

## MODIFICATION OF THE METHOD FOR ANALYSIS OF GENOME EDITING RESULTS USING CRISPR/CAS9 SYSTEM ON PREIMPLANTATION MOUSE EMBRYOS

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Genetically modified animals are an important tool for biomedical research. The CRISPR/Cas9 editing genome system is increasingly being used for production of such animals. Through microinjection, complex with guide RNA and Cas9 protein is delivered in fertilized eggs from which the animal subsequently develops with a modification in the genome. Generally, analysis of the specificity and efficiency of the system in each case is carried out after obtaining a progeny with the likely mutation. However, analysis at the preimplantation stage would allow reducing the time of the experiment, as well as understanding the reason for the birth of a small number of transgenic animals, or even lack of them in the offsprings. The paper proposes a modification of the method of preparation of total DNA from mouse blastocysts. The modification allows to easier and faster detect the results of microinjection of the CRISPR/Cas9 complex in the zygote. Having applied the method described in this paper, we successfully identified short deletions in intron 34 of dystrophin gene (*DMD*) in 12 out of 13 treated embryos and insertion in the break site in intron 8 of the *DMD* gene in 11 out of 21 samples analyzed. Using for analysis the total DNA prepared by the method proposed, you can analyze up to 20 different sites in the mouse embryo genome at the blastocyst stage without the need for full genomic amplification.

**Keywords:** genome editing, CRISPR/Cas9, short nucleotide insertions, nucleotide deletions, mouse embryos, Duchenne muscular dystrophy

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## МОДИФИКАЦИЯ МЕТОДА АНАЛИЗА РЕЗУЛЬТАТОВ РЕДАКТИРОВАНИЯ ГЕНОМА С ПОМОЩЬЮ СИСТЕМЫ CRISPR/CAS9 НА ПРЕДИМПЛАНТАЦИОННЫХ ЭМБРИОНАХ МЫШИ

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Генно-модифицированные животные — важный инструмент биомедицинских исследований. Для их получения все чаще используют систему редактирования генома CRISPR/Cas9. С помощью микроинъекции комплекс РНК-гида и белка Cas9 доставляется в оплодотворенную яйцеклетку, из которой впоследствии развивается животное с модификацией в геноме. Как правило, анализ специфичности и эффективности системы в каждом случае проводят после получения потомства с вероятной мутацией. Однако анализ на предимплантационной стадии позволил бы сократить время эксперимента, а также понять причину рождения малого числа или даже отсутствия трансгенных особей в потомстве. В статье предложена модификация метода подготовки тотальной ДНК из бластоцист мыши, позволяющая проще и быстрее детектировать результаты микроинъекций комплекса CRISPR/Cas9 в зиготу. Применив описанный в статье метод, мы успешно идентифицировали короткие делеции в интроне 34 гена дистрофина (*DMD*) в 12 из 13 обработанных эмбрионов и вставку по месту разрыва в интроне 8 гена *DMD* в 11 из 21 проанализированных образцов. Используя приготовленную предложенным способом тотальную ДНК, можно анализировать до 20 различных сайтов в геноме мышинного эмбриона на стадии бластоцисты, не прибегая к полногеномной амплификации.

**Ключевые слова:** редактирование генома, CRISPR/Cas9, короткие вставки нуклеотидов, делеции нуклеотидов, эмбрионы мыши, миодистрофия Дюшенна

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Genetically modified organisms are an indispensable tool in the study of gene functions and non-coding sequences, interactions between regulatory sequences in the genome and recombinant protein expression. They are also vital for human disease modeling. Until recently, obtaining genetically modified animals was very time-consuming and expensive, thus making the process practically inaccessible for many research groups. However, all these obstacles have been eliminated by the advent of new genome editing systems. In 2013, the first paper on the use of CRISPR/Cas9 system (clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9) was published — techniques that can be used to inactivate several genes at a go [1].

The system includes an RNA containing clustered regularly interspaced short palindromic repeats (CRISPR), transactivating RNA and Cas9 nuclease. This complex, which naturally acts as bacterial immunity against parasitic phages [2], was adapted for DNA engineering both in vitro and in mammalian cells [3]. The Cas9 protein generates double-stranded breaks at 3 nucleotides from the PAM (protospacer adjacent motif) site — NGG, which is located just behind the sequence complementary to the 19-nucleotide guide RNA [4]. The cell repair system of the genome makes short deletions or insertions in the site of the break. Addition of constructs containing sequences homologous to the region around the break leads to homologous repair and possibly insertion of the desired fragment into the genome at a particular site.

This approach is used to knock in expression cassette into a site where a break has been introduced. The discovery of CRISPR/Cas9-mediated genome engineering system has revolutionized the generation of genetically modified animals and shortened the experimental time from several years to several months. Genetically modified mice [6, 7], rats [7], monkeys [8], and other animals were obtained through pronuclear injections into zygotes by Cas9/RNA-mediated gene targeting.

The CRISPR/Cas9 system has been repeatedly applied effectively for creation of mouse model for human diseases [9–11]. In creating such models, it is important that modification is done strictly at a specific site, without disrupting other genes. The issue of non-specific modifications is a very urgent one. Despite the existence of many bioinformatics programs that can pick up guide RNAs for given sites and assess their effectiveness and specificity, there is still the probability of introducing unwanted mutations in off-target sites [6]. Predicted off-target sites are usually analyzed for the presence of mutations. The analysis is done after the birth of transgenic offspring (at least three weeks after microinjection), while analysis at the blastocyst stage would allow to in advance assess the specificity of modifications being introduced.

However, the methods most commonly used for DNA amplification of preimplantation embryos cannot analyze several regions of the genome [12]. At present, whole genome amplification is used for analysis of multiple sites in mouse embryonic genomes before staging a polymerase chain reaction (PCR) or two PCR rounds are carried out [13]. Such an approach leads to both false positive and false negative results due to the low DNA content in the original sample [14].

Moreover, the high cost of whole genome amplification reagents compels researchers to analyze fewer embryos.

In this paper, we describe a method for preparation of total DNA from one mouse embryo at the blastocyst stage, which simplifies, speeds up and reduces the cost of the process of obtaining genetically modified animals.

METHODS

In the first phase of the study, the efficiency of several blastocyst lysis methods was assessed. Experiments were carried out in triplicates from the moment fertilized eggs were obtained. At least 20 blastocysts were used for each method. We concurrently investigated the influence of blastocyst-preparation method on lysis. The minimum amount of lysate for amplification (three experiments) was also determined. Then, experiments were performed to analyze the efficiency of CRISPR/Cas9-mediated genome engineering (detection of short insertions and deletions, identification of target insertions in the genome) using the lysis method that yielded the best results in the previous experiments. Guide RNAs were selected for introns 8 and 34 of the dystrophin gene (*DMD*) mutations in which triggers Duchenne muscular dystrophy.

Selection and synthesis of guide RNAs

Guide RNAs were selected using online resource CHOPCHOP [15]. For guide RNA synthesis, two partially complementary oligonucleotides — SgR (containing the T7 promoter) and Sg31 (encodes the g31 site in the genome) were used or SgR and Sg34 (encodes the g34 site in the genome) were used. DNA research company Evrogen (Russia) synthesized the primers. Their sequences are presented in the table.

To obtain DNA matrix, oligonucleotides were mixed in equimolar ratio and amplified in thermal cycler T100 (BioRad, USA) based on the program: 95 °C — 1 min; 30 cycles of 95 °C — 30s, 65 °C — 30s, 72 °C — 30s; 72 °C — 5 minutes, using reagent kit GenPack PCR-core kit (Isogene, Russia). The matrix was purified from reaction products using reagent kit CleanUp Kit (Evrogen, Russia) based on the manufacturer’s protocol. Guide RNA was synthesized using reagent kit RiboMax express (Promega, USA) and was isolated from the reaction mixture by phenol-chloroform extraction, and was then precipitated in isopropanol [16]. Guide RNA was dissolved in free of nucleases water. The concentration was measured on NanoDrop 8000 (Thermo Fisher Scientific, USA). Guide RNA aliquots were stored at –70 °C.

In conducting experiments to detect an insertion at the break site, we synthesized repair template — a 70-nt single stranded oligonucleotide composed of two overlapping restriction sites for NcoI and BamHI enzymes, surrounded by 30-nt homology arms on each side. In the PAM site, replacements were made to prevent the binding of guide RNA to the repair template (sg31-HDRt, the sequence is shown in the table). Synthesis was performed by DNA research company Evrogen.

Oligonucleotides sequences

Name	5'-3' sequence
g31	GTGGCAGACTAGTAGTTTG
g34	GTAAGACTCGGCAGTTAAG
SgR	AAAAGCACCGACTCGGTGCCACTTTTCAAGTTGATAACGGACTAGCCTATTTTAACTTGCTATTCTAGCTCTAAAAC
sg31-HDRt	GTAGATAGAATAGTTTATTGGTGATCTCAACCATGGATCCACTACTAGTCTGCCACTGAGAAAAGAGAAG

### Microinjections into the pronucleus of zygotes and embryo cultivation

In immature female mice (C57BL6 x CBA), weighing 12–13 g, superovulation was induced by introducing at first 5 units of pregnant mare serum gonadotropin (PMSG) and then after 46–48 hours — 5 units of human chorionic gonadotropin (hCG). After injection, the female mouse was immediately placed in a cage with a male mouse for mating. Ovulation occurred within 11–14 hours after hCG injection. Fertilized eggs were surgically washed out within 12–13 hours after copulation (middle of the dark illumination period), i.e. 25–27 hours after hCG injection.

Genome editing complex was microinjected predominantly into the male pronucleus of mouse zygotes at the two-pronuclear stage. The zygotes were placed in a chamber consisting of two coverslips, fixed one above the other such that the upper and lower droplet edge of the M2 medium (MTI-GlobalStem, USA) were flat and parallel. Pronuclei were visualized by differential interference contrast microscope Axiovert 200 (Carl Zeiss, Germany). The microinjection needles were made from G100 glass capillaries (Narishige, Japan) on P97 puller (Shutter Instruments, USA), while holder capillaries were made from GD1 capillaries (Narishige) on PC-10 puller (Narishige) and MF-900 Microforge (Narishige).

For microinjection, guide RNA (50 ng/ml) was mixed with Cas9 (0.1 pM; NEB, UK) in TE buffer (10 mM Tris-HCl, pH 7.4; 0.1 mM EDTA) and, depending on the experiment – with repair matrix (3 pM; Evrogen, Russia). The components were mixed immediately prior to microinjection and incubated for 5 min at 37 °C to form a complex.

After microinjection, the zygotes were cultured for 2–3 hours in CO<sub>2</sub> incubator 150 IGO (Thermo Electron Corporation, France) at a 5 % carbon dioxide content in air and 100 % humidity. They were then assessed visually and those that were in satisfactory condition were left for 3 days in KSOM buffer (MTI-GlobalStem, USA) to form a blastocyst. Cultivation took place in Petri dishes (35 mm in diameter) in 50–60 µl droplets. The droplets were covered with embryo tested light mineral oil. All the preparatory procedures were performed in a laminar flow hood.

### Preparing total blastocyst DNA samples and PCR amplification of target fragment

Under a microscope, each embryo was transferred sequentially through 3 drops of water for PCR (Evrogen) using automatic pipette and tips with filters and placed in a 1 µl volume on a 0.2 ml tube wall. 1 µl of water from the last drop was used as the PCR negative control. When necessary, samples at this stage were frozen until further analysis and stored at –20 °C or unwashed blastocysts were used, for which embryos were transferred in a small volume of the incubation medium (less than 1 µl) using glass capillary on a tube wall for analysis.

Several methods were used for lysis of blastocysts: direct addition of blastocyst to a small volume in the reaction mixture, i.e. alkaline lysis (200 mM NaOH, 50 mM DTT) [17]; repeated freezing and thawing in water for PCR, lysis with lauryl sarcosine [18]; Proteinase K processing with detergent using the Sakurai et al. technique [19]. The last method was modified by deleting yeast tRNA from the buffer and increasing the amount of buffer to the sample to 20 µl. 20 µl of buffer was added to the test tubes for lysis. The buffer consisted of proteinase K (125 µg/ml), Tris-HCl (100 mM, pH 8.3), KCl (100 mM), gelatin 0.02 %, and Tween-20 0.45 %. The tubes were incubated for 10 min at 56 °C, then for 10 min at 95 °C to inactivate proteinase. PCR

analysis was performed immediately after sample preparation or lysate was stored at –20 °C.

The target region around the recognition site of guide RNA sg31 was amplified using primers g31-434F (5'-TC AACAAAAGGCAGAAGAGTAAG-3') and g31-434R (5'-GGTCCAAAGTAGGCCTCGTA-3'), guide RNA sg34 – using primers g34-505F (5'-CAGTGCCCCACACACATACA-3') and g34-505R (5'-AGCAAAGTTATTTTAGGGCATACT-3'). From 1 to 10 µl blastocyst lysate or the appropriate control sample was added to the reaction. Reagent kit GenePack PCR core (Isogene, Russia) was used for PCR. Amplification program: 95 °C — 1 min; 40 cycles of 95 °C — 30 sec, 60 °C — 30 sec, 72 °C — 30 sec; 72 °C — 5 min. PCR products were analyzed by electrophoresis in 2% agarose gel using intercalating dye.

### Mutational analysis using bacteriophage T7 endonuclease I

Short deletions and insertions which are formed after double-stranded break repair were detected using bacteriophage T7 endonuclease I (T7EI; NEB, UK). For this purpose, PCR product was mixed with control template, amplified under the same conditions (5 µl of reaction mix for each fragment) and with buffer NEB2 (NEB). In the final 9 µl volume, oligonucleotides were annealed at a temperature of 95 to 25 °C at a speed of 0.1 °C/sec. Then, 0.1 units of enzyme activity were added to each tube. The reaction was conducted for 1 hour at 37 °C. Reaction products were separated by electrophoresis in a 2 % agarose gel using intercalating dye. The deletion boundaries and insertion sizes were determined by Sanger sequencing of PCR products (Genome Center, Russia).

### Checking for the presence of insertion in the restriction genome at the BamHI site

Single-stranded DNA fragment used as the repair template encoded two recognition sites for two endonucleases: NcoI and BamHI. PCR products obtained by amplification of the intron 8 site of injected and control embryos were mixed with buffer FastDigest Green Buffer 10X and added with 0.1 units of activity of BamHI (Thermo Fisher Scientific, USA). The reaction was conducted for 1 hour at 37 °C. Reaction products were separated by electrophoresis in a 2 % agarose gel using intercalating dye. The presence of an insert was confirmed by Sanger sequencing of PCR products (Genome Center, Russia).

## RESULTS

### Choosing a PCR technique in blastocysts

In a series of experiments using various lysis methods, samples containing PCR product were in the greatest amount if lysate was prepared by Sakurai et al. technique [19] with modifications indicated methods (results not shown).

It was further found that preparation of blastocysts for lysis is vital to the reproducibility of PCR results since the components of the medium can inhibit lysis and polymerase later on. In parallel experiments, it was shown that with other things being equal, embryo transfer in the incubation medium worsens the reproducibility of PCR results (an average of 11 successful reactions on 20 samples) compared to the use of embryos washed from the medium components (20 successful reactions on 20 samples). In this and subsequent experiments, lysis was performed by Sakurai et al. method with modifications.

Experiments were carried out to determine the minimum amount of lysate that can be used for amplification. For 40 PCR cycles, 434 bp DNA segment was successfully amplified using 1 to 10 µl of lysate as the matrix (fig. 1).

Thus, the proposed method can analyze up to 20 different sites in a mouse embryonic genome at the blastocyst stage.

**Searching for short insertions and deletions in the g34 site of the *DMD* gene of mouse embryos microinjected with guide RNA and Cas9 protein**

Guide RNA sg34 with Cas9 protein was microinjected into the pronucleus of the zygote. 13 embryos at the blastocyst stage were lysed by Sakurai et al. method with modifications. 505 bp *DMD* gene fragment was amplified using injected and control embryos lysates as a template. PCR fragments were successfully amplified in all samples (results not shown).

Next, PCR fragments were hybridized with the control fragment and processed with endonuclease I. In 12 out of 13 samples, there was cleavage into two fragments — 300 bp and 250 bp (fig. 2, A). Four PCR fragments were selected to check for the presence of mutations in the gRNA binding region by Sanger sequencing. The results are shown in fig. 2, B. 12-nt deletion (from -5 to 7, where nucleotide N in triplet NGG of the PAM site was adopted as position +1) was found in sample 1. In samples 2 and 3, the deletion size was 17 nt (from -11 to +6), while in sample 4, the size was 38 nt (from -7 to +31).

**Detection of insertion after homologous recombination at break site**

Single-stranded oligonucleotide sg31-HDRt was injected together with the gRNA sg31/Cas9 complex into the pronucleus of zygotes. 21 embryos at the blastocyst stage were used for analysis. The intron 8 site of the 434 bp *DMD* gene was amplified with injected and control embryos that were preliminarily lysed using the chosen method. PCR products were treated with restriction endonuclease BamHI. The results are shown in fig. 3. In 11 out of the 21 treated samples, partial or complete cleavage into two fragments was detected. Sample 2 was shorter than the others by about 50 nucleotides, indicating sufficiently large deletion. Sample 5 was lost during restriction analysis (fig. 3A). Five samples (6, 12, 15, 19 and 20) were selected to confirm the presence of insertions in the sg31 recognition site by Sanger sequencing. An insertion was detected in all sequences. In samples 6, 15 and 19, apart from copies of the gene with insertion, a normal copy was identified (fig. 3, B).

**DISCUSSION**

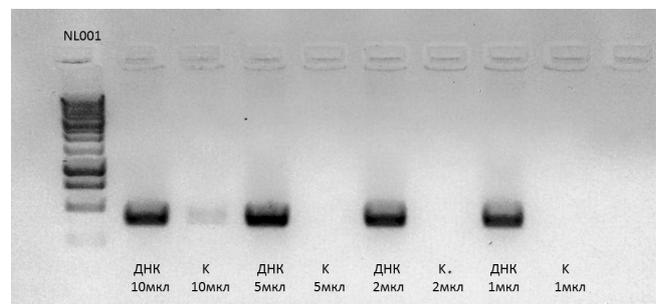
The DNA of newborn offspring is most often used to determine the success of manipulations in generating genetically modified organisms [12]. However, this approach requires replanting of fertilized embryos after microinjection into the recipients and total or partial incubation of offspring, which takes most of the time allotted for the experiment. Analysis of embryos at the blastocyst stage described by us helps to quickly determine whether it is possible to obtain genetically modified organisms using a selected guide RNA. At the same time, thanks to the fact that results are obtained rapidly, the need to test guide RNAs on cell cultures in advance is eliminated. In addition, statistical analysis is also possible. In cases where replanting of fertilized eggs after microinjection does not result in the

birth of genetically modified offspring, the proposed method can help determine the causes of fetal death by analyzing the non-specific gRNA binding sites or test the effectiveness of the gRNA-Cas9 complex.

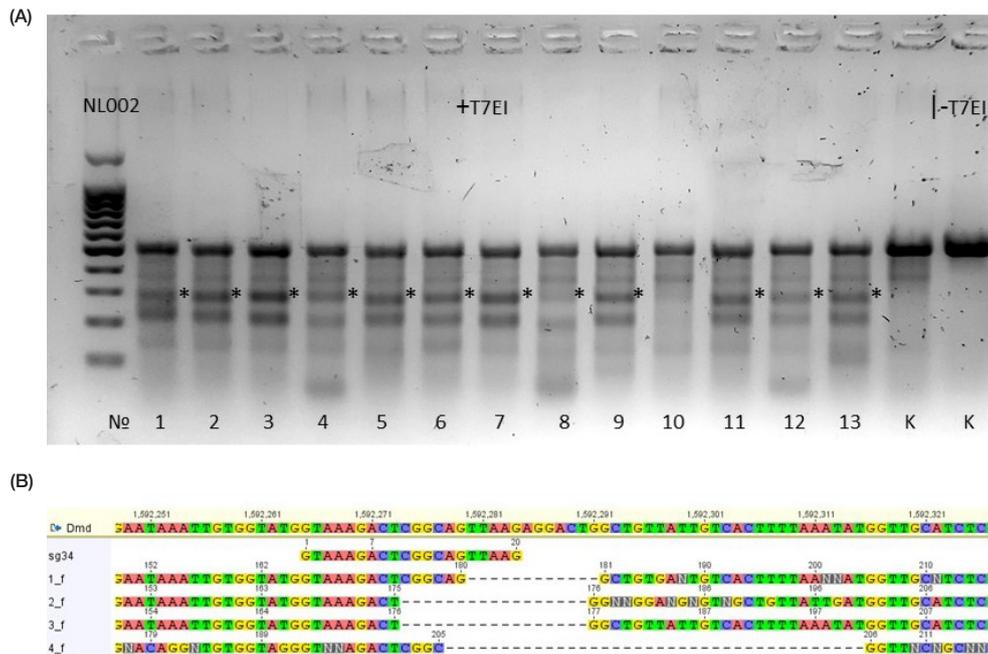
Clonal selection is characterized by the problem of insufficient amount of DNA in the starting material after transfection (using plasmid encoding the nuclease complex) of primary cell cultures, immortalized cell lines or stem cell. In this case, selection of genomic DNA using commercial reagent kits is expensive and inefficient, while the proposed method for obtaining total DNA can help to analyze a greater number of clones. This is especially essential during insertion into the genome via homologous recombination matrix since the efficiency of this process is much lower than in formation of short deletions or insertions.

The modified method of obtaining total DNA from mouse embryos eliminates the need for whole genome amplification, use of a large number of cycles or conduct of several PCR rounds. Analysis of embryos at the blastocyst stage will not only save time and money for the researcher but is also more humane than postnatal analysis of offspring since the number of animals involved in the experiment is reduced.

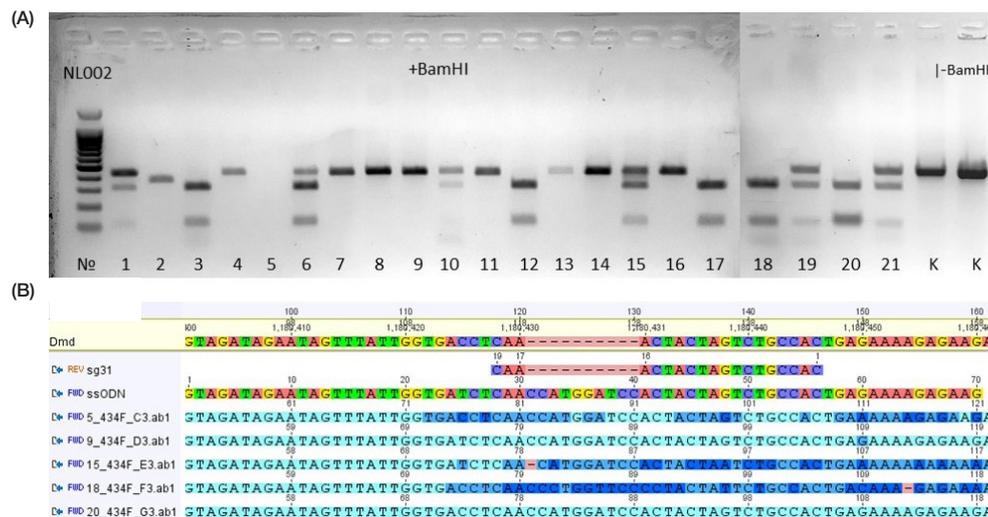
The total DNA prepared — by the proposed method — from mouse embryos at the blastocyst stage is suitable for analysis of short deletions or insertions in the Cas9 cutting site using bacteriophage T7 endonuclease I and other similar enzymes, as well as specific hydrolysis by restriction endonucleases with existence of appropriate site. Bacteriophage T7 endonuclease I recognizes and cleaves single-stranded sites in the heteroduplex composition. It can be used to detect the presence of short deletions and insertions at the double-stranded break site after hybridization of the analyzed fragment with control amplicon. Some studies question the applicability of bacteriophage T7 endonuclease I in identification of short deletions or insertions in mouse embryos [7, 12]. Using the described sample preparation method, T7EI and Sanger sequencing, it was established that CRISPR/Cas-mediated modifications in mouse genome using a single guide RNA are highly efficient. This finding is consistent with previous results [1]. Thus, it was established that amplification of the target fragment from one blastocyst, followed by processing with bacteriophage T7 endonuclease I is a reliable method for mutation detection after microinjection of gRNA/Cas9 into a fertilized egg. It was shown that the method involving sample preparation of total DNA from one blastocyst is suitable for detection of insertions at the break site via restriction analysis. It was also established



**Fig. 1.** Amplification of *DMD* intron 8 using different amounts of matrix. Blastocysts were lysed by Sakurai et al. method [19] with modifications in a final volume of 20 µl. 1 to 10 µl of total DNA solution was used for polymerase chain reaction (PCR). The appropriate volume of water from the final wash, treated similarly was used as the control for each sample. PCR products were separated in a 2 % agarose gel using intercalating dye. NL001 (Evrogen, Russia) was used as the DNA fragment length marker.



**Fig. 2.** Detection of insertions and deletions in *DMD* intron 34 after microinjection of the sg34/Cas9 complex  
 The intron fragment around recognition site g34 was amplified with injected and control embryos. PCR products were annealed in control amplicon and processed with bacteriophage T7 endonuclease I. Cleavage into two fragments occurred in samples that contained insertions and deletions. Such samples are marked in the figure with an asterisk \* (A). The boundaries of insertions and deletions in selected samples were determined via Sanger sequencing (B). PCR products were separated in a 2 % agarose gel using intercalating dye. NL001 (Evrogen, Russia) was used as the DNA fragment length marker.



**Fig. 3.** Detection of insertion at the break site in *DMD* intron 8 after microinjection of the sg31/Cas9 complex with repair matrix  
 The intron fragment around recognition site g34 was amplified with injected and control embryos. PCR products were treated with restriction endonuclease BamHI, whose recognition site was encoded in repair matrix. Cleavage into two fragments occurred in samples that contained insertions and deletions. Such samples are marked in the figure with an asterisk \* (A). The presence of an insertion in selected samples was confirmed by Sanger sequencing (B). PCR products were separated in a 2 % agarose gel using intercalating dye. NL001 (Evrogen, Russia) was used as the DNA fragment length marker.

that homologous recombination at the guide RNA recognition site occurs less efficiently than non-homologous connection of ends in a double-stranded break (11/21 vs 12/13).

CONCLUSIONS

The article describes a modification of a method for obtaining total DNA from mouse embryos at the early development stage. It was shown that sample preparation process is equally of great importance as a correctly selected lysis technique. Unlike the presently used method, the proposed method is

simple and eliminates the need for rare and expensive reagents. Using embryo lysate as the matrix at the blastocyst stage, it is possible to amplify the desired DNA fragment in a single PCR round, significantly reducing the probability of result distortion and contamination. Moreover, the mixture components do not inhibit PCR and enzymatic reactions, and the method allows for analysis of up to 20 different independent fragments of the mouse genome.

The proposed modification of the analysis method can be useful for amplification of DNA regions after using other genome engineering systems, such as TAL effector nucleases or zinc fingers nucleases.

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