

Comparison of methods for the extraction of bacterial DNA from human faecal samples for analysis by real-time PCR

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Real-time PCR analysis of bacterial DNA isolated from faecal specimens has become increasingly used for the quantification of indigenous intestinal microbiota. The success of such analysis requires effective methods for the extraction of faecal bacterial DNA. Three extraction methods were assessed for their effectiveness in extracting *Escherichia coli* and *Enterococcus* spp. bacterial DNA from human faecal samples: an in-house phenol/chloroform extraction, and two commercially available kits, ExtractMaster (Epicentre Biotechnologies) and UltraClean (Mo Bio Laboratories) faecal DNA extraction kits. Real-time PCR using the standard curve method was used to quantify the level of bacterial DNA extracted from ten faecal samples. The phenol/chloroform method required an additional dilution step before DNA could be amplified by real-time PCR. After taking into account this dilution, the three extraction methods did not differ in the level of *E. coli* bacterial DNA detected. However, for *Enterococcus*, the ExtractMaster kit resulted in significantly less DNA detected. The phenol/chloroform method reliably extracted DNA, and produced extracts with short-term stability. Extraction using phenol/chloroform produces quantities of faecal bacterial DNA comparable to commercially available kits when amplified by current real-time PCR technology. Furthermore, phenol/chloroform extraction is rapid and simple, and provides a clear cost advantage for laboratories with limited funds.

Keywords DNA extraction; Faecal samples; Real-time PCR

1. Introduction

It is well established that the indigenous microbiota that inhabit the human gastrointestinal (GI) tract play an important role in maintaining host health and normal gut function [1-4]. The majority of studies of human intestinal microbiota have been performed under the assumption that faeces contain a representative sample of the prevalent intestinal microbiota. Traditionally, most of our knowledge regarding the activity of the GI microbiota has come from conventional culture based methods, requiring knowledge of bacterial growth conditions and several days for cultivation. This, together with the fact the only between 20-50% of intestinal bacteria can be cultured, led to the development of a more sensitive, specific and rapid molecular 16S DNA detection system based on the polymerase chain reaction (PCR) [1, 3-5]. However, the application of PCR to faecal samples has several pitfalls, as faeces contain numerous species of bacteria and several PCR inhibitors, most of which have not been identified [6, 7].

The difficulty with applying PCR methods to faeces has been attributed to the difficulty of removing inhibitors [8]. The extraction and purification of DNA from faecal samples largely influences the amount of these inhibitory substances. Phenol-chloroform extraction is the classical method for preparation of faecal samples for PCR assay, and inactivates microbes very efficiently [9]. However, phenol-chloroform extraction has been shown to be ineffective in removing all types of inhibitors from faecal samples, and residual phenol can inhibit amplification [9, 10]. Several commercially available faecal DNA extraction kits have been developed to overcome these limitations. These kits operate by different principles for the efficient lysis of bacterial cells and extraction of DNA ready for PCR.

To accurately analyze bacterial DNA an efficient method is required that produces high-quality DNA with the removal of inhibitory substances, without reducing the yield of target DNA below an amplifiable level [11]. Studies comparing the efficiency of faecal DNA extraction methods have relied upon traditional PCR methods [9, 11-14]. Although conventional PCR provides a faster and more reliable method than culturing techniques, laborious post-PCR steps are required for evaluation of the amplification product. Furthermore, these end-point results are not very precise, and are only semi-quantitative [13].

In attempting to try and quantify results, studies based on conventional PCR have compared faecal DNA extraction methods by spiking faecal samples with a known dilution series of the target bacteria [9, 13, 14]. The extraction method that led to the detection of bacterial DNA over the greatest range of spiked concentrations was determined as the most effective. However, there are limitations with conventional PCR associated with the phase at which results are obtained. End-point detection means that the amount of final product may not be directly related to the initial starting quantity in the sample, making it common for replicate reactions to yield different results [15, 16].

The emergence of a modified PCR technique, real-time PCR, overcomes these pitfalls, allowing the continuous quantification of DNA as the reaction is proceeding [17]. Real-time PCR does not rely on the end-product for quantification as data is collected in the exponential phase of the reaction. This is the optimal phase for analyzing data, since it is the only stage at which amplification is reproducible [16]. Measurements taken during the exponential phase

will not be effected by limiting reagents, small differences in reaction components, or cycling parameters [15]. Analysis of data during this phase also allows a higher order of magnitude of dynamic range, with real-time PCR being able to detect as little as a two-fold change, compared to end-point agarose gel resolution at about 10-fold [16].

Studies assessing the efficiency of faecal DNA extraction methods have been largely limited to analysis by conventional post-PCR detection methods. Therefore, in the present study, the efficiency of three different faecal DNA extraction methods in producing bacterial DNA for real-time PCR amplification was compared. The efficiency of extraction was compared for both Gram-positive (*Enterococcus*) and Gram-negative (*E. coli*) species (normal inhabitants of the human GI tract) from human faecal samples, using the following extraction methods: (1) a modified phenol/chloroform extraction method; (2) the UltraClean Fecal DNA kit (Mo Bio Laboratories); and (3) the ExtractMaster Fecal DNA Extraction kit (Epicentre Biotechnologies). These commercially available kits were chosen to represent different methods of bacterial cell lysis. The time and cost of processing samples for each extraction method were also evaluated.

2. Materials and Methods

2.1. Faecal sampling

Faecal specimens utilized in this study were obtained from ten healthy adult volunteers (six females and four males). Participants were instructed to provide one faecal sample (approximately 1 g) using a sterile faecal container, at a convenient time and place. Faecal samples were stored at -80°C, and 10% w:v faecal homogenates were prepared in sterile phosphate-buffered saline (PBS, Sigma, NSW, Australia), and homogenized using a stomacher. Aliquots of each specimen were frozen at -80°C until DNA was extracted. A total of ten faecal samples were studied. The protocol was approved by the Human Research Ethics Committee of Swinburne University of Technology.

2.2. Test bacteria and culture conditions

The strains used to construct the real-time PCR standard curves in this study were *E. coli* American Type Culture Collection (ATCC) 25922 and *Enterococcus faecalis* ATCC 19433 and were obtained from the collection held by the Microbiology Laboratory, at Swinburne University of Technology, Australia. Bacteria were grown in Brain Heart Infusion broth (Oxoid) overnight at 37°C. The number of colony-forming units per ml (CFU/ml) of bacteria was determined by plating *E. coli* on Chromocult agar (Merck), and *Enterococcus faecalis* on KF agar (Oxoid), with plates incubated for 3 days at 37°C. All media were prepared according to the manufacturers' instructions with de-ionised water, and autoclaved at 121°C for 20 min before use.

2.3. Extraction of DNA from faecal samples

Each extraction method specified different recommended starting sample sizes and final elution volumes, which would affect the quantity of DNA extracted. Input/output measurements were standardized by using the same amount of starting material and elution volume, to enable the direct comparison of methods. For each extraction method a starting material of 150 µl of faecal homogenate was used, with a final elution volume of 150 µl. The two manufactures did not supply complete information regarding the composition of kit components, due to the proprietary nature of the kits. However, technical information provided indicated that with the UltraClean Fecal DNA extraction kit, DNA lysis was achieved by physical disruption using bead-beating, and an inhibitor removal solution (IRS) was used to remove inhibitors of PCR. The ExtractMaster Fecal DNA extraction kit utilized a detergent lysis process combined with a chromatography step to remove inhibitors. Apart from the modifications made to the input/output measurements, faecal DNA was prepared using the two commercial DNA extraction kits according to the manufacturers' instructions.

2.3.1 Phenol/chloroform method

The method used was modified from Gouvea et al. [18]. Faecal homogenates were thawed and clarified by centrifugation (14,500 x g for 30 s). To this, 20 µl of SDS, 20 µl of Na-acetate, and 200 µl of phenol-chloroform were added. This was homogenized by vortex mixing for 20 seconds. The mixture was then centrifuged (14,500 x g for 30 seconds), and the aqueous phase placed in a new tube. To this, 50 µl of hydroxyapatite (HA) were added and mixed by vortexing for 30 seconds. This was centrifuged (14,500 x g for 30 s), and the supernatant discarded. The HA pellet was washed by resuspending in 500 µl of washing solution (10mM potassium phosphate). This was vortexed for 30 seconds, and centrifuged (14,500 x g for 30 s), after which the supernatant was discarded. This wash was repeated for a second time, with all traces of liquid removed. The DNA was eluted by resuspending the HA in 150 µl of elution solution (200mM potassium phosphate). This was vortexed for 30 seconds, and centrifuged (14,500 x g for 30 s), and the supernatant containing DNA recovered.

2.4. Real-time PCR

2.4.1. PCR Primers

The primer sets used in this study with bacterial target species and optimized PCR conditions are summarized in Table 1. The oligonucleotides were designed and optimized for real-time PCR using SYBR green technology [19, 20] and were synthesized commercially by Invitrogen Custom Primers, VIC, Australia. The specificities of these primer sets were confirmed by amplifying genomic DNA from target and non-target bacterial strains by conventional PCR. Agarose gel electrophoresis was performed to confirm the specific PCR products. Specificity of primers was also confirmed by melt curve analysis at the end of each real-time PCR run.

Table 1 Primer sequences, optimized conditions and bacterial target species for real-time PCR.

PCR assay (amplicon size, annealing temp)	Oligonucleotide sequence (5'→ 3')
Target species ^a	
<i>E. coli</i> subgroup (340 bp, 61°C)	F: GTTAATACCTTTGCTCATTGA
<i>E. coli</i> ^b , <i>Hafnia alvei</i> and <i>Shigella</i> spp.	R: ACCAGGGTATCTAATCCTGTT
<i>Enterococcus</i> . spp. (144 bp, 61°C)	F: CCCTTATTGTTAGTTGCCATCATT
<i>Enterococcus faecalis</i> ^b , <i>E. faecium</i> , <i>E. asini</i> , <i>E. saccharolyticus</i> , <i>E. casseliflavus</i> , <i>E. gallinarum</i> , <i>E. dispar</i> , <i>E. flavescens</i> , <i>E. hirae</i> , <i>E. durans</i> , <i>E. pseudoavium</i> , <i>E. raffinosus</i> , <i>E. avium</i> , <i>E. malodoratus</i> , <i>E. mundtii</i> , <i>E. azikeevi</i> , <i>E. canis</i> , <i>E. gilvus</i> , <i>E. haemoperoxidus</i> , <i>E. hermanni</i> , <i>E. moraviensis</i> , <i>E. pallens</i> , <i>E. phoeniculicola</i> , <i>E. villorum</i> , <i>E. rottae</i>	R: ACTCGTTGTACTIONCCCATTTGT

^a Target species were obtained from the Ribosomal Database Project (Rintilä *et al.*, 2004).

^b Used to construct standard curve for Real-time PCR assay.

2.4.2. Real-time PCR assay

The quantification of DNA by real-time PCR was performed with the MyIQ Single Colour Real-Time PCR Detection System (Bio-Rad, California, USA) associated with MyIQ Interface Software (version 1.0). Each reaction was performed in duplicate in a total volume of 25 µl in 96-well optical grade PCR plates, sealed with optical-quality sealing tape (Bio-Rad). Amplification reactions were carried out with 12.5 µl of iQ SYBR Green Supermix (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM of each deoxynucleotide triphosphate, iTaq DNA polymerase, 50 units/ml, 6 mM MgCl₂, SYBR Green I, 20 nM fluoresein, and stabilizers) (Bio-Rad), mixed with the selected primer set at a concentration of 0.5 µM for each primer, 5 µl of template DNA and made up to 25 µl with distilled water. Amplification involved one cycle at 95°C for 10 min for initial denaturation, followed by 35 cycles of denaturation at 95°C for 15 s, primer annealing at 61°C for 20 s and extension at 72°C for 30 s. Melt curve analysis was performed by gradually heating the PCR mixture from 55 to 95°C (1°C per cycle of 10 s).

2.4.3. Standard curve

The standard curve for real-time PCR quantification was constructed using bacterial DNA extracted from a pure culture of a selected reference strain of target bacteria for each primer set (Table 1). Briefly, the bacterial reference strain was inoculated in BHI broth, and incubated overnight at 37°C. DNA was then extracted from 1 ml of broth culture while in logarithmic growth phase using the Wizard Genomic DNA purification kit (Promega, Madison, USA). The concentration (ng/µl) of extracted DNA was determined on a GeneQuant Pro spectrophotometer (Pharmacia, Cambridge, UK) with absorbance measured at 260nm. This DNA was used to establish a standard curve. The number of colony-forming units per ml (CFU/ml) was also calculated for this overnight broth culture, using standard laboratory methods. To confirm the correct product amplification by selected primer sets, DNA extracts from the pure cultures were amplified using conventional PCR (MyCycler Thermal Cycler; Bio-Rad). The PCR products were visualized after electrophoresis (120V for 40 min) in 1.8% agarose gels (Progen, QLD, Australia), and band sizes confirmed using the GeneRuler DNA ladder (Fermentas, Maryland, USA).

The C_T was defined as the PCR cycle at which the increase in fluorescent signal was statistically significant above the background measurement. To generate standard curves for *E. coli* and *Enterococcus*, the C_T values were plotted relative to corresponding serial 10-fold dilutions of template DNA extracted from representative cultures. A standard curve covering at least a 5-fold magnitude was used. The reaction efficiency (*E*), calculated using the slope of the standard curve ($E = 10^{-1/\text{slope}}$) was between 90-110% for all PCR assays. The results were found to be linear over the range of bacterial concentrations tested, with the correlation coefficients (*R*) ≥ 0.990. The amount of DNA measured by real-time PCR (ng/µl) was converted to CFU/ml. This approach was used because, for faecal samples, it is easier to interpret results in terms of CFU numbers rather than in DNA concentrations or copy numbers [21].

2.5. Statistical analysis

Means and standard deviations were calculated for the amount of *E. coli* and *Enterococcus* detected from faecal samples after extraction using each method. Differences between the amounts of DNA extracted using each method were tested for significance using a non-parametric Friedman one-way within subjects ANOVA. Statistical significance was defined at a *p* value of less than 0.05. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) for Windows Statistical Package Version 14.0.

3. Results

3.1. Specificity of primers

Conventional end-point PCR with gel electrophoresis confirmed the specificity of primer sets, by yielding PCR products of expected size for target species, and no product for nontarget species. This was further confirmed by real-time PCR results, showing only one specific PCR product for each set of primers, illustrated by one peak in melting curve analysis, as shown below in Figure 1. In real-time PCR, no fluorescent signal was detected from non-target bacterial DNA.

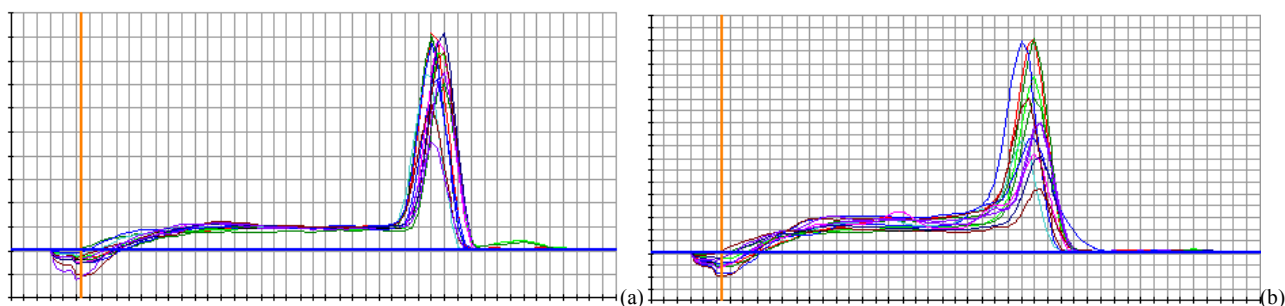


Fig. 1 Specificity of primers by real-time PCR melting curve (a) *E. coli* and (b) *Enterococci* from human faecal samples.

3.2. Quality of faecal DNA extracts

The quality of the total extracted DNA was assessed by spectroscopy using 260/280 absorbance ratios. Each extraction measurement produced spectral readings within the range specified for pure DNA. However, undiluted DNA isolated using the phenol-chloroform extraction method resulted in inhibition of real-time PCR, therefore spectrophotometric analysis of DNA purity was not considered a good indicator of the presence of PCR inhibitors.

3.3. Comparison of the amount of bacterial DNA extracted using each method

The mean amount of *E. coli* and *Enterococcus* detected from faecal samples by real-time PCR after extraction using each method is provided in Table 2. For *E. coli*, there was no significant effect for the type of extraction method used, $\chi^2 (2, N = 10) = 0.20, p > 0.05$. However, for *Enterococcus*, there was a significant effect for the type of extraction method used, $\chi^2 (2, N = 10) = 16.80, p < 0.001$. Pairwise comparisons with non-parametric *t*-tests (Wilcoxon Signed Rank tests) indicated that the amount of *Enterococcus* detected using the ExtractMaster kit was significantly less than that detected using the phenol/chloroform method ($Z = -2.40, p \leq 0.017$) and the UltraClean kit ($Z = -2.80, p \leq 0.017$). In addition, the cost of each extraction method and the time required to process a sample are shown in Table 3.

Table 2 Mean and standard deviation for amount of *E. coli* and *Enterococcus* detected from faecal samples after extraction using each method

	<i>E. coli</i>	<i>Enterococcus</i>
Extraction Method	Mean (SD)	Mean (SD)
Phenol/chloroform	$2.06 \times 10^5 (2.28 \times 10^3)$	$2.02 \times 10^5 (4.53 \times 10^3)$
UltraClean kit	$1.09 \times 10^5 (1.95 \times 10^3)$	$1.51 \times 10^7 (4.02 \times 10^7)$
ExtractMaster kit	$6.17 \times 10^4 (1.10 \times 10^3)$	$9.38 \times 10^2 (1.65 \times 10^3)$

Note: The amount of *E. coli* and *Enterococcus* are reported as CFU/ml of faecal sample. CFU/ml were calculated by using the standard curve.

Table 3 Comparison of cost and processing time between the evaluated faecal DNA extraction methods

Extraction method	Price (\$) per sample ^a	Processing time ^b (h:min)
Phenol/chloroform	0.25	0:20
UltraClean kit	5.60	0:35
ExtractMaster kit	6.84	1:15

^a Based on pricing quote (2008) from each manufacturer for larger-volume orders.

^b Determined for a single extraction. Timing began with aliquoting of first reagent or sample and concluded with recovery of extracted DNA.

^c Excluding standard laboratory equipment and reagents

^d Australian dollars

Further analysis showed the phenol/chloroform method reliably extracted DNA from faecal samples, with no significant difference between replicate extracts (χ^2 (2, $N = 5$) = 5.20, $p > 0.05$). When performing laboratory analyses, it is often required for DNA extracts to be stored prior to analysis. There was no significant difference between phenol/chloroform DNA extracts stored under different temperatures (4°C, -20°C and -80°C) after one week χ^2 (2, $N = 8$) = 4.0, $p > 0.05$.

4. Discussion and Conclusions

Real-time PCR has several advantages over conventional PCR for the quantification of faecal bacterial levels, including greater resolution and reproducibility of results. Both techniques require the efficient extraction of DNA from faecal samples, which is problematic due to the complex gut microbiota and the presence of PCR inhibitors. However studies assessing the most efficient method for the extraction of faecal DNA have relied upon conventional PCR methods, which are associated with several shortcomings. This study evaluated the most efficient extraction method for the production of faecal bacterial DNA for quantification using current real-time PCR technology.

A real-time PCR assay targeting the 16S rDNA for quantification of *E. coli* and *Enterococcus* was used to compare the efficiency of three extraction methods for the isolation of DNA from faecal samples; two commercially available kits, namely the ExtractMaster and UltraClean faecal DNA extraction kits, and an in-house phenol/chloroform extraction method. These kits were chosen to represent various methods of bacterial cell lysis. *E. coli* and *Enterococcus* make up a normal part of the human GI microbiota, and represent both Gram-negative and Gram-positive bacterial types. Faecal samples are considered to provide a representative sample of the prevalent intestinal bacteria.

Each extraction method was successful in detecting *E. coli* and *Enterococcus* in 100% of faecal samples with the commercial kits. However, when using the phenol-chloroform method to isolate DNA from faecal samples, several DNA extracts failed to amplify following real-time PCR. This finding is consistent with several other studies showing that phenol/chloroform extraction of DNA from faecal samples can result in PCR amplification failure [7, 11, 12, 22, 23]. Authors suggested that extraction using phenol-chloroform failed to completely remove all impurities present in faecal samples, resulting in inhibition of PCR amplification. It has also been proposed that residual phenol left over from the extraction method may inhibit amplification of the extracted DNA [9].

The observation that not all phenol/chloroform DNA extracts resulted in amplification failure suggested incomplete removal of faecal inhibitors, rather than inhibition from phenol itself. It was found that for phenol/chloroform extracts that failed to amplify, adequate dilution resulted in amplifiable DNA by real-time PCR. Furthermore, the dilution factor required to overcome amplification failure varied between individuals, suggesting that the concentration of PCR inhibitors among faecal samples is not consistent. These differences may be related to diet, the moisture content of faeces, or possible gender effects [13]. It was found that diluting phenol/chloroform DNA extracts by at least 10-fold was sufficient to overcome amplification failure. Although this dilution reduces the amount of target DNA present, quantification of diluted DNA samples was still possible using real-time PCR, as the technique is highly sensitive. In order to account for the possible presence of inhibitors in phenol/chloroform extracts, future studies could simply increase the final elution volume in the method described here.

The results of the present study showed that there was no significant difference in the amount of *E. coli* detected from faecal samples after extraction using each method. That is, the recovery of Gram-negative *E. coli* DNA from faecal samples for quantification by real-time PCR was similar using the phenol/chloroform method, the UltraClean kit, and the ExtractMaster kit. However, the quantity of *Enterococcus* detected after extraction of faecal DNA using the ExtractMaster kit was significantly less compared to extraction using phenol/chloroform ($Z = -2.40$, $p \leq 0.017$) and the UltraClean kit ($Z = -2.80$, $p \leq 0.017$). This difference observed for *Enterococcus* and not *E. coli* may be due to differences in cell wall structure, where Gram-positive bacteria (*Enterococcus*) have a thick layer of peptidoglycan in the cell wall, in contrast to Gram-negative bacteria (*E. coli*) which have a thin layer. This thick cell wall structure may pose problems when it comes to the effective lysis and release of bacterial DNA.

While the effective extraction of DNA is important, each protocol was also evaluated in terms of processing time and ease, and cost per extraction. Phenol/chloroform extraction offered the most economical method at just A\$0.25 per

sample, while the UltraClean kit and ExtractMaster kit were considerably more costly at A\$5.60 and A\$6.84 per sample, respectively. Furthermore, the processing time required for the phenol/chloroform method was less than the commercial extraction kits, shown in Table 3. Each method was simple to perform, and all of the methods used standard equipment commonly available in clinical laboratories. However, the UltraClean kit included a step where samples were mixed horizontally which required a specialized vortex adaptor available from the manufacturer (or securing tubes horizontally on a flat-bed vortex pad), while the ExtractMaster kit required a refrigerated centrifuge.

Commercial faecal DNA extraction kits were developed to overcome problems associated with traditional phenol/chloroform extraction, mainly the effect of residual faecal PCR inhibitors. However, this study has demonstrated that the adapted phenol/chloroform method can be successfully used for the extraction of real-time PCR amplifiable bacterial DNA from faeces in amounts comparable to that of commercial kits. Commercial faecal DNA extraction kits were developed to offer standardized, quality-controlled reagents optimized for each step of the extraction [9]. Indeed, studies have shown faecal DNA extraction using commercial kits to be highly reproducible [21]. In the current study, the phenol/chloroform extraction method was found to reliably extract DNA from faecal samples, and produce extracts with short term stability.

In summary, this study used current real-time PCR technology, together with cost and time considerations, to evaluate faecal DNA extraction methods. The results illustrate that the ExtractMaster extraction kit introduced some bias in the amount of bacterial DNA detected for Gram-positive bacteria. Phenol/chloroform extracts required further dilution to overcome the inhibitory effects of incompletely removed faecal inhibitors, with the resulting yield of diluted DNA still detectable by real-time PCR. After taking dilution factors into account, the phenol/chloroform method was shown to extract levels of faecal DNA comparable with that of the UltraClean kit. However the phenol/chloroform method provides a clear cost advantage over more expensive commercially available kits for laboratories with limited funds. Furthermore, this in-house method was found to be time-efficient, and reliably extracted DNA from faecal samples, with extracts stable over short-term storage conditions. Although precautionary measures need to be taken when handling phenol/chloroform, this extraction method has been demonstrated as an effective alternative to commercially available kits.

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