Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Assessment of sample pooling for SARS-CoV-2 molecular testing for screening of asymptomatic persons in Tunisia

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ABSTRACT

The aim of this study is to test a pooling approach for the RT-PCR test to detect low viral loads of SARS-CoV-2. We found that a single positive specimen can still be detected in pools of up to 10. Each laboratory should conduct its own evaluation and validation of pooling protocols according to its specific context.

Coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This virus emerged in the human population in December 2019 in China and has since spread across the globe. The pandemic caused by SARS-CoV-2 poses an enormous burden on society, economic, and healthcare systems worldwide (WHO, Interim Guidance, 2020). The ability to rapidly diagnose COVID-19 is crucial for evaluating the spread of disease and for tracing the contacts of infected individuals. Real-time reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive and specific assay and is considered as the gold standard (WHO, Laboratory and diagnosis, 2020). It requires expensive reagents and kits. This molecular method is based on viral RNA extraction from samples collected by nasopharyngeal (NP) swabs, followed by reverse transcription and amplification using a real time polymerase chain reaction instrument. In Tunisia, where the prevalence of COVID-19 is less than 1%, RT-PCR has been initially implemented only by the National Influenza Center (NIC), hosted at Charles Nicolle hospital of Tunis, where the capacity of testing was around 250 tests per day (1–57 positive cases per day). Then 5 other public health laboratories have been included throughout the country. On March 22, 2020 the number of positive cases in Tunisia has reached 75 with 3 deaths. Preventive strategies were implemented, particularly, lockdown during 75 days and enhancement of contact tracing around all positive cases (National Observatory of New and Emerging Diseases Ministry of Health, Tunisia, 2020a). Recently, following the lifting of lockdown measures, the need to increase the capacity testing through the country is crucial. Some ~~re-cent~~ studies have demonstrated the performance of pooled specimens testing for the diagnostic of COVID-19 (Hogan et al., 2020; Yelin et al., 2020; Lohse et al., 2020; Eis-Hübinger et al., 2020; Pouwels et al., 2020; Abdalhamid et al., 2020). This strategy is used for community monitoring of other infectious diseases such as detection of the human immunodeficiency virus and hepatitis B and C viruses blood samples in blood donors or mass detection of malaria infection (Aprahamian et al., 2016; Taylor et al., 2010). Pooling samples is a cost-saving method allows to cover a greater percentage of the population with the same amount of tests without significantly impacting the ability to detect the virus in specimens with high or medium viral loads (Ct ≥24) (Pouwels et al., 2020; Abdalhamid et al., 2020). However, group testing is a reasonable option if pooling does not reduce the sensitivity of the test. Therefor it's crucial to test the sensitivity at various group sizes for estimating optimal pool size. With smaller group sizes, additional amplification cycles, or inclusion of a re-test for negative group samples, the false negative rate could be reduced further (Pouwels et al., 2020).

To avoid shortage of reagents and to increase COVID-19 laboratory testing capacities in Tunisia, this study was conducted to establish the optimal parameters for sensitive COVID-19 RT-PCR test in multi-sample pools to detect low viral loads.

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To detect SARS-CoV-2 in NP specimens, the Hong Kong RT-PCR assay using an ABI 7500 instrument was performed (WHO, Laboratory and diagnosis, 2020). It's a qualitative real time RT-PCR TaqMan method. With this assay, a positive COVID-19 result is determined when both targets N and Orf1b-nsp14 reach a defined threshold below 0.2 and Ct value less than 40. Low Ct values indicate the presence of high viral RNA and high Ct values indicate lower viral RNA. Twenty confirmed positive specimens and 780 negative specimens that have been tested at the NIC were used in this study. The selected Ct values of positive specimens were between 25 and 34 for N and Orf1b-nsp14. Six pools of 4, 5, 8, 10, 20, and 40 specimens with each containing one of the positive patient mixed with 3, 4, 7, 9, 19, and 39 negative specimens were tested (total volume of 200 μL/pool, same volume from each sample). RNA of mixed samples was extracted from 200 μL of NP swabs using the GXT NA extraction kit for GenoXtract Instrument following the manufacturers' instructions (Hain Lifescience Gmbh, Nehren, Germany).To increase the sensitivity of the pooling technique, the number of cycles has been increased to 45.

The pooled testing application "https:// www.chrisbilder.com/shiny" was used to calculate the most efficient pool size. The following parameters were selected: the prevalence rate of COVID-19 in Tunisia (0.8%), RT-PCR sensitivity (95%), and specificity (100%), two-stage pooling algorithm and pool ranges between 4 and 40.

A total of 120 pools were screened. As the number of negative samples increase in the pool, the amplified RNA reaches the threshold later. All samples reached the threshold in 110 sample pools. All undetectable targets were in pools of 20 and 40 specimens, respectively). The Ct values for both N and Orf1ab-nsp14 genes assays in pools and individual positive samples were below 40 and categorized as positive, except sample number 12 for which the Ct values were 41.17 and 36.70 for N and Orf1ab-nsp14, respectively (pool 6). Ct value differences between pooled tests and individual positive samples were in the range of 0.24 and 12.33 [Table 1]. According to these results, a single positive specimen can be tested in pools of up to 10, with the same performance of the standard RT-PCR. However, the sensitivity was decreased to 80% and 70% when 20 and 40 specimens were pooled, respectively. The Shiny application analysis predicted that the most efficient pool size is 11 samples leading to reduce the expected number of tests by 80% when compared to individual testing.

The first phase of the COVID-19 pandemic threat management in Tunisia allowed achieving the general objective of the strategy put in

Table 1
Comparison of threshold cycles between the original and pooled COVID-19 positive samples.

Positive Specimen N°	Target	Original Ct value	Pool1 (4 specimens)		Pool 2 (5 specimens)		Pool 3 (8 specimens)		Pool 4 (10 specimens)		Pool 5 (20 specimens)		Pool 6 (40 specimens)	
			Ct value	Ct difference	Ct value	Ct difference	Ct value	Ct difference	Ct value	Ct difference	Ct value	Ct difference	Ct value	Ct difference
1	**N**	31.12	33.75	**2.63**	34.22	**3.10**	35.37	**4.25**	35.66	**4.54**	35.81	**4.69**	36.69	**5.57**
	ORF1ab	29.71	32.15	**2.44**	32.22	**2.51**	33.00	**3.29**	33.24	**3.53**	34.77	**5.06**	40.89	**11.18**
2	**N**	33.92	37.10	**3.18**	37.22	**3.30**	38.00	**4.08**	38.00	**4.08**	39.00	**5.08**	-	**ND**
	ORF1ab	31.24	34.90	**3.66**	36.41	**5.17**	37.30	**6.06**	41.00	**9.76**	43.00	**11.76**	-	**ND**
3	**N**	32.36	33.21	**0.85**	34.24	**1.88**	35.12	**2.76**	36.34	**3.98**	44.69	**12.33**	37.79	**5.43**
	ORF1ab	30.85	31.89	**1.04**	33.12	**2.27**	33.62	**2.77**	34.77	**3.92**	-	**ND**	-	**ND**
4	**N**	31.49	34.61	**3.12**	34.30	**2.81**	35.74	**4.25**	35.87	**4.38**	36.94	**5.45**	37.33	**5.84**
	ORF1ab	28.76	32.91	**4.15**	32.95	**4.19**	34.30	**5.54**	34.13	**5.37**	39.37	**10.61**	36.80	**8.04**
5	**N**	31.35	34.00	**2.65**	33.81	**2.46**	34.80	**3.45**	34.83	**3.48**	35.92	**4.57**	39.00	**7.65**
	ORF1ab	28.96	32.43	**3.47**	31.92	**2.96**	33.29	**4.33**	33.14	**4.18**	35.91	**6.95**	38.32	**9.36**
6	**N**	33.59	37.33	**3.74**	37.37	**3.78**	37.48	**3.89**	39.11	**5.52**	38.17	**4.58**	-	**ND**
	ORF1ab	30.76	37.76	**7.00**	38.84	**8.08**	39.15	**8.39**	41.20	**10.44**	-	**ND**	-	**ND**
7	**N**	35.00	36.65	**1.65**	37.64	**2.64**	38.00	**3.00**	39.63	**4.63**	-	**ND**	-	**ND**
	ORF1ab	34.00	37.18	**3.18**	37.96	**3.96**	38.64	**4.64**	39.15	**5.15**	-	**ND**	-	**ND**
8	**N**	33.10	34.20	**1.10**	34.75	**1.65**	35.25	**2.15**	37.21	**4.11**	37.49	**4.39**	36.40	**3.30**
	ORF1ab	31.24	33.73	**2.49**	33.79	**2.55**	34.71	**3.47**	37.00	**5.76**	37.52	**6.28**	36.13	**4.89**
9	**N**	28.00	29.38	**1.38**	28.97	**0.97**	30.24	**2.24**	31.55	**3.55**	31.49	**3.49**	33.00	**5.00**
	ORF1ab	26.69	28.73	**2.04**	28.75	**2.06**	29.48	**2.79**	30.10	**3.41**	31.44	**4.75**	32.90	**6.21**
10	**N**	29.47	31.78	**2.31**	29.95	**0.48**	31.13	**1.66**	31.17	**1.70**	31.40	**1.93**	33.74	**4.27**
	ORF1ab	27.20	32.61	**5.41**	30.00	**2.80**	31.41	**4.21**	30.96	**3.76**	30.93	**3.73**	33.23	**6.03**
11	**N**	28.10	30.12	**2.02**	29.96	**1.86**	31.24	**3.14**	31.89	**3.79**	33.00	**4.90**	36.88	**8.78**
	ORF1ab	27.35	30.16	**2.81**	29.63	**2.28**	30.93	**3.58**	31.70	**4.35**	32.35	**5.00**	-	**ND**
12	**N**	30.11	33.08	**2.97**	33.54	**3.43**	36.04	**5.93**	36.39	**6.28**	38.07	**7.96**	41.17	**11.06**
	ORF1ab	28.35	32.37	**4.02**	33.06	**4.71**	34.96	**6.61**	35.35	**7.00**	35.98	**7.63**	36.70	**8.35**
13	**N**	26.20	26.57	**0.37**	26.59	**0.39**	27.46	**1.26**	27.99	**1.79**	28.67	**2.47**	29.78	**3.58**
	ORF1ab	26.98	27.22	**0.24**	27.50	**0.52**	27.92	**0.94**	28.36	**1.38**	29.57	**2.59**	30.00	**3.02**
14	**N**	27.53	31.49	**3.96**	31.69	**4.16**	32.01	**4.48**	32.92	**5.39**	33.60	**6.07**	34.48	**6.95**
	ORF1ab	27.79	31.19	**3.40**	31.67	**3.88**	32.09	**4.30**	32.45	**4.66**	33.00	**5.21**	34.26	**6.47**
15	**N**	28.95	31.16	**2.21**	31.50	**2.55**	31.96	**3.01**	33.34	**4.39**	34.05	**5.10**	34.73	**5.78**
	ORF1ab	28.45	29.94	**1.49**	30.94	**2.49**	31.16	**2.71**	31.61	**3.16**	31.94	**3.49**	32.96	**4.51**
16	**N**	26.60	27.90	**1.30**	28.12	**1.52**	28.74	**2.14**	29.99	**3.39**	31.24	**4.64**	31.35	**4.75**
	ORF1ab	24.63	27.33	**2.70**	27.40	**2.77**	28.31	**3.68**	29.29	**4.66**	30.50	**5.87**	30.60	**5.97**
17	**N**	29.40	31.86	**2.46**	31.63	**2.23**	32.40	**3.00**	32.81	**3.41**	33.22	**3.82**	34.16	**4.76**
	ORF1ab	28.00	31.27	**3.27**	31.35	**3.35**	31.74	**3.74**	31.87	**3.87**	32.87	**4.87**	33.27	**5.27**
18	**N**	29.32	32.85	**3.53**	32.95	**3.63**	33.36	**4.04**	34.15	**4.83**	34.16	**4.84**	35.29	**5.97**
	ORF1ab	29.00	32.29	**3.29**	32.64	**3.64**	32.99	**3.99**	34.00	**5.00**	33.27	**4.27**	34.00	**5.00**
19	**N**	24.96	28.25	**3.29**	29.00	**4.04**	29.18	**4.22**	29.68	**4.72**	30.77	**5.81**	30.98	**6.02**
	ORF1ab	24.26	28.95	**4.69**	29.60	**5.34**	30.13	**5.87**	30.26	**6.00**	31.83	**7.57**	31.95	**7.69**
20	**N**	31.58	32.90	**1.32**	33.03	**1.45**	34.00	**2.42**	34.18	**2.60**	34.71	**3.13**	35.64	**4.06**
	ORF1ab	31.78	33.23	**1.45**	33.83	**2.05**	37.04	**5.26**	36.08	**4.3**	-	**ND**	-	**ND**

Pool 1 = one positive specimen mixed with 3 negative ones; Pool 2 = one positive specimen mixed with 4 negative ones; Pool 3 = one positive specimen mixed with 7 negative ones; Pool 4 = one positive specimen mixed with 9 negative ones; Pool 5 = one positive specimen mixed with 19 negative ones; and Pool 6 = one positive specimen mixed with 39 negative ones. (−):Undetectable; ND = not determined.

place: to slow down the progression of SARS-CoV-2 and to avoid overshooting the capacity of the health system. Since 4thMay 2020, Tunisia has initiated a progressive targeted containment, a new phase in the management of the pandemic. New challenges arise to avoid a rebound. The risk of rebound largely depends on the implementation of measures of targeted containment and on the system's capacity to early detect, isolate and treat all new cases. On 7th June, 2020, the cumulative number of confirmed COVID-19 cases is 1087 (out of a total of 55,519 samples) corresponding to a cumulative incidence of 9.17/100,000 inhabitants and an average of daily incidence around 12 cases. Among the cases detected, 5.72% are still active and constitute a risk of transmission if the preventive measures are not properly observed. The occurrence of new cases in past 2 days confirms the existence of high risk of SARS-CoV-2 transmission (National Observatory of New and Emerging Diseases Ministry of Health, Tunisia, 2020b).

All individual specimens in a negative pool are regarded as negative. Deconvoluted testing is recommended if any of the pools are positive. Key principles for successful application of group testing involve knowledge of the limit of detection, sensitivity and specificity of the assay and the prevalence of disease in the population. Abdalhamid et al. (2020) have shown that optimal pool size and prevalence rates are inversely proportional; when the prevalence rate of COVID-19 in population is low more samples can be mixed, which can lead to varying the size of the pool to be tested according to the level of circulation of the virus. According to our results, pooling can increase test capacity with existing equipment and test kits and detects positive samples with sufficient diagnostic accuracy. The RT-PCR protocol used is confirmed to have a wide dynamic range (2^{-4}-2000 viral titre$TCID_{50}$/reaction) (WHO, Laboratory and diagnosis, 2020). Pooling of more than 10 samples is not recommended to avoid the effect of dilution leading to false negatives. According to literature, the optimal specimen pool size varied from 5 to 10 (Abdalhamid et al., 2020;Hogan et al., 2020; Hanel and Thurner, 2003). However, when RNA extracts were pooled the optimal pool size is up to 32 samples. The authors suggest that additional amplification cycles increasing the pool size up to 64 and reduce the number of false negatives (Lohse et al., 2020; Yelin et al., 2020).

The pooling of samples using molecular testing for SARS-CoV-2 would allow expanding the current capacities of the available laboratories thereby enabling the expansion of detection in the community, as well as in closed settings, such as hospital staff, army or policy units, or factory shifts. However, for each laboratory, an independent evaluation of the protocols according to the reagents and the automates used is mandatory.

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Compliance with ethical standard

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