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Methodology Report

Use of Recombination-Mediated Genetic Engineering for Construction of Rescue Human Cytomegalovirus Bacterial Artificial Chromosome Clones

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Bacterial artificial chromosome (BAC) technology has contributed immensely to manipulation of larger genomes in many organisms including large DNA viruses like human cytomegalovirus (HCMV). The HCMV BAC clone propagated and maintained inside *E. coli* allows for accurate recombinant virus generation. Using this system, we have generated a panel of HCMV deletion mutants and their rescue clones. In this paper, we describe the construction of HCMV BAC mutants using a homologous recombination system. A gene capture method, or gap repair cloning, to seize large fragments of DNA from the virus BAC in order to generate rescue viruses, is described in detail. Construction of rescue clones using gap repair cloning is highly efficient and provides a novel use of the homologous recombination-based method in *E. coli* for molecular cloning, known colloquially as recombineering, when rescuing large BAC deletions. This method of excising large fragments of DNA provides important prospects for *in vitro* homologous recombination for genetic cloning.

1. Introduction

Human cytomegalovirus (HCMV), also known as human herpesvirus-5, belongs to the betaherpesvirinae subfamily of the herpesviridae family. The virus is ubiquitously found in all geographic locations and among different socioeconomic groups with seroprevalence up to 90% [1]. Though a large percentage of the human population is infected with this virus, it does not cause significant clinical illness in immunocompetent individuals; however, high mortality and morbidity rate is found in immunocompromised individuals such as AIDS patients, transplant recipients, newborns, and developing fetuses [1]. Like other herpesviruses, this virus also retains the ability to remain latent in its host for life [2]. The virus can infect many different cell types including epithelial cells, endothelial cells, monocytes, macrophages, fibroblasts, smooth muscle cells, and neurons [1]. The virus is transmitted among individuals via bodily fluids, prenatal exposure, and sexual contact [2].

The HCMV genome is the largest and most complex among human herpesviruses, with 200–235 kb nucleotide pairs encoding for more than 200 proteins [3]. The virus genome encodes 40 herpesvirus core proteins and the rest of the genes encode betaherpesvirus virus-specific protein or HCMV-specific proteins. At present, the function of all of the viral genes has not been fully understood. One method for studying gene function is to delete a specific gene and study the mutant virus phenotype in the host. Due to the large genome size of HCMV, making recombinant virus is a challenging and cumbersome task; as a result, the functional study of many viral genes is hindered.

Several approaches have been used for the construction of recombinant HCMV. One of the earlier methods was to transfect human permissive cell lines with virus genome along with a plasmid carrying a marker gene with long flanking homology to the viral region to be modified into a host cell and then selecting for recombinants [4]. Some of the drawbacks in this method were: (1) the primary

permissive cells are difficult to transfect and the probability of getting both the viral genome and the plasmid in the same cell was low; (2) efficiency of homologous recombination was low and long homology sequences (over 500 bp) are required; (3) replication cycle of the virus was slow and selecting a recombinant virus took a few days, and (4) the recombinant viruses and parental viruses were mixed and therefore isolation of the recombinant virus required extensive plaque purification. However, the development of a cosmid system did lead to a better method for construction of recombinants [5–7]. Manipulation of the viral genome could be done in one of the cosmids and cotransfection of this mutated cosmid along with other wild-type cosmids in a permissive cell would give rise to recombinant virus upon homologous recombination [5, 7]. Although the cosmid method was an improvement, it was far from perfect as HCMV has a large genome and a total of eight cosmids were required for recombinant generation [5]. This does not seem to be a problem intuitively, but the resultant virus from this method of mutagenesis would be the product of several recombination events that are not only difficult to control, but verification of the viral genome can only be done after growth and isolation of the mutant virus, leading to a great deal of lost time and effort if all of the recombination events did not occur properly.

Overcoming the aforementioned difficulties, the development of the bacterial artificial chromosome clone (BAC) of the virus was a breakthrough in the HCMV mutagenesis field. The HCMV BAC clone can be maintained stably and propagated inside a bacterial cell, which allows for easy manipulation of the viral genome. Any required mutation can easily be achieved inside the *E. coli* cell and this mutation can be verified before making recombinant virus. Recombinant viruses can be made rapidly and straightforwardly when utilizing BAC genetic methods of manipulation. The first CMV BAC was made by using the murine cytomegalovirus (MCMV) Smith strain by Messerle et al. in 1997 [6]. Shortly after the construction of the MCMV BAC, Borst et al. described the construction of first HCMV BAC clone using the AD169 strain of the virus [8]. Recombinant viruses were made inside a CBTS *E. coli* strain, which carries a *recA* amber allele and a temperature-sensitive amber suppressor, so that the cells are *recA* positive at 30°C and *recA* negative at temperatures higher than 37°C [8]. The CBTS *E. coli* carrying the virus BAC were transformed with a shuttle plasmid containing virus sequence with desired modification and upon homologous recombination, the altered virus sequence replaced the wild-type sequence from the BAC, resulting in recombinant BAC clones. The recombinant BAC was then verified, isolated, and transfected into mammalian cells in order to generate recombinant virus. Since the development of this novel method for construction of recombinant viruses, detailed functional study of virus genome has been done using the BAC mutagenesis approach [3, 9–11].

However, the methods for BAC mutagenesis we use today were not at first easily attained as the large size of BACs posed a serious obstacle for their exact manipulation, as is required for viral research. Techniques were developed that utilized the power of homologous recombination in order to create recombinant viruses with the ability to safeguard every step in the process. We can now replace, and therefore delete large DNA fragments by inserting antibiotic resistance markers for proper and accurate selection of recombinant BAC clones and insert a luciferase reporter gene in order to measure expression of the recombinant virus. These new systems circumvented the problems associated with conventional genetic engineering as there was no longer a size restriction as seen when using restriction enzymes or other previous methods [12, 13]. Various modifications have been made to increase the efficacy of the homologous recombination-based method for the construction of recombinant viruses. This method is known as recombineering, or, less colloquially, the recombination-mediated genetic engineering method in *E. coli* that uses the recombination proteins derived from λ phage [13]. Discussed below are the methods commonly used to make recombinant clones via this method.

2. Uses of Recombination-Mediated Genetically Engineered BACs in Viral Research

2.1. Construction of Deletion Mutants. Several different approaches have been utilized to make deletion clones of HCMV using the BAC clone. Use of site-directed mutagenesis in *E. coli* using positive and negative selection markers is a common method of making recombinants. The virus BAC is maintained inside a modified DH10B strain of *E. coli* such as DY380 or SW102, which are *recA* negative and harbor repressed λ prophage recombination systems. The recombination system consists of *exo*, *bet,* and *gam* genes and is under the control of temperature-sensitive *cI* repressor protein [12, 13]. A generalized summary of the straightforward procedure of using this recombination system in *E. coli* to generate deletion mutant BACs is given in Figure 1. When using a positive selection marker to replace a region of interest (ROI) by homologous recombination (Figure 1), the marker is usually a PCR-amplified antibiotic resistant gene, kanamycin-resistant gene in this case (a), containing short flanking homology to the region of interest to be deleted that was conferred by primer design (b). The recombination system of the *E. coli* harboring target DNA (d) can be induced, only when required, by inactivating the repressor protein by incubating the bacteria at 42°C for 15 minutes during electrocompetent cell preparation (e). The kanamycin cassette is transformed into electrocompetent and recombination-activated *E. coli*, in this case DY380 with its recombination system induced, harboring the wild-type virus BAC (f). The recombinants produced by homologous recombination event (g) are selected in the presence of kanamycin, which allows for the growth of only the clones containing the kanamycin resistant gene. Advantage of this method is that two genes can be deleted sequentially using two different drug resistance markers [14].

The second approach, which makes use of a positive and counterselection protocol, utilizes the SW105 (or SW102 or SW106) strain of *E. coli*, which is derived from the DY380 strain [15]. The SW105 strain contains the same λ prophage

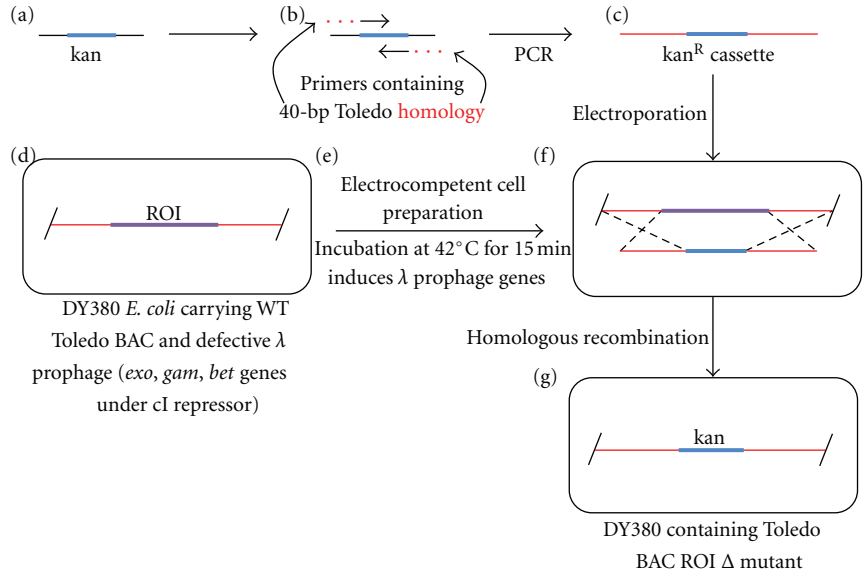

Figure 1: Toledo ROI Deletion Mutant BAC Clone Generation. A kanamycin resistant gene (a) is amplified by primers containing 40-bp homology to the sequences flanking the region of interest (ROI) in Toledo BAC (b) to create the kanamycin resistant cassette (c). In order to generate an ROI deletion mutant BAC clone (ROI Δ), DY380 *E. coli* carrying WT Toledo BAC (d) must undergo electrocompetent cell preparation and induction for recombination gene expression (e). The kanamycin resistant cassette is then electroporated into the electrocompetent DY380 harboring WT Toledo BAC (f). Upon homologous recombination, the ROI will be replaced by the kanamycin resistant gene, generating the Toledo ROI Δ mutant BAC clone (g).

recombination system as DY380, but the major difference is that the SW105 contains a galactose operon in which the galactokinase (*galK*) gene is defective, so when SW105 bacteria are incubated on minimal media with galactose as the sole source of carbon, the bacteria cannot grow. However, when the *galK* gene is provided *in trans*, it will complement the defective *galK* gene, thus allowing the bacteria to grow in minimal media with galactose. When making deletion mutants using this approach, instead of a cassette conferring drug resistance, a PCR-amplified *galK* gene is used to replace the gene of interest by homologous recombination. The bacteria containing the correct deletion clones are selected on minimal media with galactose, where positive growth indicates the presence of the inserted *galK* gene. Colonies are further verified by growing them on MacConkey agar, where the clones containing the *galK* gene produce dark pink colonies [15].

2.2. Construction of Rescue Clones. When making rescue or mutant clones by a positive selection method that takes advantage of homologous recombination in DY380, first the gene of interest is deleted by recombination with the antibiotic resistant cassette methods discussed above. Next, the gene is cloned from WT BAC and mutated (if making a point, frameshift, or nonsense mutation) either by PCR in which the primers confer the mutation, or via site-directed mutagenesis. The mutated gene is cloned into a plasmid either upstream or downstream of a drug resistance gene for positive selection (Note: antibiotic-resistant gene must be different than the gene used to construct the deletion mutant). The gene antibiotic-resistant cassette is then amplified by PCR using primers containing homology arms flanking the BAC genetic locus. The homologous sequences can be as short as 40 bp. The cassette is transformed into bacteria that contain the gene deletion virus BAC clone, and the resulting recombinant clones are selected using the new drug resistance gene marker. The clones can be further verified by confirming their sensitivity to the previous drug used to make the deletion. The drawback of this method is that the virus genome will contain a drug resistance gene. To improve the shortcomings of this method, the drug resistance gene can be flanked with loxP sites, which can be removed by addition of cre during transfection of the recombinant BAC DNA into mammalian cells. This will leave only 34 bp loxP site in the mutant BAC clone and virus [16].

When making rescue clones in SW105 using the *galK* counterselection marker method, the WT gene or mutated gene is PCR amplified along with the homology arms flanking the BAC genetic locus. This PCR cassette is electroporated into electrocompetent and recombination-activated SW105 harboring the deletion mutant BAC, and by homologous recombination, the *galK* gene will be replaced by the WT or mutated gene from the cassette. The recombinants are selected in minimal media containing 2-DOG and glycerol. The bacteria that contain *galK* genes will be unable to grow due to the toxic metabolites released from 2-DOG metabolism, whereas the clones in which the *galK* gene has been replaced by the wild-type or the mutated sequence

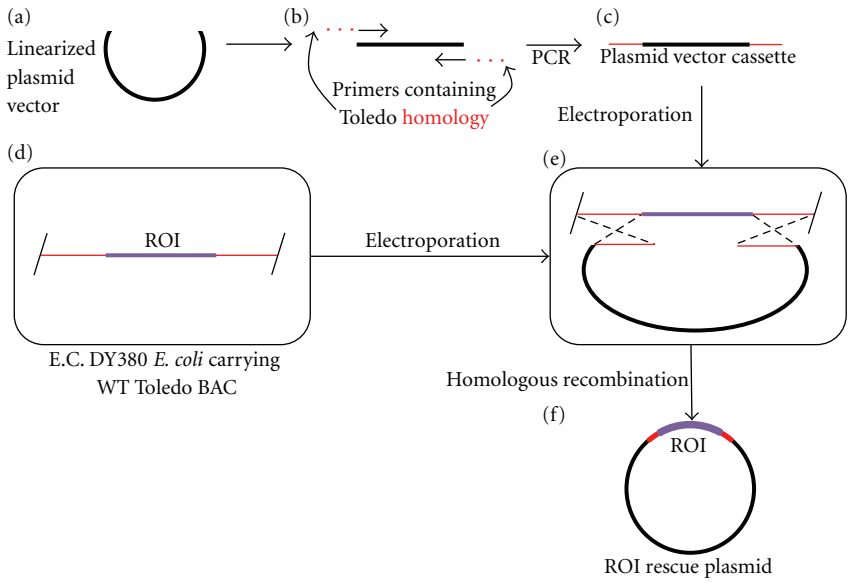

Figure 2: Gene Capture Method. (a) Linearized plasmid vector (b) is amplified by primers containing homology arms to the sequences flanking the region of interest (ROI) in Toledo to create the plasmid vector cassette (c). Electrocompetent and recombination-activated DY380 harboring WT Toledo BAC (d) are used for electroporation with the plasmid vector cassette (e). Upon homologous recombination and circularization, the ROI is captured, creating the ROI rescue plasmid (f).

will survive using glycerol as the source of carbon [15]. This method has advantages over the first one in that the rescue clones do not contain any foreign sequences in the viral BAC clone, hence in the virus. Even though this method is more advantageous over the previous one, making rescue clones in this manner is still a difficult process.

As simple as the previously cited methods sound, they are equally unsuitable when making rescue clones of large deletion mutants such as a 6-kb deletion, due to the requirement of PCR amplification of the region. In order to bypass the size restriction of PCR, a slightly different approach to making rescue clones of a large fragment deletion mutant (15-kb) was used by Wang et al. [17]. Since the 15-kb region in WT BAC DNA was flanked by two EcoRI restriction enzyme sites, the BAC DNA was digested by EcoRI and separated on the gel. From the gel, an 18-kb size product, which contains the 15-kb region fragment with 1.5-kb homology arms on either side, was extracted and transformed into the DY380 *E. coli* containing the deletion mutant BAC. It was possible to use this method because the deleted region was flanked by restriction enzyme sites in such a way that it provided long homology arms at both ends of the region of interest. The applicability of this method depends entirely upon the presence of restriction enzyme sites flanking the gene/region of interest. When working with large DNA like HCMV BAC, it is likely not to have suitable enzyme sites flanking the gene/region of interest. Furthermore, even if restriction enzyme sites flank the region appropriately, there could be additional restriction sites in other locations of the viral genome, resulting in the digested band of interest in the gel containing multiple different species of DNA fragments.

To resolve the issues associated with each of the methods described above, a gene capture method was developed and used for the first time to rescue a large 15-kb deletion mutant HCMV BAC [18]. This approach utilizes the principle of gap repair cloning in yeast with modification. Gap repair cloning is a commonly used method to repair double-strand breaks in yeast. This method has been described as the most efficient and error proof method of cloning [19]. It was shown that the plasmid was integrated into the chromosome if a crossover event was followed by the gap repair, whereas nonintegrated plasmid with repaired double-stranded break was the result of no crossover event [19]. As small as 30-bp homology arms have been shown to be sufficient for efficient gap repair cloning [20]. A modification of the method was to use oligonucleotide linkers as homology arms so that unique restriction enzyme sites can be added to the vector plasmid along with the oligonucleotide arms [21].

When making rescue clones by this gene capture method using DY380 containing the wild-type virus BAC (Figure 2), first the region of interest (ROI) is deleted by the above-mentioned method in DY380, generating an ROI deletion mutant. Next, a linearized plasmid (a) containing a positive selection marker is amplified by PCR with primers containing homologous sequences flanking the ROI in the BAC (b). In order to facilitate screening of rescue clones, a gene providing resistance to an antibiotic different than the antibiotic markers in both the BAC and rescue plasmid, flanked with loxP sites, is inserted within the captured ROI by homologous recombination. The PCR-amplified cassette (c) is transformed into electrocompetent and recombination-activated DY380 harboring the virus BAC (d). By homologous recombination, the ROI will be captured into the

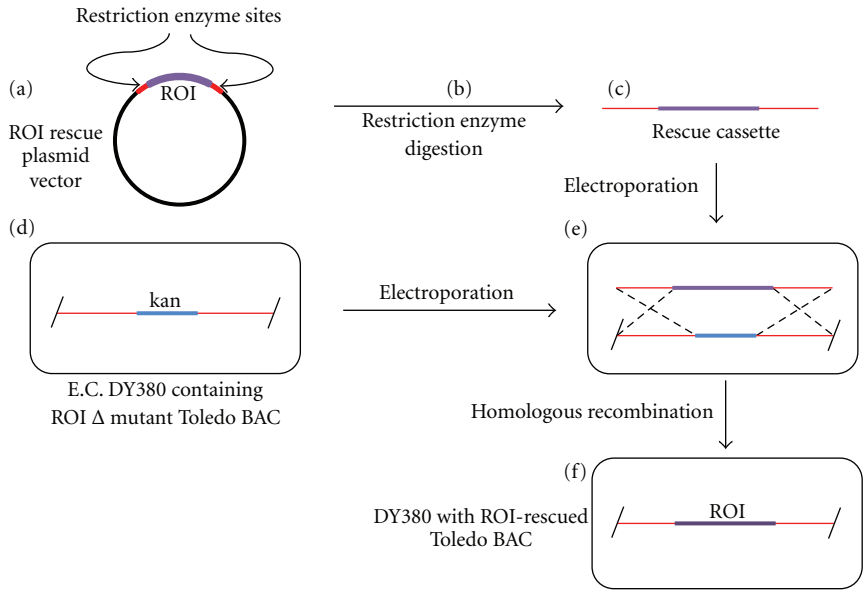

Figure 3: Toledo ROI Rescue BAC Clone Construction. Since the ROI rescue plasmid contains restriction enzyme sites (a), the plasmid is digested by restriction endonucleases (b), leading to isolation of the rescue cassette (c). Electrocompetent and recombination-activated DY380 harboring the ROI Δ mutant Toledo BAC (d) are used for electroporation with the rescue cassette (e). Upon homologous recombination, the ROI is inserted into the ROI Δ mutant Toledo BAC, generating the ROI-Rescued Toledo BAC (f).

plasmid due to gap repair cloning (e). These recombinant clones are selected using the drug resistance marker gene on the plasmid (Note: the plasmid antibiotic resistance should be separate than the antibiotic resistance of the BAC). This capture can be further verified by amplifying an open reading frame within the ROI by PCR and also by digesting the plasmid with restriction enzymes to check for the presence of the captured region.

Figure 3 provides the method for generating the rescued BAC clone after ROI capture. First, the plasmid containing the ROI (a) is digested by specific restriction enzymes with recognition sites flanking the region of interest (b). This rescue cassette (c) is then electroporated into electrocompetent and recombination-activated DY380 (d) containing the ROI deletion BAC (e). By homologous recombination, the rescue cassette will be inserted into the ROI deletion BAC, replacing the gene conferring kanamycin resistance, and thereby generating a rescued BAC clone (f). Since the ROI contains an antibiotic resistance marker different than that in the BAC, the clones are screened for resistance to that antibiotic in LB agar. The rescue clones can be confirmed further by verifying sensitivity to the previous antibiotic resistance (kanamycin) used to make the deletion.

It is important to note the subtle yet significant differences between the insertion of a cassette into the BAC DNA for creation of a deletion of mutant and the excision, or capture, of a DNA fragment from the BAC vector into a plasmid for rescue virus generation. As shown in Figure 4, directly after PCR amplification, the homology arms of the plasmid vector cassette are oriented differently than the cassette for deletion mutant construction. The forward primer sequence that generates the positive marker cassette with homology for the ROI flanking sequences in the BAC is the same sequence oriented in the same direction as the coding sequence upstream of the ROI. However, the reverse primer used in this positive marker cassette generation needs to be the reverse complement of the downstream coding sequence as to allow for proper insertion required for deletion mutant generation (a). The slight but compulsory variance between insertion and capture lies in the fact that during PCR amplification of the plasmid vector cassette, the template is linear, whereas when it is electroporated into *E. coli* for gene capture, the plasmid vector cassette becomes circular and the homology arms invert (b). This inversion necessitates the forward primer to be the reverse complement sequence of the upstream homology region coding sequence, and the reverse primer to be identical to the coding sequence downstream of the ROI in sequence and in orientation. This inversion needs to be taken into careful consideration when designing suitable primers for the gene capture method to work properly.

3. Specific Recombineering Methods in Construction of Rescue BAC Clone

3.1. Construction of 6-kb Region IV Deletion Mutant. We deleted a 6-kb region (UL132–151, referred to as region IV hereafter) from wild-type (WT) Toledo BAC by replacing it with a positive antibiotic selection marker. A kanamycin cassette with added homology arms flanking the region IV of Toledo BAC DNA was PCR amplified from pGEM-oriV/kan1 plasmid using Hotstar Taq polymerase (Qiagen Company, CA). Forward and reverse primers for

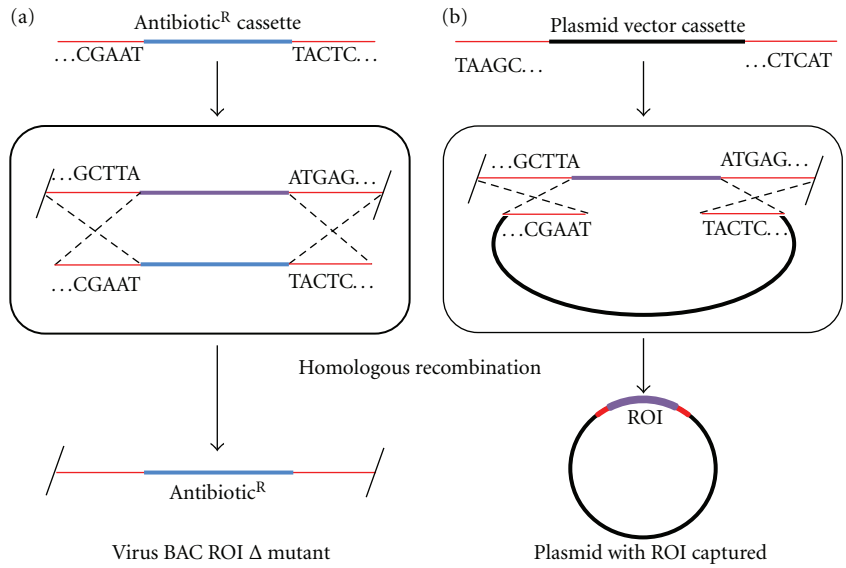

Figure 4: Variation in Gene Capture Method Recombineering. (a) Orientation of homology arms in generation of deletion mutant BAC clone. (b) Variant orientation of homology arms in linearized plasmid cassette for generation of plasmid with ROI captured.

kanamycin cassette were designed with 20-bp sequences from the kanamycin resistance gene and 40 bases homologous nucleotide sequences flanking region IV (Table 1). The amplified kanamycin cassette was treated overnight with DpnI restriction endonuclease (New England Biolab, MA) to digest any template plasmid and then purified using Qiagen's PCR purification kit (Qiagen, CA). The kanamycin cassette was used to replace region IV in the Toledo BAC. 300 ng of the purified cassette was transformed into electrocompetent DY380 cells carrying WT Toledo BAC. During electrocompetent DY380 cell preparation, the λ prophage recombination system in the cells was induced by incubating the bacteria in a 42°C water bath for 15 minutes with vigorous shaking. The presence of λ recombination proteins in the *E. coli* strain prevents degradation of the electroporated linear DNA cassette and allows for homologous recombination [13]. The transformed cells were grown at 32°C on LB agar with kanamycin (30 μg/mL). Colonies were restreaked onto LB agar with ampicillin (100 μg/mL) and LB agar with kanamycin plates, respectively, to confirm the antibiotic resistance of the colonies and since the kanamycin cassette was amplified from pGEM-oriV/kan1 plasmid containing an ampicillin resistance gene, the colonies were screened for ampicillin sensitivity in order to prevent selection of clones containing the pGEM-oriV/kan1 plasmid. The colonies that were sensitive to ampicillin and resistant to kanamycin were used for minipreparation. Miniprep of the BAC DNA was done as described by Zhang et al. [14]. After confirmation of the deletion mutant BAC, a maxiprep of the bacteria using NucleoBond BAC Maxi kit (BD Biosciences, CA) extracted the Toledo Region IV deletion (IV-Δ) mutant BAC DNA from the *E. coli* on a large scale. Deletion was once more verified by PCR and integrity of the region IV-Δ BAC DNA was analyzed by restriction endonuclease digestion with EcoRI (New England Biolab, MA) and compared with the digestion pattern of Toledo BAC DNA.

3.2. Construction of Rescue Clone through Gene Capture. Region IV was cloned into a linearized rescue vector (pUC19) with flanking homology arms, as previously described by gene capture, and the captured fragment was then put back into the region IV-Δ BAC from the rescue vector. In order to facilitate screening of rescue clones, a zeocin resistance gene flanked with loxP sites was inserted between ORF UL149 and UL130 region, which is included in the region IV ROI, by homologous recombination. The zeocin resistance gene, along with the flanking loxP sites, was amplified from pGEM-lox-zeo plasmid by PCR. The primers contained 40-bp homology flanking the BAC DNA locus where the gene was inserted (Table 1). The PCR product was treated with DpnI, as mentioned before, to remove the template plasmid and then gel purified using Qiagen's gel purification kit (Qiagen Company, CA). ~300 ng of the purified PCR product was electroporated into recombination-activated electrocompetent DY380 cells harboring WT Toledo BAC. The colonies were grown on LB agar with zeocin (50 μg/mL) and the insertion of the zeocin resistance gene was confirmed by PCR (Figure 5).

Region IV was captured into a rescue vector, pUC19, along with an extra 150 bp sequences at each end that were used as homology arms. To capture the region IV DNA fragment, the rescue plasmid was first linearized by BamHI digestion and amplified using primers containing homology flanking region IV (Table 1). Since the large size of the region being captured did not possess adequate restriction enzyme sites for digestion, unique restriction sites for AscI (upstream) and FseI (downstream) were added

Table 1: Table of primers used for PCR. ToledoUL132KanF and ToledoUL151KanR: primers to amplify kan^R cassette for insertion of positive antibiotic selection marker and simultaneous deletion mutant construction. ZeoInsertF and ZeoInsertR: amplification of zeo^R cassette for insertion of positive antibiotic selection marker insertion within region IV between UL 130 and UL 149. pUC19-IVCap_F and pUC19-IVCap_R: amplification of plasmid cassette for region IV capture for rescue clone construction.

Primer	Sequence (5′ → 3′)	Function of primers
ToledoUL132KanF	CGCGGACATAGCAAGAAATCCA	Amplify kan^{R}cassette for antibiotic selection and region IV deletion mutant construction
	CGTCGCCACATCTCGAGAGCTCT	
	TGTTGGCTAGTGCGTA	
ToledoUL151KanR	CGACCAGCGCTTTGTGCGCGCT	
	GCCTGTGCGTGTCGTCCCTCTGC	
	CAGTGTTACAACCAA	
ZeoInsertF	GTCCGGCAGGATAGCGGTTAAG	Amplify zeo^R cassette for antibiotic selection within region IV between UL 130 and UL 149
	GATTCGGTGCTAAGGCCGCATG	
	GCCGCGGATGGATCC	
ZeoInsertR	TATCTGCGTGGGTCTAATCATGG	
	GTGTCACCGTGATCGCGGCCGC	
	ACTAGTGATAGATCT	
pUC19-IVCap_F	CATTCAGGCGCGCCGGTAGTGT	Amplify plasmid cassette for region IV capture into puc19 plasmid for rescue clone generation
	GTACAAAGGGAGGCGTGCTCAC	
	GGCCCGCAACCCGGGTACCGAG	
	CTCGAAT	
pUC19-IVCap_R	TACTCAGGCCGGCCATCAAAAC	
	GCGAGCCCATATCGCCGCCATC	
	ATTGTAATCAGATGTGTGAAATT	
	GTTATCC	

to the ends of the region via these primers. The PCR product with the homology arms was purified and 300 ng of the purified PCR cassette was electroporated into DY380 carrying WT Toledo BAC with zeocin inserted into region IV. The transformed cells were grown overnight at 32°C on LB agar with zeocin and ampicillin. The colonies grown were picked from the LB agar plate and cultured overnight in 5 mL of LB with zeocin and ampicillin. Miniprep of pUC19-IV capture plasmid isolated and purified the DNA from the overnight culture using Qiagen's miniprep kit (Qiagen, CA). The captured region IV DNA fragment was confirmed by antibiotic selection and verified by PCR amplification of the first (UL132) ORF, the second to last (UL150) ORF, another ORF outside of region IV as a negative control (UL147), and zeocin resistance gene was verified as a positive control for capture (Figure 5). The plasmid was then digested with AscI and FseI restriction enzymes to separate the vector sequence from the region IV ROI. 500 ng of the purified digestion product (the rescue cassette) was transformed into DY380 carrying the Toledo IV-Δ mutant BAC by electroporation and the transformants were cultured on LB agar with zeocin and hygromycin (50 μg/mL). The resulting colonies were restreaked onto LB agar with either kanamycin or zeocin. The colonies that grew only on zeocin, and not on kanamycin plates, were used for miniprep. Region IV rescue (IV-R) BAC DNA was used for PCR verification along with WT and IV-Δ BACs (Figure 6). The IV-R BAC was also digested by EcoRI to check the integrity of the BAC DNA.

In order to produce mutant and rescue Toledo viruses, ~3–5 μg of the respective BAC DNA was electroporated into MRC-5 cells along with pCDNA-71 (pp71 expressing plasmid) and pGS403Cre (Cre expressing plasmid). The pp71 protein is a viral transactivator and is used to enhance viral growth [22]. The Cre protein is a recombinase that is used to remove the loxP flanking BAC vector and zeocin gene from the viral genome [16]. The electroporation conditions used were 260 V, 975 μF and time constant of ≤50 ms. MRC-5 cells electroporated with the IV-R BAC were grown in a 10-cm cell culture dish and split when cells reached 100% confluency, with wild-type BAC used as a control. Six-to-eight days following transfection, plaques with similar growth kinetics and characteristics were observed from both WT and rescue BAC DNA, indicating that the recombinant, deletion-rescued BAC DNA was as infectious as the wild-type BAC.

4. Discussion

BAC cloning technique of viral genome has been a useful tool, especially for mutagenesis studies of large DNA viruses such as HCMV, in which the BAC DNA has been used to mutate or delete the individual genes to understand their functions in viral replications. Its greatest usefulness was marked by studies in which viral gene functions were screened at global scale for both MCMV and HCMV [6, 9, 10]. HCMV BAC DNAs of several clinical and attenuated

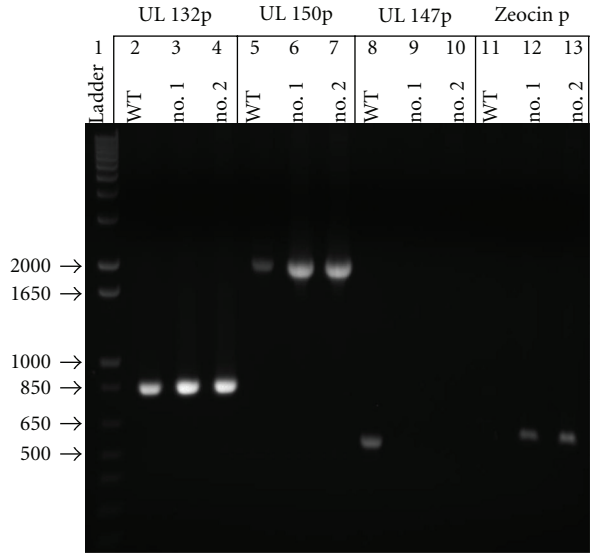

Figure 5: 1% Agarose gel electrophoresis of targeted amplification reactions in WT Toledo BAC (WT) and two pUC-19-IV capture plasmids. Lane 1: 1 kb Plus DNA Ladder. Lanes 2–4: amplification of UL 132 (~850 bases) with UL 132 F and R primers. Lanes 5–7: amplification of UL 150 (~1.9 kb) with UL 150 F and R primers. Lanes 8–10: amplification of UL 147 (negative control, not within region IV) (~500 bases) with UL 147 F and R primers. Lanes 11–13: amplification of zeocin marker (positive control for capture only) (~550 bases) with Zeocin F and R primers.

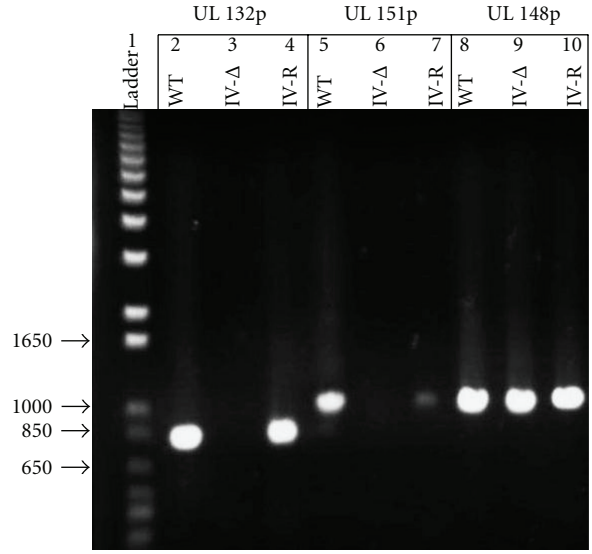

Figure 6: 1% Agarose gel electrophoresis of ORF targeted amplification reactions in WT Toledo BAC (WT), Region IV Deletion Mutant BAC (IV-Δ), and Region IV Rescued BAC (IV-R). Lane 1: 1 kb Plus DNA ladder. Lanes 2–4: amplification of UL 132 (~850 bases) with UL 132 F and R primers. Lanes 5–7: amplification of UL 151 (~1100 kb) with UL 151 F and R primers. Lanes 8–10: amplification of UL 148 (~1100 bases) with UL 148 F and R primers (positive control outside of region for deletion).

strains have been generated so that the genomic DNA can be propagated and sustained inside an *E. coli* host [3, 23]. The cloning technique using BAC DNA has several modifications over traditional cloning such as (1) using *E. coli* strains containing λ recombination proteins to enhance homologous recombination efficiency [12, 24–27]; (2) using cre/lox system so that extra DNA segments can be removed when making viruses using BAC DNA [15]; (3) inserting luciferase gene in the viral genome so that viral growth curve experiments can be more reproducible and less time consuming [12, 14, 17, 22]. These modifications have made BAC cloning easier and more efficient than before.

So far, most mutagenesis studies using the BAC system require generation of a rescue clone of the mutation to assure that no mutations occur anywhere other than the target site. The procedure of making rescue BAC DNA requires

an original DNA fragment to be inserted back into the viral genome. This can be achieved by PCR amplification of the DNA fragment along with homology arms flanking the mutated region and insertion of the amplified fragment back into the mutant genome by homologous recombination. If the DNA fragment were larger than the PCR limit, difficulties would arise in two aspects: (1) it is not easy to amplify; and (2) even if it is possible to be synthesized by currently available specific polymerases, the errors in DNA sequences are still unavoidable. To solve this problem, we have utilized the gap repair cloning method in BACs and modified it for the purpose of generating rescue BAC clones in a method called gene capture. Although the gap repair method of BACs has been utilized in yeast [19–21] and *E. coli* [13, 24–27], it has not been optimized for viral BACs in order to generate rescue viruses. It was first accomplished in HCMV when we presented the rescue of a 15-kb deletion BAC clone by the gene capture method, previously unattainable with the then current methods of PCR-based amplification of the region of interest for generation of rescue virus BAC clones [18]. It has here been presented as an optimized gene capture method for rescuing the region IV deletion mutant HCMV Toledo BAC clone with more practical methods that can be applied across many fields. By capturing the region IV DNA fragment into the rescue vector and putting it back into the mutant BAC, we successfully rescued the region IV deletion in HCMV genome. This gene capture method has three advantages: (1) no PCR was needed so that possible mutations induced by PCR can be avoided; (2) cloning of large DNA segments can be accomplished in only one step with high efficiency; and (3) different selection markers can be used during each step to ensure a higher cloning efficiency.

An additional advantage of the gene capture method is that it provides flexibility for the selection of the rescue vector. Any commercial or lab constructed plasmid can be used as a vector for capture. However, smaller plasmids are desirable, as they need to be PCR amplified with homologous regions for the capture method to proceed. As a proof of principle, we used a commonly available pUC19 plasmid as a rescue vector and successfully captured region IV from Toledo BAC. While choosing the vector plasmid, unique restriction sites flanking the capture gene are desirable. Those sites are later required to separate the captured region from the plasmid backbone for rescue cassette isolation and rescue procedure. If there are no unique restriction sites in the plasmid, they can be added while amplifying the plasmid via PCR primers, as was done in this case.

Previously, when generating a 15-kb rescue virus, we used homology arms of more than 1-kb on each side [18]. After this study, it has been shown that the size of the homology arms can be much smaller, which will reduce the total size of the rescue plasmid. In the case of this region IV deletion rescue, we used homology arms of 150-bp on each side of the rescue cassette, a significant improvement over the 1.5-kb arms used previously.

In summary, this enhanced gene capture method allows for easy cloning of large DNA fragments, previously a dubious practice when done by PCR, which can be used to rescue large deletions from any genome. By using region IV, a 6-kb DNA piece from HCMV genome as an example, we have demonstrated that the gene capture method is efficient and easier to perform than ever before for the construction of rescue clones from large deletion fragment mutants.

5. Future Directions

The potential for this method is undoubted, as it is not limited to just HCMV and can be used for mutagenesis study of other large DNA viruses as well as other organisms. Furthermore, the prospects of using the homologous recombination system *in vitro* for methods like gene capture has numerous applications, the extent of its reach has yet to be calculated in genetic and molecular cloning.

Abbreviations

BAC:	Bacteria artificial chromosome
HCMV:	Human cytomegalovirus
MCMV:	Murine cytomegalovirus
Toledo:	HCMV clinical strain
WT:	Wild type
ROI:	Genomic region of interest
region IV:	6-kb Toledo sequence (UL 132–151)
region IV-Δ:	Region IV deletion mutant clone
region IV-R:	region IV rescued clone.

Author's Contribution

K. Dulal and B. Silver contributed equally to this work.

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