Best Practice Guidance: Implications for SARS-CoV-2 variants and the monitoring of impact on antigen assay performance (including lateral flow tests)

Authors & affiliations: Nathaniel Storey[1], Jim Huggett[2], Rui Pereira[2], Denise O'Sullivan[2], Kathryn Harris[1], Rebecca Riches-Duit [3], Louise Oliver[4], Richard Vipond[4], Kirsten Kelly[4], Monee Shamsher[4], Lisa Stevens[4]

1. Department of Microbiology, Virology and Infection Prevention & Control, Great Ormond Street Hospital NHS Foundation Trust.
2. Molecular and Cell Biology Team, National Measurement Laboratory (LGC)
3. Medicines and Healthcare products Regulatory Agency (MHRA)
4. UK Health and Security Agency (UKHSA)

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1. Scope

This document outlines the procedure for manufacturers of antigen tests to address alterations in the viral genome that can cause a change in the structure of antigens recognised by antibodies used by lateral flow devices (LFDs). This guidance should be used as part of post-market surveillance requirements to routinely monitor for new mutations and variants currently circulating within the United Kingdom and to assess the impact of associated genetic changes on the performance of antigen assays.

2. Introduction

Several Variants of Concern (VOC) and Variants under investigation (VUI) have been recognised in the SARS-CoV-2 virus that are circulating globally. The term VOC is assigned to variants which are currently emerging or circulating, and for which we have confirmed or can predict a detrimental change in biological properties (changes in transmissibility, severity or immune evasion) compared to the current dominant variant(s); and have a growth rate potentially compatible with maintaining transmission and/or displacing the current dominant variant.

The nature of replication in RNA viruses leads to a wide genetic diversity of variants and selection pressure for those with an advantage to persist. This is particularly true for surface located antigens like the spike protein, and VOCs to date have exhibited multiple S gene changes. Selection pressure for mutation in the gene encoding the nucleocapsid (N), envelope (E) or RNA dependent RNA polymerase (RdRp) proteins appears to be less common when looking at frequency of changes in VOC.

Antigen testing have been used as diagnostic tools performed by enzyme linked immunosorbent assays (ELISA) and LFDs using oral, nasal, throat, nasopharyngeal and oropharyngeal swab samples and antibodies to target SARS-CoV-2 antigens within these samples. Typically, antigen tests have a single SARS-CoV-2 antigenic target; currently the majority of those available are designed to detect the virus by the antibodies binding to specific regions of the antigen (epitopes) of the highly expressed N protein in these swab samples.

The antibodies, or equivalent high affinity molecules, used to label and bind the target in the antigen tests may be selected to recognise one or multiple epitopes i.e., epitope recognition regions can be linear (continuous residues on a protein sequence) or conformational (residues that are discontinuous in the protein sequence yet come within close proximity to form an antigenic surface on the antigen's three-dimensional structure). The use of single monoclonal antibodies for both labelling and binding of the antigen, whilst favourable for test specificity and epitope mapping, is more at risk of assay failure due to VOC change in the nucleocapsid that may impact the target epitope: polyclonal solutions provide redundancy as they target multiple epitopes. Therefore, tests based on combinations of monoclonal or polyclonal antibodies would likely require larger numbers of changes across several epitopes before their performance is adversely affected.

Any alterations in the viral genome that causes a change in the structure of the antigens and hence the epitope region(s) could negatively affect performance of an antigen-based assay. Most mutations (referred to as “synonymous”) do not result in any change in viral proteins. Some mutations (“non-synonymous”) change the amino acid sequence resulting in changes in epitope regions and can have a marginal, catastrophic, or no effect on the performance, and so can potentially result in a false negative result. Therefore, it is essential for manufacturers to routinely monitor for mutations in antigenic regions (epitopes) that are targeted by the antibodies used within LFDs to minimise impact of any viral mutations on test performance. *In silico* sequence assessment can be used as a starting point to mitigate the possible reduction in assay sensitivity through virus mutation(s).

Armed with the genomic or amino acid sequence of the genetic variants in question, it is relatively easy to compare an epitope region against the nucleotide or amino acid sequences of circulating variants and determine whether any genetic change within this region may impact on the performance of an LFD. Furthermore, using suitable criteria, an assessment can be made as to whether the change may be minor or result in catastrophic failure of the diagnostic test leading to false negative results and feed into an early warning and response system. This assessment also provides crucial information for the design of the test; if a non-synonymous mutation occurs that changes the structure of an epitope and ultimately a change in the binding affinity of target antibodies resulting in changes to test performance, then the test manufacturers may need to redesign the assay to fulfil the required sensitivity/specificity.

Under regulation 34A of the Medical Devices Regulations 2002 no antigen or molecular detection COVID-19 (SARS-CoV-2) test may be placed on the UK market without first being validated against minimum performance standards through a Coronavirus Test Device Approvals (CTDA) desktop review (COVID-19 test approval: how to apply - GOV.UK (www.gov.uk)). If the assay has been re-

designed to consider a potential issue with a VOC or VUI, this assay may need to be re-validated and submitted to the CTDA for approval before it can be sold on the UK market. Tests listed on the CTDA register are approved for the instructions for use (IFU) version listed on the register only. Where a manufacturer updates their IFU they shall submit the amended IFU to CTDA for assessment as a minor or major change. If deemed a major change a new full application will need to be submitted. As CTDA is a goods regulation, only those tests (IFU versions) listed on the register are allowed to be sold on the UK market.

If a test is re-designed, changed, or an IFU is updated, to retain UKCA/CE marking the manufacturer may also need to perform a conformity assessment and possibly gain approval from its Approved Body or Notified Body, depending upon the classification of the IVD under the UK MDR 2002. To address this change to the IVD or to correct another problem, the manufacturer may also need to submit a Field Safety Notice (FSN) and Field Safety Corrective Action (FSCA) form to the MHRA, and to send the FSN to their customers to alert them of any changes and of corrective actions to mitigate risk. Please refer to section 8 for further detail on FSNs.

In this document we recommend a methodology for *in silico* analysis of sequence variation in the genetic regions targeted by the LFDs.

3. Specific requirements relating to antigen tests and antigen lateral flow devices (LFDs)

Manufacturers of antigen tests shall detail in their IFU the following product specifications information for the purpose of end user assurance:

- the target antigen(s) detected by the assay e.g., N protein or S protein used to generate/select for the antibodies/binding molecules used in the LFD
- the nature of antibodies/binding molecules used for detection/labelling of the antigen e.g., or monoclonal/polyclonal or combinations thereof
- the nature of the antibodies/binding molecules used to capture the antigen at the test line e.g., monoclonal/polyclonal or combinations thereof, or colloidal gold, etc.

Whilst some details on specific reagents, epitopes in target antigens and how these are prepared are intellectual property and/or commercially sensitive information, this can be shared with the relevant competent authority. In the UK, this is the MHRA; such confidential information shall be part of the full data set required. This information shall include the details described above, along with specific information of domain, subunit, peptides or epitopes that the antibodies were raised against; the specific combination of antibodies or high affinity binding molecules used for both labelling and test line binding. The information is required so that the MHRA can monitor any issues that might occur with a given antigen test if a predominant variant arises.

4. Frequency recommendations for *in silico* analysis

The frequency of running *in silico* analysis is of great importance. Due to the rapidly evolving distribution of SARS-CoV-2 lineages both within the UK and globally, *in silico* analysis should be conducted afresh every two months and in a timely manner. The epitope sequences should be compared against a time span of 60 days of variant sequence submissions circulating in the UK within the chosen database (see section “5. Bioinformatics recommendation for *in silico* analysis for antigen tests”), unless a new emerging variant requires immediate investigation – in this instance you will be notified by the MHRA. This would allow rapid identification of lineages that may be

affected by the genetic changes within assay target regions and allow for continuous monitoring for the stability of these genetic changes that may have arisen.

The prevalence over time can be skewed due to the lag time between sample collection, sequencing, and the submission of sequence data to a database. To address this uncertainty, a rolling window of 60 days is recommended. The frequency of analysis should remain the same to ensure comparability.

However, during periods of rapid expansion of specific lineages or increased diversity, more frequent and targeted analysis may be required.

5. Bioinformatics recommendation for *in silico* analysis for antigen tests

The analysis of sequences should be performed by a trained bioinformatician or person with significant experience in the handling of large databases and the tools used should be both peer-reviewed and version controlled. The version information and references of all tools and software should be recorded for each analysis and the use of differing tools or versions between analyses is strongly discouraged.

Considerations for the database against which the epitope (target query) sequence is to be compared against shall include the assessment of the quality of sequence deposited, diversity or number of sequences deposited for each lineage or variant and whether the data is representative of UK lineages.

Sequence quality is of particular importance and care should be taken to reduce the number of aligned sequences containing unknown or ambiguous bases. The diversity or number of sequences deposited for each variant will vary depending on the database used, so it is recommended that a global database such as GISAID (GISAID - gisaid.org) or country specific, in this case representing UK specific lineage data from COVID-19 Genomics UK Consortium (COVID-19 Genomics UK Consortium (cogconsortium.uk), be used. UK specific sequence data is available either as a single file from the COG-UK data page or international data can be searched with specific criteria from GISAID. These sequences can be downloaded from databases in any format that will allow for the analysis and sorting of the data. Epitope sequences should be compared against the lists of amino acid changes per sequence extracted directly from GISAID or COG-UK. Under normal circumstances it may be beneficial to filter sequences by using the 'Complete' definition of GISAID. However, in certain circumstances such as a rapidly emerging variant with significant divergence from previous lineages, we recommend that none of the filters are selected in order to obtain a suitable quantity of sequences for analysis. Regular updates on SARS-CoV-2 lineage prevalence and growth rates observed in the UK can be obtained from the GOV UK website (SARS-CoV-2: genome sequence prevalence and growth rate - GOV.UK (www.gov.uk)) and manufacturers should use the latest update to determine which VOCs/VUIs sequences to interrogate. Up to date information about SARS-CoV-2 genome mutations and variants of interest circulating in the UK can also be accessed through the COG-UK Mutation Explorer (COG-UK-ME) interface COG-UK/Mutation Explorer (gla.ac.uk). This latter source is updated twice a week (correct as of July 2023).

The target query sequences should be compared to all suitable sequences for the VOC or VUI within a time span of 60 days of variant sequence submissions to the database. In instances where the number of sequences available for a representative lineage circulating within the UK is limited, sequences should be analysed to yield significant and reliable interpretation of the data. If the sequence data is limited by low sequence numbers (<100 sequences), this should be caveated in the

interpretation of the results. In the latter instance where the analysis has been conducted with limited number of sequences, the manufacturer should explain this in a caveat to highlight the higher frequency of mutations (if any) for these lineages and should continue to monitor this lineage in the next analysis.

For use as the target query sequence, it is recommended that short sequences (<200bp) are extracted from complete records that correspond to regions associated with the target nucleotide sequence. This reduces the chances of non-specific alignment and allows the region of interest to be more accurately quality controlled. Any sequence containing unknown or ambiguous bases within the target query region should be removed from the database to prevent misidentification of mutations. In the GISAID database, the hCoV-19/Wuhan/WIV04/2019 (WIV04) GISAID - hCoV-19 Reference Sequences is the official reference sequence employed (EPI_ISL_402124). WIV04 was chosen because of its high-quality genome sequence and because it represented the consensus of a handful of early submissions for the beta coronavirus responsible for COVID-19. On the protein level NCBI Reference Sequence: NC_045512.2 Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, co - Nucleotide - NCBI (nih.gov) is the predominantly used reference sequence.

From full length variant sequences extracted from the appropriate database(s), multiple sequence alignments (MSA) or pairwise alignments should be performed to identify amino acid changes within target sequences, using peer-reviewed tools. For generating MSA from large sequence databases manufacturers should use an aligner such as minimap2 (https://github.com/lh3/minimap2), in order to reduce the computational requirements, it is recommended that multiple pairwise alignments are performed on consensus sequences rather than MSA-proper. From the sam files produced by the aligner of choice, further tools such as gofasta (https://github.com/virus-evolution/gofasta) may be used to convert the data into fasta format for further analysis. For the specific analysis of amino acid changes within SARS-CoV-2 genes, the use of tools such as Nextalign (https://hub.docker.com/r/nextstrain/nextalign) may offer improvements in ease of use and is available in a containerised form.

Any lineages with previously identified mutations shall be continually updated within the database so that any further acquired mutations that may result in assay failure are rapidly identified.

Information of either: all sequence identifiers (for example, COG-UK ID or accession) of sequences used in the analysis, or sufficient metadata (for example, lineage and epi week/date range) and sequence selection criteria to ensure that the data used can be independently identified, shall be made available to the MHRA should this be requested.

6. Assessment of impact on lateral flow devices

Any change in amino acid sequence in the target antigen should be considered a possible risk to assay/test performance due to its potential conformational changes in the 3D protein structure. This variant impact should be assessed and reported accordingly.

As a result of the *in silico* analysis performed, the frequency of amino acid change for each variant should be reported, and proportions calculated for regular surveillance purposes. A defined criteria to determine if the proportion of mutation(s) occurring can have an impact on the assay should be used. This resulting information from the *in silico* analysis should be formatted in an easy to review table and include the information below as a minimum:

1. Reference sequence used e.g., hCoV-19/Wuhan/WIV04/2019 (WIV04).

2. Location of target query sequence of the epitope analysed within the reference sequence.
3. Database used for mining of circulating sequences e.g., GISAID.
4. Timeframe when sequences were mined e.g., 01 March 2023 to 31 March 2023.
5. Database used to determine which variant sequences were analysed e.g., UK circulating variants.
6. Variant sequences analysed, using SARS-CoV-2 pangolin lineages as standard nomenclature e.g., BA.1.
7. Location of mismatch(es) within epitope on each variant e.g., P151S.
8. Frequency and proportion at each mutation(s) or mismatch(es) within each variant analysed. The frequency and location of all mutation(s) or mismatch(es) within all epitope targets shall be noted and percentage frequency based on the number of target sequences analysed for this lineage should be calculated for regular surveillance purposes (for example, a single SNP/SNV that effectuates an amino acid change should be recorded as: P151S, 1.5% frequency).
9. Predicted impact of mutation(s) and what criteria is used for defining impact on the assay performance including a justification of the likelihood that the mutation may impact assay performance. A further justification for any actions taken, or not taken, based on the outcome of this analysis should also be mentioned.

Evidence of impact should be based on dynamic *in silico* structural homology modelling of the mutated variant amino acid changes and assessed against predicted antibody binding affinity.

It is required that both favourable and unfavourable data should be reported to the MHRA as assurance of either positive or negative performance.

7. Wet laboratory tests

Details of any performance evaluation of tests against variants that have been conducted or how manufacturers plan to test to demonstrate performance with either recombinant protein (containing the sequence changes) or characterised viral isolates shall be clearly communicated.

Where amino acid sequence variation is detected in target sequences and there is an impact predicted due to the amino acid change, manufacturers shall conduct confirmatory wet lab testing of their product or assay against the variant of interest or mutation profile of the variant of interest. It is advisable even if prior *in silico* analysis of variants does not identify any lineages carrying mutations in the target epitopes, these *in silico* predictions should still be challenged with a variety of wet-testing methods using either recombinant protein studies, live virus studies, inactivated virus studies, or clinical studies to ensure that there is no impact on the assay due to changes in 3D structure.

Wet testing with a clinical sample with the mutation(s) within the epitope, if available, is ultimately required to show its effect on assay performance. Where clinical material is not available, due to potential difficulties in identifying and acquiring such a sample, use of synthetic recombinant target peptide with and without the amino acid mutation is acceptable although corroborative experiments using clinical material at a later date should be performed when such materials do become available. Limit of detection (LOD) testing should be performed in parallel as specified in the IFU for synthetic targets containing both the “wild-type” (no mutation) and mutation(s) representing the variant of interest.

Information from wet testing should be formatted in an easy to review table and include the information below as a minimum:

1. Type of material used in wet laboratory testing e.g., synthetic molecules (or recombinant protein) with defining mutations or clinical samples.
2. Methodology used to determine LOD.
3. LOD of the wild type (original reference sequence) as validated in IFU and when tested alongside variant sequences.
4. Number of samples used for wild type and variant sequences e.g., number of clinical samples used to conduct the experiment.
5. LOD of the variants using same standard units as used for wild type material.
6. Impact on the assay through wet testing.

If wet testing results show that the mutation negatively affects assay performance and risks the assay producing false negative results, then this shall be reported to the MHRA within 48 hours as a serious public health threat, and an FSN shall be issued to alert customers.

8. Field safety notice (FSN)

The UK medical devices regulations (UK MDR 2002) state that manufacturers must report incidents involving their device as soon as possible, and no later than 30 days after becoming aware of the incident. In line with UK MDR 2002 vigilance and field safety corrective action reporting requirements, the MHRA consider reports relating to SARS-CoV-2 Variants of Concern to be serious public health threats, therefore significant safety issues, including indirect patient harm through false negative and false positive results, should be reported within 48 hours. If the wet testing performance of any assay target is directly impacted by these new virus variant(s), a FSN shall be issued immediately to alert customers. The FSN and FSCA shall also be submitted to the MHRA. Similarly, if a manufacturer receives customer reports regarding false negative results, this shall be investigated immediately and reported to the MHRA. FSNs may also need to be issued if there are changes to the device IFU, or to the assay design, as field safety corrective actions may need to be undertaken. Further guidance on effective FSNs can be found at:

https://www.gov.uk/guidance/effective-field-safety-notices-fsns-guidance-for-manufacturers-of-medical-devices.

9. Post-market surveillance plan (PMSP)

Effective post-market surveillance shall be performed for SARS-CoV-2 screening and diagnostic assays under the UK MDR 2002 to continuously monitor, investigate and assess the performance of an assay against newly emerging variants. A post-market surveillance plan shall be in place and this plan should be provided to the MHRA. It is also good practice to apply international standards to quality management systems (ISO 13485) and risk management (ISO 14971) for medical devices, as well as participating in any available and relevant External Quality Assurance (EQA) schemes.

10. Conclusions

The diversity of genomic sequences of SARS-CoV-2 necessitates a proactive approach to assay design and monitoring. To this end these recommendations should be used to guide *in silico* assay monitoring, and to ensure that further in-depth monitoring of clinical assays is maintained. Using the available SARS-CoV-2 sequence data in this way will provide the clinical community and those tasked

with managing the COVID-19 pandemic with the foresight to avoid diagnostic assay failure in the face of viral genome evolution. Together, with manufacturers of screening and diagnostic SARS-CoV-2 assays, we can help to protect the health of patients and the public.

11. References

ISO - ISO/TS 5798:2022 - In vitro diagnostic test systems — Requirements and recommendations for detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by nucleic acid amplification methods

SARS-CoV-2: genome sequence prevalence and growth rate - GOV.UK (www.gov.uk)

Guidance for manufacturers: diagnostic assurance with SARS-CoV-2 variants in circulation

Target Product Profile: Laboratory-Based SARS-CoV-2 Viral Detection tests