Application of Next Generation Sequencing to Biological and Biotechnological Products: How to Balance Regulation and Innovation

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Outline

- Viral safety
- Applications of NGS in biological products
- Validation requirements for NGS
- Conclusion and Future Perspective
Safety of vaccines and other biological products is critical.

Safety is particularly critical for:

- live vaccines
- gene therapy viral vectors
- cell therapy medicinal products
ICH Q5A, specifically requires that a manufacturer of biological products for human use demonstrate the capability of the manufacturing process to remove or inactivate known contaminants.
Various EMA guidelines provide recommendations for validation of viral inactivation biopharmaceutical products.

These recommendations also set specific values for virus clearance levels that had to be attained.
Detection of viral contaminants in biopharmaceutical products

Live Infectious virus

Cell cultures

Infectivity Assay (Retroviruses)

Tests in animals

Non-specific test methods

- Test for reverse transcriptase
- Transmission Electron Microscopy (TEM)

Viral components

- PCRs
- in vivo Antibody production tests (MAP, RAP, HAP)

Virus-specific test methods

- 9CFR for Bovine & Porcine viruses
- Tests in animals
- In vitro Antibody production tests (MAP, RAP, HAP)

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Original concerns focused on a relatively small number of known viruses associated with the production cell lines.

Case study: PCV in Rotavirus Vaccine

Case study: Sf Rhabdovirus
Limitation of conventional assays

- Cell lines may not be permissive for the virus
- Virus replication is not visible (no CPE)
- Cytotoxicity – Neutralization - Interference
- Virus not detected by PCR primers
Regulatory expectations on viral safety of biopharmaceutical products have evolved over the past decade.

Today, the concerns are much broader, encompassing unknown and uncharacterized agents.

Increasingly stringent conditions are intended to decrease the risk of transmitting viruses.

Next generation sequencing (NGS) is a sensitive and un-biased detection method for adventitious agents.
Evolution of European Pharmacopoeia

Ph. Eur. Chapter 5.2.14: “Substitution of in vivo method(s) by in vitro method(s) for the quality control of vaccines”, implemented 1/2018, version 9.3

5.2.14. SUBSTITUTION OF IN VIVO METHOD(S) BY IN VITRO METHOD(S) FOR THE QUALITY CONTROL OF VACCINES

PURPOSE
The purpose of this general chapter is to provide guidance to facilitate the implementation of in vitro methods as substitutes for existing in vivo methods, in cases where a typical one-to-one assay comparison is not appropriate for reasons unrelated to the suitability of one or more in vitro methods. This general chapter will not discuss the details of assay validation as such, since those principles are described elsewhere.

The general chapter applies primarily to vaccines for human or veterinary use, however the principles described may also apply to other biologics such as sera.

Detection of viral extraneous agents by novel molecular methods

Detection of viral extraneous agents in cell banks, seed lots and cell culture harvests is currently conducted using a panel of in vivo and in vitro methods at different stages of the manufacturing process. Novel, sensitive molecular techniques with broad detection capabilities are available, including deep sequencing or high-throughput sequencing methods, degenerate polymerase chain reaction (PCR) for whole virus families or random-priming methods (associated or not with sequencing), hybridisation to oligonucleotide arrays and mass spectrometry. The use of these new molecular methods has highlighted gaps in the existing testing strategy by identifying previously undetected viral contaminants in final product, the cell banks from which it was produced and intermediate manufacturing stages. These new molecular methods (e.g.
5.2.3. CELL SUBSTRATES FOR THE PRODUCTION OF VACCINES FOR HUMAN USE

This general chapter deals with diploid cell lines and continuous cell lines used as cell substrates for the production of vaccines for human use; additional issues specifically related to vaccines prepared by recombinant DNA technology are covered by the monograph Products of recombinant DNA technology (0784). The testing to be carried out at the various stages (cell seed, master cell bank (MCB), working cell bank (WCB), end of production cells (EOPC) or extended cell bank (ECB) corresponding to cells at or beyond the maximum population doubling level used for production) is indicated in Table 5.2.3.1. General provisions for the use of cell lines and test methods are given below. Where primary cells or cells that have undergone a few passages without constitution of a cell bank are used for vaccine production, requirements are given in the individual monograph for the vaccine concerned.

Infectious extraneous agents. For cell lines for vaccine production, the testing for infectious extraneous agents must be carried out based on a risk assessment. The origin of the cell substrate as well as the potential extraneous agents that may be inadvertently introduced during production processes or through the use of animal or plant derived raw materials must be taken into account in the choice of suitable permissive cells. One such strategy is given in Table 5.2.3.1, but alternative strategies could focus on more extensive testing at the MCB or WCB level. In any case, any strategy must be justified and lead to the same level of safety as outlined in Table 5.2.3.1. New, sensitive molecular techniques with broad detection capabilities are available, including massive parallel sequencing (MPS) methods, degenerate polymerase chain reaction (PCR) for whole virus families or random-priming methods (associated or not with sequencing), hybridisation to oligonucleotide arrays and mass spectrometry. These methods may be used either as an alternative to in vivo or specific NAT tests or as a supplement/alternative to in vitro culture tests, in agreement with the competent authority. The capacity of the process to remove/inactivate specific viruses must take into account the origin and cultural history of the cell line and adventitious viruses that are known to persistently infect the species of origin, for example, simian virus 40 in rhesus monkeys, Flock house virus in insect cells or viruses that may inadvertently be introduced during production processes or through the use of raw materials of animal or plant origin. For cell lines of insect origin, tests for specific
Evolution of European Pharmacopoeia


2.6.16. TESTS FOR EXTRANEOUS AGENTS IN VIRAL VACCINES FOR HUMAN USE

INTRODUCTION

A strategy for testing extraneous agents in viral vaccines must be developed based on a risk assessment following the principles of viral contamination risk detailed in general chapter 5.1.7. Viral safety. This strategy includes a full package of suitable tests that are able to detect different families of extraneous agents that may infect the source of virus strains including cell substrates and raw material of animal or plant origin. It also takes into account the capacity of the manufacturing process to remove or inactivate viruses. The new, sensitive molecular methods with broad detection capabilities are available. These new approaches include high-throughput sequencing (HTS) methods, nucleic acid amplification techniques (NAT) (e.g. polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), product-enhanced reverse transcriptase (PERT) assays) for whole virus families or random-priming methods (associated or not with sequencing), hybridisation to oligonucleotide arrays, and mass spectrometry with broad-spectrum PCR. These methods may be used either as an alternative to in vivo tests and specific NAT or as a supplement/alternative to in vitro culture tests based on the risk assessment and with the agreement of the competent authority.
"New, sensitive, molecular methods, with broad detection capabilities are being developed... The new generation of massively parallel (deep) sequencing (MPS) methods may have particular utility. They can be applied to detect virions after nuclease treatment to remove cellular DNA and unencapsidated genomes. Used in this mode, MPS has been used to discover new viruses in serum and other tissues and has revealed the contamination of human vaccines by porcine circovirus."

"MPS can also be employed to screen cell substrates for both latent and lytic viruses by sequencing the transcriptome. In this mode, enormous quantities of data are generated, and robust bioinformatic methods are required to detect viral sequences by either positive selection against viral databases or negative selection to remove cellular sequences."
WHO - TRS 878, Annex 1
“It is probable that application of methods of this type will be expected or required by regulatory agencies in future.”

WHO TRS 993 Annex 2. Scientific principles for regulatory risk evaluation on finding an adventitious agent in a marketed vaccine

“WHO defined Next-generation sequencing (NGS) as “high-throughput sequencing technology that processes sequences in parallel, producing thousands or millions of sequences at once from a sample… Significant bioinformatics using curated (trusted) databases are needed to analyze the considerable amount of data generated in each sequencing run.”

“New methods and technologies, such as NGS or microarrays, are powerful tools for the detection and identification of sequences from viruses and other adventitious agents without prior knowledge of the nature of the agent. In the future such new technologies may uncover the presence of other, as yet unrecognized, adventitious agents.”
Application and Usefulness of NGS -1

- Detection method for adventitious agents.
  - Removal, supplementation, replacement, substitution of in vivo adventitious agent tests
  - Substitution of in vitro nucleic acid based tests
Application and Usefulness of NGS -2

Characterization, screening studies

Ensuring the safety of vaccine cell substrates by massively parallel sequencing of the transcriptome

D. Osorio, C. Coudé, B. Loeve, S. Koduri, A. Armstrong, A. Chang, J. Kolman

ARTICLE INFO

NARRATIVE

Ensuring the safety of vaccine cell substrates by massively parallel sequencing of the transcriptome, an important aspect of vaccine manufacturing, is described. The authors present a novel, high-throughput method for the detection of off-target expression of genes and their derived products, which is a critical aspect of the safety and efficacy of vaccines. This method allows for the identification of potential safety risks and the optimization of vaccine production processes. The study highlights the importance of comprehensive transcriptome analysis in ensuring the safety and efficacy of vaccines.

Pre Master Cell Bank / Pre Seed

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Application and Usefulness of NGS -3

- Investigational tool

For example: to clarify if an identified contaminant is replicative
Other potential applications of NGS

NGS could also be used at different stages e.g. product development, manufacturing or finished product:

- Identification and characterization of vaccine strains
- Evaluation of genetic stability of vaccine strains after successive passages
- Reversion to virulence of the attenuated vaccine strains
NGS Platforms mostly used

- Short reads
  - illumina
  - ion torrent
  - MGI

- Single molecule
  - Pacific Biosciences
  - Oxford Nanopore Technologies
Challenge to Use NGS to Detect Adventitious Agents

Major challenge ➔ Validation of NGS Method

- Diversity of viral targets and biological matrices (e.g. cell banks, viral seeds, raw materials)

- Complexity of the NGS technologies and associated bioinformatics

- Model viruses would be useful for performance evaluation, standardization and validation of NGS

- Bioinformatics analysis pipeline must be optimized

- Complete and correctly annotated database must be available
Due to the need to validate each step of NGS method, a coordinate work among specialists is important.

In 2014 the Advanced Virus Detection Technologies Interest Group (AVDTIG), gathering together Regulatory and Government agencies, Industry, Service providers, Technology developers, and Academics from all over the world, has been formed.

Efforts of the Advanced Virus Detection Technologies Interest Group (AVDTIG) for NGS applications in Biologics

2nd Conference on Next Generation Sequencing for Adventitious Virus Detection in Human and Veterinary Biologics - An IABS-EU Meeting

November 13-14, 2019 - Het Pand, University of Ghent, Belgium

Jean-Pol Cassart and AVDTIG
Validation and Standardization -1

- Preliminary consideration
  - NGS is not a quantitative analysis
  - Sample flow similar to PCR assays

- Sample and library preparation
  - Extractions and recovery of viral nucleic acids controls (accuracy of the method)
  - Extractions and recovery

Perspective

Current Perspectives on High-Throughput Sequencing (HTS) for Adventitious Virus Detection: Upstream Sample Processing and Library Preparation

Siemon H. Ng 1*, Cassandra Braxton 2, Marc Elloit 3, Szi Fei Feng 5, Romain Fragnoud 6, Laurent Mallet 7, Edward T. Mee 8, Sarmitha Sathiamoorthy 1, Olivier Vandeputte 9, and Arifa S. Khan 10

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Validation and Standardization -2

- Appropriate model viruses for spiking studies (needs for a standard).
  - Efficiency of the different steps of the methodology
  - Evaluation of total NGS workflow in different biological matrices
  - Compare NGS with current assays for virus detection (PCR, in vivo, in vitro)
  - Generation of well-characterized datasets for evaluating bioinformatics pipelines
  - Sensitivity studies

Reagent available from NIBSC catalogue
www.nibsc.org/products ref: 11/242-001
Validation and Standardization -3

➢ Specificity

❖ Demonstrated by a negative control extracted and sequenced in parallel

❖ Breadth of detection confirmation
Bioinformatics - Pipeline optimization

- Criteria for acceptable quality of reads
- Parameters for short read assembly
- Hybrid assembly to correct high error-rate in long-read sequencing
- Strategies to identify novel viruses with minimal similarity to known sequences
Validation and Standardization -5

- Development of a complete and correctly annotated, publicly available, Reference Virus Database

Database available at: https://rvdb.dbi.udel.edu/

A Reference Viral Database (RVDB) To Enhance Bioinformatics Analysis of High-Throughput Sequencing for Novel Virus Detection

Norman Goodacre, Aisha Aljanahi, Subhiksha Nandakumar, Mike Mikailov, Arifa S. Khan
**NGS positive sample: follow-up strategy**

- **Confirmation of a “true” hit**
  - Can the results be confirmed by PCR or another assay?
  - Is a complete viral genome present?

- **Determination of biological relevance and significance of a positive signal**
  - Are particles present?
  - Are the particles infectious?
  - Is there a replication-competent virus?
  - Can the nucleic acid/particles be quantified?
Summary of the steps which need validation to utilize NGS for Biological and Biotechnological Products -1

- **Sample preparation and processing**
  - Extraction efficiency of different virus structure (with/out envelope)
  - cDNA synthesis of different virus genome (Single/double strand; DNA/RNA)
  - Library preparation
  - Enrichment steps for viral nucleic acid Controls (reagents, method)

- **Sequencing platform**
  - Selection of sequencer to provide sufficient reads to detect a low level virus
  - Consider error rate of sequencing technology: short reads vs long reads
Summary of the steps which need validation to utilize NGS for Biological and Biotechnological Products -2

Bioinformatics

- Strategies for detection of known and novel viruses (nucleotide vs amino acids, programs/tools, reads vs contigs, criteria and parameters for runs)
- Databases
- Unmapped reads?
- Re-analysis?
Conclusions

➢ Evaluation of NGS platforms for virus detection

➢ Standardization of the methods, including availability of virus references representing different virus families

➢ Developing bioinformatics tools and strategies for accurate virus detection and data interpretation
Future perspective -1

- Further dialogue between researchers, developers, companies and regulators to understand current hurdles to approve the implementation of NGS.

- Improvement of experimental projects for an accurate standardization of all the steps involved in NGS and biologicals control.

- Collaboration between researchers, companies and regulators for the development of specific guidance on requirements for regulatory acceptance of NGS.
Future perspective - 2

- Coordination between regulatory bodies to harmonize requirements

- Organize collaborative studies to address technology complexity on common grounds
How to Balance Regulation and Innovation

- Absence of specific guideline
- Next Generation Sequencing could be accepted
- A strict Validation is requested
- Validation must cover all the steps
- To be easily usable by Assessors, all Validation steps must have a unique rationale
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