Head-to-Head Comparison of NGS with *In Vivo* Animal Assays and *In Vitro* Cell Culture Assays for Adventitious Virus Detection

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Project Overview

Next generation sequencing (NGS) has demonstrated capabilities for detection of known and unknown viruses in biologics, without having prior sequence knowledge

- The **Goal** of the project was to obtain NGS data for decision-making regarding suitability of NGS applications for adventitious virus detection in biologics
  - Replacement of animal-based adventitious agent testing
  - Development of tools to support novel *in vitro* adventitious agent testing
  - Identify new *in vitro* release tests to replace animal-based release tests in vaccine manufacturing quality control

- The project **Aims** were to:
  - Evaluate NGS virus detection in a complex biological material and directly compare with the *in vivo* animal assays and the *in vitro* cell culture assays using the same spiked samples for testing in all of the different assays
  - Enhance NGS bioinformatics for known and novel adventitious virus detection by refining the Reference Virus Database (RVDB) for increasing specificity and accuracy of virus detection and building automated pipelines for enhancing efficiency of NGS bioinformatics work

Project Teams+

NGS Laboratory/Bioinformatics Work

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Bioinformatics Enhancement Work

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Study Contributions

TEAM	NGS LABORATORY/BIOINFORMATICS WORK							BIOINFORMATICS ENHANCEMENTS
Organization	**Viruses & Matrix** • Testing • Characteriza-tion	**Spiked Sample Prep**	***Pre-Study*** • ddPCR	***Pre-Study:*** • *In vitro* assay for virus detection • Assay interference	**NGS**	***In Vitro* assays** • **3 cell lines**	***In Vivo* assays** • **3 rodent species**	• **Databases** • **Annotation pipeline** • **Adventitious virus pipeline** • **Enhanced Web capabilities**
FDA/CBER	+	+	+		+			+
Merck/Milli-poreSigma	+			+	+	+	+	
GSK					+	+		
U. Delaware								+

Study Considerations

- It is generally *presumed* that NGS is superior to the *in vivo* and *in vitro* assays with regard to breadth of virus detection and NGS can have a similar sensitivity of virus detection to PCR assays
- This study was specifically designed to challenge the NGS workflow and to maximize detection by the biological assays by selecting viruses known to be infectious in at least one of the target cell lines in the *in vitro* assays or one animal species in the *in vivo* assays.
- To achieve this goal, only 2 of the 5 CBER NGS Virus Reference Reagents* for adventitious virus detection were used and the breadth of NGS could not be evaluated compared to the other assays in this study.
- The two RNA viruses used in the study were human RSV (ss, enveloped virus) and Reovirus type1 (ds, non-enveloped virus). However, based on the literature and the AVDTIG 2B study, it is known that NGS could detect viruses that would not be expected to be detected in the *in vivo* and *in vitro* assays (e.g., PCV-1, EBV, FeLV).

- *Prepared by FDA/CBER. Available https://www.niaid.nih.gov/research/bei-resources-repository (BEI NR-59622)*

Study Design and Sample Preparation

- **Virus selection:** **Human RSV and Reovirus** were selected as spikes from the CBER NGS Virus Reference Reagents for adventitious virus detection in biologics
  - Already characterized for virus copies/mL, infectious titer, host cell DNA, adventitious viruses
  - In-house ddPCR assays available in CBER lab for the study viruses
- **Sample types:** **CHO cell bioreactor material (high mAb producer)** was characterized as matrix for the spiking study
  - Sterility and mycoplasma testing
  - Host cell proteins were determined by BCA and ELISA
  - ddPCR assay was established to quantify host cell DNA
  - No toxicity study needed due to previous experience
- **Six spiked samples and one unspiked control** were selected for testing in all assays by all study participants
  - 12 ten-fold serial dilutions of each virus were made and spiked into a fixed volume of matrix. Samples were **blinded** and stored at -80 C prior to distribution. Aliquots were made for one time use per experimental set up per assay, without a freeze-thaw.
  - Six samples were selected based on **a Pre-Study** to determine the range of infectious titer and genome copy number which would produce a positive result in the test assays, with at least one virus
  - For a **head-to-head comparison**, the same volume was tested by ddPCR, NGS and *in vitro* assays. The same material was tested *in vivo* but to use the minimum number of animals, a tiered strategy was used starting with the lowest dilution. Volumes for inoculation of each species were based on the protocols for the compendial assay

Virus Detection Assays

- A **28-day *in vitro* cell culture assay** was performed using Vero, MRC-5, and CHO-K1 cell lines
  - The two participating labs used the same experimental set up as per the compendial assay
  - Observations for CPE, HA and HAD were noted during the study period, as applicable
- ***In vivo* assays** were performed in suckling mice, adult mice, and embryonated hens' eggs
  - The age and number of mice and eggs, volumes and routes of inoculation, observation times and read out for clinical signs or death were done based on the compendial assay. A subpassage was included for suckling mice and embryonated hens' eggs
- **NGS (short-read)**
  - Each lab used their own protocols for the entire workflow including the bioinformatics pipelines
  - To compare results for virus detection, for the bioinformatics, all used the same accession numbers of the reference virus sequence for the targeted analysis and RVDB for non-targeted analysis
- **ddPCR**
  - Results from the Pre-Study were used to determine sensitivity of virus detection

Summary of RSV Detection

Sample ID	RSV		NGS (Targeted Bioinformatics)[a]			In Vitro (28-day assay)		In Vivo (1^0 passage/subpassage)[b]
	Viral copies (GC/mL)	Virus infectious titer ($TCID_{50}$/mL)	**Lab 1**	**Lab 2**	**Lab 3**	**Lab 2**	**Lab 3**	**Lab 3**
A	Unspiked	Unspiked	-	-	-	**-**	**-**	- / -
B	1.04×10^7	1.08×10^4	NT[c]	NT	NT	**NT**	**NT**	- / -
C	1.04×10^6	1.08×10^3	+	+	+	**+**	**+**	NT
D	1.04×10^5	1.08×10^2	+	+	+	**+**	**+**	NT
E	1.04×10^4	1.08×10^1	+	+	-	**+**	**-**	NT
F	1.04×10^3	1.08×10^0	+	-	-	**+**	**-**	NT
G	1.04×10^2	1.08×10^{-1}	-	-	-	**+**	**-**	NT
H	1.04×10^1	1.08×10^{-2}	-	-	-	**-**	**-**	NT

[a]Positive criteria for targeted analysis was > 2 reads for Lab 1 and $\geq$ 1 reads for Labs 2 and 3
[b]RSV was done with the highest spiked level in *in vivo* assays
[c]NT, Not tested

Summary of Reovirus Detection

Sample ID	REO		NGS (Targeted Bioinformatics)[a]			In Vitro (28-day assay)		In Vivo (1^0 passage/ subpassage)[b]
	Viral Copies (GC/mL)	Virus infectious titer ($TCID_{50}$/mL)	Lab 1	Lab 2	Lab 3	Lab 2	Lab 3	Lab 3
A	Unspiked	Unspiked	-	-	-	-	-	- / -
B	1.40×10^7	1.10×10^7	NT[c]	NT	NT	NT	NT	+ / -
C	1.40×10^6	1.10×10^6	+	+	+	+	+	+ / +[d]
D	1.40×10^5	1.10×10^5	+	+	-	+	+	+ / -
E	1.40×10^4	1.10×10^4	+	-	-	+	+	- / -
F	1.40×10^3	1.10×10^3	-	-	-	+	+	- / -
G	1.40×10^2	1.10×10^2	-	-	-	+	+	- / -
H	1.40×10^1	1.10×10^1	-	-	-	+	+	- / -

[a]Positive criteria for targeted analysis was > 2 reads for Lab 1 and $\geq$ 1 read for Labs 2 and 3.
[b]REO was only seen the positive results in suckling mice.
[c]NT, Not tested
[d]Individual animal sub passaged

Overall Study Results

- **NGS (Labs 1, 2, 3)**
  - All detected both RSV and Reo in the complex background of the unprocessed CHO cell matrix, although difference in sensitivity of virus detection was seen for the two viruses
  - Difference in sensitivity of virus detection was seen by the different labs.
  - Review of the protocols indicated this was due to difference in the NGS workflows (pre-treatment to reduce host cell nucleic acids in the sample may be important for enhancing virus detection)
  - NGS showed a similar sensitivity of virus detection to the ddPCR results (obtained from the pre-study)
- ***In vitro* assays (Labs 2, 3)**
  - Results of the *in vitro* cell culture assays showed similar results with Reo, using independent source of cell lines, reagents, and observation of results for the read-out assays (CPE, HA, HAD).
  - Difference in virus detection was seen with RSV
  - In general, the expected high sensitivity of virus detection was seen in the cell culture assays compared with NGS,
- ***In vivo* assays (Lab 3)**
  - A positive result was seen with Reo, which is based on subpassage. However, this was due to a modification in the subpassage for suckling mice

In vitro Infectivity Study Outcomes

- Three principle, complementary approaches, are used to control for potential viral contamination of biotechnology products, ICHQ5A(R2)
- Key method to control for contamination is *in vitro* infectivity test uses selected cell lines followed by end point analysis CPE, HAD and HA
  - *monolayer cultures of the same species and tissue type as that used for production , monolayer cultures of human diploid cells, monolayer cultures of monkey kidney cells*
- This study provides experimental data and confirms that levels of variability of detection between laboratories are likely based on choice of cell line, pre- treatment of test materials and end point analysis during the test period
  - In vitro assays
    - RSV and REO were detected in replicate samples by CPE, HA, HAD or any combination of the endpoints in any one of the three indicator cell lines
    - All positive and negative controls performed as expected
    - Both viruses were detected by CPE but only REO was detected by HA using rhesus monkey cells at two temperatures
- The study shows the limitations of virus using the traditional *in vitro* methods which further provides evidence that supplementary or replacement of this assay using NGS should be considered

NGS Study Outcomes

- This is the first study to directly compare NGS with both the *in vivo* and the *in vitro* virus detection assays, using the same sample material in all of the tests
- The results support the recommendations for using NGS in the updated guideline on *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* published by the International Council for Harmonisation on Nov 1, 2023 [ICH Q5A(R2)]
  - Replacement assay (*in vivo* assays; PCR assays)
    - *In vivo* AV assays – NGS can provide defined sensitivity and breadth of virus detection. **Aligns with the 3Rs initiative to reduce animals for testing**
    - PCR assays – NGS can have similar sensitivity than PCR assays; broader virus detection; single assay
  - Supplementary or Replacement assay (*in vitro* assays)
    - Cell substrate characterization – particularly in case where there are concerns for occult and novel viruses
    - *In vitro* AV assays – particularly in case of assay interference due to lack of effective neutralization of vaccine virus; as a read-out to reduce assay time
- The study provides head-to-head data to support the suitable use of NGS in biologics (*paper in preparation)*

Still More Work for Routine Implementation

BMGF/NIIMBL GHF-04 Project (2024-2026)

- Optimizing NGS for broad virus detection in complex matrices by spiking studies using:
  - 7 viruses in the "1st WHO international reference panel for HTS" (BEI NR-59630)
  - Well-characterized virus-infected cell lines (Khan lab – *Poster by Sandra Fuentes)*
- Optimizing NGS bioinformatics for enhancing efficiency of database generation and accuracy of data analysis for adventitious and endogenous virus detection.

Desired Outcomes

- Development of protocols, strategies, and NGS datasets for establishing the technology broadly and for routine use


Questions !