CRISPR knock-in protocol for cell lines (Ayaz Najafov)

(based on the 1% DMSO knock-in efficiency enhancement discovery by Stratigopoulos et al., PLoS One. 2018 Jun 4;13(6):e0198637.)

1) Seed HT-29 cells into 6-well plates in 3ml McCoy's medium (10% FBS + Antibiotic/Antimycotic).
2) 24 hrs later, at 80% confluence, treat with 1% DMSO (in the old medium).
3) 24 hrs after DMSO addition transfect the cells (in the old medium).

   Per well:

   - **Tube A:** 125 µl OptiMEM + 2 µg pX459-puro-sgRNA plasmid + 3 µg Mutant PCR donor + 5 µl P3000
   - **Tube B:** 125 µl OptiMEM + 7.5 µl Lipofectamine 3000
   - Mix the tubes A and B. Pipette to mix. Incubate at 25^{o}C for 15min. Deliver 250 µl per well.
4) 24 hrs later (1 day post-transfection), expand each well into a 15cm dish.
5) 24 hrs later (2 days post-transfection), add fresh medium with puromycin (2.5 µg/ml).
6) 48 hrs later (4 days post-transfection), add fresh medium with puromycin (2.5 µg/ml).
7) Single-cell sort by flow cytometry, do limited dilution or keep in 15cm plates for colony picking.
8) At 7 days post-transfection, switch to puromycin-free medium.
9) Grow for 2 weeks.
10) Make two replicates of the clones, in 12-well plates (one for propagation and one for genotyping). Spin the plates to enhance cell attachment: 1,000xg, 3min, 25^{o}C.
11) 48 hrs later:
    a. Feed with the replicates for propagation with 1ml new medium
    b. Trypsinize the replicates for genotyping and transfer the cell pellets into 8-strip PCR tubes.
12) Generate cell lysates using Tail Lysis protocol (Truett et al., BioTechniques 29:52-54 (July 2000))
    a. Add 75 µl of tail lysis buffer (25 mM NaOH, 1 mM EDTA).
    b. Heat at 95^{o}C for 1hr.
    c. Put tubes on ice for 5min.
    d. Add 75 µl of neutralization buffer (40 mM Tris-HCl, pH5, dissolve Tris-HCl, no pH adjustment).
13) Setup PCR:
    a. 5 µl 2X EmeraldAmp® GT PCR Master Mix
    b. 5 µl Nuclease-free water
    c. 0.5 µl 10 µM F+R primer stock
    d. 0.5 µl of the cell lysate.
        - 98^{o}C 2min (hot start)
        - 40 cycles:
            - 98^{o}C 15sec
            - 60^{o}C 30sec
            - 72^{o}C 1min
        - 72^{o}C 5min
14) Prepare the PCR products for sequencing:
    a. 11 µl PCR product + 1 µl ExoI + 2 µl rSAP
    b. Incubate at 37^{o}C for 30min.
    c. Incubate at 80^{o}C for 20min.
    d. Cool to 4^{o}C.
15) Send the whole reaction for sequencing with the forward primer used to generate the PCR donor.

Materials:

EmeraldAmp® GT PCR Master Mix	Clontech, RR310B
Exonuclease I (ExoI)	NEB, M0293S
Shrimp Alkaline Phosphatase (rSAP)	NEB, M0371S

Mutant PCR donor = ~150bp PCR amplicon (with the desired point mutation in the middle) purified using Qiagen's PCR purification kit.