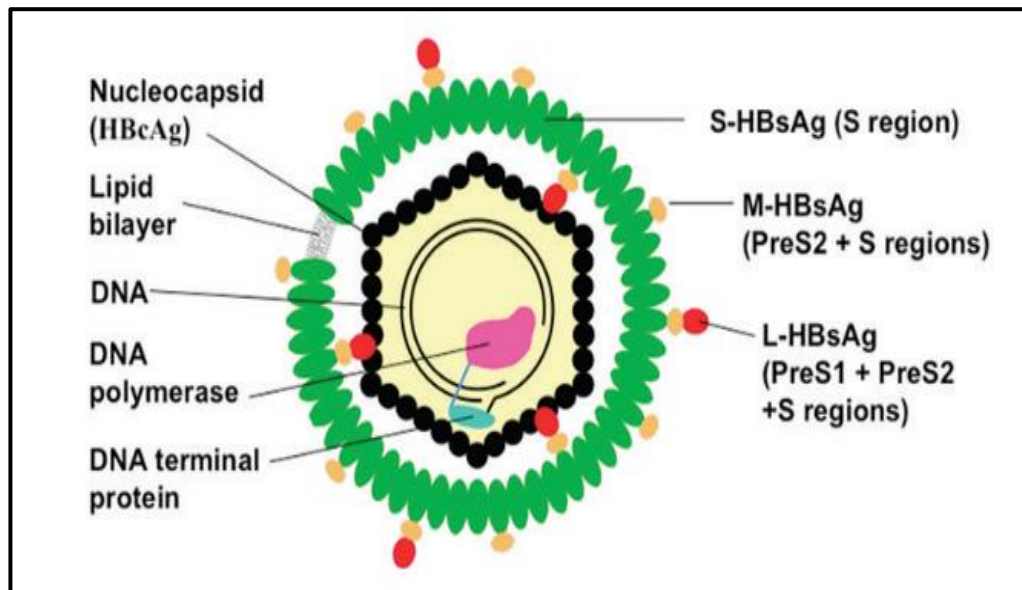


Figure 2.1: Structure of Hepatitis B virus.

2.2.2. Life Cycle of the Hepatitis B Virus

HBV life cycle briefly summarized in figure 2.2 [W. Seto et al., 2018]. The infection of HBV begins by attaching to heparan sulfate proteoglycans, and the virus binds to hepatocyte receptors, which are known as the sodium taurocholate co-transporting polypeptide (NTCP) [Noordeen, 2015]. The attachment actualizes by binding pre-S1 region of HBV to the extracellular domain of the hepatocyte receptors, followed by internalization of virus nucleocapsid by triggering endocytosis process [Fourati and Pawlotsky, 2016]. Viral genome transferred into the nucleus. Relaxed DNA is replaced with covalently closed circular DNA (cccDNA) by helping from the host materials [Maepa et al., 2015]. cccDNA in the virus genome is used as a DNA template most of the expression of subviral particles and pre-genomic RNA [Klein et al., 2003]. The polymerase of the virus is performed process of the reverse transcription for pre-genomic RNA [Bloom et al., 2018]. Thus, viral negative strand DNA was formed, followed by negative strand by using as a DNA template forms plus viral strand DNA. The nucleocapsid form can release out of the cell through endoplasmic reticulum or integrated into the nucleus for more expression cccDNA [Maepa et al., 2015].

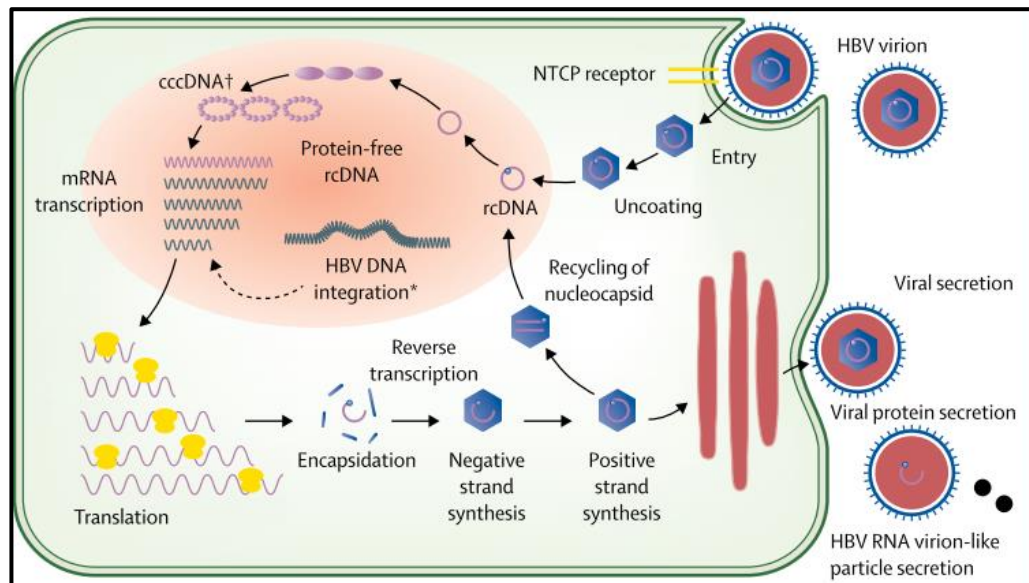


Figure 2.2: The Life cycle of the Hepatitis B Virus.

2.3. Treatments of Chronic Hepatitis B

The first plasma-derived hepatitis B vaccine is licensed in USA in 1981, Later this discovery was replaced with recombinant hepatitis B between 1986 and 1989 [W. Seto et al., 2018]. Although, vaccination provided a beneficial effect on most people nowadays, chronic hepatitis B still appears a significant health problem worldwide [Başalp et al., 2017]. Currently, chronic HB patients are treated with antiviral drugs. The aim of the drugs provides minimize of HBV DNA replication. Thus, the development of hepatocarcinoma and cirrhosis dependent on HBV replication are prevented [W. K. Seto et al., 2014]. Furthermore, it was revealed that long term antiviral therapy could provide regression of hepatocarcinoma and cirrhosis [Yuen et al., 2018]. The most commonly used antiviral drugs are seven pieces in the worldwide, two of them consist of immunomodulatory drugs which are interferon alpha formulations. They are the classic interferon alpha and pegylated version. Other drugs are nucleos(t)ide analogs, like, lamivudine, adefovir, entecavir, telbivudine, and tenofovir, respectively. [W. K. Seto et al., 2014].

The most important and common mutation are in the pre-core region. One of them is a single nucleotide change in 1896th nucleotide which produces a stop codon early. The other mutation causes a decrease of the pre-core messenger Ribosomal

Nucleotide acid (mRNA) transcription by double changing nucleotides in 1762 and 1764 positions at the core promoter region. While the mutations negatively affect the expression of Hepatitis B e antigen, the replication capacity still remains constant. In this approach, these mutations create HBeAg negative chronic HBV infection [W. K. Seto et al., 2014]. Most antiviral drugs target the reverse transcription of HBV replication [Maepa et al., 2015]. The novel drugs should be developed against cHBV patients who have drug resistance mutations [Mohebbi et al., 2018]. Current drugs for the treatment provides a decrease in HBV DNA levels, HBsAg concentrations in the serum, but they are insufficient to eradicate cccDNA, and loss of HBsAg in the patients [Karayiannis, 2003]. Beside, after the long term usage of drugs, sometimes the virus can gain resistance against the drug by getting mutation on the genome [Gomes-Gouvêa et al., 2015].

2.3.1 Immunomodulatory Drugs

Therapy with interferon shows immunomodulatory and antiviral activity. However, it has many side effects on patients like fatigue, bone marrow repression, depression, and autoimmune illnesses. According to 2013, many studies demonstrated that the interferon therapy decreased HBsAg concentration in the patient serum; however, it declined in HBsAg detected changeable parts according to HBV genotypes. The therapy also provided no considerable response to the HBeAg negative patients [W. K. Seto et al., 2014]. Pegylated interferon alfa is still limited for one year on most of the patients in the early treatment of chronic hepatitis B stage. The immunomodulatory drug obtained as long term therapy to obtain low suppression of virus DNA (80 IU/mL or less) in the only 23% of the patients [P Marcellin et al., 2009].

2.3.2. Nucleos(t)ide Analogous

Nucleos(t)ide analogous usually targets viral life cycle proteins like inhibition reverse transcription of pregenomic RNA. HBV DNA and HBsAg was not detected in significant levels in the patients, however they were having both HBeAg positive and

HBeAg negative sites to treat the nucleos(t)ide analogs targets. After one year alanine aminotransferase concentration reaches normal levels [W. K. Seto et al., 2014]. It is shown that entecavir, tenofovir disoproxil fumarate, and tenofovir alafenamide drugs provide repression of viral replication and HBV DNA amounts in most patients' having chronic hepatitis B. Furthermore, they demonstrated no major side effect, and the virus could develop no resistance against the drugs [Patrick Marcellin et al., 2013]. As a result, treatment with nucleos(t)ide analogue showed long-term suppression for viral replication and regression of cirrhosis and HCC in 71% of the patients even after more than five years. Even though the drugs are used as a reliable treatment for some diseases such as chronic renal tubular damage, Fanconi syndrome, respectively. A decline density of bone mineral revealed consequent of the long-term usage of the nucleos(t)ide analogue treatments [W. K. Seto et al., 2014].

2.3.4. Other Treatments for Providing HBsAg Inhibition

Various treatment strategies are available such as nucleotide based interfered strategies; RNA interference, gene editors, antisense oligonucleotides and ribonucleotide enzymes by using gene therapy. Most of these treatment selections started to gain importance recently, and many problems need to be resolved related to gene therapy and strategies in the future [Maepa et al., 2015].

To have more efficient immune response against HBV with full virus free with subviral particles (SVP), especially HBsAg, needs to be inhibited. Furthermore, the drugs providing inhibition of HBsAg help the immune system to eradicate HBV. [Mohebbi et al., 2018]. SVPs are responsible for progression chronic Hepatitis B to lead for hepatocellular carcinoma (HCC), cirrhosis, liver failure and effect the immunosuppression. It was shown that HBsAg positive cHBV patients increased the risk of developing HCC between 25% and 37% [Kondo et al., 2013].

Most of the researchers believed that the treatments related to HBsAg seroclearance in cHBV patients provide a promising for the regression of HBV [McCaffrey et al., 2003]. Even though most of the drugs approved by the FDA for cHBV treatment support antiviral response positively, they lead to side effects, high cost.

Moreover, appearing of drug resistance is a major obstacle during the treatment [Gomes-Gouvêa et al., 2015]. Therefore, new therapeutic drugs have searched to decrease the side effect of current drugs and stimulate more effective the immune system through cHBV treatment [Klumpp et al., 2018].

It was performed that lots of studies with natural compound to suppresses HBsAg in cHBV patients. In Chinese medicine, the effect of Osthole compound extracted from Du Huo root was searched whether providing of HBsAg inhibition or not. The study showed that Osthole inhibited both small and middle HBsAg by interfering post-transcriptional [Huang et al., 1996]. Other herbal compounds were used to provide inhibition of HBsAg. Clerodendrum inerme and lemmatis Sinensis achieved inhibition of HBsAg with percentage 85 and 60, respectively [Mehdi et al., 1997]. The active compound of OjF has demonstrated HBsAg inhibition. It was thought in that study that OjF extracts may affect HBV DNA polymerase and expression of HBsAg as a non-functionally [Wang et al., 2005]. Another study demonstrated that the Abelmoschus manihot (L) media, is known as a flower extract, and inhibits the HBsAg in cHBV patients' serum. Inhibition rate reached to 82.27 % at dose dependent. Furthermore, the extract with nontoxic dose provided regression of liver disease [Wu et al., 2007]. In addition, these natural products, Hydroxyl (OH)-substituted Alisol A compounds [Zhang et al., 2008], polypeptide PEI extracted from Jelly Fig Achenes, [Huang et al. 2013], Luteolin-7-O-glucoside extracted from leafy vegetable lettuce [Cui et al., 2017] were studied for HBsAg expression inhibition as an alternative treatment.

Consequently, all of the compounds completed the inhibition of HBsAg *in vitro* studies. However, it is still not known yet that the HBsAg inhibition exact mechanism whether provided by natural compounds or not. Moreover, most of the drugs did not pass to clinical phases. The studies should be expand and focus on the mechanisms of this molecules/or compounds more in the future, [Mohebbi et al., 2018].

2.4. Recombinant Antibodies

Monoclonal antibodies are a useful source for diagnosis of the many diseases by using biomarker [Baird et al., 2009]. Over the last ten years, recombinant DNA technologies such as chimerisation and humanization, have increased the clinical efficacy of mAbs and immunoglobulin domains [Holliger and Hudson, 2005].

Therapeutic recombinant antibodies are derived from immune mice. Mainly, the nonimmunogenic human antibodies are produced method indirectly by applying humanization or were acquired directly by using a transgenic mouse [Rader and Rader, 2001]. Hybridoma cells for obtaining monoclonal antibodies (mAb) are performed by fusion the murine B cells and myeloma cells. However, this technique is time-consuming, genetic instabilities. There are also high risks for microbial contamination [Bialon et al., 2014]. On the other hand, antibody libraries use as a valuable source for the production of monoclonal antibody. The antibody libraries are selected to produce high affinity and specificity monoclonal antibodies according to the subject studies [Rader and Rader, 2001]. The antibody libraries are constructed either hybridoma or display technology (yeast surface or phage display) after human or mouse immunization [Baird et al., 2009].

In general, most therapeutic recombinant antibodies are formed in IgG format (bivalent, Y shaped) [Holliger and Hudson, 2005]. The recombinant antibodies are use in different immunoglobulin forms as therapeutic agents, and diagnosis purposes such as a single chain variable fragment (scFv), antigen-binding fragment domains (Fab). Different antibody formats used as therapeutic agents are presented in figure 2.3 [Holliger and Hudson, 2005]. Using scFv fragment and Fab fragment as detection reagents for diagnosis of cancer and some diseases are suggested by many researchers since it contains small protein with high affinity and specificity features [Liu et al., 2003]. Fab domains are 50 kDa and they are known as a fragment of the antibody without constant region (CH2-CH3) [Rader and Rader, 2001]. scFv fragment is 25 kDa and contains variable heavy and light (VH and VL) that bind each other by a polypeptide linker [Rader and Rader, 2001]. The major advantage of them are they have a small structure especially like scFv. Therefore, they can penetrate the cell,

expressed easily in different cell line [Bialon et al., 2014]. The detection of the presence of HBsAg was demonstrated by one-step ELISA with adding the alkaline phosphatase to the scFv fragment formed against HBsAg [Erdag et al., 2017]. A complete antibody in IgG format is more stable than scFv. Fc region provides a more stable formation antibody. Detection of scFv antibody in transfected cells also is difficult in some conditions [W. Tan et al., 2013]. As a solution, the chimeric antibodies produced to provide more stable and easy detection. It was acquired chimeric rabbit/human Fab antibody against HBeAg by phage display technology in 2017. The chimeric Fab had consisted of variable domains (VH and VK) and CH1 and CL. The purification and detection of the Fab were facilitated by adding histidine sequence to carboxyl terminal of VH-CH1 region.

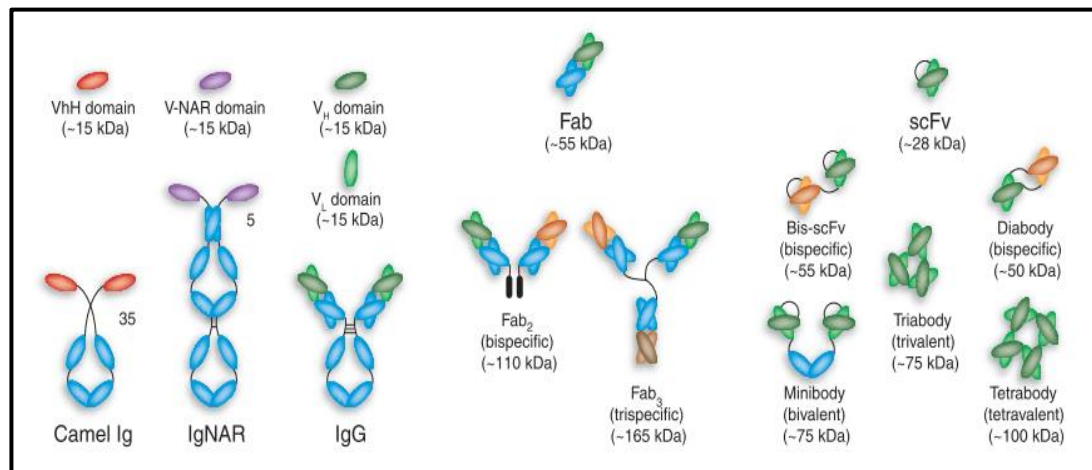


Figure 2.3: Different antibody formats.

3. MATERIALS

3.1. Equipments

Gel Imaging System (C600 Azure Biosystem), Trans-Blot SD Semi-Dry Transfer Cell (1703940 BIO-RAD), Electrophoresis Power Supply (Model 200/2.0 165-4761 BIO-RAD), PowerPac™ Basic Power Supply (1645050 BIO-RAD), Mini-PROTEAN Tetra Cell (1658000EDU BIO-RAD), GEL Doc 2000 Lab UV Transilluminator (170-8126 BIO-RAD), Nitrocellulose Membranes (10484059, BIO-RAD), Microcentrifuge (5415R Eppendorf), Microcentrifuge (5702R Eppendorf), Thermocycler (T3000 Bio-Mettra), Dri-Block Heater (DB-2D Techne), Hotplate-Stirrer (ICB302 Stuart), Speed Dry Vacuum Concentrator (RVC 2-25 CDplus Christ), Chemistry Diaphragm Pump (MZ 2C NT Vacuubrand), pH Meter (8521 Hanna Instruments), Vortex (Genie 2 Scientific Industries), Low-temperature thermostats (RE 104 Lauda), Water Bath Shaker (Innova 3100 New Brunswick), UV/Vis Scanning Spectrophotometer (DU-730 Beckman Coulter), Gel Dryer (Model 583, BIO RAD), Microplate Reader (7091000, Bio Tek), NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific), Ice Machine (AF100, Scotsman), Costar(R) Assay Plate 96 well (Corning, 3690), Inverted Microscope (CKX41 Olympus), Fluorescents Microscope (Primovert, ZEISS), 0.5 ml, 1.5 ml Microcentrifuge Tubes (311-8-08-051, Axygen), Automated Cell Counter (LUNA II, Logos Biosystem), Water Bath (My bath 2000, Benchmark Scientific), CO2 Incubator (MCO-18AC(UV), Sanyo), Clone Select Imager (Molecular Devices), Cell Imaging Multi-Mode Reader (Cytation 5, Bio Tek).

3.2. Buffers and Solutions

Table 3.1: List of buffers and solutions.

5X SDS Running Buffer	124 mM Tris-Base (pH:8.3) ,0,96 M Glycine, 0,5% SDS
1X Transfer Buffer	47 mM Tris-Base, 39 mM Glycine, 20% SDS, 20% Methanol
Staining Buffer	Brilliant Blue 0,1%, Methanol 50%, Acetic Acid 10%, dH ₂ O 39,9%
Destaining Buffer	Methanol 10%, Acetic Acid 7%, dH ₂ O 83%
10X PBS	137 mM NaCl, 15 mM KH ₂ PO ₄ , 81 mM Na ₂ HPO ₄ , 27 mM KCl
1X TPBS	1X PBS, 0.1% Tween-20
1% Blocking Solution	1 % BSA in 1X TPBS
Coating Buffer	0,1M NaHCO ₃ pH8,6
Substrate solution for alkaline phosphatase conjugate, pH:9,8	10% Diethenolamine, 0,01% MgCl ₂
Substrate solution for peroxidase conjugate, pH: 4	50mM citric acid, 0,1mg ABST
5X TAE	0,185M Tris Base, 94mM Glacial acetic acid, 5mM EDTA
0,5 X TAE	1,85mM Tris Base, 9,4mM Glacial acetic acid, 0,5mM EDTA
LB Broth Medium (for 1L)	10g Bacto Tryptone, 5g Yeast Extract, 10g NaCl
LB Agar (250mL)	10g Bacto Tryptone, 5g Yeast Extract, 10g NaCl, 15 g Bacterial agar
3M Sodium Acetate pH 5.0	27.22g Sodium Acetate, 5.75ml Acetic Acid, 75.4 ml distilled water
Freezing medium	5% DMSO, 95% the complete medium of the cell line
Complete medium for PLC/PRF/5 or CHO-K1 cell line,respectively	DMEM or Ham's F-12 Nutrient Mix, 10% FBS, 1% Penicillin streptomycin

- SDS PAGE Sample Buffer

- 4ml of dH₂O, 1 ml of 0.5M Tris-HCl pH: 6.8, 800 µl of 99% Glycerol, 800 µl of 20% SDS, 400 µl of β Mercaptoethanol, 200 µl of Bromo Phenol Blue, 60 µl of 1M NaOH were prepared and aliquoted in 1.5 ml minicentrifuge tubes and stored in – 20°C. The sample buffer solution was used in SDS-PAGE gel to denature the samples.

3.3. General Kits and Reagents

High Pure PCR Product Purification kit (Roche, Cat No.11732676001), Plasmid Isolation Miniprep Kit (ZymoPURE, D4209), DNA Sequencing Clean-Up Kit (Zymo Research, D4051), 10X T4 DNA Ligase Buffer (00057611, Fermentas), T4 DNA Ligase (00077294, Fermentas), Fermentas Taq DNA polymerase (EP0404, ThermoScientific), MyTaq™ DNA Polymerase (BIO-21105, Bioline), Taq DNA polymerase kit (Promega, M186A), Cutsmart Buffer (B7204S, NEB), BamHI enzyme (R3136S, NEB), HindIII enzyme (R3104S, NEB), PAGERuler Prestained Protein Marker (26616, Thermo Scientific), 4-Chloro-1-naphthol (C6788, Sigma), Red Safe Nucleic Acid Staining Solution (21141, Intron), 21141, Mass Ruler Low Range DNA Ladder (SM0383, Thermo Scientific), Mass Ruler High Range DNA Ladder (SM0393, Thermo Scientific), Mass Ruler DNA Ladder Mix (SM0403, Thermo Scientific), Mass Ruler DNA Loading Dye 6X (R0621, Thermo Scientific), NBT/BCIP tablet (Ref 11697471001, Sigma).

3.4. Antibodies and Antigens

- Monoclonal Anti-poly Histidine-Peroxidase clone HIS-1 (A7058, Sigma Aldrich)
- Anti-Mouse IgG (H+L) Secondary antibody (31320, ThermoFisher Scientific)
- Anti-Mouse IgG (H+L) Poly Secondary antibody HRP (32230, ThermoFisher Scientific)
- Anti-rabbit IgG (H+L) Secondary antibody, AP (31340, ThermoFisher Scientific)

- Anti-Human IgG (Fc specific) –alkaline phosphatase antibody (Sigma, A9544)
- Human IgG (I2511, Sigma Aldrich),
- Anti-Human Kappa Light Chain antibody (K3502, Sigma Aldrich)
- Recombinant Hepatitis B Surface Antigen (Adw) (Ab91276, Abcam)
- Recombinant Hepatitis B Surface Antigen (Ayw) (Ab73741, Abcam)
- Anti-Hepatitis B Surface Antigen Antibody (Ab8636, Abcam)
- Rabbit Anti-Mouse Immunoglobulins/HRP (P0260, Dako)
- Protein L-HRP (M00098, GenScript)
- Recombinant Protein L Type 1 Protein (Novus Biologicals, NBP2-35050).

3.5 Primers

➤ Primer Name	Sequence
• Rlig7	5' CGCCCGTTTGATTTCCAGCTTGGT3'
• Flig7-1	5' AGCTGTGTCCTATCCCAGGTGCAGCTGCAG AGTC3'
• FLig7-2	5' GCTCTTCTGCCTGGTGACATTCCCAAGCT GTGTCCTATCCCA3'
• FLig7-3	5' GCCGCCACCAATGGCTGTCTTGGGGCTGC TCTTCTGCCTGGTGAC3'
• FCH2	5' GCACCTGAACTCCTGGGGGGA3'
• RCH3	5' TCATTTACCCGGAGACAGGGAGAGG3'
• Lig7KRVHingLinFor	5' TCAAACGGGCGAAACGGGTTGAGCCCAA TCTTGTGAC3'
• KRVHingLinkRev	5' AGGAGTTCAGGTGCTGGGCACGGTGGGC3'
• Fc_dizileme_F	5' GGCAAGGAGTACAAGTGC3'
• Fc_dizileme_R2	5' CACCTGTGGTTCTCGG3'
• Fc_dizileme_R3	5' GGAAGAGGAAGACTG3'
• CMV-For	5' CGCAAATGGGCGGTAGGCGTG3'
• IRES2-R1	5' ACATATAGACAAACGCACACCGG3'

3.6. The vectors

- pIRES2 AcGFP1 vector Clontech Cat. No. 632435
- pBI-CMV1 vector Clontech Catalog No. 631630

3.7. Cell Culture Reagents and Solutions

Dulbecco's Modified Eagle's Medium (D5030, Sigma), Ham's F-12 Nutrient Mix (21765-029, Gibco), Fetal Bovine Serum (10500-064, Gibco), Penicillin-Streptomycin (15140-122, Gibco), Trypsin-EDTA 0.25% (25200-056, Gibco), L-glutamine (G5792, Sigma), Lipofectamine 2000 Transfection Reagent (P/N52887, Invitrogen), Lipofectamine 3000 Transfection Reagent (P/N100022050, Invitrogen), TurboFect Transfection Reagent (R0531, Thermo Scientific), Optimem (31985-062, Gibco), DMSO (SC-358801 Chemi Cruz), G-418 Solution (Ref: 04727878001, Sigma) Puromycin dihydrochloride (P8833, Sigma).

3.8. Cell Lines

- PLC/PRF/5 cell line ATCC CRL8024
- CHO-K1 cell line ATCC CCL61

4. METHOD

4.1. Cell Culture Experiments

4.1.1. Cell Thawing

- The cryovial containing the frozen cells was removed from liquid nitrogen storage tank and, was immediately placed into a 37°C water bath.
- The cells were thawed quickly by gently swirling the vial in the 37°C water bath until there was just a small bit of ice left in the vial.
- The vial containing the cells was transferred into a laminar flow hood. Before opening the vial, its outer surface was wiped with 70% ethanol.
- The desired amount of the pre-warmed complete growth media was transferred dropwise into the centrifuged tube containing the thawed cells.
- The tube was centrifuged at 1300 rpm for 5 minutes to remove the media used for freezing the cells.
- The supernatant was discarded and the pellet was resuspended in 1 ml fresh complete media.
- 10 µl of the sample was mixed with equal volume of trypan blue dye. The cell number and cell viability were calculated using hemocytometer and Luna II cell counter.
- Based on the observed cell count, the culture was transferred either to T-25 flask or 6 well plate.
- The respective T-25 flask or 6 well plate was incubated at 37°C in an incubator.

4.1.2. Cell Subculturing

PLC/PRF/5 cells or CHO-K1 cells was passaged once twice or thrice a week based on their confluency.

- Subculturing was performed, if the confluency reaches above 80%. Complete media was kept at 37°C in a water bath for pre-warming.
- Culture media were removed from the culture flasks or wells.
- Cells were washed with PBS solution (without Ca²⁺/Mg²⁺ ions) to get rid of any FBS in the residual culture media. Wash PBS solution was removed and discarded by aspirating with pipet.
- Pre-warmed Trypsin-EDTA (0,25X) solution was added to the vessel, approximately 1ml per 25 cm² of surface area. Vessel was rotated to cover the monolayer with the solution. The vessel was incubated at 37 °C in an incubator for maximum 2-10 minutes.
- The vessel was observed under an inverted microscope to ensure that all the cells got detached from the surface.
- Cells were resuspended in 3mL of pre-warmed fresh serum-containing medium to inactivate the trypsin and, sample was taken out to determine cell count.
- The total cell number and percentage viability were determined by using a hemocytometer or Luna II cell counter, following the fifth step in section 4.1.1.
- The resuspended culture in step 7 was transferred into centrifuge tubes and, it was centrifuged at 1300 rpm for 5 minutes.
- The cell suspension was diluted to the seeding density recommended for the cell line and was transferred into new cell culture vessels and, it was incubated in a CO₂ incubator.

4.1.3. Cell Freezing Protocol

The cell lines were stocked to reuse anytime using below mentioned protocol.

- Cells were detached from the tissue culture vessel following the procedure used in section 4.1.2.
- Each vial was adjusted to contain 500.000 cells.

- After the vials were labeled, freezing media containing 5% DMSO with pre-warmed complete media was prepared.
- After centrifugation, the supernatant was aspirated and the pellet was resuspended with the freezing medium.
- One ml of the cells that contained freezing media was dispensed into each vial. Vials were placed into the freezing cup in – 80 °C refrigerator for 24 hours. The next day, they were moved to a liquid nitrogen tank.

4.1.4 Transfection

Turbofect, Lipofectamine 2000 and Lipofectamine 3000 are the three different transfection reagents used in the present study. The Turbofect, Lipofectamine 2000 and Lipofectamine 3000 transfection protocol was performed for transfection in 24 well plate format.

- Turbofect Transfection Reagent Protocol:
- In each well, approximately 5×10^4 adherent cells in 1 ml of growth medium was added 24 hours before transfection.
- Adherent cells were transfected once optimal confluency reached to 70-90%.
- The vector DNA was diluted to desired concentration in 100 μ l of opti-mem media (31985-062, Gibco).
- Transfection reagent was vortexed and desired amount of the reagent was added to the diluted DNA vector.
- The vector DNA and reagent mix were incubated for 20 -30 minutes at room temperature and, 100 μ l of the mixture was dispersed well by mixing gently.
- The plate was incubated in 5% CO₂ at 37° C incubator. After 48 hours, it was analyzed with Cell Imaging Multi-Mode Reader (Cytation 5, Bio Tek) or fluorescent microscope.
- The culture supernatant was collected into microcentrifuge tubes to analyze expression of the expressed protein and were kept in +4 °C refrigerator.

- Lipofectamine 2000 Transfection Reagent Protocol:
 - Cells were seeded to have 70 to 90 percent confluency at the time of transfection into 24 well plates.
 - The next day, culture medium was replaced with serum free medium, optiMEM media.
 - The required amount of Lipofectamine 2000 Reagent (2 μ l, 3 μ l, 4 μ l and 5 μ l) was diluted in 50 μ l of optiMEM media (31985-062, Gibco) in microcentrifuge tubes.
 - The required vector DNA concentration was prepared in 50 μ l of optiMEM media in another microcentrifuge tubes.
 - Diluted vector DNA was mixed with Lipofectamine 2000 reagent solution in a ratio of 1:1.
 - The mixture was incubated for 5 minutes at room temperature. 100 μ l of the mixture was dispersed well by mixing gently.
 - The plate was incubated in 5% CO₂ at 37° C incubator. After 48 hours of incubation, it was analyzed with Cell Imaging Multi-Mode Reader (Cytation 5, Bio Tek) or fluorescent microscope.
 - The supernatant was collected into microcentrifuge tubes to analyze expression of the protein and were kept in +4 ° C refrigerator.
- Lipofectamine 3000 Transfection Reagent Protocol:
 - Cells were seeded to have 70 to 90 percent confluency at the time of transfection into 24 well plates.
 - The next day, culture medium was replaced with serum free medium, optiMEM media (31985-062, Gibco).
 - Desired amount of Lipofectamine 3000 Reagent (0,75 μ l and 1,5 μ l) was diluted in 25 μ l optiMEM media in microcentrifuge tubes.
 - The required vector DNA concentration and 1 μ l of P3000 Reagent (2 μ l/ μ g DNA) were diluted in 25 μ l of optiMEM media in another microcentrifuge tubes.
 - Diluted vector DNA was mixed with Lipofectamine 3000 Reagent solution in a ratio of 1:1.

- The mixture was incubated for 15 minutes at room temperature. 50 µl of the mixture was dispersed well by mixing gently.
- The plate was incubated in 5% CO₂ at 37° C incubator. After 48 hours, it was analyzed with Cell Imaging Multi-Mode Reader (Cytation 5, Bio Tek) or fluorescent microscope.
- The culture supernatant was collected into microcentrifuge tubes to analyze expression of the expressed protein and were kept in +4 ° C refrigerator.

4.2. Protein Analysis

4.2.1. Preparing of SDS-PAGE Gel

- Two glasses of 0,75 mm in thickness were compressed by using gel casting Mini-PROTEAN Tetra Cell (1658000EDU BIO-RAD) system.
- 12% Separating Gel Solution was prepared in a 50 ml centrifuge tube as shown below, respectively. As soon as 10% Ammonium persulfate and TEMED were added into the tube, the mix solution was poured immediately between the gel cassette

12% Separating Gel Solution (For two gel):

- ddH ₂ O	2.625 ml
- 30% Acrylamide, 0,8 Bis-Acrylamide	1.875 ml
- 4X 0,5 M Tris-HCl pH: 8.8 SDS 0.4 %	3 ml
- 10% Ammonium persulfate	80 µl
- TEMED	8 µl

- 160 µl of isopropanol was poured above the separating gel to prevent its contact air and removal of air bubbles from it.
- When separating gel became solid, isopropanol was removed from the top of the gel.

- 4% Stacking Gel Solution was prepared in a clean tube as shown below. Once, 10% ammonium persulfate and TEMED were added in the tube, mix solution was poured on the frozen separating gel immediately.

4% Stacking Gel Solution (For two gel):

- ddH ₂ O	1.538 ml
- 30% Acrylamide 0,8 Bis-Acrylamide	335 µl
- 4X 0,5 M Tris-HCl pH: 8.8 SDS 0.4 %	612.5 µl
- 10% Ammonium Persulfate	25 µl
- TEMED	2,5 µl

- The comb was placed into the stacking gel immediately. The SDS-PAGE gel was incubated until it solidifies at room temperature.
- The SDS-PAGE gel was kept with 1 X SDS buffer at + 4 °C to use for western blot and staining with Coomassie blue.

4.2.2. SDS-PAGE Gel Running Procedure

- As preliminary, comb present in the gel cassette was removed and washed with dH₂O to discard the residues over the comb. The gel was placed decently in vertical electrophoresis Mini-PROTEAN Tetra Cell (1658000EDU BIO-RAD) system.
- The space between the two gels were filled 1X SDS buffer and, the wells washed with 1X SDS running buffer by pipetting.
- In order to load the samples to SDS-PAGE gel, 4X sample buffer was diluted with the samples.
- The samples were incubated at 95°C for 3 minutes.
- After a short spin, 14µl of samples were loaded into the wells one by one. Page ruler prestained protein marker (cat: 26616 Thermo Scientific) was used as a marker to determine the size of the samples.
- 100V of electric potential was applied initially. Later, it was increased to 120V once the loading dye appeared in the separating gel.

4.2.3. Staining of the SDS-PAGE Gel

- On completion of run, the SDS gel was laid in a small plastic box containing 10 ml of Coomassie blue solution and, incubated for 45 minutes on a shaker.
- After the incubation, staining buffer was replaced with destaining buffer to detect the presence of proteins on the gels.
- Destaining step was repeated 3 times with 15 minutes of time intervals. The SDS gel was dried by using dry machine (Gel Dryer (Model 583, BIO RAD) to protect the results.

4.2.4. Western Blot Transfer

- In this step, nitrocellulose membrane (Nitrocellulose Membranes (10484059, BIO-RAD), and filter papers were cut according to the size of the SDS-PAGE gel.
- After the completion of running process, SDS-PAGE gel, nitrocellulose membrane and 6 pieces of 3mm filter papers were placed in 1X transfer buffer, separately for Western Blot.
- 3 pieces of 3mm filter papers (nitrocellulose membrane, the SDS gel and remained 3mm filter papers) were placed on Trans-Blot SD Semi-Dry Transfer Cell device (1703940 BIO-RAD), respectively. Air bubbles were removed by the help of a roller.
- It was executed for 50 minutes with 15 V. Thus, the proteins were transferred from the SDS gel to nitrocellulose membrane.

4.2.5. Western Blot

- After transfer step, the membrane was blocked with 1% BSA solution to fill remaining gaps and incubated for 2 hours at room temperature on a shaker.
- The membrane was washed thrice with TPB S for 5 minutes.

- Primary antibody was diluted to an appropriate ratio with 1% BSA and membrane was placed in this solution on a shaker for 90 minutes at room temperature.
- The washing step was repeated.
- The secondary antibody was diluted with 1% BSA according to the recommended ratio of the antibody and was poured on the membrane and incubated for 1 hour on a shaker at room temperature.
- The washing step was repeated.
- 4-Chloro-1-naphthol tablets dissolved in 10 ml methanol. 2 ml of the solution was mixed with 10 ml triethanolamine and 100 μ l H_2O_2 was used as a substrate solution for the membrane.
- Substrate solution was poured on the membrane, the membrane was incubated on the shaker for 5 minutes.

4.3 ELISA

4.3.1. Sandwich ELISA

Sandwich ELISA was applied to performed to detect the functional protein.

- The high bind microplate (Corning, 3690) was coated 50 μ l of anti-HBsAg or HBsAg (changeable testing samples) with coating buffer in 3.2 for overnight at +4°C.
- Each antibody-coated well was washed with 100 μ l of TPBS washing solution three times.
- The wells were blocked with 100 μ l of 1% Bovine serum albumin (BSA) or 4% milk powder and were incubated for 2 hours at room temperature.
- The washing step was repeated for 6 times,
- 50 μ l of each of the samples were added in the wells and were incubated for 90 minutes at room temperature.
- The wells were washed again.

- As per the requirement, 50 µl of primary antibody which was diluted at recommended ratio with the blocking buffer, were added to each well. Later, the plate was incubated for 90 minutes at room temperature.
- Washing step was repeated.
- 50 µl of secondary antibody with conjugate alkaline phosphatase or horseradish peroxidase, which was diluted to desired ratio with blocking buffer, were loaded to each well and were incubated for 90 minutes at room temperature.
- Washing step was repeated, according to a conjugate enzyme (secondary antibody) and the substrate solution. If conjugate enzyme (secondary antibody) is alkaline phosphatase, 4-Nitrophenyl phosphate disodium salt hexahydrate (pNPP tablet) were dissolved in substrate buffer for alkaline phosphatase, as given in 3.2. If conjugate enzyme (secondary antibody) is horseradish peroxidase, 100 µl H₂O₂ was added to substrate buffer for peroxidase, as given in 3.2.
- 50 µl of the substrate solution was loaded to each well for 30 minutes at room temperature. Since the substrate was light sensitive, the microplate was protected from light by covering with aluminum foil.
- The absorbance was read at 405 nm with Microplate Reader (7091000, Bio Tek).

4.3.2. One Version Ultra-ELISA HBsAg Kit (DIA-PRO)

As a preparation, kit solutions and kit microplate strips, which were already coated with anti-HBsAg antibody, were put at room temperature for 1 hour. Before used, Kit's wash buffer 20X and Enzymatic Conjugate solution 20X were diluted to 1X. The microplate was washed with 350 µl of 1X wash buffer one time. The kit positive control, negative control and the samples were loaded into each well (150 µl per well), except A1 well which was used as a blank. 100 µl of the 1X Enzymatic Conjugate solution was dispensed on the samples, except A1 well and the wells were covered with adhesive sealing foil. The microplate strips were incubated at 37 °C for 2 hours.

The samples were aspirated and the wells were washed with 350 µl of 1X washing buffer five times. 200 µl Chromogen/Substrate solution was dispersed all the wells, including A1 well. The microplate was covered with an aluminum foil to protect from the light and was incubated for 30 minutes at room temperature. 100 µl Sulphuric acid solution was added into all the wells to stop the reaction. The plate was read under Microplate Reader (7091000, Bio Tek) at 450 nm.

4.4. PCR

The PCR reaction mix was prepared in a 0.5 ml microcentrifuge tube. While preparing the PCR reaction mix, all the required solutions were kept on ice. Different PCR kits containing MgCl₂ and dNTP solution were used. The final volume of the PCR reaction mix was adjusted by changing the amount of the nuclease-free water. The reaction solutions were prepared according to PCR kit in the following order:

Table 4.1: PCR reaction's equipments and their amounts.

Equipment of PCR Kit	Amount (µl)
Nuclease free water	36 µl
Fermentase buffer 10X	5 µl
dNTP Mix (10 mM)	1 µl
MgCl ₂ (25mM)	2 µl
Forward primer (10pmol)	2 µl
Reverse Primer (10pmol)	2 µl
DNA template (100ng or 200ng)	2 µl
DNA polymerase (1U/ µl)	1 µl
Total volume per reaction	50 µl

The reaction tube was centrifuged briefly and, the tube was placed in a Thermocycler (PCR) to perform the polymerase chain reaction. The below mentioned general program of PCR steps were followed.

Table 4.2: PCR reaction general program.

Step	Temperature (°C)	Time (minute)	
Initial Denaturation	95 °C	2	
Denaturation	94 °C	1	29 Cycles
Annealing	55 °C	2	
Extension	72 °C	2	
Final Extension	72 °C	10	
Final Hold	4 °C	Infinite	

4.4.1. Joining PCR of Two DNA Fragments

The PCR reaction mix was prepared in a 0.5 ml microcentrifuge tube. While preparing the PCR reaction mix, all the required solutions were kept on ice. The PCR reaction was applied by using different PCR kits. The PCR reaction was performed at two stages. The reaction solutions for stage 1 and stage 2 were prepared according to the steps given in the PCR kit in the following order:

Table 4.3: Contents and amounts of joining PCR for first phase.

Requirements for First Phase	Amounts
Nuclease free water	Complete 50 µl
DNA template-1	50-100 ng/reaction
DNA template-2	50-100 ng/reaction
DNA template-3	25-50 ng/reaction
dNTP (10mM)	1 µl
MgCl ₂ (25mM)	2 µl
Buffer 10X	5 µl
DNA polymerase (1U)	1 µl
Total volume	50 µl

Table 4.4: First stage programme for joining PCR.

Temperature	Time	
95 °C	1 min	7 or 14 cycles
63 °C	4 min	

After the first step was completed with recommended cycle number (7-14) in the first step PCR programme that was mentioned above, PCR reaction was stopped and, the second stage tube was added into first step tube. PCR reaction was continued with the second PCR programme in the below.

Table 4.5: Contents and amounts of joining PCR for second phase.

Requirements for second phase	Amounts
Nuclease free water	Complete 50 µl
Forward Primer (10pmol)	2 µl
Reverse Primer (10pmol)	2 µl
dNTP (10mM)	1 µl
MgCl ₂ (25mM)	2 µl
Buffer 10X	5 µl
Taq Pol (1U)	1 µl

Table 4.6: Second stage programme for joining PCR.

Temperature	Time	
94 °C	1 min	29 cycles
55 °C	2 min	
72 °C	2 min	
72 °C	4 min	
4 °C	Pause	

4.4.2. PCR Purification

After the PCR, sample purification was performed by following High Pure PCR Product Purification kit (Roche, Cat No.11732676001) protocol:

1. 500 µl Binding Buffer was added to each 100 µl of the tubes containing PCR products and the mixture was mixed gently.
2. After one High Pure Filter Tube into one Collection Tube was placed into Collection Tube, the mixture was transferred into the Filter Tube.
3. The column was centrifuged for 1 minutes at 16000 x g at room temperature and flow through was removed.
4. After the Collection Tube was replaced with the Filter Tube and 500 µl Wash Buffer was added into the column.
5. Step 3 was repeated again.
6. Filter Tube was connected to Collection Tube and 200 µl Wash Buffer was added into the column.
7. After the centrifuge step was repeated, Filter tube was placed onto clean 1, 5 ml microcentrifuge tube. 50 µl Elution Buffer was added into the Filter Tube.
8. The purified PCR product was kept at -20 °C after centrifugation at 16000 x g for one minute.

4.4.3. Purification of DNA Fragment from Agarose Gel

According to agarose gel image, extraction of specific band from agarose gel was performed by using the High Pure PCR Product Purification kit (Roche, Cat No.11732676001). The below mentioned protocol was followed:

- After the sample was run on the agarose gel, desired band was cut with a clean lancet under the UV light by protecting your head.
- The band was put into a clean and pre-weighed 1,5 ml microcentrifuge tube and the weight of the band was defined.

- According to the weight, 300 µl of Binding Buffer was added to each 100 mg the gel slice into the tubes. The gel containing desired DNA fragment dissolved in the Binding Buffer by vortexing.
- The tube was incubated for 10 minutes at 56 °C, every 2 minutes, it was vortexed during the time of incubation.
- 150 µl isopropanol for every 100 mg band slice was added to the tube and was vortexed carefully.
- One High Pure Filter Tube into one Collection Tube was prepared and the mixture was transferred the column.
- The column was centrifuged for 1 minutes at 16000 x g, flow through was discarded.
- After the Filter Tube was replaced into the same Collection Tube, 500 µl Wash Buffer was added to the Filter Tube.
- Centrifugation step (7) was repeated and Filter Tube was put into the same Collection Tubes.
- After 200 µl Wash Buffer was added into Filter Tube, centrifugation step (7) was performed again.
- Collection Tube was removed and the Filter Tube was placed onto a clean 1,5 ml microcentrifuge tube.
- 50 µl Elution Buffer was added into Filter Tube and it was centrifuged for 1 min at 16000 x g.
- Eluted DNA was kept in -20 ° C refrigerator.

4.5. Enzyme Digestion

Insert DNA and vector DNA were cut with restriction enzymes. The digestion reactions were prepared in a 0.5 ml microcentrifuge tube for insert DNA and pBI-CMVI vector according to the following quantities, separately. They were incubated at 37 °C incubator for different incubation times.

After incubation, the samples were run on the 1% agarose gel. In addition, 25 µl of each sample were mixed 5 µl loading dye and loaded on the 1% agarose gel for

1 hour at 80 V. Band isolation was performed by following the Roche gel extraction kit protocol.

Table 4.7: Contents and amounts for enzyme digestion.

Requirements	Amounts
Insert DNA or Vector DNA	600 ng / 5 µg
Restriction Enzyme	1 µl
Restriction Enzyme	1 µl
Cut smart 10X buffer	5 µl
Sterilized ddH ₂ O	completed to 50 µl

4.6. Ligation

After vector DNA and insert DNA was cut with the same restriction enzymes, ligation process was performed according to quantities in the below Rates of insert DNA: vector DNA was used for the calculation with NEBioCalculator [Web 1, 2019]. It was tried rate of vector DNA: insert DNA; 1:5. For the ligation, the tubes were incubated in a water bath at 16 °C overnight.

Table 4.8: Contents and amounts for ligation.

Requirements	Amounts
Vector DNA: insert DNA	1:5
T4 DNA ligase 10X buffer (Fermentas, 00057611)	5 µl
T4 DNA ligase (Fermentas, 00077294)	1 µl
Nuclease free water	Completed to 50 µl

4.7. Competent Bacteria Preparation

E. coli DH5 α strain was used as a competent bacteria. *E. coli* DH5 α strain stored at -80 °C were seeded into 5 ml LB liquid medium. It was incubated at 37 °C at 220 rpm for overnight. Next day, 0,4 ml of the culture was taken into 40 ml LB medium. The culture was amplified in incubator 37 °C at 220 rpm until OD₆₀₀ value reached to 0,5. The culture was incubated on the ice for 10 minutes. The culture was centrifuged at 4 °C at 3000rpm for 10 minutes. The supernatant was removed and, it was resuspended in 20 ml of previously cooled 50mM CaCl₂ solution. After it was incubated on the ice for 30 minutes and later, it was centrifuged at 3000rpm for 10 minutes at 4°C. The supernatant was removed and resuspended with previously cooled 50mM CaCl₂ solution. Therefore, *E. coli* DH5 α bacteria became competent cells and ready for the transformation. The remaining competent cells were stocked with 99% glycerol to be contained 15% glycerol at -80 °C.

4.8. Transformation

The ligation reaction was completed with 50mM Tris-HCl solution (pH: 7. 2) to 50 μ l in a sterile tube. Previously prepared competent *E.coli* DH5 α bacteria cells were taken from -80 °C and 300 μ l of the competent bacteria was added into a glass tube. 50 μ l of the ligation product was mixed with 300 μ l the competent cells in the glass tube. At heat-shock process, the glass tube was incubated for 1 hour on the ice. After the incubation, it was kept at 42°C in a water bath for three minutes, then, it was incubated at room temperature for ten minutes. In order to provide both amplifying of the transformed bacteria and translation of the antibiotic resistance gene, the glass tube that was added 1 ml LB media was placed in an incubator at 37 °C at 220 rpm for one hour. 200 μ l of bacteria culture was seeded onto LB agar containing 1% of antibiotic to select the transformed bacteria which have contained the antibiotic resistance gene. In addition, the residue from the culture was added into 5 ml LB medium containing the same antibiotic to be 1%. The bacteria culture was kept in the incubator overnight at 37 °C at 220 rpm. The agar plate was put at 37 °C overnight.

Next day, positive PCR clones were isolated by using Plasmid Isolation Miniprep Kit (ZymoPURE, D4209) protocol:

- 5 ml of bacterial culture was transferred into 1,5 ml microcentrifuge tubes part by part and was centrifuged for 30 seconds at 16000 x g.
- Supernatant was removed completely and 250 µl of ZymoPURE™ P1 (Red) was added into the microcentrifuge tube and, it was resuspended by pipetting.
- 250 µl of ZymoPURE™ P2 (Green) was added into microcentrifuge tube, the mixture was mixed gently by inverting the tube for 6-8 times and, it was incubated at room temperature for 3 minutes.
- 250 µl of ice-cold ZymoPURE™ P3 (Yellow) was added and inverted 8 times. The mix will form a yellow or yellowish sediment in the microcentrifuge tube.
- After the lysate was incubated for 5 minutes on the ice, it was centrifuged for 5 minutes at 16,000 x g.
- 600 µl of the supernatant was transferred into a clean 1.5 ml microcentrifuge tube carefully.
- 275 µl of ZymoPURE™ Binding Buffer was added onto the microcentrifuge tube and was mixed gently by inverting the tube 10 times.
- A Zymo-Spin™ II-P Column was placed in a Collection Tube and, the mixture was transferred into column.
- After the mixture was incubated for 2 minutes into Zymo-Spin™ II-P/Collection column at room temperature, the column was centrifuged at 5,000 x g for 1 min., the flow through collected in the collection tubes was discarded.
- 800 µl of ZymoPURE™ Wash 1 was added into the Zymo-Spin™ II-P Column and it was centrifuged at 5,000 x g for 1 min, flow though was discarded again.
- 200 µl of ZymoPURE™ Wash 2 was added into the Zymo-Spin™ II-P Column and it was centrifuged at 5,000 x g for 1 min. The flow through was discarded.

- The Zymo-Spin™ II-P Column was centrifuged again at 10,000 x g for 1 minute to remove any wash buffer completely.
- The Zymo-Spin™ II-P Column was transferred into a clean 1.5 ml tube and 25 µl of ZymoPURE™ Elution Buffer was added to the column and, it was incubated at room temperature for 4 minutes.
- After the column was centrifuged at 10,000 x g for 1 minute. The column was removed and the isolated plasmid DNA was kept in -20°C refrigerator.

Post transformation, the colonies on agar plates were observed with the naked eye. 15 µl of sterilized distilled water were added in 0,5 ml microcentrifuge tubes. In addition, 5ml of LB broth containing 1% ampicillin were prepared in glass tubes. The colonies were chosen by using sterilized toothpicks. The toothpick with bacteria dissolved into microcentrifuge tubes and then the toothpick placed into glass tubes to grow from one colony. The samples in microcentrifuge tubes were incubated at 95 °C for 3 minutes to disrupt the bacterial membrane. They were centrifuged at 13000 rpm for 1 minute. Supernatants were used as a DNA template for colony. The polymerase chain reaction was performed by using 2 µl DNA template and PCR protocol in 4.4.

4.9. DNA Sequencing

Sequence analysis was performed using the DNA of the clones as a template to determine the nucleotide sequence in each fused Lig7_Fc clone. GenomeLab™ “Dye Terminator Cycle Sequencing” kit was used during the sequence analysis reactions.

4.9.1. Preparation of the Sequencing Reaction:

The PCR reaction mix was prepared in a 0.2 ml microcentrifuge tube. While preparing the reaction mix, all the required solutions were kept on ice. The reaction solution was prepared in the following order:

Table 4.9: Contents and amount for sequence PCR.

Requirements	Amounts
Sterilized water	5 μ l
DNA template (concentration 100ng- 200 ng per reaction)	2 μ l
Primer Forward or reverse (1.6 pmol/ μ l)	2 μ l
DTCS solution	4 μ l
Total Reaction volume	13 μ l

The reaction tube is thoroughly mixed and centrifuged. The tube was placed in a Thermocycler (PCR) to perform the polymerase chain reaction. The following programme was applied:

Table 4.10: Sequence PCR programme.

Temperature (°C)	Time	
90 °C	20 sec.	} 30 Cycles
50 °C	20 sec.	
60 °C	4 min.	
4 °C	Pause	

4.9.2. Alcohol precipitation of PCR products

- 0.5ml microcentrifuge tubes were prepared for each sample.
- 5 μ l of freshly prepared stop solution (2 μ l of 3M Sodium Acetate (pH: 5.2), 2 μ l of 100 mM Na₂-EDTA (pH: 8.0) and 1 μ l of 20mg/ml Glycogen) was added to the tubes.
- The sequencing reaction products were added.
- 60 μ l of 95% ethanol chilled (-20 ° C) was added into the tube.
- The tubes were centrifuged immediately for 15 minutes at 14000 rpm at + 4° C.
- Supernatant was discarded from tubes.

- The pellet was washed with 60 µl of 70% chilled (-20 ° C) ethanol.
- Again, the tubes were centrifuged for 2 minutes at 14,000 rpm at + 4° C.
- Step 7 and 8 were repeated again in the same manner. At last, the supernatant was removed completely and, the pellet was dried by Speed Dry Vacuum Concentrator device (RVC 2-25 CDplus Christ).
- The pellets were resolved in 30 µl sample loading solution (SLS) present in the kit and, approximately 300 µl of separation solution including the sequencing kit were added into ELISA plate.
- Samples were transferred sequentially to 96 well plate that is suitable for the CEQ 8800 instrument.
- Mineral oil was added over the top of the wells. Plate was placed at respective position in the device and analysis was made with CEQ8800 LFRa program.
- Sequence result was analyzed by pairwise sequencing alignment-Emboss needle in EMBL-EBI [Web 2, 2019].

5. RESULT

5.1. Characterization studies of PLC/PRF/5 cell line

5.1.1. Growth Curve of PLC/PRF/5 Cell Line

The PLC/PRF/5 cell line (Alexander cell) was obtained from a patient containing HBsAg in his blood, who had liver cancer [Daemer et al., 1980]. While the cell line releases hepatitis B surface antigen to the culture medium owing to the integrated HBsAg gene into its genome [Kirby et al., 1980], it contains no other HBV antigens such as HBcAg & HBeAg [Daemer et al., 1980]. Figure 5.1 shows an image of PLC/PLRF/5 cell line by using Cell Imaging Multi-Mode Reader (Cytation 5, Bio Tek). Since it releases HBsAg into culture media, PLC/PRF/5 cell line was used to show the inhibition effectiveness of anti-HBsAg scFv Lig7 in this study.

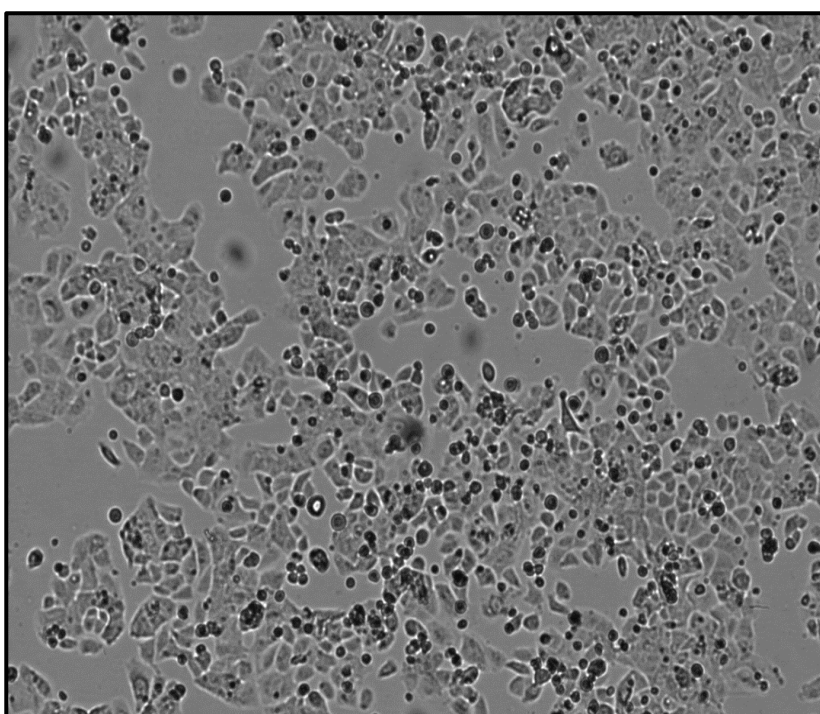


Figure 5.1: Invert microscope image of PLC/PRF/5 cell line image.

First of all, growth curve experiment was performed to determine both the doubling time and the time cell reaches 70% or 80% confluency to plan the next assays. The population doubling time is determined by defining a cell number with the exponential phase of the curve, monitoring the curve until this number doubles, and calculating the time between the two.

Doubling Time formula is:

$$DT = T \ln 2 / \ln (X_e / X_b) \quad (5.1)$$

- DT: Doubling time
- T: The incubation time in any units.
- X_b: The cell number at the beginning of the incubation time.
- X_e: The cell number at the end of the incubation time.

Growth curve assay for PLC/PRF/5 cell line was performed with three different cells quantities which were 100000, 30000 and 10000 cells per well in 24 well plates. The complete medium was changed with complete media twice a week because of nutrients depletion and drop in medium pH. The plate was observed in Clone Select Imager (Molecular Devices) for 10 subsequent days starting from the initial day. A growth curve graph was created by Clone Select Imager (Molecular Devices), as shown in figure 5.2. The doubling time (55,56 hours) of PLC/PRF/5 cell line seeded with 100000 cells was calculated by using the equation in 5.1[Web 3, 2019]. According to growth curve assay of PLC/PRF/5 cell line in figure 5.2, the cultures started with 10000 and 30000 cells per ml demonstrate insufficient growth, the cultures that were started with 100000 cells per ml revealed a growth curve. PLC/PRF/5 cell line, seeded with 100000 cells per ml, reached 70% confluency at approximately 69th hours, as shown in figure 5.2.

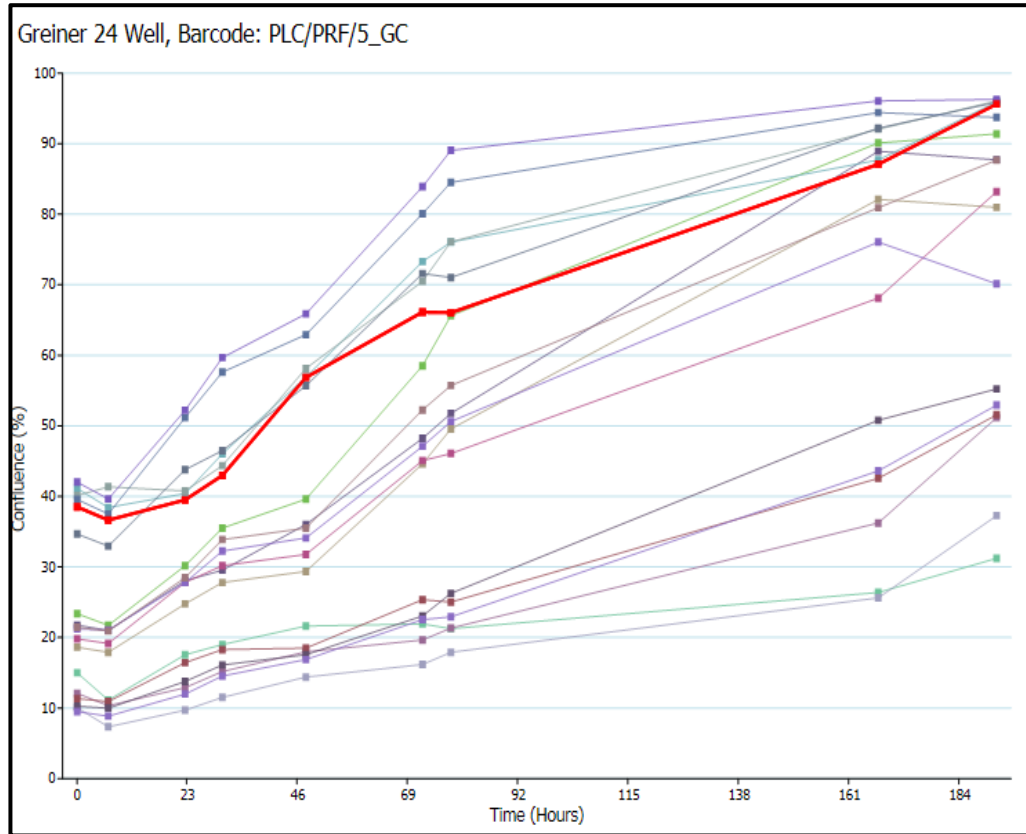


Figure 5.2: PLC/PRF/5 cell line grow curve graph through 10 day.

5.1.2. Determination of Optimal Antibiotic Concentration

Previously, anti-HBsAg scFv Lig7 [Erdag et al., 2017] gene was inserted into pIRES2 AcGFP1 vector (Clontech Cat. No. 632435) in the scope of T1007 project (project number: 113G100) at TUBITAK MAM Genetic Engineering and Biotechnology Institute. The vector provides the expression of the inserted gene and GFP1 gene by use single bicistronic mRNA, owing to the internal ribosome entry site (IRES) sequence, simultaneously. Figure 5.3 shows map of pIRES2 AcGFP1 plasmid vector.

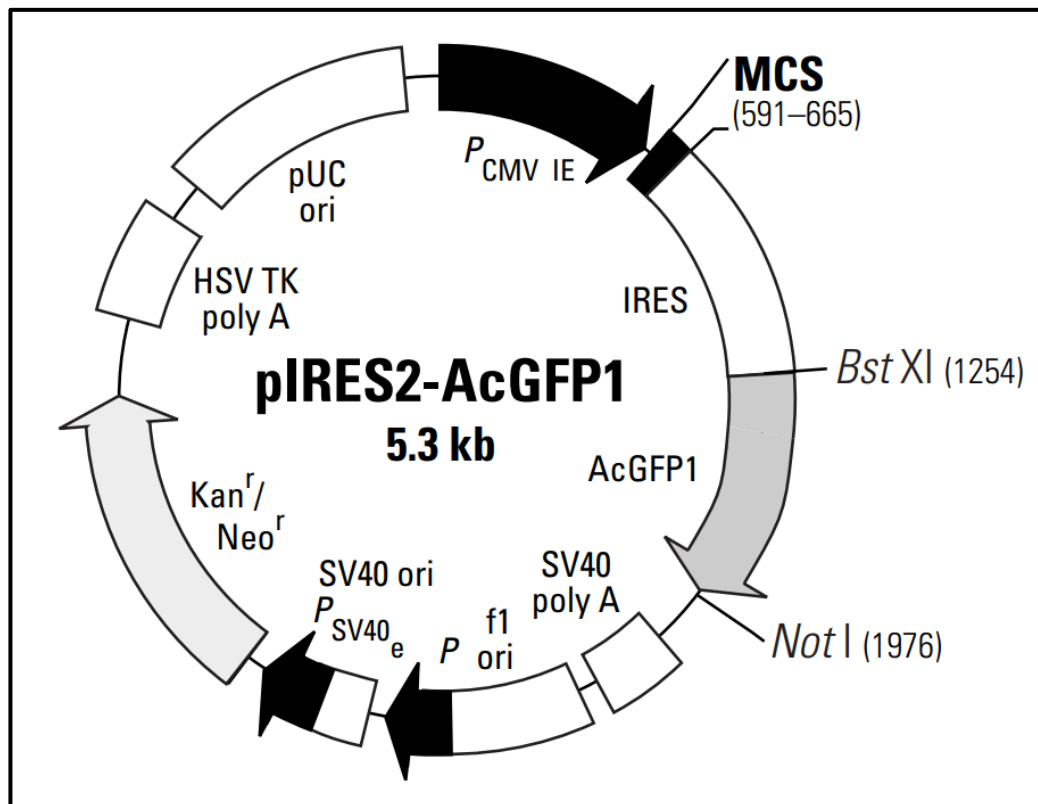


Figure 5.3: pIRES2-AcGFP1 vector map.

Antibiotic kill curve technique was utilized to determine optimal antibiotic concentration for the present cell line. At the dose-dependent experiment, minimum antibiotic concentration to kill all the cells defines as an optimal antibiotic concentration for providing stable transfection on the cell line.

Before performing stable transfection, the kill curve experiment was performed to define the optimal concentration of G-418 Solution (Geneticin) (Ref: 04727878001, Sigma) onto PLC/PRF/5 cell line since pIRES2 AcGFP1 vector contained an antibiotic resistance gene as a selective marker. Cell line was subcultured according to the protocol in section 4.1.2 into 24 well plates. The next day, different concentrations (50, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 µg/ml) of G-418 Solution (Ref: 04727878001, Sigma) were prepared and, these were added into the wells in ascending order. As a negative control, antibiotic solution was not added into 2 wells. Clone Select Imager (Molecular Devices) instrument was used to read the plate daily. Media change should be done on every third day. Consequently, optimal antibiotic

concentration to select transfected PLC/PRF/5 cell line was found to be 1000 µg/ml. This antibiotic's concentration that completely blocked the growth of cells was used in all transfection experiments to select the resistant cells. These transfected cells were further grown as stable cell lines.

5.2. Characterization of HBsAg

5.2.1. Analysis of HBsAg and scFv Lig7 Interaction with ELISA

ELISA assay was performed in order to identify interaction between HBsAg in PLC/PRF/5 culture medium and scFv Lig7. PLC/PRF/5 cell line released HBsAg into the culture medium, supernatant was collected and, ELISA assay was performed as per the protocol mentioned in section 4.3.1. High bind microplate (Corning 3690) was coated with the collected supernatant on daily basis. In addition, the plate was coated with 500 ng of HBsAg (Adw) and complete media as positive and negative controls were also kept, respectively. Milk powder (4%) was used as a blocking agent and, the blocked plate was incubated at 37 °C for 90 minutes. scFv Lig7 (1:500) was purified from *E. coli*, that contains His-tag sequence [Erdag et al., 2017], was added to bind to HBsAg for 2 hours at room temperature after the washing step. Monoclonal anti-polyhistidine–peroxidase antibody (Sigma, A7058) were added at the ratio of 1:2000 into the microplate as a secondary antibody to bind isolated scFv Lig7-His from *E. coli*. After 2 hours, it was continued with substrate solution. Consequently, commercial HBsAg (Adw) was detected, HBsAg present in the supernatant of the PLC/PRF/5 cell line could not be detected with ELISA by using bacterial produced scFv Lig7-His antibody, as shown in figure 5.4. The results are in-lined with the data presented by Berrin, Erdag et al. [Berrin et al., 2017].

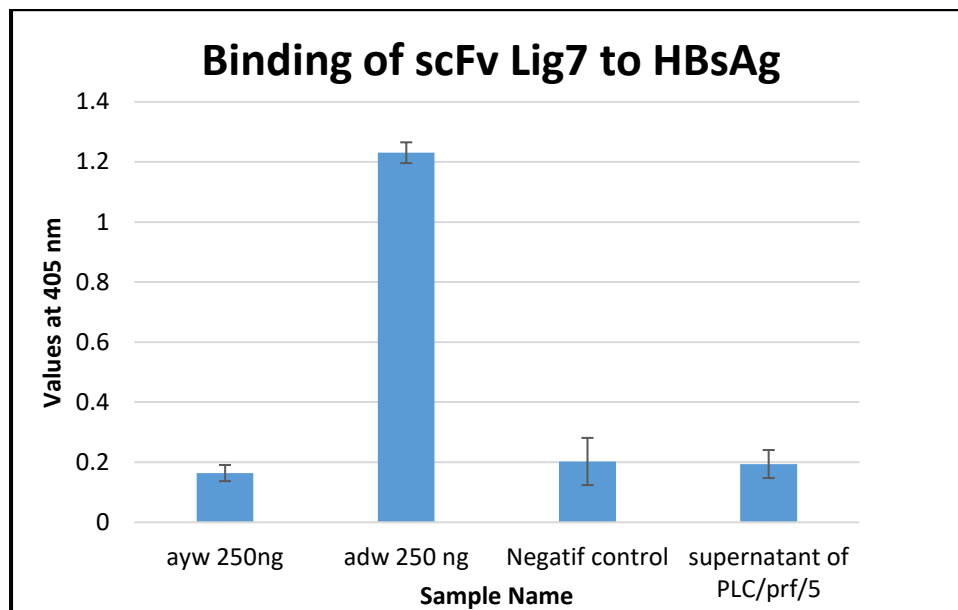


Figure 5.4: Demonstration of binding of scFv Lig7 to HBsAg.

5.2.2. Interaction of Commercial Recombinant HBsAg Types and anti-HBsAg Lig7 with Western Blot

SDS-PAGE and Western Blot Analysis techniques were used to observe the interaction of anti-HBsAg Lig7 to recombinant HBsAg (Adw, Abcam: ab91276) and HBsAg (Ayw, Abcam: ab73741) proteins. The Recombinant proteins, HBsAg (Adw, Abcam: ab91276) and HBsAg (Ayw, Abcam: ab73741), were diluted with sterilized ddH₂O to 50 ng/well. Western blot protocol was followed and, the staining of SDS-PAGE gel with Coomassie brilliant blue as mentioned in section 4.2. Page ruler pre-stained protein marker (Thermo Scientific, 26621) was used as a marker. Purified scFv Lig7-His-tag from *E. coli* (ratio of 1:500) was used as a primary antibody. Monoclonal anti-polyhistidine-peroxidase antibody (Sigma, A7058) at ratio of 1:10000 was added as a secondary antibody. According to western blot results, the interaction between the commercial HBsAg proteins and purified scFv Lig7 could not be detected. As the SDS-PAGE gel staining result, recombinant HBsAg (Ayw) was failed to be observed on the SDS PAGE gel. The weight of recombinant HBsAg (Adw) was found to be 24 kDa, as shown in the figure 5.5.

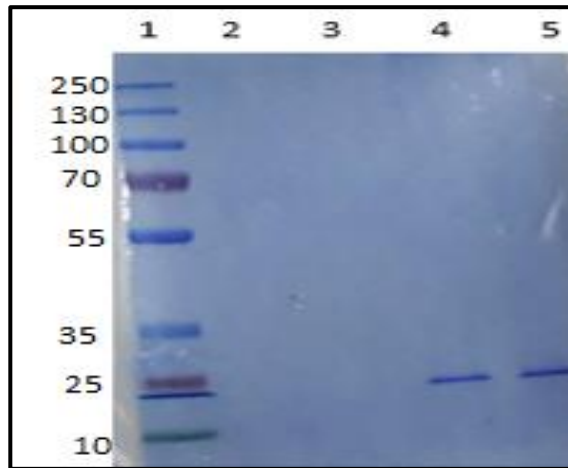


Figure 5.5: SDS-PAGE gel staining image of commercial HBsAg Adw and Ayw. 1. Pageruler presatined protein marker, 2., 3. Recombinant HBsAg (Ayw), 4., 5. Recombinant HBsAg (Adw).

5.2.3. Detection of HBsAg Produced by PLC/PRF/5 Cell Line with Western Blot

Western blot was performed to detect the presence of HBsAg released into the culture medium by PLC/PRF/5 cell line. During the subculturing, 200 μ l culture was taken and, the cells were removed by centrifugation. The supernatant was collected and stored in refrigerator to perform SDS-PAGE staining and western blot analysis. Staining of the SDS-PAGE and western blot analysis were performed as per the protocol mentioned in section 4.2. Diluted (1:1000) Anti-HBsAg (Abcam, Ab20758) and, diluted (1:1000) anti-mouse immunoglobulins/HRP (Dako, P0260) were used as primary antibody and secondary antibody, respectively. According to the results, HBsAg protein could not be detected with western blot analysis.

5.2.4. Demonstration of HBsAg in Culture Medium of PLC/PRF/5 Cell Line with ELISA

As shown by Rlrichard J. Daemer et al., 0.01 μ g/ml of HBsAg was detected by day 5 in the culture medium and, it's amount reached to a peak value of 0.6 μ g/ml from day 8 to day 15 [Daemer et al., 1980]. The amount of HBsAg present in 10- days culture media of PLC/PRF/5 cell line was analyzed by sandwich ELISA, as per the protocol mentioned in section 4.3.1. Each well of microplate was coated with 250 ng

of recombinant HBsAg (Adw). It was blocked with 1% BSA solution for 90 minutes. Anti-HBsAg (Ab20758), 2 µg/ml, was added to each well of the microplate as a primary antibody. The diluted (1:1000) rabbit anti-mouse immunoglobulins/HRP (Dako, P0260) was used as a secondary antibody. Anti-HBsAg antibody strongly bonded to commercial recombinant HBsAg (Adw), as shown in the figure 5.6. The amount of HBsAg produced by PLC/PRF/5 cells was very low and, the current methodology was not able to detect it, as shown in the figure 5.6.

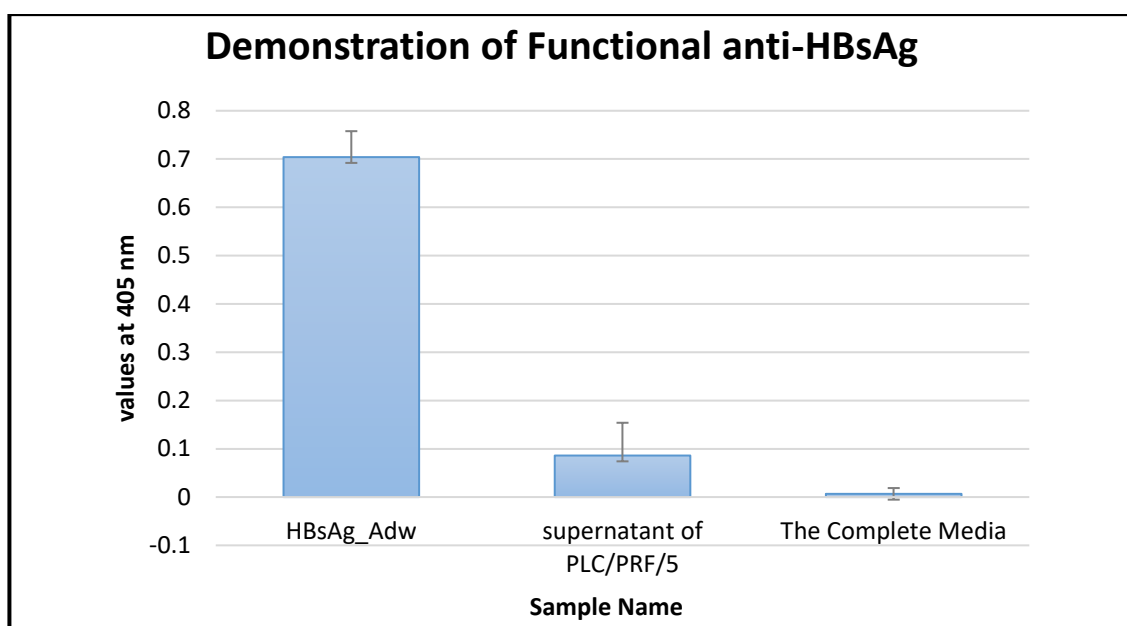


Figure 5.6: ELISA result of HBsAg from culture medium of PLC/PRF/5 cell line.

5.2.5. Detection of HBsAg with Ultra-ELISA HBsAg Kit

Furthermore, Ultra-ELISA HBsAg kit (DIA-PRO) was used to capture minimum amount of HBsAg present in culture medium of PLC/PRF/5 cell line. The Kit could capture minimum 2 ng of HBsAg protein. The Kit was used as per the standard protocol mentioned in section 4.3.2. The recombinant HBsAg (Adw) protein (Abcam, ab91276), 37ng, was used as a positive control along with the positive control present in the Kit. The recombinant HBsAg (Adw) protein showed the highest absorbance at 450 nm, figure 5.7. No absorbance was observed for the complete media, so it was proved that the complete medium of PLC/PRF/5 cell line did not contain HBsAg

protein. Furthermore, the second highest absorbance was observed for supernatant of PLC/PRF/5 cell line, figure 5.7. Thus, it was proved that the cell line released HBsAg protein into the culture medium.

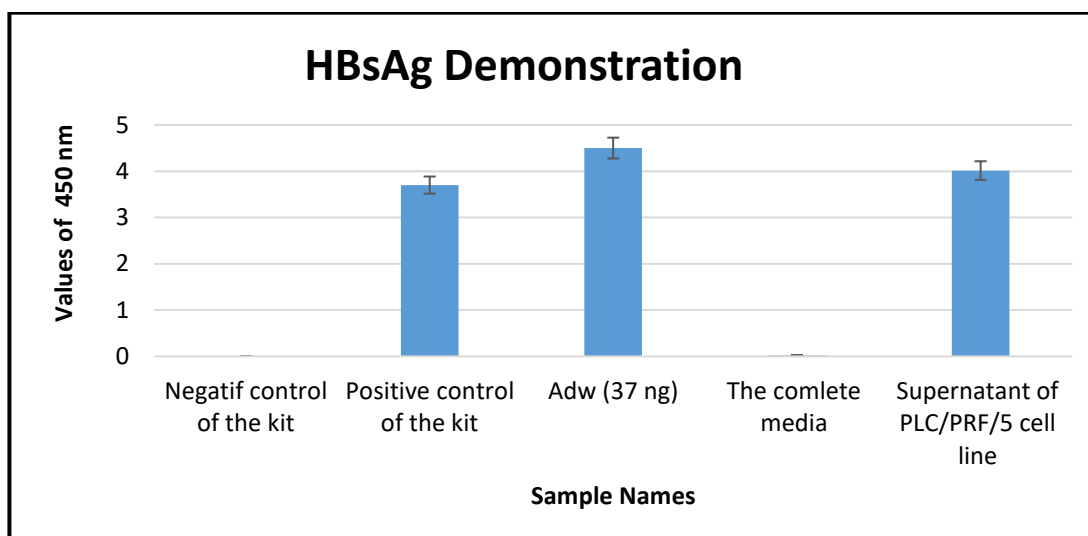


Figure 5.7: Demonstration of HBsAg in culture medium of PLC/PRF/5 with ELISA HBsAg kit.

5.3. Transfection of pIRES2 AcGFP1 Vector Containing scFv Lig7 Gene in PLC/PRF/5 Cell Line

5.3.1. pIRES2 AcGFP1 Vector Transfection Using Lipofectamine 3000

After determining optimal antibiotic concentration (section 5.1.2), transfection of pIRES2 AcGFP1 vector containing scFv Lig7 gene into PLC/PRF/5 cell line was initiated as per the protocol mentioned in section 4.1.4. Before transfection, PLC/PRF/5 cell line was subcultured with seeding density of 100000, 20000 and 300000 cells/ml in four replicates in 24 well plates. The next day, 500 ng/well of vector containing insert DNA was transfected to the cells that reached 80% confluency by using Lipofectamine 3000 protocol mentioned in section 4.1.4. Transfected cells present in optimum solution were incubated for two days in an incubator. In addition, the cells were observed under the fluorescent microscope. Figure 5.8 shows image of transfected cells (seeded with 100000 cells/well) containing construct (pIRES2 AcGFP1 vector with scFv Lig7 gene). After 48 hours of

the transfection, the culture containing cells was collected in microcentrifuge tubes to demonstrate inhibition of HBsAg with western blot analysis. In order to select stably transfected cells, 1000 µg/ml G-418 solution (Ref: 04727878001, Sigma) was added into the culture medium of transfected cells, while subculturing it for initial two weeks to eliminate non transfected cells.

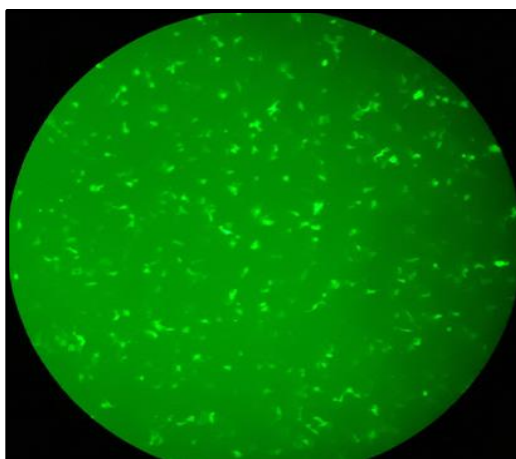


Figure 5.8: Image of the transfection under the fluorescent microscope.

Western blot analysis and SDS-PAGE staining were performed, protocol as mentioned in section 4.2, in order to prove inhibition of the HBsAg in the culture media after 48 hours of post-transfection with Lipofectamine 3000. In addition to the transfected supernatant samples, recombinant HBsAg (Adw) was used as a positive control, the complete media of the PLC/PRF/5 was used as a negative control. Purified Lig7 from *E. coli* (diluted to ratio 1:500) and monoclonal anti-polyhistidine–peroxidase antibody (diluted to ratio 1:10000) were used as a primary and secondary antibody, respectively. In western blot analysis, the presence of HBsAg protein and, its inhibition could not be detected in the culture medium of the transfected cells. After adding G-418 solution (Ref: 04727878001, Sigma) into the culture medium, PLC/PRF/5 cells died. Thus, an ELISA assay could not be performed.

5.3.2. Transfection with Turbofect Transfection Reagent Using Varied Concentration of Vector DNA

Before the day of transfection, PLC/PRF/5 cells were seeded with 100000 cells/ml of seeding density into 24 well plates. On the day of transfection, different concentrations (0, 5 µg/well, 1 µg/well and 1, 5 µg/well) of vector DNA were transfected into the PLC/PRF/5 cells using different concentration (1 µl /well, 1, 5 µl/well and 2 µl/well) of Turbofect transfection reagents as per the protocol mentioned in section 4.14. After 48 hours of post-transfection, transfection efficiency was determined with Cell Imaging Multi-Mode Reader (Cytation 5, Bio Tek). Post transfection efficiency observed was 1,5 %. Moreover, supernatant was collected into microcentrifuge tubes to demonstrate the presence of scFv Lig7 gene with western blot analysis, protocol mentioned in section 4.2. Rabbit anti-mouse immunoglobulins/HRP (Dako, P0260) was used directly at a ratio of 1:1000 as secondary antibody. According to the results of western blot analysis, scFv Lig7 gene could not be observed in culture of PLC/PRF/5 cells post-transfection.

5.3.3. Transfection with Different Transfection Agents into PLC/PRF/5 Cell Line

PLC/PRF/5 cells were seeded with 20000 cells/well of seeding density in each well of 96 well plate. On the subsequent day, three different transfection agents (Lipofectamine 2000, Lipofectamine 3000 and Turbofect) were tried at different concentration in combination to varied concentration of vector DNA, mentioned in the table 5.1. After 48 hours of post-transfection, transfection efficiency was measured with Cell Imaging Multi-Mode Reader (Cytation 5, Bio Tek). As depicted in the figure 5.9, better transfection efficiency was observed in PLC/PRF/5 cells by changing the amount of DNA used, rather than the different amounts of the transfection reagents used. Whereas, the lowest transfection efficiency was observed with Turbofect reagent and, the highest efficiency was observed with Lipofectamine 2000. In addition, after 48 hours of post transfection, the supernatants were collected for ELISA assay.

Table 5.1: Amounts of different DNA and three different transfection agents.

Amounts of vector DNA	Amounts of the reagents			
DNA 100 ng /well	0,15 μ l reagent	0,2 μ l reagent	0,3 μ l reagent	Lipofectamine 3000
DNA 175 ng /well	0,15 μ l reagent	0,2 μ l reagent	0,3 μ l reagent	
DNA 250 ng /well	0,15 μ l reagent	0,2 μ l reagent	0,3 μ l reagent	
DNA 100 ng /well	0,2 μ l reagent	0,3 μ l reagent	0,5 μ l reagent	Lipofectamine 2000
DNA 175 ng /well	0,2 μ l reagent	0,3 μ l reagent	0,5 μ l reagent	
DNA 250 ng /well	0,2 μ l reagent	0,3 μ l reagent	0,5 μ l reagent	
DNA 100 ng /well	0,3 μ l reagent	0,4 μ l reagent	0,6 μ l reagent	Turbofect
DNA 200 ng /well	0,3 μ l reagent	0,4 μ l reagent	0,6 μ l reagent	
DNA 300 ng /well	0,3 μ l reagent	0,4 μ l reagent	0,6 μ l reagent	

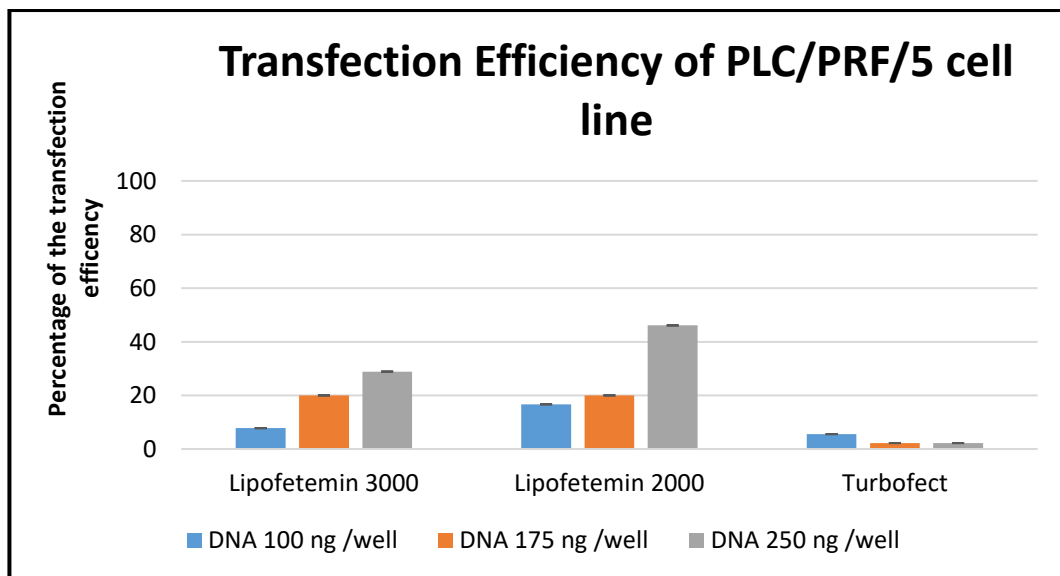


Figure 5.9: The result of transfection efficiency which was performed with three different transfection agents.

Ultra-ELISA HBsAg kit (DIA-PRO) was used to demonstrate HBsAg inhibition in the culture medium of PLC/PRF/5 cell line post transfection. The two different

concentrations (200 ng and 50 ng) of recombinant HBsAg (Adw) protein were used as positive control in the experiment. After 48 hours of post-transfection, the samples that showed the best transfection efficiencies were selected and proceeded further for ELISA assay. According to recombinant HBsAg results, concentration of the samples was calculated. As shown in the figure 5.10, the transfection with Lipofectamine 3000 ensured the best inhibition of HBsAg protein. While the transfection with Lipofectamine 2000 provided HBsAg inhibition close to transfection with Lipofectamine 30000. Least HBsAg inhibition was observed in the cells transfected with Turbofect reagent.

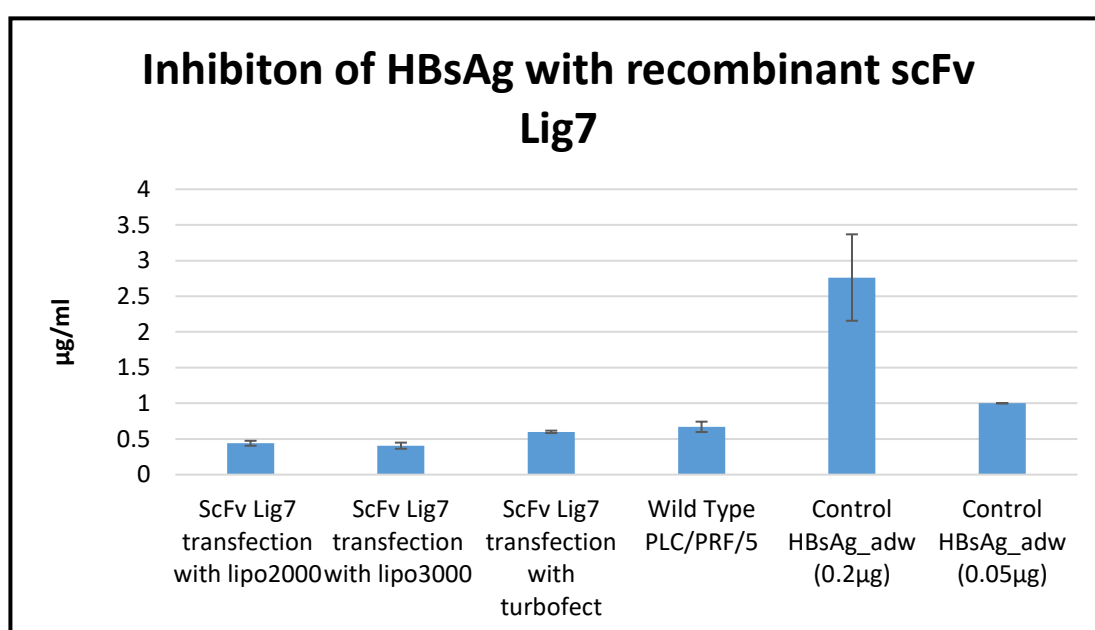


Figure 5.10: Demonstration of HBsAg inhibition after the scFv Lig7 transfection with ELISA HBsAg kit.

5.3.4. Transfection with Different DNA Amounts

PLC/PRF/5 cells were subcultured at 200,000 cells/ml in 24 well plate by following the protocol mentioned in section 4.1.2. In order to investigate the impact of different DNA amounts on transfection efficiency, different amounts (100 ng, 250 ng, 750 ng and 1000 ng per well) of pIRES2 AcGFP1 vector containing scFv Lig7 gene were used for the transfection. Lipofectamine 3000 reagent was used at a concentration of 1.5 µl per well. After 48 hours of post transfection, the culture medium was

collected to analyze by ELISA. Consequently, an increase in transfection efficiency was observed by increasing the amounts of DNA vector used as observed under Fluorescent microscope (Primovert, ZEISS), figure 5.11.

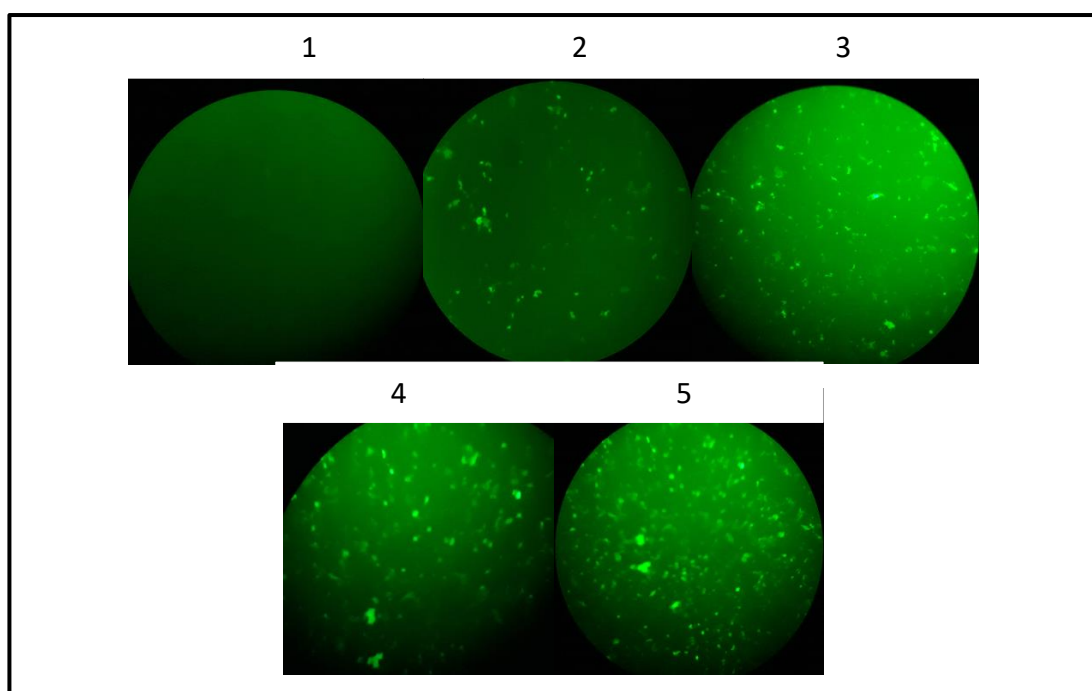


Figure 5.11: Image of scFv Lig7 transfection after 48 hours with different amounts in PLC/PRF/5. 1. Negative control, 2. 100ng/well pIRES2 AcGFP congaing scFv Lig7, 3. 250 ng/well pIRES2 AcGFP congaing scFv Lig7, 4. 750 ng/well pIRES2 AcGFP congaing scFv Lig7, 5. 1000 ng/well pIRES2 AcGFP congaing scFv Lig7.

Protocol of One Version ultra-ELISA HBsAg kit (DIA-PRO) in section 4.3.2 was used in order to the define HBsAg inhibition in the culture medium of PLC/PRF/ 5 cell line post transfection, which was performed with different DNA amounts. The sample collected from non-transfected cells was used as negative control, while PLC/PRF/5 supernatant containing DNA vector (100 ng, 250 ng, 750 ng, 1000 ng per well) was used as test samples. As a result, HBsAg inhibition was observed in all the supernatant of PLC/PRF/5 cells after the transfection of pIRES2 AcGFP1 vector containing scFv Lig7 gene into the cells. The transfected cells, containing 750 ng/well DNA, provided the highest level of inhibition of HBsAg protein. While, the transfected cells containing 250 ng/well and 1000ng/ well DNA showed almost the same level of HBsAg inhibition, figure 5.12.

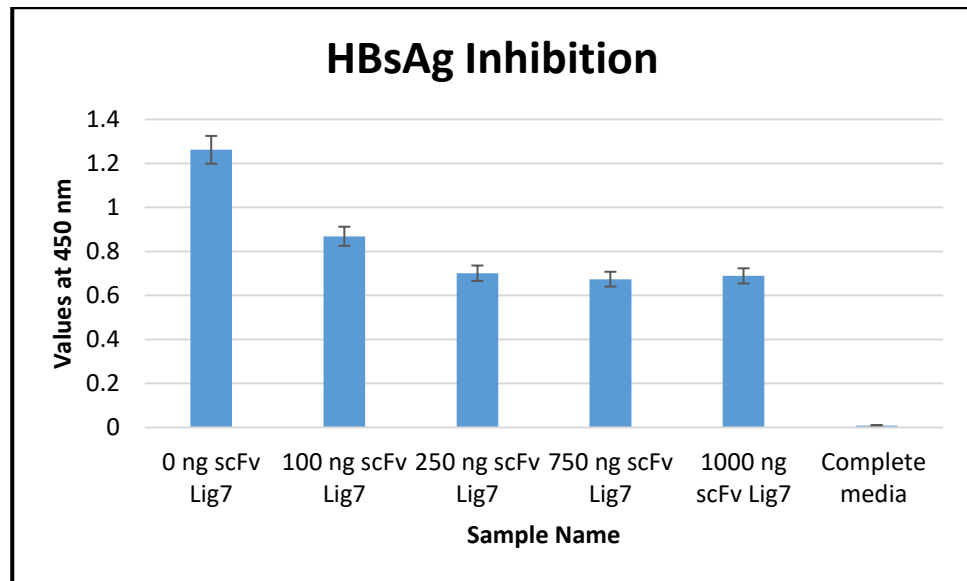


Figure 5.12: HBsAg inhibition after 48 hours from the scFv Lig7 transfection.

Sandwich ELISA was performed in order to determine the presence of scFv Lig7 gene in pIRES12 AcGFP1 vector, the protocol mentioned in section 4.3.1. The cells containing 1000 ng vector DNA was not used because of their inability to grow in the presence of G418 (Ref: 04727878001, Sigma) solution. The microplate was coated with 250 ng/well of HBsAg (Adw) protein. 1% BSA solution was used as a blocking reagent and, later, the plate was incubated for 2 hours at room temperature. Protein L-HRP (M00098, GenScript) at ratio of 1:20000 was directly used as a secondary antibody and, the plate was incubated for 90 minutes at room temperature after its addition. After substrate solution was added to the microplate and, it was incubated 1 hour. Later, it was measured with Microplate Reader (7091000, Bio Tek) at 405 nm. Consequently, while the transfected cells containing 250 ng vector DNA showed the highest levels of expression of scFv Lig7 gene, whereas, the transfected cells containing 750 ng vector DNA showed the second-highest level of expression of scFv Lig7 gene, as shown in figure 5.13.

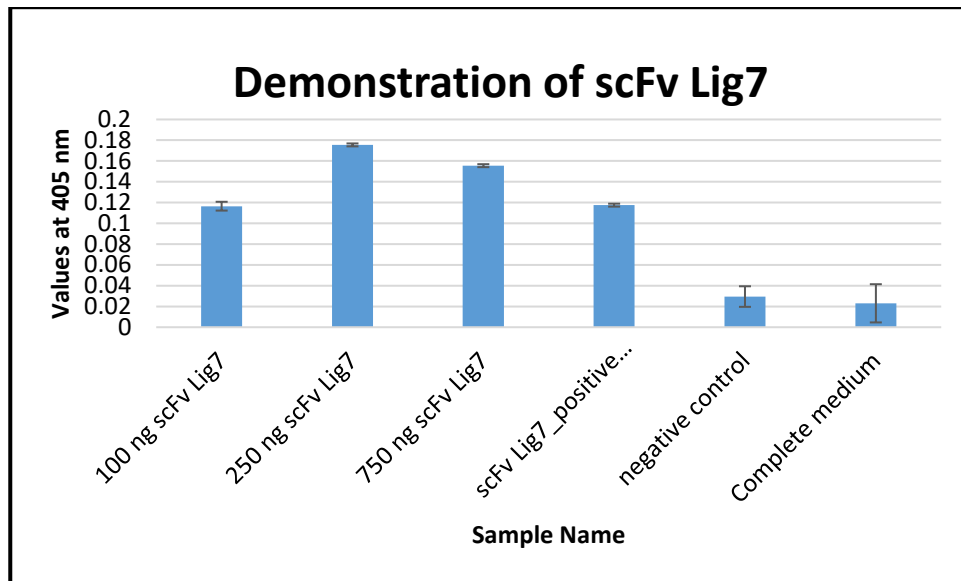


Figure 5.13: Demonstration of scFv Lig7 with ELISA after 48 hours the transfection.

5.4. Fusion of scFv Lig7 and Human Constant Fragment

After HBsAg inhibition was demonstrated with the ELISA kit, the presence of scFv Lig7 gene in transfected cells was difficult to be determined by ELISA. Therefore, in order to detect its expression easily, the human constant fragment (CH₂CH₃) was combined with scFv Lig7 gene. Furthermore, scFv Lig7 gene conjugated with human constant fragment became more stable.

Sequence manipulation suite web tool [Web 4, 2019] was used to design the primers in 3.4. Figure 5.14 shows the target sequence of the fusion Lig7_Fc antibody. scFv Lig7 fragment was extended with Flig7-1, Flig7-2 and Flig7-3 primers to add peptide leader sequence, Kozak sequence and BamHI restriction enzyme sequences, CH₂CH₃ fragment contains HindIII restriction enzyme sequence. Extended Lig7 and CH₂CH₃ fragment was joined together with a hinge region.

GAGTCATTTCGGATCCCGCCGCCACCAATGGCTGTCTTGGGGCTGCTCTTCTGCCTGGTGACATTCCCAA
 GCTGTGTCTTATCC**CAGGTGCA**GCTGCAGGAGTCAGGGGGAGCCTTAGTGAAGCCTGGAGGGTCCCTG
 AAACCTCTCCTGTGCAGCCTCTGGATTCACTTTTCAGTAACTATGCCATGTCTTGGGTTCGCCAGACTCC
 AGAGAAGAGGCTGGAGTGGGTTCGAGCCATTAATAATAATGGTGGTAGTACCTACTATCCAGACACTG
 TGAAGGACCGATTACCATCTCCAGAGACAATGTCAAGAACACCCTATATCTGCAAATGAGGAGTCTG
 AGGTCTGAGGACACAGCCGTGTATTATTGTGTAAGACAGTGGAGCTGGGCTGACTACTGGGGCCAAGG
 CACCACGGTCACCGTCTCC**TCAGGTGGA**GGCGGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGG
VL
ACATCGAGCTCACTCAGTCTCCAGCCTCCCTATCTGTCTCTGTGGGAGAGACTGTCACCATCACATGT
 CGAGCAAGTGAAAAATATTTTCCGTAGTTTAGCATGGTATCAGCAGAAACAGGGAAAAATCTCCTCAACT
 CCTGGTCTACGATGCAACAAACTTAGCAGATGGTGTGTCATCAAGGTTTCAGTGGCAGTGGATCAGGCA
 CACAGTTTTCCCTCAAGATCCACAGCCTGCAGTCTGGAGATTTTGGGACTTATTACTGTCAACATTTT
 TGGGGTACTCCGTGGACGTTTCGGTGGAGGCACCAAGCTGGAAATC**AAACGGGCG**AAACGGGTT**GAGCC**
CH2
 CAAATCTTGTGACAAACTCACACATGCCACCGTGCCCA**GCACCTGAA**CTCCTGGGGGGACCGTCAG
 TCTTCCTCTTCCCCCAAAACCAAGGACACCCTCATGATCTCCCGACCCCTGAGGTCACATGCGTG
 GTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCA
 TAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGGGTGGTCAGCGTCCTCACCG
 TCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCC
CH3
 CCCATCGAGAAAAACCATCTCCAAAGCCAAA**GGGCAGCCC**CGAGAACCACAGGTGTACACCCTGCCCCC
 ATCCCGGGAGGAGATGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCG
 ACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACACTACAAGACCACGCCTCCCGTGTCTG
 GACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAA
 CGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGT
 CTCCGGGTAAATGAAAGCTTGAATGACTC

Figure 5.14: The reference sequence of Lig7_Fc. The bold fonts represent starting sequences of VH, VL, CH2, CH3, respectively.

5.4.1. Amplification of scFv Lig7 gene present in pIRES2 AcGFP1 Vector

The first PCR was conducted to amplify scFv Lig7 gene present within pIRES2 AcGFP vector by using MyTaq DNA polymerase kit containing CMV-For forward and IRES2-R1 reverse primers. General PCR program mentioned in section 4.4 was used. Presence of scFv Lig7 gene was confirmed with 1% agarose gel as shown in the figure 5.15. The size of the scFv Lig7 gene was found to be 900 bp. Roche PCR purification kit, protocol mentioned in section 4.4.2, was applied for the PCR products, since they were used as a DNA template for the next PCR cycle as mentioned in section 5.4.2. Their concentrations were measured with NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

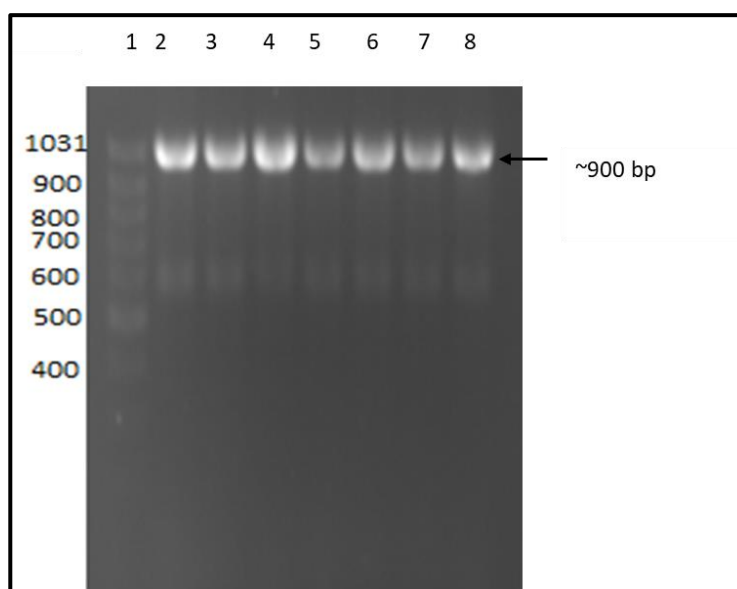


Figure 5.15: Gel image of scFv Lig7 from pIRES2 AcGFP1 vector. 1. Mass ruler low range marker (SM038), 2.,3.,4.,5.,6.,7.,8. scFv Lig7.

5.4.2. Amplification of Extended scFv Lig7 with Three-Step PCR Reactions

The purified scFv Lig7 fragment was extended with three-step polymerase chain reactions by using three different forward primers (Flig7-1, Flig7-2 & Flig7-3) and one reverse primer (Rlig7), as protocol mentioned in section 3.4. In the first PCR experiment, purified scFv lig7 gene (100ng/reaction), that was amplified with previous PCR in section 5.4.1, was used as a DNA template strand. Flig7-1 and Rlig7

were used as forward and reverse primers, respectively. The complete construct (scFv Lig7 gene & pCANTAB6 vector) was used as a positive control in the first PCR experiment. The amplified PCR product, Lig7-1 PCR product (735 bp), was observed in between 700 bp and 800 bp on the 1% agarose gel. The results were shown in the figure 5.16. The amplified PCR product, Lig-1, was purified with Roche PCR purification kit. The kit protocol followed for purification was mentioned in section 4.4.2.

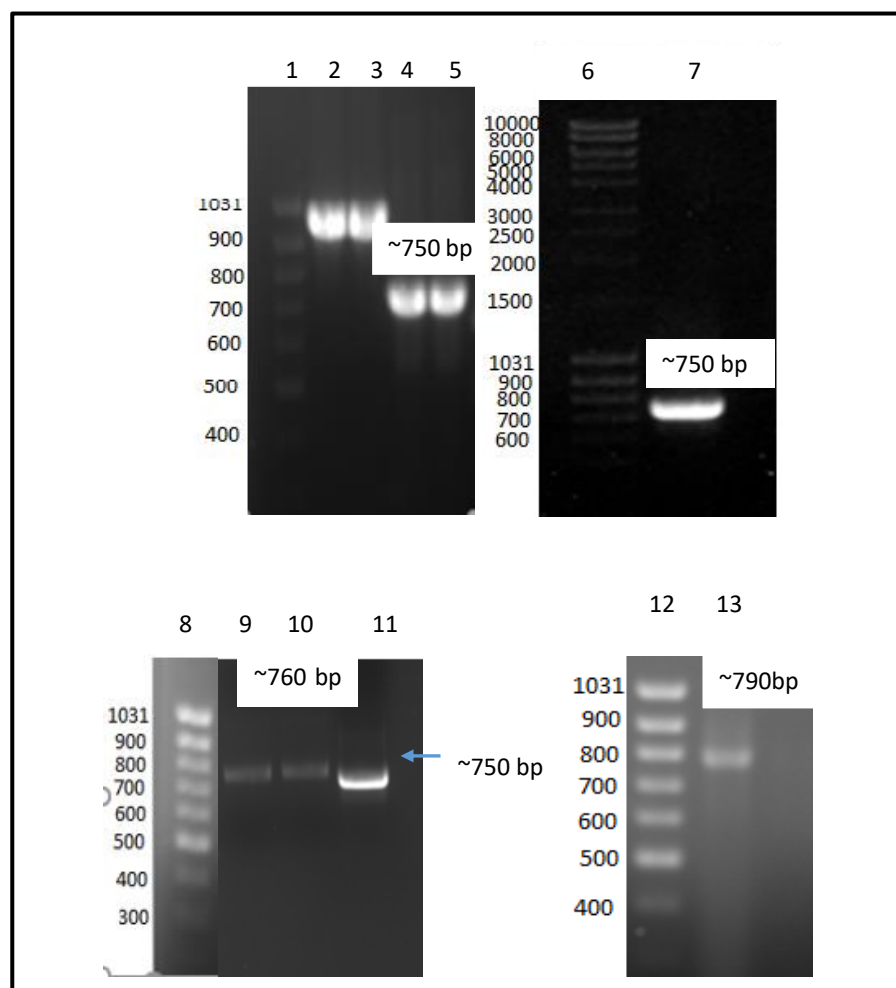


Figure 5.16: Demonstration of Lig7_1, Lig7_2 and Lig7_3 products after the first PCR on the gel. 1., 8., 12 Mass ruler low range marker (SM038), 2., 3. pCANTAB-6_Lig7 positive control, 4., 5. Lig7_1PCR-1, 6. Mix mass ruler marker (SM0403), 7. Lig7_1 after the PCR purification. 9., 10. Lig7_2 PCR, 11. Lig7_1 PCR, 13. Lig7_3 PCR.

In this section, purified product of first PCR reaction was used as a DNA template strand for the next PCR reaction. The same steps were followed for the subsequent PCR reaction. The PCR protocol mentioned in the section 4.4 was used as

such for amplifying Lig7-1, Lig7-2 & Lig7-3 PCR products. Once the PCR reaction was completed, the amplified product was run on 1% agarose gel with an appropriate weight molecular marker. The concentrations of the samples were measured with NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

The Lig7-1 product purified from the first PCR reaction was used as a DNA template strand for the second cycle of PCR reaction. Its concentration used in the second PCR reaction was 100ng/reaction. Flig7-2 and Rlig7 were used as forward and reverse primers for the second PCR reaction. The amplified product, Lig7-2, was observed approximately at a position of 760 bp on the gel, as shown in the figure 5.16. Later, it was purified by following the Roche PCR purification kit protocol as given in the section 4.4.2. Then, it was used as a DNA template strand for the third PCR reaction. As per the present results, the size of the Lig7-2 PCR product was higher than Lig7-1 PCR product.

The purified Lig7-2 (100 ng/reaction) product was used as a DNA template in the third PCR reaction. FLig7-3 and Rlig7 were used as forward and reverse primers in the third PCR reaction. Its results were shown in the figure 5.16. The amplified product, Lig7-3, was observed approximately at a position of 790 bp on the gel.

5.4.3. Amplifying of Human Constant Fragment (CH₂CH₃) and Hinge Linker with PCR

Simultaneously, human CH₂CH₃ fragment sequence was amplified with FCH₂ (forward) & RCH₃ (reverse) primers. 100ng/reaction of human Fc sequence containing pIRES plasmid was used as a DNA template (Homo sapiens clone AL-222H immunoglobulin heavy chain mRNA, partial cds) [Web 5, 2019]. The PCR protocol mentioned in the section 4.4 was followed by using MyTaq DNA polymerase kit. The size of CH₂CH₃ PCR fragments was found to be 654 bp on the 1% agarose gel. Apart from it, an unspecific band of 500 bp was also observed on the gel, as shown in the figure 5.17.

In addition, the annealing temperature of the general PCR program was changed from 55 °C to 60 °C and 52°C, step by step, to produce specific CH₂CH₃ band. The PCR protocol mentioned in section 4.4 was run using Fc plasmid (100 ng/reaction). FCH₂

(Forward) and RCH3 (Reverse) were the required primers for the reaction mix. The amplified PCR products were detected on 1% agarose gel with an appropriate weight molecular marker. According to the gel images, all the PCR products were containing the same unspecific band, as shown in the figure 5.17.

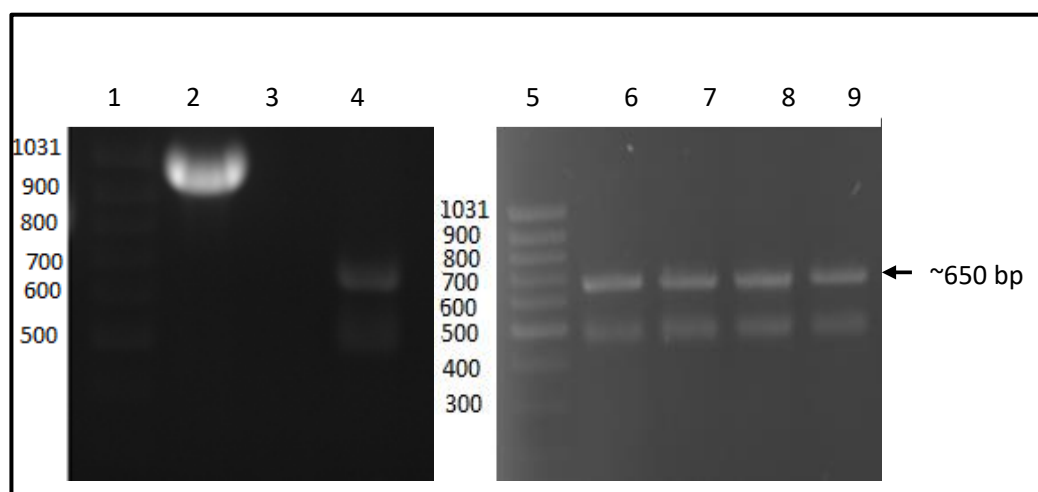


Figure 5.17: Gel image of amplifying CH₂CH₃ fragment with different annealing temperatures. 1., 5. Mass ruler low range marker (SM038), 2. pCANTAB6 _Lig7 positive control, 3., 4. CH₂CH₃_60 °C annealing PCR, 6., 7. CH₂CH₃_55°C PCR-1, 8., 9. CH₂CH₃_52°C PCR-1.

The desired band present at 650bp position, (CH₂CH₃ fragment) was cut out from the gel. Later, it was purified from the gel by using the Roche agarose gel DNA extraction kit, protocol mentioned in section 4.4.3. After the band isolation, it was run on the 1% agarose gel with an appropriate weight molecular marker. The presence of desired band was confirmed as shown in the figure 5.18.

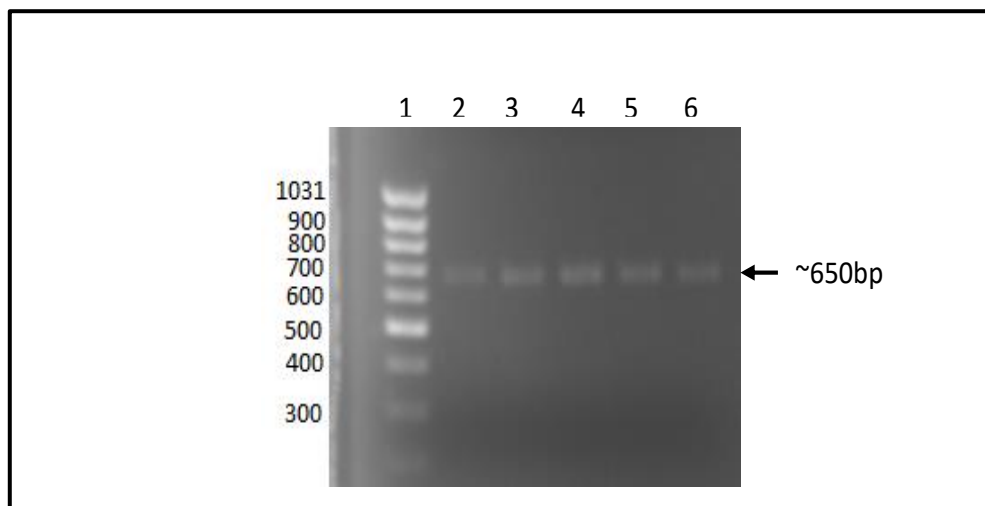


Figure 5.18: Demonstration of CH₂CH₃ fragment after band isolation. **1.** Mass ruler low range marker (SM038), **2., 3., 4., 5., 6.** CH₂CH₃_PCR after band isolation.

Furthermore, the hinge linker region was amplified by three different PCR programs, each one was formed by changing annealing temperature and extension time from the general PCR program in section 4.4. As its size was around 79 bp, therefore the extension time was decreased to 5 seconds and annealing temperature was kept 55°C in the first PCR program. In the second PCR program, extension time remained 5 seconds but the annealing temperature was increased from 55°C to 60°C. The last PCR program, annealing time was adjusted to 60°C, while the extension time was kept 30 seconds. The PCR was performed with MyTaq DNA polymerase enzyme kit. Whereas, Lig7KRV HingLinFor and KRVHing LinRev were used as primers in the PCR reaction. The Fc sequence containing pIRES plasmid (Homo sapiens clone AL-222H immunoglobulin heavy chain mRNA, partial cds) was used as a DNA template (100ng/reaction) strand [Web 5, 2019]. PCR products were run on 1% agarose gel with an appropriate weight molecular marker, as given in the figure 5.19. According to the gel image, all of the desired PCR products were observed on the gel, however, the hinge linker (79 bp) could not be identified on the gel. It might be due to weaker visibility of marker band (400 bp), as it was the last seen band on the gel. The PCR products were purified with Roche PCR purification kit protocol given in section 4.4.3 and, their concentrations were determined by using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

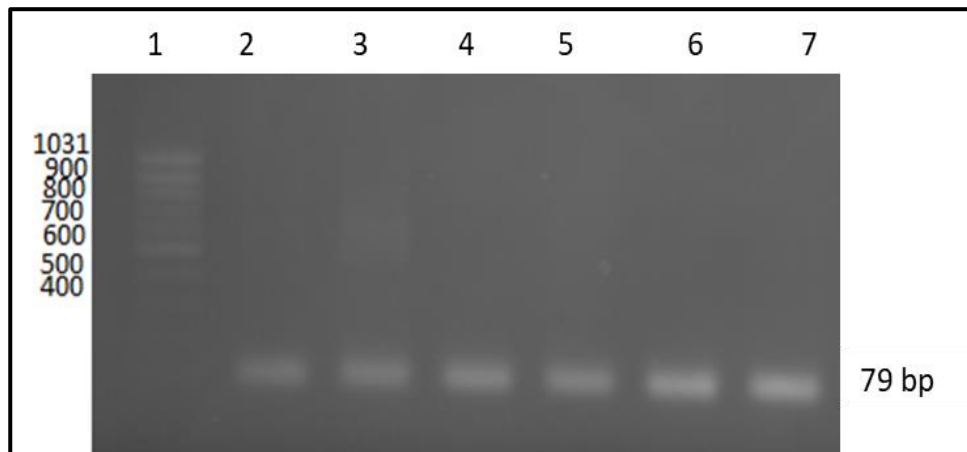


Figure 5.19: Gel image of amplifying hinge linker fragment with different annealing temperatures and extension times. 1. Mass ruler low range marker (SM038), 2., 3. Hinglinker PCR extension 5 sec annealing 55 °C, 4., 5. Hinglinker PCR extension 5 sec annealing 60° C, 6., 7. Hinglinker PCR extension 30 sec annealing 60° C.

5.4.4. Fusion of Lig7 and Human Fc (CH2CH3) with PCR

The scFv Lig7 gene and CH2-CH3 fragments of the human constant region were fused together with the help of hinge linker. The PCR protocol, 2- phases PCR reaction, was followed as mentioned in section 4.4.1. The DNA template fragments were mixed together in PCR tube to combine the fragments in the first step, as given in section 4.4.1. The first PCR reaction mix contains 50 µl of purified Lig7-3 (100 ng/reaction), 50 µl of purified CH2CH3 fragment (100 ng/reaction) and, 50 µl of the hinge linker (50 ng/reaction). No primers were used in the first step PCR. Fermentas Taq DNA polymerase and Promega Taq DNA polymerase kits were used in it. BamH1 and HindIII enzyme digestion sites containing primers were utilized to amplify united fragments in the second step in 4.4.2. The amplified product was run on 1% agarose gel with an appropriate weight molecular. The size of Lig7_Fc was found to be approximately 1500 base pairs on the gel, figure 5.20. According to the results, the Fermentas Taq polymerase kit failed to unite the DNA fragments. The fusion of Lig7_Fc was demonstrated together with unspecific bands as a smear on image with Promega Taq polymerase kit in the figure 5.22. Band isolation from the agarose gel was performed by using Roche agarose gel DNA extraction kit, protocol in section 4.4.3, to eliminate unspecific bands from the Lig7_Fc samples that were obtained

with Promega Taq DNA polymerase kit. The samples, Lig7_Fc, were run on 1% agarose gel with an appropriate weight molecular marker as shown in the figure 5.20.

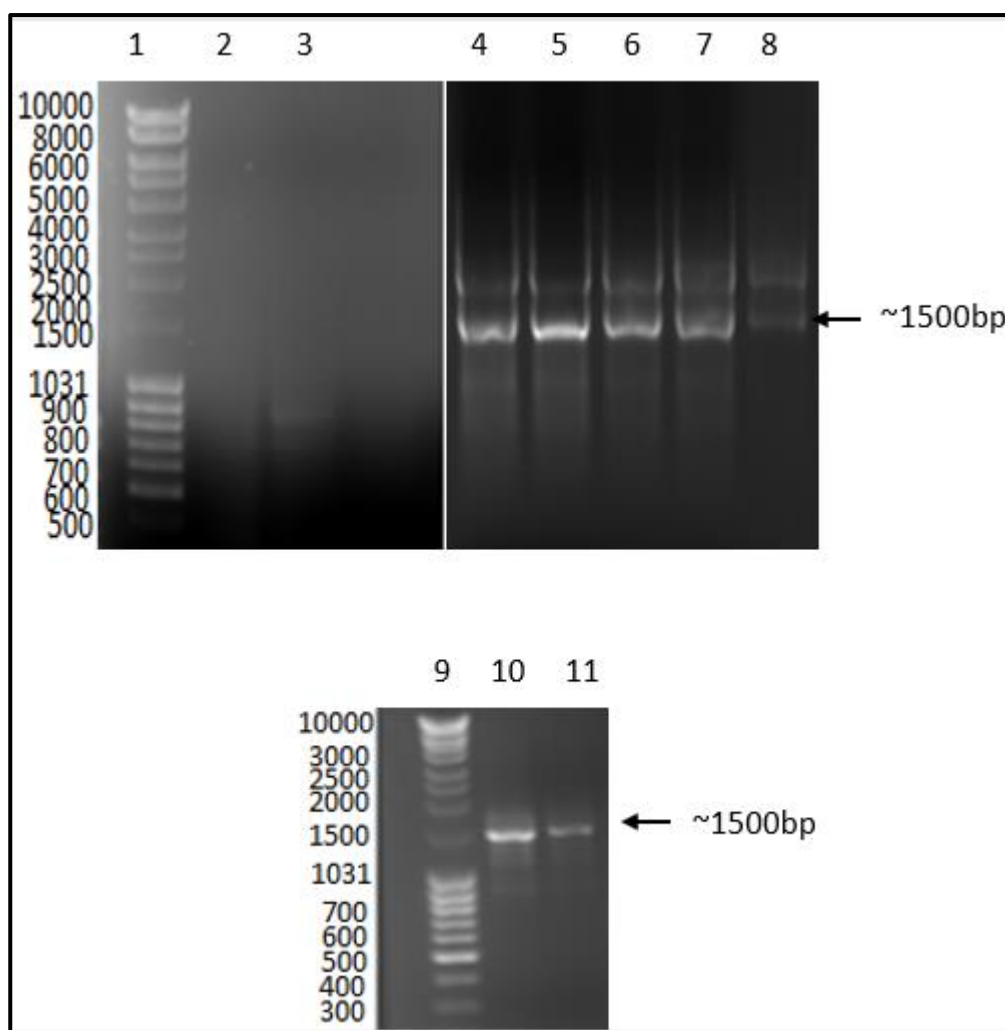


Figure 5.20: Gel image of assembling extended Lig7, hinge linker and CH₂CH₃ fragment. 1.9. Mix mass ruler marker (SM0403), 2., 3. Lig7_Fc PCR product 1 with Fermentas Taq DNA polymerase kit, 4., 5., 6., 7., 8. Lig7_Fc PCR product 5 with Promega Taq DNA polymerase kit. 10.,11. After the band isolation combined PCR product.

5.4.5. Digestion of Lig7_Fc and pBI-CMV1 Vector with Restriction Enzymes

pBI-CMV1 vector plasmid (Clontech, Catalog No. 631630) was used for cloning of Lig7_Fc fragment. The vector is a bidirectional expression vector and, it is suitable for mammalian transfection. It contains two different human cytomegalovirus

promoters (PminCMV1 and PminCMV2). The two inserted genes could be expressed in it. Its map is shown in the figure 5.21. It contains two multiple cloning sites (MCS1, MCS2). In this study, MCS 1 was used for inserting Gene of Interest.

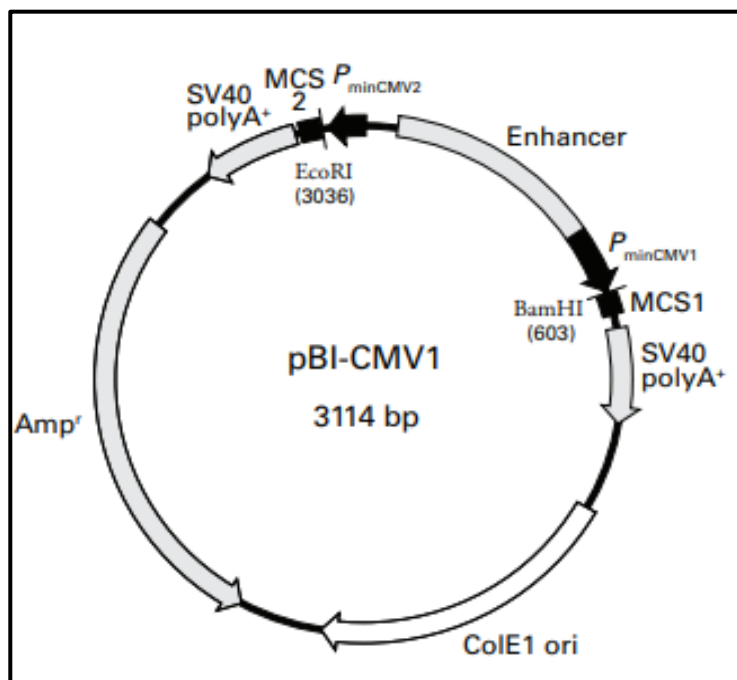


Figure 5.21: pBI-CMV1 vector map.

Restriction digestion of Lig7_Fc DNA fragment and pBI-CMV1 vector was performed with BamHI (R3136S) and HindIII (R3104S) restriction enzymes. The protocol mentioned in section 4.5 was used for restriction digestion. The prepared reaction mix contained Lig7_Fc insert (300 ng/25 μ l reaction) and pBI-CMV1 vector (5 μ g/50 μ l reaction). The reaction mix was incubated at 37°C for 30 minutes. Later, the digested samples were run on 1% agarose gel with an appropriate weight molecular marker, as shown in the figure 5.22.

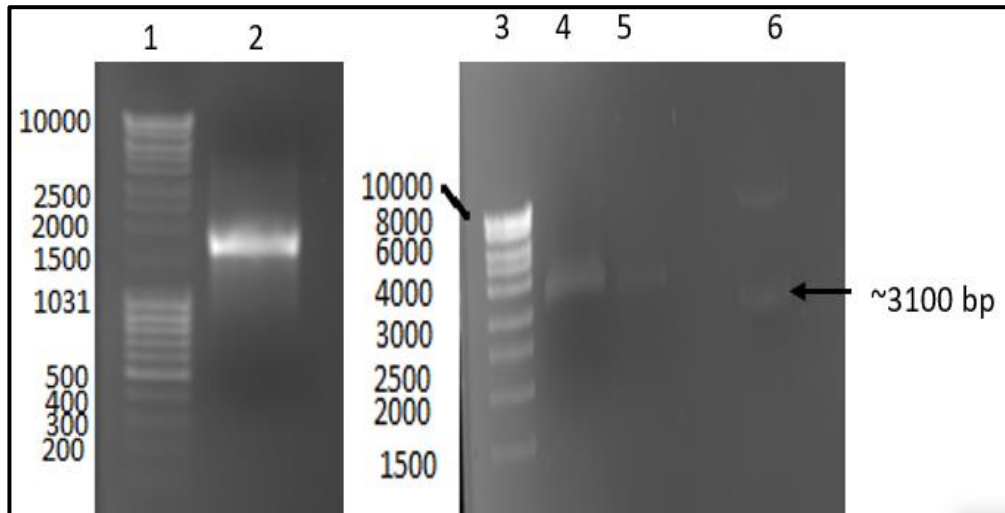


Figure 5.22: Gel image of digestion Lig7_Fc and pBI-CMV1 vector with BamHI and HindIII restriction enzymes. 1. Mix mass ruler marker (SM0403), 2. Digested Lig7_Fc, 3. Mass ruler high range marker (SM0393), 4., 5. Digested pBI-CMV1, 6. pBI-CMV1 without digestion.

5.4.6. Ligation of Lig7_Fc insert and pBI-CMV1 Vector

First of all, Lig7_Fc insert and pBI-CMV1 vector were cut with same restriction enzymes, protocol mentioned in section 4.6. Later, pBI-CMV1 vector and the Insert DNA was mixed in 0,5 ml microcentrifuge tube at the ratio of 1:5. Afterwards, it was incubated at 16 °C for overnight.

5.4.7. Transformation of the Vector DNA into *E.coli* DH5α Strain

The ligated reaction content was transformed into competent *E. coli* DH5α strain by using the transformation protocol given in section 4.8. Since, pBI-CMV1 vector contains ampicillin resistance gene, therefore, Ampicillin (100mg/ml) was used as selective marker to select the transformed bacterial cells. After the transformation step, the bacterial culture was seeded simultaneously in liquid LB and LB agar plate containing 100mg/ml ampicillin, as mentioned in the protocol given in section 4.8. Afterwards, these were incubated at 37 °C incubator in a shaker incubator at 220 rpm for overnight.

5.4.8. Colony PCR After the Transformation

In order to confirm the presence of Lig7_Fc gene in the transformed colonies grown on the agar plate, PCR was performed by choosing all the eight colonies present on the agar plate as per the protocol mentioned in the section 4.8. The general PCR program, mentioned in section 4.4, was used. The colony PCR samples were run on 1% agarose gel with an appropriate weight molecular marker, figure 5.23. As per the results, colonies 2, 3 and 7 confirms the presence of Lig7_Fc in it. It had an appropriate band size that is approximately 1500 bp. Consequently, the three transformed colonies were transferred into 50 ml LB broth containing 100mg/ml ampicillin. The next day, 300 µl of the bacterial culture, that had positive colonies (2,3 and 7), was frozen by mixing with 300 µl of 60% glycerol. The stocks were incubated at -80°C refrigerator. The Plasmid was isolated from the remaining culture by using the Plasmid Isolation Miniprep Kit (ZymoPURE, D4209) as mentioned in section 4.8. The purified plasmid concentrations were measured with Nanodrop (NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific)).

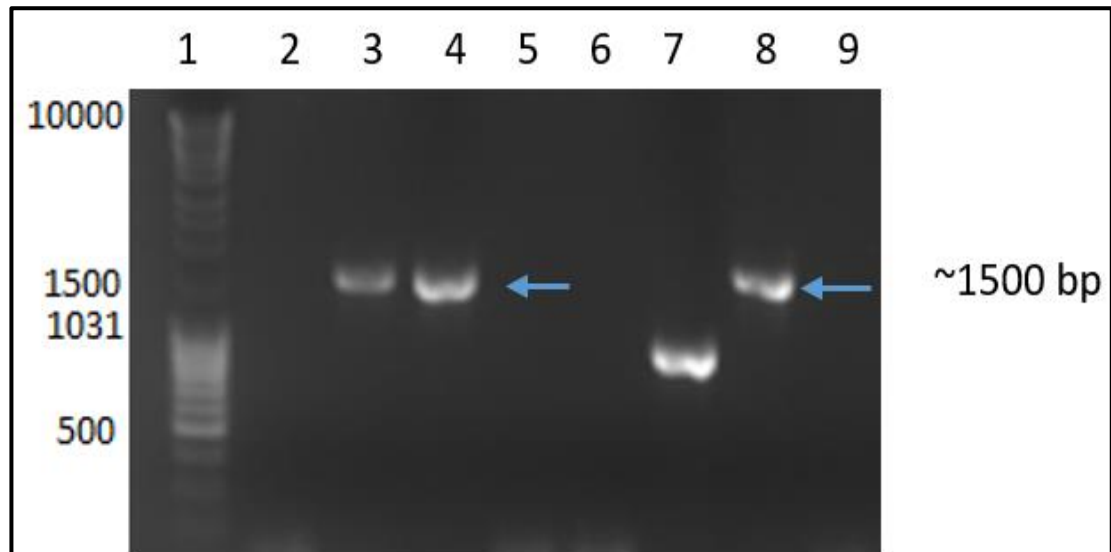


Figure 5.23: Gel image of colony PCR results after the transformation. 1. Mix mass ruler marker (SM0403), 2. Colony 1, 3. Colony 2, 4. Colony 3, 5. Colony 4, 6. Colony 5, 7. Colony 6, 8. Colony 7, 9. Colony 8.

5.4.9. Sequence Analysis of Lig7_Fc Genes

The required amount, 200ng of pBI-CMV1 vector containing Lig7_Fc, of the plasmid was isolated from the three colonies (2, 3 and 7). Later, DNA sequencing was performed by Beckman Coulter CEQ-8000 Analyzer to confirm that the colonies (2, 3 and 7) contains the desired sequence. The protocol mentioned in section 4.9 was used for DNA sequencing. The forward and reverse primers were diluted to 1,6 pmol. The sequence of Lig7_Fc were determined by using the same protocol as mentioned in the section 3.4 primers. The output data was stored in FASTA format. All the sequences were compared with the reference sequence by using EMBOSS Needle and Pairwise Sequence Alignment tool [Web 2, 2019]. The results are shown in the figure 5.24. It was concluded that no exact sequence similarity was observed with the reference sequence, as shown in the figure 5.24. However, except the human constant region (CH2CH3) and linker region sequence found similarity with the reference sequence. The results are depicted in the figure 5.24.

Colony 2:

```
-----GTTACATTTCCAAGTTGTTCT
ATCTCAGGTGCAGCTTAAGCAGTCAGGACCTGGCCTGGTGCAGCCCAGCCAGAGCCTGTCCATCACCTGCACCGTC
TCTGTTTTCTCACTGACTAACTA-CGGTGTCCACTGGGGGCGCCAGTCTCCAGGCAAGGGCCTGGAGTGGCTCGGA
G---TGATCTGGAGCGGTGGCAACACCGACTACAATACTCCTTTCACT-----TCCAGACTGAGCATCAACAAG
GACAATTCCAAGAGCCAAGTGTTTTTCAAGATGAACAGCCTGCAATCTAATGACACAGCCATCTACTATTGT----
-----GCCAGAGCCC-----TCAC---CTACTATGAT-----TAC-----GA
GTTTGCTTACTGG-----GGCCAAGGAACCTCTGGTCACTGTCTCTGCAGCCTC-----CACCAAG
GG-----CCCATCG---GTC-----TTCCC-----CCTGG-----
-----CACC-CTCCTCCAAG-----AGCA-----CCTCTGGG-----
---GGCACAGCTG---CCCT-----GGGCTGC---CTGG---TCAAGGAC---TACT-----
-----CG-GCGTGCACACC-TTCCCGGCT-----GTCCTACATCCCCGAAC---CGGTGA
CGGTGTCG---TGGAATCA---GGC-----GCC-----TGACCAG-G---TCCTC-----
-----AGGACTC-----TACTCCC---G---TCCTC-----AGGACTC-----TACTCCC-
-----TCAGCAGC---GTGGTGACCGTG-----CCC-----TCCAG-----CA
GCTTGG---GCA-----CCCAGACC TACATCTGCACAGTGAATCACAAGCCCAGCAACAC---CAAGGTGGAC
AAGAG-----AGTTGAGC-CCAAATCTTG-----TGACAAACTCACACATGC-----CCACCGTGCCCACT
CCCAGCCCCATCGAGAAAACCATCTCCAAGCCAAAGGGCAGCCCCGAGAACCACAGGTCTACACCTGCCCCCA
TCGCGGGAGGAGATGACCAAGAACCAGGTGAGCCTGACCTGCTGCTGCAAGGCTTCTATCCCAGCGACATCGCCG
TGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTT
CTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGCAACGTCTTCTCATGCTCCGTGATGCAT
GAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGAAAGCTAGTCAACAATATCT
CCAGAGGAT
```

[illegible]

Colony 7:

-----CG-CACACAATG--TGACAAGATCATAGAGTTTGTGTTGTGACTGTT-----CTGGTT
ACATTTCCAAGTATGTGTTCTATCTCAGGTGCAGCTTAAGCAGTCAGGACTCG-----CCTGGTGCAGCCAGCCA
GAGCTTGTC-----ATCACCTGCACCCTCTCTGGTTTCTCACTGACTAACTA-CGGTGTCCACTGGGTTGCGCA
GTCTCCAGGCAAGGGCCTGGAGTGCTCGGAG-----TGATCTGGAGCGGTGGCA-ACAAACCGACACTACAATA
CTCCTTTTCACTTCCAG-ACTGAGCATCAACAAGGA-CAATTC-----CAAGAGCCAAGTGTGTTTTCAAGATGA
ACAGC-----CTGCAA-----TCTAATGACACAGCCATCTACTATTGTGCCAGGACCCCTCACTTA
CTATGATTACGAGTT---TGCTTACTGGGGCAA-----AGGTGGACGGC-----
GTGGAGGTGCAGACTCCACCAAGGGCCATCGGTCTTCCCCTG-GCACCTC---CTCCAAGAGGAAACAGGGAA
AATCTCTCTCAAATC-----AGGCGCC---CTGACCAG-CGGCGTG-AACCACAGGTCTATGCTCAG-GGC
TGTGCGGGAGG-----AGATGAC---CAAGAACCAGGTCAAGCCTGACCTCCGGAGACTTGGGC ACCCAGACCTGT
-----CGCGGTGGA-----GTGGGAG-AGCAATGGGCAGCCGGAGATCACAAGCCCAGCAACAC
CAAGGTGGAG-AAGAGAGTTGAGCCCAAATCTTGTGACAAAATCACAATGCCCCACCTGCCCCAGCACCTGAACT
CCTGGGGGGGACCTCAGTCTTCTCTTCCCCCAAAACCCAAAGGACACCCCTCATGATCTCCCGGACCCCTGAGGTC
ACATGCGTGTGTGGTGGACGTGAGCCACGAAGACCTGAGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGC
ATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGGGTGGTCAAGCTCTCACCCTCTGCA
CCAGGACTGCTGAATGGCAAGGAGTACAAGTGCAGGTCTCCAACAAAGCCCTCCAGCCCCATCGAGAAAACC
ATCTCCAAAGCCAAAGGGGACCCCGAGAAACCACAGGTCTACACCTGCCCCATCGCGGGAGGAGATGACCAAGA
ACCAAGTCAAGCTGACCTGCTGCTGGTCAAAAGGCTTCTATCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCA
GCCGGAGAACTACAAGACCACGCT-----
-----CAGGTGGCAGCAGGGCAAGCTCTTCTATGCTCCGTGATGCA TGAGGCTCTGCACAACCACTAC
ACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGAAAGCTAGTCAACAATATCTCCAGA

5.4.10. Transfection of Colonies 2, 3 and 7 into CHO-K1 Cell Line

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4.1.6. In the transfection step, 4µl of the transfection reagent and 500 ng/well of the vector DNA were used. After 48 hours of post transfection, the culture medium of the cells was collected into centrifuge tubes to analyze the presence of the Lig7_Fc expression with ELISA and western blot. After 48 hours of post transfection, the medium of the cells was replaced with the complete media of the CHO-K1 in order to eliminate the non-transfected cells.

Western blot was performed to demonstrate the presence of Lig7_Fc gene in the CHO-K1 cells post transfection. 1% BSA solution was used as blocking reagent for 90 minutes. The two different types of the secondary antibodies were tried in the western blot analysis. One of them was anti-human IgG (Fc specific) –alkaline phosphatase antibody (1:50000) and, other was anti-mouse IgG (H+L) poly-
HRP (1:5000). For alkaline phosphatase conjugate, NBT/BCIP tablet (Ref 11697471001, Sigma) dissolved in distilled water was used as a substrate solution. Later, all the proteins in the culture medium was observed on the SDS-PAGE gel after the staining. Western blot analysis could not detect the presence of Lig7_Fc in the culture medium.

5.4.11. Determination of Lig7_Fc After the Transfection of colonies of 2, 3 and 7 with ELISA

Samples were collected after 48 hours of post transfection. Sandwich ELISA technique, as mentioned in section 4.3.1., was used to find the interaction between Lig7 and human constant fragment. The microplate was coated with recombinant HBsAg (Adw). Later, it was blocked with 1% BSA and incubated for 90 minutes. Samples were added to each well and, the plate was incubated for 2 hours. 50µl of conjugated secondary antibody, Anti-Human IgG (Fc specific) –alkaline phosphatase antibody (Sigma, A9544), diluted at the optimal concentration (1:50000) in blocking buffer was used. The interaction was not detected with the current antibody.

Another sandwich ELISA, protocol as mentioned in section 4.3.1., assay was performed with three different antibodies. The types of antibodies and secondary antibodies, which were used, are mentioned in the table 5.2. The color combination of coating antibody and secondary antibody, used in the experiment, are mentioned

in the table 5.2. Thus, four different conditions were tested to detect the presence of Lig7_Fc in the culture medium of the CHO-K1 cell line. The coating concentration of each antibody coating was adjusted to 250 ng per well. According to ELISA reader results, Lig7_Fc could not be defined with ELISA. In addition, it was revealed that all of the used antibodies in the sandwich ELISA assay worked functionally since positive control showed interaction between the primary and secondary antibody.

Table 5.2: Four different ELISA conditions with three different coating antibodies and secondary antibodies.

Coating	Secondary Antibodies
Anti-Human IgG (Fc specific) antibody (Sigma, I2136)	Goat anti-Mouse IgG (H+L), AP (Thermo Scientific,31320), 1: 5000
Anti-Human IgG (Fc specific) antibody (Sigma, I2136)	Anti-Human IgG (Fc specific) –alkaline phosphatase antibody (Sigma, A9544), 1:50000
Goat anti-Mouse IgG (H+L) Poly-HRP (Thermo Scientific 32230)	Anti-Human IgG (Fc specific) –alkaline phosphatase antibody (Sigma, A9544), 1:50000
Goat anti-Mouse IgG (H+L), AP (Thermo Scientific,31320)	Goat anti-Mouse IgG (H+L) Poly-HRP (Thermo Scientific 32230), 1:10000

In another sandwich ELISA assay, 250 ng of Recombinant Protein L Type 1 Protein (Novus Biologicals, NBP2-35050) was used for coating each of the microplate and, anti-Human IgG (Fc specific) –alkaline phosphatase antibody (Sigma, A9544), 1:50000 was used as secondary antibodies. The microplate was blocked with 1% BSA solution and, was incubated for 2 hours. After 48 hours of post transfection, Lig7_Fc antibodies which were isolated from the colonies 2, 3 and 7 are shown in the figure 5.28.

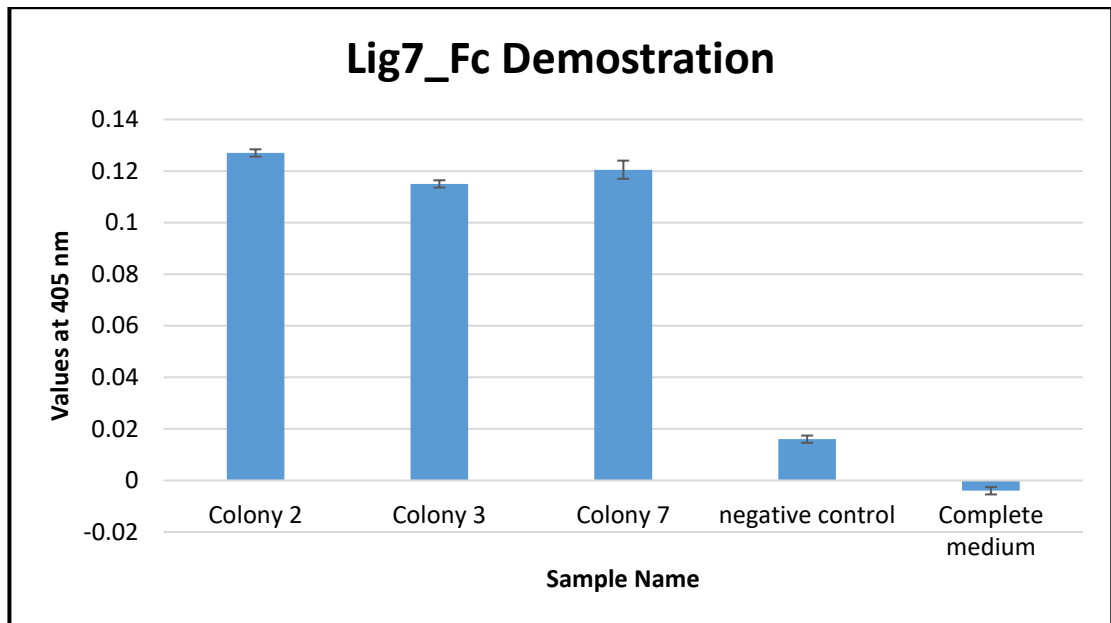


Figure 5.25: Demonstration of Lig7_Fc after 48 hours from the transfection.

6. DISCUSSION

Lig7 scFv was created against HBsAg by phage display technology in the Immunogenetics Laboratory at TUBITAK MAM Genetic Engineering and Biotechnology Institute [Erdag et al., 2017]. In this study, the therapeutic activity of scFv Lig7 in mammalian cells was investigated. Our findings demonstrated scFv Lig7 after the transfection inhibits to HBsAg in PLC/PRF/5 cell line.

PLC/PRF/5 cell line model was used to test HBsAg inhibition. First of all, in this study, we have shown that isolated His-tag labeled scFv Lig7 from E. coli could not inhibit HBsAg in PLC/PRF/5 cell culture. Studies in 5.2.1 and 5.2.2 were demonstrated that scFv Lig7 bonded to commercial HBsAg protein. However, the result of the binding capacity of scFv Lig7 to HBsAg in the culture medium of PLC/PRF/5 cell line was observed same as the negative control in figure 5.4. Furthermore, existence of HBsAg in PLC/PRF/5 cell culture was assessed in this thesis. Previously, Deamer and colleagues demonstrated the presence of HBsAg in 15-days culture medium of PLC/PRF/5 cell line by radioimmunoassay, the concentration of HBsAg was defined 90 ng per 150 μ l [Daemer et. Al., 1980]. Based on the article's results, studies in section 5.2.3 and 5.2.4 were performed to detect the presence of HBsAg in ten-days culture medium for ELISA by using commercial anti-HBsAg (Abcam, Ab20758) as a primary antibody. As a result, no significant results were obtained with the experiments (figure 5.6). However, HBsAg in three-days culture medium of the cells was defined with One version ultra-ELISA HBsAg kit (DIA-PRO). The HBsAg had been the second-highest value with approximately 30 ng per 150 μ l (figure 5.7). While protein assays demonstrated that the bacterial scFv_Lig7 could not bind to the HBsAg released by PLC/PRF/5 cell line, another ELISA result proved that HBsAg released in the culture medium of PLC/PRF/5 cell line.

We have shown HBsAg inhibition after the transfection of scFv Lig7. To demonstrate HBsAg inhibition in the culture medium of PLC/PRF/5 cell line, three different strategies with three different transfection reagents (Lipofectamine 2000, 3000 and Turbofect transfection reagents) and, three different DNA amounts were performed. After the transfection, HBsAg inhibition was demonstrated by using One

version ultra-ELISA HBsAg kit (DIA-PRO). Less amount of HBsAg was released by the transfected cells containing scFv Lig7. HBsAg inhibition was observed significantly after the transfection with Lipofectamine 2000 and 3000, however, almost ever HBsAg inhibition could not be seen after the transfection with Turbofect, in figure 5.10. In further studies, a label to identify scFv Lig7 fragment in transfected cells was needed to study this inhibition in detail.

Literature is available which provides the methods to detect scFv fragments and to produce more stable antibody fragments structure. For instances, in one study, the results pointed out that the anti-HBeAg chimeric Fab fragment was produced with high affinity and specificity to use both diagnosis and therapeutically [Zhuang et al., 2017]. In another study, fusion antibody was derived from scFv and constant fragment (CH2-CH3). It was generated specific vector pPIC9-Fc that contains the constant fragment and scFv fragment was fused the pPIC9-Fc vector. The results revealed that the fusion antibody successfully bonded with high affinity to its antigen and detected from the Fc region easily [Liu et al., 2003]. Based on the above results, human constant fragment (CH2CH3) was added to Lig7 fragment as a label to easily detect after the transfection. In this study, cloning studies were performed to identify Lig7 fragment more easily by adding human Fc (CH2CH3) fragment to scFv Lig7. As a result of cloning studies, three recombinant clones containing Lig7_Fc were obtained. Though, the sequences of these clones could not be determined fully. Although different sequence primers were used, in order to confirm the expression of Lig7_Fc of the clones, transfection study was performed to CHO_K1 cells and subsequent ELISA studies were performed in order to detect the presence of Lig7_Fc in the cell culture medium. As a result of the studies, the presence of Lig7_Fc expressions was captured only by protein L and demonstrated by detecting anti-human Fc antibody in figure 5.25. In addition, after transfection study in 5.3.4 of scFv Lig7 in the cell line, both HBsAg inhibition with ELISA kit (figure 5.12) and expression of scFv Lig7 with designed ELISA (figure 5.13) were demonstrated. scFv Lig7 was captured with recombinant HBsAg (Adw) and was detected with Protein L-HRP (M00098, GenScript). But its concentration was found to be low. Consequently, two ELISA systems have been developed to identify both scFv Lig7 and Lig7_Fc, separately.

In the last two decades, the number of death cases with cirrhosis and hepatocellular carcinoma has increased due to HBV infection. Approximately 500000 patient carrying HBV die every year despite vaccination [Vlachogiannakos and Papatheodoridis, 2018]. Committee of American Association for the Study of Liver Diseases and the European Association for the Study of the Liver in the meeting in 2017 declared that cHBV cure determines as being lost of HBsAg existence in serum completely and eradication of integrated HBV DNA and cccDNA [Lok et al., 2017]. Covering in patients containing cHBV provides sub viral particles inhibition, especially HBsAg, and elimination of HBV DNA in the serum.

There are various therapies such as nucleos(t)ide analogues, immunomodulatory drugs, interfering agents, which have been used for the treatment of cHBV. Each therapy has some advantages and disadvantages over others. Currently, nucleos(t)ide analogues as a drug use commonly, anti-viral therapy by interfering HBV DNA replication, these drugs have no effect on the cccDNA eradication and HBsAg seroclearance in most patients carrying cHBV [Fourati and Pawlotsky, 2016]. Although therapy with nucleos(t)ide analogous continues long term, just 10% of patients achieved HBsAg seroclearance. Most patients also failed to recover from hepatocellular carcinoma despite long term therapy [Testoni, Durantel, & Zoulim, 2017]. Immunomodulatory drugs, interferon alpha and derivatives drugs are being nowadays. However, therapy with immunomodulatory drugs lead to the severe side effects and was unable to obtain the main cure in most patients [Testoni et al., 2017]. Monoclonal antibodies production against specific sub viral particles can be an advanced solution [Mohebbi et al., 2018]. Understanding of the virus life cycle and advanced medical technology helped to develop the new therapeutic drugs [Maepa et al., 2015]. Despite current drugs, novel therapeutic is still under investigation worldwide to eradicate cccDNA and loss of HBsAg by itself or be combined with another therapeutic agent [Fourati and Pawlotsky, 2016].

Currently, in order to cure chronic HBV infection, some researchers focus on HBsAg inhibition with small molecules. For instance, as a potential inhibitor, HBF-0259 small molecule demonstrated inhibition of HBsAg expression in time-dependent manner in vitro studies [Dougherty et al., 2007]. Later, it was identified

that HBF-0259 interfered with HBsAg expression during post-translational modifications, but it had no effect on the virus replication [Mohebbi et al. 2016]. BM601, a Benzimidazole derivative, is capable of inhibiting HBsAg expression. It was shown that BM601 worked as an inhibitor compound for HBsAg expression and inhibited HBV viral particle formation [Xu et al., 2014]. NJK14047, Biphenyl amide non-nucleoside derivate, demonstrated HBsAg inhibition almost 70% and 90% in both integrated HBV and temporary HBV HepG2 cell lines with in vitro studies, respectively [Kim et al., 2017]. Furthermore, siRNAs targeted with HBsAg was used in some studies. As a result, HBsAg inhibition in the serum was provided with the siRNA, but it was revealed that siRNA derivatives had a short-lasting inhibition effect, [Klein et al., 2003]. HBVU6no.2, short hairpin RNA (shRNA), showed HBsAg inhibition approximately rates of 90% in vitro studies. Also, amounts of cccHBV DNA and other particles were correlated with HBsAg inhibition in the transfected mouse [Mccaffrey et al., 2003]. ARC-520 siRNA showed promising results for HBsAg inhibition. Currently, it is under Phase II trial. It was determined that the major disadvantage of ARC-520 was short half-life for the inhibition [Mohebbi et al., 2018]. Others investigate especially inhibition of HBsAg by using monoclonal antibodies. These antibodies are useful both as detection marker and therapeutic reagents. Wang et al. studied monoclonal antibodies with neutralizing capability against HBsAg. in vitro and in vivo studies demonstrated the antibody, G12, bond to HBsAg with high affinity. G12 is defined as a potential neutralizing agent [Wang et al., 2016]. In another study, a bispecific scFv antibody fragment was generated against HBsAg and red blood cells for clinical diagnosis. Binding of the bispecific scFvs to HBsAg was demonstrated by ELISA. As a result, the bispecific scFv can use for HBsAg inhibition as a therapeutic marker [Chen et al., 2006].

In conclusion, the therapeutic activity of scFv Lig7 was shown in transfected PLC/PRF/5 cell line by decreasing HBsAg in the culture medium. Moreover, in order to easy detection, the human constant fragment was added to scFv Lig7. The Lig7_Fc was transfected in CHO-K1 cell line and subsequently demonstrated that expressed the Lig7_Fc in transfected cells by ELISA. In the further studies, whole sequence of Lig7_Fc will be defined by using new designed primers. In order to obtain a high

concentration of Lig7_Fc in transfected cell culture, ELISA assays will be performed the transfected culture medium that had been concentrated. Therapeutic activity of Lig7_Fc antibody will be researched with transfection studies and ELISA assays, subsequently. Furthermore, the transfected cell line will be characterized and where the interaction of Lig7_Fc and HBsAg will be observed with immunoprecipitation studies. In the future, Lig7_Fc can be produced as a native drug for chronic HBV patients in Turkey, after it will be completed clinical studies.

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BIOGRAPHY

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APPENDIX

Appendix A: DNA and Protein Markers

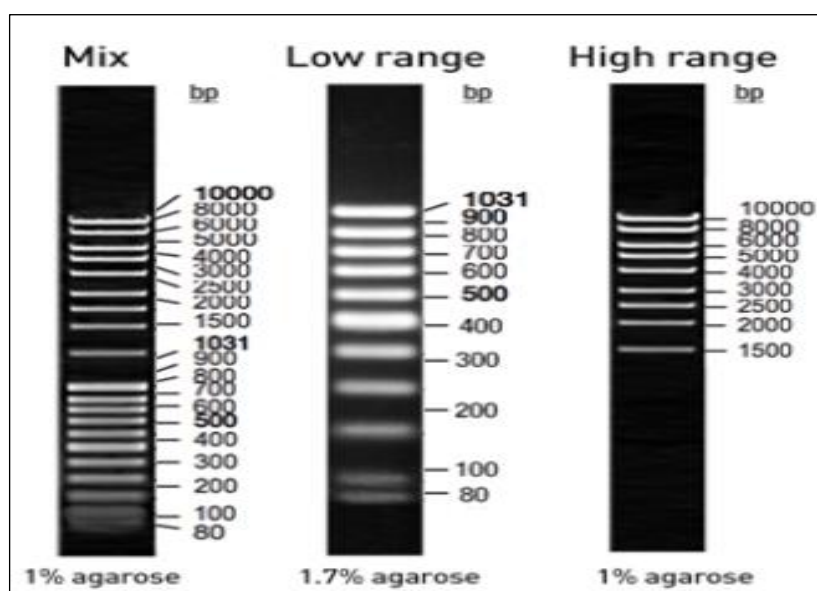


Figure A 1.1: Mass Ruler DNA Ladder Mix, Mass Ruler Low Range DNA Ladder, Mass Ruler High Range DNA Ladder, respectively. (SM0403, SM0383, SM0393, Thermo Scientific).

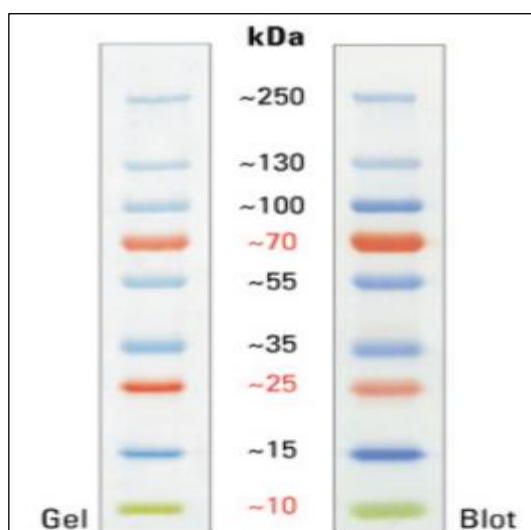


Figure A 1.2: Pageruler Prestained Protein Marker. (26616, Thermo Scientific).