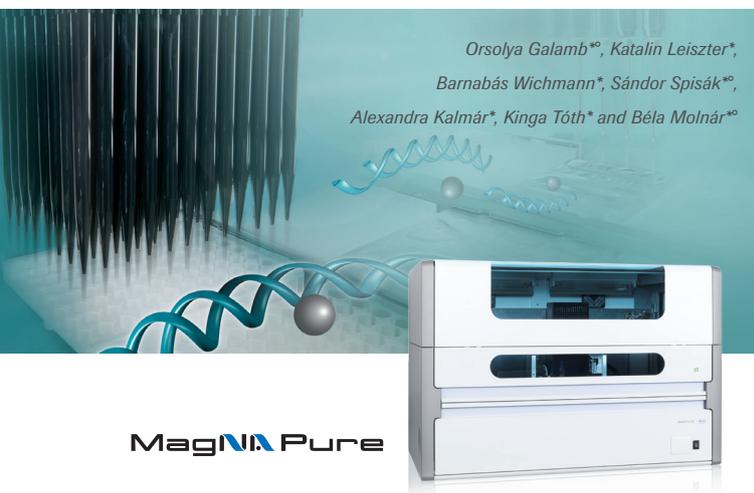


Comparison of Automated MagNA Pure 96 and Manual PAXgene RNA Isolation from Stabilized Peripheral Blood Samples

Orsolya Galamb[°], Katalin Leiszter^{*},
Barnabás Wichmann^{*}, Sándor Spisák[°],
Alexandra Kalmár^{*}, Kinga Tóth^{*} and Béla Molnár[°]



* 2nd Department of Medicine, Semmelweis University, Budapest, Hungary

° Hungarian Academy of Sciences, Molecular Medicine Research Unit, Budapest, Hungary, and 1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary

Abstract

Background: Peripheral blood based molecular diagnostics requires automated sample processing and evaluation of RNA for RT-PCR analysis. Our aims were to compare the automated and manual RNA isolation and to determine the purity, recovery, RNA integrity (RIN) and sensitivity of downstream RT-PCR applications.

Methods: Total RNA was isolated from blood samples of 25 individuals with colorectal cancer (CRC) and 19 healthy individuals, either manually using PAXgene Blood RNA Kit and in parallel, using the MagNA Pure 96 Instrument. The expression of seven CRC and one adenoma markers was analyzed using real-time RT-PCR.

Results: OD260/280 of manually isolated samples (2.09) was equal to that of MagNA Pure 96 Instrument isolated samples (2.01), while OD230/260 of MagNA Pure 96 Instrument samples (1.85) was significantly better than that of manually isolated samples (1.52). The RIN is acceptable in case of both isolation methods. The manual protocol produced slightly more intact RNA (RIN-manual: 9.19 ± 0.26 ; RIN-MagNA Pure 96 Instrument: 8.25). The MagNA Pure 96 System resulted in slightly higher yields of isolated RNA (manual: 5.65 µg/tube; MagNA Pure 96 System: 5.99 µg/tube). Marker correlation between the two batches isolated using the MagNA Pure 96 Instrument and between two PAXgene batches was high. In overall qPCR reproducibility and mRNA yield/ratio were higher with the MagNA Pure 96 System. The automated method showed less standard deviation. Genomic DNA contamination could not be detected in any of the MagNA Pure 96 System isolated RNA samples.

Conclusion: Automated RNA isolation from peripheral blood samples using the MagNA Pure 96 Instrument produces high quality, high purity and high yield total RNA. MagNA Pure 96 Instrument extraction is a fully automated, fast and reliable method with 96 extractions in 85 min, compared to the equally precise, but time consuming manual extraction.

Introduction

Studying colorectal cancer (CRC) has a number of challenges (1,2). Sample collection via colonoscopy can be invasive, and the specificity and sensitivity for CRC detection using conventional serum biomarkers (CA19-9, CEA*) have been limited. Recently, specific circulating tumor cell biomarkers for CRC were identified using mRNA microarray studies, real-time qPCR, and multiplex arrays (32, 34, 35, 36). These techniques require high quality of RNA for analysis. Fortunately, advancements of RNA stabilization reagents that prevent the degradation of free and intracellular RNA, such as PAXgene Blood RNA Tubes, have enabled studies using less evasive collection of peripheral blood. Additional advancements of high throughput nucleic acid extraction systems, such as the MagNA Pure 96 System, have chemistry compatibility

with RNA stabilization reagents and have further increased lab capacity for biomarker studies.

Experiments in this paper investigate the quality and capacity of RNA isolation from PAXgene tubes on the MagNA Pure 96 System compared to conventional manual extraction methods. Researchers used a modified RNA isolation protocol (described in the MagNA Pure 96 Cellular RNA Large Volume Kit product insert) that extracts up to 48 stabilized blood samples (or 96 aliquots) simultaneously. Attributes such as purity, recovery, RNA integrity (RIN) and sensitivity in downstream RT-PCR applications were compared and analyzed. The goal for these studies is to develop an optimized and more efficient workflow for the detection of CRC biomarkers in peripheral blood samples.

Materials and Methods

Patients and samples

After informed consent of untreated patients, 4 x 2.5 ml of peripheral blood samples from 19 healthy and 25 individuals with colorectal cancer were taken into PAXgene Blood RNA Tubes (Qiagen Inc., Germantown, USA) after endoscopic intervention, and stored at -80°C. These individuals had neither autoimmune nor inflammatory diseases. Ethics Committee approval was obtained (Nr.:69/2008. Semmelweis University Regional and Institutional Committee of Science and Research Ethics, Budapest, Hungary).

Total RNA isolation

Before RNA isolation, PAXgene Blood RNA Tubes were thawed for at least 2 hours at +15 to +25°C. Total RNA from 2 x 2.5 ml blood of each subject was extracted according to the manual protocol of PAXgene Blood Kit (Qiagen). In parallel, 2 x 2.5 ml blood of each subject was used for automated isolation with the MagNA Pure 96 System (Roche Diagnostics) and the MagNA Pure 96 Cellular RNA Large Volume Kit (Roche Diagnostics), according to manufacturers' instructions. After the first two centrifugation steps (described in the manual PAXgene Blood RNA Kit protocol), the mononuclear cell pellet was resuspended in 400 µl of RNase-free PBS for MagNA Pure 96 RNA isolation. The lysis buffer provided by the manufacturer was used for the manual isolation protocol. DNase treatment is implemented in both isolation protocols of both manufacturers, for elimination of genomic DNA. The extracted total RNA was stored at -80°C until analysis.

Quantitative and qualitative analysis of the isolated total RNA

For analyzing the possible genomic DNA contamination of the total RNA samples, cyclin A (CycA) assay was used. Previously isolated (both MagNA Pure 96 System and manually extracted) RNA samples from 2 normal and 2 CRC patients were tested with and without reverse transcription. For the genomic CycA DNA detection, after denaturation at 95°C for 5 min, 45 PCR cycles were performed (denaturation at 95°C for 1 sec, annealing at 55°C for 10 sec and extension 72°C for 18 sec), followed by a melting curve analysis. The CycA concentration was determined using a calibration curve of known human genomic DNA dilutions (1 ng/ul, 100 pg/ul, 10 pg/ul, 1 pg/ul, and 0.1 pg/ul final concentrations). In parallel, the cDNA synthesis was controlled using RT-PCR for two previously applied CRC markers (PCR conditions see in the next chapter).

Quantitative real-time PCR analysis

Ready-to-use real-time PCR assays were used for expression measuring of 7 CRC and 1 adenoma-specific markers (lifescience.roche.com). Gene specific forward and reverse primers and fluorescently labeled hydrolysis probes from Universal Probe Library (Roche Diagnostics) were used in qPCR. Using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics), 1 µg total RNA from peripheral blood samples from 19 healthy and 24 individuals with CRC, were reverse transcribed. The quality of the cDNA samples was checked using real-time PCR targeting the SDHA (succinate

Materials and Methods

dehydrogenase complex, subunit A, flavoprotein) housekeeping gene. Expression analysis of the selected genes was performed from 8.3 ng/sample cDNA template using ready-to-use real-time PCR assays and LightCycler® 480 Probes Master (Roche Diagnostics). Measurements were performed using a LightCycler® 480 Instrument (Roche Diagnostics), as described in the product's user guide (lifescience.roche.com). After enzyme activation and denaturation at +95°C for 10 min, 45 PCR cycles were performed (denaturation at +95°C for 10 sec, annealing and extension at +60°C for 30 sec and signal detection at +72°C for 1 sec). To select the most appropriate reference gene, three different housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein

L13a (RPL13A) and succinate dehydrogenase complex, subunit A, flavoprotein (SDHA)) were used for the real-time PCR array.

Relative quantification of gene expression was performed and fold change values were calculated using the ddCT method (8). The threshold cycle (CT) of the SDHA (succinate dehydrogenase complex, subunit A, flavoprotein) endogenous control was used for normalizing target gene expression (dCT) to correct for experimental variation. The correlation between manual and automated RNA isolation was evaluated with STATISTICA Software using the linear correlation option.

Results and Discussion

Total RNA quality and quantity

Total RNA was isolated from 19 healthy individuals and 25 individuals with CRC using their PAXgene blood samples. Four parallel isolations were performed from each case (44 x 4 = 176): 88 (2 x 44) isolations were done using the MagNA Pure 96 System, and 88 (2 x 44) isolations were

performed using the manual PAXgene protocol (see Figure 1). RNA quality and quantity data of each isolation (n = 176 isolations for each method) were obtained, and the data of 2-2 parallel isolations (2 manual, 2 MagNA Pure 96) were not averaged (see Table 1).

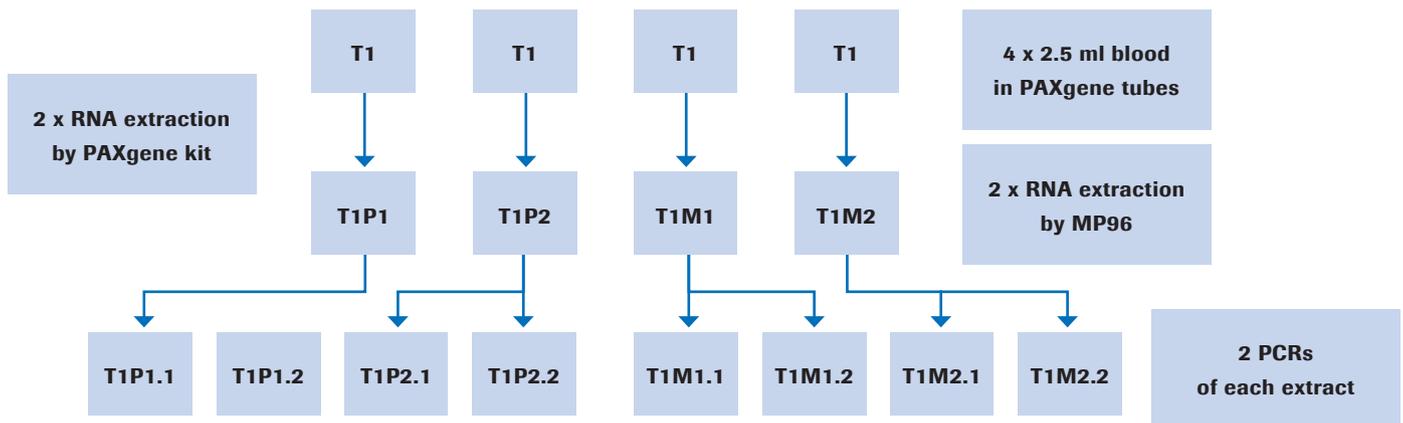


Figure 1. Extraction scheme

Volumes of 4 tubes of 2.5 ml peripheral blood were taken from each individual, and total RNA was extracted from 2 tubes using MagNA Pure 96 System. In parallel, 2 tubes of the above peripheral blood were isolated manually using the PAXgene Blood RNA Kit. PCR analysis for 8 markers was performed in duplicates for each extract (T1 = sample tumor 1 as an example).

Results and Discussion

Table 1: Comparison of the two isolation methods

Analyzed Parameter	MagNA Pure 96	Manual PAXgene	P value
Mean OD260/280 ± SD	2.01 ± 0.05	2.09 ± 0.03	2.20 x 10 ⁻¹⁶
Mean OD260/230 ± SD	1.85 ± 0.23	1.52 ± 0.57	1.87 x 10 ⁻⁶
Purity OD260/280 ± SD	44/44	44/44	
Purity OD260/230 ± SD	43/44	26/44	
Mean RIN ± SD (all samples)	8.25 ± 0.35	9.19 ± 0.26	2.2 x 10 ⁻¹⁶
RIN range	7.50 – 9.00	8.00 – 9.60	-
No. of samples RIN ≤ 8	21 (23.9 %)	1 (1.1 %)	-
No. of samples 8 < RIN < 9	66 (75 %)	10 (11.4 %)	-
No. of samples RIN ≥ 9	1 (1.1 %)	77 (87.5 %)	-
Mean RIN ± SD (samples collected in 2010)	8.28 ± 0.36	9.22 ± 0.24	-
Mean RIN ± SD (samples collected in 2007/2008)	8.16 ± 0.31	9.10 ± 0.31	-
P value (RIN2010 vs. RIN2007/2008)	0.106	0.077	-
Mean RNA recovery (µg/sample) ± SD (all isolated samples)	5.99 ± 2.04	5.65 ± 2.75	0.35
Mean RNA recovery (µg/sample) ± SD (CRC samples)	5.94 ± 2.48	5.95 ± 3.31	-
Mean RNA recovery (µg/sample) ± SD (normal samples)	6.06 ± 1.28	5.26 ± 1.75	-
P value (RecoveryCRC versus RecoveryN)	0.78	0.25	-

a. RNA quality by OD260/280 and OD260/230

The ratio of the absorbance at 260 and 280 nm (OD260/280) was used to assess nucleic acid purity. The 260:280 ratio is a measure of protein contamination in the isolated nucleic acids. Absorption at 230 nm can also be caused by contamination by phenolate ion, thiocyanates, and other organic compounds. For a pure RNA sample, the OD260/230 should be approximately 2.0.

The purity OD260/280 of PAXgene Blood RNA Kit isolated samples (2.09 ± 0.03) was equal to that of MagNA Pure 96 isolated samples (2.01 ± 0.05 , see Figure 2A). As pure RNA has an OD260/280 of approximately 2, the purity (OD260/280) of both MagNA Pure 96 System and manual PAXgene isolated total RNA fulfilled the criteria by having an OD of 1.8-2.1. **The OD (260/230) was significantly better in case of MagNA Pure 96 System isolated samples (1.85 ± 0.23) compared to manually isolated ones (1.52 ± 0.57 , see Figure 2B); $p < 0.0001$.**

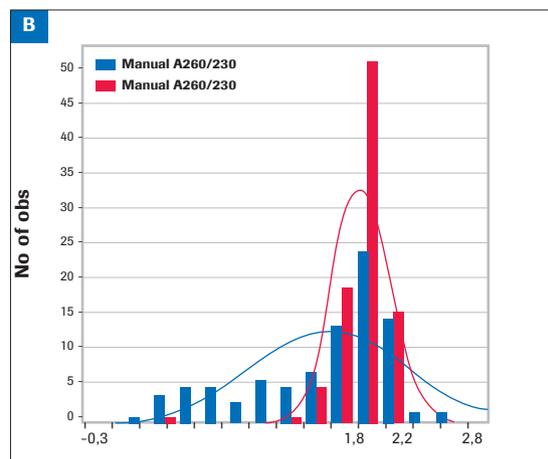
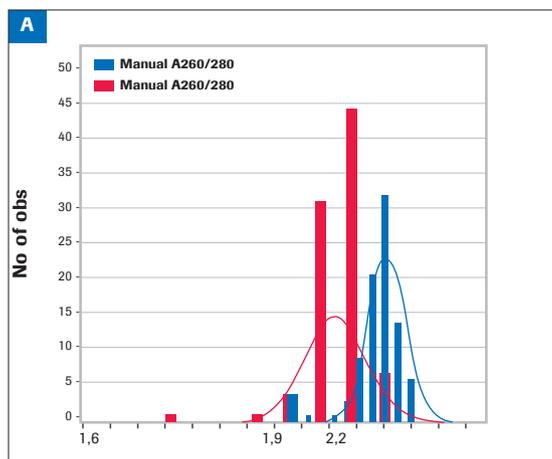


Figure 2A, 2B: Comparison of data quality and recovery of total RNA from samples isolated using the MagNA Pure 96 System or manual PAXgene Blood RNA Kit.

A. Distribution of the OD260/280 purity values. **B.** Distribution of the OD260/230 purity values.

Results and Discussion

b. RNA integrity by capillary gel electrophoresis

RNA integrity number (RIN) is another numerical assessment of RNA integrity. The RIN software algorithm allows the classification of total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded and 10 being the most intact. **The RIN was found to be acceptable for both isolation methods, but the manual PAXgene protocol provided a more intact RNA as shown by slightly higher RNA integrity numbers (RIN manual: 9.19 ± 0.26 ; RIN MagNA Pure 96: 8.25 ± 0.35 ; $p < 0.0001$, see Figure 2C).** No significant RIN differences were detected between prospective and archived samples.

c. RNA recovery by OD260

The MagNA Pure 96 System resulted in slightly higher yields of isolated RNA (MagNA Pure 96: $5.99 \pm 2.04 \mu\text{g}/\text{tube}$; manual: $5.65 \pm 2.75 \mu\text{g}/\text{tube}$), but this difference was not statistically significant (see Figure 2D). There was no significant difference between RNA recovery of CRC and of normal samples isolated by the same method. **There are several factors by which the RNA recovery can be influenced, such as: (1) features of an individual's blood (red blood cell number, leukocyte**

number etc.), (2) volume of collected blood (can differ from the theoretical collection of 2.5 ml/PAXgene tube), and (3) features of blood cell pellet resuspension, and (4) differences in the isolation protocol

d. Genomic DNA contamination

Cyclin A genomic DNA was detected in none of the MagNA Pure 96 System isolated RNA samples, while low concentrations ($< 10 \text{ pg}/\text{ul}$) of CycA could be observed in 3 of 4 manually extracted total RNA samples (Figure 2E or Figure 3.). The efficacy of the MagNA Pure 96 DNase treatment was significantly higher than that of the manual PAXgene RNA purification. This can probably be attributed to the differences of the DNase enzyme concentration, incubation buffer composition and to the longer DNase incubation time used in MagNA Pure 96 extraction.

e. Influences of sample types

CRC specimens showed the same values as specimens from healthy subjects with respect to OD260/280, OD260/230, RIN values and yield. According to the above mentioned parameters, total RNA samples from archived CRC specimens (10 from 2008) also showed similar features as the prospective collected CRC specimens (15).

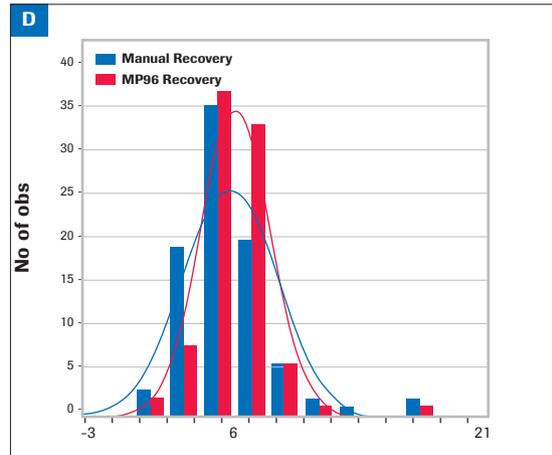
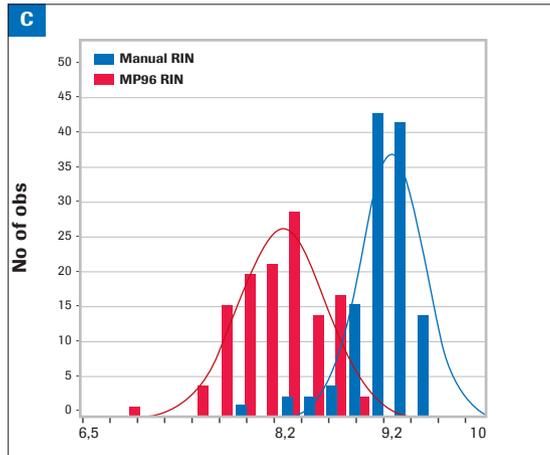


Figure 2C, 2D: Comparison of data quality and recovery of total RNA from samples isolated using the MagNA Pure 96 System or manual PAXgene Blood RNA Kit.

C. Distribution of the RNA Integrity Numbers (RIN). **D.** Distribution of the RNA recovery values ($\mu\text{g}/\text{tube}$).

Results and Discussion

II. qPCR marker correlation

II. qPCR marker correlation Comparison of the MagNA Pure 96 and manual PAXgene extraction methods, and evaluation by RT-PCR, showed a linear regression ($r = 0.98$). **For all 8 markers, the marker correlation in PCR both between two MagNA Pure 96 batches and between two PAXgene batches was high ($r = 0.99$; $r = 0.98$, see Figures 3A, B, C).** Higher correlation was found for two MagNA Pure 96 System batches for 5 of the 8 analyzed markers. The qPCR results of 3 markers proved to be more reproducible between the two PAXgene batches.

Overall, qPCR reproducibility was higher for MagNA Pure 96 System isolated samples than the manually isolated total RNA samples. The Cp values of the MagNA Pure 96 isolated samples were lower indicating higher expression, than those of the manual extraction, reflecting the higher mRNA yield/ratio of the MagNA Pure 96 System isolation procedure. The standard deviations (SD) of the Cp values showed lower variability in MagNA Pure 96 System extracted cases than in manually isolated samples.

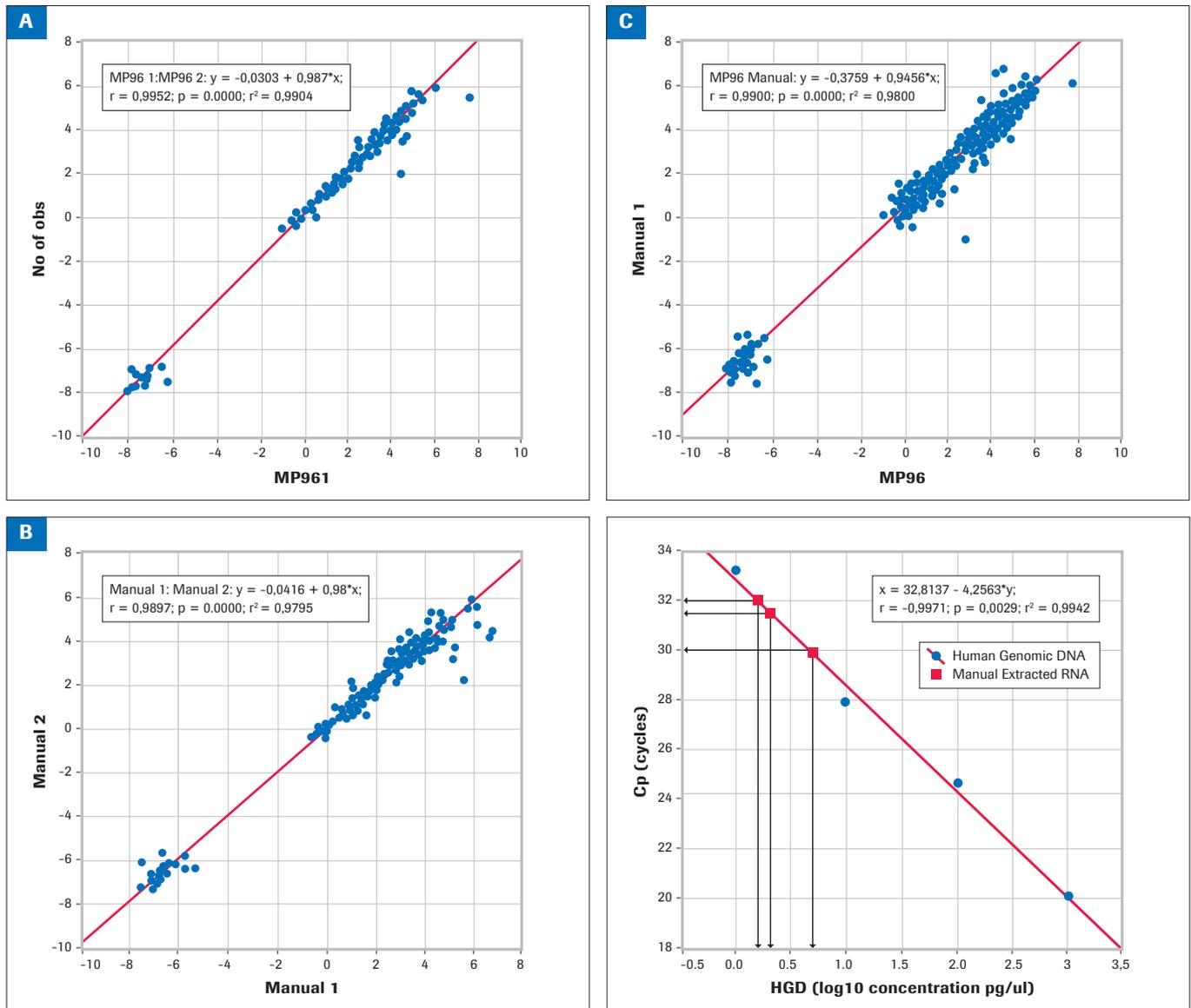


Figure 3: Genomic DNA contamination

Cyclin A genomic DNA could not be detected in any of the MagNA Pure 96 System isolated RNA samples, while low concentrations (< 10 pg/ul) of CycA were observed in 3 of 4 manually extracted total RNA samples. Due to the low genomic DNA concentration in the manually extracted RNA samples, late Cp values could be detected.

Figure 4: The CycA concentration was determined using a calibration curve using known human genomic DNA dilutions

(1ng/ul, 100pg/ul, 10pg/ul, 1pg/ul, and 0.1 pg/ul final concentrations) ($R^2=0,9942$). The degree of contamination (the detected CycA concentrations) can be seen on the X-axis showing data for the three DNA contaminated RNA samples.

Conclusion

Automated RNA isolation from stabilized peripheral blood samples using the MagNA Pure 96 Instrument produces high quality, high purity and high yield total RNA. The Cp values of the RNA extracted using the MagNA Pure System 96 were lower indicating higher expression levels

than the Cp values for manual extraction. The MagNA Pure 96 extraction is a fully automated, fast and reliable method permitting 96 RNA extractions in 85 min, compared to the equally precise, but time consuming manual extraction using the PAXgene Blood RNA Kit.

References

1. Bingham S et al. (2004) Nat Rev Cancer 4:206-215
2. Levin B et al. (2008) Gastroenterology 134: 1570-1595
3. Han M et al. (2008) Clin Cancer Res 14: 455-460
4. Solmi R et al. (2006) BMC Cancer 6:250
5. Smirnov DA et al. (2005) Cancer Res 65:4993-4997
6. Yeh CS et al. (2006) Int J Oncol 28:411-420
7. Martin KJ et al. (2001) Proc Natl Acad Sci USA 98:2646-2651
8. Livak KJ et al. (2001) Methods 25:402-408

Important Note: Regarding the specific assay(s) described, Roche was neither involved in establishing the experimental conditions nor in defining the criteria for the performance of the specific assays. Roche therefore cannot take any responsibility for performance or interpretation of results obtained for the biological target parameter(s) described by the authors or other users using a similar experimental approach.

Potential users are informed to be aware of and in accordance with local regulations for assay validation and the scope of use for the involved Roche products, and to ensure that their use is valid in the countries where the experiments are performed.

The MagNA Pure 96 Instrument (06 541 089 001) is for *in vitro* diagnostic use.

First Strand cDNA Synthesis Kit, Universal ProbeLibrary, and the LightCycler® 480 Instrument are for life science research only. Not for use in diagnostic procedures.

License Disclaimer information is subject to change or amendment.
For current information on license disclaimers for a particular
product, please refer to the Online Technical Support page
(<http://technical-support.roche.com>).

MAGNA PURE, TAQMAN and LIGHTCYCLER are trademarks of Roche.
All other product names and trademarks are the property of their
respective owners.

Published by
Roche Molecular Diagnostics
4300 Hacienda Drive
Pleasanton, California 94598
USA

© 2015 Roche Diagnostics.

©0315