Next Generation Sequencing

Validation, Regulatory Approval and Implementation of NGS as an alternative to *In Vivo* testing for Flumist / Fluenz

Alice Alston, Director, Global Quality Control

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Next Generation Sequencing – Why?

- Next Generation Sequencing can be used to characterise any adventitious agent (contaminant) sequence signatures that may be present and optionally confirm the genome identity of a virus-based test article.
- Next Generation Sequencing is seen widely as an alternative to *In Vivo* / *In Vitro* testing
- *In Vivo* testing is outdated and not sensitive / robust – regulators are slowly moving away from animal models – Ph.Eur to introduce a chapter for NGS (2.6.41)
- Aligns with 3R's (Replace / Reduce / Refine) and sustainability initiatives
- Move to a more technologically advanced analysis for adventitious agents
- Reduces Turn Around Time (TAT) for an assay which is on critical path for lot release
- High percentage of invalids with traditional *In Vivo* (due to flu sample matrix)
- AZ Liverpool did not have the lab capacity to develop and validate NGS internally – we entered into a collaboration with Merck to validate the NGS method for Flumist / Fluenz

Regulatory Guidance

Ph. Eur.	**Ph. Eur. 5.2.14. Substitution of *in vivo* method(s) by *in vitro* method(s) for the quality control of vaccines**	• Novel, sensitive molecular techniques with broad detection capabilities are available, including deep sequencing or high throughput sequencing (HTS) • Use of broad molecular methods has highlighted the gaps with the existing testing strategy by identifying previously undetected viral contaminants
Ph. Eur.	**Ph. Eur. 5.2.3. Cell substrates for the production of vaccines for human use** **Ph. Eur. 2.6.16. Tests for extraneous agents in viral vaccines for human use**	• Novel, sensitive molecular techniques with broad detection capabilities are available, including HTS and degenerate PCR • These methods may be used either as an alternative to *in vivo* or specific NAT tests in agreement with the competent authority
US. FDA	**US FDA CBER. Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines IV. Description of test methods**	• Advances in science and technology are likely to yield additional information that could lead to modification or replacement of some described tests, and except where prohibited by regulation, manufacturers may use alternative test methods, where scientifically justified • Manufacturers should consider how they can replace, refine, or reduce their use of *in vivo* tests.

Regulatory Guidance

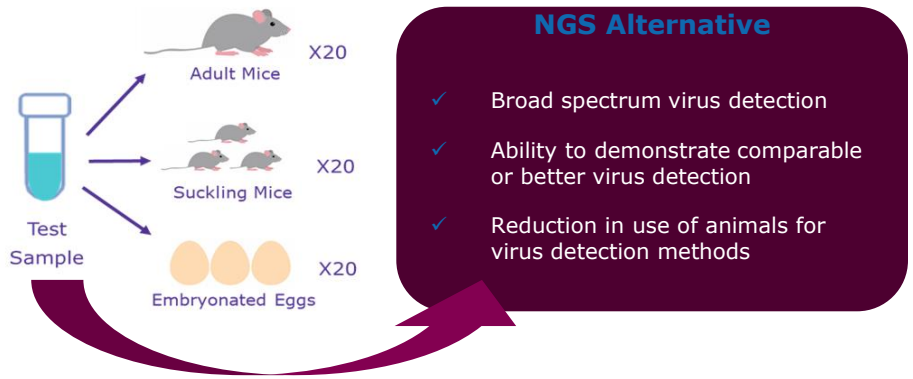
ICH

Q5A (R2) Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin

- Non targeted NGS is encouraged as a replacement for *in vivo* assays due to its breadth and sensitivity of virus detection and the limitations of the *in vivo* assays. Furthermore, this promotes the global initiative to replace, reduce and refine the use of animal testing

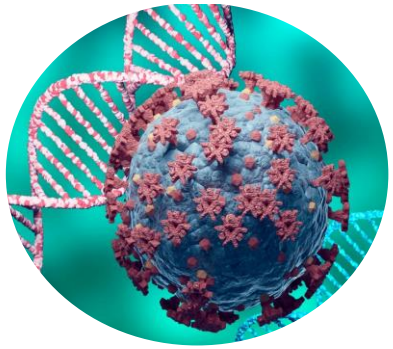
When is it appropriate to use NGS technology

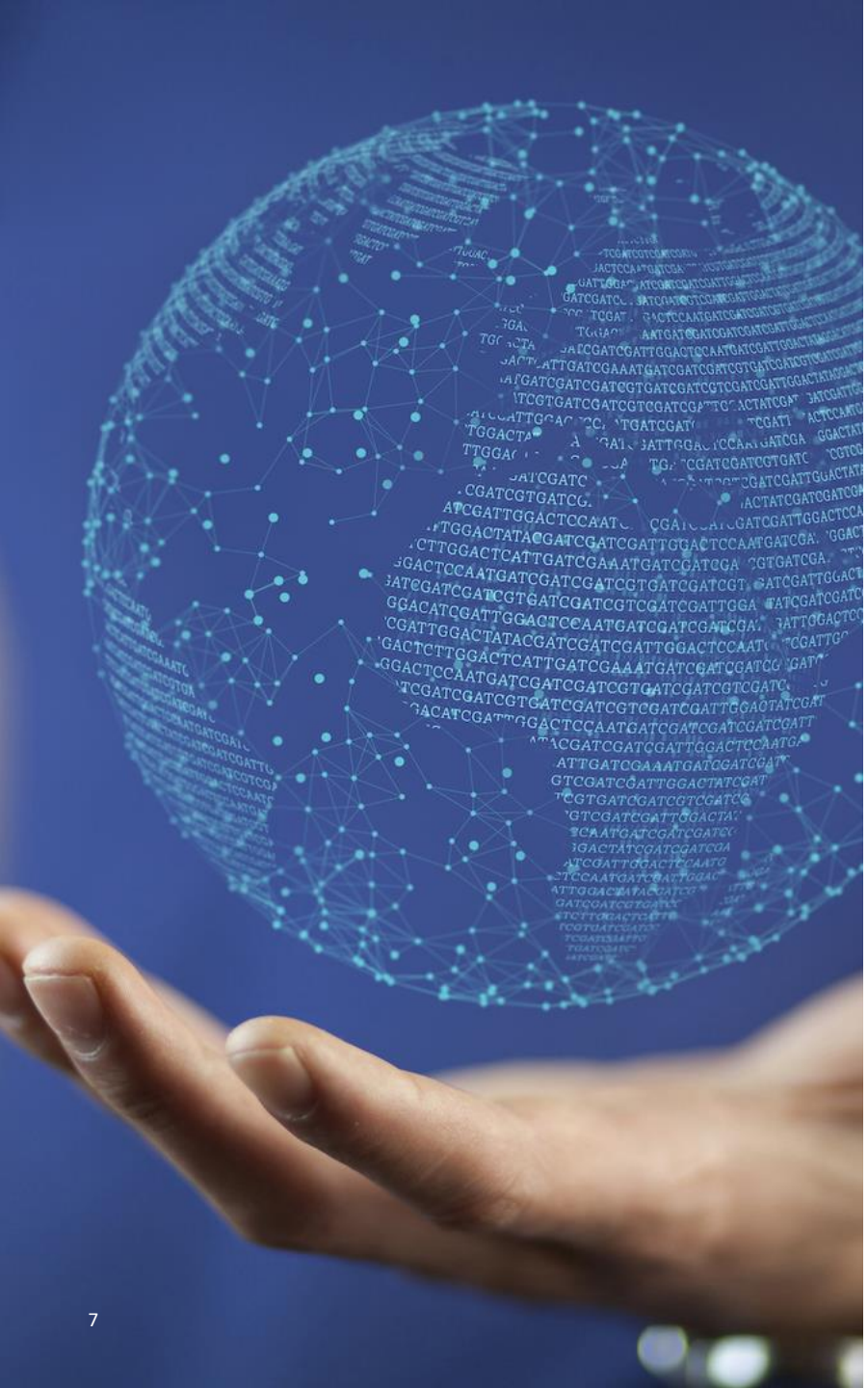
- Infectious products or intermediates where a **neutralizing antibody is unavailable** for culture-based methods
- Cell line screening in lieu of animal studies and ***in vivo* testing**
- **Broad virus screening** assay to complement or round out the safety profile of the test agent
- Product release testing as an **alternative method** to confirm safety profile
- Instances where traditional assays would not be expected to identify a potential contaminant of interest or no traditional assay exists.
- Replacement for *in vivo* and *in vitro* methods (with appropriate qualification/validation testing).
    - Consistent with 3R Principles to reduce animal use
    - Provides faster alternative to traditional testing



NGS as an alternative to *in vivo* virus testing
Considerations for use

- **Virus identification through NGS requires follow-up investigation**
  - Detects virus nucleic acids but not infectious particles
  - Information gained through sequencing may be able to guide investigation path
    - Virus identification
    - Whole or partial genome
    - Known replicative intermediates
- **Method Quality**
  - Establish criteria for method suitability and validity
    - Sample preparation
    - Assay limit
    - Process Controls
  - Data processing and storage
  - Defined bioinformatics
  - Data interpretation in the context of the sample tested



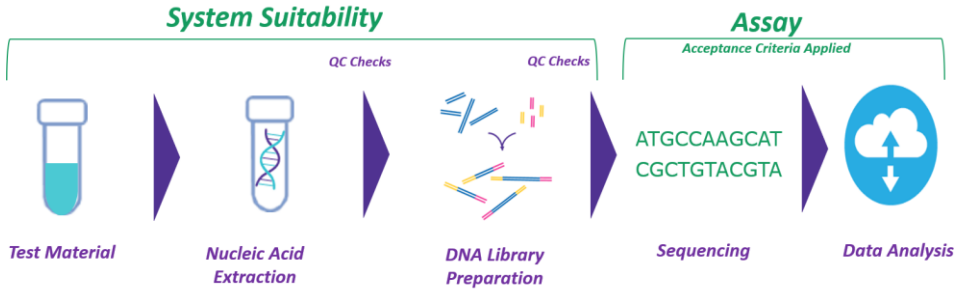


NGS as a replacement for *In Vivo* - approach

1. Validation & Sample Matrix Qualification
2. Regulatory Submission Approach
3. Health Authority Questions
4. Health Authority Approvals
5. Implementation Strategy

Validation

- NGS Assay is validated in a Modular Fashion:
  - Extraction
  - Library Preparation
  - Sequencing
  - Bioinformatics



- This approach confirms that each portion of the assay behaves as-expected.
- Limit of detection for client specific matrices are established through END-TO-END analysis with known reference material (per ICH Q5A recommendations) to achieve ICH Q2 parameters.
- Representative viruses encompass different physiochemical properties:
  - WHO Reference material: EBV, RSV, FeLV, PCV, REO, product-specific concern
- Full validation package consists of

GENERIC VALIDATION + SPIKING STUDY

Sample Matrix

- Goal: Replacement of *in vivo* animal model testing for Adventitious viruses
- Test Sample: Bulk Harvest Material for Quadrivalent Influenza Vaccine
- Matrix: Allantoic Fluid (4 separate strains / lots)
  - H1N1
  - H3N2
  - B Yamagata
  - B Victoria
- FluMist® Quadrivalent vaccine & Fluenz® Tetra vaccine are manufactured using SPF eggs as per Ph.Eur 5.2.2 (Chicken Flocks Free From Specified Pathogens for the Production and Quality Control of Vaccines – current) and USDA memorandum 800.65 (Eggs and Chickens for Production of Veterinary Biological Products – current)

FluMist Quadrivalent
Influenza Vaccine Live, Intranasal

FLUENZ
nasal spray, suspension
influenza vaccine (live attenuated)
Flu protection without the injection

Live Attenuated Influenza QC testing

- QC Lot release testing is performed on all stages throughout the manufacturing process
- The testing is designed to assess the safety, purity, identity and efficacy of the drug substance / drug product
- Testing is in compliance with Ph. Eur. 2.6.16 (Test for Extraneous Agents in Viral Vaccines for Human Use - Current) and US Guidance for Industry (Characterization and Qualification of Cells Substrates and Other Biological materials Used in the Production of Viral Vaccines for Infectious Disease Indications – 2010)

FluMist Quadrivalent
Influenza Vaccine Live, Intranasal

FLUENZ
Flu protection without the injection

Live Attenuated Influenza QC testing

- *In vivo* testing as specified in Ph. Eur. 2.6.16 was completed on every lot manufactured. This assay was subject to high invalid rates due to inherent variability within a biological model, egg hardiness etc
- A suitable alternative was validated which can deliver:
    - Deliver and scale for patients, with quality, speed and efficiency
    - Supply to market, with a repeatable, robust assay
    - Accelerate digital and technical innovation
    - Removal of animal testing in line with AstraZeneca 3R's program
- NGS was chosen as a suitable alternative to the traditional *in vivo* method as it covers all the above points and allows us to move away from traditional methods and utilise newer technologies

Results of Method Suitability

Samples- 1e6 GC/mL Spike (1 replicate)	221,942,275	244,724,659	254,744,107	254,701,834
Virus/Taxonomy	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits
Epstein Barr Virus/Human Gammaherpesvirus 4	13,228	29,599	6,157	6,024
Respiratory Syncytial Virus/Human orthopneumovirus	16,632	49,498	15,446	34,941
Feline leukemia virus	9,493	25,473	4,350	8,856
Porcine circovirus 1	1,052	1,694	467	339
Reovirus 1/Mammalian orthoreovirus 1	223	383	144	186
Bovine Viral Diarrhea Virus	4,279	14,693	2,818	6,198

Samples- 1e4 GC/mL Spike (2 Replicates)	307,816,803	327,643,993	275,381,110	260,814,058	240,638,453	473,926,241	259,239,557	281,634,344
Virus/Taxonomy	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits
Epstein Barr Virus/Human Gammaherpesvirus 4	226	527	593	544	227	421	104	177
Respiratory Syncytial Virus/Human orthopneumovirus	207	256	415	412	154	295	423	449
Feline leukemia virus	111	121	207	158	74	51	123	77
Porcine circovirus 1	5	8	21	9	1	9	7	3
Reovirus 1/Mammalian orthoreovirus 1	8	12	10	10	0	3	3	4
Bovine Viral Diarrhea Virus	30	119	118	94	35	29	42	90

Samples- 1e3 GC/mL Spike (2 Replicates)	637,879,810	231,347,132	278,514,830	212,187,679	259,587,396	227,693,457	267,231,034	248,969,183
Virus/Taxonomy	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits
Epstein Barr Virus/Human Gammaherpesvirus 4	75	48	13	4	14	16	4	21
Respiratory Syncytial Virus/Human orthopneumovirus	70	8	25	40	20	19	34	19
Feline leukemia virus	15	2	12	1	2	4	7	6
Porcine circovirus 1	0	0	3	10	0	0	0	0
Reovirus 1/Mammalian orthoreovirus 1	0	0	0	0	0	0	0	0
Bovine Viral Diarrhea Virus	27	5	10	6	0*	1*	6	7

Samples- 1e2 GC/mL Spike (2 Replicates)	293,313,165	323,142,705	235,977,997	292,019,806	301,540,390	306,656,092	269,401,807	323,268,646
Virus/Taxonomy	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits	Number of Confirmed Hits
Epstein Barr Virus/Human Gammaherpesvirus 4	0	0*	2	8	2	0*	2	0
Respiratory Syncytial Virus/Human orthopneumovirus	2	0	6	2	0	0	6	16
Feline leukemia virus	0	0	0	0	0	6	0	1
Porcine circovirus 1	0	0	0	0	1	0	0	0
Reovirus 1/Mammalian orthoreovirus 1	0	0	0	0	0	0	0	0
Bovine Viral Diarrhea Virus	0	0	0	0	0	10	3	0

- One replicate for 1e6 genomic copies per sample type
- 2 replicates for other spike levels per sample type
- Hits for each spike level were tabulated and assessed in context of total number of hits and hits per million.
- Observations:
  - High number of hits for 1e6 spiked samples
  - Decreasing number of hits as spike level decreased
  - Little to no hits seen in the 1e2 spiked samples.
- Detectability = Positive is at least 1 hit
- Detectability = Negative if no hits

Results of Method Suitability
High Level summary of results

Sample + 1e6 Spikes

EBV	RSV	FLV	PCV	REO	BVDV
+	+	+	+	+	+
+	+	+	+	+	+
+	+	+	+	+	+
+	+	+	+	+	+
4/4	4/4	4/4	4/4	4/4	4/4

Sample + 1e4 Spikes

EBV	RSV	FLV	PCV	REO	BVDV
+ +	+ +	+ +	+ +	+ +	+ +
+ +	+ +	+ +	+ +	+ +	+ +
+ +	+ +	+ +	+ +	- +	+ +
+ +	+ +	+ +	+ +	+ +	+ +
8/8	8/8	8/8	8/8	7/8	8/8

Sample + 1e3 Spikes

EBV	RSV	FLV	PCV	REO	BVDV
+ +	+ +	+ +	- -	- -	+ +
+ +	+ +	+ +	+ +	- -	+ +
+ +	+ +	+ +	- -	- -	- +
+ +	+ +	+ +	- -	- -	+ +
8/8	8/8	8/8	2/8	0/8	7/8

Sample + 1e2 Spikes

EBV	RSV	FLV	PCV	REO	BVDV
- -	+ -	- -	- -	- -	- -
+ +	+ +	- -	- -	- -	- -
+ -	- -	- +	+ -	- -	- +
+ -	+ +	- +	- -	- -	+ -
4/8	5/8	2/8	1/8	0/8	2/8

All Assays and controls passed validity criteria, as expected. No “hits” in unspiked samples.

Results of Method Suitability

Combined LOD (7/8)

Sample Tested	EBV	RSV	FLV	PCV	REO	BVDV
Sample + 1e6 Spike	+	+	+	+	+	+
Sample + 1e4 Spike	+	+	+	+	+	+
Sample + 1e3 Spike	+	+	+	-	-	+
Sample + 1e2 Spike	-	-	-	-	-	-
Sample + No Spike	-	-	-	-	-	-

	Genomic Copy/mL	TCID50 Equivalent/mL
Epstein Barr Virus	1,000	29.73
Respiratory Syncytial Virus	1,000	1.10
Feline leukemia virus	1,000	0.43
Porcine Circovirus	10,000	0.44
Reovirus	10,000	12,727.27
BVDV	1,000	8.86

LOD Results

- Final results showed Detectability of EBV, RSV, FelV and BVDV at 1,000 GC/mL.
- Final results showed Detectability of PCV and REO at 10,000 GC/mL

Conclusions from Spiking Study

1. NGS was able to detect representative virus spikes at a suitable limit of detection, in-line with previous matrices published through worldwide studies (e.g. AVDTIG).
2. Results are IN-LINE with expectations regarding limit of detection between 1e3 and 1e4 Viral Genome Copies per mL.
3. Higher Limit of Detection in REO and PCV is not unexpected given the nature of those 2 viruses
    - REO- Double-stranded RNA virus
    - PCV- Very small single-stranded DNA virus
4. Read numbers ≥100 Million reads per sample are ideal for hitting detection limit based on “Hits per million” assessment. Minimum read-count set at 130 Million for this matrix.

Regulatory Submission Approach

- Summary validation package from Merck for overall modular validation approach
- Final summary report Merck / AZ – detailing the overall product specific qualification for Flumist / Fluenz
- AZ Contamination Control assessment of manufacturing and QC labs – how adventitious agents can't be introduced during manufacturing or testing
- AZ Risk Assessment for the introduction of NGS as an alternate to *In Vivo* including*:*
    - *Contamination control of adventitious agents*
    - *Comparison of NGS vs In Vivo*
    - *Risks of switching to NGS*
    - *Assessment of safety profile and discussion of other safety tests completed*
    - *Investigation approach for a "positive hit"*
    - *Overall summary: NGS will offer a more robust / sensitive method, along with other registered safety assays for the detection of adventitious agents*

Health Authority Questions

- Questions on the submission package were received from multiple health authorities, some examples are below:
  - **The applicant should provide a detailed description and justification of the primary (algorithm) and secondary analysis criteria leading to the definition of a "hit" and "a true positive viral hit".**
  - **Please provide the details in preparing the dilutions of the virus stocks containing $10^{10\sim11}$ VGC/mL to $10^{5\sim6}$ VGC/mL (including the dilution scheme).**
  - **Please describe the PHF lots used in the spiking studies. Please clarify if the PHF lots used in the spiking studies are research materials or commercial lots.**
  - **The LOD of REO and PCV is a log higher than the rest of the viruses. Please continue to improve the LOD of these families. In the meantime, we recommend that you perform targeted bioinformatic analysis for avian reoviruses and avian circoviruses to demonstrate absence of these potential adventitious agents in the vaccine material.**
  - **Please provide the rationale to determine the following viral signals as not being considered significant:**
    - **i. Low eukaryotic viruses such as marine-, and fungi-specific viruses**
    - **ii. Plant and the hosts other than G. gallus such as Mus musculus.**
  - **Please explain the detection of influenza strains that are different from the expected LAIV strains in the blast results of PHF lots.**
  - **Please submit plans for updating the validation studies for NGS virus detection. For NGS technology, since the workflow can still be optimized and the database and bioinformatics software are evolving, please specify how often the NGS method for the detection of adventitious agents in MVS and MVB will be re-validated.**

Health Authority Approvals

- *Questions were received from EMA / Swiss Medic / FDA*
- *All questions were responded to within the boundaries of active submissions under review*
- *Markets approved*
  - *EMA (European Medicines Agency)*
  - *MHRA (UK)*
  - *Swiss Medic (Switzerland)*
  - *Ministry of Health (Israel)*
  - *Health Canada*
  - *FDA (USA)*
- *Future new markets – currently under review*
  - *Mexico*
  - *Taiwan*
  - *South Korea*
  - *Australia*
- *Japan – we are currently in discussion with Japan to understand if NGS will be accepted or if In vivo is still the chosen method for adventitious agents detection*

Implementation Strategy

- NGS is now the preferred option for adventitious agents detection for Flumist / Fluenz*
- NGS has been used routinely as part of the lot release program since April 2023
- In that time, we have had 0% invalid assays (in relation to In vivo which could be as high as 50%)
- We have had 0 positive hits which needed to be investigated
- TAT for NGS is 32 days, In Vivo was 60 days – a 28-day TAT saving
- NGS is more costly than *In vivo*, however, this is balanced by
  - Reduced TAT
  - Fewer invalid assays
  - Fewer investigations for unconfirmed Out of Specifications

* *In Vivo still used for specific lots for markets not yet approved*

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Thank you.

AstraZeneca PLC, 1 Francis Crick Avenue, Cambridge Biomedical Campus, Cambridge, CB2 0AA, UK
+44(0)203 749 5000
www.astrazeneca.com