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Quantitative bulk transcriptomics

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Livia Hecke Morais[1]

[1]California Institute of Technology

Livia Hecke Morais
California Institute of Technology

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We use this protocol and it's working

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Abstract

Quantitative bulk transcriptomics used in Morais et al. for total striatum tissue RNA sequencing.

RNA extraction

1 Homogenize the tissue in TRIzol.

2 200 ul chloroform per 1 ml of TRIzol.

3 Shake vigorously 15 sec. (DO NOT VORTEX)

4 Incubate at RT for 3 min.

5 Spin 15 min at 4C, 12,000g to separate phases.

6 Carefully transfer 400 ul of the clear, colorless supernatant to a clean sterile 1.5 ml tube. Avoid the middle, white layer. Dispose of the remaining liquid in the liquid waste.

7 To the 1.5 ml tube, add an equal volume (400 µl) of 70% ethanol to the collected supernatant,
mix well through pipetting.

8 Transfer 700 µl to an RNeasy spin column placed in a 2mL collection tube. Centrifuge at room temperature for 1 minute at 10 000 rpm.

9 Discard the flow-through and transfer any remaining solution to the column and centrifuge again.

10 Transfer the flow through column to a new collection tube.

11 Wash with 350 µl Buffer RW1.

12 Centrifuge at room temperature for 1 minute at 10 000 rpm. Discard the flow through with a pipette.

13 Separate tube, add 10 µL DNase I Stock Solution to 70 µL Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube

14 Carefully add the 80 µL of DNase I mix directly to the RNeasy spin column membrane, and place on the benchtop (20–30°C) for 15 min

15 Add 350 µl Buffer RW1 to the RNeasy spin column.

16 Add 350 µl Buffer RW1 to the RNeasy spin column.

17 Centrifuge for 1 minute at 10 000 rpm. Discard the flow through with a pipette.

18 Wash with 500 µl Buffer RPE

19 Centrifuge at room temperature for 1 minute at 10 000 rpm. Discard the flow through with a pipette

20 Centrifuge at room temperature for 1 minute at 10 000 rpm. Discard the flow through with a pipette

21 Centrifuge at room temperature for 2 minutes at 13 000 rpm. This step removes any ethanol or other washes.

22 Transfer the column to a clean, sterile 1.5 ml Eppendorf tube.

23 Elute RNA with 35 µl RNase free water. Incubate at room temperature for 5 minutes.

24 Centrifuge at room temperature for 1 minute at 13000 rpm.

25 Immediately store at -20 or -80 until further use.

Library prep using Lexogen QuantSeq 3‘ mRNA-Seq Library Prep Kit FWD HT

26 Mix 200 ng of total RNA in a volume of 5 µl, with 5 µl First Strand cDNA Synthesis Mix 1 (**FS1**) in a PCR plate or 8-well strip tubes. If necessary, adjust the total volume to 10 µl with RNase-free water. Mix well by pipetting.

27 Denature the RNA / **FS1** mix for 3 minutes at 85 °C in a thermocycler and then cool down to 42 °C.

28 Prepare a mastermix containing 9.5 µl First Strand cDNA Synthesis Mix 2 (**FS2**) and 0.5 µl Enzyme Mix 1 (**E1**) per reaction. Mix well, spin down, and pre-warm the mastermix for 2 - 3 minutes at 42 °C.

29 Quickly spin down the denatured RNA / **FS1** samples from step 2 at room temperature to make sure all liquid is collected at the bottom of the wells. Place the samples back onto the thermocycler at 42 °C.

30 Add 10 µl of the **FS2** / **E1** mastermix to each reaction, mix well, and seal the plate.

31 Quickly spin down at room temperature and incubate the reactions for 15 minutes at 42 °C.

32 Add 5 µl RNA Removal Solution (**RS**) directly to the first strand cDNA synthesis reaction. Mix well and reseal the plate.

33 Incubate 10 minutes at 95 °C, then cool down to 25 °C. Spin down the plate at room temperature and carefully remove the sealing foil.

34 Add 10 µl Second Strand Synthesis Mix 1 (**SS1**) to the reaction. Mix well by pipetting,

35 Incubate the plate for 1 minute at 98 °C in a thermocycler, and slowly cool down to 25 °C by setting the ramp speed to 10 % (0.5 °C/second). Incubate the reaction for 30 minutes at 25 °C.

36 Quickly spin down the plate at room temperature before removing the sealing foil.

37 Prepare a mastermix containing 4 µl Second Strand Synthesis Mix 2 (**SS2**) and 1 µl Enzyme Mix 2 (**E2**). Mix well.

38 Add 5 µl of the SS2 / E2 mastermix per reaction. Mix well. REMARK: Use a pipette set to 30 µl for efficient mixing.

39 Incubate the reaction for 15 minutes at 25 °C. Safe stopping point. Libraries can be stored at -20 °C at this point.

40 Add 16 µl of properly resuspended Purification Beads (PB) to each reaction, mix well, and incubate for 5 minutes at room temperature.

41 Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear (depends on the strength of your magnet).

42 Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.

43 Add 40 µl of Elution Buffer (EB), remove the plate from the magnet, and resuspend the beads properly in EB.

44 Incubate for 2 minutes at room temperature.

45 Add 56 µl of Purification Solution (PS) to the beads / EB mix to reprecipitate the library.

46 Mix thoroughly, and incubate for 5 minutes at room temperature.

47 Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

48 Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.

49 Add 120 µl of 80 % EtOH, and incubate the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step.

50 Remove and discard the supernatant.

51 Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely as traces of ethanol can inhibit subsequent PCR reactions.

52 Leave the plate in contact with the magnet, and let the beads dry at room temperature for 5 - 10 minutes or until all ethanol has evaporated only. Do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.

53 Add 20 µl of Elution Buffer (EB) per well, remove the plate from the magnet and resuspend the beads properly in EB.

54 Incubate for 2 minutes at room temperature.

55 Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

56 Transfer 17 µl of the clear supernatant into a fresh PCR plate. Make sure not to transfer any beads. Safe stopping point.

57 Prepare a mastermix containing 7 µl of PCR Mix (PCR) and 1 µl Enzyme Mix 3 (E3) per reaction.

58 Add 8 µl of this PCR / E3 mastermix to 17 µl of the eluted library.

59 Add 5 µl of the respective i7 index. Mix well by pipetting.

60 Seal the PCR plate and quickly spin down to make sure all liquid is collected at the bottom of the well.

61 Conduct 14 cycles of PCR with: Initial denaturation at 98 °C for 30 seconds, 14 cycles of 98 °C for 10 seconds, 65 °C for 20 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 1 minute, hold at 10 °C.

62 Safe stopping point. Libraries can be stored at -20 °C at this point.

63 Add 30 µl of properly resuspended Purification Beads (PB) to each reaction, mix well, and incubate for 5 minutes at room temperature.

64 Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

65 Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.

66 Add 30 µl of Elution Buffer (EB), remove the plate from the magnet, and resuspend the beads properly in EB. Incubate for 2 minutes at room temperature.

67 Add 30 µl of Purification Solution (PS) to the beads / EB mix to reprecipitate the library.

68 Mix thoroughly, and incubate for 5 minutes at room temperature.

69 Place the plate onto a magnetic plate, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

70 Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.

71 Add 120 µl of 80 % EtOH, and incubate the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.

72 Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely.

73 Leave the plate in contact with the magnet, and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated.

74 Add 20 µl of Elution Buffer (EB) per well, remove the plate from the magnet, and resuspend the beads properly in EB. Incubate for 2 minutes at room temperature.

75 Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

76 Transfer 15 - 17 µl of the supernatant into a fresh PCR plate. Do not transfer any beads.

77 Libraries are now finished and ready for quality control.

78 Libraries can be stored at -20 °C at this point.