GENERATION AND CHARACTERIZATION OF RNA APTAMER AGAINST DENGUE VIRUS 2 NS1 GLYCOPROTEIN

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GENERATION AND CHARACTERIZATION OF RNA APTAMER AGAINST DENGUE VIRUS 2 NS1 GLYCOPROTEIN

by

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LIST OF SYMBOLS AND ABBREVIATIONS

A	Adenine
APS	Ammonium persulfate
ATP	Adenosine 5'-triphosphate
Bis	N, N'-methylene bisacrylamide
bp	Base pair (s)
BPA	Bisphenol A
BSA	Bovine serum albumin
C	Cytosine
°C	Degrees Celsius
cDNA	Complementary DNA
CTP	Cytidine 5'-triphosphate
ddH_2O	Double-distilled water
DENV	Dengue virus
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
ELDENA	Enzyme-linked DENVI Based Assay
ELISA	Enzyme-Linked Immunosorbent Assay
et al.	and others
EtBr	3, 8-diamino-5-Ethyl-6-phenyl phenanthridinium Bromide
g	Gram
G	Guanine
GTP	Guanosine 5'-triphosphate
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidise

ICT	Immunochromatography assay
IPTG	Isopropyl-β-D-thiogalactopyranoside
KCl	Potassium chloride
K_d	Dissociation constant
kDa	Kilodalton
KOH	Potassium hydroxide
LB	Luria Bertani medium
M	Molar, [(Mole)/(Litre)]
Mg^{2+}	Magnesium ion
Min	Minute (s)
mL	Milliliter
mM	Millimolar
Na^{+}	Sodium ion
NaCl	Sodium chloride
NaOAc.3H2O	Sodium acetate trihydrate
NaOH	Sodium hydroxide
ng	Nanogram
nM	Nanomolar
nt	Nucleotide (s)
N-terminal	Amino-terminal
-OH	Hydroxyl
PAGE	Polyacrylamide gel electrophoresis
PBST	Phosphate buffered saline with Tween 20
PCR	Polymerase chain reaction
PDB	Protein Data Bank
RCSB	The Research Collaboratory for Structural Bioinformatics
RMSD	Root-mean-square deviation
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
RT	Room temperature
RT-PCR	Reverse transcription-PCR

s	Second (s)
SELEX	Systematic Evolution of Ligands via Exponential Enrichment
ssDNA	Single-stranded DNA
T	Thymine
TAE	Tris–Acetic Acid–EDTA
TBE	Tris-Boric Acid-EDTA
TEMED	N,N,N’,N’-Tetramethylethylenediamine
Tris	Tris-(Hydroxymethyl)-Aminomethane
tRNA	Transfer RNA
U	Units of enzymatic activity
UTP	Uridine 5‟-triphosphate
u.v.	Ultraviolet
V	Volt (s)
v/v	Volume per volume
w/v	Weight per volume
X–gal	5’-Bromo-4’-Chloro-3’-Indolyl-β-Dgalactoside
x g	Relative Centrifugal Force
µg	Microgram
µL	Microliter
µM	Micromolar
2'-F	2'-fluoro
γ32P	Gamma Phosphorus-32

PENJANAAN DAN PENCIRIAN APTAMER

RNA TERHADAP GLIKOPROTEIN NS1 VIRUS DENGGI 2

ABSTRAK

Demam denggi adalah wabak yang melanda wilayah tropika dan subtropika yang disebabkan oleh gigitan nyamuk. Menurut analisa yang dibuat oleh Organisasi Kesihatan Sedunia (WHO) baru-baru ini, adalah dianggarkan bahawa sebanyak 3.9 bilion orang daripada 128 buah negara berisiko menghadapi jangkitan virus denggi. Ini menekankan keperluan ujian diagnostik denggi yang unggul dan sesuai, iaitu ujian yang mampu membezakan demam denggi daripada penyakit lain yang mempunyai manifestasi klinikal yang serupa, sangat sensitif, pantas dan dengan kos yang efektif. Antigen NS1 mempunyai potensi untuk menjadi sasaran diagnostik bagi penyakit denggi kerana ia mampu mendorong tindak balas 'humoral' yang kuat dan mempunyai kepekatan tinggi dalam darah pesakit yang dijangkiti sehingga 9 hari selepas jangkitan primer dan sekunder. Walaubagaimanapun, kaedah diagnostik semasa bagi penyakit denggi berdasarkan antibodi mengalami beberapa kelemahan. Aptamer, jujukan tunggal DNA atau RNA yang mempunyai afiniti dan kekhususan yang tinggi terhadap sasaran ialah kelas biomolekul elegan yang berpotensi untuk mengatasi kelemahan antibodi. Kajian ini berusaha untuk memperoleh aptamer RNA yang mempunyai afiniti dan kekhususan yang tinggi terhadap glikoprotein NS1 virus denggi 2, yang merupakan 'serotype' yang paling dominan dalam penyakit denggi. Sebanyak 11 kitaran SELEX telah dijalankan. Pengklonan dan analisa jujukan telah mengenalpasti 7 kelas jujukan RNA (yang dinamakan seperti DENVI 1 sehingga DENVI 7). DENVI 1, 3, 4, dan 5 mempunyai penampilan frekuensi yang tinggi dengan peratusan 14%. DENVI 2 dan DENVI 6 mempunyai frekuensi sebanyak 7.1% manakala DENVI 7

mempunyai frekuensi yang paling kurang sebanyak 3.6%. Analisa ‘nitrocellulose filter binding’, menganggarkan nilai penceraian berterusan sebanyak 4.695±1 nM, 16.6±1 nM dan 6.305±1 nM masing-masing untuk DENVI 3, DENVI 4 and DENVI 6. Aptamer RNA yang stabil telah dihasilkan melalui penggabungan 2'-F-dCTP dan 2'-F-dUTP semasa tindak balas transkripsi *in vitro* yang mengekalkan penggabungan terhadap NS1. ‘In silico molecular docking’ meramalkan struktur tiga dimensi bagi kompleks aptamer RNA dan NS1 protein, yang membuktikan penggabungan aptamer terhadap protein sasaran. Nukleotida dalam aptamer DENVI 3, DENVI 4 dan DENVI 6 yang berinteraksi terhadap protein sasaran NS1 juga telah berjaya dikenalpasti, dan maklumat ini boleh digunakan untuk pemangkasan atau pengecilan aptamer pada masa akan datang. Penyumbang utama kepada interaksi antara aptamers dan protein sasaran, NS1 ialah ikatan hidrogen. Aptamer RNA yang dihasilkan mempunyai potensi diagnostik terhadap perkembangan pengesanan jangkitan denggi berasaskan aptamer dan juga mungkin berpotensi digunakan dalam aplikasi terapeutik.

GENERATION AND CHARACTERIZATION OF RNA APTAMER AGAINST DENGUE VIRUS 2 NS1 GLYCOPROTEIN

ABSTRACT

Dengue Fever, a mosquito borne viral disease, is a malady that plagues tropical and subtropical regions. A recent survey by WHO indicated, 3.9 billion of people in approximately 128 countries are at risk of infection with dengue viruses. The current predicament necessitates an ideal dengue diagnostic test, an assay that is able to distinguish dengue fever from other clinically similar diseases, highly sensitive, rapid and cost-effective. Antigen NS1 represents the potential diagnostic target of dengue as it was found to induce strong humoral response and is present at high concentrations in the blood up to 9 days after primary and secondary infections. However, the current diagnostic of dengue based on antibodies suffers from several drawbacks. Aptamers, ssDNA or RNA that have high affinity and specificity against the target, constitute a class of biomolecules that having the potential to alleviate the disadvantages of antibodies. This study endeavors to isolate RNA aptamer that have high affinity and specificity against NS1 of DENV 2, which is the most common serotype of dengue. A total of 11 SELEX cycles were carried out, whereby the eventual nucleic acid pool exhibited binding against the target NS1. Sequencing of the RNA pool revealed the presence of seven classes of sequences (named DENVI 1 to DENVI 7). DENVI 1, 3, 4, and 5 have the highest frequency of appearance with the percentage of 14.3%. DENVI 2 and DENVI 6 have the frequency of 7.1%, while DENVI 7 has the lowest frequency of 3.6%. Nitrocellulose filter binding assay has estimated binding affinity of 4.69±1 nM, 16.6±1 nM and 6.305±1 nM for DENVI 3, DENVI 4 and DENVI 6 aptamers, respectively. Stabilized RNA aptamers generated via the incorporation of 2′-

F-dCTP and 2′-F-dUTP during the *in vitro* transcription reaction retain binding against NS1. *In silico* molecular docking predicted the three-dimensional structure of the aptamer-NS1 protein complex, which corroborates the binding of the aptamers against the target protein. Interacting nucleotides of the aptamers DENVI 3, DENVI 4 and DENVI 6 against the target protein NS1 were also successfully identified, which could be useful for the truncation or miniaturization of the aptamers in future. The major contributor to the interaction between the aptamers and the target NS1 is the hydrogen bond. The isolated RNA aptamers harbor diagnostic potential towards the development of aptamer-based diagnostic detection of dengue infection and could also be potentially applied in therapeutic application.

CHAPTER 1

INTRODUCTION

1.1 Epidemiology of Dengue fever

Dengue is a vector-borne or an arthropod-borne viral disease and is an important public health issue among tropical and subtropical regions around the globe (Nishiura and Halstead, 2007). Dengue fever is an ancient disease (Gubler, 1997), which bears similarities to the illnesses occurred during the Chin Dynasty (AD 265 to 420) (Gubler, 2006; Bhattacharya et al., 2013). Later the breakout was reported in three continents such as Asia, Africa and North America between 1779 and 1780 (Pepper, 1941; Melvyn, 1977). At present, World Health Organization have predicted that 40% of world population is at risk of contracting dengue and it can cause an immense impact on the global economy (Montibeler and de Oliveira, 2017). In Malaysia, the first epidemic outbreak was reported in 1973, with 1487 cases and 54 deaths (Wallace et al., 1980; Mudin, 2015). Dengue cases remains the number one infectious disease with 101,357 cases and 231 deaths reported, though the cases reported declined to 71,641 cases in September 2017 (Lokman, 2017).

Dengue is transmitted among human or primates via *Aedes aegypti* and *Aedes albopictus* in sub-urban and rural areas (Singla et al., 2016), via breeding of the mosquitos in domestic water container (Pang et al., 2016). Urban areas constitute 70 to 80% of reported dengue cases. It has been proven that all gender and ethnic groups are equally vulnerable to dengue infection (Pang et al., 2016). Generally, factors that account for the occurrences of dengue cases are high density of population, rapid

developmental activities or urbanization which favours dengue transmission. In Malaysia particularly, rise of dengue cases can be ascribed to climate change such as rise of temperature, increased rainfall, higher humidity, rapid industrialization and urbanization including improper solid waste disposal account for the rise of dengue cases (Mudin, 2015).

1.2 DENV-2 serotype is the most common serotype in Malaysia

Dengue fever is engendered by Dengue virus (DENV), which is from the genus *Flavivirus* in the family *Flaviviridae.* Comprising of four distinct phenotypes (DEN-1, DEN-2, DEN-3, DEN-4), DENV consists of approximately 11 kb positive single-stranded RNA genome, which codes for three structural proteins (Capsid protein C, membrane protein M and envelope protein E) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). Additionally, a new serotype was discovered in 2013 from the serum sample collected during the 2007 dengue outbreak in Malaysia (Mustafa et al., 2015). The variance among these serotypes lies in their genetic makeup (Singla et al., 2016). All serotypes of dengue virus are found in Malaysia, but the predominant serotype changes every year. The serotypes surveillance showed that dengue cases based on DENV-2 serotype increases rapidly starting from March 2013 compared to other serotypes (Mudin, 2015).

1.3 Current diagnostics of dengue fever

The current diagnostic of dengue infection is via virus isolation (Tripathi et al., 2010) during the period of viraemia (WHO & TDR., 2009). Dengue virus is isolated from blood, serum or plasma. The method is expensive and time consuming (around 1 to 2 weeks) (WHO & TDR., 2009). Viral RNA detection is also a

diagnostic strategy for dengue detection. Serum or blood samples taken from patients in the acute phase of disease are subjected to viral RNA isolation and nucleic acid amplification test (NAAT) (Peeling et al., 2010). The method can cause false positivity due to sample contamination. Dengue is also detected via serological methods, whereby immunoglobulins (IgM, IgG, and IgA) specific for the envelope protein E are identified via ELISA or direct detection. However, detection of the signal varies, depending on whether the patient has primary or secondary infections. IgM is detectable 6 to 10 days after the onset of fever, suggesting time as the possible limitation of the serological method (Kuno et al., 1991). IgM level is low in secondary infections and this could result in false positive results (Hunsperger et al., 2009). Instead of detecting IgM, direct detection of the antigen of dengue virus is viewed as a more feasible strategy of dengue diagnosis as it can be screened as early as the first day of infection without perturbation by the presence of IgM (Xu et al., 2006).

1.4 NS1 protein as the potential diagnostic marker in the direct detection of DENV

Out of the seven non-structural proteins, antigen non-structural glycoprotein-1 NS1 is an apposite biomarker for the direct diagnostic detection of the DENV. The diagnostic value of NS1 was testified in the diagnostic tests for early infection (Pal et al., 2014). NS1 is found to induce strong humoral response and present at high concentrations in the blood up to 9 days after primary and secondary infections (Peeling et al., 2010). One interesting feature of NS1 is its excretion into the human serum after infection. The amount of excreted NS1 in the human serum of an DENV-2 infected individual was found to be in direct correlation with viremia (Libraty et

al., 2002). Another added advantage of using NS1 protein is that the presence of anti-dengue immunoglobulin M (IgM) antibodies do not hinder its detection (Xu et al., 2006).

1.5 Antibody-based detection of NS1 protein suffers from several drawbacks

Several direct detection test kits were developed based on DENV-2 NS1 such as Panbio test, InBios test, and Bio-Rad test which are antibody-based assays. The sensitivities scored were 94.1%, 100.0%, and 80.0% for Panbio test, InBios test and Bio-Rad test, respectively (Pal et al., 2014).

Though high sensitivity was scored for these antibody-based assays, antibodies as the diagnostic agent have several disadvantages. These include high cost associated with antibodies and non-specific binding of antibodies that may cause false-positive results. One amenable class of Molecular recognition element (MRE) that harbours the capacity to address the shortcomings of antibodies are aptamers.

1.6 Aptamers

Aptamers are ssDNAs or RNAs that comprise of a myriad of secondary and tertiary structures which promotes binding to a wide variety of targets with high specificity. The word ‘aptamer’ is derived from the term ‘aptus’ which means to fit in Latin (Ellington & Szostak., 1990) and the term ‘meros’ which refers to particle in Greek (Stoltenburg et al., 2007).

Aptamers are the output of an *in vitro* selection process, widely known as Systematic Evolution of Ligands by Exponential Enrichment. Systematic Evolution

of Ligands by Exponential Enrichment (SELEX) consists of 3 major steps of; (i) incubation of the randomized oligonucleotide library with the target; (ii) separation of the bound from unbound nucleic acid ligands; and (iii) amplification (Stoltenburg et al., 2007).

1.7 Advantages of aptamers over antibodies

Unlike antibodies, aptamers are easier to be modified and exhibits no batch-to-batch variations. Moreover, aptamers have high discerning features, able to discriminate very similarly structured biomolecules such as between caffeine and theophyline (Jenison et al., 1994). Aptamers are able to be selected against a wide range of targets such as ions, drugs, toxins, peptides, proteins, viruses, bacteria, cells, and even tissues (Parekh et al., 2010; Bayrac et al., 2011). The smaller size of aptamers (probably 8-25 kDa compared to ~150 kDa of antibodies) permits faster and more efficient tissue penetration (Sun et al., 2014). Aptamers are less immunogenic compared to antibodies (Eyetech study group, 2002; Eyetech study group, 2003). Aptamers have reversibly denaturation property. Aptamers can be synthesised with much more lower cost compared to that of antibodies.

1.8 Aptamer-based diagnostics (Aptanostics)

Aptamers have excellent features that qualify them as an ideal class of diagnostic agent. Aptamer isolated against Ochratoxin A (OTA), a nephrotoxic and teratogenic secondary metabolite generated by *Aspergillus* and *Penicillium* strains, was used in the Enzyme-Linked Aptamer Assay (ELAA). The detection limit achieved was 1 ng/mL. Aptamer isolated against Aflatoxin B1 was also used in the fluorescent-based aptamer assay for the detection of the metabolite in food samples

(Malhotra et al., 2014). A DNA aptamer isolated against inactivated intact H1N1 virus particles was applied in sandwich enzyme-linked oligonucleotide assay (ELONA) and in an electrochemical impedance (EIS) aptasensor. The detection limit was 0.3 ng/μL in ELONA and 0.9 pg/μL in EIS, respectively (Bai et al., 2018).

1.9 Problem statement

The disadvantages associated with antibodies evoke an effort to shift from the antibody-centric diagnostic to aptamer-based diagnostic of NS1 protein. Isolation and characterization of aptamer against NS1 are envisaged to assuage the drawbacks associated with antibodies and could effectively pave the path towards the aptasensing of NS1 for dengue detection.

1.10 Objectives

The purpose of this study is to isolate RNA aptamers against NS1 protein and to further characterize the isolated aptamers.

1) To generate RNA aptamers that have high sensitivity and specificity for DENV-2 NS1.
    i. Execution of SELEX cycles up to cycle 11.
    ii. Interrogation of binding of the nucleic acid pool from cycle 5, 8 and 11 against NS1.
    iii. Cloning, sequencing and bioinformatics analysis of the potential RNA aptamer candidates.
2) To characterize the generated RNA aptamers.
    i. Determination of the dissociation constant of the selected RNA aptamers.
    ii. Post-SELEX modifications to increase the stability of the aptamers against

nucleases.

iii. Determination of the secondary structures of the selected aptamers.

3) To simulate the aptamer-NS1 protein complex formation by *in silico* prediction.

i. To perform a rigid docking between the aptamers and the NS1 protein by PatchDock.

ii. To predict the binding site of the aptamers on the NS1 protein.

CHAPTER 2

IN VITRO SELECTION AGAINST NS1 PROTEIN

2.1 Introduction

In vitro process or also known as Systematic Evolution of Ligands by Exponential Enrichment (SELEX) is a technique used to isolate aptamers against their cognate target molecules. In the first step of SELEX, approximately 10^{12}-10^{15} of single stranded nucleic acids are incubated with the target of interest. Following incubation, the target-bound sequences are separated from the unbound sequences via a process known as partitioning. The sequence bound molecules are eluted and subjected to amplification. At this juncture, the resulting double-stranded DNA sequences were converted to single-stranded sequences in the case of DNA aptamer generation (Marimuthu et al., 2012). For RNA aptamer generation, double-stranded DNA sequences were used as the templates for the production of RNA via *in vitro* transcription (Gopinath, 2011). The resulting molecules were used for the next round of SELEX and this process was repeated for 8-11 rounds. At the end of the SELEX cycles, PCR product was cloned and subjected to sequencing. Sequence analysis reveals the potential aptamers based on the frequency of appearance (Figure 2.1).

In this study, SELEX will be carried out against the target NS1 protein. After certain cycles of SELEX, the binding of the nucleic acid pool against the target NS1 will be interrogated. Having affirmed the binding of the nucleic acid pool against the target protein, sequencing and analysis of the sequences were carried out to identify the potential aptamers.

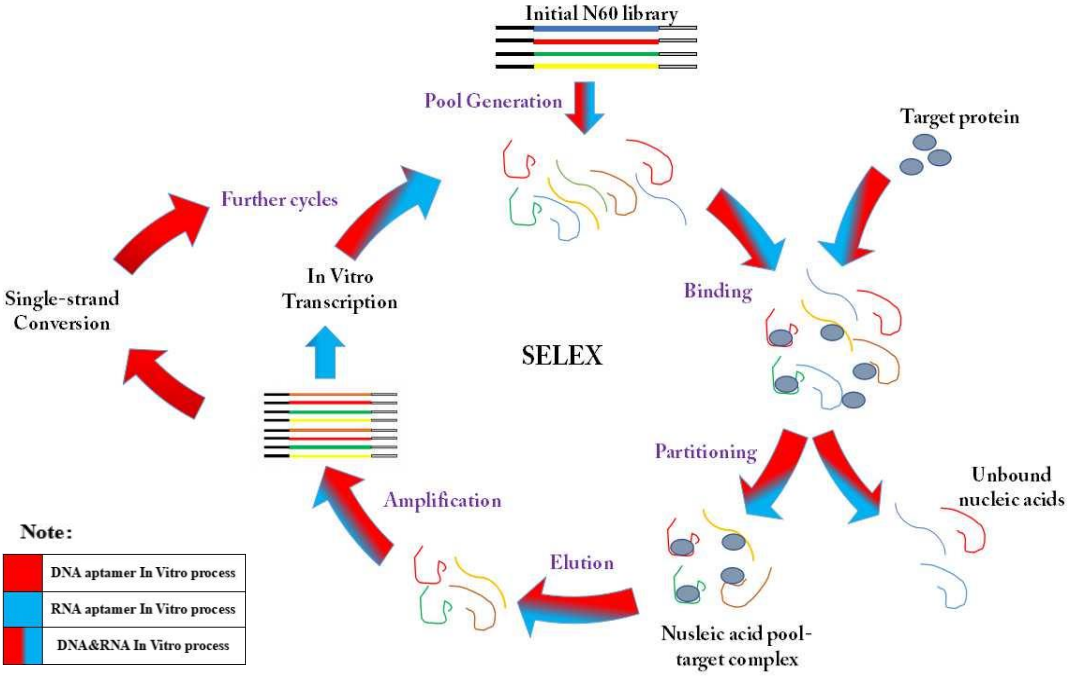

Figure 2.1: *In Vitro* selection process or SELEX for the isolation of DNA and RNA aptamers.

2.2 Materials and Methods

2.2.1 Designing random ssDNA library and primers

Random ssDNA library and the primers were purchased from Integrated DNA Technology (IDT, Iowa, USA). In this study, the random ssDNA library was designed with the length of 118 nucleotides, comprising of primer binding regions that flank the randomized region in the middle. The primer binding regions having the length of 19 nucleotides and 20 nucleotides bind to both the forward and reverse primers, respectively. The forward primer binding region has the T7 promoter sequence. The randomized region was designed with a length of 60 nucleotides, which theoretically accounts for 4^{60} of different sequences. The random ssDNA oligonucleotide sequence is shown as below:

5'- TAATACGACTCACTATAG**GGAGCTCAGCCTTCACTGC**-(N60)-**GGCACCACGGTCGGATCCAC**-3'

N60 represents the randomized region of the ssDNA library, while the underlined region is the T7 promoter sequence. 'N' can be either A, C, G, and T. For the production of RNA molecules, the random ssDNA molecules are first PCR-amplified followed by *in vitro* transcription.

2.2.2 PCR amplification

PCR amplification was carried out in a 100 µL reaction mixture containing 0.2 mM dNTPs (Promega, Madison, WI USA), 1 X PCR buffer (Yeastern Biotech Co., Taipei, Taiwan), 2 µM forward, 2 µM reverse primer and 5 U *YEAtaq* DNA Polymerase (Yeastern Biotech Co., Taipei, Taiwan). PCR amplification was executed using Biorad MyCyler Thermal Cycler and the parameters for PCR cycles

were as follow: an initial denaturation at 95°C for 60 s, amplification cycles that consist of 30 s denaturation at 95°C, 30 s annealing at 55°C, 30 s extension at 72°C and final elongation of 120 s at 72°C. The amplification was carried out starting at PCR cycle 6 and additional cycles were added till the PCR band with the correct size appears in each SELEX cycle.

2.2.3 Gel electrophoresis

For the visualization of the PCR product, 5 µL of the PCR product was mixed with 1 µL of 6X Bromophenol DNA loading dye (10 mM Tris-HCl [pH 7.5], 50 mM EDTA [pH 8.0], 0.03% bromophenol blue). This mixture was added with ethidium bromide, EtBr to a final concentration of 0.5 µg/mL (Sigma, St. Louis, USA). The mixture was loaded onto 4% agarose gel (Promega Corporation, Madison, USA) and the electrophoresis was run using an agarose mini gel apparatus (Bio-Rad Laboratories, Hercules, USA) in 1 X TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 100 V for 30 minutes. The gel was visualized with Gel Doc™ XR+ Gel documentation system (BioRad, California, USA). The PCR amplicon size with the expected size of 118 bp was confirmed with 25 bp DNA ladder (Promega Corporation, Madison, USA).

2.2.4 Ethanol precipitation

The PCR products were subjected to ethanol precipitation. One mL of absolute ethanol (Merck KGaA, Darmstadt, Germany), 40 µL of 3 M Sodium acetate (NaOAc) pH 5.2 (R & M Marketing, Essex, U.K.) and 300 µL ddH_2O were added together with the PCR product. The mixture was incubated for 30 minutes at -80°C. Subsequently, the mixture was centrifuged at 24,000 x g for 15 minutes at 4°C using

Sorvall™ ST 16R centrifuge (Thermo Fisher Scientific, Massachusetts, USA) and the supernatant was discarded. The resulting pellet was washed with 70% ethanol solution and air-dried in a vacuum concentrator (Eppendorf, Hamburg, Germany) for 5 minutes. Finally, the pellet was resuspended in 8 µL of ddH_2O.

2.2.5 *In-vitro* transcription

Four microliters of the ethanol precipitated PCR product was used as the template for an *in vitro* transcription reaction. *In-vitro* transcription was carried out using Ampliscribe™ T7-Flash™ Transcription kit (Epicentre, Wisconsin, USA). The *In vitro* transcription reaction mixture was assembled in a 20 µL reaction mixture containing 1 X AmpliScribe T7 reaction buffer (40 mM Tris-HCl [pH 7.5], 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine), 7.5 mM each of ATP, CTP, UTP, GTP, 10 mM of dithiothreitol (DTT), 20 U of AmpliScribeTM T7 Polymerase *Flash* Enzyme Solution (10 U/µL) and 20 U of RiboGuard RNase Inhibitor (40 U/µL). The mixture was then incubated at 37°C for 16 hours in a water bath (Fisher Scientific International, Inc., Hampton, USA). Followed by the incubation, treatment with 10 U of RNase-Free DNase I (Fermentas, Burlington, Canada) was done for 20 minutes at 37°C. The *in vitro* transcription reaction was stopped by adding equal volume of 2 X RNA loading dye (8 M urea, 0.05% bromophenol blue) and the mixture was heated at 95°C for 2 min in a thermomixer (Eppendorf, Hamburg, Germany).

2.2.6 Denaturing Urea PAGE

The *in vitro* transcription reaction was loaded onto 12%, 7 M denaturing-urea polyacrylamide gel in 1 X TBE (89 mM Tris-borate [pH 8.0], 2 mM EDTA) and

electrophoresed using BioRad Mini-PROTEAN 3 Electrophoresis Cell (Bio-Rad Laboratories, Hercules, USA) at 140 V for 1 hr.

2.2.7 Purification of transcribed RNA

After the completion of the electrophoresis, the gel was covered with a plastic wrap and was placed on top of the silica coated glass plate (Merck KGaA, Darmstadt, Germany). The band of interest was visualized by using a hand-held u.v. light source (UVP, LLC, Upland, USA). Band of interest which appears as a single prominent band under low-intensity u.v. shadowing (254 nm) was excised and transferred into a sterile 1.5 mL microcentrifuge tube (Eppendorf, Hamburg, Germany). The gel slices were meshed by using 1 mL pipette tip. The crushed gel particles were added with 400 µL of ddH_2O and heated at 60°C for 60 min to liberate the RNA that are 'incarcerated' in the gel matrix. One mL of absolute ethanol was added into the mixture and mixed vigorously. The mixture was centrifuged at 24,000 x g for 2 min at 4°C, followed by the collection of the supernatant into a fresh microcentrifuge tube and was added with 40 µL of 3 M Sodium acetate, NaOAc (pH 5.2). The solution was mixed and incubated at -80°C for 30 min followed by centrifugation at 24,000 x g for 15 min at 4°C. The supernatant was removed and the pellet was washed with 1 mL of 70% ethanol. Following a brief centrifugation at 24,000 x g for 2 min, supernatant was removed, the pellet was air-dried and dissolved in 25 µL of ddH_2O. The concentration of the RNA was estimated using nanodrop spectrophotometer (Implen Inc, Westlake Village, USA).

2.2.8 SELEX

The prepared RNA pool was subjected to SELEX cycles comprising of an iterative process of incubation, partitioning, elution and amplification. The sequences were identified by cloning and sequence analysis.

2.2.8(a) RNA pool-target protein complex formation

A reaction mixture that comprises of 6.25 µM of initial RNA pool, and 1 X SELEX binding buffer (150 mM NaCl and 10 mM HEPES-KOH [pH 7.4]) (GE Healthcare Bio Sciences, Uppsala, Sweden) was prepared. The mixture was heated up to 2 minutes at 95°C followed by cooling at RT for 10 minutes. The mixture was then added with 10.2 µM yeast tRNA (Invitrogen Corporation, Carlsbad, USA) as competitor followed by the addition of 2.5 µM of Dengue virus 2 NS1 glycoprotein (Abcam, Thailand). The reaction mixture was incubated at RT for 15 minutes. The concentration of RNA pool, yeast tRNA, and NS1 glycoprotein used in every SELEX cycle is varied.

2.2.8(b) Nitrocellulose filter membrane and microtiter-based partitioning

Nitrocellulose filter membrane was used for partitioning in SELEX cycle 1, 2, 4, 6, 7, 9, and 10. Nitrocellulose filter membrane (0.45 µm) was placed on top of the pop top filter and pre-wetted with 1 X SELEX buffer. The resulted mixture (from section 2.2.8(a)), was pipetted on top of the membrane filter and with the aid of the vacuum suction the unbound RNA molecules were removed. One mL of 1 X SELEX buffer was used to wash the membrane and the membrane was collected and placed into a sterile tube.

Partitioning for SELEX cycles 3, 5, 8, and 11 were aided by Xenobind plate (Xenopore, Hawthorne, NJ). First, the wells were washed with 1 X SELEX buffer for once to remove contaminants. Initially, the wells were coated with 1.5 µM of Dengue 2 Virus NS1 glycoprotein in 50 mM sodium phosphate buffer (pH 7.5) and incubated overnight at RT. After incubation, the wells were washed with 300 µL of 0.05% PBST for 3 times to remove the unbound proteins from the wells, followed by coating of the wells with 3% BSA for 3 hours at RT. Two other empty wells were also coated with 3% BSA for SELEX pre-selection step under the same condition before washing thrice to remove unbound BSA.

For the selection on the microtiter plate, RNA mixture heated at 95ºC for 2 min and cooled at RT for 10 min was incubated in the 3% BSA coated wells for pre-selection at RT for 8 min. The unbound RNA sequences, which in the form of supernatant was collected from the 3% BSA coated wells added with yeast tRNA. The reaction mixture was added into the NS1 protein-coated wells and incubated at RT for 15 min. The wells were washed with 0.05% PBST for 3 times before elution.

2.2.8(c) Elution of target-bound nucleic acid

The RNAs were recovered from the filter membrane by heating at 95°C with 400 µL of 7 M urea for 2 min. Similarly, the bound RNAs on the surface of the wells were recovered using boiled 7 M urea. The eluted RNAs were subjected to ethanol precipitation with the aid of Dr. GenTLE™ precipitation carrier.

2.2.8(d) Reverse transcription of eluted RNA molecules

The eluted RNA molecules were subjected to reverse transcription reaction. The reaction mixture was prepared to contain 1 X Revert AID reverse transcriptase reaction buffer (50 mM Tris-HCl [pH 8.5], 8 mM $MgCl_2$, 30 mM KCl, 1 mM DTT) (Thermo Fisher Scientific, Massachusetts, USA), 0.4 µM of reverse primer and ddH_2O. The ethanol precipitated RNAs were resuspended in the reverse transcription reaction mixture. The mixture was heated for 2 min at 95ºC followed by cooling to RT for 10 min. To the reaction mixture, 1.6 mM dNTPs and 10 U of RevertAID reverse transcriptase (Thermo Fisher Scientific, Massachusetts, USA) were added and was incubated at 60ºC for 1 hour. The resulting cDNA from the reverse transcription reaction mixture was PCR amplified, ethanol precipitated and used for *in vitro* transcription to prepare RNA molecules for the next round of SELEX.

2.2.8(e) Counter-selection against nitrocellulose filter membrane/prefiltration

This step is vital to minimize sequences that bind to the nitrocellulose filter membrane prior to each SELEX cycle. The step was carried out at each SELEX cycle. The purified RNA was added with 400 µL of 1 X HEPES buffer and the mixture was heated at 95ºC for 2 min. The solution was left at RT for 10 min to equilibrate to RT. Then, the RNA was gradually passed through a nitrocellulose filter membrane placed inside the pop top filter holder using a 1 mL syringe. Prefiltration was carried out thrice and the filtered RNA solution was ethanol precipitated before each SELEX cycle.

2.2.8(f) 5'- end labeling of the RNA pool with (γ-32P) ATP

Prior to the labelling of the RNA at the 5'-end with (γ-32P) ATP, dephosphorylation was carried out. The dephosphorylation mixture consists of 1 X alkaline phosphatase buffer (New England Biolabs, Massachusetts, USA), 0.26 µM of RNA and 5 U/µL of alkaline phosphatase enzyme (New England Biolabs, Massachusetts, USA). The reaction mixture was incubated at 37°C for 60 min before ethanol precipitation with Dr.GenTLE^{TM} precipitation carrier.

The dephosphorylated RNA was added to a reaction mixture containing 1 X T4 RNA ligase reaction buffer A (Thermo Fisher Scientific, Massachusetts, USA), 6.6 pmol of radiolabeled γ32P-ATP (Perkin Elmer, Massachusets, USA) and 10 U of T4 ploynucleotide kinase, PNK (Promega, Wisconsin, USA). The mixture was incubated at 37°C for 60 min in a waterbath. The radiolabelled RNA was purified using Quick spin radiolabeled RNA purification Sphadex G-25 column (Roche Diagnostics GmbH, Mannheim, Germany).

2.2.8(g) Nitrocellulose filter binding assay for assessing the binding of the RNA pools against NS1 protein

Nitrocellulose filter binding assay was carried out as reported previously (Yamamoto et al., 2000; Kumarevel et al., 2004a; Kumarevel et al., 2004b). The final concentration of 1 nM of radiolabeled RNA pool sequences in reaction mixture that contained 1 X binding buffer and ddH_2O, were heated at 95°C for 2 min followed by cooling at RT for 10 min. The RNA reaction mixture was added with 200 nM of yeast tRNA and various concentrations of NS1 glycoprotein that were prepared in the reaction volume of 50 µL were added and incubated for 15 min at RT.

Concentration of NS1 protein for the 5^{th}, 8^{th} and 11^{th} cycle of SELEX were 0, 800, and 1600 nM, respectively. The reaction mixtures were filtered through pre-wet nitrocellulose filter membranes under vacuum suction and were washed with 1 mL of 1 X HEPES buffer. Following the filtration and washing, the filter membranes were collected and exposed to X-ray film before imaging using auto-radiography.

After the completion of SELEX cycles, PCR product was subjected to cloning and transformation. The plasmids were extracted and sequenced. The sequences obtained were arranged into clusters and their binding against NS1 protein was assessed.

2.2.9 Cloning and sequencing

2.2.9(a) Cloning and transformation

RNA pool after the completion of SELEX cycles were subjected to PCR amplification with the extension time extended up to 10 min. The resulting PCR product was cloned into pCR@2.1 cloning vector using TOPO TA cloning kit (Thermo Fisher Scientific, Massachusetts, USA). The reaction mixture for cloning was set up such that it contains 2 µL of PCR product, 1 µL of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$), 2 µL ddH_2O, and 1 µL pCR@2.1 cloning vector. The mixture was incubated for 30 min at RT.

Chemically competent *Escherichia coli* (TOP 10 strain) cell was thawed on ice for a few min. The prepared ligation mixture was added into the thawed cells and the mixture was incubated on ice for 15 min. The plasmids and cells mixture were subjected to a heat shock state in a 42ºC water-bath for 45 seconds and 800 µL of LB

broth was added into the mixture immediately afterwards. Later, the mixture was incubated at 37ºC with shaking for 90 min. Meanwhile, a mixture of 50 µL of 100 mM IPTG (Fermentas, Burlington, Canada), 50 µL of 40 mg/mL X-Gal (Takara Bio, Shiga, Japan), and 25 µL of LB broth were spread on top of LB agar plate (Pronadisa, Madrid, Spain) containing 50 µg/mL kanamycin (Roche Daignoctics GmbH, Mannheim, Germany). Following the completion of the incubation at 37ºC, the cell suspension was spread on the surface of the LB agar plate. The plate was incubated overnight at 37ºC.

2.2.9(b) Plasmid extraction

A total of 30 white colonies (denotes the presence of cloned sequences) were randomly picked from the LB-kanamycin plate and inoculated into 10 mL LB broth containing 50 µg/mL kanamycin. The inoculation was incubated for overnight at 37°C with shaking. Later, plasmid extraction was done using High Pure Plasmid Isolation Kit (Roche Daignostics GmbH, Mannheim, Germany). The steps were according to the manufacturer's instructions with few modifications. One mL of overnight culture was aliquoted in 1.5 mL microcentrifuge tube and was centrifuged at 6,000 x g for 1 min. The tubes were added with 250 µL of suspension buffer/RNase (50 mM Tris-HCl and 10 mM EDTA, pH 8.0) the pellets were resuspended before the addition of 250 µL of lysis buffer (0.2 M NaOH and 1% SDS). The tubes were inverted few times slowly to mix the mixture slowly and were incubated at RT for 5 min. Afterwards, chilled 350 µL binding buffer (4 M guanidine hydrochloride and 0.5 M potassium acetate, pH 4.2) was added to the tubes and the tubes were inverted few times slowly and incubated on ice for 5 min until the flocculant can be seen. The mixture was centrifuged for 10 min at maximum speed

of 13,000 x g at 4°C. The resulted supernatant was transferred into high pure filter tubes housed in a microcentrifuge tubes and was centrifuged at 13,000 x g for 1 min. Flow-through from the collection tube was discarded and 500 µL of washing buffer I (5 M guanidine hydrochloride, and 20 mM tris-HCl, pH 6.6) were added into the high pure filter tubes and was centrifuged under the same conditions as before. The resulted flow-through was discarded before 700 µL of washing buffer II (20 mM NaCl, 2 mM tris-HCl, pH 7.5) were added and centrifuged as before. The flow-through was decanted and the previous step was repeated one more time without the addition of washing buffer. The high pure filter tube was later transferred to new sterile 1.5 mL microcentrifuge tubes and 30 µL of elution buffer (10 mM tris-HCl, pH 8.5) was added into the filter tubes before it was centrifuged for 1 min at 13,000 x g. The extracted plasmid concentrations were quantified using nanodrop and stored in -20°C prior to use.

2.2.9(c) DNA sequencing

The quality of plasmids was confirmed by 0.8% agarose gel electrophoresis. The plasmid (0.5 µg) was mixed with 20 pmol of M13 forward primer and sequencing was carried out by First BASE Laboratories Sdn. Bhd., Selangor, Malaysia.

2.2.9(d) Sequence analysis

The sequences obtained were analyzed for sequence homology using CLUSTALW (http://www.ebi.ac.uk/clustalw/). Similar sequences were clustered together and the frequency of appearance will be calculated. The most abundant

sequence clusters were subjected to binding evaluation against the target NS1 protein.

2.3 Results and Discussion

2.3.1 Eleven cycles of SELEX were successfully completed

The selection buffer used in this study was 10 mM HEPES buffer which contained 150 mM of NaCl (Carothers et al., 2010). The monovalent ion Na^+ functions to 'appease' the electrostatic repulsion between the negatively charged nucleic acids and the negatively charged target molecule. HEPES functions as the buffering agent to resists pH change.

In this study, a total of 11 cycles of SELEX were carried out. The parameters such as RNA pool concentration, target protein concentration and competitor yeast tRNA concentration that were used in this study are shown in Table 2.1. Throughout the SELEX process, selection pressure was exerted by reducing the amount of target protein and RNA pool with the concomitant increase in the amount of competitor yeast tRNA.

Table 2.1: The parameters used for eleven SELEX cycles. Nitrocellulose filter membrane-based partitioning was used for all the SELEX cycles except for the cycles with the notations '*', in which Xenobind plate-based partitioning was used.

SELEX cycle	***RNA pool (µM)***	***Competitor (µM)***	***NS1 Protein (µM)***	***PCR amplification cycles***
1	6.6	10.2	2.5	8
2	3.9	30.6	2.0	8
3*	2.1	50.9	1.5	8
4	1.6	81.6	1.0	8
5*	1.1	112.2	0.75	8
6	0.8	152.9	0.5	8
7	0.4	193.7	0.25	6
8*	0.3	203.9	0.125	6
9	0.3	203.9	0.125	6
10	0.2	203.9	0.1	5
11*	0.1	203.9	0.05	8

2.3.2 Nitrocellulose filter membrane-based partitioning as the simple and rapid approach

Nitrocellulose filter membrane is used extensively as it offers a simple and rapid retention of protein by its non-specific affinity against amino acids (Song et al., 2012). Moreover, nitrocellulose membrane does not require the immobilization of target proteins as is necessitated by other revenues such as affinity chromatography, magnetic beads and capillary electrophoresis. The principle of partitioning is based on the retention of the target protein on the surface of nitrocellulose filter membrane, which also retains nucleic acid molecules that have affinity against the protein. By means of vacuum suction, nucleic acid molecules that do not interact with the protein are discarded. Subsequently, the membrane was treated with 7 M urea. Urea, as a strong chaotropic agent denatures protein, releasing the protein-bound RNA molecules.

2.3.3 Nature of the PCR amplification in SELEX

The eluted RNA molecules were converted to first-strand cDNAs followed by PCR amplification. In each SELEX cycle, the band with the expected size of 118 bp was obtained (Figure 2.2). As the template being amplified is short, no optimization of annealing temperature for PCR amplification was needed. Standard $MgCl_2$ concentration of 1.5 mM and 0.2 mM concentration of dNTPs were used for the PCR amplification. *Taq* Polymerase was used for the enzymatic amplification despite lacking 3'-5' exonuclease proof-reading. Mutation resulting from the absence of this proof-reading activity was viewed as an added advantage in creating the diversity of the nucleic acid pool (Bartlett and Stirling, 2003).

PCR amplification for the template amplification is very crucial and sensitive step. A highly efficient amplification is needed to minimize the amplification bias and to reduce the loss of potential high affinity binders at early stages of SELEX cycle. This is also pivotal to suppress the fecundity of non-target binders. One of the most important circumspection practised throughout the SELEX process is to minimize the number of PCR cycles. The PCR cycles were added in the increments of 1 or 2 PCR cycles starting from PCR cycle 6 until the band of the expected size appears. The PCR cycles for each SELEX cycle were maintained as low as possible to diminish the accumulation of unintended or 'cryptic' PCR products. Over amplification could permit the DNA templates having randomized region in the middle to form Watson Crick base-pairing with each other. This phenomenon is known as opportunistic mispairing and is accountable for the appearance of aberrant or cryptic PCR products, which could oppress the amplification of intended target (Tuerk, 1997).

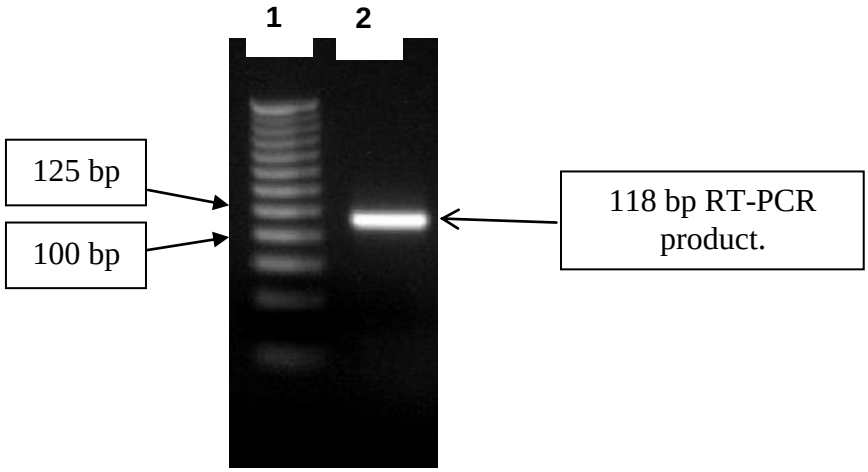

Figure 2.2: A representative of the gel electrophoresis of the PCR product from SELEX cycle 2. Eight cycles of PCR were carried out using the reverse-transcribed RNA pool from SELEX cycle 2 as the template. The product was electrophoresed on 4% agarose gel. Lane 1: 25 bp DNA ladder; lane 2: 5 µL of RT-PCR product.