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HiCAR for human iPSC-CM following enhancer perturbations

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We use this protocol and it's working

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Abstract

This protocol describes ATAC-seq methods in human iPSC-CM following growth with or without GSK3 inhibition using CHIR99021.

Troubleshooting

Matrigel coating tissue culture plates

1 Prepare Matrigel-coated vessels as needed, following the manufacturer's instructions. Use coated vessels within two weeks.

2 Thaw Matrigel aliquot and dilute 1:30 in cold DMEM/F12.

3 Add Matrigel solution to plates quickly (2 ml for a 6-well, 12 ml for a 10-cm, 32 ml for a 15-cm).

4 Incubate at 37°C for a minimum of 1 hour. Use plates before the medium evaporates or within two weeks.

iPSC to cardiomyocyte (iPSC-CM) differentiation with WTC11 cell line

5 Aspirate media and add dPBS to wash. Then, aspirate dPBS. The differentiation protocol is based on previously published work. (**1**)

6 Add AccutaseTM to cells to dissociate them (1 ml for a 6-well, 3 ml for a 10-cm). Incubate at 37°C for 3 to 5 minutes.

7 Add 1:1 warm DMEM/F12 to the AccutaseTM and pipette gently 2 to 3 times to promote a single-cell suspension. Transfer cells to a conical tube and centrifuge at 300g for 5 minutes.

8 Aspirate medium and resuspend cells in mTeSR+TM (mTeSR+TM basal medium + supplement) + Rock inhibitor (10uM final concentration).

9 Seed cells in Matrigel coated plates quickly so that in 72hrs cells are 70% to 80% confluent. (2ml for 6-well, 12ml for a 10-cm of mTeSR+**TM**)

10 Change medium to RB- (RPMI 1640 + 50x B-27**TM** supplement - insulin) + CHIR99021 (10 µM final concentration) (3 ml for a 6-well, 18 ml for a 10-cm). Volumes per tissue culture plate will remain consistent during differentiation.

11 After 48 hours, change medium to RB- + IWP2 (7.5 µM final concentration). Change media as close to 48 hours as possible, within +/- 5 minutes.

12 After another 48 hours, change medium to RB-. Change media as close to 48 hours as possible, within +/- 5 minutes.

13 After another 48 hours, change medium to RB+ (RPMI 1640 + 50x B-27™ supplement with 100 U/ml penicillin and 100 µg/ml streptomycin). Change media as close to 48 hours as possible, within +/- 5 minutes.

14 After another 48 hours, change medium to RB+. Change media as close to 48 hours as possible, within +/- 5 minutes. Check for cell beating.

15 After another 48 hours, check for cell beating. If beating has not occurred, replace with RB+ until beating occurs. If beating has occurred, change medium to NG+ (RPMI 1640 - glucose + 50x B-27™ supplement with 100 U/ml penicillin, 100 µg/ml streptomycin, 500 µg/ml recombinant human albumin, 213 ng/ml ascorbic acid, and 0.748 µl/ml 60% w/w sodium lactate solution), based on CDM3 medium. (**2**)

16 After another 48 hours, change medium to NG+.

17 After 48 hours, replate cells in an appropriate dish and density (3 × 10^6 cells for a 6-well, 1.8 × 10^7 cells for a 10-cm). To do this, aspirate media, add dPBS to wash, and then aspirate dPBS. Differentiation is complete at this point. Cells are now referred to as iPSC-CMs.

18 Add trypsin-EDTA (0.05%) to cells to dissociate them (1 ml for a 6-well, 3 ml for a 10-cm). Incubate at 37°C for 3 minutes.

19 Add a 1:1 mixture of stop media (DMEM + 5% FBS + DNase at 20 µg/ml final concentration) to quench the trypsin-EDTA (0.05%). Pipette gently to dislodge cells from the tissue culture plate and form a single-cell suspension, limiting to 3 to 5 repetitions. Transfer cells to a conical tube and centrifuge at 300g for 5 minutes.

20 Resuspend cells in RB+ + Rock inhibitor (10 µM final concentration). Seed cells in a 6-well plate over six wells (3 × 10^6 cells per well).

21 After 24 hours, change medium to RB+ (3 ml for a 6-well). Repeat every 48 hours for 144 hours.

Transfections for high-titer lentiviral production

22 Plate 1.2 × 106 or 7 × 106 HEK293T cells in a 6 well plate or 10 cm dish in the afternoon with 2 mL or 12 mL of complete opti-MEM (Opti-MEM‰ I Reduced Serum Medium

supplemented with 1x Glutamax, 5% FBS, 1 mM Sodium Pyruvate, and 1x MEM Non-Essential Amino Acids). Based on previously published protocols. (**3**)

23 The next morning, transfect HEK293T cells with 0.5 µg pMD2.G, 1.5 µg psPAX2, and 0.5 µg transgene for 6 well plates or 3.25 µg pMD2.G, 9.75 µg psPAX2, and 4.3 µg transgene for 10 cm dishes using Lipofectamine 3000.

24 Exchanged media 6 hours after transfection and collect and pool lentiviral supernatant at 24 hours and 48 hours after transfection.

25 Centrifuged lentiviral supernatant at 600g for 10 min to remove cellular debris.

26 Concentrate lentivirus to 50–100× the initial concentration using Lenti-X Concentrator (Takara Bio). Resupsend viral pellet in dPBS

27 Snap freeze virus in liquid nitrogen and store at -80C

Determining viral titer

28 Plate 4×10^6 HEK293T cells per well in a 6-well plate in complete DMEM (DMEM supplemented with 1x Glutamax, 5% FBS, 1 mM Sodium Pyruvate, 1x MEM Non-Essential Amino Acids, and100 U/ml penicillin and 100 µg/ml streptomycin). Transduce individual wells add 0ul,0ul, 1ul, 5ul, 10ul, and 20ul of concentrated virus.

29 Change media 24 hours following transduction with complete DMEM.

30 Change media 24 hours following transduction with complete DMEM + blasticidin (8ug/ml final concentration). Add complete DMEM without blasticidin to one well that received no virus. This well will be the control well for MOI calculations. Change media every 48 hour until the well that received no virus but was supplemented with blasticidin has no remaining adherent cells, usually 96 hours.

31 Count surviving cells across all wells. Plot viral load (ul) vs % survival and generate a linear fit with the y-intercept = 0.

Transduce iPSC-CMs

32 Transduce iPSC-CMs with virus 29 days following the initiation of differentiation for a desired MOI of 0.3 to reduced the negative effects of viral transduction of iPSC-CMs and maintain function.

33 Change media with RB+ 24 hours following transduction.

34 Change media with RB+ + blasticidin (4ug/ml final concentration) 48 hours following transduction.

35 Change media with RB+ + blasticidin (4ug/ml final concentration) every 48 hours for 96 hours total culture.

36 Change media with RB+ every 48 hours following.

Induce stress response and fetal gene expression using Endothelin-1

37 Culture iPSC-CMs in RB+ with or without Endothelin-1 (ET-1, 1 µM final concentration) for 72 hours. This step occurs 39 days after the initiation of differentiation and 10 days post-transduction. (**4**)

HiCAR

38 Culture of iPSC-CMs was maintained for 42 days following the initiation of differentiation, 13 days post-transduction, and 72 hours after culture with ET-1.

39 Add trypsin-EDTA (0.05%) to cells to dissociate them (1 ml for a 6-well, 3 ml for a 10-cm). Incubate at 37°C for 3 minutes.

40 Add a 1:1 mixture of stop media (DMEM + 5% FBS + DNase at 20 µg/ml final concentration) to quench the trypsin-EDTA (0.05%). Pipette cells gently to promote dissociation from the tissue culture plate and formation of a single-cell suspension, limiting to 3 to 5 repetitions. Transfer cells to a conical tube and centrifuge at 300g for 5 minutes.

41 Proceed to HiCAR NGS library generation as described in the original publication. (**5**)

42 HiCAR libraries were then pooled at equal molarity. Targeted enrichment was performed for regions of interest, including regions surrounding the MYH6 transcription start site (TSS) and gene body, the MYH7 TSS, and a distal regulatory element. Biotinylated probes and protocols from Twist Bioscience were used for targeted enrichment. (**6**)

43 Enriched libraries were sequenced using an Illumina NextSeq 1000/2000 P2 kit on an Illumina NextSeq 2000.

44 Sequencing data was processed using the nf-core HiCAR pipeline v1.0.0, with Hg38 as the reference genome.

45 A contact matrix was created from cooler files generated by the HiCAR pipeline for all reads mapping to Hg38 chr14:23,300,000–23,460,000.

46 Differential contact frequency within the region was determined using DESeq2. (**7**)

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