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

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Materials for Reverse RNP Transfection:

1.

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

2.

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

Nuclease Free Duplex Buffer **IDT**

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

TE Buffer **Contributed by users**

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

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.

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Culturing Immortalized HUVECs

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

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

1.2 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 Make a table for set of conditions on a 24 well plate-

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

3.1 To prepare the gRNA complex, combine the crRNA and tracrRNA. Take 1 uL from each component, and add 98 µL of Duplex Buffer to bring it to a final concentration of 1 µM.

3.2 Heat the mixture at 95C for 5 minutes, followed by letting it cool to room temperature on the bench top.

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4 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. Mix well and briefly centrifuge

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

5 To form crRNA:tracrRNA:Cas9 complexes in a tube and to start excision of homology casette, mix 6 µL of crRNA:tracrRNA complex (1 µM), 6 µL of Cas9 working solution (1 µM), and HDR Donor Plasmid(0.6-1.5 µg). Bring volume to 100 uL using OptiMEM. Mix the protein solutions gently by inverting the tube.

For example for 5 wells in 24 well plate
1)30µL of crRNA:tracrRNA complex (1 µM)
2)10 nM-25 nM HDR Donor Plasmid(Set two replicates with two concentrations per condition)
3)30µL of Cas9 (1 µM)
4)OptiMEM to bring to 500 µL

5.1 In a seperate tube**, per well**, mix 4.8 uL of Lipofectamine RNAiMAX and 95.2 µL Opti-MEM. Incubate for 5 minutes

6 Combine both tubes 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.

7 Wash CI-huVEC with **warm** 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 500 x g. Resuspend the cell pellet in EGM2 (-Gentamycin) and count the cells.

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

8.1 If IDT HDR Enhancer V2 is used, add 1 µM HDR Enhancer V2(Stock concentration **690 µM)** to the mixture.
IMPORTANT: IF ENHANCER IS ADDED, MAKE A CONTROL SAMPLE WITH 1% DMSO

9 After incubation of the transfection solution, pipette 200 µL of the transfection solution into

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one well of a 24-well plate. Gently mix the complexes by pipetting before adding to the well.

10 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

11 Incubate at 37 °C and 5% CO2.

11.1 After 24h change medium w/ EGM2 without antibiotics.

11.2 48 hours post transfection, change medium w/ EGM2 **with Blasticidin S.** 10 µg/mL concentration is suggested per Anna's experiments for immortalized HUVECs and HUVECs in general. Change media depending on overall cell status preferably after 48 hours.

11.3 ⚠

3-4 days after antibiotic addition, check the positive conditions on the 24 well plate under a light microscope for cell fitness, morphology and overall phenotype. Additionally, check for mKate2 fluorescence and acquire images of ~200 cell in total. From these conditions, mark the wells you would like to subculture.

NOTICE: If the fluorescent cell ratio is above 90%, continue with the expansion(step 12). For fluorescence ratio between 10-90%, use a dilution assay to produce single cell colonies or FACS sorting(different protocol).

12 When cells reach **at least** 80% confluency, wash with 500 µLof PBS and trypsinize with 200 µL of Trypsin/EDTA. Stop the reaction with 0.5 mL EGM2+0.5 mL FBS after cell detachment.

13 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.

14 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.

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Estimation of Genome Editing Efficiency

15 Determine the DNA concentration of the lysed cells DNA extract at the Nanodrop. Either do steps 16-21 OR do PCR and gel staining using primers for Genomic PCR primers. For the latter approach, have Genomic PCR primers and untreated cell culture DNA extract ready.

15.1 Following gel assay, send two components for sequencing:
1) DNA extract
2)Sequencing primer for YAP Exon 9 C-termini. The sequencing, if successful, would provide ab1 files with nucleotide sequences of Donor plasmid casette

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

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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

Immunofluorescence/Live Microscopy Testing

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