TITLE: Evaluation of CRISPR-mediated nonhomologous end-joining (NHEJ) recombination

SOP Number: D-DEZ-PRO-015 Revision Number: 0

Effective Date: 07 Apr 2015

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QA Approval: ______________________ Date: ______________

A. OBJECTIVE

This SOP is to provide information to determine if designed and cloned guide RNAs modify intended loci. For this determination, bicistronic plasmids will be transfected into HEK293T cells (n=2).

B. APPLICABILITY

This procedure shall be performed by a trained scientist or technician in the analytical lab (4156 MERF).

C. REFERENCES

D-DEZ-PRO-013 gDNA Isolation from Cultured Cells

D. MATERIALS AND EQUIPMENT

HEK293T cells (ATCC; ACS-4500)
Lipofectamine 2000 (Invitrogen; 11668-019)
Serum-free media (DMEM) (Gibco; 10566-016)
Complete media (DMEM, 10% FBS - Gibco; 26140-079, 0.2% Primocin - InvivoGen; ant-pm-1)
Bicistronic plasmids
DNeasy Blood and Tissue gDNA isolation kit (Qiagen; 69581)
target oligos* (20uM)
AccuPrime DNA Polymerase High Fidelity kit (Invitrogen; 12346086)
Surveyor Mutation Detection Kit (Integrated DNA Technologies; 706020)
2% Agarose Egels (Invitrogen; G5018-02)
100 bp Quick Load DNA Ladder (NEB; N0467S)
pCR2.1-TOPO-TA kit (Invitrogen; K4500J10)
TOP10 OneShots (Invitrogen; C4040-10)
LB-AIX plates
LB + Amp
Machery-Nagel Nucleospin Plasmid Mini prep kit (Clontech; 740588.250)
M13 -20 F and M13R sequencing primers

E. DESCRIPTION

This procedure is executed to determine if designed and cloned guide RNAs modify intended loci. For this determination, bicistronic plasmids will be transfected into HEK293T cells (n=2). Four days post-transfection, genomic DNA will be isolated and amplified with primers surrounding the target locus or loci. Following heteroduplex formation, amplicons will be treated with Surveyor S nuclease to detect InDels generated from NHEJ-mediated DSB repair. To quantify the modifications, amplicons will be subcloned and sequenced.

F. TRANSFECTION

1. 16-24 hours before transfection passage 400,000 HEK293T cells onto each well of 12-well tissue

culture plate

2. The next day dilute 500ng of each plasmid into 200ul serum-free media per condition. Include a mock transfection control (200ul serum-free media only)

3. Dilute 5ul x (n+1) Lipofectamine 2000 into 100ul x (n+1) serum-free media.

4. Add 210ul Lipofectamine dilution to each diluted plasmid reaction (+ mock) mix.

5. Incubate at RT for at least 5 min.

6. While DNA and Lipofectamine are complexing, remove media from cells and replace with 500 ul complete media.

7. Add 200ul DNA/lipofectamine to appropriate wells and incubate overnight at 37°C, 5% CO2.

8. The next day, remove complexes and replace with 1 ml complete media.

G. gDNA ISOLATION

1. 2-3 days post-transfection, isolate gDNA from each well.

2. Remove culture medium and rinse with 1X PBS.

3. Scrape cells into 1 ml of 1X PBS and centrifuge at 1000 rpm for 3 min.

4. Remove supernatant and isolate gDNA according to kit instructions – elute in 50ul sterile dH2O.

5. Determine concentrations via Nanodrop and adjust to 100ng/ul.

H. SURVEYOR PCR

1. Set up PCR reactions as follows:

   AccuPrime Reaction Buffer II5ul
   gDNA (100ng)1ul
   target oligo F (20nM)0.5ul
   target oligo R (20nM)0.5ul
   AccuPrime Taq0.25ul
   H2O42.75

2. Cycling parameters:

   1x 94°C 3m
   35x 94°C15s
   58°C 20s
   72°C 1m
   1x72°C 5m

3. Check amplification by running 5ul on a 2% Egel. Estimate concentration of each sample by

comparing to the 1000 bp band of the 100 bp NEB QuickLoad DNA ladder (95 ng). Amplicons should be at ≥ 20ng/ul.

I. HETERODUPLEX FORMATION

1. Add 20ul each amplicon to new PCR tube.
2. 2.Heteroduplex parameters:

95°C 10m
95-85°C -2°C/s
85°C 1m
85-75°C -0.3°C/s
75°C 1m
75-65°C -0.3°C/s
65°C 1m
65-55°C -0.3°C/s
55°C 1m
55-45°C -0.3°C/s
45°C 1m
45-35°C -0.3°C/s
35°C 1m
35-25°C -0.3°C/s
25°C 1m
25-4°C -0.3°C/s
4°C hold

J. SURVEYOR NUCLEASE DIGESTION

1. Set up digestions as follows:

heteroduplex DNA 20ul
MgCl2 (150mM) 2.5ul
Surveyor S Enhancer 1ul
Surveyor S Nuclease 1ul

2. Incubate reaction for 1 hr at 42°C
3. Visualize digestions on 2% Egel.

K. QUANTIFICATION OF NHEJ

1. Dilute Surveyor PCR reactions 1:10 and subclone 4ul into pCR2.1-TOPO-TA vector.
2. Transform 4ul TOPO TA reactions into TOP10 OneShots.
3. Plate 150ul outgrowth onto LB + AIX plates.
4. Submit plates to Functional Biosciences for prep and sequencing with M13 (-20) Forward primer.

L. * TARGET OLIGO DESIGN

1. When designing target oligos use PrimerQuest on IDT website
   http://www.idtdna.com/Primerquest/Home/Index
2. Choose ~400 bp on either side of gRNA target sequences to input.
3. Choose primer pairs with amplicons of ~400 bp to ~600 bp.
4. Avoid pairs with gRNA target sequences at center.

M. HISTORY

Effective Date	Revision	Change
07 Apr 2015	0	Initial issue of SOP