ADVICE ON POOLING OF SAMPLES FOR DETECTION OF SARS-COV-2 WITH RT-PCR

RAG subgroep Testing – 16 februari 2021

Note: The current recommendations are subject to change depending on new scientific data and/or the evolution of the epidemic.

CONTEXT

Pooling of specimens for RT-PCR from multiple individuals can substantially reduce the cost when the positivity rate is low. The number of tests to perform is expected to increase within the context of a broader testing in specific populations. The RAG testing was therefore requested to provide an advice on the use of pooling strategies.

PREVIOUS RECOMMENDATIONS ON POOLING

The **testing strategy update of August 2020**[1], listed the following recommendations with regards to pooling of samples for SARS-CoV-2 testing with RT-PCR:

- Currently, it is not recommended to use pooling as a diagnostic tool for symptomatic individuals or contacts, since pooling reduces sensitivity to a certain extent, increases the risk of errors/contamination and is probably not efficient in reducing the use of capacities in case of shortages (when prevalence is probably high).
- Pooling could be used for screening of large asymptomatic populations expected to have a low prevalence. When screening in low-risk asymptomatic populations (e.g. schools), a certain loss in sensitivity is acceptable, as those most contagious/superspreaders (ie lowest CT values) should still be detected. The impact of the reduced sensitivity will depend on the setting, being possibly more problematic in high-risk settings (e.g. WZC/MRS) than in low-risk settings (e.g. schools).
- It is recommended that laboratories that implement pooling use a validated protocol (extension of the RT-PCR cycles may be considered). For development of a pooling protocol, the applications and methods developed and referred to in this document can be useful to determine the best pooling method and/or the number of samples in a pool. Deconstruction of the pools should also be well described to reduce risk of cross-contamination. The experience of veterinary departments and transfusion centers in developing pooling protocols should be used, as they have a lot of experience in pooling for mass screening.

[1] TESTING STRATEGY UPDATE AUGUST 2020: POOLING, SALIVA TESTING, RT-LAMP, RAPID ANTIGEN TESTING, SELF-COLLECTED NOSE, THROAT AND NASOPHARYNGEAL SWABS AND MULTIPLEX - RAG 19/08/2020

- It is recommended to pool before RNA extraction to reduce the risk of contamination and limit the use of reagents.
- An overview of Belgian laboratories experienced with/capable of pooling for COVID-19 is needed.
- RIZIV/INAMI reimbursement of pooling is currently administratively not possible.
- In the context of platform bis, pooling for screening should be integrated in its 'own' flow.

In addition, pooling of saliva samples combines the benefits of both strategies, however, sensitivity will inevitably be further decreased. There are limited data available on the performance of the combination of these two techniques, and further operational studies are highly needed.

In the **September 2020 update**[2], pooling was mentioned as a possible strategy for a broader testing in the context of an outbreak, for example when samples from an outbreak investigation can be joined with samples from other asymptomatic people. The need to develop a protocol was repeated.

In the **October 2020 update**[3], pooling is mentioned as a possible strategy to reduce costs in repetitive testing of large populations, and in the recent RAG advice on repetitive testing in specific populations[4], it was recommended to pool samples whenever possible to reduce costs and turn-around time. The effective size of the pool should be determined per applied procedure.

DISCUSSION

- Pooling is not recommended for diagnosis in people with symptoms, nor for testing in the context of cluster outbreak investigations (because of the loss of sensitivity and the delay in obtaining positive results). It is only useful (not recommended, but can be used) for screening of large asymptomatic populations in which the prevalence rate is expected to be low.
- An important disadvantage of pooling is that it introduces a delay (one day) in obtaining the final result of the positive cases. The decision to pool or not has therefore to take this into account, and pooling might not be indicated when a result is promptly needed. Using rapid Ag tests for the second round could resolve this, but little is known about the effectiveness of rapid Ag Test in this context.

[2] TEST STRATEGIE UPDATE SEPTEMBER 2020 RAG 09/09/2020
[3] TEST STRATEGIE UPDATE OKTOBER 2020 GEBRUIK VAN SPEEKSELTESTEN EN SNELLE ANTIGEENTESTEN - RAG 12/10/2020 or MISE À JOUR DE LA STRATEGIE DE TEST OCTOBRE 2020 UTILISATION DE TESTS SALIVAIRES ET DE TESTS ANTIGÈNES RAPIDES - RAG 12/10/2020
[4] AANBEVELINGEN BETREFFENDE HERHAALDELIJK TESTEN IN SPECIFIEKE BEVOLKINGSGROEPEN or RECOMMANDATIONS SUR LE DÉPISTAGE PÉRIODIQUE DANS DES POPULATIONS SPÉCIFIQUES

- Another disadvantage is that it complicates the work at the laboratory and that there are two different protocols to be applied (one with and without pooling). These issues could, however, be addressed in the pooling protocol.
- The prevalence threshold above which it was agreed that pooling is not useful, is 10%.
- Two-rounds of testing (testing of a pool, followed by individual testing of all samples of positive pool) is the best pooling strategy.
- The most adequate pool size has to be based on the expected positivity rate, the lower the prevalence, the larger the pool. The pool sizes applied by ULiège (3 when the prevalence was around 5%, 6 now that the prevalence has decreased) are appropriate examples. It is difficult to establish exact pool sizes per prevalence, because there are other factors that have to be considered, such as the type of sample used.
- The calculator by Pilcher et al. is a useful tool, but there are several parameters to be filled out that are not known.
- Household pooling, in which the whole household is put in quarantine without a second round of testing, as has been modelled by UHasselt, is an interesting approach, but raises a number of challenges at laboratory level, and is currently not feasible.
- The next step has to be the development of a pooling protocol. This will be done within the context of the pilot project of repetitive testing of school staff.

RECOMMENDATIONS

- The recommendation not to use pooling as a diagnostic tool for symptomatic individuals or contacts, is still valid. It is also not recommended in cluster outbreak investigations.
- Pooling should only be used for screening of large asymptomatic populations expected to have a low prevalence, such as in repetitive screenings. The prevalence threshold above which pooling is no longer considered useful is a positivity rate in the tested population of 10% or more.
- Pooling is never obligatory. Each laboratory can decide, based on the available capacity, if they want to pool samples or not.
- Different pooling strategies can be used. The most straightforward strategy is two rounds of testing, where each sample of a positive pool is retested individually.
- The best pool size varies according the expected positivity rate and according the sensitivity of the specimen used (saliva versus swabs) and has therefore to be decided ad hoc. The available results of modelling studies or the use of calculators can provide guidance.

- It is recommended that laboratories that implement pooling use a validated protocol. A protocol should be developed as soon as possible in the context of the planned pooling of samples for the repetitive testing of school staff.

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BACKGROUND LITERATURE

Literature on pooling

An extensive literature review with regards to pooling is available in the testing strategy update of August 2020, and the conclusions are still valid:

- Several pooling strategies can be used:
    - two rounds of testing, where each sample of a positive pool is retested individually (most straightforward);
    - three or more rounds of testing, where pools with positive samples are further divided into smaller pools and then are tested individually or re-divided in smaller pools (not time efficient);
    - two rounds of testing (and if needed an extra round for individual testing), in the second round samples are tested in multiple overlapping groups (difficult scheme);
    - one round of testing, where samples are distributed into a matrix of overlapping groups (fast but very complex).
- Pooling is more efficient at low prevalence/test positivity ratios (although one study suggests pooling is efficient up to a prevalence of 20%). With increasing prevalence/test positivity ratio, pooling will increasingly become less efficient and may lead to delayed test results (for positive pools, due to retesting of all individual samples).
- Pooling could be used in a context of shortages of capacities (reagents, human resources in lab). However, since such shortages are more likely to occur in a context of high virus circulation (and high sero-prevalence), it will probably not be cost-effective.
- Optimal pooling size depends on:
    - Prevalence/test positivity ratio.
    - Pooling method used.
    - Ct values of positive samples.
- Optimal pooling strategy depends on:
    - Prevalence/test positivity ratio.
    - Acceptable complexity of pooling scheme.
- Sensitivity decreases with pooling, the extent (which is small according to literature) to which depends on the protocol used. A decrease of sensitivity will mainly impact samples with high Ct values (theoretically a 2-fold dilution of RNA increases the Ct with 1). Some studies suggest using an alternative cut off when pooling.
- Particular caution is required with regards to pre and post analytical errors. The risk of cross contamination is greater when using pooling approaches.
- Pooling can be done before and after RNA extraction. The impact on sensitivity of both techniques is not clear from literature. Pooling before RNA extraction will additionally save extraction reagents and decrease the risk of contamination.

- Several models and applications have been developed to determine the best pool size or pooling strategy.

Additional literature on the relationship between efficiency and prevalence

A *study by UGent* evaluated one-time (1D) pooling and two-dimensional-matrix (2D) pooling for massive, low prevalence population screening using real-life RT-PCR data from 1632 positive samples (1). It observed an inverse relationship between efficiency and prevalence over the prevalence range from 0.01% to 10%. Until a prevalence of 0.36%, 1x24 is the most efficient strategy, from 0.40% to 2.51% 16x24 becomes the most efficient, from 2.82% to 4.47% the most efficient strategy is 12x24 and from 5.01% to 10% 8x12 is the most efficient strategy. Strategies employing a larger pool size display a higher efficiency when the prevalence is low, but as the prevalence increases, there is a tipping point for each strategy at which its smaller pool size variant becomes more efficient. As a general trend, 2D pooling methods are less sensitive to changes in prevalence in comparison with 1D pooling methods. The authors conclude that the most efficient pool size very much depends on the prevalence, but 2D pooling methods generally are most efficient when prevalence is higher than 0.4%. They also observed that at a prevalence lower than 1%, there is an increased variation in sensitivity and that the increased efficiency at low prevalence comes with a low and pool size-dependent problematically variable sensitivity.

The cut-off of the prevalence rate above which pooling is no longer cost-efficient varies according studies, but is generally considered to be high. Aragón-Caqueo et al. calculated that for a prevalence of 10% of positive tests, 40.6% of tests can be saved, for a 20% prevalence, 17.9% of tests can be saved, and for higher prevalence rates, the strategy flattens and loses effectiveness (2). Abdalhamid et al. concluded that when the prevalence rate is 10% or less, pooling will result in the saving of reagents and personnel time with an overall increase in testing capability of at least 69% (3). Eberhardt et al. found that pooling is more efficient than individual testing for prevalence rates under 30% (4). For prevalence rates under 12%, multi-stage schemes had higher improvement factors than two-stage schemes.

Several mathematical models have estimated the best pool size per prevalence rate, for the most straightforward strategy of two or three rounds of testing where each sample of a positive pool is retested. The table below summarizes some of the results. Most studies recommend a pool size of +/-4 when the prevalence rate is expected to be around 10%, but higher sizes when the prevalence is lower. For a prevalence of approximately 1% the recommended pool size ranges from eight to eleven. Some authors recommend a three-stage pooling when prevalence is 1% or lower. When prevalence was 0.1%, larger pools and particularly 3-stage pools were substantially more efficient. However, some models indicated that pool sizes >25 are expected to reduce analytic sensitivity by >20%.

Pilcher et al. provide a free, publicly available web calculator to help inform laboratory decisions on SARS-CoV-2 pooling algorithms.

Table: Recommended pool size per prevalence rate

Prevalence rate	Aragón-Caqueo et al.(2)	Pilcher et al.(5)	Deckert et al.(6)	Becker et al.(7)	Abdalhamid et al.(3)	Eberhardt et al.(4)	Shani-Narkiss et al.(8)
0.1%		25:5		10		9:3	24
0.5%		25:5	14	10		9:3	16
1%	11	25:5	10	10	11	9:3	8
2%	8			8		9:3	8
3%	6			6	6	9:3	8
4%	6			6		9:3	8
5%	5	6	5	5	5	16:4	4
10%	4	4	3	4	4	16:4	4

Some studies showed that when positive cases are clustered by known social structures, such as student households, the pooling of samples by these social structures can substantially further reduce the total cost (6,9). In the modelling study by UHasselt, in which a sample pooling of individuals that belong to the same households was applied, to allow for a universal testing procedure, the size of the pools was 16 and 32 (10).

Experiences with pooling in Belgium

Uliège applied a strategy of pooled testing of three samples in the repetitive screening of staff of nursing homes. The positivity rate was 0.92%. Sensitivity was good in samples with a high viral load (Ct value <25) and only 0.3% of the positive samples was missed. Pooling of student samples was initially per three, but later increased to six because the prevalence had decreased.

International recommendations

ECDC

In its Technical Report 'COVID-19 testing strategies and objectives' of 15 September 2020, ECDC states that pooling or group testing of specimens is faster than individual testing and saves resources in situations where the proportion of positive samples is expected to be very small (up to 5%) (11). Several samples are combined and tested once, typically with a leftover or second sample kept from each individual. If the combined result is positive, which may occur rarely or more frequently depending on the epidemiological situation, the individual samples are then tested.

Alternatively, samples may be put into several pools, the results of which together identify the sample that was positive. For infection rates from 0-2.5%, binary splitting pooling seems to be the best method while others have suggested a single stage non-adaptive group-testing approach for up to 1.3% positivity without the need to subsequently test individual samples.

ECDC has also provided a methodology for estimating the point prevalence of SARS-CoV-2 infection through pooled RT-PCR testing (12).

WHO

In its Interim guidance on Diagnostic testing for SARS-CoV-2 of 11 September 2020, WHO recommends pooling of specimens for RT-PCR from multiple individuals to increase the diagnostic capacity for detecting SARS-CoV-2 when the rate of testing does not meet the demand (13).

They propose as possible strategies a two-stage testing or matrix pooling. Pooling of specimens could be considered in population groups with a low/very low expected prevalence of SARSCoV-2 infection, but not for cases or cohorts that more likely to be infected with SARS-CoV-2. Routine use of the pooling of specimens from multiple individuals in clinical care and for contact tracing purposes is not recommended.

Before any sample pooling protocols can be implemented, they must be validated in the appropriate populations and settings. An inappropriate testing strategy may lead to missed cases or other laboratory errors that may, in turn, negatively affect patient management and public health control measures. In addition, the risk of cross-contamination and the potential increase in workload complexity and volume must be considered. To perform reliable pooling, adequate automation is key (e.g. robotic systems, software supporting the algorithms to identify positive samples, laboratory information systems and middle-ware that can work with sample pooling).

CDC

CDC published an Interim Guidance for Use of Pooling Procedures in SARS-CoV-2 Diagnostic, Screening, and Surveillance Testing on 23 October 2020 (14). It states that a pooling strategy depends on the community prevalence of virus, and pool size will need to be adjusted accordingly. CDC recommends that laboratories should determine prevalence based on a rolling average of the positivity rate of their own SARS-CoV-2 testing over the previous 7–10 days. Laboratories should use a standardized methodology or calculator that factors in the sensitivity of the assay they are using and their costs of testing to determine when the positivity rate is low enough to justify the implementation of a pooling strategy. Laboratories should also understand and, where appropriate, communicate the limitations associated with pooled testing.

The prevalence of COVID-19 in a population affects the efficiency of pooled testing strategies. In general, lower disease prevalence may enable a laboratory to use a larger optimal pool size. They refer to the study by Abdalhamid et al.(3) that found that RT-PCR for SARS-CoV-2 reliably returned a positive result when one positive sample was mixed with four negatives, and could reduce the number of tests needed by >50% in certain scenarios (such as a COVID-19 prevalence of 5%). However, as the prevalence of COVID-19 increases, the cost savings of a pooling strategy decreases because more pooled tests will return positive results and those specimens will need to be retested individually.

The Netherlands

The RIVM states in their update on diagnostic testing of 8 January 2021 (15):

Pooled testing of samples from different patients is one way to increase testing capacity and is or will be applied by a number of laboratories. Only in the case of positive pools do all samples then have to be tested separately. There are also some disadvantages: open robot systems are needed for the pipetting steps, it only works with a relatively low prevalence, the pooling dilutes the samples slightly which may reduce their sensitivity slightly, the re-testing of the positive pools causes some delay and it requires an adaptation of the laboratory information management system which in most labs is only equipped for testing individual samples. By means of tenders, VWS has recruited suppliers and laboratories that will make large-scale pooling possible. The quality of pooling is also monitored by a validation panel of the RIVM.

France

The 'Haut Conseil de la Santé Publique' has recently (15 January 2021) updated its advice on pooling (16). They now recommend:

- not to practice pooling as part of an individual diagnostic procedure for a person being cared for in a health establishment or a medico-social establishment or when the person is at risk of a serious form of Covid19;
- not to practice pooling during a screening process when the prevalence of infection is more than 5% in the tested population;
- to consider pooling (from 5 to 10 samples) of samples only when the prevalence of infection among the population tested is less than 5% and provided that this practice does not lead to a lengthening of the time taken to deliver results or disruption of the operation of the laboratories;
- to conduct pilot studies to evaluate new techniques such as digital RT-PCR or high-speed sequencing (NGS).

United Kingdom

The last advice on pooling from Public Health England dates from 25 September 2020 (17). They state that sample pooling is only efficient when the expected positivity rate is low, and that no pooling strategy is effective when the positivity rates exceed 10%. Aggressive pooling can be used with low transmission; however the pool size needs to be reduced quickly when the positivity rate is above 1% e.g. for a positivity rate of around 3% the ideal pool size is 6 samples. Lab processes must be in place to successfully and reliably identify individual samples from pooled samples and retest with lower TAT and accuracy. Suggested target groups include asymptomatic elective patients and asymptomatic staff where most samples are going to be negative. Pooling should not be used on individuals who are symptomatic and likely to test positive. Pooling should not be used for individuals where a rapid confirmation of COVID-19 status is required, such as for diagnostic purposes or for testing to cohort patients in healthcare settings. The advice also contains a testing protocol.

Germany

The Robert Koch Institute issued on 7 July 2020 a report on the optimization of laboratory capacities for the direct and indirect detection of SARS-CoV-2 in the context of the management of measures, in which pooling of samples is addressed (18).

Pooling can be used as a limited procedure if the actual demand for services exceeds the available resources. The criterion is considered to be exceeding the threshold of 95% for at least 4 weeks. Pooling is possible in the context of screening and surveillance investigations (e.g. testing of asymptomatic employees in medical facilities or during occupational health examinations) and/or surveillance investigations (e.g. indicator populations) with an expected very low prevalence or regionally correspondingly low 7-day incidence. Orientation or decision limits can be set in the event of a pandemic.

Pooling should not be used for diagnostic tests in the areas of symptom-oriented testing of patients and staff, contact person testing, suspicion clarification, or admission screening. If pooling is used as a general method in connection with regional or fundamental resource scarcity, any reduction in sensitivity in relation to the individual sample should be compensated for as fully as possible by repeat measurements.

Liquid samples with a homogeneous consistency are particularly suitable as a matrix. A pool size of up to 5 samples is considered possible in principle.

REFERENCES

1. Verwilt J, Mestdagh P, Vandesompele J. Evaluation of efficiency and sensitivity of 1D and 2D sample pooling strategies for diagnostic screening purposes. medRxiv. 2020 Aug 17;2020.07.17.20152702.

2. Optimization of group size in pool testing strategy for SARS-CoV-2: A simple mathematical model - PubMed [Internet]. [cited 2021 Feb 11]. Available from: https://pubmed-ncbi-nlm-nih-gov.vdicp.health.fgov.be/32330297/

3. Abdalhamid B, Bilder CR, McCutchen EL, Hinrichs SH, Koepsell SA, Iwen PC. Assessment of Specimen Pooling to Conserve SARS CoV-2 Testing Resources. medRxiv. 2020 Apr 6;

4. Eberhardt JN, Breuckmann NP, Eberhardt CS. Multi-Stage Group Testing Improves Efficiency of Large-Scale COVID-19 Screening. J Clin Virol. 2020 Jul;128:104382.

5. Pilcher CD, Westreich D, Hudgens MG. Group Testing for Severe Acute Respiratory Syndrome-Coronavirus 2 to Enable Rapid Scale-up of Testing and Real-Time Surveillance of Incidence. J Infect Dis. 2020 Aug 17;222(6):903–9.

6. Deckert A, Bärnighausen T, Kyei N. Full title: Pooled-sample analysis strategies for COVID-19 mass testing: a simulation study. :17.

7. Becker MG, Taylor T, Kiazyk S, Cabiles DR, Meyers AFA, Sandstrom PA. Recommendations for sample pooling on the Cepheid GeneXpert® system using the Cepheid Xpert® Xpress SARS-CoV-2 assay. PLOS ONE. 2020 Nov 9;15(11):e0241959.

8. Efficient and Practical Sample Pooling for High-Throughput PCR Diagnosis of COVID-19 | medRxiv [Internet]. [cited 2021 Feb 12]. Available from: https://www.medrxiv.org/content/10.1101/2020.04.06.20052159v2

9. Hemani G, Thomas AC, Walker JG, Trickey A, Nixon E, Ellis D, et al. Modelling pooling strategies for SARS-CoV-2 testing in a university setting. medRxiv. 2021 Jan 20;2021.01.19.20248560.

10. Libin P, Willem L, Verstraeten T, Torneri A, Vanderlocht J, Hens N. Assessing the feasibility and effectiveness of household-pooled universal testing to control COVID-19 epidemics. medRxiv. 2020 Oct 6;2020.10.03.20205765.

11. COVID-19 testing strategies and objectives [Internet]. European Centre for Disease Prevention and Control. 2020 [cited 2021 Feb 12]. Available from: https://www.ecdc.europa.eu/en/publications-data/covid-19-testing-strategies-and-objectives

12. Methodology for estimating point prevalence of SARS-CoV-2 infection by pooled RT-PCR testing [Internet]. European Centre for Disease Prevention and Control. 2020 [cited 2021 Feb 12]. Available from: https://www.ecdc.europa.eu/en/publications-data/methodology-estimating-point-prevalence-sars-cov-2-infection-pooled-rt-pcr

13. Diagnostic testing for SARS-CoV-2 [Internet]. [cited 2021 Feb 12]. Available from: https://www.who.int/publications-detail-redirect/diagnostic-testing-for-sars-cov-2

14. CDC. Labs [Internet]. Centers for Disease Control and Prevention. 2020 [cited 2021 Feb 11]. Available from: https://www.cdc.gov/coronavirus/2019-ncov/lab/pooling-procedures.html

15. Aanvullende informatie diagnostiek COVID-19 | LCI richtlijnen [Internet]. [cited 2021 Feb 12]. Available from: https://lci.rivm.nl/covid-19/bijlage/aanvullend

16. HCSP. Diagnostic et dépistage du Covid-19 à l'aide du poolage : actualisation des recommandations [Internet]. Paris: Haut Conseil de la Santé Publique; 2020 Nov [cited 2021 Feb 12]. Available from: https://www.hcsp.fr/explore.cgi/avisrapportsdomaine?clefr=968

17. Coronavirus » Pooling of asymptomatic SARS COV-2 COVID-19 samples for (PCR/or other) testing [Internet]. [cited 2021 Feb 12]. Available from: https://www.england.nhs.uk/coronavirus/publication/pooling-of-asymptomatic-sars-cov-2-covid-19-samples-for-pcr-or-other-testing/

18. RKI - Coronavirus SARS-CoV-2 - Bericht zur Optimierung der Laborkapazitäten zum direkten und indirekten Nachweis von SARS-CoV-2 im Rahmen der Steuerung von Maßnahmen [Internet]. [cited 2021 Feb 12]. Available from: https://www.rki.de/DE/Content/InfAZ/N/Neuartiges_Coronavirus/Laborkapazitaeten.html