

## **Rare Allele Induction and Detection (RAID) Protocol Version 1.1**

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**Reference; Miyaoka et al., Isolation of single-base genome-edited human iPS cells without antibiotic selection, *Nature Methods* 11, 291–293 (2014)**

In this protocol, we describe main procedures A-F, from design of primers and probes to iPS cell isolation. Refer to additional files for the details of each procedure as described in this protocol.

**A. Design of TALENs/guide RNAs, oligo DNA donors, TaqMan probes, and primers.**

**B. Validation of TaqMan probes and primers.**

**C. Validation of TALEN/CRISPR/Cas9 double nickase activity in 293 cells.**

**D. Transfection of human iPS cells.**

**E. RAID-ddPCR and sib-selection.**

**F. Cloning of iPS cells and genotyping PCR.**

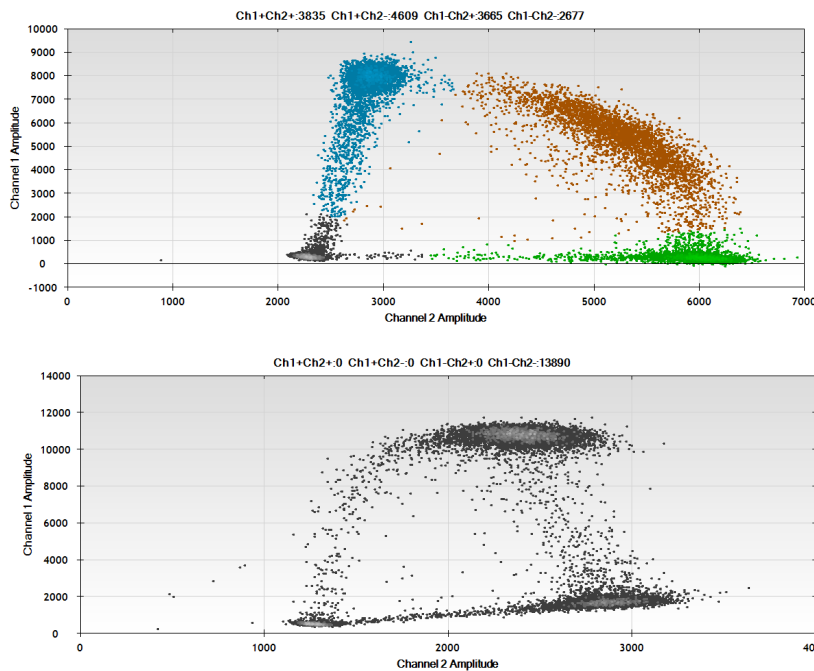
**A. Design of TALENs/guide RNAs, oligo DNA donors, TaqMan probes, and primers.**

1. Sequence your target genomic region in human iPS cells and HEK293 cells. If the sequence of either TALEN/guide RNA target sites, TaqMan probe binding sites, or primer binding sites is different between iPS cells and HEK293 cells, you have to find an easy-to-use cell line with the same sequences as iPS cells instead of HEK293 cells.
2. Design a pair of TALENs by ZiFiT or TALEN targeter. I usually use ZiFiT, but both work. For guide RNAs for the CRISPR/Cas9 double nickase system, I use CRISPR Design at MIT and pX335 (Addgene 42335). You can design several TALENs/guide RNAs that target slightly different target sequences so that you can try several different combinations of TALENs/guide RNAs. It is ideal to have your point mutation in between the pairs of TALENs/guide RNAs to maximize the efficiency.
3. Order a 60 nt oligo DNA donor that has the mutation in the right middle of it (29 nt homologous 5' part-a nucleotide substitution-30 nt homologous 3' part). Order it as a DNA pellet and reconstitute it in water to 3 ug/ml. Simple desalting purification works fine.
4. Design allele specific TaqMan probes and primers by using Primer Express 3.0 (Life Technologies). Open a new document, choose the "TaqMan® MGB Allelic Discrimination",

put your sequence, and design probes and primers with the default settings. Follow the “Designing Primers and Probes for Allelic Discrimination Assays” section of the instruction (*cms\_041902.pdf*). Make sure to have at least one of the two primers outside of the donor oligo DNA. Conjugate your mutated and wild-type allele probes to FAM and VIC, respectively. If you cannot find good probes and primers automatically, you may change parameters, design probes and primers for the complementary sequence, or you can manually design them following the “Manually Designing Primers and Probes” section in the instruction (*cms\_041902.pdf*). Order MGB-conjugated TaqMan probes (as Custom TaqMan® MGB Probes at Life Technologies) and primers (e.g. normal grade primers at IDT) separately. You can order them as a mixture (as Custom TaqMan® SNP Genotyping Assays at Life Technologies), but we do not recommend this option, because you cannot replace only one of the two probes when the assay does not work properly.

**B. Validation of TaqMan probes and primers, and TALEN activity in 293 cells.**

1. Mini-prep positive control plasmids for both a wild-type allele and a mutated allele. Make sure that the plasmids have probe and primer binding sites. Dilute them to 0.5 pg/ul and combine them to make a 1:1 mixture. You can also synthesize both alleles from vendors, e.g. gBlocks. If your DNA fragment is 300 bp, dilute it to 0.05 pg/ul. Note: the most dangerous thing in this assay is this positive control mixture. If a reagent is contaminated with this mixture, it will kill your assay. Handle positive control mixtures CAREFULLY.
2. Test probes and primers by ddPCR with 0.5 (plasmid) or 0.05 (synthesized DNA fragment) pg of the 1:1 mixture. Perform a 50 to 60C temperature gradient PCR. You are supposed to see distinct negative, FAM-positive, VIC-positive, and double-positive populations in the 2D-plot. If not, you have to redesign your probes and/or primers. See *ddPCR™ protocol.docx* for the details.
3. You can extend the temperature gradient to find the best temperature, which is the highest temperature at which the best separation of the four populations is achieved. Typically 51C to 57C.
4. Optional: You can make a dilution series of the mutated allele to see how sensitive the ddPCR assay for your target is.



**Upper: Good example.**

In this 2-D plot, negative (grey), FAM+ (blue), VIC+ (green), and double+ (brown) populations are distinguishable.

**Lower: Bad example.**

FAM+ and double+ populations are merged, so there are only three populations. We solved this problem by changing the FAM probe.

**C. Validation of TALEN activity in 293 cells.**

1. Transfect HEK293 cells with a TALEN/pX335 pair (400/200 ng each) and an oligo donor DNA (800/400 ng) by using LipofectAmine 2000. As a negative control, prepare a well for an unrelated TALEN/pX335 pair (400/200 ng each) and the oligo donor DNA (800/400 ng). Transfection is done in 12/24-well format ( $4 \times 10^5 / 2 \times 10^5$  cells/well).
2. Extract gDNA. For example, by using Qiagen DNeasy Blood & Tissue Kit.
3. Measure allelic frequency of the mutated allele by ddPCR. Include a water control and the negative/positive (1:1 mixture of plasmids) control. See *ddPCR™ protocol.docx* for the details.
4. Optional: You can try Surveyor assay to validate the TALEN activity.

**D. Transfection of human iPS cells.**

1. Transfect  $2 \times 10^6$  human iPS cells with a TALEN/pX335 pair (3 ug each) and an oligo donor DNA (6 ug) by using Nucleofector human Stem Cells Solution 1 (Lonza). As a negative control, transfect  $2 \times 10^6$  cells with an unrelated TALEN/pX335 pair (3 ug each) and the oligo DNA (6 ug). After transfection, a half of the cells for the mutagenesis are resuspended in 5 ml Essential 8 (E8) + Rock inhibitor (Ri) and plated on 48 Matrigel-coated wells of a 96-well plate. One eighth of the control cells are resuspended in 1.25 ml E8 + Ri and plated

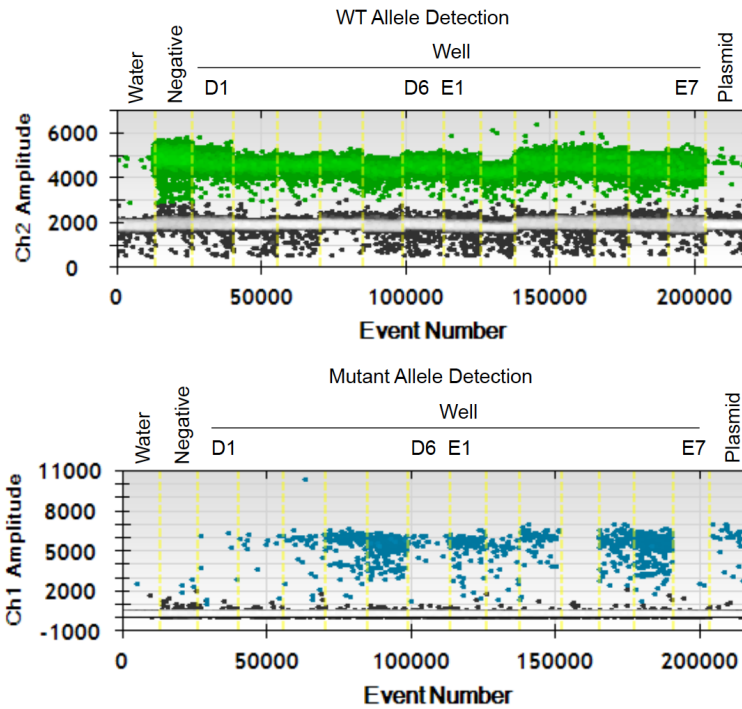
on 12 Matrigel-coated wells of a 96-well plate. See *Nucleofection.docx* for the details.

2. Exchange media to E8 2 days after transfection.
3. Usually, cells reach 80-90% confluency by 4 days after transfection. Extract gDNA from the control cells. Treat the cells for mutagenesis with 30 ul Accutase/well, transfer 15 ul of the cell suspension to 50 ul cell lysis buffer/well on a 96-well plate for gDNA extraction, and freeze another 15 ul by adding 75 ul freezing media and 75 ul mineral oil/well at -80C. See *gDNAExtraction96well.docx* and *Freezing and Thawing 96-Well Plate.docx* for the details.

#### **E. RAID-ddPCR and sib-selection.**

1. Measure the mutant allelic frequency of the transfected cells by ddPCR. Because a lot of cells are plated per well right after transfection, I usually do not see a great variability among different wells. Ten to twenty wells are analyzed. See *ddPCR™ protocol.docx* for the details.
2. Thaw the frozen plate, and transfer the cells in the first to fourth or fifth best wells to 200 ul E8 + Ri in a 1.5 ml tube to mix. Count the number of the cells from the best well by Countess (Life Technologies). Transfer the cells to E8 + Ri in a 15 ml tube and plate the cells in a 96-well plate. For our iPS cell line, WTC, we follow this guideline: when <0.5% mutant allelic frequency -> 500 cells/well, 0.5- 1% -> 350 cells/well, 1-3% -> 250 cells/well, >3% -> 150 cells/well. I usually plate 48 wells. You can plate the whole 96 wells, but we rarely go through all of them. Spin down the rest of the cells in the best well and the other wells at 5000 min for 3 min, remove the oil and medium carefully, resuspend the cells in E8 + Ri, and plate the cells on 24-wells. See *Freezing and Thawing 96-Well Plate.docx* for the details. Because the cells are plated right after the transfections, clones from these different wells are independent of each other. Therefore, it is very important to keep them, if you want to have multiple independent lines.
3. Feed the cells with E8 + Ri every other day until cells look like they are in stable clusters of 10 or more cells.
4. Switch to E8 media (without Ri) and continue feeding cells every other day until cells are about 70-80% confluent.
5. Treat the cells with 30 ul Accutase/well, transfer 15 ul of the cell suspension to 50 ul cell lysis buffer/well on a 96-well plate for gDNA extraction, and freeze another 15 ul by adding 75 ul freezing media and 75 ul mineral oil/well at -80C.
6. Go to Step 1 to measure the allelic frequency. Repeat this cycle until the allelic frequency

reaches at least 5%. We typically plate 500 cells/well when the allelic frequency is below 1%, and then 200 cells/well after the frequency reaches >1%.



### Typical sib-selection result.

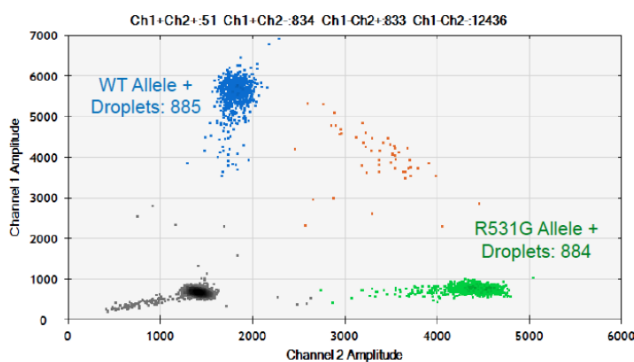
Thirteen 96-wells (D1-D6 and E1-E7) were analyzed by ddPCR to measure WT (upper, green) and mutant (lower, blue) allelic frequencies. “Negative” is cells treated with unrelated TALENs and “Plasmid” is a control plasmid mixture. The WT allele was detected in all the wells analyzed at a similar level, whereas the mutant allele was not detected in some wells and the droplet number was highly variable, indicating enrichment in some wells.

### F. Cloning of iPS cells and genotyping PCR.

1. Thaw the frozen plate, and transfer the cells in the first to third or fourth best wells to 200 ul E8 + Ri in a 1.5 ml tube to mix. Count the number of the cells from the best well by Countess. Transfer the cells to E8 + Ri in a 15 ml tube and plate the cells at 50 cells/well on half a 96 well plate and 25 cells/well on another half of the 96 well plate.
2. Feed the cells as described in steps 3-4 in RAID-ddPCR and sib-selection protocol.
4. Usually, at 14 days after the plating, ~30% of the wells have survived cells and the color of the media in these wells is yellow. Treat these wells that have yellow media with 30 ul Accutase, and transfer 15 ul of the cell suspension to 50 ul cell lysis buffer/well on a new 96-well plate for gDNA extraction. Add 75 ul freezing media to the Accutase-treated wells to resuspend the cells and transfer the cell suspension to a new 96-well plate. Overlay 75 ul mineral oil/well and freeze the plate at -80C. Rearrange the order of the clones on new 96-well plates and make sure that the plates for gDNA extraction and the frozen plates are replica plates.
5. Screen the individual wells using ddPCR to determine the composition of the well. If the

mutant:WT allele frequency is 49-51%, the clone is purely heterozygous. Sometimes, homozygous clones can be isolated (almost 100% mutant allele). If the frequency is different, subclone this cell population by repeating the Cloning Protocol.

6. Once a pure clone is isolated, PCR amplify the target gDNA region from the extracted gDNA to sequence verify the genotypes.
7. Thaw the frozen plate and plate the genotyped cells on Matrigel-coated 24-wells. During the cloning process, cells tend to partially differentiate. In this case, you can recover the cells just by repeating passaging the cells at 1:6 several times.



**Genotyping of PRKAG2 R531G heterozygous line by ddPCR as an example.**

An isolated clone was analyzed by ddPCR for the WT (FAM) and R531G mutant (VIC) alleles. From these droplet numbers, the R531G allelic frequency was estimated to be 49.97%.