Biosensors and Bioelectronics 26 (2010) 1696–1700

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Short communication

Toward specific detection of Dengue virus serotypes using a novel modular biosensor

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ARTICLE INFO

Article history:
Received 14 April 2010
Received in revised form 8 July 2010
Accepted 13 July 2010
Available online 21 July 2010

Keywords:
Nucleic acid biosensor
Enzymatic amplification
Aptamer
Nuclease
Dengue virus

ABSTRACT

Arthropod-borne diseases affect a significant portion of the world's population. Dengue fever, a viral disease carried by the *Aedes aegypti* mosquito, is one of the most wide-spread, with many fatalities evident each year. To date, Dengue viral diagnostic technologies have been too complex, time-consuming and expensive to be widely deployed, particularly in developing countries where the disease is most prevalent.

Here we demonstrate a modular biosensor that is able to rapidly identify sequences associated with the Dengue virus genome. The biosensor consists of an oligonucleotide linker module, an aptamer/restriction endonuclease signal transducer and a fluorescent signalling molecule. The linker molecule has a simple stem/loop conformation and comprises a target-complementary moiety within the loop and a trigger moiety within the stem. When bound to the target nucleic acid, the trigger strand of the denatured stem can bind to the aptamer within the signal transducer. Disruption of the aptamer releases the restriction endonuclease EcoRI from aptamer-mediated inhibition. Active EcoRI is able to rapidly cleave multiple signalling molecules to generate a detectable signal.

The biosensor was able to detect sequences derived from each of the four Dengue virus serotypes with a great degree of specificity. Along with sequences specific to each serotype, a pan-Dengue sequence, common to all serotypes, was also detected.

1. Introduction

Rapid and accurate identification of infectious diseases is essential for public health management. The detection and monitoring of arthropod-borne viral diseases is an area in which a new generation of biosensors could provide considerable advantages over existing diagnostic technology. Dengue fever, which affects a significant and ever-increasing portion of the world's population due to factors such as climate change, is an important example of one such disease (Yang et al., 2008; Beebe et al., 2009).

Symptoms of the disease range from relatively mild to lethal. Manifestation of the fatal form of the illness is likely due to a secondary infection by a virus serotype different to that of the initial infection (Gubler, 1998). Thus, in addition to identifying the presence of the Dengue virus in humans and in the mosquito vector, a biosensor that can accurately identify each virus serotype is desirable.

Currently, enzyme linked immunosorbent assay (ELISA)-based methods and other laborious techniques such as reverse-transcriptase polymerase chain reaction (RT-PCR) are used for Dengue virus detection (Poloni et al., 2010). The associated costs along with the equipment and expertise requirements of these techniques make them difficult to implement. ELISA requires antibodies that are time-consuming to produce and often unstable while RT-PCR is complex, similarly time-consuming and requires the use of an expensive thermocycler.

The biosensor described herein uses a simple discrete stem/loop-forming oligonucleotide for highly specific detection of a nucleic acid (DNA or RNA) target-of-interest. The target-complementary region of this oligonucleotide is the only portion of the biosensor that is tailored to the specific target-of-interest. The signal transducer module is based on an inhibitory-aptamer/restriction endonuclease complex that is functionally independent of the target detection step and may be used without modification for any assay target.

Other reported biosensors which use aptamer/enzyme (usually thrombin) complexes for signal transduction commonly require modification of the aptamer to achieve target specificity (e.g. Yoshida et al., 2006). Such modification by the addition of extraneous sequences can adversely impact the aptamer's binding ability due to unintended changes in the aptamer's structure. One thrombin-based biosensor, reported by Beyer and Simmel (2006), uses a complex multi-component translation system to avoid re-modifying the aptamer for each individual target.

When released from aptamer-mediated inhibition, the highly processive restriction endonuclease allows for rapid signal ampli-

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doi:10.1016/j.bios.2010.07.046

fication. Restriction endonucleases are enzymes that can rapidly and specifically cleave their nucleic acid substrate. The wide range of easily synthesised restriction endonucleases allows for simple customisation of the signal detection module to fit the individual requirements of the biosensor. It is anticipated that the robust nature of many restriction endonucleases will also aid the stability of the biosensor in field environments.

Using this biosensor, we demonstrate the highly specific, reproducible detection and identification of unique sequences derived from each Dengue virus serotype as well as a 'pan-Dengue' sequence common to all four Dengue virus serotypes. The low-cost and flexibility inherent in this technology could be an important step toward effective diagnostic technology for managing the occurrence and impact of Dengue fever and other infectious diseases.

2. Materials and methods

2.1. Materials

Desalted linker, target and non-target control oligonucleotides and the HPLC-purified molecular break-light (MBL) oligonucleotide were synthesised by Sigma–Aldrich. The PAGE-purified aptamer oligonucleotide was supplied by Operon Biotechnologies. The restriction endonuclease EcoRI and supplied bovine serum albumin (BSA) were purchased from New England BioLabs. 100 μl PCR tubes and maximum recovery 1.5 ml tubes were supplied by Axygen Scientific. All other chemicals were commercial- or laboratory-grade. Reactions were run and analysed on a Rotorgene RG-3000 real-time PCR machine (Corbett Research). The genomic DNA alignment was prepared using AlignX software supplied by Invitrogen. Secondary

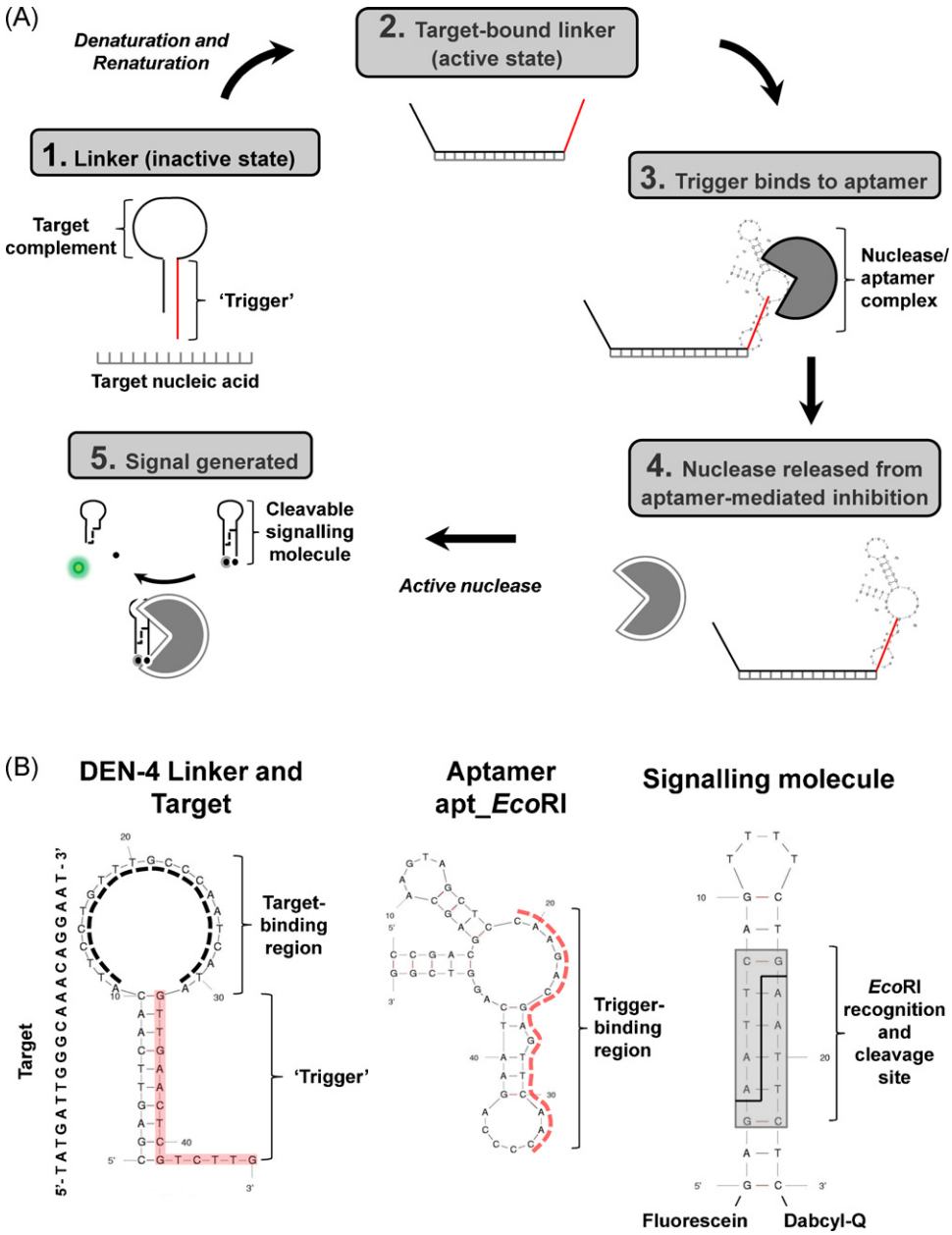

Fig. 1. Schematic diagram of the modular biosensor and the structure of its components. (A) Initially, the linker forms a stable stem/loop structure (1). During chemical denaturation and renaturation, the linker binds to its nucleic acid target (2). The linker's single-stranded trigger then binds to (3) and releases an EcoRI molecule from aptamer-mediated inhibition (4). The active nuclease rapidly cleaves multiple signalling molecules containing EcoRI's recognition sequence (5). (B) The predicted secondary structures of the DEN-4 linker, apt_EcoRI and the MBL signalling molecule.

structures of the linkers, aptamer and signalling molecule were predicted using the MFOLD software (Zuker, 2003). The aptamer apt_EcoRI sequence was identified using a SELEX-based procedure (Tuerk and Gold, 1990; Ellington and Szostak, 1990). The sequences of the oligonucleotides used in this study are shown in Table S1 (Supplementary data).

2.2. Trigger-induced biosensor assay

A master-mix was prepared containing EcoRI, apt_EcoRI, BSA and buffer, then aliquoted into 100 μl PCR tubes. In each 20 μl reaction were: EcoRI (3 units); apt_EcoRI (175 nM); BSA (20 μg) and buffer (75 mM NaCl; 10 mM $MgCl_2$; 150 mM Tris–HCl; 0.0375% Triton X-100; pH 7.5). Apt_EcoRI and EcoRI were bound in the master-mix (BSA and dH_2O) for 45 min at 37 °C prior to the addition of the remaining buffer reagents. 1 μl of the trigger oligonucleotide solution (stock: 8.3 μM; final reaction concentration: 416 nM) and MBL (stock: 1.34 μM; final reaction concentration: 66.7 nM) were added and the reactions immediately analysed on a Rotorgene operating at a constant temperature of 30 °C. Readings on the instrument's FAM channel were acquired every 30 s. Control reactions without apt_EcoRI (positive control) and a non-trigger oligonucleotide substituted for the trigger (negative control) were performed simultaneously. Four replicates of all treatments were carried out.

Mean fluorescence readings from the four replicates of each treatment were graphed using Microsoft excel, with error bars at each fluorescence-acquiring time-point representing ±1 standard deviation from the mean.

2.3. Dengue serotype target-induced biosensor assay

DEN-1, DEN-2, DEN-3 and DEN-4 Dengue virus serotype genomic sequences were obtained via GenBank (Benson et al., 2005). An alignment of the full genomic sequences was carried out using the AlignX software. Serotype-specific linkers were designed to hybridise to each serotype at regions of limited sequence identity and the pan-Dengue linker was designed to hybridise to a region of complete identity. Target oligonucleotides and control oligonucleotides representing the aligned regions of the non-target serotypes were commercially synthesised. A non-Dengue virus oligonucleotide was also used to generate a base-line background signal and ensure that an equal amount of DNA was present in all reactions.

Similarly to the trigger-induced assay, a master-mix was prepared containing EcoRI, apt_EcoRI, BSA and buffer, then aliquoted into 100 μl PCR tubes. In each 20 μl reaction were: EcoRI (3 units); apt_EcoRI (175 nM); BSA (20 μg) and buffer (75 mM NaCl; 10 mM $MgCl_2$; 150 mM Tris–HCl; 0.0375% Triton X-100; pH 7.5). Apt_EcoRI and EcoRI were bound in the master-mix (BSA and dH_2O) for 45 min at 37 °C prior to the addition of the remaining buffer reagents.

Linker/target and linker/control denaturation and renaturation was carried out by adding 1.1 μl of linker (100 μM) and 1.1 μl of target (100 μM) or control (100 μM) to 9 μl of NaOH (150 mM) for denaturation followed by neutralisation through the addition of 6 μl HCl (200 mM) and 1.1 μl Tris–HCl (1 M; pH8.0).

1 μl of the renatured linker/target or linker/control solution and 1 μl of the MBL (stock: 1.34 μM; final reaction concentration: 66.7 nM) were added and the reaction immediately analysed on a Rotorgene operating at a constant temperature of 30 °C. Fluorescence readings on the instrument's FAM channel were acquired every 30 s. In total, 6 pmol of the target or control oligonucleotide were added to the reaction to give a final concentration of 300 nM. Control reactions with targets derived from the two most similar aligned serotype sequences and the non-Dengue virus sequence were performed simultaneously. Four replicate reactions for all treatments were carried out.

For observational simplicity, relative fluorescence was graphed by subtracting the mean of the baseline signal (non-Dengue target control) from the mean of each Dengue serotype treatment. The standard deviation (SD) of the relative fluorescence of each treatment at each acquiring time point was calculated as the square root of $(SD_{treatment})^2 + (SD_{baseline})^2$. Error bars at each fluorescence acquiring time point represent ±1 standard deviation from the mean.

3. Results and discussion

3.1. The modular biosensor

In order to identify the nucleic acid targets-of-interest derived from the Dengue virus genome, a novel biosensor was developed. An outline of the biosensor and its components is shown in Fig. 1. The biosensor uses discrete modules for target nucleic acid detection and signal transduction. A tailored stem/loop linker oligonucleotide is employed for detection of the target-of-interest and a restriction endonuclease/inhibitory-aptamer complex forms the signal transducer. In the presence of the target nucleic acid, the linker can interact with this complex and liberate the restriction endonuclease EcoRI from aptamer-mediated inhibition. The active EcoRI molecule is then able to rapidly cleave multiple signalling molecules. Signal amplification is generated via the highly processive nature of EcoRI. The nature of a modular system allows for simple adaptation of the linker to the target-of-interest without modifying the signal transduction module components.

The target-detection module comprises an oligonucleotide linker with a stable stem/loop secondary structure. The loop portion is complementary to the target-of-interest. One strand of the stem duplex, the trigger, is complementary to a portion of the aptamer within the transducer module. The linker is designed such that the melting temperature of the target-complementary portion is higher than that of the stem portion. Consequently, it is thermodynamically favourable for the linker to hybridise to the target in preference to reverting to its native stem/loop structure during

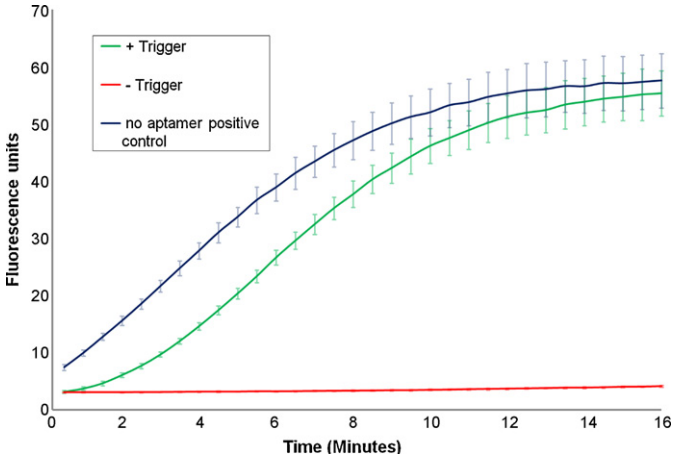

Fig. 2. The addition of the trigger oligonucleotide releases EcoRI from aptamer-mediated inhibition. Fluorescence outputs for each treatment replicate were acquired at 30 s intervals, with the mean for each treatment presented along with error bars representing ± one standard deviation from the mean. The presence of the trigger in solution was clearly discernable after 2 min of reaction time. The maximum rate of MBL cleavage as signified by the slope of each curve is similar for the 'no aptamer' positive control and the 'plus trigger' treatment, indicating that all EcoRI molecules were rapidly released from inhibition. Digestion of all the MBL molecules in solution was complete within 16 min. The background signal generated in the absence of trigger was exceedingly low, indicating strong inhibition of EcoRI's activity by the aptamer apt_EcoRI.

denaturation and renaturation. Following target/linker binding, the trigger remains single-stranded. The correct relative melting temperature of the stem was achieved by modifying the length of the strand complementary to the trigger. In addition, the target detection module can also be based on a simple nucleic acid sandwich assay, similarly to previously described sandwich-based biosensors (e.g. Baeumner et al., 2002).

3.2. The signal transduction module

The signal transduction module comprises the restriction endonuclease EcoRI in complex with an aptamer capable of inhibiting its activity. An ideal aptamer for this module should not only strongly and specifically inhibit EcoRI, but have a structure that is amenable to disruption via the hybridisation of the trigger present within the linker molecule. Such an aptamer was selected via the SELEX procedure (Tuerk and Gold, 1990; Ellington and Szostak, 1990) for use in the biosensor and synthesised with extraneous terminal portions removed (designated apt_EcoRI). The trigger moiety of the target detection module was designed to bind to a portion of apt_EcoRI. Accordingly, addition of the trigger oligonucleotide to a solution containing apt_EcoRI in complex with EcoRI resulted in the rapid release of EcoRI from inhibition (Fig. 2).

3.3. The signal detection module

To detect EcoRI activity, a modified version of a molecular break-light (MBL) (Biggins et al., 2000) was used. The MBL is a stem/loop-forming oligonucleotide with a 5′ fluorophore (e.g. Fluorescein) and 3′ quencher (e.g. Dabcyl). Within the stem duplex is an EcoRI recognition sequence. Upon cleavage by EcoRI, the dissociation of fluorophore and quencher results in a detectable signal. The intensity of the fluorescent signal produced over time is proportional to the quantity of MBLs cleaved and, therefore, the number of active EcoRI molecules in solution under a given set of reaction conditions.

3.4. The biosensor reactions

The biosensor assay is carried out as two sequential reactions. Initially, a reaction containing the linker and nucleic acid sample to be analysed is subjected to denaturing and renaturing conditions. Denaturation and renaturation can be temperature- or pH-mediated. This solution is then added to a buffered solution containing the appropriate concentrations of the aptamer/EcoRI complex and MBL, and the level of fluorescence determined at specified time intervals.

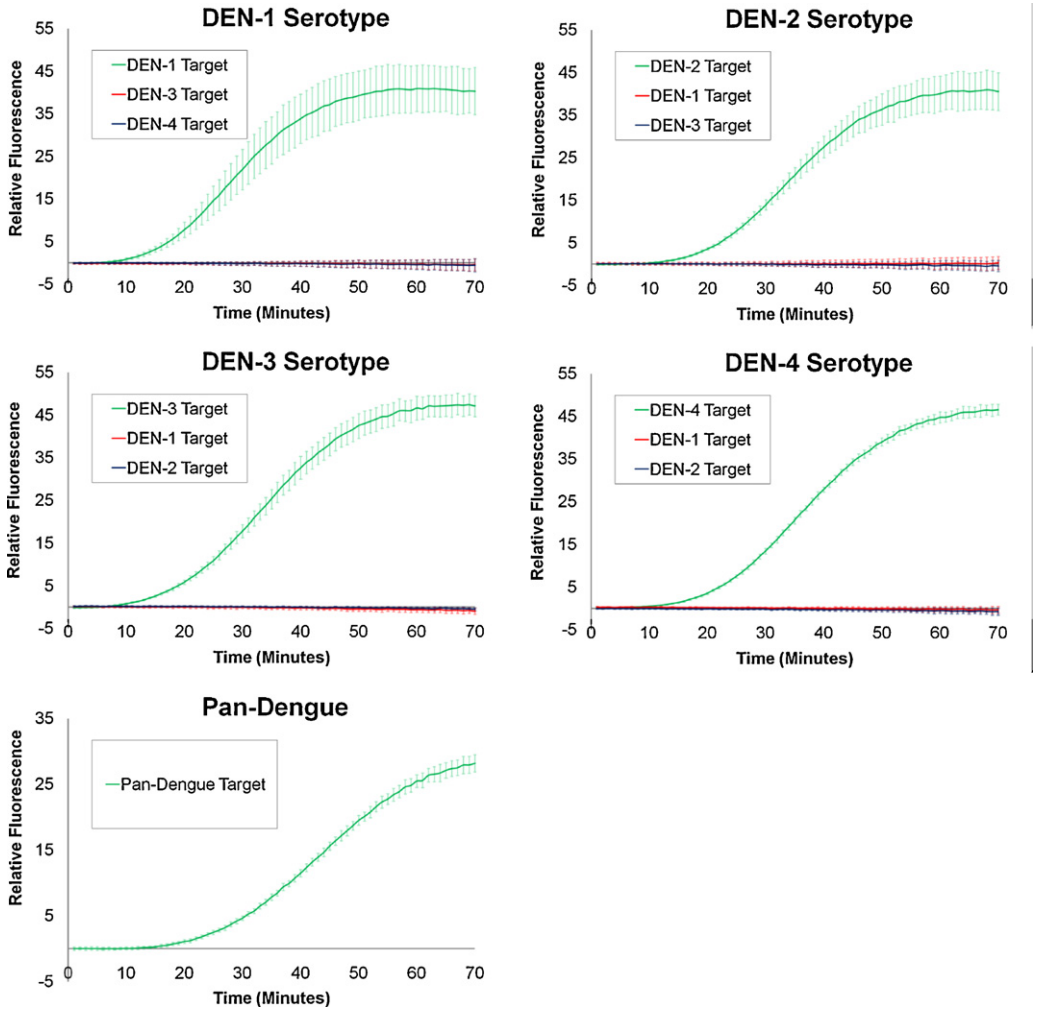

Fig. 3. Specific detection of sequences derived from each Dengue virus serotype and a pan-Dengue sequence common to all serotypes. Each curve represents the mean fluorescence of the target and control treatments relative to the non-Dengue baseline signal. Relative error bars representing ± one standard deviation are shown. Relative fluorescence levels above zero indicate the presence of the target nucleic acid. The presence of the target nucleic acid was clearly discernable for all targets following 20 min reaction time. The signals produced by the non-target-serotype controls were indiscernible from those of the non-Dengue baseline, indicating no cross-hybridisation of the detectors to non-target sequences.

3.5. Specific detection of Dengue virus serotypes

Using this novel biosensor, we sought to determine whether sequences derived from each of the four Dengue serotypes could be rapidly and specifically detected. Initially, an alignment of the DEN-1, DEN-2, DEN-3 and DEN-4 serotype genome sequences was carried out. To identify a specific serotype, heterologous regions of the alignment were identified and linkers were designed to hybridise to the optimal target sequences within those regions. The relative fluorescence data generated for each linker are displayed in Fig. 3. Sequences originating from the serotypes most similar to the target in the aligned regions were used as non-target controls. To test linker specificity, synthetic oligonucleotides were used to represent target and control sequences. An additional pan-Dengue linker was designed to specifically hybridise to a homologous portion of the Dengue alignment and thus detect all Dengue virus serotypes. A non-Dengue virus sequence was used to generate a baseline signal and ensure equal quantities of DNA were present in all reactions.

All reactions were carried out isothermally, with adjustments in pH used to denature and renature the linker/target and linker/control reactions. The fluorescence level of each reaction was acquired at 30 s intervals. For observational simplicity, the mean of each treatment relative to the baseline for each time point is presented (Fig. 3; Section 2).

Our analysis demonstrated that each linker bound to its nucleic acid target in a highly specific manner and rapidly induced a fluorescent signal. The signal generated by the presence of the target nucleic acid was clearly distinguishable from those produced by the non-target control reactions after a period of 20 min. For the serotype-specific linkers, the signals generated by the non-target-serotype controls were indiscernible from the non-Dengue baseline signal, indicating there was no cross-hybridisation between the linkers and non-target-serotype sequences. Similarly, the pan-Dengue linker designed to hybridise to all four Dengue virus serotypes was able to rapidly differentiate between the target sequence and the non-Dengue virus baseline.

4. Conclusions

Using a novel biosensor comprising an oligonucleotide detector module, an aptamer/nuclease signal transducer and a fluorescent signalling molecule, we were able to specifically detect sequences associated with each of the four Dengue virus serotypes, as well as a pan-Dengue sequence. Hybridisation of the detector oligonucleotide to the target Dengue sequence allowed for the release of an EcoRI molecule from aptamer-mediated inhibition, with the active EcoRI rapidly cleaving multiple signalling molecules and generating an easily detectable signal.

In addition to the biosensor's specificity, the robust and low-cost reagents that make up each module indicate that the biosensor may play an important role toward managing Dengue and other infectious diseases.

Acknowledgements

We would like to thank Dr. Joanne Attema for her assistance with the preparation and review of this manuscript. This work was supported with funding provided by: The Australian Government through the Commercial Ready Plus and Climate Ready programs, as well as The South Australian State Government through a Business Development Initiative Grant from Bio Innovation SA.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.07.046.

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