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Allele-specific disruption of KRAS p.G12V in colorectal cancer cells using electroporated Cas9 RNPs

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ABSTRACT

Objective(s): Colorectal cancer (CRC) is a major cause of mortality, and KRAS mutations drive tumorigenesis and therapy resistance. Although CRISPR-Cas9 enables precise genome editing, intracellular delivery of Cas9 ribonucleoproteins (RNPs) remains challenging. We evaluated direct electroporation of Cas9-sgRNA RNPs targeting KRAS p.G12V in SW480 CRC cells to assess delivery efficiency, on-target editing, off-target activity, and viability effects.

Materials and Methods: An sgRNA targeting KRAS p.G12V was designed computationally. Cas9-sgRNA RNPs were assembled and validated by *in vitro* digestion. RNPs were electroporated into SW480 cells using a square-wave electroporation protocol; pmaxGFP served as the delivery control. On-target editing and five predicted off-target loci were assessed by Sanger sequencing and TIDE analysis. Cell viability was measured by the MTT assay.

Results: Electroporation resulted in high GFP expression (~97%). TIDE analysis at the KRAS locus estimated ~27% total indels. Cells treated with KRAS-targeting RNPs exhibited significantly reduced viability compared with controls ($P<0.05$). No detectable indels were observed at the five off-target sites. While supporting feasibility, deeper sequencing and mechanistic analyses are needed to confirm specificity.

Conclusion: Direct electroporation of Cas9-sgRNA RNPs enabled efficient intracellular delivery and targeted disruption in SW480 cells, reducing viability without detectable editing at selected off-target loci. This approach is practical for KRAS-targeted studies in CRC and warrants further investigation. Direct electroporation of Cas9–sgRNA RNPs enabled efficient delivery in SW480 cells, accompanied by reduced viability, with no detectable editing at selected off-target candidates by Sanger/TIDE. These findings support RNP electroporation as a practical approach for KRAS-targeted studies in CRC and justify follow-up with amplicon deep sequencing and additional cell models.

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Introduction

Colorectal cancer (CRC) is among the most common malignancies worldwide and a leading cause of cancer mortality, with progression driven by diverse genetic and epigenetic alterations (1). Contemporary molecular taxonomies describe four consensus molecular subtypes (CMS1–4) that reflect differences in microsatellite instability, canonical WNT/MYC signaling, metabolic features, and mesenchymal/TGF-β activity, underscoring the heterogeneity of CRC and the need for genotype-tailored strategies (2). *KRAS* mutations occur in roughly 40% of CRCs and are strongly associated with resistance to anti-EGFR therapies and poorer clinical outcomes (3). The *KRAS* proto-oncogene encodes a small GTPase in the RAS/MAPK pathway that functions as a molecular switch for proliferation and survival; recurrent substitutions at codons 12 and 13 (e.g., G12D, G12V, G13D) lock *KRAS* in an active state and drive oncogenic signaling (4). *KRAS* mutations cause the protein to be constantly active. This persistently active KRAS protein acts as a molecular switch, continuously stimulating downstream signaling pathways that promote cell proliferation and survival, ultimately driving cancer development (5). Direct pharmacological inhibition of *KRAS* has long been challenging due to its biochemical properties and high intracellular abundance. While allele-specific inhibitors have recently emerged for select variants, broad and durable targeting across *KRAS* genotypes in CRC remains an unmet need. Patients diagnosed with CRC and harboring KRAS mutations tend to have a less favorable prognosis than those with wild-type KRAS (3). Genome editing offers an orthogonal approach: CRISPR–Cas9 can introduce targeted double-strand breaks in mutant alleles to disrupt oncogenic signaling, with potential allele selectivity (6).

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Among CRISPR delivery formats, ribonucleoprotein (RNP) delivery—preformed Cas9 protein complexed with guide RNA—minimizes the duration of nuclease activity, avoids vector integration, and reduces off-target risk compared with plasmid or mRNA approaches (7, 8). A persistent bottleneck, however, is the efficient and cytocompatible intracellular delivery of large RNP complexes, particularly in difficult-to-transfect lines such as SW480 (KRAS p.G12V) (9). Electroporation is a straightforward, nonviral method that can achieve high uptake of proteins and nucleic acids when parameters are optimized for a given cell type (10).

Here, we designed a single-guide RNA (sgRNA) targeting the *KRAS* p.G12V allele, validated the RNP complex *in vitro*, and delivered Cas9–sgRNA RNPs to SW480 cells by square-wave electroporation. We quantified on-target editing by Sanger sequencing with TIDE analysis, evaluated predicted off-target loci by Sanger-based decomposition /TIDE, and assessed the biological impact on cell viability using MTT assays. This work provides a practical, nonviral workflow for allele-directed *KRAS* editing in CRC cells and establishes a foundation for follow-up studies using deep sequencing and expanded functional readouts.

Thus, we aimed to establish a reproducible, allele-directed workflow for *KRAS* p.G12V disruption using RNP electroporation in CRC cells, addressing the gap between delivery efficiency and allele-specific editing.

Materials and Methods

General reagents

RPMI was purchased from Gibco (Thermo Fisher, USA). Fetal bovine serum (FBS) (Biosera, France). IVT (*in vitro* transcription) kit (New England Biolabs, ref: E2040S). 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Munich, Germany). -Green viewer and DNA ladder 1 kb were purchased from Cinnagen (Tehran, Iran). Cas9 protein New England Biolab (EnGen Cas9 NLS, S. pyogenes ref: M0646T).

Cell line and cell culture

The SW480 cell line (with KRAS G12V mutation) was purchased from the Pasteur Institute of Iran an cultured in Roswell Park Memorial Institute Medium (RPMI) (Gibco, Thermo Fisher, USA). The cell culture media contain 10% fetal bovine serum (FBS, Biosera, France), penicillin (100 µg/ml), and streptomycin (100 µg/ml) at 37 °C under 5% CO_2. The KRAS p.G12V mutation in the SW480 cell line was analyzed and confirmed using the IntelPlex PCR method, as previously described in the literature (11).

sgRNA Design for targeting mutant KRAS

KRAS gene sequences were obtained from the NCBI database. sgRNAs targeting the mutant *KRAS* allele c.35 G>T (p.G12V), but not wild type *KRAS*, were designed using CRISPOR (http://crispor.tefor.net/) and CHOPCHOP (https://chopchop.cbu.uib.no/).

Designing vector construction

The DR274 vector-containing bacterial stab was streaked onto LB plates containing 50 µg/ml kanamycin and incubated at 37 °C overnight. Several isolated colonies were selected and cultured overnight at 37 °C while being shaken at 250 rpm in 6 ml of LB liquid medium containing 50 µg/ml Kanamycin. With the use of the Favorgen Plasmid Extraction Mini Kit (FAPDE050), plasmid DNA was extracted from the bacterial pellet. DNA concentrations were quantified using a NanoDrop spectrophotometer to prepare the constructs for subsequent procedures. The DR274 vector was linearized using Eco31I (BsaI) enzyme (10 U/µl, Thermo Fisher Scientific) and analyzed on a 1% agarose gel. A gel clean-up kit was used to trim and purify the digested band. The protocol was followed in the hybridization of each sgRNA's sense and antisense sequences. Briefly, each sgRNA was prepared at a final concentration of 100 µM, and the sense and antisense oligos were mixed at a 1:1 volume ratio. The mixture was incubated at 95 °C for 5 min in a water bath, then allowed to cool at room temperature for 1 hr to complete annealing. Then, the resulting sgRNAs were cloned into the digested plasmid using the T4 enzyme. For the ligation reaction, a total volume of 10 µl was prepared by combining 1 µl of T4 DNA ligase (5 U/µl), 1 µl of 10× ligation buffer, 1 µl of the digested DR274 plasmid, and 7 µl of the annealed sgRNA. The reaction mixture was incubated at room temperature for 2 hr, then stored at 16 °C overnight. The recombinant DR274 plasmid was transformed into *E. Coli*, XL-1 Blue competent cells were plated on an LB agar plate containing 50 µg/ml kanamycin and incubated overnight at 37 °C. Ten colonies were inoculated in LB liquid medium containing 50 µg/ml kanamycin and were grown overnight at 37 °C with shaking at 250 rpm. Plasmid DNA was isolated from the bacterial pellet using the Favorgen Plasmid Extraction Mini Kit (12).

In vitro transcription of sgRNA using customized DR274

As directed by the manufacturer, sgRNAs were generated via *in vitro* transcription using the HiScribe T7 High Yield Kit (New England Biolabs, ref: E2040S). In summary, the DraI restriction enzyme broke down recombinant DR274. The reaction mixture contained 40 units of DraI enzyme, 1× DraI digestion buffer, and 20 µg of recombinant DR274, with a total volume of 50 µl. The reaction was carried out for 5 hr at 37 °C. A PCR cleanup kit was used to purify the digestion products. In a reaction mixture containing 1 µg of digested DR274, 1X reaction buffer, 5 mM final concentration of each NTP (ATP, GTP, CTP, and UTP), 1 µl RNase inhibitor, 4.5 µl $MgCl_2$ (11.25 mM), 2 µl T7 RNA Polymerase Mix reaction, and up to 20 µl RNase-free water, RNA transcription was carried out. The mixture was then incubated at 37 °C for four hours. To eliminate template DNA, a mixture of 70 µl nuclease-free water, 10 µl 10X DNase I Buffer, and 2 µl RNase-free DNase I was prepared and incubated for 30 min at 37 °C. The EZ-10 Spin Column RNA Cleanup & Concentration Kit was used to purify sgRNA.

In vitro evaluation of the (ribonucleoprotein) RNP complex

The Cas9 protein (EnGen Cas9 NLS, S. pyogenes ref: M0646T) was obtained from New England Biolabs. Cas9 protein and sgRNA were combined in a 3:1 and 2:1 ratio (5.89pmol:38.4pmol) and (3.72pmol:38.4pmol), respectively, to create RNP complexes. The mixture was then incubated for two hours at 37 °C in a 10 µl reaction volume using a 5X complex assembly buffer (50 mM Tris–HCl, pH 7.5 at 37 °C, 500 mM NaCl, 5 mM EDTA, and 5 mM DTT). Target-sequence amplification primers (Table 1) were used to perform PCR on DNA from wild-type and mutant *KRAS* (G12V). Conditions and materials for the PCR were as

Table 1. Sequences and location of sgRNAs in order to evaluate their on-target efficiency and potential off-target effects

sgRNA	Position	Strand	Sequence (5'→3')	PAM	On-target score	Specificity score	Off-target score
sgRNA1	5591	-	GTAGTTGGAGCTGTTGGCGT	AGG	63.3	61.5	12

follows: DW: up to 20 µl; 95°: 5 min, 95°: 30 s, 59°: 30 s, 72°: 1 min, 35 cycle, and 72°: 5 min; master mix amplicon 2X: 10 µl; forward primer: 10 pmol; reverse primer: 10 pmol; DNA: 50 ng; One microgram of PCR products were treated by Cas9-guide RNA(RNP) complex in 5X reaction buffer (50 mMTris–HCl pH 7.5 at 37 °C, 500 mM NaCl, 50 mM MgCl2,5 mM DTT) for 2-3 hr at 37 °C in a 100 µl reaction volume. Digestion was purified using a PCR cleanup kit and evaluated by 3% agarose gel electrophoresis (13).

RNP electroporation protocol

Sw480 cells were electroporated according to the manufacturer's instructions using an ECM 830 Square Wave Electroporation System (BTX, Holliston, MA, USA). In brief, 2×10^5 SW480 cells were washed once with Ca2+/Mg^{2+}-free PBS and resuspended in 40 µl of BTXpress electroporation solution. Then, cells were mixed with 50 pmol/µl of Cas9 protein (Spy Cas9 NLS NEB), pre-coupled with sgRNA at a 3:1 molar ratio, transferred into a 0.2-cm cuvette, and electroporated at 250 V for 5 ms. After electroporation, the cells were incubated in culture medium for 3 days for Sanger sequencing analysis and also seeded in a 96-well plate for the MTT assay. We also used 1 µg of pmaxGFP plasmid (Lonza, Basel, Switzerland) to assess electroporation efficiency in the SW480 cell line (12).

MTT assay

Cells in three groups: control (not electroporated), electroporated, and RNP electroporated, were seeded in 96 culture plates at a density of 1 × 10^4 cells per well. MTT assays were performed 24, 48, and 72 hr after cell plating (n=3 wells per condition). The MTT (3-(q 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well at a proportion of 1/10, after which plates were incubated for 4 hr at 37 °C. The medium was then removed, and 180 µl DMSO was added to each well. The absorbance at 490 nm was measured by a microplate reader. The mean cell proliferation was calculated from the absorbance units.

Off-target evaluation

To predict possible off-target locations based on sequence similarity and other factors, we performed an *in silico* genome analysis to assess the site specificity of sgRNAs using Cas-OFFinder, followed by CRISPOR, GuideScan, and e.g. We could not detect off-target sites with either none or single and double exonic mismatches and DNA bulge size = 0. However, introducing three random mismatches revealed two potential off-targets. When four random mismatches were introduced, we identified 22 potential off-targets. We focused on five off-targets among these candidates, based on their potential relevance to the KRAS gene location and function (14).

Results

sgRNA designing evaluation

The G12V *KRAS* mutation is the target of the intended sgRNA (Figure 1). The thermodynamic properties of the sgRNA were assessed using the OligoAnalyzer online tool, and the NCBI BLAST tool was used to reconfirm its sequence specificity. sgRNA was evaluated with the online tool GT-scan (https://gt-scan.csiro.au/gt- scan), Cas-OFFinder (http://www.rgenome.net/cas-offinder/), and CRISPOR (http://crispor.gi.ucsc.edu/) to determine potential off-target cleavage sites. A sgRNA exhibiting the highest efficiency and minimal off-target cleavage sites was selected. sgRNA-encoding oligonucleotides were chemically synthesized. Desired sgRNA sequence and its counterpart primers are listed in Tables 1 and 2.

Vector production for sgRNA transcription and validation

To produce guide RNA (gRNA), the T7 promoter was employed for *in vitro* transcription using the DR274 (plasmid #42250)-Addgene (http://www.addgene.org/crispr/ jounglab/) guide RNA expression vector (Figure 2A). After sgRNA cloning, PCR was performed on all minipreps using M13 forward and reverse primers (Figure 2B). Recombinant DR274 plasmids were approved using Sanger sequencing (Figure 2C). DR274 was a gift from Majid Mojarrad (Addgene plasmid # 42250; http://n2t.net/addgene:42250; RRID: Addgene_42250). They were sent for sequencing and approved.

In vitro transcription of sgRNA

After sgRNA production, the concentration was determined using a Nanodrop 2000 Spectrophotometer. The concentration was determined using a Nanodrop 2000

Table 2. Sequences of primers for mutant KRAS (p.G12V)

Primer name	Sequence (5'→3')
KRAS sgRNA *BsaI* T7 sense	GTAGTTGGAGCTGTTGGCGT
KRAS sgRNA *BsaI* T7 antisense	ACGCCAACAGCTCCAACTAC
M13F	TGTAAAACGACGGCCAGT
M13R	CAGGAAACAGCTATGAC

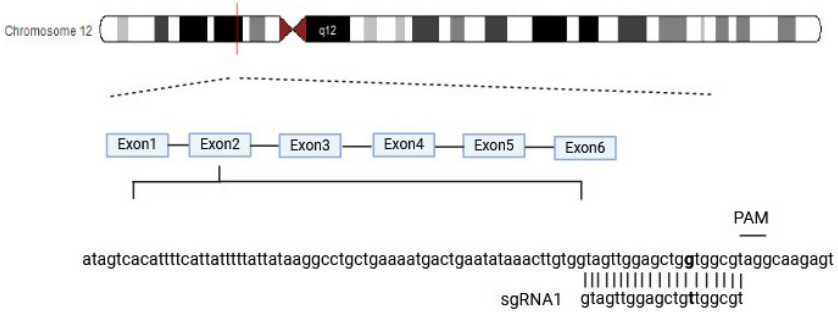

Figure 1. KRAS sequence and desired sgRNA for targeting G12V

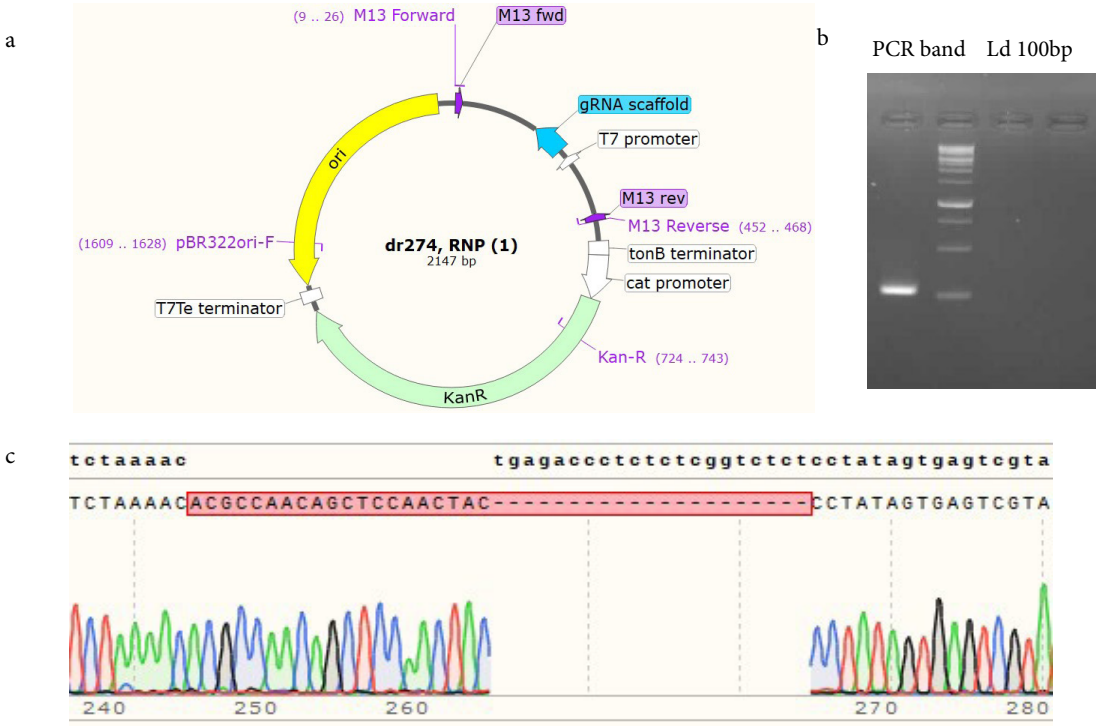

Figure 2. a) DR274 Plasmid, guide RNA (gRNA) expression vector used to create a gRNA to a specific sequence; uses T7 promoter for *in vitro* transcription. Vector backbone: sgRNA-Expression Vector. Backbone size (bp): 2147, Vector type: Worm Expression, CRISPR; sgRNA expression. 5′ sequencing primer: M13 forward. Bacterial Resistance(s): Kanamycin, 50 µg/ml. Growth Strain(s): XL-1 Blue. b) PCR of the miniprep with anti sgRNA primer and M13. c) Sanger sequencing and alignment of PCR products of inserted sgRNA in DR274 for one clone of sgRNA G12V (Snap Gene software)

Spectrophotometer, yielding an average concentration of 102 ng/µl.To verify the integrity of the RNA, 150–300 ng of it was diluted in RNase-free water, stored at 70 ℃ for 10 min, and then transferred to ice. It was then run on a 3% agarose gel and subjected to electrophoresis at 100 V (Figure 3).

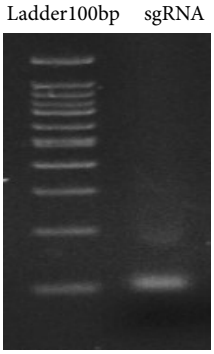

Figure 3. After diluting and heating 200 ng of RNA, the integrity of the produced RNA was checked using a 3% agarose gel. sgRNA is roughly 103 bp. Ladder size: 100 bp, band size: 103 bp

Efficiency of in vitro assessment of the RNP

Since we produced the RNP complex using two ratios of Cas9 and sgRNA, we deduced that all of the *KRAS* template was digested in a 3:1 ratio (complete cleavage of mutant vs. partial/no cleavage of WT) (Figure 4a). At the next stage, we use this ratio to evaluate how well our sgRNA digests HEK293 as wild-type *KRAS* (Figure 4b). We performed PCR for the KRAS gene using DNA extracted from the SW480 and HEK293 cell lines.

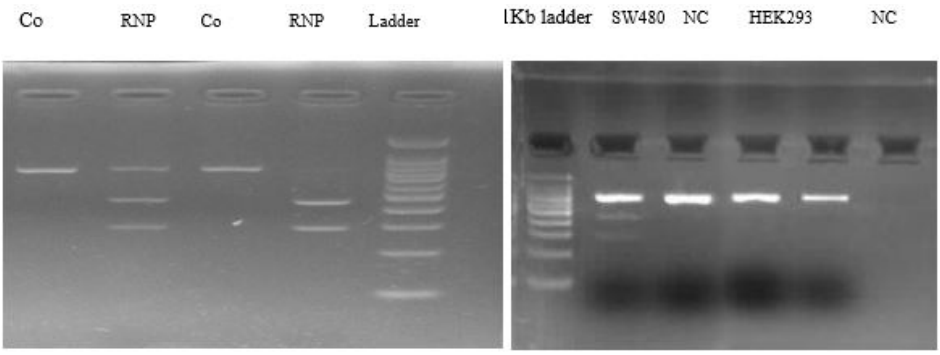

Figure 4. a) Optimization of RNP complex values. From right to left, ladder 1kb first well. Cas9, sgRNA (3:1 ratio), negative control (2:1 ratio). b) *In vitro* evaluation of the nuclease activity of the sgRNA-Cas9 ribonucleoprotein (RNP) complex was conducted using PCR-amplified wild-type DNA fragments. G12V KRAS DNA from the mutated cell lines (SW480) and the HEK293 cell line for wild-type KRAS. digested with the RNP complex.

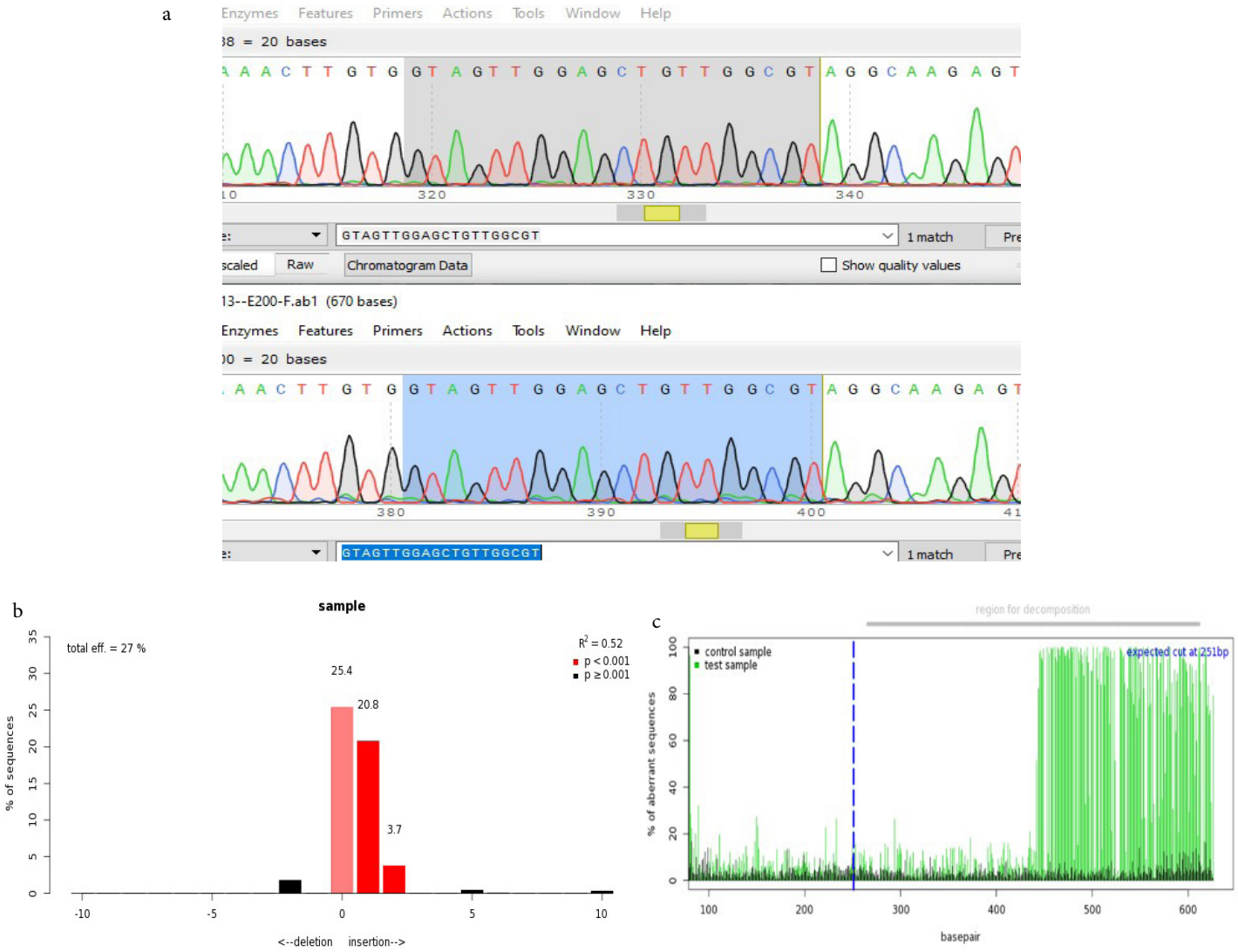

Figure 5. a) Sanger sequencing results of the SW480 cell line are shown, with the control sample displayed above and the electroporated sample below. b) Indel spectrum plot: This graph illustrates the combination of predicted trace models (indels) that best account for the observed sequence pattern in the experimental sample, based on non-negative linear modeling. The R^2 value reflects the goodness of fit, while the statistical significance of each detected indel is also calculated. According to the results, 25.4% of alleles remain unmodified. Meanwhile, 24.5% of the sequences exhibit deletions with significant R^2 values, indicating an overall editing efficiency of approximately 27%. c) For quality assessment, the altered sequence signals are shown for both the control (black) and electroporated (green) samples, along with the predicted cleavage site marked by a vertical dotted line

Detecting mutations in electroporated SW480 cell line with CRISPR/Cas9 RNP

Sanger sequencing was used to identify alterations in the CRISPR/Cas9 (RNP)-electroporated cell line. After 48–72 hr of detection of induced mutations in the electroporated cell line, DNA was extracted from the control and the RNP-electroporated cell line, and PCR was performed targeting KRAS. PCR products were used for Sanger sequencing (Figure 5A). The spectrum and frequency of the targeted mutation were ascertained using the TIDE online software.

This software provides a simple and accurate method for precisely detecting the range and frequency of targeted mutations introduced in a cell population by genome-editing technologies such as CRISPR/Cas9, Transcription Activator-Like Effector Nucleases (TALENs), and Zinc Finger Nucleases (ZFNs) (15). Also, we electroporated the pmaxGFP plasmid for positive control of our electroporation method and realized GFP was expressed in more than 97% in the cell line. These evaluations were prepared with fluorescence microscopy and flow cytometry (Figure S1).

Off-target results

TIDE (Tracking of Indels by Decomposition) analysis revealed unwanted cleavage at the predicted off-target sites (Figure 6). As illustrated, there is no off-target at the desired genes. List of primers for off-target evaluation (Figure S2).

Effects of mutant KRAS genome knock out on cell viability

We next determined whether RNP-induced disruption of mutant KRAS induced cancer cell death. First, we assessed the safety of electroporation for cell growth, and subsequently, the effect of RNP (Figure 7).

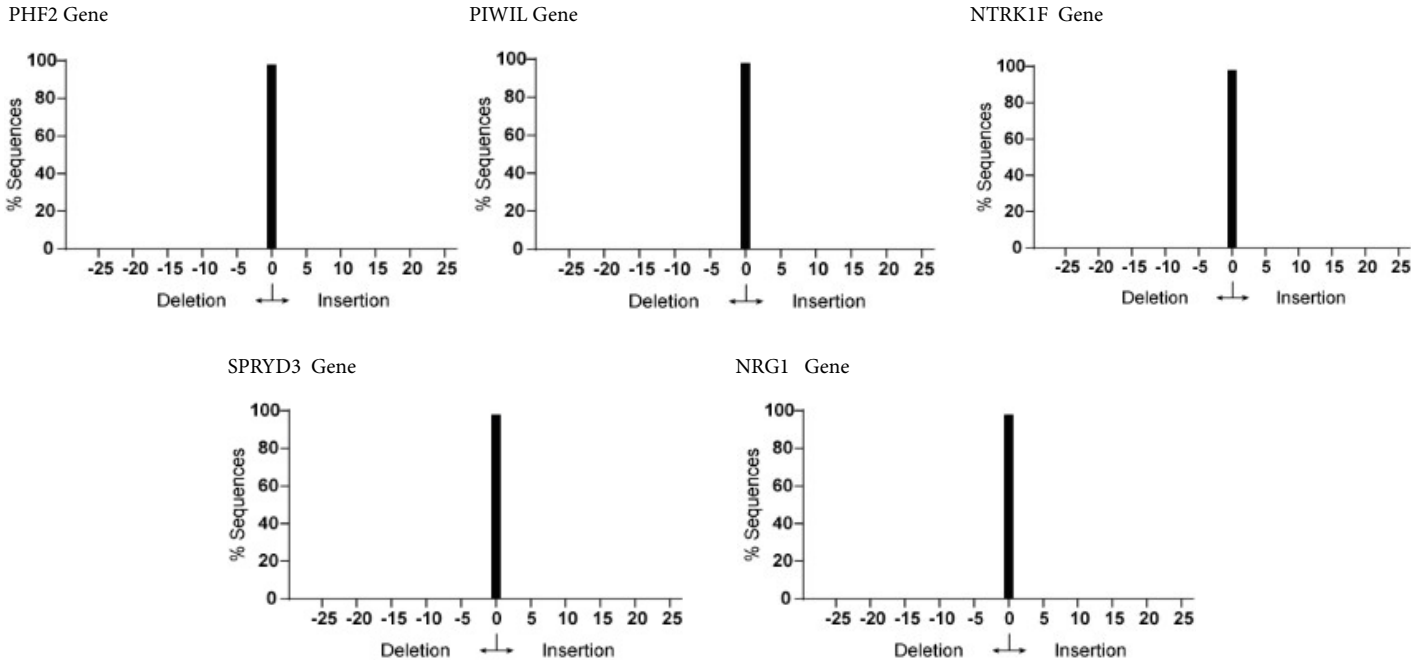

Figure 6. Graphic representation of five predicted off-target sites, evaluated by TIDE (Tracking of indels by decomposition)

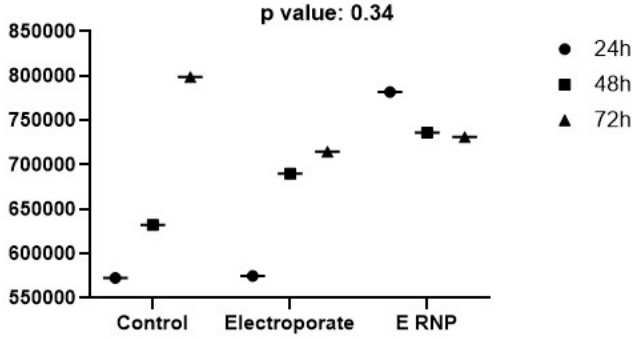

Figure 7. Cell viability of SW-480 cells after electroporation with Cas9-sgRNA ribonucleoprotein (RNP) complexes (*P<0.05, Student's t-test, compared with control (not electroporated) and just electroporated (without RNP)). Data are presented as mean±SD

Discussion

Current CRC therapies involving anti-EGFR drugs are significantly limited by their ineffectiveness in patients harboring *KRAS* point mutations. These mutations enable tumor cells to maintain uncontrolled proliferation despite EGFR inhibition(16). To address the resistance of *KRAS*-mutant CRC to anti-EGFR monoclonal antibody therapy, we introduced a well-designed sgRNA that achieves highly effective knockout of mutant KRAS using the CRISPR/Cas9 system. This allele-specific strategy leverages the single-nucleotide difference in the G12V mutation to preferentially disrupt oncogenic signaling while potentially sparing wild-type function, thereby addressing a key challenge in targeting "undruggable" oncogenes such as *KRAS* (17). In this study, we evaluated a nonviral workflow to disrupt the KRAS p.G12V oncogenic allele in CRC cells via direct electroporation of Cas9–sgRNA RNPs. We demonstrate (i) efficient physical delivery, evidenced by ~97% GFP-positive cells after control electroporation, (ii) on-target genome editing at KRAS of 27% as quantified by Sanger/TIDE decomposition, and (iii) a significant reduction in SW480 cell viability relative to mock and non-targeting controls. Screening of top in silico off-target candidates by Sanger/TIDE did not detect indels above the assay's practical sensitivity, suggesting limited off-target activity under our conditions.

Our findings align with recent studies demonstrating the feasibility of CRISPR-based *KRAS* disruption in CRC models, such as those using high-fidelity Cas9 variants to target G12D and G12C alleles, which similarly reported editing efficiencies of 20-40% sufficient to impair tumor growth in preclinical settings (18). However, unlike viral delivery approaches that achieve higher yields but raise integration concerns, our nonviral RNP method prioritizes transient activity, consistent with reports showing reduced off-target effects in cancer cells (19). Discrepancies in editing outcomes across *KRAS* variants highlight mutation-specific dependencies; for instance, G12V-driven CRC exhibits a unique reliance on metabolic enzymes such as ACSS2 for acetyl-CoA synthesis, potentially amplifying the impact of the partial disruption observed here (20).

High GFP delivery but lower editing at the genomic target highlights that intracellular uptake is not the only determinant of editing yield. Post-delivery constraints—such as RNP nuclear entry kinetics, chromatin accessibility at *KRAS* exon 2, repair pathway activity, and the transient lifetime of RNPs—likely limit maximal editing in these cells(21). Although the observed ~27–30% indel rate is moderate when compared to lentiviral-based continuous expression systems, it is highly consistent with foundational studies utilizing transient Cas9 Ribonucleoprotein (RNP) electroporation without selection enrichment, which typically report indel frequencies between 20% and 40% in mammalian cells (22, 23)

The observed editing range is nevertheless consistent with "hit-and-run" RNP editing and sufficient to elicit a measurable biological phenotype in a population context. Alternative explanations for this disparity could include cell cycle-dependent editing biases in SW480 cells, where NHEJ predominates in G1/S phases, or incomplete RNP disassembly post-electroporation, as noted in recent

nonviral delivery optimizations (24). Importantly, this level of editing proved functionally potent in our study. The significant reduction in overall cell viability at this editing threshold can be attributed to 'oncogene addiction'. SW480 cells are heavily dependent on the mutant *KRAS* pathway for survival; thus, an acute disruption of this critical driver gene in approximately one-third of the population is sufficient to effectively compromise overall tumor cell viability and unmask the essentiality of *KRAS* in this CRC model.

The decrease in viability after *KRAS* targeting is consistent with the central role of mutant *KRAS* in promoting proliferation and survival signaling. Because electroporation alone and non-targeting RNPs did not significantly change viability, the effect is unlikely to be an artifact of the delivery procedure or nonspecific RNP exposure. While these data support a *KRAS*-dependent vulnerability in SW480, mechanistic confirmation will benefit from orthogonal readouts (e.g., reduced p-ERK and downstream transcriptional signatures) and long-term growth measures (e.g., colony formation), which we outline below as next steps.

Beyond immediate viability effects, these results have broader implications for precision oncology in CRC, where *KRAS* mutations correlate with consensus molecular subtypes CMS2/3 and metabolic reprogramming. By enabling ex vivo modeling in patient-derived organoids, our workflow could facilitate screening for synthetic lethals, such as combining *KRAS* disruption with MEK or ACSS2 inhibitors to exploit G12V-specific vulnerabilities and overcome resistance (2, 20). Clinically, scalable nonviral platforms like this may support adoptive therapies or combination regimens, bridging gaps left by allele-specific inhibitors limited to G12C variants.

Our Sanger/TIDE screen of predicted off-targets did not reveal detectable indels above the method's ~2–5% sensitivity, supporting a favorable on-target specificity profile in this setting. Nonetheless, Sanger-based decomposition cannot rule out low-frequency events or unexpected off-targets. Amplicon deep sequencing (CRISPResso2 analysis) of the *KRAS* locus and top off-targets will refine the on-target fraction and indel spectra, enable allele-level quantification, and more rigorously define detection limits (24). Adoption of high-fidelity Cas9 variants and truncated or chemically modified guides could further reduce off-target risk in future iterations. Delivering pre-assembled RNPs avoids vector integration and limits nuclease exposure, which is advantageous for perturbation studies and translational workflows. Electroporation offers reproducible, scalable delivery across adherent cell lines when pulse programs and buffers are tuned, and our parameters yielded robust uptake in SW480 (25). The same framework is readily extensible to (i) additional *KRAS* codons (e.g., G12D, G13D) and (ii) multiplexed perturbations (e.g., *KRAS* + pathway nodes) to probe synthetic vulnerabilities.

Limitations: First, conclusions are based on a single *KRAS*-mutant line; generalizability should be evaluated in additional CRC models with distinct *KRAS* genotypes and, ideally, a KRAS-wild-type comparator to establish the specificity of the viability effect. This single-model approach may overlook subtype-specific responses, such as those in CMS4 tumors with stromal infiltration, thereby potentially overestimating its broad applicability. Second, editing was quantified primarily by Sanger/TIDE; although suitable for rapid estimation, it lacks the resolution of amplicon NGS for indel spectra and allele-level effects. Third, we did not include direct pathway or apoptosis markers (e.g., **p**-ERK, cleaved caspase-3, Annexin V) that would mechanistically link *KRAS* disruption to the observed phenotype. Finally, the allelic status of KRAS in the experimental cells should be confirmed by sequencing to determine whether editing preferentially disrupts the mutant allele or affects both alleles equally.

Future directions: We plan to (i) quantify on-target editing by amplicon NGS and report precise indel distributions; (ii) assess off-target activity by targeted deep sequencing of top candidates and, if warranted, unbiased detection; (iii) incorporate pathway readouts (p-ERK/ERK, p-AKT/AKT) and apoptosis/long-term growth assays (Annexin V, colony formation) to provide definitive mechanistic validation for our preliminary phenotypic observations; (iv) expand to additional *KRAS*-mutant and *KRAS*-wild-type CRC lines to benchmark genotype-specific responses; and (v) explore parameter optimization (RNP stoichiometry, pulse width/number, buffer systems) and high-fidelity Cas9 or guide chemistries to further increase on-target yield and specificity and to investigate the functional threshold of editing required to induce robust cell death in oncogene-addicted models. Additionally, integrating tumor-specific nanoparticles for RNP delivery could enhance *in vivo* translation, building on recent advances in nonviral biologics for cancer editing (19).

Conclusion

Direct electroporation of Cas9–sgRNA RNPs provides a practical, nonviral route to perturb *KRAS* in CRC cells. Despite modest editing fractions at the population level, targeted disruption of *KRAS* p.G12V was sufficient to reduce SW480 viability, supporting the functional relevance of the edit. With deeper sequencing, expanded model coverage, and mechanistic validation, this workflow can serve as a reproducible platform for allele-directed studies of *KRAS* dependence in CRC and for preclinical evaluation of combination strategies.

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Ethics Statement

The study followed the Declaration of Helsinki principles and was approved by the Medical Ethics Committee of Azad University of Shahrekord (registration number: IR.IAU.SHK.REC.1403.281).

Data Availability

The datasets generated and analyzed in the current study are not publicly available but can be obtained from the corresponding author upon request.

Authors' Contributions

M A and S SH were responsible for conception and

design; F M handled data collection and analysis; S SH and M P interpreted the data; M L also contributed to data interpretation; M S and F M were involved in drafting and revising the article.

Conflicts of Interest

The authors declare no competing interests.

Declaration

We acknowledge the use of QuillBot AI solely to improve English and correct the grammar in the introduction section. No text or content was generated by this artificial intelligence tool.

References

1. Houseini ST, Nemati F, Sattari A, Azadeh M, BishehKolaei R. Design of crRNA to regulate microRNAs related to metastasis in colorectal cancer using CRISPR-C2c2 (Cas13a) technique. Cell J 2023; 25:354-362.
2. Meng H, Nan M, Li Y, Ding Y, Yin Y, Zhang M. Application of CRISPR-Cas9 gene editing technology in basic research, diagnosis and treatment of colon cancer. Front Endocrinol 2023; 14: 1148412.
3. Zhu G, Pei L, Xia H, Tang Q, Bi F. Role of oncogenic KRAS in the prognosis, diagnosis and treatment of colorectal cancer. Mol Cancer 2021; 20:143.
4. Timar J, Kashofer K. Molecular epidemiology and diagnostics of KRAS mutations in human cancer. Cancer Metastasis Rev 2020; 39:1029-1038.
5. Meng M, Zhong K, Jiang T, Liu Z, Kwan HY, Su T. The current understanding on the impact of KRAS on colorectal cancer. Biomed Pharmacother 2021; 140:111717.
6. Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. Science 2014; 346:1258096.
7. Hille F, Richter H, Wong SP, Bratovič M, Ressel S, Charpentier E. The biology of CRISPR-Cas: Backward and forward. Cell 2018; 172:1239-1259.
8. Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, *et al.* Evolution and classification of the CRISPR–Cas systems. Nat Rev Microbiol 2011; 9:467-477.
9. Ju E, Li T, Ramos da Silva S, Gao S-J. Gold nanocluster-mediated efficient delivery of Cas9 protein through pH-induced assembly-disassembly for inactivation of virus oncogenes. ACS Appl Mater Interfaces 2019; 11:34717-34724.
10. Wiesinger M, März J, Kummer M, Schuler G, Dörrie J, Schuler-Thurner B, *et al.* Clinical-scale production of CAR-T cells for the treatment of melanoma patients by mRNA transfection of a CSPG4-Specific CAR under full GMP compliance. Cancers 2019; 11:1198.
11. Nouri M, Sharif S, Soltani E, Mozaffari-Jovin S, Abbaszadegan MR. Performance of KRAS mutation detection in plasma exosomes from patients with early-stage colorectal cancer. J Transl Med 2025; 23:730.
12. Mirzaei F, Eslahi A, Karimi S, Alizadeh F, Salmaninejad A, Rezaei M, *et al.* Generation of zebrafish models of human retinitis pigmentosa diseases using CRISPR/Cas9-mediated gene editing system. Mol Biotechnol 2024; 66:2909-2919.
13. Li C, Psatha N, Gil S, Wang H, Papayannopoulou T, Lieber A. HDAd5/35++ adenovirus vector expressing anti-CRISPR peptides decreases CRISPR/Cas9 toxicity in human hematopoietic stem cells. Mol Ther Methods Clin Dev 2018; 9:390-401.
14. Cattaneo C, Enzo E, De Rosa L, Sercia L, Consiglio F, Forcato M, *et al.* Allele-specific CRISPR-Cas9 editing of dominant epidermolysis bullosa simplex in human epidermal stem cells. Mol Ther 2024; 32:372-383.
15. Brinkman EK, Kousholt AN, Harmsen T, Leemans C, Chen T, Jonkers J, *et al.* Easy quantification of template-directed CRISPR/Cas9 editing. Nucleic Acids Res 2018; 46:e58-e58.
16. Ryu J-Y, Choi YJ, Won E-J, Hui E, Kim H-S, Cho Y-S, *et al.* Gene editing particle system as a therapeutic approach for drug-resistant colorectal cancer. Nano Res 2020; 13:1576-1585.
17. Hu Y, Liu L, Jiang Q, Fang W, Chen Y, Hong Y, *et al.* CRISPR/Cas9: A powerful tool in colorectal cancer research. J Exp Clin Cancer Res 2023; 42:308.
18. Álvarez-Pérez JC, Sanjuán-Hidalgo J, Arenas AM, Hernández-Navas I, Benitez-Cantos MS, Andrades A, *et al.* High-fidelity Cas9-mediated targeting of KRAS driver mutations restrains lung cancer in preclinical models. Nat Commun 2025; 16:7080.
19. Tompkins J, Ray RM, Scott TA. Toward an age of CRISPR delivery with non-viral biologics. Mol Ther Nucleic Acids 2026; 37.
20. Budagyan K, Cannon AC, Chatoff A, Benton D, Kurimchak AM, Araiza-Olivera D, *et al.* KRAS G12V mutation-selective requirement for ACSS2 in colorectal adenoma formation. Cell Rep 2025; 44:115444
21. Javaid N, Choi S. CRISPR/Cas system and factors affecting its precision and efficiency. Front Cell Dev Biol 2021; 9:761709.
22. Kim S, Kim D, Cho SW, Kim J, Kim JS. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome Res 2014;24:1012-1019.
23. Liang X, Potter J, Kumar Sh, Zou Y, Quintanilla R, Sridharan M, *et al.* Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. J Biotechnol 2015:208:44-53.
24. Assa G, Kalter N, Rosenberg M, Beck A, Markovich O, Gontmakher T, *et al.* Quantifying allele-specific CRISPR editing activity with CRISPECTOR2. 0. Nucleic Acids Res 2024; 52:e78-e78.
25. Karp H, Zoltek M, Wasko K, Vazquez AL, Brim J, Ngo W, *et al.* Packaged delivery of CRISPR–Cas9 ribonucleoproteins accelerates genome editing. Nucleic Acids Res 2025; 53:gkaf105.