Procedure

In silico design

1. sgRNA, primer
    a. via SnapGene, CRISPOR, NCBI Primer Blast, UCSC Genome Browser

Growth of Human iPSCs (Cell Culture)

2. K562 cell lines are used
    a. Stored in liquid nitrogen
3. Growth Medium for K-562 cell line
    a. RPMI1640
    b. 10% FBS
    c. Anti-Anti
        i. mL per 500 mL of culture medium

Amaxa Nucleofector System

4. Electroporation using the Amaxa kit
    a. 2x35mm plates
    b. Set the CRISPR/Cas9 components on ice
        i. sgRNA
        ii. Cas9
    c. Wash $1x10^6$, sgRNA and Cas9 in PBS
        i. Incubate at room temperature
        ii. Transfer appropriate amount into reaction tube
    d. Make Cas9/gRNA RNP master mix
    e. Harvest cells in a flask by trypsinization
        i. Aspirate media from cultured cells and wash once with PBS
        ii. Add trypsin solution to cells and incubate until cells release from flask making sure a single-cell suspension is achieved
        iii. Neutralize trypsin by adding culture media
    f. Count trypsinized cells
    g. Transfer cells into a sterile tube
        i. Centrifuge cells at room temperature
        ii. Remove as much supernatant with disturbing the pellet   wash cells in PBS
        iii. Centrifuge cells at room temperatures
        iv. Remove as much supernatant with disturbing the pellet
    h. Resuspend cells with supplement
    i. Add RNP master mix
    j. Transfer solution into Amaxa transfection cuvette
        i. Make sure there are no air bubbles
    k. Electroporate
    l. Transfer cells into 2x35mm plated

        i. 1 plate for analysis
        ii. 1 plate for monoclonal isolation

Validation of deletion

5. Use 1 of the 35mm plates for validation
    a. Detach the cells
        i. Aspirate medium
        ii. PBS wash
        iii. Incubate
    b. Isolate genomic DNA (gDNA)
    c. Purify DNA
    d. Test for presence of positive clones
    e. If the cell pool contains no positive clones
        i. Repeat transfection   adjust Cas9/gRNA RNP master mix
6. Further incubation of second 35mm plate
    a. Check for appropriate colony size

Identification of positive clones

7. Use one of the plates to identify positive clones
    a. Freeze the other well plate
    b. Detach cells
        i. Aspirate medium
        ii. PBS wash
        iii. Incubate
    c. Isolate each gDNA
8. Thaw second 96 well plate
    a. Discard negative clones
    b. Transfer each clone to 35mm plate
    c. Grow the positive clones
        i. Passage the cells

Test for expression

9. TSHR expression
    a. Immunohistochemistry/immunostaining
    b. qPCR
    c. Western blot
10. Off-targets
11. Karyotypic changes (reveals changes in chromosome number associated with aneuploid conditions)

Differentiation of Human iPSCs into Definitive Endoderm and of TFCs

- As thyroid development progresses via definitive endoderm

    - Human iPSCs were cultured in medium supplemented with Activin A
- TSH is important for thyroid development
    - After TSHR mRNA expression was observed in cells check if the cells have the potential to differentiate into TFCs at this time point with TSH treatment
- Cultivate human iPSCs obtain the deletion in these and differentiate them subsequently into TFCs using the compounds