Cell culture protocol for sc-Tiling: High-resolution characterization of gene function using single-cell CRISPR tiling

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Method Article

Keywords: CRISPR, sc-Tiling, gene scan, sc-RNAseq

Posted Date: December 5th, 2022

DOI: https://doi.org/10.21203/rs.3.pex-1544/v1

Abstract

Identification of novel functional domains and characterization of detailed regulatory mechanisms in cancer-driving genes is critical for advanced cancer therapy. Here, we present the cell culture protocol for “sc-Tiling,” which integrates a CRISPR gene-tiling screen with 10X Genomics single-cell RNAseq (sc-RNAseq) workflow.

Introduction

Reagents

LentiCas9-Blast (Addgene; #52962)

Blasticidin S (Gibco; A1113903)

Puromycin (Gibco; A1113803)

psPAX2 (Addgene; #12260)

pMD2.G (Addgene; #12259)

HEK293 cells (ATCC; CRL1573)

Fetal Bovine Serum (FBS) (Omega Scientific; FB-02)

Penicillin-Streptomycin (Gibco; 15140-122)

GlutaMAX (Gibco; 35-050-061)

Plasmocin (InvivoGen; NC9886956)

10X GEM v3.1 kit (10X Genomics; PN-1000121)

Equipment

Standard tissue culture equipment including a tissue culture room, a CO2 incubator, a biosafety cabinet, and a cell centrifuge are required.

Chromium Controller (10X Genomics; PN-120223)

Procedure

A. Pre-titrate the parental cells with:

(1) Blasticidin (Blast) for LentiCas9-Blast selection: titration range 0, 0.625, 1.25, 2.5, 5, 10, 20, 40 ug/ml final x 3-5 days, find the minimal killing dose.

(2) Puromycin (Puro) for sgRNA library selection: titration range 0, 0.05, 0.1, 0.2, 0.5, 1, 2, 4 ug/ml final x 2-3 days, find the minimal killing dose.

B. Establish the monoclonal spCas9-expressing cells for CRISPR screen:

We use LentiCas9-Blast stably transduced cells for a constitutive spCas9 expression. We suggest single cloning these cells and identify monoclonal $Cas9^+$ cells that exert the best CRISPR editing activity for the CRISPR library screen. For this, we developed a “self-inactivation RFP system” for a flow cytometric assay to select the best CRISPR editing clones.

(1) Transduce the cells with LentiCas9-Blast at ~15-20% infection. Blasticidin selects for 3-5 days.

(2) Single cloning by seeding the cells in Blast-medium in 96-well-plates x 3, each contains 300, 100, and 30 cells in the plate.

(3) Select wells that contain monoclonal cells and expand the culture. It typically talks 2 – 4 weeks to expand from a single cell to get enough cells for frozen stocks and the functional test.

(4) Test the CRISPR editing efficiency of each monoclonal Cas9-expressing cell using the sgRNA targeting RFP (sgRFP; 5’-GTCACCACATACGAAGACGG-3’). Select the best CRISPR editing clones for CRISPR screens.

C. Preparation of CRISPR library virus:

Make library virus using HEK293 cells in P100 dishes. We suggest making 10 dishes of virus per 1,000 sgRNA constructs in the library for a typical one condition, 1,000x coverage, triplicated screen. The real dish # of viral production required for the screen depends on the infection potential of the target cells, and the screen designs such as coverage-fold, # of replicates, # of conditions (e.g., drug treatments, etc.). A pilot culture to calculate the required viral amount is highly recommended.

(1) Day 1: Seed HEK293 cells at 3.4 x 10^6 cells per P100 dish in DMEM medium with 10% FBS, penicillin (100 units/ml), streptomycin (100 µg/ml), and plasmocin (5 µg/ml).

(2) Day 2: Transfect the HEK293 cells with a mixture of pPAX2 plasmid: pMD2 plasmid: CRISPR library plasmid = 5.6 µg: 5.6 µg: 5.6 µg (Total DNA = 17 µg).

(3) Day 3: Replace medium 18-24 h after transfection.

(4) Day 5: Harvest the viral containing supernatant from the HEK293 cultures. Spin at 2,000 g x 10 min to remove the cell debris. Aliquot the viral supernatant and store @ -80℃.

D. Pilot library virus test infection:

(1) Setup a viral titration in a 12-well plate scale. Typical seeding density: 1 – 2 million cells per 12-well. Titrate the virus with a 2-fold gradience. Each well contains 10ug/mL polybrene (final concentration).

(2) Read the RFP+ cell % at 48hr post-infection. Identify the well with 15 – 20 % RFP^+ % (this will be the titer for the real screen). Using this condition, we expect to have ~ 90% of the infected cells receiving only one sgRNA construct.

E. Pilot library test culture (mimic the real screen condition but in a smaller scale):

(1) Transduce the Cas9-expressing cells with the sgRNA library virus using the condition for 15 – 20 % RFP^+ infection and add puromycin 24hr post-infection (and maintain puromycin during the whole course of the screen).

(2) After 24hr in puromycin, check the RFP^+ % (usually expect to reach > 60% RFP^+ after 24hr puromycin treatment). This is the START of the test screen culture.

(3) Seed cells in the culture conditions for the screen. If planning for a drug response screen, use this Pilot test culture to titrate for the drug concentrations as needed.

(4) Use the pilot library test culture to determine the large-scale screen culture protocol (days, seeding density, drug conditions…). Calculate the reagents required for the large-scale screen culture (virus, medium, compounds, FBS, cytokines, etc.) to ensure sufficient materials to complete the screen.

F. Large-scale screen culture:

(1) Transduced at 15 – 20% RFP^+ as previously titrated.

(2) Initial sgRNA library infection needs to get 1,000x coverage to get enough cells to start.

· Example for a 1,000 sgRNA library: Start from 10 million cells per replicate at 15~20% infection rate. Expecting at least 1 million independently infected RFP^+ cells.

(3) Add puromycin 24hr after infection.

(4) At the defined time point (e.g., day 3 post-transduction), submit freshly harvested screen cells for 10X Chromium single-cell isolation using the 10X GEM v3.1 kit.