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Total RNA Protocol (extraction, quantification and Illumina library preparation)

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1 *Works for me* dx.doi.org/10.17504/protocols.io.457gy9n

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ABSTRACT

This experimental procedure was designed to isolate total the RNA aiming to obtain intact and high-quality integrity (RIN>7) for further next generation sequencing analysis. The protocol was developed to analyse the environmental microbial active community providing a clear overview of the microbial transcriptional activity in a specific time or after a different condition exposition sets, such as the investigation of thermal adaptation after heat-shock. The procedure is specifically designed to extract total-RNA from water samples although it can be used for various environmental samples as done in Schostag et al. and Bang-Andreasen et al. 2019 in soil with similar accuracy results. The total-RNA yield was >1000 ng/mL with a RIN ranging from 7 to 8.5. The final Illumina library preparation yield ranged between 15 and 31 ng/µL.

This protocol is a compiled by using the Qiagen RNeasy® PowerSoil® Total RNA Kit with additional steps according the DNeasy Max®Kit (Qiagen), NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina® (New England Biolabs, Inc), KAPA Library Quantification Kit for Illumina® Platforms (Kapa Biosystems), Qubit 2.0 (Thermo Fisher Scientific, Hvidovre, Denmark), Bioanalyzer® (Agilent Technologies, Inc.) sample Purification Beads (New England Biolabs) and Liquid G2 DNA/RNA Enhancer (Ampliqon) manufacturer protocols.

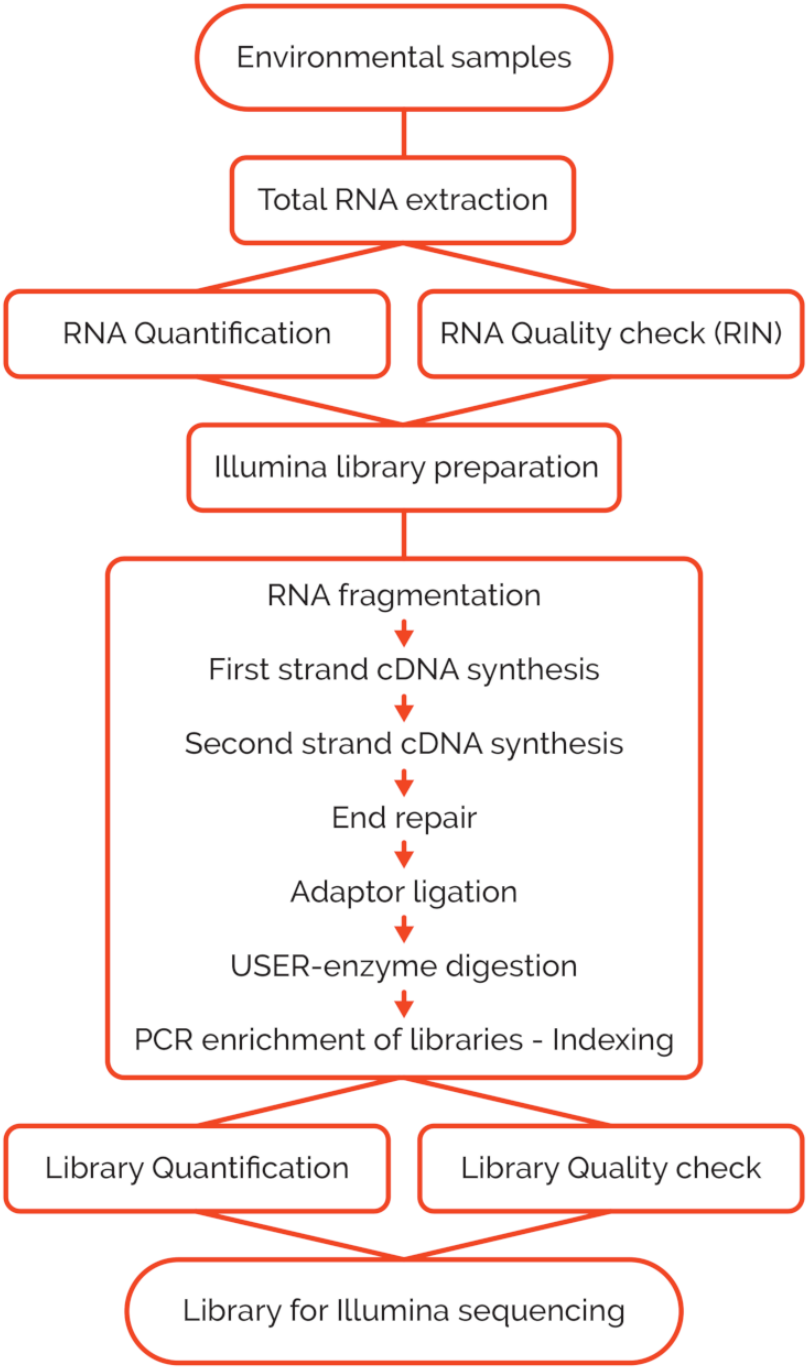

Figure 1: Workflow Scheme

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

1) Schostag M, Priemé A, Jacquiod S, Russel J, Ekelund F, Jacobsen CS. Bacterial and protozoan dynamics upon thawing and freezing of an active layer permafrost soil. The ISME journal. 2019 May;13(5):1345. 2) Bang-Andreasen T, Anwar MZ, Lanzén A, Kjøller R, Rønn R, Ekelund F and Jacobsen CS (2019) Total RNA-sequencing reveals multi-level microbial community changes and functional responses to wood ash application in agricultural and forest soil. DOI: https://doi.org/10.1101/621557 3) Schostag MD, Anwar MZ, Jacobsen CS, Larose C, Vogel TM, Maccario L, Jacquiod S, Faucherre S and PrieméA (2019) Transcriptomic responses to warming and cooling of an Arctic tundra soil microbiome. DOI: https://doi.org/10.1101/599233

GUIDELINES

Perform all the work under a microbiological hood and change gloves frequently.

MATERIALS

NAME	CATALOG #	VENDOR
NEBNext Multiplex Oligos for Illumina (Index Primers Set 1) - 24 rxns	E7335S	New England Biolabs
Liquid nitrogen		
UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v)	15593031	Thermo Fisher Scientific
NEBNext Ultra II Directional RNA Library Prep Kit for Illumina - 24 rxns	E7760S	New England Biolabs
Disposable Latex Gloves, Medium, 100/Box	GL002M.SIZE.1PK	Bio Basic Inc.
Qubit dsDNA HS Assay kit	Q32854	Thermo Fisher Scientific
RNase Zap	R2020-250ML	Sigma Aldrich
Bioanalyzer chips and reagents (DNA High Sensitivity and small RNA kit)	5067-1548	Agilent Technologies
80% Ethanol		
NEBNext Sample Purification Beads	E7767	New England Biolabs
Ice & ice bucket		
Qubit RNA HS Assay Kit	Q32852	Thermo Fisher Scientific
RNeasy® PowerSoil® Total RNA Kit	12866-25	Qiagen
S-Pak Filters 0.22µm 47mm white gridded	GSWG047S6	Merck Millipore
DNeasy Max®Kit	15200-50	Qiagen
KAPA Library Quantification Kit for Illumina® Platforms	KK4835	Kapa Biosystems
Liquid G2 DNA/RNA Enhancer	A420015	Ampliqon

MATERIALS TEXT

1. Microbiological Hood
2. Filtering system
3. Vacuum pump
4. Vortex-Genie® 2 Vortex
5. Vortex Adapter for 4 (15 ml) tubes (cat. no. 13000-V1-15)
6. Pipette tips with filter (20 µL – 1000 µL)
7. Thermoblock (set at 45°C)
8. Incubator for -20 and 4°C
9. 96-well 0.2 ml PCR Plates and Microseal® 'B' Adhesive Sealer (BioRad MSB-1001) or 0.2 ml RNase-free tube
10. 15 mL tubes and 1.5 mL vials racks
11. Qubit Invitrogen
12. Biology Tweezer 115mm, Ideal Tak
13. Bioanalyzer, Agilent G2991AA
14. SimpliAmp Thermal Cycler PCR, Applied Biosystems
15. Benchtop Centriguge
16. Mini-centrifuge
17. Magnetic Stand

SAFETY WARNINGS

When using Phenol Chloroform work under the fume hood, when not possible, seal the tubes properly with parafilm

BEFORE STARTING

Remove RNases cleaning the surfaces, pipettes/forceps and gloves using RNaseZAP according to the manufacturer protocol. Pre-cool all the centrifuges.

Water samples filtration

1 Assemble the filtration system and place the 0.22 µm MF-membranes filters using RNase free forceps

2 Once completed the filtration, transfer the filters in the 15 mL tubes with beads (provided in the RNeasy®kit).

Transfer the filters carefully folding it to fit in the 15mL tubes using two forceps and letting the surface with visible filtered particles inward the tube. Push carefully down to the beads.

No more than 2 filters can be added to a single tube

RNA Isolation

3 Add G2 and snap freeze immersing the bead tubes 15 seconds in liquid nitrogen keeping the lid slightly open

Add 500µL of G2 each filter. If a thick sediment layer accumulates on the filter, it has to be weighed and 500 µL of G2 added each 0.25 grams of sediments. No more than 1 gram can be added to the tube. Addition of more soil/sediments can reduce the lyse cells efficiency. For more info on G2 test see appendix 1 go to step #133 *. Alternatively, to liquid nitrogen, tubes can be freeze O/N at -20°C*

4 Immediately add 2.5 ml of PowerBead Solution

5 Add 0.25 ml of Solution SR1

6 Add 0.8 ml of Solution IRS

7 Add 3.5 ml of phenol/chloroform/isoamyl alcohol (pH 6.5) and vortex until the solution is homogeneous.

After vortexing the solution should have a muddy consistency. At this stage the tubes have to be placed outside the Microbiological hood and to avoid phenol chloroform diffusion in the air is preferably to seal the tubes using parafilm

8 Place the PowerBead tube horizontally on a Vortex Adapter (cat. no. 13000-V1-15) and vortex at maximum speed for 20 minutes at **Room temperature**

This vortexing time was specifically adapted for tubes containing filters with the addition of Phenil chloroform and previously snap freezed in Liquid Nitrogen. For different samples vortex for 15 minutes according to the manufacturer protocol

9

Remove PowerBead tube from the shaker and centrifuge **Room temperature** for 10 minutes at 2,500 x g

For different centrifuge unit see appendix 2 **go to step #134**

10 Transfer the upper aqueous phase (avoid the interphase and lower phenol layer) to a clean 15 ml Collection Tube (provided) using a 1000µL pipette. Ensure to take only the top layer (Approx. 3 mL) leaving some aqueous phase to not disturb the interphase.

If the filters, after centrifuge, are interfering with normal procedure of pipetting or have formed agglomerates, pour the tube content (leaving behind the filters) in a new 15 mL tube and repeat the centrifugation at step 9.

11 Add 1.5 ml of Solution SR3 to the tube containing the aqueous phase and vortex to mix until the solution gets homogeneous

12 Incubate at **4 °C** for 10 minutes

13 Centrifuge at 2,500 x g **Room temperature** for 10 minutes

14 Transfer the supernatant, without disturbing the pellet if present (usually not present), to a new 15 ml Collection Tube (provided)

Its preferred to leave behind few microliters

15 Add 5 mL of Solution SR4 to the supernatant in the Collection Tube using a serological 5 mL pipette

16 Vortex until the biphasic layer disappear (2 cycles of 10 seconds)

17 Incubate at **Room temperature** for 30 minutes

18 Centrifuge at 2500 x g at **Room temperature** for 30 minutes

19 Decant the supernatant

20 Quickly spin down the tubes to collect remaining drops on the tubes wall

21 Remove the collected liquid without disturbing the pellet

22 Allow the tubes to dry for 5 minutes

Do not let the pellet over dry (more than 10 minutes) or will result hard to resuspend it.
Pellet colors were observed in a range of light grey and more often light/dark brown.

RNA Purification

23 Shake Solution SR5 and add 1 ml to the 15 ml Collection Tube.

24 Resuspend the pellet completely by repeatedly pipetting.

Usually is hard to be resuspended and using a P1000 pipette just remove the pellet from the wall and proceed to the next step.

25 Incubate the tubes at **45 °C** in a preheated thermoblock. Check on the tube every 5 min and vortex the solution. Visual inspection should reveal the total resuspension.

26 Repeat step 25 until the pellet is fully resuspended

27 Once the pellet is resuspended place the JetStar Mini Column (provided capture column) in a new 15 ml tube

28 Shake SR5 solution and add 2 mL to the RNA capture column

From this step on never allow to completely gravity flow through the column.A dry column will reduce the downstream RNA yield.Add warning here

29 Once the SR5 solution has reached the top of the resin (1 mm thickness) in the column add the dissolved pellet to the column and allow it to gravity flow through the column

30 When dissolved pellet has reached the top of the resin (1 mm thickness) add 1 ml of shacked SR5 solution to the capture column and allow it to gravity flow through it

31 Once the SR5 solution has reached the top of the resin shake solution SR6 and add 1 mL to the column

32 Immediately transfer the RNA capture column to a new clean 15 mL tube

33 Allow solution SR6 to flow through the column. Here wait until all the column is completely dry

34 Transfer the eluted RNA to a 2.2 mL Collection Tube (provided)

35 Shake solution SR4 and add 1mL to the 2.2 mL collection tube. Close gently the lid and invert tube to mix 10 times.

36 Incubate tube at **-20 °C** for 30 minutes

37 Centrifuge the 2.2 vials at **4 °C** for 15 minutes at 13,000 x g

After centrifugation the pellet may have a light brown color or white. If the final RNA yield is low may be barely visible and in this case is recommendable to place the vials in the centrifuge memorizing the position where the centrifugation force may deposit the pellet in order to have a proper final RNA resuspension.

38 Decant the supernatant

39 Quickly spin down the tubes to collect remaining drops on the tubes wall

40 Remove the supernatant using a smaller pipette (P20 or P100) without touching the pellet

41 Air dry the pellet for 5 minutes

42 Resuspend the RNA pellet in 40 µL of Solution SR7

43 Split the resuspended RNA in clean RNase free 200µL PCR tubes as follows.
Final Volume (FV) 40 µL

Tube 1 – 17 µL
Tube 2 – 17 µL
Tube 3 – ~ 6 µL

Tube 3 will be used for RNA quantification and RIN assessment while tube number 1 will be used for the downstream DNase treatment. Tube 2 can be stored at -80°C as a back-up sample in case of problems during the downstream treatments.
If a low RNA yield is occurring (according to preliminary tests) resuspend the RNA pellet (step 42) in a 20 µL final volume and split the sample as follows:
Tube 1 – 17 µL
Tube 2 – ~3 µL

44 Place the PCR tubes on ice and proceed to step 45 or place the samples at **-20 °C** for short storage and **-80 °C** for longer

RNA yield assessment

45 Place the PCR tubes containing the eluted RNA on ice

46 Determine concentration of RNA using Qubit. Take 2-4 µL from tube #3

DNase treatment using DNase Max Kit

47 Thaw on ice the tube #1 containing 17µL total RNA extraction from step 43 **go to step #43**

48 Add 2µL of 10X DNase Buffer

49 Add 1 µL of DNase I enzyme (10 units)

50 Mix by gently pipetting

Don't vortex the DNase I enzyme or the final mixture. It will denature the enzyme

51 Incubate the mixture, using a thermocycler, at **37 °C** for 20 minutes and proceed to the next step, the DNase removal

52 Resuspend the DNase Removal resin by vortexing until the slurry is homogeneous

53 Add 5 µL of DNase removal resin to the mixture (step 51) go to step #51

54 Incubate for 10 minutes at **Room temperature** and check on the tube every 2 minutes with resuspending the resin by gently pipetting the solution

55 Centrifuge the tubes at 13.000 x g for 1 minute to pellet the resin

56 Transfer the supernatant containing the RNA target to a new clean 200µL tube without touching or removing the resin

To avoid contaminant carryover pipette approximately 15µL

57 Determine the successful DNA removal procedure using Qubit or for higher detection limit perform a qPCR control. Use 1 to 5 µL from the previous step (56)

58 Determine concentration of RNA using Qubit. Use 1-2 µL

59 Proceed to the RIN assessment number or store the RNA at **-80 °C**

RNA quality assesment (RIN)

60 Allow all reagents to equilibrate to room temperature for 30 minutes before use and protect dye and dye mixture from light.

61 Set up the priming station for Bioanalyzer® chip adjusting the base plate in C position and sliding the syringe clip up to the top position

Be sure of the clip position. Different position will lead to a negative output

62 Prepare the RNA ladder and the gel

Ladder and gel preparation described in appendix 3 go to step #135

63 Vortex RNA dye concentrate (blue cap vial) for 10 seconds, spin down and add 1 µL of dye into a 65 µL aliquot of filtered gel

64 Vortex solution well. Spin tube at 13000g for 10 minutes at **Room temperature**

Use prepared gel-dye mix within one day

65 Place a new RNA chip on the chip priming station

Always insert the pipette tip to the bottom of the well when dispensing the liquid. Placing the pipette at the edge of the well may lead to poor results.

66 Pipette 9 µL of gel-dye mix in the well marked

67 Make sure that the plunger is positioned at 1 mL and then close the chip priming station

68 Press plunger until it is held by the clip

69 Wait for exactly 30 seconds then release clip

70 Wait for 5 seconds and slowly pull back plunger to 1 mL position

71 Open the chip priming station and pipette 9 µL of gel-dye mix in the wells marked with

72 Pipette 9 µL of the RNA conditioning solution (vial with white cap) into the well marked CS

73 Pipette 5 µL of RNA marker (green cap vial) in all 11 sample wells and in the well marked

74 Pipette 1 µL of the heat denatured and aliquoted ladder in the well marked

75 Pipette 1 µL of sample in each of the 11 sample wells. Pipette 1 µL of RNA Marker (green cap vial) in each unused sample well

It is recommended to heat denature all RNA samples before use for 2 min and 70 °C (once) and keep them on ice

76 Place the chip in the Agilent 2100 Bioanalyzer and run the instrument within 5 min

Set up the Bioanalyzer instrument prior loading the chip. Keeping loaded gel for more than 5 minutes will allow the liquid in it to evaporate leading to a poor or non-acceptable analysis.
For a clear overview of the RNA integrity electropherogram output from the bioanalyzer see appendix 4
go to step #137

77 Place the chip horizontally in the IKA vortexer and vortex for 1 min at 2400rpm.

DO NOT VORTEX FOR MORE THAN 1 MINUTE AND MORE THAN 2400rpm

78

This procedure was used for libraries without any enrichment or depletion of total RNA with RIN scores > 7 and a concentration of 100 ng purified RNA. RNA was prepared in 5 µL final volume of Nuclease-free water, to start with the first protocol step. The protocol is optimized for approximately 200 nt RNA inserts. To generate libraries with longer RNA insert sizes or lower RIN number, refer to Neb manufacturer protocol.

RNA Fragmentation and Priming

Assemble all the reaction on ice using a nuclease-free tubes and in a dedicated clean PCR hood. The Illumina library preparation requires 7 steps using the thermocycler with different incubation times/cycles and temperatures; it is highly recommended to create a dedicated folder in the thermocycler with saved programs as suggested in the protocol in a chronological way. See appendix 5 go to step #138 to set up the thermocycler

Thaw the RNA samples from step 58 on ice go to step #58

79 Perform dilutions, if necessary, to obtain a 100ng of total RNA in 5µL Final Volume using new clean 200µL PCR tubes

80 Set up the reaction (using the tubes from the previous step) as follows:

81 Set a 20 µL pipette to 5 µL and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube

82 Place the sample in a thermocycler, upload and start program #1

For program #1 see appendix 5 go to step #138

83 Once the incubation is ended, immediately transfer the tube to ice

84 Keeping the tubes on ice, assemble the following reaction:

85 Set a 20 µL pipette to 10 µL and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube

86 Place the sample in a thermocycler, upload and start program #2

For program #2 see appendix 5 go to step #138

87 Once the incubation is ended, immediately transfer the tube to ice and proceed to next step

88 Keeping the tubes on ice, assemble the following reaction:

89 Set a 100 µL pipette to 40 µL and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube

90 Place the sample in a thermocycler, upload and start program #3

For program #3 see appendix 5 go to step #138

91 Once the incubation is ended proceed to the first Purification step (91.1)

Purification of Double-stranded cDNA

91.1

Before starting, prepare 80% ethanol; use a daily freshly prepared one. It is preferably to process no more than 6 samples at once; this will reduce the risk of over-dry the beads with a lower DNA target recovery. In this protocol the purification was performed usingNEBNext Sample Purification Beads

Resuspend the NEBNext Sample Purification Beads by vortexing until the slurry is homogeneous

91.2 Add 144 µL (1.8X) of resuspended beads to the tubes from step 89 (second strand synthesis, ~80 µl) go to step #89

91.3 Mix well on a vortex mixer

91.4 Incubate for 5 minutes at Room temperature

Briefly spin the tube in a microcentrifuge to collect any sample on the sides of the tube

91.5

91.6 Place the tube on a magnetic rack for ~5 minutes to separate beads from the supernatant

91.7 Once the solution is clear, carefully remove and discard the supernatant without either removing or touching the beads

Be careful with the beads, they retain the target DNA

91.8 Add 200 µL of 80% ethanol to the tube while in the magnetic rack

91.9 Incubate at **Room temperature** for 30 seconds and then carefully remove and discard the supernatant

91.10 Repeat from Step 91.8 once for a total of 2 washing steps

91.11 Briefly spin the tubes and put the tubes back on the magnetic rack

91.12 Remove the residual ethanol pipetting and air dry the beads for up to **5 minutes** while the tube is on the magnetic rack with lid open

Do not over-dry the beads. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry

91.13 Remove the tube from the magnetic rack

91.14 Add 53µL of 0.1X TE Buffer (provided) to elute the DNA from the beads

91.15 Mix well on a vortex mixer

91.16 Quickly spin the tube and incubate for 2 minutes at **Room temperature**

91.17 Place the tube on the magnetic rack for ~5 minutes, until the solution is clear

91.18 Remove 50µL of the supernatant and transfer to a clean 200µL nuclease free PCR tube without disturbing the beads pellet

If you need to stop, this is a safe stop point. Samples can be stored at -20°C

Illumina library preparation

92 Place the tube from step 91.18 on ice and assemble the following reaction:

Here is possible to prepare a mastermix. If a master mix is made, add 10µL of master mix to 50µL of cDNA for the End Prep reaction

93 Set a 100 µL pipette to 50 µL and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube

It is important to mix well. The presence of a small amount of bubbles will not interfere

94 Place the sample in a thermocycler, upload and start program #4.
Immediately proceed to the next step

For program #4 see appendix 5 go to step #138

95 During the incubation, perform a 5-fold dilution of the (red) NEBNext Adaptor in Adaptor Dilution Buffer and keep the diluted adaptors on ice

The NEBNext adaptor is provided in NEBNext oligos

96 Once both the Adaptor dilution and incubation are done, assemble the ligation reaction on ice by adding the following components

Follow the order given. We do not recommend a premix

97 Set a 100 µL pipette to 80 µL and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube

The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance

98 Place the sample in a thermocycler, upload and start program #5

For program #5 see appendix 5 go to step #138

99 Add 3 µL (blue cap vial) USER Enzyme to the ligation mixture after the incubation, resulting in total volume of 96.5 µL

100 Set a 100 µL pipette to 80 µL and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube

101 Place the sample in a thermocycler, upload and start program #6

For program #6 see appendix 5 go to step #138

102 Once the incubation is ended proceed to the second Purification step

Purification of the Ligation Reaction

102.1 Resuspend the NEBNext Sample Purification Beads by vortexing until the slurry is homogeneous

102.2 Add 87µL (0.9X) of resuspended beads to the tubes from step 100 (Ligation reaction, ~96.5µL)

102.3 Mix well on a vortex mixer

102.4 Incubate for 10 minutes at **Room temperature**

102.5 Briefly spin the tube in a microcentrifuge to collect any sample on the sides of the tube

102.6 Place the tube on a magnetic rack for ~5 minutes to separate beads from the supernatant

102.7 Once the solution is clear, carefully remove and discard the supernatant without either removing or touching the beads

Be careful with the beads, they retain the target DNA

102.8 Add 200 µL of 80% ethanol to the tube while in the magnetic rack

102.9 Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant

02.10 Repeat from Step 102.8 once for a total of 2 washing steps

02.11 Briefly spin the tubes and put the tubes back on the magnetic rack

02.12 Remove the residual ethanol pipetting and air dry the beads for up to **5 minutes** while the tube is on the magnetic rack with lid open

Do not over-dry the beads. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry

02.13 Remove the tube from the magnetic rack

02.14 Add 17µL of of 0.1X TE Buffer (provided) to elute the DNA from the beads

02.15 Mix well on a vortex mixer

02.16 Quickly spin the tube and incubate for 2 minutes at **Room temperature**

02.17 Place the tube on the magnetic rack for ~5 minutes, until the solution is clear

02.18 Remove 15µL of the supernatant and transfer to a clean 200µL nuclease free PCR tube without disturbing the beads pellet

If you need to stop, this is a safe stop point. Samples can be stored at -20°C

Illumina library preparation

103 Place the tube from step 102.18 on ice and assemble the following reaction:

The following PCR Enrichment was performed specifically using NEBNext Multiplex Oligos for Illumina (Index Primers set 1) (NEB #E7335S) with the oligos concentration of 10 µM.
The PCR cycles are recommended using an initial RNA concentration of 100 ng; limit the PCR cycles to avoid overamplification with a secondary unwanted peak (~1000 bp) which will appear on the Bioanalyzer

The Universal PCR Primer should be added to all the samples while the indexing should be unique for each sample. See appendix 6 go to step #139 *for an example of assembling reaction*

104 Place the sample in a thermocycler, upload and start program #7

For program #7 see appendix 5 go to step #138

105 Once the PCR amplification is ended proceed to the last Purification step

Purification of the PCR Reaction

105.1 Resuspend the NEBNext Sample Purification Beads by vortexing until the slurry is homogeneous

105.2 Add 45µL (0.9X) of resuspended beads to the tubes from step 100 (PCR reaction, ~50µL)

105.3 Mix well on a vortex mixer

105.4 Incubate for 5 minutes at Room temperature

105.5 Briefly spin the tube in a microcentrifuge to collect any sample on the sides of the tube

105.6 Place the tube on a magnetic rack for ~5 minutes to separate beads from the supernatant

105.7 Once the solution is clear, carefully remove and discard the supernatant without either removing or touching the beads

> *Be careful with the beads, they retain the target DNA*

105.8 Add 200µL of 80% ethanol to the tube while in the magnetic rack

105.9 Incubate at **Room temperature** for 30 seconds, and then carefully remove and discard the supernatant

05.10 Repeat from Step 105.8 once for a total of 2 washing steps

05.11 Briefly spin the tubes and put the tubes back on the magnetic rack

05.12 Remove the residual ethanol pipetting and air dry the beads for up to **5 minutes** while the tube is on the magnetic rack with lid open

> *Do not over-dry the beads. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry*

05.13 Remove the tube from the magnetic rack

05.14 Add 23µL of 0.1X TE Buffer (provided) to elute the DNA from the beads

05.15 Mix well on a vortex mixer

05.16 Quickly spin the tube and incubate for 2 minutes at **Room temperature**

05.17 Remove 20µL of the supernatant and transfer to a clean 200µL nuclease free PCR tube without disturbing the beads pellet

106 Determine concentration of DNA (Step 105.17) using Qubit. Use 1 µL

107 Store the DNA at -20°C or proceed to the library quality assessment on Bioanalyzer DNA chip

Library Average Fragment Length Assessment on an Agilent Bioanalyzer DNA Chip

108 Allow all reagents to equilibrate to room temperature for 30 minutes before use and protect dye and dye mixture from light

109 Set up the priming station for Bioanalyzer® chip adjusting the base plate in C position and sliding the syringe clip up to the bottom position

Be sure of the clip position. Different position will lead to a negative output

Preparing the Gel-Dye Mix

110 Add 15 µL of High Sensitivity DNA dye concentrate (blue) to a High Sensitivity DNA gel matrix vial (red)

111 Vortex solution well and spin down. Transfer to spin filter

112 Centrifuge at 2240 g ± 20 % for 15 min. Store at 4 °C.

Protect solution from light. Use prepared gel-dye mix within 6 weeks of preparation

Chip loading

Always insert the pipette tip to the bottom of the well when dispensing the liquid. Placing the pipette at the edge of the well may lead to poor results

113 Place a new DNA chip on the chip priming station

114 Pipette 9 µL of gel-dye mix in the well marked

115 Make sure that the plunger is positioned at 1 mL and then close the chip priming station

116 Press plunger until it is held by the clip

117 Wait for exactly 60 seconds then release clip

118 Wait for 5 seconds and slowly pull back plunger to 1 mL position

119 Open the chip priming station and pipette 9 µL of gel-dye mix in the wells marked with

120 Pipette 5 µL of DNA marker (green cap vial) in all 11 sample wells and in the well marked

121 Pipette 1 µL of High Sensitivity DNA ladder (yellow cap vial) in the well marked

122 Pipette 1µL of sample in each of the 11 sample wells. Pipette 1µL of DNA Marker (green cap vial) in each unused sample well

123 Positionate the chip horizontally in the IKA vortexer and vortex for 1 min at 2400rpm

DO NOT VORTEX FOR MORE THAN 1 MINUTE AND MORE THAN 2400rpm

124 Place the chip in the Agilent 2100 Bioanalyzer and run the instrument within 5 min

Set up the Bioanalyzer instrument prior loading the chip. Keeping loaded gel for more than 5 minutes will allow the liquid in it to evaporate leading to a poor or non-acceptable analysis.
For a clear overview of the DNA Average Fragment Length electropherogram output from the bioanalyzer see appendix 7
go to step #140

qPCR quantification of the library prepared for Illumina sequencing

125 Place all the reagents of the KAPA Library thawing on ice

If the kit is used for the first time, add the Primer Premix (10X) (1 mL) to the bottle of KAPA SYBR® FAST qPCR Master Mix (2X) (5 mL). Mix thoroughly using a vortex mixer

126 Prepare the appropriate library dilutions (using DNA dilution buffer). Depending on the expected concentration of the library, 1:1,000 – 1:100,000 dilutions may be appropriate

All DNA Standards and library dilutions have to be assayed in triplicate.

Each sample concentration should fall within the dynamic range of given standards.Check appendix 8
go to step #141 *to see examples of dilution calculations*

127 Setup the following reaction on ice using 96-well 0.2 ml PCR Plates

128 Perform qPCR with the following cycling protocol:

129 Perform the results analysis to check the library quantification

KAPABIOSYSTEMS provides a spreadsheet premade designed for the analysis of NGS library quantification data generated

KAPABIOSYSTEMS provides a spreadsheet premade designed for the analysis of NGS library quantification data generated with the KAPA Library Quantification Kit for Illumina®. You can download it at the following link: https://www.google.it/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=2ahUKEwjAyPrV2s_hAhWxlYsKHW-mB9gQFjAAegQIARAC&url=https%3A%2F%2Fwww.kapabiosystems.com%2Fdocument%2Fkapa-library-quantification-data-analysis-template%2F%3Fdl%3D1&usg=AOvVaw0cKEl4Os4HF1F8a

130 Normalize all the DNA samples to the lowest amount of DNA

131 Pool the samples for the Illumina sequencing

132 Store the samples at -80°C

Appendix 1

133 **G2 test**

"G2 DNA/RNA Enhancer is convenient to use, when optimal DNA and or RNA extraction yield is required from especially clay. The primary function of G2 DNA/RNA Enhancer is to relieve inhibitory DNA-clay particle formations. G2 DNA/RNA Enhancer should be used in combination with either standardized extraction methods or commercial kits intended for DNA & RNA extraction from soil and clay" (Ampliqon).

For this test were processed 500 mL and 1 mg of debris/soil for each sample with the addition of G2. According to protocol were added 2 mL of G2, to check the effect on the RNA extraction. A control without G2 was used.

Graphic representation of the higher RNA yield using G2

Appendix 2

134 Relative Centrifugal Force (**RCF**) is the term used to describe the amount of accelerative force applied to a sample in a centrifuge. **RCF** is measured in multiples of the standard acceleration due to gravity at the Earth's surface (x g). This is why **RCF** and "x **g**" are used interchangeably in centrifugation protocols.

RCF or G = 1.12 x Radius (mm) x $(rpm/1000)^2$

Appendix 3

135 **Preparing the RNA Ladder**

135.1 Spin the ladder down and pipette in an RNase-free vial

135.2 Heat denature the ladder for 2 min at **70 °C**

135.3 Immediately cool the vial on ice

135.4 Add 90µL of RNase-free water and mix thoroughly

135.5 Prepare aliquots in recommended 0.5 mL RNase-free vials with the required amount for typical daily use

135.6 Store aliquots at **-80 °C** . After initial heat denaturation, the frozen aliquots should not require repeated heat denaturation

135.7 Before use, thaw ladder aliquots on ice

Avoid extensive warming

136 **Preparing the Gel**

136.1 Pipette 550 µL of RNA gel matrix (red) into a spin filter

136.2 Centrifuge at 1500g ± 20 % for 10 min at **Room temperature**

136.3 Aliquot 65µL filtered gel into 0.5 mL RNase-free microcentrifuge tubes.Use filtered gel within 4 weeks.
Store at **4 °C** .

Appendix 4

137 In the following section can be understood how the bioanalyzer works and the regions evaluated to check the final quality score

Electropherogram detailing the regions that are indicative of RNA quality

Sample electropherograms used to assess the RNA Integrity Number (RIN) software. Samples range from intact (RIN 10), to degraded (RIN 2)

Appendix 5

138 PCR Tables

Appendix 6

139 Examples of how to perform PCR enrichment (Indexing) with 4 samples:

The only difference in the mixture is the Indexing choice (**Highlighted in Bold**). NebNext Multiplex Oligoes for Illumina (Index Primers Set 1) contains 12 index primers and they can all be used following the previous example for 12 samples.

Appendix 7

140 Assessment of DNA library fragment length average

Figure showing an example of correct RNA library size distribution on a Bioanalyzer. The electropherogram should show a narrow distribution with a peak size approximately 300 bp.

If a peak at ~ 80 bp (primers) or 128 bp (adaptor-dimer) is visible in the Bioanalyzer traces; Bring up the sample volume (from Step 104.18) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 105.1).

A dilution may be necessary for running on a Bioanalyzer High Sensitivity DNA Chip

Appendix 8

141 **qPCR dilutions calculation**

Prepare the appropriate library dilutions (using DNA dilution buffer). Depending on the expected concentration of the library, 1:1,000 – 1:100,000 dilutions may be appropriate.
It is recommended to perform a ten-fold serial dilution to obtain a more reliable dilution.

Follow the empiric calculation:

1 ng/µL from the library with a 300 bp length it's equal to say 3.5 nM in concentration

So, 1ng/µL = 3.5 nM in concentration

Practical calculation:

If we have a sample with 20 ng/µL from the library, multiply 20 ng/µL for 3.5 (conversion value) :

20 ng/µL x 3.5 = 70 nM

The value in µM has to be transformed in pM and diluted a proper number of times to fall in the following standard table provided by the KAPABiosynthesis.

70 nM = 70.000 pM

Possibly we should dilute the samples to fall between standard 3 and 5.

70.000 pM has to be diluted at least 100.000 times in order to obtain a final concentration of 0.7 pM. In this case an additional 10-fold dilution of each sample is recommended.