Easy and Reliable qRT-PCR Analysis of Total RNA Isolated from Fresh Frozen and FFPE Tissue Samples

Yuli Sun, Tanja Scheuermann, Susanne Sagner, Nina Lassonczyk and Barbara Rueger

Research and Development, Roche Applied Science, Roche Diagnostics GmbH, Penzberg, Germany

INTRODUCTION

The use of tissue sections for molecular analysis of pathogenic states in mammalian tissue has become an indispensable approach for understanding of molecular mechanisms in etiology and disease progression.
With the identification of each new gene expression profile, one step forward is achieved, to develop therapeutic treatments tailored to a given individual.
In the biomedical research routine, a variety of different sample materials exist potentially interesting for gene analysis. Especially tumor genesis, as a very local process requires individual sample preparation and conservation.

Most commonly, dissected tissue samples are prepared as fresh frozen or even more often as formalin-fixed paraffin embedded (FFPE) samples. Both techniques show both, advantages and disadvantages.
Especially degradation of RNA in FFPE samples is the most severe drawback for this particular technique. However, the worldwide well established FFPE tissue banks have become an invaluable source for conserved tissue material.
On the other hand, despite the fact that fresh frozen sample material better reflects the pathological state of a tissue, the challenge to economically organize the required logistical chain necessary for fresh frozen tissue samples have not been satisfactorily solved. (1, 2, 3).

Here, we present data for two independent workflows in gene expression analysis.
RNA samples were isolated from HeLa xenograft tissue, FFPE or fresh frozen, using either the MagNA Pure LC RNA Isolation Kit III (Tissue) together with the MagNA Pure LC 2.0 Instrument or the High Pure FFPE RNA Micro Kit. Isolated RNA was subjected to cDNA synthesis using the Transcriptor First Strand cDNA Synthesis Kit. Roche's LightCycler® 480 Instrument was used for RT-PCR analysis together with SYBR Green I and the Universal Probe Library formats.
Our results show that a robust workflow leading to excellent data sets suitable for gene expression analysis can be established by a simple convinient combination of Roche products.

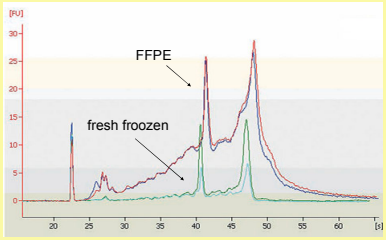
EXPERIMENTAL DETAILS

FFPE tissue

High Pure FFPE RNA Micro Kit FFPE

Step 1. RNA isolation

RNA isolation from fresh frozen tissue
Approx. 10 mg fresh frozen sample from HeLa xenograft tissue were prepared using a scalpel and transferred into a tube of MagNA Lyser Green Beads pre-cooled on dry ice. Immediately before starting tissue homogenization, 800 µl Lysis Buffer from MagNA Pure LC RNA Isolation Kit III (Tissue) were added. Tubes were placed in a MagNA Lyser Instrument and processed four times for 30 seconds at 6500 rpm. Between two processing steps, samples were cooled in a pre-cooled sample rack for one minute. Subsequently samples were incubated for 30 minutes on a Roller Mixer at +15 to +25°C. After centrifugation for two minutes at 8000 x g, 350 µl lysate were used for the RNA isolation, following the protocol for the MagNA Pure LC RNA Isolation Kit III (Tissue) on MagNA Pure LC 2.0 system.
RNA isolation from FFPE tissue
Ten micrometer thick slices from a FFPE block prepared from HeLa xenograft tissue were cut using a microtome. Each slice was deparaffinized and the air dried deparaffinized section was used for RNA isolation following the protocol given in the pack insert of the High Pure FFPE RNA Micro Kit.



FFPE fresh froozen

Sample Source	A_{260}/A_{280}	RNA Integrity Number (RIN)
fresh froozen tissue	1.96	7.1
FFPE tissue	2.12	8.9

Electropherogram and gel view of RNA preparations. One microliter of each preparation were analyzed by an Agilent Bioanalyzer and a NanoDrop Instrument.

RNA Integrity Number (RIN) and absorption ratio at 260 nm versus 280 nm are summarized in the table below.

Fresh froozen tissue

MagNA Lyser Green Beads

MagNA Lyser Instrument

MagNA Pure LC RNA Kit III (Tissue)

MagNA Pure LC 2.0

Transcriptor First Strand cDNA Synthesis Kit

Step 2. Reverse transcription of the isolated RNA

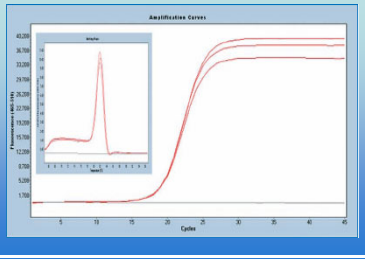
cDNA was sythesiszed using the Transcriptor First Strand cDNA Synthesis Kit according to the protocol given in the pack insert. One microgram of total RNA and anchored oligo(dT) primers were used for each reaction.

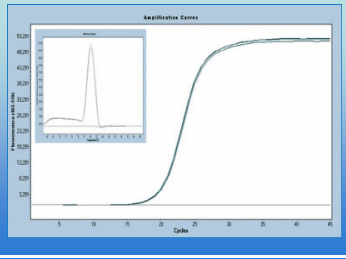
Transcriptor First Strand cDNA Synthesis Kit

LightCycler 480 Instrument and Universal Probe Library

Step 3. cDNA quality check

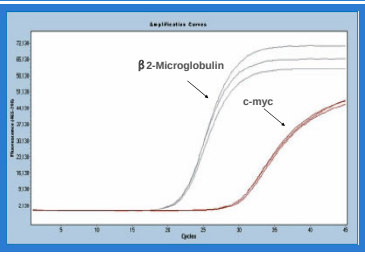
In order to check the quality of cDNA generated from either fresh frozen tissue or FFPE material, a qPCR using SYBR Green I detection format was performed on the LightCycler® 480 Instrument. The real-time PCR mixture contained 10 µl 2x LightCycler® 480 SYBR Green I Master, 0.4 µmol/L of each primer and 2 µl of cDNA. The cycling conditions included an initial incubation step at 95°C for 5 minutes followed by 45 cycles of amplification with 10 seconds at 95°C, 10 seconds at 60°C, and 10 seconds at 72°C (single acquisition). The melting analysis consisted of 95°C for 30 seconds followed by cooling to 65°C for 30 seconds before the temperature was raised to 95°C at a rate of 0.1°C/s with continuous fluorescence acquisition. Two housekeeping genes, β2-Microglobulin and GAPDH were used to check cDNA from fresh frozen tissue and FFPE material, respectively.

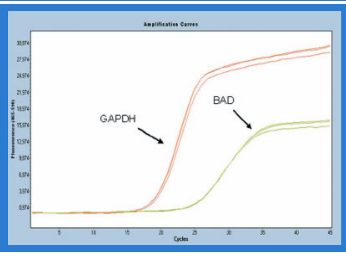




Step 4. qRT-PCR analysis

A qPCR using the Universal ProbeLibrary detection format was performed on the LightCycler® 480 Instrument to analyze gene expression in both tissues. Two sets of genes, including one gene of interest and one housekeeping gene, were quantified. The real-time PCR mixture contained 10 µl 2x LightCycler® Probe Master, 0.4 µmol/L of each primer, 0.1 µmol/L of UPL probe and 2 µl of cDNA (1:4 dilution). The cycling conditions included an initial incubation step at 95°C for 10 minutes followed by 45 cycles of amplification with 10 seconds at 95°C, 30 seconds at 60°C (single acquisition), and 1 seconds at 72°C. The final cooling step was 40°C for 30 seconds. Two housekeeping/target gene sets, β2-Microglobulin/c-myc for FFPE material and GAPDH/BAD for fresh frozen tissue were recorded, respectively.





LightCycler 480 Instrument and Universal Probe Library

CONCLUSION

The most crucial point for the performance of qRT-PCR analysis of archived tissue samples is the quality of the RNA preparation used as a template.

The quality characteristics of the RNA preparation are strongly dependent on the quality of the sample material as well as on the selected technique and good manufacturing practice applied for their preservation.

FFPE material is certainly one of the most challenging sample materials available. Within recent years, due to the development of new protocols and optimization of existing techniques, it is now possible to perform valuable gene expression analysis irrespective of the starting material as long as it is in general of the highest possible quality.

Here, we show two optimized workflows for gene expression analysis from both FFPE and fresh frozen sample material. Both workflows are built of easy to establish procedures, linked together to achieve reproducible, highly reliable results.

For our experiments, we have selected a rather recent sample material from xenografted HeLa tissue (09/2008) prepared in our local routine lab for tissue sample preparation. Results with FFPE samples may vary in addition to the already mentioned factors also depending on the time of storage.

Both starting materials resulted in the highly reproducible and reliable detection of two low copy genes in comparison to a housekeeping gene.

We have selected two different gene sets for the analysis of the RNA on purpose. It might be quite tempting to compare the expression rate in both sample preparations. This can certainly be achieved, if the processes are strongly validated and placed on a solid statistical basis. However, as our main goal was to show that it is technically possible to get highly reliable data, we did not focus on creating the statistics for such a comparison. If the research focus is positioned on the direct comparison of expression rates in fresh frozen versus FFPE material with respect to a specific gene set, a statistical validation is mandatory and we strongly recommend using matched samples and respective controls, *e.g.,* healthy versus diseased or treated versus untreated tissue material.

REFERENCES

1. A High Frequency of Sequence Alterations Is Due to Formalin Fixation of Archival Specimens. C. Williams et. al, American Journal of Pathology, Vol. 155: 14767–1471, 1999
2. Effect of Fixatives and Tissue Processing on the Content and Integrity of Nucleic Acids. M. Srinivasan et. al, American Journal of Pathology, Vol. 161: 1961–1971, 2002
3. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. N. Masuda et. al, Nucleic Acids Res, Vol. 27: 4436–4443, 1999