Modular design of a chimeric dengue antigen elicits tetravalent neutralizing antibodies

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Abstract

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Full Text

Preamble

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Abstract

Dengue virus comprises four antigenically related serotypes, and an effective vaccine should induce balanced immunity against all four. Current recombinant and subunit strategies remain constrained by the difficulty of integrating multiserotype antigenic information while maintaining structural consistency and expression feasibility. We developed a structure-guided workflow that combines boundary-constrained modular recombination, computational maturation and experimental selection to design single-molecule dengue antigens. From a constrained design space, we identified A6, which was expressed in mammalian cells, retained flavivirus envelope protein-related antigenicity, induced high bindingantibody titres against dengue virus serotypes 1–4, and elicited tetravalent neutralizing activity in mice. Here, we show that boundary-constrained modular recombination coupled with computational maturation supports the design of candidate tetravalent dengue antigens.

Keywords

Dengue virus (DENV), Modular mosaic assembly, Broad-spectrum vaccine, Antibody-dependent enhancement (ADE)

1 Introduction

Dengue virus (DENV) is a major mosquito-borne viral pathogen of global importance, causing widespread infections annually and remaining a persistent public health challenge[1, 2]. DENV exists as four antigenically related serotypes, rendering vaccine development more complex than for most viruses with a single serotype[2]. A primary infection usually induces relatively robust protection against the homologous serotype, yet the cross-reactive antibodies generated against heterologous serotypes are not invariably sufficiently neutralizing[3]. Accordingly, the central objective of dengue vaccine development is not simply to provoke antibody responses, but to establish a balanced and functional immune response against all four serotypes[4]. How to simultaneously achieve coverage of all four serotypes while ensuring immunological safety has become a central issue in DENV vaccine design[5].

In response to this challenge, DENV vaccine development has advanced along several technological routes, including live-attenuated, inactivated, and recombinant subunit vaccines. Clinical studies of existing tetravalent vaccines have demonstrated the feasibility of inducing protection against all four serotypes simultaneously, yet the replication fitness, immunogenicity, and magnitude of serotype-specific responses among different components are not always well balanced[5]. By contrast, subunit antigens provide advantages in safety and dose control, but their development is constrained by a central limitation: soluble E proteins often cannot stably preserve the native-like dimeric conformation, which may compromise the presentation of protective quaternary epitopes and is frequently associated with suboptimal expression yield and poor production stability[6, 7]. Previous studies have shown that structural stabilization of the E protein can improve dimer preservation and expression properties[8, 9]; however, how to integrate antigenic information from all four serotypes into a single candidate molecule while preserving both structural and expression feasibility remains an unresolved design challenge.

On this basis, we sought to exploit the modular recombination principles that are commonly observed in protein evolution. The classical view holds that biological evolution produces new functions through the modification and recombination of pre-existing parts[10–12], and structural biology studies further indicate that domain duplication, combination, and rearrangement constitute major sources of functional expansion in multidomain proteins[13–15]. Recent analyses of viral proteins further suggest that persistent reshuffling of protein fragments across distinct functional contexts can markedly expand the diversity of host interaction-related functions[16, 17].

Therefore, for a DENV antigen comprising four related serotypes, we hypothesized that constrained recombination of corresponding fragments from different serotypes, while preserving structural boundaries and fragment order, may be preferable to unconstrained engineering of the full-length E protein[6]. This strategy may help preserve the structural compatibility within native fragments while also enabling the integration of

antigenic information from all four serotypes into a single candidate molecule[18–20].

Because the initial recombinants do not inherently guarantee proper folding, stability, or expression, we further introduced a computational maturation step for stability optimization and structural screening of the candidates.

Here, we present a structure-guided modular recombination and computational maturation strategy for the design of broad-spectrum DENV antigens. We divided the E protein antigens from the four serotypes into corresponding structural fragments and constructed 24 chimeric candidates while preserving fragment order; we then progressively converged on representative candidates A1–A8 by combining PROSSbased stability design[21, 22], Rosetta energy evaluation[23], AlphaFold 3 structural filtering[24], and sequence clustering. Among

these candidates, A6 performed best in eukaryotic expression, antigenicity assessment, and structural characterization, and elicited high-titer antibody responses together with neutralizing activity against all four DENV serotypes in mice. These results indicate that a design strategy integrating native fragment recombination with computational maturation can yield candidate DENV subunit antigens with potential tetravalent coverage.

2 Results

2.1 Modular recombination expands the dengue antigen design space To generate an initial candidate space for single-molecule integration of all four serotypes, we first selected the E proteins of DENV-1 to DENV-4 as reference antigen sequences. Multiple sequence alignment with secondary-structure annotation showed that, despite marked sequence divergence among the four serotypes, the overall structural scaffold characteristic of flaviviral E proteins is preserved[6] (Fig. 1a [Figure 1: see original paper], b). The DENV E protein is a class II fusion protein whose ectodomain is composed of three predominantly β -sheet domains linked to the membrane-anchoring region by the stem region[6]. Therefore, we used UniDoc to structurally segment the reference antigens rather than dividing the full-length sequences into equal-length parts[25]. UniDoc identifies structural boundaries from inter-residue distance information and is thus well suited for defining segment boundaries for subsequent module assembly[25]. For each serotype, four contiguous fragments were obtained and used as the basic units for subsequent modular recombination (Tab. S1).

After defining four reference fragments for each serotype, we further imposed module-assembly constraints as follows: (1) the four fragments in every candidate were kept in the fixed order of Part 1 to Part 4, with no fragment rearrangement. (2) each candidate was composed of four fragments originating from four different serotypes, so that every design integrated local sequence information from DENV-1 to DENV4 within a single molecule. (3) junctions were confined to positions near predefined structural boundaries and were designed to avoid key modified and conserved structural sites in the E protein, including the N-linked glycosylation sites N67 and N153 as well as conserved cysteine residues, thereby minimizing disruption of local conformation and the disulfide-bond network[6, 26, 27]. Under these constraints, the feasible

1 Design of chimeric DENV sequences. (a) Sequence alignment of DENV serotypes 1–4. (b) Structural superimposition of the DENV 1–4 E proteins. (c) Segment boundaries defined by UniDoc parsing, alongside the design rules and the 24 possible combinations for modular assembly.

allocation of the four serotypes to the four fragment positions formed a complete permutation space, resulting in 24 chimeric candidates (Fig. 1b; Tab. S2).

These 24 chimeras were not produced by arbitrary concatenation, but instead defined an initial design space built under constraints imposed by native fragments and structural boundaries. Each candidate consisted of local native frag-

ments derived from the four serotypes, with recombination restricted to regions near the boundaries defined by UniDoc, thereby integrating information from all four serotypes within a single molecule while preserving, as much as possible, the local sequence context within each fragment. Rather than simply mixing four antigens, this order-preserving modular assembly compressed information from all four serotypes into a single candidate molecule and provided a common starting point for subsequent comparison of structural feasibility and stability within a unified molecular framework.

2.2 Computational maturation prioritizes structurally plausible candidates The 24 mosaic sequences generated in the previous section satisfied the structural-boundary and assembly rules, but these initial chimeras should still be regarded primarily as a screenable starting design space rather than as optimized candidate molecules. To improve stability and downstream expression feasibility, we established a progressively tightened computational maturation pipeline (Fig. 2a [Figure 2: see original paper]). We first applied PROSS stability design to the 24 mosaic scaffolds[21, 22]. PROSS combines phylogenetic information with atomic-level design calculations to optimize protein stability and heterologous expression, making it well suited for first-round optimization of the initial chimeras[21, 22]. While preserving key functional sites, we obtained 62 stabilized variants in total (Tab. S3); together with the original 24 mosaic sequences, these formed a candidate set of 86 members.

2 Computational screening and evaluation. (a) The in silico maturation workflow. (b) Rosetta energy scores. (c) AlphaFold ipTM scores. (d) Sequence grouping of the final candidates.

We then applied a two-step structure-based filtering procedure to the 86 candidates. First, we evaluated the relative energies of all candidates using Rosetta relax together with the ref2015 all-atom energy function, using the four native DENV E

proteins as reference baselines (Fig. 2b)[23, 28]. The Rosetta ref2015 all-atom energy function is widely used in protein modeling and design, making it suitable for comparing the relative energy states of different candidates after relaxation[23, 28]. Based on this criterion, we excluded sequences with unfavorable relative energies, thereby narrowing the candidate set from 86 to 36. We next subjected the energy-filtered sequences to AlphaFold3 monomer structure prediction and used pTM together with PAE to further assess the confidence of their overall conformations and relative fragment arrangements (Fig. 2c)[24]. AlphaFold3 provides confidence-related structural metrics, with pTM indicating the reliability of the predicted global topology and PAE indicating the uncertainty in relative positioning among different parts[24]. Through this step, we further removed sequences with insufficient structural confidence, ultimately retaining 30 candidates that satisfied both the energy and structural criteria.

After the energy- and structure-based filtering steps, we further applied MMseqs2 to dereplicate the remaining 30 sequences[29, 30]. MMseqs2 provides

efficient sequenceclustering and dereplication tools, making it well suited for the final diversitycompression step of the candidate set[29, 30]. At thresholds of 95% sequence identity and 80% coverage, the 30 sequences were partitioned into 8 sequence clusters (Fig. 2d). The aim of this step was not to perform another round of simple ranking, but to reduce highly similar redundant candidates while preserving sequence diversity.

We then selected one representative sequence from each cluster by jointly considering Rosetta energy and AlphaFold3 structural metrics, ultimately yielding 8 candidate molecules, A1–A8.

2.3 Biophysical profiling and expression identify A6 as the lead candidate To assess the sequence-level physicochemical developability of the eight final candidate molecules, A1–A8, we first analyzed them using ExPASy ProtParam and SoluProt[31, 32]. ProtParam calculates sequence-based physicochemical properties, including the theoretical pI, instability index, aliphatic index, and GRAVY, whereas SoluProt provides an auxiliary indicator of soluble-expression propensity at the sequence level[31, 32]. As shown in Fig. 3a [Figure 3: see original paper], the designed group and the native control group did not differ significantly in solubility, theoretical pI, instability index, aliphatic index, GRAVY, or number of amino acids. These results indicate that the overall sequence-level physicochemical properties of A1–A8 are broadly comparable to those of native DENV E proteins, with no evident increase in hydrophobicity, length deviation, or instability.

We next cloned A1–A8 individually into eukaryotic expression vectors and compared their transient expression in FreeStyle ™293-F cells. FreeStyle ™293-F is a mammalian expression system compatible with serum-free suspension culture and recombinant protein production, making it suitable for preliminary assessment of candidate-expression feasibility. Under uniform expression and purification conditions, the products of each candidate were examined by SDS-PAGE (Fig. 3b). A6 showed the most distinct protein band near the expected molecular weight, whereas the remaining candidates displayed weaker bands or no evident accumulation.

3 Characterization and expression of the designs. (a) Physicochemical property comparison of designed versus natural sequences. (b) SDS-PAGE of eukaryotic expression for candidates A1–A8. (c) Western blot analysis of A1–A8 using the conformation-specific 4G2 antibody.

To further evaluate whether the expressed products retained antigenic features associated with flaviviral E proteins, we subjected A1–A8 to 4G2 Western blot analysis[33, 34]. 4G2 is a flavivirus group cross-reactive antibody that recognizes the fusion loop region at the distal end of E protein domain II and can therefore serve as a probe for E protein-associated antigenicity[33]. As shown in Fig. 3c, A4, A6, A7, and A8 all displayed detectable 4G 2 reactivity under our assay conditions, with A6 showing the strongest signal, in agreement with its superior expression observed in results, this indicates that A6 not only has

better expression feasibility in the eukaryotic system, but also retains flaviviral E protein-related antigenic features recognizable by 4G2 in its expression product. Therefore, we designated A6 as the lead candidate for subsequent immunological evaluation.

2.4 A6 induces antibody responses against all four dengue serotypes To assess the humoral immunogenicity of A6, we implemented a multi-dose immunization and sequential serum-sampling scheme in BALB/c mice (Fig. 4a [Figure 4: see original paper]). After formulation with aluminum hydroxide adjuvant, A6 protein was administered at 0.4, 2, or 10 µg per mouse as a prime followed by three booster immunizations, and sera were collected after the second, third, and fourth immunizations for subsequent analyses.

4 In vivo immunogenicity of the A6 antigen. (a) Schematic overview of the mouse immunization protocol and blood collection schedule. (b) Binding antibody titers against the four DENV serotypes. (c) Analysis of IgG subclass distribution (IgG1, IgG2a, IgG2b, and IgG3) in mouse sera.

As shown in Fig. 4b, sera from A6-immunized mice exhibited detectable binding antibody responses to DENV1–4. After the second immunization, all three dose groups showed clear ELISA signals against all four serotypes, although the differences among doses remained relatively limited. As the number of booster immunizations

increased, antibody titers against all four serotypes rose further, with the effect being more evident in the high-dose group. After the fourth immunization, the 10 µg group achieved the highest binding antibody titers across all four serotypes, with the strongest response against DENV-1 at above 2×106 , DENV-3 and DENV-4 at comparable levels above 5×105 , and DENV-2 relatively lower but still above 2×105 , meeting the requirements for subsequent evaluation. Overall, A6 was able to induce dose-dependent and booster-enhanced humoral binding antibody responses against DENV1–4.

In addition, we profiled the IgG subclass distribution in sera from immunized mice to further characterize the humoral immune response elicited by A6 (Fig. 4c). In mice, IgG1, IgG2a, IgG2b, and IgG3 are the commonly assessed IgG subclasses and can be used to further describe the composition of vaccine-induced antibody responses[35].

The results showed that the antibody response induced by A6 was dominated by IgG1, with IgG1 titers increasing further as both the immunization dose and the number of booster immunizations increased; IgG2a and IgG2b were also detectable and showed upward trends in the high-dose group and after subsequent booster immunizations, whereas no evident IgG3 signal was observed. These results indicate that the humoral response induced by A6 was not restricted to a single antibody class, but was dominated by IgG1 and accompanied by subclass responses of IgG2a and IgG2b. Given that aluminum hydroxide was used as the adjuvant in this study, this distribution is consistent with the commonly

reported alum-associated humoral response pattern in mice, which is typically Th2 biased and characterized by higher IgG1 levels[36–38].

2.5 A6 elicits tetravalent neutralizing antibody responses To assess the functional activity of antibodies induced by A6, we performed plaque reduction neutralization tests (PRNTs) using sera from the high-dose group collected after the fourth immunization to measure neutralization against DENV1–4 (Fig. 5a [Figure 5: see original paper],b)[39]. PRNT is a widely used experimental assay for the measurement of DENVneutralizing antibodies and enables quantitative assessment of the capacity of serum to inhibit viral infection[39]. The results showed that sera from A6-immunized mice displayed detectable neutralizing activity against all four serotypes, indicating that A6 elicited functional antibody responses to DENV1–4. There were some differences in neutralization levels among the four serotypes, with higher neutralizing titers against DENV-2 and DENV-4, both above 1:600, whereas DENV-1 and DENV-3 were relatively lower but still above 1:300, indicating that the response was not restricted to a single serotype. Overall, these results indicate that A6 can elicit neutralizing activity against all four dengue virus serotypes.

Beyond its neutralizing activity, we further characterized the basic biophysical properties of A6. First, thermal stability analysis on the Uncle platform showed that A6 had a Tm of more than 70 ∘ C (Fig. 5c). To further support A6 from a structural perspective, we predicted its three-dimensional conformation using AlphaFold3 (Fig. 5d)[24]. The prediction indicated that A6 adopts a continuous and compact overall fold, with structural features consistent with the predominantly β -sheet architecture of flaviviral E proteins[6]. For comparison, we performed a sequence-level comparison between A6 (colored) and the reported tetravalent recombinant DEN-80E candidate

5 Neutralization and structural evaluation of A6. (a) PRNT visualization for DENV 1–4. (b) Serum neutralizing antibody titers. (c) Thermal stability of A6. (d) AlphaFold3 predicted structure.

V180 (gray); V180 has been described in the literature as a subunit vaccine candidate in development[42, 43]. A6 and V180 shared approximately 260 identical residues out of 394 positions, corresponding to about 66%, with the differing sites marked as spheres in the figure.

3 Discussion

This study establishes and validates a modular recombination and computational maturation workflow for dengue antigen design. By integrating information from all four serotypes within structural-boundary constraints and combining this with downstream computational screening, in vitro expression, and immunological evaluation, we converged on candidate A6 from a constrained starting design space. A6 was expressed in a eukaryotic system, retained antigenic features associated with flaviviral E proteins, elicited high-level binding antibody responses against DENV1–4 in mice, generated tetravalent neutraliz-

ing activity, and showed acceptable baseline biophysical properties. Thus, the advance of this work lies not merely in the identification of a single candidate molecule, but in showing that structure-boundary-guided modular recombination followed by computational maturation can provide an actionable route for tetravalent DENV candidate-antigen design.

Compared with existing DENV subunit-vaccine studies, the value of this work lies not in demonstrating the feasibility of the recombinant-protein route itself, but in introducing a single-molecule design strategy that integrates information from multiple serotype sources. Previous studies have shown that DENV subunit vaccine candidates can induce humoral immune responses using recombinant E proteins or truncated derivatives thereof[44]. For example, V180 (DEN-80E), a tetravalent recombinant subunit candidate under development, has shown favorable safety and measurable immunogenicity in phase I clinical trials[42, 43]. Overall, however, DENV

subunit vaccines still face two central challenges: achieving the most balanced possible immune response across the four serotypes, and simultaneously reconciling immunological balance, expression feasibility, and structurally stable antigen presentation[44].

Recent RSV tri-epitope design studies further suggest that computational methods can simultaneously present multiple discontinuous epitopes on a single scaffold[45, 46].

In contrast, this study addresses a different but equally critical problem: under the immunological constraints of four-serotype DENV, how to integrate information from multiple serotype sources into a single candidate molecule through structure-boundary-constrained modular recombination, and ultimately obtain a candidate antigen with expression feasibility, tetravalent binding-antibody responses, and tetravalent neutralizing activity. Methodologically, these findings further underscore the strong design value of structural priors learned from evolution. Our strategy was not an unconstrained sequence search, but instead relied on native E-protein fragments, modular recombination under structural-boundary constraints, and subsequent convergence through stability design and structural screening. Recent studies similarly indicate that design workflows centered on native fragments and physical constraints can still perform strongly in multisite functional engineering and highaccuracy structural design[47]. Accordingly, the more promising path forward may lie not in advancing any single method in isolation, but in integrating native fragments, physical models, and generative models[19, 20].

This study still has several boundaries that should be made explicit. First, we have not yet performed viral challenge experiments, and the current data are therefore insufficient to directly establish the in vivo protective efficacy of A6. Second, ADE has not yet been specifically evaluated in this study. Although A6 induced relatively balanced four-serotype neutralizing responses under the conditions used here, which is encouraging from a design perspective, balanced

neutralization cannot replace direct experimental assessment of ADE risk[48, 49]. DENV vaccine development has long emphasized the importance of four-serotype immune balance, but the relevant safety boundaries still require further evaluation in dedicated models[48, 50]. In addition, the neutralization results reported here were generated under the present mouse immunization conditions and the PRNT system used in this study, and because PRNT itself varies across experimental settings, its numerical interpretation should remain cautious[51]. Finally, only a subset of the A1–A8 candidates showed detectable expression, indicating that both the current design space and the screening criteria still have room for further optimization.

From a broader translational perspective, the modular recombination and computational maturation workflow proposed here could in principle be extended to antigen design for other flaviviruses. The potential generalizability of this approach does not depend on DENV and other flaviviruses having identical immunological constraints, but instead on its use of native fragments as structural priors to define an initial design space under structural-boundary constraints, followed by progressive convergence through downstream computational screening. For other flaviviruses, such as Zika virus and West Nile virus, this strategy may offer a useful framework for single-molecule antigen integration, improved expression feasibility, and structural optimization. However, protective epitopes, cross-reactivity patterns, and safety

boundaries are not identical across flaviviruses; prior studies have shown that crossreactive flaviviral antibodies can lead either to cross-neutralization or to ADE risk, and the extension of this approach must therefore be evaluated separately in each system for its immunological consequences and safety[49, 50].

4 Methods

4.1 Sequence Retrieval and Initial Structure Prediction The amino acid sequences of the dengue virus (DENV) envelope (E) proteins were retrieved from the National Center for Biotechnology Information (NCBI) database using the following accession numbers: NP 059433.1 (DENV-1), NP 056776.2 (DENV2), YP 001621843.1 (DENV-3), and NP 073286.1 (DENV-4).

To define the structural modules for mosaic assembly, we required structure-based domain parsing. Because the UniDoc algorithm specifically requires PDB-formatted input files, we utilized LocalColabFold rather than AlphaFold 3 to generate the initial monomeric structures, thereby avoiding potential mmCIF format incompatibility. The predictions were executed locally using the following command parameters: colabfold batch –amber –templates –num-recycle 3 –use-gpu-relax –model-type auto [input.fasta] [output dir].

For each of the four serotypes, the top-ranked energy-relaxed model (rank 0001.pdb) was selected for downstream analysis. The structural domain boundaries of the E protein extracellular regions were subsequently parsed using the UniDoc web server (https://yanglab.qd.sdu.edu.cn/UniDoc/). To ensure the

reliability of the domain partitioning, the Predicted Aligned Error (PAE) matrices generated by LocalColabFold were used as an auxiliary reference to cross-validate the UniDoc-defined boundaries.

4.2 Stability Optimization via PROSS

To improve the thermodynamic stability of the 24 mosaic scaffolds, we utilized the PROSS (Protein Repair One-Stop Shop) stability design server. As PROSS requires high-confidence structural inputs, the initial models for all 24 designs were generated using LocalColabFold with the following parameters: colabfold batch –amber –templates –num-recycle 3 –use-gpu-relax –model-type auto. The top-ranked relaxed models (rank 0001.pdb) were used as the starting structures for the design process. optimization performed PROSS server (https://pross.weizmann.ac.il/). During the configuration, the protein structures were uploaded as user-defined models. To preserve the biological efficacy of the immunogens, we applied strict structural constraints: known EDE (Envelope Dimer Epitope) broad-spectrum neutralizing epitopes and critical N-linked glycosylation sites (N67 and N153) were designated as fixed residues to prevent mutation. The calculations were executed using the Rosetta ref2015 energy function. All other parameters, including the MSA (Multiple Sequence Alignment) generation settings, were maintained at their default values. This process yielded a library of stabilized variants with varying mutation loads for further screening.

4.3 Rosetta Energy Minimization and Scoring To evaluate the thermodynamic stability of the candidate library, we performed energy minimization on the 4 wild-type DENV E proteins, the 24 initial mosaic scaffolds, and the 62 PROSS-optimized variants (totaling 90 sequences). The calculations were executed using the locally installed Rosetta 3.14 (Linux binaries). Each structure was refined using the Rosetta FastRelax protocol with the ref2015 scoring function. To preserve the backbone and sidechain geometry of the design models during minimization, we applied coordinate constraints to the starting positions. The refinement was performed using the following command-line parameters: relax.default.linuxgccrelease -s [input.pdb] -nstruct 5 -in:file:fullatom -ignore unrecognized res -relax:constrain relax to start coords relax:coord constrain sidechains -relax:ramp constraints false -relax:fast -ex1 -ex2 -use input sc.

For each candidate, five independent relaxation trajectories were generated. The trajectory with the lowest total energy (Total Score) was selected as the representative value for the energy landscape analysis. The resulting energy scores were used to rank the designs against the wild-type benchmarks to identify candidates with enhanced thermodynamic stability.

4.4 Structural Validation via AlphaFold 3 To rigorously validate the folding certainty of the 36 top-ranking candidates from the Rosetta energy filter, we performed high-confidence structural predictions using a local installation of AlphaFold 3. This orthogonal validation step aimed to ensure that the artificial

mosaic sequences could successfully adopt the target fold, particularly at the engineered splice seams.

Input files were prepared in standard JSON format. To ensure exhaustive sampling of the conformational space, the prediction parameters were configured with a high emphasis on stochastic diversity, as recommended in the AlphaFold 3 literature.

The predictions were executed using the following parameters: -num recycles=10, -num seeds=200, and -num diffusion samples=5. The full command was as follows: python3 run alphafold.py -json path=[input.json] -model dir=[model path] -db dir=[db path] -output dir=[output path] -num recycles=10 -num seeds=200 -num diffusion samples=5.

Candidates were evaluated based on the Predicted Template Modeling (pTM) score and the Predicted Aligned Error (PAE) matrix. Only designs demonstrating high structural confidence (pTM ¿ 0.8) and minimal error at the modular junctions were retained. This high-resolution sampling strategy ensured that the final selected candidates possessed both thermodynamic stability and high structural determinacy.

4.5 Sequence Clustering and Final Candidate Selection To minimize sequence redundancy and ensure structural diversity in the experimental validation phase, the 30 candidates that passed the AlphaFold 3 structural filter were

clustered using MMseqs2. We applied a sequence identity threshold of 95% and a minimum coverage of 80% using the following command: mmseqs easy-cluster [input.fasta] [output prefix] [tmp dir] -min-seq-id 0.95 -c 0.8 -cov-mode 0.

This procedure grouped the 30 sequences into 8 distinct clusters based on their primary sequence similarity. To select the most promising lead from each cluster, we employed a ” best-in-class” strategy. Within each cluster, candidates were ranked by integrating their Rosetta total energy and AlphaFold 3 pTM scores. The sequence with the lowest energy and highest structural confidence from each group was selected as the representative, resulting in a final set of 8 candidates, designated A1–A8, for subsequent in vitro expression and functional assays.

4.6 Virus Cultures and Cell Lines

The dengue viruses used in this study were Dengue virus type I (DENV I), strain GE27; Dengue virus type II (DENV II), strain New Guinea C (NGC); Dengue virus type III (DENV III), strain YN01; and Dengue virus type IV (DENV IV), strain 30.All viruses were cultured and produced in C6/36 cells (ATCC, Manassas, VA, USA). Culture supernatants from the virus-infected cells were centrifuged at 4000×g to remove cellular debris. The viruses were then dispensed into cryopreservation tubes and stored at -80 ∘ C.

The C6/36 cells were cultured in the Roswell Park Memorial Institute (RPMI)-1640 medium with 10% fetal bovine serum (FBS; Excel, New Taipei City, Tai-

wan). BHK-21 (ATCC) cells were cultured in Dulbecco's modified Eagle medium (DMEM) medium with 10% FBS serum. FreeStyle™ 293-F cells(Thermo Fisher Scientific, Waltham, MA, USA) used for protein expression were cultured in FreeStyle 293 Expression Medium 12338 (Gibco, Grand Island, NY, USA). C6/36 cells were cultured at 28 ∘ C without CO2 . For producing DENV, C6/36 cells were cultured in the same way, except for FBS, which was adjusted to 2%. BHK-21 cells were cultured at 37 ∘ C with 5% CO2 .

4.7 Immunization of animals and ethics statement Female Balb/c mice (6-8 weeks old, weighing 16-18 g) were purchased from Beijing SPF Biotechnology Co., Ltd. The mice were randomly assigned to different groups as experimental animals and housed under specific pathogen-free (SPF) conditions at the Laboratory Animal Center of the Academy of Military Medical Sciences in Beijing, China. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Animal Welfare and Ethics Committee of the Academy of Military Medical Sciences (Approval No. IACUC-DWZX-2024-A022) and conducted in accordance with ethical guidelines and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Female Balb/c mice with specific pathogen free (SPF) aged 6-8 weeks were randomly divided into different groups (n = 10 per group). The mice were immunized with purified A6 molecule as the antigen. The subunit vaccine was prepared by diluting a single antigen (0.4, 2, or 10 μg per dose) in sterile PBS and mixing with 10 % (w/w) aluminum hydroxide gel, with a PBS antigen + adjuvant group serving as the negative control. Each mouse received a 100 μL intramuscular injection of the prepared

subunit vaccine, for a total of four immunizations administered at two-week intervals.

Then, two weeks after the second immunization, 200 μL of blood was collected from each mouse, with subsequent blood samples obtained in the same manner two weeks after each following immunization.

4.8 Preparation and identification of protein The amino acid sequences of A1-A8 were synthesized by custom gene synthesis. Each fragment was cloned into the eukaryotic expression vector pTSE-His via double restriction enzyme digestion. For expression, the constructed plasmids were co-transfected with FectoPRO transfection reagent (Polyplus-transfection®, France; #116-001) into FreeStyle 293-F cells. Seventy-two hours post-transfection, the cell suspension was centrifuged at 8,000 × g for 10 min, and the supernatant was collected. The supernatant containing the target protein was purified using a HisTrap HP column (Cytiva, USA), followed by buffer exchange into phosphate-buffered saline (PBS, pH 7.4) via a HiTrap Desalting column (Cytiva, USA). Finally, the purified proteins were analyzed under both reducing and non-reducing conditions by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (GenScript, China; #M00668), stained with QuickBlue (Biolinked, China; #BF06152), and quantified by measuring the absorbance at 280 nm

(A280).

The structural size and antigenicity of the purified protein were determined by western blotting. First, the molecular weight of the protein was assessed by 10% SDSPAGE, and the proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane using a transfer apparatus. The membrane was blocked with 5% skimmed milk at 37 ◦ C for 2 h. It was then incubated overnight at 4 ◦ C with diluted detection antibody 4G2 (final concentration 1 µg/mL) and washed with Tris-buffered saline containing 0.1% Tween-20 (TBST). Finally, the membrane was incubated with a 1:4000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) antibody, followed by washing with TBST. The blots were developed by adding western blotting solution A and B, and the signals were captured using a gel imaging system (Bio-Rad, Hercules, CA, USA).

4.9 Analysis of physical and chemical properties of proteins The thermal stability of the proteins was assessed using the Uncle platform. Candidate molecules were analyzed based on their melting temperature (Tm) combined with an optional dynamic light scattering (DLS) program. Specifically, the test proteins were placed in UniTubes in duplicate. The intrinsic fluorescence of the protein samples was excited at 266 nm, and spectral data were collected. The protein melting temperature (Tm) was determined from changes in the spectral profile. Finally, the spectral data were analyzed to generate protein aggregation-denaturation curves.

The secondary structure of the protein and the reference standard was analyzed in this experiment by collecting their circular dichroism (CD) absorption spectra in the far-ultraviolet (190–260 nm) and near-ultraviolet (250–340 nm) regions, followed by software-based analysis. First, the cuvette was soaked overnight in 2 M HNO3 , thoroughly rinsed with deionized water, and air-dried. The background signal was

recorded, followed by a scan of the blank buffer. Subsequently, the test sample was added to the cuvette, and a far-ultraviolet scan from 190 to 260 nm was performed under the aforementioned parameters to collect the corresponding data.

4.10 Detection of the immune efficacy of serum from immunized mice Detect the antibody titer in the mouse serum by enzyme-linked immunosorbent assay (ELISA).The antigen was diluted to 2 ng/µL in carbonate-bicarbonate buffer and coated onto ELISA plates (Costar #9018, Corning, Washington, DC, USA), with control wells left uncoated. After overnight incubation at 4 ◦ C, each well was blocked with 200 µL of phosphate-buffered saline (PBS) containing 2% skimmed milk for 2 h at 37 ◦ C to prevent nonspecific binding. Immune mouse sera were serially diluted twofold in blocking buffer, and 100 µL of each dilution was added to duplicate wells, followed by incubation at 37 ◦ C for 1.5 h. The plates were then washed six times with PBS containing 0.1% (v/v) Tween-20 (PBST) and incubated with horseradish peroxidase (HRP)-conjugated goat

anti-mouse immunoglobulin G (IgG) antibody (1:4000, v/v) for 1 h at 37 ∘ C. After another six washes with PBST, color development was performed using o-phenylenediamine (OPD) substrate, and the absorbance was measured at 492 nm. Wells containing only blocking buffers served as negative controls (N). A well was considered positive if the OD492 value of the immune group (P) exceeded 0.5 and the P/N ratio was $ $2; the corresponding serum dilution was recorded as the antibody titer.For antibody isotype analysis, the secondary antibody was replaced with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G1, G2a, G2b and G3 (IgG1, IgG2a, IgG2b, IgG3) antibodies, while all other steps remained identical to those described for antibody titer determination.

4.11 Determination of neutralizing antibody titers in mouse serum Neutralizing antibody titers in serum were determined by plaque reduction neutralization assay. BHK-21 cells (8 × 105 cells) were seeded into 6-well plates (2 mL per well) and incubated for 24 h at 37 ∘ C under 5% CO2 . Serum samples were serially diluted twofold in DMEM, mixed with approximately 100 plaque-forming units (PFU) of dengue virus, and incubated at 4 ∘ C for 1 h. The resulting serum–virus mixtures were added to BHK-21 monolayers in 6-well plates and incubated at 37 ∘ C for 1 h.

After removal of the supernatant, 2 mL of semi-solid overlay medium (prepared by mixing equal volumes of 2× DMEM, 4% (v/v) fetal bovine serum, and 2% (w/v) low-melting-point agarose, equilibrated to room temperature) was added to each well.

Following solidification, the plates were incubated at 37 ∘ C with 5% CO2 for 4–5 days until plaques became visible. Nonlinear curve fitting was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) to determine the serum dilution that reduced plaque counts by 50%, which was reported as the neutralizing antibody titer.

4.12 Statistical analysis

Data are presented as the means ± standard deviation (SD). Statistical significance was determined using GraphPad Prism 8.0 software.

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L.P., Manuscript review & editing: Z.Y., R.W. and J.L.; All authors have read and approved the article.

Declaration of Interests. Z.Y., R.W., J.L,J.G., L.P., and H.S. are listed as inventors on a pending patent application for A6. The other authors declare no competing interests.

Data Availability Statement. The data supporting the findings of this study can be obtained from the corresponding authors upon reasonable request.

References

[1] Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P.: The global distribution and burden of dengue. Nature 496(7446), 504–507 (2013) [2] Guzman, M.G., Gubler, D.J., Izquierdo, A., Martinez, E.: Dengue infection.

Nature Reviews Disease Primers 2, 16055 (2016) [3] Dejnirattisai, W., Jumnainsong, A., Onsirisakul, N., Fitton, P.: Cross-reacting antibodies enhance dengue virus infection in humans. Science 328(5979), 745–748 (2010) [4] Shukla, R., Ramasamy, V., Shanmugam, R.K., Ahuja, R.: Antibody-dependent enhancement: A challenge for developing a safe dengue vaccine. Frontiers in Cellular and Infection Microbiology 10, 572681 (2020) [5] Halstead, S.B.: Delivering safe dengue vaccines. The Lancet Global Health 12(8), 1229–1230 (2024) [6] Kuhn, R.J., Zhang, W., Rossmann, M.G., Pletnev, S.V.: Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell 108(5), 717–725 (2002) [7] Rouvinski, A., Dejnirattisai, W., Guardado-Calvo, P., Vaney, M.-C.: Covalently linked dengue virus envelope glycoprotein dimers reduce exposure of the immunodominant fusion loop epitope. Nature Communications 8, 15411 (2017)

[8] Kudlacek, S.T., Metz, S., Thiono, D., Payne, A.M.: Designed, highly expressing, thermostable dengue virus 2 envelope protein dimers elicit quaternary epitope antibodies. Science Advances 7(42), 4084 (2021) [9] Phan, T.T.N., Hvasta, M.G., Kudlacek, S.T., Thiono, D.J.: A conserved set of mutations for stabilizing soluble envelope protein dimers from dengue and zika viruses to advance the development of subunit vaccines. Journal of Biological Chemistry 298(7), 102079 (2022) [10] Jacob, F.: Evolution and tinkering. Science 196(4295), 1161–1166 (1977) [11] Moore, A.D., Björklund, A.K., Ekman, D., Bornberg-Bauer, E.: Arrangements in the modular evolution of proteins. Trends in Biochemical Sciences 33(9), 444–451 (2008)

[12] Liu, Y., Di, Z., Gerlee, P.: Ladderpath approach: How tinkering and reuse increase complexity and information. Entropy 24(8), 1082 (2022) [13] Bashton, M., Chothia, C.: The generation of new protein functions by the combination of domains. Structure 15(1), 85–99 (2007) [14] Bornberg-Bauer, E., Albà, M.M.: Dynamics and adaptive benefits of modular protein evolution. Current Opinion in Structural Biology 23(3), 459–466 (2013) [15] Zhang, Z., Liu, C., Zhu, Y., Peng, L.: Evolutionary tinkering enriches the hierarchical and interlaced struc-

tures in amino acid sequences. Physical Review Research 6(2), 023215 (2024) [16] Smug, B.J., Szczepaniak, K., Rocha, E.P.C., Dunin-Horkawicz, S.: Ongoing shuffling of protein fragments diversifies core viral functions linked to interactions with bacterial hosts. Nature Communications 14(1), 7460 (2023) [17] Odai, R., Leemann, M., Al-Murad, T., Abdullah, M.: The viral AlphaFold database of monomers and homodimers reveals conserved protein folds in viruses of bacteria, archaea, and eukaryotes. Science Advances 11(40), 8560 (2025) [18] Li, S., Peng, L., Chen, L., Que, L.: Discovery of highly bioactive peptides through hierarchical structural information and molecular dynamics simulations. Journal of Chemical Information and Modeling 64(21), 8164–8175 (2024) [19] Lipsh-Sokolik, R., Khersonsky, O., Schröder, S.P., Boer, C., Hoch, S.-Y., Davies, G.J., Overkleeft, H.S., Fleishman, S.J.: Combinatorial assembly and design of enzymes. Science 379(6628), 195–201 (2023) [20] Listov, D., Vos, E., Hoffka, G., Hoch, S.Y.: Complete computational design of high-efficiency kemp elimination enzymes. Nature 643, 1421–1427 (2025) [21] Goldenzweig, A., Goldsmith, M., Hill, S.E., Gertman, O.: Automated structure18

and sequence-based design of proteins for high bacterial expression and stability.

Molecular Cell 63(2), 337–346 (2016) [22] Weinstein, J.J., Goldenzweig, A., Hoch, S., Fleishman, S.J.: Pross 2: a new server for the design of stable and highly expressed protein variants. Bioinformatics 37(1), 123–125 (2021) [23] Leaver-Fay, A., Tyka, M., Lewis, S.M., Lange, O.F.: Rosetta3: An object-oriented software suite for the simulation and design of macromolecules. Methods in Enzymology 487, 545–574 (2011) [24] Abramson, J., Adler, J., Dunger, J., Evans, R.: Accurate structure prediction of biomolecular interactions with alphafold 3. Nature 630(8016), 493–500 (2024)

[25] Zhu, K., Su, H., Peng, Z., Yang, J.: A unified approach to protein domain parsing with inter-residue distance matrix. Bioinformatics 39(2), 070 (2023) [26] Mondotte, J.A., Lozach, P.R., Amara, A., Gamarnik, A.V.: Essential role of dengue virus envelope protein n glycosylation at asparagine-67 during viral propagation. Journal of Virology 81(13), 7136–7148 (2007) [27] Yap, S.S.L., Rathinam, S.G., John, J.M.S., Soh, R.P.S.S.: Dengue virus glycosylation: What do we know? Frontiers in Microbiology 8, 1415 (2017) [28] Alford, R.F., Leaver-Fay, A., Jeliazkov, J.R., O' Meara, M.J.: The rosetta all-atom energy function for macromolecular modeling and design. Journal of Chemical Theory and Computation 13(6), 3031–3048 (2017) [29] Steinegger, M., Söding, J.: Mmseqs2 enables sensitive protein sequence searching for the analysis of massive data sets. Nature Biotechnology 35(11), 1026–1028 (2017) [30] Steinegger, M., Söding, J.: Clustering huge protein sequence sets in linear time.

Nature Communications 9, 2542 (2018) [31] Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I.: Expasy: The proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Research 31(13), 3784–3788 (2003) [32] Hon, J., Marusiak, M., Martinek, T., Kunka, A.: Soluprot: prediction of soluble protein expression in escherichia coli. Bioinformatics 37(1), 23–28 (2021) [33] Crill, W.D., Chang, G.-J.J.: Localization and characterization of flavivirus envelope

glycoprotein cross-reactive epitopes. Journal of Virology 78(24), 13975–13986 (2004) [34] Henchal, E.A., Gentry, M.K., McCown, J.M., Brandt, W.E.: Dengue virusspecific and flavivirus group determinants identified with monoclonal antibodies

by indirect immunofluorescence. The American Journal of Tropical Medicine and Hygiene 31(4), 830–836 (1982) [35] Vidarsson, G., Dekkers, G.H., Rispens, T.: Igg subclasses and allotypes: From structure to effector functions. Frontiers in Immunology 5, 520 (2014) [36] Hogenesch, H.: Mechanism of immunopotentiation and safety of aluminum adjuvants. Frontiers in Immunology 3, 406 (2013) [37] Weeratna, R.D., McCluskie, M.J., Xu, Y., Davis, H.L.: Cpg dna induces stronger immune responses with less toxicity than other adjuvants. Vaccine 18(17), 1755–1762 (2000)

[38] Cribbs, D.H., Ghochikyan, N., Vasilevko, V., Tran, M., Petrushina, I., Sadzikava, M., Kesslak, M., Kieber-Emmons, T., Cotman, C.W., Agadjanyan, M.G.:

Adjuvant-dependent modulation of th1 and th2 responses to immunization with beta-amyloid. International Immunology 15(4), 505–514 (2003) [39] Thomas, S.J., Endy, R.G.: Critical issues in dengue vaccine development. Current Opinion in Infectious Diseases 24(5), 442–450 (2011) [40] Greenfield, N.J.: Using circular dichroism spectra to estimate protein secondary structure. Nature Protocols 1, 2876–2890 (2006) [41] Miles, A.J., Janes, R.W., Wallace, B.A.: Tools and methods for circular dichroism spectroscopy of proteins: a tutorial review. Chemical Society Reviews 50(15), 8400–8413 (2021) [42] Manoff, S.B., Sausser, M., Russell, A.F., Martin, J.: Immunogenicity and safety of an investigational tetravalent recombinant subunit dengue vaccine in flavivirusnaive adults: a phase i randomized study. Human Vaccines & Immunotherapeutics 15(9), 2195–2204 (2019) [43] Durbin, A.P., Pierce, K.K., Kirkpatrick, B.D., Grier, P.: Immunogenicity and safety of a tetravalent recombinant subunit dengue vaccine in adults previously vaccinated with a live attenuated tetravalent dengue vaccine: Results of a phase-i randomized clinical trial. The American Journal of Tropical Medicine and Hygiene 103(2), 855–863 (2020) [44] Lee, M.F., Long, C.M., Poh, C.L.: Current status of the development of dengue vaccines. Vaccine X 22, 100604 (2025) [45] Castro, K.M., Watson, J.L., Wang, J., Southern, J., Ayardulabi, R., Georgeon, S., Rosset, S., Baker, D., Correia, B.E.: Accurate single-domain scaffolding of three nonoverlapping protein epitopes using deep learning. Nature Chemical Biology (2025)

[46] Wang, J., Lisanza, S., Juergens, D., Tischer, D., Watson, J.L., Castro, K.M.:

Scaffolding protein functional sites using deep learning. Science 377(6604), 387–394 (2022) [47] Braun, M., Tripp, A., Chakatok, M., Kaltenbrunner, S., Fischer, C., Stoll, D., Bijelic, A., Elaily, W., Totaro, M.G., Moser, M., Hoch, S.Y., Lechner, H., Rossi, F., Aleotti, M., Hall, M., Oberdorfer, G.: Computational enzyme design by catalytic motif scaffolding. Nature 649(8095), 237–245 (2026)

[48] Parra-González, M., Nájera-Maldonado, L., Peralta-Cuevas, E., Gutierrez-zOnofre, A.J.: Overcoming dengue vaccine challenges through next-generation virus-like particle immunization strategies. Frontiers in Cellular and Infection Microbiology 15 (2025) [49] Uhuami, A.O., Lawal, N., Bello, M.B., Imam, M.U.: Flavivirus cross-reactivity:

Insights into e-protein conservancy, pre-existing immunity, and co-infection. The Microbe 4, 100105 (2024) [50] Flores, H.E., Burgos, E.F.P., Ortega, S.C., Heredia, A.: From antibodies to immunity: Assessing correlates of flavivirus protection and cross-reactivity. Vaccines 13(5), 449 (2025) [51] Roehrig, J.T., Hombach, J., Barrett, A.D.T.: Guidelines for plaque-reduction neutralization testing of human antibodies to dengue viruses. Viral Immunology 21(2), 123-132 (2008)

Supplementary information

Table S1 Sequence parts of DENV 1-4 defined by UniDoc.

Serotype

Part 1

Part 2

Part 3

Part 4

DENV-1

MRCVGIGNRD FVEGLSGATW VDVVLEHGSC VTTMAKDKPT LDIELLKTEV TNPAVLRKLC IEAKISNT

TTDSRCPTQG EATLVEEQDT NFVCRRTFVD RGWGNGCGLF GKGSLIT

CAKFKCVTKL EGKIVQYENL KYSVIVTVHT GDQHQVGNET TEHGTTATIT PQAPTSEIQL TDYGALTLDC SPRTGLDFNE MVLLTMKKKS WLVHKQWFLD LPLPWTSGAS TSQETWNRQD LLVTFKTAHA KKQEVVVLGS QEGAMHTALT GATEIQTSGT TTIFAGHLKC RLKMD

KLILKGMSYV MCTGSFKLEK EVAETQHGTV LVQVKYEGTD APCKIPFSSQ DEKGVTQNGR LITANPIVTD KEKPVNIEAE PPFGESYIVV GAGEKALKLS WFK

DENV-2

MRCIGMSNRD FVEGVSGGSW VDIVLEHGSC VTTMAKNKPT LDFELIKTEA KQPATLRKYC IEAKLTNT

TTESRCPTQG EPSLNEEQDK RFVCKHSMVD RGWGNGCGLF GKGGIVT

CAMFRCKKNM EGKVVQPENL EYTIVITPHS GEEHAVGNDT GKHGKEIKIT PQSSITEAEL TGYGTVTMEC SPRTGLDFNE MVLLQMENKA WLVHRQWFLD

LPLPWLPGAD TQGSNWIQKE TLVTFKNPHA KKQDVVVLGS QEGAMH-TALT GATEIQMSSG NLLFTGHLKC RLRMDK

LQLKGMSYSM CTGKFKVVKE IAETQHGTIV IRVQYEGDGS PCK-IPFEIMD LEKRHVLGRL ITVNPIVTEK DSPVNIEAEP PFGDSYIIIG VEPGQLKLNW FK

DENV-3

MRCVGVGNRD FVEGLSGATW VDVVLEHGGC VTTMAKNKPT LDIELQKTEA TQLATLRKLC IEGKITNI

TTDSRCPTQG EAVLPEEQDQ NYVCKHTYVD RGWGNGCGLF GKGSLVT

CAKFQCLEPI EGKVVQYENL KYTVIITVHT GDQHQVGNET QGV-TAEITPQ ASTTEAILPE YGTLGLECSP RTGLDFNEMI LLTMKNKAWM VHRQWFFDLP LPWASGATTE TPTWNRKELL VTFKNAHAKK QEVVVLGSQE GAMHTALTGA TEIQNSGGTS IFAGHLKCRL KMDK

LELKGMSYAM CTNTFVLKKE VSETQHGTIL IKVEYKGEDA PCK-IPFSTED GQGKAHNGRL ITANPVVTKK EEPVNIEAEP PFGESNIVIG IGDNALKINW YK

DENV-4

MRCVGVGNRD FVEGVSGGAW VDLVLEHGGC VTTMAQGKPT LD-FELTKTTA KEVALLRTYC IEASISNI

TTATRCPTQG EPYLKEEQDQ QYICRRDVVD RGWGNGCGLF GKG-GVVT

CAKFSCSGKI TGNLVQIENL EYTVVVTVHN GDTHAVGNDT SNHGV-TAMIT PRSPSVEVKL PDYGELTLDC EPRSGIDFNE MILMKMKKKT WLVHKQWFLD LPLPWTAGAD TSEVHWNYKE RMVTFKVPHA KRQD-VTVLGS QEGAMHSALA GATEVDSGDG NHMFAGHLKC KVRMEKLRIK GMSYT

MCSGKFSIDK EMAETQHGTT VVKVKYEGAG APCKVPIEIR DVNKEKVVGR IISSTPLAEN TNSVTNIELE PPFGDSYIVI GVGNSALTLH WFR

Table S2 24 Possible combinations for modular assembly of chimeric DENV sequences.

Design Name

Part 1

Part 2

Part 3

Part 4

Mosaic-1 Mosaic-2 Mosaic-3 Mosaic-4 Mosaic-5 Mosaic-6

DENV-1 DENV-1 DENV-1 DENV-1 DENV-1 DENV-1

DENV-2 DENV-2 DENV-3 DENV-3 DENV-4 DENV-4

DENV-3 DENV-4 DENV-2 DENV-4 DENV-2 DENV-3

DENV-4 DENV-3 DENV-4 DENV-2 DENV-3 DENV-2

Mosaic-7 Mosaic-8 Mosaic-9 Mosaic-10 Mosaic-11 Mosaic-12

DENV-2 DENV-2 DENV-2 DENV-2 DENV-2 DENV-2

DENV-1 DENV-1 DENV-3 DENV-3 DENV-4 DENV-4

DENV-3 DENV-4 DENV-1 DENV-4 DENV-1 DENV-3

DENV-4 DENV-3 DENV-4 DENV-1 DENV-3 DENV-1

Mosaic-13 Mosaic-14 Mosaic-15 Mosaic-16 Mosaic-17 Mosaic-18

DENV-3 DENV-3 DENV-3 DENV-3 DENV-3 DENV-3

DENV-1 DENV-1 DENV-2 DENV-2 DENV-4 DENV-4

DENV-2 DENV-4 DENV-1 DENV-4 DENV-1 DENV-2

DENV-4 DENV-2 DENV-4 DENV-1 DENV-2 DENV-1

Mosaic-19 Mosaic-20 Mosaic-21 Mosaic-22 Mosaic-23 Mosaic-24

DENV-4 DENV-4 DENV-4 DENV-4 DENV-4 DENV-4

DENV-1 DENV-1 DENV-2 DENV-2 DENV-3 DENV-3

DENV-2 DENV-3 DENV-1 DENV-3 DENV-1 DENV-2

DENV-3 DENV-2 DENV-3 DENV-1 DENV-2 DENV-1

Table S3 62 variants obtained after PROSS antigen stability optimization.

Variant

Mutations

Variant

Mutations

Variant-1-1

Mosaic-1

T68I, N83P

Variant-14-5

Mosaic-14

Variant-1-2

Mosaic-1

T68I, N83P, S95T

Variant-14-6

Mosaic-14

Variant-1-3

Mosaic-1

T68I, N83P, S95T, S66T

Variant-14-7

Mosaic-14

Variant-3-1 Variant-3-2 Variant-3-3 Variant-5-1

Mosaic-3 Mosaic-3 Mosaic-3 Mosaic-5

I65L, T236M I65L, T236M, K122L T236M

Variant-15-1 Variant-16-1 Variant-16-2 Variant-16-3

Mosaic-15 Mosaic-16 Mosaic-16 Mosaic-16

Variant-6-1

Mosaic-6

Variant-16-4

Mosaic-16

Variant-6-2 Variant-6-3

Mosaic-6 Mosaic-6

Variant-17-1 Variant-17-2

Mosaic-17 Mosaic-17

Variant-6-4

Mosaic-6

Variant-18-1

Mosaic-18

Variant-7-1 Variant-7-2 Variant-7-3 Variant-8-1 Variant-8-2 Variant-8-3

Mosaic-7 Mosaic-7 Mosaic-7 Mosaic-8 Mosaic-8 Mosaic-8

Variant-18-2 Variant-18-3 Variant-19-1 Variant-19-2 Variant-19-3 Variant-19-4

Mosaic-18 Mosaic-18 Mosaic-19 Mosaic-19 Mosaic-19 Mosaic-19

Variant-9-1 Variant-10-1 Variant-10-2

Mosaic-9 Mosaic-10 Mosaic-10

Variant-20-1 Variant-20-2 Variant-20-3

Mosaic-20 Mosaic-20 Mosaic-20

I65L, G63A I65L, K83N I65L, T236M I65L, T236M, S66T I65L, T236M, S66T, T81S, K122L V83P, F96Y, A171V V83P, F96Y, A171V, S64K

Variant-11-1

Mosaic-11

K83P, R231H, G112S K83P, R231H, G112S, V96Y, S66T K83P, R231H, G112S, V96Y, S66T, N388H, G180T V83P, T81Y, I114V V83P, T81Y, I114V, F96Y T68I, S122L T68I, S122L, T251V, S262T, S170T, S100Q V81Y, S122V, S120T, G123T K120E, L125M

V83K, G63A, T81Y, Y233H V83K, G63A, T81Y, M237L, Y233H, F96Y V83K, G63A, T81Y, M237L, Y233H, F96Y, V91I, S262T, S122L, S120T, N399H V91I, K93R, G63A V91I, K93R, G63A, S95D V91I, K93R, G63A, S95D, V91I, K93R, G63A, S95D, N83D, Y233H, K64S, G112S G112S, N389S

Variant-20-4

Mosaic-20

Variant-11-2

Mosaic-11

K120E

Variant-20-5

Mosaic-20

Variant-11-3 Variant-11-4 Variant-11-5 Variant-12-1 Variant-13-1

Mosaic-11 Mosaic-11 Mosaic-11 Mosaic-12 Mosaic-13

K120E, L125M, K64R K120E, K64R

Variant-21-1 Variant-22-1 Variant-22-2 Variant-22-3 Variant-22-4

Mosaic-21 Mosaic-22 Mosaic-22 Mosaic-22 Mosaic-22

Variant-14-1 Variant-14-2

Mosaic-14 Mosaic-14

Variant-23-1 Variant-24-1

Mosaic-23 Mosaic-24

Variant-14-3

Mosaic-14

Variant-24-2

Mosaic-24

I65L, V91I

Variant-14-4

Mosaic-14

V83K, G63A V83K, G63A, T81Y, M237L V83K, G63A, T81Y, M237L, I65L V83K, G63A, T81Y, M237L, Y233H 24

V83P, F96Y, A171V, S64K, S66T V83P, F96Y, A171V, S64K, S66T, V91I, A311S N83P, S64K N83P, S64K, S95T, S66T N83P, S64K, S95T, S66T, V379I S64K, R233Q

Variant-24-3

Mosaic-24

I65L, V91I, V323I

Note: Figure translations are in progress. See original paper for figures.