


RESEARCH ARTICLE

Highly multiplex guide RNA expression units of CRISPR/Cas9 were completely stable using cosmid amplification in a novel polygonal structure

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Abstract

Background: Genome editing using the CRISPR/Cas9 system is now well documented in basic studies and is expected to be applied to gene therapy. Simultaneous expression of multiplex guide RNA (gRNA) and Cas9/Cas9 derivative is attractive for the efficient knockout of genes and a safe double-nicking strategy. However, such use is limited because highly multiplex gRNA-expressing units are difficult to maintain stably in plasmids as a result of deletion via homologous recombination.

Methods: Lambda *in vitro* packaging was used instead of transformation for the construction and preparation of large, cos-containing plasmid (cosmid). Polymerase chain reaction fragments containing multiplex gRNA units were obtained using the Four-guide Tandem method. Transfection was performed by lipofection.

Results: We constructed novel cosmids consisting of linearized plasmid-DNA fragments containing up to 16 copies of multiplex gRNA-expressing units as trimer or tetramer (polygonal cosmids). These cosmids behaved as if they were monomer plasmids, and multiplex units could stably be maintained and amplified with a lack of deletion. Surprisingly, the deleted cosmid was removed out simply by amplifying the cosmid stock using lambda packaging. The DNA fragments containing multiplex gRNA-units and Cas9 were transfected to 293 cells and were found to disrupt the X gene of hepatitis B virus by deleting a large region between the predicted sites.

Conclusions: We present a simple method for overcoming the problem of constructing plasmids stably containing multiplex gRNA-expressing units. The method may enable the production of very large amounts of DNA fragments expressing intact, highly-multiplex gRNAs and Cas9/Cas9 derivatives for safe and efficient genome-editing therapy using non-viral vectors.

KEYWORDS

cosmid, double nicking, lambda packaging, multiplex guide RNA, non-viral vector, nucleic acid therapy

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1 | INTRODUCTION

Genome editing using the CRISPR/Cas9 system is now well described in basic studies and is expected to be applied to gene therapy.^{1,2} The Cas9 enzyme recognizes 5'-terminal 20-nucleotide sequences of guide RNA (gRNA) and cleaves the specified position in the target gene. A gRNA expression unit (hereafter referred to as gRNA unit) consists of an approximately 250-nucleotide U6 promoter, a 20-nucleotide target sequence and a gRNA scaffold of around 100 nucleotides; the total length of one gRNA expressing unit is approximately 350 nucleotides.

Multiplex gRNA expression is often desired for several reasons. First, the simultaneous modification of several loci/genes in the same cell is useful in basic research fields and can be valuable for genome editing therapy. Genomes of cancer cells usually contain multiple mutated genes, which accelerates abnormal growth. Therefore, the simultaneous disruption of multiple genes is desirable to stop the development and growth of cancer. Second, although a single cleavage in the cell genome is immediately repaired by the end-joining mechanism, two simultaneous cleavages in one target gene achieve effective gene knockout via irreversible deletion.^{3,4} Third, the strategy of double nicking using Cas9 nickase, a mutated Cas9 introducing a nick instead of a cleavage, drastically increases cleavage specificity and reduces off-target activity, which contributes to safety.^{5,6} This strategy requires two gRNAs for one cleavage. Finally, multiple cleavage in one target gene guarantees disruption even if cleavage efficiency is low. Together, if the simultaneous expression of four or more multiplex gRNAs was available, the efficient and safe knockout of multiple target genes would become possible.

Although multiplex genome editing can be achieved by the co-transfection of multiple gRNA units, cells receiving all of the expressing units are limited, and the copy numbers of respective gRNA units transduced are different. The problem could be solved if the multiplex units are connected in one molecule: the same number of copies of each expressing unit can be introduced into a single cell. However, direct repeat sequences in plasmids tend to be deleted even in the commonly-used *RecA* hosts of *Escherichia coli* because of homologous recombination.⁷⁻⁹ Therefore, multiplex-gRNA units consisting of the U6 promoter, target sequences and gRNA scaffolds could be unstable via homologous recombination in plasmids sequentially amplified using DH5 α because all expression units are identical (except the approximately 20-nucleotide target sequences). It was also proposed that inverted repeats led to abortive replication structures.¹⁰ Thus, all identical DNA fragments are necessary for connection in the same orientation.¹¹

The technique of Golden Gate Assembly¹² was used for the construction of plasmids containing multiplex DNA fragments. Plasmids containing seven and 30 multiplex-gRNA units have been reported using this method.^{13,14} In this process, the construction of seven and 30 plasmids for each gRNA unit containing U6 promoter, a 20-nucleotide target sequence and a gRNA scaffold in this order is initially needed, following further construction for the assembly of these plasmids.

We recently developed a method of Four-guide Tandem (Nakanishi T, Saito I, unpublished data). In this method, a deleted gRNA scaffold and a deleted U6 promoter sequence in this order are ligated with a 20-nucleotide target oligo tagged with compensating nucleotides. All segments consisting of four gRNA-expressing units are specifically connected to produce an array, which is amplified by a polymerase chain reaction (PCR) and cloned into a cosmid utilizing lambda *in vitro* packaging (hereafter referred to as lambda packaging). By contrast to transformation, lambda packaging is size-selective and only transfers very large plasmids containing lambda cos sequences, known as cosmids, which are typically 36–52 kb in size.^{15,16} Consequently, unwanted deleted cosmids generated by homologous recombination are not packaged and thus are removed. Furthermore, because the transduction efficiency of lambda packaging is higher than that of transformation, less duplication of *E. coli* occurs and the chance of deletion is minimized. Together, cosmids containing four and eight multiplex-gRNA units can be amplified without deletion using DH5 α (Nakanishi T, Sato K, Saito I, unpublished data). However, because these cosmids must exceed 36 kb, they need to contain large, meaningless spacer DNAs to maintain this size, except that the spacer is utilized for adenovirus vector construction (Nakanishi T, Saito I, unpublished data). Therefore, these spacer-bearing (s-b) cosmids are not suitable for transfection, electroporation or nucleic acid therapies using multiplex gRNAs for genome editing.

In the present study, we demonstrate that DNA segments bearing multiplex-gRNA units lacking the spacer of s-b cosmids can be lambda-packaged after ligation, even though they are much smaller than the packaging limit of 36 kb. We found that the obtained "polygonal cosmids", multimers of the DNA segments, containing up to 16 multiplex-gRNA units, were stably amplified without deletion using DH5 α . Notably, they practically behaved as if they were monomer plasmids. Our findings suggest that they could be used for nucleic acid therapies utilizing genome editing.

2 | MATERIALS AND METHODS

2.1 | Construction of multiplex-gRNA units

Multiplex-gRNA units were constructed according to the Four-guide Tandem method (Nakanishi T, Saito I, unpublished data). Briefly, each target oligonucleotide was treated with T4-polynucleotide kinase and annealed together. To obtain four gRNA-unit fragments, ligation was performed by mixing five cassette fragments consisting of U6 promoter and gRNA scaffold and four annealed target oligos. All nine DNA fragments have specific termini generated by *Bsa*I. The ligated DNA was electrophoresed in an agarose gel, excised, and used for DNA extraction with the Wizard SV Gel and PCR Clean-Up kit (Promega Corp., Madison, WI, USA). DNA then underwent PCR amplification using Tks Gflex DNA polymerase (Takara Bio, Inc., Shiga, Japan) and was used for cosmid cloning. The detailed procedure for handling the cosmids is described by Sambrook *et al.*¹⁶). Normally,

approximately 200–300 µg of cosmid DNAs in 200 µL of TE buffer were obtained from 50 mL of full-growth culture.

We always checked the initial numbers of *E. coli* containing cosmid DNA added in 50 mL of culture by counting the number of colonies obtained from one sixtieth of the *E. coli* solution. If the number of colonies was small, the *E. coli* obtained in the full-growth culture was duplicated many times and the chance of deletion of the cosmids could be high. If the colony numbers using 1 µL of miniprep DNA was less than 100, the 50 mL of culture was discarded and another 50 mL of culture was prepared after scaling-up the packaging step or concentrating the miniprep DNA. Using a cosmid DNA of the second or additional lots, several thousand colonies were usually obtained in this assay.

2.2 | Cosmid preparation

Escherichia coli DH5α was used in all of the experiments. The cosmids consisted of a spacer (an approximately 30-kb adenovirus vector genome derived from pAxc4wit2),¹⁷ eight gRNA units, the Cas9 expression unit and a lambda-cos sequence. The expression unit of Cas9 was excised from pX330³ obtained from Addgene (Watertown, MA, USA), except that CB promoter was replaced by CAG promoter.¹⁸ The lambda *in vitro* packaging kit (Lambda Inn) was purchased from Nippon Gene (Tokyo, Japan). In total, 10 µg of s-b cosmid was digested with *Sall* (Figure 1, asterisks) and electrophoresed overnight in a 14-cm long 0.8% Tris-acetate-ethylenediaminetetraacetic acid (TAE) agarose gel at 35 V. The DNA fragment was purified using the Wizard SV Gel and PCR Clean-up kit as described above and was self-ligated. It was then packaged *in vitro* using DH5α to obtain polygonal cosmids. Miniprep-derived DNA was proceeded to 50 mL of culture via direct lambda-packaging of the circular DNA to minimize doubling times and devoid deletion of multiplex-gRNA units.

2.3 | Transfection experiments

The 293 cells are human embryonic fibroblasts derived from human kidney¹⁹ and were cultured in Dulbecco's modified Eagle medium (Kohjin Bio, Inc., Saitama, Japan) supplemented with 10% fetal calf serum. psCM103G,²⁰ a plasmid expressing pregenome RNA of hepatitis B virus (HBV) under the control of cytomegalovirus promoter and inducing HBV genome replication, was transfected with polyg cosmids of 4 g, 8 g and 12 g, digested by *SacI*. Transfection was performed using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in accordance with the manufacturer's instructions. Total cellular DNA was prepared 3 days post-transfection and amplified by PCR with Tks Gflex DNA polymerase (Takara Bio, Inc.) using a primer set HBV-X F primer (5'-AACTGGATCCTGCGGGGACGTCCCTTTGTC-3') and β-globin poly(A) R primer (5'-CTTTATTAGCCAGAAGTCAGATGCTCAAGG-3'). The PCR cycling conditions were: 94°C for 1 minute, followed by 30 cycles at 98°C for 10 seconds, 65°C for 15 seconds and 68°C for 30 seconds. The samples were analyzed on agarose gels.

3 | RESULTS

3.1 | Construction of “triangle” cosmids consisting of three identical copies of the fragments containing eight multiplex-gRNA units

We recently showed that the cosmids containing four and eight multiplex-gRNA units constructed using Four-guide Tandem method were able to be amplified stably maintaining the four and the eight multiplex-gRNA units. We first constructed the s-b cosmid of “all-in-one” type containing a Cas9-expression unit and eight multiplex-gRNA units (simply called s-b 8 g, hereafter) targeting the DR2 region of the HBV genome (Figure 1, top, and Figure 2A, third row; the eight multiplex units are shown in different colors from blue to brown.).

Next, the large spacer region was removed by cleaving this cosmid using the enzyme indicated by asterisks and a broken line (Figure 1, top), and approximately 13 kb of DNA containing both eight multiplex-gRNA units and Cas9 unit was isolated by gel electrophoresis (Figure 1, middle), followed by ligation and lambda packaging. Although the size of the 13-kb fragment is far below the packagable size of between 36 and 52 kb, thousands of colonies were obtained, and the restriction-enzyme patterns of the DNAs were in agreement with them comprising truly circularized 13-kb plasmids (Figure 1, bottom right; the result of digestion with *FspI* is shown later). However, examination of the uncut DNAs showed that their sizes were not 13 kb but instead approximately 38 kb (Figure 3A, lane 8 g), suggesting that the cosmid is the trimer of the 13-kb fragments, which contains 24 (8 × 3) gRNA units. We term these “triangle” cosmids (Figure 1, bottom left) and refer to cosmids of this type as “polygonal (polyg)” cosmids.

3.2 | Construction of s-b cosmids and polyg cosmids containing up to 16 multiplex-gRNA units

We attempted to examine the stability of such cosmids containing fewer and more multiplex-gRNA units both for s-b cosmids and for polyg cosmids. First, we newly constructed the s-b 4 g cosmid containing four gRNA-expression units targeting the HBV DR1 region and Cas9-expressing units (Figure 2A, second row; the four units are shown from red to purple). Then, to obtain the s-b 12 g cosmid (Figure 2A, fourth row), the block fragment of 4 g tagged with a small surrounding DNA of the 4 g cosmid was inserted into the s-b 8 g cosmid at the top of the eight-gRNA units. By chance, we obtained not only the desired s-b 12 g cosmid, but also a cosmid containing 16 gRNA units bearing a dimer of the 4 g block fragment, in which one set of four gRNA units was duplicated (s-b 16 g cosmid; fifth row). Then, we constructed polyg cosmids using cosmids of s-b 4 g, s-b 12 g and s-b 16 g using the method shown in Figure 1; finally, we obtained the whole set of s-b cosmids and polyg cosmids both containing arrays of 4, 8, 12, and 16 multiplex-gRNA units.

To examine the structures of all s-b cosmids, they were cleaved with *SspI*, which excises the DNA fragment containing the array of

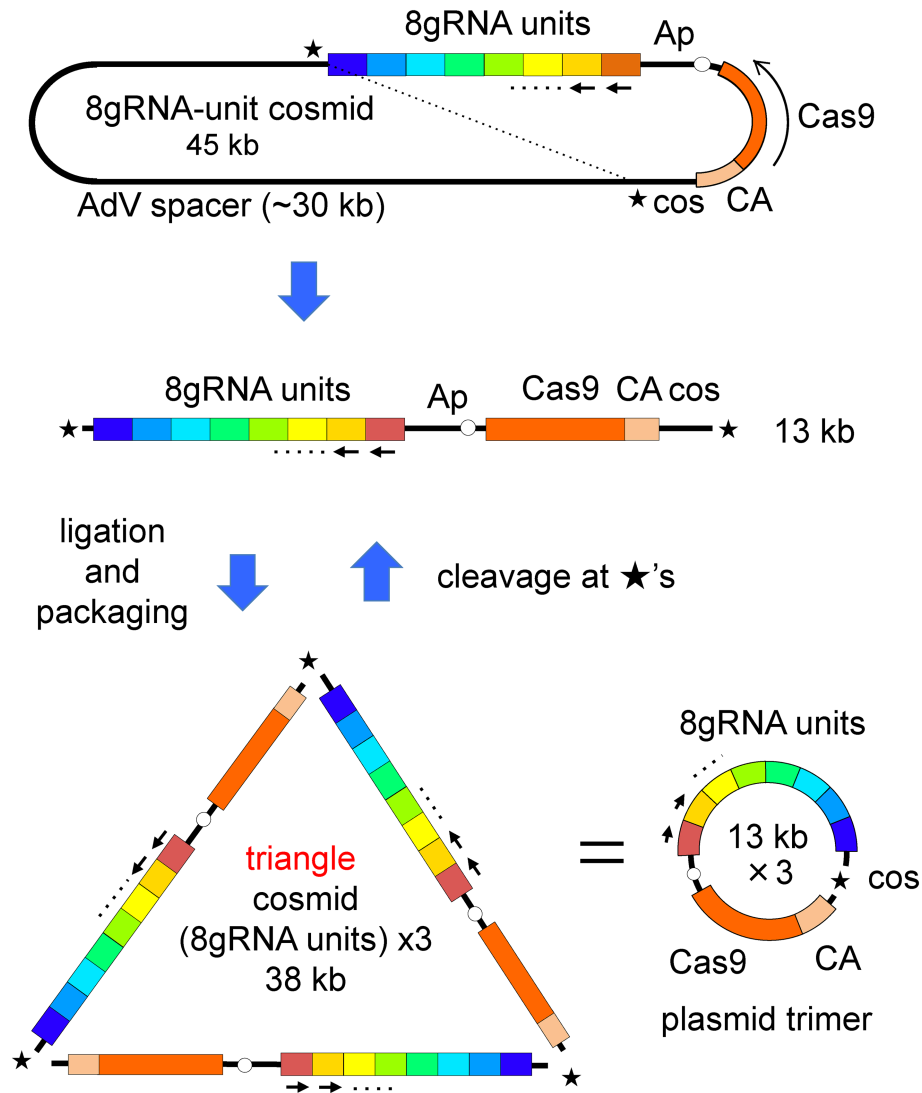


FIGURE 1 Construction of the triangle cosmids consisting of three blocks, each containing eight gRNA units and a Cas9 expression unit. Top: the structure of a cosmid containing eight gRNA units and a Cas9 expression unit. The stars (*Sall* sites) and the broken line indicate cleavage of the cosmid and removal of the large spacer DNA. Several *Sall* sites are present in the spacer, although only two sites are shown in the top row. Small arrows beneath the units show the direction of transcription of the U6 promoter. CAG promoter (CA) and Cas9 are shown as regions of light brown and brown, respectively. Small white circles near Ap (ampicillin) represent the plasmid replication origin. Middle: the structure of the excised “all-in-one” *Sall* fragment. Bottom left: the structure of the triangle cosmid consisting of three linear DNAs shown in the middle. Bottom right: a virtual plasmid corresponding to the circularized one side of the triangle cosmid. The triangle cosmid is the trimer of this plasmid and behaves as if this is a monomer plasmid because all of the DNA fragments generated from this cosmid by cleavage using any restriction enzyme are the same as those produced from this vertical monomer plasmid (see text; see also Figure 3B, right)

all gRNA units (Figure 2B, left). The excised fragments containing from 4 to 16 gRNA units maintained their intact sizes and accurately lined stepwise (Figure 2B, left, lanes 4 g to 16 g). No apparent ladder pattern as a result of deletions by homologous recombination was observed. However, some deletions appeared to occur especially for s-b 16 g because the intensity of the 8.0-kb band (lane 16 g), which contains 16 gRNA units, was considerably lower than that of the 9.5-kb band of the vector backbone. In addition, from s-b 4 g to s-b 16 g (lanes 4 g to 16 g), the intensity of the bands decreased as the sizes of the bands increased. *NdeI*, which cleaves a gRNA unit at one site present in the U6 promoter (Figure 2A, right), produced only the expected bands: a 0.38-kb band of unit length with increased intensity from s-

b 4 g to s-b 16 g (lanes 4 g to 16 g) and slightly higher bands in the lanes s-b 12 g and s-b 16 g as a result of the tags of adjacent DNA (Figure 2A; four gRNA units included in “12 guides” and “16 guides” were tagged with short bold black lines at both sides of four units of red to purple). Therefore, no apparent rearrangement was detected.

To examine the total sizes of all polyg cosmids, the uncut cosmid DNAs of polyg 4 g, 12 g and 16 g were electrophoresed using 0.4% agarose gel (Figure 3A). The cosmid sizes of polygs 12 g and 16 g were approximately 42 and 47 kb, respectively (lanes 12 g and 16 g). Therefore, they were probably triangle cosmids because the calculated lengths of the three fragments of the multiplex-gRNA units with a Cas9-expressing unit of 14.1 and 15.8 kb are 42.4 and 47.4 kb,

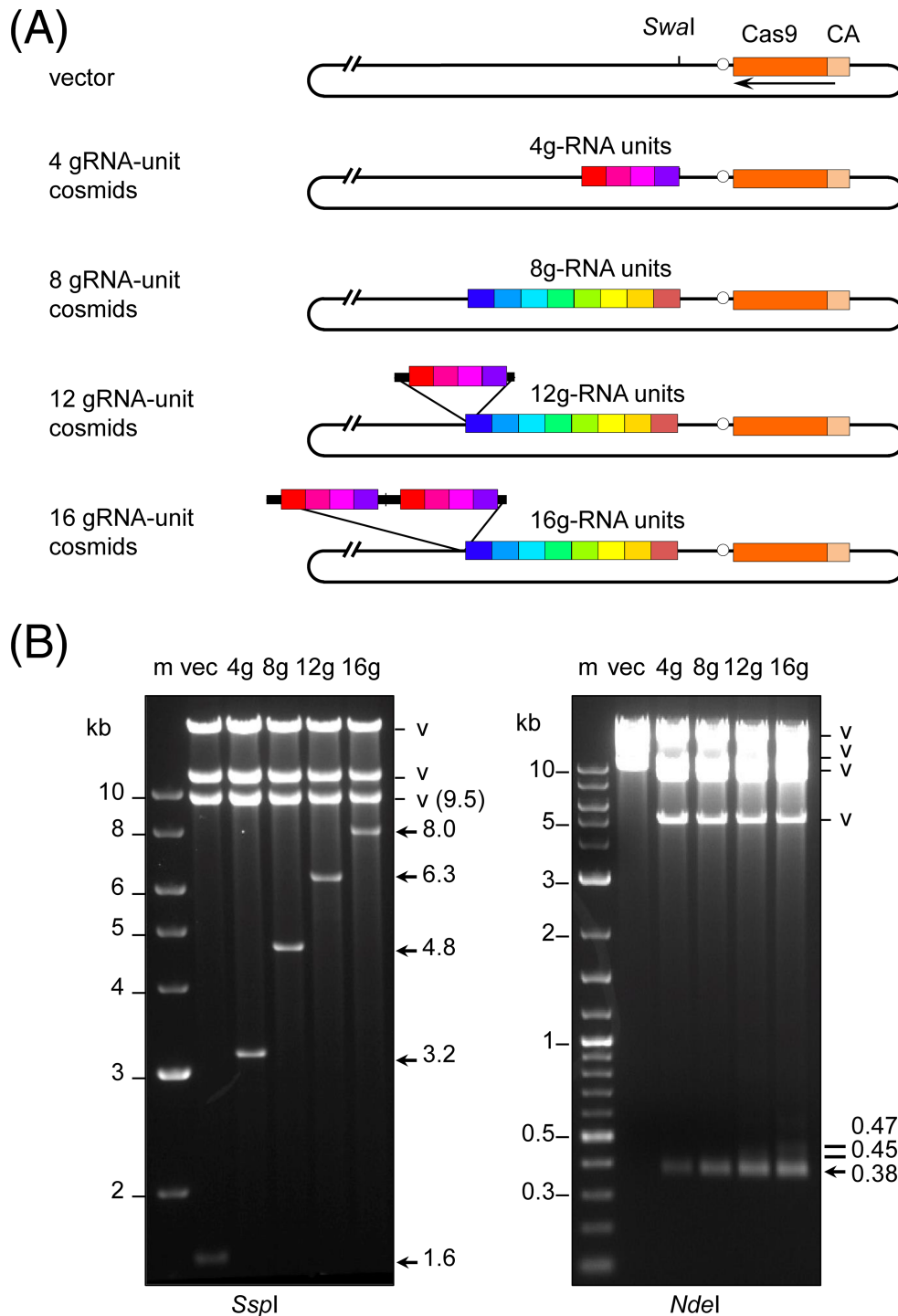


FIGURE 2 Structure of spacer-bearing (*s-b*) cosmids containing multiplex-gRNA units. (A) The whole set of *s-b* cosmids. First row: cosmid vector lacking gRNA unit. CA stands for the CAG promoter and the direction of transcription is indicated by an arrow. The blocks of four gRNA units (red to purple) inserted in the 12 g and 16 g cosmids are flanked with short DNAs (bold black lines), yielding bands corresponding to slightly higher molecular weights of 0.45 and 0.47 kb produced by *NdeI* digestion (Figures 2B and 3B, lanes 12 g and 16 g). (B) Stability of the multiplex-gRNA units in *s-b* cosmids. Left: *SspI*-cleaved *s-b* cosmids. *SspI* excises the fragments containing multiplex-gRNA units. Lane m, size marker; vec, vector cosmid lacking gRNA units. V, bands derived from vector backbone. The bands of 1.6, 3.2, 4.8, 6.3 and 8.0 correspond to the fragments containing 0, 4, 8, 12 and 16 gRNA units, respectively. Right, *NdeI*-cleaved *s-b* cosmids. *NdeI* cleaves the gRNA units at a single site and yields no bands (lane vec), intense bands of gRNA units of 0.38 kb (lanes 4 g to 16 g), and weak, higher bands of gRNA units tagged with the small DNA (lanes 12 g and 16 g). The intensity of the 0.38-kb band corresponds to copy numbers (the copy numbers of 0.38 kb must be 3, 7, 10 and 13 in the lanes of 4 g, 8 g, 12 g and 16 g and two higher bands consist of the right and left halves of the unit and tagged small DNAs) (Figure 2A)

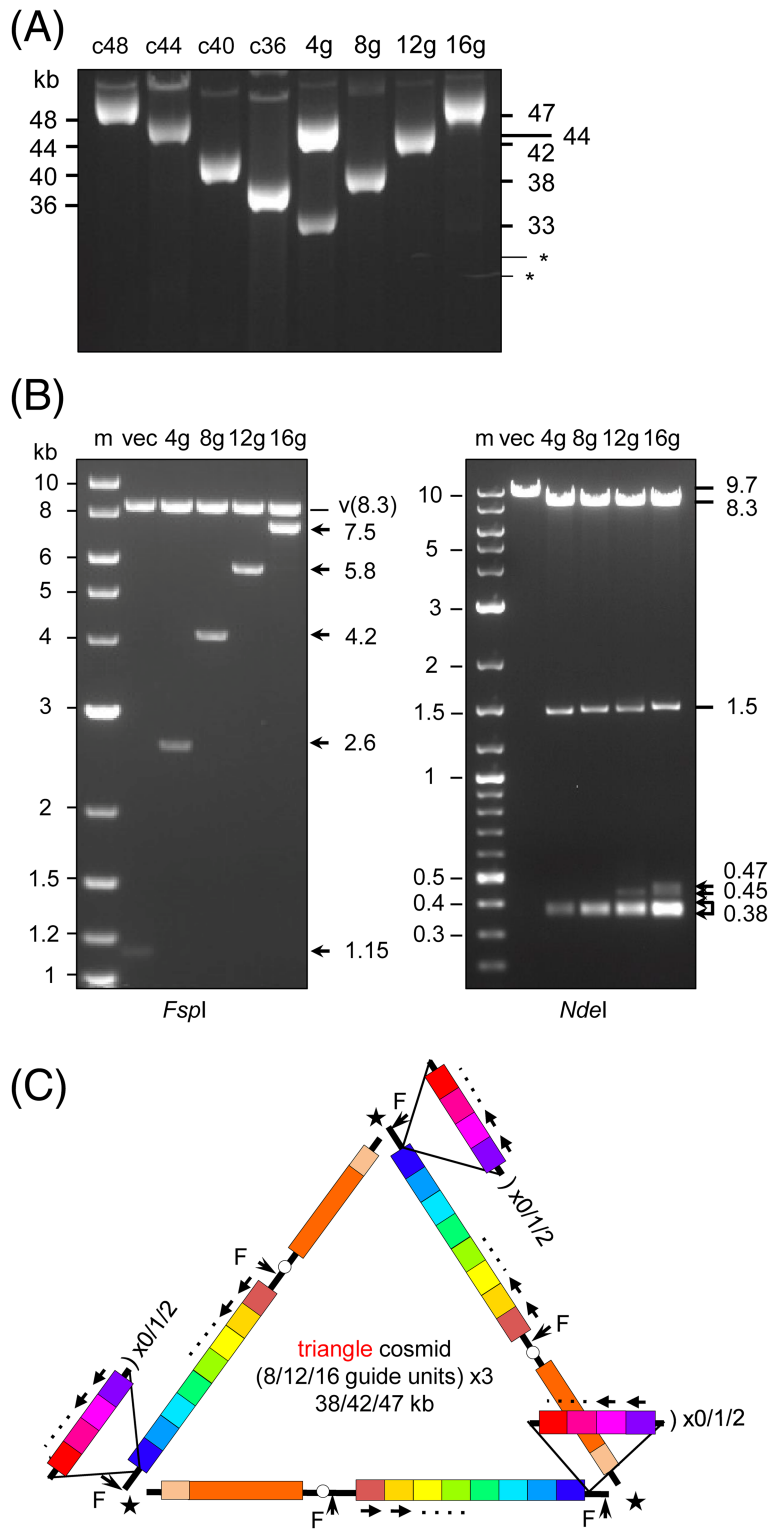


FIGURE 3 Structure of polygonal (polyg) cosmids containing multiplex-gRNA units. (A) The gel electrophoresis pattern showing the whole size of the polyg cosmids of closed circular form. Uncut polyg cosmid DNA was electrophoresed using 0.4% agarose gel and TAE buffer at 60 V for 20 hours. c48 to c36, size markers of uncut charomid DNAs.¹⁵ Asterisks, faintly deleted cosmids in lanes 12 g and 16 g. (B) Stability of the multiplex-gRNA units in polyg cosmids. Left: *FspI*-cleaved polyg cosmids. *FspI* divides the DNA fragments containing multiplex-gRNA units and the vector backbone of 8.3 kb common in lanes from 4 g to 16 g. Right: *NdeI*-cleaved polyg cosmids. The positions and intensities of gRNA units excised from the plasmid backbone are the same as in Figure 2B. Vec, the linearized vector plasmid of 9.7 kb lacking a gRNA unit. The bands of 1.5 and 8.3 kb contain the right and left halves of the unit, respectively. (C) Structure of the triangle cosmids containing 8, 12 and 16 multiplex-gRNA units in three blocks. Note that the total copy numbers of the units are 24, 36 and 48, respectively. The additional four multiplex units present in polyg 12 and polyg 16 are shown as insertions present near the three apices. The other presentations are the same as in Figure 1

respectively, which fall within the preferable packagable size range from 36 to 52 kb, as well as polygs 8 g. The structures of polygs 8 g, 12 g and 16 g are shown in Figure 3C. The vast majority of the three cosmids maintained their intact sizes, indicating that they were considerably stable. The reason why only trimers (i.e. triangular cosmids) were observed is that the dimers and tetramers are either too small

or too large for packaging in the construction of polygs of 8 g, 12 g and 16 g. By contrast, the uncut polyg 4 g cosmid yielded two bands of approximately 44 kb and less than 36 kb (lane 4 g). Because the calculated length of the four gRNA units plus the Cas9-expression unit is 11 kb, this result suggested that the polyg 4 g cosmid is a mixture of triangle and quadrangle cosmids of 33 and 44 kb, respectively (see

Supporting information, Figure S1). The reason why there was a significant amount of triangular cosmids of 33 kb, which is smaller than the normal lower limit for packaging, may be that the triangular cosmid was initially a very minor component but became a major one because of its growth advantage, or that more DNA fragments generated by tri-molecular ligation were present than those by tetra-molecular ligation in a low concentration of DNA fragments.

Next, we investigated whether deletion or rearrangement occurred in these polyg cosmids. Note that, if the polygs 8 g, 12 g and 16 g are triangle cosmids, they contain 24, 36 and 48 multiplex-gRNA units in one molecule (Figure 3C). All polyg cosmids of 4 g (a mixture of triangle and quadrangle cosmids), 8 g, 12 g and 16 g were digested with *FspI* (Figure 3B, left). *FspI* separates the fragment containing the array of multiplex-gRNA units from the common vector backbone of 8.3 kb. In the lanes of polygs of 4 g, 8 g, 12 g and 16 g, the expected bands of 2.6, 4.2, 5.8 and 7.5 kb, respectively, were observed and they were stepwisely lined, as observed for s-b cosmids. Surprisingly, in contrast to the results of s-g cosmids, the intensity of the 7.5-kb band of 16 g was very similar to that of the 8.3-kb band of the vector backbone, and, from 4 g to 16 g, the intensity of the bands increased together with the band sizes. This result shows that the multiplex-gRNA units in polyg cosmids were much more stable than those of s-b cosmids. *NdeI* yielded the expected 0.38-kb bands of the excised gRNA units and their intensities corresponded to the copy numbers of the multiplex-gRNA units. Altogether, the deduced structures of triangle cosmids consisting 8, 12, and 16 multiplex-gRNA units together with Cas9-expressing units were very stable and deletion and rearrangements were scarcely detected.

3.3 | The deleted cosmid was removed by further amplification using the lambda package

We also examined the stability of cosmids containing many identical 0.4-kb fragments derived from HBV DR1 region, the size of which is similar to gRNA units. In these experiments, an excess amount of the fragments and a small amount of cosmid vector were cleaved using enzymes such as *AvaI*¹⁵ or *BstXI* (present study), which produce nonpalindromic ends that can be ligated only in the same direction. Surprisingly, one of the obtained cosmids contained 75 copies of identical 0.4-kb fragments (Figure 4A, left): the cosmid consisted of two plasmid backbones and two large inserts of 22 and 16 kb, which correspond to the lengths of arrays consisting of 55 and 20 copies, respectively (Figure 4A, middle). The structure was confirmed by *BstXI* digestion, which excised the 0.4-kb fragment. In addition to vector-derived bands, we observed a very dense band of 0.4 kb that consisted of 75 copies of the fragment with no detectable rearrangement (Figure 4A, right, lane 5). A similar pattern was observed for another clone, which appeared to contain two arrays of approximately 25 and 40 copies of the 0.4-kb fragment (lane 6). Therefore, even 75 copies of 0.4-kb fragments were stably maintained using lambda packaging.

Normally, we obtained such cosmids with no detectable deletion but, occasionally, these were contaminated with a small amount of deleted cosmids (Figure 4A, right, lane 3, asterisk). During a similar

experiment using a cosmid containing 60 copies of 0.5-kb fragment from HBV S gene (see Supporting information, Figure S2), the cosmid was contaminated with a considerable amount of deleted cosmid in the first lot of the 50-mL culture preparation (Figure 4B, 1st, lane *Sfil*, red arrow). This was a rare accident in our experience. We prepared the second lot from the 50-mL culture, which corresponded to 200-fold amplification. Unexpectedly, the band of the deleted cosmid disappeared in this second lot (Figure 4B, 2nd, lane *Sfil*, red arrow), suggesting that the deleted cosmid had been removed, probably because its size was below the packaging limit. These results appear to be important because the problem of deletion could be avoided simply by amplifying further using lambda packaging, and a large number of intact cosmids could be prepared by repeating the amplification.

3.4 | Disruption of a target gene via a large deletion by expression of multiplex gRNAs

To examine whether multiplex gRNAs disrupt a target gene at multiple sites, the DNA fragments containing expression units of four, eight or twelve multiplex gRNAs and Cas9 (e.g. Figure 1, center) were prepared from polyg cosmids of 4 g, 8 g and 12 g, respectively, by digesting the cosmids with a restriction enzyme, shown as stars. The 4 g, 8 g and 12 g fragments included 4gRNA, 8 gRNA, and both 4 and 8 gRNA, respectively. The 293 cells were transfected with these fragments together with pSCM103G,²⁰ a plasmid inducing HBV genome replication and containing DR1 and DR2 regions within the target HBV X gene (Figure 5A). Three days after transfection, the structures of X gene in the target plasmid were examined by PCR (Figure 5B). Initially, we extracted total cellular DNA for the experiment but only the unprocessed DNA of 0.6 kb was observed, probably because transfected DNAs were attached to the cell surface.²¹ Therefore, we isolated cell nuclei, although transfected DNA substrate still appeared to be contaminated in this experiment. The 4 g fragment is expected to cause three possible cleavages of guide 1, 2 and 3 (Figure 5A, seventh and eighth rows) and to produce the fragments lacking the regions among the cleavage sites by end-joining mechanism in 293 cells. In fact, the 0.53-kb band was observed (Figure 5B, lane 4 g), suggesting the double cleavage occurred at guide 1 and guide 3 (Figure 5C, second low of 4 g). The cleavage efficiency of guide 2 cannot be examined in this experiment. In the lane of 8 g, the 0.48-kb band was observed together with a weaker 0.51-kb band, probably indicating that the former was produced by cleavage of both guide 5 and guide 12 (third low). The latter may be yielded by cleavage of both guide 7 and guide 12 (fourth low), suggesting that the cleavage of both guides 5 and 6 was not efficient so that the fragment derived from cleavage by guide 7 was detected. In the lane of 12 g, 0.29-kb band was detected together with a 0.34-kb band; the former may be produced by cleavage occurring between 8 gRNA 5 and 4gRNA 3, whereas the latter produced between 8 gRNA 5 and 4gRNA 2 (fifth and sixth lows). In a short exposure of 0.6-kb bands (Figure 5B, lower), the intensity of the bands of 4 g, 8 g and 12 g decreased while the number of guides increased, which might suggest that additional multiplex guides can disrupt targets more efficiently.

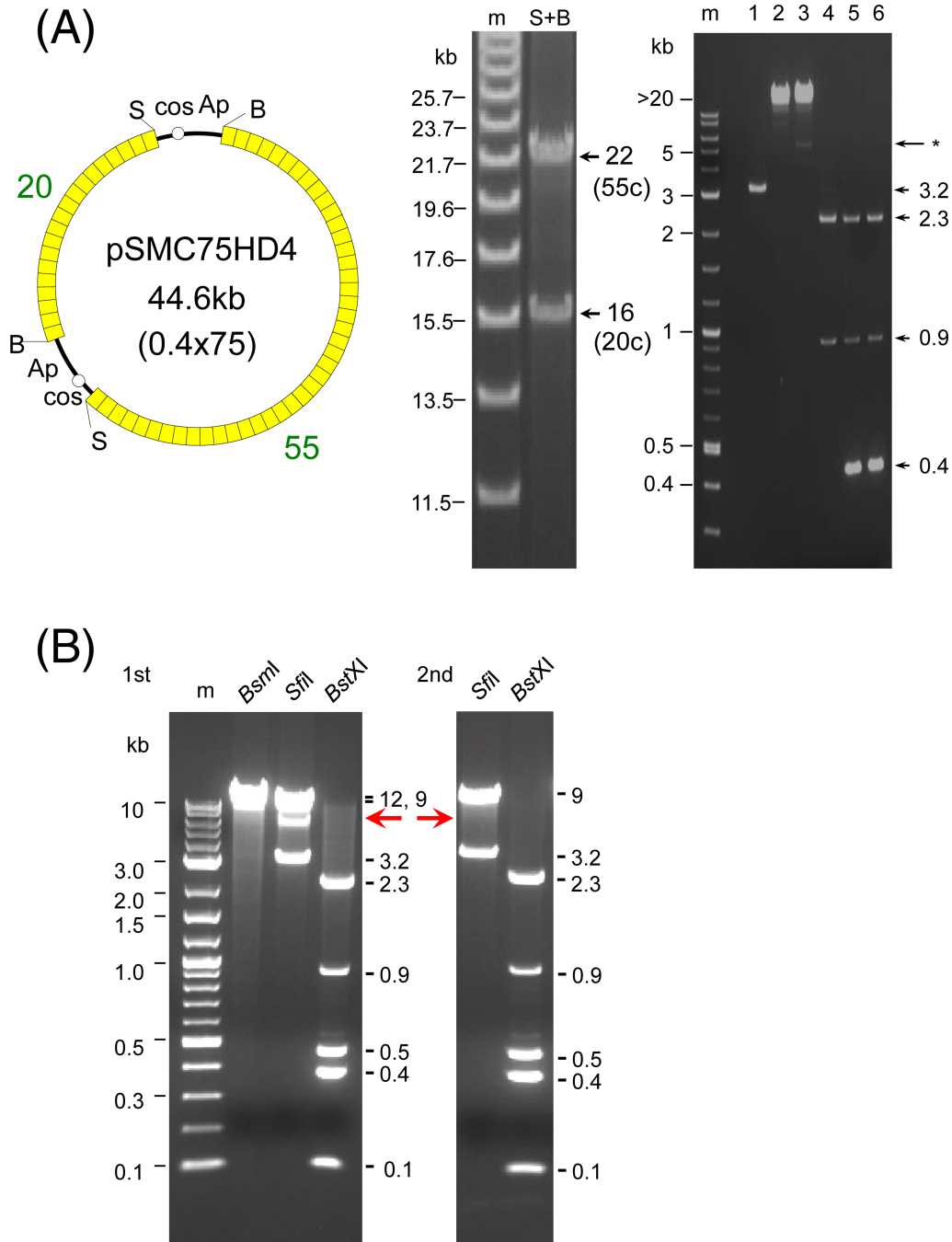


FIGURE 4 Cosmid containing 75 copies of the 0.4-kb identical fragments. (A) Left: the structure of the cosmid. Cos, lambda cos sequence; Ap, ampicillin; small circle, replication origin of the plasmid; S, *Sall*; B, *Bam*HI. Middle: the gel electrophoresis pattern showing the sizes of the arrays of 0.4 kb. The size marker (m) was a 2-kb ladder of linearized charomid-vectors.¹⁵ 55c and 20c; 55 copies and 20 copies. Right: *Bst*XI excised 75 copies of the 0.4-kb fragments. Lanes 1 and 4: the original cosmid vector of 3.2 kb; lanes 2 and 5, pSMC75HD4; lanes 3 and 6, a similar cosmid of a different clone. Lanes 1, 2 and 3, *Bam*HI-cleaved cosmids; lanes 4, 5 and 6, *Bst*XI-cleaved cosmids. The cosmid vector of 3.2 kb contains not a single site, but two *Bst*XI sites, and produces two fragments of 2.3 and 0.9 kb; only one site was selectively used in the tandem cloning because all of the *Bst*XI sites were only specifically ligated and the non-cloning site was restored. Asterisk, an extra band in lane 3 showing a deleted cosmid. (B) Disappearance of the deleted cosmid by the passage of the first lot. This cosmid contains 60 copies of 0.5-kb fragments in total (three blocks each containing 20 copies; see Supporting information, Figure S2). *Bsm*I separates each block and *Sfi*I separates the array consisting of 0.5-kb fragments and the 3.2-kb vector. The red arrow shows the position of the deleted cosmids seen in lane *Sfi*I of the first lot. In the lanes of *Bst*XI of the first and second lots, the bands of 2.3 and 0.9 kb are derived from the vector. The inserted 0.5-kb fragment has a dam-methylated *Bst*XI site, which is cleaved only partially (see catalog; New England Biolabs, Beverly, MA, USA) and produces 0.4, 0.1 and uncut 0.5-kb bands

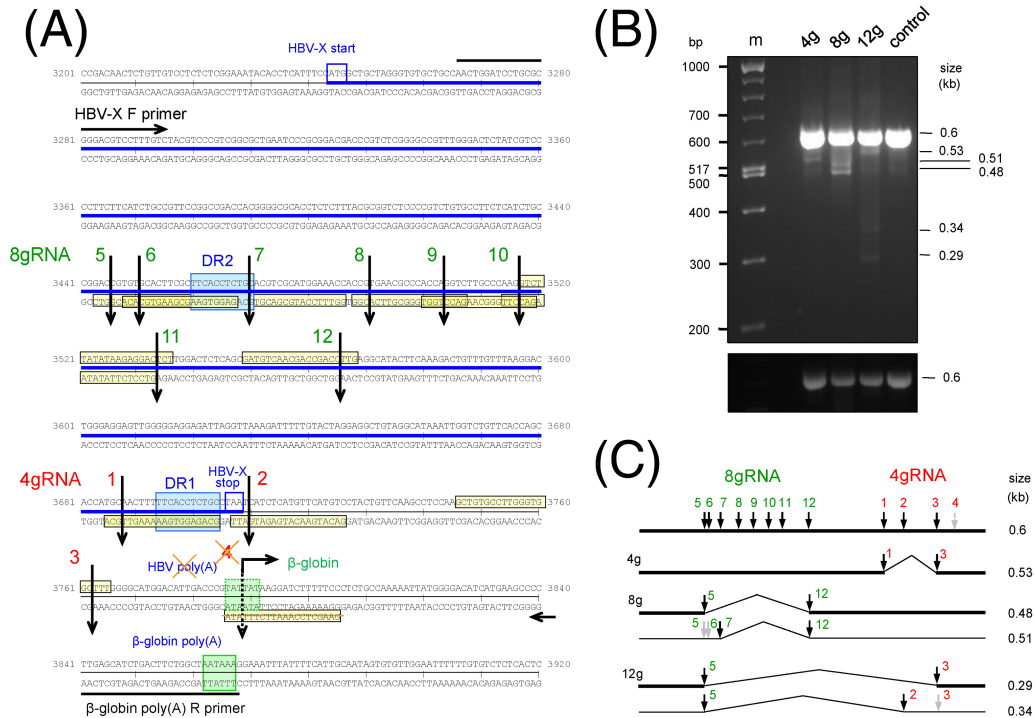


FIGURE 5 Disruption of HBV X gene via deletion using DNA fragments derived from 4 g, 8 g and 12 g cosmids in 293 cells. (A) Cleavage sites of gRNAs included in the 4 g (4gRNA, red) and 8 g (8gRNA, green) fragments. The 12 g fragment includes both 4gRNA and 8 gRNA cleavage sites. The 20-nucleotide recognition sequences of individual guide RNAs are boxed. The coding region is indicated by thick blue lines. HBV poly(a) sequences are disrupted and replaced by chicken β-globin poly(a) sequences to elongate the half-life of HBV mRNAs.^{18,21} (B) Specific cleavages using 4 g, 8 g and 12 g DNA fragments. The 293 cells were transfected with the above fragments together with the target plasmid psCM103G and the nuclear DNAs were amplified using HBV-X F and β-globin poly(a) R primers (shown in A), 3 days post transfection. A short exposure of the photograph of the unprocessed PCR product of 0.6 kb is also shown. Control, 293 cells transfected only with psCM103G. (C) Schematic representation of possible gRNA cleavage sites in the PCR products. Cleavage sites of 4gRNA and 8 gRNA are shown in red and green, respectively

4 | DISCUSSION

In the present study, we have described a method for the construction of “polygonal cosmids”, which behave as ordinary plasmids in digestion with restriction enzymes and in measurement of the DNA amount using the usual optical density. Most importantly, they appeared to be completely stable and were amplified without deletion using the commonly-used DH5α *E. coli* strain. Besides the lambda packaging, another difference from plasmids is that the polyg cosmids require linearization by a single-cut enzyme before transformation or electroporation because their large sizes probably decrease efficiency.

The polyg cosmids constructed in the present study could be used for large-scale production, which would be sufficient for nucleic acid therapy using multiplex gRNA-expressing units together with Cas9- or Cas9-derivative-expressing units. Particularly, the deleted cosmid generated during the first amplification was removed by the second amplification using lambda packaging, suggesting that polyg cosmids can be amplified further without deletion. However, preliminary experiments suggested that, when the cosmid of Figure 4B was intentionally used for transformation instead of lambda packaging in the preparation of the first lot, a possible dimer of the deleted cosmid of the packageable size was produced, which was difficult to remove using

subsequent lambda packaging. The difference might be explained by transformation not being size-selective.

Because the deletion could be reduced by minimizing the number of duplication times of *E. coli* used for production, the monitoring of *E. coli* numbers added in the 50-mL culture described in the Materials and methods is probably important. Because the concentration of miniprep DNA used for preparation of the first lot is low, the number of the *E. coli* containing the cosmids transduced by lambda packaging is limited. Consequently, it duplicates many times and the chance of deletion is high. However, once the cosmid of the first lot with lack of deletion was obtained, the generation of deleted cosmids during the amplification in the second and further preparations could highly be reduced because the concentration of the cosmid DNAs is much higher than the miniprep DNA and, hence, low duplication times can be obtained. Because the lysate of lambda packaging can be produced on a large scale, a large amount of intact polyg cosmids sufficient for practical use could be prepared, although further examinations are needed to test this possibility.

In the genome-editing therapy, the off-target problem hampers its safe application. Although the double-nicking strategy using Cas9 nickase, a mutated Cas9 that introduces a nick instead of a cleavage, drastically increases the cleavage specificity,^{5,6} this method has not

been used frequently because two gRNAs are needed for one cleavage. The polyg cosmids described in the present study may offer one solution and even multiple cleavages using this strategy via the expression of multiplex gRNAs. In addition, lambda packaging is not necessarily expensive compared to transformation because only one-fifth or less is sufficient, since one packaging of commercially available product intends to generate whole genomic library.

We have shown that even 16 multiplex gRNA-expressing units were stably amplified, indicating that eight simultaneous double-nicking cleavages are possible. We also observed that 16 g polyg cosmids, which contain 48 gRNA units (and not 16 gRNA units), were stable and, moreover, 75 and 60 copies of identical DNA fragments of approximately 0.4 kb were stably maintained. These results suggest that cosmids containing up to 75 multiplex-gRNA units are likely to be stable, and that construction could be achieved by assembling multiplex-gRNA fragments. Cosmids containing "super-multiplex" gRNA units would be useful for screening the best gRNA by comparing cleavage efficiency in the same condition.

The results of the transfection experiment suggest that the DNA fragments of 4 g, 8 g and 12 g containing the expression units of multiplex gRNAs and Cas9 in one molecule caused deletions between the predicted sites and disruption of the target gene in 293 cells. Because the efficiency of both first and last cleavages, which causes deletion of whole cleavage region, appeared to sufficiently high, the cleavage efficiency of individual guides except the first and last cannot be estimated in this experiment. However, because the last guide worked efficiently, the activity of U6 promoter appeared not to be suppressed by upstream promoters. Therefore, most, if not all, of the internal promoters might work efficiently. Note that, although our PCR assay only showed deletions, small insert/deletions causing knockout of the gene function could occur at individual gRNA sites in multiplex-gRNA approaches.

The large-scale preparation of plasmid DNA is particularly important for nucleic acid therapy. Although multiplex gRNA-expressing units are unstable in plasmids, the present study shows that polyg cosmids were completely stable and could be amplified without deletion. Therefore, polyg cosmids could offer the possibility of safe and efficient non-viral gene therapy vectors that simultaneously target highly multiplex genes.

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AUTHOR CONTRIBUTIONS

TN developed the methods, especially for constructing cosmids containing 4 to 16 multiplex gRNA. AM analyzed the structure of polygonal cosmids. TY and ZP carried out the experiments about removal of deleted cosmids and construction of cosmids bearing fragments of extremely many copies, respectively. HT, KS, MM and MK constructed cosmids containing 4 to 16 multiplex gRNA units and polygonal cosmids. IS designed the project and wrote the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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