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Quantify the Cas9 level of expression after transfection in human cells

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The use of RNA-guided Cas9 nuclease associated with the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), derived from microbial immunity, has become a novel promising method for targeted gene editing in any species including humans. Its efficiency highly depends on the Cas9 delivery system in cells, directly correlated with its expression pattern. Uncertainties remain towards the duration of Cas9 expression within the cell following its plasmidic delivery. The aim of our study is to evaluate the expression level of Cas9 protein over time following its transient transfection by a plasmid-based method via a cationic polymer-based molecule in HeLa cells. Cas9 RNA extracted by a previous year team was used as a measure due to poor result of RNA extraction this year. The Cas9 RNA cell content appeared equivalent at 48h and 72h post-transfection. A Coomassie Blue and a Western Blot were performed to quantify the Cas9 protein compared to a BSA range by using the Gelix One®G230 Version 4:1 (biostep®, Burkhardtsdorf, Germany) software. The Cas9 protein was directly detected in cells after 24h and 48h in a similar way, suggesting a long-lasting Cas9 expression. More experiments have to be realized in the future as the measure of the Cas9 expression on a wider time range (from 24h to 7 days for example) to allow a more precise insight on the Cas9 expression compared to other delivery techniques and evaluate which appears optimal for an efficient genome editing.

Keywords

CRISPR; Cas9; recombinant plasmid; cell transfection; gene expression; protein synthesis

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Upon more than a decade of basic research into the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) repetitive elements, derived from a microbial adaptive immune system, the CRISPR-Cas9 tool was finally fully developed for application in mammalian cells in 2013 (Cong L. et al, 2013 & Mali P. et al, 2013). The CRISPR-based adaptive immune systems in prokaryotes rely on the insertion of an invader sequence copy within the CRISPR loci, and the subsequent synthesis of short transcripts named crRNA. These non-coding RNAs serve as guides for targeting the CRISPR-associated RNA-guided Cas endonucleases to invading nucleic acids and silencing them at the DNA or RNA level (Terns M.P. and Terns R.M., 2011).

Among the diverse Cas proteins existing in microbial immune systems, the site-specific Cas9 nuclease recently appeared as a promising basis for developing a novel genetic engineering tool. By replacing the guide sequence by a particular sequence of interest matching the target, genomic modifications can be performed at any precise location in the genome of numerous eukaryotic organisms

including humans. After the targeted gene is silenced due to the ability of Cas9 to induce a Double Strand Break (DSB), the activation of a DNA damage repair pathway allows a re-ligation process, resulting either in the gene knock-out or the replacement of a defective gene by its functional analogue. The presence of errors in this repairing process results in the gene modification (Zhang F. et al, 2013). The ease and efficiency of genome editing allowed by this technique was the leading reason for its global adoption by the scientific community, and constituted a significant turning point in genetic engineering (Mali P. et al, 2013).

The reconstitution of the RNA-guided nuclease function of Cas9 in mammalian cells requires the delivery of two essential elements: Cas9 protein and RNA components including the guide RNA (Zhang F. et al, 2013). The growing interest towards this technology justifies the search for optimized heterologous Cas9 delivery and expression methods, constituting a limiting step in genome editing efficiency (DeWitt MA et al, 2017). Two parameters must be taken into account: the Cas9 format (plasmid DNA, mRNA or protein) and the delivery method (electroporation, ribonucleoprotein complex, lipid-mediated transfection, lentiviral delivery...). Transient transfection using chemical compounds is the most common strategy due to its low toxicity for the cells. Precedent studies have suggested that Cas9 nuclease remained longer in the cell when delivered in the form of a plasmid, and therefore induced more off-target mutations (OT) (Liang X. et al, 2015). Indeed, Cas9 appearance in cells transfected in those conditions followed an increasing pattern over at least 72h. On the contrary, when delivered as RNA or protein, Cas9 expression was globally lower and reached a maximum as early as four hours post transfection. However, uncertainties remain towards the exact duration of Cas9 expression within the cell following its plasmidic delivery (Liang X. et al, 2015).

In the present study, we investigated the expression level of Cas9 after a DNA transfection to precise its persistence in human cells and further determine the optimal time for a guide RNA parallel delivery. We measured Cas9 RNA and protein levels after 24h, 48h and 72h following its transient transfection by a plasmid-based method via a cationic polymer-based molecule.

MATERIALS AND METHODS

Materials. HeLa cells, pSpCas9(BB)-2A-GFP (PX458), pEBV-YFP(2347), Gibco® 10% Fetal Bovine Serum (FBS), Microdrop™ Plate and water bath from Thermo Fisher Scientific, Dulbecco's Phosphate Buffered Saline (PBS), Trypsin-Versene EDTA, 10cm Culture dish, 12-well plate, Zoe™ Fluorescent Cell Imager, Thermal cycler, BioRad iScript™ cDNA Synthesis kit, Mini-PROTEAN Tetra Cell, PrecisionPlus Protein Kaleidoscope Standard and 1kb ladder from Biorad, Malassez Cell and lamella, JetPrime® kit from polyplus transfection, Agarose gel 1%, Buffer 3.1 (100 mM NaCl, 50 mM Tris-HCl , 10 mM MgCl2 , 100 µg/ml BSA , pH 7.9@25°C) from NEB, BglII restriction enzyme, PvuI restriction enzyme, EcoRV restriction enzyme, NotI restriction enzyme, Loading dye, RNeasy Mini Kit from Qiagen, Dimethylsulfoxyde (DMSO), Taq'Ozyme kit, 4X Laemmli Buffer, Centrifuge MiniSpin from Eppendorf, β-mercaptoethanol, Heat Blocker from Stuart, (Bovine Serum Albumine powder (BSA) 96%, Acrylamide/Bis-Acrylamide 30%, Tris Base, TEMED, Coomassie Blue, Decoloration solution, Nitrocellulose

membranes, Whatman blotting papers, Western-Blot grade skimmed-milk powder, Tween 20, Anti-CRISPR/Cas9 antibody, Mouse Monoclonal clone, 3-3' Diaminobenzidine, HRP conjugated Anti-mouse IgG concentrate, Dulbecco's Modified Eagle Medium (DMEM) and Non-Essential Amino Acid solution from Sigma-Aldrich, Gelix One®G230 Version 4:1 (biostep®, Burkhardtsdorf, Germany).

Methods.

Mammalian cell cultures. The cell line was obtained from ATCC. HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS, Gibco^{TM}, ThermoFisher Scientific), 1% Non-Essentials Amino Acids (100X-M7145, Sigma) and 1% L-Glutamin-Penicilin-Streptomycin solution (G1146, Sigma) in 10 cm culture dishes. Cells were passaged 1 time and seeded in a 12-well plate 24h prior to transfection.

Cell transfection. HeLa cells were transfected using the cationic polymer-based jetPRIME transfection kit (PolyPlus-transfection) 24 hours after the first passage, at a density of 200 000 cells per well. For one well of a 12-well culture plate, 0.75µg of DNA was diluted into 75µL of jetPRIME buffer and 1,5µL of jetPRIME reagent was added before a 10min incubation at RT. 200µL of transfection mix was distributed in each well and replaced by growth medium after 4h. Cells were transferred to -80°C either 24h or 48h post-transfection and stored until the extraction step.

Plasmids used. pSpCas9-2A-GFP plasmid was used and provided by Addgene as the Cas9 expression vector in mammalian cells (Zhang F. et al, 2013).

pEBV-YFP(2347) plasmid was also used, as a positive transfection control and a negative Cas9 expression control. This plasmid has been given by Dr. Denis Biard (CEA/DRF/Jacob/SEPIA).

2.5 Plasmid digestion. The Cas9 plasmid was digested with 3 different restriction enzymes. The digestion mix was prepared as follows (Table 1).

Table 1: Composition of digestion mixes

Digestion	Mix digestion
Digestion 1	Buffer 3.1, BglII restriction enzyme, PvuI HF restriction enzyme, native plasmid and distilled water.
Digestion 2	Buffer 3.1, EcoRV restriction enzyme, NotI restriction enzyme, native plasmid and distilled water.
Digestion 3	Buffer 3.1, NotI restriction enzyme, PvuI restriction enzyme, native plasmid and distilled water.

After 1 hour of incubation at 37°C water bath, loading mixes were prepared as follows (Table 2).

Table 2: Composition of loading mixes for digestion product migration along with the molecular weight of the bands theoretically expected.

Samples	Mix	Expected bands*
Ladder 1kb	Ladder, loading dye, distilled water.	
Native plasmid	Native plasmid, loading dye, distilled water.	9289 bp
Digestion 1	Digestion mix 1, loading dye.	6406 bp and 2883 bp
Digestion 2	Digestion mix 2, loading dye.	5964 bp and 3325 bp
Digestion 3	Digestion mix 3, loading dye.	7809 bp and 1480 bp

*The expected sizes were predicted using the ApE software.

Mixes were loaded onto the 1% agarose gel, and the migration was run at 110 volts for 30 minutes.

RNA extraction. 200 000 transfected cells were thawed on ice. Total RNA was extracted using the silica membrane-based RNeasy Mini Kit (Qiagen) technology. Cell samples were lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate-containing buffer (RLT). 70% ethanol was added to the homogenized lysate, and 700µL of the sample was applied to the RNeasy Mini spin column placed in a 2mL collection tube. Three washing steps were performed using RW1 and RPE buffers, along with two 15s and one 2min centrifugation at 10 000 rpm. Attached RNA was eluted in RNase-free water and stored at -80°C until the reverse-transcription step.

Spectrophotometry measurements. RNA content and quality were assessed by spectrophotometric measurements using a µdrop plate (Thermo Fisher Scientific). Either 2µL or 5µL of samples were pipettes onto the plate and absorbances were measured at 230 nm, 260 nm and 280 nm on a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific). RNase-free water was used as a blank, and its values were automatically subtracted from the absorbance obtained for the samples. A260/A230 and A260/A280 ratios were automatically calculated, and the concentration in µg/mL was calculated using the formula A260*20*40.

Cas9 cDNA amplification by RT-PCR. Reverse transcription (RT) of RNA has been performed with BioRad iScript™ cDNA Synthesis kit. A mix of RNA template, Nuclease-free water, 5X iscript reaction mix, iscript Reverse Transcriptase has been prepared. The mix has been run in a thermocycler (BioRad) according to the following program (Table 3).

Table 3: Conditions for reverse-transcription of RNA

Annealing	25°C 5 minutes
Extension	42°C 30 minutes
Reverse transcriptase's stop	85°C 5 minutes
Conservation	10°C Infinite

Polymerase Chain Reaction (PCR) has been performed with Taq'Ozyme kit. The mix was prepared with Nuclease-free water, Reaction Buffer 10X, $MgCl_2$, dNTP PreMux, Upstream primer, Downstream primer, Taq' Ozyme and DNA matrix. The mix has been run in a thermocycler (BioRad) according to the following program (Table 4).

Table 4: PCR conditions for amplification of cDNA

Initial Denaturation		95°C 2 minutes
Cycle X35	Denaturation	95°C 30 secondes
	Annealing	55 or 58°C 30 secondes
	Extension	72°C 1 minute
Final Extension		72°C 5 minutes
Conservation		4°C Infinite

The couple of primers was used from a previous study to amplify the Cas9 gene (Senturk S. et al, 2017). One primer couple was used to amplify the TBP domestic gene. An overview of the primers used along with the lengths of the subsequent fragments generated are presented in Table 5.

Table 5: Sequences of primers to amplify Cas9 and TBP regions of cDNA expression products, along with the corresponding fragment length.

Name	Sequence	%GC	TM (°C)	Amplicon length (bp)
SpCas9-F	5'-CCCAAGAGGAACAGCGATAA-3'	47	53	220
SpCas9-R	5'-TTGGCTTCCAGAAAGTCGAT-3'	45	55	
TBP-F	5'-CTCACAGGTCAAAGGTTTAC-3'	45	52	90
TBP-R	5'-GCTGAGGTTGCAGGAATTGA-3'	50	57	

RNA/DNA electrophoresis on agarose gel. The 1% agarose gel was prepared in Tris Acetate-EDTA (TAE) buffer and heated for 2 min 30. 3µL Ethidium Bromide solution (BET) (Sigma-Aldrich) was added in 100 mL. For each RNA/DNA extract, 5 volumes were added with 1 volume of 6X gel loading dye (NEB). Migration was run at 110V for 30 min and revelation was made under UV-light.

Protein isolation and Western Blot analysis. Proteins were extracted using Laemmli Buffer. Cell lysates were resuspended in 2X Laemmli Buffer (1% Bromophenol Blue, 1.5M Tris-HCL pH 6.8, glycerol, SDS) and heat at 95°C during 5 minutes in a Heat Blocker. Then, the tube has been centrifuged at maximum speed for 1 minute. The supernatant containing proteins have been transferred in a new tube.

The goal was to load 25 µg of total protein cell lysate in each well. Due to the quantification measurements problems connected to Laemmli buffer we could not obtain a precise quantification of the proteins. We had to estimate the proteins quantification by BSA range (Figure 7). Eventually, 20 µL of sample was loaded per well to SDS PAGE for the electrophoresis corresponding to the equivalent of the protein content of 200000 cells. Proteins were then transferred onto a nitrocellulose membrane. Afterword the amount of the protein was quantified from the picture (Figure 7) by software Gelix One®G230 Version 4:1 (biostep®, Burkhardtsdorf, Germany). In comparison with a BSA range the amount of protein was calculated with a calibration curve and mathematical equations (Figure 8).

Western blot procedure was conducted under a specific condition for 2 hours. Immunodetection was made by incubation with Primary antibodies (Cas9 (7A9-3A3) Mouse mAb #14697 AbCam) at a 1:1000 dilution in blocking buffer consists 5% skimmed milk powder in PBS + Tween 20 for 1 hour. After washing, the membrane was incubated for another 1 hour with Secondary antibodies (HRP-conjugated Anti-Mouse IgG Concentrate (Item I2)) that were diluted to 1:1000 in blocking buffer consists 5% skimmed milk powder in PBS + Tween 20.
The secondary antibodies conjugated with Horseradish peroxidase (HRP) were used in order to obtain good bands visualization. The final coloration was made by the activation of HRP by the 3, 3'-diaminobenzidineHydrogen (DAB), a substrate of HRP that yields to a brown precipitate in presence of HRP and peroxide.

RESULTS

Cas9 plasmid verification. Before transfecting HeLa cells with the pSpCas9-2A-GFP plasmid, a selective digestion using pre-identified restriction enzymes was performed to verify its integrity. The digestion products were migrated on an agarose gel and revealed under UV-light (Figure 1).

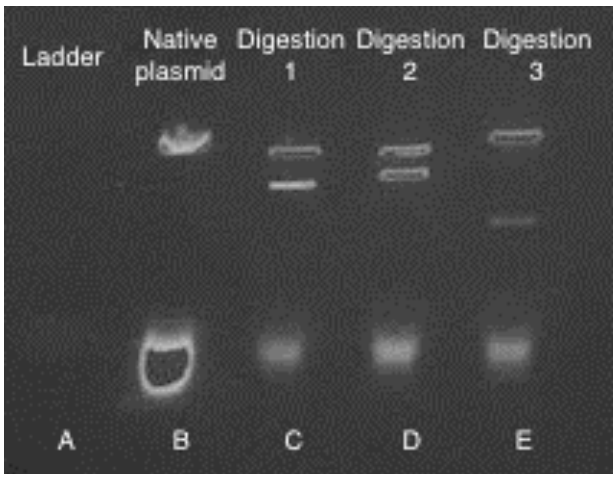


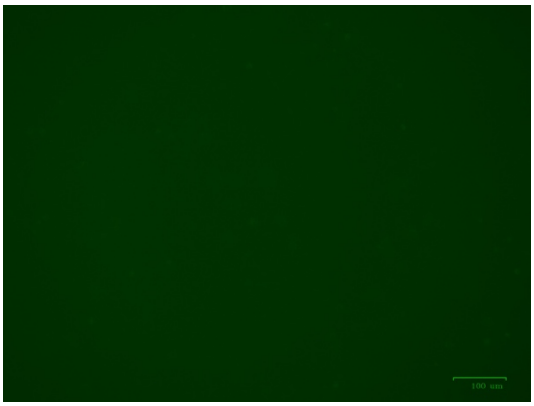
Fig 1: Digestion of the pSpCas9-2A-GFP plasmid using several restriction enzyme couples. (A) 1kb ladder (B) Native pSpCas9-2A-GFP plasmid (C) Native plasmid digested with BglII and PvuI-HF (Digestion 1) (D) Native plasmid digested with EcoRV and NotI (Digestion 2) (E) Native plasmid digested with NotI and PvuI (Digestion 3).

The ladder was not observable on the gel so the exact size of the bands obtained could not be determined. However, the relative heights of the bands present for each sample appeared consistent with the different sizes theoretically expected. For the native plasmid, the single band obtained appeared thicker than those of the digestion fragments suggesting a proper plasmid integrity before digestion. Based on this assumption, and given the plasmid

molecular weight is known to be 9288 bp, this band could serve as a reference for the approximation of digestion fragment sizes. The digestion 1 gave 2 bands close to those given by the digestion 2 as expected. (6406 bp and 2883 bp for Dig.1 and 5964 bp and 3325 bp for Dig.2). The two fragments obtained following digestion 3 were respectively higher (7809 bp) and lower (1480 bp) than those obtained with the previous digestions matching the theoretical expectations.

HeLa cells transfection. HeLa cells were transfected with the pSpCas9-2A-GFP plasmid and observed under a fluorescent microscope to verify the proper plasmid insertion and expression compared to a positive control (pEBV-YFP(2347)).

Non-transfected HeLa cells were not visible under the Zoe™ Fluorescent Cell Imager due to the absence of the GFP according to the experiment.



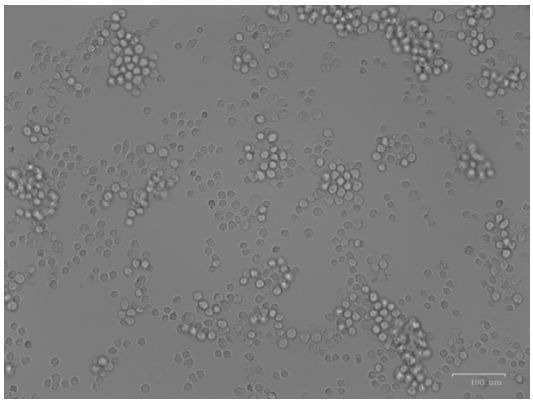


Fig 2: Non-transfected HeLa cells observed under Zoe™ Fluorescent Cell Imager.

Visual aspect of HeLa cells was observed 48h post-transfection with the pSpCas9-2A-GFP plasmid. Some cells clearly appeared fluorescent, indicating that the Cas9-GFP plasmid was properly inserted and expressed within those cells.

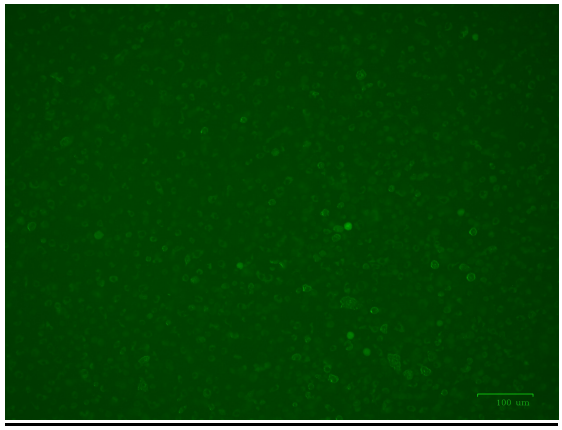


Fig 3: HeLa cells 48h post-transfection with pSpCas9-2A-GFP observed under Zoe™ Fluorescent Cell Imager.

Visual aspect of HeLa cells was observed 48h post-transfection with the pEBV-YFP(2347) plasmid. Some cells appeared fluorescent, indicating that the pEBV-YFP plasmid was properly inserted and expressed within the cells.

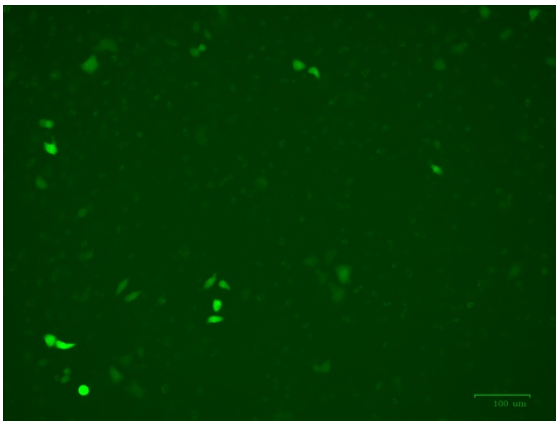


Fig 4: HeLa cells 48h post-transfection with pEBV-YFP(2347) observed under Zoe™ Fluorescent Cell Imager.

Verification of extracted RNA aspect.

- **Determination of extracted RNA quantity and quality**

Total RNA content was extracted from HeLa cells either 24h or 48h post-transfection in order to further assess the Cas9 level of expression at those times. Before amplifying the Cas9 gene, these extracts were submitted to a spectrophotometer analysis to verify the respective RNA concentrations obtained, as well as the eventual presence of protein and solvent contaminations. The absorbance was measured at 230nm, 260nm and 280nm. Sample quality was assessed based on the calculation of different ratios, and RNA concentration was deduced from the A_{260} value. Results from RNA extracts obtained in 2017 in comparison with those obtained in 2016 by another team are presented in Table 6.

Table 6: Determination of RNA content and quality by spectrophotometer measurements (NT = Non-transfected cells)

Sample	Average A260nm/A230 nm	Average A260nm/A280nm	Concentration (µg/mL)	
p2347	0,12	2,51	27,06	2017 samples (N=2)
NT	0,68	2,05	23,58	
Cas9 24h	0,21	2,51	104,28	
Cas9 48h	0,36	2,38	15,88	
NT	1,74	1,94	603,20	2016 samples (N=1)
Cas9 48h	2,13	1,97	651,52	
Cas9 72h	1,53	1,88	353,52	

The A_{260} values obtained for the 2017 samples were too low (<0,15) to allow a relevant RNA concentration calculation. The values obtained were therefore not significant. These low A_{260} values obtained suggest a poor content in nucleic acids which means that the RNA was contaminated. On the contrary, the 2016 samples show a more satisfactory RNA concentration in accordance with the higher A_{260} values obtained.

The A260/A280 ratio values should be around 2,0 (+/- 0,1) to qualify an RNA extract as pure. We obtained good values for the 2016 samples, but only one 2017 sample value was comprised in this range over the four measured (Ctrl 48h). The others are slightly higher but still acceptable.

The A260/A230 ratio values should be equal to 2,0. In our samples, it is the case for only one 2016 sample measured (Cas9 48h). Globally, the 2016 samples displayed a value closer to this range whereas the 2017 samples are drastically lower. This strongly suggests the presence of a compound absorbing at 230 nm and therefore a solvent contamination.

- **Verification of RNA integrity on 1% agarose gel**

Due to the unexpected results obtained for the RNA content verification by spectrophotometry, all 2017 extracts were migrated on the agarose gel to visually evaluate the aspect of the total nucleic acid content. For each sample, an estimated quantity of 300 ng of RNA was loaded on the gel. Results are presented in Figure 5.

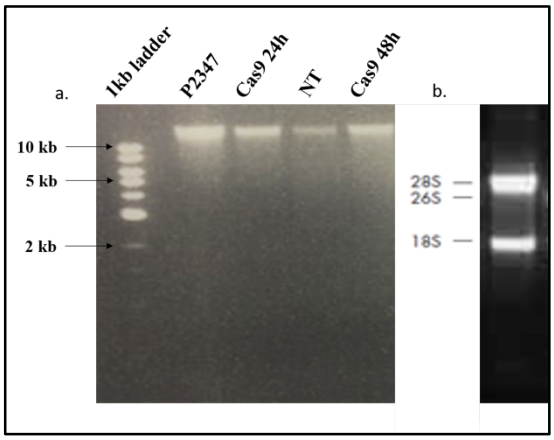


Fig 5: Molecular weight distribution of nucleic acids contained in RNA extracts a. 2017 samples; b. Theoretical results expected.

The migration results did not correspond to the theoretical expectations. Total human RNA should include two bright bands at 5 kb and 1.9 kb, respectively corresponding to the 28S and 18S ribosomal RNA. Only one band was visible for all samples, at a molecular weight above 10kb. The bands obtained were bright and well-defined; no smear was significantly observable. This molecular weight seems to indicate the presence of DNA, rather than RNA, in our samples. In addition to this important DNA contamination, almost no RNA was obtained which means that our RNA extraction did not work properly.

Cas9 gene amplification by RT-PCR. In order to estimate the quantity of Cas9 expression in the transfected mammalian cells at different times, the corresponding gene was amplified from the RNA extracted using a RT-PCR strategy. This test could not be performed with our RNA extracts, due to the absence of RNA in our samples associated with an important DNA contamination. RNA extracted by another team in 2016 were submitted to selective RT-PCR for Cas9 gene and the resulting products were migrated on agarose gel for a subsequent expression estimation by comparison with TBP housekeeping gene (Figure 6).

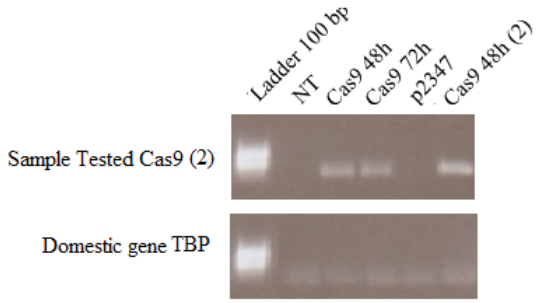


Fig 6: RT-PCR results after Cas9 amplification on 1% agarose gel compared to the domestic gene TBP.

Once band was observable in each well containing RT-PCR products of the cells transfected with the Cas9 containing plasmid. The ladder was highly compact and did not allow a precise molecular weight determination for the bands obtained. However, their molecular weight appeared to approximately match the theoretical expectation of 220 bp, corresponding to the size of the expected amplicon. This result shows the expression of Cas9 in transfected cells. The band thickness and fluorescence did not seem to significantly differ between 48h and 72h post-transfection. Both NT and p2347 controls were negative. Despite the presence of TBP amplification products, the results obtained were not clear enough to allow a relevant estimation of the relative Cas9 gene expression compared to TBP.

Coomassie Blue. A Coomassie Blue was performed in order to verify the presence of Cas9 protein in our samples. Our samples were in presence with Laemmli buffer which prevents us from colorimetric dosage such as Bradford dosage because of the blue color of this buffer. That's why, a BSA range in five dilutions was performed as a reference in order to obtain accurate protein measurements. We performed the protein quantification from the picture (Figure 7) by software Gelix One®G230 Version 4:1 (biostep®, Burkhardtsdorf, Germany). The protein quantity was calculated with a standard curve and a mathematical equation in two repetitions after a densitometric analysis (Figure 8). The average amount of the

protein in our samples from the two mathematical equations are equal to 0,1982 mg/ml. For the BSA range, we saw on the gel that the higher the concentration, the larger was the band. According to the gel, we obtained one band in each well for the BSA range between 50 and 75kDa which is in agreement with the expected size for BSA (66,430kDa). The results obtained for the Cas9 and control samples indicate the total input of the proteins extracted through the presence of multiple bands at different weights along the migration axis. The Cas9 protein, which molecular weight is 158,441 kDa, is not clearly visible on the gel. However, a particularly large band is present for all transfected and non-transfected samples at around 60 kDa. This should correspond to an over-expressed protein encoded within the HeLa cell genome. We could suppose that it corresponds to tubulin, which is implicated in the cell division process and is therefore highly expressed in cancerous cell lines.

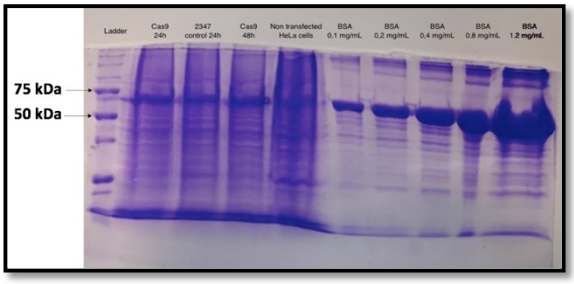


Fig 7: Coomassie Blue of Cas 9 protein expression compared to BSA range.
Well 1: PrecisionPlus Protein Cat#161-0363 Biorad, Well 2: Cas9 protein expression in HeLa cells after 24 hours transfection, Well 3: HeLa Cells after 24 hours transfection with pEB-GFP (2347) plasmid, Well 4: Cas9 protein expression in HeLa cells after 48 hours transfection, Well 5: Non transfected HeLa cells, Well 6: BSA at the concentration of 0,1mg/mL, Well 7: BSA at the concentration of 0,2 mg/mL, Well 8: BSA at the concentration of 0,4mg/mL, Well 9: BSA at the concentration of 0,8mg/mL, Well 10: BSA at the concentration of 1,2mg/mL.

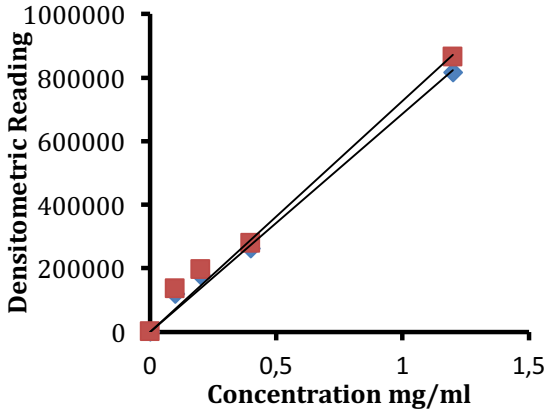


Fig 8: Curves of densitometric readings of the same BSA range gel in two repetitions. Mathematical equations: red squares curve y = 685499x, blue dots curve y = 726545x.

Western Blot analysis. In order to total intracellular proteins visualization before the Western Blot assay we decided to perform a Coomassie Blue (figure 9, A). It is hard to observe any band on the electrophoresis Gel after Coomassie Blue by the naked eyes. To obtain precise indication of Cas9 protein we decided to do a Western Blot analysis, which is an immunodetection technique using a specific antibody against Cas9 protein. The Cas9 protein weight is approximately 160 kDa. (Figure 9, Both of the Cas9 proteins bands are clearly visible, line 2 and 4) which confirms an efficient plasmid transformation and expression. However, band 4 that indicates the Cas9 protein level in the HeLa cells after 24 hours of the expression is thicker. This can prove that the 24 hours post-transfection expression of Cas9 is more effective than after 48 hours.

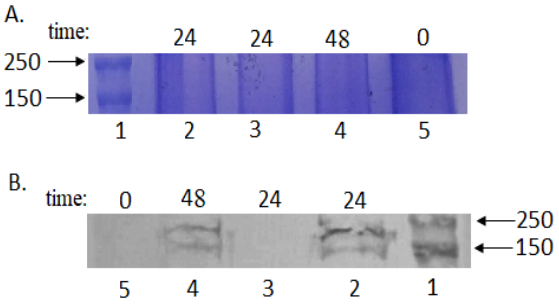


Fig 9: Detection of the Cas9 protein from the extracted total intracellular proteins. A - Coomassie Blue gel colouration analysis and B - Western Blot analysis. Line 1 was protein ladder (Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards cat #161-0375), line 2 was the Cas9 protein level in the HeLa cells after 24 hours of the expression, line 3 shows control sample, HeLa cells transfected by pEB-GFP (2347) plasmid without Cas9 sequence after 24 hours, line 4 shows the Cas9 protein level in the HeLa cells after 48 hours of the expression, line 5 shows our negative control sample, corresponding to non-transfected HeLa cells.

DISCUSSION

The pSpCas9-2A-GFP plasmid was successfully used to deliver the Cas9 protein into human cells. The observation of fluorescence under the ZOE Fluorescent Imager proved the success of the transfection step as well as the proper plasmid expression. The gene expression level and the quantity of protein synthesized were evaluated at different times post-transfection.

The total RNA content was extracted from the transfected cells either 24h or 48h post-transfection. The results obtained for the RNA analysis showed a poor RNA content along with a high DNA contamination, suggesting that the extraction did not work properly. The RNA extraction was performed twice year-on-year by different operators, and the same poor results were obtained. Various factors could explain this unexpectedly low RNA content. The extraction protocol followed is efficient for isolating RNA with a size superior to 200 bp. Both ribosomal 18S and 28S RNAs, as well as the Cas9 RNA, are largely above this limit. The presence of degraded RNA in the final extracts is unlikely as no smear was visible at the bottom of the migration gel. It therefore appears that simply no RNA was extracted. According to the different steps performed, the problem could come from the homogenization step which is supposed to induce the inactivation of RNases. An abnormal RNase activity due to an inefficient lysis buffer could result in RNA degradation in the extracts obtained. Once degraded, the resulting RNA fragments could possibly be too small to be retained in the silica membrane leading to their leakage in the flow-through. The abnormal presence of DNA could come from a contamination of the centrifuge tube with the remaining flow-through. We decided for the rest of our experiment to work on the RNA extracted by the group from the previous year as the amount of RNA obtained then was exploitable.

The Cas9 amplification results were consistent with our expectations. The presence of bands for Cas9 transfected cells confirms the presence of Cas9 RNA and therefore the Cas9 gene expression at every time post-transfection tested. It also validates both Cas9-specific primers. For the housekeeping genes, we can identify a band in every well for TBP. Our primers were validated for TBP only. After 30 cycles, we must be at saturation, yet the diffuse aspect of the bands disallows us to quantify for sure the expression of Cas9. This might be due to an inadequate gel texture, or to an old TAE, and could not

lead to a precise and relevant expression quantification. However, we were able to evaluate the relative expression of Cas9 over time. The expression did not seem to significantly differ between 48h and 72h post-transfection. Despite the absence of results for its expression 24h post-transfection, the results strongly suggest the long-lasting Cas9 expression until 72h in cells.

The Cas9 protein was detected by our Western Blot analysis. The presence of bands at the expected size of 160 kDa corresponding to the Cas9 protein (158,441 kDa) was confirmed by the protein extraction and the immunologic detection using Primary antibodies. Moreover, we obtained no band for our two controls (24 hours post-transfection with pEB-GFP (2347) plasmid, and HeLa cells without transfection. The two bands obtained at 24 hours and 48 hours post-transfection appeared globally similar, despite a slightly thicker band at 24h. This suggests a better protein synthesis after 24h, but duplicates have to be performed to confirm this hypothesis. Furthermore, in our Western Blot analysis we observed 2 bands in both 24 and 48 hours samples. We suggest that the higher band, around 200 kDa, illustrates a result of post-transcriptional attachment to the Cas9 protein. Above mentioned attachment could be a reason for higher molecular weight and in the consequence of the slower protein migration rate.

Altogether, those results confirm that Cas9 delivery through a recombinant plasmid can be efficiently used for Cas9 expression in mammalian cells. The protein appears equally expressed from 48h to 72h post-transfection, with a slightly lower synthesis at 24h. In the context of CRISPR-Cas9 use for gene editing, the nuclease is intended to perform its activity quickly and in a limited period of time in order to prevent OT mutations. The results obtained in the present study for the Cas9 expression pattern suggest that delivering Cas9 by a plasmid transfection in human cells, when used in combination with a guide RNA inserted into this pSpCas9-2A-GFP backbone, does not appear as an optimal method for this use. Indeed, this method allows an optimal Cas9 expression after 48h only and the long-lasting of the protein over time leading to potential off-target effects. If used prior to the parallel delivery of a guide RNA, this should be injected at 48h post-transfection, when the Cas9 expression appears maximal. However, further experiments should be driven in order to precise those assumptions and better evaluate the Cas9 expression pattern in comparison with other delivery strategies.

FUTURE EXPERIMENTS

All steps have been realized only once, so more experiments about the quantification of Cas9 protein should be done for the repeatability of our results. The transfection of HeLa cells with the pSpCas9-2A-GFP plasmid have to be performed again at 24, 48 and 72 hours to precisely quantify Cas9 expression at those times after plasmid transfection and try to assess any potential difference among time. It would also be interesting to measure the Cas9 expression on a wider time range (from 24h to 7 days for example) using a different type of cell lines. It could help to find the most

efficient time duration of the expression and the most optimal cell line for that kind of experiments. Here, the protein expression only was measured. Cas9 efficiency in genome editing should also be monitored over time in parallel, as its active state only raises potential off-target issues.

Moreover, the plasmid verification could be improved using other restriction enzymes.

The RNA extraction step was the most important difficulty encountered. Improvements should be done in the design of this experiment by adopting several strategies: avoid using a kit already used, working in all sterile necessary conditions, and eventually performing an additional DNase digestion after RNA purification.

Once those steps are optimized, we should compare the expression duration of Cas9 when delivered in a plasmid, and as the protein directly. This could be a great help in determining the optimal Cas9 delivery method for use in genetic engineering, depending on the editing and the cell type targeted.

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