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A Comparative Study on the Efficiency of CRISPR-Cas9 in Human Embryonic Kidney 293 Cells and Peripheral Blood Mononuclear Cells for Disruption in Programmed Cell Death Protein 1

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ABSTRACT

CRISPR-Cas9 is the most important tool in genome engineering in recent years. The efficiency of this instrument on active and non-active genes is variable. Programmed cell death protein 1(PD-1) is a surface acceptor on T cells, B cells, and dendritic cells. This protein has an important role in the production of inducing tolerance in lymphocytes. Nowadays, this characteristic is used in cell therapy and immunotherapy of cancer. In the present study, the peripheral blood mononuclear cells and HEK293 cells were selected as expression and non-expression cells of the *PD-1* gene. Six pairs of sgRNA were designed for the *PD-1* gene. The transfected cells were sorted by the FACS machine. A common pair of primers were used for amplification of cute regions. Px458 was used as an expressional vector for the transfection of PBMCs and HEK293. Transfection was done using lipofectamine and electroporation methods. In PBMCs, 2 guides, sgRNA (3+1) and sgRNA (3+5) were able to disrupt the *PD-1* gene. In contrast, in HEK293, none of the 6 guides were able to disrupt it. According to the results obtained, the *PD-1* gene cutting in HEK293 cells was failed. However, it was successful in PBMCs. Therefore, it can be told that the heterochromatin region or other genome remodeling mechanisms such as epigenetic remodeling inhibit the *PD-1* gene cutting by CRISPR-Cas9 in HEK293 cells.

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Introduction

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is an effective tool in genome engineering, which uses a specific 20-nucleotide guide RNA (sgRNA) to cleave the genome. These sgRNAs guide Cas9 nucleases to desired locations, to which they bind (Cong *et al.,* 2015). In its natural form, Cas9 protein associates with a duplex of two RNA molecules: the CRISPR RNA (crRNA), which recognizes a short section of target DNA, and a transactivating RNA, which acts as a scaffold to link the crRNA and Cas9 endonuclease. After the target DNA cleavage, mutations arise through the action of cellular DNA repair pathways (Alambeladi *et al.,* 2021). Non-Homologous End Joining (NHEJ) can yield short insertions and deletions for gene knockout, whereas Homology-Directed Repair (HDR) pathways utilize exogenous donor templates to introduce precise sequence changes (Cong *et al.,* 2015; Alambeladi *et al.,* 2021). So far, many efforts have been made to determine the effect of CRISPR-Cas9 on all cells, i.e., the effect of CRISPR-Cas9 on the cleavage of active and non-active genes.

Programmed cell death protein 1 (PD-1) is a human protein that belongs to the

immunoglobulin superfamily and is a cell surface receptor (Keir *et al.,* 2008; Pen *et al.,* 2014). The *PD-1* gene is naturally expressed on T-cells and pro-B-cells. It has two ligands called PD-L1 and PD-L2. It plays its regulatory role through two mechanisms: increasing apoptosis in antigen-specific T-cells, and simultaneously, reducing apoptosis in suppressor T-cells. Except for T-cells, B-cells, and dendritic cells, PD-1 is not naturally expressed in most human cells (John *et al.,* 2013; Lioyd *et al.,* 2013; Sus *et al.,* 2016). Checkpoint blocking is considered a revolutionary method in cancer treatment. This method eliminates cancer cells by maintaining the sensitivity of immune cells. Nowadays, cell therapy through checkpoint blocking is known as the most efficient method of cancer control (Alambeladi *et al.,* 2021).

The programmed cell death protein 1 is the B and T-cells surface receptor that binds two ligands named PD-L1 and PD-L2. PD-1, as an immune check protein, has a vital role in weakening the immune responses by reducing the number of stimulated T-cells. In normal situations, a decline in the immune responses can cause self-induced tolerance and prevent autoimmune diseases (Luke *et al*., 2015; Bally *et al*., 2016). Inducing tolerance in T-cells activated by the binding of PD-L1 to PD-1 occurs in two different ways: an increase in apoptosis of activated T-cells against cancer cells and a decrease in apoptosis of regulatory T-cells (Alambeladi *et al.,* 2021). In addition to the surface of the activated T-cells, PD-1 can be found on regulatory T-cells. Also, in addition to macrophages and dendritic cells, PD-L1 is produced by cancer cells in large quantities (Alambeladi *et al.,* 2021). The expression of PD-L2 is more restricted than PD-L1 and this ligand has been proven to be expressed only in dendritic cells and some cancer cells (Alambeladi *et al.,* 2021). HEK293 cells originate in human embryonic kidney cells. *PD-1* is not naturally expressed in this type of cell. These cells are widely used in laboratories of cell biology (Alambeladi *et al.,* 2021).

Peripheral Blood Mononuclear Cells (PBMCs) have a round nucleus. These cells consist of lymphocytes and monocytes. In humans, lymphocytes make up the majority of the PBMCs population. These cells can be extracted from the blood, using ficoll, a hydrophilic polysaccharide that separates layers of blood (Alambeladi *et al.,* 2021).

Although the reducing effects of heterochromatin and other expressional regulating factors such as epigenetic remodeling are not clear on the efficacy of CRISPR-Cas9, many of the researchers believe that these factors have decreasing effects on cutting genome by CRISPR-Cas9 (Kalimasioti *et al*., 2018).

In this study, according to the fact that the PD-1 is only expressed in some immune cells such as lymphocytes, the efficacy of CRISPR-Cas9 was compared on two different human cells, HEK293 cells as a non-expression PD-1 cell and peripheral mononuclear cells as a PD-1 expression cell.

Materials and Methods

Guide RNAs and primers

Six double-stranded polynucleotides specific to the *PD-1* gene were designed for px458 and annealed as a guide for RNAs (Table 1). Then, using the Bbs1 enzyme, they were integrated into a vector. To ensure the entrance of inserts into the vector, PCR and sequencing were carried out using the forward primer of the vector and the reverse strand related to guide RNA.

Table 1. Polynucleotides and target locations on the *PD-1* gene.

Segment No.	Polynucleotide (5′→3′)	Site of target bases
1	GCAGTTGTGTAACACGGAAG	241852756 - 241852775
2	GACAGCGGCACCTACCTCTG	241852708 – 241852689
3	ACCCTGGTGGTTGGTGTCGT	241852288 – 241852269
4	TCTCTTTGATCTGCGCCTTG	241852648 - 241852667
5	GGCGTGACTTCCACATGAGCG	241852729 - 241852749
6	GGGCCCTGACCACGCTCATG	241852717 - 241852736
Out-Fwd	GGTCTTAGTCCAGGGGCCTT	241852043 - 241852062
Out-Rev	ACCTCTCTCCATCTCTCAGACT	241852978 - 241852999

*Fwd: forward primer; Rev: reverse primer.

In this study, to increase the efficiency and reduce the off-target events, two different sgRNAs were simultaneously entered into the cells. Hence, the sgRNAs were designed to target different locations within the *PD-1* gene. Furthermore, a pair of primers were designed with a 956-bp PCR product to examine the presence of cleavage bands (Table 1).

px458 is an expression vector (addgene, 48138) containing Green Fluorescent Protein (GFP) genes, which is resistant to ampicillin. It also contains a recognition sequence for the Bbs1 enzyme. The vector is 9288 bp in length and is designed in a way that a 20-nucleotide sequence, scaffold RNA, and Cas9 have been located immediately after a U6 promoter-driven guide RNA cassette.

Transformation of competent cells

For quick transformation, the Stbl3™ *Escherichia coli* strain was used as the competent cell. The transformation was performed following its protocol by heat shock (Cong *et al*., 2015).

The HEK 293FT cells were kept under the conditions recommended by the manufacturing company, and a DMEM medium with 10% Fetal Bovine Serum (FBS) was used for culture. Moreover, the cells were incubated at 37 °C with 5% CO_2. Between 16-24 hours before transfection, 5×10^5 cells were placed in a 6-weel plate in DMEM medium without any antibiotics. During transfection, confluency was set at 70-90%.

For the preparation of PBMCs cells, 10 ml of fresh blood mixed with heparin was prepared, and using Ficoll, these cells were separated immediately. Using trypan blue, the number of living cells per milliliter was calculated and a mixture of 50,000 living cells was added to six-well plates, and a sufficient RPMI medium was added instantly. Transfection was performed with 70% confluency.

For transferring the guides to the HEK293FT cells and PBMCs, the method of "simultaneous entering of two sgRNAs" has been used.

Lipofectamine

In the plasmid extraction stage, the required vector volume was measured based on optical density. Then, 2.5 µg sgRNA (1.25 µg of each), were calculated for 5 x 10^5 cells in each well and mixed with 10µg of lipofectamine. The formed structure was slowly added to the cells and incubated at 37 °C and 5% CO_2. After 5 hours, the cells were washed by phosphate-buffered saline and cultured in DMEM medium without fetal bovine serum.

For evaluation of efficiency, the cells were examined by fluorescence microscope after 48 hours of transfection. These cells were sorted based on GFP through the Fluorescence-Activated Cell Sorting machine (FACS). To separate the transformed cells 3, 5, and 7 days after transfection, the cells were collected immediately and DNA extraction was carried out. The presence of cleavage bands was inspected for each sgRNA pair separately through PCR

Electroporation

A total of 5×10^5 cells underwent electroporation in GT porator buffer with 2 mm cuvette, 960µF capacitance, the voltage of 220V, and 25 ms pulse time in 3, 5, and 7µg amounts as paired plasmids, and DNA extraction and PCR were conducted after 3, 5, and 7 days. To prevent the vector's enzymatic degradation, an ice-cold container was used throughout all the stages. The cells were immediately transferred to a 25ml flask containing DMEM for HEK293 and RPMI for PBMCs medium with fetal bovine serum and pen-strep. The mixture was incubated at 37 °C in a 5%-carbon-dioxide atmosphere. At the end of each step, to confirm its documentation by fluorescent imaging, DNA sequencing, and gel electrophoresis were conducted.

Results

Different sgRNAs were designed for different locations within the *PD-1* gene, and the guides were entered into HEK293 and PBMC cells using dual-transfection simultaneously and two different methods (Table 1). The regions of the genome that were expected to be altered, were amplified by appropriate primers using PCR. Each stage was repeated eight times and no cleavages were observed in HEK293FT. However, two pairs of sgRNA, (3+1) and (3+5), cut the genome successfully in PBMCs. The length of deletion for (3+1) was 487bp and for (3+5) was 461bp.

Comparison of the sgRNAs

Table 2 provides a comparison of the simultaneous entrance of sgRNA3 and sgRNA4 by lipofectamine and electroporation, as well as the length of cleaved fragments and genomic alteration. As can be observed, there is no significant relationship between the distance of the two guides and the presence of cleavage sites in HEK293ft. However, in PBMCs, two pairs of sgRNA, (3+1) and (3+5) with suitable distances (461 and 487 bp) were able to cleave the genome successfully.

Table2. Comparison of lipofectamine and electroporation.

	Transfection by lipofectamine			Transfection by Electroporation		
sgRNA	**Length of deletion**	**Indel (HEK293ft)**	**Indel (PMBCs)**	**Length of deletion**	**Indel (HEK293ft)**	**Indel (PMBCs)**
sg (3+1)	487	-	+	487	-	+
sg (3+2)	420	-	-	420	-	-
sg (3+4)	379	-	-	379	-	-
sg (3+5)	461	-	+	461	-	+
sg (3+6)	448	-	-	448	-	-
sg (4+1)	108	-	-	108	-	-
sg (4+2)	41	-	-	41	-	-
sg (4+5)	82	-	-	82	-	-
sg (4+6)	109	-	-	109	-	-

Genome cleavage observation

Table 3 shows genome cleavage by lipofectamine and electroporation. There was no DNA double-stranded break after 2, 3, and 5 days in HEK293ft while in all three days the DNA double-stranded break was observed in PBMCs. This result shows that CRISPR-Cas9 has high efficiency in PBMCs to knockout the *PD-1* gene.

Table 4 presents the existence of genome cleavage in 3, 5, and 7µg of px458 transfected by lipofectamine and electroporation. In PBMCs, each of the two sgRNAs, (3+1) and (3+5) could knock out the *PD-1* gene in all of the vector amounts. However, there was no DNA double-stranded break in HEK293 in all amounts of the vector.

Confirmation of vector entrance

The confirmation of vector entrance into competent cells was done through PCR. In both procedures, the vector's forward primer was utilized, and the insert's complementary strand was used as the reverse primer (Table1). Three colonies of *Escherichia coli* Stbl3™ were used as competent and transfected cells in the colony PCR method. In this regard, a 141-bp band was observed, which confirmed the entrance of inserts into the vector (Fig. 1).

GFP expression and sgRNAs entrance

Our results showed that HEK293 and PBMCs cells had almost similar (~60%) GFP expression after 48 hours (Fig. 2). Simultaneous entrance of sgRNAs and *PD-1* knockout in PBMCs was shown in Fig. 3. The size of deletion regions was 487bp and 461bp. Since electroporation had more efficiency than lipofectamine, the cleavage band in Figure 3E is sharper than Fig. 3D. Also, DNA sequencing showed indels are exhibited.

Forward and reverse primers mentioned in Table 1 were used for the *PD-1* gene sequencing (Fig. 4). Isolation of transfected PBMCs by FACS showed 7.55% of PBMCs produced GFP while in non-transfected cells, GFP was not observed (Fig. 5).

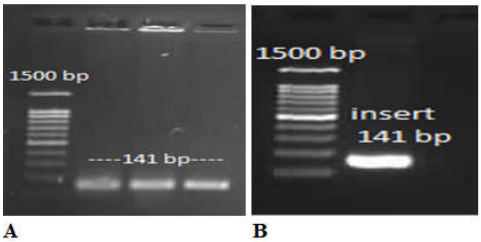

Fig. 1. Gel electrophoresis (Agarose 2%): A) Confirmation of vector entrance into competent cells Stbl3™ *Escherichia coli* (Colony PCR); B) Confirmation of insert entrance into the vector. (Size of insert: 141bp); 1500bp= DNA Size Marker, 100 bp Ladders (100-1,500 bp), BioAcademia Co.

Table 3. Comparison of genome cleavage after lipofectamine and electroporation transfection in the different days.

sgRNA	Transfection by lipofectamine						Transfection by electroporation					
	HEK293ft			PBMCs			HEK293ft			PBMCs		
	2 D	3 D	5 D	2 D	3 D	5 D	2 D	3 D	5 D	2 D	3 D	5 D
sg (3+1)	-	-	-	+	+	+	-	-	-	+	+	+
sg (3+2)	-	-	-	-	-	-	-	-	-	-	-	-
sg (3+4)	-	-	-	-	-	-	-	-	-	-	-	-
sg (3+5)	-	-	-	+	+	+	-	-	-	+	+	+
sg (3+6)	-	-	-	-	-	-	-	-	-	-	-	-
sg (4+1)	-	-	-	-	-	-	-	-	-	-	-	-
sg (4+2)	-	-	-	-	-	-	-	-	-	-	-	-
sg (4+5)	-	-	-	-	-	-	-	-	-	-	-	-
sg (4+6)	-	-	-	-	-	-	-	-	-	-	-	-

*D= Day

Table 4. Comparison of the existence of cleavage after transfection of HEK293ft and PBMCs cells by different DNA concentrations via lipofectamine and electroporation.

sgRNA	Transfection by lipofectamine						Transfection by electroporation					
	HEK293ft			PBMCs			HEK293ft			PBMCs		
	3 µg	5 µg	7 µg	3 µg	5 µg	7 µg	3 µg	5 µg	7 µg	3 µg	5 µg	7 µg
sg (3+1)	-	-	-	+	+	+	-	-	-	+	+	+
sg (3+2)	-	-	-	-	-	-	-	-	-	-	-	-
sg (3+4)	-	-	-	-	-	-	-	-	-	-	-	-
sg (3+5)	-	-	-	+	+	+	-	-	-	+	+	+
sg (3+6)	-	-	-	-	-	-	-	-	-	-	-	-
sg (4+1)	-	-	-	-	-	-	-	-	-	-	-	-
sg (4+2)	-	-	-	-	-	-	-	-	-	-	-	-
sg (4+5)	-	-	-	-	-	-	-	-	-	-	-	-
sg (4+6)	-	-	-	-	-	-	-	-	-	-	-	-

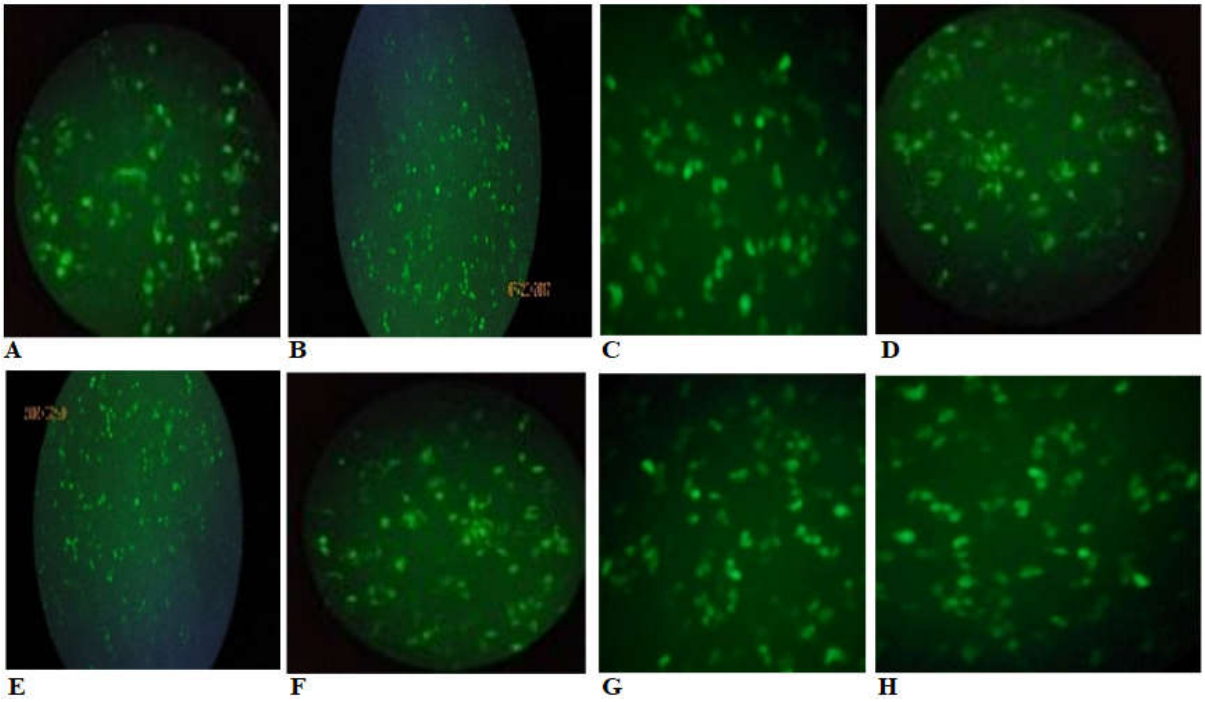

Fig. 2. GFP expression. Transfection by lipofectamine and electroporation in HEK293 & PBMCs: A) Sg (3+1), B) Sg (3+2); C) Sg (3+5); D) Sg (3+6); E) Sg (4+1); F) Sg (4+2); G) Sg (4+5); H) Sg (4+6); Almost 75% of cells were transfected. (Fluorescent imaging. Scale: ×1000).

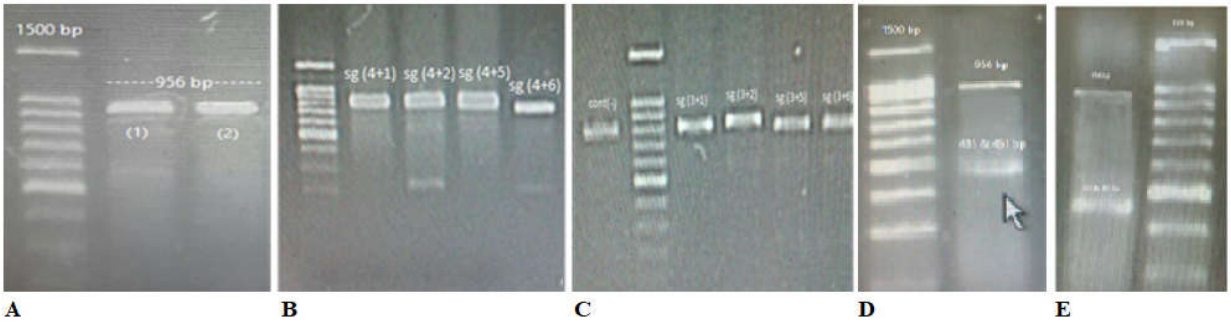

Fig. 3. Evaluation of gene cleavage: A) PBMCs transfection by vector backbone (Negative control); B and C) Lack of cleavage bands after transfecting HEK293 cells by lipofectamine and electroporation methods; D) & E) PD-1 knockout by sg (3+1) and sg (3+5) through lipofectamine and electroporation methods; 1500bp= DNA Size Marker, 100 bp Ladders (100-1,500 bp), BioAcademia Co.

A) Negative control

GGTCTTAGTCCAGGGGCCTTCATCAGGGACTTAGCCTGGCGGGGAGATGGGGGGAGGTGGGGTGGGGTGA
GGGAAGGGTGGAGGAAGGGGAGGCGGGAGTGAGGGCCGCCAGCAGGGTTAGGGCAGGGCAGGCCGAGGGG
CTGGGATGACGTTACCTCGTGCGGCCCGGGAGCAGATGACGGCCAGGACCCAGACTAGCAGCACCAGGCT
GCCCAGCAGGCCGCCCACGACACCAACCACCAGGGTTTGGAACTGGCCGGCTGGCCTGGGTGAGGGGCTG
GGGTGGGCTGTGGGCACTTCTGCCCTTCTCTCTGGAAGGGCACAAAGGTCAGGGGTTAGGACGGGGTCAG
GGTGGAGGGTCAGGGTCAGGGGTGAGGGCAGACTAGAGGGGCTGGGGTGCTTCCAGAGCTAGAGGACAGA
GATGCCGGTCACCATTCCCCAGGTGCAGGACAGAGCCCTGGACTGGAGCTGGGGGGTCCCTGCCCTACGA
CCCTGGAGCTCCTGATCCTGTGCAGGAGGGGACACCCACCCCAGGACCGGCTCAGCTCACCCCTGCCCCG
GGGCCTCCGAGGCCGCACCTGTCACCCTGAGCTCTGCCCGCAGGCTCTCTTTGATCTGCGCCTTGGGGGC
CAGGGAGATGGCCCCACAGAGGTAGGTGCCGCTGTCATTGCGCCGGGCCCTGACCACGCTCATGTGGAAG
TCACGCCCGTTGGGCAGTTGTGTGACACGGAAGCGGCAGTCCTGGCCGGGCTGGCTGCGGTCCTCGGGGA
AGGCGGCCAGCTTGTCCGTCTGGTTGCTGGGGCTCATGCGGTACCAGTTTAGCACGAAGCTCTCCGATGT
GTTGGAGAAGCTGCAGGTGAAGGTGGCGTTGTCCCCTTCGGTCACCACGAGCAGGGCTGGGGAGAAGGTG
GGGGGGTTCCAGGGCCTGTCTGGGGAGTCTGAGAGATGGAGAGAGGT

B) sgRNA (3+1)

GGTCTTAGTCCAGGGGCCTTCATCAGGGACTTAGCCTGGCGGGGAGATGGGGGGAGGTGGGGTGGGGTGA
GGGAAGGGTGGAGGAAGGGGAGGCGGGAGTGAGGGCCGCCAGCAGGGTTAGGGCAGGGCAGGCCGAGGGG
CTGGGATGACGTTACCTCGTGCGGCCCGGGAGCAGATGACGGCCAGGACCCAGACTAGCAGCACCAGGCT
GCCCAGCAGGCCGCCCACGACACCAACCACCAGGG.....
.....GAAGGCACAATGTGTTGACGGCGGCAGTCCTGGCCGGGCTGT
CCTCGGGCTGCGGGGAAGGCGGCCAGCTTGTCCGTCTGGTTGCTGGGGCTCATGCGGTACCAGTTTAGCA
CGAAGCTCTCCGATGTGTTGGAGAAGCTGCAGGTGAAGGTGGCGTTGTCCCCTTCGGTCACCACGAGCAG
GGCTGGGGAGAAGGTGGGGGGGTTCCAGGGCCTGTCTGGGGAGTCTGAGAGATGGAGAGAGGT

C) sgRNA (3+5)

GGTCTTAGTCCAGGGGCCTTCATCAGGGACTTAGCCTGGCGGGGAGATGGGGGGAGGTGGGGTGGGGTGA
GGGAAGGGTGGAGGAAGGGGAGGCGGGAGTGAGGGCCGCCAGCAGGGTTAGGGCAGGGCAGGCCGAGGGG
CTGGGATGACGTTACCTCGTGCGGCCCGGGAGCAGATGACGGCCAGGACCCAGACTAGCAGCACCAGGCT
GCCCAGCAGGCCGCCCACGACACCAACCACCAGGG.....
.....GCGAGTACACCTTCAGTGCGGGCGGCAGTCCTGGCCGGGCTGGCTGCGGTCCTCGGGGA
AGGCGGCCAGCTTGTCCGTCTGGTTGCTGGGGCTCATGCGGTACCAGTTTAGCACGAAGCTCTCCGATGT
GTTGGAGAAGCTGCAGGTGAAGGTGGCGTTGTCCCCTTCGGTCACCACGAGCAGGGCTGGGGAGAAGGTG
GGGGGGTTCCAGGGCCTGTCTGGGGAGTCTGAGAGATGGAGAGAGGT

Fig. 4. PCR product sequencing: A) Negative control without transfection; B) Indel produced by sgRNA (3+1) (lipofectamine); C) Indel produced by sgRNA (3+5) (Electroporation).

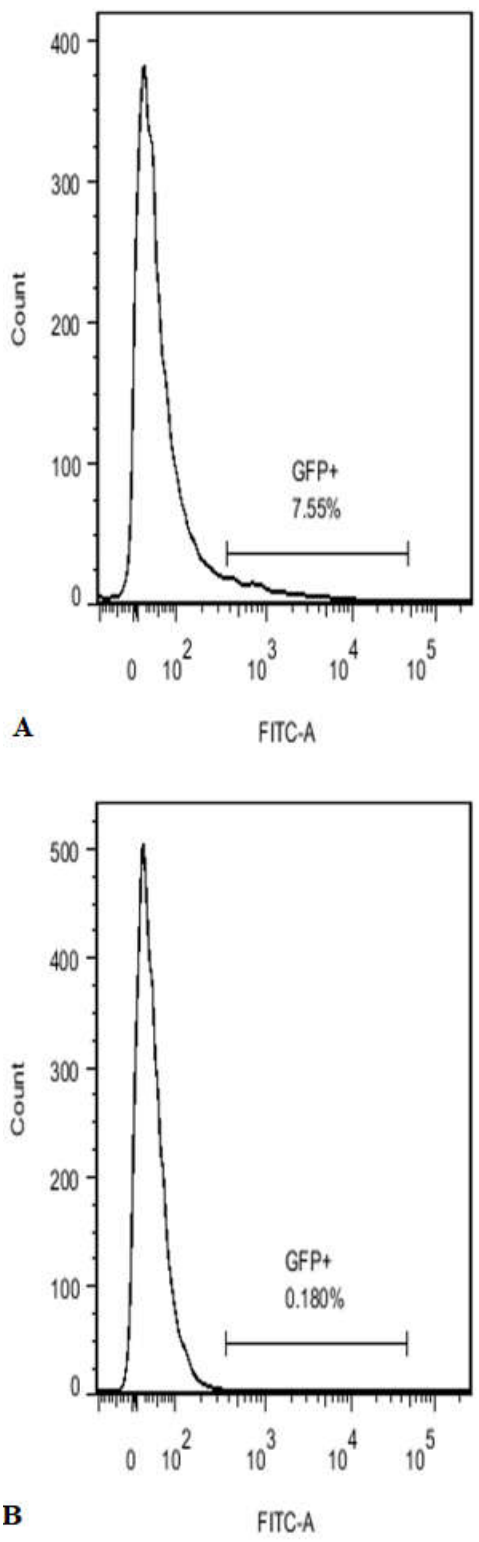

Fig. 5. Isolation of transfected PBMCs by FACS: A) 3-5 days after transfection. It shows that 7.55 % of GFP produced by PBMCs was sorted by machine. B) Negative control, no produced GFP.

Discussion

It is well established that loci location has an important effect on the efficiency of indel production by CRISPR-Cas9. However, Cas9 has a dependent evolution in prokaryotic cells. The previous studies have indicated that epigenetic properties of the genome, such as histone modification or nucleosome position, affect the sgRNA and Cas9 bound to the target site. The inhibitory effect of heterochromatin on sgRNAs and Cas9 binding and cleaving genome is well known (Kalimasioti *et al*., 2018; Cong *et al*., 2015; Makarova *et al*., 2011; Hsu *et al*., 2013).

It has been reported that many guides show less activity within heterochromatin while other sgRNAs do not. The reasons are not completely clear. Furthermore, the findings show that double-stranded break repairing is influenced by heterochromatin and epigenetic remodeling (Mali *et al*., 2013; Kalimasioti *et al*., 2018). DNA double-stranded break repairing induced by Cas9 has been influenced by the chromatin environment at the site of cleavage. Similarly, the epigenetic modification of DNA and histone protein influence the outcome of CRISPR-Cas9 mutagenesis, particularly the frequency of indel arising from NHEJ (Cong *et al*., 2013; Mali *et al*., 2013; Kalimasioti *et al*., 2018).

Previous studies have indicated a significant relationship between the creation of genomic alteration by CRISPR-Cas9 and the presence of heterochromatin regions. Using cellular models, recent studies have demonstrated that under alteration in the heterochromatin regions, due to epigenetic remodeling, some instruments such as CRISPR-Cas9 are less efficient (Kalimasioti *et al*., 2018).

It seems that in HEK293 cells, heterochromatin inhibits Cas9 complexes to cleave the *PD-1* gene. Considering that no cleavage band was observed after designing six guides for different regions within the PD-1 gene and transfecting them through two different methods, the following conditions are proposed:

An array of cis-DNA elements, transcription factors, and epigenetic components, including DNA methylation and histone modification, control the *PD-1* expression. Given that the *PD-1* is not naturally expressed in HEK293 cells, it

is possible that the presence of heterochromatin regions would prevent the guides or Cas9 binding to specific sites on the genome. In this study, every stage was repeated eight times, and no genetic alteration was observed in the genome of HEK293 in any repetition. The successful knockout of the *PD-1* gene in human cells that express it using CRISPR-Cas9 technology, such as PBMCs, verifies these results. It is possible that epigenetics effects such as DNA methylation can directly influence Cas9 binding and/or cleavage. Future studies can clarify these assumptions.

Since the *PD-1* gene is expressed naturally in T cells, B cells, and dendritic cells, to knock it out, six different guides were designed and transfected in PBMCs.

Two guides were transfected simultaneously (dual-transfection) by applying electroporation and lipofectamine methods (Wang *et al*., 2009; Qils *et al*., 2013). After transfection with the px458 vector, the inspection of PBMCs by fluorescent microscope showed that in both electroporation and lipofectamine methods, vector entrance into lymphocytes with different guides was satisfactory (Fig. 3).

Given the cleavage bands observed in the simultaneous use of sgRNA (1+3) and sgRNA (3+5), it can be said that the distance between the targets is an important factor in cutting the PD-1 gene. In other pairs of sgRNAs, we did not observe cleavage bands which can be the result of the conformational inhibition caused by the two Cas9 proteins reaching proximity, or due to the presence of heterochromatin areas or epigenetic remodeling (Wang *et al*., 2009; Qils *et al*., 2013).

In the case of guides 1 and 3, 3 and 5, the appropriate distance between the two guides (487 and 461 nucleotides) prevents the formation of conformational inhibition and therefore, indels. In the simultaneous use of guides 3 and 2, it is impossible to make an indel. Thus, it seems that the minimum distance between the two sgRNAs must be more than 448 nucleotides for cutting in the *PD-1* gene. The difficulty of cutting the simultaneous use of other guides can be due to the spatial restraint caused by Cas9 proteins, binding area, or other factors such as heterochromatin regions, epigenetic remodeling, and polymorphism (Wang *et al*., 2009; Qils *et al*., 2013).

Guides 3 and 1, 3 and 5, were able to cut the *PD-1* gene in both transfection methods while the other pairs were not. As can be seen in Figure 3, the cleavage band created with the electroporation method is thicker than the lipofectamine method. It shows that the electroporation method has higher efficiency in plasmid transfer to PBMCs compared to the lipofectamine method. This has also been mentioned in previous studies (Wang *et al*., 2009; Qils *et al*., 2013; Tumeh *et al*., 2014).

In this study, it was shown that by the simultaneous entrance of the two guides into the T cell, it is possible to knock out the *PD-1* gene and create T cells resistant to induced tolerance resulting from the binding of PD-L1 ligand to the PD-1 receptor.

Our study shows that in both electroporation and lipofectamine methods, the entrance of guide RNAs into the PBMCs was successful. The minimum distance between the two sgRNA must be more than 448 nucleotides for cutting in the *PD-1* gene.

The appropriate distance between the two guides was 487 and 461 nucleotides. In other distances, some factors such as conformational inhibition, heterochromatin remodeling, epigenetics, and polymorphism may prevent the *PD-1* gene cutting.

Moreover, the results show that the use of the dual-transfection method knocks out the *PD-1* gene in PBMCs, enhances the efficiency of gene cutting, and comforts the detection of cleavage bands.

Conflicts of Interest

The authors declare no conflicts of interest.

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