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1 **Comparison of simple RNA extraction methods for molecular diagnosis of hepatitis C**
2 **virus in plasma**
3
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17 # Abstract

18 We evaluated the efficacy of four simple RNA extraction methods for the detection of
19 hepatitis C virus (HCV) in plasma samples: silica-membrane-based, magnetic beads-based,
20 boiling with Diethyl Pyrocarbonate (DEPC)-treated distilled water or a commercial lysis
21 buffer. HCV RNA was detected using both real time reverse transcription polymerase chain
22 reaction (RT-PCR) and reverse transcription loop mediated isothermal amplification (RT-
23 LAMP). The magnetic beads-based extraction can be used as an alternative RNA extraction
24 method for on-site HCV detection. Boiling with DEPC-treated distilled water was not
25 appropriate for low HCV load samples and boiling with a lysis buffer was not recommended.

26

27 **Keywords:** RNA extraction; HCV; molecular diagnosis; RT-LAMP; RT-PCR; plasma

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28 # Introduction

29 Hepatitis C virus (HCV) is a leading cause of chronic liver disease, cirrhosis, and liver
30 cancer. The current oral direct-acting antiviral (DAA) combinations can cure 95-99% patients
31 [1], but the majority of HCV-infected people are unaware of their infection status. Testing for
32 antibody to HCV (anti-HCV) is insufficient to diagnose a current HCV infection or ongoing
33 HCV replication. HCV RNA testing is needed. Real time reverse transcription polymerase
34 chain reaction (RT-PCR) is commonly used for HCV RNA detection/quantification in clinical
35 practice. However, this test is rarely available for an on-site diagnosis, especially in remote
36 settings since it requires specific reagents and instruments with high costs. The development
37 of reverse transcription loop-mediated isothermal amplification assay (RT-LAMP) can
38 facilitate the access to molecular testing of HCV. Indeed, the clinical sensitivity and specificity
39 of RT-LAMP has been previously reported as high as 90-100% for HCV detection [2-4]. RT-
40 LAMP also stands out in terms of rapidity, simplicity, cost-effectiveness, and accessibility,
41 making it ideal for field or point-of-care use in remote settings where sophisticated and
42 expensive equipment are not available. However, this technique still requires an effective
43 nucleic acid extraction and purification steps. Currently, available commercial nucleic acid
44 extraction kits, mostly based on a silica-based column extraction methodology, are expensive
45 and require a centrifugation process, limiting the use of RT-LAMP for on-site diagnosis or in
46 the field. In this study, we evaluated the efficacy of various simple RNA extraction methods
47 from plasma samples for HCV detection using RT-LAMP and confirmed the presence of HCV
48 RNA in extracted samples with real time RT-PCR.

49 # Materials and methods

50 This study used leftover plasma samples of 50 HCV-infected individuals who had HCV
51 viral load testing and genotyping for clinical care at the Faculty of Associated Medical

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52 Sciences, Chiang Mai University. HCV viral load was initially measured using a commercial
53 real-time RT-PCR assay (COBAS AmpliPrep/COBAS TaqMan HCV Test). The study was
54 approved by the Human Experimentation Committee (Number 17/64) and the Institutional
55 Biosafety Committee (Number CMUIBC0363004) of Research Institute for Health Sciences,
56 Chiang Mai University, Thailand.

57 Viral RNA was extracted from plasma samples using four different methods: 1)
58 NucleoSpin RNA Virus (Macherey-Nagel, Germany) and; 2) NucleoMag Virus kit (Macherey-
59 Nagel, Germany) following the manufacturer's recommendations; 3) Boiling with water, 100
60 µL plasma were mixed 1:1 with Diethyl Pyrocarbonate (DEPC)-treated distilled water
61 (Invitrogen, USA) and boiled at 95°C for 10 min [5], the mixture was centrifuged at 8,000 xg
62 for 1 min and supernatant was collected; 4) Boiling with lysis buffer, as above but 100 µL
63 plasma were mixed 1:1 with a commercial lysis buffer (Lysis Buffer RAV1, Macherey-Nagel,
64 Germany). Yield and purity of RNA extracted according the four methods were measured using
65 the NanoDrop™ 2000/2000c Spectrophotometer.

66 For RNA detection by real time RT-PCR, four µL of viral nucleic acid extract were
67 amplified with 400 nM of each HCV forward and reverse primers, and 100 nM of HCV probe
68 [6] of the sensiFAST Probe Lo-ROX One-Step kit. Amplification was performed on the
69 Applied Biosystems 7500 instrument as follows: 45°C for 10 min; 95°C for 2 min; 40 cycles
70 of 95°C for 5 sec and 60°C for 20 sec. The fluorescence signal was measured at 60°C of each
71 cycle. For RNA amplification by RT-LAMP, five µL of nucleic acid extract were used as
72 previously described [2]. Briefly, RT-LAMP reaction was processed at 65°C for 60 min. The
73 result was visualized with the naked eye based on the color change of reaction mixture induced
74 by pre-added hydroxynaphthol blue. The samples that turned sky blue were considered as
75 positive, while those that remained purple were considered as negative. To evaluate the
76 analytical sensitivity of the four extraction methods, 10-fold serial dilutions of plasma with

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77 HCV viral load of 10^6 IU/mL in 1X phosphate buffer saline were prepared. Aliquots of each
78 dilution were extracted by the four extraction methods. All RNA extracts were tested for HCV
79 RNA by both real time RT-PCR and RT-LAMP. Each reaction was performed in triplicate.

80 Results

81 The four extraction methods showed considerably variable quantities of RNA, boiling
82 plasma samples with a commercial lysis buffer provided the highest yield of RNA
83 concentration 695.37 ng/µL and boiling with DEPC-treated distilled water the lowest yield, 0
84 ng/µL. The purity ranged from 0.14 to 3.46 and was the highest with the silica membrane-
85 based extraction (Table 1). Analytical sensitivity on triplicate of serial dilutions of HCV RNA
86 extracts from silica-based membrane and magnetic beads were all detected by real-time RT-
87 PCR with similar cycle threshold (Ct) and by RT-LAMP at the 10^6 concentrations. The
88 numbers of triplicates detected by both techniques decreased when initial concentrations were
89 10^5 or lower. RNA extracts obtained after boiling in DEPC-distilled water were less well
90 detected at higher Ct than the silica-membrane and magnetic beads RNA extracts. Though
91 boiling with a commercial lysis buffer gave the highest RNA yield, none of the RNA extracts
92 gave a signal by real-time RT-PCR, independently of the initial HCV RNA concentration. The
93 results from RT-LAMP could not be interpreted since the color turned from purple to sky blue
94 immediately after adding the extracted RNA to the reaction mix. Thus, boiling with a
95 commercial lysis buffer was not further evaluated.

96

97 **Table 1. Efficacy of four different extraction methods for molecular diagnosis of HCV in**
98 **plasma.**

Plasma		Extraction methods

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HCV RNA level (IU/mL)		Silica-membrane based	Magnetic beads-based	Boiling with DEPC-treated distilled water	Boiling with a commercial lysis buffer
10^6	concentration (ng/µL)	345.80 ± 11.44	6.87 ± 4.03	125.63 ± 16.91	695.37 ± 13.62
	purity (260/280)	3.45 ± 0.04	1.57 ± 0.12	0.87 ± 0.02	1.01 ± 0.04
	Real time RT-PCR, Ct[a] (mean±SD)	24.91 ± 0.29	24.11 ± 1.17	29.86 ± 0.77	Undetectable
	RT-LAMP, no. of detectable samples (%)	3/3 (100)	3/3 (100)	2/3 (67)	Uninterpreted
10^5	concentration (ng/µL)	347.73 ± 9.20	1.97 ± 0.83	28.30 ± 34.75	636.37 ± 1.19
	purity (260/280)	3.44 ± 0.01	1.78 ± 0.90	0.99 ± 0.28	1.07 ± 0.04
	Real time RT-PCR, Ct (mean±SD)	28.27 ± 1.25	27.42 ± 0.78	32.48 ± 0.98	Undetectable
	RT-LAMP, no. of detectable samples (%)	1/3 (33)	1/3 (33)	0/3 (0)	Uninterpreted
10^4	concentration (ng/µL)	326.90 ± 47.56	2.80 ± 0.87	0.20 ± 0.14	620.47 ± 10.98
	purity (260/280)	3.43 ± 0.04	1.97 ± 0.45	1.85 + 2.06	1.08 ± 0.01
	Real time RT-PCR, Ct (mean±SD)	32.84 ± 0.42	32.80 ± 1.18	34.87 ± 1.01	Undetectable
	RT-LAMP, no. of detectable samples (%)	0/3 (0)	0/3 (0)	0/3 (0)	Uninterpreted
10^3	concentration (ng/µL)	318.33 ± 26.13	2.57 ± 0.72	0 ± 0.10	551.37 ± 126.27
	purity (260/280)	3.46 ± 0.01	2.48 ± 0.73	0.14 ± 0.90	1.10 ± 0.01
	Real time RT-PCR, Ct (mean±SD)	Undetectable	Undetectable	Undetectable	Undetectable
	RT-LAMP, no. of detectable samples (%)	0/3 (0)	0/3 (0)	0/3 (0)	Uninterpreted

99 [a] Ct: Cycle threshold.

100 Using real time RT-PCR as measurement, RNA extracted from plasma samples using
101 the silica-membrane-based or magnetic beads-based extraction methods gave positive scores
102 in 50/50 (100%) (Table 2 and S1 Table). The RNA extracted from boiling with DEPC-treated
103 distilled water gave 76% detectable rate (38/50). Using RT-LAMP for HCV detection, RNA

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104 extracted with the silica-membrane method showed the best results with 66% detectable rate
105 (33/50), while the magnetic beads-based method showed 62% (31/50). Boiling samples with
106 DEPC-treated distilled water provided the less number of samples with HCV RNA detected;
107 only 7 of 50 (14%) tested positive (Table 2 and S1 Table). RT-LAMP results according to the
108 RNA extraction methods, are shown in Fig 1. The analysis of correlation between Ct values
109 of RNA extracted from the three different extraction methods showed an excellent correlation
110 between Ct values of samples extracted with silica-membrane based vs magnetic beads-based
111 methods (R^2 = 0.88, Fig 2A). The correlation was less good between Ct values of samples
112 extracted using silica-membrane based vs boiling with DEPC-treated distilled water methods
113 (R^2 = 0.60, Fig 2B).
114
115 **Table 2. Clinical efficacy evaluation of the viral RNA extraction methods for HCV**
116 **detection using real time RT-PCR and RT-LAMP.**

HCV viral load (log_{10} IU/mL)	**Extraction methods**					
	Silica-membrane based		**Magnetic beads-based**		**Boiling with DEPC-treated distilled water**	
	Real time RT-PCR, Ct (mean ± SD)	**RT-LAMP , no. of detectable samples (%)**	**Real time RT-PCR, Ct (mean ± SD)**	**RT-LAMP, no. of detectable samples (%)**	**Real time RT-PCR, Ct (mean ± SD)**	**RT-LAMP, no. of detectable samples (%)**
6.01-7.00 (N=10)	24.95 ± 1.64	10/10 (100)	23.19 ± 1.35	10/10 (100)	29.50 ± 1.27	3/10 (30)
5.01-6.00 (N=14)	26.76 ± 1.13	13/14 (93)	24.59 ± 0.90	12/14 (86)	30.86 ± 0.93	4/14 (29)
4.01-5.00 (N=14)	29.89 ± 1.37	9/14 (64)	27.89 ± 1.03	9/14 (64)	33.83 ± 1.07	0/14 (0)
3.01-4.00 (N=12)	32.60 ± 1.44	1/12 (8)	31.21 ± 1.23	0/12 (0)	34.11 ± 0.72	0/12 (0)
Total (N=50)	50 (100)	33 (66)	50 (100)	31 (62)	38 (76)	7 (14)

117
118

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119 **Fig 1. RT-LAMP results of all 50 clinical samples tested, according to the RNA extraction**
120 **methods.**
121
122 **Fig 2. Correlation analysis between cycle threshold (Ct) values obtained by real time RT-**
123 **PCR using extracted RNA (n =50) from silica-membrane based and magnetic beads-**
124 **based extraction methods (A) and boiling using DEPC-treated distilled water extraction**
125 **methods (B).**
126
127

128 # Discussion

129 Our study showed variable sensitivities of molecular HCV detection in plasma samples
130 depending on the RNA extraction method used. The silica-membrane based extraction method
131 combines the selective binding properties of a silica-based membrane with the speed of
132 microspin technology which employs a simple bind-wash-elute process. As noted, the eluates
133 of this kit contain both viral nucleic acids and carrier RNA which may exceed the amount of
134 authentic nucleic acids of virus when quantified by photometric method. However, the
135 extracted RNA from this method is suitable for HCV molecular diagnosis by real time RT-
136 PCR, and by RT-LAMP when using samples with a high viral load. Previous studies also
137 showed that this method was suitable for RNA isolation from plasma and ready-for-use in
138 subsequent reactions for viral detection [7, 8]. This extraction method is fast but the most
139 expensive of the four extraction methods tested. Furthermore, it requires a centrifuge for the
140 separation steps. The magnetic beads-based extraction method is based on the reversible
141 adsorption of nucleic acids to paramagnetic beads. This method uses only magnetic separator
142 for separation magnetic beads containing nucleic acids and solution. It does not need a

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143 centrifugation step but requires careful pipetting to remove the solution from the beads. In this
144 method, 200 µL plasma sample was used, compared to only 100-150 µL of sample in the other
145 three methods which may lead to an increase in the RNA extraction capacity. Previous studies
146 also recommended the magnetic bead technology for viral RNA extraction from serum or
147 plasma [9, 10]. Our results showed that Ct values from HCV samples extracted by magnetic
148 beads-based method and silica-membrane based method correlated well. Thus, it can be used
149 as an alternative extraction method. However, samples with low viral loads may cause a false
150 negative result when used with RT-LAMP detection. Boiling is a simple and rapid method to
151 release viral RNA from samples, it takes about 15 min. Previous studies suggested that simple
152 or direct boiling without any additional purification steps can be used as an alternative RNA
153 isolation method to detect viral infections in clinical samples [5, 11]. However, this method
154 yielded the lowest RNA concentration and purity, as compared to others. This might be
155 explained by a degradation of RNA during boiling and no additional step for concentration.
156 Plasma samples showed a slightly decreased sensitivity when processed by boiling prior to
157 amplification. When using real time RT-PCR for detection, the Ct values from HCV samples
158 extracted by boiling were slightly higher than those of RNA extracts by silica-membrane and
159 magnetic beads-based extraction methods and did not strongly correlate. Although the boiling
160 method could be used as a cost-effective alternative to expensive extraction methods, it can
161 only be used when samples have a high viral load. Boiling samples with a commercial lysis
162 buffer provided the highest RNA concentration, a quite low purity of RNA . This might be due
163 to the RNA carrier contained in the lysis buffer and the absence of additional step to remove
164 the lysis buffer components or purification prior to amplification. We were unable to detect
165 any HCV RNA in all RNA extracts and the results of RT-LAMP could not be interpreted. This
166 may be due to an effect of inhibitors created or released by boiling. Thus, boiling plasma
167 samples with a commercial lysis buffer cannot be used for RNA extraction in viral detection.

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168 The limitation of this study may be the relatively low number of samples and the diversity of
169 HCV genotypes tested.
170 In summary, the magnetic beads-based extraction method can be used as an alternative
171 method of plasma RNA extraction for HCV detection. This method is simple, rapid,
172 inexpensive, and does not require a centrifugation process which makes it suitable for on-site
173 diagnosis or in the field when combined with RT-LAMP technique. This approach will
174 contribute to identify new HCV viremic cases and to reaching the long-term goal of HCV
175 eradication.
176

177 ## Acknowledgements

178 We would like to thank the Division of Clinical Microbiology, Faculty of Associated
179 Medical Sciences, Chiang Mai University for specimen processing.

180 ## Funding

181 This study was supported by Chiang Mai University, Thailand.

182 **Competing interests:** The authors have declared that no competing interests exist.

183 ## Author contributions

184 **Conceptualization:** Sayamon Hongjaisee, Woottichai Khamduang
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196

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245

246 # Supporting information

247 **S1 Table. Clinical efficacy evaluation of the viral RNA extraction methods for HCV**
248 **detection using real time RT-PCR and RT-LAMP by samples**

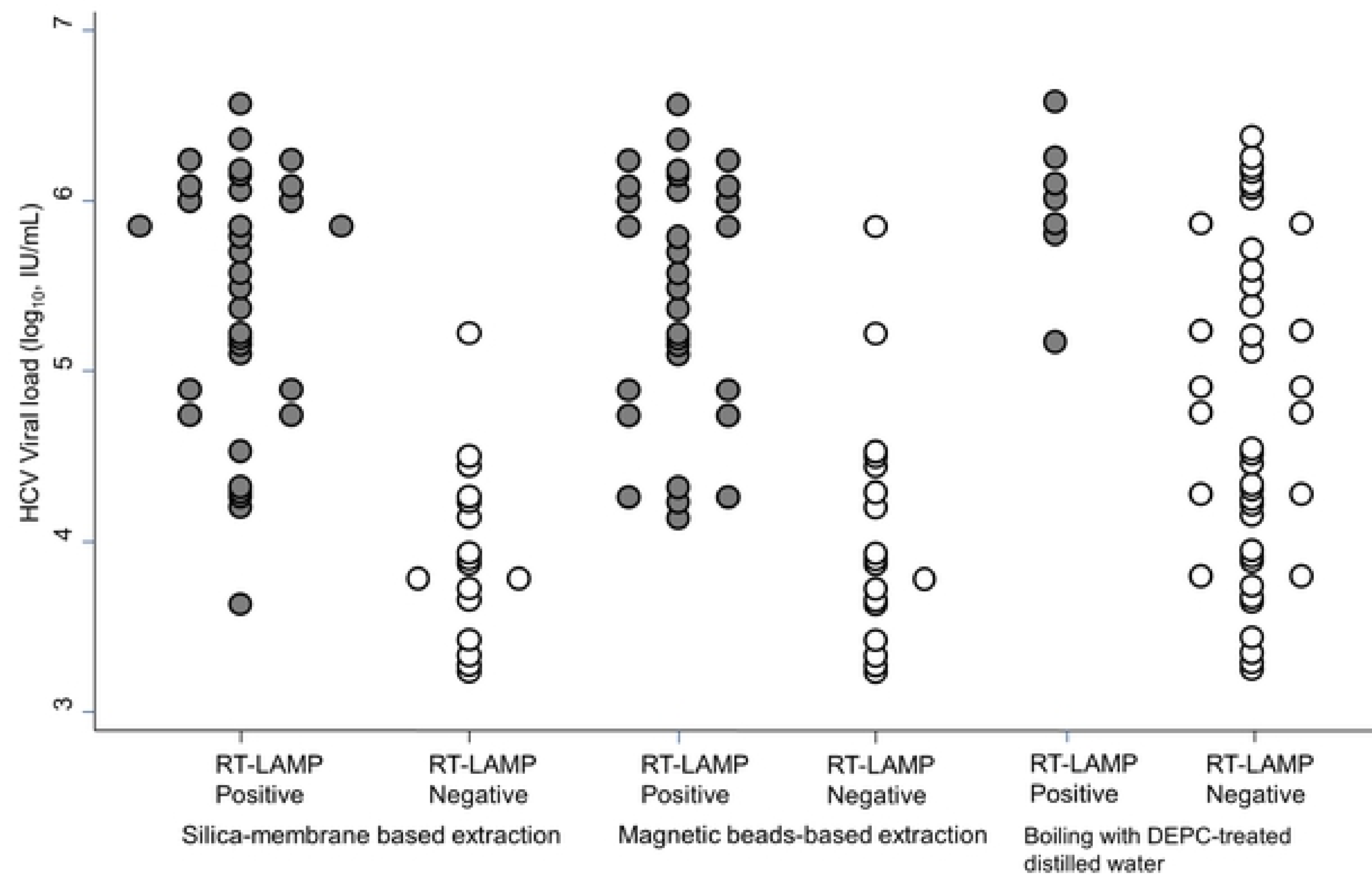
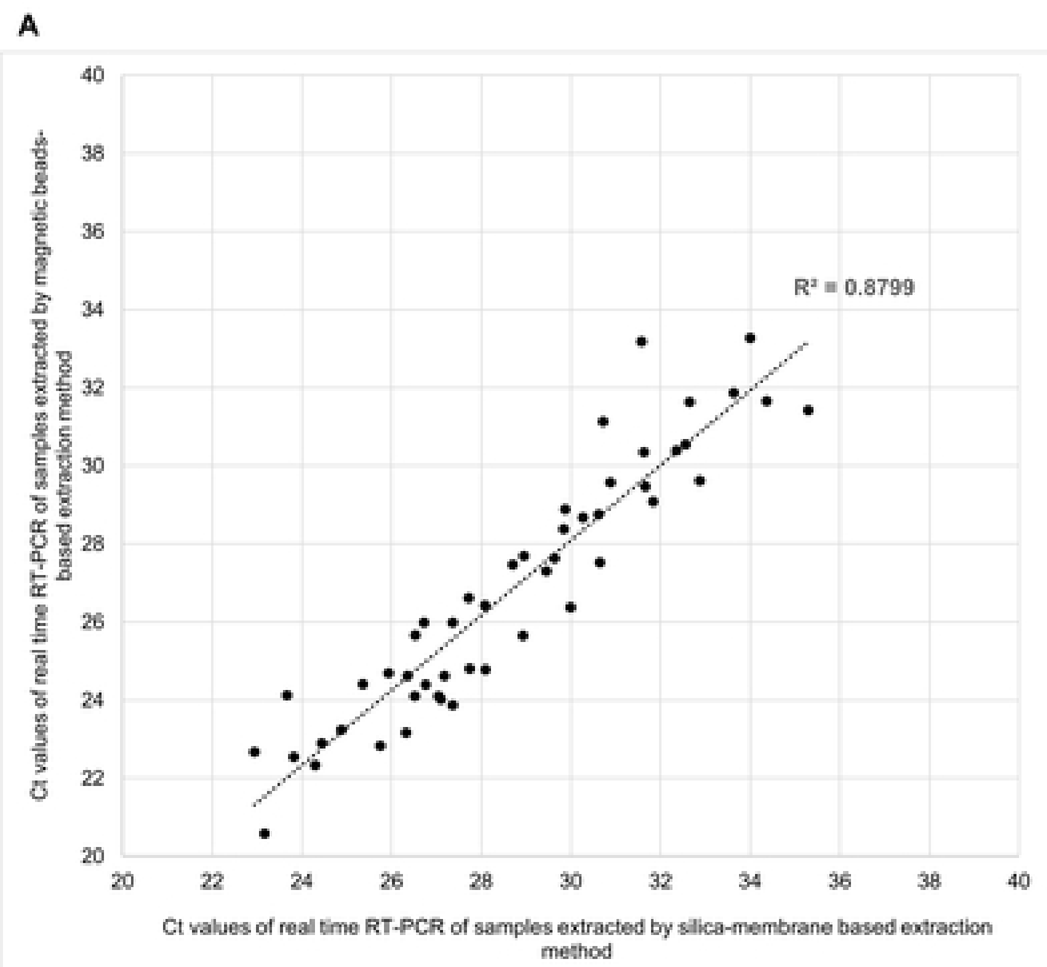


Figure 1



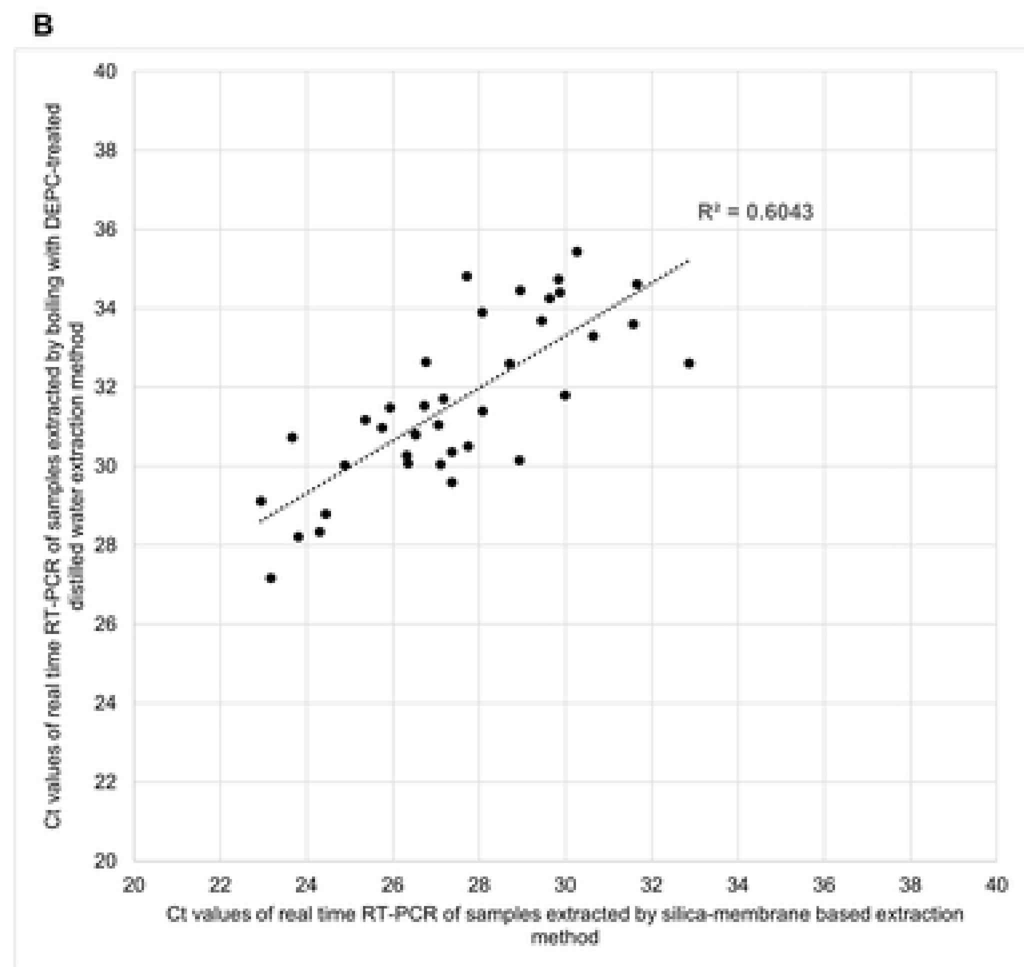


Figure 2