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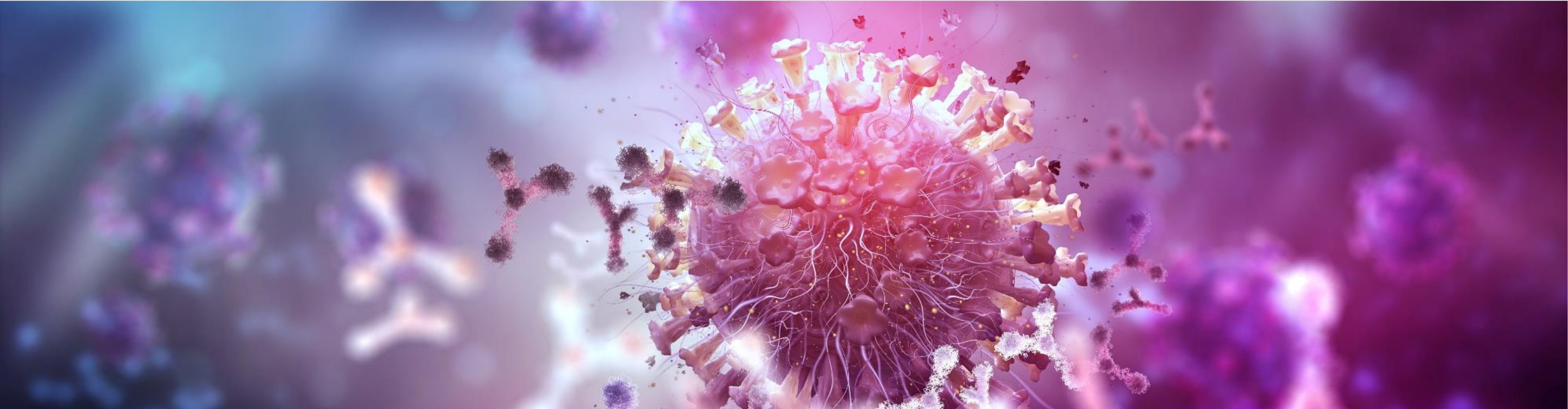

NGS as a tool to replace traditional pathogen safety testing strategies

ViruSure Workshop; April 8th 2024

Hans-Peter Fasching, Deputy Head of Molecular Biology

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Introduction to NGS

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What is NGS?

Difference between NGS and Sanger Sequencing

Sanger Sequencing	NGS
Sequence has to be known	Sequence does not need to be known upfront
Around 5000 bp can be sequenced per run	Complete genomes can be sequenced in a single run
Region of interest has to be amplified before sequencing	No amplifcation needed
Each read is generated in a single reaction (max 48 reads per run)	Millions of reads can be generated each run
Max read length around 800 bp	Theroatically reads of any size can be sequenced
Best suitable for resequencing of genetic inserts of max 5000 bp	Can be used for a variety of applications (detection of contaminations, resequencing of viral vectors and genetic inserts, etc.)

What is NGS?

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Long read vs Short read sequencing

Long-read Sequencing (e.g. Oxford Nanopore)	Short-read Sequencing (e.g. Illumina)
Reads of any length can be generated	Read length fixed of 200-250 bp
Long reads provide easier genome resolution, making it possible to analyse difficult sequences (e.g. repetitive regions)	Due to short reads-lengths repetitive regions can often not be resolved
Ability to perform native DNA sequencing without PCR amplification	Necessity for PCR amplification leading to possible PCR biases (e.g. influence of GC-rich regions)
Long reads reduce the background signal Additionally, it is possible to cover the complete genome of contaminants in a single read, giving stronger evidence that an observed hit is a possible contamination	Short reads increase the background noise when testing for the presence of adventitious agents, as the likelihood of random matches in a database is increased due to the short read length
Longer reads make genome assemblies easier, e.g. when resequencing complete genomes	More computational work is necessary to generate genome assembles with short reads. Also repetitive regions may not be completely covered.
Accuracy > 99.9%	Accuracy > 99.9%

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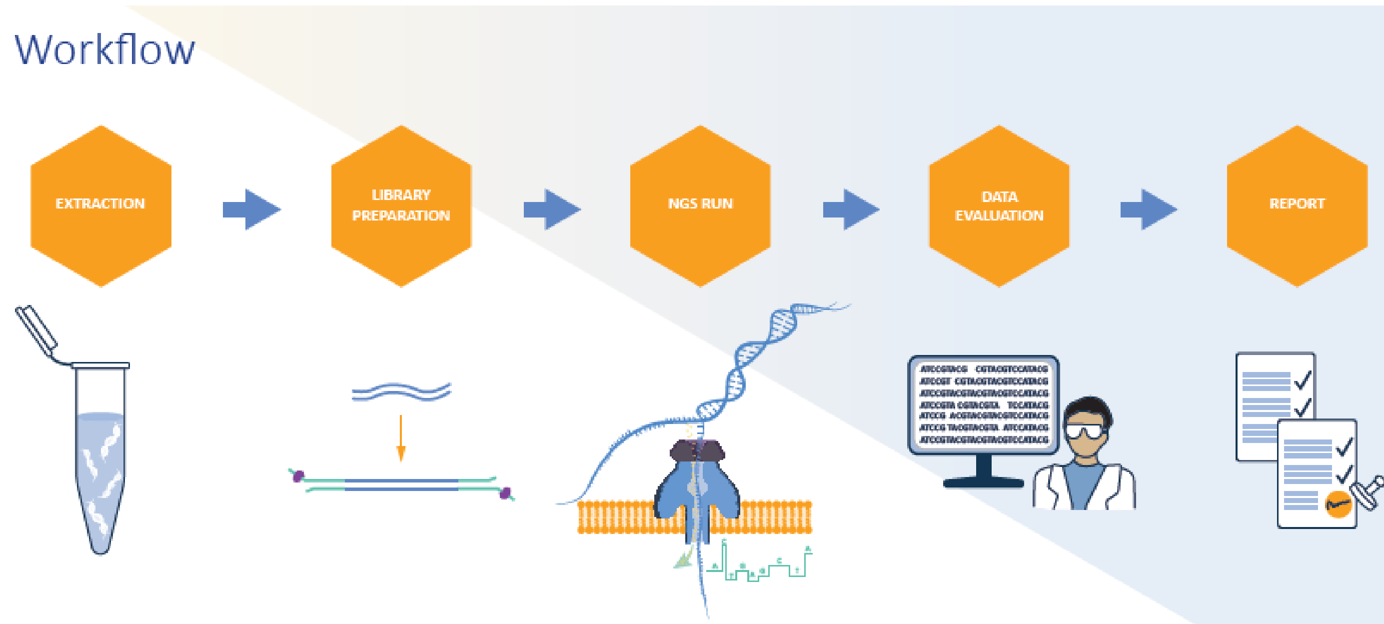
The NGS workflow



- Extraction DNA/RNA/total nucleic acids
- (Additional sample preparation steps)
- Library preparation
- NGS run
- Data Analysis
- Report generation

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Replacement of traditional safety testing strategies

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ICH Q5A Revision 2 – Where are we at?

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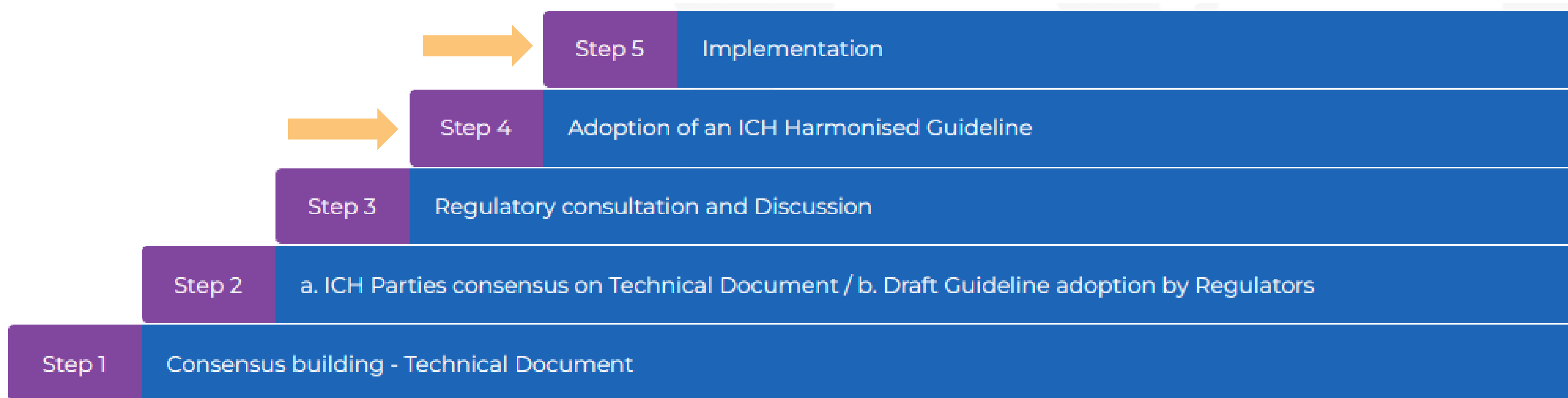
ICH Guideline Q5A(R2) on viral safety evaluation of biotechnology products derived from cell lines of human and animal origin

1997 – Implementation

23 years

1999 – Revision 1

2022 – Revision 2 *(release for public consultation)*



https://ich.org/page/formal-ich-procedure

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Adventitious Viruses

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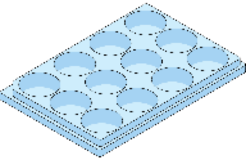
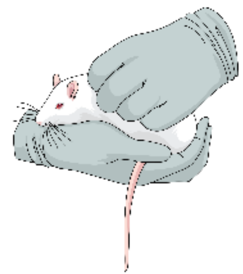
Assay	MCB	WCB	Cells at the LIVCA
Adventitious Viruses			
In vitro assays			+
In vivo assays (in vivo assays with suckling and adult mice, embryonated eggs)			
Antibody production test (MAP/HAP)			-

These can now be supported or replaced by
NGS



***In vitro* assay –** Test Sample incubated on three different cell lines (origins: bovine, human and species of the TS). Cells are observed for the occurrence of CPE. Very good sensitivities



***In vivo* testing –** Test Sample is inoculated into animal models. Animals are observed for the duration of the study for any pathogenic effects

MAP/HAP – animals are inoculated with the Test Sample. At the end of the study blood samples are used to look for the production of antibodies against specific viruses

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Comparison of NGS with current testing portfolio

Parameter	In vivo AAT	MAP/HAP	In vitro AAT	9 CFR	Retroviruses (PG-4)	TEM	RT activity	qPCR	NGS
Detection of unknown virus	✓	-	✓	-	✓	✓	✓	-	✓
Detection of replicating virus	✓	✓	✓	✓	✓	-	-	-	✓/ -
Identification of virus	-	✓	-	✓	-	-	-	✓	✓
Specificity of Detection	unspecific	specific	unspecific	specific	unspecific	unspecific	unspecific	specific	Unspecific
Sensitivity	+	+	+++	+	+	-	+++	+++	++

NGS combines the a lot of the advantages of the current testing portfolio:

- Non-targeted approach → detection and identification of all contaminants (including silent infections)
- High sensitivity
- Depending on the set-up it can already give an indication on whether virus is actively replicating or not
- Less prone to interference

Different testing principles → No head-to-head comparison between NGS and other methods is required

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New Classes of Biotechnology Products – Extended Scope

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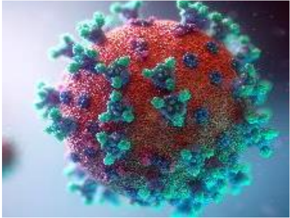
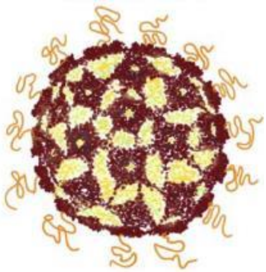


Genetically-engineered Viral Vectors



Mohsen, Mo et all, Vaccines, 2018

Viral Vector–derived Products

New classes of biotechnological products made a revision of the guideline necessary:

- Virus clearance cannot be performed for all of them, as the actual product could be removed, making them rely even more heavily on testing to reduce the contamination risk
- Short shelf-lives of these products make a different testing strategy necessary

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NGS at ViruSure

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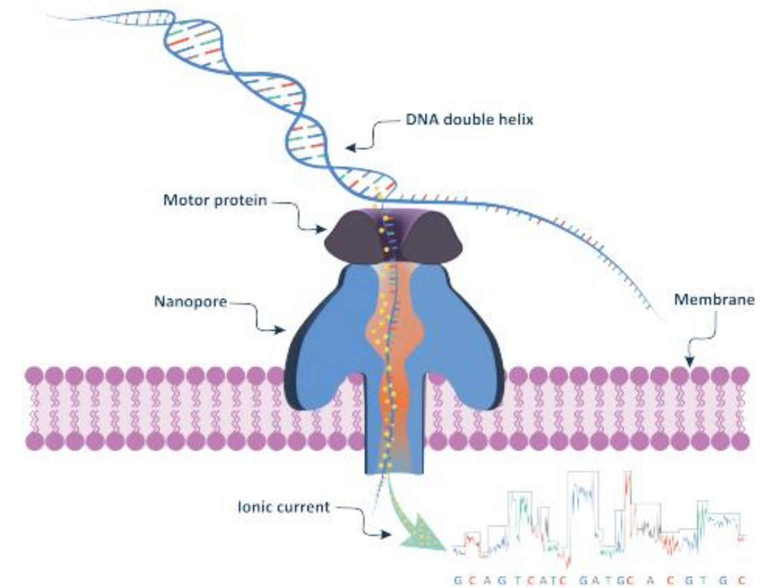
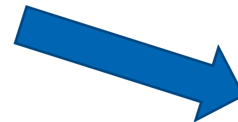
NGS at ViruSure

The technology

- Oxford Nanopore Sequencing
- Decision based on a comparability study:
  - Cellular Background spiked with DNA virus
  - Sequencing with Nanopore MinION and Illumina Miseq

→ recovered more of the viral genome in the smaller MinION Data set
→ Long-reads make virus detection in a lot of background easier





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Which biological samples and products can be tested by NGS?

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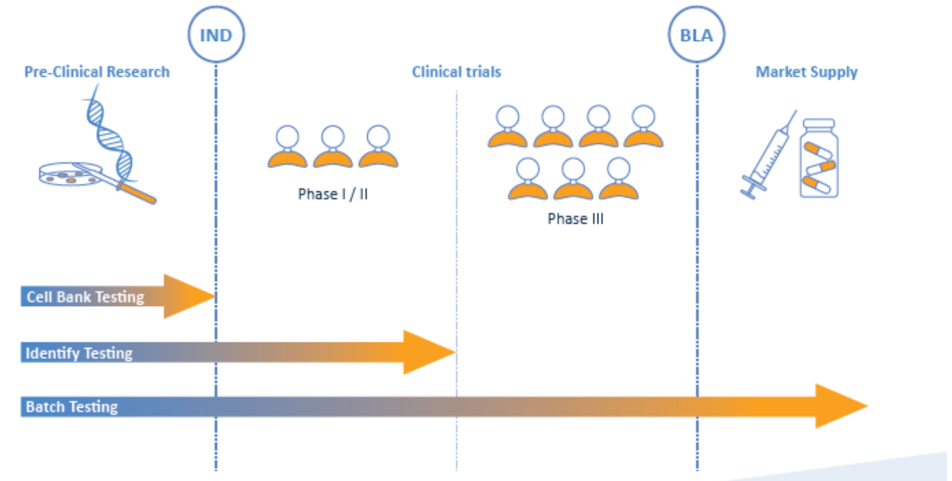
- Which products can you test by NGS?
  - Vaccines
  - Cell & Gene Therapies
  - Recombinant Proteins

- Which biological samples can you test by NGS?
  - Cell Banks (RCB, MCB, WCB, EOPCB)
  - Virus Banks (MVS, WVS)
  - Bulk Harvests



Any sample that contains nucleic acid sequences can be submitted to NGS analysis for different types of experiments

- Which tests can you perform by NGS?
  - Detection and identification of contaminants
  - Confirmation of sequence identities
  - Evaluation of integration sites

  - Also other applications might be possible with the correct set-up (e.g. testing for residual host-cell DNA)

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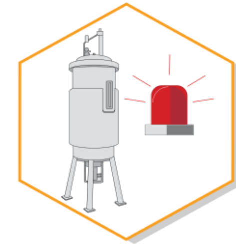
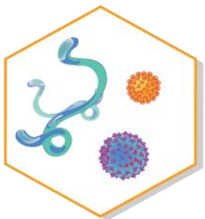
Tests for contaminants

- Mycoplasma and Sterility testing
  - Targeted analysis for highly conserved regions in bacterial and fungal genomes
  - Region of interest is amplified by PCR
  - If present, amplicons are sequenced to identify the contaminant
  - Advantage of target approach:
    - Higher sensitivity (no host sequences are amplified)
    - Faster TAT
  - Also whole genome approach possible to use data set for multiple analysis (e.g. combine with adventitious virus detection or resequencing)

- Species identification in contamination events
  - After discovery of a contamination event, the contaminant can be identified in <u>less than a week</u>



- Detection of adventitious viruses
  - Agnostic (no prior sequence knowledge necessary)
  - Complete nucleic acid content of a sample is compared against a viral database to scan for similarities to detect contaminations



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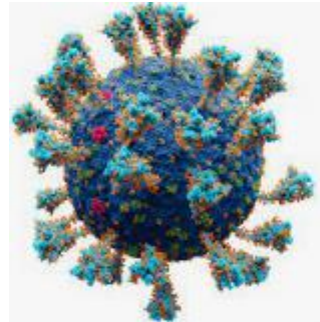
Adventitious Viruses

- For adventitious virus detection, different approaches can be used:
  - Genomics:
    - In the genomics approach, the complete sample is used for nucleic acid extraction. Therefore, all the signals that are present are picked up giving the advantage that nothing is missed. But received hits have to be checked on whether they are indeed derived from replicating virus
  - Transcriptomics:
    - The transcriptomics approach focuses on RNA only present in the sample. All viruses have at least RNA intermediates during their life cycles. Picking up a signal in a transcriptomics approach it already tells that the virus is also replicating
  - Viromics:
    - The viromics approach focuses only on intact viral particles. Before the actual extraction of viral genomes, nucleic acid from host cells as well as other free DNA/RNA is removed by nuclease treatments. After the treatment, only intact viral particles should be left for extraction and sequencing, also giving already an indication that a pick-up signal might be from a life virus.



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Sequence confirmation approaches

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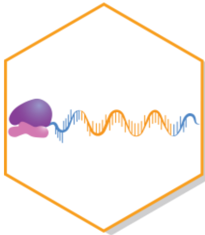
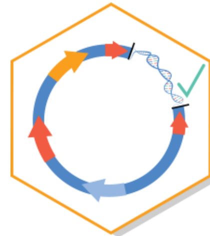
Sequence confirmation is necessary for a lot of different samples/approaches

- mRNA vaccines
  - With Nanopore sequencing RNA can be analysed in its native form
  - No reverse transcription is necessary, avoiding the introduction of biases
  - With direct RNA sequencing, you can also determine the integrity of the RNA, as you can sequence the complete mRNA in one read
  - Could also be sequenced as cDNA, to be combined with other experiment types (e.g. adventitious agent detection)



- Genetic stability
  - Gene copy number determinations
  - Integration site analysis
  - Scan for Off-Target integration events



- Identity confirmation of viral vectors
  - Complete viral genomes can be sequenced, potentially also in one single read

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Summary – Principles of main types of analysis

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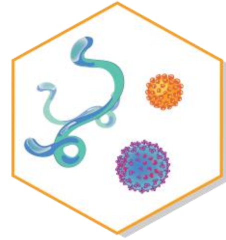

Adventitious Agent Testing	Sequence confirmation approaches
Detection of contaminants within the complete data set	Comparison of sequencing data against provided reference sequence
Removal of host sequences, then comparison of reads for similarities against viral database	Screen for discrepancies between data and reference sequence
Limit test: Necessary to demonstrate Sensitivity and Specificity for each sample matrix	
Negative Control included to determine sequences originating from used reagents	

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Validation of NGS assays

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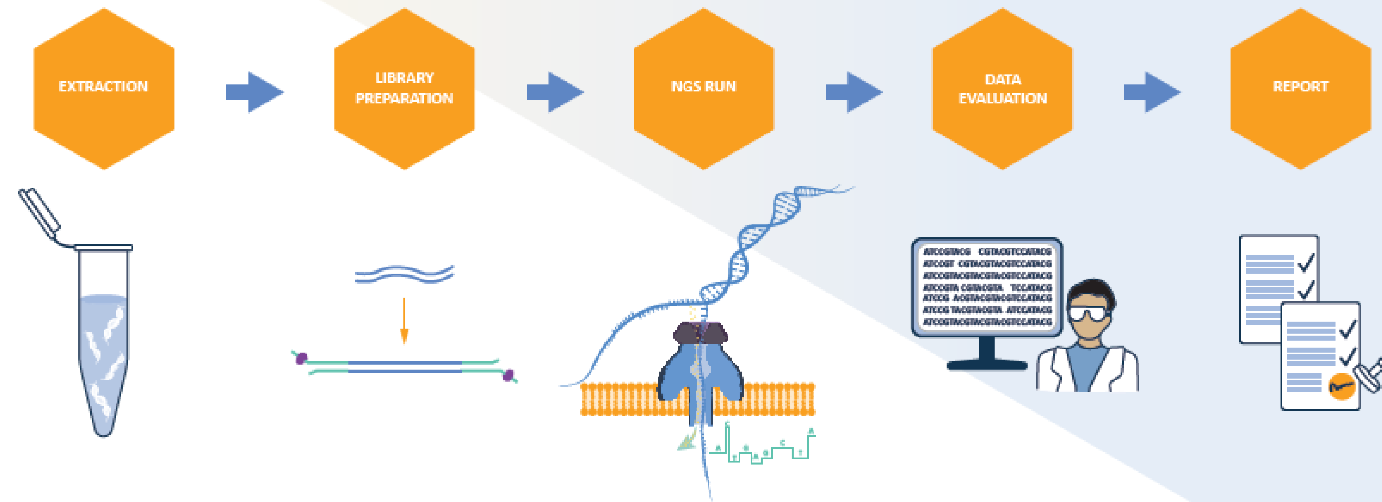
NGS device

- ✓ Qualified (Eudralex Volume 4, EU Guidelines for Good Manufacturing Practice, Annex 11 Computerized Systems, 2011)
- ✓ 21 CFR part 11 compliant

NGS workflow

A modular validation approach is used. Therefore, each step in the workflow will be validated separately, leaving the flexibility to combine the validated steps as needed and generate a workflow customized for the underlying sample type.



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Validation of NGS for Adventitious Virus Testing

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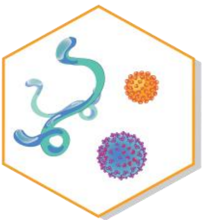
- ✓ NAT should be appropriately validated for its intended use (method and matrix validation)
  - → Genomics approach
    - sequencing of all nucleic acids within the sample
    - Benefit of not missing any viral sequences (e.g. Due to damaged viral particles during a nuclease treatment)
- ✓ **NGS is classified as a limit test** → for each sample LOD and specificity (breadth of virus detection) need to be considered
  - Each test sample matrix is different. The effects of the matrix on the recovery of viral particles has to be evaluated by performing matrix validation
- ✓ **Validation of NGS includes**
  - → suitable reference materials (5 WHO reference viruses (EBV, FeLV, HRSV, MRV-1, PCV-1))
  - → comprehensive database (e.g. URVDB, NCBI virus)
  - → Matrix specific verification (Spiking Studies)
- ✓ **No head-to-head comparison between NGS and in-vivo/in-vitro testing necessary**
  - → Different testing principles



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Recent developments at ViruSure

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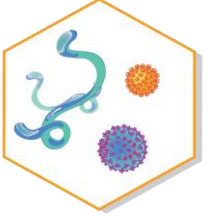
- Sequence identity confirmation for haploid organisms (viruses, bacteria) → nearly GMP ready

- Adventitious virus detection → under R&D (validation tasks ongoing)



- In the pipeline
  - mRNA control sequencing
  - AAV identity pipeline
  - Genetic Stability
  - Sterility and mycoplasma testing
  - ...

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감사합니다 Gracias Danke

Благодаря 谢谢 Tack धन्यवाद Dziękuję

Спасибо Thank You Obrigado

Děkuju Grazie Ευχαριστώ Merci Köszönöm

ありがとうございました Teşekkür ederim

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