Manual

CRISPRcraft S.p. Cas9 Nuclease
CRISPRcraft S.p. Cas9 Control Kit

For Research Use Only. Not for use in diagnostic procedures.

IMPORTANT
-20 ºC storage required
immediately upon receipt

BIOSEARCH™ TECHNOLOGIES
GENOMIC ANALYSIS BY LGC

Manual

CRISPRcraft S.p. Cas9 Nuclease

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2. Technical support

LGC, Biosearch Technologies™ is dedicated to the success and satisfaction of our customers. Our products are tested to assure they perform as specified when used according to our recommendations. It is imperative that the reagents supplied by the user are of the highest quality. Please follow the instructions carefully and contact our technical service representatives if additional information is necessary. We encourage you to contact us with your comments regarding the performance of our products in your applications. Thank you.
Email: techsupport@lgcgroup.com
Phone: +1 608 831 9011

Product guarantee: Biosearch Technologies guarantees that CRISPRcraft™ S.p. Cas9 Nuclease will perform as specified for 6 months from the date of shipment, and the CRISPRcraft S.p. Cas9 Nuclease Control Kit will perform as specified for 1 year from the date of shipment.

3. Product description and kit contents

CRISPRcraft S.p. Cas9 Nuclease contains one C-terminal nuclear localisation signal (NLS) sequence and one C-terminal 6xHis tag and is provided as a 10 mg/mL (62 µM) solution or a 3.2 mg/mL (20 µM) solution.

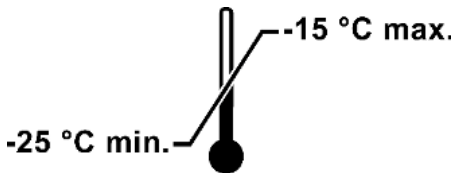
Product description	Size	Catalog number
CRISPRcraft S.p. Cas9 Nuclease	40 µg, 3.2 mg/mL (20 µM)	70020-0
	400 µg, 10 mg/mL (62 µM)	70020-2

CRISPRcraft S.p. Cas9 Nuclease Control Kit contains positive controls for testing enzyme activity *in vivo* and *in vitro*. The control kit is sold separately from CRISPRcraft S.p. Cas9 Nuclease.

Product description	Component	Part number	Size
CRISPRcraft S.p. Cas9 Nuclease Control Kit, Catalog # 70030-1	10X RGEN Buffer	F883960-1	1 mL
	Cas9 Control HPRT Guide crRNA	F824344-1	10 µL at 100 µM
	Cas9 Universal tracrRNA	F824347-1	10 µL at 100 µM
	Control HPRT Substrate DNA	F824045-1	30 µL at 35.5 ng/µL (20 nM)
	Human HPRT Forward PCR Primer	F814345-1	10 µL at 100 µM
	Human HPRT Reverse PCR Primer	F814348-1	10 µL at 100 µM

4. Storage conditions

Store all kits and components at -20 °C



5. Quality control

CRISPRcraft S.p. Cas9 Nuclease is tested to be free of detectable DNA, as well as RNase, exonuclease and nonspecific endonuclease activities. The protein is >95% pure by SDS-PAGE and endotoxin levels are less than 10 EU/mg by LAL assay.

CRISPRcraft S.p. Cas9 Nuclease and components of the CRISPRcraft S.p. Cas9 Nuclease Control Kit are tested in a 30 µL *in vitro* cleavage assay in which enzyme activity at the following specification is verified: 80% of 2 nM Control HPRT Substrate DNA is digested after a 10 minute incubation at 37 °C in 1X RGEN Buffer with 20 nM CRISPRcraft S.p. Cas9 Nuclease and equimolar Cas9 Control HPRT Guide crRNA:Cas9 Universal tracrRNA duplex.

6. Introduction

Cas9 nuclease from *Streptococcus pyogenes* is an RNA-guided endonuclease (RGEN) belonging to the Class 2 CRISPR/Cas family. An RGEN performs targeted DNA cleavage, creating a double stranded break at a specific locus by forming a ribonucleoprotein (RNP) complex with a small guide RNA (gRNA) that directs the RGEN to the desired locus. Natural Cas9 gRNAs consist of two parts: a CRISPR RNA (crRNA), and a transactivating CRISPR RNA (tracrRNA)[1] (see **Designing Cas9 Guide RNA Sequences**). Delivery of RNP complexes for *in vivo* editing is efficiently performed via lipid-based transfection, electroporation or microinjection.

Cas9 binding and cleavage requires an NGG protospacer adjacent motif site (PAM site) immediately 3' to the target sequence within the target DNA. Cas9 cleaves target DNA approximately 3 bp upstream from the PAM site leaving a blunt, double-stranded break.[1]

When used *in vivo*, the break is then repaired through cellular repair pathways. In the presence of a donor DNA repair template, the HDR (homology-directed repair) pathway can generate sequence insertions or substitutions. In the absence of a donor, repairs are made by the NHEJ (non-homologous end-joining) pathway. The default NHEJ pathway is prone to the generation of insertions or deletions (indels) at the break site, which frequently results in gene knock-outs[2-4]. Cells can be analysed for gene editing efficiency using standard methods, such as Enzyme Mismatch Cleavage (EMC), RNA-guided Endonuclease-Restriction Fragment Length Polymorphism (RGEN-RFLP), High Resolution Melt Analysis (HRMA), and DNA sequencing methods.

CRISPRcraft S.p. Cas9 Nuclease contains a C-terminal 6xHis tag and a C-terminal nuclear localisation signal (NLS) sequence to ensure efficient delivery to the nucleus. Positive controls for testing enzyme activity *in vivo* and *in vitro* are available as a separate kit (CRISPRcraft S.p. Cas9 Nuclease Control Kit, Cat # 70030-1). These controls may be used to test enzyme function in the *in vitro* or *in vivo* assays described in this product manual.

The CRISPRcraft S.p. Cas9 Nuclease Control Kit, sold separately from the nucleases, contains positive controls for testing enzyme activity *in vivo* and *in vitro*. Control guide RNA (Cas9 Control HPRT Guide crRNA:Cas9 Universal tracrRNA duplex) targeting human HPRT1 can be used as positive controls for *in vivo* gene editing experiments in human cells. The provided HPRT PCR Primers can be used to amplify the HPRT target region from genomic DNA for any mutation detection assay requiring PCR, such as the T7E1 assay described in this manual (Appendix A). The Control guide RNAs can also be used in conjunction with the Control HPRT Substrate DNA and 10X RGEN Buffer to test the enzyme function *in vitro*.

Component	Use with *in vitro* assays	Use with *in vivo* assays
Cas9 Control HPRT Guide crRNA and Universal tracrRNA	X	X
10X RGEN Buffer	X	
Control HPRT Substrate DNA	X	
Human HPRT Forward / Reverse PCR Primers		X

7. Designing Cas9 Guide RNA sequences

When targeting a gene of interest, we recommend designing and testing at least three different guide RNA sequences for each target to increase the probability of a gene modification event. The guide sequence consists of a crRNA sequence and a tracrRNA sequence. The crRNA and tracrRNA can be generated as two separate RNA molecules and duplexed before use. The guide RNA provided with the CRISPRcraft S.p. Cas9 Nuclease Control Kit (Cas9 Control HPRT Guide crRNA and Cas9 Universal tracrRNA) is an example of a guide RNA of this type. Alternatively, the crRNA and tracrRNA can be combined into a single guide RNA molecule (sgRNA).

In both cr:tracr and sg RNA formats, the 5’ end of the crRNA determines where the RNP complex will bind through complementarity to the target DNA. This is the portion of the guide that is specific to the target and should be designed carefully. When designing your target-specific sequence:
• Ensure that the crRNA sequence matches the target DNA sequence (same target site, same organism)
• The NGG PAM site must be located directly 3’ to the target sequence in the context of the target DNA.
• The PAM site is not contained within the crRNA sequence.

Dual guide format

The following sequences are what was reported in the first publication detailing the requirements of the Cas9 crRNA and tracrRNA. Truncations of the sequences are common. When ordering guides from a nucleic acid synthesis vendor, often only the target-specific portion is required to place an order. The remainder of the sequence may be dictated by the vendor. The N's in the sequences listed represent the target-specific portion of the crRNA.

crRNA sequence:

Duplexes with tracrRNA

5'-NNNNNNNNNNNNNNNNNNNNguuuuagagcuaugcuguuuug-3'

The portion of the crRNA that duplexes with the tracrRNA is indicated and is as reported in Jinek *et al*. The sequence in the blue box is the minimal sequence required for annealing to tracrRNA and target DNA[1]. Truncated versions of the crRNA that maintain activity have been reported[5].

tracrRNA sequence:

Duplexes with crRNA

5'-ggaaccauucaaaacagcauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugcu-3'

The tracrRNA sequence is as reported in Jinek *et al*. with the portion of the tracrRNA that duplexes with the crRNA indicated. The sequence in the blue box is the minimal sequence required for crRNA annealing and Cas9 binding[1]. Truncated versions of the tracrRNA have been reported[5].

The guide RNA provided with the CRISPRcraft S.p. Cas9 Nuclease Control Kit are provided as separate crRNA and tracrRNA. The lengths of each have been optimised for maximal activity. The target-specific portion of the Cas9 Control HPRT Guide crRNA used to target the HPRT1 gene is CCCAAGGAAAGACUAUGAAA.

Single guide format

sgRNA sequence:

Duplexes with tracrRNA

5'-$(N)_{20}$guuuuagagcuaGAAAuagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc-3'

Duplexes with crRNA

A single guide RNA format is often used and combines the crRNA and tracrRNA into a single oligoribonucleotide[6]. The portions of the sequence that form the cr:tracr duplex are indicated. The GAAA sequence forms a loop between crRNA and tracrRNA. The remainder of the tracrRNA forms secondary structure that is important for binding to Cas9.

Guide RNAs for use with Cas9 can be purchased from various providers or made using an *in vitro* transcription kit like the AmpliScribe™ T7-Flash™ Transcription Kit (Cat # ASF3257).

Store guide RNA according to the manufacturers recommendations, or according to the recommendations of the *in vitro* transcription kit used to synthesise the guide RNA.

Additional resources for Cas9 guide RNA design can be found online:
http://crispr.mit.edu/
https://www.atum.bio/eCommerce/cas9/input
http://chopchop.cbu.uib.no/index.php
http://crispor.tefor.net/
http://www.e-crisp.org/E-CRISP/

Many of these resources include algorithms that predict the probability of a designed guide to cut at an unintended locus, known as off-target sites. It is important to balance the on-target efficiency with the probability of predicted off-target cleavage events.

8. *In vivo* use protocols: additional materials required

The following two transfection protocols may be used to deliver Cas9 RNP (ribonucleoprotein) complexes consisting of CRISPRcraft S.p. Cas9 Nuclease and either a user-supplied guide RNA or the Control HPRT Guide RNAs provided in the CRISPRcraft S.p. Cas9 Nuclease Control Kit.

Material and equipment required	Vendor
Nuclease-Free Water	Biosearch Technologies, Cat #W7350M
Low TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5)	Various
1X Dulbecco's Phosphate Buffered Saline or equivalent	ThermoFisher Scientific, Cat # 14190144
Opti-MEM I	ThermoFisher Scientific, Cat # 11058021
Trypsin-EDTA (0.25%), phenol red	ThermoFisher Scientific, Cat # 25200056
Lipofectamine RNAiMAX Transfection Reagent	ThermoFisher Scientific, Cat # 13778030
*Trans*IT-X2® Dynamic Delivery System	Mirus Bio, Cat #MIR 6003
Gene Pulser Xcell™ Electroporation System	Bio-Rad, Cat # 1652660 or # 1652661
Ingenio® Electroporation Solution	Mirus Bio, Cat # MIR 50111

9. Lipid-based transfection recommendations

The following protocol may be used to form Cas9 RNP (ribonucleoprotein) complexes and deliver them to HEK293T cells using TransIT-X2® Dynamic Delivery System or Lipofectamine® RNAiMAX Transfection Reagent. The protocol below details a lipid-based transfection protocol using a reverse transfection method in a 96-well plate. The transfection complexes are added to the plate first, followed by the cell suspension. Optimisation may be required when using alternative transfection reagents and cell lines.

Step 1: Prepare guide RNA
Note: RNA is prone to degradation. Use good laboratory practices (e.g., wear gloves, clean work surfaces) when handling guide RNA.

1. Determine volume of guide RNA needed. See Step 2, 3.
2. Prepare guide RNA dilution
   Single guide format (cr and tracr RNA as single oligoribonucleotide):
   a. Dilute guide RNA to 1 µM in low TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5).
   Dual guide format (cr and tracr RNA as two separate oligoribonucleotides):
   1. If cr and tracr RNA are lyophilised, resuspend in low TE to 100 µM.
   2. Dilute the crRNA and tracrRNA to a final concentration of 1 µM prior to RNP formation as follows. Total volume needed may change depending on user requirement.

Component	Volume (µL)
crRNA, 100 µM	1
tracrRNA, 100 µM	1
Low TE	98
Total	**100**

3. Heat to 95 °C for 5 minutes to duplex crRNA and tracrRNA. Cool at room temperature.

   Note: The crRNA:tracrRNA guide duplex generated from controls included in the CRISPRcraft S.p. Cas9 Nuclease Control Kit (Catalog #70030-1) are stable for at least 6 months with no loss in activity when stored at -20 °C at a concentration of ≥1 µM. Follow the manufacturer's recommendation when using crRNA:tracrRNA from other sources.

Step 2: RNP Formation

1. Keep CRISPRcraft S.p. Cas9 Nuclease on ice.
2. Dilute S.p. Cas9 Nuclease to 1 µM in Opti-MEM I or 1X DPBS.
   Note:
   • CRISPRcraft S.p. Cas9 Nuclease is supplied at 10 mg/mL (62 µM) or 3.2 mg/mL (20 µM).
   • The nuclease solution is viscous, use caution when pipetting small volumes.
3. Form RNP complexes by combining S.p. Cas9 Nuclease and guide RNA in Opti-MEM I.

Component	Volume per well of 96-well plate (µL)	
	RNAiMAX	*Trans*IT-X2
Opti-MEM I	20.5	6.5
Diluted S.p. Cas9 Nuclease, 1 µM	1.5	1.1
Guide RNA, 1 µM	3	2.2
Total	**25**	**9.8**

4. Mix gently by pipetting up and down 10 times.
5. Incubate at room temperature for 10 minutes.
6. Proceed directly to transfection of the RNP complexes. Do not store the RNP complexes before use; make fresh RNP for each transfection.

Step 3: Transfection

1. Combine RNP complex and transfection reagent:

Component	Volume per well of 96-well plate (µL)	
	RNAiMAX	*Trans*IT-X2
Opti-MEM I	20.5	6.5
Diluted S.p. Cas9 Nuclease, 1 µM	1.5	1.1
Guide RNA, 1 µM	3	2.2
Total	**25**	**9.8**

3. Mix gently by pipetting up and down 10 times.
4. Incubate 15 minutes at room temperature.
5. During incubation, wash cells with 1X DPBS and trypsinise.
6. Count cells and dilute to 400,000 cells/mL in complete media without antibiotics.
7. Following incubation period, add 50 µL RNP/RNAiMAX or 10 µL RNP/TransIT-X2 complex to each well in 96-well plate.
8. Add 100 µL cells (40,000 cells/well) to the transfection complex in each well. Final RNP concentration is 10nM, consisting of a 2:1 guide-to-Cas9 molar ratio.
   Note: The final RNP concentration and guide-to-Cas9 molar ratio defined here are a starting point. Further optimisation may be required. Changes to the Cas9 concentration and/or guide to Cas9 ratio may also require optimisation of the transfection reagent volume.

9. Incubate the transfection plate in tissue culture incubator (37 ºC, 5% CO_2) for 48 hours. A culture medium change may be necessary for longer incubations.
10. Harvest DNA from cells to analyse gene modification. Appendix A presents methods for harvest of genomic DNA using QuickExtract™ DNA Extraction Solution and analysis by Enzymatic Mismatch Cleavage (EMC) using T7 Endonuclease I (T7EI).

10. Electroporation transfection recommendations

The following protocol may be used to form Cas9 RNP (ribonucleoprotein) complexes and deliver them to HEK293T cells using the Bio-Rad Gene Pulser Xcell instrument. Optimisation may be required when using alternative electroporation devices and cell lines.

Step 1: Prepare guide RNA
Note: RNA is prone to degradation. Use good laboratory practices (e.g., wear gloves, clean work surfaces) when handling guide RNA.

1. Determine volume of 50 µM guide RNA needed. Volume per electroporation in the table below is based on desired final concentration (see Step 3, 8).

Component	Volume per electroporation (µL)	x	=
0.2 cm cuvette	3.0	Number of electroporations	Minimum Total Volume 50 µM guide RNA (µL)
0.4 cm cuvette	7.5		

2. Prepare guide RNA dilution
   Single guide format (cr and tracr RNA as single oligoribonucleotide): Dilute guide RNA to 50 µM in low TE.
   Dual guide format (cr and tracr RNA as two separate oligoribonucleotides):
   a. If cr and tracr RNA are lyophilised, resuspend in low TE to 100 µM.
3. Duplex the crRNA and tracrRNA to a final concentration of 50 µM by mixing the 100 µM stocks 1:1, keeping in mind the minimum total volume calculated above.
4. Heat to 95 °C for 5 minutes to duplex crRNA and tracrRNA. Cool at room temperature.

Step 2: Prepare Ingenio Solution/RNP/cell mixture
Note: Prepare RNP solution immediately before electroporation.

1. Warm Ingenio Electroporation Solution, trypsin-EDTA (if needed) and complete growth medium to room temperature.
2. Wash cells with 1X DPBS. Harvest cells by trypsinisation. Count cells to determine harvested cell density/mL.
3. Determine the total electroporation volume required to perform the desired number of electroporations.

Cuvette size	x	=
0.2 cm cuvette	# of electroporations x 0.10 mL	= total electroporation volume
0.4 cm cuvette	# of electroporations x 0.25 mL	

4. Calculate the cell volume required for all electroporations according to the formula:

Cells	Final cell density/mL	+	x	=
adherent	5 × 10^6 cells/mL	harvested cell density/mL	total electroporation volume	= Cell volume (mL)
suspension	10 × 10^6 cells/mL			

5. Pipette the determined cell volume into a new tube and centrifuge at 800 × g for 5 minutes. Aspirate the supernatant.
6. During centrifugation, add pre-warmed complete culture medium to a new culture dish to accept cells following electroporation.
7. Prepare the Ingenio® Solution/cell mixture by resuspending cells in Ingenio® Electroporation Solution using electroporation volume calculated above.
8. Prepare the RNP complex by combining guide RNA and Cas9 in a microcentrifuge tube. The table below lists volumes for guide RNA and Cas9 final concentrations of 1.5 and 0.75 µM respectively, in the cuvette.

   Notes:
   - CRISPRcraft S.p. Cas9 Nuclease is supplied at 10 mg/mL (62 µM) or 3.2 mg/mL (20 µM).
   - The nuclease solution is viscous, use caution when pipetting small volumes.
   - The final concentration and guide-to-Cas9 molar ratio defined here are a starting point. Further optimisation may be required.

CRISPRcraft S.p. Cas9 Enzyme Concentration:	10 mg/mL, 62 µM (Catalog # 70020-2)		3.2 mg/mL, 20 µM (Catalog # 70020-0)	
	Volume per 0.2 cm cuvette (µL)	Volume per 0.4 cm cuvette (µL)	Volume per 0.2 cm cuvette (µL)	Volume per 0.4 cm cuvette (µL)
S.p. Cas9 Nuclease	1.2	3.0	3.8	9.4
Guide RNA, 50 µM	3.0	7.5	3.0	7.5

9. Incubate the gRNA/Cas9 protein mixture at room temperature for 5 minutes.
10. Add the Ingenio/cell mixture to the RNP complex.
11. Mix gently but thoroughly. Do not create air bubbles in the mixture.

Step 3: Electroporation

1. Aliquot the Ingenio/RNP/cell mixture into cuvettes for electroporation. Pipet 100 µL per 0.2 cm cuvette and 250 µL per 0.4 cm cuvette.
2. Electroporate the cells at room temperature with settings as follows; 160 V, 950 uF capacitance, exponential decay.
   Note: The optimal pulse conditions vary depending on the cell type and electroporator used. Refer to the Ingenio® Electroporation Solution Full Protocol for recommended pulse conditions. If your cell type is not listed, the correct settings should be determined experimentally.
3. Transfer the electroporated cells into the previously prepared culture dish.
   Example: Transfer 100 µL electroporated cells to one well of a 12-well plate or 10 µL electroporated cells to one well of a 96-well plate
   Note: Users should determine their own post-electroporation best cell culture density depending on the cell type and post-electroporation incubation period.
4. Incubate the electroporated cells at 37 °C, 5% CO_2, in appropriate culture medium for 48 hours or as required. A culture medium change may be necessary for longer incubations.
5. Harvest cells and assay as required. Appendix A presents methods for harvest of genomic DNA using QuickExtract DNA Extraction Solution and analysis by Enzymatic Mismatch Cleavage (EMC) using T7 Endonuclease I (T7EI).

11. *In vitro* use protocols: additional materials required

The following guidelines can be used to test the function of CRISPRcraft S.p. Cas9 Nuclease in an *in vitro* activity assay. In this assay, CRISPRcraft S.p. Cas9 Nuclease is complexed with a guide RNA and tested for the ability to cleave a linearised plasmid substrate. Results are visualised by band intensity after agarose gel electrophoresis.

This assay can be performed with the controls included in the CRISPRcraft S.p. Cas9 Nuclease Control Kit (Catalog #70030-1). The control kit contains Control HPRT Substrate DNA, a linearised plasmid containing a fragment of the human HPRT1 gene, as well as Cas9 Control HPRT Guide crRNA, SpCas9 Universal tracrRNA, and 10X RGEN Buffer.

Material and equipment required	Vendor
CRISPRcraft S.p. Cas9 Nuclease	Biosearch Technologies
10X RGEN buffer	User supplied or as supplied in CRISPRcraft S.p. Cas9 Nuclease Control Kit
Guide RNA	User supplied or as supplied in CRISPRcraft S.p. Cas9 Nuclease Control Kit
Nuclease-free water	Various
0.5 M EDTA	Various
Agarose Gels, Electrophoresis Buffers	Various
Gel documentation equipment	Various
Image analysis software	Various

12. *In vitro* cleavage assay

Notes:
- Dilute fresh enzyme for each experiment. Do not store the diluted enzyme for later use.
- RNA is prone to degradation. Use good laboratory practices (e.g., wear gloves, clean work surfaces) when handling guide RNA.

1. If CRISPRcraft S.p. Cas9 Nuclease Control Kit is not available, prepare 10X RGEN Buffer (0.5 M NaCl, 0.5 M Tris-HCl, 0.1 M $MgCl_2$, 10 mM DTT, pH 7.5).
2. Prepare guide RNA dilution
   Single guide format (cr and tracr RNA as single oligoribonucleotide):
   Dilute guide RNA to 1 µM in low TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5).
   Dual guide format (cr and tracr RNA as two separate oligoribonucleotides):
   a. If cr and tracr RNA are lyophilised, resuspend in low TE to 100 µM.
3. Dilute the crRNA and tracrRNA to a final concentration of 1 µM prior to RNP formation as follows. Below preparation is for 100 µL volume. Prepare more if needed.

Component	Volume (µL)
crRNA, 100 µM	1
tracrRNA, 100 µM	1
Low TE	98
Total	**100**

4. Heat to 95 °C for 5 minutes to duplex crRNA and tracrRNA. Cool at room temperature.
5. Dilute CRISPRcraft S.p. Cas9 Nuclease to 200 nM by adding the following components to a chilled 1.5 mL Eppendorf tube in order:
   Note: the nuclease solution is viscous, use caution when pipetting small volumes.

CRISPRcraft S.p. Cas9 Nuclease Stock Concentration	Volume Nuclease-free Water (µL)	Volume 10X RGEN Buffer (µL)	Volume CRISPRcraft S.p. Cas9 Nuclease (µL)	Final Concentration, CRISPRcraft S.p. Cas9 Nuclease
S.p. Cas9 Nuclease	89	10	1	200 nM
Guide RNA, 50 µM	278	31	1	200 nM

6. Mix gently by pipetting up and down 10 times.
7. Assemble the in vitro cleavage reactions by combining the following components in 0.2 mL PCR tubes. If testing additional guide RNA sequences of interest, set up a negative control reaction for each substrate DNA to be tested.

Component	Volume (µL) per reaction
Nuclease-free Water	20.7
10X RGEN Buffer	2.7
1 µM Guide RNA **(Step 2)**	0.6
200 nM CRISPRcraft S.p. Cas9 Nuclease, **(Step 3)**	3.0
Total	**27.0**

8. Mix gently by pipetting up and down 10 times.
9. Add 3 µL substrate DNA. The reaction will proceed quickly once substrate is added.
10. Incubate the reaction tubes in a thermal cycler, set to 37 °C, for 10 minutes.
11. During the incubation, make 100 µL of 250 mM EDTA by mixing 50 µL Nuclease-free Water and 50 µL 0.5M EDTA.
12. Following the 10 minute incubation, remove the tubes from the thermal cycler and add 3 µL 250 mM EDTA to each tube to stop the reactions. If there are many reactions, use a multichannel pipette to stop all reactions simultaneously.
13. Mix gently by pipetting up and down 10 times.
14. Incubate the reaction tubes in a thermal cycler, set to 65 °C, for 20 minutes to dissociate the RNP from the substrate.
15. Remove the reaction tubes from the thermal cycler. Continue to the next step or store the reactions at -20 °C before proceeding.
16. Run 15 µL of each reaction on agarose gel to visualise the results. After electrophoresis, three bands should be present in each lane: a parent fragment, and two digest fragments.
    Notes:
    • If the reaction went to completion, no parent fragment will be visible.
    • Fragments created when using CRISPRcraft S.p. Cas9 Nuclease Control Kit (Catalog #70030-1) are easily resolved by a 1.5% agarose gel.
17. Calculate the reaction efficiency by quantitating the intensity of all three bands using gel analysis software.
    a. Add the intensity of all 3 fragments to obtain the total intensity:
       (Fragment 1) + (Fragment 2) + (Parent Fragment) = Total Intensity
    b. Calculate the fraction cleaved:
       $$\frac{\text{(Fragment 1) + (Fragment 2)}}{\text{Total Intensity}} \times 100 = \%\ \text{DNA Cleaved}$$

    Note: Banding pattern created when using CRISPRcraft S.p. Cas9 Nuclease Control Kit (Catalog #70030-1) is as follows: 2.9 kb (parent fragment), 1.9 kb (fragment 1), and 1 kb (fragment 2). Expected results using controls in the control kit will indicate >80% cleavage of the control substrate DNA. If 100% cleavage was achieved, no parent band will be visible.

Appendix A: Mutation detection by T7E1 Assay

Gene editing efficiency can be analysed using the T7E1 Assay Protocol provided here, or other standard methods. This protocol is designed to evaluate gene editing efficiency for cells transfected in 96-well plates.

The assay utilises the ability of T7 Endonuclease 1 to recognise and cleave mismatches, or “bubbles”, in double-stranded DNA. Target site PCR amplicons from an experimental cell population are denatured and reannealed, resulting in a mixed population of duplexed amplicons, including heteroduplexed amplicons containing both edited and wild-type sequence. T7E1 endonuclease recognises and cleaves both strands at the site of the mismatch, and resulting fragments are visualised by gel electrophoresis. The relative intensities of the cut fragments to the total is used to determine the percentage of gene modification within the amplicon population.

If RNP made with Control HPRT Guide RNA has been used as a positive control in your experiment, use the Human HPRT Forward and Reverse PCR Primers to amplify the HPRT locus in Step 2: PCR. The expected size of the HPRT amplicon is 1083 bp.

Use these guidelines to prepare a PCR product for T7E1 assay:

- Target amplicon should be ~ 700-1000 bp.
- The target site should be contained within the amplicon, and in such a way that cleavage by T7E1 endonuclease creates fragments that are resolvable by gel electrophoresis.

If desired, a positive control for the T7E1 assay may be prepared by combining a PCR amplicon from a wild-type template with the equivalent amplicon from a known mutant template. When the WT and mutant amplicons are combined in a 1:1 ratio, melted, and reannealed, the resulting mixture should contain 50% heteroduplexes. Performing Step 3: Mutation Detection by T7E1 Assay (below) should therefore result in cleavage of 50% of the duplexes.

Material and equipment required	Vendor
Dulbecco’s Phosphate Buffered Saline or equivalent	ThermoFisher Scientific, Cat #14190144
QuickExtract DNA Extraction Solution	Biosearch Technologies, Cat #QE09050
PCR Plate and caps	Various
Thermal cycler with 96-well plate block	Various
Nuclease-free water	Various
Phusion™ HF polymerase with 5X HF buffer, 2 U/µL or equivalent	M0530S or various
Target-specific forward and reverse PCR primers	Various
NEBuffer™ 2	NEB, Cat #B7002S
T7E1 Enzyme, 10 U/µL	NEB, Cat #M0302
Agarose Gels, Electrophoresis Buffers and Equipment	Various
Gel documentation equipment	Various
Image analysis software	Various

Step 1: Extract genomic DNA (gDNA)

1. 24 to 48 hours post-transfection, remove cell culture media from wells.
2. Wash cells with 100 µL 1x DPBS. Remove 1X DPBS.
3. Add 50 µL QuickExtract DNA Extraction Solution per well to lyse cells.
4. Transfer lysed cells to PCR plate, seal with caps, and incubate at 65 °C for 15 minutes, followed by 95 °C for 15 minutes.
5. Add 100 µL nuclease free water to dilute genomic DNA and mix.
6. Proceed directly to Step 2: PCR, or store the plate at -20 °C.

Step 2: PCR

The following conditions were found to be successful using Phusion-HF polymerase. Other polymerases and their associated protocols may also be used.

1. Make a PCR master mix according to the following table.

Reagent	Volume for each 20 µL reaction (µL)
Forward Primer (100 µM)	0.1
Reverse Primer (100 µM)	0.1
dNTP's (2.5 mM)	1.6
5X HF Buffer	4.0
Nuclease-free water	10.8
Phusion-HF polymerase, 2U/µL	0.4

2. For each sample, transfer 3 µL extracted genomic DNA from Step 1 to a well of a PCR plate.
3. Using repeat pipettor, aliquot 17 µL master mix into each well with genomic DNA.
4. Cap the reaction tubes and centrifuge the plate briefly to collect liquid at the bottom of the plate.
5. Cycle using the following parameters for an approximately 1 kb amplicon or as specified by manufacturer of polymerase.

   Below are example cycling parameters that can be used with the Human HPRT Forward and Reverse PCR Primers found in the CRISPRcraft™ S.p. Cas9 Nuclease Control Kit. Annealing temperature and extension time may have to be adjusted for user-specific amplicons.

Temperature	Time	
98 °C	2 minutes	
98 °C	10 seconds	× 30 cycles
65 °C	15 seconds	
72 °C	30 seconds	
72 °C	5 minutes	

6. Run 1 µL of each sample or of a representative test set of samples on a 1% agarose gel to check yield and specificity of PCR reactions. The reaction should yield a single band at the desired molecular weight. Extraneous bands may confound interpretation of the assay results. *Note:* The Human HPRT Forward and Reverse PCR Primers in the CRISPRcraft S.p. Cas9 Nuclease Control Kit will produce a 1083 bp amplicon.
7. Determine the average total yield in nanograms (ng) of PCR product per µL and multiply by 5. The yield will be used to determine T7E1 assay incubation time.

Step 3: Mutation detection by T7E1 Assay

1. Make a T7E1 Buffer assay master mix according to the following table.

Reagent	Volume for each 20 µL Reaction (µL)
NEBuffer 2	1.8
Nuclease-free Water	11.2

2. For each sample, transfer 5 µL PCR product from Step 2 to a well of a new PCR plate.
3. Using a repeat pipettor, aliquot 13 µL master mix into each well with PCR product.
4. Cap and centrifuge the plate briefly to collect the liquid at the bottom of the wells. *Note:* We have found that cap strips prevent evaporation more effectively than sealing tape.
5. Cycle according to the following table to create PCR product heteroduplexes.

Temperature	Time/ramp speed
95 °C	10 minutes
95-85 °C	-2 °C/second
85-25 °C	-0.3 °C/second

6. Dilute T7E1 Nuclease to 1 U/µL in 1x NEB buffer 2. For each well, prepare 2 µL of diluted enzyme.
7. Aliquot 2 µL of diluted T7E1 (1 U/µL) to each well containing heteroduplexed amplicons. Mix by gently pipetting up and down.
8. Incubate at 37 °C according to the table below. Incubation time is dependent upon the PCR product yield.

Yield of PCR product (per 5 µL)	Incubation time
10-50 ng	30 min
50-150 ng	60 min
150-250 ng	120 min

Note: T7E1 enzyme exhibits some non-specific activity against perfect duplexes, which can become significant with incubation for too long or with a ratio of T7E1 enzyme to DNA that is too high. This non-specific activity can result in overdigestion and smearing of bands, leading to inaccuracy in estimating mutation frequency. On the other hand, incomplete mismatch cleavage may result if the ratio of enzyme to DNA is too low. The guidelines above are intended to provide a proper balance between enzyme activity and DNA input.

9. Immediately store at -20 °C or add 2 µL 10X loading dye and load 15 µL onto a 2% agarose gel.
10. After running the gel, use a gel imaging station and gel analysis software to determine the intensity of each band visible in each lane.

Step 4: Calculate % modification

1. Three bands are potentially present in each lane. The intensity of each band is dependent on the extent of gene editing.
   - The largest band is the Parent Fragment, representing undigested PCR product.
   - Other bands, Fragment 1 and Fragment 2, are digest products. These are smaller than the parent fragment and their size depends on the location of the Cas9 target site within the PCR amplicon.

   Note: Use imaging software that enables band quantitation with a chromatogram so that integration of band peaks can be viewed and adjusted as needed. Examples of available software include Image Lab™ (Bio-Rad) and ImageJ (open source, NIH). Both are available as free downloads.
2. For each reaction, calculate % gene modification:
   Total Intensity = Parent Fragment intensity + Fragment 1 intensity + Fragment 2 intensity
   Fraction Cleaved = (Fragment 1 +Fragment 2) / Total Intensity
   % Modification = 100 x $(1-(1\text{-Fraction Cleaved})^{0.5})$

Note: The above equation assumes that each editing event produces a unique mutation, and therefore that heteroduplexes formed from different editing events are cleavable by T7E1. However, repair of Cas9-mediated cleavage by the NHEJ pathway often results in a non-random mutation profile, with one or a few mutations sometimes dominating the population7. In such cases, a high proportion of mutant heteroduplexes will not be cleavable by T7E1. Thus, the equation given above will often produce an underestimate of the editing frequency within a population.

Note: The banding pattern created when using human HPRT controls in the CRISPRcraft S.p. Cas9 Nuclease Control Kit (Catalog #70030-1) is as follows: Parent band is 1083kb, fragment 1 is ~800bp, and fragment 2 is ~280bp.

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Troubleshooting

Potential cause resulting in no or low enzyme activity detected	Potential solution
• Non-optimal guide RNA design • Guide RNA is degraded	• Check guide RNA design, ensure that the guide was designed for the correct target site in the desired organism's target DNA. • Test at least three guide RNA sequences per target. • Order fresh guide RNA sequence, and store according to manufacture recommendations. • Handle guide RNA using good laboratory practices for working with RNA. • Test guide RNAs with an in vitro cleavage assay on a synthetic target, before proceeding to use them in vivo
• Mutation detection assay is not optimised *(in vivo* only)	• Run positive controls with the mutation detection assay to aid in interpretation of results. A heteroduplex of a WT:mutant mixed 1:1 should result in 50% fraction cleaved. • If smearing is present then overdigestion by T7E1 may have occurred. Reduce incubation time using Table in Step 3: Mutation Detection by T7E1 Assay. • Try an alternative mutation detection assay.
• Incorrect RNP formation	• Ensure that the RNP is formulated with at least a 1:1 guide:Cas9 molar ratio. • Form the guide RNA complexes in low TE buffer. High levels of NaCl in the buffer may inhibit target binding in vitro. • Do not store RNP complex of diluted Cas9 for later use. Form fresh RNP for each experiment using a fresh dilution of Cas9 enzyme.
• No magnesium present (in vitro assay only)	• Ensure there is a source of magnesium present in the *in vitro* reaction buffer. Optimal final concentration is 5-10 mM.
• Inefficient RNP delivery *(in vivo* only)	• Use positive controls to determine transfection efficiency (e.g., a fluorescently-labelled guide RNA or a GFP-expressing plasmid)

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