Characterization of Conserved RNA Elements Using Dengue Virus Infectious Clones

TBIOMD 495

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Background

- Dengue virus (DENV) is a mosquito-borne pathogen that infects humans and is mainly transmitted by *Aedes aegypti* and *A. albopictus* mosquitoes (CDC 2019).
- There are four serotypes (DENV 1–4), but the family Flaviviridae also includes other important public health pathogens such as yellow fever virus, hepatitis C virus, and West Nile virus (Guzman et al. 2016).
- To better understand viral replication strategies and help with future vaccine development, we conducted a molecular cloning experiment to characterize putative RNA sequence elements, **Conserved Protease Coding Region 1 (CPCR-1)** and **Methyltransferase RNA-Dependent Coding Region 1 (MRdCR-1).**

Regions of Interest

1. Mutant-29 = Conserved Protease Coding Region 1 (CPCR-1), located in NS3-coding region (14 bp long)
2. Mutant-30 = Methyltransferase RNA-Dependent Coding Region 1 (MRdCR-1), located in NS5-coding region (27 bp long)

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          CPCR-1
DENV2 CATTTCCTCAGAGCAATGC
DENV1 CCTTTCCACAGAGCAATGC
DENV3 CCTTTCCTCAGAGCAACGC
DENV4 CCTTTCCCCAGAGCAACAG
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          MRdCR-1
DENV2 TGAGTGGAGTGGAAGGAGAAGGGCTGCAC
DENV1 TCAGTGGAGTGGAAGGAGAAGGACTCCAC
DENV3 ACAGTGGAGTAGAAGGAGAAGGACTGCAC
DENV4 GGAGTGGAGTGGAAGGGGAAGGTCTGCAC
       ********.*****.***** ** ***
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Figure 1. Conserved DENV coding regions selected for mutagenesis.

Sequence alignments of DENV 1–4 generated using Clustal Omega illustrating the conserved target regions: CPCR-1 (14 bp; NS3-coding region) and MRdCR-1 (27 bp; NS5-coding region). Proposed ROIs are highlighted in yellow, and asterisks (*) indicate nucleotide positions conserved across all four serotypes. These regions were targeted during generation of the mutant constructs pAG0029 (CPCR-1 mut) and pAG0030 (MRdCR-1 mut).

Sequence Conservation

Table 1. Sequence conservation of candidate RNA elements, CPCR-1 and MRdCR-1, in DENV 1-4, JEV and TBEV serogroups.
Percent identity for CPCR-1 and MRdCR-1 across DENV 1–4, JEV and TBEV serogroups based on Clustal Omega sequence allignments. Percentages indicate sequence homology in ROI compared to background (70% threshold).

Serogroup	**CPCR-1** (Background %)	**MRdCR-1** (Background %)
DENV 1-4	86.7% (53.5%)	85.2% (61.2%)
JEV	53.3% (19.5%)	63% (33.4%)
TBEV	66.7% (20.9%)	70.4% (29.4%)

Experimental Plan

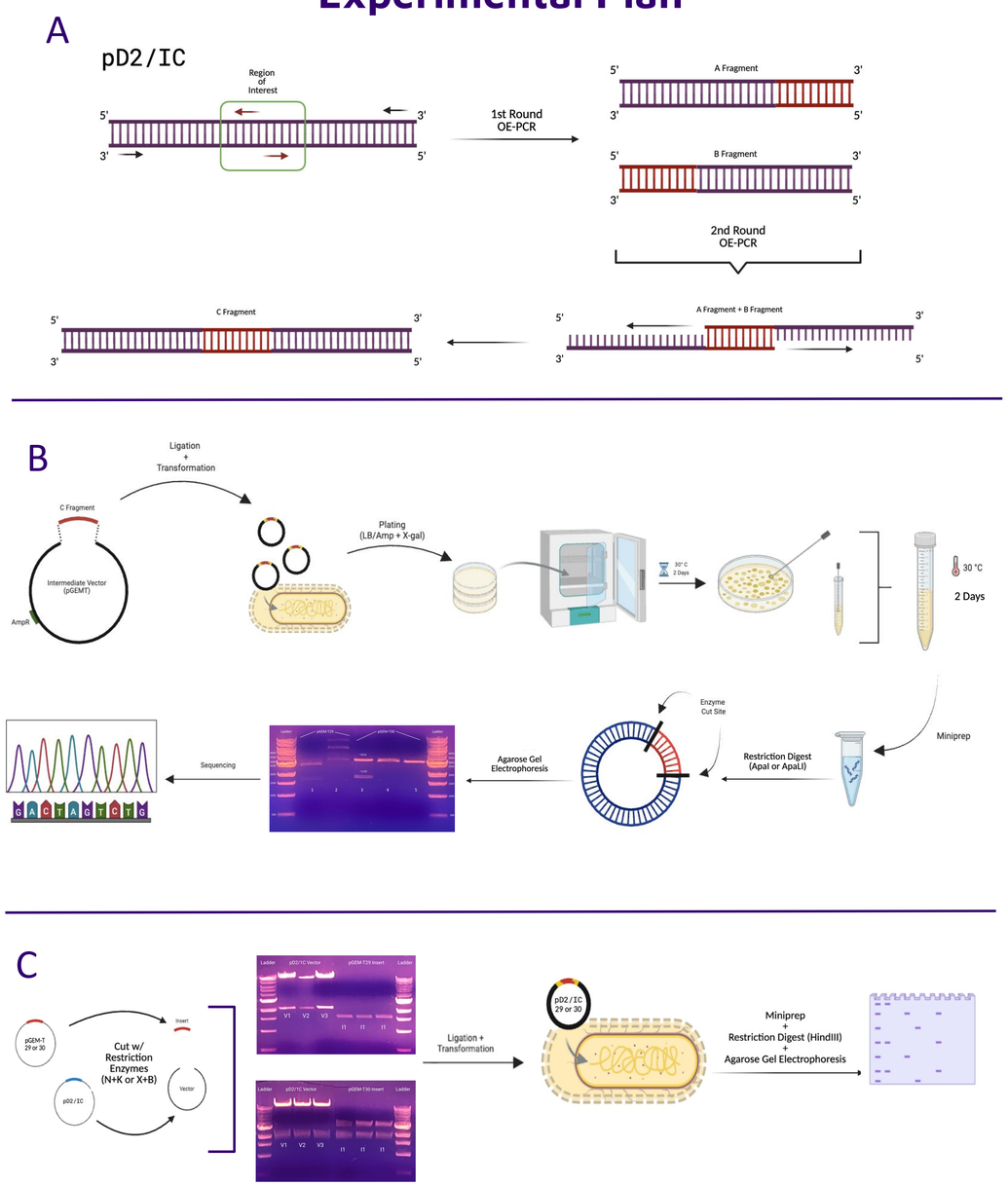

Figure 2. Experimental workflow for generating mutant infectious clones (pAG0029/pAG0030) from a wild-type pD2/IC plasmid.

(A) Two rounds of mutagenesis OE-PCR were used to generate CPCR-1 and MRdCR-1 mutant C fragments, **(B)** which were cloned into pGEM-T and transformed into *Escherichia coli*. White colonies were selected, cultured, and prepared for plasmid isolation. Constructs were screened by diagnostic restriction digestion and agarose gel electrophoresis alongside a 1 kb DNA ladder to verify the expected fragment sizes, then validated by Sanger sequencing. For CPCR-1, ApaLI digestion produced diagnostic fragments of 2542, 1449, and 1246 bp (forward) or 2317, 1674, and 1246 bp (reverse). For MRdCR-1, ApaI digestion produced fragments of 1729 and 3153 bp (forward) or 236 and 4646 bp (reverse). **(C)** Sequence-confirmed mutant inserts were excised from pGEM-T via subcloning restriction digests using enzymes KpnI and Nhe for CPCR-1, BsrGI and XbaI for MRdCR-1. Mutant ROIs were then ligated into the linearized DENV2 infectious clone vector (pD2/IC) to generate recombinant CPCR-1 and MRdCR-1 mutant infectious clones (pAG0029/pAG0030) for downstream applications like virus production and characterization. Illustration created using BioRender.

Cloning Problems

First 3 rounds of pGEM-T cloning did not provide sufficient growth of bacteria or correct integration of mutant amplicon.

Possible Reasons

- **Technique:** Pipetting errors along with not thoroughly mixing tube contents can allow for religation of vector leading to blue colonies; improper spreading and sterilizing technique can lead to cell death or growth of other microbes.
- **Power Outage:** Temperature fluctuations of competent *E. coli* cells stocks while frozen led to mucoidal phenotypes that disrupted workflow.
- **Selection of Satellites:** Accidental inoculation of satellites in overnight liquid cultures led to no visible bacterial pellet or no plasmid DNA isolated during miniprep.

Troubleshooting

Improved handling technique:

- Spin tubes down before/after setting up reactions.
- Precise pipetting.
- Improved spreading on agar plate.

Budget Miniprep → Promega kit

- Higher yield and better purity of DNA (ng/µL & A260/A280).

New batch of competent *E. coli* cells and T4 DNA ligase.

- Higher transformation competency and avoid mucoidal phenotype.
- Improves ligation transformation efficiency.

Future Work

- Ongoing validation of pGEM-T29 and pGEM-T30 mutant constructs.
- Subcloning verified for CPCR-1 and MRdCR-1 mutants, which means they can be subcloned into the DENV2 infectious clone (pD2/IC).
- Generation of recombinant DENV2 viruses.
- Use functional assays to assess the impact of mutations on viral replication and fitness.
- Evaluate role of CPCR-1 and MRdCR-1 in DENV2 replication cycle.

References