THE EUROPEAN DIRECTORATE FOR THE QUALITY OF MEDICINES & HEALTHCARE (EDQM)

EDQM/Ph. Eur. perspectives on NGS/HTS

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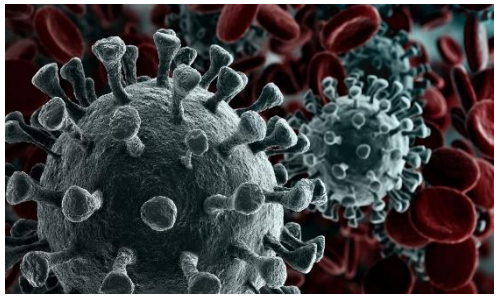


Outline

- Council of Europe & EDQM
- European Pharmacopoeia (Ph. Eur.)
- NGS/HTS in the Ph. Eur.
- Elaboration of a Ph. Eur. general chapter on NGS/HTS
- Conclusion

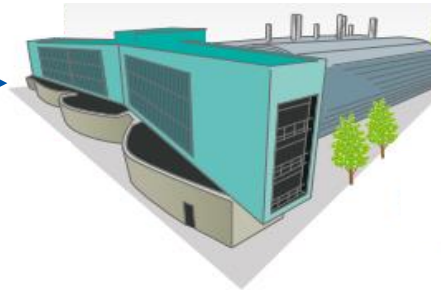
The EDQM, a Directorate of the COUNCIL OF EUROPE

COUNCIL OF EUROPE

- Founded in **1949**
- **Intergovernmental** organisation, Strasbourg
- **46** Member States
- More than **700 Million** of Citizens

COMMITTEE OF MINISTERS | PARLIAMENTARY ASSEMBLY | CONGRESS OF LOCAL AND REGIONAL AUTHORITIES | EUROPEAN COURT OF HUMAN RIGHTS | COMMISSIONER OF HUMAN RIGHTS | CONFERENCE OF INGOS

The **European Directorate for the Quality of Medicines & HealthCare** (EDQM)



edqm
European Directorate for the Quality of Medicines & HealthCare | Direction européenne de la qualité du médicament & soins de santé

COUNCIL OF EUROPE
CONSEIL DE L'EUROPE

- Founded in 1964
- Work in the framework of a **Partial Agreement, 39 Members & the EU**
- Contribute to **Public Health and access to good quality medicines and healthcare in Europe**

EDQM 60 1964 - 2024 | COUNCIL OF EUROPE 75 1949 - 2024 CONSEIL DE L'EUROPE

European Pharmacopoeia (Ph. Eur.)

Binding in the **39** signatory states of the Ph. Eur. Convention and used as a reference worldwide; **33** observers from all continents

► More than **2 800 documentary standards** for the quality control of medicines

- Cover the whole manufacturing process (*e.g. excipients, medicinal products*)
- All stages of the life cycle of a medicine from development through to production and market surveillance
- Methods verified & standardised

► **About 3000 reference standards shipped to 132 countries**

European Pharmacopeia Commission - treaty-based body - and its expert groups

Biological Standardisation Steering Committee

Laboratory, production, storage and distribution

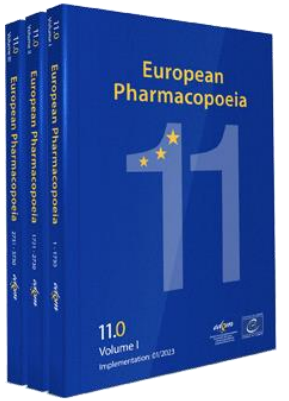
PUBLIC HEALTH IMPACT

- **Ensure equivalent quality and safety of medicinal products throughout Europe and facilitate their free movement in Europe and beyond**

NGS/HTS in the Ph. Eur.

	Ph. Eur. Chapter 5.2.3	Ph. Eur. Chapter 2.6.16	Ph. Eur. Chapter 5.2.14
Scope	**Testing of cell substrates** (including extraneous agent testing)	Extraneous agent **testing of viral seed lots / harvests**	Concept of **Substitution to replace *in vivo* methods**
Test and method	**Tests for viruses** using broad molecular methods (e.g. HTS) - As an alternative to *in vivo* tests and specific NAT, or - In addition/as an alternative to *in vitro* cell culture tests		Test for extraneous agents: Considerations for the substitution of *in vivo* methods by broad molecular methods (e.g. HTS)

→ The use of NGS/HTS is foreseen in the Ph. Eur. since 2017!



Background to the elaboration of a Ph. Eur. chapter on NGS/HTS

- Ph. Eur. chapters 5.2.3 & 2.6.16 mention HTS and foresee its use as part of the testing strategy for adventitious/extraneous agents
- However, no description of these methods or any guidance for their validation is provided

- The availability of regulatory standards including validation guidelines in the Ph. Eur. will serve as a reference for regulators and manufacturers, while:
  - HTS was recently introduced in the revised ICH Q5A guideline (*Viral safety evaluation of biotechnology products*) *(revision R2 adopted in Nov 2023)*
  - A panel of model viruses developed by FDA was also recently adopted by WHO as WHO international reference panel for HTS

Elaboration of a Ph. Eur. chapter on NGS/HTS

- *"High Throughput Sequencing for the detection of viral extraneous agents* (2.6.41)"

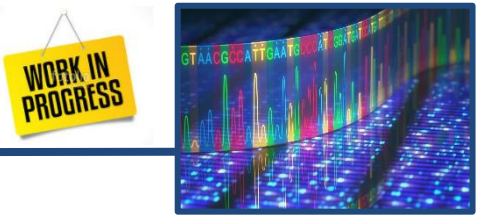
- General chapter
- Content: description of the technology/methods and of the HTS workflow, **guidelines for validation of HTS methods**

  - Elaborated by Ph. Eur.'s HTS Working Party
    (international group of regulators, OMCLs and industry from Europe, US, Canada)

  - **Draft chapter** published for public consultation in Pharmeuropa
    https://pharmeuropa.edqm.eu/home

  - Public consultation ended on 30 June 2024
  - **Stakeholder comments are being examined** by the HTS WP
    → All the elements presented should be considered in this context (i.e. work in progress)

Draft Ph. Eur. chapter 2.6.41 – Overview



- Draft chapter describes HTS methodologies used for the detection of viral extraneous agents in biological products including e.g. vaccines, recombinant proteins, viral vectors used for gene therapy, and cell-based preparations for cell therapy

- It outlines the different steps of the HTS workflow, the design of the method, analysis approaches, and the controls used in the routine test

- It also provides guidelines for HTS method validation, including recommendations for the selection of the spiking material for validation and the evaluation of the relevant performance characteristics for HTS

- Considerations on the replacement of *in vivo* and *in vitro* tests by HTS are provided by ICH guideline Q5A (R2)

Draft Ph. Eur. chapter 2.6.41 – Structure

Reference: PA/PH/Exp. 15/T (21) 27 ANP

2.6.41. HIGH-THROUGHPUT SEQUENCING FOR THE DETECTION OF VIRAL EXTRANEOUS AGENTS

1. INTRODUCTION & SCOPE

Viral extraneous agents (also referred as viral adventitious agents) can be introduced unintentionally in biological products at various stages in the manufacturing process. To ensure product quality and safety, a comprehensive strategy is established following the principles of viral safety risk assessment detailed in general chapter *5.1.7*. This strategy includes testing for viral contamination and may require a panel of suitable tests that are able to detect diverse viruses that may be present in the starting material such as the cell substrate and virus seed, or in raw materials of animal or plant origin. These tests are conducted at various stages of manufacture which may include cell banks, virus seeds, unprocessed bulks (viral or protein harvests), cells for cell therapy, and production viruses (helper viruses and viral vectors for protein expression).

High-Throughput Sequencing (HTS) (also referred to as Next-Generation Sequencing/NGS, Massively Parallel Sequencing/MPS, Deep Sequencing) can be introduced in the viral extraneous agent testing package to fill gaps identified through the risk assessment, to replace *in vivo* tests for extraneous agents and nucleic acid amplification techniques, or to replace or supplement *in vitro* methods using cell cultures. HTS is especially useful for the testing of new cell lines to detect known and unknown viruses, but also of virus seeds and harvests particularly when there is interference in conventional tests due to lack of neutralisation of the vaccine/vector virus or to sample toxicity.

HTS can be used to detect all genomic viral nucleic acids (DNA and RNA) (genomics), viral RNAs (transcriptomics), or encapsidated viral genomes (viromics). The analysis approach may be selected based on the nature of the sample to be tested. HTS may be more complex for the detection of retroviruses in cases where the host sequences containing endogenous retroviral sequences are filtered out by bioinformatics. In such cases, a dedicated analytical strategy should be designed for retrovirus detection.

1. INTRODUCTION AND SCOPE
2. DESCRIPTION OF METHODS
   - 2.1 General considerations
   - 2.2 Sample pre-treatment
   - 2.3 Extraction of nucleic acids
   - 2.4 Post-nucleic acid extraction treatment (enrichment)
   - 2.5 Library preparation
   - 2.6 Sequencing
   - 2.7 Bioinformatics analysis
   - 2.8 Scientific evaluation of the results
   - 2.9 Follow-up investigation
   - 2.10 Controls in the routine assay
3. HTS METHOD VALIDATION
   - 3.1 General considerations for validation
   - 3.2 Selection of spiking material for validation
   - 3.3 HTS method validation (generic method validation)
   - 3.4 Product-specific validation
4. TARGETED HTS

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2.6.41. HIGH-THROUGHPUT SEQUENCING FOR THE DETECTION OF VIRAL EXTRANEOUS AGENTS

1. Introduction & scope

- To ensure product quality and safety, a comprehensive strategy is established following the principles of viral safety risk assessment detailed in Ph. Eur. chapter 5.1.7
- HTS can be introduced in the viral extraneous agent testing package to fill gaps identified through the risk assessment, to replace *in vivo* tests [...] and nucleic acid amplification techniques, or to replace or supplement *in vitro* methods using cell cultures.
- HTS is especially useful for the testing of new cell lines to detect known and unknown viruses, but also of virus seeds and harvests particularly when there is interference in conventional tests (lack of neutralisation of the vaccine/vector virus or sample toxicity)
- Different HTS technologies: short-read or long-read sequencing technologies with different read lengths and throughput (number of reads generated)
- The design of the method may allow:
  - detection of a broad spectrum of viral extraneous agents (known and unknown viruses) with a non-targeted approach using a comprehensive database with viral sequence diversity, or
  - detection of a range of known viruses (or unknown viruses related to known viruses) with a targeted approach using a selective capture or amplification of viral sequences, or using reference virus genomes for bioinformatic analysis

Draft Ph. Eur. chapter 2.6.41

2. Description of methods – *2.1 General considerations*

- The selection of the most appropriate HTS approach for detecting viral extraneous agents in a given product should take into account the potential contaminating viruses and the sample type to be analysed
- Consideration should also be given to the detection of cell-associated and cell-free viruses
- Different approaches exist for sample preparation depending on the test material for detection of:
  - All genomic viral nucleic acids (DNA and RNA) (genomics),
  - Viral RNAs (transcriptomics), or
  - Encapsidated viral genomes (viromics)
- The workflow applied should have sufficient sensitivity in the relevant sample matrix and for the potential level of contamination at the stage of manufacturing

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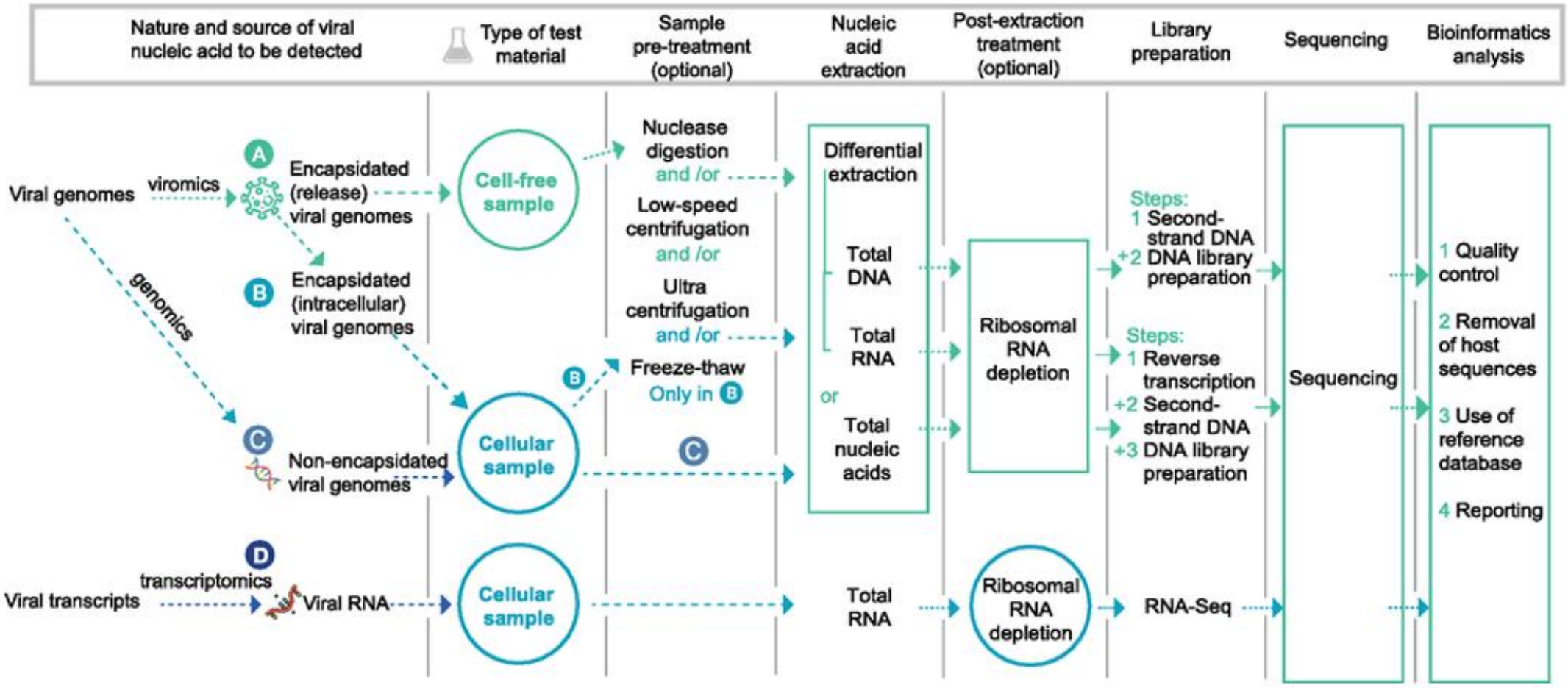

Figure 2.6.41.-1 – *Examples of HTS workflow*

Note: Figure subject to change

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2.6.41. HIGH-THROUGHPUT SEQUENCING FOR THE DETECTION OF VIRAL EXTRANEOUS AGENTS

2.2 Sample Pre-treatment

- Sample pre-treatment may increase the sensitivity of HTS by concentrating viral particles or by reducing background cellular nucleic acids
- Nuclease digestion, Freeze-thaw, centrifugation

2.3 Extraction of Nucleic Acids

- Extraction may be performed by isolating/purifying RNA and DNA simultaneously or separately
- Different types of extraction methods (e.g. silica, magnetic beads, or precipitation-based techniques) may be used based on the sample material (e.g. cellular vs cell-free sample)

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2.6.41. HIGH-THROUGHPUT SEQUENCING FOR THE DETECTION OF VIRAL EXTRANEOUS AGENTS

2.4 Post-nucleic acid extraction treatment

- An enrichment step may be applied to reduce host-cell nucleic acid content and thus increase method sensitivity and facilitate detection of low-level viral contamination
- Ribosomal RNA depletion allows highly abundant ribosomal RNA species to be removed / Degenerative oligonucleotides to capture and remove host-cell DNA

2.5 Library preparation

- Library preparation ensures that the library generated, and the resulting data, are suitable for the sequencing approach and representative of the targeted nucleic acid

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2.6.41. HIGH-THROUGHPUT SEQUENCING FOR THE DETECTION OF VIRAL EXTRANEOUS AGENTS

2.6 Sequencing

- The selection of the most appropriate sequencing technology and platform depending on the intended use should take into account the sample type (e.g. high or low-level of contamination expected), required sequencing depth and coverage, sequencing accuracy and read length
- In the context of virus detection, generating more reads in a sequencing run may increase the probability of detecting the presence of extraneous viruses.
- If multiplexing is performed, care should be taken to avoid any sample-to-sample or run-to-run contamination.
- An appropriate internal control is added to the sample prior to loading the sample into the sequencer

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2.7 Bioinformatics Analysis

- The bioinformatics analysis for virus detection involves building a pipeline that generally includes initial processing of the input raw reads to obtain quality reads, which in some cases may be followed by *de novo* assembly to generate contigs from overlapping reads, prior to mapping or aligning the reads / contigs using a reference virus or a database of viral sequences

Note: Figure subject to change

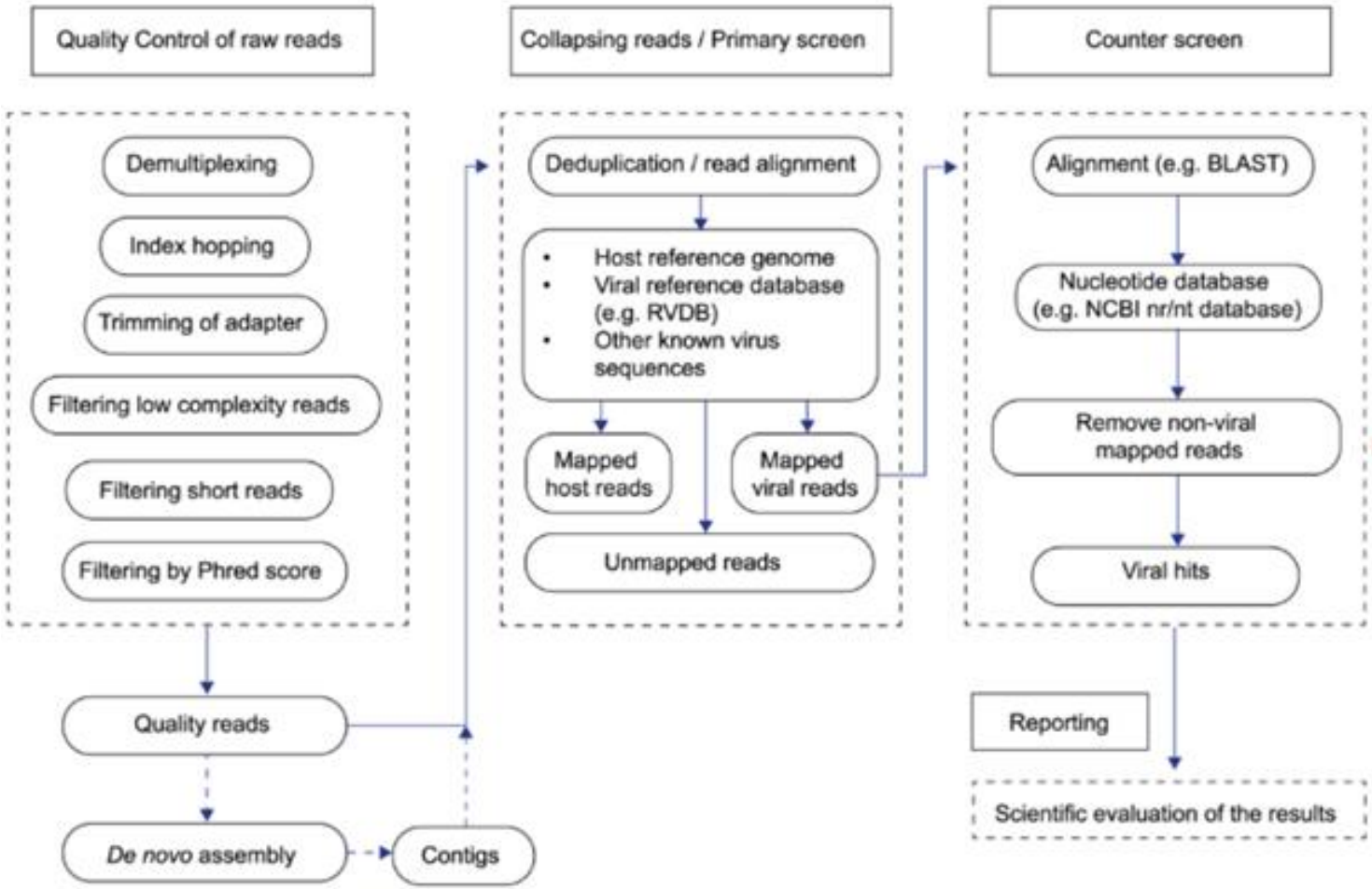

Figure 2.6.41.-2 – *A general bioinformatics analysis workflow*

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2.6.41. HIGH-THROUGHPUT SEQUENCING FOR THE DETECTION OF VIRAL EXTRANEOUS AGENTS

2.8 Scientific evaluation of the results

- The validity of the HTS run should be based on the recovery of one or more internal controls or on expected results from a control sample run in parallel
- The interpretation of the results obtained by the bioinformatics analysis should take into account pre-defined criteria for distinguishing true positives and false positive viral signals
- Positive results should be reported at the viral species level
- In addition, non-viral and background sequences can occur and should also be documented
- Appropriate controls to capture any cross-contamination from the facilities or instruments

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2.6.41. HIGH-THROUGHPUT SEQUENCING FOR THE DETECTION OF VIRAL EXTRANEOUS AGENTS

2.9 Follow-up investigation

- When a positive result has been identified, a laboratory-based follow-up is needed to assess whether the viral sequence is associated with an infectious virus

Table 2.6.41.-3 – *Follow-up investigation*

Question to be addressed	**Example approaches**
Are the nucleic acids that were found associated with full-length, intact genomes?	– Long-range or overlapping PCR, – Sequencing with long-read technology
Are the nucleic acids particle-associated?	Nuclease digestion and/or ultracentrifugation followed by virus testing: – PCR, – Transmission electron microscopy (TEM), – Detection by antibodies (e.g. immunoprecipitation, immunostaining, immunoblot, ELISA), – Mass spectrometry
Are the particles infectious in cell cultures?	Infectivity assay including readouts such as: – Cytopathic effect, haemadsorption, haemagglutination, – Viral replication by qPCR, – Transmission electron microscopy (TEM), – Additional HTS (e.g. transcriptomics), – Stranded sequencing, – Detection by antibodies (e.g. immunoprecipitation, immunostaining, immunoblot, ELISA), – Product-enhanced reverse transcriptase assay (PERT)

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2.6.41. HIGH-THROUGHPUT SEQUENCING FOR THE DETECTION OF VIRAL EXTRANEOUS AGENTS

2.10 Controls in the routine assay

- **Control of the whole workflow**
  - In order to ensure that the performance of the test is adequate, an external or internal positive control is included in each HTS test run
- Control of the library preparation step
- Control of the sequencing step

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2.6.41. HIGH-THROUGHPUT SEQUENCING FOR THE DETECTION OF VIRAL EXTRANEOUS AGENTS

3. Validation (Guidelines for Method Validation)

3.1 General Considerations for method validation

- The validation of an HTS method for the detection of extraneous viruses must demonstrate that the method is suitable for the intended purpose, based upon the sample type (e.g., cell substrate / cell bank, virus seed, harvest) and testing approach
- HTS is principally used as a qualitative limit test, as such, specificity (including breadth of virus detection) and limit of detection are the parameters to be assessed
- Requires an end-to-end assay validation and may include subdivision of the workflow into modules corresponding to sample and library preparation, the sequencing instrument, and bioinformatics analysis
- This modular validation approach provides more flexibility, potentially reducing the effort required for re-validation when updating individual modules

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2.6.41. HIGH-THROUGHPUT SEQUENCING FOR THE DETECTION OF VIRAL EXTRANEOUS AGENTS

3.2 Selection of Spiking Material for Validation

- The spiking material used for validation of the HTS method must be relevant for the intended purpose and the chosen detection strategy
- The use of model viruses as spike is relevant for the viromics and genomics approaches, whereas the use of infected cells is more appropriate for the transcriptomics approach
- For genomics and viromics approaches in testing cell substrates / cell banks, virus seeds and harvests, the reference standards should be viruses representing viral diversity in terms of structure (enveloped, non-enveloped), nucleic acid type (RNA or DNA genome, single-stranded or double-stranded genome, linear or circular or segmented), genome size, virus morphology and chemical resistance. These reference model virus stocks should be characterised for genome copy number, infectious titre, viral genome sequence(s) including any variants, and any additional expected background signals. The WHO *International Reference Panel for Adventitious Virus Detection in Biological Products by HTS* provides diverse virus families and is recommended as the minimum panel of model viruses for validation
- For transcriptomics approach in testing cell substrates / cell banks, a suitable spiking material can be cells infected with different viruses. These model infected cells should be characterised for infected vs non-infected cell count, confirmation of the viral expression, viral sequence and background host sequences

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2.6.41. HIGH-THROUGHPUT SEQUENCING FOR THE DETECTION OF VIRAL EXTRANEOUS AGENTS

3.3 HTS method validation (generic method validation)

- **Limit of detection** (LOD) is defined as the sensitivity at which viral contaminants would be detectable by the method. The sample should be spiked with a known amount of material to mimic different levels of potential virus contamination [...], added before any treatment of the sample and the HTS method applied end-to-end [...]. A minimum of three independent replicates of the end-to-end method is required to demonstrate an appropriate assay LOD unless otherwise justified. The LOD should be reported at the level where all spiked-in viruses are detectable in all replicates to indicate the worst-case detection limit and be reported as genome copies per millilitre for harvests or virus seeds, or genome copies per cell for the genomics approach, or by the amount of transcripts per cell or ratio of infected cells and non-infected cells for the transcriptomics approach
- **Specificity**: demonstrate the breadth of detection for different types of viruses as well as the correctness of the identification. The identity of the spiked viruses should be as expected. No false positive viral signal should be detected to confirm specificity
- **Re-validation** may be necessary when part of the method is modified. Can be facilitated by a modular approach

3.4 Product-specific validation

- Comparison of the matrix used during the generic validation vs the product-specific matrix to evaluate the potential impact on the LOD and specificity
- At least one end-to-end validation run is required to verify that changes to the matrix do not impact the LOD or specificity

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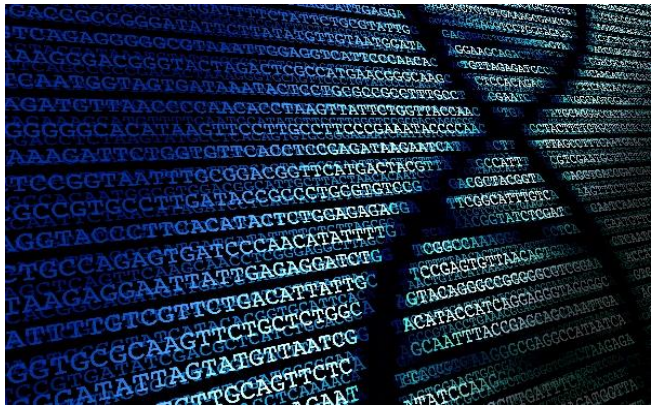
2.6.41. HIGH-THROUGHPUT SEQUENCING FOR THE DETECTION OF VIRAL EXTRANEOUS AGENTS

4. Targeted HTS

- Targeted HTS is a viral detection method using HTS in which nucleic acids of known and closely related viruses are enriched prior to sequencing. The targeted HTS methods are amplicon-based sequencing, which involves the use of PCR, and hybridisation capture, which uses complementary probes to capture the targeted sequences. The use of targeted HTS should be based on a risk evaluation as the enrichment techniques rely on the availability of known virus sequences. In addition, the design of the enrichment oligos or probes should ensure that a broad specificity is maintained. The bioinformatics analysis discussed in section 2.7 can be modified for targeted analysis using specific virus sequences as reference genomes

- Validation of a targeted HTS assay should demonstrate the sensitivity for viral detection as described for the non-targeted HTS approach (*see section* 3) but using relevant viruses as the spiking material (characterised as for non-targeted HTS approach validation)

Conclusion and next steps



- Since 2017, HTS has been successfully introduced in the Ph. Eur. for vaccines and gene therapy viral vectors
- The concept of substitution in Ph. Eur. chapter 5.2.14 can be applied to the replacement of *in vivo* tests for the detection of extraneous agents by HTS without a head-to-head comparison
- The future Ph. Eur. general chapter 2.6.41 on NGS will provide a detailed description of the technology together with validation guidelines, to support users implementing this new technology

The Ph. Eur.'s HTS WP is examining the stakeholder comments received during the public consultation

→ Stay tuned!

Acknowledgement

HTS Working Party and its Chair, Dr Johannes Blümel (PEI)

Dr Thuy Bourgeois (EDQM)

Thank you for your attention

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