

1 **Comparison of three TaqMan Real-Time Reverse Transcription-PCR**
2 **assays in detecting SARS-CoV-2**

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4 Running title: Comparison of 3 qRT-PCR assays detecting SARS-CoV-2

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34 **Abstract**

35 Quick and accurate detection of SARS-CoV-2 is critical for COVID-19 control.
36 Dozens of real-time reverse transcription PCR (qRT-PCR) assays have been
37 developed to meet the urgent need of COVID-19 control. However, methodological
38 comparisons among the developed qRT-PCR assays are limited. In the present study,
39 we evaluated the sensitivity, specificity, amplification efficiency, and linear detection
40 ranges of three qRT-PCR assays, including the assays developed by our group
41 (IPBCAMS), and the assays recommended by WHO and China CDC (CCDC). The
42 three qRT-PCR assays exhibited similar sensitivities, with the limit of detection (LOD)
43 at about 10 copies per reaction (except the ORF 1b gene assay in CCDC assays with a
44 LOD at about 100 copies per reaction). No cross reaction with other respiratory
45 viruses were observed in all of the three qRT-PCR assays. Wide linear detection
46 ranges from 10^6 to 10^1 copies per reaction and acceptable reproducibility were
47 obtained. By using 25 clinical specimens, the N gene assay of IPBCAMS assays and
48 CCDC assays performed better (with detection rates of 92% and 100%, respectively)
49 than that of the WHO assays (with a detection rate of 60%), and the ORF 1b gene
50 assay in IPBCAMS assays performed better (with a detection rate of 64%) than those
51 of the WHO assays and the CCDC assays (with detection rates of 48% and 20%,
52 respectively). In conclusion, the N gene assays of CCDC assays and IPBCAMS
53 assays and the ORF 1b gene assay of IPBCAMS assays were recommended for
54 qRT-PCR screening of SARS-CoV-2.

55 **Key words:** SARS-CoV-2; qRT-PCR; methodological evaluation; Limit of Detection;
56 reproducibility, clinical performance

57 **Introduction**

58 Since the first detection in late 2019, severe respiratory syndrome CoV-2
59 (SARS-CoV-2) caused Corona Virus Infectious Disease in 2019 (COVID-19) has
60 widely spread in the world. By April 11, 2020, more than 1.7 million patients infected
61 by SARS-CoV-2 has been reported from 185 countries (1). Given the quick increase
62 in confirmed cases and asymptomatic infections, there are increasing demands in
63 diagnostic tools for quick and accurate detection of the virus (2, 3). Several real-time
64 reverse transcription-Polymerase Chain Reaction (qRT-PCR) for the detection of
65 SARS-CoV-2 has been developed to meet the demands, including the assays by this
66 group (IPBCAMS assays), and the assays by WHO (WHO assays), and the assays by
67 China CDC (CCDC assays).

68 Because SARS-CoV-2 usually infected the lower respiratory tract, it is not easy to
69 detect the viral nucleic acids from throat swabs with relatively lower viral load (4).
70 Thus, qRT-PCR assays with higher sensitivity and better performance in the detection
71 of SARS-CoV-2 is recommended in aiding the diagnosis of COVID-19 (2). However,
72 most of the current available qRT-PCR assays were developed for emergency, a
73 comprehensive methodological comparison among these assays remains unfulfilled.
74 To comprehensively compare the performance of currently available qRT-PCR assays
75 for detection of SARS-CoV-2, we evaluated the sensitivity, specificity, amplification
76 efficiency, and linear detection ranges among IPBCAMS assays, WHO assays and
77 CCDC assays.

78 **Materials and methods**

79 **Nucleic acid extraction**

80 Nucleic acids were extracted from a volume of 200 μ l clinical samples by using
81 NucliSens easyMag apparatus (bioMe'rieux, MarcyL'Etoile, France) according to the
82 manufacturer's instructions. A volume of 50 μ l total nucleic acid eluate for each
83 specimen was recovered and transferred into a nuclease-free vial and either tested
84 immediately or stored at -80°C.

85 **Primers and probes**

86 Sequences of primers and probes for the IPBCAMS assays were recently developed
87 (5), while those for the WHO assays were obtained from the website of WHO
88 (https://www.who.int/docs/default-source/coronavirus/protocol-v2-1.pdf?sfvrsn=a9ef618c_2), and those for the CCDC assays were obtained from the website of China
90 CDC

91 (http://www.chinacdc.cn/jkzt/crb/zl/szkb_11803/jszl_11815/202003/W020200309540843062947.pdf) (Table 1). Primers and probes were synthesized by standard phosphoramidite chemistry techniques at Qingke biotechnology Co. ltd (Beijing, China). TaqMan probes were labeled with the molecule 6-carboxy-fluroscein (FAM) at the 5' end, and with the quencher Blackhole Quencher 1 (BHQ1) at the 3' end. Optimal concentrations of the primers and probes were determined by cross-titration of serial two-fold dilutions of each primer/probe against a constant amount of purified RNA of SARS-CoV-2.

99 **TaqMan real-time RT-PCR assay**

100 The TaqMan real-time RT-PCR assays were performed by using TaqMan Fast Virus
101 1-Step Master Mix (Thermo Fisher Scientific, MA, USA). Each 20 μ l reaction mix
102 contained 5 μ l of 4 \times Fast Virus 1-Step Master Mix, 0.2 μ l of 50 μ M probe, 0.2 μ l each
103 of 50 μ M forward and reverse primers, 12.4 μ l of nuclease-free water, and 2 μ l of
104 nucleic acid extract. Amplifications were carried out in 96-well plates by using
105 Bio-Rad instrument (Bio-Rad CFX96, CA, USA). Thermo-cycling conditions are as
106 follows: 15 min at 50 $^{\circ}$ C for reverse transcription, 4 min at 95 $^{\circ}$ C for pre-denaturation,
107 followed by 45 cycles of 15 sec at 95 $^{\circ}$ C and 45 sec at 60 $^{\circ}$ C. Fluorescence
108 measurements were taken at 60 $^{\circ}$ C of each cycle. The threshold cycle (Ct) value was
109 determined by the point at which fluorescence exceeded a threshold limit set at the
110 mean plus 10 stand deviations above the baseline. A result was considered positive if
111 two or more of the SARS-CoV-2 genome targets exhibited positive results (Ct \leq 35).
112 A result of 35 \leq Ct \leq 40 was considered suspected and a repeat test was performed for
113 result confirmation.

114 **Preparation of RNA transcripts**

115 RNA transcripts for N gene and ORF 1b of SARS-CoV-2 were prepared with a
116 plasmid pEasy-T1 (TransGen Biotech, Beijing, China) with T7 promoter before the
117 multiple cloning sites. The plasmids inserted with viral gene regions of N and Orf1b
118 were linearized with the restriction enzyme, BamHI, and transcribed *in-vitro* by using
119 RiboMAXTM Large Scale RNA Production Systems (Promega, WI, USA),
120 respectively. The concentrations of the RNA transcripts were determined by using
121 NanoDrop (Thermo Fisher Scientific, CA, USA).

122 **Results**

123 **Comparison of the sensitivities, reproducibility and linear detection ranges of the**
124 **three qRT-PCR assays.**

125 To determine the sensitivity of the three qRT-PCR assays, we measured the limit of
126 detection (LOD) for each assay by using RNA transcript of the corresponding gene in
127 ten-fold dilution as template (RNA transcript alone). A LOD of 10 genomic copies per
128 reaction was observed for both the N gene assay and the ORF 1b gene assay of all the
129 three qRT-PCR assays, although the Ct values for N gene assay of WHO assays and
130 ORF 1b gene assay of CCDC assays were higher than 35 cycles (Table 2).

131 The linear detection ranges of the three qRT-PCR assays were determined by using a
132 ten-fold dilution of the RNA transcript as template. It showed that the Ct values
133 increased with the RNA transcript from 10^6 to 10^1 copies in the reaction in all of the
134 three qRT-PCR assays (Table 2). Strong linear correlations were observed between Ct
135 values and quantity of RNA transcripts with $r^2=0.9926, 0.9750, 0.9987$ in the N gene
136 assay, and $r^2=0.9953, 0.9897, 0.9941$ in the ORF 1b assay of IPBCAMS assays, WHO
137 assays, and CCDC assays, respectively. These results suggested that all of the three
138 qRT-PCR assays exhibited linear detection ranges from 10^6 to 10^1 copies per reaction,
139 while the WHO assays showed lower coefficient of linear correlation.

140 The reproducibility of the three qRT-PCR assays was assessed by measuring
141 coefficient of variation (CV) of mean Ct values in the intra- and inter- assay. For the
142 N gene assay, the CVs of mean Ct values from 10^6 to 10^1 copies of RNA transcript per
143 reaction were 0.20%-1.33%, 0.46%-5.09%, 0.27%-1.97% in intra-assay, and
144 1.06%-2.45%, 0.96%-7.59%, 1.00%-5.51% in inter-assay of IPBCAMS assay, WHO
145 assay, and CCDC assay, respectively. For the ORF 1b gene assay, the CVs of mean Ct
146 values were 0.26%-4.45%, 0.29%-1.76%, 0.71%-6.52% in intra-assay, and
147 2.17%-5.12%, 0.30%-1.57%, 2.63%-4.34% in inter-assay of IPBCAMS assays, WHO
148 assays, and CCDC assays, respectively.

149 Because co-infections of respiratory viruses are common, we prepared a (v:v=1:1)
150 mixture of the RNA transcript and a pooled total nucleic acid extract from respiratory
151 specimens (RNA transcript + other extract) as template, to evaluate the effect of
152 co-existed viral nucleic acids on the performance of the assays. No effect of the
153 co-existed other viral nucleic acids on the LOD and the linear detection range was
154 observed, although higher Ct values were generated than those of RNA transcript
155 alone as template in all of the three qRT-PCR assays. However, the co-existed other
156 viral nucleic acids put some effect on the efficiencies of the three qRT-PCR assays.

157 For the N gene assays, the efficiencies were moved from 105.82%, 107.23%,
158 102.21% to 110.17%, 124.32%, 119.43% in IPBCAMS assays, WHO assays, CCDC
159 assays, respectively. For the ORF 1b assays, the efficiencies were moved from
160 107.71%, 121.83%, 93.80% to 109.18%, 138.43%, 100.92% in IPBCAMS assays,
161 WHO assays, CCDC assays, respectively.

162 **Comparison of the specificities of the three qRT-PCR assays**

163 To evaluate the potential cross-reactions with other human respiratory viruses, the
164 three qRT-PCR assays were examined by using human respiratory samples as
165 templates, which were positive for human coronaviruses (OC43, NL63, 229E, or
166 HKU1), or Influenza viruses (A or B), or respiratory syncytial virus, or parainfluenza
167 virus (1-4), or human metapneumovirus, or rhinovirus, or adenovirus, or bocavirus.
168 No cross reaction was observed in all of the three qRT-PCR assays (data not shown),
169 suggesting high specificity of the three qRT-PCR assays in detecting SARS-CoV-2.

170 **Assay evaluation with clinical specimens**

171 The three qRT-PCR assays were evaluated with 25 clinical specimens (including 13
172 throat swabs and 12 sputum) from 25 suspected COVID-19 patients. SARS-CoV-2
173 was detected from 92% (23/25), 60% (15/25), 100% (25/25) by the N gene assay, and
174 from 64% (16/25), 48% (12/25), 20% (5/25) of all enrolled clinical specimens by the
175 ORF 1b gene assay in IPBCAMS assays, WHO assays, CCDC assays, respectively
176 (Table 4). With respect to the sputum, SARS-CoV-2 was detected from 100% (12/12),
177 75% (8/12), 100% (12/12) of specimens by the N gene assay, and from 100% (12/12),
178 75% (8/12), 41.7% (5/12) of specimens by the ORF 1b gene assay in IPBCAMS
179 assays, WHO assays, CCDC assays, respectively. About the throat swabs,
180 SARS-CoV-2 was detected from 84.6% (11/13), 53.8% (7/13), 100% (12/12) of
181 specimens by the N gene assay, and from 30.8% (4/13), 30.8% (4/13), 0% (0/13) of
182 specimens by the ORF 1b gene assay in IPBCAMS assays, WHO assays, CCDC
183 assays, respectively. These results demonstrated that the N gene assay performed
184 better than the corresponding ORF 1b gene assay of all the three qRT-PCR assays, the
185 N gene assay in CCDC assays and ORF 1b gene assay in IPBCAMS assays
186 performed better than the other assays.

187 **Discussion**

188 Rapid and accurate detection of SARS-CoV-2 represent a fast-growing global demand,
189 which could be met by TaqMan real time RT-PCR (qRT-PCR). However, the current
190 available TaqMan qRT-PCR assays for SARS-CoV-2 are varied in performance,

191 including sensitivity, specificity, reproducibility, linear detection ranges, etc. Due to
192 that relative lower viral load in upper respiratory tract, reliable qRT-PCR assays for
193 the detection of SARS-CoV-2 are required. We thus compared the performance of
194 three currently wide-applied qRT-PCR assays in the detection of SARS-CoV-2.
195 Sensitivity is the primary demand in the detection of respiratory viruses (6). All of the
196 three qRT-PCR assays could provide a LOD of 10 genomic copies per reaction with a
197 detection range from 10^6 - 10^1 genomic copies per reaction. The Ct value at 10
198 genomic copies per reaction in the ORF 1b gene assay of CCDC assays was higher
199 than 35. These results suggested that most of the three qRT-PCR assays provide high
200 sensitivity and wide linear detection range in detecting SARS-CoV-2, except a
201 relative lower sensitivity observed in the ORF 1b gene assay of CCDC assays.
202 Specificity is also essential in the detection of SARS-CoV-2, because of common
203 co-infections with other respiratory viruses and high host DNA background in throat
204 swabs (7-9). We evaluated the specificity of the three qRT-PCR assays with
205 respiratory specimens positive for other common respiratory viruses. No cross
206 reaction was observed, demonstrating high specificity of the three qRT-PCR assays in
207 detection of SARS-CoV-2.
208 We next evaluated the reproducibility of the three qRT-PCR assays by measuring
209 coefficient of variation (CV) of mean Ct values in intra- and inter- assay (10). The N
210 gene assay in IPBCAMS assays and ORF 1b gene assay in WHO assays exhibited a
211 relative better reproducibility with lower intra- and inter- assay CVs, which were not
212 affected by the co-existed nucleic acids of other respiratory viruses.
213 Efficiency is another key parameter of qRT-PCR, reflecting the binding efficiency of
214 primers & probe to template and the amplification efficiency of the PCR system(11).
215 Most of the qRT-PCR assays provided good efficiency, except an abnormal efficiency
216 of 121.83% observed in the ORF 1b gene assay of WHO assays. An exceptionally
217 high efficiency indicates an increased risk of false positive (12). The co-existed
218 nucleic acids of other respiratory viruses increased the efficiency of all the three
219 qRT-PCR assays, suggesting potential increased risk of cross-reactions between the
220 primers & probe and background nucleic acids.
221 We finally evaluate the performance of the three qRT-PCR assays with clinical
222 specimens from suspected SARS-CoV-2 infected patients (13). Possibly because of
223 the lower viral load in upper respiratory tract (4), the detection rate of SARS-CoV-2
224 was lower in throat swabs than in sputum by all of the three assays. Meanwhile, the N

225 gene assay performed better than the corresponding ORF 1b gene assay in all of the
226 three qRT-PCR assays. For the N gene assay, IPBCAMS assays and CCDC assays
227 performed better than WHO assays, both of which could detect SARS-CoV-2 from
228 more than 90% of the suspected specimens. For the ORF1b gene assay, IPBCAMS
229 assays performed better than WHO assays and CCDC assays, with a detection rate of
230 64%.

231 In conclusion, we performed methodological evaluations on three widely-applied
232 qRT-PCR assays for the detection of SARS-CoV-2. Although most of the evaluated
233 assays exhibited good sensitivity, specificity, reproducibility and wide linear detection
234 range, performance test with clinical specimens from suspected COVID-19 patients
235 suggested that the N gene assay in IPBCAMS assays and CCDC assays, and the ORF
236 1b gene assays in IPBCAMS assays were the preferred qRT-PCR assays for accurate
237 detection of SARS-CoV-2.

238

239 **Data availability**

240 The original data will be available upon request.

241

242 **Conflict of interest**

243 The authors declare that there are no conflicts of interest regarding the publication of
244 this paper.

245

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Table 1. Primers and probes of the three qRT-PCR assays

	Assay	Primer/probe	Sequence (5'-3')	Genomic location*	Amplicon
N gene assay	IPBCAMS assays	Forward	AACACAAGCTTCGGCAGAC	29083-29102	
		Reverse	ACCTGTGTAGGTCAACCACG	29278-29259	195 bp
		Probe	CAGCGCTTCAGCGTTCTCGGAATGTCGC	29200-29228	
	WHO assays	Forward	CACATTGGCACCCGCAATC	28706-28724	
		Reverse	GAGGAACGAGAAGAGGCTTG	28833-28814	127 bp
		Probe	ACTTCCTCAAGGAACAAACATTGCCA	28753-28777	
	CCDC assays	Forward	GGGGAACTTCTCCTGCTAGAAT	28881-28902	
		Reverse	CAGACATTTGCTCTCAAGCTG	28979-28958	98 bp
		Probe	TTGCTGCTGCTTGACAGATT	28934-28953	
ORF 1b gene assay	IPBCAMS assays	Forward	ACGGTGACATGGTACCAACAT	13760-13779	
		Reverse	CTAAGTTGGCGTATAACGCGT	13975-13956	215 bp
		Probe	TACACAATGGCAGACCTCGTCTATGC	13804-13829	
	WHO assays	Forward	GTGARATGGTCATGTGTGGCGG	15431-15452	
		Reverse	CARATGTTAAASACACTATTAGCATA	15530-15505	99 bp
		Probe	CAGGTGGAACCTCATCAGGAGATGC	15470-15494	
	CCDC assays	Forward	CCCTGTGGGTTTACACTTAA	13342-13362	
		Reverse	ACGATTGTGCATCAGCTGA	13460-13442	118 bp
		Probe	CCGTCTGCGGTATGTGGAAAGGTTATGG	13377-13404	

Table 2. Reproducibility (Coefficient of Variation, %) of the three qRT-PCR assays

	Assay	Copy number of RNA transcript					
		1×10^6	1×10^5	1×10^4	1×10^3	1×10^2	1×10^1
N gene assay	IPBCAMS assays	Intra-assay	0.52*	1.33	0.37	0.46	0.20
		Inter-assay	1.06	2.45	1.49	1.32	1.37
	WHO assays	Intra-assay	1.08	1.19	1.12	0.87	0.46
		Inter-assay	7.59	2.94	2.78	6.60	0.96
	CCDC assays	Intra-assay	0.52	0.54	0.27	0.74	0.41
		Inter-assay	1.56	1.20	5.51	1.00	1.40
ORF 1b gene assay	IPBCAMS assays	Intra-assay	0.73	0.26	1.10	1.30	4.45
		Inter-assay	4.66	3.85	2.77	2.17	5.12
	WHO assays	Intra-assay	0.57	0.47	0.88	0.41	0.29
		Inter-assay	1.57	0.30	0.87	0.69	0.55
	CCDC assays	Intra-assay	1.66	0.78	0.71	0.92	2.45
		Inter-assay	0.52	0.54	0.27	0.74	0.41

259 The coefficient of variation was calculated by standard deviation of the Ct values of a RNA dilution divided by the mean Ct values of the same
 260 RNA dilution.
 261
 262
 263
 264

Table 3. Efficiency of the three qRT-PCR assays

Assay	Template	Mean Ct values at quantified copy number of RNA transcript						Slope ^a	Efficiency (%) ^b	
		1×10 ⁶	1×10 ⁵	1×10 ⁴	1×10 ³	1×10 ²	1×10 ¹			
N gene assay	IPBCAMS assays	RNA transcript ^d alone	17.63±0.09 ^c	21.99±0.29	24.08±0.09	28.25±0.13	31.00±0.06	33.73±0.25	-3.19	105.82
		RNA transcript + other viruses	19.40±0.19	22.40±0.04	26.38±0.09	29.98±0.07	32.17±0.28	34.51±0.26	-3.10	110.17
	WHO assays	RNA transcript alone	18.44±0.19	22.65±0.27	26.78±0.32	29.60±0.26	32.68±0.15	33.97±1.73	-3.16	107.23
		RNA transcript + other viruses	19.51±0.15	24.83±0.36	26.59±0.29	29.62±0.54	32.62±0.70	34.19±0.51	-2.85	124.32
	CCDC assays	RNA transcript alone	17.17±0.09	20.71±0.11	23.94±0.07	27.57±0.20	30.37±0.12	33.53±0.50	-3.27	102.21
		RNA transcript + other viruses	18.93±0.16	23.79±0.20	25.66±0.23	29.58±0.52	31.92±0.16	33.81±0.87	-2.93	119.43
	IPBCAMS assays	RNA transcript alone	18.64±0.14	22.20±0.06	25.73±0.28	28.83±0.37	31.90±1.42	34.22±1.15	-3.15	107.71
		RNA transcript + other viruses	19.45±0.06	22.98±0.13	25.88±0.17	29.37±0.12	32.83±0.40	34.65±2.12	-3.12	109.18
1b gene assay	WHO assays	RNA transcript alone	18.51±0.11	21.60±0.10	25.05±0.22	28.27±0.12	30.78±0.09	32.57±0.57	-2.89	121.83
		RNA transcript + other viruses	19.46±0.09	22.58±0.13	25.75±0.19	28.20±0.20	30.03±0.70	33.04±0.14	-2.65	138.43
	CCDC assays	RNA transcript alone	18.80±0.31	21.96±0.17	24.76±0.18	28.06±0.26	32.47±0.79	36.16±2.36	-3.48	93.80
		RNA transcript + other viruses	18.67±0.04	21.54±0.11	24.79±0.03	28.28±0.04	31.09±0.98	35.33±0.59	-3.30	100.92

265 ^aSlope was generated by fitting of the scatter with Excel 2010.

266 ^bEfficiency = $10^{(-1/\text{slope})} - 1$.

267 ^cValues shown are the mean of triplicate samples ± standard deviation.

268 ^d“RNA transcript” represents the *in vitro* transcribed RNA of the corresponding genes of SARS-CoV-2. “other viruses” represents the pooled
269 RNA extracted from 15 human respiratory specimens by using Trizol. “RNA transcript + other viruses” represents a 1:1 (v/v) mixture of these
270 two components.

Table 4. Evaluation of the three qRT-PCR assays with clinical specimens

Specimen	Specimen	N gene assay			ORF 1b gene assay		
		ID	type	IPBCAMS	WHO	CCDC	IPBCAMS
TS98	Throat swab	35.79	NA	35.42	NA	NA	NA
TS101	Throat swab	33.48	NA	34.24	NA	NA	NA
TS103	Throat swab	NA	NA	34.68	NA	NA	NA
TS105	Throat swab	31.5	35.76	31.64	NA	NA	NA
TS108	Throat swab	33.35	NA	32.11	33.36	NA	NA
TS110	Throat swab	29.99	31.73	29.1	33.57	NA	NA
TS165	Throat swab	27.34	30.46	28.14	31.06	27.84	NA
TS168	Throat swab	NA	NA	34.97	NA	NA	NA
TS169	Throat swab	33.34	NA	34.04	NA	34.2	NA
TS187	Throat swab	34.5	39.2	33.03	NA	NA	NA
TS188	Throat swab	35.03	35.9	33.57	NA	24.07	NA
TS189	Throat swab	31.16	35.43	31.21	34.04	30.92	NA
TS190	Throat swab	32.84	34.02	32.56	NA	NA	NA
TY1	Sputum	27.35	29.44	27.6	30.98	27.33	NA
TY2	Sputum	29.38	31.26	29.06	32.32	28.72	NA
TY3	Sputum	31.85	NA	31.3	35.84	NA	NA
TY4	Sputum	22.99	25.57	22.08	27.42	24.12	35.99
TY6	Sputum	25.51	27.52	25.58	29.03	25.58	41.54
TY7	Sputum	26.9	30.21	27.4	30.05	27.3	45.26
TY8	Sputum	29.21	31.87	30.06	33.65	29.84	NA
TY9	Sputum	26.29	28.45	26.34	30.69	26.03	46.34
XT1	Sputum	25.74	27.26	25.3	29.82	26.34	45.9
XT2	Sputum	31.57	NA	30.95	34.19	NA	NA
XT3	Sputum	31.14	NA	32.02	35.02	NA	NA
XT4	Sputum	32.67	NA	31.71	34.26	NA	NA
account (%) of positive		23 (92%)	15 (60%)	25 (100%)	16 (64%)	12 (48%)	5(20%)

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