Circular viral copy DNA of Dengue virus (DENV-2) isolated from infected mosquito cell cultures

Warachin Gangnonngiw [1], Wilawan Thongda[1], Nipaporn Kanthong[2], T.W. Flegel[1]

[1]Centex Shrimp, Faculty of Science, Mahidol University, Rama 6 Road,Bangkok 10400, and Thailand, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Klong 1, Klong Luang, Pratum Thani 12120, Thailand

[2]Dept. Biotechnology, Faculty of Science and Technology, Rajamangala University of Technology Tawan-ok, Sriracha, Chonburi 20110, Thailand

Correspondence

Nipaporn Kanthong; E-mail: Nipaporn_ka@rmutto.ac.th

T.W. Flegel; tim.flegel@gmail.com

ABSTRACT

Dengue virus serotype 2 (DENV-2) is a mosquito-borne disease in the family *Flaviviridae*. We have previously shown that DENV-2 can infect C6/36 mosquito cells and cause initial cytopathic effects that dissipate upon serial split-passage to yield persistently infected cultures with normal growth and morphology. In other words, the cell line accommodated persistent DENV-2 infections. It has recently been found that insect viral infections induce production of viral copy DNA (vcDNA) fragments via host reverse transcriptase (RT). The vcDNA occurs in both linear (lvcDNA) and circular (cvcDNA) forms and produces small interfering RNA (siRNA) transcripts that can result in an immediate protective RNA interference (RNAi) response. The vcDNA can also result in host acquisition of endogenous viral elements (EVE) in genomic DNA. Thus, we hypothesized that DENV-2 cvcDNA and DENV-2-EVE would arise in C6/36 insect cells challenged with DENV-2 virus *in vitro*. Here we describe the successful isolation and characterization of cvcDNA constructs homologous to DENV-2 from laboratory challenges with C6/36 cells. At least 1 of these appeared to arise from a DENV-2-EVE. We also show that a linear vcDNA preparation derived from the DENV-2-EVE sequence by PCR significantly reduced DENV-2 replication when applied to

naive C6/36 cells prior to DENV-2 challenge. This is preliminary work designed to lay the groundwork for further studies on use of the C6/36 cell model for screening and characterization of protective EVE against both insect and shrimp viruses.

Keywords: Anti-viral immunity; Dengue virus; C6/36 mosquito cells, circular viral copy DNA (cvcDNA); endogenous viral element(s)(EVE);

INTRODUCTION

It was formerly believed that invertebrates were incapable of specific adaptive immune responses and that they depended solely on generic responses via innate immune mechanisms (1). More recently, it has been shown that, although insects and crustaceans lack a mechanism for a specific adaptive immune response via antibodies, they are capable of a specific adaptive immune response against viruses via nucleic acids. A major feature of this response is persistent, relatively innocuous viral infections. This response has been called viral accommodation and has been recently reviewed (2, 3), as has the work related specifically to insects (4). In brief summary, endogenous host reverse transcriptase (RT) recognizes and binds with viral RNA to produce viral copy DNA (vcDNA) in both linear (lvcDNA) and circular (cvcDNA) forms. This leads to the production of small interfering RNA (siRNA) that results in individual cell and systemic RNA interference (RNAi) responses. At the same time, some of the vcDNA fragments may be integrated into the cell genomic DNA as endogenous viral elements (EVE). The EVE may also produce antisense RNA leading to an RNAi response. In addition, the EVE may give rise to cvcDNA, a feature that allows for the detection of EVE in uninfected individuals because of the relative ease of isolating and sequencing it (5).

We previously showed that C6/36 insect cells can be sequentially infected with *Aedes albopictus* densovirus (AaDNV), Dengue virus serotype 2 (DENV-2) and *Japanese encephalitis* virus to result in individual cells with innocuous, persistent triple infections that can be maintained by serial split passage (6). The mechanism underlying the ability of the cells to accommodate 3 viruses without signs of cytopathic effects (CPE) or other signs of disease was unknown at that time. More recent work described in the previous paragraph suggested that induction of host RT and production of vcDNA might be involved. Thus, in this communication, we show that cvcDNA homologous to DENV-2 can be isolated and characterized from C6/36 cells that have been challenged with DENV-2. We also show that

DENV-2-EVE arose in the challenged, cultured cells and that a linear DNA copy of one of these cvcDNA fragments produced *in vitro* by PCR could significantly reduce the replication of DENV-2 in pre-exposed, naive C6/36 cells subsequently challenged with DENV-2.

MATERIALS AND METHODS

Circular DNA (cirDNA) extraction

DENV-2 persistent cultures were established as previously reported (6, 7). Briefly, 80% confluent monolayers of naive C6/36 cells in 6-well plates were challenged with DENV-2 stock at 10^5 copy/well. After incubation at room temperature for 2 hours with gentle shaking, the medium was removed and 2 ml fresh medium containing 2% FBS was added for further incubation at 28°C. The DENV-2 infected cultures were sub-cultured every 2 days for 7 passages.

Total DNA extraction was done using a DNA Extraction kit (GeneAll, Korea) on cells and culture media from each of passages 1-7. Thereafter, the total DNA extracts from each passage were pooled and processed to prepare circular DNA (cirDNA). Briefly, exonuclease V was used to digest the linear DNA in the sample prior to isolation of the residual putative cirDNA using a DNA clean-up kit (NucleoSpin gel and PCR clean-up kit, Macherey-Nagel, Germany). The final cirDNA preparation was dissolved in TE buffer, and the DNA concentration was measured using a nanodrop (Thermo sciencetific, USA).

A linear cDNA, RT-PCR product (1736 bp) was prepared from a *Macrobrachium rosenbergii nodavirus* (MrNV) genomic RNA as a template to serve as a linear DNA positive control, while pGEM plasmid (cirDNA) served as a negative control for Exonuclease V enzyme activity in control digestion processes. For C6/36 cirDNA extracts, the positive control for cell linear DNA was PCR for the presence of the *Aedes* elongation factor gene using primers EF165_*Aedes*_F/R while the negative control was PCR for the presence of C6/36 cell mitochondrial DNA using primers for CytochromeC/Aedes_125F/R and ATPSynthase_150F/R (Table1). The pooled cirDNA preparation was used directly for library construction and sequencing without any PCR amplification step.

cirDNA library construction and sequencing

DNA library preparation and sequencing were performed by GENEWIZ, Inc. Suzhou, China. A TruSeq DNA Library Preparation Kit (Illumina) was used for cDNA library preparation,

following the TruSeq protocol. Briefly, DNA was fragmented by sonic disruption using a Covaris S220 apparatus (Covaris). Subsequently, blunt-end fragments were created, and dA-base was tailed to each 3'side. Dual indexes within P5 and P7 adapters were ligated to the fragments. The final products were purified. Then the purified DNA was size selected before PCR amplification for library construction. Initial DNA concentration was quantified using Qubit 3.0 and fragment size was determined using Agilent 2100. The final library samples were additionally examined by Agilent 2100. Afterward, amplified libraries were sequenced on an Illumina HiSeq 2000 instrument with 150 bp pair-end (PE).

The raw reads were generated in FASTQ format. The company moreover cleaned up raw reads by removing the adapter sequences. The reads with low-quality scores and the reads containing ambiguous nucleotides (N) greater than 10 percent were removed. Clean reads were obtained for further bioinformatics analysis. The clean raw reads were *de novo* assembled into contigs using QIAGEN CLC Genomics Workbench (version 12; CLC Bio, a QIAGEN company Aarhus, Denmark). Subsequently, the assembled contigs were used as query sequences to search TBLASTN against the Dengue virus serotype 2 complete genome database (accession no. M29095.1). All assembled contigs were annotated using a local database of *A. albopictus* (host) proteome with ID of UP000069940 derived from UniProtKB.

Variation of putative DENV-2 cvcDNA in persistently infected cultures

To study the prevalence of each cvcDNA in the persistently infected DENV-2 cultures, specific primers were designed from selected contig sequences using Primer-Blast at NCBI (Table 1). PCR amplification with each primer using a portion of the original, pooled cirDNA sample as a template were carried out using KOD FX Neo polymerase (Toyobo, Japan), following the manufacturer's instructions for PCR conditions. The PCR products were examined by 1.5% agarose gel electrophoresis and subjected to nucleotide sequencing using BTSeqTM Contiguous Sequencing (Celemics, Korea). All sequence results were aligned with either the *Aedes albopictus* genome (6 assemblies for this genome, 2 RefSeq GCF_006496715.1 and GCF_001876365.2) or the DENV-2 complete genome (accession no. M29095.1).

Amplicon bands of interest, including DNA fragments encoding *Aedes albopictus* or DENV-2 sequences, were cut and purified using QIAquick Gel Extraction Kit (Qiagen, USA) before

134 cloning into pGEM-T easy vector (Promega, USA). Plasmids prepared from selected clones
135 were sent for sequencing using T7/SP6 primer (Macrogen, Korea).
136 **Table 1.** Primers used to amplify DENV-2 sequences in the cirDNA preparation.
137

Primer name	Sequence (5'-3')	expected amplicon size
C6/36 cell linear DNA, elongation factor		
EF165_Aedes_F/R	CCCCAGTAAGGAGCAAGCAG CCAGCGGGATCGGATTGTAG	165
C6/36 cell mitochondrial DNA		
CytochromeC/Aedes_125F/R	TGCTACAGGATTTCATGGACTT ATCAAGCTGCTGCTTCAAAACC	125
ATPSynthase_150F/R	CCTCAAGGCACCCCTCCTAT AGGGCCGGTATTTCCTAAAAGA	150
DEN-2/NS4A,NS4B region		
DEN-2 Contig A1 F/R	CACAAGGAGTATCGTCGGCA CAGGTGGAAGGGCGTACAAT	234
DEN-2 Contig A2 F/R	CGGCAGTTCACTGAGAGCAT TGGGTTTTTCATGATGCCCG	210
DEN-2/Contig A3-NS4A_273_F/R	TCACAGAAATGGGCAGGCTTC CGTACCATAGGAGAATACTAGCCG	273
cvDEN2_300F/R	TCCCTATACCCCTTCCGCTC GGGTTTTTCATGATGCCCGC	300
cvDEN2_320F cvDEN2_320R	ACAAGGAGTATCGTCGGCAG TCACAGAAATGGGCAGGCTT	320
DEN-2 NS 5 region		
DEN-2 Contig B1 F/R	TTCTTGTGTCCCATCCTGCG GCGTCGCTTTTCATTCGGAG	213
DEN-2 Contig B2 F/R	GCACCACCTTGTTTTGGTACG AAGGAGAAGGGCTGCACAAG	213
cvDEN2_292 F/R	GGCCTCGGCTAGTTTCTTGT TTGCGTCGCTTTTCATTCGG	292
cvDEN2_297F/R	AATGGCCTCGGCTAGTTTCTT GCTTGCGTCGCTTTTCATTC	297
DEN-2 qPCR primer		
D2S	GTTCGTCTG CAAACACTCCA	
D2C	GTGTTATTTTGATTTCCTTG	

138

139 **Anti-DENV-2 activity tests using linear vcDNA (lvcDNA) produced by PCR**

140 A PCR product of 273 bp with 99% sequence identity to the DEN-2-NS4A gene in GenBank
141 record (accession no. M29095.1) was obtained by using primers (A3-NS4A_273_F/R)

designed from DEN-2 Contig A3 (Table 1) with the crude cirDNA extract as a template. Thus, it was an *in vitro*, linear DNA construct that matched a specific region of DENV-2 that we called an artificial, linear viral copy DNA (lvcDNA) fragment obtained using the cirDNA preparation as a template.

For the anti-DENV-2 activity test (see **Fig. 1** for a diagram of the protocol), naive C6/36 cells were seeded into 6-well plates at a density $5x10^6$ cells/well and incubated for 24 h at 28°C to produce 80% confluent monolayers before transfection using liposome Escort IV Transfection Reagent (Sigma) according to the manufacturer's instructions. Briefly, 10 and 100 ng of lvcDNA in 100 µl medium mixed with 2 µl Escort IV Transfection Reagent were each added to 100 µl of medium and allowed to form complexes for 45 minutes at room temperature. Then the mixtures were added dropwise (200 µl) to the cell monolayers in 800 µl fresh media. After 5 hours swirling the plates gently, they were incubated at room temperature for 16 h. Then they were challenged with DENV-2 stock at 10^5 copy/well. After incubation for 2 h with gentle shaking at room temperature, the medium was removed and 2 ml fresh medium containing 2% FBS was added for further incubation at 28°C. At 48 h post challenge, cells and culture medium from each well was collected separately and total RNA extraction was carried out using a QIAamp Viral RNA kit (Qiagen, USA). The DENV-2 infected cultures in this experiment were sub-cultured and new lvcDNA was added by repeated transfection steps, every 2 days for 3 passages. Naive and negative controls (no lvcDNA treatment) were run in parallel in the same manner.

DENV-2 numbers were quantified by realtime RT-PCR using DENV-2 specific primers, D2S/D2C (Table 1). The reaction mixtures contained 100 ng RNA template per sample diluted 2-fold with qPCR solution (iTaq Universal SYBR Green One-Step Kit, Bio-Rad) containing the primers D2S and D2C. DNA fluorescence was monitored in real-time using CFX Connect Real-Time System (Bio-Rad). The C6/36 cultures challenged with DENV-2 and naive C6/36 cultures served as positive and negative controls, respectively.

RESULTS

Verification of cvcDNA isolation

To verify the isolation of cvcDNA from DENV-2 by our isolation process, the final cirDNA preparation and the parallel controls were subjected to agarose gel electrophoresis before DNA sequencing and library construction. The total amount of undigested DNA in the pooled

extract was 29.1 ng/µl in a total of 30µl (total 873 ng) prior to linear DNA digestion and 14.3 ng/µl in a total of 20 µl (total 286 ng) after digestion. The parallel control test results demonstrated that each step of the exonuclease V enzyme activity was capable of eliminating 1 µg linear DNA from the DNA sample (PCR product) while retaining the 1 µg circular DNA (pGEM marker) unaffected (data not shown). These parallel control tests confirmed the absence of linear DNA but presence of circular DNA in the control exonuclease treatments.

We confirmed purity of cvcDNA again by PCR amplification using specific primers for host linear DNA (negative amplification confirming absence) and mitochondrial genes (positive amplification confirming presence). The cytochrome c oxidase and ATP Synthase, genes in mitochondrial DNA gave amplicons with the cvcDNA sample while no amplicons were obtained for elongation factor (**Fig 2**). These results suggested that only host linear DNA was eliminated while cirDNA forms still remained. In addition, to measure the number of host mitochondrial gene sequences in the cvcDNA preparation a total of 58,365,954 clean raw reads were compared to *Aedes albopictus* mitochondrion complete genome sequence (accession no. NC_006817). The blastN results found 7,287,271 raw reads (12.49%), 70-274 bp in length (mean length 150bp) with sequences intermittently ranging over the whole mitochondrial genome. The quality and quantity of cvcDNA (0.30 µg) in the final preparation was considered enough to generate at least one library.

Overall sequencing results from the cirDNA extract

The sequence results from the cvcDNA sample (**Table 2**) show that a total of 56,018 contigs were recovered during *de novo* assembly. Mean contig size was 872, with lengths ranging from as small as 300 bp to as a large as 625,625 bp. The N50 length was estimated to be 1,012 bp, and almost 99% of the final assemblies were >300 bp.

Table 2 Summary of contig measurements for cvcDNA

	Length
Percentile 75	464
Percentile 50	1,012
Percentile 25	45,813
Minimum	300
Maximum	625,625
Average	872
Count	56,018
Total	48,852,228

To evaluate the number and variety of DENV-2 gene sequences in the cvcDNA preparation, a total of 58,365,954 clean raw reads were analyzed for similarities to DENV-2 sequences. The blastN results found a total of 122 raw reads ranging from 60-289 bp in length (mean length 150 bp). These short reads and their frequency of occurrence were mapped against the whole DENV-2 genome in **Fig. 3**. The distribution spanned the whole genome but in an uneven manner.

To characterize gene composition of cvcDNA, all contig sequences were compared to find similarities between *A. albopictus* and DENV-2 sequences (**Table 3**). Excluding host transposable element sequences such as reverse trancriptase, endonuclease, integrase, retrotransposon gag domain and helicase, the TBLASTN results gave 9,895 contigs (17.66%) with homology to the sequences of *A. albopictus* (host) and 2 contigs with homology to the cDENV-2 sequence. These were DENV-2 Contig A (contig no. 43905) with 400 bp DENV-2 content (100% identity) and DENV-2 Contig B (contig no. 54355) with 307 bp DENV-2 content (100% identity). These accounted for less than 0.01% of the total sequencing results. The other contigs (82.33%) encoded unknown sequences. The sequences of DENV-2 Contig A and B are mapped against the relevant portion of the DENV-2 genome in **Fig. 4**. It can be seen that Contig A could not have arisen directly from a DENV-2 genome sequence because of the disjunctive and opposing reading directions of the NS3 and NS4 portions of the viral genome that are connected in the contig. In addition, both contigs contained portions of the host genome linked to the DENV-2 sequences. These features suggest that this circular DNA entity may have arisen from an EVE of DENV-2 that was acquired after DENV-2 infection.

Table 3. Contig descriptions.

Contig description	Contig Length (bp)	Interesting gene fragments in the contigs
Total contig	56,018	
DENV-2 gene contigs	2	Contig A containing NS4A/NS4B genes Contig B containing NS5 gene
A. albopictus genes in contigs	9,895	1,108 contigs containing reverse transcriptase gene 853 contig containing endonuclease gene 419 contig containing integrase gene 61 contig containing retrotransposon gag domain 42 contig containing helicase gene
Unknown sequence contigs	46,121	

Confirmation of DENV-2 contigs A & B in the cirDNA preparation

Based on the sequences of DENV-2 contigs A and B, primers were designed to specifically amplify the portion of their respective contigs that were 100% identical to the DENV-2 reference sequence (primer set DEN2/NS4A_273 in Table 1) using a portion of the final putative cirDNA preparation as a positive control template. The negative control template was DNA extracted from naïve C6/36 cells. The positive control cirDNA gave the expected band of 273 bp. However, the cvcDNA template gave no visible band at 273 bp but instead multiple bands greater than 273 bp (**Fig. 5A**). The obvious amplicon bands (example, 1,000 bp, lane 3) were cloned and sequenced but yielded *A. albopictus* genome sequences together with unknown DNA sequences. Because of number of DENV-2 genes in the clean raw reads indicated low copy numbers of cvcDNA in the cirDNA preparation, nested PCR tests were carried out using the PCR product from the first round as the template for the second. In this experiment, we obtained the expected band (**Fig. 5B**). The visible bands were purified and sequenced and showed 99% identity to the matching region of the NS4A gene of DENV-2 GenBank record (accession no. M29095.1). These results supported the presence of DENV-2-cvcDNA in the cvcDNA preparation, but a complete library of the circular forms it contained remains to be explored.

DENV-2 tolerance in C6/36 cells accelerated by lvcDNA treatment

In viral accommodation, vcDNA arises in linear (lvcDNA) and circular (cvcDNA) forms via endogenous host RT activity leading to a viral-tolerant, persistent infection state. Here, a 273 bp linear, DENV-2 lvcDNA amplicon obtained using the DENV-2 cvcDNA preparation as a template was used in pre-exposure tests with naive C6/36 cells prior to DENV-2, challenge. The consistent outcome for naive cells challenged with DENV-2 is always a significant occurrence of CPE in subculture Passage 1 followed by a progressive decrease in CPE until the 3rd or 4th passage onward, by which time viral infection progresses to the persistent state characterized by normal cell morphology (7). This is shown in **Fig. 6** (top row) in which CPE can be readily observed at Passage 1 (**Fig. 6C**) but has been resolved by Passage 2 (**Fig 6E**) in the untreated cells. This contrasts with the cells treated with DENV-2-lvcDNA at Passage 1 (**Fig. 6D**) where no CPE has occurred. By Passage 2, cells in both the treated and untreaded groups show no distinguishable morphological differences. In summary, lvcDNA treatment blocked the expression of CPE upon challenge with DENV-2.

DENV-2 replication was unaffected by lvcDENV-2 DNA treatment

264 At 48 hours post DENV-2 challenge, the copy numbers of DENV-2 were determined in both
265 cells and media of cultures treated with DENV-2-lvcDNA as shown in **Fig. 7**. Overall,
266 DENV-2 copies in the cells and media of the treated cells did not differ from those in the
267 untreated cultures at 48h after DENV-2 challenge. However, within both the treated and
268 untreated cultures the relative copy numbers were always higher in the cells than they were in
269 the medium for both the lvcDNA treated and untreated cell cultures. This did not correspond
270 with the situation at Passage 1 where the untreated cells showed CPE while the treated cells
271 did not. One might have expected that the untreated cultures showing CPE at Passage 1
272 would have shown significantly higher DENV-2 loads in the cells and medium than the
273 treated cells. Instead, the results revealed that the difference in CPE occurrence in the
274 untreated cells was not associated with a difference in DENV-2 viral load when compared to
275 the treated cells. This is an unexpected result that suggests the existence of a cell death
276 pathway that is associated with DENV-2 infection but is apparently not induced by a viral
277 threshold load. Whatever this pathway might be, it was apparently blocked by treatment with
278 DENV-2 lcvDNA. This phenomenon is worthy of further investigation.
279
280 **DISCUSSION**
281 We have confirmed that DENV-2 infections in C6/36 cell cultures, like infections of DENV-
282 1 described in earlier work (8), also result in a specific, adaptive immune response which
283 involves production of vcDNA by endogenous host reverse-transcriptase. We have shown
284 that this vcDNA includes circular forms that can be differentially isolated. Sequencing of
285 these circular forms revealed that one type had likely arisen from a host EVE rather than
286 directly from the infecting virus, similar to what was previously reported for a parvovirus in
287 shrimp (5).
288
289 Our previous work using C6/36 cells was carried out to test hypotheses regarding the shrimp
290 response to viral pathogens because no immortal crustacean cell lines existed then or even
291 now to do such work (6, 7, 9-11). Those earlier studies revealed the ability of C6/36 cells to
292 individually support three different shrimp viruses (WSSV, TSV and YHV) that remained
293 infectious for shrimp up to 5 passages but not thereafter. On the other hand, the insect cells
294 did remain immunopositive for those 3 viurses for up to 30 split passages and they were able
295 to transfer immunopositive status to hemocytes of challenged shrimp. This suggested the
296 possibility of using insect cells as a source of attenuated viurses for injection into shrimp to
297 protect (i.e., vaccinate) against disease caused by cognate viruses. A first attempt to do this

with yellow head virus (YHV) failed (12). However, the hemocytes of the injected shrimp in the failed vaccination attempt were converted to immunopositive status for YHV, and there was a significant delay in their mortality. Since the "attenuated vaccine" comprised a crude extract from whole insect cells, it is possible, based on the results described herein, that vcDNA from YHV in those extracts contributed to this delay in mortality. In addition, we have revealed here that lvcDNA prepared *in vitro* by PCR targeting an EVE of DENV-2 can reduce CPE but not reduce DENV-2 replication in naive cells challenged with DENV-2. This contrasts with a previous report with shrimp showing that a crude cvcDNA extracted from shrimp infected with the parvovirus IHHNV could result in a significant decrease in IHHNV replication (5). The specific prevention of CPE by lvcDNA treatment without an accompanying decrease in DENV-2 replication is worthy of further investigation.

These results have shown that C6/36 cells may be used as a convenient model to study the detailed mechanisms by which cvcDNA is produced directly from viral RNA and to study whether these mechanisms differ from those that give rise to cvcDNA from EVE. We hypothesize that C6/36 cells or other immortal insect cell lines may also produce cognate cvcDNA and EVE when challenged with shrimp viruses. If so, they might provide a convenient tool to develop EVE-clone libraries to facilitate screening for the most protective EVE or combinations thereof for use with shrimp. This is already possible, of course, for insect viruses in general using their matching immortal cell lines.

ACKNOWLEDGEMENTS

This research project was supported by Rajamangala University of Technology Tawan-ok (Basic Research Fund: fiscal year 2021,project no.50063) and by the National Research Council of Thailand (NRCT): High-Potential Research Team Grant Program, grant no. (N42A650869).

Figure caption

Figure 1. Diagram of the protocol for the lvcDNA activity test.

Figure 2. Verification of the cirDNA preparation via linear DNA and mitochondrial gene amplicons. Agarose gel showing positive PCR amplicons for elongation factor primers prior

to linear DNA digestion (Lane 1) but a negative PCR result after linear DNA digestion (Lane 4). For mitochondrial genes, positive PCR amplicons were obtained for ATP synthase and cytochrome c oxidase prior to linear DNA digestion (lane 2, 3) and after linear DNA digestion (lane 5,6). M is a GeneRuler DNA ladder mix.

Figure 3. Diagram showing the number, relative length and distribution of 122 raw reads obtained from the cvcDNA sequencing results mapped against the reference DENV-2 genome.

Figure 4. Scale diagram of the DENV-2 content of Contigs A and B mapped against the relevant portions of the DENV-2 genome. (A) Contig A consisting of a leading host sequence of 46 bases that gave no significant hit to the *A. albopictus* genome sequence at GenBank. (B) The 307 base Contig B with 100% identity to the matching region of the DENV-2 GenBank record M29095.1. The portions of the contig sequences in green highlight are host genome sequences.

Figure 5. Photographs of agarose gels showing PCR amplicons obtained using primer set DEN2/NS4A_273 with the cvcDNA preparation as the template. (A) One-step PCR results showing in Lane 1 no amplicon with the negative control while Lane 2 (positive control) shows the expected amplicon of 273 bp. Lane 3 shows one-step PCR results from the cvcDNA template with no band at 273 bp, but multiple bands for longer amplicons, including one at 1000 bp (red box). Lane M is a GeneRuler DNA Ladder Mix. (B) Nested PCR results show an expected amplicon of 273 bp (green box) plus a distinct band (1,000 bp) (red box) from the first round of PCR in Lane 1. Lane M is a GeneRuler DNA Ladder Mix.

Figure 6. Cell morphology of DENV-2 infected cells treated or not with DENV-2 lvcDNA (100 ng) prior to and continuing after DENV-2 challenge. Note the lack of CPE at 48h in both control cells and lvcDNA treated cells. However, the untreated cells show significant CPE at Passage 1 while those treated with lvcDNA do not. At Passage 2, both cultures show normal morphology typical for cells persistently infected with DENV-2.

Figure 7. DENV-2 copy numbers for naive C6/36 cells challenged with DENV-2 followed by treatment with DENV-2 lvcDNA. (A) Copy numbers in cell extracts. (B) Copy numbers in

the cell culture medium. Note about 100 times lower copy numbers in the medium than in the cells but no significant differences between cells treated or untreated with lvcDNA.

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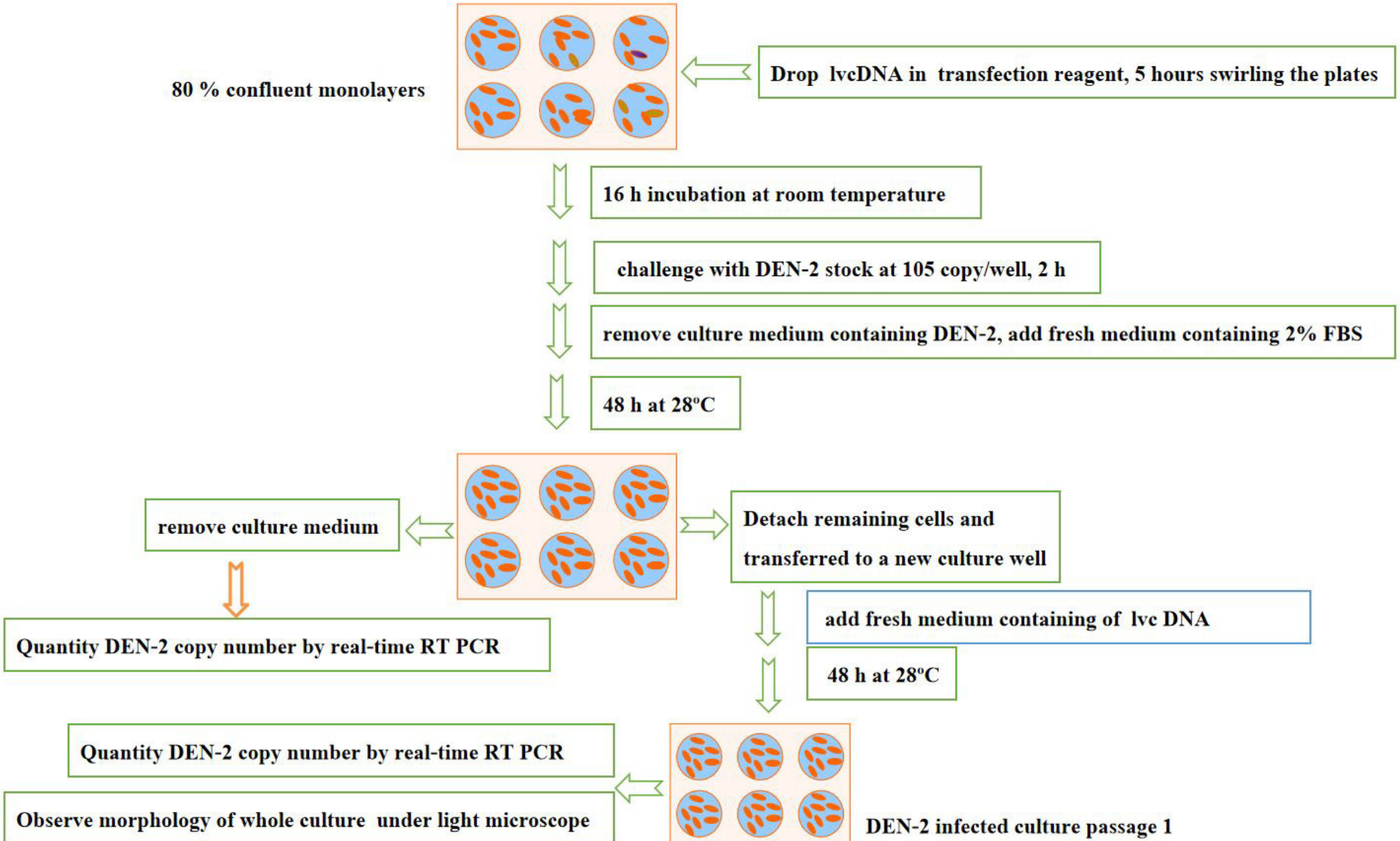
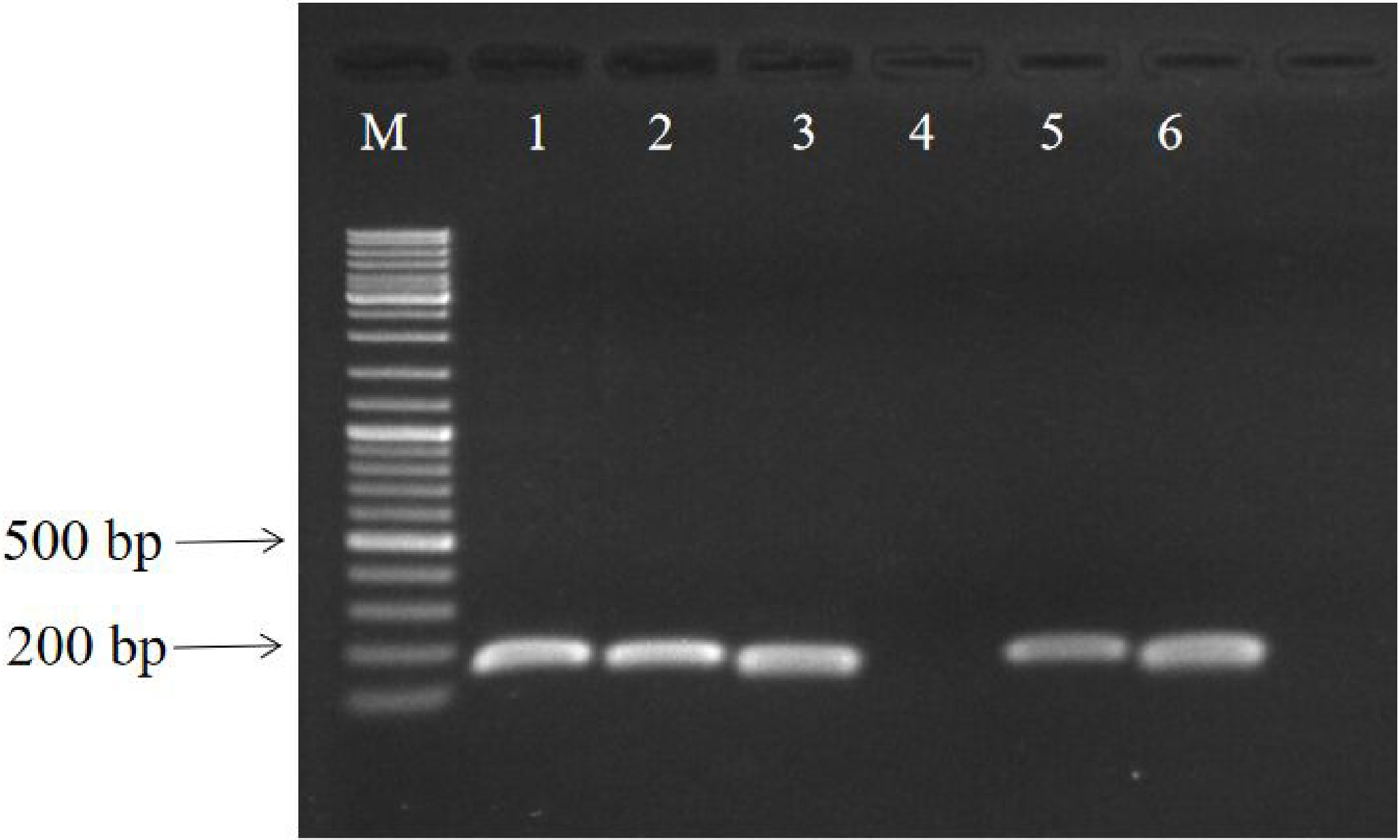

80 % confluent monolayers
Drop lvcDNA in transfection reagent, 5 hours swirling the plates
16 h incubation at room temperature
challenge with DEN-2 stock at 105 copy/well, 2 h
remove culture medium containing DEN-2, add fresh medium containing 2% FBS
48 h at 28°C
remove culture medium
Quantity DEN-2 copy number by real-time RT PCR
Detach remaining cells and transferred to a new culture well
add fresh medium containing of lvc DNA
48 h at 28°C
Quantity DEN-2 copy number by real-time RT PCR
Observe morphology of whole culture under light microscope
DEN-2 infected culture passage 1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1
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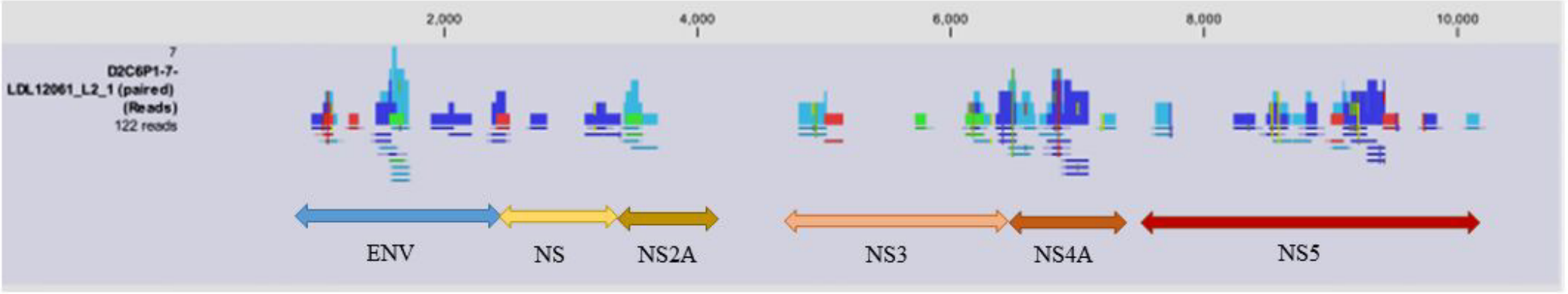
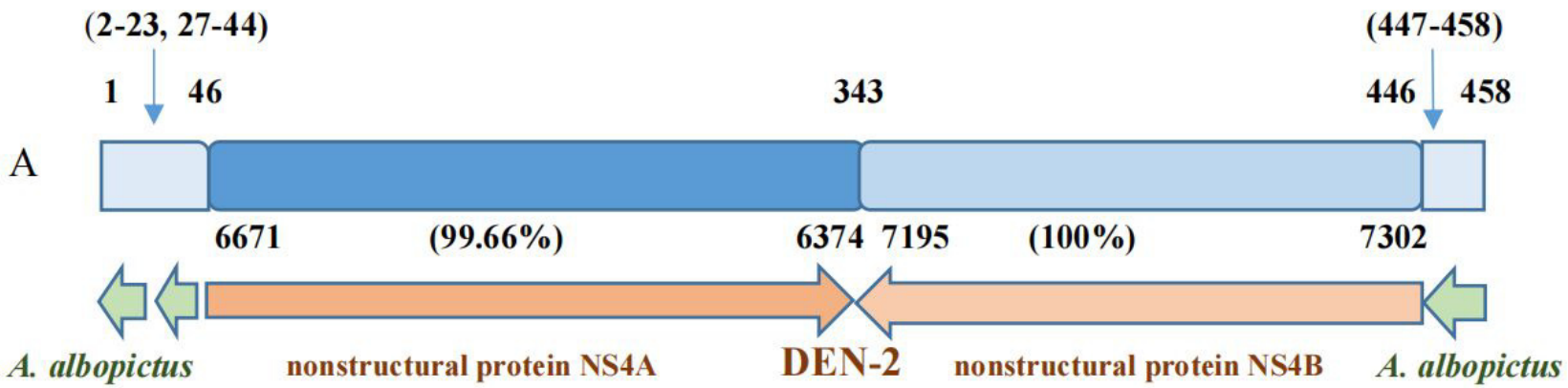
500 bp
200 bp


2,000
4,000
6,000
8,000
10,000
7
D2C6P1-7-
LDL12061_L2_1 (paired)
(Reads)
122 reads
ENV
NS
NS2A
NS3
NS4A
NS5

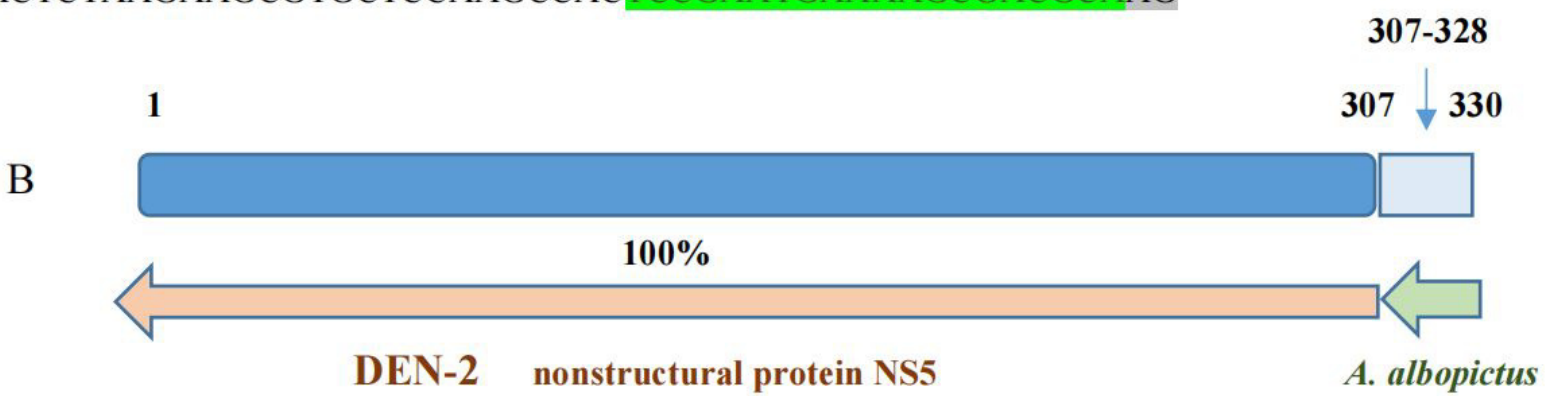
>D2C6P1-7_contig_A(43905) (458 bp)

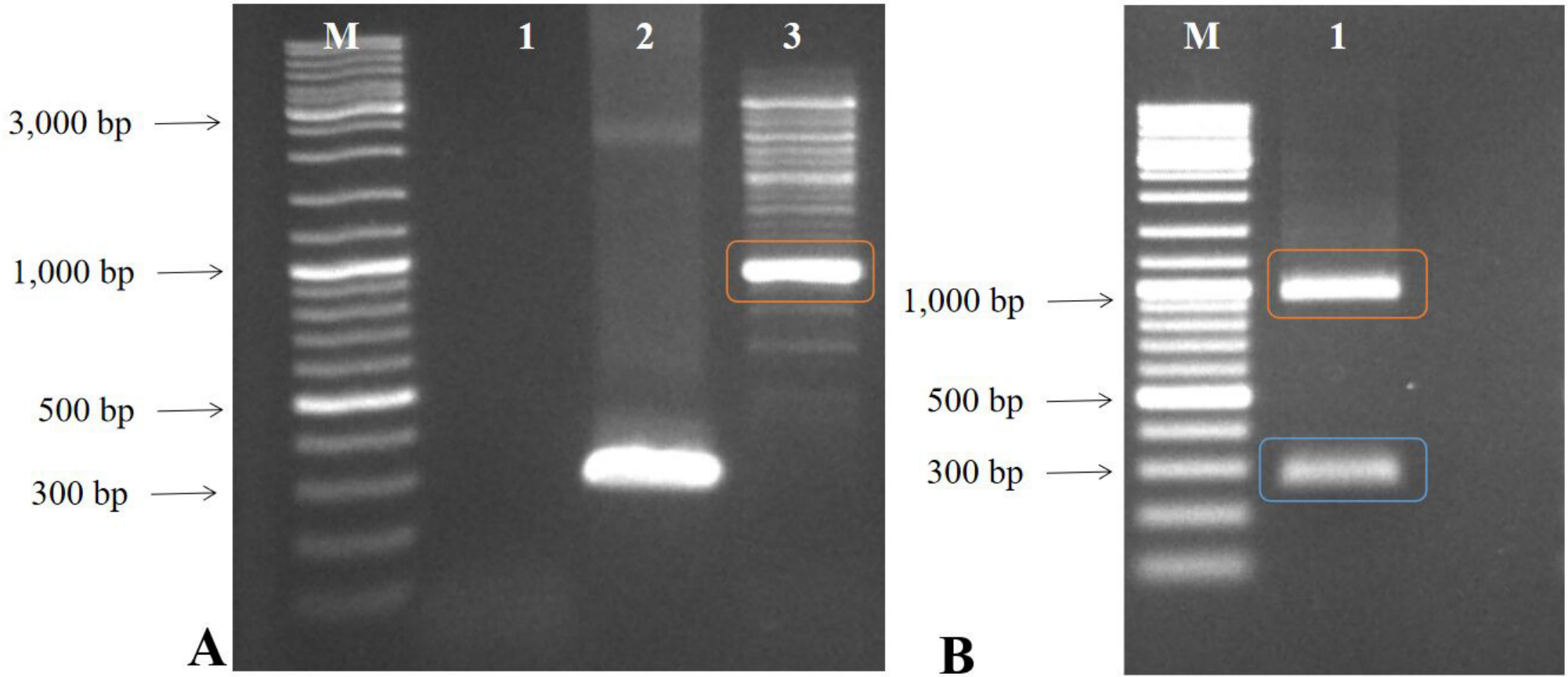
ACACAAGGAGTATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGTGCGTACCATAGGAGAATACT
AGCCGTGATTATGCAGCACATTCCCAGGGTCATCTTCCCTATACCCCTTCCGCTCATCAAGAATAAAAA
GATTCCTCCTGTGACTGTAGCCAGAAGTGTCAGTAAAAGCAATGTCTCCAGGGTCTCCGGCAGTTCACT
GAGAGCATGATTGTACGCCCTTCCACCTGCTTCAGCCGTGTGCAGCACTGCTAAGTTGTCCAGTGCGTC
TCTTGCCTTCTGAGTCATGAAAGTTGGAAGCCTGCCCATTTCTGTGATTAGGTTCAGGGTCAGGGACTC
CAAGCAAAAGCAACCAGAGAAGCTCAGAAAAGAGCAGCAGCGGGCATCATGAAAAACCCAACTGTCG
ATGGAATAACAGTGATTGACCTAGATCCAATACCCCTGTCTCTTATA

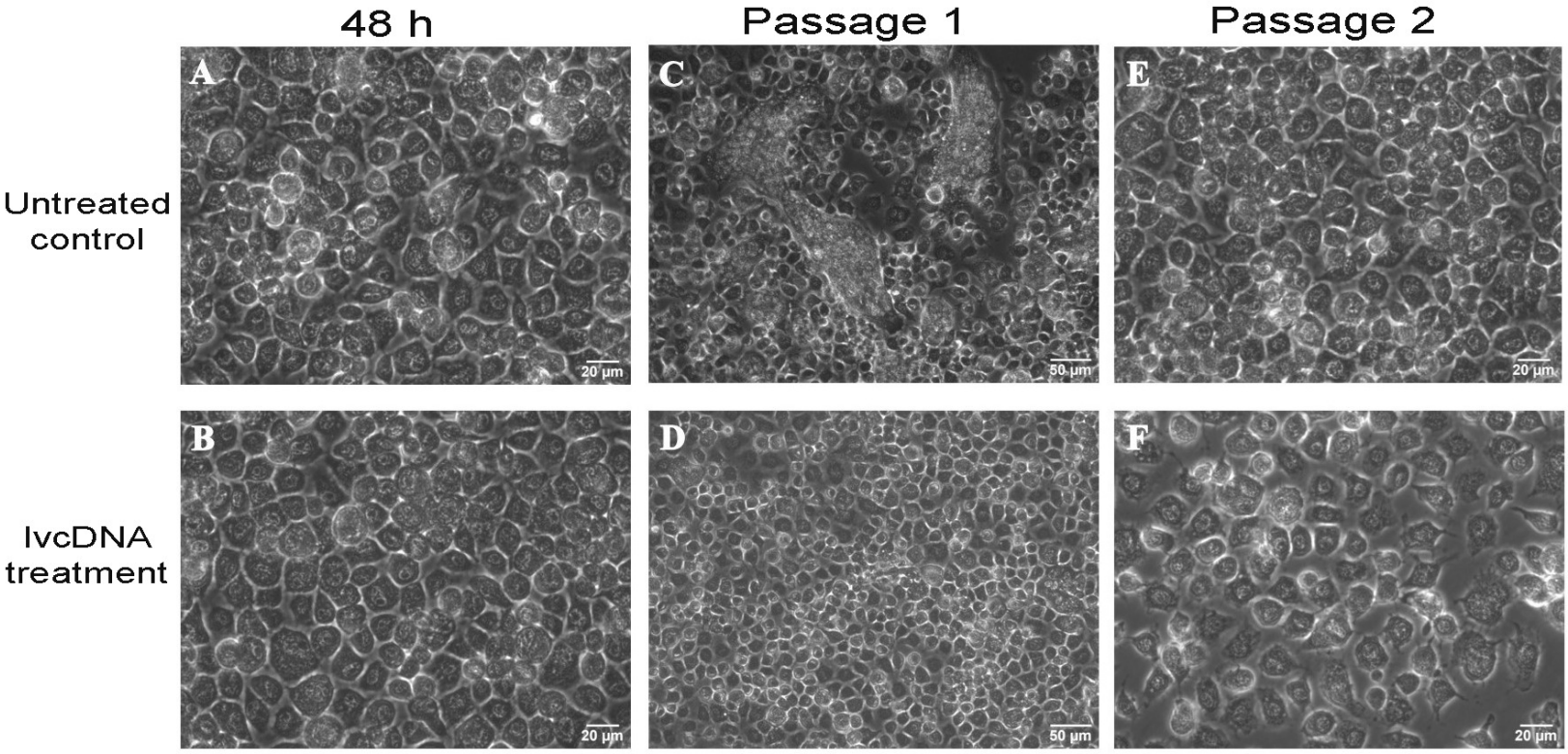


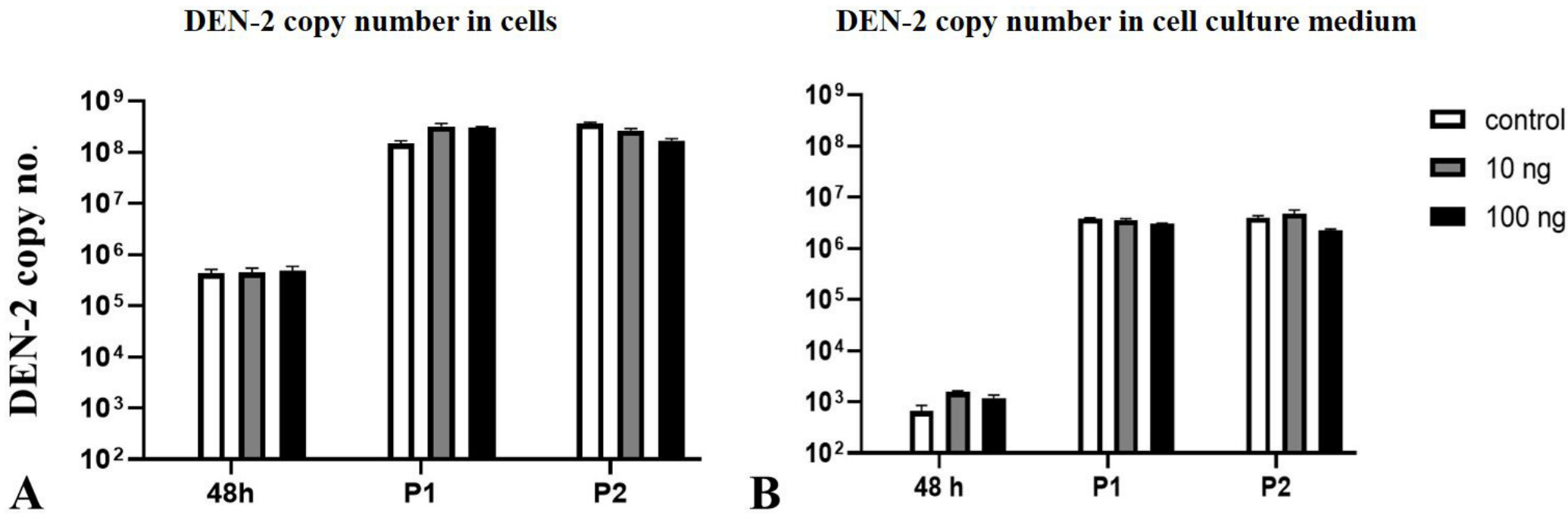
>D2C6P1-7_contig_B(54355)(330 bp)

ACGCACCACCTTGTTTTGGTACGTTAATTTGAAAATGGCCTCGGCTAGTTTCTTGTGTTCTCCTTCCATG
TGGTTTGTTACCATTTCCTCATTTTTTAGGTCTTCTAGTGTGATTCTTGTGTCCCATCCTGCGGTGTCATC
GGCATACATTGCTCCTCCCTCTTTCTTGCTCACGTCTCTTAAAATGTAACCTAGCTTGTGCAGCCCTTCT
CCTTCCACTCCACTCAAGGAGTTCTCTCTGGAGAACCAGTGATCTTCATTCAAGAATCCTAGGGCTTCA
AACTCTAAGAAGCGTGCTCCAAGCCACTCCGAATGAAAAGCGACGCAAG




M
1
2
3
3,000 bp
1,000 bp
500 bp
300 bp
A
M
1
1,000 bp
500 bp
300 bp
B


48 h
Passage 1
Passage 2
Untreated control
IvcDNA treatment
A
B
C
D
E
F
20 µm
50 µm
20 µm
20 µm
50 µm
20 µm


DEN-2 copy number in cells
DEN-2 copy no.
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
48h
P1
P2
A
DEN-2 copy number in cell culture medium
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
48 h
P1
P2
B
control
10 ng
100 ng