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Points to Consider for Replacement of Conventional Tests for Viruses with Next Generation Sequencing (NGS) Assays (Early Consideration)

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1. Introduction

To analyze human and animal genetic information, a technique for sequencing genomic nucleic acids called the Sanger method[1)] has been used traditionally. Recently developed next generation sequencing (NGS) technologies[2)] have advanced and accelerated sequencing operations, enabling one-day completion of a project equivalent to the human genome project, which took 10 years with the Sanger method. The term, next generation sequencing technologies, just means technologies emerging after the Sanger method and does not indicate a specific platform. That is, manufacturers of NGS-technology-based sequencing devices employ different platforms, and their test methods are not standardized. Standard NGS technologies typically employ massive parallel sequencing platforms in which short fragments of DNA of interest are sequenced simultaneously, and the obtained sequences or reads lengthening from <100 to <1000 bases are combined by computer software to obtain the entire sequence of the DNA of interest (short-read sequencing), but recently emerging ones enable direct reading DNA of interest >1000 bases long in a single run (long-read sequencing).

The NGS technologies have realized very powerful sequencing techniques, which are also known as high-throughput sequencing (HTS) techniques because of their high processing capacity. Based on the above advantages, the NGS technologies were expected to be applicable to detection of adventitious viruses contaminated in human or animal cells but initially had difficulties in the aspect of cost, etc. Recent advancement of the NGS technologies has reduced the cost, making their application to virus detection practical. Thus, the second version of the "Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin" (PSB/PED Notification No. 0109-3, dated January 9, 2025, hereinafter referred to as "ICH Q5A(R2)")[3)] includes additional guidance for tests for viruses using NGS technologies (hereinafter referred to as "NGS assay") and potential use of an NGS assay in place of a conventional test for viruses. The guidance concerned in the ICH Q5A(R2), however, is intended to recommend use of new technologies in place of traditional ones, aiming to reduce use of experimental animals, and use of the NGS assay in place of a conventional test for viruses itself needs to be further investigated according to the test to be replaced. At present, there is no globally agreed-upon NGS assay procedure or validation studies for analytical procedures to replace conventional tests for viruses. Appropriate procedures and evaluation are being sought by international collaboration groups and research teams in and outside Japan.

This document is intended for developers and marketing authorization holders who are considering use of an NGS assay in place of a test for viruses under the above circumstances and aims to present current regulatory consideration based on PMDA's recent experiences, discussions held among international study groups, guidelines issued by regulatory authorities outside Japan, scientific papers, etc. The consideration presented in this document is based on the knowledge available to date. Please note that it is subject to change in response to progress in research by groups in and outside Japan and new knowledge that may become available in future.

2. Scope of application

This document applies to cases where an NGS assay is used in place of nucleic acid amplification tests (hereinafter referred to as "NAT assay"), antibody production tests (hamster antibody production [HAP], mouse antibody production [MAP], or rat antibody production [RAP] test), *in vivo* tests for viruses, or *in vitro* tests for viruses for biopharmaceuticals or regenerative medical products falling within a scope of application of ICH Q5A(R2). It does not apply to cases for biopharmaceuticals or regenerative medical products falling outside the scope of application of ICH Q5A(R2), but its essential concept may be applicable as well. If an NGS assay is used as a part of comprehensive virus control strategy because of a product-specific problem but not just as a replacement for a particular test, consultation with PMDA may be available for justification for its use on a case-by-case basis.

3. Validation of analytical procedures for NGS assay

As described in Section 4, validation of analytical procedures for an NGS assay should be designed to account for its capability of meeting the expected performance standard in view of the test being replaced by the NGS assay. In this section, points for validation of analytical procedures described in ICH Q5A(R2) are discussed. In ICH Q5A(R2), NGS assay is classified as a limit test, defined in the second revision of the "Validation of Analytical Procedures" (PSB/PED Notification No. 1009-1, dated October 9, 2025, hereinafter referred to as "ICH Q2(R2)").[4)] Characteristics to be evaluated in a validation of analytical procedures should be specified in view of ICH Q2(R2).

It should be noted that development of technologies and advancement of understanding may direct a validation of analytical procedures to attach weight to factors other than those described in ICH Q5A(R2). Developers should carefully examine if there are any factors required for the validation of analytical procedures in addition to ones listed here.

3.1 Selection of reference viruses

A validation of analytical procedures is required to present the detection limit, specificity, and range (type of virus, etc.) of the target virus. To do so, an appropriate virus should be selected as the reference material (hereinafter referred to as "reference virus"). In an NGS assay, physical, chemical, and

genomic properties of the target virus may affect efficiency of extraction of nucleic acids and library preparation, described below.

When NGS assays are used to detect specific viral groups, the reference viruses should possess physical, chemical, and genomic properties similar to the target analytes. In contrast, for the purpose of comprehensive detection of unspecified viruses, multiple reference viruses with different physical, chemical, and genomic properties should be selected to cover all types of viruses.

A report of the World Health Organization (WHO)[5] and the Advanced Virus Detection Technologies Working Group (hereinafter referred to as "AVDTWG"[6,7]; the Advanced Virus Detection Technologies Interest Group was renamed in 2022) led by the Food and Drug Administration (FDA) proposed 7 viruses as reference virus candidates, which are deemed as the most common reference viruses at present. It should be noted that this reference virus panel is subject to change. Actually, 2 viruses were added between 2020 and 2024.[5),8] Developers should explain suitability of these reference viruses, even if they are the most common, according to an objective of the test.

3.2 Selection of specimens

Test specimens to be used in an NGS assay should be carefully selected based on the nature of the target virus. As described in ICH Q5A(R2), common test specimens are expected to primarily include total genomic nucleic acids extracted from cells (genomics), mRNA extracted from cells (transcriptomics), and nucleic acids prepared from cell culture supernatants (viromics). The type of detectable viruses may vary among these specimen types. A transcriptomic analysis performed on mRNA may be suitable for detection of viruses with their mRNA replication ongoing but can be less sensitive to latent viruses. A viromic analysis performed on nucleic acids prepared from cell culture supernatants may excel in detecting viruses that shed into the culture medium, but it may lack sensitivity for latent viruses or those that spread via cell-to-cell infection without budding viral particles. A genomic analysis performed on genomic nucleic acids is unlikely to have such bias risks but potentially misidentifies virus-like sequences within the chromosomal DNA. Developers should explain suitability of the test specimens in view of these advantages and limitations.

Test specimens contain a substantial amount of nucleic acids derived from cell substrates, which include endogenous virus-like sequences. In addition, test specimens collected from manufacturing process involving viral vectors or products containing viruses/vectors as an active ingredient are supposed to contain a high concentration of viral nucleic acids derived from these viruses/vectors. As an NGS assay is required to be capable of detecting true virus sequences from a wide variety of nucleic acids, an appropriate validation of analytical procedures may be performed with test specimens that are collected from the actual manufacturing process and spiked with reference viruses.

3.3 Nucleic acid extraction and library preparation

The method of nucleic acid extraction is critical. Specifically, since extraction efficiency greatly depends on specimen type and virus characteristics, a system capable of extracting target viruses appropriately should be established. In general, nucleic acid extraction kits using columns, magnetic beads, etc. are employed. A protocol of using the kit should be carefully optimized because amounts of specimens and nucleic acids applied to the column or magnetic beads, presence or absence of added carrier DNA/RNA, and a final elution volume can impact the extraction efficiency of the target nucleic acids.

Library preparation methods vary depending on a platform of NGS technology used in the assay. The protocol should be evaluated in the context of the specific platform. Quality of the library should be comprehensively evaluated based on the ability to detect the reference viruses.

3.4 Selection of NGS technology platform

Although several NGS technology platforms are available, which platform is suitable for virus detection remains unclear at present. The available NGS technology platforms are categorized into short-read and long-read sequencing platform groups, which are supposed to have greatly different characteristics. The short-read sequencing platforms determine the sequence by assembling short fragments and are thus expected to deliver highly accurate base sequences. However, they struggle with specific regions, such as repetitive sequences, which may lead to incomplete coverage. In contrast, the long-read sequencing platforms are capable of accurately determining repetitive sequences, that are challenging for the short-read sequencing platforms, but they may be prone to higher error rates due to the challenges of accurately reading many bases in a single pass. Developers should understand these characteristics and select the platform in view of the potential impact on detection of target viruses.

3.5 Selection of database and informatics analysis software

In assessment of NGS assay performance, significant importance should be placed on database coverage and performance of the bioinformatics analysis software. In addition, parameter values (number of reads, etc.) in the software should be carefully determined.

The database commonly used is the Reference Viral Database (RVDB)[9] that is published by the Center for Biologics Evaluation and Research (CBER), FDA and covers an extensive range of viruses. The database covering an extensive range of viruses, on the other hand, also excessively includes viruses without documented human pathogenicity and endogenous virus-like sequences potentially existing as remnants from ancient viruses, which can cause substantial “noise” in detection operations and may compromise the ability to detect viruses that should be truly detected. To eliminate such concerns, the database only including viruses infectious to humans, such as “Human viruses” of Virus-Host DB,[10] may be used. Developers should select the database that has been demonstrated to have

performance that meets the objective of the test.

Version control for both databases and analysis software is also critical. The versions used in a validation study of analytical procedures should be continuously used in principle, but periodic update of the database, retirement of obsolete software versions due to technological advancements, etc. may occur. Whenever the version is changed, developers should evaluate its impact and perform a validation study of analytical procedures again as necessary.

4. Replacement of conventional tests for viruses

The NGS technology is just a tool, and for what purpose it is used determines strategy of the validation study of analytical procedures. If a conventional test for viruses is replaced with an NGS assay, the "Analytical Procedure Development" (PSB/PED Notification No. 1009-2, dated October 9, 2025; hereinafter referred to as "ICH Q14")[11] serves as a key reference. According to the study objective, the strategy of the validation study of analytical procedures should be carefully determined after establishing the analytical target profile (ATP) presented in ICH Q14. In this section, points of potential great importance in the study are presented on the assumption that an NGS assay is used in place of a conventional test for viruses.

Notably, molecular biological approaches including the NGS method examine samples for presence of virus-derived nucleic acids, and detection of virus-derived nucleic acids does not necessarily indicate presence of infectious viral particles. Protocols for handling detected nucleic acids (whether a sample is provisionally deemed positive or remains under investigation pending further analysis, etc.) should be predetermined in view of positioning of the test. Supplemental investigation following the detection of viral nucleic acids typically include infectivity assay, lengths of detected nucleic acids, a coverage rate of the detected nucleic acids relative to the full length of viral nucleic acids, type of viral nucleic acids (DNA or RNA), NGS platform, and identification of a contamination route of the viral nucleic acids. If the decision is made in view of results of the additional investigation, presence or absence of viruses that hold infectivity should be carefully evaluated by comprehensively integrating all relevant factors.

4.1 Replacement of NAT assays

In general, to detect specific viruses of concern, an NAT assay is performed as a test for viruses. If an NGS assay is performed in place of the NAT assay, its ability to detect the target specific viruses must be demonstrated. Because the NAT and NGS assays share certain underlying principles but not differ in their overall methodologies, the head-to-head comparison of detection sensitivity may be of limited practical significance. Developers should collect information to explain that the NGS assay is expected to have an ability of virus detection comparable to the NAT assay, justifying its use as a replacement, and to be capable of detecting the specific viruses of concern with sufficient sensitivity,

thereby meeting the expected performance criteria.

4.2 Replacement of antibody production tests

Antibody production tests are performed in animals to detect predetermined specific viruses when rodent cell substrates are used in manufacture. As described in ICH Q5A(R2), antibody production tests are recommended to be replaced with molecular biological NGS or NAT assays for reasons such as animal welfare.

In antibody production tests, viruses listed in Table 3, ICH Q5A(R2) are detected based on elevated blood antibody titers against them, which are determined using antigen-antibody reaction as an indicator. If an NGS assay (or NAT assay) is implemented as a replacement, it must be demonstrated to be capable of detecting these viruses. Because the antibody production test and NGS assay (or NAT assay) use fundamentally different platforms, a head-to-head comparison is not required. Developers should collect information to explain that the NGS assay (or NAT assay) is capable of detecting the specific viruses of concern with sufficient sensitivity, thereby meeting the expected performance criteria.

4.3 Replacement of *in vivo* tests for viruses

In vivo tests for viruses are performed in animals to detect unspecified adventitious viruses. As described in ICH Q5A(R2), *in vivo* tests for viruses are recommended to be replaced with molecular biological NGS or NAT assays for reasons such as animal welfare, as with antibody production tests. In addition, well-characterized rodent cells (such as CHO cells) are not required to be subjected to *in vivo* tests for viruses, and thus replacement of *in vivo* tests for viruses with NGS assays would offer limited practical significance if such cell substrates are used.

The *in vivo* test for viruses utilizes fundamentally different principles from that of the NGS assay and is intended to detect unspecified viruses, and thus the head-to-head comparison is impractical. *In vivo* tests for viruses have been performed to complement *in vitro* tests for viruses. In view of this, an NGS assay may be accepted as an alternative to an *in vivo* test for viruses on the assumption that an *in vitro* test for viruses is performed, if it is demonstrated to have a detection coverage wide enough to complement *in vitro* tests for viruses.

4.4 Replacement of *in vitro* tests for viruses

In vitro tests for viruses are conducted using cultured cells to detect unspecified adventitious viruses. As described in ICH Q5A(R2), an NGS assay may be used as an alternative to *in vitro* tests for viruses. However, *in vitro* tests for viruses have historically played the central role in detection of adventitious viruses in biotechnology products, strategies for validation studies to replace them with NGS assays completely have not been internationally agreed upon. To address international consensus on this issue,

the WHO[5] and AVDTWG[6-7] have continued investigations through global studies[5] involving some Japanese national laboratories. For the time being, with close attention paid to these international activities, efforts to collect information should be made. Therefore, PMDA considers it premature to use NGS assays in place of *in vitro* tests for viruses at present. If an NGS assay is used as a part of the integrated virus safety strategy throughout the manufacturing process but not in place of an *in vitro* test for viruses, its appropriateness would be judged in a product-specific manner. Consultation with the applicable reviewing office should be considered.

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6. References

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