NGS for Adventitious viruses - Key resources for regulatory approach to HTS/NGS

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Extraneous agents testing for vaccines and viral vectors used as gene therapy products

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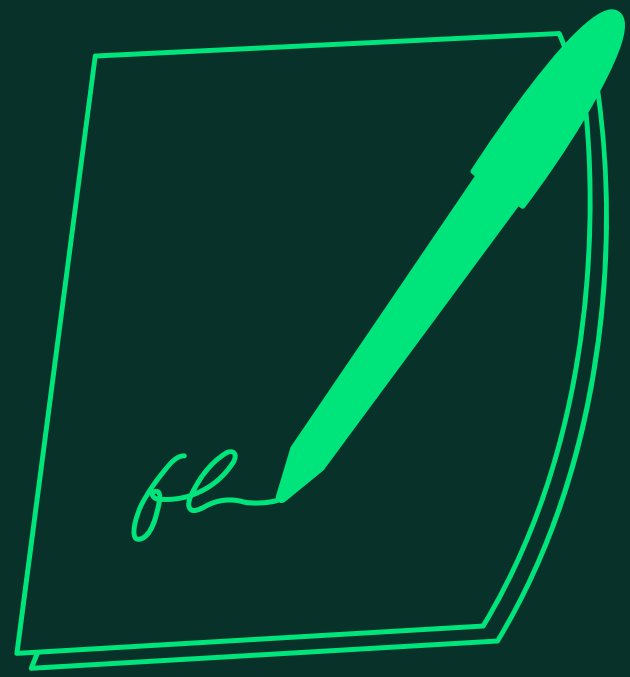
NGS/HTS regulations: EurPh 2.6.41 new Chapter



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Introduction to NGS/HTS

NGS (Next Generation Sequencing) = HTS (High Throughput Sequencing)

= Massively Parallel Sequencing (MPS)

What is NGS (Next Generation Sequencing) or HTS (High Throughput technology) ?

- Sequencing technology **capable of sequencing all the nucleic acids extracted from a biological sample**
  - million of reads (sequences), from a library of nucleotide sequences, whether they come from DNA, RNA, or a mixture,
  - **extremely fast**
  - **powerful tool for identifying known and unknown viruses**
  - **large number of applications**
  - **several technologies** (Illumina, Ion torrent, Oxford Nanopore, PacBio …):
    - short reads and long reads
    - Reads length from a hundreds of nucleotides to 50+ Kb
- Able to **detect any potential viral contaminants** in a biological sample

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NGS/HTS sample preparation workflow

- **Different steps to put under control :**

Sample

- DNA/RNA extraction

Enrichment & library preparation

- cDNA synthesis
- High length fragment constitution
- Isothermal random amplification

Next Gen Sequencing

- **e.g. Illumina technology** (Next Seq 500)

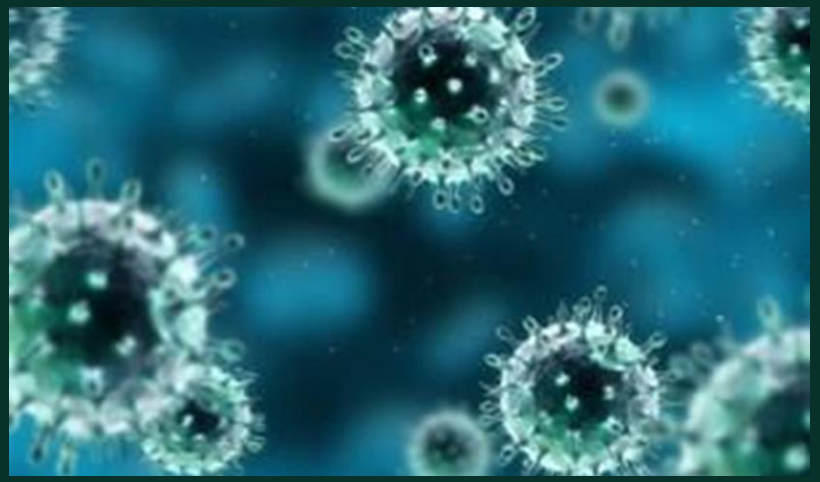
Bioinformatic analysis

- Number of reads, million paired-end reads/sample (sequencing depth)
- Reads length and quality

For Master seed testing, a random sequencing of all nucleic acids (**DNA and RNA**) is performed

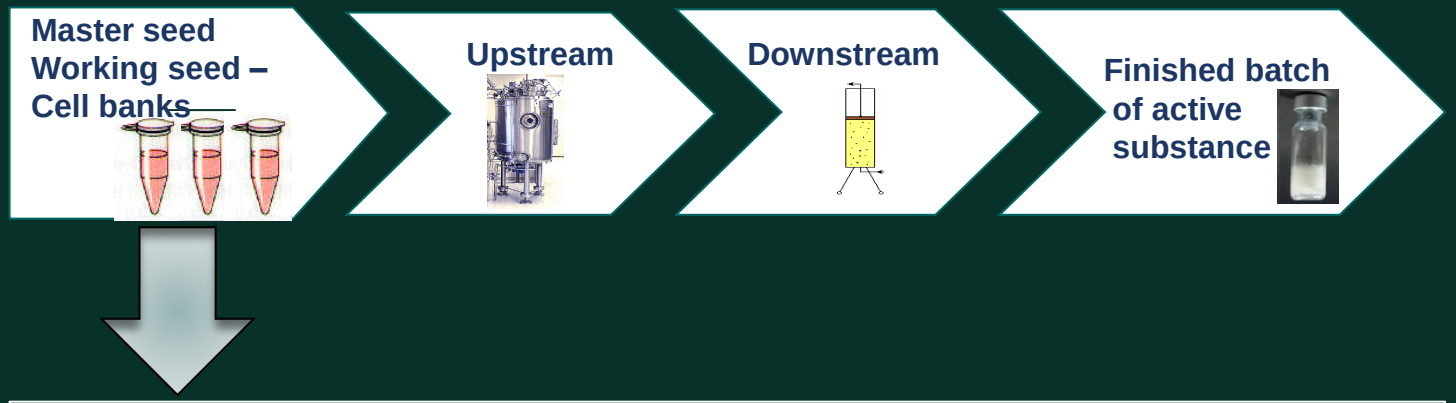
Current adventitious agents testing

Scope vaccine and viral vectors used as gene therapy products



Vaccine development & controls

Vaccine workflow



Final product

Starting materials should be controlled for the **absence of contaminants:**

- **Master cell bank**, working cell bank
- **Master virus seed** (MSV), working seed
- Raw materials

Present assays to detect contaminants:

- **Bacteria & fungi** detection
- **Mycoplasma** assays
- **Virus assays**

These assays are submitted to regulatory agencies for vaccine registration.

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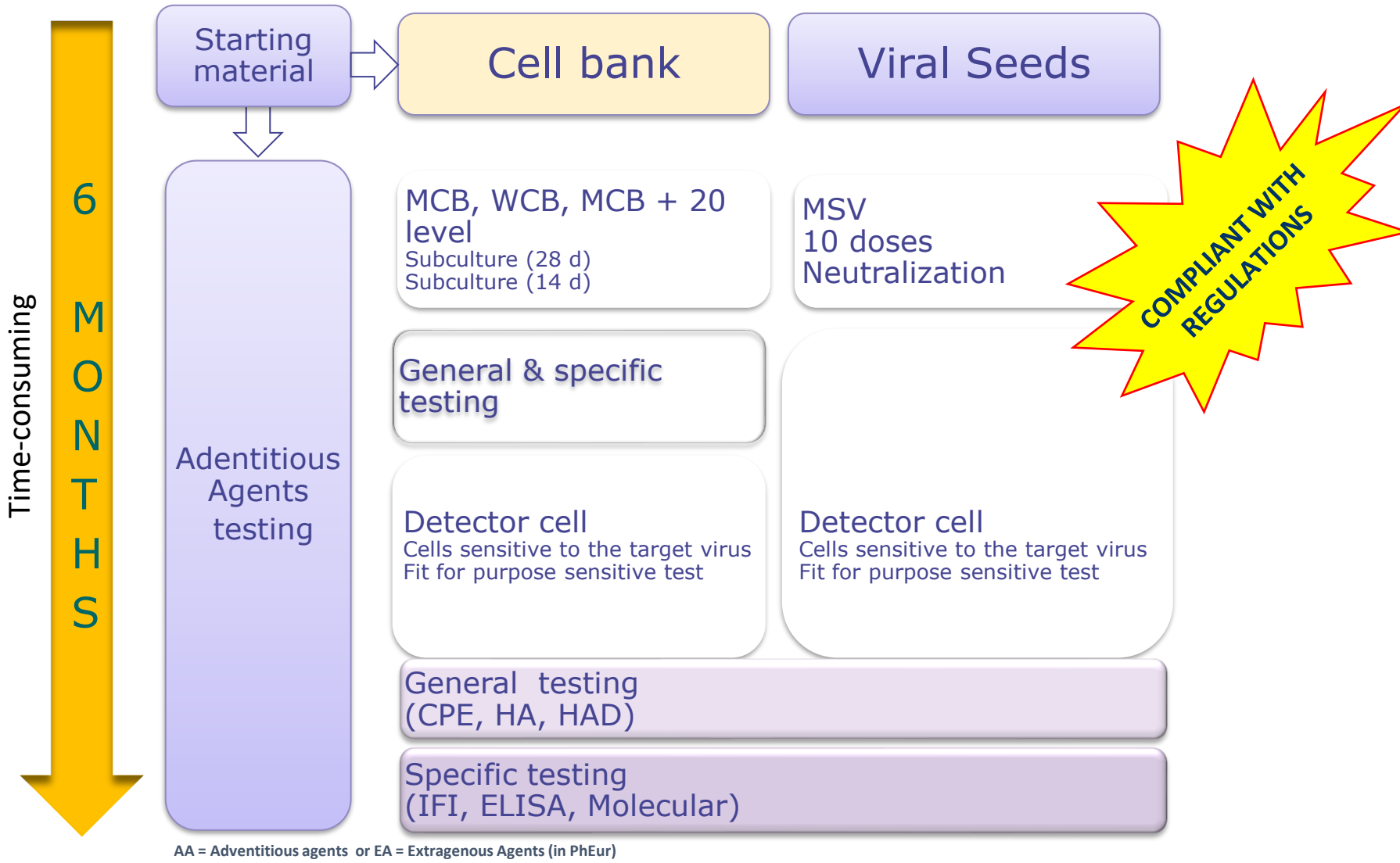
Complexicity of classical seeds purity testing

- **Challenges for veterinary vaccine seeds controls**
  - Several species, huge diversity of viruses: cumbersome testing
- For seeds testing combination of :
  - **non-specific methods** (broad range of detection)
  - **virus -specific methods** (eg PCR)

- Well-established method; high sensitivity on average

- Limitations of the method:
  - need of **reliable neutralizing antibodies**
  - cell lines **may not be permissive** for some known or novel viruses



AA = Adventitious agents or EA = Extragenous Agents (in PhEur)

The way to approach extraneous agents is changing

NEW PhEur texts for purity testing

- Revision of requirements for extraneous agents testing
Chapter 5.2.5 :
General chapters and monographs finalized.
- Date of implementation: **1st July 2020**

- **Step 1 : viral risk assessment**
  - **Justification for not conducting testing for relevant AE based on risk assessment**
  - Importance of good knowledge on production process

  Raw material (origin, SAO, treatment…) and production process (dilution, treatment…)

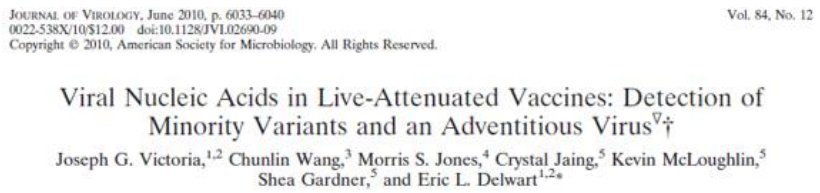
  - Certified/tested animal-derived material (e.g. serum, trypsin…)
- **Step 2 : Testing for those agents that cannot be ruled out by risk assessment**
  - ➢ Reference list of relevant EA by species to be considered

AA = Adventitious agents or EA = Extragenous Agents (in PhEur)

NGS/HTS Emergence as a Powerful New Technology

- High-throughput **Next Generation Sequencing** allowed us to consider the extremely powerful method in characterization of seeds
- Potential to identify **identifying known** and **unknown adventitious viruses** by sequencing all the nucleic acid within a sample **without needing prior knowledge** of the contaminating agents whatever the virus.

- The method **allowed to identity contaminants in biologicals**:
  - Finding porcine circovirus type 1 (PCV1) in a licensed rotavirus vaccine (Victoria *et al.*, 2010)
  - Discovery of a novel rhabdovirus in the Sf9 insect cell line used for baculovirus-expressed products (Ma *et al.*, 2014)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Viral Nucleic Acids in Live-Attenuated Vaccines: Detection of Minority Variants and an Adventitious Virus▽†

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Identification of a Novel Rhabdovirus in *Spodoptera frugiperda* Cell Lines

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HTS/NGS regulations: new Chapter 2.6.41 in EurPh

Scope vaccine and viral vectors used as gene therapy products

Elaboration of a Ph. Eur. chapter on HTS/NGS (EDQM)

- Ph. Eur. chapter 5.2.5 mentions HTS/NGS and foresees its use as part of the **testing strategy for adventitious/extraneous agents**
- No description of HTS/NGS methods or any guidance for their validation is provided

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

(international group of regulators, OMCLs and industry from Europe, US, Canada)

- The **availability of regulatory standards** including validation guidelines in the Ph. Eur. will serve as a reference for regulators and manufacturers, while:
    - Appropriate reference viruses developed by FDA; adopted as WHO international reference panel for NGS/HTS
- **Appropriate viral database** (RVDB Reference Virus Database)
- **Draft: public consultation** in Pharmeuropa (30th June 24) https://pharmeuropa.edqm.eu/home

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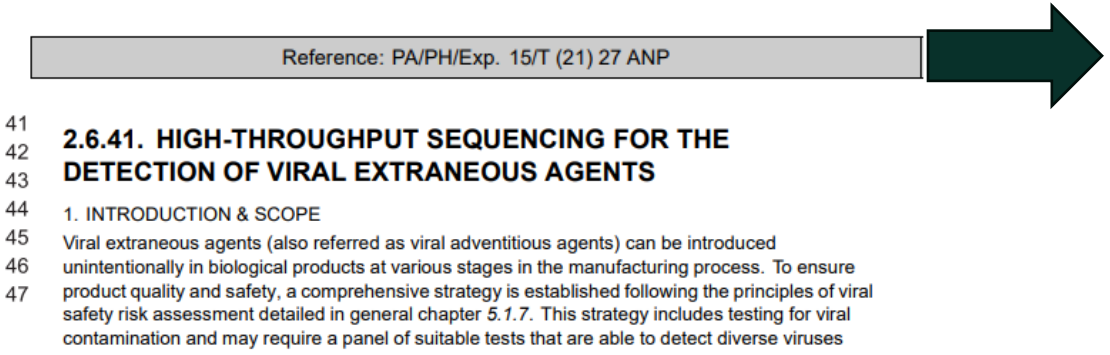
Ph. Eur. chapter 2.6.41 contents

- Description of HTS/NGS methodologies used for:

  - ***the detection of viral extraneous agents in biological products***

  (ex. vaccines, recombinant proteins, viral vectors used for gene therapy, cell-based preparations for cell therapy).

- **Guidelines for the validation of the HTS/NGS methods for viral extraneous agent detection**



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

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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Ph. Eur. chapter 2.6.41 – Description of the method

- The **strategy** for HTS viral extraneous agents testing depends on the **choice of sample type and production stage** to be tested:

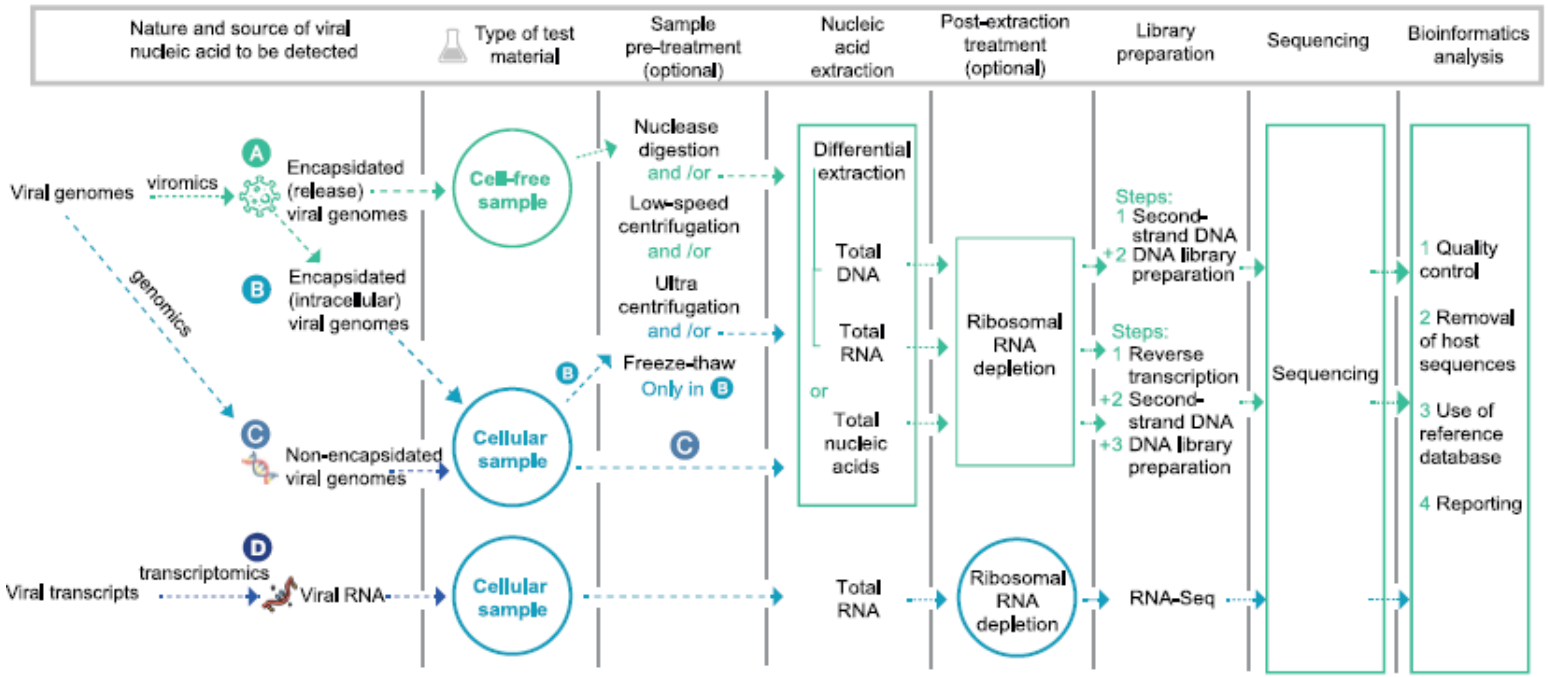


Figure 2.6.41.-1 – *Examples of HTS workflow (note: each step is explained in the following sections)*

- Different approaches for sample preparation depending on the test material:
  - **Genomics**: All genomic viral nucleic acids (DNA and RNA)
  - **Transcriptomics**: Viral RNAs
  - **Viromics**: Encapsidated viral genomes
- Sample pre-treatment (may increase sensitivity):
  - Single method or combination of method
  - Nuclease digestion, freeze-thaw, centrifuge, filtration

Ph. Eur. 2.6.41 – Sequencing & Bioinformatics

- Selection of the **most appropriate sequencing technology and platform**

  - Take account the sample type, sequencing depth and coverage, accuracy and read length

- **Bioinformatics analysis**

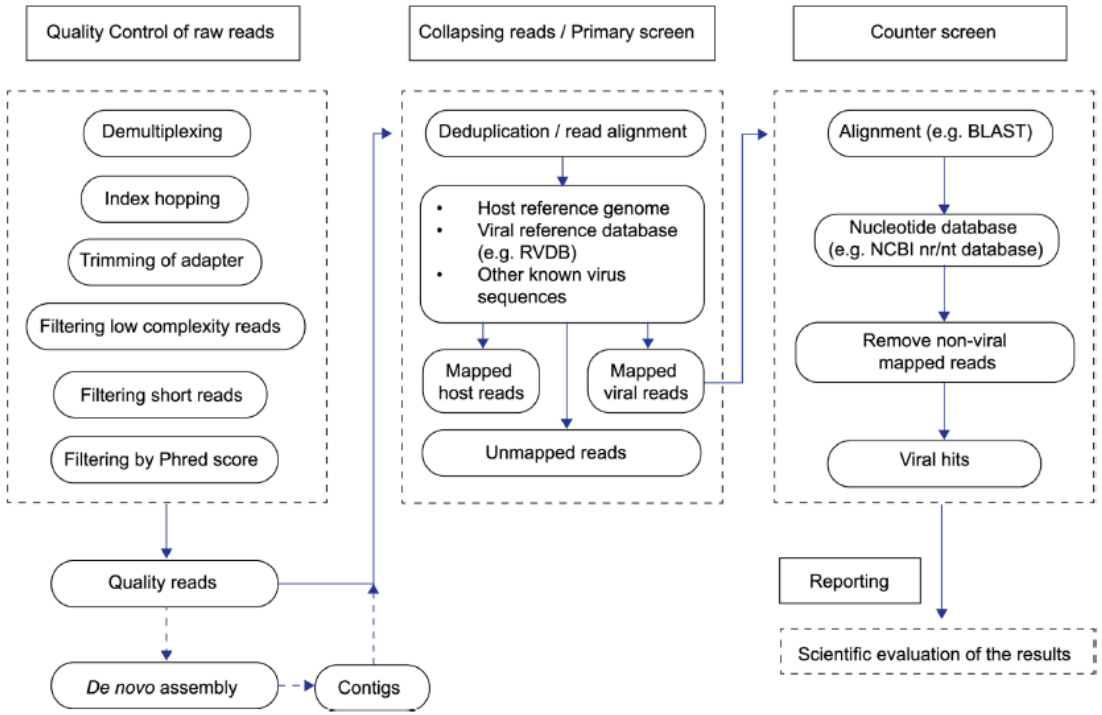


Figure 2.6.41.-2 – *A general bioinformatics analysis workflow*

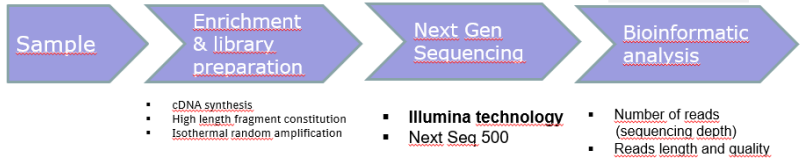
- Importance of quality controls of raw reads:
  - One sample per run
  - or Multiplexed samples (bar-coded)
- Strategies for reducing dataset and primary screen:
  - e.g.: removing identical reads (deduplication)
  - Reads of the expected sequences (host, vector, known virus or products related sequences) can be subtracted.
- Mapping against a reference virus database
  - e.g. the Reference Viral Database RVDB
  - In some cases, followed by de novo assembly to generate contigs
- Analysis pipeline
  - Usually custom-made, using a combination of commercially or in-house tools

Ph. Eur. 2.6.41 – Evaluation of results and follow-up investigation

- 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** (e.g. genome coverage, sequencing depth)
- Appropriate **controls** to capture any **cross-contamination** from the facilities or instruments
- When a true positive hit has been identified, **a laboratory-based follow-up** is needed to assess whether the viral sequence is associated with **an infectious virus.**

Ph. Eur. 2.6.41 – Validation (guidelines)

- 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 used as qualitative limit test;** parameters to assess
  - **Specificity (including breath of virus detection) & limit of detection**
- Require an **end-to-end assay validation** and may include **subdivision** of the workflow into **modules**



- Importance of **spiking material for validation** (for genomics and viromics approaches, reference standard should be **viruses representing viral diversity** in terms of structure, nucleic acid type, genome size, morphology… and **well characterized**

- **Modules** corresponding to
  - sample and library preparation,
  - sequencing instrument,
  - bioinformatics analysis
- The modular validation approach provides **more flexibility** (e.g. less effort required for re-validation)

- Well characterized and available reference standards:
  - Enveloped, non-enveloped, RNA or DNA genome, single-stranded or double-stranded genome…, size, virus morphology…
  - Genome copy number, infectious titer, viral genome sequence(s)
  - WHO international Virus Ref. Panel

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Conclusion & next steps

- More and more companies **used NGS or plan to use NGS for purity testing**
  - huge boost with mRNA vaccines and new therapeutics
  - Most of the assays concerned Illumina platform

- HTS/NGS could be part of the **testing strategy for adventitious/extraneous agents** (Ph. Eur. chapter 5.2.5*);* substitute/replace multiple *in vitro/in vivo* tests (3R)

- The future Ph. Eur. general **chapter 2.6.41 on HTS/NGS** will provide a **detailed description of the technology** together with **validation guidelines**, to support users implementing this new technology

- New versions of several regulatory texts to add NGS
  - EDQM, WHO
  - Coming Chapter EurPh 2.6.41 (2025): guidance for validation

- Implementation of NGS for Adventitious/extraneous Virus Testing of Biologics
  - Increased efficiency (time)
  - Reduce animal-derived use; 3Rs
  - Sensitivity, specificity, repeatability, accuracy

- The availability of regulatory standards will serve as **reference for regulators and manufacturers**

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Upcoming events on NGS for adventitious virus detection in Biologics

International Alliance for Biological Standardization

Frankfurt, Germany

December, 3-5 2024

4th Conference on Next Generation Sequencing for Adventitious Virus Detection in Biologics for Humans and Animal

2024 is a key year for globally considering NGS implementation with the recent publication of the revised ICHQ5A(R2) guideline and the expected publication of the EDQM/European Pharmacopoeia chapter for comments.

Scientific Committee :

- **Arifa S. KHAN,** co-chair FDA-CBER (FDA), U.S.A.
- **Laurent MALLET,** co-chair, EDQM, France
- **Pieter NEELS,** IABS Human Vaccine Committee Chair, Belgium
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- **Marie MURPHY,** Eli Lilly, Ireland
- **Siemon NG,** Notch Therapeutics Canada
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Thank you for your attention