

## A novel approach for preparation of minicircle HSV amplicons by adenovirus mediated Cre-loxP recombination in mammalian cells

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**Abstract:** Amplicon is a plasmid backbone based helper-dependent pseudovirion vector of HSV-1 and has been used as a powerful and versatile gene delivery vehicle due to its unique features. However, like plasmid-based vectors, the major drawback of conventional amplicon for gene delivery is its transient transgene expression, which has been demonstrated that the bacteria elements in the vectors are responsible for the transgene loss event, as evidenced, minicircle DNA and minicircle amplicon devoid of bacteria sequences mediated higher and sustained gene expression both in vitro and in vivo. Nevertheless, current techniques for MC DNA preparation by inducing MC producer plasmid intra-molecular recombination in bacteria have critical limitations, including their labor-intensive, time-consuming procedure, and high contamination with input plasmids and mini plasmids. We thus herein described a novel simple approach for MC-amplicon preparation by utilizing adenovirus mediated Cre-loxP site-specific recombination and HSV helper virus supplied replication and package function in mammalian cells. This technique allows for production of MC-amplicons free of bacteria elements, making it feasible to use MC amplicon instead of conventional amplicon in gene delivery studies.

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### 1. Introduction

Amplicons are helper-dependent pseudovirions of HSV-1 identical to wild type HSV-1 particles from the structural, immunological and host range points of view, but carry concatemeric form of plasmid DNA instead of the viral genome (1-2). Amplicon was originally developed by Spaete and Frenkel 1982 via the incorporation of a single origin of replication (oriS) and a single packaging/cleavage signal (pac) from the wild-type HSV-1 genome into a bacterial plasmid, which were termed "amplicon" (3). Therefore, conventional HSV-1 amplicon is composed of an Escherichia coli plasmid backbone carrying one origin of virus replication (OriS), one packaging signal (pac) from HSV-1 in addition to the transgenic expression cassette(s) of interest. When transfected into HSV-permissive cells and supplied with full HSV helper function, amplicons can be replicated and packaged into infectious HSV pseudovirions as a 150-kb linear vector DNA genome comprising "head-to-tail" concatenated structures of the original plasmid (4-5). Amplicon vectors possess unique features, including large transgene capacity of up to 150 kb of foreign DNA, the ability to transduce a wide variety of cell types of the most proliferating and non-dividing mammalian cells across a broad range of species, the ease of vector construction, and limited cytotoxicity and immunogenicity, thus making these vectors very appealing for preventive or therapeutic gene transfer as well as for upstream fundamental

studies (1, 6-8). Since it lacks virus coding genes and is unable to integrate into host chromosomes, the conventional amplicon does not cause insertional mutagenesis, and strongly reduces the risk of reactivation, and complementation or recombination with latent or resident HSV-1 genomes in the transduced organisms. However, before amplicon vectors can be safely and efficiently applied to human beings in gene therapy or vaccine protocols, there are still technique obstacles, such as producing large and high-titer stocks of vector particles without helper virus contamination (2), and their transient transgene expression limitation have to be overcome. Like plasmid mediated gene delivery, rapid transgene silencing, even in non-dividing cells, is the most critical shortcoming of conventional HSV amplicon vector (1, 9). Since Amplicon backbone DNA harbors multiple copies of bacteria elements (e.g.colE1 origin and antibiotic resistance gene), depending on the amplicon plasmid size, it may share the same mechanisms underlying the transgene loss with the plasmid mediated gene delivery. It has been demonstrated that the bacteria sequences in the plasmid are responsible for the rapid transgene expression suppression, as evidenced by that the gene expression mediated by a novel supercoiled minimal circular transgene cassette(s) known as the minicircle (MC) DNA devoid of bacteria sequences deriving from conventional plasmid DNA by site-specific recombination in Escherichia coli achieved a 10- to

1,000-fold enhancement compared with regular plasmids in long-term transgene expression even in quiescent tissues both in vitro and in vivo(10-11). For MC DNA preparation, several approaches have been developed by engineering different recombinases, such as the bacteriophage  $\lambda$  integrase(12), Flp(13-14), Cre recombinase (15-17) and phage phiC31 integrase(10, 11, 18) into the MC producer plasmid or into the bacteria genome to achieve inducible intra-molecular site specific recombination and generate recombinant MC molecules in bacteria. By adopting the phiC31 integrase mediated method, Suzuki et al 2006 generated minicircle amplicon vectors (MC amplicon) devoid of bacterial sequences and exhibited significant higher and more persistent expression at transcriptional level than conventional amplicons both in vitro and in vivo and revealed a involved molecular mechanism that the bacterial sequences in the conventional amplicon DNA triggered the whole vector chromatin inactivation (9). The findings suggested that MC amplicon might be an excellent ideal gene delivery vehicle super to the conventional amplicon and hold a promising for future gene therapy and vaccine development. However, the main shortcomings of currently used techniques for minicircle DNA preparation in bacteria have been their time-consuming, labor-intensive procedure, low-yield production and more important, high contamination with MC-producer plasmid and plasmid backbone circle (19). Therefore, the MC amplicon preparation techniques should be further improved so as to completely eliminate plasmid-amplicon contamination, simplify the preparation procedure and be capable of scale up production.

We herein describe a novel approach for the MC-amplicon preparation by adenovirus mediated Cre-loxP site-specific recombination in mammalian cells that allows for a simple and rapid production of MC-amplicons without any bacteria sequence and donor vector contamination.

## 2. Materials and methods

### 2.1 Cells, plasmids and virus

African Green monkey kidney (Vero) and 293A cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The plasmid C223(Fig.1-A), a bacteria F plasmid, named bacteria artificial chromosome (BAC) plasmid carrying GFP and puromycin resistant gene cassettes and loxP sequences, and pGEM-T-Cre were gifted from Dr Yu, Washington University in St. Louis. The HSV-1 amplicon plasmid pHSVA-DsRed (Fig.1-C) with red fluorescent reporter gene was constructed by our laboratory as described previously (20). The pAd/BLOCK-iT<sup>TM</sup>-DEST and pENTR<sup>TM</sup>/U6, a replication-incompetent adenoviral vector system for generation of adenoviral RNAi vectors were purchased

from invitrogen, (USA). The HSV-1 strain F and HSV-2 strain HG52 were obtained from Wuhan Institute of Virology, Chinese Academy of Sciences.

### 2.2 Construction of pAdv-loxP-OPD-loxP and generation of adenovirus

#### 2.2.1 Construction of pENTR-MCS

Two DNA oligonucleotides as the following sequences, 5'-TCGAAGTACTACCCGCGGACGAAT TCCAGCGGCCGCACCTCGAGACGGATCC-3', and 5'-TCGAAGTACTACCCGCGGACGAATTCCAGCGGCCGCACCTCGAGACGGATCC-3' were synthesized and annealed to create a multiple cloning sites (MCS), with SacI/SacII/EcoRI/NotI/XhoI/BamHI sites, and introduced into the Sall and XbaI sites flanked by attL1 and attL2 sites of pENTR/U6 to result in pENTR-MCS.

#### 2.2.2 Construction of pENTR-loxP-loxP

A 1.5Kb fragment containing two loxP sites in same orientation acquired from XmnI/SacI digestion of C223 plasmid was inserted into the corresponding sites of pENTR-MCS to construct an intermediate vector pENTR-loxP-loxP (Fig 1-B).

#### 2.2.3 Construction of pENTR-loxP-OPD-loxP

A 3Kb fragment containing oriS, pac elements and DsRed expressing cassette (OPD) from AvaII/NotI cutting pHSVA-DsRed was inserted into the corresponding sites of pENTR-loxP-loxP by replacing all the DNA sequences between two loxP sites except AvaII/NotI sequences to generate the pENTR-loxP-OPD-loxP, which was served as an entry vector for the adenovirus recombination. By the same way, the 1.5Kb DsRed expressing cassette from pDsRed2-C1 was cloned to the same site of pENTR-loxP-loxP to construct a pENTR-loxP-D-loxP as the control vector and verified its orientation consistent with that in pENTR-loxP-OPD-loxP as analyzed by EcoRI digestion.

#### 2.2.4 Construction of pAdv-loxP-OPD-loxP adenovirus plasmid

To transfer the loxP-OPD-loxP structure into plasmid adenovirus vector pAd/BLOCK-iT-DEST, a LR recombination was performed following the manufacturer's instruction. Briefly, in a 0.5 Eppendorf tube, 100ng plasmid DNA of pENTR-loxP-OPD-loxP and 150ng pAd/BLOCK-iT-DEST in 8ul TE buffer were mixed with 2ul LR Clonase II enzyme (2ug/ul) and incubated at 25°C for 1 hour, then 1ul of the Proteinase K solution was added into the reaction for another 10 minutes at 37°C to inactivate the LR Clonase II enzyme. 2ul of the LR recombination reaction was used to transform DH5 $\alpha$  E.coli by a hot shock transformation method and the correct clone was identified by EcoRI enzyme digestion analysis. Also, a LR recombination between pENTR-loxP-D-loxP and pAd/BLOCK-iT-DEST was carried out to generate a negative control adenovirus plasmid

pAdv-loxP-D-loxP devoid of oriS and pac elements in the same procedure ( Fig 1-D).

### 2.2.5 Generation of adenovirus Adv-loxP-OPD-loxP

To generate adenovirus , the plasmid DNA of pAdv-loxP-OPD-loxP and pAdv-loxP-D-loxP were digested with PacI respectively to remove the bacterial sequence and expose ITRs of adenoviral genome and then transfected into 293A cells with the Lipofectamin2000 (Invitrogen, USA) following the manufacturer's protocol. Adenovirus Adv-loxP-OPD-loxP and Adv-loxP-D-loxP were harvested after the transfected cells completely appearing CPE. The obtained adenovirus were amplified in 293A cells to get enough amount needed and the titers were determined by plaque formation assay.

### 2.3 Construction of pAdv-Cre vector and generation of corresponding adenovirus

The plasmid pGEM-T-Cre was digested by SacII and EcoRI to get a 2.2Kb fragment, which contained a

Cre recombinase expressing cassette driven by SV40 promoter and was introduced into the corresponding sites of pENTR-MCS to generate the pENTR-Cre. The subsequent LR recombination and Adv-Cre virus generation was carried out as the same procedure of Adv-loxP-OPD-loxP production described above (Fig 1-D).

### 2.4 HSV helper virus preparation

To provide recombinant MC amplicon the replication and package function, several infectious HSV virus were used as helpers, including recombinant BAC-HSV-1 strain HF, wildtype HSV-1 strain F and HSV-2 strain HG52. The recombinant BAC-HSV-1 strain HF helper virus were generated by transfecting Vero cells with BAC-HSV-1 strain HF plasmid, whereas other two wildtype HSV helper virus, HSV-1 strain F and HSV-2 strain HG52 were propagated in the Vero cells directly. All helper virus were tittered by a plaque formation assay.

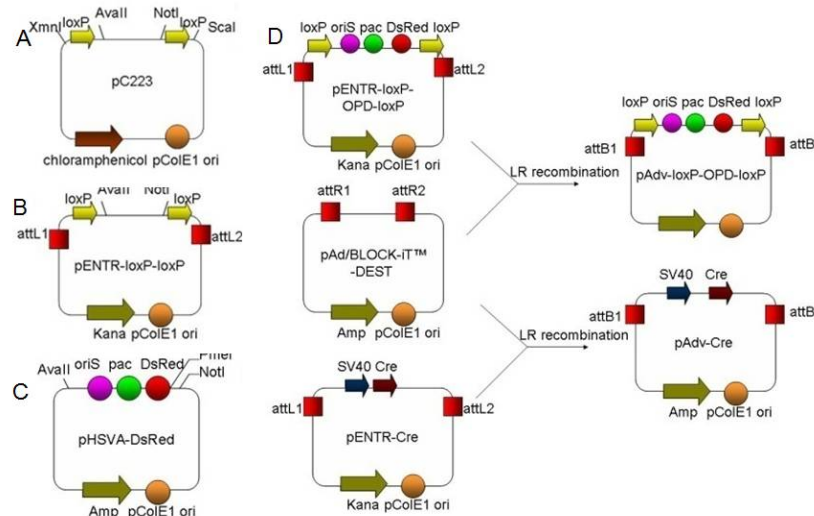


Figure 1 Flowchart of pAdv-loxP-OPD-loxP and pAdv-Cre Construction. A: The structures of plasmid pC223; B: The structures of plasmid pENTR-loxP-loxP; C: The structures of plasmid pHSVA-DsRed; D: The construction of pAdv-loxP-OPD-loxP and pAdv-Cre.

## 2.5 Generation and identification of mini-circle (MC) amplicons

### 2.5.1 Generation of MC amplicons

To generate recombinant MC amplicons and demonstrate their generation was in a recombination specific and helper-dependent manner, four different combinations were designed: Combination A., adenovirus Adv-loxP-OPD-loxP and Adv-Cre plus BAC-HSV-1-HF helper virus; Combination B., adenovirus Adv-loxP-D-loxP and Adv-Cre plus BAC-HSV-1-HF helper virus ( without oriS and pac ); Combination C., adenovirus Adv-loxP-OPD-loxP plus BAC-HSV-1-HF helper virus ( without adenovirus Adv-Cre); Combination D., adenovirus

Adv-loxP-OPD-loxP and Adv-Cre (without HSV helper virus) respectively. The brief experimental procedure was as below: The overnight sub-cultured Vero cells grown in 6 well plate at 90% confluent were transduced with adenovirus from each combination at MOI=1. Twenty-four hours later, the cells were washed with PBS for three times to remove the residual adenovirus and infected with HSV helper virus in fresh medium at MOI=0.01 in combination A, B, and C, but in combination D, just replaced with fresh medium instead. 48 hours after HSV helper infection or fresh medium replacement, the cells together with the medium were collected and processed by three freeze/thaw cycles at  $-80^{\circ}\text{C}/37^{\circ}\text{C}$  followed by a

5minutes centrifugation at 3000g and filtration with a 0.45um filter to remove the cell debris (Fig2).

### 2.5.2 DsRed fluorescence observation

The obtained supernatants from different combinations were 1000X diluted and transduced into vero cells. 24h later, the cells were examined by observing DsRed fluorescence on the inverted phase contrast fluorescence microscope (Olympus, IX71-A12FL/PH, Japan).

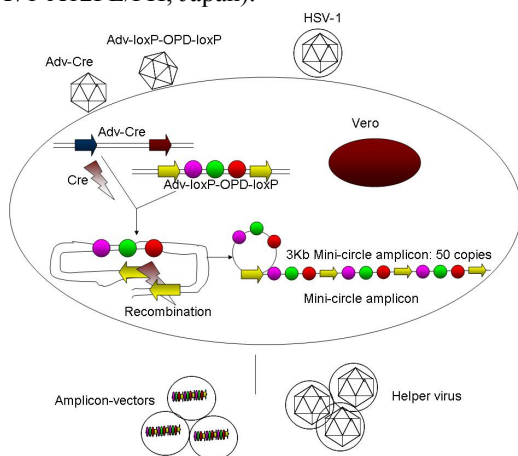


Figure 2 Schematic diagram of MC amplicon generation under the presence of HSV helper virus.

### 2.5.3 RT-PCR analysis of DsRed expression

In order to further identify the recombinant MC amplicons were successfully generated by adenovirus mediated Cre-loxP recombination and to verify their transducing ability and transgene expression function, we transduced vero cells with recombinant MC amplicon seed stock derived from the first round and performed RT-PCR analysis of DsRed expression at mRNA level in the transduced cells. Briefly, the obtained supernatants from different combinations were diluted 1000X and transduced into Vero cells. 12h later, the cells were washed with PBS for three times and collected for RNA extraction. The total RNA from the cells was extracted by the RNeasy mini kit (Qiagen) and treated with DNase I prior to RT reaction so as to remove any possible contaminated DNA sequence. The reverse transcription was achieved by using of SuperScript III kit (Invitrogen, USA) to synthesize cDNA with oligo-deoxythymidine primers and 1 ug RNA from each RNA preparation was used as template. After RT reaction, 200 ng of cDNA was amplified by PCR for a 336 bp band using the following primers: DsRedP1, 5'- ACGGCTGCTTCATCTACAA-3' and DsRedP2, 5' - ATCTCAGGAACAGGTGGTG-3' , with the program of 95°C 4', followed 95°C 30', 53°C 30 s ,72°C 30' for 35 cycles and the final extension at 72°C for 10 minutes. In order to exclude any possible DNA contamination, a RNA PCR without DNase I digestion and without RT reaction were performed as a negative control. Beta-actin was used as an internal

control standard by amplifying a 139bp band by using of the primers:  $\beta$ -actinP1, 5'-GGACTTCGAGCAGGAGATGG-3' and  $\beta$ -actinP2, 5'-CAGGAAGGAAGGTTGGAAGAGA-3'. The PCR products were electrophoresed in a 1% agarose gel and visualized by ethidium bromide staining.

In addition, the recombinant MC amplicon derived from the first round adenovirus mediated Cre-loxP recombination process were also used to transduce Vero cells again under the presence of HSV helper virus but absence adenovirus mediated Cre-loxP recombination to demonstrate their reproducibility. We called the generated MC amplicons from this round the reproduced MC amplicons.

### 2.5.4 Testing the replication/package function of MC amplicons to different HSV helper virus

To test whether the recombinant MC amplicons had a universal replication and package function to different strain of HSV helpers, we performed an adenovirus Adv-loxP-OPD-loxP and Adv-Cre mediated MC amplicon DNA recombination in Vero cells, but supplied different HSV virus as helpers, including the infectious BAC-HSV-1 strain HF, HSV-1 strain F and HSV-2 strain HG52. The procedure for generation and fluorescence identification of MC amplicons was the same as that described above.

## 3. Result

### 3.1 Fluorescence identification of recombinant MC amplicon generation

By co-transduction of Vero cells with Adv-loxP-OPD-loxP and Adv-Cre and supply BAC-HSV-1-HF helper virus, we successfully generated recombinant MC amplicons in the first round recombination process, we called the recombinant MC amplicon seed stock, which were demonstrated to possess transducing ability, mediating transgene expression function and reproducing ability when supply with full HSV helper function. As shown in Fig 3, the DsRed fluorescence was observed in Vero cells transduced with obtained supernatants from combination A, but not in the cells treated with that from any other combinations, including B, C, and D, indicating that successful generation of MC amplicons was dependent on the Cre-loxP mediated site specific recombination and HSV helpers provided replication and package function, whereas without oriS/pac in combination B, without Cre in combination C and without HSV helpers in combination D could not generate any MC amplicons. By transducing Vero cells with the recombinant MC amplicons derived from the first round recombination process and providing BAC-HSV-1-HF helper virus, but no giving adenovirus, as no more adenovirus mediated Cre-loxP recombination needed, we demonstrated that the recombinant MC amplicons were reproducible under the presence of HSV helper function and called the

generated MC amplicons in this way the reproduced MC amplicons.

### 3.2 RT-PCR analysis of recombinant MC amplicon generation

To verify the observed DsRed fluorescence under fluorescent microscope was a results of MC amplicon mediated DsRed expression, we performed a RT-PCR analysis to examine DsRed expression at mRNA level in Vero cells with supernatant from different combinations. In Fig 4, the RT-PCR results showed that the DsRed gene expression at mRNA level was only found in Combination A, not in any other combinations, completely consistent with the fluorescence observation results and thus further demonstrated that generation of MC amplicons was Cre-loxP site-specific recombination required and HSV helper-dependent. Since RNA direct PCR failed to amplify the DsRed sequences, we thus concluded MC amplicons mediated a functional DsRed transgene expression.

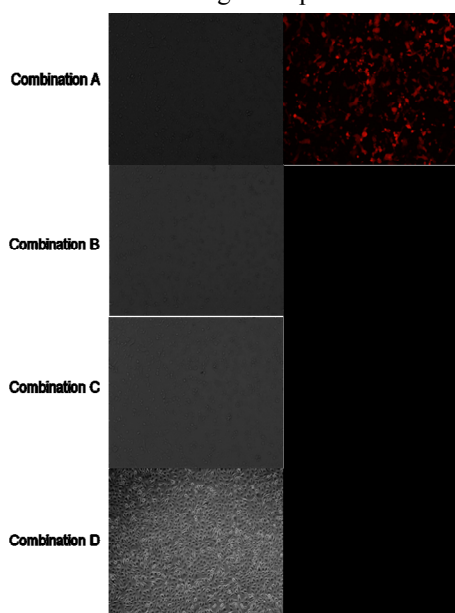


Figure 3. DsRed fluorescence observation in Vero cells treated with 1000X diluted supernatants derived from different combinations under inverted phase contrast fluorescent microscopy, the left panel was Vero cell images taken in the Bright-field and the right panel was Vero cell images taken in the Dark-field (X100). The DsRed fluorescence was observed in combination A.

### 3.3 The replication and package capacity of MC amplicon to different HSV helpers

By using the MC amplicon derived from BAC-HSV-1 strain HF and recombinant BAC-HSV-1-HF virus as helpers, we successfully generated MC amplicons by adenovirus mediated Cre-loxP recombination. To test the replication and package capacity of recombinant MC amplicon to

different HSV helpers, we performed the experiments for MC amplicon generation with the same recombination procedure by co-transduction of Vero cells with Adv-loxP-OPD-loxP and Adv-Cre adenovirus, but supply different HSV-1, and HSV-2 virus as helpers. As shown in Fig 5, we achieved the similar success in MC amplicon generation and demonstrated their transducing ability and transgene expression ability by fluorescence observation of functional DsRed gene expression under fluorescence microscope. These results indicated that our recombinant MC amplicon possessed universal replication and package function to different genotype and immuno-type HSV helper strains.



Figure 4. RT-PCR analysis of DsRed gene expression in Vero cells treated with 1000X diluted supernatants derived from different combinations in the first round. A-D represent Combination A, B, C, and D respectively. The upper gel image showed RT-PCR results of DsRed expression except in lane A1, a RNA PCR from Combination A as a negative control. The bottom gel image was the RT-PCR results showing of β-actin expression.

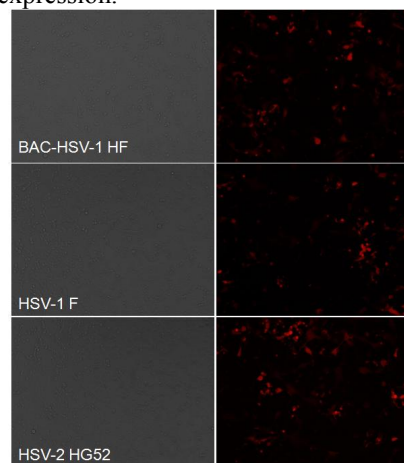


Figure 5. DsRed fluorescence observation under inverted contrast fluorescent microscopy in vero cells transduced with 1000X diluted recombinant MC amplicons generated in the first round with different HSV helper virus. the left panel showing bright-field and the right panel showing Dark-field (X100). The DsRed fluorescence was observed in Vero cells transduced with MC amplicons generated by adenovirus mediated Cre-loxP recombination but with different HSV virus as helpers.

#### 4. Discussions

HSV amplicon has been regarded as a versatile gene delivery vehicle and holds great promise in clinical gene therapy and vaccine development (1-2, 9). Due to carrying concatemeric bacteria elements in the vector, which can result in transgene silencing and trigger chromatin inactivation of the whole vector, the conventional amplicon mediates only transient transgene expression, even in non-dividing tissue culture cells (1, 9). Therefore, MC DNA-based HSV amplicon (MC amplicon) lacking bacteria sequences represents an ideal and excellent pseudovirion gene delivery platform superior to conventional amplicon as it mediated high and persistent gene expression both in vitro and in vivo. Actually, the bacterial sequences in the gene delivery vectors are dispensable for gene transfer application, but just necessary for plasmid vector production in bacteria (21). Furthermore, the antibiotic resistance genes in the vector may also cause serious biological safety problems and the regulatory agencies thus recommend totally avoiding the use of antibiotic resistance markers (22-23). In order to achieve a higher and long-term transgene expression and reduce the possible safety risk, the best way is to completely remove the bacteria elements from the gene delivery vectors, including an antibiotic resistance gene and an origin of replication.

To realize the purpose addressed above, several methods have been employed in the MC DNA preparation by using different integrases to induce intra-molecular recombination in MC producer plasmid in bacteria and followed by a isolation procedure to eliminate the contamination of minicircle producer plasmid and circular plasmid backbone from bacteria lysates, however, all of these methods have disadvantages, including labor-intensive, time-consuming preparation procedure, low yield, and high contamination of plasmid and mini plasmid. Although Chen et al very recently improved the MC DNA preparation technology by engineering a bacteria strain with inducible integrase phi-C31 and restriction I-saI, that allowed for more simple and fast to generate MC DNA like a routine plasmid preparation, however, it still remained a low level contamination of minicircle producer plasmid and circular plasmid backbone (19). As a fact, it is very difficult and even attainable to completely remove the contamination by digestion and purification method, as all of them are circular DNA molecules except different sizes. Since HSV amplicon is reproducible during propagation process under the presence of HSV genome or helper virus, any contamination, even very small amount, will be amplified round by round during the scale up steps. In this study, we have successfully developed a novel approach for MC amplicon preparation by adenovirus mediated Cre-loxP recombination in mammalian cells.

Cre recombinase is a bacteriophage P1-derived integrase catalyzing site-specific recombination between direct repeats of 34 bp loxP sites (24). In our MC amplicon preparation system, the oriS, pac and reporter gene or/and transgene cassette(s) were flanked with two loxP sites in the same orientation in the adenovirus genome, and when adenovirus delivered Cre was expressed, a recombination event was achieved between two loxP sites and resulted in two independent DNA molecules, one was recombinant mini circles DNA carrying a oriS sequence, a pac signal, a transgene expression cassette(s) and a single 34 bp lox footprint, and another was a linear adenovirus genomic DNA. The recombinant MC DNA was then replicated in a mono-directional, rolling circle-like fashion to generate a head-to-tail concatemer of DNA composed of tandem repeats of the MC DNA and cleaved/packaged around 150 kb linear fragment into MC amplicons under the presence of HSV helper virus. We developed a one-step MC-amplicon preparation techniques, that is MC DNA recombination and subsequent MC amplicon replication/package were achieved in the same mammalian cells, instead of two-step procedure, MC amplicon DNA recombination in bacteria first, and then MC amplicon replication/package in mammalian cells separately. In our system, an automatic selection mechanism is involved for the MC amplicon package, that is only MC amplicon DNA can be replicated and packaged into MC amplicons, whereas linear adenovirus vector is unable to be packaged, excluding the possibility of bacteria DNA and adenovirus DNA contamination.

While MC DNA was replicated and packaged into MC amplicons, in theory, sustained expressing Cre post MC DNA recombination might also mediate intra-molecular recombination between loxP sites within the replicated concatemeric MC DNA sequences randomly and result in more MC DNA molecules or large circular DNA harboring multiple MC DNA repeats entering mono-direction rolling circle like replication again. It is not clear if persistent Cre expression has any affection on MC amplicon packaging, however, as a fact, we achieved a  $10^6$ /ml TUs of recombinant MC amplicons in the first round recombination process indicating that Cre seemed to have no ruin effect on the MC amplicon packaging, but perhaps have an amplification effects as more MC DNA entering replication cycles, consequently leading to more and more MC amplicons generation. To further investigate the effects of Cre sustained expression on MC amplicon production, we next will engineer a tightly controlled Cre expression system and MC amplicon loxP system in the same adenovirus vector to study the Cre expression dynamic and related yield of MC amplicons. Once the recombinant MC amplicons were produced in the first round, no more adenovirus

mediated recombination process was needed as recombinant MC amplicons like seeds, were able to be reproduced during the roundly propagation process under the presence of HSV helper function.

Adenovirus has been widely used as a gene delivery vector for gene therapy and vaccine developments with advantages such as high transduction efficiency, extremely high viral titers ( $10^{10}$ - $10^{13}$ ) and capable of generation on large scale (25). Taking the advantages of adenovirus vectors, we have demonstrated the feasibility by adenovirus mediated Cre-loxP in generation of MC amplicons. Due to the limited capacity of replication-defective adenovirus, the maximum insertion size of MC amplicon DNA is less than 8 kilobases. For large size MC amplicon DNA recombination, an alternative selection is to use helper-dependent adenoviral vectors (or named gutless adenoviral vectors) as donor vectors instead of replication-defective adenovirus, which is capable of delivering up to ~37 kb size of insertion (26). Actually, it is rare to deliver transgene large than 37 kb size for regular gene transfer studies.

In this study, we used replication competent HSV-1 virus as a helper for MC amplicon generation to demonstrate this technique the feasibility and to verify the universal replication/package function of recombinant MC amplicon to different genotype and immune-type of HSV helper virus, HSV-1 HF, HSV-1 strain F, and HSV-2 strain HG52. Although helper free amplicons could be generated by transfection of HSV permissive cells with pac sequence deleted HSV genomic DNA or oversized BAC-HSV genome (27), however, the critical obstacle is the difficulty in producing high titer helper free amplicons in large scale. Due to replication defective adenovirus vectors and the gutless adenovirus vectors with replication defective adenovirus as helpers are able to be produced in large scale, therefore, a simple and large production of the adenoviral vectors in our system will replace the complicated minicircle DNA preparation procedure for MC amplicon production.

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