

1 Evaluation of commercial qPCR kits for detection of SARS-CoV-2 in pooled samples

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4

5 **Abstract:** Due to the current pandemic, global shortage of reagents has drawn interest in  
6 developing alternatives to increase the number coronavirus tests. One such alternative is sample  
7 pooling. Here we compared commercial kits that are used in COVID-19 diagnostics, in terms of  
8 sensitivity and feasibility for use in pooling. We showed that pooling of up to 60 samples did not  
9 affect the efficiency of the kits. Also, the RNA dependent RNA polymerase (RdRp) is a more  
10 suitable target in pooled samples than the Envelope (E) protein. This approach could provide an  
11 easy method of screening large number of samples and help adjust different government  
12 regulations.

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## 32 **Introduction**

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34 The recent emergence of the novel severe acute respiratory syndrome coronavirus 2  
35 (SARS-CoV-2) in December 2019 from Wuhan, China, has caused more than 5 million cases  
36 with an estimate of more than 300 000 deaths associated with coronavirus disease (COVID-19)  
37 (1). Clinical manifestation of COVID-19 infection is variable ranging from asymptomatic to  
38 severe disease, with symptoms including respiratory distress, fever, cough, dyspnea, and viral  
39 pneumonia (2). Since there is currently no targeted therapeutics against SARS-CoV-2 and  
40 clinical manifestations are not disease specific, diagnostic screening and implementation of strict  
41 biosecurity measures issued by governments are limiting the spread of the disease (3). The most  
42 widely used molecular method approved by the World Health Organization (WHO) and Centers  
43 for Disease Control and Prevention (CDC) to detect SARS-CoV-2 is the real-time reverse  
44 transcription-polymerase chain reaction (qRT-PCR) (4). In the case of a public health  
45 emergency, most of diagnostic laboratories worldwide can rely on this technology to routinely  
46 perform diagnostic services until standardized tests are widely available. Different PCR assays  
47 were rapidly developed to target the ORF1a/b, ORF1b-nsp14, RdRp, S, E, or N gene of SARS-  
48 CoV-2 and other related betacoronaviruses, such as the closely related SARS-CoV (3, 5). The  
49 majority of qPCR tests are using different sample matrixes, represented by either swabs or  
50 sputum, since they contain relatively high titer virus, due to the initial viral replication in the  
51 upper respiratory tract (6). However, the global need for a new surveillance approach reflects the  
52 requirement to adapt to the increased demand of number of molecular tests to adjust the  
53 lockdown policies.

54 Diagnostic pooling has been already shown to be effective both in veterinary medicine,  
55 detecting various diseases induced by swine influenza, African swine fever virus or foot-and-  
56 mouth disease virus (7; 8; 9) and in human medicine for human immunodeficiency virus (HIV)  
57 and other transfusion-transmittable diseases (10; 11). Recently, the same approach showed  
58 encouraging results for SARS-CoV-2 when a pool of up to 7 samples was used before the  
59 extraction and up to 60 samples could be pooled after (12).

60 Therefore, our main goal was to evaluate and compare some commercial kits currently  
61 used for COVID-19 diagnostics, using the sample pooling approach. We also showed that the

62 high sensitivity of RNA dependent RNA polymerase (RdRp) compared to other targets, for  
63 detection of SARS-CoV-2 in pooled samples.

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## 65 **Materials and methods**

### 66 *Sample collection and processing*

67 Samples included in this study consisted of swabs collected from both nostrils and the  
68 throat or sputum, following the WHO recommendations by healthcare providers and sent to the  
69 Molecular laboratory VetWork Diagnostics, Tulcea, Romania and Personal Genetics, Bucharest,  
70 Romania. A volume of 200  $\mu$ l of the transport swab buffer was mixed with 500  $\mu$ l lysis buffer and  
71 RNA was extracted using Power Prep Viral DNA/RNA Extraction kit, (Kogene Biotech, Seoul,  
72 Republic of Korea). Sputum samples were mixed with equal volume of PBS and processed as  
73 described above. We obtained samples tested between April 20-27, 2020. Samples collected from  
74 24 confirmed COVID-19 patients were extracted, aliquoted and stored at  $-80^{\circ}\text{C}$  until use. Negative  
75 samples were collected from 60 healthy volunteers with no COVID-19 associated symptoms.

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### 77 *RNA extraction*

78 Extraction of RNA was performed with Power Prep Viral DNA/RNA Extraction Kit  
79 (Kogene biotech, Seoul, Korea) according to the manufacturer's instruction. After lysis, samples  
80 were incubated at room temperature for 10 minutes, after incubation 700  $\mu$ l of binding buffer was  
81 added, followed by vortex and spin. Supernatant was passed through the Binding Column. The  
82 columns were washed two times with 500  $\mu$ l Wash Buffer A first and Wash Buffer B second. At  
83 the final 50  $\mu$ l of Elution Buffer was added and incubated for 1 minute at room temperature,  
84 followed by a spin at 13.000 rpm for one minute and eluted RNA collected.

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### 86 *Real Time PCR analysis*

87 The RNA samples were treated with PowerCheck 2019-nCoV Real-time PCR Kit (Kogene  
88 biotech, Seoul, Korea), COVID-19 PCR Diatheva Detection Kit (Diatheva, Cartoceto, Italy) and  
89 2019 nCoV CDC EUA KIT (IDT DNA, Coralville, Iowa, USA) mixed with FastGene Probe One  
90 Step Mix (Nippon Genetics Europe GmbH, Duren, Germany). The high specificity of the  
91 commercial assays is based on the unique sequence of the primers specific for the SARS-CoV-2

92 genomic sequence along with optimal PCR conditions used for amplification. This assay does not  
93 cross-react with other respiratory viruses.

94 PowerCheck 2019-nCoV Real-time PCR Kit provides testing solution for Wuhan  
95 coronavirus, specifically targeting the E gene for beta Coronavirus and the RdRp gene for 2019-  
96 nCoV in bronchoalveolar lavage fluid, sputum, nasopharyngeal swab and oropharyngeal swab. Kit  
97 contains: RT-PCR mix, Primer/Probe mix 1 (E gene), Primer/Probe mix 2 (RdRp gene), control 1  
98 (E gene) and control 2 (RdRp gene). Protocol for PowerCheck 2019-nCoV Real-time PCR Kit is:  
99 11 ul are mixed with 4 ul of each primer/probe mix and 5 ul of template RNA with a total volume  
100 of 20 ul.

101 COVID-19 PCR Diatheva Detection Kit allows the qualitative detection of SARS-CoV-2  
102 RNA in upper and lower respiratory samples, is a One-step real-time reverse transcription  
103 multiplex assay based on fluorescent-labelled probe used to confirm the presence of RdRp gene  
104 and E gene. The assay includes also RNase P target as internal positive control (IC). Protocol for  
105 COVID-19 PCR Diatheva Detection Kit used is: 5 ul of mix 1 are mixed with 0.625 ul of mix 2,  
106 9.375 ul of primer/probe mix and 5 ul of RNA template with a total volume of 20 ul.

107 2019-nCoV CDC EUA Kit mixed with Fast Gene Probe One Step Mix allows detection of  
108 N1 gene, N2 gene and RNase P gene. Protocol for 2019-nCoV CDC EUA Kit mixed with Fast  
109 Gene Probe One Step Mix used is: 10 ul of Fast Gene Probe One Step mix, 1 ul of Fast Gene  
110 Scriptase, 1.5 ul of each primer/probe 2019 n-CoV CDC EUA Kit, 2.5 ul ultrapure water and 5 ul  
111 of RNA template with a total volume of 20 ul.

112 PCR reactions were performed on a Real-time PCR System (7500 Real-time PCR System,  
113 Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA) following the programs:  
114 reverse transcription for 30 minutes at 50<sup>0</sup>C, initial denaturation 10 minutes at 95<sup>0</sup> C, 40 cycles of  
115 denaturation 15 seconds at 95<sup>0</sup> C followed by an extension of 60 seconds at 60<sup>0</sup> C for PowerCheck  
116 2019-nCoV Real-time PCR Kit; reverse transcription for 30 minutes at 48<sup>0</sup>C, initial denaturation  
117 10 minutes at 95<sup>0</sup> C, 50 cycles of denaturation 15 seconds at 95<sup>0</sup> C followed by an extension of 30  
118 seconds at 58<sup>0</sup> C for COVID-19 PCR Diatheva Detection Kit; reverse transcription for 10 minutes  
119 at 45<sup>0</sup>C, initial denaturation 2 minutes at 95<sup>0</sup> C, 40 cycles of denaturation 5 seconds at 95<sup>0</sup> C  
120 followed by an extension of 30 seconds at 55<sup>0</sup> C for 2019-nCoV CDC EUA Kit mixed with Fast  
121 Gene Probe One Step Mix. Positive to negative cutoff was set at a Ct > 40 for all the kits assayed.

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123 *Ethical considerations*

124           The study was conducted as part of surveillance program for COVID-19 implemented by  
125 the Romanian government, with no disclosure regarding name, physical, economic, cultural,  
126 social status of the patients, therefore did not require individual patient consent or ethical  
127 approval.

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129 **Results**

130           1. Evaluation of commercial SARS-CoV-2 qPCR kits

131           To determine the analytical sensitivity of the COVID-19 commercial assays used in  
132 Romanian hospitals (PowerCheck Kogene 2019-nCoV, COVID -19 PCR Diatheva Detection Kit  
133 and 2019-nCoV CDC EUA), we first evaluated their limit of detection (LOD) using the positive  
134 controls provided by the kit. Assay reproducibility was tested in duplicate using 10-fold serial  
135 dilutions of the controls and intra- and inter-assay variability evaluated for each dilution point in  
136 triplicate on two different PCR machines. The LOD of RdRp and E (end point at  $10^{-7}$  fold  
137 dilution) was similar between the assays from Kogene and Diatheva. However, for the CDC  
138 EUA kit, LOD for N1 and N2 was 2 log unit lower ( $10^{-4}$  fold dilution) than that of E and RdRp.  
139 Therefore, for the future experiments, we decided to use Kogene and Diatheva kits.

140           2. Evaluation of different clinical specimens collected from COVID-19 infected patients

141           Samples were collected from patients with laboratory-confirmed COVID-19, ranging  
142 from 22 to 80 years old and consisted of swabs (from throat and/or nostrils) or sputum. Clinical  
143 presentation was either asymptomatic or mild in 14/24 patients; the rest of 10 patients either with  
144 moderate or severe COVID-19 outcome associated with co-morbidities (Table 2). Seven patients  
145 were tested negative by PCR for the initial screening, but were positive when the PCR was  
146 repeated after 2 weeks interval. Mild or asymptomatic patients did not have any other co-  
147 morbidities, and clinical signs were limited to either fever, cough and/or shortness of breath, as  
148 shown in Table 2. PCR results revealed an average Ct value for E gene of  $25.78 \pm 1.16$  and for  
149 RdRp,  $26.05 \pm 1.18$ , with no variation between sample matrix used (either nasal/throat swabs or  
150 sputum).

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152 3. Sample pooling and comparative performance of targets

153 In order to test the pooling approach, we decided to use a sample with an average Ct  
154 value of  $26.25 \pm 2.1$  to spike into seven negative pools containing equal volumes (200 ul/sample)  
155 of 5, 10, 15, 20, 30, 40 and 60. No optimization is required if using same volumes. Pools were  
156 then processed and extracted as previously described. Each reaction contained the undiluted  
157 sample used for pooling to assess for sample degradation of variation between assays. Finally,  
158 5ul of the extracted RNA was added to the RT-qPCR reagent mix from Kogene or Dyatheva. We  
159 repeated the experiment two times and all the pooled samples were ran in triplicate (Figures 1  
160 and 2). As shown in Figure 1, all the pools were positive for RdRp gene, which is consistent with  
161 other reports selecting molecular targets for COVID-19 diagnostics (13). As the sample pool  
162 increased signal for E gene was lost after pooling 20 negative samples. However, Diatheva kit  
163 managed to detect both targets in the pool of 30 samples (Figure 2).

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165 **Discussion**

166 As some countries are lifting up lockdown measures implemented through February and  
167 March, economies have to open quickly and safely. The initial steps to resume economical  
168 activities have to prioritize public health (14). Therefore massive scaling up COVID-19 testing is  
169 the temporary solution until immunity levels are achieved. One of the approaches that can be  
170 easily applied in order to increase the number of tests represents sample pooling. We showed that  
171 using a range of negative sample matrixes with one representative sample, ranging from 5 to 60  
172 pools only leads to only an incremental increase in the Ct values, for the RdRp target. This is  
173 consistent with the initial report for SARS-CoV-2 pooling, however the number of samples used  
174 for pooling before RNA extraction, was limited to 7 (12). This approach would be feasible for  
175 laboratories that are performing large-volume testing and considering screening with the  
176 commercial kits evaluated in this study. Moreover, laboratories may consider testing as many as  
177 60 samples using different sample matrixes, using the standard protocols, as an option for cost  
178 savings without compromising the capacity to detect SARS-CoV-2. However, there are several  
179 limitations that might arise using the pooled sample approach.

180 One limitation of this approach is that it seems only the RdRp gene is suitable for  
181 detection of SARS-CoV-2 in pools larger than 30 samples, which might arise in false negative  
182 results due to equipment variation and sample handling. However, this could be easily

183 circumvented by integrating additional SARS-CoV-2 specific targets. Moreover, the complexity  
184 of the disease can influence the sensitivity and specificity of the assay (15). Our results are  
185 showing that RdRp would be the ideal target for sample pooling, rather than E gene. This agrees  
186 with the initial development of molecular tests for SARS-CoV-2 detection, when RdRp gene  
187 assays 3.6 copies per reaction for the RdRp assay (4; 13).

188 In this research, we showed that sample pooling for SARS-CoV-2 diagnostic is a feasible  
189 measure using commercial kits widely available.

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#### 191 Competing Interest Statement

192 The authors have declared no competing interest.

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<i>Kit</i>	<i>Kogene 2019-nCoV</i>		<i>COVID -19 Diatheva</i>		<i>2019-nCoV CDC EUA</i>	
<i>Gene</i>	RdRp	E	RdRp	E	N1	N2
<i>Undil.</i>	20.25±0.25	21.61±0.31	24.68±0.47	26.63±0.22	24.63±0.25	25.05±0.33
$10^{-1}$	22.12±0.45	23.45±0.62	26.02±0.42	28.65±0.74	26±0.44	28.02±0.12
$10^{-2}$	25.45±0.78	25.78±0.46	29.35±0.78	30.45±0.54	30±0.66	31.02±0.87
$10^{-3}$	27.88±0.23	29.95±0.22	31.44±0.38	31.86±0.33	33±0.72	-
$10^{-4}$	30.66±0.77	32.12±1.13	33.78±0.12	34.74±0.71	-	-
$10^{-5}$	33.35±0.66	34.13±1.52	36.25±0.46	36.67±0.39	-	-
$10^{-6}$	35.85±0.89	35.94*	38.34±1.25	38.63±1.19	-	-
$10^{-7}$	-	-	-	-	-	-

212 **Table 1.** Limit of detection using the positive controls provided by the manufacturer of three  
 213 commercial kits. Results are presented as average cycle threshold (Ct) of two independent  
 214 experiments ± standard deviation (SD).

215 Key: “-“ negative

216 \*only 1 replicate

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Gender	Age	Classification			Ct	
		status	Matrix	Co-morbidities	E	RdRp
Male*	35	Asymptomatic	swab	-	26.21±1.12	26.72±0.88
Male*	70	Severe	sputum	Hypertension, diabetes	25.95±0.81	23.67±0.45
Female	55	Moderate	sputum	Hypertension, obesity	26.18±0.65	26.92±0.75
Female	42	Mild	swab <sup>a</sup>	-	25.95±0.35	26.26±0.45
Male	66	Asymptomatic	swab <sup>a</sup>	-	26.32±0.78	26.14±0.23
Female*	48	Moderate	swab	Hypertension, asthma	25.87±0.45	26.38±0.44
Male	65	Moderate	swab	Pneumonia	25.75±0.56	26.54±0.57
Male	71	Severe	sputum	Hearth disease	20.43±0.87	21.56±0.98
Female	23	Moderate	sputum	Diabetes, kidney disease	25.91±0.93	26.37±0.65
Female	38	Asymptomatic	swab	-	26.13±0.21	26.98±0.74
Male	61	Severe	swab	Obesity, hearth disease	25.87±0.08	26.56±0.44
Male	45	Asymptomatic	swab	-	25.89±0.65	26.48±0.36
Male*	47	Asymptomatic	swab <sup>a</sup>	-	26.51±0.47	26.56±0.34
Female	38	Mild	swab <sup>a</sup>	-	26.27±0.24	25.93±0.22
Female	50	Severe	swab	Immunocompromised	25.87±0.89	26.13±0.71
Male	22	Mild	swab	-	26.01±1.12	26.48±0.52
Male*	80	Moderate	swab	Dementia	25.65±1.23	26.38±1.3
Male*	77	Moderate	sputum	Diabetes	26.29±0.69	24.67±0.44
Female	62	Mild	swab <sup>b</sup>	-	26.37±0.77	26.52±0.29
Male*	66	Mild	swab <sup>b</sup>	-	26.01±0.35	26.64±0.86
Female	50	Mild	swab	-	25.87±0.62	26.59±0.41
<b>Male</b>	<b>53</b>	<b>Asymptomatic</b>	<b>swab</b>	-	<b>26.03±0.21</b>	<b>26.48±0.36</b>
Male	58	Mild	swab <sup>b</sup>	-	25.73±0.15	26.12±0.55
Female	48	Asymptomatic	swab	-	25.87±0.44	26.48±0.22

225 **Table 2.** Different sample matrixes collected from COVID-19 confirmed patients.

226 Key: Bold represents the sample used for pooling

227 <sup>a</sup> swab taken only from nostrils

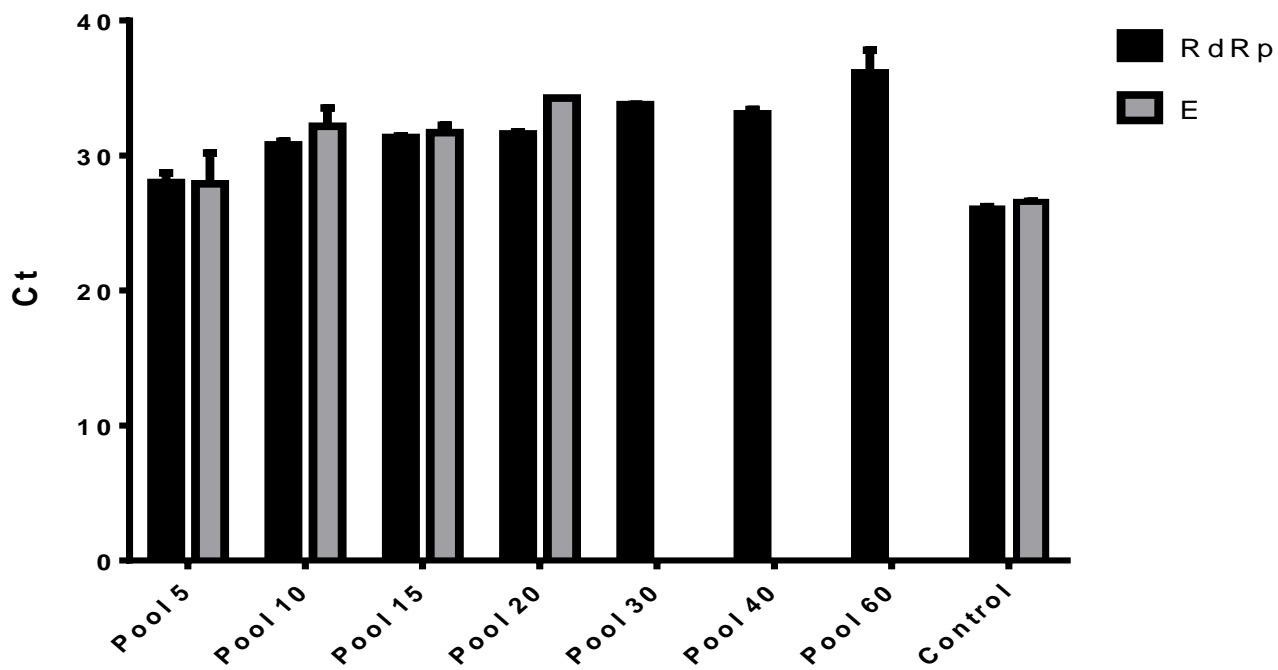
228 <sup>b</sup> swab taken only from throat

229 \*negative for the first PCR, positive after follow-up

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231 **Figure 1.** PCR results using the pools of 5, 10, 15, 20, 30, 40 and 60 using Kogene for RdRp and  
232 E genes. Results are presented as average Ct from two independent experiments.

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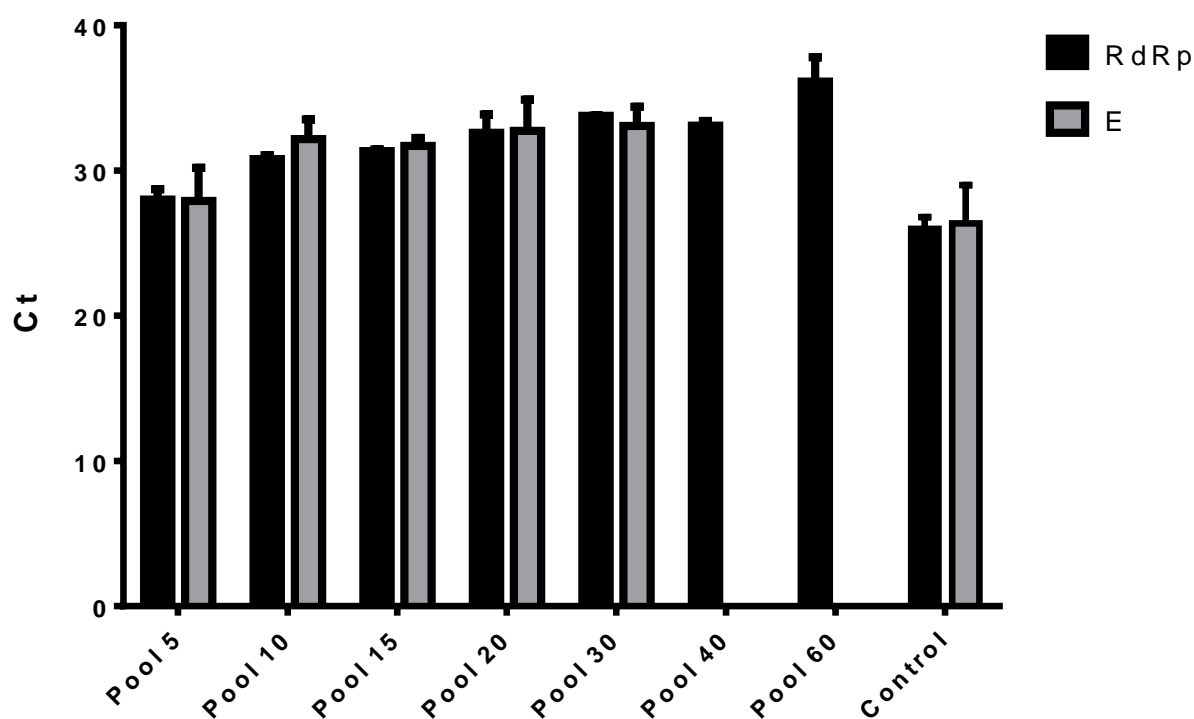
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250 **Figure 2.** PCR results using the pools of 5, 10, 15, 20, 30, 40 and 60 using Diatheva kit for RdRp  
251 and E genes. Results are presented as average Ct from two independent experiments



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