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1 **Sensitive detection of human adenovirus from small volume of primary**

2 **wastewater samples by quantitative PCR**

3

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14 Running title: Quantitative detection of adenoviruses in wastewater

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31 **ABSTRACT**

32 An accurate quantitative detection of enteric viruses from the primary wastewater requires,
33 sample concentration followed by extraction of nucleic acid with high purity. A highly
34 efficient and sensitive method was developed for the concentration and quantitative detection
35 of human adenovirus (HAdv) from wastewater samples. The two-step method which
36 combines concentration of virus from 10 mL sample with centrifugal filters followed by
37 extraction and purification of DNA with commercially available nucleic acid extraction kit
38 resulted in high purity DNA for downstream quantitative PCR (qPCR). The results obtained
39 on analytical sensitivities of five commercial nucleic acid extraction kits show that they differ
40 in their ability for DNA yield and purity. Nevertheless, despite variable analytical sensitivities
41 extracted nucleic acid was found to be relatively PCR inhibition free. The genomic copy
42 numbers of HAdv detected from the same concentrated wastewater sample was significantly
43 higher ($P < 0.01$) when Qiagen Blood and Tissue kit (1.54×10^6 L^{-1}) was used as compared to
44 MoBio PowerSoil kit (5.30×10^5 L^{-1}) which suggests that the nucleic acid extraction kit can
45 influence the sensitivity of qPCR assays. The method developed in this study is simple, rapid,
46 sensitive, and can be applicable for the qPCR detection of adenovirus and other DNA virus in
47 wastewater.

48

49 **Keywords:** Wastewater, adenovirus, virus concentration, PCR inhibitors, quantitative PCR

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51 **1. Introduction**

52 Population growth in urban areas and frequent droughts has led to the necessity of water
53 reclamation to alleviate water shortages. Alternative water sources such as storm water and
54 treated wastewater are increasingly considered as viable options to produce potable and non-
55 potable standard water (Toze et al., 2009; Page et al., 2010). The most significant issue in
56 relation to alternative water reuse is the public health risks associated with the potential
57 presence of viral, protozoan and bacterial pathogens.

58 Human adenoviruses (HAdv) are ubiquitous viral pathogens that cause a broad range of
59 diseases including, upper and lower respiratory tract infections, gastroenteritis, conjunctivitis,
60 and kerato-conjunctivitis (Swenson et al., 2003; Ko et al., 2005). HAdv serotypes 40 and 41
61 are among the leading causes of childhood diarrhea and occur in wastewater frequently (He
62 and Jiang 2005; Ko et al., 2005; Fong et al., 2010). HAdv has become highly important in the
63 drinking water and wastewater industries as they are among the most thermally stable and are
64 resistant to Ultraviolet light (Meng and Gerba, 1996; Gerba et al., 2002). In order to ensure
65 the production of high quality recycled water, monitoring of process indicators such as
66 adenovirus are increasingly advocated (Carducci et al., 2008; Kuo et al., 2010). HAdv and
67 polyomaviruses have been proposed as indicators for the presence of human viral pathogens
68 in the environment (Bofill-Mas et al., 2006; Fong et al., 2010).

69 PCR based methods have been used to detect and quantify enteric viruses in sewage,
70 recreational and drinking waters (Pina et al., 1998; Cho et al., 2000; Lee et al., 2004; Fong et
71 al., 2005). The PCR detection and quantitation of enteric virus in most environmental matrix
72 requires concentration and purification of the sample due to low virus numbers and presence
73 of PCR inhibitors. A wide range of enteric virus concentration techniques from wastewater
74 and environmental waters have been reported in the literature (Wyn-Jones and Selwood,
75 2001). The most commonly used techniques are ultrafiltration (Olszewski et al., 2005;

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76 Rodriguez-Diaz et al., 2009), ultracentrifugation (Nordgren et al., 2009) or adsorption elution
77 based protocols with glass wool (Gantzer et al., 1997, Lambertini et al., 2008), positively
78 charged membranes (Brassard et al., 2005; Bennett et al., 2010), negatively charged
79 membranes (Katayama et al., 2008) and immunomagnetic capture (Casas and Sunen, 2002). It
80 has to be noted that most of these methods have been developed to concentrate enteric viruses
81 from potable and non-potable quality water. Hence clogging of membranes due to high
82 turbidity is one of the major issues in the quantitative detection of enteric virus from
83 wastewater.

84 Generally, 40 mL to 1 L of primary effluent is concentrated for the quantitation of
85 enteric viruses (da Silva et al., 2007; Haramoto et al., 2008; Katayama et al., 2008). The
86 concentration of larger sample volume to improve enteric virus detection sometimes, prove
87 counterproductive for certain sample types, as PCR inhibitors are co-concentrated (Gregory et
88 al., 2006). Consequently, optimization of sample volume is required for sensitive detection of
89 enteric virus from the wastewater samples.

90 Complex matrices, such as wastewater, treated effluent and surface water contain
91 numerous organic and inorganic substances with the potential to inhibit PCR reaction
92 (Wilson, 1997; Abbaszadegan et al., 1999). Removal of PCR inhibitors from the concentrated
93 water samples is essential for successful qPCR detection of enteric viruses. A number of
94 techniques such as gel filtration (Abbaszadegan et al., 1993; Schwab et al., 1995), protein
95 coagulation (Jaykus et al., 1996), and guanidine purification (Shieh et al., 1995) have been
96 developed and used to overcome PCR inhibition with a varying degree of success.
97 Adsorption-elution methods are favoured over ultrafiltration as they result in less
98 concentration of PCR inhibitors. However, they require significant sample preparation time
99 and their efficiencies can vary due to variability in virus capsid charge and also due to cation
100 concentration in the source water (Katayama et al., 2008; Lambertini et al., 2008).

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101 Human adenovirus numbers ranging from 10^5 to 10^8 L^{-1} have been reported in
102 primary effluent from around the world (Bofill-Mas et al., 2006; Katayama et al., 2008; Fong
103 et al., 2010; Kuo et al., 2010). The reported variation in the adenovirus numbers in literature
104 could possibly be due to the variation in sample volume, virus concentration methodologies
105 and the efficiency of DNA extraction methods used. The effectiveness of methodology used
106 for the quantitative detection of enteric virus from wastewater depends upon the efficiency of
107 virus concentration procedure and reduction or removal of PCR inhibitors that inhibit the
108 activity of DNA polymerase enzyme (Lemarchand et al., 2005).

109 Commercially available DNA/RNA extraction and purification kits employ either
110 column-based techniques or include precipitation and centrifugation steps to obtain nucleic
111 acid for downstream qPCR. However, the relative efficiency and effectiveness of the DNA
112 extraction kits may vary from kit to kit (Holland et al., 2000; Tomaso et al., 2010).
113 Furthermore, a number of factors including sample volume, DNA yield and purity may also
114 affect the qPCR assays. Consequently, virus concentration and DNA purification
115 methodology need to be optimized for the reliable quantitation of enteric virus numbers in
116 wastewater.

117 The main aim of this study was to determine the optimum methodology for the
118 quantitative detection of DNA virus such as adenovirus from small volumes of primary
119 wastewater samples. In order to achieve this we investigated: (i) if low sample volume (10
120 mL) could be used for the consistent quantitation of adenovirus from the primary effluent (ii)
121 the influence of DNA extraction methods on the quantitative detection of human adenovirus
122 in the wastewater samples (iii) evaluate performance of five commercial DNA extraction kits
123 for removing PCR inhibitors from the concentrated primary wastewater samples.

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127 **2. Material and methods**

128 *2.1. Wastewater sample collection and concentration*

129 Grab samples of primary influent were collected from two (A and B) wastewater
130 treatment plants (WWTPs) in Brisbane, Australia on five different occasions. On each
131 sampling occasion 100 mL influent sample was collected after the grit screens in sterile
132 carboy containers (Nalgene). Collected samples were transported on ice to the laboratory and
133 stored at 4ºC prior to virus concentration.

134 Each primary effluent sample was split into three replicates (10 mL each) and
135 concentrated with Amicon® Ultra centrifugal filters (Ultracel - 50K) (Millipore, Billerica,
136 USA) device in triplicate. Ten mL replicates from sample was added to three Amicon®
137 column and centrifuged at 1,500 g for 10 min to obtain a final volume of 750 µL. The final
138 concentrate from each replicate sample was aliquoted into five equal volumes of 150 µL each.
139 All aliquots (15) from each sample were then stored at -80 ºC.

140

141 *2.2. Viral DNA extraction, purification and yield*

142 Viral DNA was extracted from each of three replicates using five commercial DNA
143 extraction kits. The starting volume was adjusted from 150 µL aliquots to the recommend
144 volume for each DNA extraction kit with Ultra pure water (Invitrogen, Carlsbad, USA) so
145 that starting adenovirus numbers in the primary effluent remain same for each extraction kit.
146 The kits used in this study were Macherey-Nagel Nucleospin (NS) (Düren, Germany),
147 Epicentre MasterPure complete DNA and RNA purification kit (EC) (Madison, USA),
148 Qiagen DNeasy Blood and Tissue kit (BT) (Qiagen, Valencia, USA), QIAamp® DNA Stool
149 Mini kit (ST) (Qiagen, Valencia, USA) and Mo-Bio PowerSoil DNA isolation kit (MB)
150 (MOBIO, Carlsbad, USA).

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151 BT and NS kits use similar process for the extraction and purification of DNA
152 whereby, chaotropic properties of guanidine thiocyanate along with proteinase K are used for
153 cell lysis and inactivation of cell nucleases followed by silica-column based isolation of DNA.
154 The EC kit uses proteinase K lysis, protein precipitation step followed by DNA precipitation
155 (isopropanol) and collection of DNA pellet after removal of RNA. The ST kit uses proteinase
156 K lysis proprietary along with proprietary InhibitEX tablets, for the removal of PCR inhibitors
157 followed by a silica column-based isolation of DNA. The MB kit uses bead beating along
158 with proprietary DNA elution method followed by silica-column based capture of DNA.
159
160 *2.3. DNA yield*
161 After extraction of DNA as per manufactures instructions, resulting DNA was tested
162 for yield and purity. The final extraction volumes were 50 µL buffer RE (NS), 35 µL TE
163 buffer (EC), 200 µL buffer AE (BT and ST), and 100 µL solution C6 (MB). DNA from all
164 samples was quantified using NanoDrop ND-1000 spectrophotometer (Wilmington, DE) and
165 then stored at -80 ºC for quantitative detection of HAdv. To prevent false positive results,
166 method and reagent blanks were included for each batch of wastewater samples.
167
168 *2.3. Cultures used in this study*
169 *Pseudomonas aeruginosa* (ATCC 15692) host was cultured in the nutrient broth at 37
170 °C overnight in shaking platform incubator. The host culture was inoculated with
171 bacteriophage PP7 (ATCC 15692-B2) and further incubated overnight at 37°C in static
172 incubator. Bacteriophage PP7 was purified by centrifugation at 3,000 g for 5 min and
173 resulting suspension was passed through 0.2 µm membrane filter and stored at 4°C.
174 Adenovirus strain 41 (ATCC VR-930) was cultured in LLK cell line by the Pathology Centre,
175 WA. The virus was then harvested and frozen at -80ºC.

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176 *2.4. PCR primers and cloning of target gene sequences*

177 The PP7 primer set was designed in this study targeting coat protein gene (GenBank
178 number NC 001628) using Primer3 software. A homology search was performed against the
179 GenBank database sequence similarity using BLAST program
180 (http://www.ncbi.nlm.nih.gov/BLAST/). The analysis indicated that designed primer pair was
181 specific for the bacteriophage PP7. Wastewater samples (*n* = 5) were also tested for the
182 presence of bacteriophage PP7 with the newly designed primer set and were PCR negative.
183 The primer sequences used for the bacteriophage PP7 qPCR were: forward primer 5´- CCA
184 AAA CCA TCG TTC TTT CG - 3´ and a reverse primer 5´- GTC CGC CTG ATC CAG TTT
185 TA - 3´ (location: Forward; 1433-1453, Reverse; 1620-1640). HAdv qPCR was carried out
186 by amplifying hexon gene (location: Forward;18971-18993, Reverse;18861-18885) using
187 previously published primer set; forward primer 5´- GCC ACG GTG GGG TTT CTA AAC
188 TT - 3´ and reverse primer 5´- GCC CCA GTG GTC TTA CAT GCA - 3´ (Heim et al., 2003).
189 This primer set detects all 51 types of human adenovirus.

190 The PCR amplified products (i.e., cDNA/DNA) for both PP7 and adenovirus were
191 purified using the QIAquick PCR purification kit (Qiagen), and cloned into the pGEM®-T
192 Easy Vector System (Promega Madison, WI, USA), transferred into *E. coli* JM109 competent
193 cells and plated on LB agar ampicillin, IPTG (isopropyl-β-D-thio-galactopyranoside) and X-
194 Gal (5-bromo-4-chloro-3-indolyl-β- D-galactopyranoside) as recommended by the
195 manufacturer. Plasmid was purified using plasmid mini kit (Qiagen). DNA sequencing was
196 carried out at the Australian Genome Research Facility (St. Lucia, Queensland, Australia).
197 The cloned material from both PP7 and adenovirus were used for preparing standards for
198 qPCR.

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201 *2.5. Preparation of standard curves*

202 Purified plasmid DNA containing the PP7 and adenovirus inserts were quantified
203 using a spectrophotometer (NanoDrop ND-1000). Plasmid copies were calculated, and a ten-
204 fold serial dilution was prepared in DNase and RNase free water to a final concentration
205 ranging from 10^0 to 10^6 copies μL^{-1} and aliquots were stored at -80ºC until use. Three
206 microliter, template from of each dilution was used to prepare standard curve for qPCR.

207

208 ***2.6.*** *Assessment of PCR inhibitor removal efficiency*

209 In order to determine the effectiveness of DNA extraction kits to remove PCR
210 inhibitors from concentrated wastewater samples, DNA isolated using five DNA extraction
211 kits were spiked with known number (10^3 gene copies) of bacteriophage PP7 cDNA. Same
212 number of PP7 cDNA was also seeded into Ultrapure pure water (Invitrogen, Carlsbad, CA)
213 to determine the threshold cycle (C_T) value for 10^3 gene copies in PCR inhibition
214 environment. The threshold cycle (C_T) values of these spiked DNA samples were compared
215 with the C_T values from the Ultrapure pure water samples spiked with the PP7.

216

217 **2.7.** *PCR amplification*

218 Quantitative PCR assays were carried out to determine the gene copy numbers of
219 seeded PP7 cDNA and adenovirus numbers in the primary wastewater samples collected from
220 the WWTP A and B. Quantitative PCR reaction were performed on Bio-Rad iQ5 (Bio-Rad
221 Laboratories, California, USA), using iQ Supermix with SYBR green (Bio-Rad). Each 25µL
222 PCR reaction mixture contained 12.5 µL of SuperMix, 120 nM of each primer, and 3 µL of
223 template DNA. Bovine serum albumin (BSA) was added to each reaction mixture to a final
224 concentration of 0.2 µg μL^{-1} to relieve PCR inhibition (Kreader, 1996). For each PCR run,
225 corresponding positive (i.e., target DNA) and negative (sterile water) controls were included.

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226 Thermal cycling conditions for PP7 were: initial denaturation at 95ºC for 5 min, followed by
227 40 cycles at 95ºC for 10 s, 60ºC for 10 s, 72ºC for 10 s with a final extension of 5 min at 72ºC.
228 Thermal cycling conditions for HAdv were as outlined in Sidhu et al., (2010). A melt curve
229 analysis was performed after the PCR run to differentiate between actual products and primer
230 dimmers, and to eliminate the possibility of false-positive results. The melt curve was
231 generated using 80 cycles of 10 s each starting at 55°C and increasing in 0.5°C intervals to a
232 final temperature of 95°C. The Tm for each amplicon was determined using the iQ5 software
233 (Bio-Rad).
234

235 **2.8.** *PCR reproducibility and limit of detection*

236 The reproducibility of the qPCR was assessed by determining intra-assay repeatability
237 and inter-assay reproducibility. The Coefficient of Variation (CV) was calculated using six
238 dilutions (10^6 to 10^0 gene copies) of the PP7 and adenovirus plasmid DNA. Each dilution was
239 tested in triplicate. The CV for evaluation of intra-assay repeatability was calculated based on
240 the C_T value by testing the six dilutions six times in the same experiment. The CV for inter
241 assay reproducibility was calculated based on the C_T value of six dilutions on six different
242 days. To determine the qPCR limit of the detection, known gene copies (i.e., 10^6 to 10^0) of the
243 PP7 and adenoviruses were tested with the qPCR. The lowest numbers of gene copies that
244 were detected consistently in replicate assays was considered as the qPCR limit of detection.
245

246 **2.9. Statistical analysis**

247 Analysis of variance (ANOVA) was performed to determine the differences in DNA
248 yields obtained with different DNA extraction kits and differences between the C_T values
249 obtained for distilled water and wastewater samples spiked with known gene copies of
250 bacteriophage PP7. In all cases, significance level was set at 95% confidence. In case a

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251 significant difference was obtained by ANOVA, additional test (Tukey's significant
252 difference test) was performed.
253
254
255 **3. Results**
256
257 **3.1.** ***Comparison of DNA extraction efficiencies***
258 The yield of genomic DNA extracted from the concentrated wastewater samples was
259 determined using the conventional method of absorbance at 260 nm. The five commercial kits
260 successfully extracted DNA from all wastewater samples with DNA yield varying between
261 three to 500 ng μL^{-1} (Table 1). The DNA yields obtained by using NS and EC kits were
262 significantly higher ($P < 0.001$) from BT, ST and MB kits. The data on the DNA yields
263 obtained from all samples (WWTP A and B) for each kit was pooled as they were not
264 significantly different within each kit ($P > 0.05$). An ANOVA was performed to determine if
265 there was a significant difference between DNA yields obtained from different extraction kits.
266 No significant ($P > 0.05$) difference in the DNA yields was observed between BT, ST and
267 MB kits. The final extraction volume for EC, NS and MB kits was lower (35-50µL) as
268 compared to ST and BT kits (200 µL) (Table 2), therefore, for a direct comparison, DNA
269 yield obtained from all kits was normalized to 200 µL. Despite the normalization, the NS kit
270 produced approximately five times more DNA than EC and BT, and 20-30 times more DNA
271 than ST and MB kits (Table 1). The A260/A280 ratio for EC and BT was 1.78 ± 0.04 and
272 1.85 ± 0.05 respectively, suggesting high purity of DNA. Whereas, A260/A280 ratios for NS,
273 ST and MB kits were above 1.80 indicating the presence of impurities in the extracted DNA.
274
275 *3.2. PCR inhibition removal efficiency of kits*

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276 The PP7 cDNA C_T values from qPCR assays with wastewater samples were compared
277 to those obtained from the same concentration of cDNA spiked into the PCR mixture
278 containing ultrapure water. Comparative C_T values data obtained from DNA extraction with
279 five different extraction kits is presented in Table 2. The mean C_T value for the spiked
280 Ultrapure water was 25.9. Whereas, C_T values for the wastewater samples extracted with
281 different kits varied between (25 to 27). The results of one-way ANOVA performed to
282 determine the difference between the C_T values obtained for Ultrapure water and those
283 obtained for DNA isolated from wastewater using various kits suggest no significant
284 differences ($P > 0.05$) between the C_T values.
285

286 *3.3. PCR reproducibility and limit of detection*

287 Ten-fold serial dilutions of plasmid DNA containing adenovirus insert were analysed
288 in order to determine PCR reaction efficiencies. The amplification efficiencies were > 95%
289 and the correlation coefficient (r^2) was > 0.99. The reproducibility of the qPCR assay was
290 determined by assessing intra-assay and inter-assay coefficient of variation (CV) of the
291 standards. The mean CV values for intra-assay and inter-assay were: less than 4% and 3%,
292 respectively indicating high reproducibility. The PCR limit of detection for assays were
293 performed by analysing purified plasmid DNA. To determine the reproducibility of the assay,
294 serial dilution of DNA ranging from 10^7 to 10^0 were tested in triplicate. Quantitation of the
295 target gene was sensitive and specific over a 7 log dynamic range. The PCR detection limit
296 was found to be between one and ten gene copies.

297 *3.4. Quantification of HAdv in wastewater samples*

298 HAdv were consistently detected in high numbers from the primary wastewater
299 samples collected from both WWTPs (Figure 1). The HAdvs numbers ranged between 3.34 x
300 10^5 to 4.72 x 10^6 (mean 1.54 x 10^6 L^{-1}) with NS kit, 7.72 x 10^5 to 9.49 x 10^6 (mean 3.35 x 10^6

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301 L^{-1}) with EP kit, 9.50 x 10^5 to 1.77 x 10^7 (mean 5.35 x 10^6 L^{-1}) with BT kit, 2.31 x 10^5 to 1.74
302 x 10^6 (mean 1.01 x 10^6 L^{-1}) with ST kit and 1.04 x 10^5 to 1.36x 10^6 (mean 5.30 x 10^5 L^{-1}) with
303 MB kit. In general, BT kit provided higher HAdv numbers as compared to the other kits used
304 in this study. The data on the HAdv numbers from both WWTP's for each kit was pooled and
305 one way ANOVA was performed to determine if there was a significant difference between
306 the kits for the HAdv numbers. The difference between the HAdv numbers detected with BT
307 kit and MB kit was highly significant ($P < 0.01$). Similarly, BT kit resulted in significant ($P <$
308 0.05) higher detection in HAdv as compared to ST and NS kit. No significant ($P > 0.05$)
309 difference was observed between the HAdv numbers obtained from the NS, EP, ST and MB
310 kits.
311

312 **4. Discussion**

313 The detection of enteric virus in the wastewater is mainly hampered by low virus numbers
314 and the presence of substances inhibitory to PCR. Consequently, concentration of a smaller
315 sample volume combined with recovery of high quality nucleic acid from the wastewater
316 samples is a critical step for the quantitative detection of enteric virus. Concentration of large
317 volume of wastewater to improve detection of enteric virus could be counterproductive due to
318 co-isolation of inhibitors, which is shown to increase with larger sample volume (Gregory et
319 al., 2006). Albinana-Gimenez et al., (2009) reported a reduction in the numbers of adenovirus
320 and polyomavirus detected from river water with an increase in sample volume. By reducing
321 sample volume, the capture of inhibitory compounds could be reduced, improving purity of
322 the extracted DNA, which subsequently improves detection limit of qPCR. In this study, a
323 smaller sample volume (10mL) was used to improve the quality of extracted DNA, which was
324 successfully used for downstream quantitative detection of adenovirus in the primary effluent
325 samples.

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326 DNA extraction kits have been widely used because of the rapidity of DNA recovery
327 and reproducibility. However, their efficiencies regarding DNA yield, DNA quality and
328 downstream performance in PCR is still questioned (Holland et al., 2000; Tomaso et al.,
329 2010). Performance of five different DNA extraction kits was evaluated for their relative
330 efficacy in extraction and purification of nucleic acid from the wastewater samples. No
331 significant difference ($P > 0.05$) in DNA yield was observed between samples (from both
332 WWTP's) extracted with each kit which suggests that all kits performed consistently with
333 primary wastewater samples. The total DNA yields obtained with ES and NS kits were
334 significantly higher (i.e., 100 to 400 ng μL^{-1}) than the MB, ST and BT kits (4 to 20 ng μL^{-1}).
335 A wide variation in the DNA yields was observed between the kits after normalization of final
336 extraction volume. The NS kit yielded four fold higher DNA than EC, 20 fold higher than
337 BT, and 100 fold higher than ST and MB kits. Our findings are consistent with the previously
338 reported results whereby three to eight fold variation in DNA yield have been reported
339 (Scupham et al., 2007; Ariefdjohan et al., 2010).

340 The DNA is generally considered to be of adequate quality when the A260/A280 ratio
341 is between 1.70 and 2.0. EP and BT kits provided DNA within the ideal range whereas,
342 extraction with NS, ST kits consistently resulted in A260/A280 ratio higher than 1.80.
343 Although, NS kit provided highest amount of DNA however, it also had high impurities with
344 A260/A280 ratio consistently higher than 2.8 (Table 1). Whereas, ST and MB kits provided
345 DNA of intermediate quality with ratio between 1.8 to 2.64. These findings corroborate with
346 the observations made by the other researchers while isolating DNA from environmental
347 samples with different extraction procedures and extraction kits (Martin-Laurent et al., 2001;
348 Lemarchand et al., 2005; Dauphin et al., 2010; Urakawa et al., 2010).

349 Our qPCR method was sensitive and specific over a seven log dynamic range for the
350 detection of PP7 coat protein gene (cDNA). The PCR detection limit was found to be between

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351 one and ten gene copies. A comparison between C_T values obtained from PP7 qPCR assays
352 with wastewater samples and those obtained from the same concentration of cDNA spiked
353 into ultrapure water suggest that all kits provided good quantitative detection of PP7 cDNA,
354 as no significant difference ($P > 0.05$) in the C_T values was observed between ultrapure water
355 and wastewater samples extracted with different kits. The lowest detection limit for PP7
356 cDNA was determined based on the lowest amount that can be consistently and accurately
357 detected, which was between one to ten gene copies per reaction from the wastewater
358 samples. This detection limit was comparable to six gene copies reported previously for PP7
359 (Rajal et al., 2007).

360 In this study, adenovirus was detected in wastewater from small sample (10 mL) with
361 all tested DNA extraction kits. The average adenovirus numbers observed (10^6 L^{-1}) in this
362 study correspond well to numbers reported previously from 10^5 to 10^8 L^{-1} in the wastewater
363 where sample volumes ranging from 10mL to 1L were used (He and Jiang, 2005; Carducci et
364 al., 2009; Fong et al., 2010). However, selection of the DNA kit had a significant influence
365 on the sensitivity of qPCR assays for the detection of adenovirus from the wastewater
366 samples. As average genomic copy numbers of HAdv detected from the same primary
367 wastewater sample was significantly higher ($P<0.01$) with BT kit (1.54×10^6 L^{-1}) as
368 compared to MB (5.30×10^5 L^{-1}).

369 Sensitivity of real time qPCR assays could be influenced by the quality of the
370 extracted DNA. Commercial extraction kits have reported to provide variable DNA purity
371 and subsequently influence sensitivity of PCR assays (Queipo-Ortuno et al. 2008; Dauphin et
372 al. 2010). This certainly appears to be the case in this study, the BT kit yielded DNA with
373 highest purity which then resulted in highest numbers of adenovirus detected in primary
374 wastewater (Figure 1). The NS kit provided highest total DNA yield with high impurities,
375 however, it still resulted in the detection of high adenovirus numbers which were

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376 comparatively higher than the MB and ST kits. Conversely, the two kits that yielded the least
377 amount of DNA (MB and ST) with on an average A260/A280 ratio of 2, resulted in lower
378 detection level. It appears that total DNA yield did not influence the results of qPCR for all
379 kits, which suggests that DNA purity has also significant influence on the sensitivity of
380 detection of DNA virus from wastewater samples.

381 The adenovirus concentration and quantitation approach used in this study consistently
382 detected low numbers of virus from 10 mL wastewater samples. However, wastewater is
383 known to have variable consistency and composition, which could result in low DNA yield or
384 purity if higher sample volumes are used. Consequently, extracted nucleic acid needs to be
385 checked for the presence of PCR inhibition. Although, EC kit provided good results, it
386 required careful purification of DNA pellet, increasing the chances of partial loss of pellet
387 along with reduced sensitivity.

388 In conclusion, virus concentration and nucleic acid purification method used in this study
389 is rapid, reliable and consistently provided PCR inhibition free DNA which could be
390 applicable for the quantitative detection of other DNA virus such as polyomaviruses and
391 torqueteno viruses from the wastewater. Application of this method will make it possible to
392 reliably quantify DNA virus load in the primary wastewater samples and hopefully lead to
393 better risk assessment. Further investigations could be carried out to assess the applicability
394 of the method used in this study to detect accurately RNA virus such as norovirus and
395 rotavirus from primary wastewater.

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405 **5. References**

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Table 1. DNA yields (ng µL^{-1}) from wastewater samples collected from two wastewater treatment plants (WWTPs) using five DNA extraction kits

Sampling dates	Mean ± SD of DNA yields (ng/µL) and DNA purity (260/280 ratios)									
	Nucleospin (NS)		Epicentre (EP)		DNeasy Blood and Tissue kit (BT)		QIAamp DNA Stool kit (ST)		Mo-Bio soil DNA kit (MB)	
	yield	Purity	yield	Purity	yield	Purity	yield	Purity	yield	Purity
WWTP A										
S1	379 ± 29.5	3.06 ± 0.06	120 ± 66.5	1.72 ± 0.04	16.0 ± 3.21	1.76 ± 0.14	3.91 ± 0.93	2.15 ± 0.31	6.41 ± 0.62	1.62 ± 0.28
S2	442 ± 40.6	2.95 ± 0.03	85.6 ± 10.6	1.80 ± 0.02	22.2 ± 2.40	1.88 ± 0.10	4.56 ± 0.55	1.94 ± 0.38	4.73 ± 1.43	1.87 ± 0.25
S3	401 ± 33.1	2.90 ± 0.08	109 ± 20.6	1.82 ± 0.02	27.2 ± 0.83	1.88 ± 0.05	5.10 ± 0.41	2.22 ± 0.23	4.76 ± 0.41	2.14 ± 0.40
S4	486 ± 68.8	2.88 ± 0.08	114 ± 17.2	1.81 ± 0.03	37.4 ± 10.3	1.91 ± 0.03	5.75 ± 0.63	2.27 ± 0.38	3.80 ± 0.46	2.20 ± 0.19
S5	373 ± 49.8	3.07 ± 0.12	108 ± 37.4	1.75 ± 0.03	20.1 ± 8.16	1.78 ± 0.06	3.00 ± 0.44	1.83 ± 0.28	3.35 ± 0.21	2.68 ± 0.84
WWTP B										
S1	362 ± 15.6	3.04 ± 0.09	85.3 ± 16.2	1.73 ± 0.03	18.4 ± 7.40	1.84 ± 0.05	4.32 ± 1.00	2.05 ± 0.45	5.84 ± 0.53	1.75 ± 0.35
S2	448 ± 35.5	2.90 ± 0.10	130 ± 37.2	1.84 ± 0.09	25.7 ± 7.63	1.88 ± 0.03	5.53 ± 1.25	2.03 ± 0.23	4.66 ± 1.68	1.82 ± 0.22
S3	432 ± 15.7	2.84 ± 0.14	164 ± 41.3	1.81 ± 0.03	29.4 ± 2.68	1.92 ± 0.03	6.21 ± 1.65	2.06 ± 0.27	4.61 ± 0.44	2.34 ± 0.46
S4	419 ± 55.7	3.03 ± 0.08	101 ± 11.8	1.80 ± 0.02	25.8 ± 5.84	1.87 ± 0.05	4.50 ± 0.12	2.14 ± 0.33	4.10 ± 0.80	2.64 ± 0.55
S5	398 ± 41.7	3.05 ± 0.14	136 ± 1.53	1.72 ± 0.03	20.2 ± 2.85	1.84 ± 0.06	4.53 ± 0.53	2.00 ± 0.48	3.57 ± 0.33	2.29 ± 0.91

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Table 2. A comparison between threshold cycle (C_T) value obtained with seeded bacteriophage PP7 in DNA extracted from wastewater with different DNA extraction kits.

Sampling dates	**Threshold cycle (C_T) (Mean ± SD)**				
	Nucleospin (NS)	**Epicentre (EP)**	**DNeasy blood and tissue kit (BT)**	**QIAamp DNA stool kit (ST)**	**Mo-Bio soil DNA kit (MB)**
WWTP A					
S1	26.0 ± 0.05	25.0 ± 0.17	25.3 ± 0.16	26.4 ± 0.39	26.3 ± 0.13
S2	25.7 ± 0.03	25.9 ± 0.13	25.2 ± 0.10	26.3 ± 0.33	26.3 ±0.16
S3	26.1 ± 0.05	28.2 ± 0.11	25.2 ± 0.03	26.5 ± 0.11	25.6 ±0.27
S4	27.2 ± 0.15	26.6 ± 0.93	26.4 ± 0.16	26.3 ± 0.16	26.1 ±0.11
S5	26.0 ± 0.12	26.5 ± 0.06	26.4 ± 0.26	26.1 ± 0.09	25.9 ±0.88
WWTP B					
S1	26.4 ± 0.30	26.0 ± 0.33	25.3 ± 0.08	26.5 ± 0.17	26.2 ±0.17
S2	26.4 ± 0.39	29.8 ± 0.60	25.3 ± 0.08	26.2 ± 0.16	26.4 ±0.10
S3	26.4 ± 0.11	28.1 ± 0.63	26.1 ± 0.04	26.5 ± 0.14	26.2 ±0.07
S4	26.2 ± 0.11	29.4 ± 0.17	26.3 ± 0.11	26.1 ± 0.22	26.0 ±0.24
S5	26.5 ± 0.07	29.8 ± 0.44	26.7 ± 0.12	26.3 ± 0.11	25.9 ±0.31

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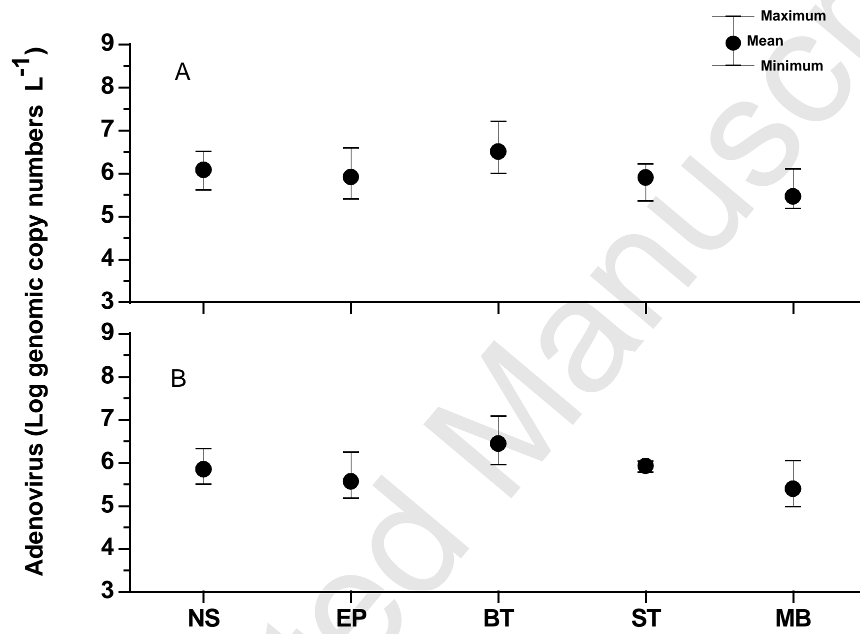

Figure 1. Adenovirus numbers detected in wastewater treatment plants (WWTPs) A and B with commercial DNA extraction kits. NS= Nucleospin, EP= Epicentre, BT= Qiagen, DNeasy Blood and Tissue, ST= QIAamp DNA Stool and MB= Mo-Bio soil DNA

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* Efficient method for sensitive and reproducible quantification of adenovirus.
* Ten mL wastewater concentrated and purified with centrifugal filters.
* Sensitivity of qPCR depends upon nucleic acid extraction method used.
* Method applicable for the qPCR detection of other DNA virus in wastewater.

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