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Easy and robust electrotransfection protocol for efficient ectopic gene expression and genome editing in human B-cells

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ABSTRACT

B-cell lines and primary PBMCs are notoriously hard to transfect, thus making genome editing, ectopic gene expression or gene silencing experiments particularly tedious. Here we propose a novel efficient and reproducible protocol for electrotransfection of lymphoblastoid, B-cell lymphoma, leukaemia cell lines and B-cells from PBMCs. The proposed protocol requires neither costly equipment nor expensive reagents; it can be used with small or large plasmids. Transfection and viability rates of about 79% and 58%, respectively, have been routinely achieved by optimizing the salt concentration in the electrotransfection medium and the amount of plasmid used. A validation of the protocol was obtained *via* the generation of a *TP53*$^{-/-}$ RPMI8866 lymphoblastoid cell line which should prove useful in future hematological and blood cancer studies.

B-cell lines and primary peripheral blood mononuclear cells (PBMCs) are notoriously hard to transfect, thus making genome editing and even simple ectopic gene expression or gene silencing experiments particularly tedious. Cell lines derived from lymphocytes, lymphoma and leukaemia cells belong to the so called hard-to-transfect cells (1,2). Both viral and non-viral (chemical and physical) transfection methods have been tested previously on lymphoid cell lines (3). Retroviral transfection methods allow for relatively high transfection efficiencies, however they carry biosafety risks and international guidelines recommend that they be performed in a biosafety level 2 or 3 laboratory (4). Another concern is that the integration of the genome carried by the virus can produce insertional mutagenesis (5). Vectors based on non-integrating viruses (such as adenovirus or herpesvirus) have shown transfection rates comparable to non-viral methods but they can produce inflammatory-like responses (6).

Electrotransfection is a technique whereby cells are exposed to an electric field which changes the permeability of their membrane allowing the entry of any molecule e.g. drugs, chemicals or nucleic acids (7). This technique was successfully used with different difficult-to-transfect cells (8). Many electrotransfection protocols were described for EBV-transformed lymphoblastoid cell lines (LCLs) and EBV-negative leukaemia and lymphoma cell lines. Most successful protocols involve the use of the Nucleofector II® device (Lonza) which does not allow users to control the parameters of electrotransfection, and result in transfection efficiencies highly variable (10% to 50%) between studies (9–12). Moreover, these rates are

attained only using small plasmids, the transfection efficiency being much lower when using larger plasmids such as those carrying CRISPR/Cas9. We here report an improved electrotransfection protocol which ensures consistent transfection efficiency with high cell viability using a large variety of lymphoblastoid and malignant B cell-lines and PBMCs.

We used the Cliniporator^{TM} (Igea) with 1-mm electroporation cuvettes (Cell Projects) and the pCMV-GFP plasmid (3.5 Kbp, PF463, Plasmid Factory) to optimize the electrotransfection parameters for the RPMI8866 LCL. Cells were grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 2% Glucose, 1mM Sodium pyruvate and 1% penicillin/streptomycin antibiotics (complete medium, Gibco®, Thermo Fisher Scientific) at 37°C in a humidified 5% CO_2 atmosphere. Forty-eight hours before electrotransfection, cells were transferred to the fresh medium to induce them to proliferate and make them more susceptible to transfection (13). On the day of electrotransfection, $5x10^5$ cells were resuspended in a total of 100 µL electrotransfection mixture composed of the Minimum Essential Medium Eagle with Spinner Modification (S-MEM, Sigma Aldrich), water and plasmid (1µg/µl, dissolved in water). The cells were then transferred into 1 mm cuvettes (Cell Projects) and electrotransfected at 1300 V/cm, with eight 100 µs pulses at 1 Hz. Electrotransfection efficiency and viability rates were evaluated 48 hours after electrotransfection. The viability rate of the electrotransfected cells was calculated with respect to that of non-electrotransfected cells set as 100% using forward *vs*. side scatter gating on a BD Accuri^{TM} C6 Flow Cytometer (BD Biosciences) as shown in the representative FACS plots (**Figure 1a**). Transfection rates were calculated as percentages of GFP-expressing cells *vs.* total live cells.

We first tested electrotransfection efficiency and cell viability using the above parameters in 10µl electrotransfection medium containing 10µg pCMV-GFP plasmid (3.5 Kbp, PF463, Plasmid Factory) with an addition of 90µl of S-MEM. The electrotransfection efficiency and viability rates (**Figure 1bc**) did not prove significantly better than other protocols used in previous studies. To improve the transfection rate, the salt concentration of the electrotransfection medium was decreased as reported earlier (14). To do so, the 10µl containing the 10µg of pCMV-GFP plasmid were diluted into 10, 40 or 70 µL water with 80, 50, 20 µL S-MEM added, respectively, to complete the 100 µl volume required for electrotransfection. This resulted in a significantly increased electrotransfection efficiency (up to 73.5±2.7%) but with a gradual decrease in cell viability. Nevertheless, with 50% S-MEM,

the transfection rate was as high as 55.3±2.3% with a very reasonable cell viability (68±3%) (**Figure 1bc**).

We next experimented with increased amounts of pCMV-GFP plasmid. Using 50µg rather than 10µg in 50% water and 50% S-MEM further improved the electrotransfection efficiency (79.2±4.6 *vs.* 55.3±2.3%) with an only slight decrease in viability (58.3±4.8% *vs.* 68±3%) (**Figure 1bc**). Altogether, this represents a significant improvement when compared to previously reported protocols.

The described protocol was also suitable for stable expression of the transgene (**Supplementary Figure 2**). We electrotransfected RPMI-8866 cells with the pCMV-GFP plasmid as described and sorted GFP+ cells after 48 hours. We then sorted cells every seven days during four weeks to obtain a cell population containing 93% GFP-positive cells.

Applying the protocol thus established to other B-cell lines, namely the Priess and Remb1 LCLs, the BL2 Burkitt lymphoma line, and the RS4;11 acute lymphoblastic leukemia cell line, yielded similar results (transfection efficiency of 41 to 73%; viability rates of 20 to 55%) (**Figure 1d**). We thus suggest that the electrotransfection parameters here defined can be applied successfully to many if not all B-cell lines.

The same electrotransfection protocol was also applied to transfect the pCMV-GFP plasmid into PBMCs purified by Pancoll (PAN biotech) density gradient centrifugation from whole blood samples from healthy donors of different sex and age purchased from the 'Etablissment Français du Sang', Hôpital Saint-Louis, Paris, France in accordance to the French legislation. As the obtained viability and transfection rate were low, we modified the protocol for transfection of PBMCs as follows: we used 400 µL of total volume of electrotransfection mixture composed of 50% SHM medium (19), instead of the S-MEM, in 4 mm cuvettes (Cell Projects); we also increased the electric field to 1500 V/cm at 24 pulses. Twenty-four hours after transfection, a 1.5±0.5% transfection rate with 30.4±1.9% of viability was obtained (**Figure 1e**). Among the transfected and living cells, 83.7±3.0% were CD3+ T cells and 7.1±2.0%, CD19+ B-cells (**Supplementary Figure 1**).

Most methods currently used for genome editing require the use of large plasmids; unfortunately increasing plasmid sizes negatively affect both transfection efficiency and cell

viability (15,16). We next tested whether the protocol established for the pCMV-GFP plasmid would also function with a 13.5 Kbp Cas9-GFP plasmid (Addgene #57818). Electrotransfection of 10µg of the Cas9-GFP plasmid resulted in a good cell viability (64.3±4.3%), but a much lower electrotransfection rate (1.1±0.1%). We then diluted the S-MEM medium twofold and used the same electrotransfection parameters adding either 10 µg of plasmid and 40 µl of water or 50 µg of plasmid. While the viability remained virtually unchanged (66.5±2.5% and 61.7±1.2%, respectively), the transfection rate reached 4.6±0.8% when using 50 µg of plasmid (**Figure 1f**). To summarize, cells should be placed in a fresh medium 48 hours before electrotransfection (1300 V/cm, 8 pulses of 100 µs each, 1 Hz frequency) performed in 50% S-MEM and 50% plasmid diluted in water. This protocol can be adapted to any device that can deliver similar pulses (e.g. the ELECTROcell B10 HVLV (Betatech), the BTX Gemini X2 (BTX Harvard Apparatus) or others) and can be scaled to transfect up to $8x10^6$ cells using 50 µg of plasmid.

We next used the electrotransfection protocol thus optimized to generate *TP53*$^{-/-}$ RPMI 8866 cells. Two gRNAs targeting sequences 7.5 kbp apart in exons 2 and 11 of the *TP53* gene were designed (**Figure 2a** and **Supplementary Table 1**). They were cloned into the phU6 plasmid (Addgene #53188) and tested using the previously reported ENIT method (17). RPMI 8866 cells ($8x10^6$) were then electrotransfected with 30 µg Cas9-GFP and 20 µg of phU6 plasmids encoding the two gRNAs (10µg/each) in 400 µL electrotransfection mixture in 4-mm electroporation cuvettes (Cell Projects). After 48 hours, individual GPF-positive cells were sorted at one cell per well into five 96-well plates, and left to grow in complete medium at 37°C in 5% CO_2 atmosphere. Sixty individual clones collected four weeks after transfection were transferred to 24-well plates for clonal expansion. After one further week in culture, the clones were screened using PCR primers covering exons 2 and 11 of the TP53 gene (**Supplementary Table 1**) and located 8 kbp apart on genomic DNA (**Figure 2a**). Upon successful deletion of the region, the primer binding sites would be now located ~400 bp apart. The KO1 and KO2 clones had PCR amplicons of 350 bp and 400 bp (**Figure 2b**), indicating the successful deletion of a part of p53 gene located between exons 2 and 11.

The two clones expressed neither *TP53* mRNA transcripts as tested by qRT-PCR (data not shown) nor the p53 protein as tested by Western Blotting (**Figure 2c**). That the *TP53* gene had been successfully knocked out was confirmed when the expression of the *P21* gene, a downstream target of p53 (18), was found significantly reduced as compared to the control

(**Figure 2d**). Using the above described method, we have thus been able to successfully and stably knock-out or delete the *TP53* gene in a hard-to-transfect B-cell line without using any viral vectors. We believe that the present electrotransfection method will prove instrumental in future cancer research using lymphoma and leukemia B-cell lines and primary blood cells.

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Declarations

The authors declare no conflict of interest.

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Figure legends

Figure 1. **Efficient electrotransfection of human B-cell lines and PBMCs.** Effect of different electrotransfection parameters on transfection rate (calculated as GFP expressing cells/living cells, grey) and cell viability (calculated as number of living cells *vs.* living cells in the untransfected controls, black). Cells were electrotransfected with a pCMV-GFP (**a-e**) or with a Cas9-GFP plasmids (**f**) using the Cliniporator^{TM}. Forty eight hours after electrotransfection, the viability and transfection rates were measured using the BD Accuri^{TM} C6 Flow Cytometer.

(**a**) Representative cytometry graphs showing the strategy used for gating the viable (upper plots) and transfected (GFP+) (lower plots) LCLs after electrotransfection. Forward scatter area (FSC-A) versus side scatter area (SSC-A) plot was used to gate the cell population and remove the debris. Forward scatter height (FSC-H) *vs*. area (FSC-A) plot was used to identify the single cell population by choosing a diagonal gating. FITC-channel versus PE-channel plot was used to gate the GFP positive cells where the gating was based on the control cells to ensure 0 % of GFP positive cells in non-electrotransfected control.

(**b,c**) RPMI8866 cells were electrotransfected at 1300 V/cm, 8 pulses of 100µs length each and 1Hz of frequency in the electrotransfection medium composed of the 10-50 µg pCMV-GFP plasmid, different amounts of water and S-MEM in a total volume of 100µL. Graphs show electrotransfection (**b**) and viability (**c**) rates.

(**d**) Electrotransfection and viability rates of different B-cell lines transfected with 50µg of pCMV-GFP plasmid.

(**e**) Electrotransfection and viability rates of PBMCs transfected with 50µg of pCMV-GFP plasmid. PBMCs were purified from blood of four different healthy donors by Pancoll (PAN biotech) density gradient centrifugation and electrotransfection and viability rates were tested 24 and 48 hours after electrotransfection.

(**f**) Effect of plasmid amount and water content on transfection efficiency and viability rates in the RPMI8866 cells transfected with a large Cas9-GFP plasmid.

Every experiment was performed at least in two biological replicates. Data are represented as means ± SEM. ***: P<0.001; **: 0.001<P<0.05; *: P<0.05, ns: non-significant. Comparisons between more than two averages were performed using the Bonferroni's post-test based on the results generated using the one-way Anova test.

Figure 2. Efficient generation of *TP53*$^{-/-}$ RPMI8866 cells. Electrotransfection was used to

create RPMI-8866 cells stably knocked-out for the *TP53* gene. (**a**) Gene map showing the designed gRNAs and PCR primers within the *TP53* gene. The effective knock-out was confirmed by PCR of genomic DNA (**b**), western blotting (**c**) and RT-qPCR for p53 downstream target p21 (**d**) from one control and two KO lines. Red arrows, gRNAs targeting the exons 2 and 11 of the TP53 gene; blue arrows, the annealing sites of the PCR primers used.

(**b**) Genomic DNA was amplified using primers annealing next to the exons 2 and 11. M, the molecular weight marker. Successful deletion should generate a PCR amplicon of ~400 bp in size.

(**c**) Western blot analysis of the p53 protein. GAPDH was used as a loading control.

(**d**) Analysis of *p21* transcription in two control and two KO RPMI-8866 lines. The transcription of the *p21*, a direct p53 target, was measured by RT-qPCR. The graph shows fold change *vs.* the controls after normalization *vs. GAPDH*.

Every experiment was performed at least in two biological replicates. Data are represented as means ± SEM. *: $P<0.05$. Comparisons between more than two averages were performed using the Bonferroni's post-test based on the results generated using the one-way Anova test.

Supplementary Figure 1. Electrotransfection of human PBMCs. A. Representative cytometry graphs showing the gating strategy to determine the viability, transfection rates and percentage of CD3+/CD19+ positive cells after electrotransfection. Forward scatter area (FSC-A) vs. side scatter area (SSC-A) plot was used to gate the cell population and remove the debris. Since PBMCs are not homogenous, we used large gating and eliminated the events with low FSC-A and SSC-A, and those with low FSC-A and high SSC-A to ensure the gating of the majority of viable cells. Forward scatter height (FSC-H) vs. area (FSC-A) plot was used to identify cell populations by choosing a diagonal gating. FITC-channel vs. PE-channel plot was used to gate GFP positive cells, the gating was based on control cells to ensure 0 % of GFP positive cells in the non-electrotransfected control. PE-channel versus APC-channel plot was used to gate CD3+/CD19- T-cells and CD3-/CD19+ B-cells in the total population and in the population of GFP-positive cells after staining with CD19-PE and CD3-APC antibodies. The automatic color compensation on single-stained controls was done by FlowJoTM 10 software (FlowJo LLC, BD, USA). **B**. PBMCs were electrotransfected at 1500 V/cm, 24 pulses of 100 µs length each and 1Hz of frequency in the electrotransfection medium composed of the 50 µg pCMV-GFP plasmid, 50% of water and the SHM medium in a total volume of 400 µL. T- and B-cell populations were evaluated by CD3-APC and CD19-PE

staining, respectively, 24 hours after electrotransfection by flow cytometric analysis in the whole population and in the population of transfected (GFP-positive) cells. Experiments were performed in three biological replicates with PBMCs from four different donors. Data are represented as means ± SEM.

Supplementary Figure 2. Fluorescent photomicrographs of RPMI8866 cells 30 days after electrotransfection with the pCMV-GFP plasmid. GFP+ RPMI8866 were initially sorted 48 hours after electrotransfection, and then were re-sorted every seven days for four weeks.

Supplementary Table 1. gRNAs and primers sequences.

Function	gRNA/ PCR primers	Sequence (5' to 3')
gRNA targeting *TP53* gene exon 2 and 11	p53-ex11	TCCCTCTCCCTGTTGGTCGG
	p53-ex2	CTCAGAGGGGGCTCGACGCT
PCR primers *TP53* DNA	p53-ex11-F	AAGCGAGACCCAGTCTCAAA
	p53-ex2-R	CAGCCATTCTTTTCCTGCTC
PCR primers *GAPDH* DNA	GAPDH DNA-F	GCTGGCACCACTACTTCAGAG
	GAPDH DNA-F	GCCAACAGCAGATAGCCTAG G
PCR primes *p21* mRNA	P21-F	CTGCCGAAGTCAGTTCCTTGT
	P21-R	CATGGGTTCTGACGGACATC
PCR primes *GAPDH* mRNA	GAPDH mRNA-F	CTGCACCACCAACTGCTTAG
	GAPDH mRNA-F	AGGTCCACCACTGACACGTT

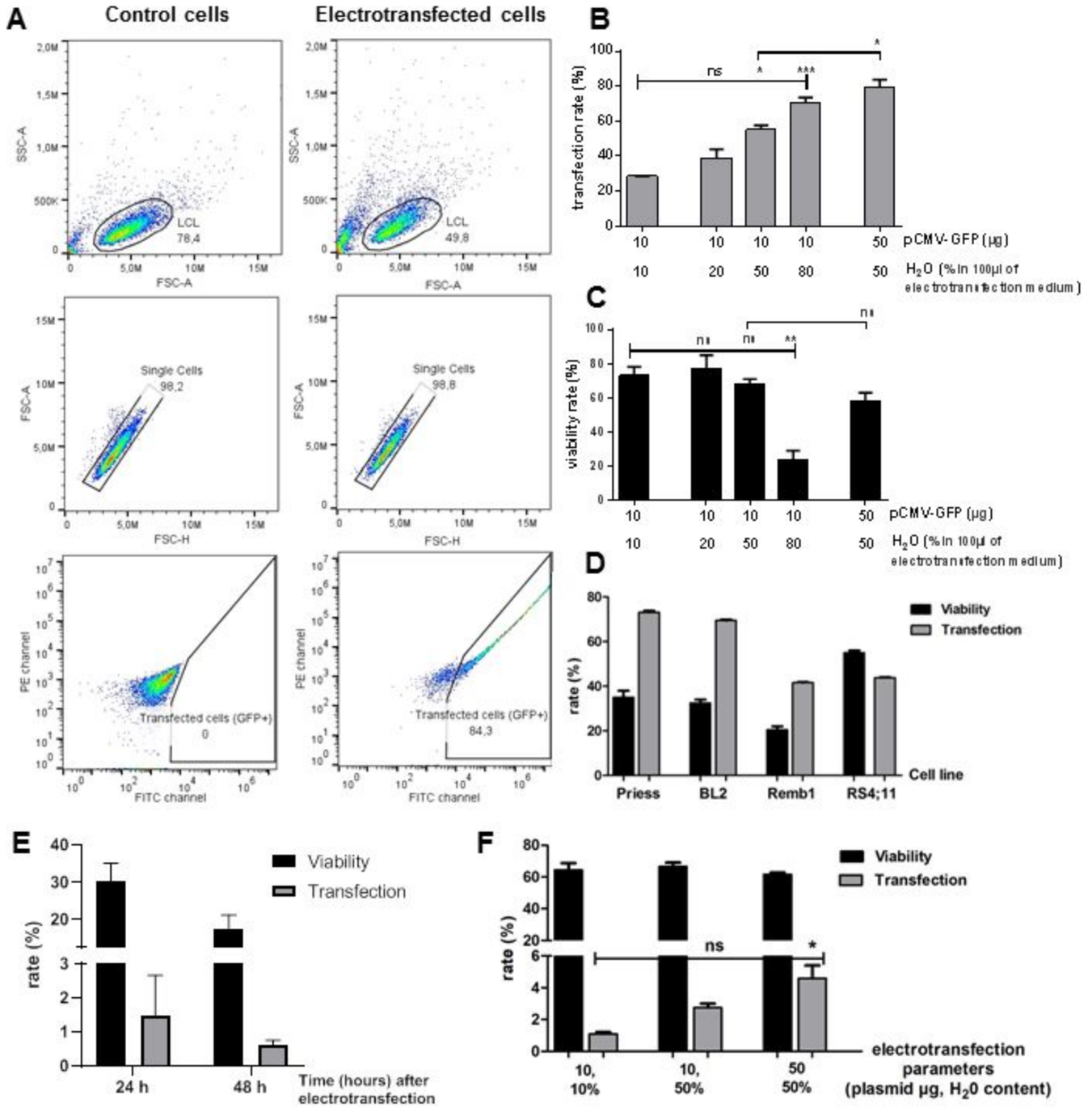

A
Control cells
Electrotransfected cells
SSC-A
FSC-A
LCL
78,4
LCL
49,8
Single Cells
98,2
Single Cells
98,8
FSC-H
PE channel
FITC channel
Transfected cells (GFP+)
0
Transfected cells (GFP+)
84,3
B
transfection rate (%)
ns
*
***
*
pCMV-GFP (µg)
H2O (% in 100µl of electrotransfection medium)
C
viability rate (%)
ns
ns
**
ns
pCMV-GFP (µg)
H2O (% in 100µl of electrotransfection medium)
D
Viability
Transfection
rate (%)
Priess
BL2
Remb1
RS4;11
Cell line
E
Viability
Transfection
rate (%)
24 h
48 h
Time (hours) after electrotransfection
F
Viability
Transfection
rate (%)
ns
*
10, 10%
10, 50%
50 50%
electrotransfection parameters (plasmid µg, H20 content)

Figure 1

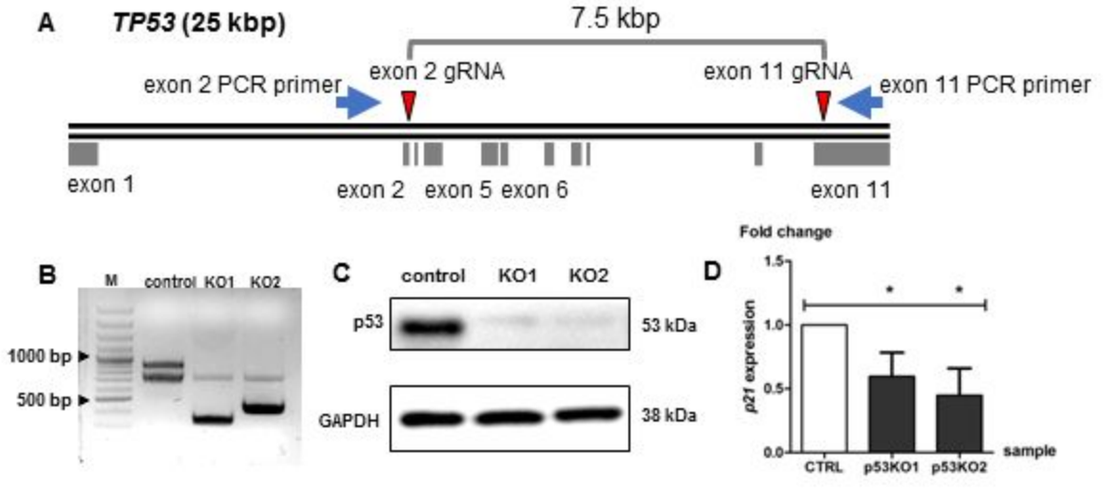

A
TP53 (25 kbp)
7.5 kbp
exon 2 PCR primer
exon 2 gRNA
exon 11 gRNA
exon 11 PCR primer
exon 1
exon 2
exon 5
exon 6
exon 11
B
M
control
KO1
KO2
1000 bp
500 bp
C
control
KO1
KO2
p53
53 kDa
GAPDH
38 kDa
D
Fold change
p21 expression
1.5
1.0
0.5
0.0
*
*
CTRL
p53KO1
p53KO2
sample

Figure 2