Clinical Chemistry 55:9
1719–1727 (2009)

RNA Extraction from Archival Formalin-Fixed Paraffin-Embedded Tissue: A Comparison of Manual, Semiautomated, and Fully Automated Purification Methods

Kerstin Bohmann,[1†] Guido Hennig,[1†] Uwe Rogel,[2] Christopher Poremba,[3] Berit Maria Mueller,[4] Peter Fritz,[5] Stephan Stoerkel,[6] and Karl-L. Schaefer[2*]

BACKGROUND: Formalin-fixed paraffin-embedded (FFPE) tumor material represents a valuable resource for the analysis of RNA-based biomarkers, both in research laboratories and in routine clinical testing. A robust and automated RNA-extraction method with a high sample throughput is required.

METHODS: We evaluated extraction performance for 4 silica-based RNA-extraction protocols: (*a*) a fully automated, bead-based RNA-isolation procedure; (*b*) its manual counterpart; (*c*) a semiautomated bead-based extraction system; and (*d*) a manual column-based extraction kit. RNA from 360 sections (90 sections per extraction method) of 30 FFPE tumor blocks up to 20 years of age was purified and analyzed by quantitative reverse-transcription PCR for *ESR1* (estrogen receptor 1), *PGR* (progesterone receptor), *ERBB2* [v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)], and *RPL37A* (ribosomal protein L37a).

RESULTS: The semiautomated protocol gave the best yield. The 3 bead-based methods showed good across-method correlations in both yield and relative mRNA amounts ($r = 0.86–0.95$ and 0.98, respectively). In contrast, correlations between any of the bead-based methods and the manual column-based method were worse ($r = 0.77–0.95$ and 0.96, respectively). The fully automated method showed the lowest variation from section to section (root mean square error, 0.32–0.35 Cq, where Cq is the quantification cycle) and required the least hands-on time (1 h).

CONCLUSIONS: The fully automated RNA-purification method showed the best reproducibility in gene expression analyses of neighboring sections of tissue blocks between 3 and 20 years of age and required the least overall and hands-on times. This method appears well suited for high-throughput RNA analyses in both routine clinical testing and translational research studies with archived FFPE material.

The use of expression profiling for identifying new molecular markers to aid diagnosis and guide treatment of cancer has been an intensive field of research in recent years, but only a few markers have found their way into routine clinical practice (e.g., inclusion of Onco*type* DX in the National Comprehensive Cancer Network's NCCN Clinical Practice Guidelines in Oncology™) *(1)*. A main reason for the slow adoption of RNA as an analyte for routine testing is that many RNA markers have been identified for fresh frozen tumor material, which harbors good-quality RNA. This material is not routinely obtained in hospitals, however, and only a very few centers can afford the logistical challenge of collecting, processing, and banking fresh frozen tissue.

In contrast, formalin-fixed paraffin-embedded (FFPE)[7] tissue has been used routinely in clinical diagnosis for decades. The procedures for collecting, preparing, analyzing, and storing such samples are well established, and no alternatives are likely to challenge this approach. The process preserves the morphology of the tissue and is ideal for histologic examinations.

[1] Siemens Healthcare Diagnostics Products, Molecular Research Germany, Cologne, Germany; [2] Institute of Pathology, Heinrich-Heine University Duesseldorf, Duesseldorf, Germany; [3] Center for Histology, Cytology and Molecular Diagnostics, Trier, Germany, [4] Institute of Pathology, Charité Hospital, Campus Mitte, Berlin, Germany; [5] Institute of Pathology, Robert Bosch Hospital, Stuttgart, Germany; [6] Institute of Pathology, HELIOS Klinikum Wuppertal, Wuppertal, Germany.
† These authors contributed equally to this work.

* Address correspondence to this author at: Institute of Pathology, Universitaetsklinikum Düsseldorf, Bldg. 14.79, Moorenstr. 5, D-40225 Duesseldorf, Germany. Fax +49-211-811-8353; e-mail L.Schaefer@med.uni-duesseldorf.de.
Received December 17, 2008; accepted June 29, 2009.
Previously published online at DOI: 10.1373/clinchem.2008.122572
[7] Nonstandard abbreviations: FFPE, formalin-fixed paraffin-embedded; RT-qPCR, reverse-transcription quantitative PCR; Cq, quantification cycle.

For translational research, very substantial archives of FFPE tissue samples for all kinds of clinical specimens have been established in many centers and linked to clinical and follow-up databases.

Nevertheless, RNA extraction from FFPE samples remains challenging: The RNA is heavily degraded, nucleic acids are cross-linked to proteins, and base modifications are introduced during the fixation process *(2)*. Several methods have been described for extracting RNA samples from FFPE tissues and for the subsequent analyses, mostly with PCR techniques *(3–8)*. In addition, several comparisons of these methods, as well as comparisons with RNA purified from snap-frozen material, have been published *(9–12)*. Although the results of these studies vary, a common conclusion is that the yield is much lower for RNA extracted from FFPE tissue than from fresh tissue. Furthermore, the RNA yield decreases with increasing age of the blocks. No consensus or standardized isolation method has emerged thus far.

An additional aspect that has contributed to the limited application of FFPE material for analyzing biomarkers is the low level of automation available to aid this process. A reproducible and high-throughput extraction method is necessary, not only for large retrospective studies with archived material but also for routine clinical use. In both cases, the introduction of an automated process would be highly desirable. Two major obstacles need to be overcome, however. The first is the laborious and difficult process of automating the elimination of paraffin from the sample. This step is currently accomplished with xylene/ethanol washing steps combined with centrifugation. The second obstacle is the standardization of lysis conditions. The requirements for this step vary substantially, owing to the different conditions that have been used during fixation and cross-linking processes, variation in the size of the tissue sample, and the heterogeneous morphologic composition of the tissue. Either extended incubation times for lysis or harsher lysis conditions (e.g., increased temperatures or repeated addition of fresh proteinase K) are required to avoid persistent unlysed tissue debris. Alternatively, the tissue debris can be eliminated from the sample before any subsequent processing step. Typically, such debris is eliminated by centrifugation, a step that is very hard to automate. A fully automated extraction method based on silica-coated magnetic particles has been developed to address these issues.

In this study, we have compared this fully automated extraction procedure and its manual equivalent with both a semiautomated bead-based method and a manual column-based extraction method.

Materials and Methods

FFPE TISSUE SAMPLES

This study included 30 FFPE blocks of breast cancer tissue of different ages collected at 3 pathology laboratories in Germany: 13 blocks from HELIOS Clinics, Wuppertal (embedded in 2005), 14 blocks from Robert Bosch Hospital, Stuttgart (embedded in 1988), and 3 blocks from Charité, Berlin (embedded in 2003). The samples had been irreversibly anonymized: No clinical or patient-related information was available.

SAMPLE PREPARATION

Eighteen consecutive 5- or 10-μm sections (only 5-μm blocks were available from Wuppertal) were cut from each patient block on a standard microtome (Reichert-Jung Hn40; Leica Instruments), placed into individual 1.5-mL microcentrifuge tubes (Sarstedt), and stored at 4 °C for about 1 month until extraction.

RNA EXTRACTION

We compared 4 methods for extracting RNA from FFPE tissue sections. We extracted 3 sections from each of the 30 blocks per method. Two methods were developed at Siemens Healthcare Diagnostics: a fully automated method and a manual method (method 1 and method 2, respectively). The fully automated method 1 runs on a Hamilton MICROLAB STARlet liquid-handling robot. Unless otherwise indicated, the robot, buffers, and chemicals are part of the VERSANT® kPCR Molecular System (Siemens Healthcare Diagnostics; not commercially available in the US). The robot was equipped with additional hardware, including a heater/shaker module for 1.5-mL reaction tubes and sample carriers with tube-specific ring magnets (currently in development at Siemens Healthcare Diagnostics). FFPE sections were lysed, and the paraffin was melted in 150 μL Buffer FFPE (10 mmol/L Tris-HCl, 0.1 mmol/L EDTA, 5 g/L SDS, pH 8.0) for 30 min at 80 °C with shaking. After cooling, 50 μL VERSANT 3.0 Assay (bDNA) Lysis Reagent (Siemens Healthcare Diagnostics) was added and incubated for 30 min at 65 °C with shaking. Residual nonlysed tissue debris was removed by a negative-selection step in which 40 μL of silica-coated iron oxide beads were added and incubated for 15 min at 65 °C. The beads with surface-bound tissue debris were then separated to the ring magnet, while the melted paraffin simultaneously separated to the inner wall of the sample tube. Lysates were then transferred to a 96-well plate with deep, 2-mL wells. Nucleic acids were added to 40 μL of fresh beads with 600 μL of added chaotropic Lysis Buffer and were bound to the beads with shaking at room temperature. The supernatants were discarded after magnetic separation, and surface-bound nucleic acids were washed 3

Table 1. Overall comparison of the 4 protocols for extracting RNA from FFPE samples, including a summary of the methods' primary characteristics.

Characteristic	Method 1	Method 2	Method 3	Method 4
Sample input	One 5- or 10-μm FFPE section			
Batch size, n	48	12	6	12
Runs required for 90 samples, n	2	8	15[a]	8
Level of automation	Fully automated	Manual	Semiautomated	Manual
Nucleic acid–binding method	Silica beads	Silica beads	Silica beads	Silica columns
Deparaffinization	No xylene/ethanol step[b]	No xylene/ethanol step[c]	2 Xylene/ethanol steps	1 Xylene/ethanol step
Lysis conditions	80 °C/65 °C for 1 h	80 °C/65 °C for 1 h	56 °C overnight	55 °C/80 °C for 30 min
Additional proteinase K step	—	—	1 h	—
DNase I digest	In eluate	In eluate	On beads	On column
Centrifugation steps, n	—	1	5	12
Elution volume, μL	100	100	100	25

[a] One batch of 6 samples was lost because of robot failure (pipette crash).
[b] Hydrophobic binding and adsorption to inner tube wall after lysis.
[c] Separated by centrifugation step after lysis.

times (850, 450, and 850 μL of Wash Buffer 1, 2, and 3, respectively) with magnetization, aspiration, and disposal of supernatants after each wash step. Nucleic acids were eluted by incubation with 100 μL Elution Buffer for 10 min at 70 °C with shaking. The supernatant obtained after magnetic separation was incubated for 30 min at 37 °C with 12 μL DNase I (2 μL RNase-free DNase I and 10 μL 10× DNase I Buffer; Ambion/Applied Biosystems). The DNA-free solution of total RNA was manually aliquoted into 1.5-mL reaction tubes and stored at −80 °C.

The manual extraction method (method 2) used identical reagents and the same workflow as described for method 1. The essential difference was a new centrifugation step (5 min at 15 000*g*) after the lysis step to eliminate both the tissue debris and the paraffin. Supernatants were carefully transferred into new 1.5-mL reaction tubes; the target was then bound to silica-coated beads under chaotropic conditions.

In addition, we evaluated 2 commercial methods available from Qiagen: the semiautomated silica bead–based method with the EZ1® RNA Tissue Mini Kit that is performed on the BioRobot EZ1 Workstation® (method 3) and a manual isolation method based on silica columns that uses the RNeasy® FFPE Kit (method 4). Both Qiagen methods were performed according to the manufacturer's instructions. The semiautomated method 3 contains an optional step of incubation with fresh proteinase K for 1 h at 56 °C in the event that the overnight lysis was judged by visual inspection not to be complete. This additional step had to be performed for 62 of the 90 sections. DNase I digestion for the manual method 4 was performed as recommended by a Qiagen representative. This procedure used Qiagen's RNase-Free DNase Set according to the manufacturer's instructions in Appendix D of the RNeasy Mini Handbook (http://www1.qiagen.com/HB/RNeasyMiniKit_EN; page 69 following). Qiagen buffer RWT replaced buffer RW1.

To prevent any potential cutting-plane effects, we distributed the 3 analyzed sections evenly in each block. We analyzed sections 1, 7, and 13 with method 1; sections 2, 8, and 14 with method 2 (both processed at Siemens' molecular research facility in Cologne); sections 4, 10, and 16 with method 3; and sections 5, 11, and 17 with method 4 (the latter 2 methods were both processed at the Institute of Pathology at the University of Duesseldorf).

Table 1 provides a comprehensive overview of all 4 methods. For each extraction method, we included at least 2 positive controls (10-μm sections of the MaxArray™ Breast Receptor Control Cell Block; Invitrogen) and at least 1 negative control (empty tube).

GENE EXPRESSION ANALYSIS WITH QUANTITATIVE PCR

Expression of the *ESR1*[8] (estrogen receptor 1), *PGR* (progesterone receptor), and *ERBB2* [v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/

[8] Human genes: *ESR1*, estrogen receptor 1; *PGR*, progesterone receptor; *ERBB2*, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (also known as *HER-2*); *RPL37A*, ribosomal protein L37a; *PAEP*, progestagen-associated endometrial protein.

A

Detected signals	Method 1	Method 2	Method 3	Method 4
RPL37A	90	90	84	90
PGR	77	83	80	76
ESR1	90	89	84	88
ERBB2	88	88	81	84

B

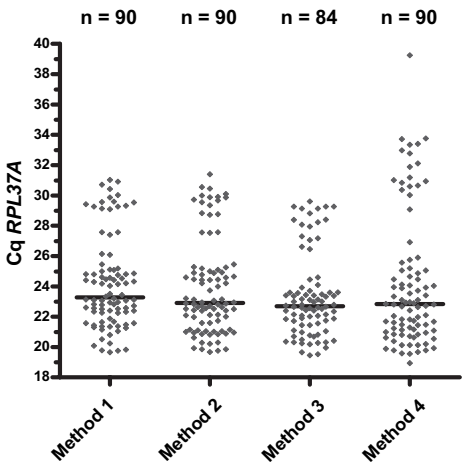

Fig. 1. Detectable genes and mRNA yields of the extractions.

(A), The number of detectable RT-qPCR results for the 4 genes and 4 methods. (B), Plots of Cq values for reference gene *RPL37A*, used as a surrogate for mRNA yield. Black bars indicate the median Cq values of 23.3 (method 1), 22.9 (method 2), 22.7 (method 3), and 22.8 (method 4).

glioblastoma derived oncogene homolog (avian); also known as *HER-2*] genes and the gene used for normalization (*RPL37A*, ribosomal protein L37a) was assessed by quantitative 1-step reverse-transcription PCR (RT-qPCR). The SuperScript® III Platinum® One-Step Quantitative RT-PCR System with ROX (Invitrogen) was used according to the manufacturer's instructions. Experiments were performed on an ABI PRISM® 7900HT Fast Real-Time PCR System (Applied Biosystems) with 30 min at 50 °C, 2 min at 95 °C, and 40 cycles of 15 s at 95 °C and 30 s at 60 °C. Relative expression of the genes of interest (i.e., *ESR1*, *PGR*, *ERBB2*) were calculated as the change in the quantification cycle, ΔCq [$\Delta Cq = 20 - (Cq_{GOI} - Cq_{RPL37A})$, where GOI is the gene of interest]. ΔCq values positively correlate with relative gene expression.

To assess DNA contamination in RNA preparations, we performed a qPCR analysis specific for the *PAEP* gene (progestagen-associated endometrial protein) without the preceding reverse-transcription step and with the reagents from the SuperScript® III Platinum® One-Step Quantitative RT-PCR System with ROX kit and *Taq* DNA polymerase (Invitrogen). The DNase I treatments should be repeated for samples with a Cq value below 35 to prevent effects on bispecific PCR assays.

Human breast adenocarcinoma (MCF-7) total RNA (Ambion/Applied Biosystems) was used as a positive control for RT-qPCR, and human genomic DNA (Roche Diagnostics) was used as a positive control for qPCR. All PCR assays were performed in triplicate, and the mean of the triplicates was reported. Primer and probe sequences are shown in Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue9.

STATISTICAL EVALUATION

For statistical analyses, Prism® 4 software (GraphPad Software) and Microsoft Excel 2003 were used. Arithmetic means, medians, and SDs were calculated. For correlation analyses, the Spearman correlation coefficient was calculated. Section-to-section variation between the 3 sections was calculated as the root mean square error.

Results

YIELD OF RNA EXTRACTION

Except for one prematurely aborted run with the semi-automated method 3 that led to the loss of 6 samples, 354 extractions were successful, as judged by a detectable RT-qPCR signal for *RPL37A* (Fig. 1A). Fig. 1B plots the distribution of Cq values for *RPL37A*, which was used as a surrogate marker for the mRNA yield of the extraction. Method 3 delivered the best yield (lowest Cq value).

The signals could be separated into 2 groups in all 4 methods, an observation that we found striking. The majority of the signals produced Cq values ≤26, but there was also a clearly separated group of results with

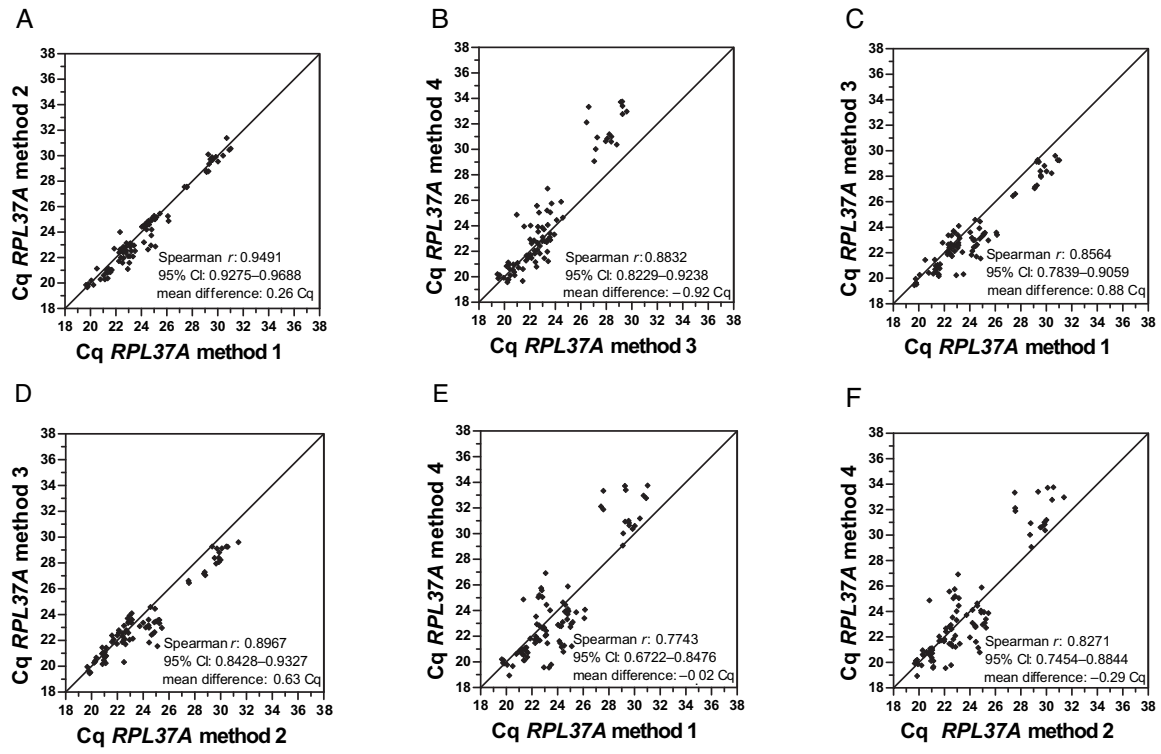

Fig. 2. Correlation of yields between the tested methods.

Cq *RPL37A* values determined for RNA purified by the 4 methods are correlated for all possible pairwise combinations (A–F). Given are Spearman correlation coefficients (*r*), 95% CIs, and mean differences in Cq for the respective method comparisons. Line of identity is indicated.

higher Cq values. In method 4, this latter group of signals varied essentially from the results for the other 3 methods, as judged by an appreciable separation from the line of identity (Fig. 2, B, E, and F). The signals belonged to 6 FFPE blocks (18 samples) from the 20-year-old cohort. Fig. 2 shows all possible correlations of yield for the 4 methods. The best correlation was observed between methods 1 and 2 (Spearman correlation coefficient, 0.949). Interestingly, these 2 methods showed a pairwise difference of 0.26 Cq in favor of method 2, although the isolation principle was the same. This difference was measured with an extremely low variance (0.40, compared with 1.04–5.19 for all other possible pairs; data not shown).

Methods 1 and 2 performed the DNase I digestion in a separate step after the elution, whereas methods 3 and 4 integrated the DNase I step with an "on bead" and an "on column" DNase I digestion, respectively. The DNA control assay showed a less efficient DNase digestion for the latter 2 methods. No sample showed a detectable *PAEP* signal with method 1, whereas 3 of 90 samples gave a Cq between 39 and 40 for method 2. Method 3 produced detectable *PAEP* signals for 34 samples, 13 of which had Cq values <35. Thirty-nine of the 90 samples in method 4 produced Cq values between 35 and 40 (data not shown). From our experience, we recommend an additional DNase I digestion for samples with *PAEP* DNA signals <35; however, a loss in RNA yield often accompanies the additional digestion step.

NORMALIZED EXPRESSION OF BREAST CANCER–RELEVANT GENES

In a next step, we analyzed and compared the methods with respect to the relative production of RNA for the *ESR1*, *PGR*, and *ERBB2* genes. In the low-yield blocks, the expression of these genes was occasionally below the limit of detection (see Fig. 1A), leading to different numbers of data points in the diagrams. Adjacent sections purified with the respective methods were compared, and a correlation could be calculated only if the gene was detectable in both sections. Therefore, the number of data points in the graph varies. Fig. 3 shows that the correlation coefficients were generally better

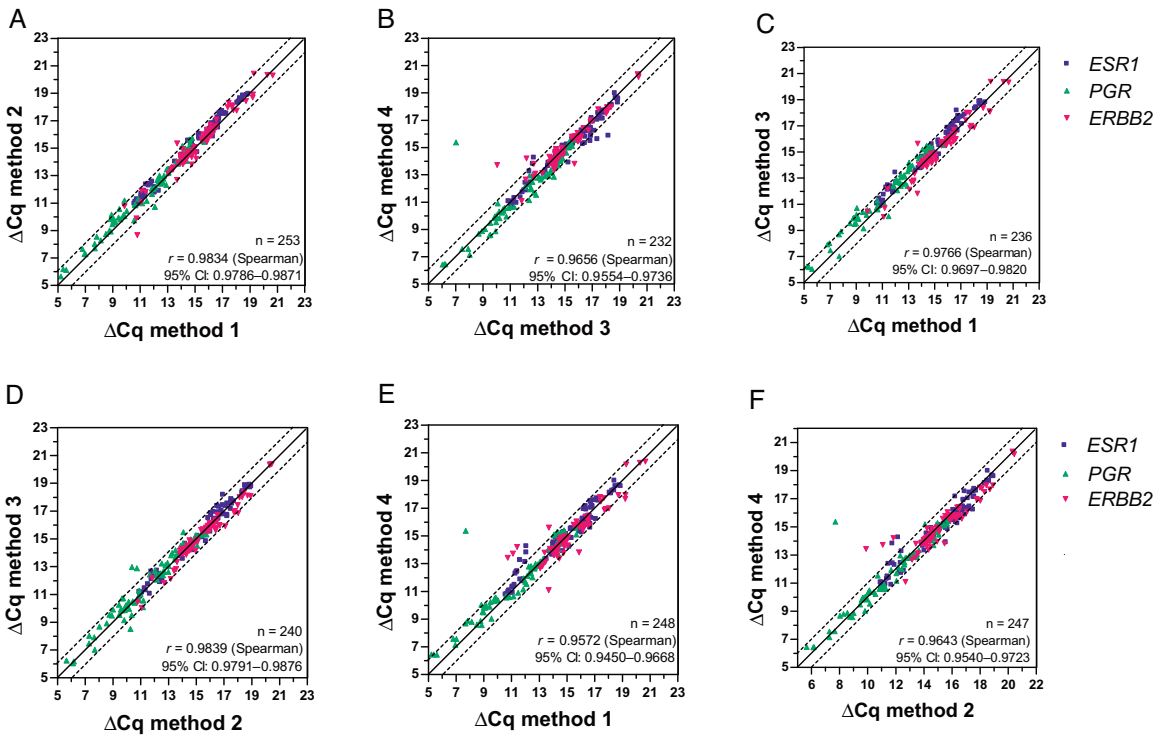

Fig. 3. Correlations of normalized expression values for breast cancer–relevant genes for all possible pairwise comparisons of the 4 tested methods (A–F).
The line of identity and the dashed lines for ±1 ΔCq are indicated.

for ΔCq than for yield (*RPL37A* Cq in Fig. 2). This feature was especially striking for the correlation between methods 1 and 3 (cf. Figs. 2C and 3C: Cq *RPL37A* correlation of 0.86 vs ΔCq correlation of 0.98), indicating that the ΔCq is quite robust against variations in yield. All correlations were good, with those for comparisons of the bead-based methods (Fig. 3, A, C, and D) being slightly better than for comparisons of a bead-based method with the column-based method 4 (Fig. 3, B, E, and F). This result was due to several signals being well beyond the line indicating a deviation of 1 ΔCq from the bisecting line.

SECTION-TO-SECTION VARIABILITY

We next analyzed the variability between all possible combinations of the 3 sections purified per method from each block. We have calculated the root mean square error for the 3 sections within all methods with respect to both *RPL37A* Cq and ΔCq. Method 1 showed very good reproducibility for both yield and normalized expression, indicating that this method is robust and highly reproducible (Fig. 4). The other methods showed similarly good section-to-section variation, but less reproducibility with respect to yield and normalized expression.

TOTAL TIME AND HANDS-ON TIME

The tested purification methods varied in their level of automation. The highest level of automation and hence the least time required for extraction was achieved with method 1. The only manual steps required for method 1 are to load the FFPE samples into tubes, load extraction reagents and consumables onto the robot, and start the program (total time for 2 runs, 8 h 40 min; hands-on time, 60 min). A much lower level of automation was achieved with method 3, for which the deparaffinization and tissue-lysis steps are performed manually before robot loading (total time, 27 h plus 3 overnight lysis incubations; hands-on time, 12 h). Of note is that an updated version of the method 3 protocol is now available on the Qiagen Web site (MA64, September 2007; http://www1.qiagen.com/literature/render.aspx?id=23670&tp=9). The elimination of an overnight lysis with this updated method substantially reduces the hands-on time and appreciably shortens the time requirements for the method; however, we

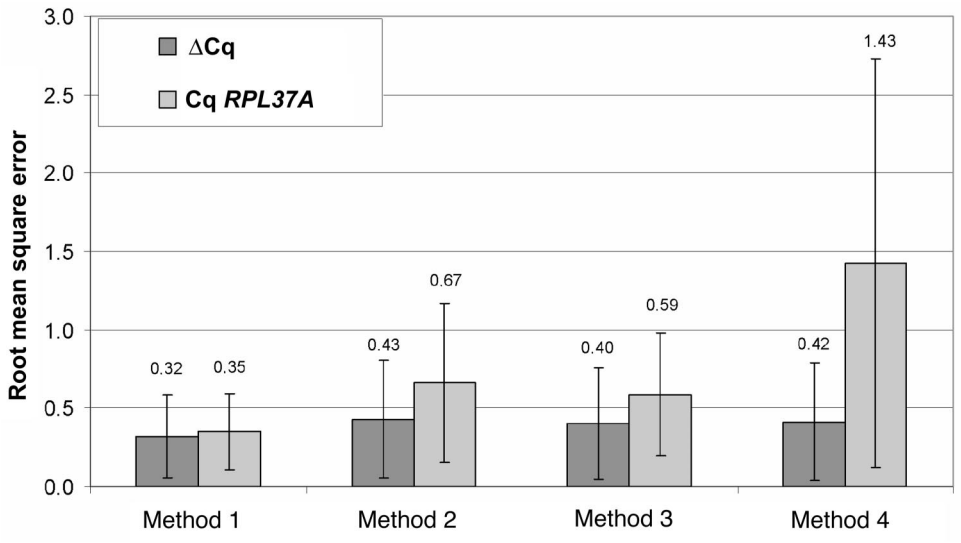

Fig. 4. Section-to-section variability.

The root mean square error (RMSE) for 3 sections analyzed from each block is plotted for all 4 methods. The RMSE is calculated for Cq *RPL37A* (n = 90, 90, 72, and 90 for methods 1–4, respectively) and for ΔCq of all 3 normalized breast cancer–relevant genes (n = 249, 255, 204, and 240 for methods 1–4, respectively).

have experienced problems with this newer protocol for certain PCR-based analyses and so have followed the older protocol (MA33, October 2005; www1.qiagen.com/literature/protocols/pdf/MA33.pdf). The other 2 methods (methods 2 and 4) are completely manual methods and therefore require more time (total time, 18 h 45 min each; hands-on time, 7 h 30 min for method 2 and 11 h 15 min for method 4) (Fig. 5).

Discussion

In this study, we conducted a comprehensive and well-controlled comparison of 4 methods for purifying total RNA from FFPE breast cancer tissue: a fully automated high-throughput purification method, its manual counterpart, a semiautomated method, and a commonly used manual method. To our knowledge, no

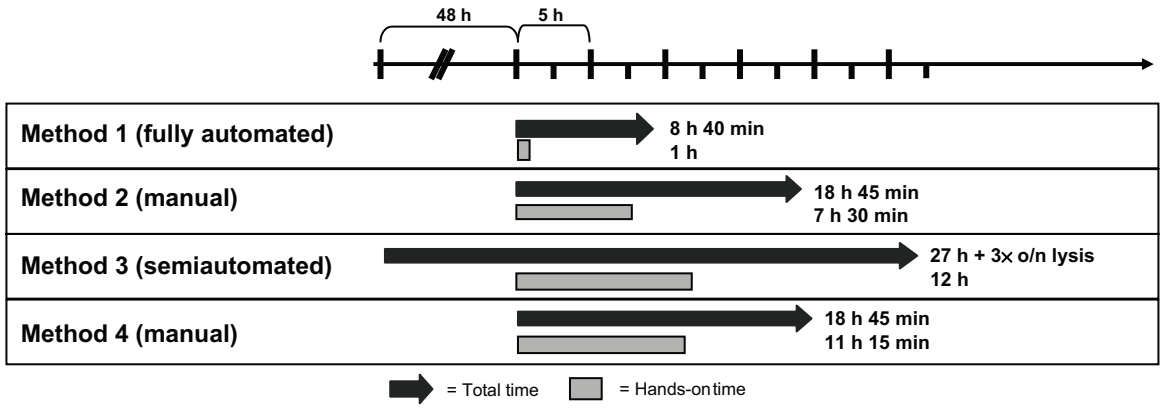

Fig. 5. Total and hands-on times required for the purification of 90 FFPE sections.

The actual times required to purify RNA from 90 samples are depicted for each of the 4 methods. o/n, overnight.

other kits for fully automated nucleic acid purification are commercially available.

Except for 1 failed run with 6 samples for method 3, we successfully isolated total RNA from 354 sections. To quantify the yield of purified RNA, we used an RT-qPCR assay of the reference gene *RPL37A*. RT-qPCR for *RPL37A* from RNA purified with method 1 produces data comparable to that for RNA quantification with Invitrogen's Quant-iT™ RiboGreen® assay (unpublished results). RT-qPCR Cq values for *RPL37A* showed that the semiautomated method 3 gave the best median yield. One possible explanation for the slightly higher yield of method 3 might be the substantially longer lysis time (Table 1), which is known to have an effect on yield *(12)*. Interestingly, methods 1 and 2, which use basically the same isolation protocol and reagents but in an automated and manual way, respectively, show a mean difference over all blocks of 0.26 Cq in favor of the manual method 2. This finding is most likely due to a difference in shaking speed during the lysis step (data not shown), suggesting that a similar performance will be obtained in an updated automated protocol under development at Siemens. (While this manuscript was under review, we found that intensifying the shaking procedure during lysis indeed leads to a reduction of Cq values for *RPL37A* of about 0.5.) Nevertheless, the variance between these 2 methods was extremely low and smaller than that observed between all of the other method comparisons. These results indicate a very robust and reliable isolation procedure. A group of 6 FFPE blocks belonging to the cohort stored for 20 years produced distinctively lower yields with all methods (Fig. 1B). In these blocks, method 4 produced the lowest yield of the 4 methods and isolated disproportionately less RNA from FFPE blocks with a low amount of preserved RNA than the other methods (Fig. 1B and Fig. 2, B, E, and F). In general, a low yield contributes to problems in detecting genes of interest with low expression. Hence, the results for RT-qPCR assays for 22 samples distributed over 5 low-yield blocks and prepared with method 4 were below the limit of detection. Even for genes that were still detectable, method 4 was associated with more analytical outliers. These outliers in the correlation of ΔCq between methods were mainly linked to these low-yield blocks (Fig. 3, B, E, and F). Whether this finding can be attributed to the difference between bead-based and column-based purification approaches remains unclear.

The correlations between the 3 bead-based methods in quantitative RNA values reflecting the expression of breast cancer–relevant genes showed excellent Spearman correlation coefficients of approximately 0.98, with narrow 95% CIs (Fig. 3, A, C, and D). Some data points were outside of the range of ±1 ΔCq, which is represented in Fig. 3 by the area between the dotted lines parallel to the line of identity, but only in the region of low expression. The correlations involving the column-based method were all slightly lower (Spearman correlation coefficients, 0.96–0.97; Fig. 3, B, E, and F) because of several outliers outside of the ±1 ΔCq interval. The majority of these signals were for samples from the low-yield blocks discussed above.

Next, we analyzed the variation in yield and normalized expression of 3 genes from section to section. Tumors are nonhomogeneous cell aggregates in which the relative proportions of cancer cells and nonpathologic cells often vary quite substantially. Therefore, sections that are hundreds of microns apart might represent different parts of the tumor and thus give different results in yield or expression analyses. In this study, the maximum distance between analyzed sections in any tumor block was 120 μm. The small differences detected for correlation of yield and normalized expression values within and between the 4 tested methods suggest that RNA composition varies only slightly over a distance of this magnitude and that all bead-based methods allow robust and reproducible extraction.

A prerequisite for an FFPE-based extraction method to be used in high-throughput routine diagnostic testing or for research studies of large FFPE archives is an automated purification method. Automation is required to ensure consistent quality, reproducibility, and cost-effectiveness. Fig. 5 shows that the fully automated method 2 required the least hands-on and total times. This result was possible only because the deparaffinization step, which is based on the hydrophobic separation of paraffin to the inner wall of the tube, was integrated into the automated workflow. All of the other currently available methods for RNA extraction require laborious and difficult-to-automate steps to eliminate the paraffin from FFPE sections. Another important aspect with respect to automation is the elimination of tissue debris. Because FFPE blocks are nonhomogeneous with respect to numbers of cells and tissue composition (cell types and extracellular matrix), as well as fixation conditions, the efficiency of lysis is extremely variable, and lysis is sometimes incomplete. Tissue or cell debris has the potential to clog pipette tips and is therefore extremely problematic for automation. The semiautomated method 3 deals with this problem by the use of a visual inspection of the sample after overnight lysis and performing an additional incubation with fresh proteinase K if necessary. In contrast, the technique for eliminating tissue debris in method 1 permits a standardized procedure without operator intervention. A caveat lies in the potential for a somewhat reduced yield with this method, which is produced by eliminating tissue debris that could still contain RNA.

In conclusion, this study of 4 RNA-purification methods for FFPE tissue demonstrated that the fully automated method 1 showed the best reproducibility in gene expression analyses of neighboring sections of tissue blocks of 3–20 years of age and outperformed the other 3 tested methods with regard to total and hands-on times. Yield was highest with the semiautomated method 3, partially because of a nonstandardized, prolonged overnight lysis. The manual method 4 was the worst of the 4 methods with respect to yield from FFPE blocks with a low RNA content.

A fully automated RNA-purification method paves the way for extensive retrospective biomarker studies of archived FFPE material with long-term clinical follow-up data. Furthermore and importantly, a fully automated method sets the stage for routine analysis of this material in the clinical laboratory.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures of Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:*

Employment or Leadership: K. Bohman, Siemens Healthcare Diagnostics; G. Hennig, Siemens Healthcare Diagnostics.
Consultant or Advisory Role: None declared.
Stock Ownership: K. Bohman, Siemens; G. Hennig, Siemens.
Honoraria: None declared.
Research Funding: None declared.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We thank Ruth Koehler, Claudia Windbergs, and Torsten Acht for excellent technical assistance; Christian von Toerne and Karsten E. Weber for advice about statistical analyses; and Ralf Kronenwett and Christoph Petry for critical review of the manuscript.

References

1. Harris L, Fritsche H, Mennel R, Norton L, Ravdin P, Taube S, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J Clin Oncol 2007;25:5287–312.
2. Masuda N, Ohnishi T, Kawamoto S, Monden M, Okubo K. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res 1999;27:4436–43.
3. Specht K, Richter T, Müller U, Walch A, Werner M, Höfler H. Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. Am J Pathol 2001;158:419–29.
4. Rupp GM, Locker J. Purification and analysis of RNA from paraffin-embedded tissues. Biotechniques 1988;6:56–60.
5. Cronin M, Pho M, Dutta D, Stephans JC, Shak S, Kiefer MC, et al. Measurement of gene expression in archival paraffin-embedded tissues: development and performance of a 92-gene reverse transcriptase-polymerase chain reaction assay. Am J Pathol 2004;164:35–42.
6. Stanta G, Schneider C. RNA extracted from paraffin-embedded human tissues is amenable to analysis by PCR amplification. Biotechniques 1991;11:304–8.
7. Mizuno T, Nagamura H, Iwamoto KS, Ito T, Fukuhara T, Tokunaga M, et al. RNA from decades-old archival tissue blocks for retrospective studies. Diagn Mol Pathol 1998;7:202–8.
8. Godfrey TE, Kim SH, Chavira M, Ruff DW, Warren RS, Gray JW, Jensen RH. Quantitative mRNA expression analysis from formalin-fixed, paraffin-embedded tissue using 5′ nuclease quantitative reverse transcription-polymerase chain reaction. J Mol Diagn 2000;2:84–91.
9. Ribeiro-Silva A, Zhang H, Jeffrey SS. RNA extraction from ten year old formalin-fixed paraffin-embedded breast cancer samples: a comparison of column purification and magnetic bead-based technologies. BMC Mol Biol 2007;8:118–28.
10. Li J, Smyth P, Cahill S, Denning K, Flavin R, Aherne S, et al. Improved RNA quality and TaqMan pre-amplification method (PreAmp) to enhance expression analysis from formalin fixed paraffin embedded (FFPE) materials. BMC Biotechnol 2008;8:10.
11. Oberli A, Popovici V, Delorenzi M, Baltzer A, Antonov J, Matthey S, et al. Expression profiling with RNA from formalin-fixed, paraffin-embedded material. BMC Med Genomics 2008; 1:9.
12. Abramovitz M, Ordanic-Kodani M, Wang Y, Li Z, Catzavelos C, Bouzyk M, et al. Optimization of RNA extraction from FFPE tissues for expression profiling in the DASL assay. Biotechniques 2008; 44:417–23.