

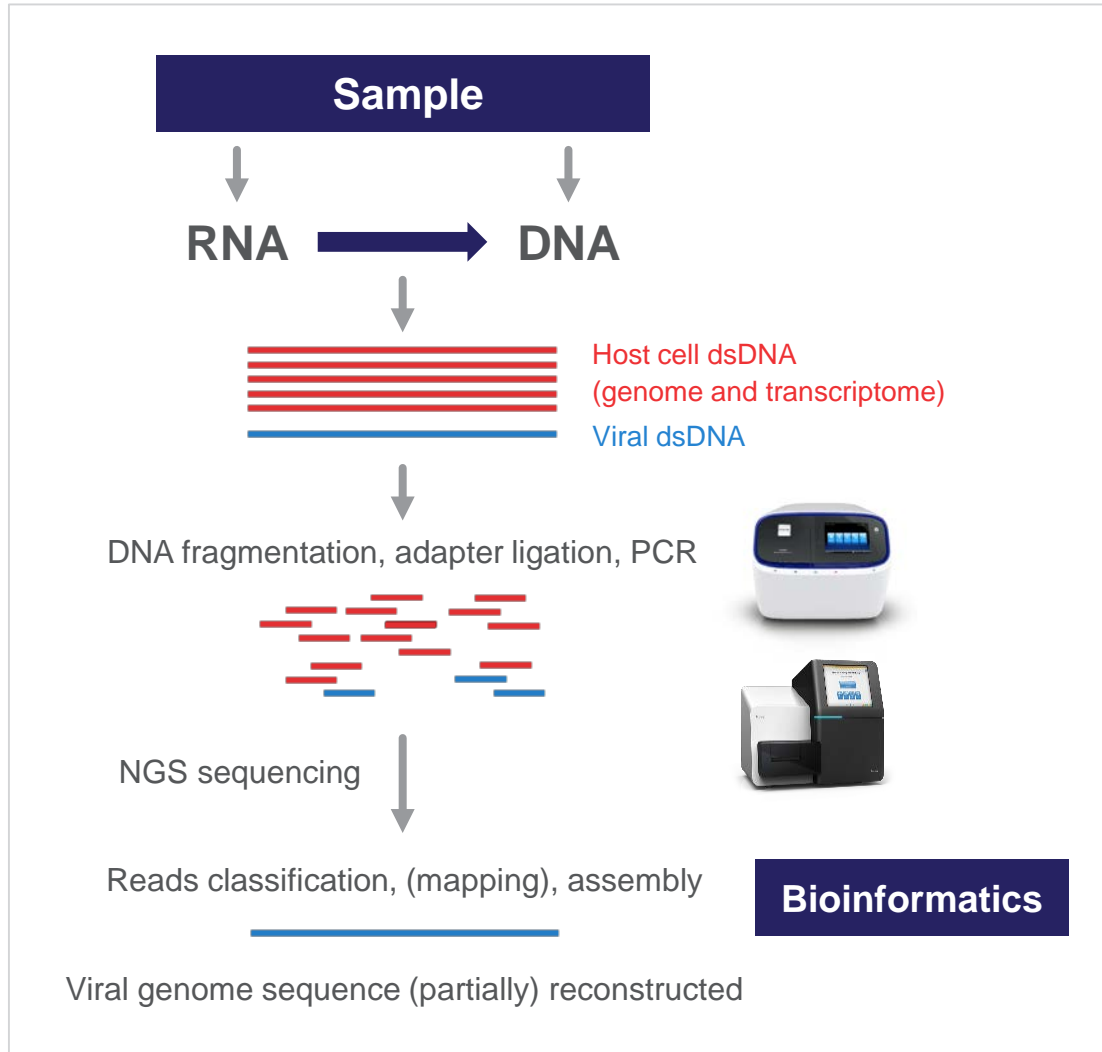


Sensitivity of NGS Based Viral Detection Methods using Viral Stocks Characterized for Genome Copy Number

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NIST/FDA workshop on Standards for NGS Detection of Viral Adventitious Agents in Biologics and Biomanufacturing, 9/18-19/2019

Shotgun NGS Sequencing for Viral Detection



Challenges

ssDNA viruses

ssRNA viruses

dsRNA viruses

Conversion into
dsDNA for
fragmentation in
preparation for
sequencing

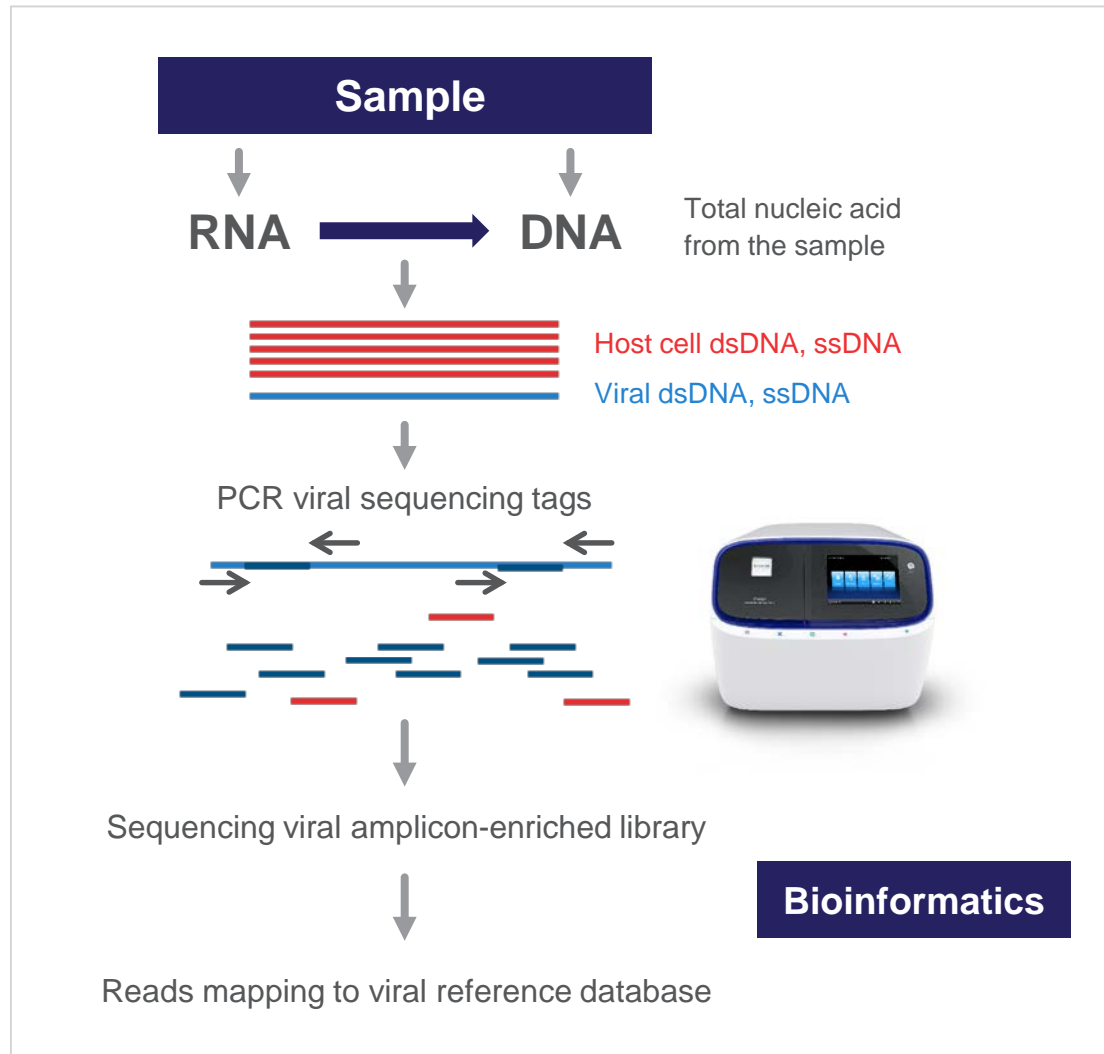
High and variable amounts of
host cell nucleic acids in
extracted material

Prevalence of host nucleic
acid sequences: "Needle in a
haystack challenge"

Challenging to
interpret results

Intensive bioinformatics
needed for analysis

Targeted NGS for Viral Contamination Detection



One workflow for all viruses

ssDNA viruses

ssRNA viruses

dsRNA viruses

Conversion into ssDNA

dsDNA fragmentation is not required

PCR amplifies viral genetic material, improving detection sensitivity

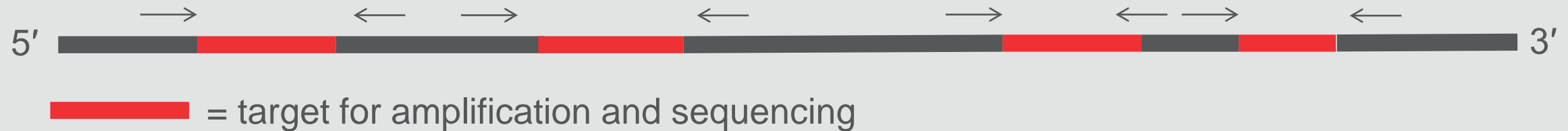
Insensitive to variable host nucleic acids content

Easy-to-interpret results

Simplified bioinformatics mapping to the reference database

Viral Targeting Strategy: Multiple Amplicons Across Viral Genomes

Illustration of panel design:



Primer sets design considerations:

- NCBI reference sequences associated with taxonomic ID for viral species
- Select primer pairs targeting multiple regions (amplicons) across the target viral genomes and strains
- Primers should be specific to target, minimal to no amplification of non-targets (human, mouse, CHO)

119 viral species targeted

Panel A: RNA

RNA viruses

- 58 RNA viral species
- 952 primer pairs

Panel B: DNA

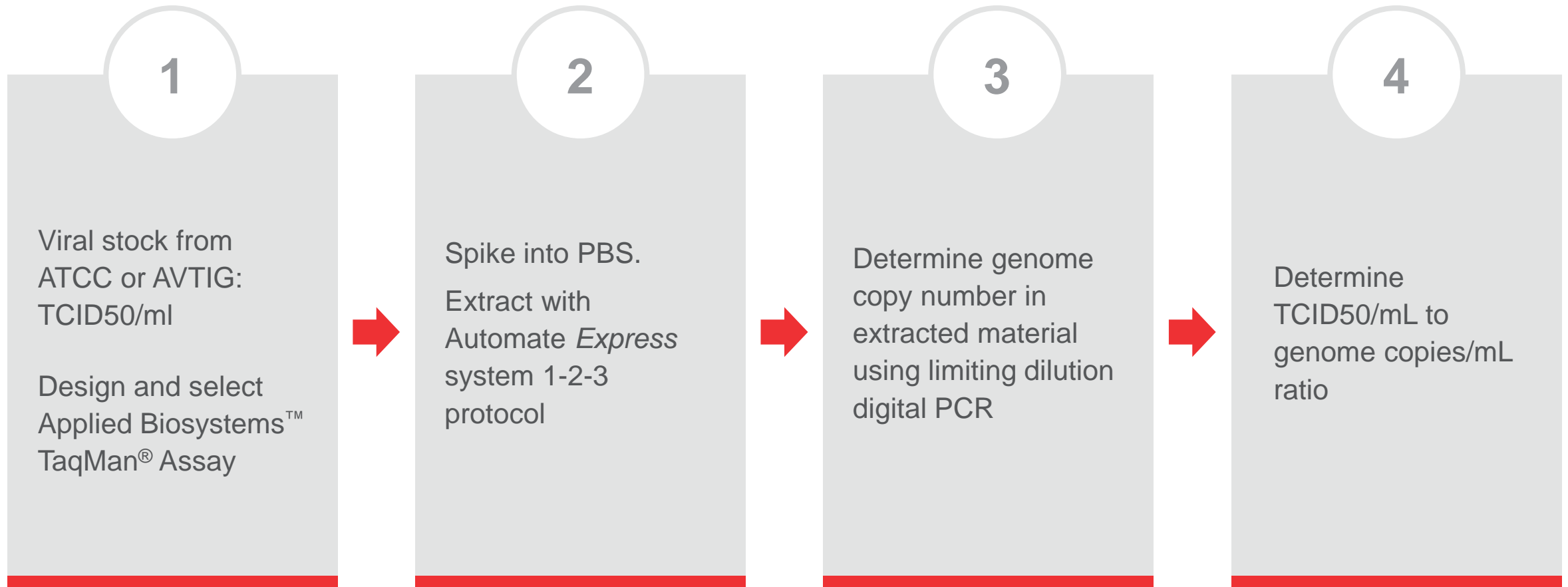
DNA viruses

- 61 DNA viral species
- 423 primer pairs

Controls

- Extraction control (specific primers)
- RT control (vesivirus primers)
- PCR control (MMV primers)

Converting TCID₅₀/mL Into Viral Genomes/mL



10 TCID 50/mL Spike Delivers Highly Variable Input of Viral Genomes Per Reaction

CBER (AVTIG) Viral Stock reference materials (Arifa Khan's group)

Genome structure	Virus name	Virus family	Strain	Infectious titer (TCID50/mL)	GC/mL	GC/Reaction (10 TCID/mL spike into media)
ssDNA	Porcine Circovirus (PCV1)	Circoviridae	Type 1	1.60E+07	1.20E+11	298.5
ssRNA	Feline Leukemia Virus (from ATCC CRL-8012)	Retroviridae	Thielen	1.60E+07	9.70E+10	241.3
ssRNA	Human Respiratory Syncytial Virus A (from ATCC VR-1540)	Pneumoviridae	A2	1.60E+06	3.00E+09	74.6
dsDNA	Human Herpesvirus 4 (Epstein-Barr Virus from ATCC VR-1492)	Herpesviridae	B95-8	1.10E+07	4.20E+08	1.5
ATCC stocks						
dsRNA	Mammalian Orthoreovirus 1	Reoviridae	Lang	1.58E+08	1.17E+09	0.3
dsDNA	Human Adenovirus 2	Adenoviridae	Adenoid 6	2.81E+07	5.34E+09	7.6
ssRNA	Coxsackievirus B3	Picornaviridae	Nancy	2.80E+06	6.76E+09	96.1
ssRNA	Vesicular Stomatitis Virus (VSV)	Rhabdoviridae	Indiana Lab [V-520-001-522]	2.51E+06	3.09E+10	490.0
ssDNA	Minute Virus of Mice (MMV) (Rodent Protoparvovirus 1)	Parvoviridae	Prototype (p)	1.58E+07	9.37E+10	236.0
ssRNA	Calicivirus Isolate 2117 (Vesivirus 2117)	Caliciviridae	Not available	Not available	9.65E+08	199.0

Reovirus1 and HHV4 have low copy per reaction when spiked at 10 TCID 50/mL (equivalent of 74 GC/mL and 384 GC/mL respectively). Detection is still possible because of multiple targets per genome and genome structure of Reovirus—multi-segmented dsRNA.

Infective Viral Titer, TCID₅₀/mL vs Genome Copies/mL

Genome	Virus Name and Type	Strain	Infectious Titer (TCID ₅₀ /mL)	Measured GC/ml	Ratio GC/TCID ₅₀
dsRNA	Bluetongue Virus	Serotype 2	2,811,707	86,500,000	30.76
dsRNA	Epizootic Hemorrhagic Disease Virus Type 2 (EHDV)	Alberta	316,228	503,900,000	1593.47
dsDNA	Human Herpesvirus 1	F	158,113,883	413,350,000	2.61
dsDNA	Human Herpesvirus 2	MS	15,811,388	1,133,625,000	71.70
dsDNA	Human herpesvirus 3 (Varicella-zoster virus)	Ellen	9,200	745,943	81.08
dsDNA	Human herpesvirus 4 (Epstein-Barr virus)	B95-8	>5000	2,800,045	560.01
dsDNA	Human Herpesvirus 5, CMV	AD-169	233,868	32,829,375	140.38
ssDNA	MMV, Rodent Protoparvovirus	Prototype (p)	15,811,388	93,714,600,000	5927.03
dsRNA	Mammalian Orthoreovirus 1 (Reo1)	Lang	158,113,883	435,000,000	2.75
dsRNA	Reovirus 2	D5 (Jones)	100,000,000	2,772,600,000	27.73
dsRNA	Reovirus 3	Dearing	280,000,000	28,770,000,000	102.75

- Ratio of GC/TCID₅₀ varies between different viral species.
- For many species, targeting nucleic acid provides a sensitivity advantage over infectivity based tests.
- Genome copy number based testing will be used to assess limit of detection of the assay.

Experimental Design:

- A panel of viruses were selected and genome copies measured by qPCR or dPCR
 - DNA Viruses: Ad5C, Ectromelia virus, HHV2, MMV, KRV, H-1 Parvovirus
 - RNA Viruses: hPIV3, Vesivirus 2117, Reo1, Reo2, Reo3
- The viral panel was spiked at a concentration of $\sim 10^4$ genome copies/mL per virus into a CHO bioreactor harvest sample containing 1.2×10^7 cells/mL. (total viral spike: 110,000 GC/mL)
- Nucleic acid was extracted and prepared for NGS analysis using either targeted or unguided approach

Results: Unguided NGS vs Targeted ViralSEQ NGS

Viral Targets	Unguided NGS (reads/M total reads)	Targeted NGS (reads/M total reads)
Adenovirus 5	-	+
Ectromelia Virus	+	+
H1 Parvovirus	-	+
Human Herpesvirus 2	+	+
Kilman Rat Virus	-	+
Mouse Minute Virus	-	+
Human Parainfluenza Virus 3	-	+
Reovirus 1	-	+
Reovirus 2	-	+
Reovirus 3	-	+
Vesivirus	-	+

- Only larger genome size dsDNA viruses : *Ectromelia* (209,771 bp) and HHV2 (164,576 bp) showed specific reads using unguided NGS
- Targeted NGS generated high number of reads per million total reads for all viral targets

Summary of Targeted vs “Shotgun” Sequencing Comparison Study

- All spiked viruses with different genome structures : dsDNA, ssDNA, ssRNA and dsRNA were confidently detected using the targeted NGS automated workflow.
- Only large genome size dsDNA viruses : Ectromelia (209,771 bp) and HHV2 (164,576 bp) showed some specific reads. Majority of the reads are short and low complexity. Only 9 reads over 90 bp can be confidently mapped to HHV2 (Herpesvirus group in NCBI) and 2 reads over 90 bp to Ectromelia.
- Unguided or “Shotgun” manual workflow used will require additional development to improve sensitivity and enable sensitive and confident detection of viruses with variety of genome structures.

Summary And Take-Home Message



Targeted NGS method offers simple and easy solution for detection of viral contaminants in CHO bioreactor samples



Viruses of all genomic structures—dsDNA, ssDNA, ssRNA, and dsRNA can be detected using same workflow with less than 2 hours hands-on time



Workflow controls are included for high confidence negative results



Thank you!

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