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Research Article

Utilization of paramagnetic microparticles for automated isolation of free circulating mRNA as a new tool in prostate cancer diagnostics

Determination of serum mRNA gained a lot of attention in recent years, particularly from the perspective of disease markers. Streptavidin-modified paramagnetic particles (SMPs) seem an interesting technique, mainly due to possible automated isolation and high efficiency. The aim of this study was to optimize serum isolation protocol to reduce the consumption of chemicals and sample volume. The following factors were optimized: amounts of (i) paramagnetic particles, (ii) oligo(dT)$_{20}$ probe, (iii) serum, and (iv) the binding sequence (SMPs, oligo(dT)$_{20}$, serum vs. oligo(dT)$_{20}$, serum and SMPs). RNA content was measured, and the expression of metallothionein-2A as possible prostate cancer marker was analyzed to demonstrate measurable RNA content with ability for RT-PCR detection. Isolation is possible on serum volume range (10–200 μL) without altering of efficiency or purity. Amount of SMPs can be reduced up to 5 μL, with optimal results within 10–30 μL SMPs. Volume of oligo(dT)$_{20}$ does not affect efficiency, when used within 0.1–0.4 μL. This optimized protocol was also modified to fit needs of automated one-step single-tube analysis with identical efficiency compared to conventional setup. One-step analysis protocol is considered a promising simplification, making RNA isolation suitable for automatable process.

Keywords:
Metallothionein / Microseparation / Paramagnetic microparticles / Prostate cancer / Serum mRNA
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1 Introduction

Extracellular mRNA was found in the circulation [1]. Such RNA can be detected in plasma, serum, and other body fluids, as well as from cell-free supernatants of in vitro cultivated cells [2, 3]. Based on the fact that the ubiquitous concentration of RNases is relatively high, and that RNA-degrading enzymes are extremely stable, it seems likely that RNA is complexed with other molecules, which makes it resistant to digestion [4].

The extracellular mRNA is thought to be released into the circulation from intact and viable cells as well as necrotic cells [5]. An increasing amount of evidence suggests that liberation of cell-free nucleic acids into plasma from organs or compartments is likely due to cell death [6, 7]. The formation of apoptotic bodies, which contain either DNA or RNA, was observed in tumor patients, especially during and after therapy [8].

The biological roles of circulating mRNA are still unclear, although its physiological significance has been investigated during the last several years. Cell-free DNA, mRNA, and microRNA seem to be promising molecular biomarkers for clinical applications, and also signaling molecules for intercellular communication. The secretion and transfer of macromolecular RNA between mammalian cells has been described [9–11]. Garcia-Olmo et al. even concluded that metastases are the result of a transformation of susceptible cells by circulating nucleic acids [12, 13]. According to their observations, the hematogenous dissemination is closely associated with the detection of circulating nucleic acids than with circulating tumor cells [14, 15].

Prostate cancer is one of the most common cancers [16]. To date, prostate specific antigen (PSA) is routinely used biomarker for this disease. However, contradictory results were published regarding the PSA screening in 2009. While European Randomized Study of Screening for Prostate Cancer reported 20% reduction of risk [17], no such benefit of screening was determined in Prostate, Lung, Colorectal

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Abbreviations: **FBS**, fetal bovine serum; **MT2A**, metallothionein-2A; **PSA**, prostate specific antigen; **ROC**, receiver operating characteristic; **SMP**, streptavidin-modified paramagnetic microparticle

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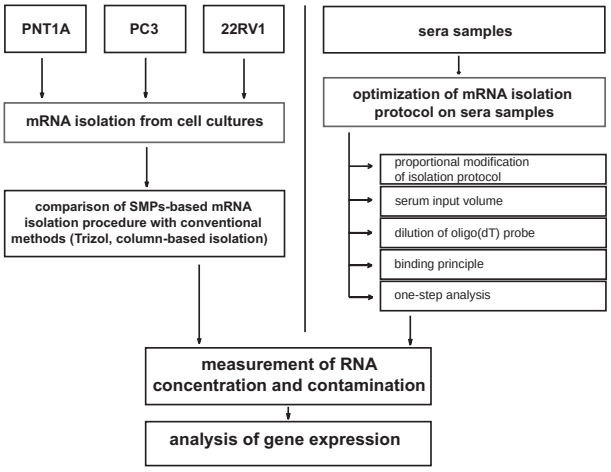

Figure 1. Scheme of study design. RNA contamination by proteins measured by absorbance ratio 260/280.

and Ovary Study [18]. Therefore, development of alternative biomarkers is desirable. Due to the fact that nucleic acids in plasma/serum may be a suitable source for the development of noninvasive diagnostic, prognostic, and follow-up tests for cancer and other types of diseases, we focused on isolation and detection of mRNA as well as determining the level of metallothionein-2A (MT2A) expression by RT-PCR. MT can indeed serve as a new prognostic marker of cancer progression, as it is clear from numerous recent publications [19–22]. The detection of circulating RNA offers certain advantages over the detection of circulating DNA [23]. First, if both plasma RNA and DNA were derived from the same cell population, the released RNA would likely be quantitatively more abundant than DNA [24]. This is because multiple copies of an RNA transcript may be present in each cell, depending on the gene's expression, whereas each cell contains only a single diploid genome equivalent of DNA. Second, some cancer researchers reported that a greater proportion of cancer cases was positive for the investigated plasma RNA markers than DNA markers [25]. The number of protocols applied for the isolation of extracellular mRNA is probably as numerous as the laboratories using them. Even if most researchers use commercially available kits, there is no accepted "standard" method so far. Fast, easily automatable, and simple procedure for isolating circulating mRNA from limited amounts of plasma/serum is highly required. Promising in this regard could be the use of paramagnetic microparticles, streptavidin-modified paramagnetic microparticles (SMPs) in particular. SMPs can be modified with biotinylated oligo(dT) fragment and therefore can bind mRNAs. In this study, we compared the isolation of mRNA made by SMPs with methods commonly used for mRNA isolation (Trizol Reagent, silica columns) and assessed the applicability of these methods for the isolation of circulating MT2A mRNA transcript for prostate cancer diagnostics. The basic scheme of the study is shown in Fig. 1.

2 Materials and methods

2.1 Chemical and biochemical reagents

RPMI-1640 medium, Ham's F12 medium, fetal bovine serum (FBS, mycoplasma-free), penicillin/streptomycin, and trypsin were purchased from PAA Laboratories (Pasching, Austria). PBS was purchased from Invitrogen Corporation (Carlsbad, CA, USA). EDTA and all other chemicals of ACS purity were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless noted otherwise.

2.2 Cell cultures and culture conditions

Three human prostatic cell lines were used in this study: (i) PNT1A human cell line established from normal adult prostatic epithelium; (ii) 22Rv1, human prostatic epithelial cell line derived from androgen-dependent primary tumor; and (iii) PC-3 human cell line established from a grade 4 androgen-independent cancer from metastatic site in bone. All cell lines used in this study were purchased from Health Protection Agency Culture Collections (Salisbury, UK).

PNT1A and 22RV1 cells were cultured in RPMI-1640 medium with 10% FBS. PC-3 cells were cultured in Ham's F12 medium with 7% FBS. All media were supplemented with penicillin (100 U/mL) and streptomycin (0.1 mg/mL), and the cells were maintained at 37°C in a humidified incubator (Sanyo, Japan) with 5% CO_2.

2.3 Cell-content quantification

Cell content was analyzed using Casy model TT system (Roche Applied Science, USA) using protocol recommended by manufacturer. For each cell type, new calibration was prepared and all subsequent measurements were performed on 100 × diluted 100 μL cell suspension. All samples were measured in duplicates.

2.4 Set of patients with diagnosed prostate carcinoma

Serum samples of patients with histologically verified prostate adenocarcinoma (35 samples) were used in our study and compared to 14 controls. Average age of patients was 62.1 years, range 53–71 years. Tumors were classified histologically with Gleason score, ranging 6–9. Pathological staging of samples varied from T2a to T4, all patients were without nodal or metastatic dissemination. In the control group, all probands were without neoplastic disease. Age of control group of volunteers varied 23–37 years, mean age was 30.1 years. Tested serum samples were obtained from Urology clinic, St. Anne's University Hospital in Brno, Czech Republic. Inclusion of patients into realized clinical study was approved by the Ethic commission of the Faculty of

Medicine, Masaryk University, and St. Anne's University Hospital, Brno, Czech Republic.

2.5 RNA isolation from cell cultures

Three different procedures were used to extract RNA from cell cultures to evaluate isolation efficiency and RNA yield. For this purpose, we used High Pure RNA Isolation Kit (Roche, Basel, Switzerland) employing silica columns (further referred to as "column-based"), Trizol RNA Isolation using TriPure reagent (Roche), and mRNA Isolation Kit (Roche) utilizing streptavidin-modified paramagnetic microparticles (SMPs). The medium was removed and samples were washed with 5 mL of ice-cold PBS twice. Cells were scraped off, transferred to clean tubes, and centrifuged at 20 800 × *g* for 5 min at 4°C. A total of 5×10^6 cells were used for each isolation procedure. Column-based isolation and isolation utilizing SMPs were used according to manufacturer's instruction starting the lysis step. Trizol isolation was performed using TriPpure reagent (monophasic solution of phenol and guanidine isothiocyanate) by incubating samples for 5 min at 25°C to permit the complete dissociation of nucleoprotein complexes. Then, 40 μL of chloroform per 200 μL of TriPure was added. Samples were centrifuged at 10 600 rpm for 15 min at 2–6°C. Following centrifugation, RNA remains exclusively in the aqueous phase. The aqueous phase was transferred into a clean tube. RNA was precipitated by mixing with isopropyl alcohol (0.1 mL of isopropyl alcohol per 200 μL TriPure). Samples were incubated at 25°C for 10 min and centrifuged at 10 600 rpm for 10 min at 4°C. Then, the supernatant was removed and RNA pellet was washed once with 75% ethanol. Two hundred microliters of ethanol per 200 μL of TriPure reagent was added. Samples were vortexed and subsequently centrifuged at 8400 rpm for 5 min at 4°C. Then, dry RNA pellet was dissolved in 50 μL of RNase-free water for 18 min at 58°C.

2.6 Extracellular RNA isolation from serum samples

All three above-mentioned isolation methods were used in order to extract extracellular mRNA from serum samples. Since the SMPs isolation protocol is not primarily intended by manufacturer for this purpose, individual steps were optimized to find out what volumes of reagents are the most effective for obtaining extracellular mRNA. Briefly, basic SMPs isolation was carried out by resuspending microbeads in 70 μL of PBS and removed by magnetic separator. Total 0.5 μL of oligo$(dT)_{20}$ probe was added to 200 μL of serum to form hybridization mix. It was mixed with SMPs and incubated for 5 min at 37°C. SMPs with bound mRNA were then separated and washed three times with SMP washing buffer. To dissociate mRNA from SMPs, 10 μL redistilled water was added and samples were incubated for 3 min at 65°C. Optimizations of isolation protocol were then carried out (Table 1).

First, volumes of reagents were proportionally modified and serum volume was gradually decreased. Consequently, effectiveness of two different binding principles was evaluated. Oligo$(dT)_{20}$ probe was first mixed with serum sample, then it was added to SMPs and incubated 5 min at 37°C. The second binding principle was based on hybridization oligo$(dT)_{20}$ probe first with SMPs; subsequently, after 5-min incubation, serum sample was added.

Dilution of oligo$(dT)_{20}$ probe was also carried out, since the amount of extracellular mRNA in serum sample is definitely much lower than in cell cultures. Probe was 10 × diluted for easy handling and its volume was gradually decreased.

2.7 One-step analysis

One-step analysis was performed using 50 μL SMPs and 200 μL serum sample. Elution step was skipped and reverse transcription followed by analysis of gene expression was carried out in the presence of SMPs. For details of individual optimization steps, see Section 3.

2.8 RNA content and purity measurement

RNA and mRNA concentrations and purities were determined by NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Optical density ratio at $\lambda = 260/280$ nm was calculated to evaluate protein contamination of RNA. While product of column-based isolation and Trizol isolation protocols is total RNA, SMPs isolation enable to obtain mRNA directly. In order to compare efficiency of these isolation methods, mRNA concentrations were calculated with an assumption that mRNA content in typical mammalian cell is 5% of total RNA at most. The same recount was applied on serum samples, where we assumed 50% of total RNA to be mRNA [26].

2.9 RNA reverse transcription

Isolated RNA was used for cDNA synthesis. From all isolation protocols, 6 μL (of total 10 μL isolated using SMPs, 50 μL using Trizol, and 50 μL using column-based isolation) of RNA was used for each transcription. RNA was transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. Five microliters of prepared cDNA (of total 20 μL transcribed) from total RNA (High Pure Isolation Kit, Trizol RNA Isolation) and mRNA (mRNA Isolation Kit) was subsequently analyzed by 7500 real-time PCR system (Applied Biosystems).

2.10 Quantitative PCR (q-PCR)

After reverse transcription, q-PCR was performed with 5 μL of cDNA from each sample using the TaqMan gene

Table 1. Optimization steps for serum RNA isolation using SMPs

Optimization step	SMPs (μL)	Serum (μL)	Oligo(dT)$_{20}$ probe (μL)	cRNA (ng/μL)	$A_{260/280}$	Threshold cycle (C_t)
Proportional modification	5	20	0.05	1.5	1.31	–
	10	40	0.10	3.8	1.14	–
	15	60	0.15	4.3	1.26	–
	20	80	0.20	8.3	1.11	–
	30	120	0.30	5.2	1.30	–
	40	160	0.40	9.7	1.31	–
	50	200	0.50	12.9	1.20	–
Serum volume	20	10	0.20	6.5	1.10	39.38
	20	30	0.20	6.6	1.15	38.65
	20	50	0.20	6.8	0.94	38.81
	20	80	0.20	7.7	1.08	40.01
	20	100	0.20	7.6	1.10	38.40
	20	150	0.20	6.3	1.06	38.86
Binding principle "A"	20	80	0.20	8.6	1.17	–
Binding principle "B"	20	80	0.20	7.6	1.10	–
Oligo(dT)$_{20}$ probe	20	80	0.40	5.3	0.91	38.44
	20	80	0.30	4.9	0.94	37.94
	20	80	0.20	3.1	1.18	37.06
	20	80	0.10	5.0	1.08	37.29

Volumes of reagents isolated RNA content, RNA contamination and metallothionein gene expression analysis by qRT-PCR. $A_{260/280}$ ratio designates protein contamination of RNA. Threshold cycle represents metallothinein qRT-PCR measurement. Binding principle "A" indicates first oligo(dT)$_{20}$ binding to sample following binding to SMPs; "B" indicates first oligo(dT)$_{20}$ binding to SMPs, following incubation with sample.

expression assay with the 7500 real-time PCR system. The primer and probe set for MT2A (Hs02379661_g1) was selected from TaqMan gene expression assay libraries (Life Technologies, USA). q-PCR was performed under the following amplification conditions: total volume of 20 μL, initial incubation 50°C/2 min followed by denaturation 95°C/10 min, and then 45 cycles at 95°C/15 s, 60°C/1 min.

2.11 Statistical analysis

To reveal differences between groups, one way and factorial ANOVA were employed. Pearson correlations were performed to reveal dependencies between continuous variables. Unless noted otherwise, p level <0.05 was considered significant. Receiver operating characteristic (ROC) analysis was used to calculate sensitivity and specificity. Software Statistica 10 (StatSoft, USA) and MedCalc 12 (MedCalc Software, Belgium) were used for analysis.

3 Results

3.1 Comparison of RNA isolation assays on cell lines

First, the RNA isolation efficiency of three widely used isolation assays as column-based (High Pure Isolation Kit), paramagnetic micro particle based (mRNA Isolation Kit, Roche, further designated as SMPs), and Trizol-based (TriPure, Roche) were compared. Because of the lack of standardized RNA isolation procedures for serum or plasma, this optimization step was performed on well-characterized prostate cell lines as PNT1A, PC-3, and 22Rv1 according to manufacturer's recommended protocol. While column-based and TriPure isolation assays isolate total RNA content indiscriminately (i.e. including mRNAs, tRNAs rRNAs, miRNAs, and other forms), SMPs isolate only mRNA molecules selectively. Inasmuch as mRNA comprises at most 5 and 50% of total cellular and extracellular RNA, respectively, detected amounts were converted to this percentage (see Section 4 for details).

Using SMPs, significantly largest amount of total mRNA from an equal number of cells (500 000 cells) was isolated (mRNA ranged 17.6–59.5 ng/μL; up to sevenfold, $p = 0.035$ and threefold, $p = 0.066$, for column- and Trizol-based isolation assays, respectively, Fig. 2A). However, the isolation using SMPs led to significantly lowest purity (mean 260/280 = 1.74 vs. 1.90 and 2.10 for TriPure and column-based isolation assays). Although distinct differences were observed between cell lines, it is beyond the scope of this study and is not widely discussed. To verify these results, the expression of MT2A level was analyzed (Fig. 2B). Insignificantly higher C_t values (i.e. lower MT2A level) were detected using paramagnetic particle assay. In addition, no significant differences were observed between cell lines.

3.2 Comparison of RNA isolation assays on serum samples

Consequently, mRNA was isolated from 200 μL of sera from prostate cancer patients. Three random samples were analyzed, all three isolation protocols were used under identical conditions as for cellular RNA isolation (30 μL SMPs,

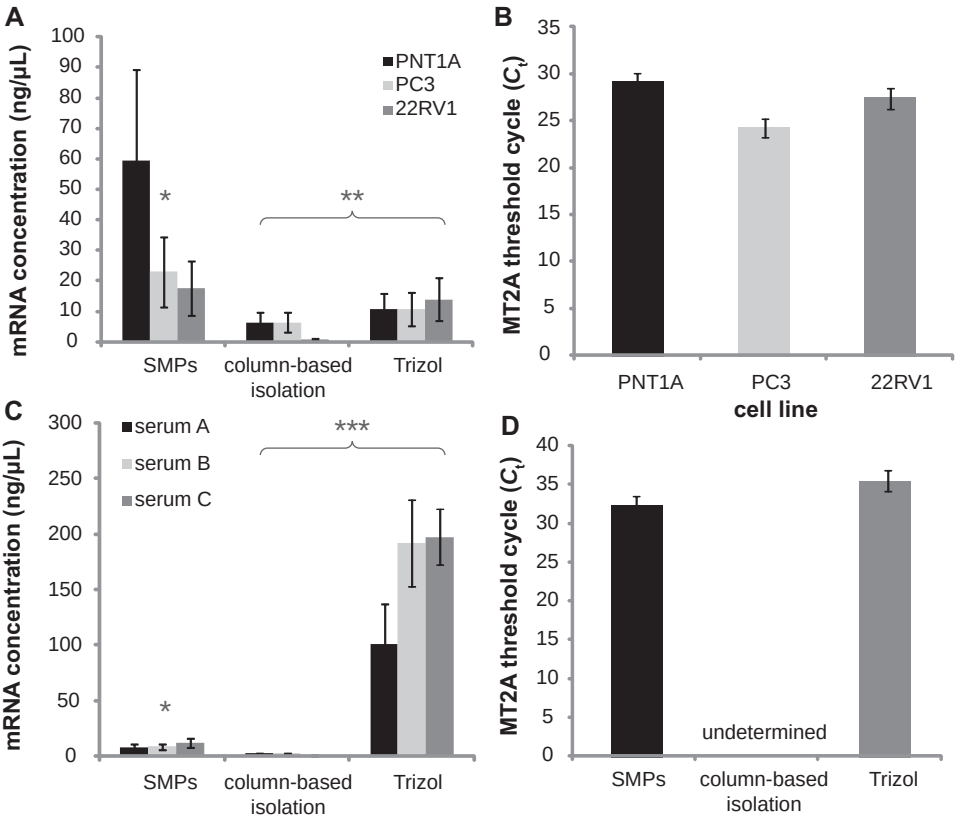

Figure 2. Comparison on isolation protocols on cellular and extracellular RNA. (A) Comparison of RNA isolation efficiency from prostate cell lines. (B) MT2A gene expression using SMP isolation protocol isolated from prostate cell lines. (C) Comparison of RNA isolation efficiency from serum. *, SMPs isolate mRNA selectively, other protocols isolate whole RNA content. **, An approximate calculation, mRNA comprises up to 5% of total cellular RNA. ***, Approximate extracellular mRNA content, up to 50% of RNA. (D) Analysis of MT2A gene expression using SMPs, Trizol- and column-based isolation from serum from prostate cancer patients. No significant difference between SMP and Trizol-based isolation. Data are displayed as mean ± SD.

0.5 μL oligo(dT)$_{20}$). Compared to cellular isolation, more distinct differences between methods were observed (Fig. 2C). Column-based method did not lead to satisfactory results with insufficient RNA content isolated. Seemingly high Trizol efficiency is caused by approximate "50% recalculation." In the next step, serum MT RNA level was detected (Fig. 2D). However column-based detection was below detection limits, no significant difference was observed between Trizol- and SMP-based isolation.

3.3 Optimization of serum RNA isolation

Based on the Section 3.2, we proved that RNA isolation from sera was possible using Trizol- and SMP-based technique. In this step, the aim was to optimize serum isolation protocol using SMPs and to reduce the need of chemicals and sample volume. Thus, the amounts of (i) paramagnetic particles, (ii) oligo(dT)$_{20}$ probe, (iii) serum volume, and (iv) the binding sequence (first anchoring paramagnetic particles with oligo(dT)$_{20}$ probe following serum addition vs. first oligo(dT)$_{20}$ probe + serum binding following addition of paramagnetic particles) were manipulated. Consequently, RNA content was measured, the expression of MT2A was analyzed to demonstrate measurable RNA content and ability for real-time PCR detection.

First, the paramagnetic particle amount was optimized. The following SMP amounts were used: 50 (recommended by manufacturer), 40, 30, 20, 15, 10, 10, and 5 μL. Together with decreasing SMP content, all components were changed ratiometrically (Table 1). RNA was isolated using whole listed SMP volume range, significant strong correlation between SMP and mRNA concentrations was detected ($r = 0.91$, $p = 0.004$, Fig. 3A). Using 5 μL SMPs, 1.5 ng/μL mRNA was isolated from 20 μL serum volume. With regard to purity of isolated mRNA, no correlation was observed; mean 260/280 ratio was 1.22 ± 0.08. To evaluate the use of this volume for qRT-PCR detection, the PCR efficiency of MT expression was calculated [27]. Thus, five dilutions were prepared and the efficiency 1.96 ± 0.12 (of max. 2.0) was determined ($r = -0.97$, Fig. 3B). Thus, isolation using 5 μL SMPs and 10 μL serum is feasible. Nevertheless, isolation using 20 μL SMPs showed slight increase in effectiveness. Thus, this concentration was used for further optimization steps.

Second, amount of serum amount was optimized (Fig. 3C). The following volumes of serum were used: 10, 30, 50, 80, 100, and 150 μL. Although relatively low purity of isolated samples was observed (260/280 nm = 1.07 ± 0.06), no significant correlation between SMP volume and mRNA concentration was observed ($r = 0.13$ at $p = 0.81$). Thus, whole concentration range may be used for RNA isolation.

In the next step, the effectiveness of binding sequence of individual components (sample, MP, oligo(dT)$_{20}$) was

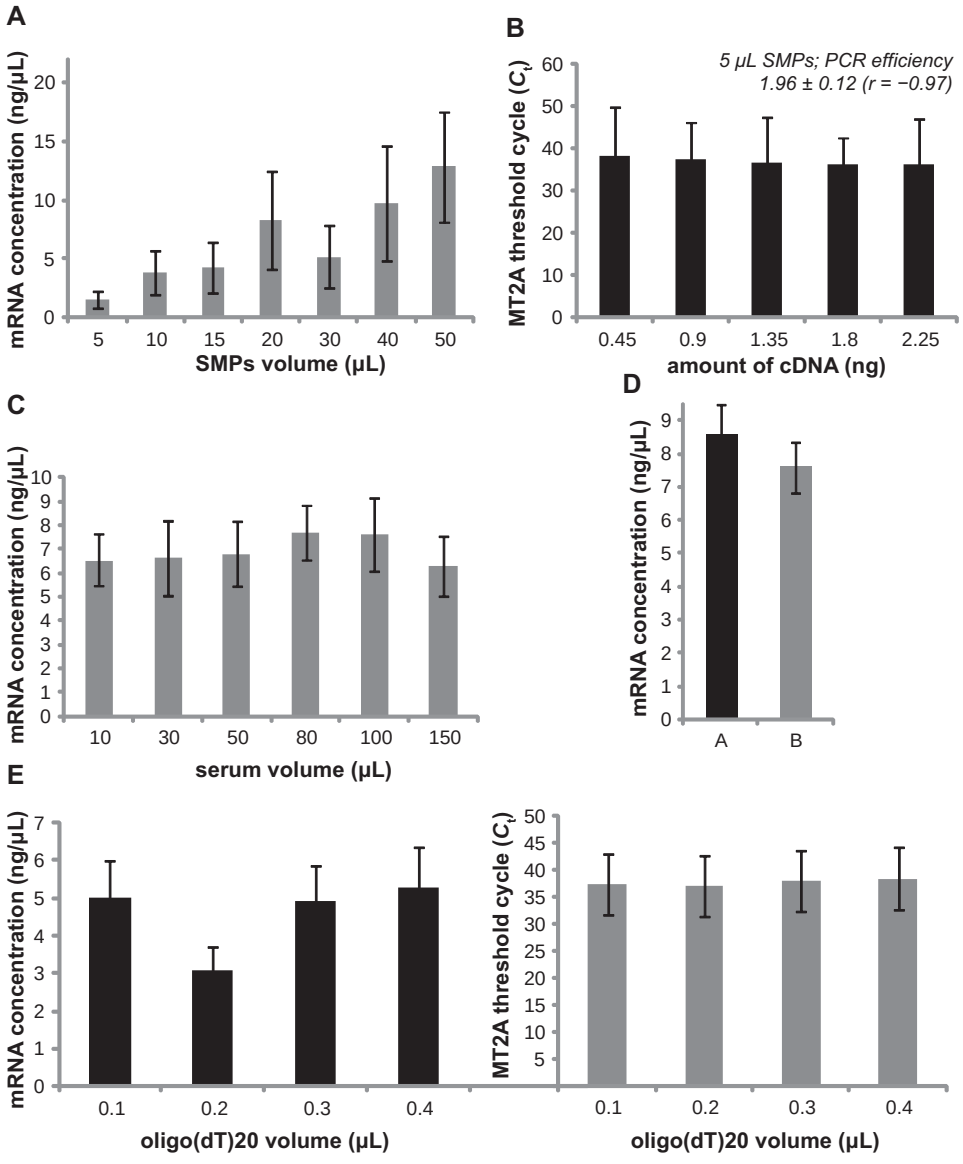

Figure 3. Optimization steps of serum RNA isolation using SMPs. (A) Amount of isolated mRNA versus volume of SMPs. (B) PCR efficiency calculation prepared from RNA isolated using lowest used (5 µL) SMP volume. (C) Optimization of sample volume, no relation was observed. (D) Optimization of binding principle. Binding principle "A" indicates first oligo(dT)$_{20}$ binding to sample following binding to SMPs; "B" indicates first oligo(dT)$_{20}$ binding to SMPs, following incubation with sample. No significant difference was observed. (E) Optimization of oligo(dT)$_{20}$ volume. Left: mRNA content, right: MT2A expression. For details, see Table 1. Data are displayed as mean ± SD.

analyzed. Using three randomly selected sera, the following variants were tested: (i) oligo(dT)$_{20}$ was first incubated with sample and this complex was subsequently bound to SMPs (also recommended by manufacturer); (ii) oligo(dT)$_{20}$ was first bound to SMPs, following incubation with sample. No significant difference between those binding sequences was detected (8.56 ± 2.05 and 7.6 ± 2.06 for first and second binding principle, Fig. 3D). Consequently, amount of oligo(dT)$_{20}$ probe was optimized (Fig. 3E). Based on the previous steps, 20 µL SMPs and 80 µL serum were used, and oligo(dT)$_{20}$ ranged 0.1–0.4 µL. No significant correlation was determined ($r = 0.35$ at $p = 0.65$). In addition, although low sample purity was detected, no significant relation with oligo(dT)$_{20}$ volume was detected (absorbance ratio 1.02 ± 0.12 for 260/280). Despite this fact, MT expression is detectable in all oligo(dT)$_{20}$ volumes, with its qRT-PCR threshold cycle (C_t) ranging 36.2–39.2. Thus, the oligo(dT)$_{20}$ volume is not an important factor influencing the effectiveness of isolation.

3.4 One-step analysis

Simple approach is desirable for automated RNA isolation, in which all the steps take place in a single tube. Therefore, previously optimized protocol was modified where SMPs were left in same tube for subsequent analyses (for details, see Section 2) and those variants were compared on three randomly selected sera (Fig. 4A).

Although lower threshold cycle values for MT expression were detected using modified one-step analysis, differences were below the level of statistical significance ($p = 0.77$, $C_t = 36.2 \pm 1.6$ and 34.7 ± 1.4 for conventional and one-step analysis, Fig. 4B).

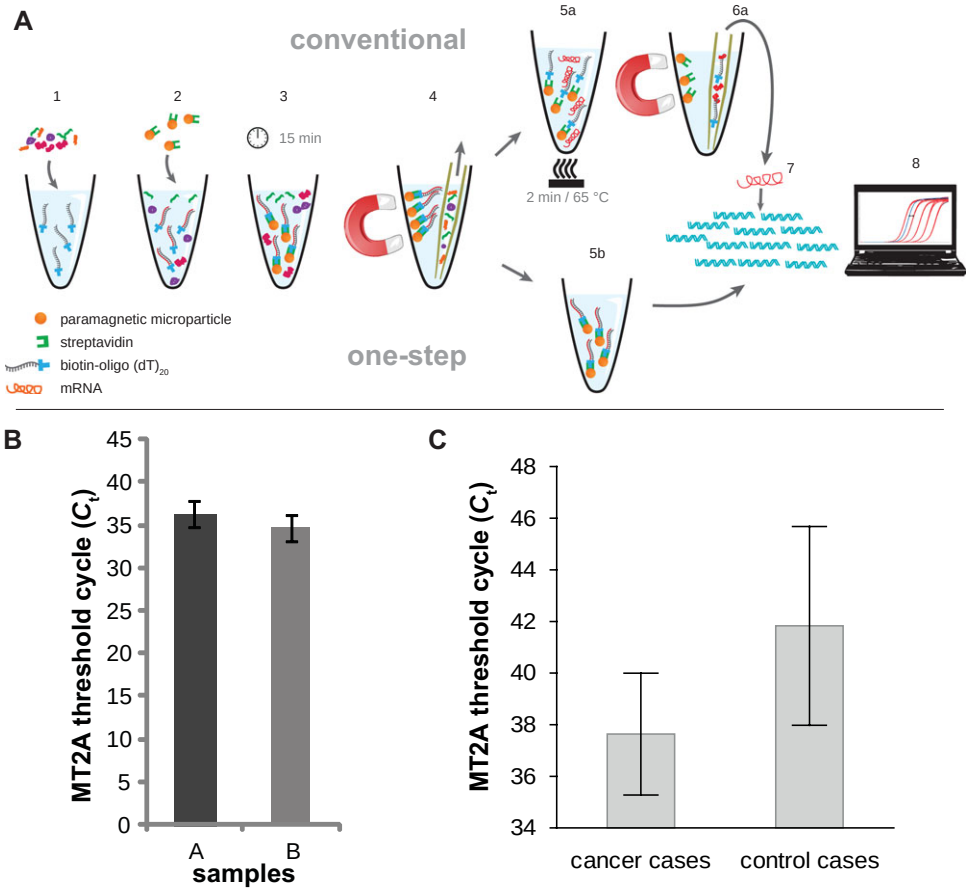

Figure 4. One-step analysis and application for prostate cancer detection. (A) Comparison of conventional and one-step isolation. (1) Addition of sample, anchorage of biotinylated oligo(dT)$_{20}$ to serum mRNA; (2) addition of streptavidin-modified paramagnetic particle; (3) incubation of RNA–oligo(dT)$_{20}$ SMP complex; (4) 3× washing; (5a) Conventional isolation, releasing streptavidin-biotin binding; (6a) Transferring RNA and oligo(dT)$_{20}$ to PCR reaction; (7) cDNA transcription and amplification using PCR; (8) Real-time PCR detection; (5b) One-step analysis, direct transfer of SMP–oligo(dT)$_{20}$–RNA to PCR reaction. (B) Comparison of one-step versus conventional analysis, expression of MT2A. No significant difference was observed. (C) Analysis of serum metallothionein 2A mRNA level in healthy individuals and prostate cancer patients isolated using one-step analysis. Significantly lower MT levels observed in healthy individuals ($p < 0.001$). Data are displayed as mean ± SD.

3.5 Serum MT mRNA as a cancer marker

To outline possible applications of serum RNA detection, serum MT2A expression was analyzed using qRT-PCR on 35 patients suffering from prostate cancer and compared to 14 healthy controls. Using optimized protocol, a significantly higher MT level was observed in patients suffering from cancer ($p < 0.001$, mean $C_t = 41.83 \pm 3.85$ vs. 37.64 ± 2.36 for controls and cases, respectively; Fig. 4C). Based on ROC analysis, 65.7% sensitivity and 85.7% specificity were determined on a threshold $C_t = 37$.

4 Discussion

In this study, we subjected serum samples to RNA extraction protocols in order to analyze their benefits in extracellular RNA isolation and in subsequent determination of free circulating mRNA transcript of MT2A in prostate cancer patients. In particular, column-based isolation, Trizol RNA isolation, and protocol employing SMPs were confronted for the first time. Consequently, an isolation protocol was optimized and the optimization efficiency was analyzed using RNA content and RNA purity measurement and verified by real-time PCR. Though relative gene expression analysis calls for standardization using housekeeping gene, no such step was employed in this study and raw C_t values are shown. It was assumed that serum composition is relatively constant and thus the predictive value of raw C_t values is sufficient for this report. In addition, reliable and unambiguous housekeeping gene for standardization of MT2A expression or appearance of MT2A transcript in serum samples is still missing.

Initially, these methods were applied on cell cultures to determine their efficiency by RNA content measurement. Afterward, identical protocols were compared on serum samples. Because SMPs isolate mRNA directly, it was necessary to convert amount of (total) RNA extracted by column-based and Trizol method to mRNA in order to compare RNA yields. It is generally known that mRNA covers 5% of total RNA in cell cultures at most. On the other hand, some reports indicate that the mRNA proportion of the total RNA extracted from serum samples could be considerably bigger (around 50%) [26]. These percentages were used to provide rough estimates.

4.1 Optimized serum isolation protocol

Moreover, our goal was also to optimize isolation protocol using SMPs on sera, because there are few reports describing utilization of SMPs in extracellular RNA isolation. Circulating RNA detection is particularly promising from the perspective of disease markers; higher percentage of detectable tumors

found with DNA markers was repeatedly reported [25]. According to our results, SMPs appear to be highly effective method for mRNA isolation from cell cultures. Compared to Trizol, isolation from serum samples is seemingly not as effective as from intracellular material. However, SMP specificity to mRNA must be taken into account compared to other isolation protocols. Furthermore, with purification being affinity-based, cDNA contamination by genomic DNA is avoided [28], and no DNase I treatment is necessary.

Due to the fact that SMP isolation protocol is designed for mRNA isolation from cell cultures with distinctly higher RNA content compared to serum, pronounced reduction of reagent volumes is possible. Optimizations validate that analysis of gene expression can be performed from 20 μL of sample using 5 μL SMPs, or even from 10 μL of serum using 20 μL of SMPs (i.e. tenfold reduction). Moreover, fivefold reduction of oligo$(dT)_{20}$ probe volume is possible. Volume reductions described herein are still far from detection limits and further reductions are feasible.

Efficiency of two different binding principles was compared. Isolation utilizing SMPs is grounded on highly specific and strong streptavidin-biotin interaction. Streptavidin is coated on the surface of microbead, whereas oligo$(dT)_{20}$ probe is labeled with biotin. Conventional binding principle (marked as principle "A") lies in initially mixing biotinyled oligo$(dT)_{20}$ probe with serum sample and following incubation of this hybridization mix with streptavidin-coated microparticles. On the other hand, modified principle (marked as "B") was based on the incubation of oligo$(dT)_{20}$ probe first with microbeads. Serum sample was added after incubation. Since in principle "A" is reduction of mRNA yield more likely owing to lesser steric constraints, this principle was assumed to be more effective. However, distinctions between two principles were not statistically significant.

In order to examine potential for further automation, one-step analysis was performed, where all procedures from extracellular mRNA isolation, reverse transcription up to gene expression analysis are run in one flow without removing SMPs. Our results are in agreement with previous reports [28] that prove no significant influence on fluorescence signal during RT-PCR analysis. Data also indicate that SMPs do not cause quenching of fluorescence signal and that their autofluorescence is negligible [28]. Here, we show that this method is reliable, sensitive and exhibit at least equally well results as conventional methods. In combination with reduction of reagents volumes, this analysis represents reproducible and economic high-throughput method for gene expression analysis from extracellular mRNA. Although there are several commercial isolation kits, adjusted for extracellular RNA extraction, they mostly require high input volume of serum (range, 250 μL–5 mL). Our goal was, therefore, to minimize input volume of biological material, which might in the upshot lead to reduction of screening invasivity. Here, we demonstrated isolation of extracellular RNA even from 10 μL of serum with no undesirable effect on analysis of gene expression by RT-PCR. The extra benefit of this method is direct mRNA isolation, whereas other procedures provide total RNA.

However, this protocol has certain disadvantages. Compared to other isolation protocols, RNA isolation using SMPs distinctly reduces purity of isolated nucleic acids. Plasma and serum are biospecimens that have a very high concentration of proteins that could potentially interfere with sample preparation and detection assay. Similar findings were also demonstrated by study dealing with optimization of microRNA isolation protocol from serum samples [29]. It was demonstrated that certain proteins and proteolipid complexes might even directly protect extracellular RNA from degradation by serum RNases [30]. More specifically, circulating RNA may be packed into apoptotic bodies, which considerably decrease susceptibility to nucleases in serum. These protein structures are obviously not removed by SMPs isolation protocol and additional purification of RNA should be performed. Despite this fact, no changes in qRT-PCR were observed.

4.2 Circulating miRNAs as cancer biomarker

Due to the controversy of prostate cancer screening using PSA [17], novel prostate tumor biomarkers are desirable. To date, various compounds and protocols were evaluated, including DNA sequence variations, genetic aberrations, and various tissue-, blood-, and urine-based markers [31–33]. From this perspective, MT seems to be a promising tool for cancer diagnostics. Use of MT as a prostate cancer marker was evaluated on both serum and protein level. Immunohistochemical analyses reported both significant decrease [21] and elevation [34] with no associations with tumor grade [21, 35–37]. On serum level, we previously reported significantly higher MT protein level [22, 38]. It should be noted that proper selection of controls is crucial when assessing the suitability of MT as a cancer biomarker. It was reported that MT levels differ between benign hyperplasia and cases significantly [39]. In addition, histological hyperplasia increases linearly with age, being detectable in 50% of 40-year-old male subjects [40]. Taken together, selection of controls younger than 40 years is desirable when assessing the difference between "healthy" and "tumorous" cases. Additionally, sera of cancer patients appear to be valuable source of useful molecular markers on both extracellular RNA and DNA level. Amplification of extracellular mRNA from serum samples may offer a new approach to cancer diagnostics, monitoring, and pharmacogenomic evaluation. There are numerous reports describing the connection of extracellular mRNA in connection with cancer diagnostics. For instance, Koperski et al. described detection of tumor-related tyrosinase mRNA in melanoma patients [41], Fleischhacker et al. referred similar findings with hnRNP-B1 and HER2/neu-specific mRNA from sera of patients with malignant lung tumor [42], and results of study of March-Villalba et al. indicate that hTERT mRNA is an effective molecular marker for the diagnosis of prostate cancer [43]. Based on our results, although serum MT2A mRNA appears to be a prospective marker for the diagnosis of

prostate cancer, further studies including higher number of patients are required to validate its diagnostic value.

To conclude, taking into consideration specific mRNAs as specific disease markers, automatable SMP-based mRNA isolation is a promising tool for high-throughput analyses. This study outlines the possibility of serum mRNA isolation using this technique, compares it with other isolation protocols, points to its relative easy miniaturization, automation, and shows possible utilization in the detection of prostate cancer.

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