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Review Article

Gene expression analysis of normal and colorectal cancer tissue samples from fresh frozen and matched formalin-fixed, paraffin-embedded (FFPE) specimens after manual and automated RNA isolation

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ABSTRACT

Although RNA isolation is a routine process in gene expression analysis studies, the applicability of most widely available formalin-fixed, paraffin-embedded (FFPE) samples is still limited compared to fresh frozen tissue samples due to the lower quality of the isolated RNA. Recently, novel automated isolation methods were developed in order to reduce manual sample handling and increase RNA quality and quantity. Here we present a comparison of the performance of fresh frozen and matched FFPE tissue samples obtained from the same surgically removed colonic specimens (10 normal, 10 CRC) in RT-PCR experiments. RNA isolations were performed with the automated MagNA Pure 96 Cellular RNA Large Volume Kit (Roche) compared to the manual RNeasy FFPE Mini Kit (Qiagen). Gene expression analysis of a colorectal cancer-specific marker set (with 7 genes: COL12A1, CXCL1, CXCL2, GREM1, IL1B, IL8, SLC7A5) was performed with array real-time PCR using Transcriptor First Strand cDNA Synthesis Kit (Roche) and Real-Time ready assays on LightCycler® 480 System (Roche). On the basis of the gene expression of the analyzed markers, fresh frozen tumorous and normal samples could be distinguished with 100% sensitivity and 100% specificity after both isolation methods. The FFPE samples could be distinguished by similarly high specificity and sensitivity with the MagNA Pure 96 isolated samples (sensitivity: 90,0%; specificity: 90,0%) and the samples isolated with manual Qiagen method (sensitivity: 85,0%; specificity: 70,0%). According to these results, FFPE samples isolated by automated methods can serve as valuable source for retrospective gene expression studies in the field of biomarker discovery and development.

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1. Introduction

Recently we have identified a discriminatory set of transcripts, whose altered expression can distinguish between fresh frozen colorectal cancer and normal colonic biopsy samples with high sensitivity and specificity [1]. As a validation of the markers, we aimed to extend the analysis and test the applicability of archive formalin-fixed, paraffin-embedded (FFPE) tissue samples based on their performance in RT-PCR reaction compared to results obtained with fresh frozen material.

Fresh frozen tissue samples are considered to be the most reliable source of archival materials for molecular investigations; however the sample collection possibility may be limited in certain centers. In contrast, formalin-fixed, paraffin-embedded (FFPE) tissue samples of a broad range of tissue types are collected routinely, can be stored at room temperature and are commonly available, making them a suitable starting material for retrospective analyses investigating high sample numbers in parallel. Despite of the advantage of high availability, the applicability of this conserved tissue type is still limited in gene expression based studies due to the effect of the fixation procedure on the subcellular level, resulting in degraded RNA. Due to the continuous protocol development and optimization of RNA isolation methods for manual [2] and also automated workflow, highly reproducible results can be achieved with both type of samples [3,4]. One of the commercially available automated nucleic acid isolation system is the MagNA Pure 96 Instrument (Roche) processing up to 96 samples at the same time within approximately 90 min making it perfect for high throughput analyses.

Here we present a comparison of the applicability and the performance of fresh frozen and matched FFPE tissue samples

Abbreviations: FFPE, formalin-fixed, paraffin-embedded tissue; CRC, colorectal cancer; ROC, receiving operating characteristic analysis; RIN, RNA integrity number.

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obtained from the same surgically removed colonic specimen (10 normal, 10 CRC) in the gene expression analysis of the selected colorectal-specific marker set using automated MagNA Pure 96 Cellular RNA Large Volume Kit on MagNA Pure 96 Instrument (Roche) and the manual RNeasy FFPE Mini Kit (Qiagen).

2. Materials and methods

2.1. Sample collection

Tissue specimens were obtained from surgically removed colon tumors (left-sided, moderately differentiated, Dukes B–C stages) and from the histologically normal adjacent tissue that originated from the area farest available from the tumor. Fresh frozen samples were snap frozen and stored at −80 °C, in parallel formalin-fixed, paraffin-embedded blocks were also prepared. Clinicopathological diagnosis was made by pathologists. Written informed consent was provided by all patients and the study was approved by the local ethics committee.

2.2. RNA isolation and cDNA synthesis

After 5 years of storage of the collected tissue specimens, approximately 20 mg fresh frozen tissue samples were cut and transferred into MagNA Lyser Beads with 800 µl MagNA Pure LC RNA Isolation Tissue Lysis Buffer and were homogenized with MagNA Lyser Instrument (Roche Applied Science, Penzberg, Germany) with 6500 rpm for 50 s twice. The tissue lysates were divided and total RNA isolation was performed with two different methods. The manual isolation was performed with RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) and the automated method with MagNA Pure 96 Cellular RNA Large Volume Kit (Roche Applied Science) on the MagNA Pure 96 Instrument with RNA Tissue FF Standard LV protocol [5] in duplicates according to manufacturers' instructions. From formalin-fixed, paraffin-embedded blocks 10 µm thick sections were cut and each section was transferred into microcentrifuge tube. Deparaffinization was performed by adding 1 ml xylene for 10 min twice and 1 ml absolute ethanol for 10 min twice. Total RNA isolation was performed from the air dried deparaffinized sections with RNeasy FFPE kit (Qiagen) and the automated MagNA Pure 96 Cellular RNA Large Volume Kit with RNA Tissue FFPE LV protocol [5] in duplicates.

RNA concentration was measured using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and RNA quality was evaluated with RNA 6000 Pico LabChip kit by using BioAnalyzer 2100 microcapillary electrophoresis system (Agilent Technologies, Inc., Santa Clara, CA USA). Reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) from 250 ng of total RNA and the combination of anchored-oligo(dT) and random hexamer primers.

2.3. Real-time PCR

A discriminatory set of 7 colorectal cancer-specific transcripts were selected on the basis of previous experiments [1]. Commercially available RealTime ready assays [6] for the seven selected markers (COL12A1, CXCL1, CXCL2, GREM1, IL1B, IL8, SLC7A5) and for the 18 ribosomal RNA housekeeping gene were obtained lyophilized in 384 well PCR plates with forward and reverse primers (400 nM) and fluorescently labeled hydrolysis probes (200 nM) from Universal Probe Library (Roche Applied Science). Gene expression analysis was performed with real-time PCR reactions in a final volume of 10 µl using 5 µl LightCycler® 480 Probes Master and 5 µl diluted sample (5 ng cDNA/well). Pipetting was performed with epMotion 5070 liquid handling robot (Eppendorf).

The following thermal cycling conditions were applied on the LightCycler® 480 Instrument: enzyme activation and denaturation at 95 °C for 10 min, 45 cycles of amplification: 95 °C for 10 s, 60 °C for 30 s and signal detection at 72 °C for 1 s with detection and cooling at 40 °C for 30 s.

2.4. Statistical evaluation of RT-PCR results

For relative quantification of gene expression 18S ribosomal RNA was applied as endogenous control. Normalization was carried out in order to correct any experimental variations of the examined genes. Multiple logistic regression analysis was applied to analyze dependence of binary diagnostic variables in case of fresh frozen and FFPE samples. In both cases modifier equations were applied as result of multiple logistic regression analysis, which can be defined as:

$$X = \mathrm{logit}(P) = \ln(P/(1-P)) = b_0 + b_1\Delta Ct_1 + b_2\Delta Ct_2 + \ldots + b_n\Delta Ct_n$$

For receiving operating characteristic (ROC) curve analysis MedCalc software was applied to evaluate the discriminatory power of examined genes [7]. Interactive dot diagrams represent differences on a scale and indicate specificity and sensitivity values of the analyzed markers.

3. Results and discussion

3.1. Quality and quantity of the isolated RNA with different methods

In order to evaluate the quantity and quality of the isolated RNA; recovery, OD 280/260, OD 280/230 ratios and RNA Integrity Number (RIN) values were determined for both sample types isolated with the different methods and statistical analysis was performed in order to specify which sample types differ in the aspect of isolation methods and disease condition (Figs. 1 and 2).

Similarly high recovery could be achieved with MagNA Pure 96 (average and standard deviation of recovery in fresh frozen normal: 8.3 ± 4.16 µg RNA, fresh frozen CRC: 15.6 ± 10.28 µg RNA, FFPE normal: 2.7 ± 1.7 µg RNA and FFPE CRC: 6.2 ± 2.4 µg RNA) and with Qiagen (average and standard deviation of recovery in fresh frozen normal: 7.88 ± 4.32 µg RNA, fresh frozen CRC: 16.28 ± 12.38 µg RNA, FFPE normal: 1.87 ± 1.55 µg RNA and FFPE CRC: 2.8 ± 1.51 µg RNA) methods.

OD 260/280 results differed between sample types both in case of MagNA Pure 96 (average and standard deviation of OD 260/280 in fresh frozen normal: 2.04 ± 0.11, fresh frozen CRC: 2.08 ± 0.03, FFPE normal: 1.65 ± 0.08 and FFPE CRC: 1.74 ± 0.08) and Qiagen (average and standard deviation of OD 260/280 in fresh frozen normal: 1.92 ± 0.03, fresh frozen CRC: 2.1 ± 0.01, FFPE normal: 1.9 ± 0.06 and FFPE CRC: 1.9 ± 0.07) methods.

OD 260/230 results differed between sample types both in case of MagNA Pure 96 (average and standard deviation of OD 260/230 in fresh frozen normal: 2.14 ± 0.05, fresh frozen CRC 2.2 ± 0.03, FFPE normal: 0.67 ± 0.27 and FFPE CRC 0.92 ± 0.42) and Qiagen (average and standard deviation of OD 260/230 in fresh frozen normal: 1.92 ± 0.36, fresh frozen CRC: 1.95 ± 0.3, FFPE normal: 1.88 ± 0.21 and FFPE CRC: 1.94 ± 0.28) methods.

High RNA integrity could be observed in the fresh frozen tissue samples, in contrast the FFPE sample-derived RNA were found to be more degraded. Both MagNA Pure 96 (average and standard deviation of RIN number in fresh frozen normal: 6.1 ± 0.05, fresh frozen CRC: 6.9 ± 0.03, FFPE normal: 2.27 ± 0.2 and FFPE CRC 2.18 ± 0.4) and Qiagen (average and standard deviation of RIN number in fresh frozen normal: 6.6 ± 0.35, fresh frozen CRC: 7.7 ± 0.62, FFPE normal: 1.9 ± 0.5 and FFPE CRC: 2.1 ± 0.01)

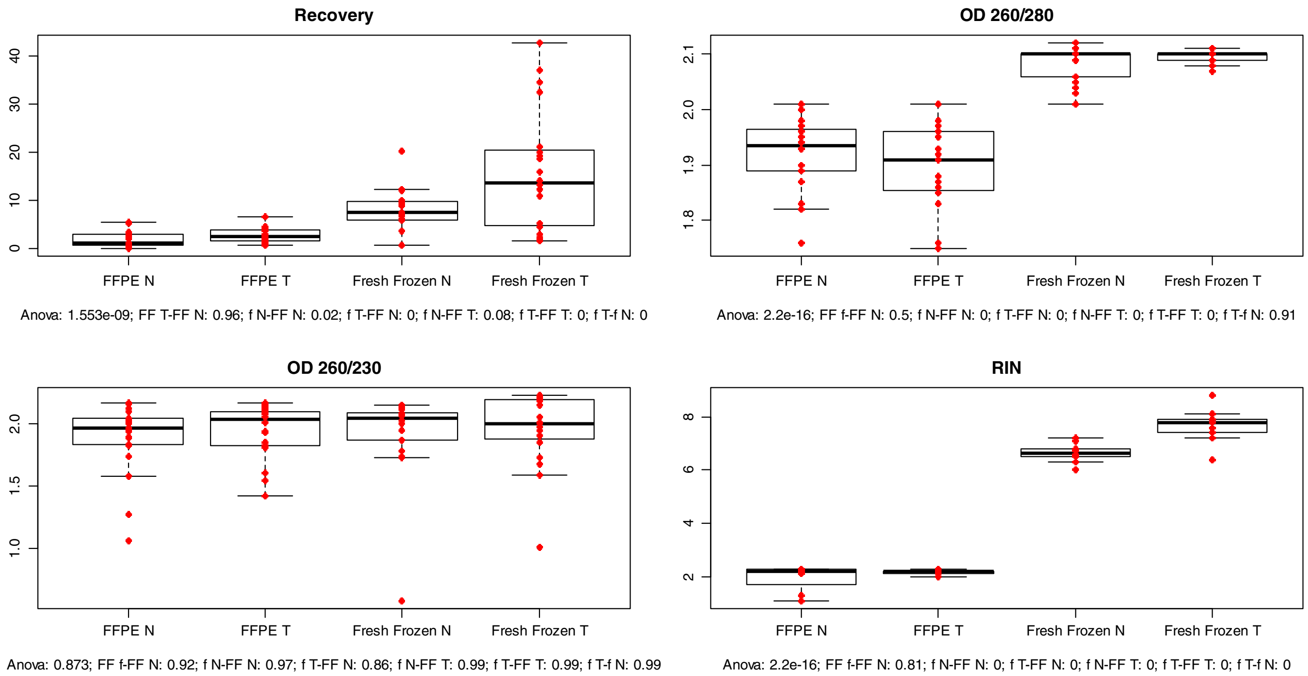

Fig. 1. Quality and quantity of the RNA samples isolated from different tissue types with Qiagen method. Red dots are the quality and quantity values, boxplots represents the median and standard deviation of the data. (N: Normal; T: colorectal tumor; FFPE: formalin-fixed, paraffin-embedded samples; RIN: RNA integrity number).

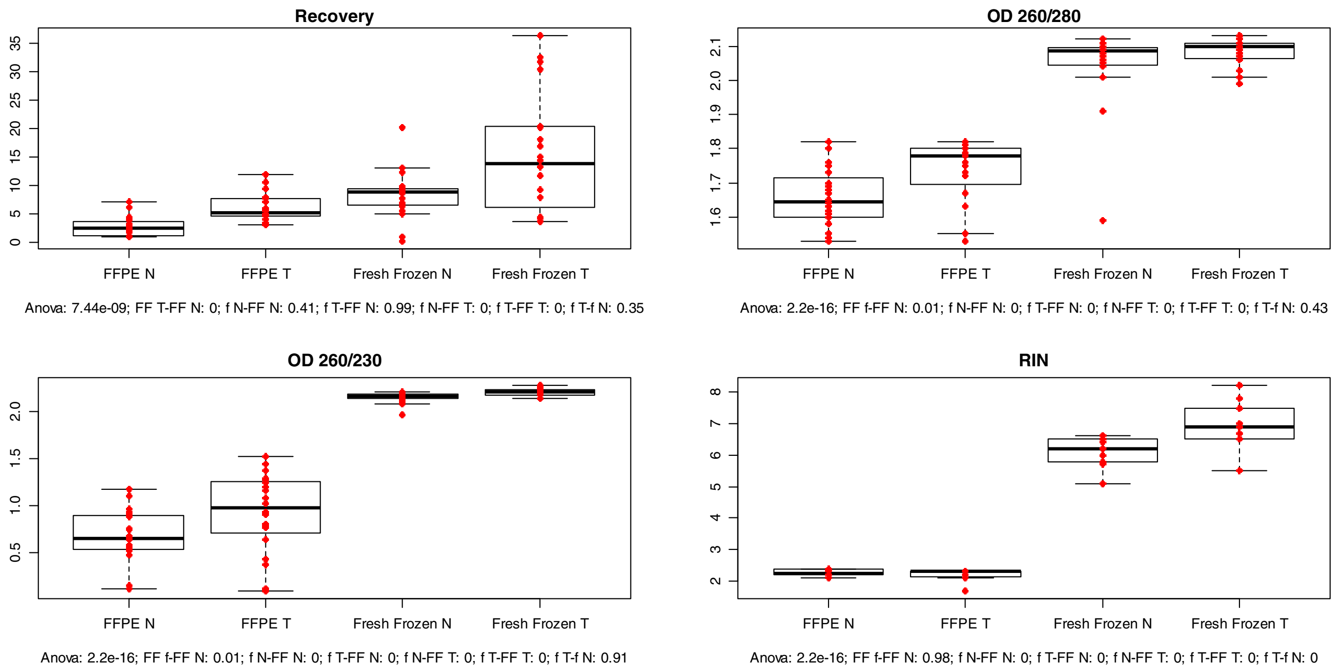

Fig. 2. Quality and quantity of the RNA samples isolated from different tissue types with MagNA Pure 96 method. Red dots are the quality and quantity values, boxplots represents the median and standard deviation of the data. (N: Normal; T: colorectal tumor; FFPE: formalin-fixed, paraffin-embedded samples; RIN: RNA integrity number).

methods showed significant variance between fresh frozen normal and CRC samples (Figs. 1 and 2).

3.2. Gene expression analysis of RT-PCR results

Receiver operating characteristic (ROC) analysis results were compared from 7 markers in case of fresh frozen and FFPE archive samples. From different isolation methods (MagNA Pure 96 and Qiagen methods) 10 normal and 10 CRC samples were compared as these samples originated from the same patients. In case of fresh frozen samples, both MagNA Pure 96 and Qiagen methods resulted in effective differentiation of the tumorous and normal tissue samples (Qiagen: sensitivity: 100%, specificity: 100%; MagNA Pure 96: sensitivity: 100%, specificity: 100%). MagNA Pure 96 threshold was 0.52, while Qiagen threshold was 0.49 (Fig. 3). The FFPE samples could be distinguished by similarly high specificity and sensitivity

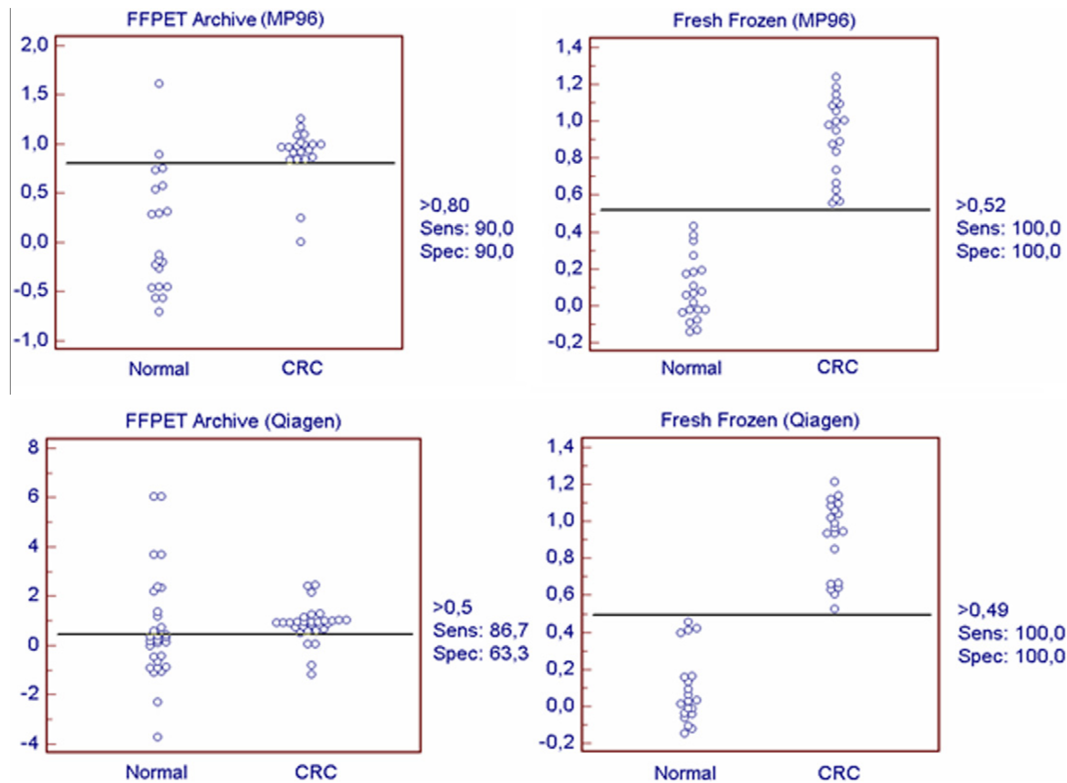

Fig. 3. ROC (Receiver operating characteristic) plots of gene expression analysis of RT-PCR results. Dots represent the results of multiple logistic regressions.

with the MagNA Pure 96 isolated samples (sensitivity: 90,0%; specificity: 90,0%) and the samples isolated with Qiagen method (sensitivity: 85,0%; specificity: 70,0%), that accuracy is lower compared to the fresh frozen tissue samples, but still providing satisfactory differentiation between the sample types. MagNA Pure 96 threshold was 0.8, while Qiagen threshold was 0.5 (Fig. 3). Linear correlation analyses were carried out in both sample types with the two different isolation methods. Between fresh frozen samples both correlation coefficient (r: 0.98) and determinate coefficient was very high (r^2: 0.97). Between FFPE archive samples correlation coefficient was relatively high (r: 0.82) while determinate coefficient was moderate (r^2: 0.67). In the comparison of the sample types correlation and determinate coefficient were weak (r: 0.45–0.5, r^2: 0.2–0.21).

4. Conclusion

Here we presented a technical comparison of the performance of fresh frozen and matched FFPE tissue samples in a gene expression analysis of a selected colorectal-specific marker set after RNA isolation with the automated MagNA Pure 96 Cellular RNA Large Volume Kit on MagNA Pure 96 Instrument (Roche) and the manual RNeasy FFPE Mini Kit (Qiagen).

Although the different isolation methods provide lower quantity and quality RNA from the archive FFPE samples than from the fresh frozen samples, possibly as a result of tissue conservation and the 5-year storage time, their applicability in this gene expression study was confirmed as colorectal cancer tissue samples could still be distinguished with high sensitivity and specificity with the automated MagNA Pure 96 isolated samples (sensitivity: 90,0%; specificity: 90,0%) and the samples isolated with Qiagen method (sensitivity: 85,0%; specificity: 70,0%) from formalin-fixed, paraffin-embedded tissue samples.

According to these results, FFPE samples can serve as valuable source for retrospective gene expression studies as isolation using high throughput nucleic acid isolation instruments can be performed within approximately 90 min with reduced sample handling time, furthermore the isolated RNA from fresh frozen or FFPE samples can provide highly reproducible data. The recent improvements on the isolation methods and the subsequent quantitative techniques may enhance the application of the challenging FFPE samples in growing number of gene expression studies in the future.

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References

[1] O. Galamb, S. Spisák, F. Sipos, K. Tóth, N. Solymosi, et al., Br. J. Cancer 102 (2010) 765–773.
[2] J.Y. Chung, T. Braunschweig, S.M. Hewitt, Diagn. Mol. Pathol. 15 (4) (2006) 229–236.
[3] Yuli Sun, Tanja Scheuermann, Susanne Sagner, Nina Lassonczyk, Horst Donner, Barbara Rueger, Easy and Reliable qRT-PCR Analysis of Total RNA Isolated from Fresh Frozen and FFPE Tissue Samples, Roche Diagnostics GmbH, Roche Applied, 2009.
[4] L. Mittempergher, J.J. de Ronde, M. Nieuwland, R.M. Kerkhoven, I. Simon, et al., PLoS ONE 6 (2) (2011) e17163.
[5] http://www.roche-applied-science.com/sis/magnapure96/index.jsp?id=magnapure96_080000, 2012.05.30.
[6] http://www.roche-applied-science.com/sis/realtimeready/index.jsp, 2012.05.30.
[7] M. Han, C.T. Liew, H.W. Zhang, S. Chao, R. Zheng, et al., Clin. Cancer Res. 14 (2008) 455–460.