QS3D DIGITAL PCR SYSTEM FOR MICROBIAL SOURCE TRACKING

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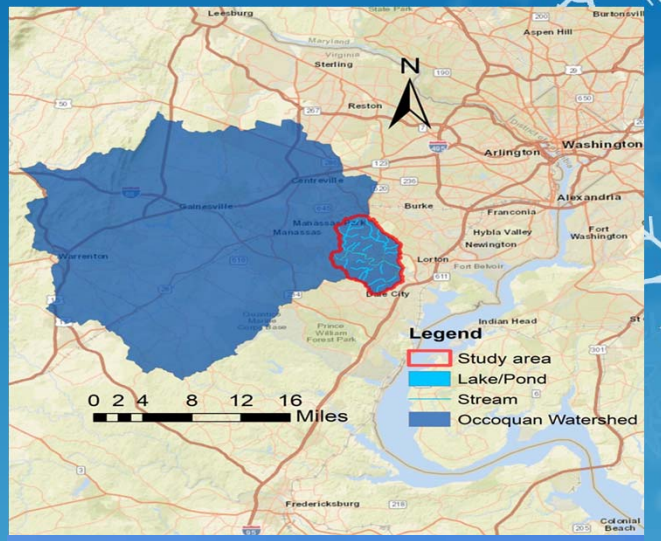
Occoquan Laboratory

- Located at Washington DC capital region
- Virginia Chapter 46 Accreditation: Commercial Environmental Laboratory

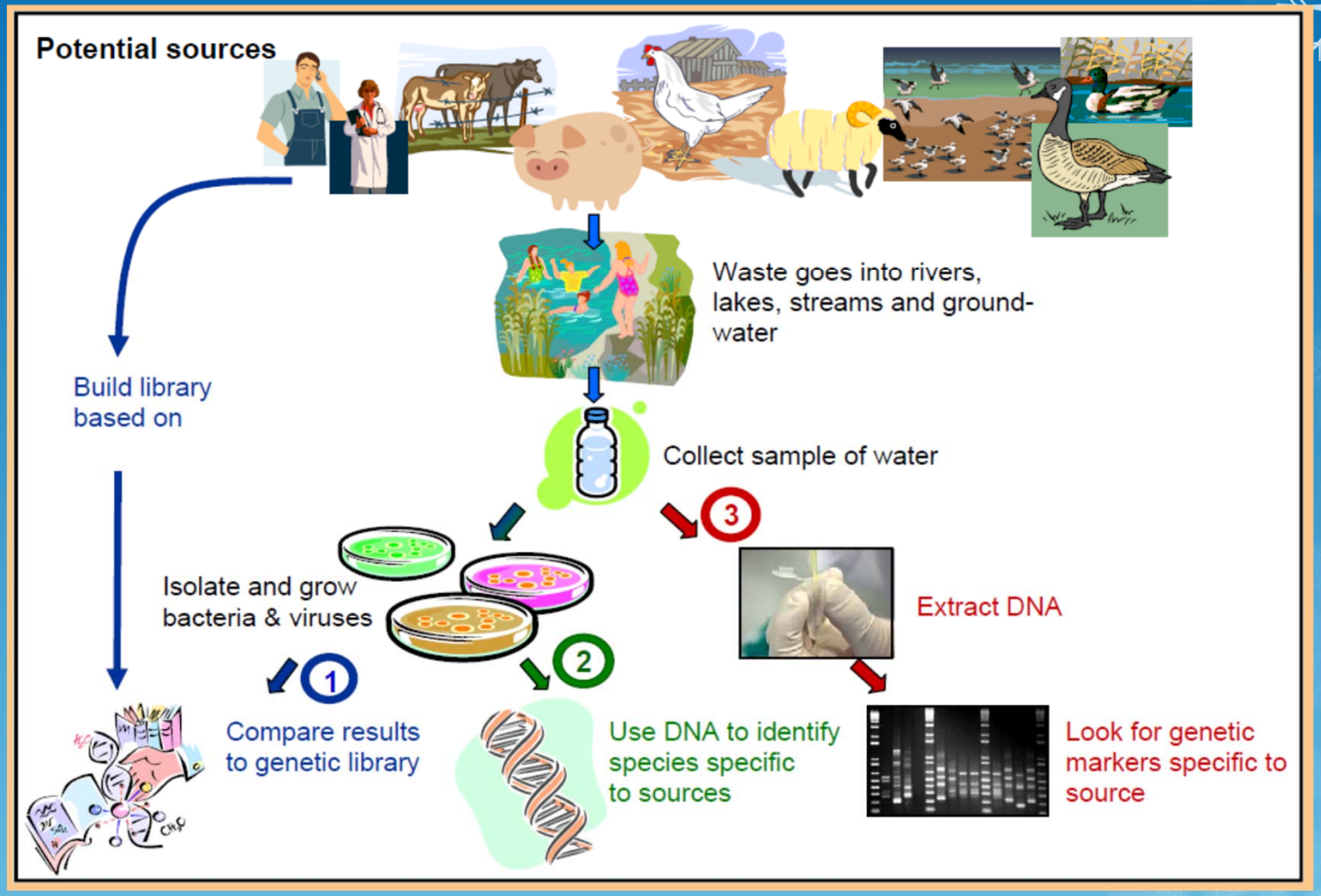


Presentation Overview

- Introduce microbial source tracking (MST)
- Digital PCR (dPCR)
- Quality control and method optimization
- Monitoring results

Background

- Series of testing to differentiate sources (such as human, domestic animals, or wildlife) of fecal coliforms in waterbody
- Based on assumption that fecal bacteria from a particular host poses its unique characteristics
- Target genes can be “fingerprinted” or tied to a particular type of mammal, human or bird
- Support bacteria TMDL development and implementation
- Identify sources and implement target controls

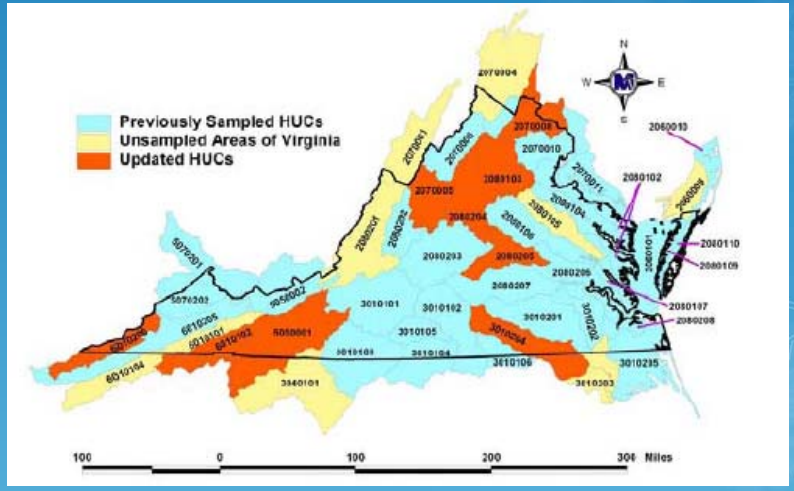


Source: Pathogen Workshop Series Fact Sheet #4. http://www.cws.msu.edu

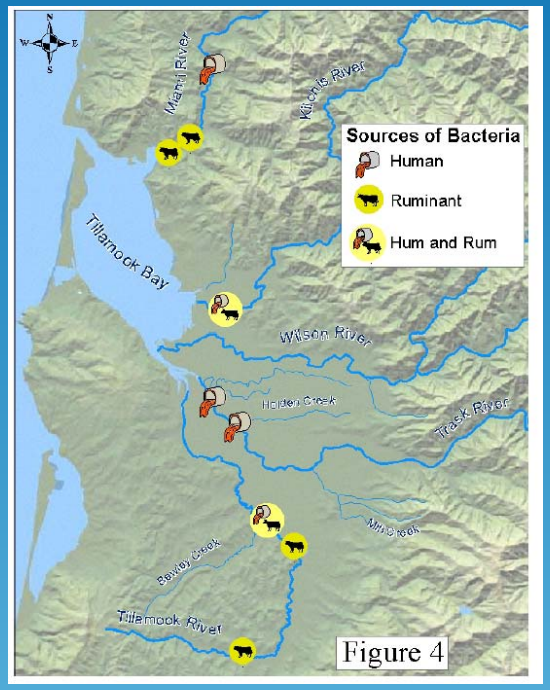
Library dependent		Library independent	
Molecular (genotypic)	Biochemical (phenotypic)	Molecular or biochemical (genotypic or phenotypic)	Molecular (genotypic)
Rep-PCR • **REP primer pair** • **Box A1R primer** **Ribotyping** • **HindIII restriction enzyme** • **EcoR1 and PvuII restriction enzymes** **PFGE**	ARA and MAR CUP and NUP Cell wall fatty acid methylester (FAME) F-specific coliphage typing	Bacteriophage F+RNA	Host-specific bacterial PCR Host-specific viral PCR Host-specific quantitative PCR Host-specific digital PCR
Cultural dependent			Cultural independent

Case Studies

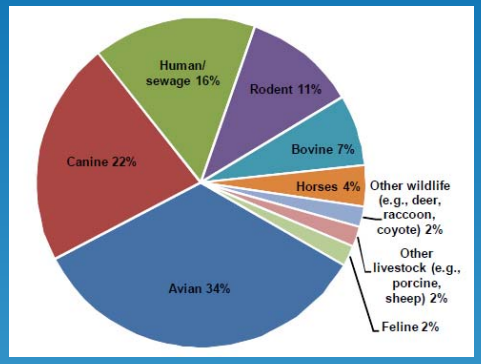
- Virginia DEQ began using MST to identify fecal bacteria sources in the 1990s and has since implemented a statewide MST program to support TMDL development
- Page Brook Watershed: average of 94% fecal coliform reduction (cattle) from pre-fencing to post-fencing (Hagedorn et al. 1999)

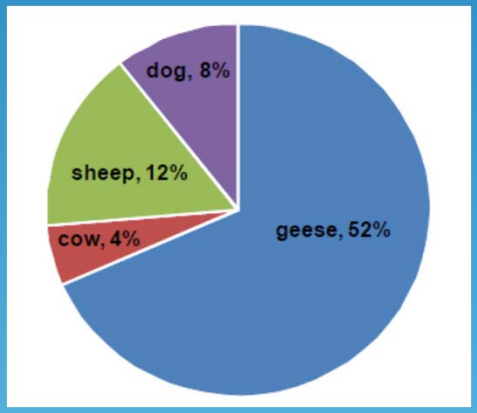


Source: Using Microbial Source Tracking to Support TMDL Development and Implementation (2011)



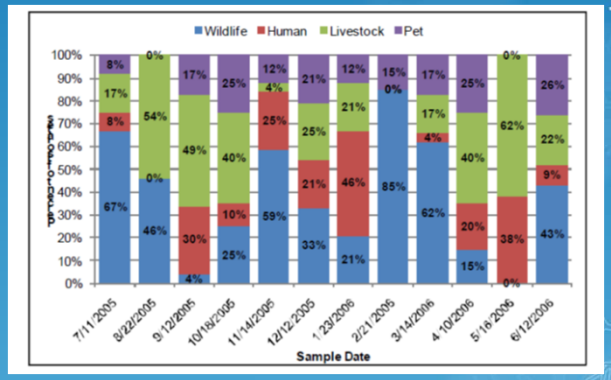
Tillamook Bay
(2001)





Sand Dam Village
Pond Town Beach,
New Hampshire
(2005)

Middle Rio Grande (MRG),
New Mexico (2002)

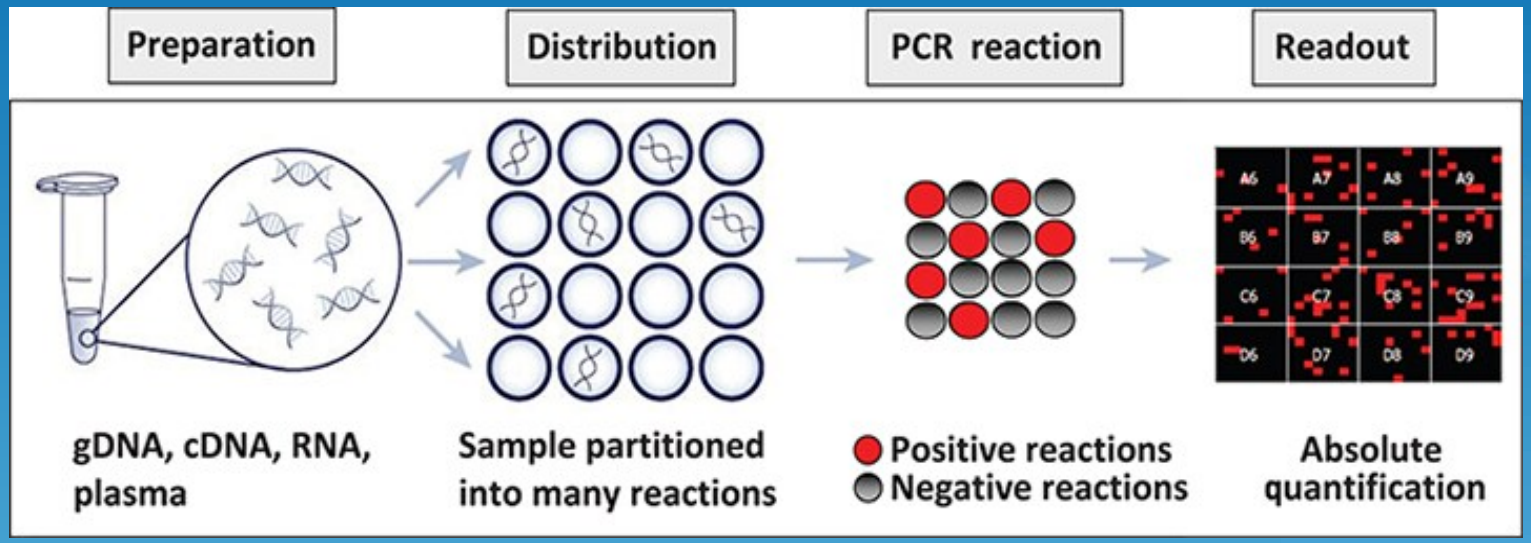


Hays Creek, Virginia
(2005)

Standardized Methods

- Method 1696: Characterization of Human Fecal Pollution in Water by HF183/BacR287 TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay. March 2019
- Method 1697: Characterization of Human Fecal Pollution in Water by HumM2 TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay® March 2019
- Method B: Bacteroidales in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay. June 2010
- Draft Method C: *Escherichia coli* qPCR method

Digital PCR Basics

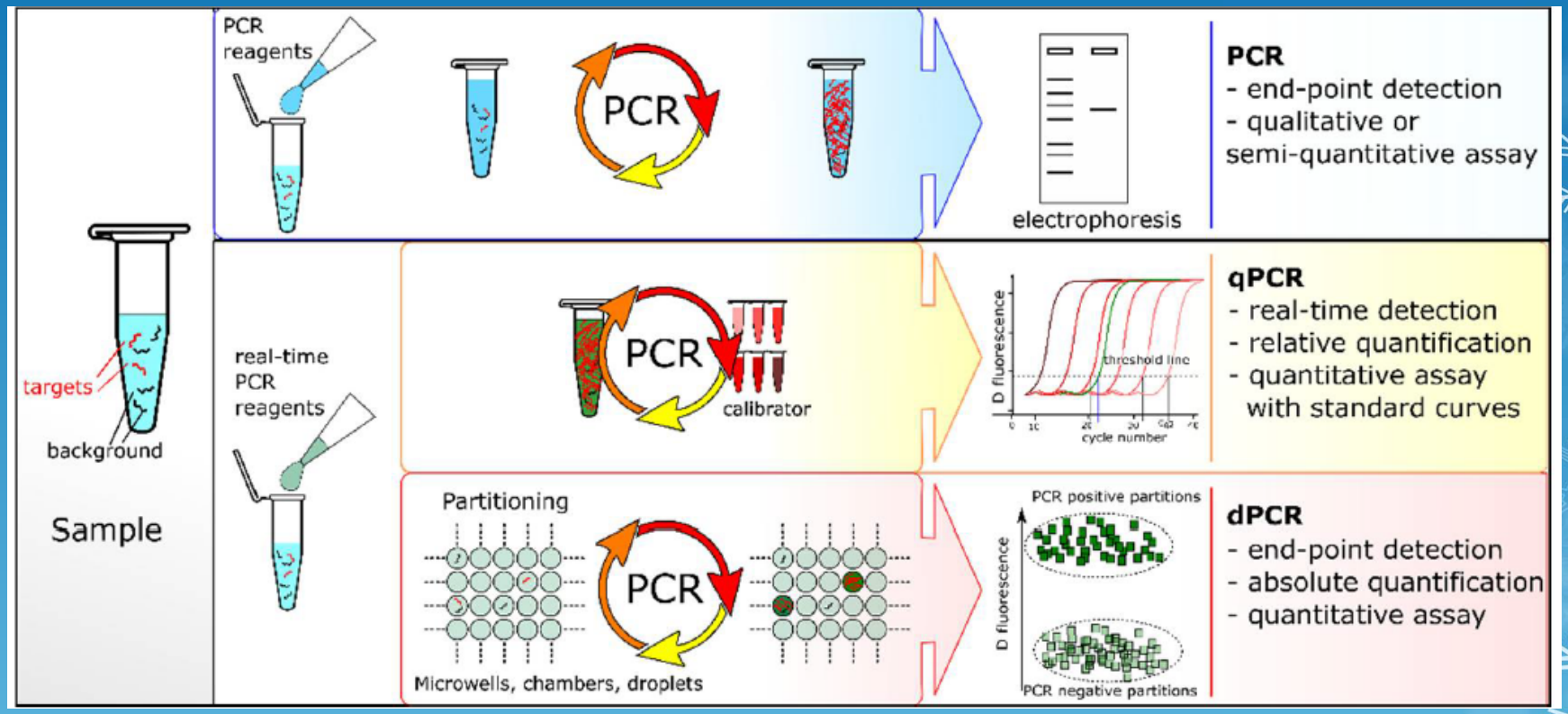


- absolute quantification
- partition of target genes
- Poisson distribution

Comparison with conventional-PCR

	Digital PCR	Real-time PCR	Traditional PCR
Overview	Measures the fraction of negative replicates to determine absolute copies.	Measures PCR amplification as it occurs.	Measures the amount of accumulated PCR product at the end of the PCR cycles.
Quantitative?	Yes, the fraction of negative PCR reactions is fit to a Poisson statistical algorithm.	Yes, because data is collected during the exponential growth (log) phase of PCR when the quantity of the PCR product is directly proportional to the amount of template nucleic acid.	No, though comparing the intensity of the amplified band on a gel to standards of a known concentration can give you 'semi-quantitative' results.

Source: https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center/real-time-pcr-basics/real-time-vs-digital-vs-traditional-pcr.html

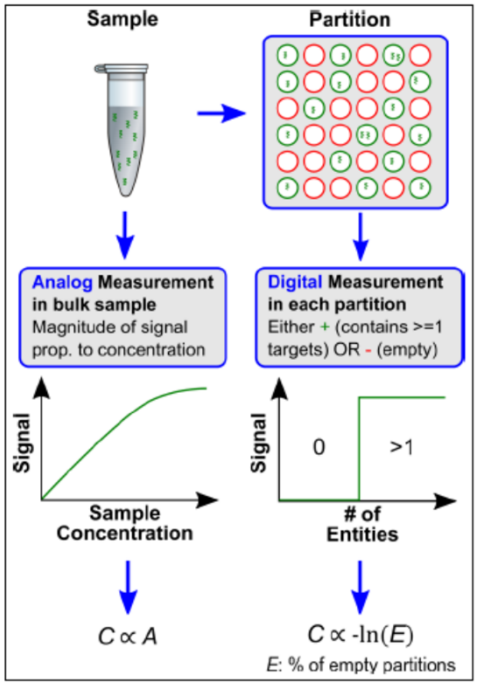


Source: Phenix-Lan Quan , Martin Sauzade, and Eric Brouzes. dPCR: A Technology Review. Sensors 2018, 18, 1271

Advantages of dPCR

- no known standards needed
- increases the ratio of the target of interest versus the background
- binomial probability well translated by using Poisson distribution
- improved sensitivity and reproducibility
- enhanced inhibitor resistance
- ease of use

Workflow of QS3D dPCR

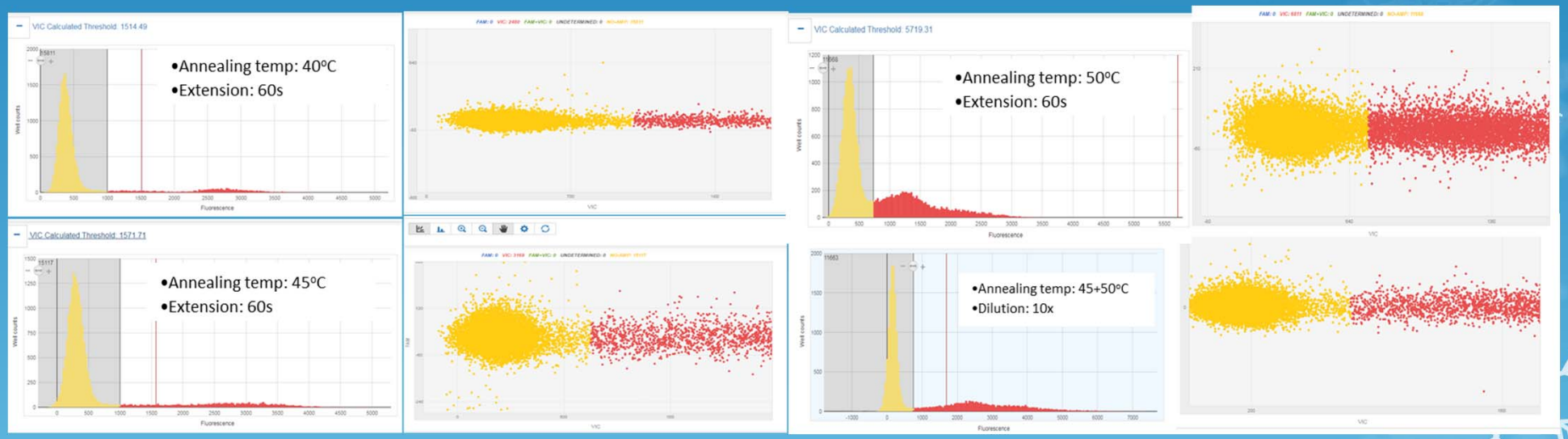


Primer sets

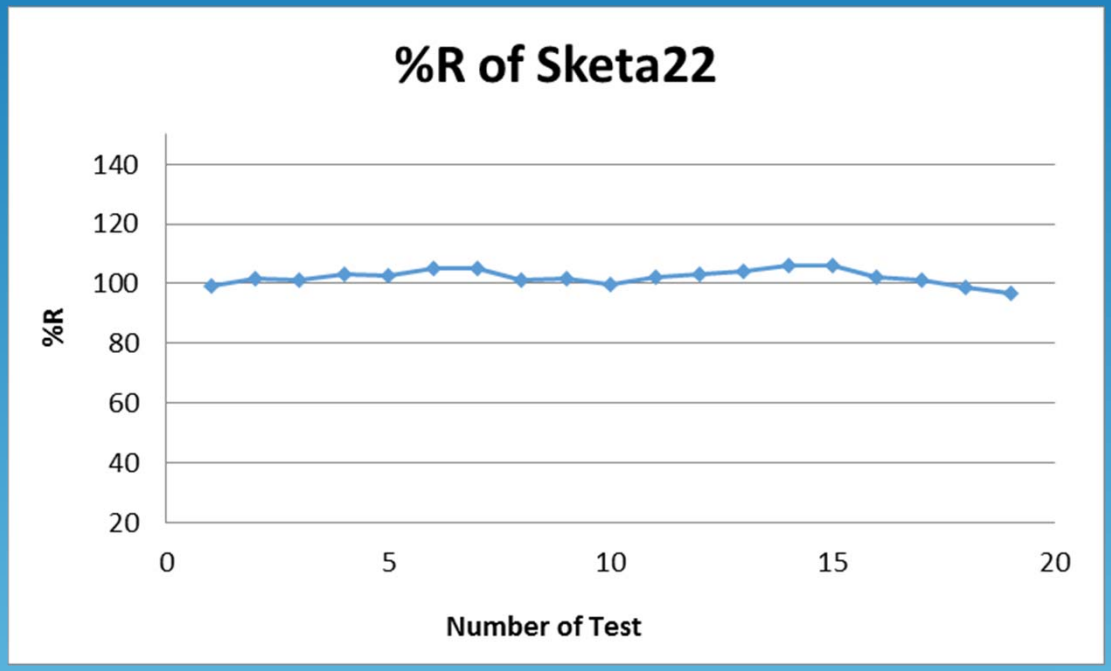
Target	Assays	Sequence	Annealing Temperature (°C)
Human-associated Bacteroides 16S rRNA	BacH	Forward Primer: 5'CTTGGCCAGCCTTCTGAAAG3' Reverse Primer 5' to 3': 5'CCCCATCGTCTACCGAAAATAC3' Probe: 5'TCATGATCCCATCCTG3'	two step 45 and 50
Ruminant: cows, buffalo, sheep, deer, goat, elk	BacR	Forward primer: GCGTATCCAACCTTCCCG Reverse Primer 5' to 3': CATCCCCATCCGTTACCG Probe: CTTCCGAAAGGGAGATT	two step 45 and 50

Assay Optimization

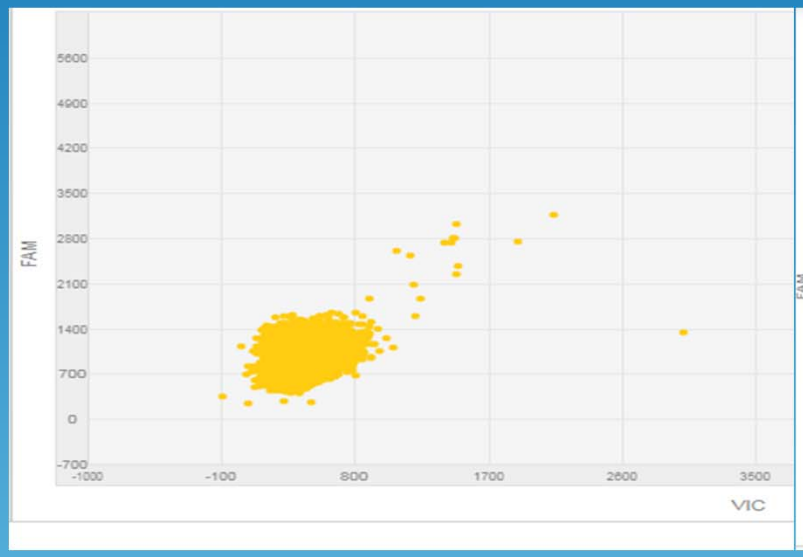
- Annealing temperature
- Amount of DNA, primers, and probe in assay
- Dilutions

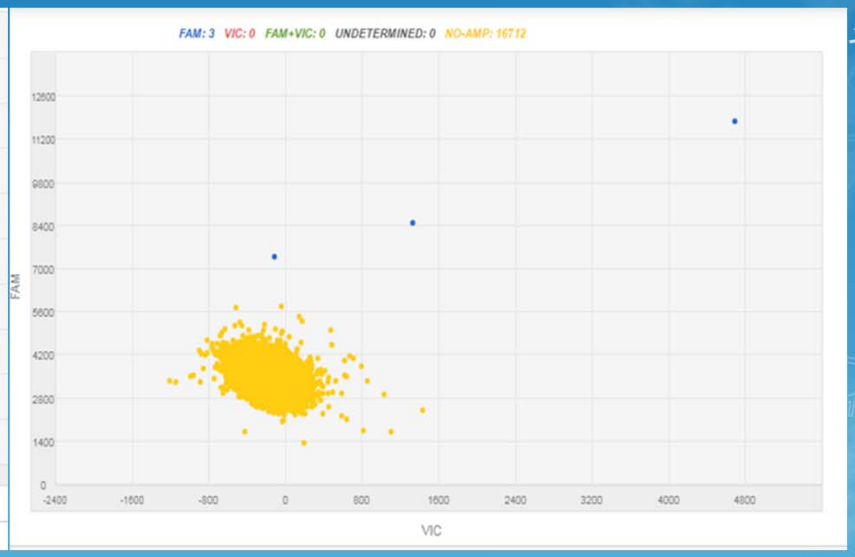


Sample Processing Control (SPC): Sketa22



Laboratory blank and non-template control

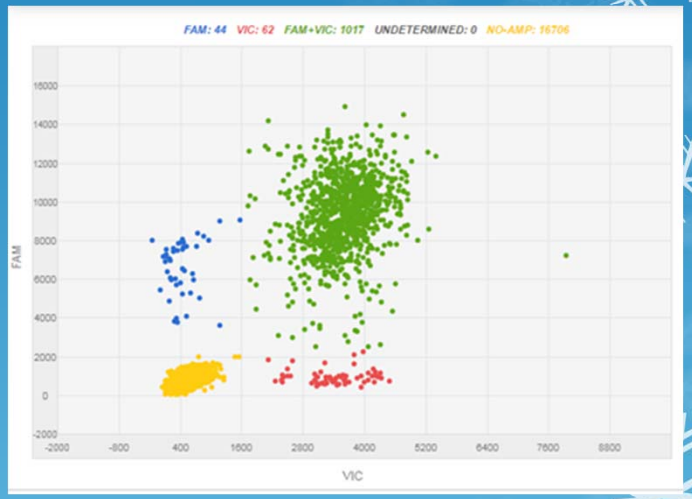




Sensitivity: detect single gene copy, relative percentage difference (RPD)<10%

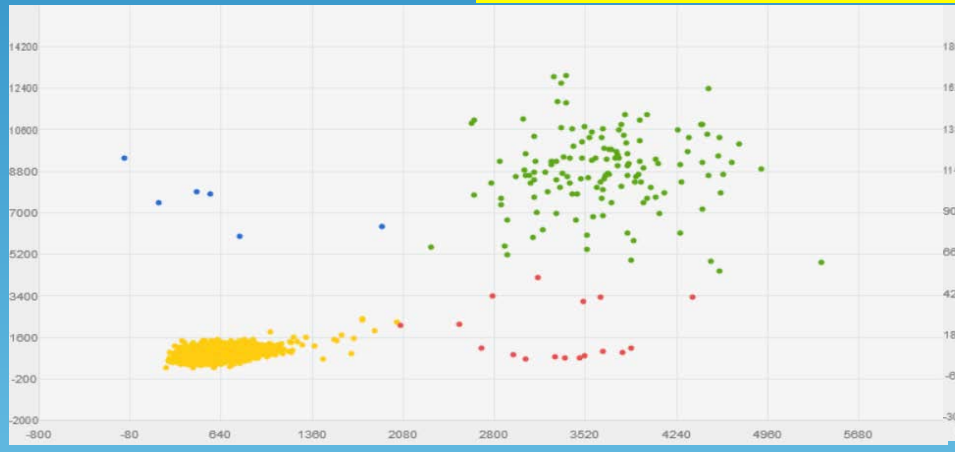
- Four replicates of diluted positive control

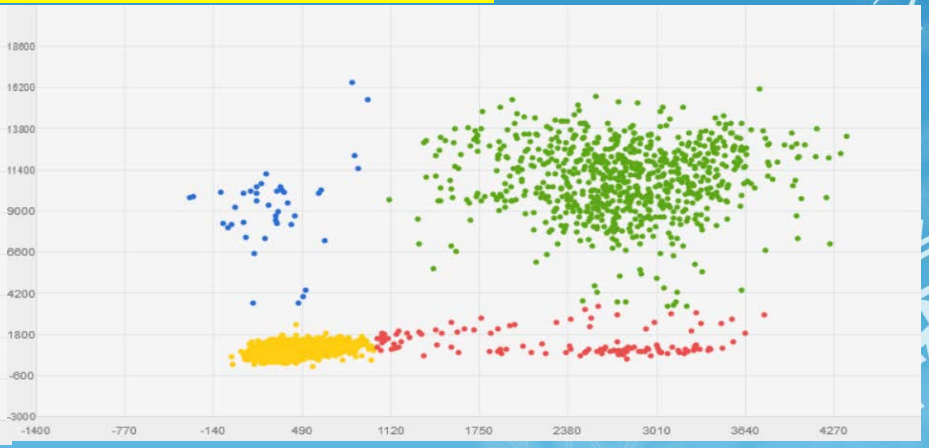
  at <10 copies/µL
- Standard deviation < 1.0 copies/µL
- Relative percent difference (RPD) <10%



Spike Raw Sewage

	copies/uL	spiked	%R
BacH	10.2	62.5	105
	10.7	67.8	114
BacR	12.8	68.9	112
	11.5	69.5	116

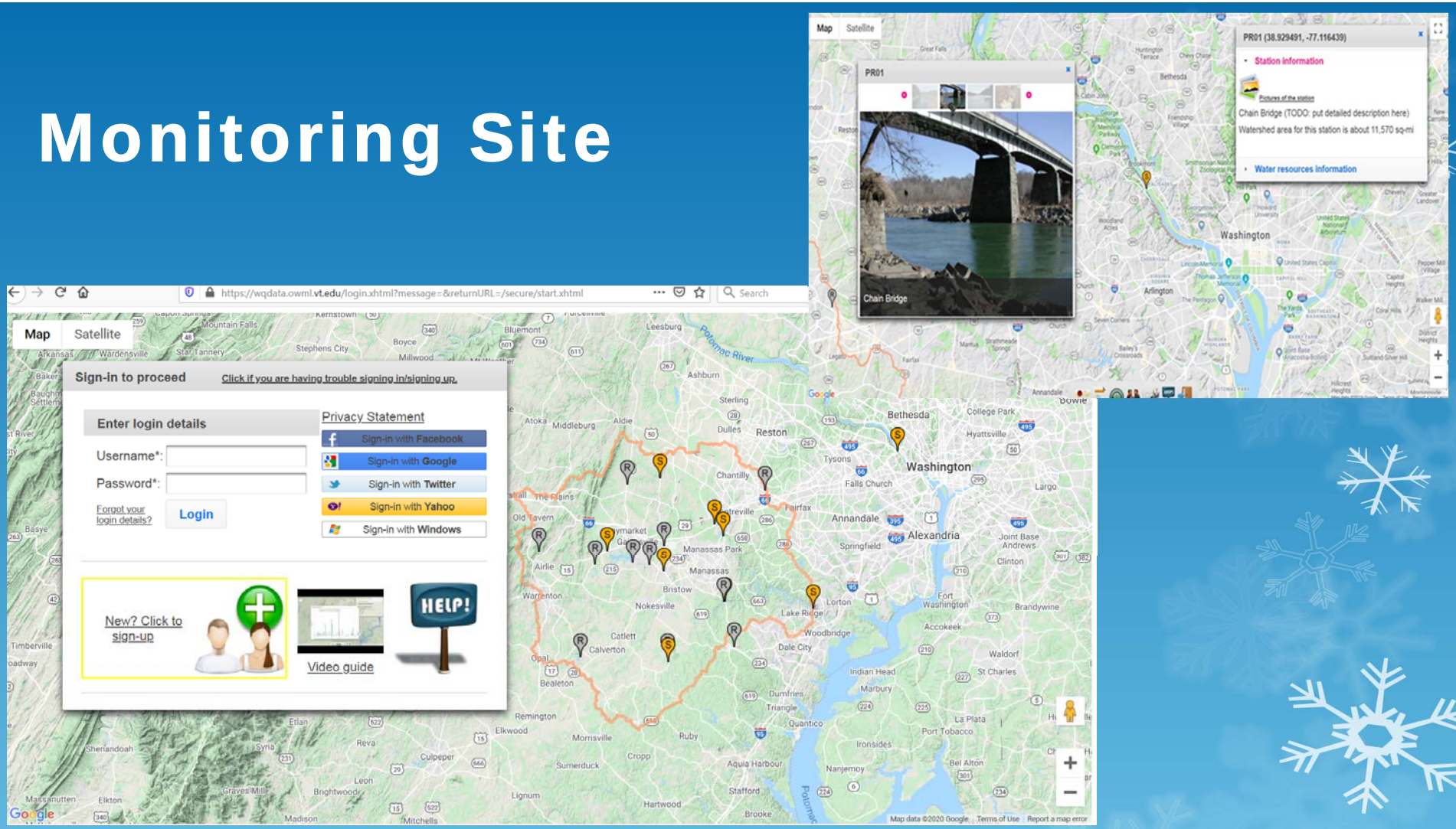




Inhibition Resistance

Dilutions	Chip reading, copies/uL	Scale up, copies/uL	Average	R, %
3X	2019	6057	6307	96
5X	1383	6915		110
10X	595	5950		94

Monitoring Site

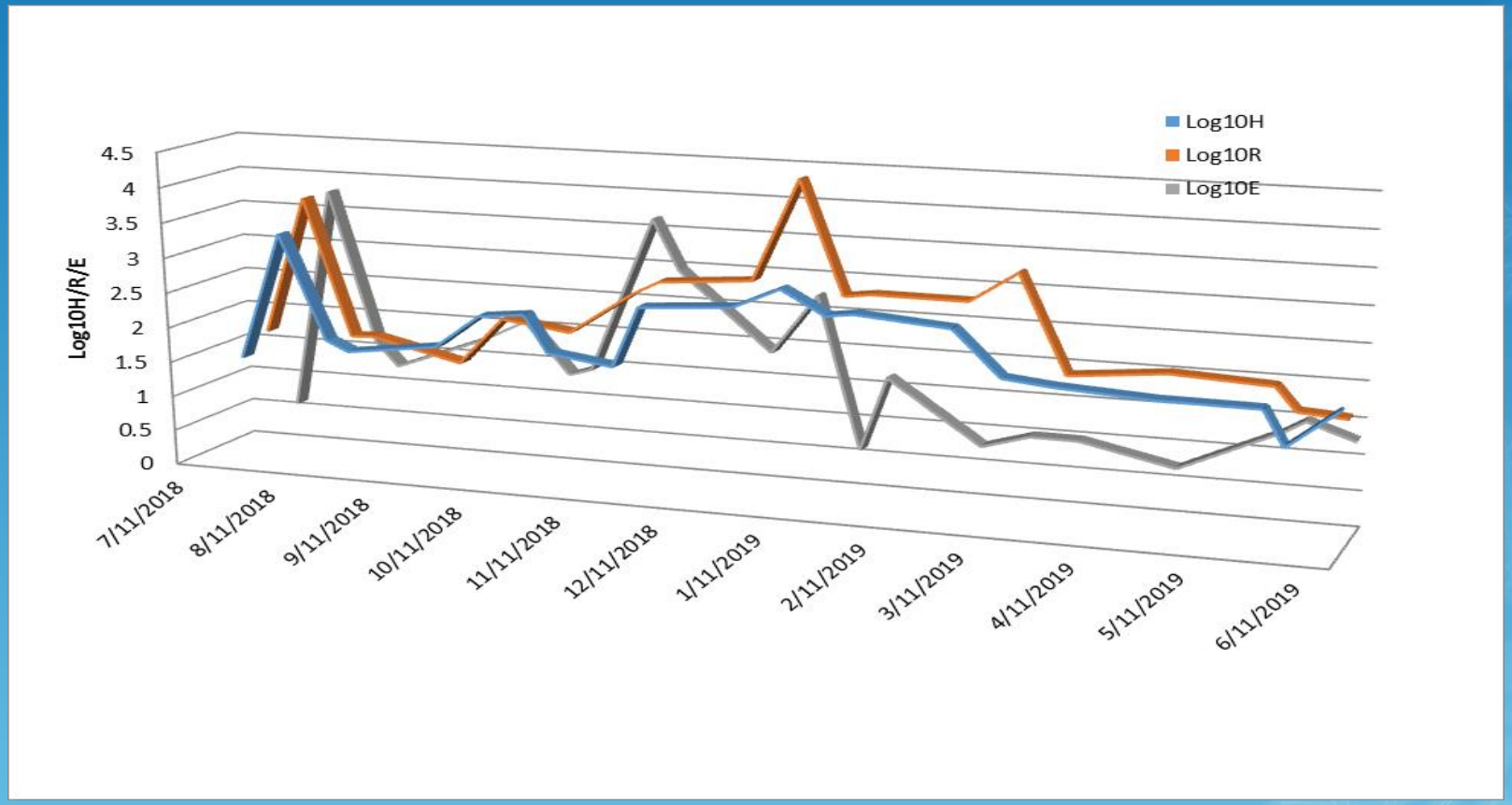


Monitoring Data

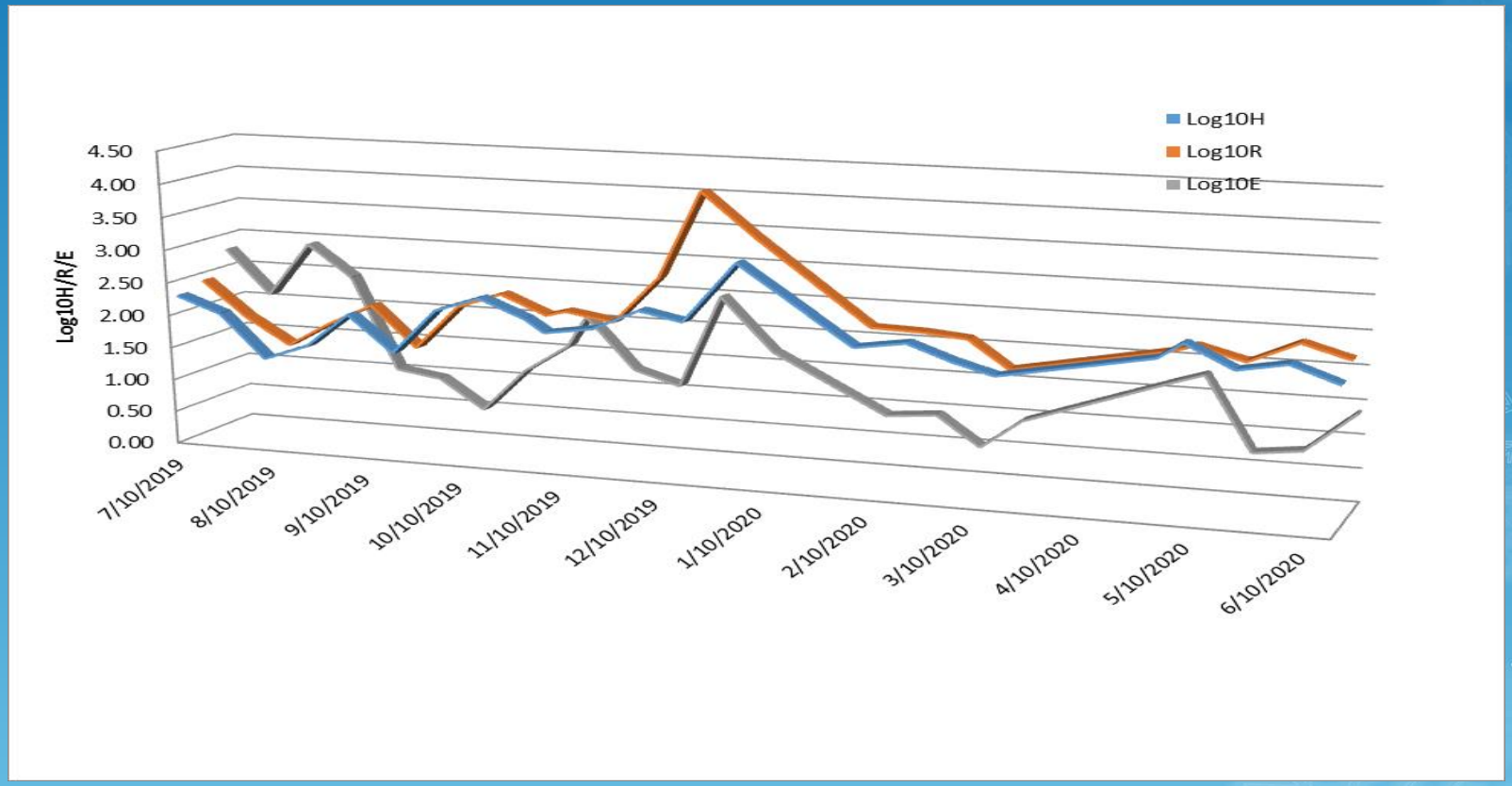
Date	HumanSource copies/100mL	RuminantSource copies/100mL	E.coli mpn/100mL
7/11/2018	39.9	69.7	4.1
8/1/2018	2537	6287	6130
8/15/2018	86.9	72.4	42.2
8/29/2018	63	75	19.7
9/5/2018	92	39	59.1
10/3/2018	273	182	122
10/17/2018	320	153	23.1
10/31/2018	98	138	29.2
11/7/2018	74	561	4610
11/27/2018	522	859	921
12/5/2018	638	1083	79.4
1/2/2019	1239	27977	548
1/16/2019	592	802	4.1
1/29/2019	661	895	45.7
2/6/2019	524	884	6.3
3/6/2019	128	2350	9.8
3/20/2019	110	110	9.7
4/3/2019	93	147	5.2
5/1/2019	91	126	21.3
5/30/2019	30	61	31.3
6/5/2019	100	55	18.7
6/19/2019	51	40	25.9

Date	HumanSource copies/100mL	RuminantSource copies/100mL	E.coli mpn/100mL
7/10/2019	186	244	579
7/24/2019	108	72	122
8/7/2019	24	29	816
8/21/2019	42	70	272
9/4/2019	141	146	10.7
9/18/2019	41	36	8.5
10/2/2019	199	170	3.1
10/16/2019	337	290	14.2
10/30/2019	183	154	38.4
11/5/2019	119	184	114
11/20/2019	153	142	19.3
12/4/2019	313	722	12.2
12/17/2019	234	14200	308
1/2/2020	1881	3380	50.4
2/5/2020	146	209	7.3
2/20/2020	189	199	8.5
3/4/2020	113	174	3.1
3/16/2020	78	69	8.6
4/29/2020	198	178	48.8
5/6/2020	339	226	63.1
5/20/2020	160	150	5.2
6/3/2020	215	307	6.3
6/17/2020	124	197	24.6

2018-19 Trend of Human/Ruminant Source, and E.coli by Colilert



2019-20 Trend of Human/Ruminant Source, and E.coli by Colilert



Conclusions

- Assay optimization is key
- Design experiment with consideration of all necessary quality control components
- Assess assay specificity
- Normalize biomarker and E. coli data
- dPCR can be a viable tool for microbial risk assessment

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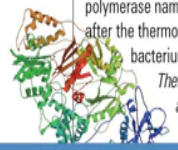
PCR through the ages

1953 The discovery of the DNA double helix structure

1967 Thomas Brock reports on the isolation of a extremophilic bacterium *Thermophilis aquaticus*

1971 Kleppe and co-workers first describe a method using an enzymatic assay to replicate a short DNA template with primers *in vitro*

1976 *Taq* polymerase is isolated (a thermostable DNA polymerase named after the thermophilic bacterium *Thermus aquaticus*)



1977 Frederik Sanger and colleagues introduce the "dideoxy" chain-termination method for sequencing DNA (also known as 'Sanger sequencing'). It utilizes DNA polymerase, nucleotide precursors and one oligonucleotide primer

1982 The first cited use of microarray technology (Augenlicht LH, Kobrin D. "Cloning and screening of sequences expressed in a mouse colon tumor.". *Cancer Research* 42 (3): 1088–1093. PMID 7059971, 1982. http://cancerres.aacrjournals.org/content/42/3/1088.long

1983 Working for Cetus, Kary Mullis discovers that using two oligonucleotides instead of one – on opposite strands- enables DNA to be synthesized from a single, specific, location in the genome. Technique for PCR was created

1985 PCR technique is described in an article published in *Science* (Saiki *et al.*)

1988 Patent for *Taq* DNA polymerase is filed by Mullis *et al.*

The first automated PCR cycler is introduced to the market by Perkin Elmer and Cetus (joint venture)

1989 Science Magazine names *Taq* polymerase its first "Molecule of the Year"

1991 The first high-fidelity DNA polymerase is characterized by Mattila *et al.*

Hot start PCR by wax

1993 Kary Mullis received the Nobel Prize

The first real-time PCR instrument is described

1994 Antibody based hot start technology described

1995 The first complete genome of a free-living organism is sequenced by Venter and colleagues *(Haemophilus influenzae)*

1996 Genome of the first eukaryotic organism, *Saccharomyces cerevisiae*, is sequenced

Two commercial real-time PCR instruments are launched to market

2000 Lynx Therapeutics publishes and markets "MPSS" - a parallelized, adapter/ligation-mediated, bead-based sequencing technology, launching "next-generation" sequencing

2003 Phusion High-Fidelity DNA Polymerase, the first PCR enzyme based on fusion protein technology, is launched by Finnzymes Oy

2007 The first complete human genome is sequenced by Levy *et. al.*

2009 The MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) are published by Bustin *et. al.*

2010 Gibson *et al.* create the first bacterial cell controlled by a chemically synthesized genome (using Phusion High Fidelity DNA Polymerase)

1950 1955 1960 1965 1970 1975 1980 1985 1990 1995 2000 2005 2010

Source: https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/spotlight-articles/history-pcr.html