

Chapter 35

Yields of Viral and Circulating Cell-Free Nucleic Acids Using the QIAamp[®] Circulating Nucleic Acid Kit

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Abstract Fragmented DNA and RNA circulate as cell-free nucleic acids in plasma, serum, urine and other body fluids. Access to these molecules for analysis may allow for detection of certain disease states based on a blood sample. In this study the extraction efficiency of a large volume nucleic acid extraction kit for circulating and viral nucleic acids was assayed with different downstream qPCR assays. For circulating DNA in plasma the yield was 7- to 9-fold higher with the large volume kit compared to the QIAamp DNA Blood Mini protocol with improved recovery of short DNA fragments. The microRNA extraction protocol of the large volume kit was found to deliver significantly increased microRNA yields (ΔC_t from 3 to 4) compared to the kit's standard protocol. For viral nucleic acids, 95% detection rates were: HBV DNA 0.25 IU/ml and HIV-1 RNA 14.2 IU/ml. The results demonstrate that the QIAamp Circulating Nucleic Acid Kit can serve as a sample preparation solution for processing up to 5 ml cell-free body fluid, it allows for small elution volumes, and can extract and concentrate circulating nucleic acids, including microRNA, and viral nucleic acids up to 250 fold.

Keywords QIAamp[®] Circulating Nucleic Acid Kit · Cell-free nucleic acids · Viral nucleic acids · MicroRNA · Plasma · Urine

Abbreviations

ccfDNA	Circulating, cell-free DNA
GE	Genome equivalent
GFP	Green fluorescent protein
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
miRNA	MicroRNA

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PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal RNA name sequence 5'-3'

Introduction

It is now well established that circulating, cell-free nucleic acids, which are present in plasma, serum, and other body fluids can serve as biomarkers for the blood-based detection of malignancies and other diseases. Tumor-specific circulating nucleic acids are promising targets for minimally-invasive early detection of tumors and, therefore, provide tools for improved cancer screening and hence open the opportunity for early and more successful treatment options (Tsang and Lo 2007). Also, cell-free nucleic acids found in the plasma and serum of pregnant women contain DNA and RNA molecules of fetal genetic origin, and analysis of these circulating, cell-free fetal nucleic acids allows for non-invasive detection of fetal genetic anomalies (Lo and Chiu 2007; Puszyk et al. 2008). The analysis of circulating nucleic acids has proven to be difficult because both circulating DNA and RNA found in plasma or serum are of low concentration; cell-free nucleic acids are also highly fragmented – circulating, cell-free DNA (ccfDNA) is present in plasma mostly as nucleosomal fragments of less than 500 bp (Diehl et al. 2005; Deligezer et al. 2008). Circulating mRNA molecules have been shown to be truncated, with 5' ends being more intact than mRNA 3' ends (Wong et al. 2005).

In order to overcome these technical challenges, we have developed an improved method for nucleic acid extraction from plasma, serum, and urine with these key features:

- Process larger sample volumes of up to 5 ml in order to isolate sufficient absolute amounts of ccfDNA and RNA.
- Enable a low elution volume (20–100 μ l) for a high concentration factor.
- Efficiently recover small DNA and RNA fragments (down to 75 bp or lower).

Here we present results on the extraction of circulating, cell-free DNA, circulating microRNA and viral nucleic acids from human plasma, as well as data on circulating DNA yields from urine samples.

General Methodology

Sample materials: Plasma and serum samples were obtained from healthy donors after Review Board approval and were processed by two-step centrifugation ($1900\times g$ and $16,000 g$) to remove all cellular material (Chiu et al. 2001). In order to obtain plasma, whole blood was collected in 10 ml Becton Dickinson Vacutainer K₂EDTA tubes; serum was obtained from whole blood collected in 8.5 ml Becton

Dickinson Vacutainer SST II Advance tubes and centrifuged after blood clotting was completed (30–60 min after blood draw). Plasma and serum was stored in 5–10 ml aliquots below –80°C until nucleic acid extraction. Urine samples were processed 60 min or less after urine collection. After centrifugation at 16,000g for 10 min and 4°C, the urine supernatant was removed to new tubes and stored below –80°C until nucleic acid extraction.

Nucleic acids were extracted using the QIAamp Circulating Nucleic Acid Kit (QIAGEN) according to the manufacturer’s instructions unless stated otherwise. The sample material was lysed in presence of detergent and chaotropic salt, followed by adjustment of binding conditions to favor binding of fragmented DNA and RNA to a silica membrane. After passing the sample lysate through the silica membrane (QIAamp Mini column) by vacuum force, the bound nucleic acids were washed in presence of high-salt and low-salt buffers and finally eluted in a low-salt elution buffer that is compatible with PCR enzymes and buffers.

Protocol variants used for microRNA extraction from plasma and circulating nucleic acid extraction from urine samples were followed as laid out in the handbook of the QIAamp Circulating Nucleic Acid Kit. As an internal control for the nucleic acid extraction efficiency, DNA marker fragments were generated by preparative PCR from the plasmid pEGFP-C1 (Clontech) using the HotStarTaq MasterMix Kit (QIAGEN) according to the kit instructions. The fragments were designed to contain different parts of the Green Fluorescent Protein (GFP) coding sequence in order to allow for subsequent detection and quantification of these DNA fragments by real-time PCR assays targeting a GFP sequence and, hence, a target which is absent from the human genome. The primers used to amplify the marker fragments are documented in Table 35.1. After synthesis by PCR, the DNA fragments were purified using the QIAquick PCR Purification Kit (QIAGEN) and quantified by UV absorbance reading. As an internal extraction control, 200,000 copies of each DNA fragment were added to samples before starting the lysis incubation step of the respective nucleic acid extraction protocol.

Real-time PCR assay details: For quantification of circulating DNA, a duplex quantitative PCR (qPCR) assay specific for the human 18S rRNA coding region was used. Two targets were amplified, with an amplicon length of 66 bp (Horlitz et al. 2009) and 500 bp, respectively. The quantification of DNA fragments added as an internal control was done by a triplex qPCR assay targeting distinct regions within

Table 35.1 Primers used for preparative amplification of DNA fragments

Name	Sequence 5'-3'
75 bp prep forward	ACCGCATCGAGCTGAAGG
75 bp prep reverse	TGTAGTTGTACTCCAGCTTGTGCC
200 bp prep forward	CCGGCAAGCTGCCCCGT
200 bp prep reverse	GCCCTCGAACTTCACCTCG
1,000 bp prep forward	GTCTATATCATGGCCGACAAGCAG
1,000 bp prep reverse	GGTACGCGCAGCGTGAC

Table 35.2 Primer and probes used in Real-Time PCR assays

Name	Sequence 5'-3'	Probe labels
18S 66 bp forward	GCCGCTAGAGGTGAAATTCTTG	5' Cy5 – BHQ2 3'
18S 66 bp reverse	CATTCTTGGCAAATGCTTTCG	
18S 66 bp probe	ACCGGCGCAAGACGGACCAGA	
18S 500 bp forward	GTGCTCGCTCCTCTCCTACTT	5' FAM – BHQ1 3'
18S 500 bp reverse	GGCTGCTGGCACCAGACTT	
18S 500 bp probe	CTAATACATGCCGACGGGCGCTGAC	
“75 bp” forward	ACCGCATCGAGCTGAAGG	5' Quasar705 – BHQ2 3'
“75 bp” reverse	TGTAGTTGTACTCCAGCTTGTGCC	
“75 bp” probe	TCGACTTCAAGGAGGACGGCAACA	
“200 bp” forward	CACATGAAGCAGCACGACTTC	5' FAM – BHQ1 3'
“200 bp” reverse	GGTGCGCTCCTGGACGTA	
“200 bp” probe	TCAAGTCCGCCATGCCCCAAG	
“1,000 bp” forward	CTGCTGCCCCGACAACCA	5' TexasRed – BHQ2 3'
“1,000 bp” reverse	TGTGATCGCGCTTCTCGTT	
“1,000 bp” probe	TACCTGAGCACCCAGTCCGCCCT	

the GFP coding sequence as part of the 75 bp, 200 bp, and 1,000 bp fragments. The assays were performed using the QuantiTect Multiplex PCR Kit (QIAGEN) according to the manufacturer’s instructions on a Rotor-Gene Q instrument. For absolute quantification of target copies/genome equivalents, quantification standards consisting of 10-fold dilutions of high molecular weight male genomic DNA (Horlitz et al. 2009) and 4-fold dilutions of the three marker fragments, respectively, were used. Primer and probe sequences are documented in Table 35.2. For microRNA quantification, commercially available miScript Assays (QIAGEN) and TaqMan microRNA assays (Applied Biosystems) were performed according to the respective handbook instructions.

Experiments and Discussion

Variability in ccfDNA Yields from Different Individuals

In order to assess the ccfDNA extraction performance, we compared the ccfDNA yields obtained from plasma samples from eight healthy individuals using the QIAamp Circulating Nucleic Acid Kit and the QIAamp DNA Blood Mini Kit. To check for the extraction efficiency as a function of DNA fragment size, we measured the yields of DNA fragments (75, 200, 1,000 bp) added to the samples during the extraction procedure as an internal control. The results showed that the ccfDNA yields varied from about 8,000 to more than 80,000 genome equivalents (GE) per sample as detected by the qPCR assay specific for a 66 bp target of the 18S rRNA coding sequence (Fig. 35.1) Running a qPCR quantification of the same samples

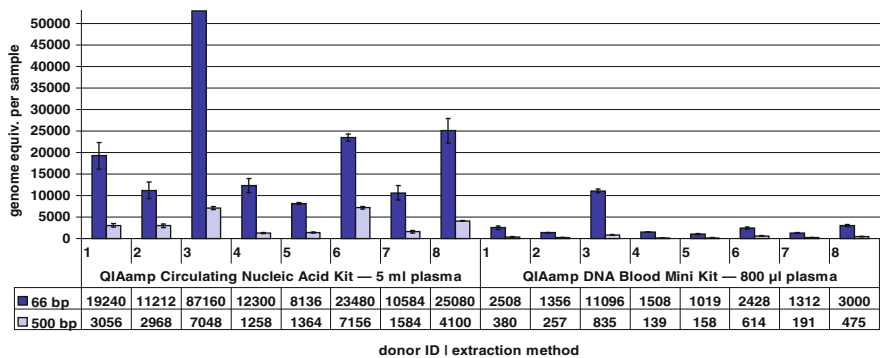


Fig. 35.1 ccfDNA extracted from plasma. Circulating cell-free DNA was extracted from plasma samples from 8 individuals using the QIAamp Circulating Nucleic Acid Kit (5 ml plasma) and the QIAamp DNA Blood Mini Kit (800 µl plasma). The QIAamp DNA Blood Mini Kit is optimized for genomic DNA extraction from cellular materials. Elution volume was 50 µl. DNA yield was quantified in triplicate by duplex, real-time PCR targeting a 66 bp and a 500 bp amplicon within the 18S ribosomal RNA coding sequence using the QIAGEN QuantiTect Multiplex PCR Kit

targeting a 500 bp region of the 18S rRNA coding sequence showed between 3-fold and 13-fold lower genome equivalents per sample. This is consistent with the fragmented nature of ccfDNA – most of the DNA fragments are shorter than 500 bp and, hence, cannot be detected by the 18S 500 bp assay (Diehl et al. 2005; Horlitz et al. 2009). The individual differences in the 66–500 bp copy number ratios among the eight donor samples also indicate that the size distribution and concentration of ccfDNA differ among individuals.

The qPCR-based quantification of added DNA marker fragments showed reproducible extraction efficiency across the plasma samples from different individuals (Fig. 35.2) It also demonstrated that the QIAamp Circulating Nucleic Acid Kit extraction procedure was more efficient in extracting short DNA fragments as evidenced by the recovery of added 75 bp fragments – which was significantly higher with the QIAamp Circulating Nucleic Acid Kit (mean: $96 \pm 15\%$) compared to the QIAamp DNA Blood Mini Kit (mean: $34 \pm 11\%$).

ccfDNA Extracted from Urine

We measured the ccfDNA recovery from pooled urine samples after extraction using the QIAamp Circulating Nucleic Acid Kit’s urine protocol. Equal volumes of morning urine from four individuals were pooled and 4 ml urine was subjected to nucleic acid extraction. Purified nucleic acids were eluted in 50 µl. The ccfDNA yield was quantified using the 18S-rDNA specific real-time PCR assay (66 and 500 bp amplicons) as described above. Two urine pools were analyzed for ccfDNA yield in six replicates. The 66 bp 18S assay detected 115,000 and 139,000 genome equivalents of DNA whereas the 500 bp 18S assay detected only about 30,000 and 34,000 GE

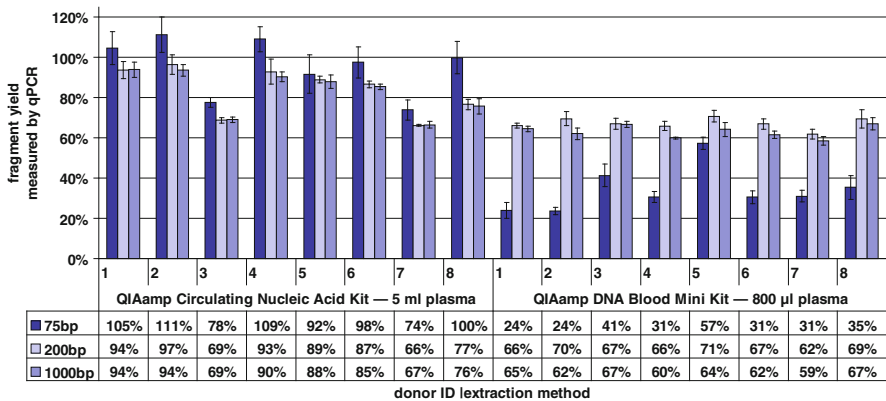
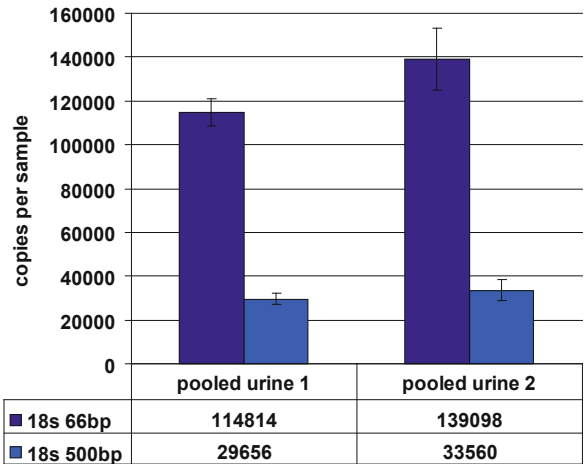


Fig. 35.2 Size-selectivity of ccfDNA extraction. In order to track the size-selectivity of the extraction procedure, DNA fragments (75, 200, 1,000 bp) were added to plasma samples at 200,000 copies/sample after the lysis step. Circulating cell-free DNA was purified using the QIAamp Circulating Nucleic Acid Kit (5 ml plasma) and the QIAamp DNA Blood Mini Kit (800 µl plasma). Elution volume was 50 µl. DNA yield was quantified in triplicate by triplex, qPCR targeting regions within the 75, 200 and 1,000 bp fragment using the QIAGEN QuantiTect Multiplex PCR Kit

per 4 ml urine sample (Fig. 35.3). This indicates that in urine, just as in plasma, the circulating cell-free DNA is mostly present as fragments of less than 500 bp. The recovery of added DNA fragments (75, 200, 1,000 bp) at 200,000 copies/sample showed similar yields for all three fragments in the range of 73–87% (data not shown); this indicates that the DNA extraction efficiencies in urine were comparable to those observed with plasma samples.

Fig. 35.3 ccfDNA was purified from pooled urine using the QIAamp Circulating Nucleic Acid Kit (4 ml urine in 6 replicates) with elution in 50 µl. DNA yield was quantified in triplicate per extraction by duplex, qPCR specific for 18S rDNA (66 and 500 bp targets; see above)



Extraction of microRNAs from Plasma

As microRNAs in plasma and serum are becoming increasingly important as potential diagnostic biomarkers (Mitchell et al. 2008; Chim et al. 2008; Gilad et al. 2008), we compared the standard extraction protocol of the QIAamp Circulating Nucleic Acid Kit and a supplementary protocol for this kit which is optimized for microRNA extraction from plasma, serum, or urine. For the standard kit protocol, 5 ml plasma from 12 individual donors was processed and the nucleic acids were eluted in 80 μ l. For the microRNA protocol, 3 ml plasma from the same 12 donors was used which is the recommended sample volume for this protocol; the purified nucleic acids were eluted in 80 μ l. We used a set of commercially available assays to determine the yields of six different human microRNA species. Both QIAGEN miScript and Taqman assays (Applied Biosystems) were used: miR-16 (miScript & TaqMan), miR-10a (miScript), miR-10b (miScript), miR-29a (miScript), miR-103 (miScript), miR-30b (TaqMan). The results (Fig. 35.4) indicate that all interrogated miRNA targets were readily detectable in microRNA extracted using the standard protocol as evidenced by mean Ct values (averaged across 12 donors) between 27.0 (miR-16/miScript) and 35.6 (miR10a). The microRNA protocol, however, delivered higher microRNA yields which were – averaged across 12 donors – by a Δ Ct of 3.3 (range: 2.2–4.6) higher compared to the standard protocol. The Δ Cts were calculated per microRNA species and donor and then averaged across the 12 donors. This shows that the microRNA protocol of the QIAamp Circulating Nucleic Acid Kit yields 10-fold more microRNA compared to the standard protocol – despite a lower sample input volume.

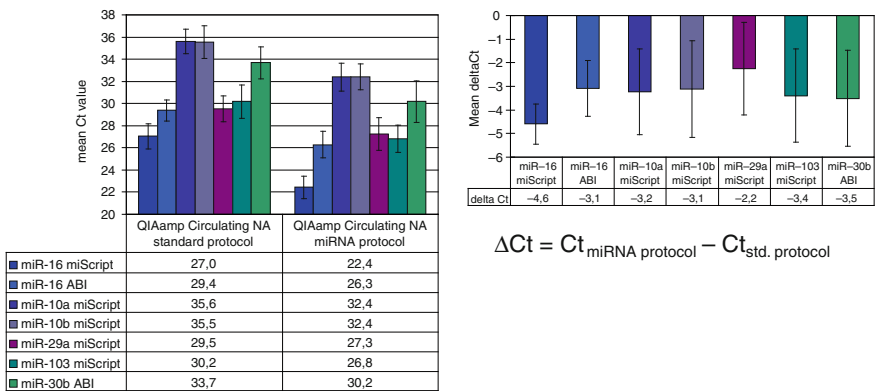


Fig. 35.4 microRNA extracted from plasma. Circulating cell-free RNA from plasma samples from 12 individuals was purified using the QIAamp Circulating Nucleic Acid Kit's standard protocol (5 ml plasma input) and miRNA protocol (3 ml plasma input). Elution volume was 80 μ l. microRNA yield was assayed by QIAGEN miScript PCR Assays and TaqMan® microRNA assays (Applied Biosystems) as indicated. Error bars correspond to mean $\pm$ 1 $\times$ standard deviation of 12 individuals with 3 PCR replicates

Viral Nucleic Acids Extracted from Plasma

We tested the performance of the QIAamp Circulating Nucleic Acid Kit with respect to the extraction of viral nucleic acids for both a DNA and an RNA virus by adding serial dilutions of HBV and HIV particles (WHO standard material) to pooled EDTA plasma from healthy donors, followed by nucleic acid extraction using the standard kit protocol. 5 ml plasma was used for viral nucleic acid isolation with elution of the extracted nucleic acids in 30 μ l, representing a theoretical concentration factor of 167. We then calculated the virus titer in plasma which still allows for detection of the specific virus at 95% probability (“limit of detection”) using the *artus*[®] HBV RG PCR Kit (QIAGEN) and the *artus*[®] HIV-1 RG RT-PCR Kit (QIAGEN), respectively. The qPCR assays were run according to the handbook instructions on a Rotor-Gene Q instrument (see Table 35.3 for detailed results). For HBV, the limit of detection was reached at 0.25 IU/ml plasma (95% confidence interval: 0.14–1.25), for HIV, the limit of detection was 14.2 IU/ml (95% confidence interval: 9.7–30.0).

Conclusions

The presented results show that the QIAamp Circulating Nucleic Acid Kit is a suitable sample preparation solution for the silica-based extraction of circulating nucleic acids which are usually present at low concentrations in the sample material. This is achieved in part by allowing for up to 5 ml sample per extraction while maintaining a small size of the nucleic acid binding silica surface and, consequently, a low elution volume of 20–150 μ l; but also by an optimized extraction chemistry which improves the recovery of short nucleic acid fragments compared to established extraction kits such as the QIAamp DNA Blood Mini Kit. The latter was developed for extracting and purifying DNA from blood cells and, hence, from material that contains high molecular weight DNA at relatively high concentrations – therefore, the extraction protocol is not optimized for recovering short DNA fragments present at low concentrations in the starting material. The QIAamp Circulating Nucleic Acid Kit showed to be superior at extracting fragmented DNA. This large volume QIAamp Kit also enables the recovery of microRNAs from cell-free body fluids (up to 3 ml) through a modified protocol that is even further optimized for recovering small nucleic acid fragments.

Our results suggest that the QIAamp Circulating Nucleic Acid Kit provides a sample preparation method for circulating, cell-free DNA and RNA for discovery and investigation of potential nucleic acid-based biomarkers from human cell-free body fluids.

Table 35.3 Detection limits of viral nucleic acids extracted using the QIAamp Circulating Nucleic Acid Kit. The efficiency of viral nucleic acid extraction was analyzed by adding serial dilutions of HBV and HIV particles (WHO standard material) to pooled HBV- and HIV-negative EDTA plasma. 5 ml plasma was used for viral nucleic acid isolation with elution of the extracted nucleic acids in 30 µl, of which 20 µl were used in a HBV- and HIV-specific *artus* qPCR assay (QIAGEN)

HBV-specific PCR				HIV-1 RT-PCR			
Input titer (IU/ml)	Replicates	Positives	Total hits (%)	Input titer (IU/ml)	Replicates	Positives	Total hits (%)
1.0	8	8	100	80	8	8	100
0.5	16	16	100	40	16	16	100
0.25	15	14	93	20	16	15	94
0.125	16	13	81	10	16	15	94
0.0625	16	13	81	5	16	10	63
0.03125	16	8	50	2.5	16	4	25
0	8	0	0	1.25	8	0	0
—	—	—	—	0	10	0	0
95% probit		0.25		95% probit		14.2	
Confidence interval		0.14–1.25		Confidence interval		9.7–30.0	

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