DEVELOPMENT OF AN ENZYME-LINKED APTASORBANT ASSAY IN DETECTING DENGUE VIRUS 2 NS1 BIOMARKER

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

BSA	Bovine serum albumin
°C	Degree Celsius
DENV	Dengue virus
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
dH_2O	Distilled water
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
et al.	and others
EtBr	3, 8-diamino-5-Ethyl-6-phenyl phenanthridinium Bromide
g	Gram
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
M	Molar, [(Mole)/(Litre)]
Mg^{2+}	Magnesium ion
Min	Minute (s)

ml	Millilitre
mM	Millimolar
Na^+	Sodium ion
NaCl	Sodium chloride
NaOAc.3H2O	Sodium acetate trihydrate
NaOH	Sodium hydroxide
ng	Nanogram
nM	Nanomolar
nt	Nucleotide (s)
PAGE	Polyacrylamide gel electrophoresis
PBST	Phosphate buffered saline with Tween 20
LINAT	LINA buffer with Tween 20
PCR	Polymerase chain reaction
PDB	Protein Data Bank
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
RT	Room temperature
RT-PCR	Reverse transcription-PCR
s	Second (s)
STDENV	Stabilized truncated dengue aptamers
ssDNA	Single-stranded DNA
TAE	Tris–Acetic Acid–EDTA
TBE	Tris-Boric Acid-EDTA
TEMED	N,N,N’,N’-Tetramethylethylenediamine
Tris	Tris-(Hydroxymethyl)-Aminomethane
TDENV	Truncated dengue aptamers
UV	Ultraviolet

V	Volt (s)
v/v	Volume per volume
w/v	Weight per volume
x g	Relative Centrifugal Force
µg	Microgram
µl	Microliter
µM	Micromolar
2'-F	2'-fluoro

PEMBANGUNAN UJIAN APTASORBANT BERKAITAN ENZIM DALAM MENGESAN BIOPENANDA NS1 VIRUS DENGGI 2

ABSTRAK

Denggi adalah ancaman yang membimbangkan kepada kesihatan dan ekonomi pelbagai negara di seluruh dunia. Walau bagaimanapun, pendekatan semasa yang digunakan dalam aplikasi diagnostik jangkitan Denggi adalah terhad kepada antibodi yang mengalami beberapa kelemahan. Aptamers ialah kelas helai asid nukleik terlipat yang mampu mengikat molekul sasaran serumpun yang berbeza dengan pertalian tinggi dan selektiviti. Aptamers mengurangkan kelemahan antibodi dengan memudahkan sebagai alat analisis yang kos efektif dan cekap dalam mengenal pasti jangkitan Denggi. Pemangkasan rasional aptamer panjang penuh yang luas kepada bentuk yang lebih padat menjana struktur aptamerik yang dioptimumkan dengan pertalian mengikat sasaran yang berpotensi dipertingkatkan, menyumbang kepada kehebatan diagnostik yang lebih baik. Oleh itu, beberapa aptamer terpenggal disediakan menggunakan aptamer RNA khusus Denggi panjang penuh yang menyasarkan antigen NS1, sasaran virus yang berfungsi sebagai biomarker yang sangat baik untuk pengesanan DENV. Analisis pengiraan digunakan untuk membandingkan prestasi pengikatan dalam siliko bagi aptamer panjang penuh yang dipotong dan sepadan. Aptamer yang dipotong telah diperiksa menggunakan fungsi pemarkahan AutoDock Vina, HADDOCK dan dok Patchdock dalam kombinasi dengan simulasi dinamik molekul. Analisis dalam siliko mendedahkan keupayaan mengikat yang lebih baik bagi aptamer terpotong ke atas jujukan panjang penuhnya. Aptamer yang

dipotong ini selanjutnya diperiksa secara in-vitro menggunakan ELASA langsung untuk memastikan pertalian mengikatnya terhadap protein NS1. Antara urutan yang diuji, aptamer terpenggal TDENV-3 dan TDENV-6a memaparkan pertalian mengikat sasaran yang dipertingkatkan dalam julat nanomolar bawah ~ 25 nM berbanding aptamer DENV-3 dan DENV-6 panjang penuh masing-masing. Aptamers juga menunjukkan kekhususan tinggi serta selektiviti sasaran yang tinggi di bawah persekitaran serum yang kompleks terhadap sasaran NS1. Oleh itu, kedua-dua aptamer telah diterima pakai untuk membangunkan platform ELASA sandwich berprestasi tinggi untuk diagnosis tepat antigen NS1 denggi. Ujian sandwic aptamerik yang direka menunjukkan had pengesanan pada kepekatan analit serendah 1 nM dalam 0.05% serum manusia yang diikat dengan sasaran NS1. Penemuan kajian ini mencadangkan bahawa aptamer TDENV yang dioptimumkan diguna untuk membangunkan sandwich ELASA menghasilkan platform yang serba boleh dan berkebolehan untuk aplikasi diagnostik dan prognosis klinikal jangkitan denggi.

DEVELOPMENT OF AN ENZYME-LINKED APTASORBANT ASSAY IN DETECTING DENGUE VIRUS 2 NS1 BIOMARKER

ABSTRACT

Dengue is an alarming threat to both the health and economy of multiple nations worldwide. However, the current approaches used in the diagnostic application of dengue virus infections are limited to antibodies-based techniques which concurrently possess several disadvantages. Aptamers are a class of folded nucleic acid strands capable of binding to different cognate target molecules with high affinity and selectivity. Aptamers alleviate the drawbacks of antibodies by facilitating as cost-effective and efficient analytical tools in identifying dengue infections. Rational truncation of extensive, full-length aptamers to more compact forms generates optimized aptameric structures with potentially improved target-binding affinity, contributing to better diagnostic prowess. Therefore, several truncated aptamers were prepared using full-length Dengue-specific RNA aptamers targeting NS1 antigens, viral targets which serve as excellent biomarkers for the detection of dengue virus. Computational analysis was employed to compare *in-silico* the binding performance of the truncated and the corresponding full-length aptamers. The truncated aptamers were examined using the scoring functions of AutoDock Vina, HADDOCK and Patchdock dockings in combination with molecular dynamic simulations. The *in-silico* analysis revealed better binding capabilities of truncated aptamers over their full-length sequences. These truncated aptamers were further examined *in-vitro* using direct Enzyme-Linked Aptasorbent Assay (ELASA) to ascertain their binding affinity

towards the NS1 protein. Among the tested sequences, truncated aptamers TDENV-3 and TDENV-6a displayed enhanced target-binding affinity in the lower nanomolar range of ~ 25 nM compared to their respective full-length DENV-3 and DENV-6 aptamers. The aptamers also demonstrated high specificity as well as high target-selectivity under complex serum environments against NS1 targets. Hence, the two aptamers were adopted to develop a high-performance sandwich ELASA platform for the accurate diagnosis of dengue NS1 antigens. The devised aptameric sandwich assay displayed a detection limit at analyte concentrations as low as 1 nM in 0.05 % human serum spiked with NS1 targets. The findings of this study suggest that the optimized TDENV aptamers utilized to develop the sandwich ELASA yield a versatile and capable platform for the diagnostic application and clinical prognosis of dengue infections.

CHAPTER 1
INTRODUCTIONS

Dengue remains a persisting threat to the communal health of many countries up until today. However, the current approaches used in the diagnostic application of dengue virus infections are limited to antibodies-based techniques which concurrently possess several disadvantages. Aptamers are a class of folded nucleic acid strands capable of binding to different cognate target molecules with high affinity and selectivity. Aptamers alleviate the drawbacks of antibodies as cost-effective and efficient molecular tools for the diagnostic application of dengue infections. In this study, we have developed an aptamer-based sandwich enzyme-linked immunosorbent assay (ELISA) which demonstrates sensitive and specific detection of Dengue-2 NS1 antigens, the biomarkers which help identity the onset of common dengue infections or dengue viral fever. The developed aptameric assay has the potential to serve as an efficient diagnostic utility, enabling a more cost-effective and equally efficient early detection platform for dengue infections in the attempt of combating the global spread of Dengue.

CHAPTER 2

LITERATURE REVIEWS

2.1 Impact of dengue in Malaysia

Dengue remains a persisting threat to the communal health of many countries up until today. dengue fever is globally caused by the dengue virus (DENV), a mosquito-borne disease (Fig.2.1) that can progress to even more severe state known as dengue hemorrhagic fever, leading to prominent bleeding, low blood counts, fever shocks, and the looming certainty of death (Rajapakse, 2011; Ralapanawa et al., 2018). Dengue fever has limited treatment options and poor to medium prognosis. The first severe case of the dengue fever epidemic was first reported in Malaysia around 1973, following a rise in infections of 1487 cases and 54 death reports (Wallace et al., 1980). The disease progressed by an yearly increasing pattern, with its largest outbreak being reported in 1966, where the country was burdened with 14 500 cases (George & Lam, 1997).

As of 9 March 2022, 6229 cases of dengue infections, have been reported in Malaysia. This is an increase of 458 cases compared to the same period in 2021 (WHO WPRO, 2022). The number of dengue cases spiked by 11.1 % in Malaysia during February 2022 compared to the same month in 2021 (CPRC Kebangsaan, 2022). According to studies performed, almost 390 million dengue virus infections per year are reported globally, among which 96 million manifest clinical symptoms (S. ; Bhatt et al., 2013). A study by Brandy *et al*., indicates that there are 3.9 billion people at risk of dengue infections, with 70% of the worldwide cases being in Asian regions alone (Brady et al., 2012). Although the diseases, unlike COVID-19, has a seasonal, low occurrence rate and transmissibility, the degree of

fatality of DENV infections is far greater without treatments (WHO, 2021). The Malaysian government is keeping an eye on both the DENV and COVID-19 infections concurrently to avoid any one of the viral diseases getting an upper hand during times of uncertainty.

A study conducted by Ong et al., reported that due to the lockdowns imposed by the government in attempts to reduce the spread of COVID-19 has had a negative impact on DENV controls (Ong et al., 2021). The study implied that due to more people being restricted of their movements to closed-home areas, the spread of non-vector-borne viruses such as SARS-CoV-2 can be curbed. However, vector-borne viruses such as dengue have higher possibilities of re-surfacing as it increases the *Aedes* mosquito populations at local, confined host areas. This in turn increases the DENV infections. Furthermore, the recent heavy-flood occurrences at densely populated cities such as Selangor or Melaka posed an even greater risk of DENV infections owing to all the muddy and stagnant, diseases bearing river waters which can drastically increase both the breeding of the vector populations and the susceptibility of the community towards a higher DENV viral transmissions.

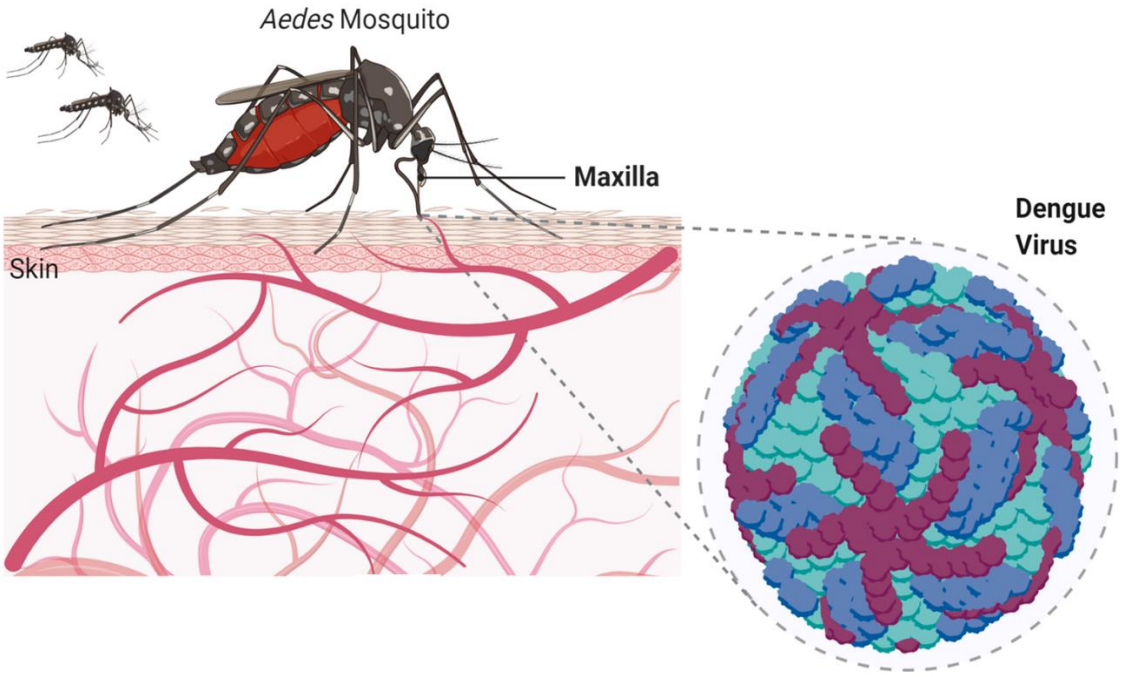


Figure.2.1: Diagram illustrating the spread of the dengue virus through mosquitos feeding of human hosts.

2.2 Virology of dengue virus

Dengue is a viral species categorized under the genus *Flavivirus* of the family *Flaviviridae*. Dengue consists of four different serotypes; DENV-1, DENV-2, DENV-3 and DENV-4. The serotypes share 65 – 70% sequence homology between each other (Azhar et al., 2015). Among them, DENV-2 is known as the most virulent and predominant of the variants found in Malaysia (Nani Mudin, 2015; Vicente et al., 2016), in comparison to the other serotypes. The RNA viral genome of DENV comprises of 10 essential genes (E.g. Capsid, PrM, Env, NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5). These genes express the structural (Capsid, PrM and Env) and non-structural (NS) viral proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) (Idrees & Ashfaq, 2012).

2.3 Current approaches in detecting dengue virus

2.3.1 Nucleic-acid amplification or RT-PCR assays:

Nucleic-acid amplification techniques (NAAT) such as reverse transcription PCR (RT-PCR) is a common method implemented in identifying DENV. The system relies on using primers targeting the viral RNA region specific for each different viral strain. Systems such as the CDC DENV-1-4 rRT-PCR multiplex assay or CDC Trioplex rRT-PCR assay are examples of such techniques applied for the detection of DENV (CDC, 2019). Several authors such as Gurukumar et al., Alm et al., and Tandel et al., have also applied RT-PCR in diagnosing DENV infections (Alm et al., 2015; Gurukumar et al., 2009; Tandel et al., 2021). Positive NAAT/RT-PCR test results indicate DENV infections while negative tests do not necessarily rule out infection as additional sub-validation (e.g. IgM or IgG antibody

test) must be conducted (CDC, 2019; Grobusch et al., 2006). The assay boasts sensitivity and specificity, however, lacks in practicality due to the need for RNA isolations steps and expensive instruments.

2.3.2 Antibody-based techniques

Antibody-based detection assays are the common diagnostic tests carried out at most medical and clinical testing facilities for the detection of DENV infections. Model techniques include serological antibody-based tests that detect the presence of dengue infections by capturing immunoglobins such as IgM or IgG, found at high levels following the onset of dengue infections (Palabodeewat et al., 2021; Tran et al., 2006). Plaque reduction neutralization tests (PRNT) are also effective approaches in detecting the humoral immune response during DENV infections (Rainwater-Lovett et al., 2012). The technique employs the detection of specific neutralizing antibodies against DENV and even other flaviviruses. The assay measures the titer of the neutralizing antibodies in the serum of the DENV infected individuals, specifically of IgM-positive patients. PRNT offers better confirmation on DENV diagnosis but is labour intensive, time-consuming and relatively costly. There exist several ELISA techniques designed for detecting DENV infections by capturing viral antigens such as the Env or NS1 proteins using DENV-specific antibodies (Narayan et al., 2016; Prommool et al., 2021; Tran, 2006). A summary of the available approaches in detecting dengue infections are provided in Table 2.1.

2.3.2(a) NS1 as excellent biomarker in identifying dengue infections

The viral NS1 glycoprotein is a major DENV pathogenic virulence factor which orchestrates numerous cascades of biochemical reactions and immune responses following the point of infections (P. Bhatt et al., 2021; Diamond & Peirson, 2012; Lin et al., 2011). The NS1 glycoprotein facilitates as prime biomarkers for both the detection of DENV-2 infections and the diagnosis of dengue fever. DENV-2 NS1 presents at high concentrations in patient serum within the initial 6 – 7 days of infections, allowing for early detection of DENV invasions (Libraty et al., 2002; Pal et al., 2014). NS1 glycoproteins are also antigens that are secreted into patient sera, enabling their direct detection without the need for tedious pre-treatment of patient samples (Flamand et al., 1999). The NS1 levels of DENV patients also correspond to the degree of infections or viremia (Libraty, 2002), which enables us to determine the severity of the viral diseases among patient groups. Moreover, the presence of IgM or IgG antibodies in DENV positive patients do not negatively affect the direct detection of circulating NS1 from patient serum samples (Xu et al., 2006).

2.3.2(b) Drawbacks of antibodies in detecting dengue NS1 antigens

The application of antibodies in the diagnostics of dengue requires research-intensive selection protocols that will incur a higher cost of operations/synthesis. Furthermore, inherent limitations such as low-temperature stability, higher batch-to-batch variations, and the need for animal models complicate the application of antibodies as feasible components of diagnostics systems (Albert, 1985; Chames et al., 2009). Moreover, antibody-based DENV diagnostic kits or the synthesized anti-DENV antibodies alone often amount to high market prices that make their applicability significantly lower.

2.4 Aptamers as the next 'ethos' in dengue diagnostic applications

Aptamers serve as excellent replacement candidates to antibodies with the potential to be ideal molecular detection elements for any diagnostic platform. Aptamers are a class of single-stranded DNA/RNA molecules that bind targets with high specificity. Generated through Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Ellington & Szostak, 1990; Gold & Tuerk, 1990), aptamers are known to bind to a broad spectrum of targets such as proteins, dyes (Bernard & Costa, 2012; De Acha et al., 2019; Wilcox et al., 2008; Zhang et al., 2017), and even microbial and animal cells or tissues (Mallikaratchy, 2017; Zhai et al., 2017). The choice of using aptamers instead as the detection element in dengue diagnostics helps circumvent the flaws associated with the use of anti-DENV antibodies. Aptamers possess high binding affinity and selectivity towards their respective target molecules on a level comparable to antibodies (Gopinath et al., 2016). They also offer lower batch-batch variations, high programmability, ease of modifications (e.g base modifications, fluorophore/biotin functionalization), and less expensive *in-vitro* synthesis processes (Jayasena, 1999; Kaur et al., 2018; Sun et al., 2014; Thevendran et al., 2020).

2.5 Application of aptamers in dengue diagnostics

Several authors have successfully applied the use of aptamers in detecting viral antigenic targets. Lee et al., developed an aptamer-based ELISA platform for the detection of NS1 proteins of Zika virus (Lee & Zeng, 2017). The system demonstrated high specificity and sensitivity of 0.1 pg/ml of NS1 targets. Zou et al., also utilized aptamers in detecting the capsid protein VP1 of enterovirus EV-A71 down to 200 ng/ml (Zou et al.,

2021). Similarly, Chen et al., also utilized aptamer-based ELISA systems in detecting the SARS-CoV-2 nucleocapsid protein, where the system exhibited detection limits at 10 ng/ml of viral analyte (Chen et al., 2020).

2.6 The commercial potential of 'aptanostic' assays in dengue detection

The dengue testing market was valued at USD 455.77 million in 2020 and is expected to reach USD 597.78 million by 2026, registering a CAGR of 4.74 % during the forecast period, based on the reports of Mordor Intelligence Inc. (Fig.2.2(A)). The application of aptamers is capable of expanding the dengue testing market owing to the low cost of synthesis and operations involving aptamers. The development aptamer-based diagnostic systems for DENV detection can overall power the dengue test market and boost its economic growths, especially in Malaysia. Aptamer-based diagnostic (aptanostic) assay can be designed as a simple-to-use commercial kit that enables users to identify dengue infections from patient samples with formulated reagents at a competitive cost. The incorporation of aptamers can potentially lower the selling price of the assays, therefore, increasing the number of units being purchased at testing sites. This maximizes the testing capacity of clinical facilities and reduces the fiscal burdens on investment capitals allocated for disease diagnostic purposes. The efficiency and low-cost properties of aptamer-based assays will boost the demand of the kit, potentially doubling their return of investments (ROI) and reduing the cost of stockpiling compared to expensive antibody-based diagnostic assays.

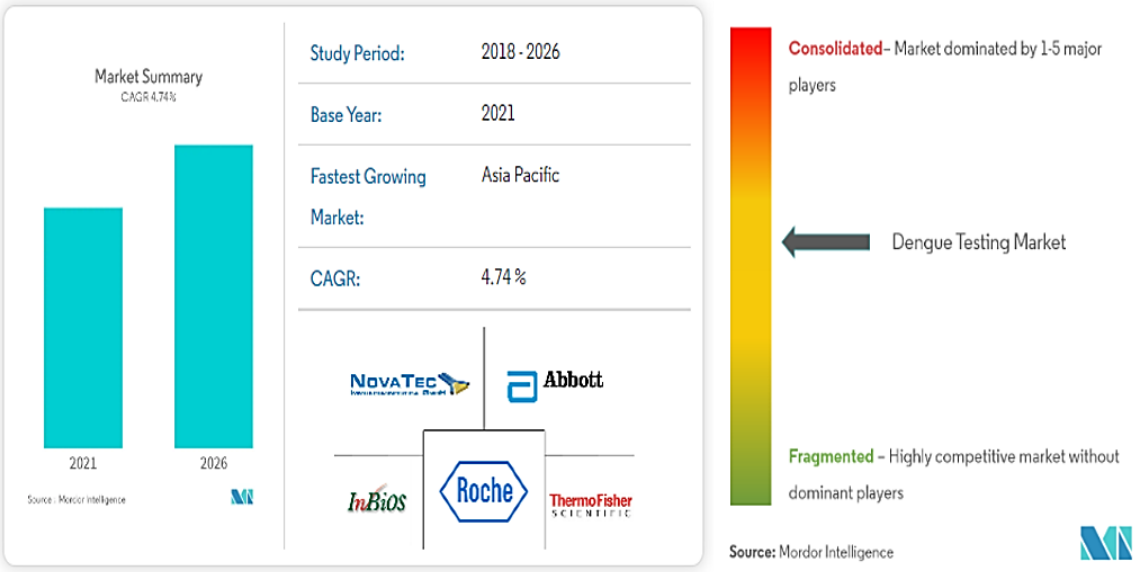
2.7 The novelty of aptamer-based diagnostic assays in dengue detections

A patent search was also done at lens.org with the following keywords; aptamer AND ELASA AND diagnostic AND kit AND dengue AND NS1. No patents or search hits were seen for the aptamer-based assays against Dengue, indicating the novelty of aptameric diagnostic system for DENV identifications (Fig.2.2(B)).

Table.2.1. An overview of the diagnostic technologies available for DENV diagnosis

Technologies	Examples	Targets	Benefits	Drawbacks
Rapid ELISA lgM test kits	Panbio DENGUE Novatec Dengue Virus IgG	IgM or IgG Ab's	Provides rapid test results. Direct patient serum testing	Uses expensive synthesis of DENV antibodies. Requires the use of animal-models for antibody derivation.
ELISA-NS1 test	DENV Oefecf NS1 ELISA Kit, MedEx Neotest Dengue Combo	DENV NS1 proteins	Provides rapid test results. Direct patient serum testing	Uses expensive synthesis of DENV antibodies. Requires the use of animal-models for antibody derivation.
RT-PCR assays	cobas™CHIKV/DENV, CDC DENV-1-4 rRT-PCR Multiplex and Trioplex rRT-PCR Assays	Viral RNA	High sensitivity. Provides serotype characterizations and identification	Requires technical expertise and expensive instruments, time-consuming

A.



B.

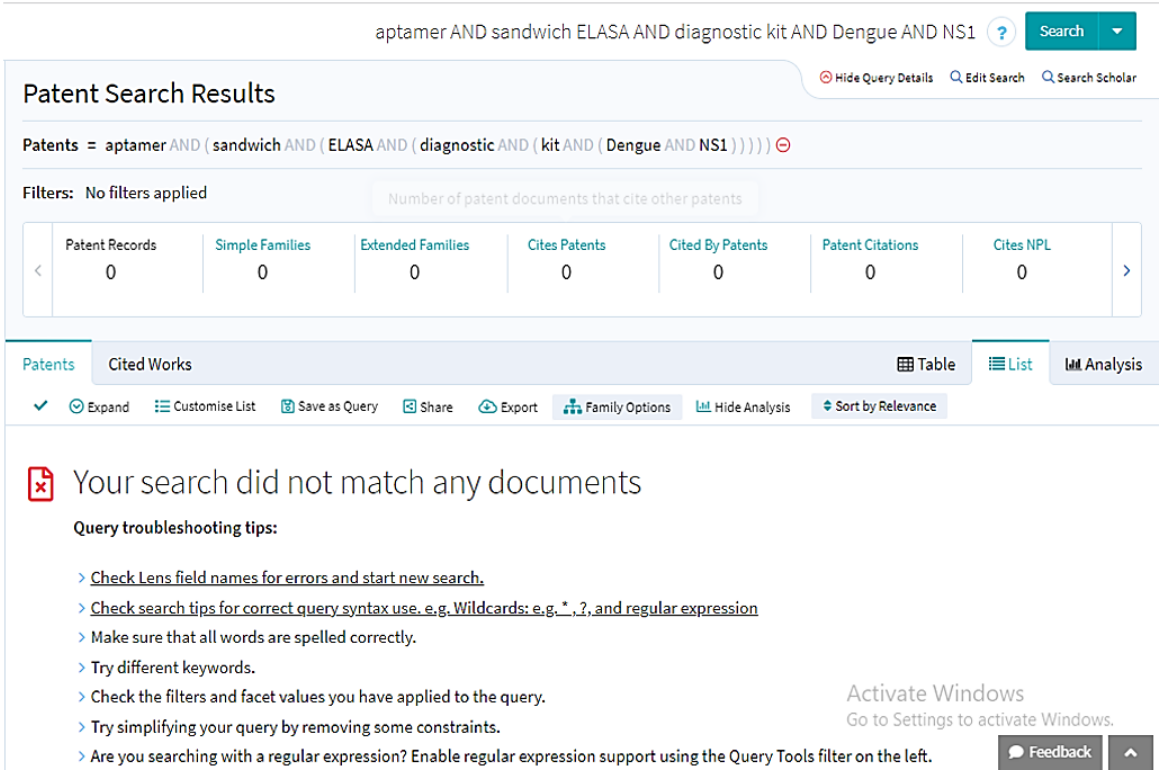


Figure.2.2: A) Market forecast report for dengue test kits 2021-2026. B) Patent search report for the aptamer-based diagnostic assay from Lens.org.

2.8 Problem statement

The techniques utilized in detecting DENV infections are largely limited to antibodies. However, the downfalls of antibodies raise the need to utilize aptamers for dengue diagnostics. Thus, developing an aptamer-based diagnostic platform for the detection of DENV could pave the way to both less expensive and equally efficient analytical systems.

2.9 Objectives

The purpose of this study is to develop an aptamer-based sandwich ELASA system for the sensitive and selective detection of dengue NS1 biomarkers.

1. To optimize RNA aptamers against DENV-2 NS1 to increase their target-binding capability:
    a. Rational truncation of DENV-specific aptamers
    b. *In-silico* assessment of truncated aptamer binding towards NS1 targets
2. To experimentally validate the performance of truncated aptamers:
    a. Determination of the binding affinity of the designed truncated aptamers
    b. Determination of the aptamer specificity and selectivity against NS1 targets
3. To develop a sandwich ELASA in detecting dengue NS1 proteins
    a. Determination of the limit of detection of the aptameric sandwich assay
    b. Analysis of NS1 detection in human serum using the sandwich ELASA

CHAPTER 3

RATIONALE TRUNCATION OF APTAMERS AND VALIDATIONS OF TARGET-BINDING IN-SILICO

3.1 Introduction

Previously, our group had successfully generated several dengue NS1 specific RNA aptamers using conventional SELEX protocols. The aptamers were designated as DENV-3, DENV-4 and DENV-6 RNA aptamers. These aptamers have an average length of 100 base pairs (bp). In this study, we hypothesized that the truncation/shortening of the full-length DENV aptamers into shorter, compact versions that still retain the core secondary structures may improve their target-binding affinities. This can be possible due to the elimination of excessive sequence regions that potentially contribute to electrostatic repulsions which can prevent strong aptamer-target interactions. As a result, optimized aptameric conformations are generated, constituting only the sequence motifs that participate in target binding which increase their binding affinity against the NS1 analyte. Several candidates of the truncated DENV-NS1 aptamers were prepared. Prior to *in-vitro* assays, we analyzed the binding of the truncated aptamers against NS1 using *in-silico* evaluations. The *in-silico* measured performance of the truncated aptamers was compared with the performance of their respective full-length versions to determine whether the new, shortened sequence display any improvements in their target-binding properties.

3.2 Materials and Methods

3.2.1 Truncation of the aptamer sequence

The secondary (2D) structure of the full-length DENV aptamers was predicted using RNAfold in the Mfold programme with default settings using the Zuker algorithm (Zuker, 2003). The secondary structure with the lowest Gibbs free energy (dG) was chosen as the conformation for each full-length RNA aptamer. The DENV RNA sequences were truncated or shortened individually at different sequence lengths and regions. The nucleotides (nt) number from the n^{th} – n^{th} position for DENV-3, 4 and 6 were picked by referring to the binding motif regions predicted by Mfold.

3.2.2 Obtaining the 3D structure of NS1 protein

The 3D structure of the NS1 protein was obtained from PDB coordinates files acquired from the Protein Data Bank with ID: 4O6B. The downloaded PBD file was manually deleted by removing unnecessary portions of the file (e.g. SOURCE and REMARKS) while keeping the atomic coordinate portion (ATOM). Additional ligands (HETATM) were also deleted.

3.2.3 Structural modelling of RNA sequences from 2D to 3D structures

The tertiary (3D) structures of truncated RNA aptamers were modelled by first re-identifying and confirming their 2D structures again using the RNAfold of Mfold. The secondary structure with the lowest Gibbs free energy (dG) was chosen as the most accurate/preferred conformation. Each folded sequence was converted to viena format, which translates the formation of the secondary structures in the form of dots and brackets. The viena formats were entered into RNAComposer webservers using the default settings

to convert the 2D structures to 3D PBD coordinate files (Barquist et al., 2012). RNAComposer can be accessed at rnacomposer.cs.put.poznan.pl.

3.2.4 Molecular dynamic (MD) simulation of the 3D RNA aptamer structures

MD simulations were conducted using the GROMACS program (downloaded at gromacs.org), in which Amber99SB-ILDN force field parameters for RNA aptamer simulations were applied. The complexes were solvated using TIP-3 water models in a cubic box, maintaining a centric position and keeping a distance of 1.0 nm between the complex and the edge of the solvated box. Sodium and chloride ions were added to neutralize the charge of the entire system. Particle Mesh Ewald (PME) method was applied to simulate electrostatic interactions. Energy minimization steps were followed through using the steepest descent algorithm with the tolerance of 1000 kJ mol-1nm-1. Verlet cutoff scheme was chosen, and periodic boundary conditions were assigned to all three dimensions (XYZ). Both thermal (NVT) and subsequent pressure equilibration (NPT) phases were simulated for 0.1 ns with a 2 fs integration/time step. NVT was conducted at room temperatures (300K) while NPT was performed using the Berendsen pressure coupling scheme. Both equilibrations were done using velocity-rescale temperature coupling by assigning the RNA and water-ions as individual groups. All bond lengths were constrained by employing LINCS algorithms. Final MD simulations of each RNA aptamer strand were performed for 10 ns at equilibrated temperature and pressure. The best possible structure or trajectory frame for each truncated aptamer of MD simulation was chosen using cluster command analysis of the coordinate file by selecting the structure among the time frames (ns) with the largest cluster size and lowest RMSD values.

3.2.5 Rigid molecular docking

Three different docking software were utilized. The aptamers were treated as the ligands for each docking submission, while the NS1 was assigned as the receptor molecules. AutoDock Vina (downloaded at vina.scripps.edu) was done under high-performance computing (HPC) environments with defaults parameters and the grid box with the size of 126, 126, 126 encompassing the entire NS1, centered at -8.7, -29.33, -27.58, corresponding to the x, y, and z dimensions respectively. HADDOCK (available at alcazar.science.uu.nl/services/HADDOCK2.2) was also performed with default settings, using the active and passive residues of the NS1 protein predicted by Cport (available at alcazar.science.uu.nl/services/CPORT) beforehand. PatchDock webserver (available at bioinfo3d.cs.tau.ac.il/PatchDock) was applied using the respective PDB ligand and receptor files with the default values of 4.0 for clustering RMSD and default complex type. Accordingly, the ten best PatchDock results of docked structures were further refined using FireDock options provided on the docking results page.

3.2.6 Molecular dynamic (MD) simulation of RNA-NS1 protein complex

MD simulations were conducted using the GROMACS program (downloaded at gromacs.org), in which Amber99SB-ILDN force field parameters for RNA-protein complex simulations were applied. The complexes were solvated using TIP-3 water models in a cubic box, maintaining a centric position and keeping a distance of 1.0 nm between the complex and the edge of the solvated box. Sodium and chloride ions were added to neutralize the charge of the entire system. Particle Mesh Ewald (PME) method was applied to simulate electrostatic interactions. Energy minimization steps were followed through

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3.3 Results and Discussions

3.3.1 Rationally trimming full-length DENV aptamers to truncated forms

Rational truncation of the full-length DENV aptamers was performed by careful probing of both the sequence and the binding domain of each aptamer. The 2-dimensional (2D) structures of the full-length aptamers were predicted using the high accuracy of Zuker energy minimization algorithms in RNAfold (Mfold) webservers. RNAfold works by predicting different nucleic acid structures and ranking them based on their computed free energy (dG) values. Structures with the lowest energy (higher negative dG values) are preferred as the most accurate and stable among the software-predicted RNA structures.

Based on the predicted secondary structures of the full-length DENV aptamers, truncation of the sequences was carried out by retaining the regions of secondary structures/motifs that have a predilection for binding to the targeted protein while removing the other sequences that are less likely to bind. Secondary structures such as hairpins or stem-loops have been identified as favourite spots of interaction for aptamers with the targeted analytes (Lebars et al., 2008; Zimbres et al., 2013).

The full-length DENV-3 aptamers were truncated by retaining the sequence region from the 8^{th} to the 69^{th} base position while removing the remaining parts (Fig.3.1). This shortened sequence was again re-examined in RNAfold to determine whether they are able to maintain the same structure as seen in their full-length versions. The resultant aptameric construct is chosen as the ideal truncated form. The same concept was also applied to DENV-4 and DENV-6. For DENV-4, the nts from the $12^{th} – 79^{th}$ position were selected as the sequence region to be retained after truncations (Fig.3.2). While, for DENV-6, nts from $2^{th} – 75^{th}$ and $71^{th} – 99^{th}$ positions were chosen to derive two different truncated versions, designated as TDENV-6a and TDENV-6b respectively (Fig.3.3). This led to a total of 4 truncated aptamer candidates; TDENV-3, TDENV-4, TDENV-6a and TDENV-6b (Table.3.1), subjected to further validations.

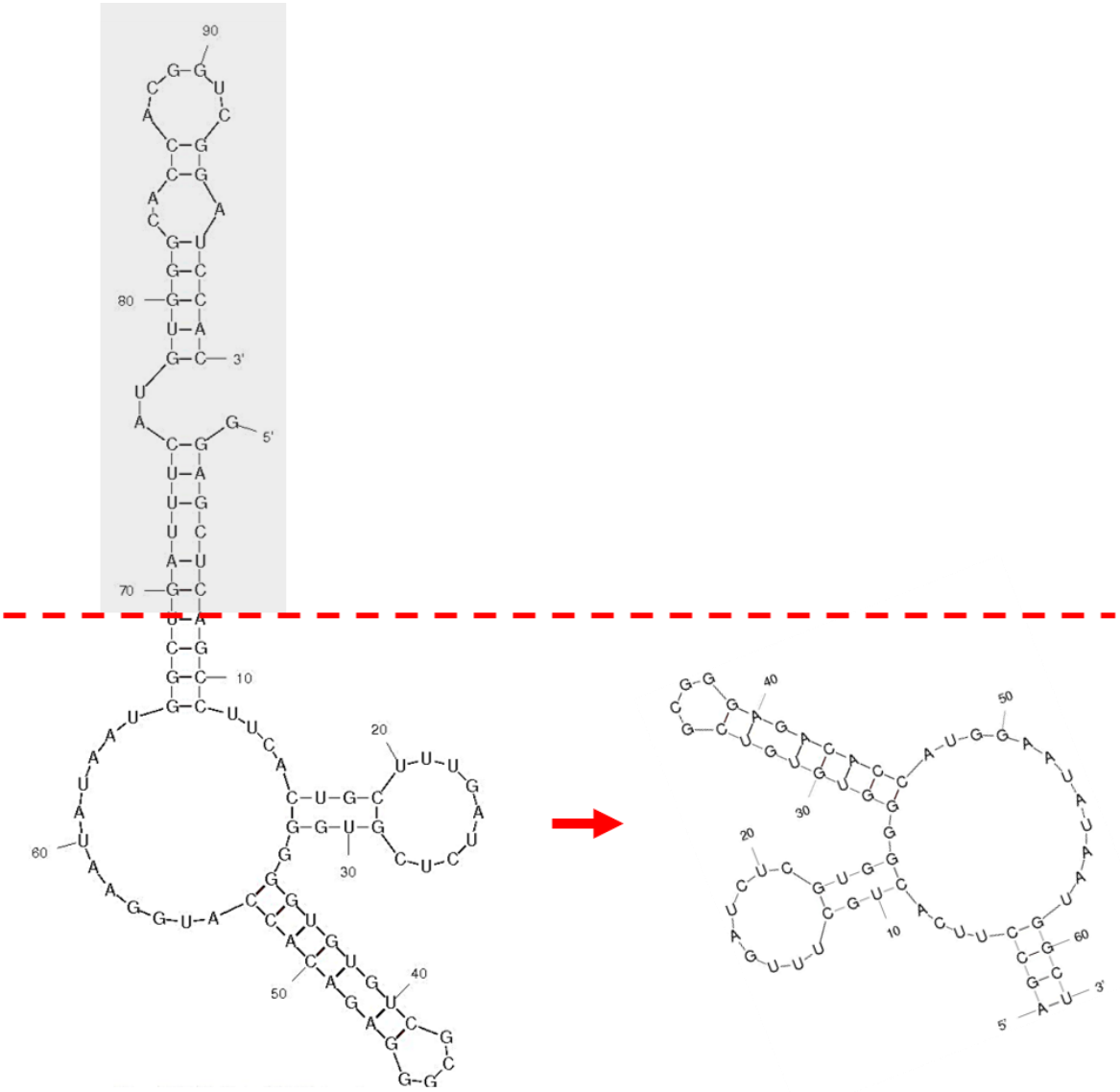

Figure.3.1: Rationale truncation of full-length DENV-3 aptamer to TDENV-3 aptamer. The grey box highlights the region of the aptamer which is excluded during truncations. The red dotted line separates the region of the aptamer strand which is chosen following rationale truncation

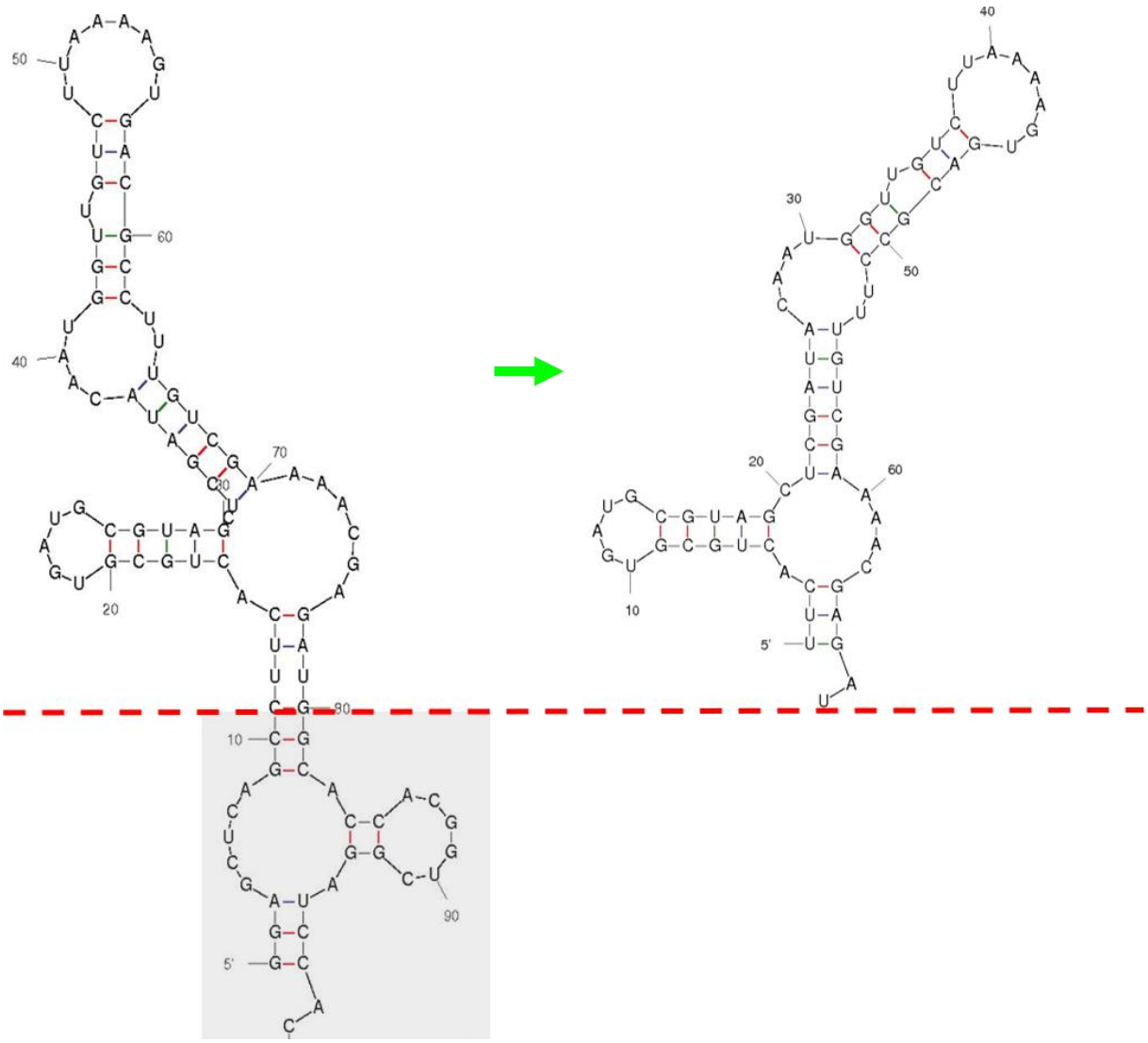

Figure.3.2: Rationale truncation of full-length DENV-4 aptamer to TDENV-4 aptamer. The grey box highlights the region of the aptamer which is excluded during truncations. The red dotted line separates the region of the aptamer strand which is chosen for truncation

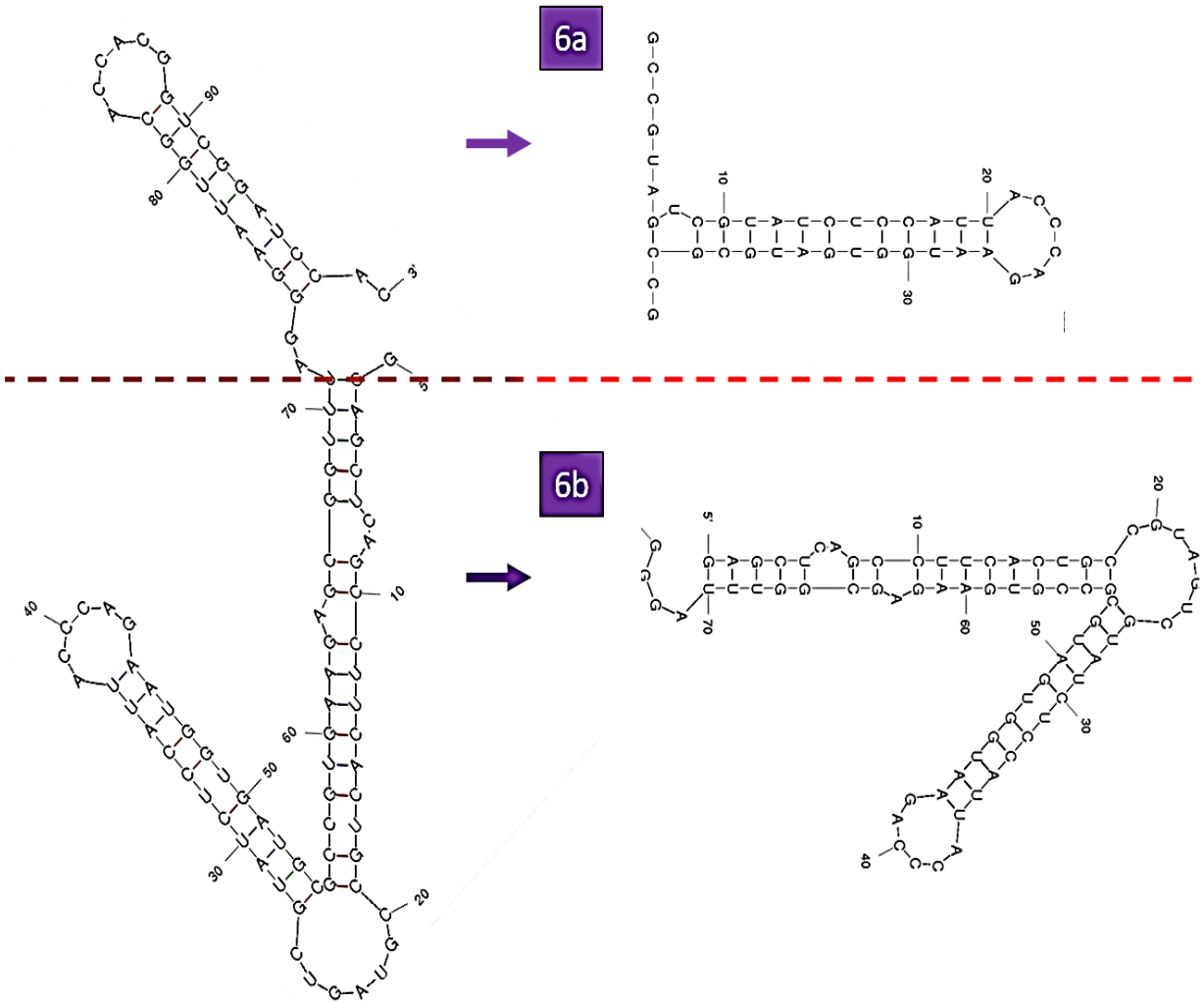

Figure.3.3: Rationale truncation of full-length DENV-6 aptamer to TDENV-6a and TDENV-6b aptamer. The red dotted line separates the region of the DENV-6 aptamer strand which is chosen for truncation. Above the dotted line is the region chosen to derive the TDENV-6a while the region of the sequences below the dotted line is chosen to generate TDENV-6b.

Table.3.1. The list of the truncated and Full-length aptamer sequences

TDENV	**Sequences (5’ – 3’)**
3	GGCCUUCACUGCUUUGAUCUCGUGGGGGUGUGUCGCGGGAGACACCAUGGAAUAUAAUGGCC
4	UUCACUGCGUGAUGCGUAGCUCGAUACAAUGGUUGUCUUAAAAGUGACGCCUUUGUCGAAAACGAGAU
6a	UCUAAUACGACUCACUAUAGGCCGUAGUCGUAUCUCCAUUACCCAGAAUGGUGAUGCGCCG
6b	GAGCUCAGCCUUCACUGCCGUAGUCGUAUCUCCAUUACCCAGAAUGGUGAUGCGCCGUGAAGAGCGGUUU
Full-Length DENV	**Sequences (5’ – 3’)**
3	GGAGCUCAGCCUUCACUGCUUUGAUCUCGUGGGGGUGUGUCGCGGGAGACACCAUGGAAUAUAUGGCUGAUUUCAUGUGGGCACCACGGUCGGAUCCAC
4	GGAGCUCAGCCUUCACUGCUUUGAUCUCGUGGGGGUGUGUCGCGGGAGACACCAUGGAAUAUAUGGCUGAUUUCAUGUGGGCACCACGGUCGGAUCCAC
6	GGAGCUCAGCCUUCACUGCCGUAGUCGUAUCUCCAUUACCCAGAAUGGUGAUGCGCCGUGAAGAGCGGUUUAGGGAAUUGGCACCACGGUCGGAUCCAC

3.3.2 *In-silico* assessments of truncated aptamer candidates

Analyzing the interaction of the truncated aptamers against NS1 *in-silico* may give a picture of their target-binding aptitudes in simulated conditions before conducting experimental tests. To enable this, a 3D model of the DENV-2 NS1 protein target is acquired from PDB. Here, a dimeric form of the NS1 is utilized throughout the *in-silico* assessments since the existence of NS1 proteins as monomers *in-vivo* host or cellular enviroments are less likely to occur where dimerization of NS1 occurs more prominently (Flamand, 1999; Rosales Ramirez & Ludert, 2018; Yap et al., 2017). Moreover, the aptameric interactions *in-silico* were performed without the help of 'feed' components such as ions or ligands to avoid skewing the accuracy of virtually analyzing the aptamer-NS1 interactions.

On the other hand, the 3D structures of the TDENV RNA candidates were modelled based on techniques adopted from Jeddi et al., and Torabi et al., (Jeddi & Saiz, 2017; Torabi et al., 2016). Each sequence was folded using the RNAfold of Mfold webservers. The most thermodynamically favoured (lowest ΔG) configuration for each TDENV sequence was chosen among the predicted secondary structures. RNAComposer was utilized in converting these 2D structures to tertiary structures given as molecular coordinate or PDB files. Subsequently, each modelled 3D structure was subjected to a 10 ns MD simulation to factor in their dynamic molecular natures under solvent effects or solvated conditions. The generated, optimum 3D structures of RNA TDENV sequences and the obtained 3D model of the NS1 were used as the input ligand and receptor models, respectively, for the following docking analysis and MD simulations (Fig.3.4)