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ORIGINAL ARTICLE

Comparative *in silico* design and validation of GPS™ CoVID-19 dtec-RT-qPCR test

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Abstract

Aims: Providing a ready-to-use reverse transcriptase qPCR (RT-qPCR) method fully validated to detect the SARS-CoV-2 with a higher exclusivity than this shown by early published RT-qPCR designs.
Methods and Results: The specificity of the GPS™ CoVID-19 dtec-RT-qPCR test by analysis of sequence alignments was approached and compared with other RT-qPCR designs. The GPS™ CoVID-19 dtec-RT-qPCR test was validated following criteria of UNE/EN ISO 17025:2005 and ISO/IEC 15189:2012. Diagnostic validation was achieved by two independent reference laboratories, the Instituto de Salud Carlos III, (Madrid, Spain), the Public Health England (Colindale, London, UK), and received the label CE-IVD. The GPS design showed the highest exclusivity and passed all parameters of validation with strict acceptance criteria. Results from reference laboratories 100% correlated with these obtained by using reference methods and showed 100% of diagnostic sensitivity and specificity.
Conclusions: The CE-IVD GPS™ CoVID-19 dtec-RT-qPCR test, available worldwide with full analytical and diagnostic validation, is the more exclusive for SARS-CoV-2 by far.
Significance and Impact of the Study: Considering the CoVID-19 pandemic status, the exclusivity of RT-qPCR tests is crucial to avoid false positives due to related coronaviruses. This work provides of a highly specific and validated RT-qPCR method for detection of SARS-CoV-2, which represents a case of efficient transfer of technology successfully used since the pandemic was declared.

Background

Last 30th January, the Emergency Committee of the World Health Organization (WHO) under the International Health Regulations (IHR) declared an outbreak of pneumonia, lately named Corona Virus Disease 2019 (COVID-19), as a 'Public Health Emergency of International Concern' (PHEIC). The disease is caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and the first genome was rapidly provided (http://virological.org/t/novel-2019-coronavirus-genome/319). SARS-CoV-2 is a *Betacoronavirus* subgenus *Sarbecovirus* of group 2B, with similar characteristics than SARS-CoV, Bat-SARS-CoV and other Bat SARS-like-CoV (Ceraolo and Giorgi 2020; Jiang and Shi 2020; Lai *et al.* 2020; Lu *et al.* 2020; Zhou *et al.* 2020; Li *et al.* 2020). A few weeks later, this novel coronavirus spread worldwide and forced WHO to declare a Pandemic on March 11, when more than 118 000 positives and 4291 deaths were already registered in 114 countries. Today, 06th July, the number of positive cases globally surpasses 11.5 million people with more than 530, 000 deaths. The aggressiveness of this global alarm has overwhelmed any forecast. A massive, reliable and rapid diagnosis, is undoubtedly vital and foremost priority for decision-making, which will facilitate public health interventions.

Current molecular diagnostic tools for viral detection are typically based on the amplification of target-specific genetic sequences using the polymerase chain reaction (PCR). In acute respiratory infection, real-time PCR (so-called quantitative PCR; qPCR) is the gold-standard method, routinely used to detect causative viruses because it is the most sensitive and reliable method (Mackay *et al.* 2002; Drosten *et al.* 2003; Poon *et al.* 2004; Corman *et al.* 2012a; Corman *et al.* 2012b). On the 17th January, WHO published the very first primers and probes for reverse transcriptase qPCR (RT-qPCR) developed by Corman *et al.* (2020). They used known genomic data from SARS-CoV and SARS-CoV related (Bat viruses) to generate a non-redundant alignment. The candidate diagnostic RT-qPCR assay was designed upon the first SARS-CoV-2 sequence released, based on the alignment with known SARS-CoV sequences. Because only a single SARS-CoV-2 genome was available, the two monoplex PCR protocols (ORF1ab and N genes) designed to detect SARS-CoV-2 are also reactive to SARS-CoV and Bat SARS-like-CoV. A few days later, 23rd January, the same laboratory together with reference laboratories from the Netherlands, Hong Kong, France, United Kingdom and Belgium, added a third monoplex RT-qPCR (Corman *et al.* 2020). Many laboratories worldwide are currently using this RT-qPCR protocol (Chan *et al.* 2020) and it has been the basis to develop many commercial kits. Almost simultaneously, other primers and probes were designed and published by scientists from the Institut Pasteur, París; Centers for Disease Control and Prevention (CDC), Division of Viral Diseases, Atlanta, USA; National Institute for Viral Disease Control and Prevention (CDC), China; Hong Kong University; Department of Medical Sciences; Ministry of Public Health, Thailand; the National Institute of Infectious Diseases, Japan (Corman *et al.* 2020; Chan *et al.* 2020; Institut Pasteur 2020; Centers for Disease Control and Prevention from Atlanta 2020; National Institute for Viral Disease Control and Prevention from China 2020; Chu *et al.* 2020; Department of Medical Sciences of Thailand 2020; Shirato *et al.* 2020). The Respiratory Viruses Branch, Division of Viral Diseases, CDC, Atlanta, recently (4th February) updateda manual of Real-Time RT-PCR Panel for detection of this 2019-Novel Coronavirus (SARS-CoV-2), which was modified 15th March. The SARS-CoV-2 primer and probe sets were designed for the universal detection of SARS-like coronaviruses (N3 assay) and for specific detection of SARS-CoV-2 (N1 and N2 assays). Finally, authors from the Institut Pasteur, Paris, based on the first sequences of SARS-CoV-2 available on the GISAID database (Global Initiative on Sharing All Influenza Data), published a protocol for the detection of SARS-CoV-2 for two RdRp targets (IP2 and IP4) (Institut Pasteur 2020).

Some biotechnology-based companies recently developed kits for detection of SARS-CoV-2, based on RT-qPCR and provided easy transfer of technology to laboratories worldwide. A fully SARS-CoV-2-specific RT-qPCR thermostable kit was early launched on 27th January by Genetic PCR Solutions™ (GPS™), a brand of Genetic Analysis Strategies SL. (Alicante, Spain). The alignments used at that time included 13 SARS-CoV-2 genome sequences released by six different laboratories, deposited in GISAID and available since 19th January 2020. With the purpose to discriminate this new SARS-CoV-2 of present outbreak from previous related SARS, a second independent monoplex RT-qPCR test to detect any other non-SARS-CoV-2 was also produced and provided (not shown). On this study, we performed a deep analytical and diagnostic validation of the GPS™ COVID-19 dtec-RT-qPCR test, following the UNE/EN ISO 17025:2005 and ISO/IEC 15189:2012, respectively. A comparative analysis of the specificity (inclusivity and exclusivity) of the designed primers and probes with most previously published RT-qPCR methods is also here reported.

Materials and methods

Genome sequences alignment and phylogenetic analysis

Partial alignments of 10 SARS-CoV-2 genomic sequences and these from strains of Bat-CoV, Bat SARS-like-CoV, SARS-CoV, Pangolin-CoV (ca. 18, 141 bp) were done. The corresponding phylogenetic tree (Fig. 1) was obtained by Neighbour-Joining method (Saitou and Nei 1987), with bootstrap values for 1000 replicates, using the MEGA 5.2.2 software (Tamura *et al.* 2011).

In silico comparative analysis of primers/probes specificity

The primers and probes of GPS COVID-19 dtec-RT-qPCR Test and the RT-qPCR designs recently published (Corman *et al.* 2020; Chan *et al.* 2020; Institut Pasteur 2020; Centers for Disease Control and Prevention from Atlanta 2020; National Institute for Viral Disease Control and Prevention from China 2020; Chu *et al.* 2020; Department of Medical Sciences of Thailand 2020; Shirato *et al.* 2020) were aligned to the corresponding homologous region of 63 SARS-CoV-2 strains and closely related *Betacoronavirus* using the Basic Local Alignment Search Tool (BLAST) software available on the National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi) website databases (Bethesda, MD, USA). This *in silico* analysis was periodically updated with new entries currently available. Number of mismatches of the primers and probes sequences of the

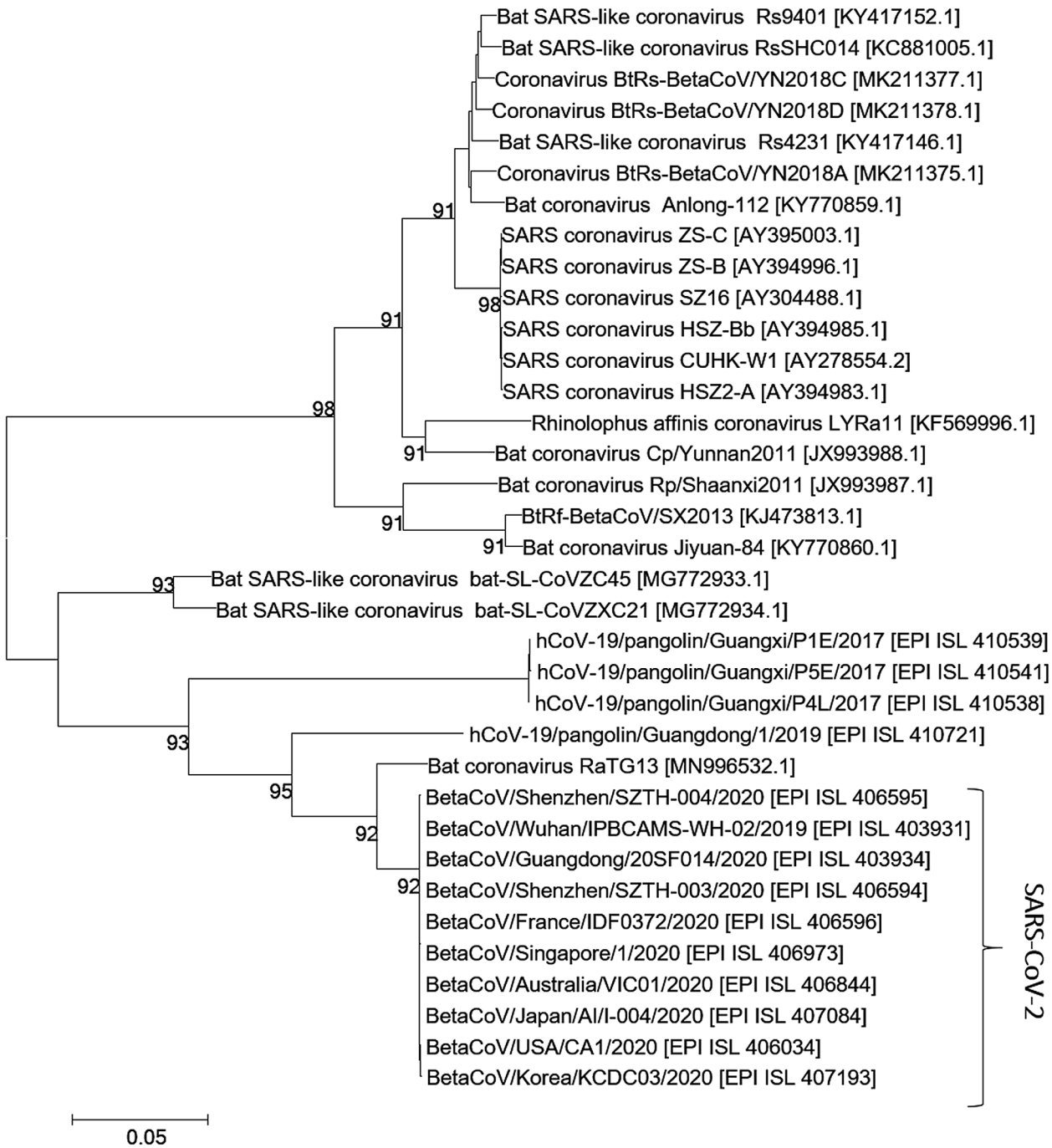

Figure 1 Phylogenetic Neighbour-Joining tree showing relationships of SARS-CoV-2 and the most related strains of some *Betacoronavirus*, including SARS-CoV, Bat-CoV, Bat SARS-like-CoV and Pangolin-CoV. The analysis was derived from the alignment of 18,141 nucleotides. Numbers at nodes indicate bootstrap values (percentage of 1000 replicates).

GPS kit and recently published designs were calculated to evaluate the *in silico* specificity (Table 1). An illustration of the mismatching of primers/probe sequences of the GPS CoVID-19 dtec-RT-qPCR Test, respect of the SARS-CoV-2, Bat SARS-like-CoV, SARS-CoV, Bat-CoV, and Pangolin-CoV groups is shown in Fig. 2.

GPS™ COVID-19 dtec-RT-qPCR test

Assays using the GPS™ COVID-19 dtec-RT-qPCR kit (Alicante, Spain) were prepared and reaction mixtures were subjected to qPCR in a QuantStudio3 (ABI) as described in the manual provided. Internal, positive and

Table 1 Number of mismatches found in the primers/probes sets of the GPS™ COVID-19 dtec-RT-qPCR Test and other RT-qPCR designs recently published, from the comparative *in silico* analysis with Bat-CoV, Bat SARS-like-CoV, SARS-CoV and Pangolin-CoV. Numbers in bold show the sum of the mismatches found in the primers/probes of the RT-qPCR designs. Numbers in brackets show the mismatches found in forward primer (FP), probe (P) and reverse primer (RP) following this format: [FP / P / RP]

			Total mismatches									
Reference	Institution	Target	SARS-CoV-2		Bat coronavirus		Bat SARS–like coronavirus		SARS coronavirus		Pangolin coronavirus	
	Genetic PCR solutions™ (Spain)	–	**0–1**	[0 / 0–1 / 0]	**37–48**	[8–11 / 17–23 / 12–14]	**26–37**	[6–9 / 12–17 / 8–11]	**36–38**	[7–8 / 19 / 9]	**19–31**	[5–9 / 4–11 / 10–11]
Institut Pasteur (2020)	Institut Pasteur (Paris)	RdRp (IP2)	**0**	[0 / 0 / 0]	**6–9**	[1–2 / 2–3 / 3–4]	**10–11**	[4 / 2–3 / 4]	**7–12**	[1–4 / 3 / 3–5]	**4–8**	[0–4 / 2 / 2]
		RdRp (IP4)	**0**	[0 / 0 / 0]	**12–13**	[1 / 6 / 5–6]	**12–17**	[1–2 / 5–7 / 6–8]	**14**	[2/ 6/ 6]	**4–11**	[0–1/ 2–3/ 2–7]
Centers for Disease Control and Prevention from Atlanta (2020)	Centers for Disease Control and Prevention (Atlanta)	N (1)	**0–1**	[0–1/ 0/ 0]	**8–10**	[3–4/ 2/ 3–4]	**8–10**	[3–4/ 2/ 3–4]	**11**	[7/ 2/ 2]	**2–11**	[1–7/ 0–1/ 1–3]
		N (2)	**0**	[0/ 0/ 0]	**6**	[0/ 5/ 1]	**6**	[0/ 5/ 1]	**7**	[0/ 5/ 2]	**4**	[1/ 3/ 0]
		N (3)	**0–1**	[0/ 0–1/ 0]	**3–5**	[1–2/ 1/ 1–2]	**2–5**	[1–3/ 1/ 0–1]	**4**	[1/ 1/ 2]	**1–3**	[0–1/ 1–2/ 0]
Corman *et al*. (2020)	Charité (Berlin)	RdRp_P1	**3**	[0/ 2/ 1]	**2**	[0/ 2/ 0]	**2–3**	[0–1/ 1/ 1]	**1**	[0/ 1/ 0]	**2–3**	[0–1/ 1/ 1]
		RdRp_P2	**1**	[0/ 0/ 1]	**4**	[0/ 4/ 0]	**3–5**	[0–1/ 2–3/ 1]	**2–3**	[0/ 2–3/ 0]	**2–5**	[0–1/ 1–3/ 1]
		E	**0**	[0/ 0/ 0]	**0–3**	[0–1/ 0–1/ 0–1]	**0**	[0/ 0/ 0]	**0–5**	[0–3/ 0–1/ 0–1]	**0**	[0/ 0/ 0]
National Institute for Viral Disease Control and Prevention from China (2020)	National Institute for Viral Disease Control and Prevention (China)	ORF1ab	**0**	[0/ 0/ 0]	**7–8**	[2/ 1/ 4–5]	**7–9**	[1–2/ 1–2/ 5]	**7–8**	[2/ 1/ 4–5]	**2–4**	[0–1/ 0–1/ 2]
		N	**0**	[0/ 0/ 0]	**8–10**	[2/ 4–5/ 2–3]	**5–10**	[0–3/ 3–4/ 2–3]	**8**	[2/ 4/ 2]	**3–8**	[1/ 1–4/ 1–3]
Chu *et al*. 2020	Hong Kong University Faculty of Medicine (Hong Kong)	ORF1b	**0**	[0/ 0/ 0]	**0–1**	[0/ 0–1/ 0]	**0–1**	[0/ 0–1/ 0]	**0–6**	[0/ 0–2/ 0–4]	**2**	[1/ 0/ 1]
		N	**4**	[0/ 4/ 0]	**4**	[0/ 4/ 0]	**4**	[0/ 4/ 0]	**5**	[1/ 4/ 0]	**4–5**	[0–1/ 4/ 0]
Department of Medical Sciences from Thailand (2020)	Ministry of Public Health (Thailand)	N	**0**	[0/ 0/ 0]	**6**	[1/ 2/ 3]	**6–7**	[1–2/ 2/ 3]	**6**	[2/ 2/ 2]	**2–6**	[0–1/ 1–3/ 1–2]
Shirato *et al*. 2020	National Institute of Infectious Diseases (Japan)	N	**1**	[0/ 0/ 1]	**9–12**	[2–4/ 1/ 6–7]	**10**	[2/ 1/ 7]	**11**	[3/ 1/ 7]	**3–7**	[2–4/ 0/ 1–3]
Chan *et al*. 2020	Hong Kong University State Key Laboratory of Emerging Infectious Diseases (Hong Kong)	RdRp/Hel	**1**	[0/ 0/ 1]	**12–18**	[1–2/ 8–12/ 3–4]	**11–15**	[2/ 8–9/ 1–4]	**11**	[1/ 10/ 0]	**4–6**	[1/ 2–3/ 1–2]
		S	**0**	[0/ 0/ 0]	**14–22**	[6–8/ 6–9/ 2–5]	**24–25**	[8–9/ 9/ 7]	**23**	[8/ 7/ 8]	**11–19**	[3–7/ 4–8/ 4]
		N	**0–1**	[0/ 0/ 0–1]	**10–13**	[2–3/ 7/ 1–3]	**10–11**	[1–2/ 7/ 2]	**11–12**	[2–3/ 7/ 2]	**2–7**	[1/ 0–3/ 1–3]

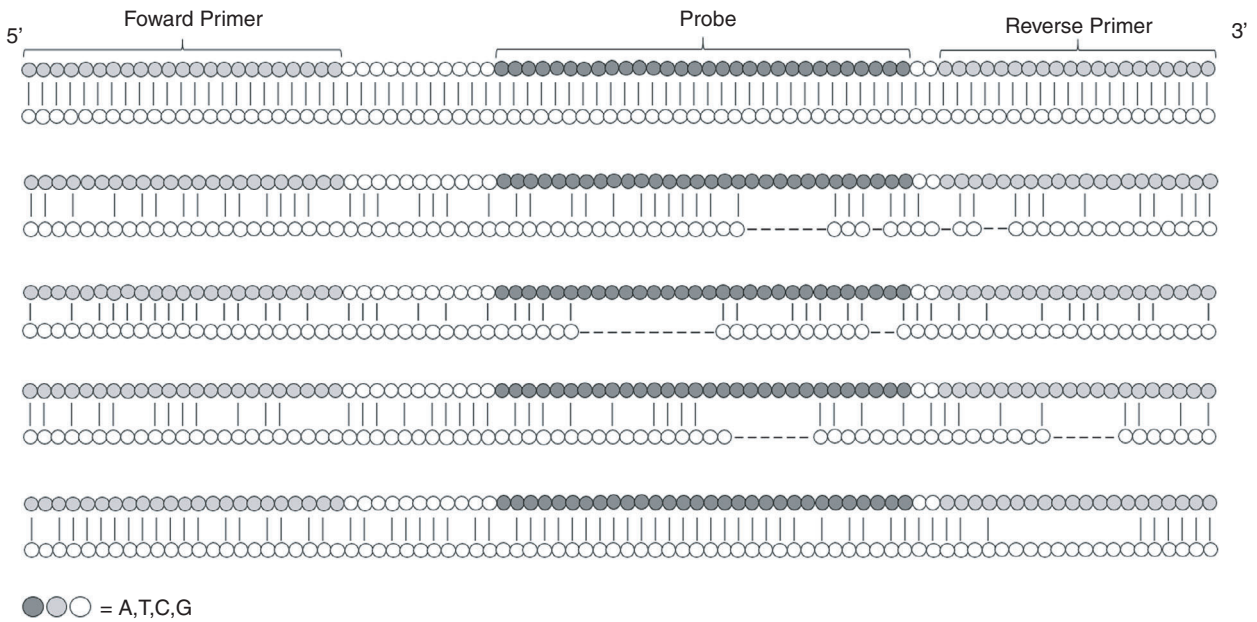

Figure 2 Illustrative alignment representation of the primers/probes sequences of GPS™ CoVID-19 dtec-RT-qPCR Test with (a) SARS-CoV-2 (MN975262.1); (b) bat SARS-like-CoV (MG772934.1); (c) SARS-CoV (AY304489.1); (d) Bat-CoV (KY770859.1); and e) Pangolin-CoV (EPI_ISL_410539).

negative PCR controls were included. Standard curve calibration of the qPCR was performed by preparing 10-fold dilution series containing 10^6 to 10 copies of standard template provided in the kit, but also using $5{\cdot}10^6$ to 5·10 copies of two complete synthetic RNA genomes from SARS-CoV-2 isolate Australia/VIC01/2020 (GenBank no.: MT007544.1) and isolate Wuhan-Hu-1 (GenBank no.: MN908947.3), provided by Twist Bioscience (South San Francisco, CA).

Analytical and diagnostic validation of the GPS™ CoVID-19 dtec-RT-qPCR test

The SARS-CoV-2 detection method of GPS™ was subjected to strict validation according to guidelines of the UNE/EN ISO/IEC 17025:2005 and ISO/IEC 15189 (UNE/EN ISO/IEC 17025 2005; UNE/EN ISO/IEC 15189 2012), as previously described in detail (Martínez-Murcia *et al.* 2018). Validation terms included were repeated 10–15 times and the acceptance criteria are shown in Table 2. Diagnostic validation was a service performed by the Instituto de Salud Carlos III (ISCIII), reference laboratory for biomedical investigation and Public Health (Madrid, Spain). A total of 80 breath specimens from the anonymous biobank of Centro Nacional de Microbiología (CNM, Madrid, Spain) were tested, and characterized by a reference protocol (Corman *et al.* 2020). The GPS™ kit was also evaluated by the Public Health England (PHE; Colindale, London, UK) with a sample panel of 195 specimens, including negative respiratory clinical specimens for SARS-CoV-2, as determined by the validated in-house PHE PCR assay (RdRP gene). Three dilutions of SARS-CoV-2 were used as positive material.

Results

The phylogenetic relationships of selected SARS-CoV-2 genomes and other betacoronavirus as SARS-CoV, Bat SARS-like-CoV, Bat-CoV and Pangolin-CoV are shown in Fig. 1.

The analysis indicated that Bat-CoV RaTG13 and a sequence of Pangolin-CoV showed the highest sequence similarity to SARS-CoV-2 (96.70 and 90.74% respectively), while other Pangolin-CoV sequences available showed a lower homology (85.21%). For *in silico* specificity analysis, the sequences of primers and probes of all RT-qPCR designs available (Corman *et al.* 2020; Chan *et al.* 2020; Institut Pasteur 2020; Centers for Disease Control and Prevention from Atlanta 2020; National Institute for Viral Disease Control and Prevention from China 2020; Chu *et al.* 2020; Department of Medical Sciences of Thailand 2020; Shirato *et al.* 2020), including the GPS™ kit, were aligned to SARS-CoV-2 and the other betacoronavirus sequences. The number of mismatches are annotated in Table 1. In order to illustrate the extent of mismatching, an alignment of primers/probe sequences of the GPS™

Table 2 Summarized results of CoVID-19 dtec-RT-qPCR Test validation according with the guidelines of the UNE/EN ISO/IEC 17025:2005 and ISO/IEC 15189:2012, and acceptance criteria adopted

Term of validation	Obtained values		Acceptance criteria	Result
Specificity	Positive: SARS-CoV-2 isolate Australia/VIC01/2020 (GenBank no.: MT007544.1) and isolate Wuhan-Hu-1 (GenBank no.: MN908947.3)		Inclusiveness: Positive for both SARS-CoV-2 strains	Accepted
	Negative: 39 negative specimens from ISCIII, previously characterized by reference protocol (Corman *et al*. 2020)		Exclusiveness: Negative for all negative specimens	Accepted
Standard curve	$Y = -3.534 \cdot m + 37.534$		$-3.587 < a < -3.103$	Accepted
	$a = -3.534$			
	$R^2 = 0.9986$			
	$F_{assay} = 0.014$		$F_{assay} < F_{fisher}$	Accepted
	$F_{fisher} = 5.318$		$90\% < e < 110\%$	Validated
	Efficiency (e) = 93.1%			
Reliability	Repeatability			
	Conc.	CV (%)		
	10^6 copies	1.18		
	10^5 copies	1.08		
	10^4 copies	0.68	CV < 10%	Repeatable
	10^3 copies	0.53		
	10^2 copies	0.54		
	10 copies	1.31		
	Reproducibility			
	Conc.	CV (%)		
	10^6 copies	1.13		
	10^5 copies	0.91		
	10^4 copies	0.93	CV < 10%	Reproducible
	10^3 copies	0.59		
	10^2 copies	0.66		
	10 copies	1.83		
Limit of detection (LOD)	10 copies	Positive = 15/15 (100%)	Positives ≥ 90%	Accepted
Limit of quantification (LOQ)	10 copies	$t_{value} = 0.582$ $t_{student} = 2.145$	$t_{value} < t_{student}$	Accepted
Diagnostic validation	Diagnostic specificity: 100% Diagnostic sensitivity: 100% Diagnostic efficiency: 100%		≥90%	Accepted

CoVID-19 dtec-RT-qPCR Test, respect to the SARS-CoV-2 and selected sequences of Bat SARS-like-CoV, SARS-CoV, Bat-CoV and Pangolin-CoV is shown in Fig. 2. The total number of mismatches were, 31 for Bat SARS-like-CoV (Fig. 2b), 37 for SARS-CoV (Fig. 2c), 40 for Bat-CoV (Fig. 2d) and 20 for Pangolin-CoV (Fig. 2e), including considerable indels in probe and reverse primer sequences.

Analytical and diagnostic validation of the GPS™ kit, according to the guidelines of the UNE/EN ISO/IEC 17025:2005 and ISO/IEC 15189 (UNE/EN ISO/IEC 17025 2005; UNE/EN ISO/IEC 15189 2012), was undertaken and the results are summarized in Table 2. Empirical validation terms were evaluated for a minimum of 10 assays (15 in the case of LOD and LOQ) and results were subjected to stablished criteria for acceptance (Table 2). The standard curve calibration of the qPCR was performed from 10-fold dilution series containing 10^6 to 10 copies of standard template (Fig. 3a,b).

The *in vitro* inclusivity was assessed by testing two complete SARS-CoV-2 synthetic RNA genomes from isolate Australia/VIC01/2020 (GenBank no.: MT007544.1) and isolate Wuhan-Hu-1 (GenBank no.: MN908947.3). Results obtained from $5{\cdot}10^6$ to $5{\cdot}10$ copies are shown in Fig. 3c,d. The slope and coefficient (R^2) values obtained were −3.534 and 0.9986, respectively, the F_{assay} (0.014) was lower than the F_{fisher} (5.318), and efficiency obtained was 93.1%, considering these values acceptable. The CV values, which assess the reliability of the method, ranged

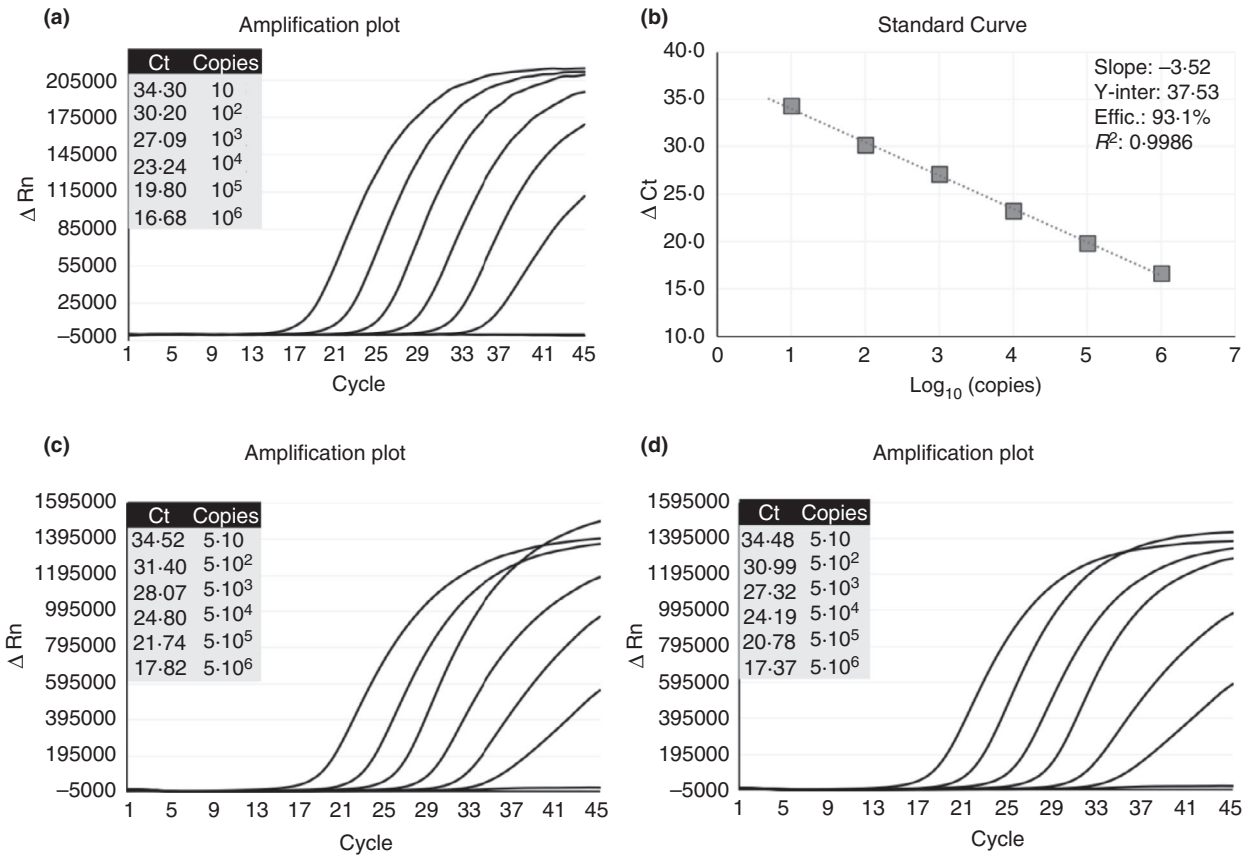

Figure 3 Quality control of the GPS™ CoVID-19 dtec-RT-qPCR test with data of six ranges of decimal dilution from 10^6 copies to 10 copies, and negative control. (a) Amplification plot and (b) a representative calibration curve with stats. Inclusivity of the GPS™ CoVID-19 dtec-RT-qPCR test using six ranges of decimal dilution from $5{\cdot}10^6$ copies to 5·10 copies, and negative control. Amplification plot of synthetic RNA of (c) Australian strain of SARS-CoV-2 (MT007544.1); and (d) Wuhan-Hu-1 strain of SARS-CoV-2 (MN908947.3).

from 0.53 to 1.31% for repeatability, and 0.59 to 1.83% for reproducibility. LOD for 10 copies was 100% reproducible and accuracy of LOQ for 10 copies was acceptable, as the t_{value} (0.582) was lower than the theoretical value from a Student table ($t_{Student}$ = 2.145). Finally, the results obtained in the diagnostic validation of the GPS™ CoVID-19 dtec-RT-qPCR Test, carried out by the Instituto de Salud Carlos III (ISCIII), are shown in Table 3. Full agreement was obtained by comparing data resulting from this commercial kit and these from the reference qPCR used by ISCIII. Consequently, 100% of diagnostic sensitivity and 100% of diagnostic specificity was assigned. The GPS™ kit was also evaluated by the Public Health England (PHE; Colindale, London, UK) and results completely correlated (100%) with these determined by the PHE in-house assay targeting the RNA-dependent RNA polymerase (RdRP) of SARS-CoV-2 (data not shown). Furthermore, the GPS™ kit obtained the CE-IVD label (*in vitro* diagnostics) under the corresponding sanitary licence from the Spanish Agency for Medicines and Health Products (Ministry of Health, Spain).

Discussion

Only 3 months ago, an outbreak of severe pneumonia caused by the novel coronavirus SARS-CoV-2 started in Wuhan (China) and rapidly expanded to almost all areas worldwide. Due to the urgent need of getting tools for SARS-CoV-2 detection, several laboratories developed RT-qPCR methods. Primers and probes sequences were designed from the alignment of a single-first SARS-CoV-2 genome sequence with known SARS-CoV, and some of these protocols were published at WHO website (Corman *et al.* 2020; Chan *et al.* 2020; Institut Pasteur 2020; Centers for Disease Control and Prevention from Atlanta 2020; National Institute for Viral Disease Control and Prevention from China 2020; Chu *et al.* 2020;

Table 3 Results obtained with GPS™ CoVID-19 dtec-RT-qPCR Test in 80 breath specimens compared with the Ct values determined by using a reference protocol (Corman *et al*. 2020), at the Instituto de Salud Carlos III (Madrid)

CNM code	CNM result	CNM Ct GEN1	CNM Ct GEN2	CoVID-19 dtec-RT-qPCRT test	Ct CoVID-19 dtec-RT-qPCR test
#01	NEG	0.00	0.00	NEG	0.00
#02	NEG	0.00	0.00	NEG	0.00
#03	POS	24.00	28.00	POS	29.43
#04	POS	24.00	28.00	POS	23.06
#05	POS	23.19	26.10	POS	27.56
#06	NEG	0.00	0.00	NEG	0.00
#07	NEG	0.00	0.00	NEG	0.00
#08	POS	27.16	30.41	POS	32.18
#09	NEG	0.00	0.00	NEG	0.00
#10	POS	20.19	25.17	POS	21.42
#11	POS	28.00	32.00	POS	30.63
#12	NEG	0.00	0.00	NEG	0.00
#13	POS	28.00	31.00	POS	30.06
#14	NEG	0.00	0.00	NEG	0.00
#15	POS	23.19	26.10	POS	24.72
#16	NEG	0.00	0.00	NEG	0.00
#17	POS	27.48	31.16	POS	19.15
#18	NEG	0.00	0.00	NEG	0.00
#19	NEG	0.00	0.00	NEG	0.00
#20	POS	23.00	25.00	POS	16.07
#21	POS	23.00	25.00	POS	19.32
#22	NEG	0.00	0.00	NEG	0.00
#23	NEG	0.00	0.00	NEG	0.00
#24	POS	25.00	29.00	POS	24.37
#25	POS	20.00	22.00	POS	26.47
#26	NEG	0.00	0.00	NEG	0.00
#27	POS	23.00	25.00	POS	24.86
#28	NEG	0.00	0.00	NEG	0.00
#29	NEG	0.00	0.00	NEG	0.00
#30	POS	24.00	27.00	POS	23.45
#31	NEG	0.00	0.00	NEG	0.00
#32	POS	24.29	27.08	POS	16.20
#33	NEG	0.00	0.00	NEG	0.00
#34	NEG	0.00	0.00	NEG	0.00
#35	POS	27.16	30.41	POS	29.22
#36	NEG	0.00	0.00	NEG	0.00
#37	NEG	0.00	0.00	NEG	0.00
#38	POS	31.00	34.00	POS	33.41
#39	NEG	0.00	0.00	NEG	0.00
#40	POS	23.13	26.49	POS	25.00
#41	POS	16.61	19.06	POS	16.58
#42	NEG	0.00	0.00	NEG	0.00
#43	POS	22.14	25.35	POS	24.01
#44	POS	26.47	29.47	POS	27.17
#45	NEG	0.00	0.00	NEG	0.00
#46	POS	25.59	28.03	POS	27.23
#47	POS	24.16	26.44	POS	25.96
#48	NEG	0.00	0.00	NEG	0.00
#49	POS	24.27	26.48	POS	25.99
#50	NEG	0.00	0.00	NEG	0.00
#51	NEG	0.00	0.00	NEG	0.00
#52	NEG	0.00	0.00	NEG	0.00

(Continued)

Table 3 (Continued)

CNM code	CNM result	CNM Ct GEN1	CNM Ct GEN2	CoVID-19 dtec-RT-qPCRT test	Ct CoVID-19 dtec-RT-qPCR test
#53	POS	24.40	26.61	POS	26.37
#54	NEG	0.00	0.00	NEG	0.00
#55	POS	25.33	26.75	POS	25.66
#56	NEG	0.00	0.00	NEG	0.00
#57	NEG	0.00	0.00	NEG	0.00
#58	POS	25.69	28.39	POS	27.08
#59	NEG	0.00	0.00	NEG	0.00
#60	NEG	0.00	0.00	NEG	0.00
#61	POS	25.73	28.61	POS	27.24
#62	POS	25.91	28.43	POS	27.46
#63	NEG	0.00	0.00	NEG	0.00
#64	POS	26.11	28.20	POS	27.98
#65	POS	25.29	28.17	POS	27.84
#66	NEG	0.00	0.00	NEG	0.00
#67	POS	24.24	27.33	POS	26.19
#68	NEG	0.00	0.00	NEG	0.00
#69	NEG	0.00	0.00	NEG	0.00
#70	POS	24.25	26.87	POS	26.81
#71	POS	26.50	29.08	POS	26.35
#72	POS	25.29	28.17	POS	26.91
#73	POS	26.11	28.20	POS	26.45
#74	NEG	0.00	0.00	NEG	0.00
#75	POS	24.24	27.33	POS	26.41
#76	POS	24.19	26.67	POS	26.65
#77	NEG	0.00	0.00	NEG	0.00
#78	POS	25.23	30.18	POS	31.72
#79	NEG	0.00	0.00	NEG	0.00
#80	POS	24.03	26.88	POS	26.43

Department of Medical Sciences of Thailand 2020; Shirato *et al.* 2020). As the number of genomes available rapidly expanded during last January, the GPS™ CoVID-19 dtec-RT-qPCR test was based on a more specific target for SARS-CoV-2 detection, being this company one of the pioneers marketing a PCR-kit for the CoVID-19 worldwide.

The phylogenetic analysis indicated that SARS-CoV-2 sequences showed a high sequence homology (over 99.91–99.97%), and the closest relatives were strains of several betacoronaviruses, with a considerable sequence identity to Pangolin isolates, (Fig. 1) which confirms previous results (Ceraolo and Giorgi 2020; Zhou *et al.* 2020; Paraskevis *et al.* 2020; Xu *et al.* 2020; Zhang *et al.* 2020; Lam *et al.* 2020). We have found that the single genome sequence of the Bat coronavirus RaTG13 isolated from *Rhinolophus affinis* in Wuhan, showed the highest homology level (96.70%) to SARS-CoV-2, as previously described (Ceraolo and Giorgi 2020; Jiang and Shi 2020; Zhou *et al.* 2020; Lam *et al.* 2020; Li *et al.* 2020a; Li *et al.* 2020). However, because only a single Bat-CoV sequence with this high identity is available, and it was deposited after the outbreak started (27th January), the possibility of RNA contamination during genome sequencing should be ruled-out before take further conclusions. During the design of the GPS™ kit, one of the purposes of the present study was the *in silico* comparison (Table 1) with designed primers and probes so far published (Corman *et al.* 2020; Chan *et al.* 2020; Institut Pasteur 2020; Centers for Disease Control and Prevention from Atlanta 2020; National Institute for Viral Disease Control and Prevention from China 2020; Chu *et al.* 2020; Department of Medical Sciences of Thailand 2020; Shirato *et al.* 2020). In overall, all qPCR designs were inclusive for SARS-CoV-2 as primers and probes showed a good matching. Only the probe for N gene designed by Chu *et al.* (2020) showed four mismatches, which may affect to its binding, particularly considering its short primary structure. In some cases, single nucleotide mismatching was observed in some primers, but none of them were located close to primer 3′-end. Considering all updated alignments, only the Australia/VIC01/2020 sequence

showed a single mismatch with the GPS™ probe. Therefore, a full calibration was run using synthetic RNA genomes from Australia/VIC01/2020 isolate and the resulting Ct values correlated with this obtained from Wuhan-Hu-1 synthetic RNA genome (Fig. 3), indicating that the mismatch in the probe has no effects on the amplification.

In silico analysis for exclusivity was more complex, showing a wide range of discriminative power for the methods subjected to analysis (Table 1). For instance, the two RT-qPCR designs IP2 and IP4 developed by Institut Pasteur seem to discriminate well between SARS-CoV-2 and other respiratory viruses as confirmed for a panel of specimens (Institut Pasteur 2020). The CDC from Atlanta (USA) designed three different primer/probes sets named N1, N2 and N3 (Centers for Disease Control and Prevention from Atlanta 2020). We found a low exclusivity in the N3 primer/probe, but a few weeks ago, this set was removed from the panel (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance). Both N1 and N2 showed a good level of mismatching with most coronaviruses except for some Pangolin-CoV sequences, which showed very few nucleotide differences. The RT-qPCR proposed by Corman *et al.* (2020), designed to detect SARS-CoV-2, SARS-CoV and Bat SARS-like-CoV, is probably the most used worldwide. They suggest the use of an E gene assay as the first-line screening tool, followed by confirmatory testing with the two probes P1 and P2 in the RdRp gene assay. While P1 probe should react with both SARS-CoV-2 and SARS-CoV, P2 probe was considered specific for SARS-CoV-2. Although our *in silico* results confirmed that purpose for P1 (Table 1), the RdRp_P2 assay may also react with some other coronaviruses. The CDC in China developed two RT-qPCR assays for ORF1ab and N genes (National Institute for Viral Disease Control and Prevention from China 2020). Both showed a good overall mismatching to consider them as exclusive, except for some Pangolin-CoV sequences. A similar conclusion may be taken for the N-gene RT-qPCR at the Ministry of Public Health of Thailand (Department of Medical Sciences from Thailand 2020). Data of Table 1 indicated that primer/probe of Chu *et al.* (2020), may be reactive with SARS-CoV-2, SARS-CoV and Bat SARS-like-CoV. The exclusivity of the RT-qPCR design developed by Shirato *et al.* (2020) clearly resided in the reverse primer as showed seven mismatches with all SARS-related coronaviruses. Finally, Chan *et al.* (2020) developed three RT-qPCR assays targeting RdRp/Hel, S and N genes of SARS-CoV-2. They selected the RdRp/Hel assay as considered to give the best amplification performance and was tested in parallel with the RdRp-P2 from Charité-Berlin (Corman *et al.* 2020). All positive patients with the RdRp-P2 assay were positive with the RdRp/Hel design. However, 42 patients negative for the RdRp-P2 assay were positive with RdRp/Hel and they found that only RdRp-P2 assay, but not RdRp/Hel, cross-reacted with SARS-CoV culture lysates (Chan *et al.* 2020). Above findings agreed with expected exclusivity derived from the present study. Additional comparative *in vitro* analysis was performed (Etievant *et al.* 2020; Jung *et al.* 2020; Nalla *et al.* 2020; Vogels *et al.* 2020). Jung *et al.* (2020) indicated that primer/probes of ORF1ab from the CDC-China seems the most sensitive, and the N2 and N3 assays from the CDC-Atlanta were the most recommended. This partially disagrees with our findings as the N3 design may react with other coronaviruses different from SARS-CoV-2 (moreover, the N3 design was recently removed from the CDC panel). In the study by Nalla *et al.* (2020), the specificity of methods from Charité-Berlin and CDC-Atlanta was tested finding no false positive results but differences in the sensitivity. The most sensitive were N2 (CDC-Atlanta) and E (Charité-Berlin). However, the present study indicates the RT-qPCR for E target may react with different SARS coronavirus. Finally, the kit GPS™ COVID-19 dtec-RT-qPCR Test showed the highest number of mismatches (i.e. 19-48) for all coronavirus sequences described so far, including these of Pangolin-CoV, which showed a range of 19–31 mismatches. In addition, considerable indels were discerned which enlarge even more the exclusivity of this design.

The GPS™ kit passed the analytical and diagnostic validation according to criteria of the UNE/EN ISO/IEC 17025:2005 and ISO/IEC 15189 (Table 2). The analysis standard curve was repeated a minimum of 10 times and average values for all parameters were optimum according to standard limits. For reliability, the coefficient of variation obtained in all cases for both, repeatability and reproducibility, was always much lower than 10%. The LOD was tested with the usual protocol for 10 copies repeated 15 times with a positive result in all cases (100%). LOQ assays were performed in two sets of 15 tests for both, 100 and 10 copies of standard template. The LOQ measurement in both cases was validated with a t-Student test with a confidence interval of 95%. The kit received diagnostic validation by two different reference laboratories, ISCIII, Madrid, and PHE, London. The results shown in Table 3 indicated 100% of diagnostic sensitivity and 100% of diagnostic specificity was assigned. Currently, the kit is being used in several Spanish hospitals and diagnostic laboratories.

Obviously, at the time of designing the published RT-qPCR assays (Corman *et al.* 2020; Chan *et al.* 2020; Institut Pasteur 2020; Centers for Disease Control and Prevention from Atlanta 2020; National Institute for Viral Disease Control and Prevention from China 2020; Chu *et al.* 2020; Department of Medical Sciences of Thailand

2020; Shirato *et al*. 2020), a lack of SARS-CoV-2 genomes available may explain the relatively scarce exclusivity found in some cases. Despite the greater or lesser *in silico* specificity of these primers and probes, the host specificity of Bat-CoV, Bat SARS-like CoV and Pangolin-CoV, together with the fact of that no human-SARS have been reported since 2004, all positive results obtained would be considered as SARS-CoV-2 infections (Chu *et al*. 2020; National Institute of Allergy and Infectious Diseases 2020). However, RNA viruses may exhibit substantial genetic variability. Although efforts were made to design RT-qPCR assays in conserved regions of the viral genomes, variability resulting in mismatches between the primers and probes and the target sequences can result in diminished assay performance and possible false negative results. Primers and probes should be reviewed and updated according to new data, which will increase exponentially during the next few weeks/months.

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Conflict of Interest

No conflict of interest declared.

Author contribution

All authors contributed equally to the work.

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