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PROTOCOL

CATS RNA-seq v2 library preparation protocol on RNA from FFPE-samples

INTRODUCTION

The following protocol offers a streamlined solution for whole transcriptome sequencing studies from human/mouse/rat FFPE tissues.

The extraction and purification of the RNA from the FFPE tissue is recommended with Qiagen's AllPrep® DNA/RNA FFPE kit. This protocol allows the researcher to purify in parallel the total RNA and genomic DNA from the sample of origin. After that, the total RNA is processed in the CATS total RNA-seq Kit v2 (C05010042) workflow starting with the depletion of cytoplasmic and mitochondrial ribosomal RNA. Afterwards, the Illumina® compatible library can be further processed for sequencing.

Before starting, it is **strongly advised to read thoroughly** both **Qiagen's AllPrep DNA/RNA FFPE handbook** and CATS total RNA-seq Kit v2 user manual.

STEP A: RNA EXTRACTION FROM FFPE SAMPLES

All Prep DNA/FFPE extraction and purification protocol

Storage

RNase-free DNase I, RNeasy MinElute spin columns and QIAamp MinElute spin columns should be immediately stored at 2-8°C upon arrival. Buffers can be stored at room temperature (RT, 15-25°C). Under these conditions, the kit components can be kept for at least 9 months without any reduction in performance.

Proteinase K is supplied in a specifically formulated storage buffer and is stable for at least 1 year after delivery when stored at RT. If longer storage is required or if ambient temperature often exceeds 25°C, Qiagen recommends storage at 2-8°C.

Equipment and reagents to be supplied by user

- Sterile, RNase-free pipet tips with aerosol barriers
- 1.5 ml microcentrifuge tubes
- 2.0 ml microcentrifuge tubes
- Microcentrifuge with rotor for 2.0 ml tubes
- Vortexer
- 96-100% absolute ethanol

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- 96-100% isopropanol
- Deparaffinization solution (Qiagen, Cat. No. 19093)
- Thermal mixer, heated orbital shaker, heating block, or water bath capable of incubation at 56°C, 80°C and 90°C

Starting material and general recommendations

Standard formalin-fixation and paraffin-embedding procedures always result in significant fragmentation and crosslinking of nucleic acids. To limit the extent of nucleic acid fragmentation and crosslinking, be sure to:

- Fixate tissue samples in 4-10% formalin as quickly as possible after surgical removal
- Use a maximum fixation time of 24 hours (longer fixation times lead to over fixation and more severe nucleic acid fragmentation, resulting in poor performance in downstream assays)
- Thoroughly dehydrate samples prior embedding, as high temperature during embedding can cause nucleic acid fragmentation
- Store FFPE samples at low temperature (2-8°C); storage at RT (15-25°C) can lead to nucleic acid degradation

The starting material for nucleic acid purification should be freshly cut sections of FFPE tissue, each with a thickness of 10-20 µm. Thicker section may result in lower nucleic acid yields, even after prolonged incubation with proteinase K. Thinner sections can be used but more difficult to pellet. Up to 4 sections, each with a thickness of 10 µm and a surface of up to 150 mm^2, or 2 sections each with a thickness of 20 µm and a surface area of up to 150 mm^2 can be combined in one preparation.

Important points before starting

- If using the AllPrep DNA/RNA FFPE kit for the first time, read the handbook available on Qiagen's website
- **Buffer RLT, buffer FRN, buffer AL, and buffer AW1 contain guanidine salt and are therefore not compatible with disinfecting reagents containing bleach**. See safety information in the AllPrep DNA/RNA FFPE kit handbook.
- Unless otherwise indicated, perform all steps of the procedure at RT (15-25°C). During the procedure, work quickly.
- Perform all centrifugation steps using a microcentrifuge, set the temperature to 20-25°C, otherwise significant cooling below 15°C may occur.
- In the procedure below, TOTAL indicates specific steps for the purification of total RNA that **does not** include small RNAs, and +**SMALL** indicates specific steps for purification of total RNA that **does** include small RNAs.

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Things to do before starting

- If using buffer FRN, buffer RPE, buffer AW1, buffer AW2, and RNase-free DNase I for the first time, **reconstitute them as described in "preparation of buffers" in AllPrep DNA/RNA FFPE kit handbook**
- If necessary, warm and gently agitate buffers RLT, ATL, AL to re-dissolve any precipitates that may have formed
- Equilibrate all buffers to RT (15-25°C). Mix reconstituted buffer FRN, buffer RPE, buffer AW1, and buffer AW2 by shaking
- Set a thermal mixer or heated orbital incubator at 56°C for use in step 6. Incubation at 56°C and at 80°C can be done with or without agitation.

Protocol RNA extraction from FFPE samples

The following protocol describes the complete procedure to isolate and purify both genomic DNA and total RNA.

Please note: CATS total RNA-seq v2 method only allows **total RNA** as starting material. Therefore isolated genomic DNA must be further separately processed.

1. Using a scalpel, trim excess paraffin off the sample block
2. Cut section 10-20 µm thick
3. Immediately place the sections in a 1.5 ml microcentrifuge tube and close the lid
4. Remove the paraffin using the Qiagen's deparaffinization solution

   A. Add deparaffinization solution: for 2X 10 µm sections or one 20 µm section, add 320 µl of deparaffinization solution; for more sample material add 640 µl of deparaffinization solution

   B. Vortex vigorously for 10s, and centrifuge briefly to bring the sample to the bottom of the tube

   C. Incubate at 56°C for 3 minutes, and then allow cooling at RT (15-25°C) and centrifuging at full speed for 2 minutes

   D. Carefully remove the supernatant by pipetting without disturbing the pellet. Carefully remove any residual deparaffinization solution using a fine pipet tip

   E. Keep lid open and incubate for 10 minutes at 37°C to dry the pellet
5. Resuspend the pellet by adding 150 µl buffer PKD and flicking the tube to loosen the pellet. Add 10 µl proteinase K, and mix by vortexing
6. Incubate at 56°C for 15 minutes.

   Depending on the sample material, the sample may not be completely lysed. This does not affect the procedure. Proceed to step 7.
7. Incubate on ice for 3 minutes.
8. Centrifuge for 15 minutes at 20.000g

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9. Carefully transfer the supernatant, without disturbing the pellet, to a new (**TOTAL**) 1.5 ml tube or (+**SMALL**) 2.0 ml microcentrifuge tube for RNA purification in steps 10-24. Keep the pellet for DNA purification in steps 25-35.

   Note: Depending on the amount and nature of the FFPE sample, the pellet may be very small or difficult to see. If the pellet is aspirated with the supernatant, allow the pellet to drop slowly to the bottom of the tube and use the pipet tip to reattach the pellet to the tube. Alternatively, centrifuge the supernatant again. The DNA-containing pellet can be stored for 2h at RT, for up to 1 day at 2-8°C, or for longer periods at -15 to -30°C.

Purification of TOTAL RNA

10. Incubate the supernatant from step 9 at 80°C for 15 minutes.

    This incubation step partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperature may result in more fragmented RNA.

    If using only one heating block, keep the supernatant at RT until the heating block has reached 80°C. To ensure maximum RNA yields, the supernatant MUST be incubated at 80°C only for exactly 15 minutes.

11. Briefly centrifuge the tube to remove drops from the inside of the lid.
12. Add 320 µl buffer RLT to adjust binding conditions, and mix by vortexing or pipetting.
13. Add (TOTAL) 720 µl or (+SMALL) 1120 µl ethanol (96-100%), and mix well by vortexing or pipetting. Proceed immediately to step 14.

    Note: Precipitate may be visible after addition of ethanol. This does not affect the procedure.

14. Transfer 700 µl of the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a provided 2 ml collection tube. Close the lid gently, and centrifuge for 15s at ≥ 8000g. Discard the flow-through. Reuse the collection tube in step 15.

    Note: the flow through contains buffer RLT and is therefore not compatible with bleach. Consult Qiagen's AllPrep DNA/RNA FFPE kit handbook for safety information.

15. Repeat the step 14 until the entire sample has passed through the RNeasy MinElute spin column. Reuse the collection tube in step 16.
16. Add 350 µl buffer FRN to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15s at ≥8000g. Discard the flow through.

    Note: the flow through contains buffer FRN and is therefore not compatible with bleach. Consult Qiagen's AllPrep DNA/RNA FFPE kit handbook for safety information.

    Reuse the collection tube in step 17.

17. Add 10 µl DNaseI 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.

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Note: DNaseI is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.

18. Add the DNaseI incubation mix (80 µl) directly to the RNeasy MinElute spin column membrane, and place on the benchtop (20-30°C) for 15 minutes.

    Note: Be sure to add the DNaseI incubation mix directly to the membrane. DNase digestion may be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

19. Add 500 µl buffer FRN to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15s at ≥8000g. **Save the flow through for use in step 20.**

    Note: do not discard the flow through, as it contains the RNA including small RNAs.

20. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Apply the flow through from step 19 to the spin column. Close the lid gently, and centrifuge for 15s at ≥8000g. Discard the flow through. Reuse the collection tube in step 21.

    Note: the flow through contains buffer FRN and is therefore not compatible with bleach. Consult Qiagen's AllPrep DNA/RNA FFPE kit handbook for safety information.

21. Add 500 µl buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15s at ≥8000g to wash the spin column membrane. Discard the flow through. Reuse the collection tube in step 22.
22. Add 500 µl buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15s at ≥8000g to wash the spin column membrane. Discard the collection tube with the flow-through.
23. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid, and centrifuge at full speed for 5 minutes. Discard the collection tube with the flow-through.

    Note: to avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counter-clockwise). It is important to dry the spin column membrane, since residual ethanol may interfere with downstream applications. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

24. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14-30 µl RNase-free water directly to the spin column membrane. Close the lid gently, and incubate for 1 minutes at RT. Centrifuge at full speed for 1 minutes to elute the RNA.

    Note: the dead volume of the RNeasy MinElute spin column is 2 µl: elution with 14 µl RNase-free water results in 12 µl eluate.

    Up to 12 µl/rxn can be used in the following rRNA depletion process.

It is recommended quantifying the RNA yield using a Qubit™ fluorimeter with the associated HS RNA assay (ThermoFisher, Q32852) as well as assessing the RNA integrity after extraction/purification from the FFPE tissue. To do so, please use the RNA 6000 Pico assay (Agilent, 5067-1513) in total RNA mode with the Agilent's 2100 BioAnalyzer®.

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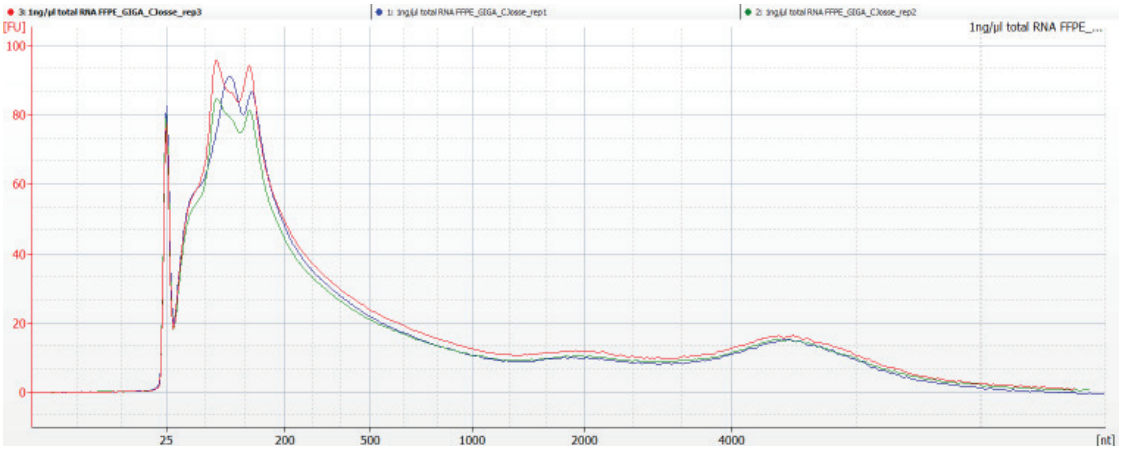

Figure 1: Three technical replicates RNA electropherograms (RIN = 2.4) of extracted/purified RNA from a 9 year-old FFPE triple negative breast cancer tissue biopsy.

Purification of genomic DNA

This section of the protocol is described for matters of continuity in the extraction/purification process but the purified gDNA will not be used any further in this method. Optionally, should gDNA be of interest as for DNA library preparation and sequencing, we recommend Diagenode MicroPlex Library Preparation Kit v2 (C05010012) for low DNA inputs starting at 50 pg.

25. Resuspend the pellet from step 9 in 180 µl of buffer ATL, add 40 µl proteinase K, and mix by vortexing.
26. Incubate at 56°C for 1h.
27. Incubate at 90°C for 2h without agitation.

    Note: this incubation step partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher temperature may result in more fragmented DNA. If using only one heating block, keep the sample at RT until the heating block has reached 90°C. Agitation during this step may lead to lower DNA yields.

28. Briefly centrifuge the microcentrifuge tube to remove drops from the inside of the lid.

    Optional: If RNA-free genomic DNA is required, allow the sample to cool to RT and then add 4 µl RNaseA (100 mg/ml) (not supplied). Incubate for 2 minutes at RT before proceeding to step 29.

29. Add 200 µl buffer AL, and mix thoroughly by vortexing. Then add 200 µl ethanol (96-100%), and mix thoroughly again by vortexing or pipetting.

    Note: It is essential that the sample, buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples. A white precipitate may form on addition of buffer AL and ethanol. This precipitate does not interfere with the AllPrep procedure.

30. Transfer the entire sample to a QIAamp MinElute spin column placed in a 2 ml collection tube (supplied).

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Close the lid gently, and centrifuge for 1 minutes at ≥8000g. Discard the collection tube with the flow through.

Note: The flow through contains buffer AL and buffer AW1 and is therefore not compatible with bleach. If the sample has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute spin column is empty.

31. Place the QIAamp MinElute spin column in a new 2 ml collection tube (supplied). Add 700 µl buffer AW1 to the spin column. Close the lid gently, and centrifuge for 15s at ≥8000g to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 32.

    Note: The flow through contains buffer AL and buffer AW1 and is therefore not compatible with bleach.

32. Add 700 µl buffer AW2 to the QIAamp MinElute spin column. Close the lid gently, and centrifuge for 15s at ≥8000g to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 33.

33. Add 700 µl ethanol (96-100%) to the QIAamp MinElute spin column. Close the lid gently, and centrifuge for 15s at ≥8000g. Discard the collection tube with the flow-through.

34. Place the QIAamp MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 minutes. Discard the collection tube with the flow-through.

    Note: to avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counter-clockwise). It is important to dry the spin column membrane, since residual ethanol may interfere with downstream applications. Centrifugation with the lids open ensures that no ethanol is carried over during DNA elution.

35. Place the QIAamp MinElute spin column in a new 1.5 ml collection tube (supplied). Add 30-100 µl buffer ATE directly to the spin column membrane. Close the lid gently, and incubate for 1 minutes at RT. Centrifuge at full speed for 1 minutes to elute the DNA.

    Important note: ensure that buffer ATE is equilibrated to RT. If using small elution volumes (< 50 µl), pipet the buffer ATE onto the centre of the membrane to ensure complete elution of the bound DNA. QIAamp MinElute spin columns provide flexibility in the choice of elution volume. Choose a volume according to the requirements of the downstream applications. The volume of eluate will be up to 5 µl less than the volume of buffer ATE applied to the column.

    Incubating the QIAamp MinElute spin column loaded with buffer ATE for 5 minutes at RT before centrifugation can increase DNA yield.

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STEP B: CATS RNA-SEQ V2 LIBRARY PREPARATION PROTOCOL ON RNA FROM FFPE SAMPLES

General recommendations

Recommended starting inputs of total RNA: 10 ng- 1 µg

Recommended RIN range ≥2

Fragment range: do not apply heat to the solution to already degraded RNA as it is surely already very fragmented (see point 1) page 12 in the below stated "Whole transcriptome library preparation on RNA from FFPE samples using CATS total RNA-seq Kit v2", Figure 1).

If the majority of the fragments are below 500 nt, it is not necessary to apply heat to the solution. If, for some reasons, the fragments are longer than 500 nt, a fragmentation time course at 94°C for a couple of minutes can be conducted.

rRNA depletion of RNA derived from FFPE samples using CATS total RNA-seq Kit v2

Required materials not provided

- Gloves
- Single channel pipettes and corresponding filter tips: 10 µl, 20 µl, 200 µl, 1000 µl
- Crushed ice
- RNase AWAY™ decontamination reagent (ThermoFisher Scientific, 10328011)
- Table top centrifuge with strip rotor
- Tube holder for 0.2 ml tubes
- DiaMag 0.2 ml tube magnetic rack (Diagenode, B04000001)
- Thermal cycler
- Agencourt RNAClean XP beads (Beckman Coulter Inc., #A63987)

Remarks before starting

The RNA sample should be free of salts (Mg^{2+}, guanidinium salts) or organic compounds (phenol, ethanol).

The actual yield of the process is dependent on the quality of the input RNA, the rRNA content of the sample and the method used to purify the total RNA. Typical recoveries are in between 10 to 20% of the starting amount of total RNA.

We recommend whenever possible to use total RNA inputs higher than 10 ng in order to increase the final library complexity and reduce sequencing duplication rate.

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Starting material: 10 ng – 1 µg of total RNA in a 12 µl total volume.

I. Hybridize the probe to the RNA

1. Prepare a RNA/probe master mix as follows:

Components	Volume
rRNA Depletion Solution	1 µl
Probe Hybridization Buffer	2 µl
Total Volume	**3 µl**

2. Add 3 µl of the above mix to 12 µl total RNA sample.
3. Mix by pipetting up and down.
4. Spin down briefly in a tabletop centrifuge, and immediately proceed to next step.
5. Place samples in a thermocycler, and run the following program which will take approximatively 15-20 minutes to complete:

Temperature	Time
95°C	2 min
95-22°C	0.1°C/sec
22°C	5 min hold

6. Spin down the samples in a table top centrifuge and place on ice. Proceed immediately to the next step.

II. RNase H digestion

7. On ice, prepare a master mix according to the following table, and mix by pipetting up and down. Use immediately

Components	Volume
RNase H	2 µl
RNase H Reaction Buffer	2 µl
Nuclease-free Water	1 µl
Total Volume	**5 µl**

8. Add 5 µl of the above mix to the RNA sample from step 6.
9. Mix by pipetting up and down.
10. Place the samples in a thermocycler (lid = 40°C) and incubate at 37°C for 30 minutes.

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11. Spin down the samples in a table top centrifuge and place on ice. Proceed immediately to the next step tp prevent non-specific degradation of RNA.

III. DNase I digestion

12. On ice, prepare a DNase I Digestion Master Mix according to the following table and mix by pipetting and down. Use immediately.

Components	Volume
DNase I Reaction Buffer	5 µl
DNase I (RNase-free)	2.5 µl
Nuclease-free Water	22.5 µl
Total Volume	**30 µl**

13. Add 30 µl of the above mix to the RNA sample from step 11 and mix by pipetting up and down.
14. Place samples in a thermocycler (lid = 40°C) and incubate at 37°C for 30 minutes.
15. Spin down the samples in a tabletop centrifuge and place on ice. Proceed immediately to the next step.

IV. RNA purification after rRNA depletion

16. Add 2.2X (110 µl) Agencourt RNAClean XP beads to the RNA sample from step 15 and mix by pipetting up and down.
17. Incubate samples on ice for 15 minutes.
18. Place the tube on an appropriate magnetic rack to separate the beads from the supernatant.
19. When the solution is clear, discard the supernatant.
20. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at RT for 30 seconds and then carefully remove and discard the supernatant.
21. Repeat step 20 once for a total of 2 washes.
22. Briefly spin the tube and put the tube back in the magnetic rack.
23. Completely remove the residual ethanol and air dry the beads.
24. Remove the tube from the magnetic rack. **Elute RNA from the beads with 7 µl of nuclease-free water.**
25. Mix well by pipetting up and down, wait for 2 minutes at RT and then put the tube in the magnetic rack until the solution is clear.
26. Transfer the 7 µl of the supernatant to a clean PCR tube.

    Optional: Assess the yield and size distribution of the sample using the BioAnalyzer® 2100 instrument with the RNA 6000 Pico kit. If quantification and sizing is not possible due to limited amount of material, the assumption can be made that 10% of the starting sample amount is recovered after rRNA depletion using this module.

27. Place the sample on ice and proceed with CATS library preparation kit or, alternatively, store the sample at -80°C until further use.

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WHOLE TRANSCRIPTOME LIBRARY PREPARATION ON RNA FROM FFPE SAMPLES USING CATS TOTAL RNA-SEQ KIT V2

Required materials not provided

General:

- Gloves
- Single channel pipettes and corresponding RNase-free **filter** tips: 10 µl, 20 µl, 200 µl, 1000 µl
- RNase AWAY™ decontamination reagent (ThermoFisher Scientific, 10328011)
- RNase-free tubes: 0.2, 0.5, 1.5 ml
- Table top centrifuge with strip rotor
- Centrifuge with a speed of 16000 g
- Vortex agitator
- Tube holder for 0.2, 0.5, 1.5 ml tubes
- DiaMag 0.2 ml tube magnetic rack (Diagenode, B04000001)
- DiaMag 1.5 ml tube magnetic rack (Diagenode, B04000003)
- Thermal cycler **with heated lid at 105°C (except for incubation at 4°C)**

For post-PCR libraries purification and clean-up:

- Agencourt® AMPure® XP Beads (Beckman Coulter, A63881)
- Absolute ethanol (VWR, 20821.310)
- 1X TE buffer (Sigma, 93283-100 ml)

For DNA libraries size and yield estimation:

- Agilent 2100 BioAnalyzer® and Agilent High Sensitivity DNA Kit (Agilent, 5067-4626)
- Qubit® Fluorometer (Thermo Fisher Scientific); Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32851)

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Protocol

Important note for points 8 and 15 in this section: The amounts of RNA to which we refer in this section are for the library preparation. Therefore, if quantification after rRNA depletion has not been possible because of limited amount of material, a recovery of 20% can be taken into account to estimate the quantity of RNA used into library preparation.

Example: starting material = 10 ng → rRNA depletion (20%) = 2 ng rRNA depleted RNA for library preparation.

1. Add 1 µl of RNA fragmentation buffer to the 7 µl of the template RNA (10 ng – 100 pg) and keep the sample on ice

   Note: It is important to add the fragmentation buffer to the reaction because it contains Mg^{2+} and it is essential for the enzymes to be active. However, do not apply heat to the solution to fragment the RNA as it is surely already very fragmented (Figure 1). If the majority of the fragments are below 500nt, it is not necessary to apply heat to the solution. If, for some reason, the fragments are longer than 500nt, a fragmentation time course at 94°C for 1-5 minutes depending on fragments size. (please contact Diagenode customer.support@diagenode.com *for further information).*

2. Prepare dephosphorylation master mix (DMM) by mixing 2 µl Dephosphorylation Buffer (DB) (yellow cap) and 0.3 µl Dephosphorylation reagent (DR) (yellow cap).
3. Add 2 µl DMM to 8 µl of RNA solution. Mix by pipetting up and down 5 times.
4. Incubate 15 minutes at 37°C on thermal cycler (lid = 105°C).
5. Meanwhile, prepare tailing master mix (TMM) by mixing 1 µl of Tailing Buffer PLUS (TBP) (red cap) and 0.5 µl Tailing Reagent PLUS (TRP) (red cap).
6. Add 1.5 µl TMM to the reaction tube. Mix by pipetting up and down 5 times.
7. Incubate 40 minutes at 37°C + 20 minutes at 65° on thermal cycler (lid = 105°C). Then, the sample can be kept on ice afterwards.
8. Add (purple cap)
   1 µl RT primer H (RTPH) for **10 ng – 500 pg** RNA
   or 1 µl RT primer M (RTPM) for **500 pg – 100 pg** RNA
   and mix by pipetting up and down 5 times.
9. Incubate 2 minutes at 72°C + 2 minutes at 42°C on thermal cycler (lid = 105°C).
10. Add 6 µl Reverse Transcription Reagent (RTR) (purple cap) and mix by pipetting up and down 5 times.
11. Incubate on thermal cycler at 42°C for 15 minutes (lid = 105°C).
12. Add 2 µl Template Switching Reagent (TSR) (purple cap) and mix by pipetting up and down 5 times.
13. Incubate 120 minutes at 42°C + 10 minutes at 70°C on thermal cycler (lid = 105°C).

    After reverse transcription, the sample can be kept at 4°C overnight or frozen until further use.

14. Add 10 µl of 10 µM CATS Index (24 indexes) (blue cap) and 70 µl PCR Master Mix (PMM) (green cap). Mix by pipetting up and down 5 times (with the same tip after adding PMM).

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15. PCR reaction (lid of the thermal cycler = 105°C):
    Initial denaturation: 98°C for 30 sec
    Amplification cycles: + from 12 (10 ng RNA input) to 18 (100 pg RNA input)
    Cycle:

Temperature	Time
(denaturation) 98°C	10 sec
(hybridization) 62°C	30 sec
(elongation) 72°C	30 sec
Final extension 72°C	**10 min**

Hold at 4°C or store at -20°C until further use

16. Optional: run a 2% agarose gel electrophoresis with 1/5th (20 µl) of the library to assess whether the library has been amplified enough
17. Clean up the library with 0.8X AMPure® XP beads (80 µl) and elute the product in 50 µl of water

    Note: If 20 µl has been taken to run a gel electrophoresis, adapt the volume of beads to add to keep the ratio at 0.8X.

18. Repeat the 0.8X clean-up procedure (45 µl) with AMPure® XP beads but elute the purified library in 20 µl 1X TE buffer
19. Perform quality check of the library with Qubit® + BioAnalyzer®
20. Load to Illumina sequencing platform.

For paired-end sequencing, use Illumina standard sequencing primer for read1 and Diagenode CATS paired-end sequencing primer (for read2) (C17011050).

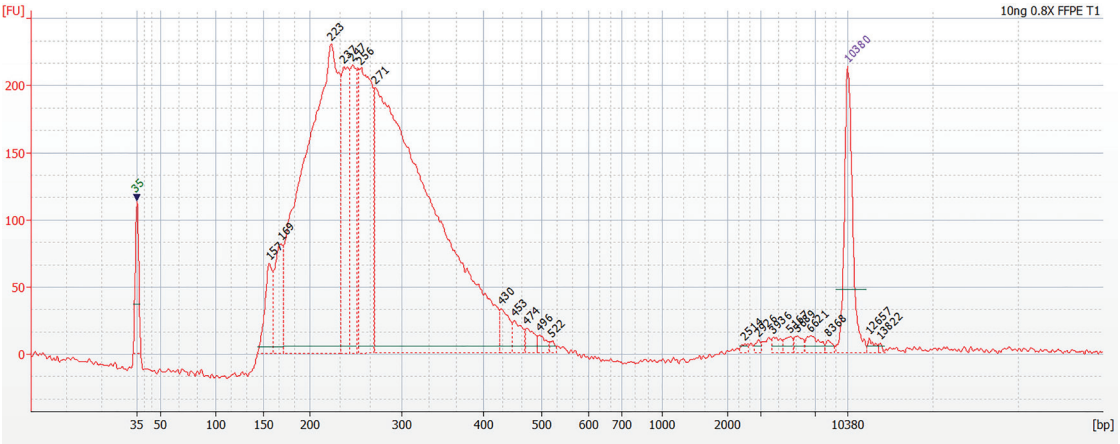

Figure 2: DNA electropherogram of CATS total RNA-seq v2 library made from 10 ng of total RNA extracted from 9 year-old FFPE sample.

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