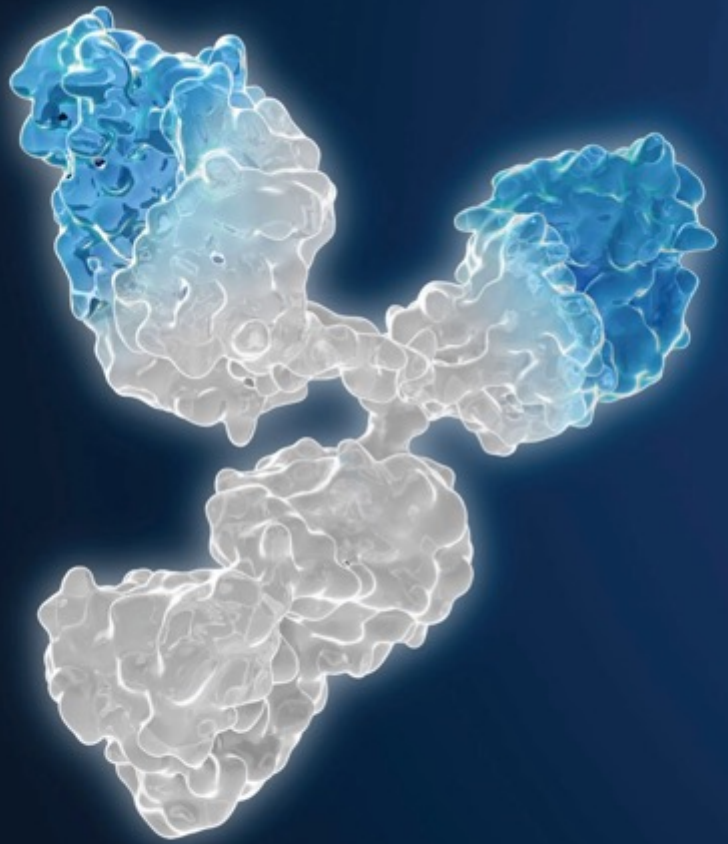

BACKGROUND NGS DATA FOR SAMPLE MATRIX AND REFERENCE CONTROL STANDARDS USING NANOPORE SEQUENCING

NIST CONFERENCE SEPTEMBER 18TH AND 19TH 2019

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SCIENCE TO MEDICINE®

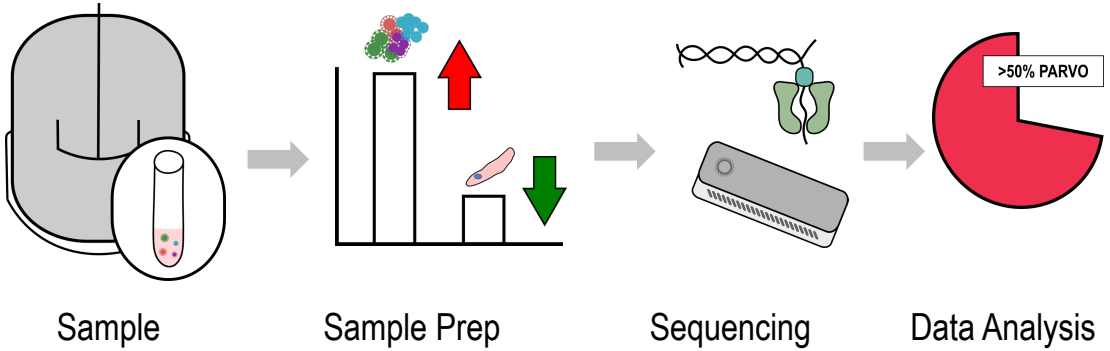
CURRENT USE OF NGS

- **Testing Requirement for MAb Lot Release**
  - In Vitro Virus Screening assay (14 Day)
  - Mycoplasma Screening (28 Day)
  - PCR Rodent Parvovirus (for known contaminant not optimally detected during In Vitro Virus Assay)

- **NGS is currently used:**

As Investigational Tool	For Adventitious Virus Screening Method Development
- OOS investigation during adventitious virus testing - Abnormal Bioreactor Events (cell crash) investigation	- Direct NGS Method development - Hybrid Method (Culture + NGS) development

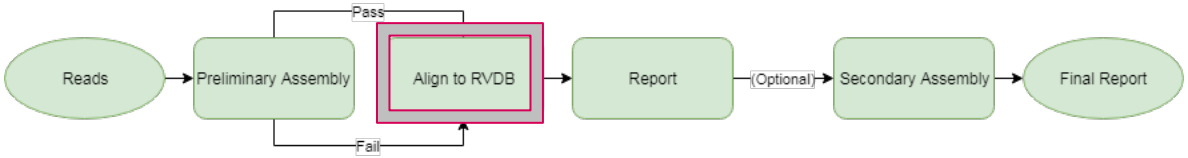
SEQUENCING TECHNOLOGY USED



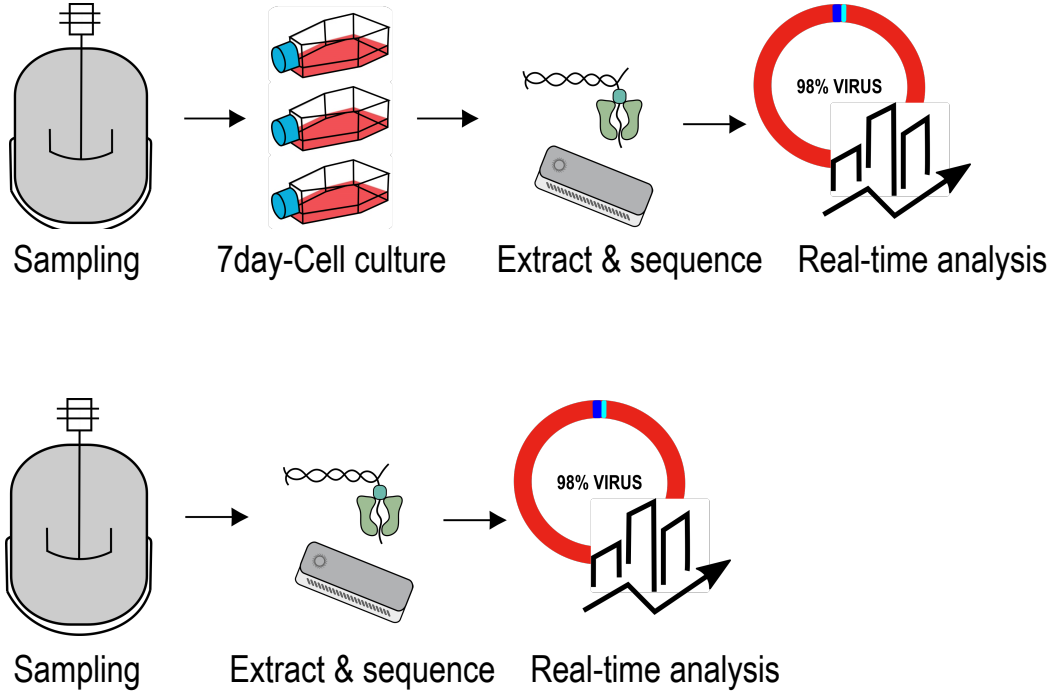
Oxford Nanopore MinION

- Direct DNA sequencing (no amplification)
- Long reads technology
- Real time streaming of sequence data

Data Analysis Workflow



DATA ANALYSIS CHALLENGES



Challenges:

- Nucleic acids in the bioreactor
- Nucleic acid or dead virus in Cell culture reagents e.g. FBS (even gamma irradiated)
- Nucleic acids or virus from indicator cells
- Nucleic acids from nucleic acid purification reagents
- Nucleic acids from sequencing reagents
- Nucleic acids from the internal controls

Generate **background signal** to be distinguished from the real contaminant

NGS BACKGROUND EXAMINATION THROUGH 3 STUDIES

1. CELL LINES & PBS 
   - CHO-K1
   - MRC-5
   - VERO
   - PBS
   - Water

2. FBS 
   - Nuclease-treated, enriched
   - Enriched
   - Unenriched

3. SPIKED ASSAY CONTROLS
   - M13
   - MS2 
   - In host background at 1E7 pfu/µl

→ **Sample Prep** → **Nanopore Sequencing** → **Align to RVDB**

STUDY-1: NGS BACKGROUND IN PBS, WATER, AND CELL LINES CHO, VERO, MRC-5

1. CELL LINES & PBS 
   - CHO-K1
   - MRC-5
   - VERO
   - PBS
   - Water

2. FBS 
   - Nuclease-treated, enriched
   - Enriched
   - Unenriched

3. SPIKED ASSAY CONTROLS 
   - M13
   - MS2
   - In host background at 1E7 pfu/µl

Sample Prep → **Nanopore Sequencing** → **Align to RVDB**

STUDY-1: NGS BACKGROUND IN PBS AND WATER

PBS			
Findings	Reads	Average Length	Coverage %
Total Reads	7,131		
Unaligned	5,379 (75%)		
CHO	1,190 (17%)		
Human	407 (6%)		
SV40	129 (2%)	379	81%
Semliki_forest_virus	1 (~0%)	424	2%
Guanarito_mammarenavirus	3 (~0%)	391	6%

PBS			
Findings	Reads	Average Length	Coverage %
Hum Picobirnavirus	1 (~0%)	485	11%

Water			
Findings	Reads	Average Length	Coverage %
Total Reads	1,474		
Unaligned	1,473 (~100%)		
Human	1 (~0%)	245	

STUDY-1: NGS BACKGROUND IN INDICATOR CELL LINE CHO-K1

CHO-K1			
Findings	Reads	Average Length	Coverage %
Total Reads	3,173,164		
Unaligned	497,674 (17%)		
CHO	2,675,490 (84%)	524	
Human	30,766 (1%)	508	
Guanarito_mammarenavirus	18 (~0%)	887	11%
White_spot_syndrome_virus	6 (~0%)	637	<1%
RVLP	708 (~0%)	870	95%

CHO-K1			
Findings	Reads	Average Length	Coverage %
BVDV	1 (~0%)	1943	15%
Bosavirus	97 (~0%)	786	92%

STUDY-1: NGS BACKGROUND IN INDICATOR CELL LINE VERO

VERO			
Findings	Reads	Average Length	Coverage %
Total Reads	3,130,681		
Unaligned	1,151,406 (37%)		
CHO	1,170 (~0%)	634	
Human	1,193,298 (38%)	1,090	
Guanarito_mammarenavirus	206 (~0%)	504	13%
White_spot_syndrome_virus	4 (~0%)	250	<1%
Endogenous retrovirus	2,598 (~0%)		

VERO			
Findings	Reads	Average Length	Coverage %
BVDV	3 (~0%)	1453	54%
Bosavirus	98 (~0%)	832	92%
SV40	725,093	671	90%
Shamonda	1 (~0%)	273	6%

STUDY-1: NGS BACKGROUND IN INDICATOR CELL LINE MRC-5

MRC-5			
Findings	Reads	Average Length	Coverage %
Total Reads	527,568		
Unaligned	130,000 (25%)		
CHO	218 (~0%)	264	
Human	396,643 (75%)	405	
Guanarito_mammarenavirus	177 (~0%)	470	11%
White_spot_syndrome_virus	6 (~0%)	194	<1%

MRC-5			
Findings	Reads	Average Length	Coverage %
BVDV	1 (~0%)	666	6%
Bosavirus	457 (~0%)	492	93%
Hum Hep B	6 (~0%)	281	66%

STUDY-1: SUMMARY

Findings	Reads	Coverage
Unaligned	Expect 10-40% of reads unalligned	
Indicator Cells (host)	Expect 40-85% of reads	
Guanarito_mammarenavirus	[3-206]	[6%-13%]
White_spot_syndrome_virus	[4-6]	<1%
SV40	[129-725,093]	[80%-90%]
Hum Picobirnavirus	1 read	11%
RVLPs	708	95%
Semliki_forest_virus	1	2%

Findings	Reads	Coverage
BVDV	[1-3]	[6%-54%]
Bosavirus	[97-457]	[92%-93%]
Hum Hep B	6	66%
Endogenous retrovirus	2,598	
Shamonda	1	6%

Example of acceptance criteria:
>10Reads and >60%Coverage

- Real Contaminants

-Artifacts

STUDY-2

1. CELL LINES & PBS 
   - CHO-K1
   - MRC-5
   - VERO
   - PBS
   - Water

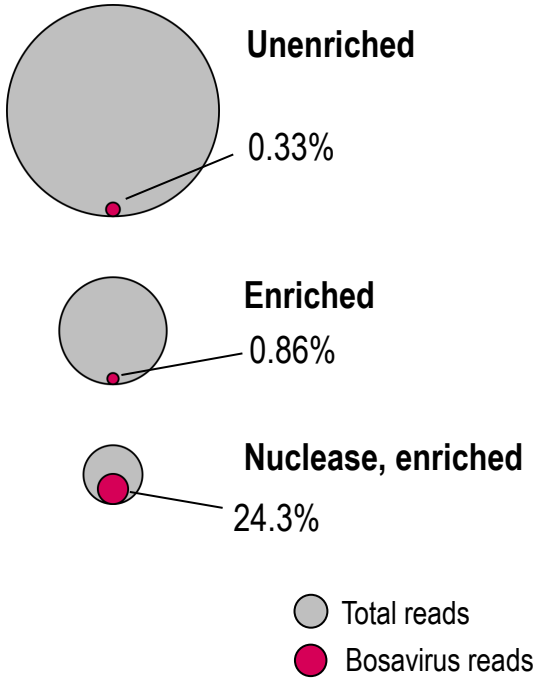
2. FBS 
   - Nuclease-treated, enriched
   - Enriched
   - Unenriched

3. SPIKED ASSAY CONTROLS
   - M13 
   - MS2
   - In host background at 1E7 pfu/µl

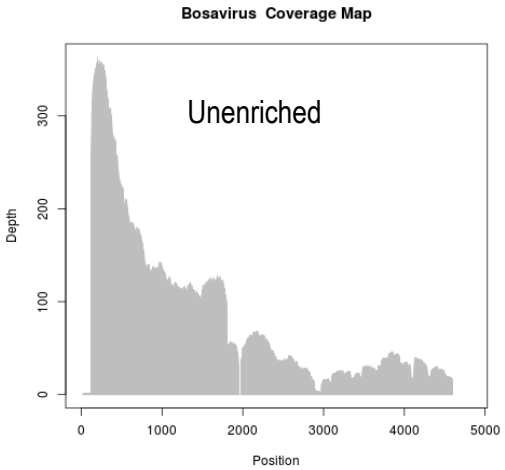
→ **Sample Prep** → **Nanopore Sequencing** → **Align to RVDB**

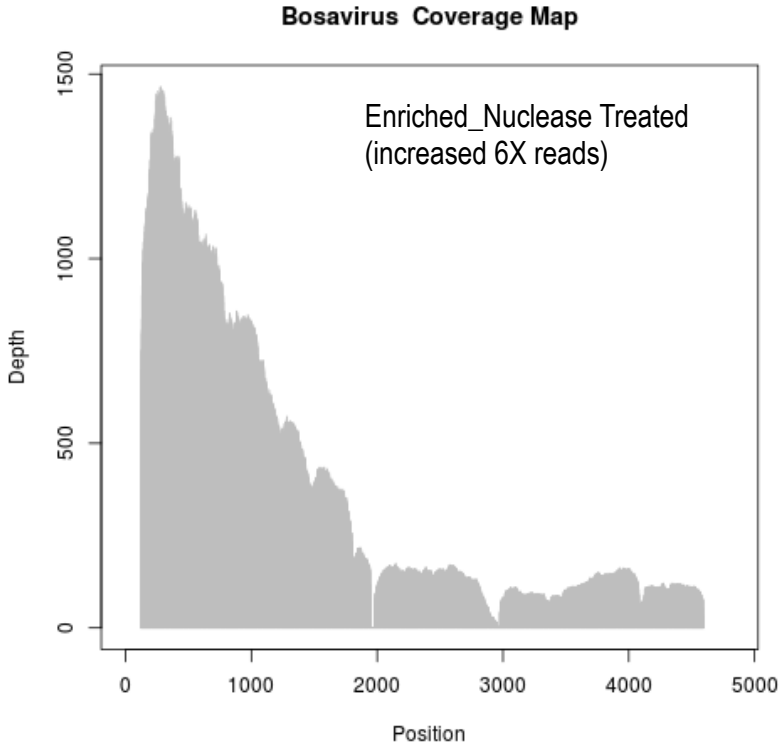
STUDY-2: FBS (GAMMA IRRADIATED)

	Unenriched			Enriched			Enriched_Nuclease Treated		
	Reads	Average Length	Coverage %	Reads	Average Length	Coverage %	Reads	Average Length	Coverage %
Host	82,877			19,471			4,868		
Endogenous elements/provirus	4	419	87.74	1	755	73.57	6	304	5.36
Bosavirus	1087	528	94.15	707	894	92.62	6090	389	92.62
BVDV	4	379	9.68	1	1568	13.01	7	439	15.32
Stealth Virus 1	34	707	67.82	7	1008	47.07	29	580	60.33
% (Bosavirus/Total Reads)	0.33			0.87			24.39		
% (Stealth Virus 1/Total Reads)	0.01			0.01			0.12		



STUDY-2 BOSAVIRUS COVERAGE MAP





STUDY-2 SUMMARY

Considering our criteria of :
>10Reads and >60%Coverage = Real Contaminant

Gamma Irradiated FBS				
	Findings	Reads	Average Length	Coverage
Real Contaminants	Bosavirus	6090	389	93%
	Stealth Virus 1	29	580	60%
Artifacts	BVDV	7	439	15%
	Endogenous retrovirus elements	6	304	5%

STUDY-3

1. CELL LINES & PBS 
   - CHO-K1
   - MRC-5
   - VERO
   - PBS
   - Water

2. FBS 
   - Nuclease-treated, enriched
   - Enriched
   - Unenriched

3. SPIKED ASSAY CONTROLS
   - M13 
   - MS2
   - In host background at 1E7 pfu/µl

→ **Sample Prep** → **Nanopore Sequencing** → **Align to RVDB**

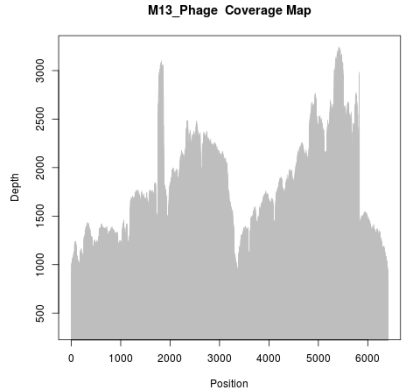
STUDY-3: INTERNAL CONTROL PHAGES ADDED TO CHO-K1 SAMPLES AND PBS

	Sample (n=3)			PBS Control		
	Reads	AVG length	% coverage	Reads	AVG length	% coverage
M13	22,879	394	100	9,926	397	100
MS2	3,533	449	97	9,128	514	99.66
CHO-K1	1,207	337		1	378	
Human	315	376		83	370	
Semliki Forest Virus	2,203	498	8	980	537	7.79
Hamster Retroviral sequence	3	637	77	0		
CHO RVLP	5	621	28	0		
Macacine betaherpesbvirus 3	2	440	<1	1	375	0.13
Stealth_virus_1_clone_3B43	10	684	45	5	623	43.95
Stealth_virus_1_clone_3B43_T3.	4	366	42	5	623	43.95
Guanarito mammarenavirus isolate	1	520	<1	0		
Herpes simplex virus (type 1 /strain RH2)	5	363	<1	4	363	0.71
Total Sequenced Reads	52,332			33,146		
Unaligned reads	22,144			13,015		

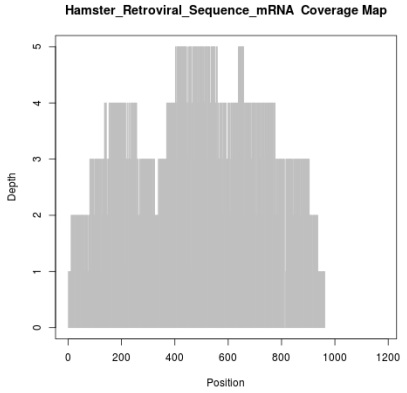
Reads as a percentage of total reads	**Spiked Sample**	**PBS**
M13	43.72%	29.95%
MS2	6.75%	27.54%
CHO-K1	2.31%	0.00%
Human	0.60%	0.25%
Semliki Forest Virus	4.21%	2.96%

STUDY-3: READS COVERAGE MAPS

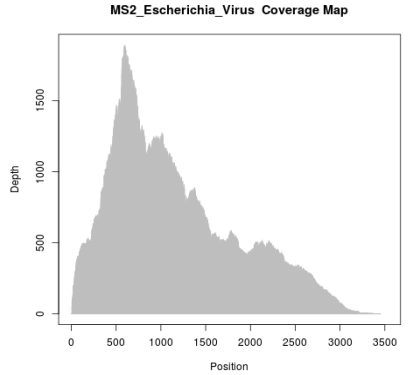
M13 6,407 bp



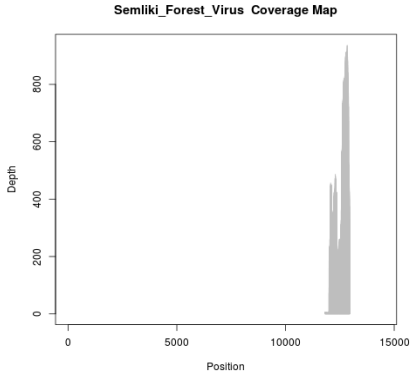
Retrovirus 1,184bp



MS2 3,534 bp



Semliki Forest Virus 14,529bp



STUDY-3 SUMMARY

Considering our criteria of :
>10Reads and >60%Coverage = Real Contaminant

- Recovery of the internal control phages
- No real contaminant was noted
- New artifacts such as Macacine betaherpesbvirus 3 and Herpes simplex virus (type 1 /strain RH2)
- MS2 recovery seems impacted by the sample matrix
- M13 is not impacted
- High reads very low coverage for Semliki Forest Virus
- Unaligned reads is between 39% to 42% of total reads (suspecting host of the phages)

CONCLUSIONS AND FUTURE DIRECTIONS

- Identified real contaminants of our reagents
  - SV40
  - Bosavirus
  - RVLPs
  - Simian Endogenous retrovirus elements
  - Stealth Virus 1
- Identified several artifacts and the most prominent being
  - BVDV
  - Semliki_forest_virus
- These findings are first steps in determining criteria for real contaminants vs. artifacts

Future Directions:

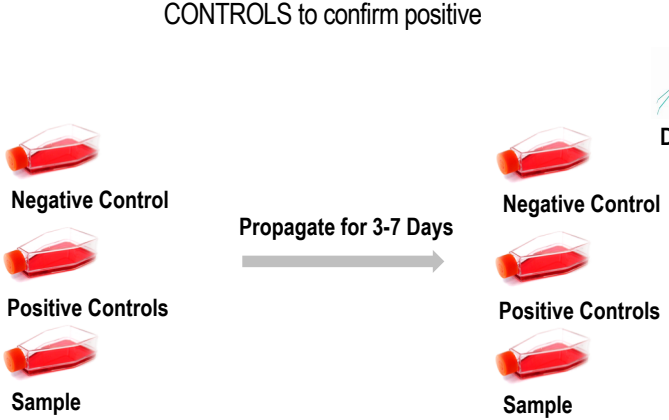
- Continue to investigate and gather sequencing data from all reagents and materials used in NGS assays.
- Optimize the data analysis with machine Learning to characterize background and artifacts
- Based on current LOD of MinION, virus amplification on cell culture will be considered

FUTURE DIRECTION: HYBRID ASSAY (CULTURE + MINION)

- **Assay system suitability**
  - Analyze NEGATIVE CONTROL to determine if different from expected background.
  - NEGATIVE CONTROL reads SUBTRACTED from POSITIVE CONTROLS to confirm positive

- **Sample Suitability**
  - Analyze INTERNAL CONTROLS spiked into all samples to determine if recovery acceptable

- **Sample results**
- Analysis includes:
  - Review of historical background (AI potentially)
  - NEGATIVE CONTROL read analysis subtracted
  - Internal Controls SUBTRACTED from SAMPLE reads
  - Remaining reads are ALIGNED to Viral database



QUESTIONS?