Construction of Self-replicating RNA for Vaccine-based Cancer Immunotherapy

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Abstract

Cancer immunotherapy is a new generation cancer treatment that employs therapeutic agents such as antibodies, cells, peptides, and nucleic acids to activate immune cells to target cancer cells and eliminate them. Our interest has focused on the polyneoepitope-encoding RNA vaccine. Neoepitopes are mutated peptides restricted to only cancer cells, and are bound to major histocompatibility complex (MHC) molecules on the cell surface to be recognized by T-cells. Polyneoepitopes have been shown to be good targets against cancer in mouse models and human trials, being clinically feasible, safe, and able to induce anti-tumor activity and immunity. The drawback to this vaccine is the instability of the RNA molecule; therefore, patients have to receive multiple injections over the duration of their treatment. In this study, we present a solution by exploiting the self-replicating ability of the RNA genome of dengue virus (DENV) to allow RNA to maintain its population in host cells. To provide a proof-of-concept study, we designed and constructed a new RNA vaccine containing the untranslated regions and non-structural protein (NS) genes from DENV, which are linked to a polyneoepitopes from MC38, a murine colon adenocarcinoma. We have almost completed the cloning processes using the In-Fusion cloning technique. Future work includes synthesis of replicon RNA *in vitro* and its transfection into murine macrophages to confirm self-replication and neoepitope presentation via Western blot and mass spectrometry. Feasibility of this study allows for the replicon RNA to be further examined in mouse models to potentiate a better alternative for cancer immunotherapy in the future.

Keywords**:** *Neoepitope, Cancer, RNA vaccine, Self-replicating RNA, Replicon RNA, Dengue virus*

1. Introduction

More than 170,000 new cancer cases were recorded in Thailand by the World Health Organization in the year 2018, and nearly 115,000 died of the disease (World Health Organization, 2019). While the most standard cancer treatments such as chemotherapy, radiation, and surgery are available, they do not discriminate between cancerous or healthy cells, thus damaging healthy cells as well (Li, Goedegebuure & Gillanders, 2017). Not only do cancer patients receiving these treatments face negative side effects, including compromised immune system, nausea, and fatigue, it should also be taken into account that individual patients may react differently to the same treatment (Fitch, McAndrew & Harth, 2015; Hu, Ott & Wu, 2018). To combat these issues, new-generation treatments that not only target precisely the cancer cells, but are also tailored to each individual patient, are emerging.

Such an example is cancer immunotherapy, which has been shown to be safe with potent results. This approach employs antibodies, peptides, cells, and nucleic acids as therapeutic agents that are administered to the patient through vaccination and function by boosting the patient's immune system to fight cancer (Zugazagoitia et al, 2016). While cells, antibodies, and peptides may be effective in inducing specific immune response, they are prone to degradation by the host's innate immune response, and some patients have developed resistance to the agents (Yu & Cui, 2018). DNA vaccines are also effective in combatting cancer, but concerns have been raised surrounding its safety, specifically its integration into the genome of host cells (Lundstrom, 2018a). An alternative to this is RNA vaccines - since RNA is extranuclear, this eliminates the possibility of chromosomal integration (Lundstrom, 2018a). It has also been shown to be safer and give formidable results in terms of immunogenicity, entry into cells, prolonged effects and lowered production cost, when compared to their cell, DNA, and protein counterparts (Pastor et al, 2018). The principle of an RNA vaccine involves introduction of tumour antigen-encoding RNA into antigen-presenting cells, such as dendritic cells or macrophages, so that the tumour antigens can be expressed and presented on the cell surface as antigens, or epitopes, by major histocompatibility complex (MHC) molecules, to be

targeted by T cells, thus eliciting antigen-specific immune responses (Pastor et al, 2018). This can give rise to long-term immunity against the specific tumour antigens by T memory cells (Pastor et al, 2018).

RNA vaccines can be divided into two types: mRNA vaccines and self-amplifying RNA replicons. The structure of mRNA vaccines typically consists of a 5′ methyl-guanosine cap, 5′- and 3′-untranslated regions (UTRs), an open reading frame encoding the tumour antigens, and a 3′-terminal tail of 100 to 250 adenosine residues (Geall, Mandl & Ulmer, 2013). In contrast, RNA replicons are engineered from positive-stranded RNA viruses, such as alphaviruses and flaviviruses, with majority of the viral structural genes being replaced with sequences of the tumour antigens (Brito et al, 2015; Lundstrom, 2016; Pepini et al, 2017). Self-replication of the RNA replicon is dependent on the RNA replicase or the RNA-dependent RNA polymerase enzyme that is part of the viral non-structural proteins (NSs) (Lundstrom, 2016, 2018b). Additionally, a sub-genomic promoter can be inserted upstream of the sequence of interest to allow strong expression of tumor antigens, independent of viral mechanism (Kümmerer, 2018). While mRNA vaccines are less stable, RNA replicons are more prone to degradation by host innate immunity (Kato & Hishiki, 2016; Ng, Soto-acosta, Bradrick, Garcia-blanco & Ooi, 2017; Pastor et al, 2018). However, some viral RNA replicons are still able to escape detection and degradation in host cells without ability of infection to other cells due to exclusion of the viral coat (Kato & Hishiki, 2016; Ng et al, 2017; Pastor et al, 2018).

For an RNA vaccine to efficiently target cancers, the encoded tumor epitopes should be highly expressed and well-presented on the cell surface by MHC molecules and elicit a strong, specific immune response by T cells (Kreiter et al, 2015). Accordingly, the exploitation of neoepitopes have been found to be useful in developing RNA vaccines. Neoepitopes are tumor-specific epitopes that are derived from somatic changes in the genome, and while these mutations cannot be predicted, recent advancement in bioinformatics has led to the development of algorithms that enable prediction of epitope binding to MHC molecules (Li et al, 2017). Coupled with current high-throughput RNA sequencing and mass spectrometry, these immunogenic neoepitopes can be determined and engineered into RNA vaccines as a target for elimination by the immune cells (Efremova et al, 2018; Kreiter et al, 2015; Yadav et al, 2014).

While numerous studies have confirmed the potential of mRNA vaccines, there has, however, been limited studies on utilizing RNA replicons to treat cancer (Lundstrom, 2018b). In this study, we propose to construct a novel polyneoepitope-encoding RNA vaccine with the ability of self-amplification in host cells by exploitation of dengue virus (DENV) components. DENV is a flavivirus possessing ~10.7k nt RNA genome in which its replication depends on the viral 5′- and 3′-UTRs and NS5 RNA polymerase. The neoantigens used in this study were selected from MC38, a murine colon adenocarcinoma, which was based on extensive studies by Yadav *et al*. and Efremova *et al*. (Efremova et al, 2018; Yadav et al, 2014). This replicon vaccine, once validated to be feasible *in vitro*, has potential for further examination and application in mouse models.

2. Objectives

To design and construct a novel polyneoepitope-encoding RNA vaccine utilizing the DENV replication system.

3. Materials and Methods

3.1 Cell line

Murine RAW Lucia 264.7 cells will be used for transfection of polyneoepitope-encoding RNA replicon to assess self-replication of RNA as well as neoepitope presentation. Cells were cultured in DMEM with 2 mM L-glutamine and 10 % FBS supplemented with 100 µg/mL Normocin and 200 µg/mL of Zeocin, at 37°C and 5 % CO_2.

3.2 Reverse transcription and PCR

Total RNA was extracted from DENV serotype 3 and reverse-transcribed to cDNA, to be further used as template for amplification of viral components: 5′ and 3′-UTRs, first 24 codons of capsid protein (C24), last 30 codons of envelope protein (E30), and all non-structural proteins (NS1, NS2, NS3, NS4, and

NS5). A *SnaBI* restriction site was added immediately downstream of the 3´ UTR for linearization of the final replicon plasmid. The sequences of the internal ribosome entry site (IRES) from encephalomyocarditis virus (ECMV), secretion signal (Sec) and MHC class I trafficking domain (MITD) were obtained from NCBI (Kreiter et al, 2008; Kümmerer, 2018) (**Table 1**). These fragments and the MC38 polyneoepitopes were synthesized by GENEWIZ (China), and all primers (Table 2) used in this study were designed using In-fusion Cloning Primer Design Tool (TAKARABIO, Japan) and synthesized by INTEGRATED DNA TECHNOLOGIES (Singapore).

Table 1 DNA fragments used in this study.

Elements	Sequences
IRES	CCCCTCTCCCTCCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGGCCGGTGT GCGTTTGTCTATATGTTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCCCGGA AACCTGGCCCTGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTCGCCAAAGGAAT GCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAAC AACGTCTGTAGCGACCCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTG CGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGCCACG TTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGG GGCTGAAGGATGCCCAGAAGGTACCCCATTGTATGGGATCTGATCTGGGGCCTCGGTGCA CATGCTTTACATGTGTTTAGTCGAGGTTAAAAAAACGTCTAGGCCCCCCGAACCACGGGG ACGTGGTTTTCCTTTGAAAAACACGATGATAAGGATCCTCTAGA
Sec	ATGCTGGTCATGGCGCCCCGAACCGTCCTCCTGCTGCTCTCGGCGGCCCTGGCCCTGACCG AGACCTGGGCCGGCTCC
MITD	ATCGTGGGCATTGTTGCTGGCCTGGCTGTCCTAGCAGTTGTGGTCATCGGAGCTGTGGTCG CTGCTGTGATGTGTAGGAGGAAGAGTTCAGGTGGAAAAGGAGGGAGCTACTCTCAGGCT GCGTGCAGCGACAGTGCCCAGGGCTCTGATGTGTCTCTCACAGCTTGA
Polyneoep -itope	GCTAGCGGGGGAGGCGGGTCTGGCGGCGGAGGCTCTTCACTGGTAATTAGTGCTTCCATA ATAGTGTTTAACCTCCTCGAACTGGAAGGTGATTATCGCGATGATCACATCTTTTCAGGGG GAGGCGGGTCTGGCGGCGGAGGCTCTAACGGGCGGGTCCTTGAATTGTTTAGGGCAGCCC AACTCGCAAATGATGTTGTTCTTCAAATAATGGAGCTGTGTGGGGCCGGGGGAGGCGGGT CTGGCGGCGGAGGCTCTAGGCCTGGCATCCCTGTTCACCTGGAACTCGCAAGCATGACCA ACATGGAATTGATGAGCTCTATAGTACACCAACAAGTATTCCCCGGGGGAGGCGGGTCTG GCGGCGGAGGCTCTACTCCCACATCTGATACCAGCAGCGAATCATCCGCCACTAAAGAGG AAGACGATGAAAAAACCAGGATGCACAGTACAGCAGGGGGAGGCGGGTCTGGCGGCGG AGGCTCTACCGATATGACAAAGACCGTTAAGTCTTTCGTGGGCCCCCCCCCTGCCCCCGCC CCTGCTCCAAGGACTCTCCCAGCCGCCGGGGGAGGCGGGTCTGGCGGCGGAGGCTCTACT AGT

Table 2 Primers used in this study.

Amplified Fragments	Primer Sequences (5′-3′)
DENV 5′-UTR – C24	Forward: gactcactatagggcgaattagttgttagtctacgtggaccg Reverse: gggagaggggttaattaagcggccgctgacacacggtttctcacgc
DENV 3′-UTR	Forward: atcctctagaaagcaggaggcaaactgtca Reverse: cactatagaatactcaagcttacgtagaacctgttgattcaaca
IRES	Forward: gcttaattaacccctctccctcccccc Reverse: cctcctgctttctagaggatccttatcatcgtg
Sec	Forward: gagctcggtacccggggatcatgctggtcatggcgccc Reverse: ccccgctagcggagccggcccaggtctcg
MITD	Forward: ctctactagtatcgtgggcattgttgctgg Reverse: tgcctgcaggtcgactctagtcaagctgtgagagacacatcag
Polyneoepitope	Forward: ggccggctccgctagcgggggaggcggg Reverse: tgcccacgatactagtagagcctccgccgc
Sec, MITD, polyneoepitope	Forward: acgatgataaggatcatgctggtcatggcgccc Reverse: cctcctgctttctagtcaagctgtgagagacacatcagag
E30-NS5	Forward: gaaaccgtgtgtcagcggcctagggttgaattcaaaaaacacttc Reverse: ggggggagggagagggggttattaccaaatggctccctctgac

3.3 Molecular cloning

The pGEM3Z vector (PROMEGA) was used for cloning the RNA vaccine in this study. Amplified DNA fragments either were gel- or column-purified and cloned into their respective vectors using In-Fusion cloning kit (TAKARABIO, Japan) or via enzymatic ligation. 10 µl of the In-Fusion cloning reaction or 5 µl of ligation reaction was transform into 100 µl of competent *E. coli* cells and correct clones were screened by colony PCR, and subsequently grown in LB broth overnight. The vectors were extracted and verified by restriction digestion and Sanger DNA sequencing.

To generate the replicon construct, initially, DENV 5′- and 3′-UTRs and an internal ribosome entry site (IRES) were inserted into pGEM3Z. The resulting plasmid, named p4, was linearized prior to the insertion of Sec, MITD, and MC38 polyneoepitopes with glycine-serine linkers into between IRES and DENV 3′-UTR to yield the plasmid p5m.

4. Results and Discussion

We have designed a novel RNA vaccine as illustrated below in **Figure 1**. Most of the DENV structural genes including capsid (C), membrane (prM) and envelope (E) were omitted to remove viral infectivity. The first 24 codons of the capsid protein (C24) and the last 30 codons of the envelope protein (E30) have been left with the viral NS genes since they contain cis-acting elements necessary for efficient RNA replication and proper NS1 configuration, respectively. An IRES from ECMV have been introduced for strong expression of polyneoepitopes, independent from viral polyprotein expression, downstream of the stop codon of the viral open reading frame (ORF) (Kümmerer, 2018). The polyneoepitopes, with glycine-serine linkers flanking and in between every neoepitope, were flanked by the secretion signal (Sec) and the MHC I trafficking domain (MITD) to allow polyneoepitopes to reach the cell membrane via trafficking of the polyneoepitope into the MHC I antigen-processing pathway (Kreiter et al, 2008). The entire construct was under the control of the viral 5′- and 3′-UTRs that allowed cyclization of the whole replicon, essential for RNA replication in host cells.

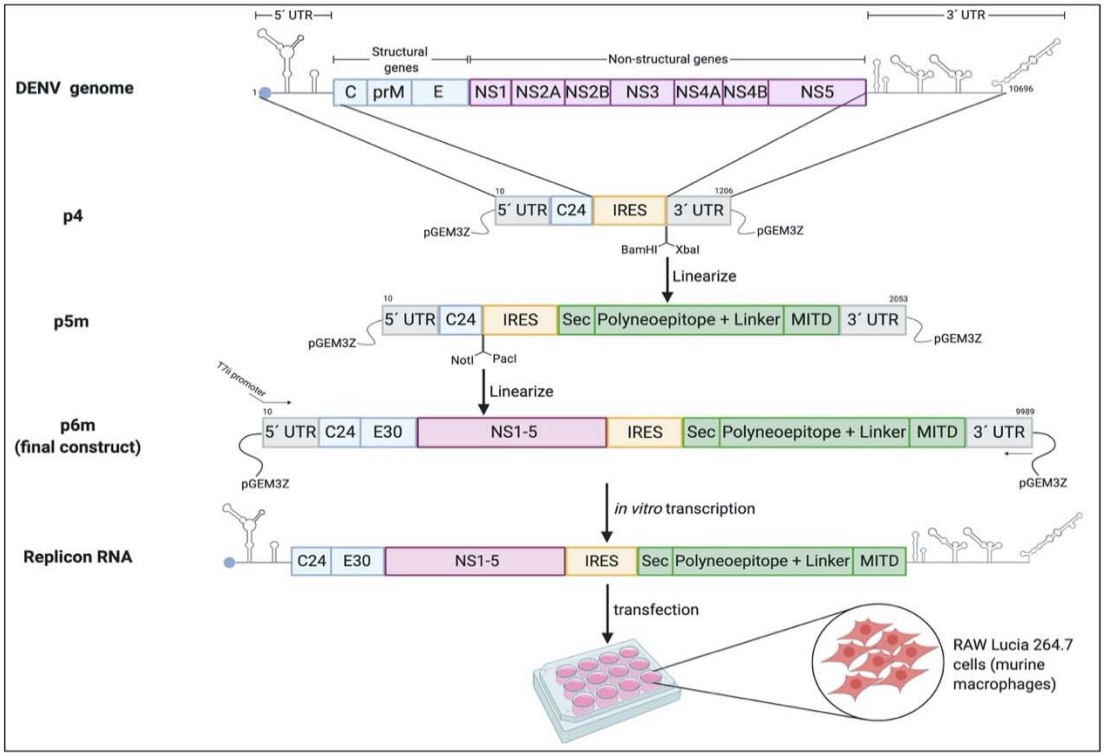

Figure 1 Schematic diagram of the construct of self-replicating RNA vaccine in this study

The first step was to generate the plasmid p4 (**Figure 1**). The fragments of 5′-UTR-C24, IRES, and 3′-UTR were amplified by PCR, gel purified and cloned into pGEM3Z linearized by *Eco*RI and *Hind*III). The overall processes of p4 cloning via the In-Fusion technique is illustrated in **Figure 2**. Briefly, each amplified fragment carried 20 bp of sequences homologous to the corresponding neighbouring fragments. The In-Fusion enzyme mix generated single-stranded 5′ overhang at the termini of both inserts and vector. These overhangs annealed at their complementary sites and the reaction was directly transformed into *E. coli* to complete the ligation of the vector. Positive colonies carrying p4 plasmids were screened by colony PCR of the insert (1242 bp) (**Figure 3).**

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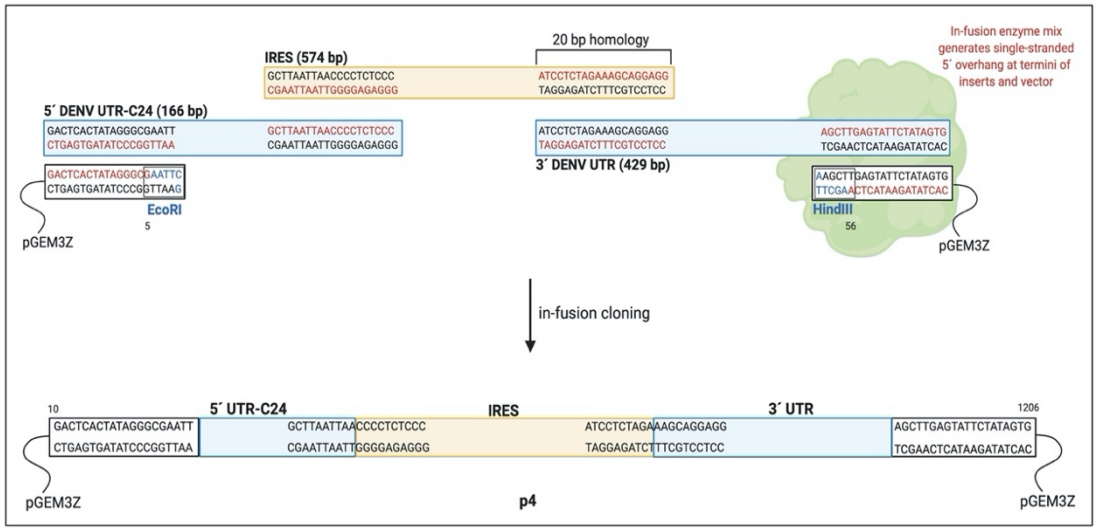

Figure 2 Cloning of the p4 construct using the In-Fusion technique

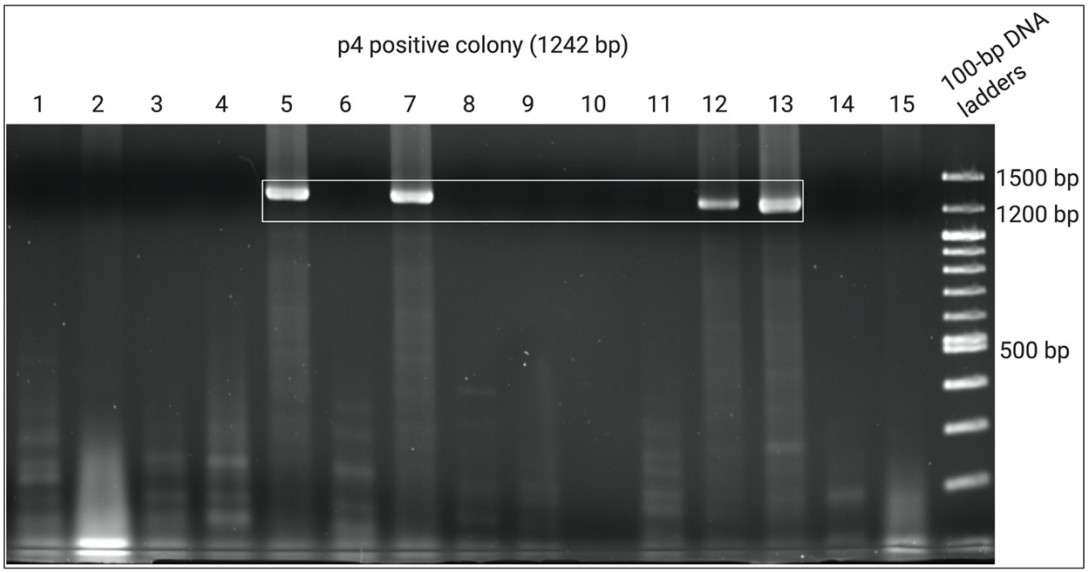

Figure 3 Colony PCR of p4 inserts (1242 bp) (indicated by white box)

DNA sequencing of the resulting p4 plasmid showed that there was a single nucleotide replacement from G to U in the 5´ variable region of the DENV 3´-UTR, which has lower conservation among 4 serotypes of DENV (**Figure 4**). We, therefore, concluded that this mutation might derive from the DENV strain used for cloning in this study, and not affect viral RNA replication mechanism.

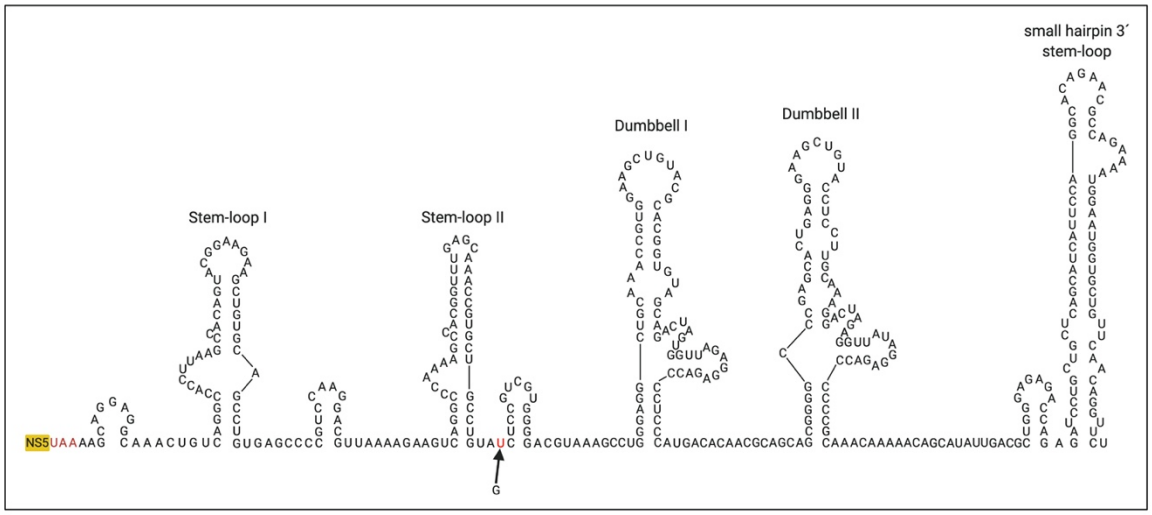

Figure 4 Secondary structures of DENV 3´-UTR and a G-to-U mutation (black arrow) found in the sequence of p4

Next, the three fragments of Sec, polyneoepitopes, and MITD were cloned into pGEM3Z, and their sequence validated (not shown). Then they were amplified as a single fragment (878 bp) by PCR (**Figure 5**), gel-purified, and inserted into the linearized p4 vector, downstream of IRES, via *BamH*I and *Xba*I sites. The clones were selected via colony-PCR and DNA sequencing. This process yielded the p5m vector.

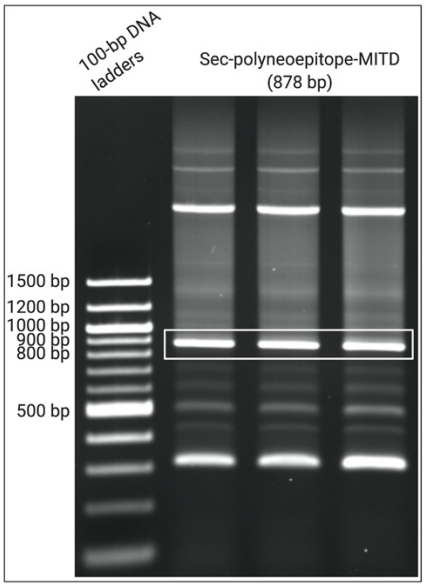

Figure 5 Agarose gel image of PCR-amplified DNA fragment of Sec-polyneoepitope-MITD

The final step of constructing the vector of self-replicating RNA vaccine is an insertion of the fragment of DENV NS genes into the linearized p5m vector between *Not*I and *Pac*I sites (**Figure 1**). The long fragment of E30-NS5 was successfully amplified from the DENV-3 genome (**Figure 6**), and its insertion into p5m is underway.

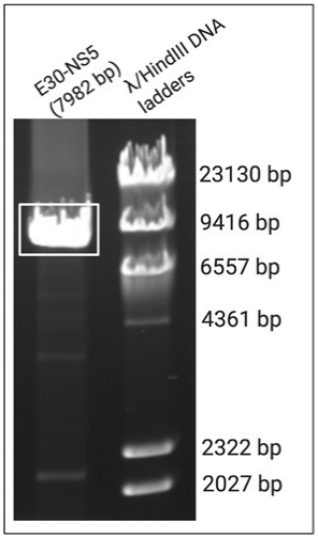

Figure 6 Successful amplification of the DENV-3 NS genes (E30-NS5) fragment

5. Conclusion

The novel concept of replicon RNA cancer vaccine has been highlighted, in that it utilizes the self-replication ability of the flavivirus, DENV. We used the In-Fusion technique to quicken the cloning of the long replicon. Currently, the insertion of E30-NS5 fragment into p5m is underway and after completion of the final p6m vector (**Figure 1**), the *in vitro* run-off transcription will be carried out, using the *SnaB*I-treated p6m as template to synthesize the RNA vaccine molecules. We will transfect this RNA into murine RAW Lucia 264.7 macrophage cells to investigate self-replication of replicon RNA and expression of polyneoepitopes on cell surface. Our results, if practical, may allow further investigation in mouse models, the success of which may open a new avenue for personalized cancer immunotherapy in the future.

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7. References

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