otogenetics corporation
4553 Winters Chapel Road, Ste 100
Atlanta, GA 30360
USA
www.otogenetics.com sales@otogenetics.com
Tel: 1-855-oto-gene (1-855-686-4363)

RNA-Seq of FFPE Samples: Recommendations and Expectations

Description

This summary discusses (1) important notes for RNA extraction from FFPE tissues, (2) QC standard of FFPE RNA employed by Otogenetics that correlates with (3) RNA-Seq outcomes. Key conclusions and recommendations are listed below.

Summary of Conclusions and Recommendations (Based on data compiled by Otogenetics)

- 1-2 x 10 µm FFPE sections provide adequate sample volumes for NGS sequencing for most tissue sections (Fig. 1). (Additional sections may be combined if necessary depending on individual sample.)
- DNAase treatment is required during RNA isolation.
- FFPE RNA often peaks at <200nt with a RNA Integrity Number (RIN)<2.5 and an rRNA ratio =0. RNA from fresh samples often have a RIN number of 8 (grade 1), or 5-8 (grade 2) (Fig. 2)
- FFPE RNA can be classified to Grade 3 (higher grade) or Grade 4 (lower grade) based on size distribution. Grade 3 FFPE RNA peaks at <200nt, but show minor distribution >200nt, while Grade 4 FFPE RNA only have a peak<200nt (Fig. 2).
- rRNA-depletion is to be used for RNA-Seq.
- With sufficient amount of RNA as shown in the following data of >432 ng (at > 10 ng/µl), RNA-Seq is usually successful for FFPE RNA using rRNA depletion approaches.
- Fresh RNAs of Grade1 and Grade 2 perform well in RNA-Seq. Mapping rates and duplication rates of Grade 3 FFPE RNA-Seq are slightly inferior than that of Grade 2 fresh RNA and are overall comparable to that of Grade 2 fresh RNA. For Grade 4 FFPE RNA, mapping rates are lower and duplication rates are higher (Table 1). Increased amounts of reads are recommended for Grade 4 FFPE RNA, and successful RNA-Seq can be achieved with increased read numbers.

4553 Winters Chapel Road, Ste 100
Atlanta, GA 30360
USA
www.otogenetics.com sales@otogenetics.com
Tel: 1-855-oto-gene (1-855-686-4363)

(I) RNA extraction from FFPE tissues

General Introduction

RNA can be isolated from FFPE tissues or or FFPE fixed sections using the RNeasy FFPE Kit (Qiagen). The RNeasy FFPE Kit isolates all RNA molecules longer than 70 nucleotides from FFPE samples, providing usable RNA fragments with adequate sample volumes for NGS sequencing. RNA yields were determined by measuring A_{260} using Nanodrop or Agilent RNA-Chip. If A_{260} is to be used, the reading should be great than 0.15 to ensure significance above background noise. The yield of RNA depends on the nature of samples, such as tissue type and species (Fig. 1). Pure RNA has an A_{260}/A_{280} ration of 1.9-2.1. Due to the fragmented nature of FFPE samples, random primers should be used instead of oligo-dT primers for cDNA synthesis.

Notes for RNA extraction from FFPE Tissues regarding the starting material

- 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, it is encouraged to:
    - Use tissue samples less than 5 mm thick to allow complete penetration by formalin and fixate tissue samples in 4–10% neutral-buffered 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 to embedding
    - Use low-melting paraffin for embedding
- The starting material for RNA purification should be freshly cut sections of FFPE tissue, each with a thickness of up to 20 µm. Thicker sections may result in lower nucleic acid yields, even after prolonged incubation with proteinase K.
- Up to 4 sections, each with a thickness of up to 10 µm and a surface area of up to 250 mm^2, can be combined in one preparation. More than 4 sections can be combined if the total sum of the thickness of the sections is 40 µm or less (e.g., eight 5 µm thick sections), or if less than 30% of the surface area consists of tissue and the excess paraffin is removed using a scalpel prior to starting the protocol.
- For tissues with particularly high DNA content (such as thymus) fewer sections per preparation are recommended in order to avoid DNA contamination of the purified RNA. If there is no information about the nature of your starting material, no more than 2 sections are recommended per preparation. Depending on RNA yield and purity, it may be possible to use up to 4 sections in subsequent preparations.
-

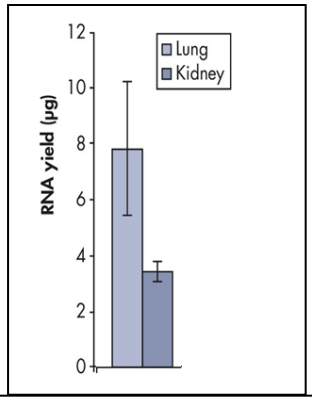

Figure 1. RNA yield varies depending on the nature of the tissues. Included are rat FFPE Lung and Kidney tissues (1 x 10 µm), from which the yield differs ~2 fold.

4553 Winters Chapel Road, Ste 100
Atlanta, GA 30360
USA
www.otogenetics.com sales@otogenetics.com
Tel: 1-855-oto-gene (1-855-686-4363)

(II) QC of FFPE RNAs

Otogenetics uses Agilent regular or high sensitive RNA-Chip to assess the quantity and integrity of total RNA.

High quality RNA from fresh tissues/cells has peaks at 18s and 28s with a RIN (RNA Integrity Number) >8.0 and an rRNA ratio (28s/18s) of >1.5 is identified as shown (Fig. 2A). Such RNA samples are top grade or Grade 1 RNA samples. RNA samples are often partially degraded, demonstrating area under the curve below the 18s peak with RIN values between 5-7 and rRNA ratios <1.5. These are identified as Grade 2 (Fig. 2B).

RNA samples isolated from FFPE samples are often mostly degraded, with RIN values < 2.5 (Fig. 2C,D). The RNAs are peaked at <200nt, but Grade 3 demonstrate some area under the curve >200nt (Fig. 2C) compared to Grade 4 with no distinguishable area under the curve (Fig. 2D).

Due to degradation of FFPE RNAs, polyA RNA-Seq cannot be used. rRNA depletion followed by random priming is the method to proceed with RNA-Seq of FFPE RNA samples.

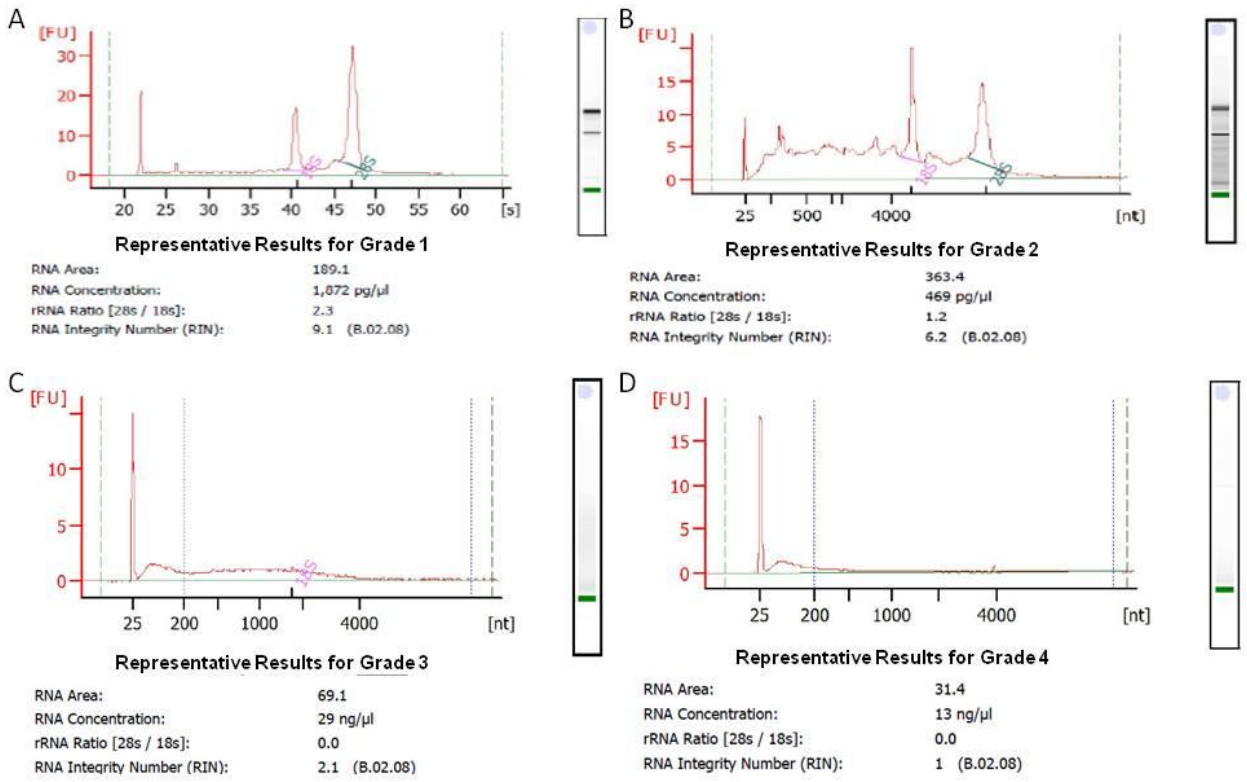

Fig. 2. Comparison of RNA QC for Fresh and FFPE RNA. Fresh tissue/cell (non-FFPE) RNA are categorized as high quality Grade 1 (**A**) and medium quality Grade2 (**B**). FFPE RNA are graded into Grade 3 (**C**) or Grade 4 (**D**).

4553 Winters Chapel Road, Ste 100
Atlanta, GA 30360
USA
www.otogenetics.com sales@otogenetics.com
Tel: 1-855-oto-gene (1-855-686-4363)

(III) Correlation of RNA sequencing outcome with initial QC

The outcome of RNA sequencing quality was assessed based on mapping and duplication rates mapping rates. Sequencing data quality itself (Q30) is not affected by the quality of RNA from FFPE tissues.

Mapping rates decrease and duplication rates increase for lower quality FFPE samples.
The mapping rate and duplication rate correlated with the quality of the initial samples, as shown in Table 1 below.

Table 1. Correlation of RNA-Seq sequencing data quality with initial RNA quality

Sample QC	Starting Amount (ng, average)	Mapping Rate (average)	Duplication (average)	Q30 (average)
Grade 1 (Fresh)	4447	85	15	92
Grade 2 (Fresh)	2807	87	26	94
Grade 3 (FFPE)	1584	80	25	93
Grade 4 (FFPE)	432	49	58	86