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Synthesis of in vitro transcribed RNA from whole bacterial transcriptome

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Identifying RNA modifications across the transcriptome has been made possible by direct RNA sequencing using nanopore sequencing from Oxford Nanopore Technologies. This is due to voltage changes happening when each nucleotide passes through the pore. Hypothesis is that since each nucleotide passing through the pore has a distinct change in voltage, nucleotides with epigenetic modifications (e.g.methylation) also has distinct voltage changes when passing through. This is identified by errors in basecalling.

Several software have been developed to identify locations of modifications using machine learning, deep learning models. In order to make highly accurate predictions, there is the necessity of comparing the signals from native RNA sequencing with RNA without any modifications. A few recent research publications get RNA through *in vitro* transcription but only for ribosomal RNAs (rRNA). This is easier since there are only 7 different ribosomal RNAs for each type i.e., 16S, 23S and 5S.

Getting IVT RNA for entire transcriptome is not trivial since bacterial transcriptome is complex and depends on the condition the bacteria has been grown in. We use the method of strand switching during cDNA synthesis to get cDNA for whole bacterial transcriptome which can then be used as a template for IVT.

Bhargava Reddy Morampalli, Olin Silander, Bhargava Reddy Morampalli, Olin Silander 2021. Synthesis of in vitro transcribed RNA from whole bacterial transcriptome. **protocols.io** https://protocols.io/view/synthesis-of-in-vitro-transcribed-rna-from-whole-b-brbmm2k6

in vitro transcription, direct RNA sequencing, RNA modifications

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Great precautions should be taken when handling RNA.

HiScribe™ T7 Quick High Yield RNA Synthesis Kit - E2050S
RNA Clean & Concentrator-5 - R1013
RNaseOUT
Primers: oligo-d(T)-22 primer, T7-Strand Switch Primer, PR2/T7 promoter primer (primer sequences commented in the protocol)
10 mM dNTP solution
Nuclease-free water (Thermofisher - #AM9937)
0.2 mL thin-walled PCR tubes
1.5 mL Eppendorf LoBind tubes
Maxima H Minus Reverse Transcriptase (200 U/uL) with 5x RT buffer (Thermofisher - #EP0751)
Pre-chilled freezer block at -20 C
LongAmp Taq 2X MasterMix (NEB - #M0287)
RNase Cocktail Enzyme Mix (Thermofisher - #AM2286)
RNAclean Agencourt XP beads

Clean the bench surfaces and pipettes using RNase ZAP to inhibit all RNases. Always use RNase free centrifuge tubes, PCR tubes and pipette tips for all steps wherever required.

Addition of poly(A) tail to E. coli RNA

1 A reaction is setup in a PCR tube using the following components

A	B	C	D	E	F	G	H
Components	**Volume (µL)**						
RNA	1 - 10 µg						
10x E. coli poly(A) polymerase reaction buffer	2						
ATP (10 mM)	2						
RNase inhibitor	1						
E. coli poly(A) polymerase	1						
Water	upto 25 µL						
Total	25						

2 Incubate the reaction mixture for **00:15:00** at **37 °C** in a thermocycler followed by **00:20:00** at **65 °C** for inactivation. 35m

3 Transfer the reaction mixture into a RNase free tube for cleanup step. Add **45 µL (1.8 volumes of reaction)** of RNAClean AMPure XP beads and **105 µL (4.2 volumes)** of 100 % ethanol to the reaction mixture

4 Flick the tube for mixing and incubate at **Room temperature** for **00:05:00** . 5m

5 Keep the tube on a magnetic rack and allow the beads to pellet towards the magnet

6 Prepare **500 µL** of 80% ethanol during this time. Pipette the solution out of the tube taking care to not disturb the pellet

7 Wash the beads by pipetting **150 µL** of 80% ethanol and repeat the step. Remove the ethanol, spin and put the tube back in the magnetic rack to remove any left over solution

8 Allow the beads to air dry for **00:00:30** making sure not to over dry. Add **20 µL** of RNase free water to the beads and incubate at **Room temperature** for **00:05:00** to elute RNA. 5m 30s

9 Place the tube back on the magnetic rack allowing the beads to separate and pipette the poly(A) tailed RNA in water into a new RNase free tube and keep it **On ice**

cDNA first strand synthesis 1h 45m

10 Prepare a reaction with the following components in a PCR tube

A	B
Reagent	**Volume**
poly A+ RNA	1 µg (x µL)
anchored oligo d(T)22	1 µL
10 mM dNTPs	1 µL
RNase free water	9 - x µL
Total	11 µL

11 Incubate the reaction mixture at **65 °C** for **00:05:00** in a thermocycler and then snap cool on a pre-chilled freezer block 5m

12 In a separate tube, mix together the following:

A	B
Reagent	**Volume**
5x RT Buffer	4 µl
RNaseOUT	1 µl
Nuclease-free water	2.5 µl
T7 - Strand-Switching Primer (SSP)	0.5 µL
Total	8 µl

13 Mix gently by *flicking the tube*, and *spin down*.

14 Add the strand-switching buffer to the snap-cooled, annealed mRNA, mix by *flicking the tube* and *spin down*

15 Incubate at **42 °C** for **00:02:00** 2m

16 Add 1 µl of Maxima H Minus Reverse Transcriptase. The total volume is now 20 µl.

17 Mix gently by *flicking the tube*, and *spin down*.

18 Incubate using the following protocol: 1h 35m

A	B	C	D
Cycle Step	**Temperature**	**Time**	**No. of Cycles**
Reverse transcription and strand-switching	42° C	90 mins	1
Heat inactivation	85° C	5 mins	1
Hold	4° C	∞	

RNA strand degradation and second strand synthesis 58m

19 Add **1 µL** of RNase Cocktail Enzyme Mix (ThermoFisher, AM2286) to the reverse transcription reaction.

20 Incubate the reaction for **00:10:00** at **37 °C** . 10m

21 Resuspend the AMPure XP beads by vortexing.

22 Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

23 Add **17 µL** of resuspended AMPure XP beads to the reaction and mix by *flicking the tube*.

24 Incubate on a *Hula mixer* (rotator mixer) for **00:05:00** at **Room temperature** . 5m

25 Prepare **500 µL** of fresh 70% ethanol in nuclease-free water.

26 *Spin down* the sample and *pellet on a magnet*. Keep the tube on the magnet, and pipette off the supernatant.

27 Keep the tube on the magnet and wash the beads with **200 µL** of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

28 Repeat the previous step.

29 *Spin down* and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~ **00:00:30** , but do not dry the pellet to the point of cracking. 30s

30 Remove the tube from the magnetic rack and resuspend pellet in **20 µL** nuclease-free water.

31 Incubate on a *Hula mixer* (rotator mixer) for **00:10:00** at **Room temperature** . 10m

32 *Pellet beads* on magnet until the eluate is clear and colourless.

33 Remove and retain **20 µL** of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

34 Prepare the following reaction in a 0.2 ml thin-walled PCR tube:

A	B
Reagent	**Volume**
2x LongAmp Taq Master Mix	25 µl
PR2 Primer (PR2) - T7 promoter primer (IDT)	3.75 µl
Reverse-transcribed sample from above	20 µl
Nuclease-free water	1.25 µl
Total	50 µl

35 Incubate using the following protocol: 17m

A	B	C
Temperature	**Time**	**Cycle**
94 °C	1 mins	1
50 °C	1 mins	1
65 °C	15 mins	1
4 °C	∞	

36 Resuspend the AMPure XP beads by vortexing.

37 Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

38 Add **40 µL** of resuspended AMPure XP beads to the reaction and mix by *flicking the tube*.

39 Incubate on a *Hula mixer* (rotator mixer) for **00:05:00** at **Room temperature** . 5m

40 Prepare **500 µL** of fresh 70% ethanol in nuclease-free water.

41 *Spin down* the sample and *pellet on a magnet*. Keep the tube on the magnet, and pipette off the supernatant.

42 Keep the tube on the magnet and wash the beads with **200 µL** of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

43 Repeat the previous step.

44 *Spin down* and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~ **00:00:30** , but do not dry the pellet to the point of cracking. 30s

45 Remove the tube from the magnetic rack and resuspend pellet in 21 µl nuclease-free water.

46 Incubate on a *Hula mixer* (rotator mixer) for **00:10:00** at **Room temperature** . 10m

47 *Pellet beads* on magnet until the eluate is clear and colourless.

48 Remove and retain **21 µL** of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

49 Measure the concentration of the double stranded cDNA obtained after this step.

in vitro transcription 2h 15m

50 100 ng of double stranded cDNA synthesized is used for *in vitro* transcription.

51 New England Biolab's HiScribe™ T7 Quick High Yield RNA Synthesis Kit (E2050s) is used for

RNA synthesis using cDNA as a template.

52 Thaw the necessary kit components, mix and pulse-spin in microfuge to collect solutions to the bottoms of tubes. Keep on ice.

53 Assemble the reaction at room temperature in the following order:

A	B
Components	**Volume**
Nuclease-free water	used to make up the volume to 20 µL
NTP Buffer Mix	10 µL (10 mM each NTP final)
Template DNA (cDNA)	X µL (100 ng - 1 µg)
T7 RNA Polymerase Mix	2 µL
Total Reaction Volume	20 µL

54 Mix thoroughly by pipetting and pulse-spin in a microfuge.

55 Incubate at **37 °C** for **02:00:00** . Incubation over 2 hours will no negatively affect the RNA synthesis 2h

56 **30 µL** of Nuclease-free water to the reaction mixture and **2 µL** of DNase I (included in the kit) is added after that.

57 Mix the reaction mixture by pipetting and incubate for **00:15:00** at **37 °C** 15m

58 Following the incubation, RNA is purified using any RNA cleanup kit. I used the RNA Clean & Concentrator-5 kit (R1013) and finally eluted in **20 µL** of RNase-free water.

59 Concentration is checked using Qubit RNA kit.