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RESEARCH ARTICLE

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Utilizing CRISPR/Cas9 technology to prepare lymphoblastoid cell lines harboring genetic mutations for generating quality control materials in genetic testing

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Abstract

Background: To meet the requirements of the rapidly progressing genetic testing technologies in clinical laboratories, assuring the quality of genetic tests by utilizing appropriate quality control materials is of paramount importance. The CRISPR/Cas9 technology was used to prepare quality control materials because genome-edited human cell lines are one of the major resources for quality control materials.

Methods: In this study, in vitro transcribed sgRNA were transfected into a Cas9-expressing lymphoblastoid cell line (LCL)—by electroporation—to simulate the SEA-type deletion observed in α-thalassemia. The edited positive cell line was screened and identified by polymerase chain reaction (PCR) followed by Sanger sequencing. The whole-genome sequencing was also performed to show evidence of predicted mutation.

Results: The results showed that electroporation of the in vitro transcribed gRNAs into stable Cas9-expressing LCL was a more efficient gene-editing technique as compared to plasmid-mediated transfection, and that the positive rates could reach up to 35.9%. The predominance of indel sizes relative to the predicted deletion length was clustered between 10 and 0 bp. The results of whole-genome sequencing also demonstrated the existence of SEA-type deletion of α-thalassemia.

Conclusions: Gene-editing based on Cas9-expressing LCL by electroporation of sgRNA was a more efficient approach to introduce mutations for generating quality control materials for genetic testing. The edited lymphoblastoid cell lines were feasible to serve as quality control materials in genetic testing.

KEYWORDS

CRISPR/Cas9, genetic testing, in vitro transcribed gRNAs, lymphoblastoid cell line, quality control material, stably Cas9 expressing cell line

1 | INTRODUCTION

With the progressive development of molecular genetic testing in clinical laboratories, assuring the quality of human genetic testing has been recognized as a major need. The lack of appropriate quality control (QC) materials in genetic tests is the key reason to prevent the development of quality assurance in genetic testing, QC materials refer to the materials intended for use in quality assurance, test validation, external quality assessment, etc[1-3] The current QC materials used in genetic tests can be classified into the following three categories: (a) non-cell line-based QC materials, such as artificially synthesized materials; (b) establishment of stably transformed cell lines by using residual patient samples; and (c) modified human cell lines with target mutations. Those three QC materials have their unique strengths and weaknesses. The modified human cell lines are technically inhibited due to the complexity of introducing a targeted mutation into the wild-type cell lines.[1] It is imperative to figure out an efficient approach to introduce the desired mutation into the cell lines.

Recently, with the rapid expansion of the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated endonuclease Cas9 (Cas9) gene-editing technique, this technique has been applied in preparing QC materials by introducing a targeted mutation into wild-type cell lines in several studies. Peng et al[4] used CRISPR/Cas9 technology to edit non-small cell lung cancer (NSCLC) cell lines to introduce EML4-ALK rearrangement and prepare reference materials for EML4-ALK testing. Jia et al[5] generated a novel KRAS G12V mutant HEK293T cell line utilizing the CRISPR/Cas9 technique to serve as quality control material for the next-generation sequencing (NGS) of KRAS mutations. Lin et al[6] efficiently integrated exogenous sequences containing the target mutations into the ALK locus in HEK293T and A549 cells by CRISPR/Cas9-mediated non-homologous end-joining. However, the basic strategies in those studies were to introduce target mutations into the adherent cells by delivering reconstructed plasmids with or without donor templates by lipofection. On one hand, although the mutation can be successfully integrated into the cell lines, it is a very labor-intensive and time-consuming process to construct and identify the reconstructed plasmids. On the other hand, the plasmid-based methods involving lipofection were merely suitable for the adherent cells because of the extremely low transfection efficiency of the plasmids in the suspension cells.[7-9] Therefore, it is necessary to establish a more efficient method to employ the CRISPR/Cas9 technology for preparing quality control materials in the suspension cells, such as lymphoblastoid cells. This is especially important in genetic testing where most of the parent-child cell lines housed in the Coriell cell repository was lymphoblastoid cell lines. Highly efficient delivery of sgRNA or Cas9 vectors into the cells is a pre-condition for successful genome editing. The transfection efficiency of the plasmids is quite low in the suspension cells,[10,11] which makes the subsequent experiments more complicated. In recent years, some studies have indicated that CRISPR/Cas9-based gene-editing by utilizing synthetic guide RNAs in the Cas9-expressing cells can produce results similar to those obtained with the stably expressed sgRNAs introduced by viral transduction.[12-14] In light of the short length of sgRNA (98-mer), we aimed to observe if the editing efficiency could be improved by directly transfecting in vitro transcribed sgRNA into the Cas9-expressing lymphoblastoid cell line (LCL). The SEA-type deletion mutation of α-thalassemia was selected as target mutation for its high frequency in α-thalassemia and due to its potential application in subsequent research on non-invasive prenatal testing (NIPT).

This study aimed to utilize CRISPR/Cas9 technology to develop lymphoblastoid cell lines containing α-thalassemia genetic mutations that could serve as quality control materials for genetic testing. This research sheds new light on generating cell lines with defined mutations in LCL by transfecting in vitro transcribed sgRNA into Cas9-expressing LCL. The mutations were verified using different methods. The results showed that gene-editing based on Cas9-expressing LCL by directly transfecting sgRNA was a more efficient approach for introducing mutations into the lymphoblastoid cell lines, and the edited lymphoblastoid cell lines were feasible to serve as quality control materials in genetic testing.

2 | MATERIALS AND METHODS

2.1 | Cell culture

GM12878 cells stably expressing Cas9 and mCherry were kindly gifted by the Wensheng Wei Laboratory. The cells were cultured in RPMI 1640 medium supplemented with 15% FBS, 100 μg/mL streptomycin, and 100 IU penicillin (all from Thermo Fisher Scientific) at 37°C in an atmosphere of 5% CO_2.

TABLE 1 The primers for PCR to amplify gRNA DNA ahead of in vitro transcription

	Name	Sequence (5′ to 3′)
Primer	sgRNA-F-A1	GAAATTAATACGACTCACTATA**G**ACAAACGCCCGTCCGACTCAGTTTTAGAGCTAGAAA
Primer	sgRNA-F-B1	GAAATTAATACGACTCACTATA**G**GCTGTCGGCGCCAAACTAGCGTTTTAGAGCTAGAAA
Primer	sgRNA-F-A2	GAAATTAATACGACTCACTATA**G**GGTCGTCCCCACTGTCGTCGGTTTTAGAGCTAGAAA
Primer	sgRNA-F-B2	GAAATTAATACGACTCACTATA**G**CGGCGCCAAACTAGCTGGATGTTTTAGAGCTAGAAA
Primer	sgRNA-Rev	AAAAGCACCGACTCGGTGCC

Note: The base "G" was added in front of the primer to increase in vitro transfection efficiency.

2.2 | sgRNA design and in vitro transcription

sgRNA oligo sequences were chosen based on CHOPCHOP (http://chopchop.cbu.uib.no/#). According to the mutation of α-thalassemia, chr16:165301-165501 and chr16:184401-185001 were used as input to simulate the SEA-type α-thalassemia mutation (Figure S1). The higher score was selected to be the most appropriate sgRNA for simulating the SEA-type α-thalassemia mutation. Two sgRNAs were selected for two sites and were termed sgRNA A1/A2 and sgRNA B1/B2, respectively. T7 RNA polymerase promoter and the specific target sequence were added to the gRNA sequences by PCR using the synthetic plasmid that contained scaffold associated region of the full gRNA as templates. The specific forward primers and universal reverse primer used for PCR were listed in Table 1. The PCR products were used as templates for in vitro transcription using the HiScribe™ T7 High Yield RNA Synthesis Kit (New England Biolabs). The obtained gRNAs were purified utilizing the MEGAclear kit (Life Technologies) and eluted in nuclease-free water and stored at−80°C until use.

2.3 | Electroporation

The electroporation machine, pressured electroporation tubes, and electroporation buffer were obtained from Celetrix LLC, Manassas VA. The cells (2×10^6 cells/mL) were resuspended in an electroporation buffer. Each in vitro transcribed guide RNAs (12 μg) was added to the cells. Electroporation was completed according to the manufacturer's instructions. The electroporation conditions were as follows: 400 V for 30 ms using a 20 μL tube electroporator machine. After electroporation, the cells were immediately transferred into a warm medium to continue culture.

2.4 | T7EI assay

T7EI assay was performed according to the manufacturer's instructions and then run on a 1.5% agarose gel. The densities of cut and uncut bands were calculated using Image J software. We used F_{cut} = (cut1 + cut2)/(uncut + cut1 + cut2) and Indel% = $1 - \sqrt{(1 - F_{cut})}$ formulas to get the efficiency of cleavage activity.

2.5 | Fluorescence-activated cell sorting and genotype identification of single clones

After 96 hours of transfection, the single cells were sorted in round-bottom 96-well plates (Corning) using a MoFlo® XDP-SX (XDP, Beckman Coulter). The clones were screened for CRISPR/Cas9-mediated deletion after 2 − 3 weeks of culture. The genomic DNA was extracted by Quick Extract DNA extraction solution (Epicenter) according to the manufacturer's instructions. PCR conditions using Prime STAR Max DNA Polymerase (Takara Bio Inc) were as follows: 98°C for 5 minutes followed by 35 cycles at 98°C for 10 seconds, 65°C for 5 seconds, 72°C for 5 seconds; and 72°C for 4 minutes. The PCR products of the positive clones were subjected to Sanger sequencing by Sango Biotech.

2.6 | Genomic DNA isolation and whole-genome sequencing

Genomic DNA was extracted from 3 cell lines: two WT cell lines (blank control: Blank-1 express Cas9, Blank-2 does not express Cas9), one Cas9-edited biallelic deletion cell lines (Homo) using the QIAamp DNA Mini Kit (QIAGEN, Cat No./ID: 51304) following their protocol. For each sample, at least 1 μg DNA was used to construct a sequencing library and the final libraries were sequenced on Gene + Seq-2000 (Paired-end 150 bp) with an average 35 × sequencing depth at the Gene+ company. The sequences were aligned using BLAST between Cas9-edited and WT cell lines. On-target site mutations are showed through WGS data in Figure 5 using Integrative Genomics Viewer (IGV) tools.[15]

3 | RESULTS

3.1 | Electroporation of in vitro transcribed gRNAs into stable Cas9-expressing LCL is highly efficient and expedite gene-editing technique

CRISPR/Cas9 components can be delivered in the form of plasmid or the Cas9/gRNA complex. Considering the published reports regarding the high cost of the RNP system and the low transfection efficiency of plasmids into LCL,[7-9] and the results of our preliminary experiments, we performed gene-editing by transfecting in vitro transcribed gRNAs into stably Cas9-expressing LCL by electroporation. Almost no indels were detected in the plasmid transfection groups (data not shown), however, the gRNAs transfection group showed 10 − 30% indels in T7EI assay (Figure 1) and the editing efficiency reached up to 35.9% (28/78), which indicates the latter is a more efficient gene-editing way. Further, the time needed for the workflow was shortened as the transcription of the specific sgRNA had been completed based on the PCR product including T7 promoter and target sequence (Figure S1). For different sgRNAs, the template and the reverse primer were common. The entire process from ordering the primer to identifying the genotype of a single clone took about 3 weeks. I also calculated the cost during the experiment and looked up the related data to estimate the cost of directly using synthetic sgRNA. The former cost about $18.05 while the latter cost at least $600 during one experiment,[16] which showed the latter was much more expensive. Those results suggested that electroporation of in vitro transcribed gRNAs into stable Cas9-expressing LCL is an efficient and cost-effective approach.

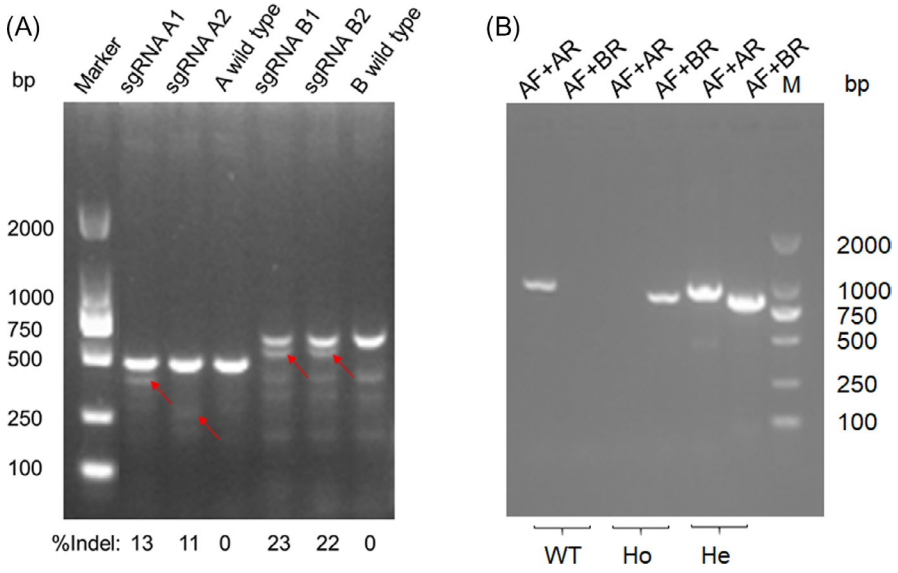

FIGURE 1 T7EI assay results and identification of the mutant genotype by traditional PCR. A, A wild type and B wild type mean the blank control group. The indel rates of each group were marked at the bottom of each electrophoresis band. The specific cutting bands caused by CRISPR/Cas9 cleavage was marked with red arrows. B, The genotype was identified by PCR using two sets of primers (AF/AR or AF/BR)

3.2 | Identification of SEA-type α-thalassemia cell line generated by the CRISPR/Cas9 system

The individual editing efficiency of the two pairs of sgRNA (sgRNA A1/ B1 and sgRNA A2/ B2) is summarized in Table 2. The common editing efficiency (percentage of positive clones) of sgRNA A1 and sgRNA B1 was 35.9% (28/78) which was much higher than that of sgRNA A2 and sgRNA B2 (19.2%, 27/141). This finding was coincident with the predicted data obtained from the CHOPCHOP website. The genotype was identified by PCR using two sets of primers. The non-deletion band was the amplification product of the primer AF and primer AR. The deletion band was the amplification product of the primer AF and primer BR. The clones that had PCR product of the deletion band and lack the non-deletion band were defined as biallelic deletion(homozygotes). The clones that had both the non-deletion band and deletion band were defined as monoallelic deletion clones (heterozygotes). The non-deletion clones (wild type) were identified as clones that had the PCR product of the non-deletion band and did not have the deletion band. All three cell types were successfully identified (Figure 1).

3.3 | Analysis of the edited mimetic SEA-type α-thalassemia cell line by Sanger sequencing

The PCR products of the positive clones were subjected to Sanger sequencing and then aligned to the human genome sequence (GRCh38/hg38). Figure 2 showed the Sanger sequencing results for all the positive clones. The number of base pairs inserted or deleted relative to the predicted deletion length was calculated as indel sizes. The range of indel sizes was from −437 to +483 bp in the mutant homozygotes and from −100 to +1 bp in the mutant heterozygotes. The representative Sanger sequencing results of the mutant clones whose cleavage sites were exactly the same as those of the predicted cut sites are presented in Figure 3. The preponderance of the indel sizes was clustered between −10 and 0 bp irrespective of whether they were present in mutant homozygotes or heterozygotes (Figure 4). Notably, the precise predicted deletion on α globin gene cluster occurred in 15.8% (6/38) of the alleles in the biallelic and 41.2% (7/17) in the monoallelic deletion clones. Sanger sequencing of the biallelic deletion clones revealed 14/38 (36.8%) of the single clones to be not completely homozygous based on the differing indels at the predicted deletion sites on each allele. The remaining 24/38 (63.2%) of the biallelic deletion clones had only a single deletion junction identified by sequencing.

3.4 | Identification of on-target mutations at the genome-wide scale by whole-genome sequencing

We tested on-target site mutations in one CRISPR/Cas9-edited and two WT cell lines by WGS. WGS of three cell lines genomic DNAs yielded a total of 536 Gb of raw data, and produced between 255.20 and 393.46 million sequence reads per clones. Over 94.85% of the generated sequence reads were mapped and sequencing depth per clones ranged from 47 to 72 × genome coverage after alignment to the reference genome, suggesting that it is a rigorous data set that covers the genome in sufficient depth to detect sequence variants. Analysis of the WGS data clearly uncovered specific deletion in the CRISPR/Cas9-edited cell lines but not in WT. The representative result revealed a homozygous deletion of 19 kb in chr16(Figure 5), which were consistent with the former PCR identification conclusions.

4 | DISCUSSION

In this study, we sought to prepare SEA-type α-thalassemia lymphoblastoid cell line by CRISPR/Cas9 technology in a simple and highly efficient way. The Cas9-expressing lymphoblastoid cell line was

TABLE 2 Biallelic deletion frequency and monoallelic deletion frequency among the different sgRNA combinations after single-cell sorting

Deletion type	sgRNA A1 and sgRNA B1	sgRNA A2 and sgRNA B2	Total
Biallelic deletion	19 (24.4%)	18 (12.8%)	37 (16.9%)
Monoallelic deletion	9 (11.5%)	9 (6.4%)	18 (8.2%)
Non-deletion	50 (64.1%)	114 (80.8%)	164 (74.9%)
Total	78	141	219

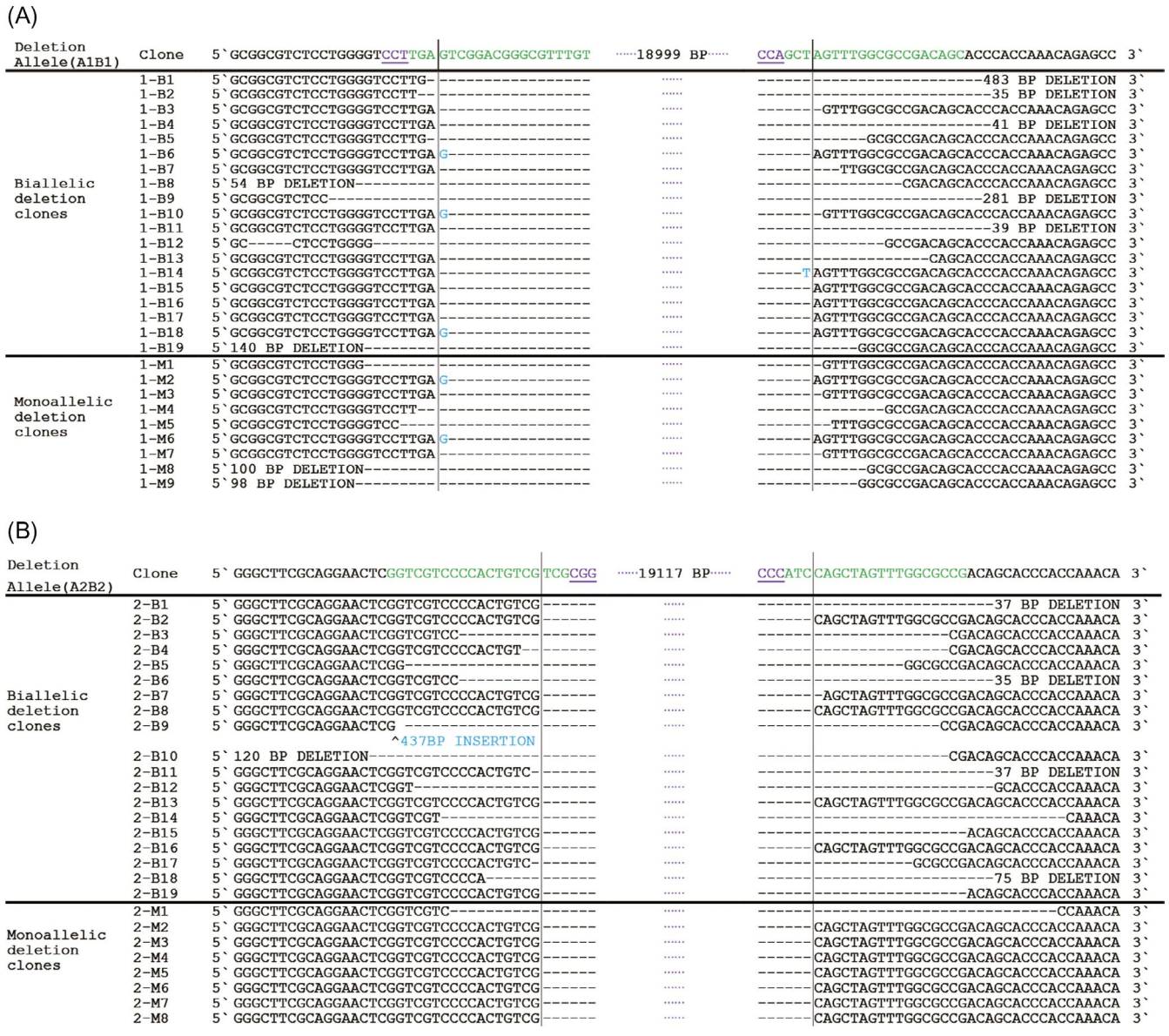

FIGURE 2 Indels at the non-deletion/non-inversion allele and indels at the predicted deletion junction at the α globin gene locus. A,B, Sequencing of the monoallelic deletion allele in the compound deletion clones and biallelic deletion clones at the α globin gene locus by sgRNA A1/B1 and sgRNA A2/B2 are shown. The top row indicates the sequence of the unmodified allele. The sgRNA sequences are shown in green and the PAM sequences in purple and underlined. The deletion events are shown by an equivalent number of dash marks and insertions are highlighted in blue. The vertical lines indicate the predicted cleavage site between positions 17 and 18 of the sgRNA. The representative Sanger sequencing results of the mutant clones whose cleavage sites were exactly the same as those of the predicted cut sites

used in this research by transfecting in vitro transcribed sgRNA into it. Other studies have also reported transfection of chemically synthetic sgRNAs or crRNA:tracrRNA complexes into Cas9-expressing cells to assess the result of gene-editing.[13] However, chemical synthesis of gRNA for each target sequence is quite expensive if different kinds of mutant cell lines are needed to be prepared. Compared

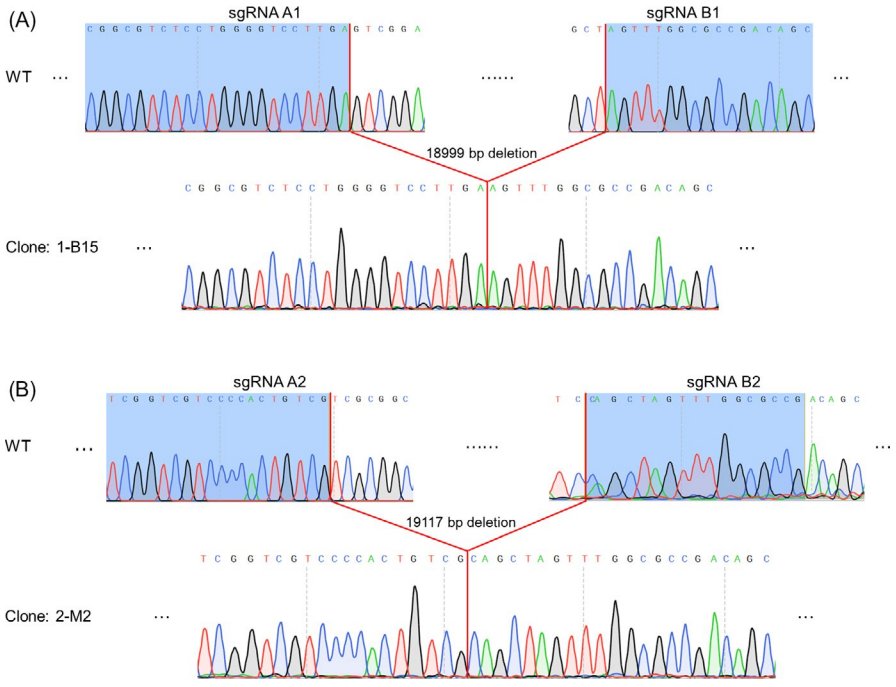

FIGURE 3 The representative Sanger sequencing results of the mutant clones whose cleavage sites were the same as those of the predicted cut sites. A, showed an exact 18 999-nt deletion existed at the PAM motifs junction generated by sgRNA A1/B1. B, showed an exact 19 117-nt deletion existed at the PAM motifs junction generated by sgRNA A2/B2

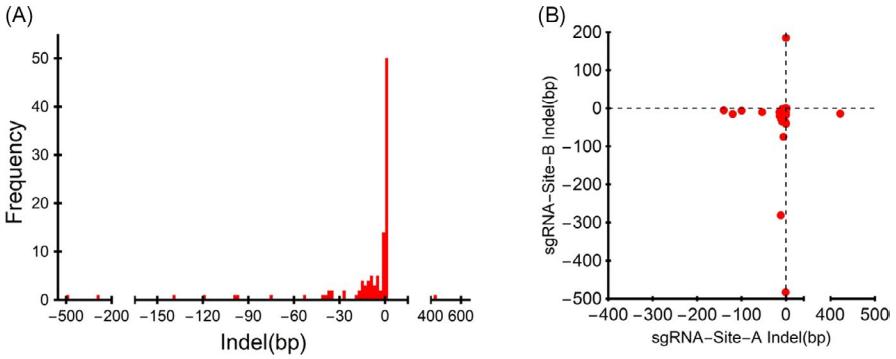

FIGURE 4 Indels at the α globin gene locus. A, The frequency distribution of indel formation at the predicted deletion junctions from the deleted alleles of monoallelic and biallelic deletion clones across the four loci was examined in detail. B, The distribution of indel formation at the predicted deletion junctions from the deleted alleles of deletion clones produced by sgRNA A and sgRNA B across the four loci was examined

to transfection by recombinant RNPs,[17-19] our approach just requires synthesis of different forward primers for different target mutations as the gRNA DNA template and the reverse primer are universal. Thus, our approach reduces the variation among the batches associated with Cas9 nuclease generation and the expenditure on the synthesis of specific sgRNA. Compared to transfection using recombinant plasmids,[20-23] it took at least 5 days to prepare and verify the recombinant plasmids. Hence, our method was more cost-effective and time-saving.

The results of analysis by Sanger sequencing revealed that both the monoallelic and biallelic deletion clones showed subjects of indel clustering between −10 and 0 bp, which were in fact a quite insignificant part of difference for the nearly 19 kb deletion. Therefore, the edited cell lines could serve as QC materials in genetic testing of SEA-type α-thalassemia. The edited cell lines were also identified by whole-genome sequencing, which confirms that the edited cell lines could serve as QC materials. In addition, the GM12878 employed in our investigation was used in ENCODE projects, and hence, information regarding its genome is quite clear. Besides, GM12878 cell line was derived from the mother of the CEPH reference family, the same mutation could be created in the lymphoblastoid cell line originating from the offspring and the father. Our group has recently found an approach that could produce matched maternal and fetal cfDNA (cell-free DNA) that could be employed for the preparation of QC materials for non-invasive prenatal testing (NIPT) of trisomy.[24] Considering that >6600 kinds of monogenic diseases have been found in the world.[25] It is impossible that in every common disease, the parent-child cell lines could be purchased. Therefore, the parent-child genetically mutant cell lines prepared by the CRISPR/Cas system could compensate for the shortage of parent-child cell lines for some familiar monogenic diseases, which may play an important role in the NIPT of single-gene genetic diseases.

Much is still being learned about the limitations of this approach. First of all, the "off-target" effects, which may result in false-positive and negative results, need to be considered. Continuous expression of Cas9 and sgRNA in the cells could result in accumulation of off-target sites over time.[26] Some studies showed that the off-target effects could be ameliorated by controlling the expression duration or concentration of the gRNA or

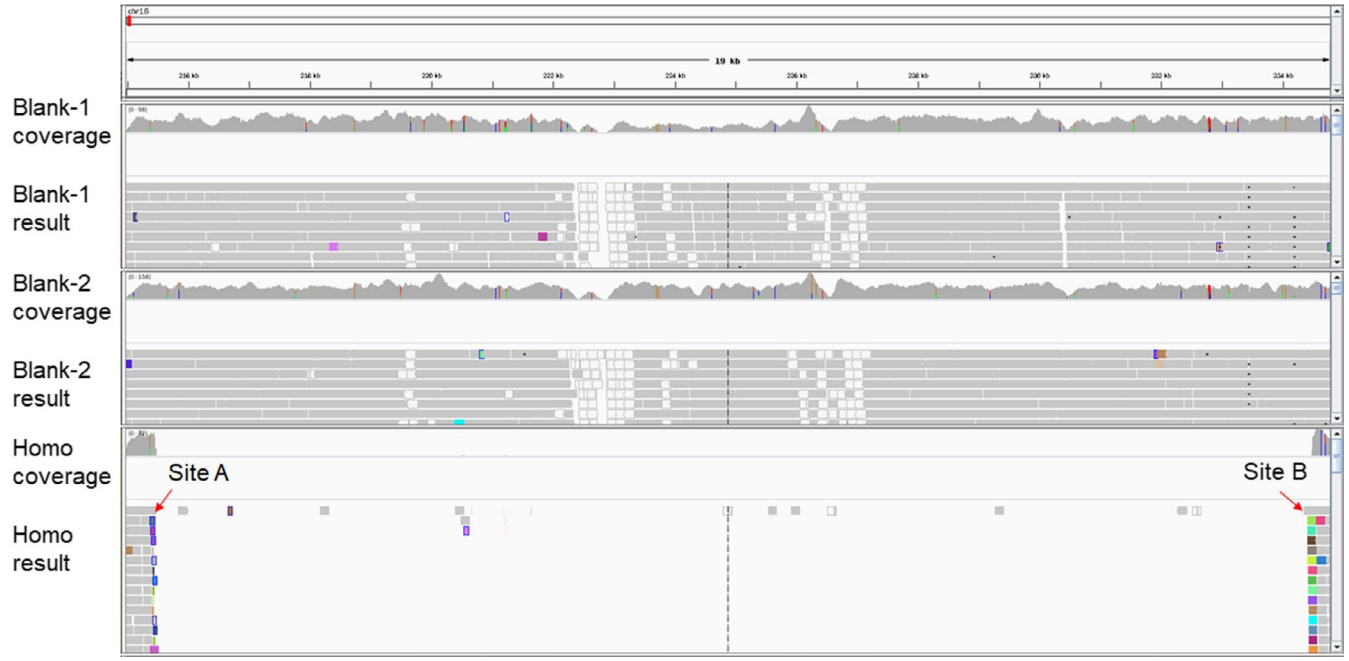

FIGURE 5 Whole-genome sequencing analysis at sgRNA A/B target region of chr16 in two wild type and representative Cas9-edited cell lines by Integrative Genomics Viewer (IGV). The pileup strip represents Cas9-edited a homozygous deletion of 19 kb in chr16. Red arrows indicate DNA cleavage sites

Cas9 ribonuclease.[27,28] Chemically synthetic or in vitro transcribed gRNAs or can be utilized in CRISPR-Cas9 experiments to provide a perfect solution to circumvent the long-term existence of sgRNA. It was worth mentioning that tolerance for the level of off-targeting effect would vary depending on the application of genome editing. For instance, the therapeutic applications may have a much lower endurance for off-target effects as compared to our research where CRISPR-Cas9 technology was applied to prepare QC materials that would not be used until the QC materials were verified. The other concern was that the Cas9 gene sequence was inserted into the genome of the Cas9-expressing LCL, which might have an impact on DNA detection or genomic stability. Nevertheless, we all knew that the CRISPR/Cas system was a naturally occurring adaptive immune defense system first found in bacteria.[29] Therefore, this would not influence the detection of mutation in the human genome. In regard to genomic stability, the function of CRISPR/Cas system required two components (Cas9 nuclease and gRNA) to exist at the same time, thus the genomic stability of Cas9-expressing LCL and LCL was comparable.

In conclusion, we have implemented a Cas9-expressing cell line and in vitro transcribed gRNA-based CRISPR platform to generate cell lines containing genetic mutations that was proved to be feasible for QC materials. This method will reduce the technological hurdles for the application of CRISPR/Cas9 in developing QC materials for genetic testing, especially when cells are hard to transfect. The edited cell lines were verified by various methods to show the evidence of predicted mutation and the feasibility for QC materials. This could provide a very efficient method to rapidly generate large panels of isogenic cell lines with different genetic mutations for preparing QC materials used in genetic testing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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