# Mutagenesis of Putative RNA Sequence Elements within the Dengue Virus Genome

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## Background

- Dengue virus (DENV) is an enveloped virus with a positive-sense, single-stranded RNA (ssRNA) genome. The DENV serogroup is part of the *Flaviviridae* family, consisting of four distinct serotypes (DENV1-4) (Guzman et al. 2016).
- *Flaviviridae* family includes viruses from the Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBEV) serogroups (Clyde and Harris 2006).
- Reported cases of DENV are increasing in the US (814 cases in 2021 to 10,298 cases in 2024) (CDC 2025).
- Also known as breakbone fever, symptoms range from a febrile illness to severe disease (Guzman et al. 2016).
- No specific antiviral treatment is currently available so it is important to understand viral replication to unlock novel drug targets.
- Recent studies show conserved RNA structural elements within the DENV genome can have important regulatory functions in viral replication (Groat Carmona et al. 2012).
- Conserved Protease Coding-Region 1 (CPCR-1) and Methyltransferase RNA Dependent Coding-Region 1 (MRdCR-1) are two conserved RNA sequence elements located in the NS3-coding region and NS5-coding region of DENV genome, respectively (Table 1).
- Silent mutations introduced into regions of interest (ROI, CPCR-1 and MRdCR-1) within an infectious clone of DENV2 were used to define the functional role of these RNA sequence elements in viral replication (Figure 1).

**Table 1.** Percent nucleotide homology of two putative RNA sequence elements, CPCR-1 and MRdCR-1, compared to background genomic regions (30 nucleotides upstream and downstream of ROI) across the DENV1-4, JEV, and TBEV serogroups. Sequence positions for ROI in the DENV1-4 serogroup were used as references to identify homologous regions in the other serogroups; however, MRdCR-1 was not detected in the JEV serogroup (Falen 2025).

| | Serogroup | Region of Interest | Background Sequence (+/- 30 nt) |
|---|---|---|---|
| CPCR-1 | DENV1-4 | 86.70% | 46.7% (28/60) |
| | JEV | 53.30% | 35% (21/60) |
| | TBEV | 60.00% | 58.3% (35/60) |
| MRdCR-1 | DENV1-4 | 85.20% | 53.3% (32/60) |
| | JEV | --- | 26.7% (16/60) |
| | TBEV | 70.40% | 63.3% (38/60) |

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                    CPCR-1
DENV4     .tccctttccccagagcaacagc 5513
DENV2     .CCCATTTCCTCAGAGCAATGCA 5511
DENV3     .tgcctttcctcagagcaacgct 5506
DENV1     .ggcctttccacagagcaatgca 5512
            * ***** ********
DENV2
Proposed  .CCCAttCccAcaATCAaacGCA 5511
Mutant      * ** ** **     **
                   MRdCR-1
DENV4     GGAGTGGAGTGGAAGGGGAAGGTCTGCAC 9101
DENV2     TGAGTGGAGTGGAAGGAGAAGGGCTGCAC 9108
DENV3     ACAGTGGAGTAGAAGGAGAAGGACTGCAC 9100
DENV1     TCAGTGGAGTGGAAGGAGAAGGACTCCAC 9109
            ******** ***** ***** ** ***
DENV2
Proposed  TGtcaGGcGTcGAgGGcGAgGGcCTcCAt 9108
Mutant      ** ** ** ** ** ** ** ** **
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**Figure 1. WT vs. mutant RNA sequence elements (CPCR-1 and MRdCR-1).** Sequence alignment of ROI (underlined) in the DENV1–4 serogroup, highlighting sequence homology (*) for the proposed RNA sequence elements, CPCR-1 and MRdCR-1. Alignments were generated using Clustal Omega. In DENV2, the introduction of proposed silent mutations (lowercase) reduces homology within these regions (highlighted in yellow). Mutagenic primers were designed to introduce these mutations using overlap extension (OE) PCR (Falen 2025).

## Experimental Road Map

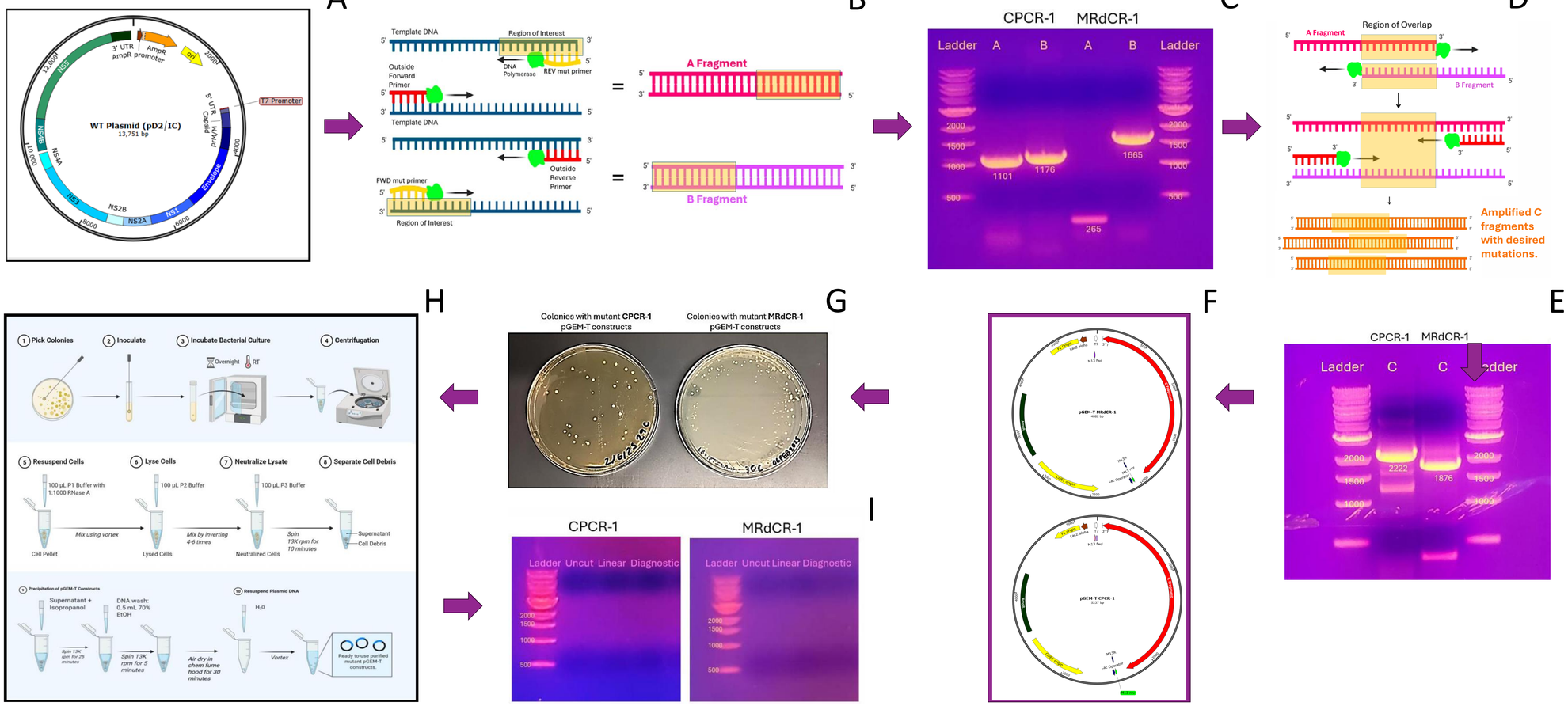


**Figure 2. Experimental Road Map**.
(A) Mutant clones were designed to introduce silent mutations into the wild-type DENV2 infectious cloning plasmid (pD2/IC) using targeted OE-PCR mutagenesis.
(B) A and B fragments for the two mutant variants were amplified using mutagenic and flanking outside primers targeting the ROI in DENV2 during the first step of OE-PCR.
(C) PCR products (fragments A and B) for both mutants were analyzed using agarose gel electrophoresis. Amplicons of the expected sizes (indicated on gel) were isolated and purified.
(D) A second round of OE-PCR was performed to fuse the A and B fragments into a single C fragment. C fragments were prepared for ligation into an intermediate vector (pGEM-T) by adding 3' adenine overhangs using Taq DNA polymerase.
(E) C fragments were analyzed using agarose gel electrophoresis and amplicons of expected sizes (indicated on gel) were isolated and purified.
(F) Mutant C fragments were ligated into pGEM-T and propagated in competent *E. coli* cells.
(G) Transformed *E. coli* were plated on LB/ampicillin agar. Plates were incubated at room temperature for 3–4 days to allow colony growth.
(H) Colonies presumed to contain the desired mutant pGEM-T constructs were selected for propagation and plasmid isolation.
(I) A diagnostic restriction digest (enzyme KpnI for CPCR-2 and XbaI for MRdPCR-1) was performed to confirm the orientation of the inserted C fragments. No bands were visible except for the 1kb DNA ladder (representative image shown). Expected fragment sizes for CPCR-1 for uncut (4882 bp supercoiled), linearized (4882 bp), forward insertion (2542 bp, 1449 bp, 1246 bp), and reverse insertion (2317 bp, 1674 bp, 1246 bp) were not present. Expected fragment sizes for MRdCR-1 for uncut (4882 bp supercoiled), linearized (4882 bp), forward insertion (3153 bp, 1729 bp), and reverse insertion (4646 bp, 236 bp) were not present. Figure was generated using BioRender (Falen 2025, Fulginiti and Rezk 2025).

## Conclusion

- Potential loss of mutant C fragments during pGEM-T cloning step.
- Attempts to desalt pGEM-T constructs with ethanol precipitation or modifying plasmid isolation protocol (additional centrifuge time, longer neutralization spin, and altering incubation temperatures) were unsuccessful.
- Low transformation efficiency and poor DNA quality likely hampered attempts at plasmid isolation of pGEM-T constructs from transformed *E. coli*.
- Suggest remaking mutant pGEM-T constructs using competent *E. coli* cells.
- Confirm insertion of mutant C fragments into pGEM-T with diagnostic restriction digest and sequencing.
- Transfer mutant C fragments into a DENV2 infectious cloning vector (pD2/IC) to generate vRNA.
- Transfect mutant vRNAs into mammalian host cells to generate viruses that can be tested using functional assays.
- This work will contribute to broader efforts to identify conserved RNA elements that are important to viral survival and pave the way for the development of novel antiviral treatments for dengue fever.

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Bibliography