Aug 08, 2024

RNA-seq of primary human fibroblasts cultured on soft and stiff ECM

DOI
dx.doi.org/10.17504/protocols.io.e6nvwd447lmk/v1

Brian D. Cosgrove[1], Lexi Bounds[1], Carson Key Taylor[1], Alan L. Su[1], Anthony J. Rizzo[1], Alejandro Barrera[1], Andrea R Daniel[1], Gregory E. Crawford[1], Brenton D. Hoffman[1], Charles A. Gersbach[1]

[1]Duke University

Andrea R Daniel: This protocol was adapted from the work of Brian Cosgrove and Lexi Bounds in the Gersbach lab at Duke University.

Andrea R Daniel
Duke University

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Protocol Citation: Brian D. Cosgrove, Lexi Bounds, Carson Key Taylor, Alan L. Su, Anthony J. Rizzo, Alejandro Barrera, Andrea R Daniel, Gregory E. Crawford, Brenton D. Hoffman, Charles A. Gersbach 2024. RNA-seq of primary human fibroblasts cultured on soft and stiff ECM. **protocols.io https://dx.doi.org/10.17504/protocols.io.e6nvwd447lmk/v1**

Manuscript citation:
Mechanosensitive genomic enhancers potentiate the cellular response to matrix stiffness
Brian D. Cosgrove, Lexi R. Bounds, Carson Key Taylor, Alan L. Su, Anthony J. Rizzo, Alejandro Barrera, Gregory E. Crawford, Brenton D. Hoffman, Charles A. Gersbach
bioRxiv 2024.01.10.574997; doi: https://doi.org/10.1101/2024.01.10.574997

Protocol status: Working
We use this protocol and it's working

Created: December 20, 2023

Last Modified: August 08, 2024

Protocol Integer ID: 92562

Keywords: RNA-seq, enhancer, extracellular matrix, mechanoresponsive, matrix stiffness

Funders Acknowledgement:
NIH
Grant ID: HG012053

Abstract

This protocols describes methods for RNA-seq to characterize the cellular gene expression responses to changes in extracellular matrix stiffness cues.

Materials

Cells: ATCC CRL-2097
Hydrogel dishes: 35 and 150 mm PetriSoft EasyCoat dishes (Matrigen)
RNA isolation: Norgen Total RNA Purification Kit (#17250)
library prep: TruSeq Stranded Library Prep Kit (Illumina #RS-122-2101)

Cell culture and soft hydrogel processing

1 Culture primary human neonatal fibroblasts (HFF cells, ATCC CRL-2097) in DMEM with 10% FBS, 1% AntiAnti, and 1% NEAA (Sigma) on tissue culture plastic (TCP).

2 Use polyacrylamide hydrogel 35 and 150 mm PetriSoft EasyCoat dishes (Matrigen) with an Elastic Modulus of 1 kPa (“soft”) and 50 kPa (“stiff”).

3 Incubate dishes for 5 minutes with sterile PBS, rinse two more times with sterile PBS, followed by the addition of 10 ug/mL fibronectin (Sigma) for 30 minutes at room temperature.

4 Remove fibronectin and rinse dishes twice with sterile PBS, followed by a 20 minutes incubation with complete growth media.

5 Remove media from the dishes and add cell suspensions. Allow cells to attach overnight.

Bulk RNA-Seq

6 Seed 40,000 HFF cells on 50 kPa dishes and TCP dishes, seed 70,000 HFF cells on 1 kPa dishes to achieve the same effective plating density due to slightly reduced HFF attachment rates (and spreading) on 1kPa hydrogels.

7 20 hours after seeding, trypsinize cells and spun down at 300g for 5 minutes. Isolate RNA from the cells using the Norgen Total RNA Purification Kit (#17250) according to the manufacturer's protocol.

8 Run RNA samples on a RNA TapeStation (Agilent) to verify all samples had a RIN score > 8.

9 Build cDNA libraries from RNA inputs using the TruSeq Stranded Library Prep Kit (Illumina #RS-122-2101) according to manufacturer’s instructions.

10 Perform quality control by running the amplified libraries out on a High Sensitivity D1000 Tapestation (Agilent) to confirm expected size.

11 Perform Qubit dsDNA HS (High Sensitivity) assays to determine a final concentration.

12 Dilute libraries to 10nM and pool them together in equal volumes. Perform sequencing on an Illumina HiSeq 2500 using a 50bp PE RapidRun kit.

13 Subject resulting reads to adapter trimming using Trimmomatic v0.32 (1), align them to GRCh38 with the STAR v2.4 aligner (2), and retrieve counts using featureCounts (3) from subread version 1.4.6p4 with Gencode v22 gene annotations used as reference.

14 Perform differential expression analysis using edgeR quasi-likelihood methodology (4) and visualize data using Degust (5) and Rstudio. Determine genes with significant differential expression using a threshold of FDR < 0.05 and absolute value of Log2(FC) > 1.

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