PEP-NET

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Predictive Epigenetics: Fusing Theory and Experiment

Title of the protocol: Targeted knock-in into mouse embryonic stem cells (mESC) using CRISPR-Cas9

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Introduction:
The RNA-guided Cas9 nuclease from the microbial clustered regularly interspaced short palindromic repeats (CRISPR) is a powerful and precise tool for genome engineering. It can target 20-nt sequences adjacent to a protospacer adjacent motif (PAM) introducing double or single strand breaks followed by nonhomologous end joining (NHEJ) or homology-directed repair (HDR). This protocol describes a targeted knock-in strategy using CRISPR-Cas9 in E14 mESC, including transfection, single cell sorting, and clone screening using PCR.
The following points must be considered in advance:
First, CRISPR-Cas9 binding sites must be determined at the genomic position of interest, Various CRISPR-Cas9 guide RNA (gRNA) design tools exist online, e.g., from IDT (https://eu.idtdna.com/site/order/designtool/index/CRISPR_SEQUENCE).
Second, a targeting vector containing the knock-in sequence flanked by 0.5-1 kb homology arms surrounding the genomic position of the gRNA recognition site must be designed.
And third, PCR primer pairs must be designed for a 3', 5' and wild-type (WT) screening PCR. The 3' and 5' PCR indicate the presence of the knock-in sequence, whereas the WT PCR provides information about a homo- or heterozygote insertion. For the 3' and 5' PCRs, one primer should be located inside the knock-in sequence and one primer should be located on genomic DNA outside the homology arms. The WT PCR primers are located on genomic DNA outside the homology arms.

Description of the protocol

CRISPR-Cas9 knock-in using Lipofection 3000
Reagents and consumables

- Targeting vector
- gRNA/Cas9 vector (e.g., Addgene Plasmid #42230)
- E14 medium (Glasgow's MEM (GMEM), 15% FBS, 1x MEM Non-Essential Amino Acids Solution, 1 mM Sodium pyruvate, 2 mM L-Glutamine, 0.1 mM β-Mercaptoethanol, 20 U/mL LIF)
- Accutase® Cell Detachment Solution
- 1x Phosphate-buffered saline (PBS)
- Lipofectamine™ 3000 Transfection Reagent
- Gibco Opti-MEM I Reduced Serum Media
- Rock Inhibitor (Y-27632)
- Primocin™

- PenStrep (100 IU/ml penicillin, 100 µg/ml streptomycin)
- 96-well tissue culture plates
- 6-well tissue culture plates
- 0.1% Gelatin in 1x PBS

Protocol

Harvesting cells (6-well tissue culture plate)

1. Wash cells once with 1x PBS and add accutase. Leave for 10 min at 37°C
2. Add double the amount of medium and resuspend
3. Spin down at 950 rpm for 3 min
4. Resuspend cells in 1 ml media
5. Count cells and seed $1.5*10^6$ cells in a gelatinized 6-well plate (prepared the day before)

Lipofection of freshly seeded cells (Lipofectamine 3000)

1. Prepare Solution 1 (125 µL OptiMEM, 5 µL Reagent 3000, 2-20 µg targeting vector, 1 µg gRNA/Cas9 vector) and
   Solution 2 (125 µL OptiMEM, 5 µL Lipofectamine 3000)
2. Add Solution 2 to Solution 1 and incubate for at least 15 min
3. Drop Lipofection Solution on freshly seeded cells

Selection

If the knock-in sequence included a resistance marker, cells can be selected as follows:

1. For 48 h after Lipofection, keep cells in medium not containing corresponding antibiotic
2. Start selection by changing the medium to medium containing corresponding antibiotic until cells in a control well are dead
3. Let cells recover in medium not containing corresponding antibiotic for at least 2 and up to 4 days before sorting

If Cas9 was linked to a resistance marker, cells can be selected as follows:

1. For 24 h after Lipofection, keep cells in medium not containing corresponding antibiotic
2. Start selection by changing the medium to medium containing corresponding antibiotic for 24 h
3. Let cells recover in medium not containing corresponding antibiotic for at least 2 and up to 4 days before sorting

Single-cell sort

1. Prepare gelantinized 96-well plates the day before
2. Remove gelantine and add 100 µL medium containing 100 µg/mL primocin and 10 µM ROCK inhibitor, keep warm
3. Harvest and sort cells
4. Two days after the sort, replace medium with medium containing PenStrep
5. Let cells grow until they are confluent (8-14 days) and split one plate for maintenance and one plate for genomic DNA extraction

Clone Screening

Reagents and consumables

- Lysis buffer (100 mM Tris pH 8.0, 5 mM EDTA, 0.2% SDS, 50 mM NaCl, add 1 mg/mL Proteinkinase K and 0.05 mg/mL RNAse A freshly)
- Isopropanol
- 70% Ethanol
- Nuclease-free water
- Hot Start polymerase (e.g., Phusion Hot Start II)

- 96-well PCR plate
- 1% Agarose gel

Protocol

Genomic DNA extraction

Cells on plate should become confluent before extracting genomic DNA

1. Remove medium
2. Add 100 µL lysis buffer
3. Agitate for 10 min at RT, incubate over night at 37°C
4. Add 100 µL isopropanol, agitate for at least 2 h or over-night at RT
5. Spin plate at 3000 rpm, 30 min, 4°C
6. Remove supernatant by flipping the plate
7. Add 100 µL 70% Ethanol
8. Spin plate at 3000 rpm, 30 min, 4°C
9. Remove supernatant by flipping the plate
10. Air dry plate at RT for at least 15 min, no ethanol drops should be visible anymore
11. Add 50 µL nuclease-free water
12. Agitate at RT for 1 h
13. Measure concentration of a couple of wells and dilute till concentrations around 10 ng/µL
14. Store at 4°C

Genotyping PCR

For PCR screening, always use a Hot Start polymerase, e.g. Phusion Hot Start II, and set up the PCR in small volume (e.g. 10 µl) in order to save reagents. As a positive control and to set up the genotyping PCR, genomic DNA from the pool of cells of the knock-in can be used. In an initial screen, use either the 3' or 5' PCR set-up. After, expand positive clones, extract genomic DNA, and verify those clones using all set-up PCRs.

Example plate PCR for Phusion Hot Start II:

1. Prepare PCR master mix

	10 µL reaction	**Final concentration**
5x Phusion GC buffer	2 µL	1x
10 mM dNTP mix	0.2 µL	200 µM each
Forward primer	X µL	0.5 µM
Reverse primer	X µL	0.5 µM
Phusion Hot Start II DNA Polymerase (2 U/µL)	0.1 µL	0.02 U/µL

2. Mix 7 µL master mix and 3 µL genomic DNA per well in a 96-well PCR plate
3. Run PCR

	Temperature	**Time**	**Cycle**
Initial denaturation	98°C	30 s	1
Denaturation	98°C	5-10 s	
Annealing	X°C	10-30 s	25-35
Extension	72°C	15-30 s/kb	
Final extension	72°C	5-10 min	1
	4°C	hold	

4. Analyse using a 1% Agarose Gel
5. Expand positive clones
6. Verify expanded clones using all genotyping PCRs

References:

Ran, F. A., et al. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature Protocols, 8*(11), 2281-2308. Pubmed