bio-protocol

Published: May 20, 2022

CRISPR/Cas9 Gene Editing of HeLa Cells to Tag Proteins with mNeonGreen

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Abstract

Subcellular localization dynamics of proteins involved in signal transduction processes is crucial in determining the signaling outcome. However, there is very limited information about the localization of endogenous signaling proteins in living cells. For example, biochemical mechanisms underlying the signaling pathway from epidermal growth factor (EGF) receptor (EGFR) to RAS-RAF and ERK1/2/MAPK are well understood, whereas the operational domains of this pathway in the cell remain poorly characterized. Tagging of endogenous components of signaling pathways with fluorescent proteins allows more accurate characterization of their intracellular dynamics at their native expression levels controlled by endogenous regulatory mechanisms, thus avoiding possible tainting effects of overexpression and mistargeting. In this study, we describe methodological approaches to label components of the EGFR-RAS-MAPK pathway, such as Grb2, KRAS, and NRAS, with the fluorescent protein mNeonGreen (mNG) using CRISPR/Cas9 gene-editing, as well as generation of homozygous single-cell clones of the edited target protein.

Keywords: mNeonGreen, EGF receptor, KRAS, NRAS, CRISPR-Cas9

This protocol was validated in: J Cell Biol (2021), DOI: 10.1083/jcb.202107103

Background

The epidermal growth factor (EGF) receptor (EGFR) is activated by EGF or other ligands at the cell surface, which triggers the RAS-RAF-MAPK/ERK1/2 signaling pathway involved in cell proliferation, differentiation, survival, and motility (Karnoub and Weinberg, 2008). Activated EGFR is endocytosed and accumulated in early endosomes, where it is either recycled back to the plasma membrane or targeted for lysosomal degradation. Whether active EGFR continues to signal through the RAS-ERK1/2 axis from endosomes to sustain ERK1/2 activation remains controversial and is under debate in the literature (reviewed in von Zastrow and Sorkin, 2021). Evidence for the endosomal signaling to ERK1/2 is based on the detection of components of the ERK1/2 pathway in endosomes in cells stimulated with EGF (Pol *et al.*, 1998; Howe *et al.*, 2001; Jiang and Sorkin, 2002; Lu *et al.*, 2009; Schmick *et al.*, 2014). However, these observations were made either with overexpressed recombinant proteins or with chemically fixed cells or by subcellular fractionation, approaches that may not correctly report subcellular localization of endogenous proteins. We initially studied the spatio-temporal dynamics of the endogenous tagged EGFR and components of the ERK1/2 pathway by fluorescently tagging these proteins using gene editing techniques utilizing zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN) (Pinilla-Macua *et al.*, 2017, 2016). Subsequently, we switched to using the CRISPR-Cas9 system (Surve *et al.*, 2019; Surve *et al.*, 2021) for gene-editing. We selected for these studies a subvariant of HeLa cells that we consistently used in the laboratory because they express EGFR at levels similar to those in many mammalian cells in vivo, and because we have rigorously characterized the EGFR endocytic trafficking system in these cells.

CRISPR-Cas9 has proven to be versatile and efficient method of gene editing compared to ZFNs and TALEN, and is widely used for gene knockouts, gene activation, gene inhibition, and knock-ins. The fascinating discovery and evolution of CRISPR technology is beyond scope of this article, and excellent reviews on this topic can be found elsewhere. We used this technology to tag KRAS, NRAS, and Grb2. Generally, there are two main components of CRISPR-Cas9 technology: i) Cas9—a RNA guided endonuclease, ii) a short non-coding guide RNA consisting of a target complementary CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA) that shuttles Cas9 to a specific site. These components are delivered inside the cells via plasmids or via direct delivery of an *in vitro* generated complex of purified Cas9 and purified sgRNA (Ran *et al.*, 2013). Following the delivery, gRNA through Watson-Crick base pairing binds to its target DNA sequence, and Cas9 makes a double stranded break (DSB) near the PAM site of gRNA. The DSB is either repaired by the error prone non-homologous end joining (NHEJ) mechanism or by precise homology dependent repair (HDR) mechanism. In case of gene tagging, an additional component is provided in the form of a repair template, *i.e.*, a donor DNA containing sequences of a fluorescent protein or small tags such as His, HA, or MYC, which through HDR allows the insertion of the tag of interest. HDR mechanism of repair is inherently inefficient, resulting in lower yields of clones of cells with the in-frame incorporated tag compared to generation of knockout clones, which generally involves NHEJ. The protocol here describes the variations (Table 1) in the approach by which gene tagging (knock-in) can be achieved in transformed cell lines like HeLa.

Materials and Reagents

1. CellTrics 50 µm sterile Disposable Filters (Sysmex, catalog number: 04-004-2327)
2. 96-well plate (Ibidi, 15 µ-Plate 96 Well Black)
3. 12-well cell culture plate (Corning, catalog number: 3513)
4. 25 or 75 cm^2 cell culture flasks (Corning, catalog number: 430639 or 430641U)
5. 15 mL tube conical base (Sarstedt, catalog number: 62.554.205)
6. DNA oligonucleotide primers from IDT
7. GeneArt Precision gRNA Synthesis kit (Invitrogen, catalog number: A29377)
8. Tracr Fragment + T7 Primer Mix (contains the universal PCR amplification primers and the 80-nt constant region of the crRNA/tracrRNA) (Invitrogen, catalog number: A29377)
9. Phusion™ High-Fidelity PCR Master Mix (2×) (Invitrogen, catalog number: A29377)
10. Nuclease free water
11. dNTP mix (10 mM each of dATP, dGTP, dCTP, dTTP) (NEB, catalog number: N0447)
12. 5× TranscriptAid™ Reaction Buffer (Invitrogen, catalog number: A29377)

13. TranscriptAid™ Enzyme Mix (Invitrogen, catalog number: A29377)
14. DNase I (Invitrogen, catalog number: A29377)
15. Single stranded DNA (ssDNA) oligos from IDT
16. NEBuilder® HiFi DNA Assembly Master Mix (NEB, catalog number: E2621)
17. pSpCas9(BB)-2A-Puro (PX459) plasmid (Addgene plasmid # 62988)
18. BbsI (NEB, catalog number: R0539)
19. NEB Buffer 2 (NEB, catalog number: B7002S)
20. NEB® 5-α Competent *E. coli* (High Efficiency) (NEB, catalog number: C2987)
21. Ampicillin Agar plates and Kanamycin Agar plates
22. NEB buffer 2.1 (NEB, catalog number: B7202)
23. QIAquick PCR Purification Kit (Qiagen, catalog number: 28104)
24. Synthetic double stranded donor DNAs from IDT
25. Phusion High-Fidelity DNA Polymerase (NEB, catalog number: M0530)
26. Phusion GC buffer 5× (NEB, catalog number: B0519)
27. Zero Blunt™ TOPO™ PCR Cloning Kit (Invitrogen, catalog number: 45-1245)
28. EcoRI-HF (NEB, catalog number: R3101)
29. CutSmart Buffer 10× (NEB, catalog number: B6004)
30. QIAquick Gel Extraction Kit (Qiagen, catalog number: 28704)
31. XbaI (NEB, catalog number: R0145)
32. Easy-Fusion Halo plasmid (Addgene plasmid # 112850)
33. EcoRI-HF (NEB, catalog number: R3101)
34. AvaI (NEB, catalog number: R0152)
35. HincII (NEB, catalog number: R0103)
36. DMEM (Gibco, catalog number: 11965-092) with 10% FBS (Invitrogen, catalog number: 16140071)
37. DPBS (without Ca^{2+} or Mg^{2+}) (Gibco, catalog number: 14190-144)
38. Trypsin (Gibco, catalog number: 25200-056)
39. Neon Transfection System (Invitrogen, catalog number: MPK5000)
40. Neon Transfection System 10 µL Kit (Invitrogen, catalog number: MPK1025)
41. Buffer R
42. Puromycin (Sigma-Aldrich, catalog number: P8833)
43. Platinum Cas 9 (Invitrogen, catalog number: A36498)
44. BD FACSAria III sorter fitted with 488 100 mW Trigon laser and 100 µm nozzle (BD Biosciences)
45. Nocodazole (Sigma-Aldrich, catalog number: M1404)
46. Lipofectamine 3000 (Invitrogen, catalog number: L3000001)
47. OptiMEM (Invitrogen, catalog number: 31985062)
48. Recombinant KRAS rabbit monoclonal antibody (Thermo Fisher Scientific, catalog number: 703345)
49. Cell Sorting buffer (see Recipes)
50. TGH buffer (see Recipes)

Software

1. https://www.benchling.com/. Benchling is a cloud-based informatics platform that is free to academic researchers. It provides design tools for DNA, oligonucleotides, and amino acids.
2. https://portals.broadinstitute.org/gppx/crispick/. The website is hosted by the Genetic Perturbation Platform of the Broad institute, MA, USA.
3. http://chopchop.cbu.uib.no/. CHOPCHOP is a web-based tool, designed by the Valen group at the University of Bergen, Norway. The website is for non-profit and academic use only.
4. https://nebuilder.neb.com. The web tool can be used to design primers for HiFi DNA assembly or Gibson assembly.

Procedure

Experimental Design

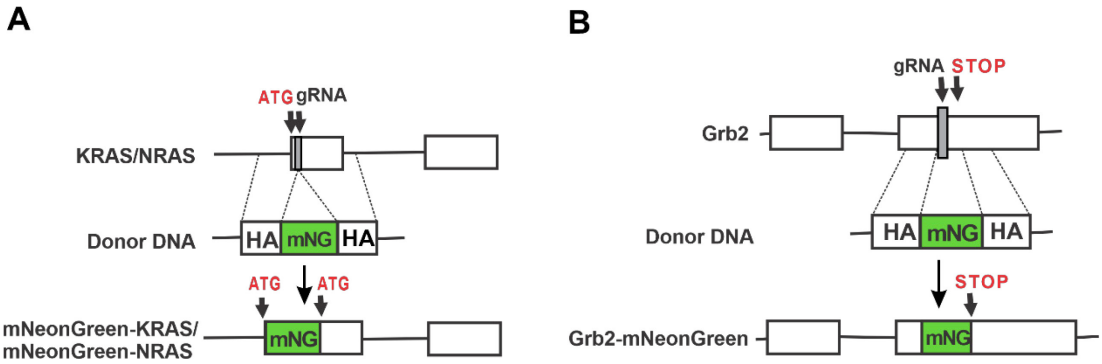

Figure 1. Schematics of insertion of mNG in *KRAS*, *NRAS*, and *Grb2*.
mNG sequence was inserted at amino terminus of *KRAS* and *NRAS* genes (A) and at carboxy terminus of *Grb2* gene (B). Donor DNAs were generated for all three genes with homology arms ranging from 500 to 1,000 bp. ATG, Start codon; STOP, Stop codon; HA, Homology Arm; mNG, mNeongreen; gRNA-guide RNA; empty boxes represent exons of *KRAS*, *NRAS* and *Grb2*.

Table 1. Sources of the components used to tag the genes

	KRAS	*NRAS*	*Grb2*
Cas9	Commercial purified enzyme	Commercial purified enzyme	Plasmid -PX459
gRNA	*In vitro* transcribed	*In vitro* transcribed	Cloned in Plasmid- PX459
Donor	Synthetic gene fragment	Synthetic gene fragment	Homology arms and mNG fragments cloned in a plasmid
Delivery method	Electroporation	Electroporation	Transfection via Lipofectamine

A. Generation of gRNAs for *KRAS, NRAS*, and *Grb2*

1. *In-vitro* Synthesis of gRNAs for tagging *KRAS* and *NRAS*
   a. Identification of the target gRNA
   We used amino termini of both *KRAS* and *NRAS* genes to tag with mNeonGreen fluorescent protein sequence because all RAS proteins are processed at their carboxy termini (Figure 1). Several guide RNA design tools are available online. We routinely use Benchling, Broad Institute GPP (now CRISPick), and CHOPCHOP, among others, to design target gRNAs. Using these tools, two targets for both *KRAS* and *NRAS* were identified, which were very close to the Start codons of both the genes. Order two complementary primers for each target (Table 2).

Table 2. Primers for in vitro translation of gRNAs

Primer	Sequence 5'--- 3'	
Kras_1F	TAATACGACTCACTATAGGAATATAAACTTGTGGTAGT	Target 1
Kras_1R	TTCTAGCTCTAAAACACTACCACAAGTTTATATTC	Target 1
Kras_2F	TAATACGACTCACTATAGAATGACTGAATATAAACTTG	Target 2
Kras_2R	TTCTAGCTCTAAAACCAAGTTTATATTCAGTCATT	Target 2
Nras_1F	TAATACGACTCACTATAGGACTGAGTACAAACTGGTGG	Target 1
Nras_1R	TTCTAGCTCTAAAACCCACCAGTTTGTACTCAGTC	Target 1
Nras_2F	TAATACGACTCACTATAGAATGACTGAGTACAAACTGG	Target 2
Nras_2R	TTCTAGCTCTAAAACCCAGTTTGTACTCAGTCATT	Target 2

i. Dilute target primers to a stock solution 100 µM in 1× TE buffer.
ii. Prepare a mixture of target primers of 10 µM stock solution by adding 10 µL each of the 100 µM forward and reverse target primer to 80 µL of nuclease-free water.
iii. Prepare the 0.3 µM target primer mix working solution by diluting 3 µL of the 10 µM target primer mix stock solution in 97 µL of nuclease-free water.

b. Assembly of the gRNA DNA templates

This step generates the DNA template required for the *in vitro* transcription of the gRNAs. Thaw the reagents from GeneArt Precision gRNA synthesis kit on ice. Mix and centrifuge contents of all the vials. Set up the following PCR assembly reaction in a 25-µL volume, adding the reaction components in the order given (Table 3).

Table 3. Components to make gRNA DNA template

Phusion™ High-Fidelity PCR Master Mix (2×)	12.5 µL
Tracr Fragment + T7 Primer Mix	1 µL
0.3 µM Target F/R oligonucleotide mix	1 µL
Nuclease free water	10.5 µL

Since the PCR product is expected to be very short (120 bp), perform a two-step assembly PCR using following parameters (Table 4).

Table 4. PCR parameters to generate gRNA DNA template

Cycle Step	Temperature	Time	Cycles
Initial denaturation	98°C	10 s	1×
Denaturation	98°C	5 s	32×
Annealing	55°C	15 s	32×
Final extension	72°C	1 min	1×
Hold	4°C	Hold	1×

c. *In vitro* transcription (IVT) of gRNAs

Use the gRNA DNA template generated in step A.1.b for *in vitro* transcription of gRNAs. Set up the reaction as follows in the order given (Table 5).

Table 5. Components of IVT reaction

NTP mix (100 mM each of ATP, GTP, CTP, UTP)	8 µL
gRNA DNA template (from PCR assembly)	6 µL
5× TranscriptAid™ Reaction Buffer	4 µL
TranscriptAid™ Enzyme Mix	2 µL

Mix the contents thoroughly and centrifuge. Carry out the IVT reaction for 3 h at 37°C.

d. Removal of the DNA template by DNase I digestion
It is helpful to remove DNA from IVT reaction so that it does not interfere with the subsequent applications of the RNA transcript.
   i. Incubate the IVT reaction mix with 1 µL of DNase I (1 U/µL) immediately after the IVT reaction and incubate at 37°C for 15 min.

e. Purification of gRNAs using the columns and the buffers supplied in the kit
   i. Dilute the IVT reaction to 200 µL with nuclease-free water and add 100 µL of Binding Buffer. Mix by pipetting.
   ii. Add 300 µL of ethanol (>96%) and mix by pipetting.
   iii. Transfer the mixture to the GeneJET™ RNA Purification Micro Column (preassembled with a collection tube) and centrifuge for 30–60 s at 14,000 × *g*. Discard the flow-through
   iv. Wash the bound RNA with 700 µL Wash Buffer 1 and Wash Buffer 2.
   v. Centrifuge the empty purification column for an additional 60 s at 14,000 × *g* to completely remove any residual Wash Buffer.
   vi. Transfer the purification column to a clean 1.5-mL Collection Tube.
   vii. Add 10 µL of nuclease-free water to the center of the purification column filter, and centrifuge for 60 s at 14,000 × *g* to elute the RNA.
   viii. Store eluted gRNA at –20 °C until use. For prolonged storage, store the gRNA at -80°C.
   Note: Follow all the necessary precautions while working with RNA to avoid its degradation or contamination and refer to the manufacturer's instructions for detailed guidelines for generating gRNAs.

2. Cloning of gRNAs for tagging *Grb2*
   a. Identification of the target gRNA
   Grb2 was tagged with mNG at its carboxy terminal. Cloning of Grb2 gRNAs was performed using NEBuilder HiFi DNA assembly mix which allows assembly of a linear plasmid and a single stranded DNA fragment, just as multiple DNA fragments assembly. Similar to RAS proteins, two target gRNAs for Grb2 were identified using Benchling and Broad Institute GPP (now CRISPick). The following target gRNA sequence oligos were ordered as ssDNA from IDT (Table 6). Underlined sequences represent the actual target gRNA sequence flanked by overlapping sequences around BbsI restriction enzyme cut site from PX459.

Table 6. ssDNA oligos for Grb2 gRNA

grb2-RNA_T1	ATCTTGTGGAAAGGACGAAACACCGGGTTAGACGTTCCGGTTCAC GGGTTTTAGAGCT AGAAATAGCAAGTT
grb2- gRNA_T2	ATCTTGTGGAAAGGACG AAACACCGGGCTTAG ACGTTCCGGTTCACGGTTTTAGAGCTAGAAATAGCAAGTT

   b. Digestion of PX459 with BbsI
   Linearize PX459 plasmid by digestion with BbsI at 37°C for 4 h (Table 7). Purify the digested plasmid using QIAquick PCR purification kit.

Table 7. PX459 digestion set up

PX459 Plasmid (500 ng/µL)	10 µL
10× Buffer 2.1	2 µL
BbsI (10 U/µL)	1 µL
ddH_2O	7 µL

c. Ligation of ssDNA into digested PX459
Centrifuge ssDNA oligo pellet and resuspend it in 1× TE to a final concentration of 100 µM, which is further diluted to a working stock of 0.4 µM in NEB Buffer 2. Set up the ligation reaction of linearized PX459 and ssDNA oligos in the order given below (Table 8).

Table 8. HiFi DNA assembly set up

Linear PX459 plasmid (50 ng)	1 µL
0.4 µM ssDNA stock	5 µL
ddH_2O	4 µL
NEBuilder® HiFi DNA Assembly Master Mix	10 µL

Incubate assembly reaction at 50°C for 1 h and transform 2 µL of the reaction mix into NEB 5-α Competent *E. coli* cells. Select the ampicillin resistant colonies by plating transformed *E. coli* cells onto Ampicillin agar plates.

d. Screening and confirmation of the positive clones
Select a few colonies from the ampicillin plates for plasmid isolation. Confirm the positive clones containing gRNA sequences by Sanger sequencing.

B. Generation of donor DNAs for tagging *KRAS, NRAS,* and *Grb2*

1. Cloning of donor DNA for tagging KRAS and NRAS
Donor DNAs with mNG sequence flanked by 5' and 3' homology arms of approximately 500 b each were obtained from IDT as gene fragments. The fragments were amplified using primers and cloned into pCRBluntII-TOPO plasmid using TOPO PCR cloning kit.
   a. Amplification of the donor DNAs
   Centrifuge vials containing gene fragments to collect the precipitate in 50 µL 1× TE. Amplify the synthetic DNA using Phusion polymerase (Tables 10 and 11) and primers mentioned below (Table 9).

Table 9. Primers for amplification of KRAS and NRAS synthetic donor DNA

Primers	**5'----------3'**
KRas_5HAF	AGGTGGGGGTCCACTAGGAA
KRas_3HAR	CATATGATGTCACAATACCAAGAAAC
NRas_5HAF	CAGAGGCAGTGGAGCTTG
NRas_3HAR	ACAATCAGACAGTCTCGCTACTAT

Table 10. PCR set up for amplification of KRAS and NRAS synthetic donor DNA

	KRAS donor DNA	NRAS Donor DNA
Donor gene fragment	5 µL	5 µL
Phusion GC Buffer 5×	10 µL	10 µL
10mM dNTP	1 µL	1 µL
5HAF	2.5 µL	2.5 µL
3HAR	2.5 µL	2.5 µL
Phusion Polymerase	1 µL	1 µL
Nuclease free water	28 µL	28 µL

Table 11. PCR parameters for amplification of KRAS and NRAS synthetic donor DNA

Cycle Step	Temperature		Time	Cycles
	KRAS donor	NRAS donor		
Initial denaturation	98°C	98°C	1 min	1×
Denaturation	98°C	98°C	15 s	30×
Annealing	61.6°C	64.7°C	15 s	30×
Extension	72°C	72°C	1 min	30×
Final extension	72°C	72°C	10 min	1×
Hold	4°C	4°C	Hold	1×

Perform gel purification of 1.68 kb of PCR products from KRAS and NRAS amplified donor DNAs.

b. Ligation of amplified donor DNAs and pCRBluntII-TOPO plasmid
Set up ligation reaction of gel eluted KRAS and NRAS donor DNAs and pCRII-Blunt-TOPO plasmid in a 6 µL volume as follows (Table 12).

Table 12. Donor DNA and pCRBluntII-TOPO ligation set up

	KRAS Donor	NRAS Donor
Gel eluted fragment	2 µL	2 µL
Salt Solution	1 µL	1 µL
Nuclease free water	2 µL	2 µL
pCR-II-Blunt-TOPO	1 µL	1 µL

Incubate the ligation reaction at room temperature for 5 min. Transform 1–2 µL of the ligation mixture into competent *E. coli* and plate on kanamycin containing plates for selection.

c. Screening and confirmation of donor DNAs
Select few colonies from kanamycin plates and grow them to isolate plasmid. Confirm the positive clones by EcoRI digestion and by Sanger sequencing to make sure there are no changes in the donor sequence.

d. Digestion and gel elution of donor DNA sequences from positive clones
The fragments are inserted in pCRII-Blunt-TOPO in such a way that EcoRI sites lie at the ends of the insert, allowing recovery of the insert by EcoRI digestion. Digest the positive clones containing donor sequences with EcoRI as follows (Table 13).

Table 13. Digestion of pCR-II-Blunt-TOPO-Donor plasmid

	KRAS	NRAS
pCR-II-Blunt-TOPO-Donor (1 µg/µL)	20 µL	20 µL
CutSmart buffer 10×	5 µL	5 µL
EcoRI-HF (10 U/µL)	3 µL	3 µL
Nuclease free water	22 µL	22 µL

Carry out the digestion for 3 h at 72°C. Gel elute the 1.68 kb bands from both the samples. Store the eluted donor fragments at -20°C.
Note: The donor PCR fragments were cloned to facilitate the confirmation of correct donor sequence using Sanger sequencing and to avoid repeated amplification. Purified PCR fragments can be used directly as donor DNAs, thus reducing the time of cloning.

2. Cloning of donor DNA for tagging *Grb2*
To clone donor DNA for Grb2 gene editing, 5' and 3' homology arms (HA) were amplified from HeLa genomic DNA using primers designed by the NEbuilder algorithm (NEB), whereas KRAS donor DNA was used as a template to amplify mNG ORF. The primers add overlapping sequences to the resulting PCR fragments and these fragments are then assembled with Easy-Fusion Halo plasmid using NEBuilder® HiFi DNA Assembly Master Mix (Table 14).

Table 14. Component Fragments for HiFi DNA assembly

Name	Length (bp)	Produced by
5HA fragment	793	PCR
3HA fragment	743	PCR
mNG fragment	755	PCR
EasyFusion halo plasmid as backbone vector	2,927	Restriction Digestion

a. Amplification of homology arms (HA) and mNG ORF
Design primers with overlapping sequences using NEBuilder algorithm (Table 15). Amplify all three fragments required for the construction of the donor DNA as follows (Tables 16, 17, and 18).

Table 15. Primers for amplification of Grb2 homology arms and mNG ORF

Primer	5'……..3'
Grb2-5HA_f	TATCGATAAGCTTGATATCGAGAGGCAGTGTGTAGCCAG
Grb2-5HA_r	CTCCTCCTAAGACGTTCCGGTTCACGGG
Grb2-Neon_f	CCGGAACGTCTTAGGAGGAGGAGGATCAG
Grb2-Neon_r	TTGACTCTTACTTGTACAGCTCGTCCATG
Grb2-3HA_f	GCTGTACAAGTAAGAGTCAAGAAGCAATTATTTAAAG
Grb2-3HA_r	CCACCGCGGTGGCGGCCGCTTCTCGAACTCCTGACCTTG

Table 16. PCR set up for amplification of Grb2 Has

	5HA Fragment	3HA Fragment
HeLa genomic DNA (100 ng/μL)	3 μL	3 μL
Phusion GC Buffer 5×	10 μL	10 μL
10 mM dNTP	1 μL	1 μL
Grb2-5/3HA_f	2.5 μL	2.5 μL
Grb2-5/3HA_r	2.5 μL	2.5 μL
Phusion Polymerase	1 μL	1 μL
Nuclease free water	30 μL	30 μL

Table 17. PCR set up for amplification of mNG ORF

	mNG Fragment
mNG-KRAS donor (100 ng/μL)	3 μL
Phusion GC Buffer 5×	10 μL
10mM dNTP	1 μL
Grb2-Neon_f	2.5 μL
Grb2-Neon_r	2.5 μL
Phusion Polymerase	1 μL
Nuclease free water	30 μL

Table 18. PCR parameters for amplification of Grb2 HAs and mNG ORF

Cycle Step	Temperature			Time	Cycles
	5HA	3HA	mNG		
Initial denaturation	98°C	98°C	98°C	1 min	1×
Denaturation	98°C	98°C	98°C	15 s	30×
Annealing	64.4°C	60°C	59.6°C	15 s	30×
Extension	72°C	72°C	72°C	25 s	30×
Final extension	72°C	72°C	72°C	10 min	1×
Hold	4°C	4°C	4°C	Hold	1×

Gel elute the PCR products using QIAquick Gel Extraction Kit and estimate the DNA concentration of the eluted fragments.

b. Restriction enzyme digestion of EasyFusion-Halo plasmid.
Digest EasyFusion-Halo plasmid with EcoRI and XbaI to remove the Halo insert at 37°C for 4 h (Table 19).

Table 19. Digestion of EasyFusion-Halo plasmid to remove Halo insert

EasyFusion-Halo (5 μg)	10 μL
CutSmart buffer 10×	2 μL
EcoRI	2 μL
XbaI	2 μL
Nuclease free water	2 μL

Gel elute the 2.9 kb fragment of the digested plasmid using QIAquick Gel Extraction Kit and estimate the DNA concentration of the eluted fragment.

c. HiFi DNA assembly ligation reaction
Mix all four fragments required for the generation of Grb2 donor DNA to a final volume of 10 µL. Thaw HiFi DNA assembly mix on ice and vortex thoroughly. Add equal volume of HiFi DNA assembly master mix to the fragments (Table 20).

Table 20. Ligation set up for the generation of Grb2 donor DNA

0.1 pmol of 5HA	50 ng	0.8 µL
0.1 pmol of mNG	50 ng	0.9 µL
0.1 pmol of 3HA	50 ng	1.1 µL
0.1 pmol of digested vector	185 ng	2 µL
HIFi DNA assembly master mix		10 µL
Nuclease free water		5.2 µL

Carry out the ligation reaction at 50°C for 1 h. Transform 2 µL of the reaction mix into competent *E. coli.* Screen several colonies for positive clones containing all three fragments (5HA-mNG-3HA) using restriction enzyme digestion and Sanger sequencing.

C. Delivery of gRNAs and Donor DNAs in HeLa cells

1. Electroporation of RNP complex to tag *KRAS* and *NRAS*
   a. Grow HeLa cells in DMEM with 10% FBS to 90% confluency in a 75 cm^2 flask. Treat the cells with 100 ng/mL Nocodazole for 12 h.
   b. On the day of the electroporation, thaw and store buffer R, KRAS and NRAS specific *in vitro* transcribed gRNA, and donor DNAs on ice. Dilute gRNAs and donor DNA to appropriate concentrations in nuclease free water.
   c. Wash away Nocodazole with 1× DPBS. Split the cells with Trypsin and inactivate it by the addition of FBS containing DMEM. Centrifuge the single cell suspension at 300 × *g* for 5 min. Resuspend the pellet in 10 mL of DMEM with 10% FBS. Perform cell counting using hemocytometer.
   d. Prepare RNP mixture as follows (Table 21), one for each gene-editing reaction in a 1.5 mL microcentrifuge tube.

Table 21. Components of RNP mixture for electroporation

Buffer R	1 µL
Platinum Cas9 (1 µg)	0.5 µL (1 µg)
gRNA (500 ng)	2 µL

   e. Incubate RNP complex at room temperature for 20 min.
   f. While the RNP is incubating, prepare electroporation set up and keep cells ready for the electroporation by centrifuging them at 300 × *g* for 5 min. Aspirate DPBS and resuspend the cell pellet to a final cell density of 8×10^7 cells/ml in buffer R.
   g. Add 3 mL of Electrolytic buffer (Buffer E) into the Neon Pipette station.
   h. Add 5 µL of cells (final 4×10^5 cells) and 1 µg of respective donor DNA fragments to the RNP mixture. Make sure the volume of the final mixture is 10 µL. Aspirate the final mix in Neon Tip using Neon Pipette without incorporating air bubbles and electroporate at pulse voltage of 1,005 V, pulse width of 35 ms for 2 pulses.
   i. Transfer the electroporated cells in a 12-well plate containing prewarmed DMEM with 10% FBS without antibiotics and continue incubating the plate at 37°C in a CO_2 incubator.

2. Transfection of gRNA and donor to tag *Grb2*
   a. Grow HeLa cells in DMEM with 10% FBS to 90% confluency in 12-well plates. Treat the cells with 100 ng/mL Nocodazole for 12 h.

b. On the day of the transfections, dilute PX459-Grb2-gRNAs and Grb2-mNG donor DNA plasmids to appropriate concentrations in nuclease free water.
c. Wash away Nocodazole with 1× DPBS. Add 1 mL of fresh FBS containing DMEM.
d. Prepare DNA-lipid mixture as follows (Table 22), in a 1.5 mL microcentrifuge tube in the order given.

Table 22. Components of transfection mixture to tag Grb2

	Tube 1
OptiMEM	50 µL
PX459-Grb2-gRNA (250 ng)	0.5 µL
Grb2-mNG donor (750 ng)	1 µL
P3000 reagent	2 µL
	Tube 2
OptiMEM	50 µL
Lipofectamine 3000 Reagent	1.5 µL

e. Add contents from Tube 1 to Tube 2. Incubate the mixture at RT for 10 min.
f. Add plasmid-lipid mixture to the cells dropwise.
g. Next day after the transfection, treat transfected cells with 2 µg/mL of puromycin for 48 h to enrich transfected cells by killing un-transfected cells.
h. Remove puromycin containing DMEM, wash the cells with DPBS twice and allow the cells to recover. Split the surviving cells and expand them for flow sorting analysis.

D. Flow Sorting of mNG positive cells

1. Expand tagged HeLa cells in DMEM with 10% FBS in 25 or 75 cm^2 flasks.
2. On the day of sorting, split the cells using Trypsin and inactivate it with FBS containing DMEM. Centrifuge the cells and wash once with PBS. Resuspend the final pellet in Cell sorting buffer.
3. Pass the cell suspension through disposable filters to get rid of cell aggregates just before the sorting. Use positive (parental HeLa cells transiently or stably expressing mNG) and negative (parental untagged HeLa cells) controls to calibrate the sorting instrument.
4. Collect single sorted cells in 96-well plate or in a 15 mL conical tube as a pooled population with prewarmed DMEM with 10% FBS and antibiotics to prevent contamination.

E. Confirmation of positive clones

1. Freeze at least one vial of single cell clones as a backup before using them for confirmation.
2. Clones can be confirmed by Western blot analysis using protein-specific antibody or mNG antibody. The correct fusion protein should have an apparent molecular mass ~25 kD larger than the parental protein. It is also important to sequence the region around the site of mNG sequence insertion to make sure there are no unwanted insertions or deletions during homology repair.
   Note: Head-to-head comparison of efficiency of different delivery methods was not performed. In all the cases, the yield of mNG positive cells as determined during flow sorting was 0.1% to 0.5%. Since we were interested in single cell colonies for screening, one 96 well plate of single sorted cells was sufficient to screen them. It should be noted that flow sorting could inadvertently result in clones expressing higher yields of fusion protein.

Data analysis

1. Characterization of the tagged cell line clones

Select several colonies arising from flow sorted population. Process the colonies for DNA isolation for Sanger sequencing and for making lysates for Western blot analysis. To generate PCR products for Sanger sequencing, design primers that can amplify the region beyond the homology arms. The sequencing should reveal the correctly inserted mNG sequence as well as linkers and start/stop codons. Western blot should reveal the correct molecular weight of the fusion protein. For example, a screen of HeLa/mNG-KRAS clones using Western blot is shown in Figure 2. The colonies were lysed in TGH buffer, and the lysates were electrophoresed and transferred onto nitrocellulose membrane. We detected two homozygous clones in 24 colonies screened. Load lysates from parental cell line as control for the WT untagged protein. It is essential to make sure that off target changes occurring during Cas9 mediated gene editing do not affect the cells in such a way that they are unfit for the study. Since our focus is to study EGFR-RAS-MAPK pathway, we chose the clones that exhibited similar ERK1/2 phosphorylation kinetics in HeLa parental and tagged KRAS and NRAS cells in presence of EGF (Surve *et al.*, 2021).

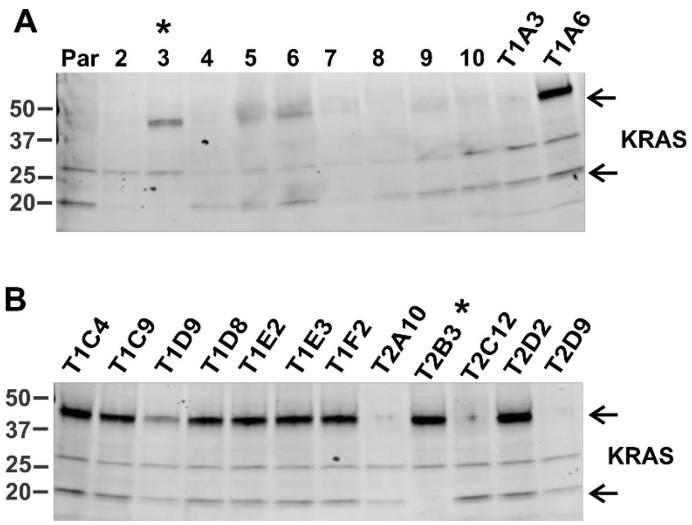

Figure 2. Screening of HeLa/mNG-KRAS single cell clones.
The single cell colonies were obtained either from a 10-cm plate where transfected cells were diluted or from a flow cytometer sorted population in a 96 well plate. First screening was done by checking cell fluorescence using a confocal microscope. Second screening was performed by probing lysates of parental (Par) HeLa and single-cell clones of HeLa/mNG-KRAS cells using Western blotting (WB) with KRAS antibodies to determine the zygosity of the clones expressing mNG-KRAS. Clones from 2 to 10 (in ***A***) were obtained from 10 cm plate, whereas remaining clones were obtained via flow sorting. Clone names starting with T1 are from Target 1 gRNA, whereas those with T2 are the results of Target 2 gRNA transfection. Top arrow in ***A*** and ***B*** indicate mNG-KRAS fusion protein, whereas lower arrow indicates untagged KRAS protein. Clones with asterisk are homozygous clones as they are missing a band corresponding to the untagged native KRAS.

2. Examples of localization of endogenous tagged KRAS, NRAS and Grb2

After the screening and confirmation of the tagged cell lines, we imaged living tagged cell lines with spinning disc confocal microscope (Figure 3).

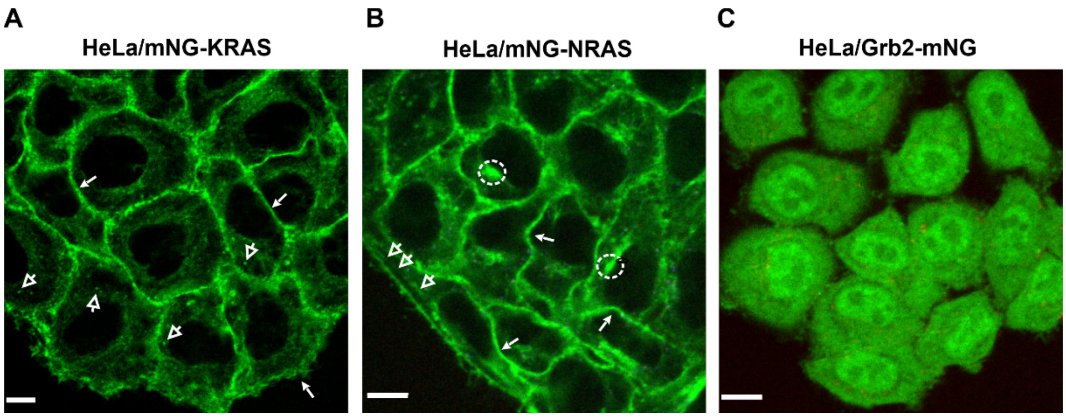

Figure 3. Localization of endogenous tagged KRAS, NRAS, and Grb2 with mNG in HeLa cells. Live cell imaging was performed using a spinning disc confocal microscope through 488 nm (green; mNG) channel. Single confocal sections are shown. **A.** mNG-KRAS localized mostly at the plasma membrane (closed arrows), and a small proportion also localized in vesicles (open arrows). **B.** Majority of mNG-NRAS localized at the plasma membrane. mNG-NRAS was also in the juxta-nuclear region (dotted circle) and tubular shaped structures (open arrows). **C.** Grb2-mNG localized to cytoplasm and nucleus.

Recipes

1. **Cell Sorting buffer**
   1× PBS (Ca/Mg++ free)
   2% heat-inactivated FBS
   1 mM EDTA

2. **TGH buffer**
   1% Triton X-100
   10% glycerol
   50 mM HEPES
   2 mM EGTA
   Phosphatase and protease inhibitors

Acknowledgments

Authors thank Drs Feng Zhang, Broad Institute, Cambridge, MA, and Janet Rossant, Peter Gilgan Centre for Research and Learning, The Hospital for Sick Children, Toronto, ON, Canada, for plasmids. This study was supported by NIH grants CA089151 and GM124186. S. Surve was also supported by the fellowship from National Cancer Center. This protocol was adapted from Surve *et al.* (2021).

Competing interests

The authors declare no competing financial interests.

References

Howe, C. L., Valletta, J. S., Rusnak, A. S. and Mobley, W. C. (2001). NGF signaling from clathrin-coated vesicles: evidence that signaling endosomes serve as a platform for the Ras-MAPK pathway. *Neuron* 32(5): 801-814.

Jiang, X. and Sorkin, A. (2002). Coordinated traffic of Grb2 and Ras during epidermal growth factor receptor endocytosis visualized in living cells. *Mol Biol Cell* 13(5): 1522-1535.

Karnoub, A. E. and Weinberg, R. A. (2008). Ras oncogenes: split personalities. *Nat Rev Mol Cell Biol* 9(7): 517-531.

Lu, A., Tebar, F., Alvarez-Moya, B., Lopez-Alcala, C., Calvo, M., Enrich, C., Agell, N., Nakamura, T., Matsuda, M. and Bachs, O. (2009). A clathrin-dependent pathway leads to KRas signaling on late endosomes en route to lysosomes. *J Cell Biol* 184(6): 863-879.

Pinilla-Macua, I., Grassart, A., Duvvuri, U., Watkins, S. C. and Sorkin, A. (2017). EGF receptor signaling, phosphorylation, ubiquitylation and endocytosis in tumors *in vivo*. *Elife* 6: e31993.

Pinilla-Macua, I., Watkins, S. C. and Sorkin, A. (2016). Endocytosis separates EGF receptors from endogenous fluorescently labeled HRas and diminishes receptor signaling to MAP kinases in endosomes. *Proc Natl Acad Sci U S A* 113(8): 2122-2127.

Pol, A., Calvo, M. and Enrich, C. (1998). Isolated endosomes from quiescent rat liver contain the signal transduction machinery. Differential distribution of activated Raf-1 and Mek in the endocytic compartment. *FEBS Lett* 441(1): 34-38.

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A. and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 8(11): 2281-2308.

Schmick, M., Vartak, N., Papke, B., Kovacevic, M., Truxius, D. C., Rossmannek, L. and Bastiaens, P. I. H. (2014). KRas localizes to the plasma membrane by spatial cycles of solubilization, trapping and vesicular transport. *Cell* 157(2): 459-471.

Surve, S., Watkins, S. C. and Sorkin, A. (2021). EGFR-RAS-MAPK signaling is confined to the plasma membrane and associated endorecycling protrusions. *J Cell Biol* 220(11).

Surve, S. V., Myers, P. J., Clayton, S. A., Watkins, S. C., Lazzara, M. J. and Sorkin, A. (2019). Localization dynamics of endogenous fluorescently labeled RAF1 in EGF-stimulated cells. *Mol Biol Cell* 30(4): 506-523.

von Zastrow, M. and Sorkin, A. (2021). Mechanisms for Regulating and Organizing Receptor Signaling by Endocytosis. *Annu Rev Biochem* 90: 709-737.