DEVELOPMENT OF SURFACE PLASMON RESONANCE BASED ASSAY FOR SIMULTANEOUS DETECTION OF HEPATITIS C AND B VIRAL INFECTIONS

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By

MORVARID AKHAVAN REZAEI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

May 2015

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DEDICATION

To my lovely family, my late Father, my Mother, my Sister and my Brother in law

I appreciate for all your endless and constant support, encouragement and sacrifices you made during the challenges of graduation.

I am truly thankful for having you all in my life and would like to give my deepest expression of love to you.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Doctor of Philosophy

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May 2015

Chair : Assoc. Prof. Zeenathul Nazariah Allaudin,PhD
Faculty : Veterinary Medicine

Hepatitis C (HCV) and Hepatitis B virus (HBV) infections are major public health issue worldwide. These two hepatotropic viruses share same ways of transmission and also coinfection with these two viruses is not unusual, especially in areas with a high prevalence of HBV infection and among people at high risk for parenteral infection (Liu and Hou 2006). It is imperative that effective and reliable techniques for hepatitis C virus (HCV) and hepatitis B (HBV) infection diagnosis be devised. Additionally, non-reactive and misdiagnosis, often demanding iteration of tests for confirmation. Biosensors based on surface plasmon resonance (SPR) detection assay provides a solution for these limitations. Additionally, the SPR can overcome the contamination susceptibility of molecular methods that rely heavily on the purity of the template nucleic acid and that would result in false positives.

This study proposes an optimized SPR protocol for large-scale Hepatitis C and B samples screening. HCV and HBV detection chips were set up separately. The HCV detection chip was established by immobilization of HCV Core genotype 1, HCV Core genotype 3a and HCV NS5 genotype 3a antigens for hepatitis C genotype screening. On the other hand, HBs Ag and HBsAg antibody were used to establish the HBV screening chip. The limit of detection (LOD) was calculated for each immobilized flow cell of each established chip used 20 human donor serums were each spiked with HCV antibody for HCV established chip and 20 human donor serums were each spiked with HBsAg antibody for HBV established chip. LOD of HCV detection chip was in the range of 14.4 – 33.48 pg/ ml followed by a range of 18- 25.2 pg/ml for HBV detection chip used .

A total 137 HCV positive and negative samples were collected. All of the 137 tested as positive or negative HCV samples, were analyzed for genotype 1 and genotype 3a with established HCV screening chip. 37 samples were tested positive for HCV antibody, showed 100% positivity for genotype 1. Consequently, 6.6 % tested positive for core genotype 3a, while 16% tested positive for NS5 genotype 3a.

Among the genotype 3a samples tested positive, only one sample showed positive results for both core 3a and NS5 3a.

A total 400 HBV positive and negative samples were collected. All 100 positive HBsAg, 100 negative HBsAg and 200 negative HBsAg antibody samples were confirmed serologically by the HBV established chips.

Finally, the HBsAg, HCV core genotype 1 and HBsAg antibody were immobilized to establish the dual detection chip. Based on the international distribution of HCV genotype 1, it was chosen to establish the dual detection chip. The LOD for this dual detection chip was in the range of 12.6–26.4 pg/ml. To determine the LOD for this established chip, 20 human donor serums were used and each sample spiked with HCV antibody and HBsAg antibody individualy and analysed with established chip.

Out of total 137 samples, 37 samples tested positive for HCV antibody and 100 tested negative for HCV antibody. Among oof 400 samples, 100 samples tested positive for HBsAg, 100 samples tested negative for HBsAg and 200 samples tested HBsAg antibody negative.

A comparison was made between the SPR and ELISA techniques in the diagnosis of HCV and HBV infection. It was found that there was a strong correlation between SPR and ELISA test when used to detect HCV and HBV in the samples. The correlation coefficient obtained with the two techniques approached 1 ($P< 0.01$). The range of linear dose was observed for each immobilized flow cell with a coefficient of determination between 98 to 99%.

The novelty of this study is the ability to serologically detect HCV and HBV antigens and antibody, as well as HCV genotypes mixture simultaneously. LOD for developed SPR chips (observed between 14.4 – 33.48 pg/ml) showed higher efficiency when compared to HCV and HBV commercially used enzyme-linked immunosorbent assay (ELISA) and chemiluminescent immunoassay (ChLIA) (ng/ml). Due to the low detection limit of SPR compared to ELISA and ChLIA, it can detect false negative samples caused by lower level of antibody and antigen than ELISA and ChLIA detection limit, a greater efficiency lacking in the later mentioned application.

In conclusion, the optimized SPR approach can serve as a standard operating procedure (SOP) for national blood screening centers. Taken together, data indicate that the assays developed are highly reproducible, specific and sensitive , accuracy, precision, repeatability, linearity, range and robustness.. This biosensor-based assay is a more efficient tool for accurate screening of antibody and antigen in HCV and HBV infected patient serum, while retaining the advantages of ELISA. Moreover, the inbuilt robotic automated system with reusable chip is on the top of novelty for this dual antigen and antibody detection assay for suspected co-infected samples.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PEMBENTUKAN BERASASKAN SUMBER PERMUKAAN PLASMON UNTUK MENGESAN JANKITAN HEPATITIS B DAN C SECARA SERENTAK

Oleh

MORVARID AKHAVAN REZAEI

Mei 2015

Pengerusi : Prof. Madya Zeenathul Nazariah Allaudin, PhD
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Jangkitan daripada virus Hepatitis C (HCV) dan virus Hepatitis B (HBV) merupakan isu-isu kesihatan awam yang utama secara global. Tambah lagi, jangkitan kedua-dua virus ini pada waktu yang sama adalah biasa berikutan dengan perkongsian cara penghantaran yang sama. Oleh kerana itu, keperluan untuk teknik yang lebih sensitif dan yang boleh dihasilkan semula adalah penting di dalam pengesanan jangkitan HCV dan HBV. Di samping itu, isu-isu semasa seperti sampel keputusan ujian yang tidak reaktif dan yang memberikan keputusan ujian negatif yang palsu semasa penggunaan esei-esei immuno seperti ChLIA dan ELISA, sering mengakibatkan diagnosis yang salah dan disebabkan demikian pelbagai ujian-ujian lain diperlukan untuk pengesahan. Oleh yang demikian, penggunaan esei pengesanan berdasarkan plasmon permukaan resonans (SPR) dapat menawarkan satu kaedah penyelesaian terhadap had tersebut. Di samping itu, SPR juga boleh mengatasi kelemahan utama pengesanan molekul HCV RNA yang mudah terdedah kepada pencemaran.

Kajian ini mencadangkan penggunaan protokol SPR yang optima untuk pengesanan hepatitis C dan B di dalam sampel secara skala besar. Pada mulanya, cip pengesanan HCV dan HBV telah ditubuhkan secara berasingan. Antigen-antigen HCV Teras, HCV teras genotip 3a dan HCV NS5 genotip 3a telah digunakan sebagai ligan dalam pembentukan cip pemeriksaan HCV dan HBs Ag dan HBsAg antibodi telah digunakan untuk pembentukan cip pemeriksaan HBV. Had pengesanan (LOD) telah dikira bagi setiap sel aliran pada setiap cip yang dibentuk. LOD yang dicip pengesanan HCV adalah di dalam rangkaian pengesanan 14.4-33.48 pg/ml diikuti oleh rangkaian pengesanan 18 dan 25.2 pg/ml untuk cip pengesanan HBV.

Dalam kajian ini, sebanyak 137 sampel HCV positif dan negatif telah dianalisis untuk genotip 1 dan genotip 3a. Daripada itu, didapati 37 HCV antibodi sampel yang positif menunjukkan 100% positif untuk genotip 1. Seterusnya, 6.6% adalah positif bagi genotip 3a teras manakala 16% untuk NS5 genotip 3a. Antara sampel-sampel genotip 3a yang positif, hanya satu sampel menunjukkan keputusan yang positif

untuk kedua-dua 3a teras dan NS5 3a. Sementara itu, semua sampel HBs Ag yang positif dan yang negatif dapat disahkan secara serologi oleh cip pengesanan HBV yang telah dibentuk.

Selain itu, cip dwi-pengesanan telah dibentuk dengan pengtetapan HBsAg, HBsAg antibodi dan HCV teras di atas permukaan cip. LOD untuk cip dwi-pengesanan ini adalah di dalam rangkaian pengesanan 12.6-26.4 pg/ml. Didapati daripada jumlah 537 sampel, 37 sampel adalah positif terhadap HCV antibodi dan 100 adalah negatif terhadap HCV antibodi manakala 100 sampel menunjukkan kepututsan yang positif terhadap HBsAg, 100 sampel negatif untuk HBsAg dan 200 sampel menunjukan keputusan yang negative terhadap HBsAg antibodi.

Perbandingan yang dibuat di antara teknik SPR dan ELISA dalam diagnosis jangkitan HCV dan HBV menunjukkan korelasi yang kuat antara kedua-dua ujian tersebut dalam pengesanan HCV dan HBV daripada sampel. Pekali korelasi yang diperolehi dengan kedua-dua teknik ini didapati menghampiri 1 ($P < 0.01$) manakala rangkaian kelurusan bagi setiap sel aliran adalah di antara 98-99%.

Kebaharuan kajian ini adalah di dalam keupayaannya untuk mengesan HCV dan HBV antigen dan antibodi dengan serentak di samping campuran genotip secara serologi. LOD untuk SPR (pg/ml) menunjukkan pengesanan yang lebih baik jika dibandingkan dengan ELISA komersial dan ChLIA (ng/ml). Oleh kerana had pengesanan yang lebih rendah ini, cip dwi-pengesanan yang telah dibentuk boleh diaplikasikan untuk mengesan sampel negatif palsu pada masa hadapan.

Kesimpulannya, pendekatan SPR telah dioptima boleh berfungsi sebagai prosedur operasi standard (SOP) untuk pusat-pusat pemeriksaan darah negara. Esei pengesanan ini yang berdasarkan biosensor berpotensi berfungsi sebagai alat yang sensitif untuk pemeriksaan segera antibodi dan antigen jangkitan HCV dan HBV daripada sampel darah pesakit, sementara mengekalkan kelebihan ELISA. Selain itu, sistem terbina robot automatik dengan cip yang boleh diguna semula merupakan ciri-ciri kelebihan untuk cip dwi-pengesanan yang telah dibentuk dalam pengesanan sampel yang disyaki dijangkiti dengan HCV dan HBV.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

% CV	Percentage coefficient of variance
A	Absorbance
A/C.O.	Absorbance/ Cut off
ANOVA	Analysis of variance
Anti-HBsAg	Anti-HBsAg mouse monoclonal antibody
Anti-HCV Core	Anti-HCV Core mouse monoclonal antibody
B	Buffer
BSA	Bovine serum albumin
CHC	Chronice Hepatitis C
CM	Carboxymethyl
C.O.	Cut off
DAI	Diagnostic Automation
DL	Detection limit
DNA	Deoxyribonucleic acid
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELISA	Enzyme linked immunosorbent assay
FC	Flow cell
GST	Glutothione S-transferase
H	Hydrophobic core
HBS-EP	HEPES-buffered saline with EDTA and surfactant P 20(Tween 20)
HBS-N	HEPES-buffered saline with Nacl
HBS-P	HEPES-buffered saline with surfactant P 20
HBV	Hepatitis B Virus
HBsAg	Hepatitis B surface Antigen
HCV	Hepatitis C Virus
HCV 3a	Hepatitis C Genotype 3a
HLD	Higher ligand density

HTJS	Hospital Tuanku Jaafar seremban
IFC	Integrated microfluidic channel
IEC	Ion Exchange Chromatography
Ig	Immunoglobulin
IS	International Standard
IU	International unit
LOD	Limit of Detection
LOQ	Limit of Quantification
M	Method
MAb	Monoclonal antibody
Mrna	Messenger ribonucleic acid
Mut	Methanol utilization pathway
MW	Molecular Wieght
N	Amino terminus
NAT	Nucleic Acid Test
NBBM	National Blood Bank Malaysia
Nc	The mean absorbance value for 3 negative control
NCx	Mean absorbance of negative control
ND	Not determined
NHS	N-hydroxysuccinimide
No	Number
NP	Not provided
Nt	Nucleotides
OD	Optical density
PBST	Phosphate-buffered Saline with Tween 20
PCx	Mean absorbance of Positive control
QL	Quantification limit
RIA	Radio Immuno Assay
RIBA	Recombinant Immuno Blot Assay

RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse Transcriptase PCR
RU	Resonance unit
S	Sample
SCF	Standard Cubic Foot
S/C.O.	Sample optical density/ Cut off
SOP	Standard Operation Procedure
SPR	Surface Plasmon Resonan
Std	Standard
STD ID	Standard identification
STDEV	Standard deviation
UTR	Untranslated region
VMD	Volumetric mean diameter
WHO	World Health Organization
X	Any amino acid

CHAPTER 1

INTRODUCTION

1.1 Background of study

Approximately 400 million people worldwide are infected with Hepatitis B virus (HBV) (Brouwer 2015, Lavanchy and Kane 2016). The World Health Organization (WHO) estimates the number of people infected with HCV to be approximately 170 million (Crockett *et al*., 2005, Liu *et al*., 2006).

Hepatitis C and B are amongst the most challenging diseases in the world. Hepatitis C virus is responsible for chronic liver diseases, such as cirrhosis, end-stage liver disease, and hepatocellular carcinoma (Yao *et al*., 2014, WHO, 2015). Due to the absence of vaccines for Hepatitis C, it is critical to detect infection at an early stage (Lauer *et al*., 2014). The Hepatitis C does not present any observable symptom, due to which chronic liver disease can progress to cirrhosis and hepatocellular carcinoma undetected (WHO, 2014a).

However, in endemic areas, a large number of patients are infected with both viruses, mainly because of exposure through common routes of transmission (Konstantinou *et al*., 2015). Among the Malaysian population aged between 15-64 years, the prevalence of HCV infection is 2.5% and HBV infection is 4.7% (McDonald *et al*., 2014). In 75% of chronic liver disease detected in Malaysians, hepatitis B virus is the major causative agent. In 2013, the incidence rate of chronic HCV was 6.8 per 100,000 populations (McDonald *et al*., 2014). Around 5% of healthy blood donors in Malaysia are chronic carriers for hepatitis B virus. Furthermore, the most prevalent HCV genotype is genotype 3 (54.5%) and subtype 3a (94%) followed by genotype 1 (40%). Hepatitis C virus subtypes 3a, 1a, and 1b are detected frequently in patients, while subtype 3a being the most prevalent amongst them (Mohamed *et al*., 2013).

Serological and virological tests have become essential in the management of Hepatitis infection in order to diagnose infection, plan treatment, and assess the virological response to antiviral therapy (Uliana, Riccardi *et al*. 2014). A range of different technologies have been developed for screening and detection of hepatitis infection, such as serological assays, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) (Gupta, Bajpai *et al*. 2014) . Currently, ELISA is the most widely used method for screening and detection of HCV. The assay is performed for the detection of anti-HCV antibodies using recombinant HCV genome proteins (Ijpelaar et al., 2005). Detection of anti-HCV antibodies is unable to distinguish between a current or past infection, because people will retain anti-HCV antibodies for life once they have been exposed to HCV (Pei, Zhang *et al.* 2013). In addition, the testing for anti-HCV antibodies might provide false negative results, because of window period (Hofmann, Dries *et al*. 2005). As such the nucleic acid testing (NAT) methods are currently being used as a supplementary test for ELISA

instead of PCR because it is not only expensive to perform but also susceptibility to contamination (Dufour et al., 2003). However, detection of HCV RNA could provide false positive results due to contamination. It is also too expensive and labor-intensive for routine use. It has been reported that the HCV core antigen can be detected in the serum of most patients during the acute infection (Gaudy, Thevenas *et al*. 2005). HCV core antigen levels correlate well with HCV RNA levels, and may consequently be used as an indirect marker of HCV replication, in a low-cost alternative technique for diagnosis of acute HCV infection (Krishnadas, Li *et al*. 2010, Park, Lee *et al*. 2010Moscato, Giannelli *et al*. 2011) . The HCV core antigen has been reported to appear earlier than the anti-HCV antibody, and may be detected within 1 or 2 days following the appearance of HCV RNA in serum (Uliana, Riccardi *et al*. 2014). Despite heigh sensitivity and specificity, it has limitations. Firstly, the viruses cannot be detected during the early stages of infection. Secondly, antibodies against HCV antigens are not produced in the early stages of infection. Thus, detection of HCV infection at an early stage is not possible with ELISA. Additionally, the immune-suppressed patients during their post transplantation period, and immune-compromised patients infected by human immunodeficiency virus (HIV), while infected by HCV, they may not produce any perceptible antibodies (Lee et al., 2007).

The Surface Plasmon Resonance (SPR) method has more advantages compared to the conventional ELISA. It is real-time, requires shorter duration for detection, label-free and can be highly specific and sensitive with greater simplicity (Lee *et al*., 2007b; Kumbhat *et al*., 2010). Surface plasmon resonance is a sensitive optical detection method for characterizing interactions between macromolecules (Biacore Hand Book, 2012).

Application of SPR-based biosensor is popular in wide variety of fields such as proteomics, drug-screening, medical diagnostics, and food safety. It offers capability to determine the specificity, kinetics, and affinities of macromolecule interactions high throughput and low sample consumption (Spindel & Spsford, 2014). Currently, SPR technology has been applied in virology because it is a sensitive and reliable diagnostic tool that is performed through the immobilization of DNA, RNA, protein (usually antibody-antigen), virus particles, and whole viruses. It has been used for the detection of influenza (Bai *et al*, 2012), Rabies (Xu *et al*., 2012) and dengue viruses (Jahanshahi *et al*., 2014).

Unlike currently available viral assays such as ELISA, cell culture, PCR, reverse transcriptase PCR (RT-PCR) and radioimmunoassay (RIA), SPR makes it possible to monitor interactions in real-time and quantify at low limit of detection. Although SPR technology has been widely used in recent years, there is no specific report on SPR application in the detection of hepatitis infection. Furthermore, there is no record on direct immobilization of enveloped virus on SPR chips. There is a demand for dual detection of hepatitis B and C through a fast, reliable and sensitive technique such as SPR.

Hepatitis C and hepatitis B viruses are responsible for many severe ailments. The hepatitis C core Ag and hepatitis B surface Ag were chosen for the development of a new chip-based detection assay. A reliable assay is expected to differentiate positive and negative samples without relying on supplementary assays. There is currently no dual detection method for screening and detecting hepatitis C and B or determining prevalence of hepatitis C and B co-infections.

1.2 Hypothesis

The SPR-based technique for simultaneously detecting hepatitis C and B viral infections in serum is sensitive and specific.

1.3 Objectives of study

1.3.1 General objective

To develop a detection assay for the diagnosis of the C and B viral infection simultaneously using SPR technology

1.3.2 Specific objective

1. To establish ligand proteins for SPR- based detection of hepatitis C and B viral infections.
2. To develop and optimize an assay for simultaneous detection of hepatitis C and B viral infection.
3. To validate the specificity and sensitivity of the simultaneous hepatitis C and B virus detection assay.
4. To compare the detection limits of hepatitis C and B viral infections between the SPR- based assay, ELISA and chemiluminescent immunoassay (ChLIA).

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NATIONAL INSTITUTES OF HEALTH APPROVAL FOR CONDUCTING RESEARCH IN THE MINISTRY OF HEALTH MALAYSIA

PENGESAHAN INSTITUSI PENYELIDIKAN NEGARA UNTUK MENJALANKAN PENYELIDIKAN DI KEMENTERIAN KESIHATAN

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Unique NMRR Registration ID : *[Nombor Pendaftaran]*	NMRR-12-588-12199
Research Title : *[Tajuk]*	Development of a surface plasmon resonance chip-based system for Hepatitis B and C diagnostics
Protocol Number if available : *[Nombor Protokol jika ada]*	

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I have reviewed the above titled research, and approve of its design and conduct.

Saya telah menyemak kajian yang bertajuk seperti di atas dan meluluskan rekabentuk dan perlaksanaannya.

Name of Director : *[Nama Pengarah]*	Dr. Shahnaz Murad
NIH Institute (IMR, CRC, IPH, IHM, IHSR and IHBR) *[Nama Institusi di bawah NIH]*	Institute for Medical Research (IMR)
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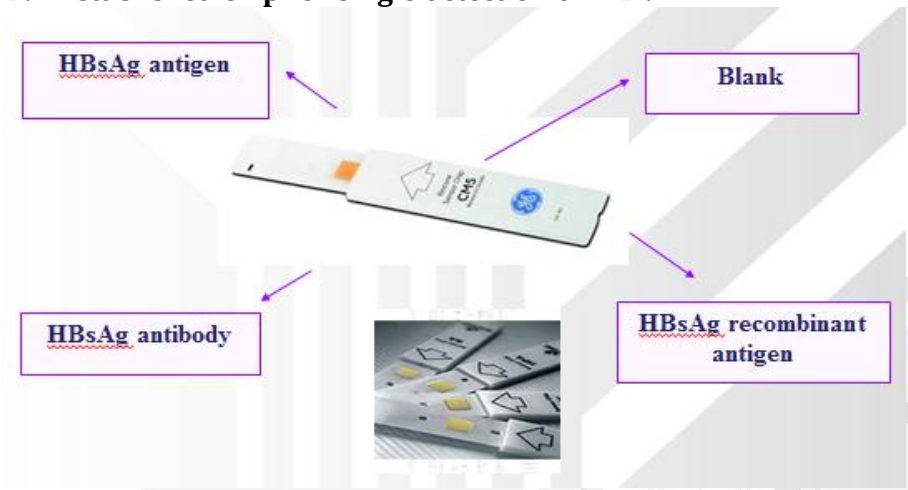
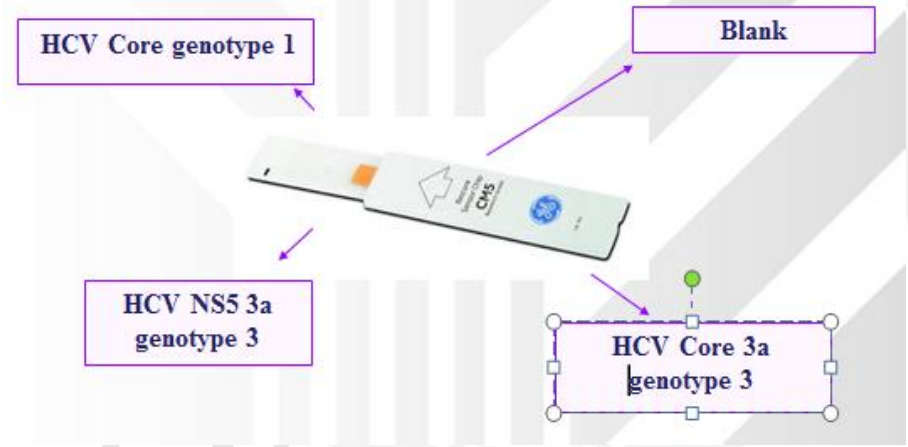
Appendix B

Chip Establishment

B.1 Established chip for single detection of HBV



B. 2 Established chip for single detetion and genotyping of HCV



B.3 Established chip for dual detection of HCV and HBV

Appendix C

Standard Curves

C.1 ELISA standard curve

ELISA HBsAg kit was used in Chapters 4 to calibrate the amount of rHBsAg standard. Its procedure is described in section 3.2.2.
A typical standard curve is shown in Figure F1.

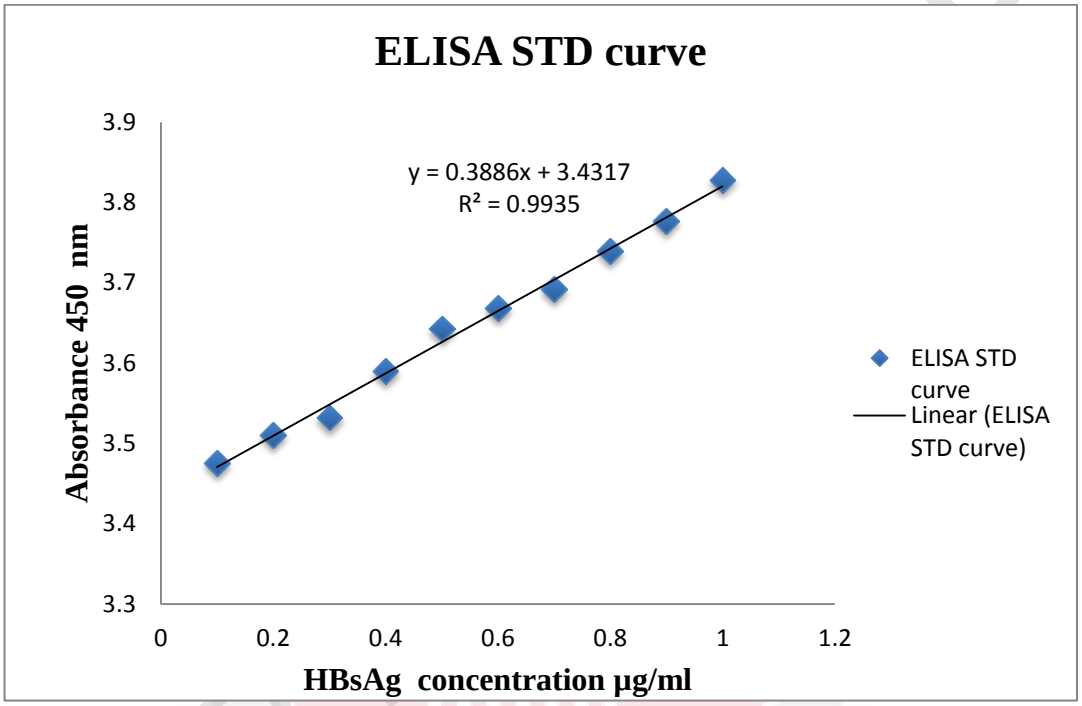


Figure F1: Standard curve for ELISA kit
Note: The error bar (present in the symbol) represents the standard deviation between the duplicates. The linear correlation and its R2 are depicted in the figure. If R2>0.9, the standard generated is good.

C.2 Protein determination (Brad Ford) standard curve

The procedure for protein determination is described in section 3.2.3. BSA was used as a standard throughout the study to determine the concentration of total protein and BSA. A typical standard curve is shown in Figure F2.

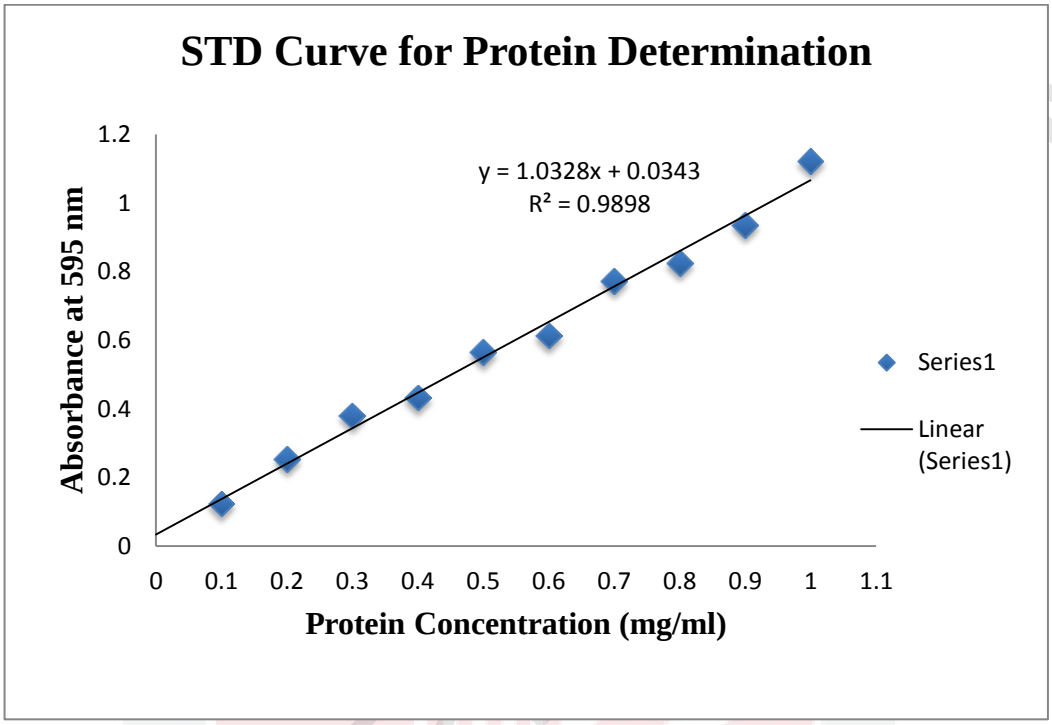


Figure C2: Standard curve for protein determination (Brad Ford)

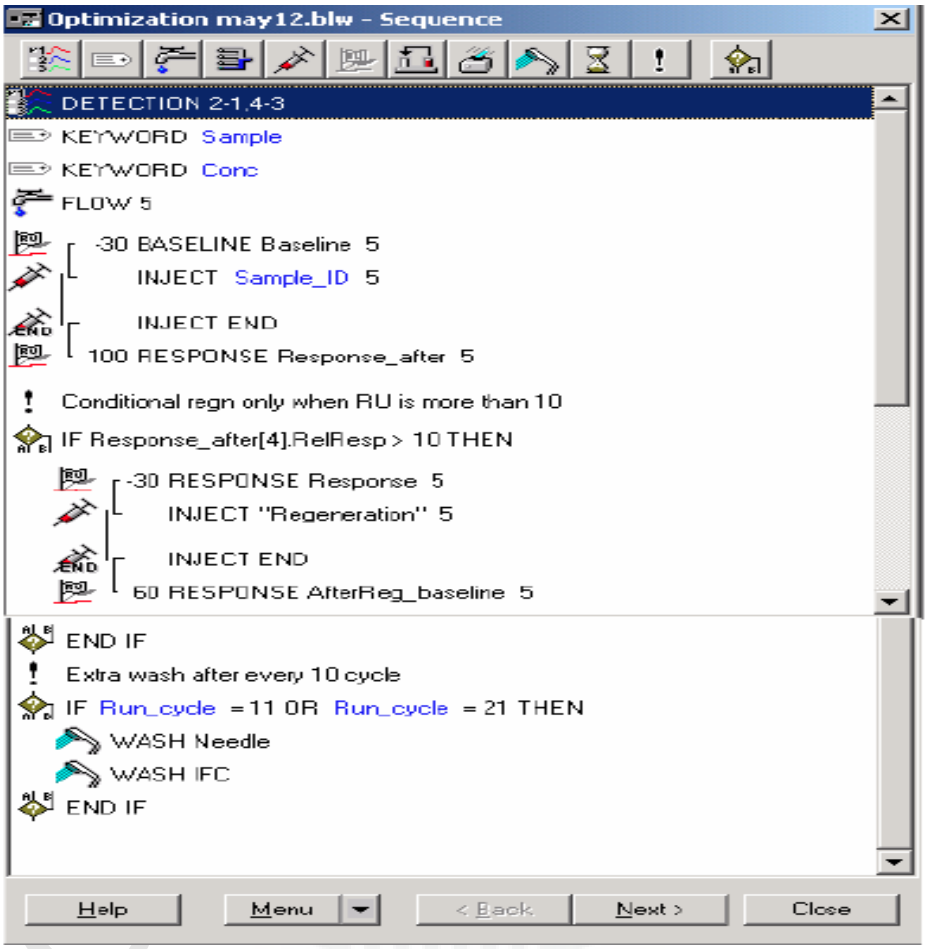
Note: The error bar (present in the symbols) represents the standard deviation between the triplicates. The linear correlation and its R2 are depicted in the figure. If R2>0.9, the standard generated is good.

Appendix D

Program

D.1 Customized program to run samples in Biacore 3000

Program is created using Biacore control software to run samples in an automatic fashion. A model of a program is shown below.



Appendix E

Refrence Flow Cell

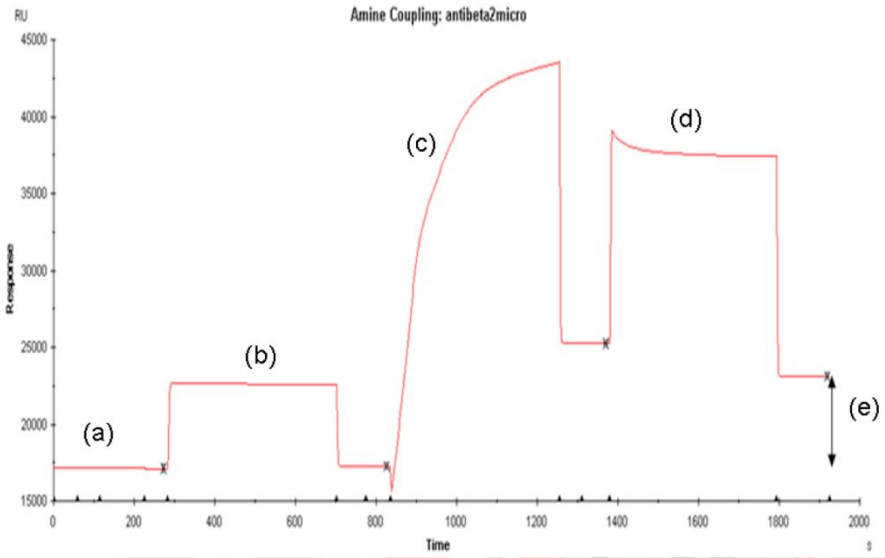


Figure D1 Model of Immobilization sensorgrams

a = baseline response, b = activation of surface chip, c = injection of samples (ligand), d = inactivation of the remaining active site on the surface e = difference in RU compared between the final response compared to baseline which reflects the amount of proteins immobilized

Appendix F

Equation

F.1 Calculations the coefficient of variation

The coefficient of variation (CV) is a normalized measure of reliability expressed in percentage. It has the advantages to be a dimensionless number enabling the user to compare the CV between different data sets without taking into consideration the mean value. When the mean value is closer to zero the CV is very sensitive to small changes and are therefore not as useful. CV is normally presented in percentage and with the number of data in the set as n. CV is calculated with Equation below.

$$CV(\%) = \frac{\sigma}{\mu}.100$$

Where σ = standard deviation and μ = mean.

F.2 Calculations for limit of detection, limit of quantification

F.2.1 Limit of detection (LOD)

$$LOD = 3 \times SD\ (Blank)$$

F.2.2 Limit of quantification (LOQ)

$$LOQ = 10 \times SD(Blank)$$

BIODATA OF STUDENT

The student was born on August 26, 1973 in Rasht, North of IRAN. After her primary education was received from Azarm School, middle and high school educations were obtained from Effat and Forogh high school in Rasht. Morvarid went on to obtain a degree on Microbiology (1994-1998) from the Islamic Azad University, Lahijan, IRAN. Subsequently, she also received her Master degree on Microbiology (1999-2001) in the Islamic Azad University, Lahijan.

Morvarid's work experience includes nine years (1999- 2005) for 9 years in Blood Transfusion Service in Quality Control Laboratores (Virology, Bacteriology, Serology and Biochemistry). In December 2006, she enrolled for a fulltime Ph.D. program.

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