Feb 15, 2022

YAP1 mKate:BSD HDR Knock-in (via Cas9 RNP lipofection)

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Emir Bora Akmeriç 2022. YAP1 mKate:BSD HDR Knock-in (via Cas9 RNP lipofection). **protocols.io** https://protocols.io/view/yap1-mkate-bsd-hdr-knock-in-via-cas9-rnp-lipofecti-b4sbqwan

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Materials for Reverse RNP Transfection:

1.

Opti-MEM (Reduced Serum Medium) **Thermo Fisher Scientific Catalog #31985062** Step

2.

Lipofectamine™ RNAiMAX Transfection Reagent **Thermo Fisher Catalog #13778030** Step

Nuclease Free Duplex Buffer **IDT**

3. **Technologies Catalog #11-01-03-01** Step or

TE Buffer **Contributed by users** Step

4. Alt-R® CRISPR-Cas9 crRNA (Integrated DNA Technologies [IDT] for YAP1 (HDR_YAP_Pref 5'-/AltR1/rUrUrA rGrArA rUrUrC rArGrU rCrUrG rCrCrU rGrArG rUrUrU rUrArG rArGrC rUrArU rGrCrU /AltR2/ -3'))

5.CRISPR-Cas9 tracrRNA (IDT)

6. MDC Homebrew eSpCas9 in HEPES buffer(pH 7.5)(41 µM)
6b. 20 mM Hepes pH 7.5, 0.15 M KCl, 1 mM DTT

7. As negative CRISPR/Cas control: Alt-R® CRISPR-Cas9 Negative Control crRNA #1

Culturing Immortalized HUVECs 20m

1 In a t25 flask, add 10 mL of EGM2 (with antibiotics). Incubate flask for 20 minutes 20m

1.1 Thaw a 500k/1M CI-huVEC tube in a 37C water bath and add the cells into the flask.

1.2 Change media 6-8 hours later or when cells have adhered to the flask surface to remove excess DMSO.

1.3 Culture until confluent. Prior to 80% confluency, change media every other day. After 80% confluency, change media daily.

RNP Complex Formation and Reverse RNP Transfection

2 Materials for Reverse RNP Transfection:

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☒Nuclease Free Duplex Buffer **IDT**

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2.1 Resuspend lyophilized crRNA and tracrRNA in nuclease-free TE buffer to stock concentrations of 100 µM.

2.2 **For each condition:**
Mix 1 µL of each stock solution (crRNA and tracrRNA) with 98 µL nuclease-free duplex buffer to form 1 µM crRNA:
tracrRNA complexes. Incubate at 95 C for 5 min and allow the complexes to cool down to room temperature

3 Prepare 10 µM eSpCas9 in HEPES buffer. For example: 7.32 µL of 41µM eSpCas9 in 22.86 HEPES buffer to have a final Volume of 30µL 10µM eSpCas9.

Prepare a working solution of 1 µM eSpCas9 in Opti- MEM™.
Always prepare fresh Cas9 nuclease V3 working solution before complexing with crRNA:tracrRNA.

4 Mix 6 µL of crRNA:tracrRNA complex (1 µM), 6 µL of Cas9 working solution (1 µM), and 88 µL of Opti-MEM™ I per replicate to form crRNA:tracrRNA:Cas9 complexes in a tube. Incubate at room temperature for 5 min. Mix the protein solutions gently by inverting the tube.

For exapmle for 5 wells in 24 well plate
30µL of crRNA:tracrRNA complex (1 µM)
440µL of Opti-MEM™

5 Combine 100 µL of the crRNA:tracrRNA:Cas9 complexes , 4.8 µL of Lipofectamine™ RNAiMAX, and 95.2 µL of Opti-MEM™ I per replicate to get the final transfection solution. Incubate at room temperature for 20 min. Mix properly by pipetting up and down or by inverting the tube.

6 Wash CI-huVEC with PBS during incubation of the transfection solution. Trypsinize the cells, stop the reaction with FBS and EBM 1:1:1, after detachment and centrifuge for 5 min at 300 x g. Resuspend the cell pellet in EGM2 (-Gentamycin) and count the cells.

7 Use EGM2(-Gentamycin) to adjust the cell concentration to 90,000 cells per mL.

8 After incubation of the transfection solution, pipette 200 µL of the transfection solution into one well of a 24-well plate. Gently mix the complexes by pipetting before adding to the well.

9 Add 400 µL of the cell suspension to the transfection complexes to obtain a final concentration of 10 nM RNP and 36,000 cells/well. Gently mix by pipetting and swirl the plate to ensure sufficient distribution of cells and complexes

10 Incubate at 37 °C and 5% CO2. Change medium (EGM2) every 2–3 days.

11 When cells reach **at least** 80% confluency, wash with 500 µLof PBS and trypsinize with 200 µL of Trypsin/EDTA. Stop the reaction with 1 mL of EGM2 after cell detachment.

12 Subculture 800 µL (2/3 of the cells) to one well of a 6-well plate, add 1 mL EGM2, and culture the cells at 37 °C and 5% CO2 .

13 Centrifuge the remaining cells (1/3) for 5 min at 300 x g. Aspirate and discard the supernatant. The supernatant should be removed completely, as any residual medium can inhibit PCR

efficiency. Resuspend and lyse the cell pellet in 50 µL of our DNA Extraction Solution and transfer the lysate to a PCR tube.

Estimation of Genome Editing Efficiency

14 **Materials for Estimation of Genome Editing Efficiency:**

NEBuffer 2 - 5.0 ml **New England**

1. **Biolabs Catalog #B7002S** or NEBuffer™2 10x (NEB): 500mMNaCl, 100mMTris–HCl, 100 mM MgCl2, 10 mM DTT, pH 7
2. Nuclease-free H2O

T7 Endonuclease I - 250 units **New England**

3. **Biolabs Catalog #M0302S**
4. Agarose gel
5. Gel loading buffer (6x)
6. TBE Buffer 10x: 890 mM Tris, 890 mM boric acid, 20 mM Titriplex III

Incubate extracted DNA at 65 °C for 15 min and for another 15 min at 95 °C in a thermal cycler. Store at 4 °C.

15 Determine the DNA concentration of the lysed cells DNA extract at the Nanodrop.

16 Amplify the target region by PCR using 2–20 ng of genomic template DNA regarding the PCR protocol.

17 Following PCR, form heteroduplexes by combining 5 µLof PCR product with 1.5 µL of NEBuffer™ 2 (10x) and 6.5 µL of nuclease-free H2O. Use 1.5 µL of T7EI (10 U/µL) and NEBuffer™2 (10x) stock solutions and fill up to 15 µL with nuclease-free H2O to dilute T7EI to 1 U/µL. Volumes can be adjusted as required

18 Heat to 95 °C in a thermal cycler for 10 min and cool down to 85 °C with a ramp rate of -2 °C/s. Next, cool down to 25 °C with a ramp rate of -0.3 °C/s.

19 Prepare a 2% agarose gel with 1x TBE in a gel tray.

20 Add 3 µL of 6x loading dye and separate the digested products by gel electrophoresis. A unique band pattern indicates successful CRISPR/Cas9 genome editing.

During T7EI digestion, mismatch positions in the hetero duplexed PCR products are specifically cleaved, whereas homo- duplex DNA will not. This results in three specific bands after separation by gel electrophoresis. Measuring their intensities enables an estimation of the CRISPR/Cas9-induced indel rate.

Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F (2013). Genome engineering using the CRISPR-Cas9 system.. Nature protocols.
https://doi.org/10.1038/nprot.2013.143

21 Determine the volume intensities of the fragments and estimate indel rates as described in Ran FA et al before.

$$\text{Indel}(\%) = 100 * \left(1 - \sqrt{1 - \frac{\text{digested fragment 1} + \text{digested fragment 2}}{\text{digested fragment 1} + \text{digested fragment 2} + \text{undigested fragment}}}\right)$$

Calculation for Indel rates

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