

Recovery efficiencies of nucleic acid extraction kits as measured by quantitative LightCycler™ PCR

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Abstract

Aims—To compare the efficiency of five nucleic acid extraction kits for processing clinical material for the diagnosis of infection.

Methods—Five nucleic acid extraction kits for processing clinical material for the diagnosis of infection were compared for their relative efficiencies in purifying and recovering either viral DNA or RNA from serum samples. Quantitative polymerase chain reaction (PCR) assays for hepatitis B virus (HBV) DNA and hepatitis C virus (HCV) RNA were performed on the LightCycler™ instrument to determine the relative concentrations of the viral nucleic acids recovered by the various protocols.

Results—Large differences between kits in recovery efficiencies were observed for HBV DNA, with those protocols using enzyme digestion in addition to chaotropic solutions performing better than those using chemical disruption alone.

Conclusions—There were large differences between the kits and it appeared that those extraction kits containing a proteolytic enzyme and carrier nucleic acid (and that are supplied RNase free) have the widest potential application in the routine microbiology laboratory.

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Keywords: quantitative polymerase chain reaction; LightCycler™; hepatitis B virus DNA; hepatitis C virus RNA

The detection of microbial nucleic acid for the diagnosis of infection is dependent on the successful separation of nucleic acid from clinical material. Clinical material usually contains numerous molecules that are inhibitory for the enzymes used in nucleic acid amplification techniques, such as Taq polymerase in the polymerase chain reaction (PCR). Some inhibitory compounds are well recognised and may be inherent—for example, haemoglobin in whole blood—or introduced to the clinical material, as with heparin and nucleic acid extraction reagents, which might not be removed efficiently from the extracted nucleic acid.

Early nucleic acid amplification protocols often used the classic technique of phenol chloroform separation of nucleic acid from protein, or described crude sample preparation techniques, such as boiling of clinical material to denature proteins and disrupt virions.^{1 2} These methods require hazardous chemicals or are limited in their abilities to process diverse types of clinical materials, and are generally

unsuited to routine use in clinical laboratories. The growth of nucleic acid detection in diagnostic laboratories has relied on the use of commercial kits for specimen preparation. These may be designed and marketed for specific applications, such as the isolation of hepatitis C RNA from cell free blood, or be more general in that protocols are supplied for the processing of a range of biological material for viral RNA or DNA.

Many of the most popular kits use spin column technology because, in general, this approach is easily integrated into laboratories with standard equipment. These protocols and reagents can also be adapted to allow high throughput testing with the use of automated robotic liquid handling. In general, nucleic acid is released from virus particles and also, in some cases, from cells and tissue using chaotropic solutions to denature proteins and release nucleic acid; this chemical process is in some cases augmented by digestion with proteinases. The separation of nucleic acid from other components is achieved by temporary non-specific adsorption to a matrix of silica particles held within a disposable plastic column. High salt concentration washes remove proteins and low molecular weight compounds, and the subsequent application of a low salt concentration buffer elutes the purified nucleic acid in a form suitable for enzymatic processes.

Some applications of nucleic acid detection require very sensitive techniques and the efficiency, in terms of recovery rate of extraction and purification of nucleic acid becomes crucial. For example, PCR has made possible the rapid laboratory diagnosis and immediate management of some viral infections. Viruses are often present in very low concentrations in biological fluids, such as cerebrospinal fluid during central nervous system infection, or in plasma during the treatment of chronic infections, when quantitative measurement of viral load may be required. In addition to good recovery efficiency, a kit with a flexible protocol, able to process different types of clinical material for both DNA and RNA with effective separation of potential enzyme inhibitors, is considered to be an advantage for a routine diagnostic laboratory.

The LightCycler™ instrument (Roche Molecular Biochemicals, Lewes, Sussex, UK) is an integrated thermal cycler and fluorimeter that allows real time monitoring of amplicon accumulation during PCR with fluorescence chemistry for the accurate determination of starting target copy number. This study was designed to compare five nucleic acid extraction kits for

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clinical material using quantitative PCR assays to measure their relative viral DNA and RNA recovery efficiencies from serum samples.

Materials and methods

Viral nucleic acid extraction kits from Qiagen Ltd (Crawley, UK; QIAamp blood DNA and QIAamp viral RNA), Roche Molecular Biochemicals (High Pure viral nucleic acid and High Pure viral RNA), and Biogene Ltd (Kimbolton, UK; NucleoSpin virus) were compared. Nucleic acid extraction was performed without modification according to the manufacturers' instructions contained in the kit inserts, except that for standardisation of the serum volume/eluate volume ratio the elution buffer volume used was equal to half the original volume of serum used in each case. Optional procedures mentioned in some of the kit instructions—for example, double nucleic acid elution and longer incubation times—were not used in our study.

For the comparison between kits of DNA extraction, aliquots of a pool of serum samples containing 25 million genome equivalents/ml of hepatitis B virus (HBV) DNA were processed using the kits, followed by quantitative HBV LightCycler™ PCR of the eluates. This serum sample pool had been tested previously by Amplicor HBV Monitor (Roche Diagnostics Ltd) for the measurement of HBV DNA. HBV PCR was performed in a solution with a total volume of 20 µl containing 10 µl of the eluates from the test kits, 2 µl 10× PCR buffer (Qiagen Ltd), each deoxynucleotide triphosphate (dNTP) (Life Technologies Ltd, Paisley, UK) at a concentration of 0.25 mM, 3.0 mM MgCl₂, 0.5 µM each oligonucleotide primer (MWG Biotech Ltd, Milton Keynes, UK), 1 µl of 10× Sybr Green 1 solution (Sigma Ltd, Poole, Dorset, UK), 1 µl of 10 mg/ml acetylated bovine serum albumin (BSA; Sigma Ltd) solution, and 1.25 U of Taq polymerase (manufacturer's units; Qiagen Ltd). The oligonucleotide primers (MWG Biotech) were designed for this study and target the core open reading frame of the HBV genome (sense: ATGCAACTTTTTTCACCTCTG and antisense: GAAGGAAAGAAGTCAGAAGG) and are based on a consensus of sequences in the EMBL database (accession numbers: D00630, D12980, M12906, M38454, M38594, M38636, M54923, M57663, and X04615).³

The HBV PCR thermal cycling and amplicon detection incubations used were as follows: initial denaturation at 95°C for five seconds, followed by 33 cycles of incubation at 96°C for zero seconds, 50°C for five seconds, 72°C for 15 seconds, and 81°C for five seconds. Measurement of the specific PCR amplicon concentration at the end of the 81°C incubation period was achieved by measurement of the fluorescent signal at 530 nm generated by double stranded PCR amplicon bound Sybr Green 1 using the LightCycler™ fluorimeter. At this temperature, it had been determined using the LightCycler™ melting curve analysis software that only specific PCR amplicons remained double stranded (data not shown).

For the comparison of RNA extraction, aliquots of a pool of serum samples containing 2.5 million genome equivalents/ml of hepatitis C virus (HCV) RNA (measured by Amplicor HCV Monitor) were processed using the kits, followed by quantitative HCV LightCycler™ PCR. HCV PCR was performed in a solution with a total volume of 20 µl containing 5 µl of the eluates from the test kits, 2 µl 10× LightCycler™ reverse transcription-PCR (RT-PCR) reaction mix Sybr Green 1 (LightCycler™ RNA amplification kit Sybr Green 1; Roche Molecular Biochemicals), 1.5 mM MgCl₂, 0.5 µM each oligonucleotide primer, and 0.5 µl LightCycler™ RT-PCR enzyme mix (LightCycler™ RNA amplification kit Sybr Green 1). The primers (sense: CCCTGTGAGGAAC-TWCTGTCTTCA and antisense: GGTGAC GGTCTACGAGACCT) were modified from a previous report⁴ and target the 5' non-translated region of the HCV genome.

The HCV PCR thermal cycling and amplicon detection incubations used were as follows: reverse transcription and initial denaturation at 55°C for 10 minutes and 95°C for five seconds, respectively, followed by 40 cycles of 96°C for zero seconds, 55°C for five seconds, 72°C for 15 seconds, and 88°C for five seconds. Measurement of the PCR amplicon concentration at the end of the 88°C incubation was achieved as for the HBV assay.

Two experiments were performed for both DNA and RNA. First, three replicates of the serum sample pools were processed in parallel with each extraction protocol and PCR performed to determine which protocol had the best recovery efficiency. Each of the replicates was amplified within a single experiment. Second, the nucleic acid from the best performing extraction protocol was diluted to make a log₁₀ series of quantitation standards by which the relative efficiencies of the other extraction protocols could be measured using the LightCycler™ software. An additional experiment was performed for DNA to determine the relative ability of each kit to process haemolysed serum. For this, HBV DNA negative EDTA treated whole blood was added (10% vol/vol) to aliquots of the serum sample pools before processing with the various kits.

Results

The relative concentration of HBV DNA and HCV RNA recovered from serum by the kits was measured by determining the PCR cycle at which amplicon specific fluorescence became detectable by crossing a noise band positioned above background values of fluorescence using the LightCycler™ software (fig 1).

The most efficient kits for HBV DNA extraction were the High Pure viral nucleic acid and QIAamp blood DNA kits, which gave approximately equivalent noise band crossing points (table 1). The remaining kits had later, approximately equivalent, noise band crossing points of HBV DNA amplification. For HCV RNA extractions all of the kits gave amplifications with approximately equivalent noise band crossing points, with the exception of the NucleoSpin virus kit which had a later noise

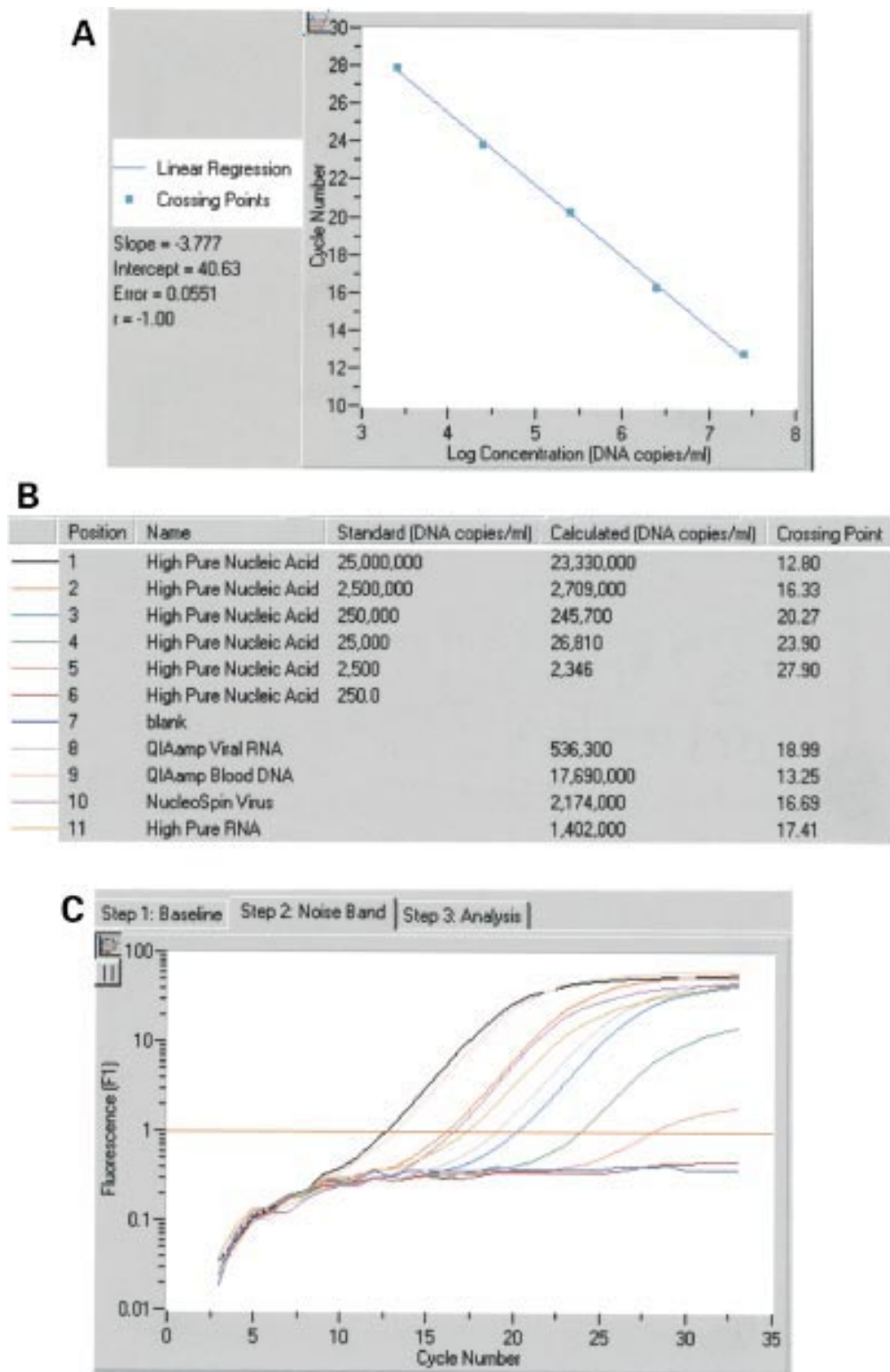


Figure 1 Demonstration of real time quantitative PCR using the LightCycler™ instrument and software. (A, B) Relative concentrations of hepatitis B virus (HBV) DNA recovered by the kits were measured using DNA extracted with the High Pure viral nucleic acid kit as quantitation standards. A similar comparison was performed for hepatitis C virus (HCV) RNA. The PCR cycle at which the amplicon specific fluorescent signal becomes detectable above background level is recorded by the software as a noise band crossing point. (C) Samples with a higher starting target concentration will cross sooner than more dilute samples.

Table 1 Noise band crossing points for LightCycler™ PCR amplification of HBV DNA or HCV RNA at concentrations of 2.5 million copies/ml processed by the nucleic acid extraction kits

Virus	Extraction kit	Noise band crossing points			Mean
HBV	QIAamp viral RNA	21.57	20.55	21.86	21.33
	QIAamp blood DNA	16.43	16.29	15.94	16.22
	NucleoSpin virus	20.19	18.72	18.42	19.11
	High Pure viral RNA	19.98	19.79	19.94	19.90
	High Pure viral nucleic acid	15.13	16.14	15.25	15.51
HCV	QIAamp viral RNA	27.98	28.08	28.23	28.10
	QIAamp blood DNA	27.57	27.74	27.65	27.65
	NucleoSpin virus	36.23	35.23	35.05	35.50
	High Pure viral RNA	29.37	30.05	28.60	29.34
	High Pure viral nucleic acid	27.23	27.00	27.94	27.39

HBV, hepatitis B virus; HCV, hepatitis C virus.

Table 2 Comparison of amplification crossing points, calculated values for DNA and RNA concentration, and relative percentage recoveries for the kits using nucleic acid extracted by the high pure nucleic acid kit as the standard

Virus	Extraction kit	Noise band crossing point	Calculated copies/ml (millions)	Relative recovery
HBV	High Pure viral nucleic acid	12.80	23.3	100%
	QIAamp viral RNA	18.99	0.5	2.2%
	QIAamp blood DNA	13.25	17.7	76%
	NucleoSpin virus	16.69	2.2	9.3%
	High Pure viral RNA	17.41	1.4	6.0%
HCV	High Pure viral nucleic acid	28.03	2.5	100%
	QIAamp viral RNA	28.80	1.6	64%
	QIAamp blood DNA	28.23	2.2	89%
	NucleoSpin virus	36.23	0.02	0.9%
	High Pure viral RNA	29.36	1.2	46%

HBV, hepatitis B virus; HCV, hepatitis C virus.

band crossing point (table 1). For both HBV and HCV the nucleic acid extracted by means of the High Pure viral nucleic acid kit was used to make a log₁₀ dilution series of standards by which the nucleic acids extracted using the other kits were quantified for their relative concentrations of HBV DNA or HCV RNA (fig 1; table 2).

In the experiment in which whole blood was mixed with the serum samples before processing with the extraction kits, no difference in noise band crossing points compared with unadulterated serum was noted (data not shown).

Discussion

The aim of this study was to discover which of these column based nucleic acid extraction kits for clinical material has the best recovery rate from serum for two commonly detected viral nucleic acids: one DNA and one RNA. Extraction kits marketed for processing a variety of viral nucleic acids such as those tested here need to be capable of efficient nucleic acid extraction from a variety of virion types. Ideally, a kit would also be capable of good recovery of nucleic acid, and effective removal of enzyme inhibitors from diverse clinical material, allowing the flexibility necessary in a clinical diagnostic laboratory.

Five nucleic acid extraction kits were used; two (NucleoSpin virus and High Pure viral nucleic acid) are marketed for the extraction both of viral DNA and RNA, two for the extraction of viral RNA only (QIAamp viral RNA and High Pure viral RNA), and the remaining kit for DNA only (QIAamp DNA blood). The kit inserts for the QIAamp kits state that the RNA kit will co-purify DNA from

cell free material and that the DNA kit will co-purify RNA, but that it is not manufactured under RNase free conditions. The QIAamp viral RNA kit is recommended by the manufacturer for the preparation of DNA from urine samples because of the high concentration of inhibitors which, it is claimed, are more efficiently removed by this kit.

Large differences were seen in the recovery rates of HBV DNA between the five kits used. The best performing kits were the High Pure viral nucleic acid and the QIAamp DNA blood kits, which both use proteolytic enzymes (proteinase K and protease, respectively) in addition to chemical denaturants. The kits using chemical denaturation alone for the release of DNA all recovered less than 10% of the HBV DNA when compared with the enzyme protocols (table 2).

The HCV RNA recovery efficiencies for four of the kits were similar, with the most successful kit performing approximately twice as efficiently as the least successful kit. The remaining kit, NucleoSpin virus, had a lower recovery efficiency despite using RNase free water heated to 80°C, as recommended in the kit insert for the elution of RNA from the columns. The finding of a lower HCV RNA recovery rate for this kit was confirmed by performing additional extractions where similar results were obtained (data not shown). It is possible that the NucleoSpin virus kit did not completely remove unidentified PCR inhibitors from the HCV RNA because recent modifications to the wash buffers supplied with the other kits used in this study are described in the kit inserts as improving the potential for the removal of inhibitors. Again, the two best performing protocols were those that used proteolytic enzymes.

The HBV virion contains a protein nucleocapsid largely comprising core antigen, which is thought to interact with the viral nucleic acid.⁵ The nucleocapsid is surrounded by a further layer of surface antigen. Little is known about the biology of the HCV virion but related viruses in the Flaviviridae contain a nucleocapsid surrounded by a lipid membrane, which makes up 17% of the viral weight.⁶ It is possible that efficient nucleic acid recovery from virions such as HBV with a high protein content, or when the nucleic acid is in close association with structural proteins, is more dependent on the proteolytic capability of the extraction procedure than less structured virions, where a large proportion of the viral particle comprises lipid membrane.

The utility and "hands on" time of the five extraction protocols is similar. In the experiment to mimic the processing of haemolysed blood all the kits were successful in removing haemoglobin from the purified nucleic acid; however, there are many more potential enzyme inhibitors and a more thorough investigation would be required to confirm that these kits have equivalent performance in purifying nucleic acids. In terms of nucleic acid recovery, the two protocols using enzyme digestion in addition to chemical denaturation performed better than the protocols using

denaturation alone for both of the viruses used. On the basis of these data, the High Pure viral nucleic acid kit is used in this laboratory for the extraction of a variety of viral nucleic acids from various body fluids and tissue samples. The QIAamp blood DNA kit has similar recovery efficiencies but is not supplied RNase free and, for the extraction of low copy number targets (< 10 000 genomes/ml), the manufacturer recommends the use of a carrier nucleic acid, which is not included in the kit. Carrier RNA is included in the other kits and in each case is used regardless of the copy number expected in a sample, which cannot necessarily be anticipated. The High Pure viral nucleic acid kit uses proteinase K and has been used in this laboratory to digest whole blood and biopsy tissue; however, for the processing of difficult to lyse organisms—such as Gram positive bacilli, mycobacteria and fungi—proteolysis is supplemented by digestion with other types of lytic enzymes.

Nonetheless, these data do indicate that those extraction kits containing a proteolytic enzyme and carrier nucleic acid, and that are supplied RNase free, have the widest potential application in the routine microbiology laboratory, which is increasingly reliant on the sensitive detection of pathogen nucleic acid.

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