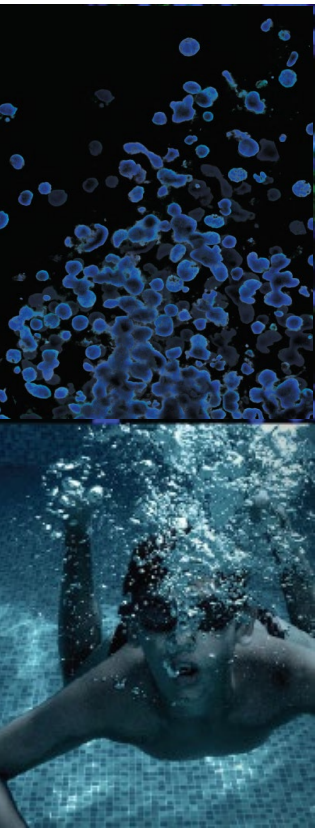
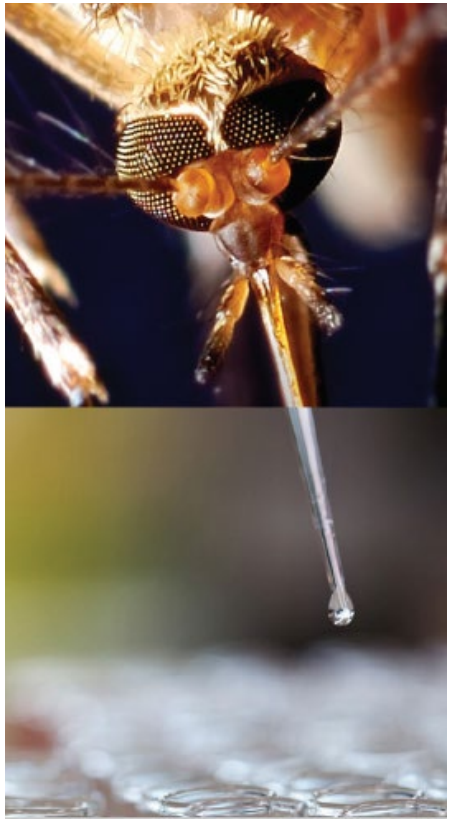
Simplifying assay development with molecular standards: Remove culturing from the equation

Kyle Young, BS, MBA
Product Specialist, ATCC

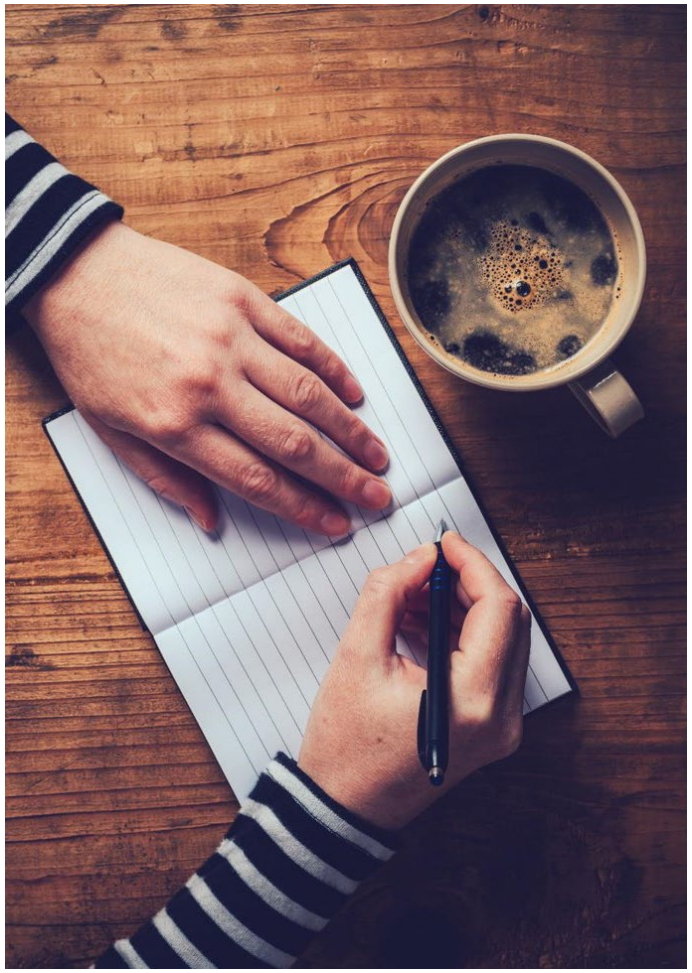
Credible Leads to Incredible™





Agenda

- ✓ How ATCC molecular standards accelerate assay development
- ✓ Development process for ATCC's synthetic molecular standards
- ✓ Validation data = materials you can trust

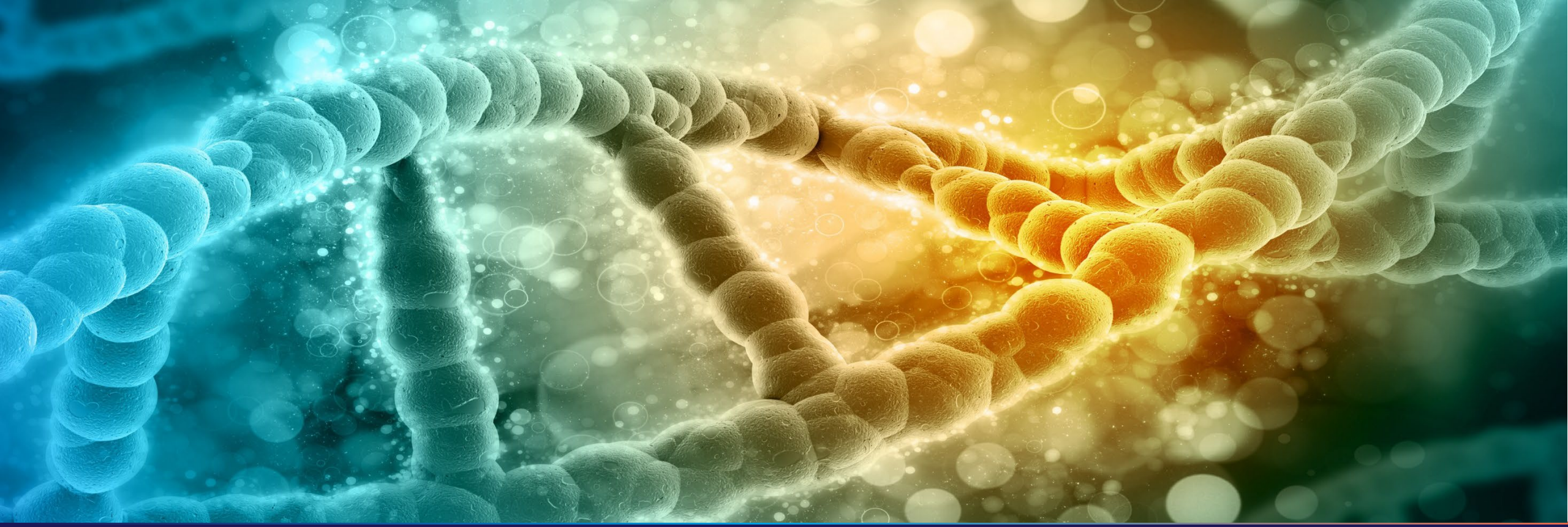


About ATCC

- Founded in 1925, ATCC is a non-profit organization with HQ in Manassas, VA, and an R&D and Services center in Gaithersburg, MD
- World's premier biological materials resource and standards development organization
  - 5,000 cell lines
  - 80,000 microorganisms
  - Genomic & synthetic nucleic acids
  - Media/reagents

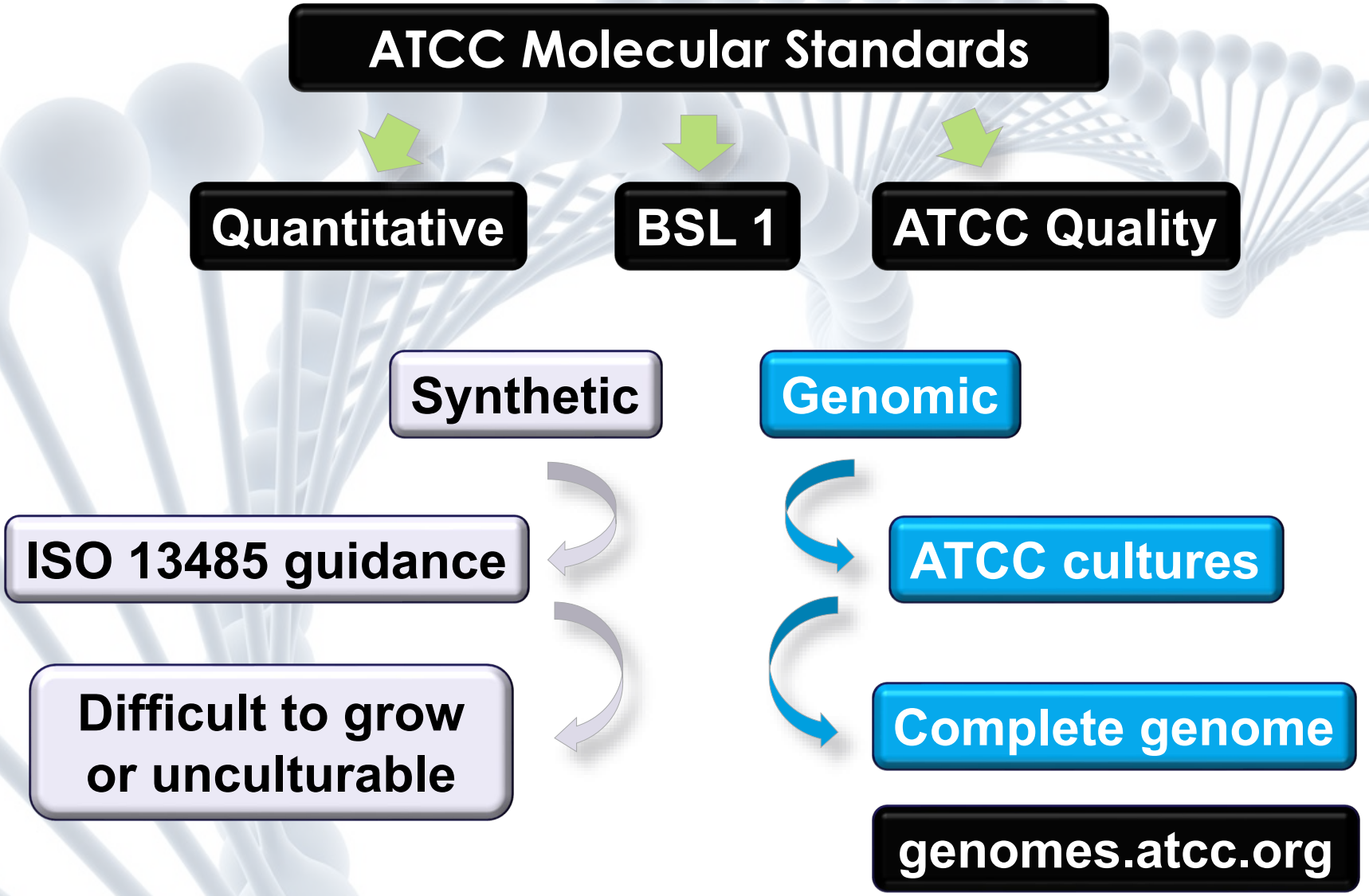
- ATCC collaborates with and supports the scientific community with industry-standard biological products and innovative solutions
- Growing portfolio of products and services
- Sales and distribution in 150 countries, 19 international distributors
- Talented team of 500+ employees, over one-third with advanced degrees

ATCC®



Molecular Standards

ATCC molecular standards



Specifications

Synthetic Standards

Authentication	NGS to verify synthetic sequence
Functionality & Identity	qPCR amplification, 3.32 cycles between Cq threshold
Genome copy number by ddPCR™	1 x 10^5 to 1 x 10^6 construct copies/ µL
Fill Volume	100 µL per vial
Format	Frozen

Genomic Standards

Authentication	Amplicon sequencing
Integrity	High molecular weight DNA by gel electrophoresis
Genome copy number by ddPCR™	1 x 10^5 to 1 x 10^6 genome copies/ µL
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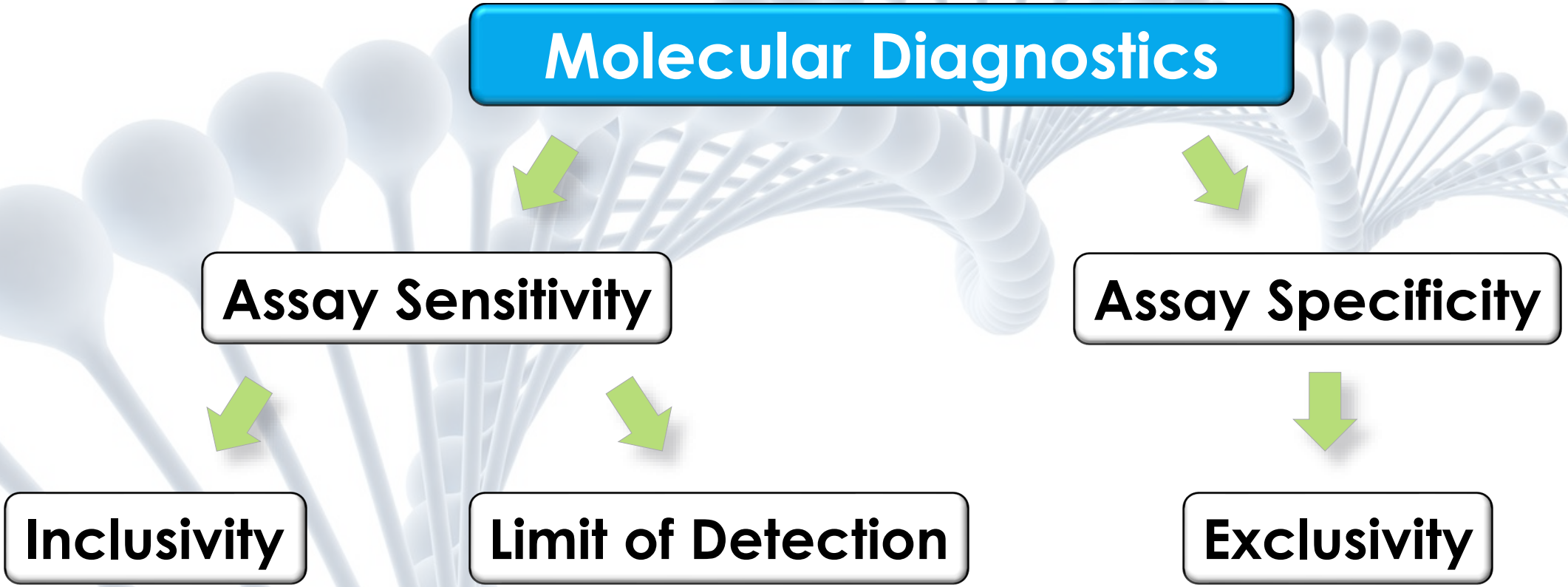
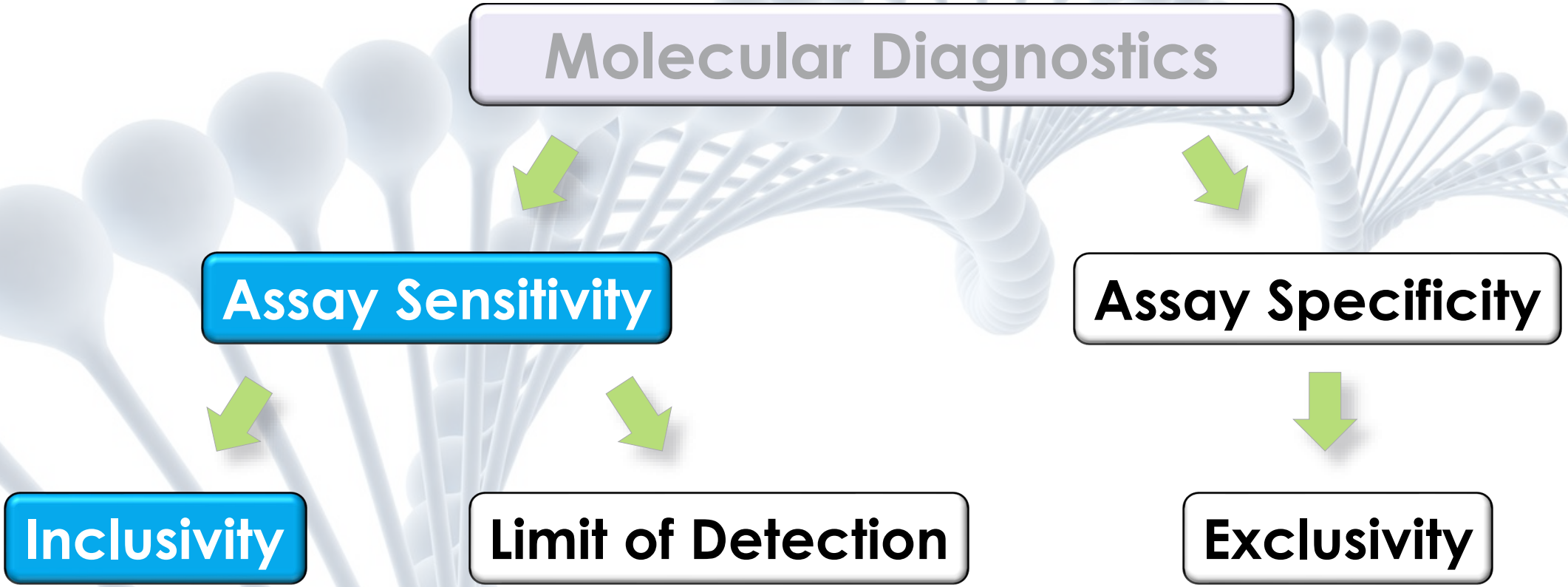
Assay development

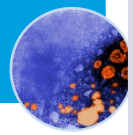


Assay Sensitivity - Inclusivity testing



A menu for assay design

Blood-borne disease



- BK virus
- Hepatitis B virus
- Hepatitis C virus
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- Human immunodeficiency virus 1
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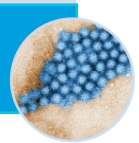
- *Neisseria meningitides*
- *Plasmodium malariae*
- *Human parechovirus 3*

Gastro-Intestinal disease



- Astrovirus
- *Cyclospora cayetanensis*
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- Sapovirus
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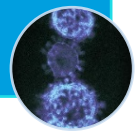
Respiratory disease



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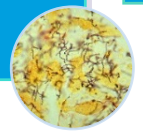
Sexually transmitted infections



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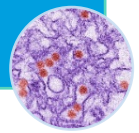

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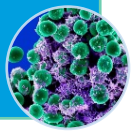
Epidermal & Nosocomial disease



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- *Streptococcus pyogenes*
- *Candida albicans*
- *Pseudomonas aeruginosa*
- *Candida krusei*

ATCC

Assay scope – strain selection for safety testing

Water safety

- *Enterococcus faecalis*
- *Vibrio cholerae*
- *Cryptosporidium parvum*
- Rotavirus
- *Pseudomonas aeruginosa*
- *Escherichia coli* serotype O157:H7

Food safety

- Norovirus
- Big Six *Escherichia coli*
- *Campylobacter jejuni*
- *Salmonella enterica*
- *Listeria monocytogenes*
- Sapovirus

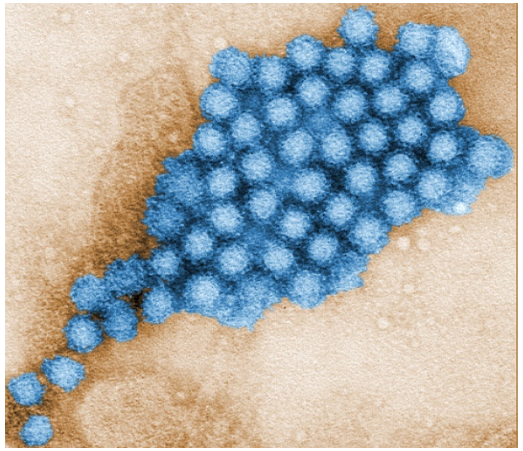


Photo credit: CDC, Dr. Charles D. Humphrey

SARS-CoV-2 molecular standards

BSL 1

ATCC® No.	Description	Compatible assays
VR-3276SD™	Quantitative Synthetic SARS-CoV-2 RNA containing portions of ORF1ab, N, E, nsp12 (RdRp), and ORF1b-nsp14 genes	•China CDC Primers and probes for detection 2019-nCoV (24 January 2020) •Diagnostic detection of Wuhan coronavirus 2019 by real-time RT-PCR – Charité, Berlin Germany (17 January 2020) •Detection of 2019 novel coronavirus (2019-nCoV) in suspected human cases by RT-PCR – Hong Kong University (23 January 2020) •PCR and sequencing protocol for 2019-nCoV - Department of Medical Sciences, Ministry of Public Health, Thailand (Updated 28 January 2020) •US CDC Real-Time RT-PCR Panel for Detection 2019-Novel Coronavirus (28 January 2020) •US CDC panel primer and probes– U.S. CDC, USA (28 January 2020)
VR-3277SD™	Quantitative Synthetic SARS-CoV-2 RNA: containing a portion of Spike 5' end gene.	•Detection of WN-Human1 sequence from clinical specimen. – National Institute of Infectious Diseases Japan (17 January 2020)
VR-3278SD™	Quantitative Synthetic SARS-CoV-2 RNA: containing a portion of Spike 3' end gene.	•PCR and sequencing protocols for 2019-nCoV- National Institute of Infectious Diseases Japan (24 January 2020)
VR-3279SD™	Quantitative Synthetic SARS-CoV-2 RNA containing portions of the nsp9 and nsp12 (RdRp) genes	•RT-PCR assays for the detection of SARS-CoV-2 with RdRp - Institut Pasteur, Paris (2 March 2020) •Diagnostic detection of Wuhan coronavirus 2019 by real-time RT-PCR – Charité, Berlin Germany (17 January 2020)

BSL 2

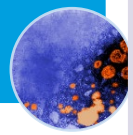
ATCC® No.	Genomic RNA from isolate
VR-1986D™	USA-WA1/2020 lineage A
VR-1991D™	Hong Kong/VM20001061/2020 lineage A
VR-1992D™	Italy/INMI1 lineage B
VR-1994D™	Germany/BavPat1/2020 lineage B (D614G mutation)
VR-3326D™	USA/CA_CDC_5574/2020 lineage B.1.1.7
VR-3327D™ *	USA/MD-HP01542/2021 lineage B.1.351
VR-3338D™ *	Japan/TY7-503/2021 lineage P.1

* In development

A menu for assay design

Inclusivity candidate

Blood-borne disease



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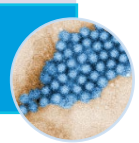

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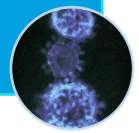

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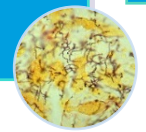
Sexually transmitted infections



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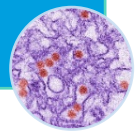
Vector-borne disease



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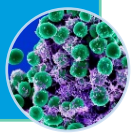
Epidermal & Nosocomial disease



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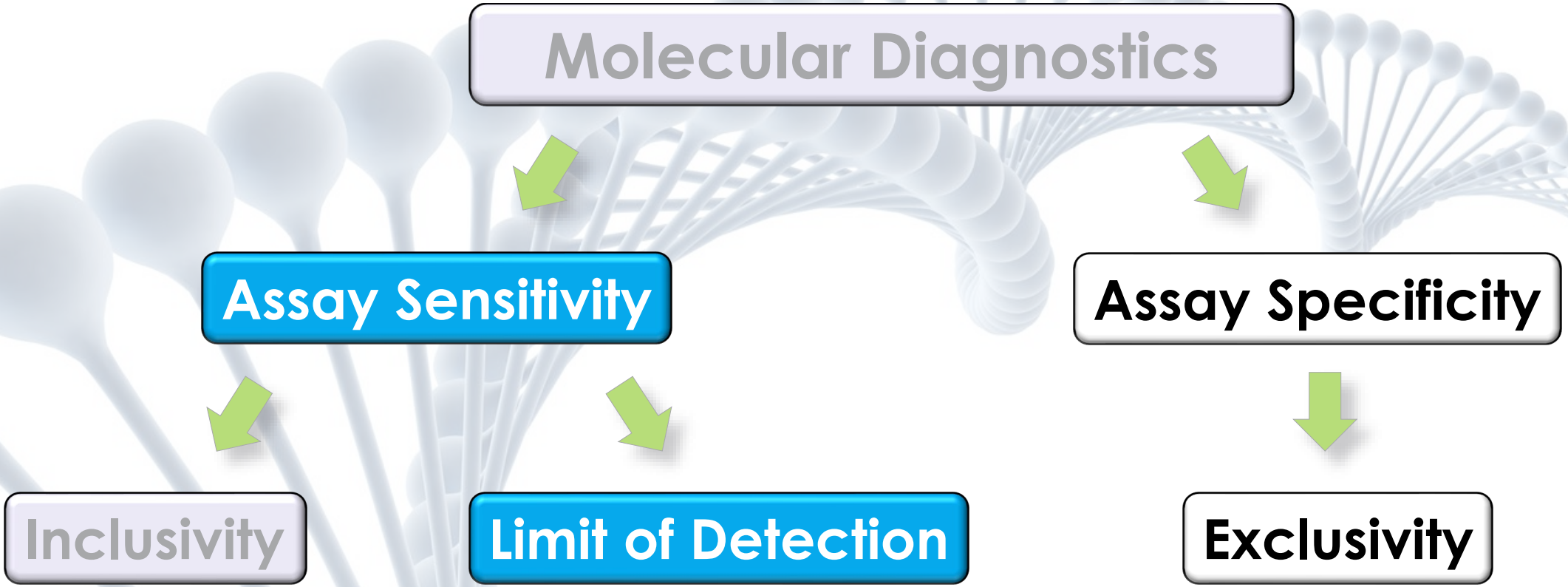
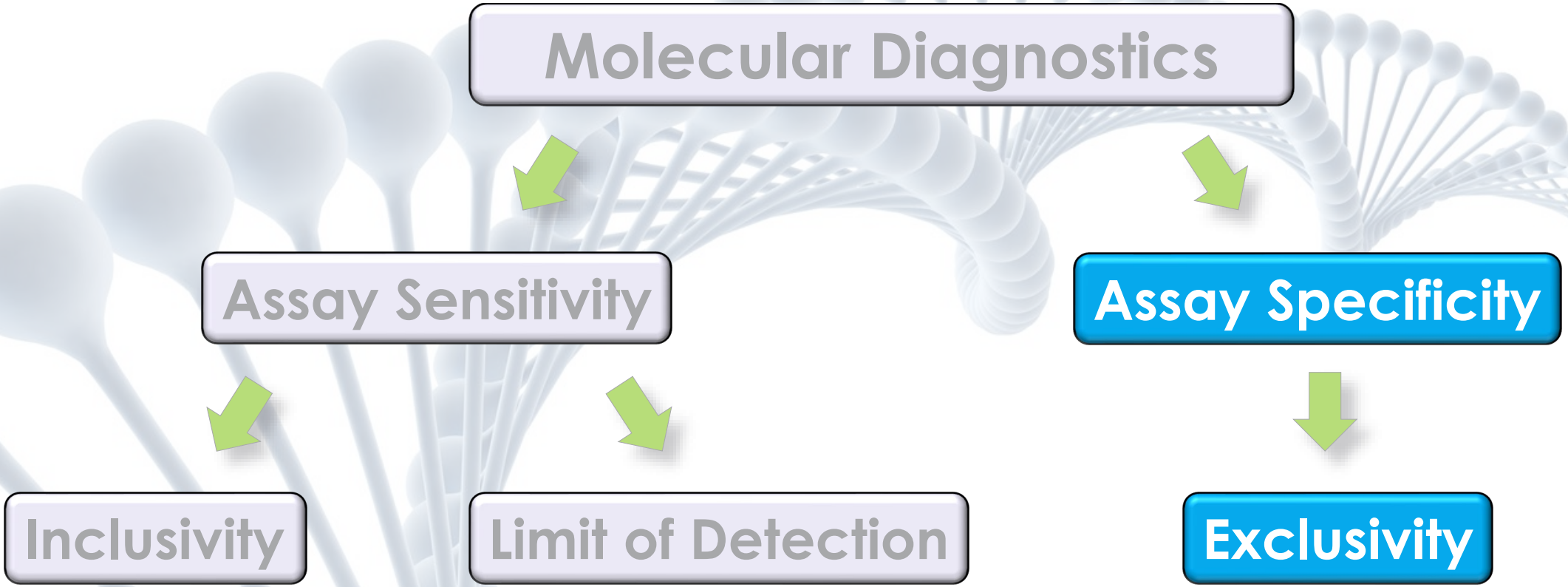
ATCC

Assay sensitivity - limit of detection



Assay specificity - exclusivity testing



Resources for SARS-CoV-2 inclusivity/exclusivity

List of recommended pathogens for assay design in the FDA's emergency-use-authorization letter.

Viral pathogen formats	Inactivated / Live	Quantitative Genomic	Synthetic
SARS-CoV-2	2 (heat-killed)	5	4
Human coronavirus 229E	1	1	
Human coronavirus OC43	1	1	
Human coronavirus HKU1			1
Human coronavirus NL63			1
SARS-CoV			1
MERS-CoV			1
Adenovirus	68	7	
Human metapneumovirus			1
Parainfluenza virus 1-4	4	3	
Influenza A & B	82	14	
Enterovirus	118	3	1
Respiratory syncytial virus	9	3	
Rhinovirus	132	6	

Non-viral pathogen formats	Inactivated / Live	Quantitative Genomic	Synthetic
Chlamydia pneumoniae	7	2	
Haemophilus influenzae	73	1	
Legionella pneumophila	35	1	
Mycobacterium tuberculosis	30	2	
Streptococcus pneumoniae	170	1	
Streptococcus pyogenes	155	2	
Bordetella pertussis	30	3	
Mycoplasma pneumoniae	16	2	
Candida albicans	225	2	
Pseudomonas aeruginosa	328	4	
Pneumocystis jirovecii			1*
Staphylococcus epidermis	33	1	
Streptococcus salivarius	11	1*	

*In development and planning stages.

ATCC

A menu for assay design

Inclusivity candidate

Exclusivity candidate

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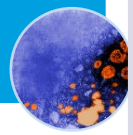

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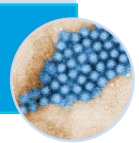

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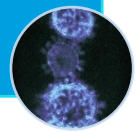

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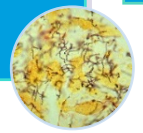

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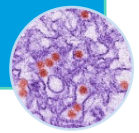

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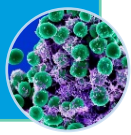
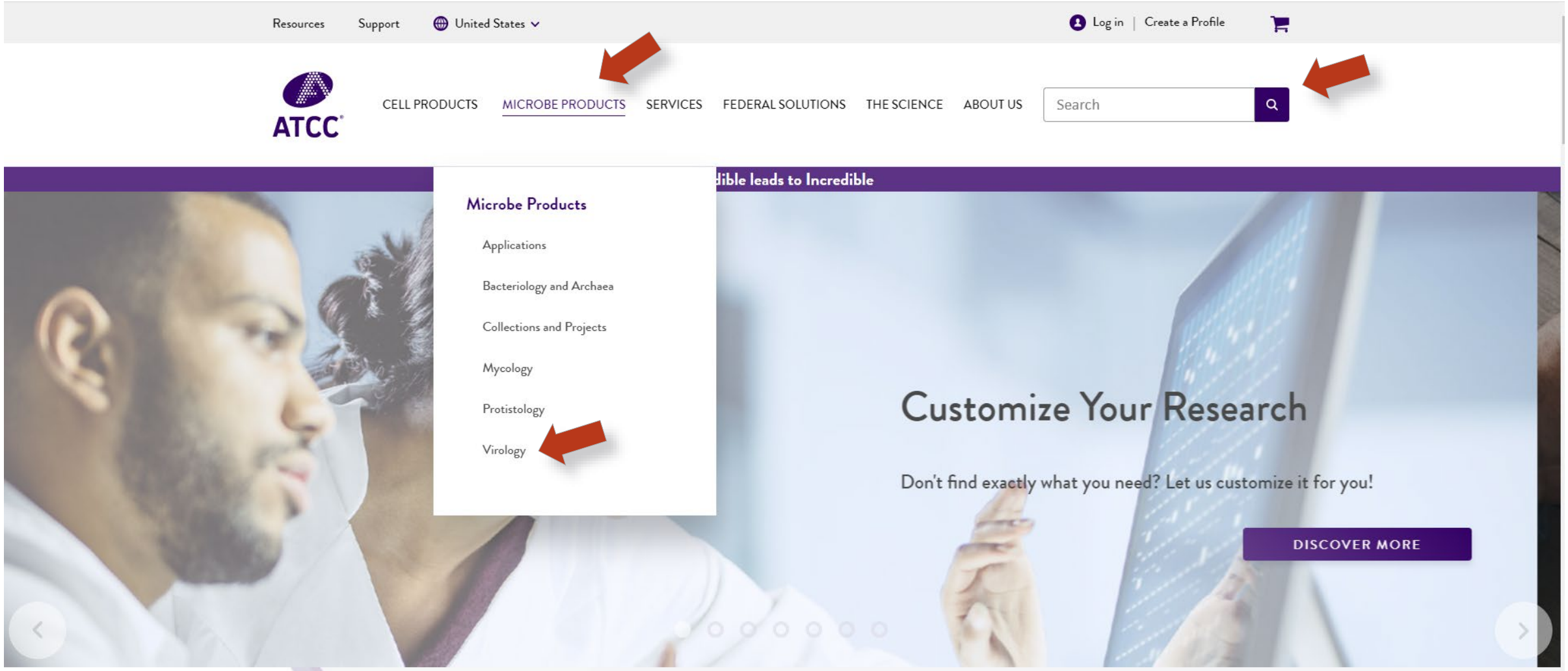
- *Pseudomonas aeruginosa*
- *Candida krusei*



ATCC

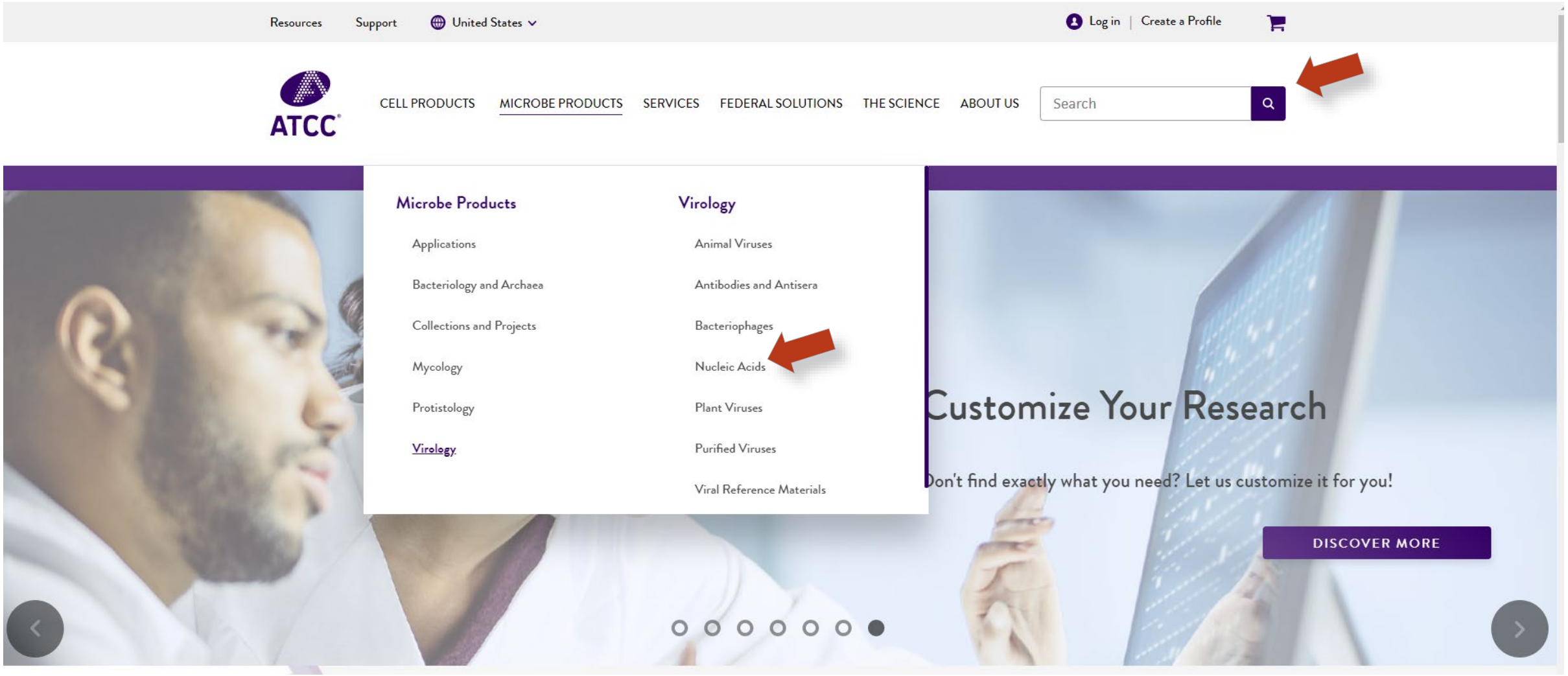
Get the materials

How to find the strains you need



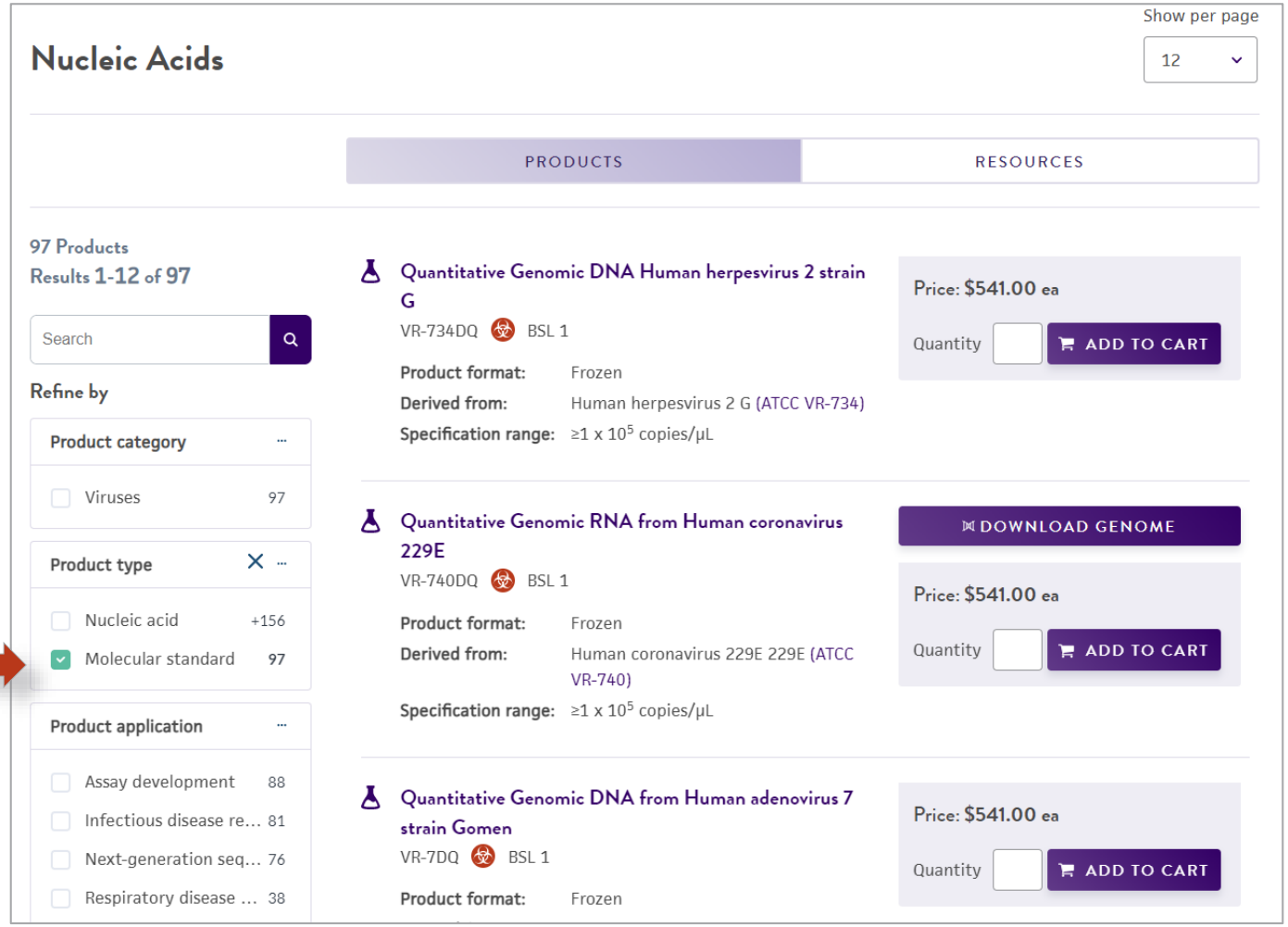
Get the materials

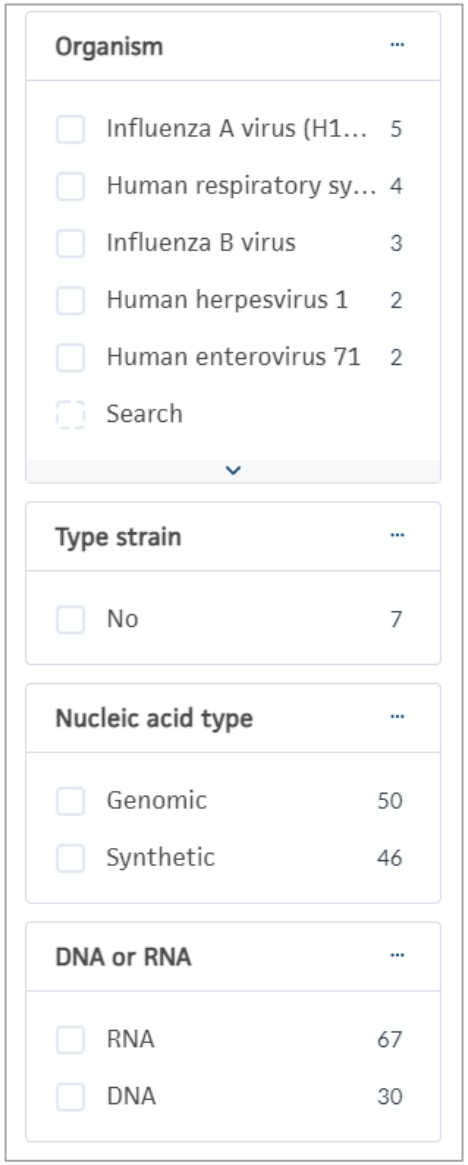
How to find the strains you need



Get the materials

How to find the strains you need

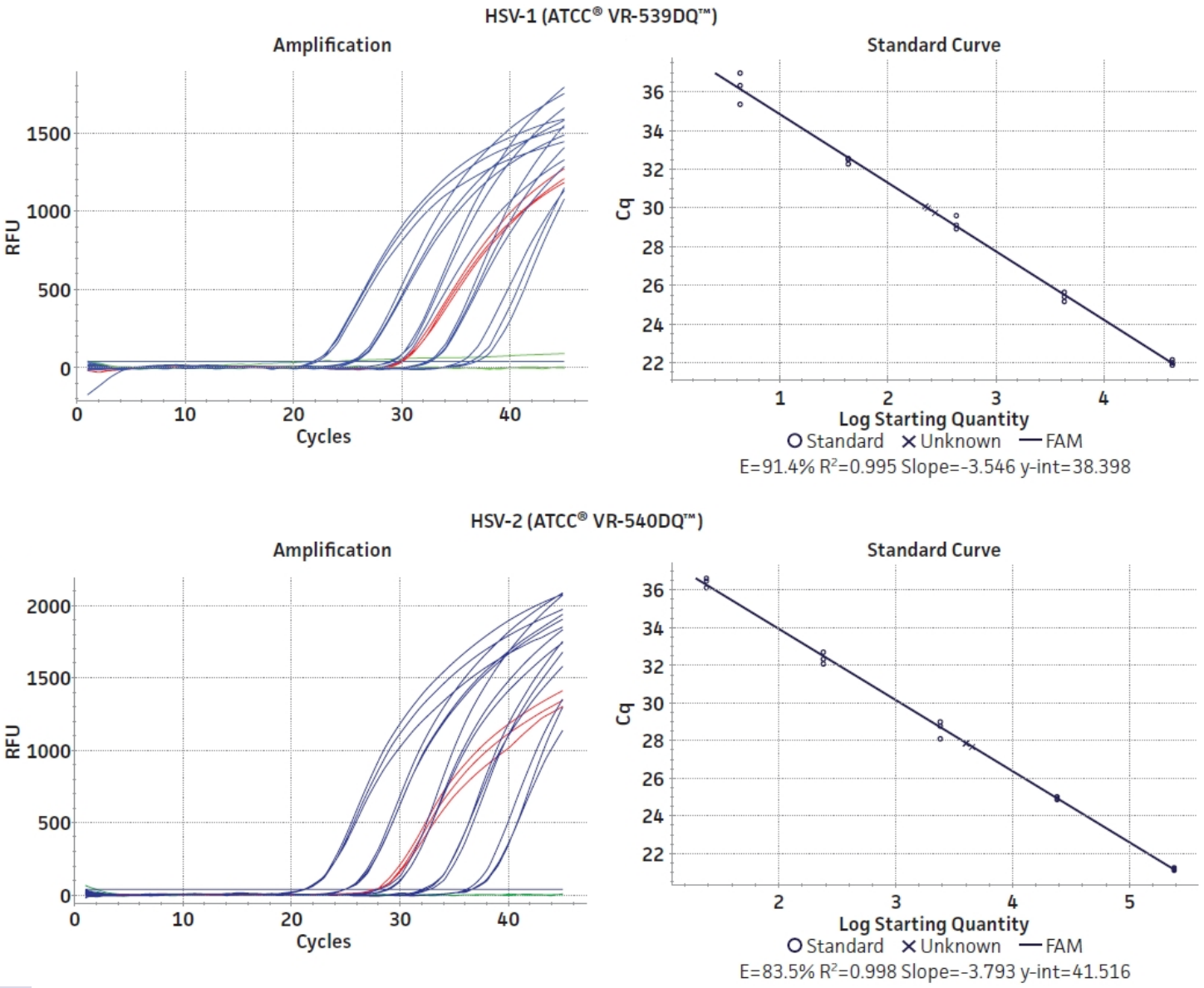


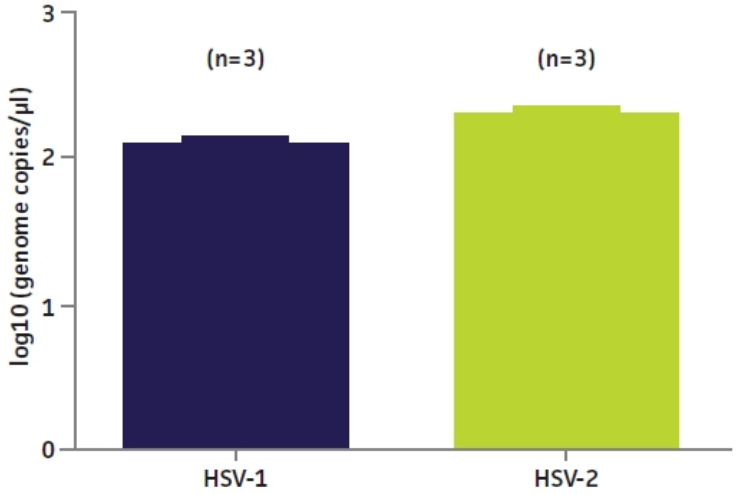


Ongoing and future efforts

- Quality control strains – pharmacopeia, CLSI, or other citations
- Panels for pathogens
  - Respiratory
  - STI
  - Enteric
  - Oncoviruses
  - Opportunistic
  - Anti-microbial resistant
- What do *you* need?
  - ATCC exists to be a resource for scientists.

Assay validation - Human herpes viruses

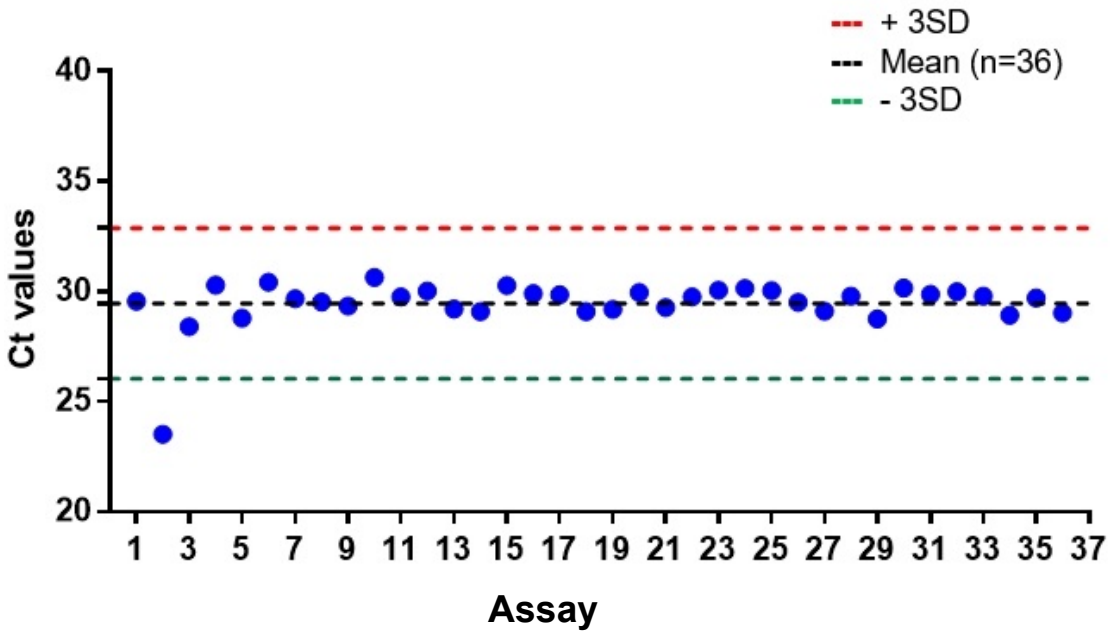




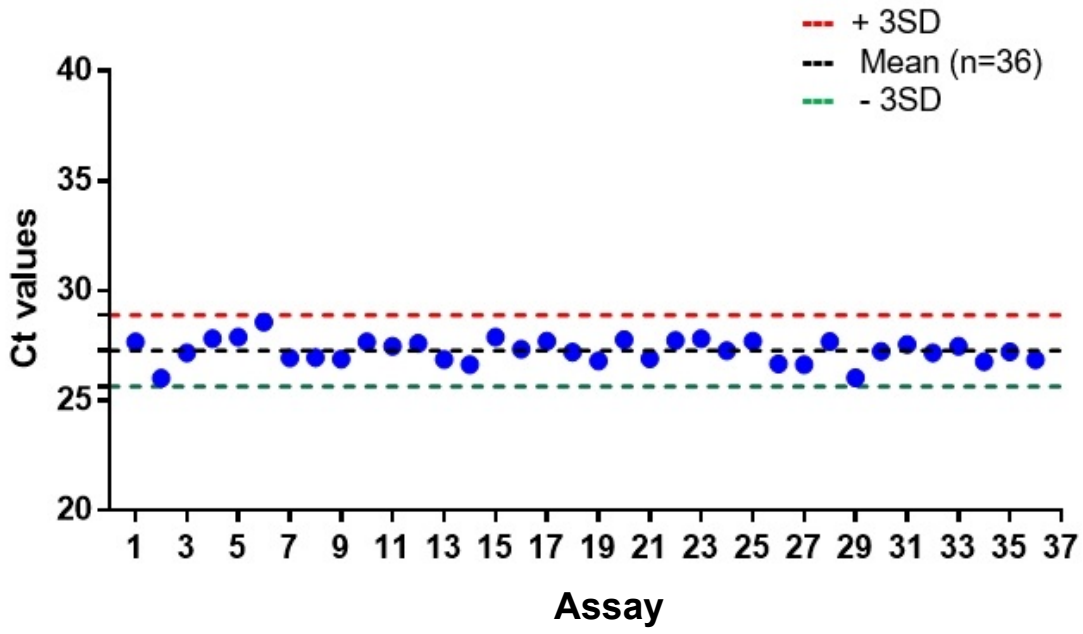
Ryncarz AJ, *et al.* Development of a high-throughput quantitative assay for detecting herpes simplex virus DNA in clinical samples. J Clin Microbiol 37(6): 1941-1947, 1999. Pubmed: 10325351

Assay control - Human herpes viruses

HSV-1 (ATCC® VR-539D™)



HSV-2 (ATCC® VR-540D™)



	Average Ct	Standard Deviation	Coefficient of Variation
HSV-1 (ATCC® VR-539D™)	29.46	1.14	3.9%
HSV-2 (ATCC® VR-540D™)	27.27	0.54	2.0%

Synthetic Molecular Standard Design

Norovirus standards – feedback and adjustment

Interest in the first standards was high, but feedback showed the synthetic constructs had room for improvement. ATCC modified the design and production processes, presenting the following changes at CVS in 2015.

Stability

Changed from dried to frozen

Stability

Added RNA stabilizer

Quantification

Added ddPCR™ to specifications

ISO 13485 guidance for manufacturing

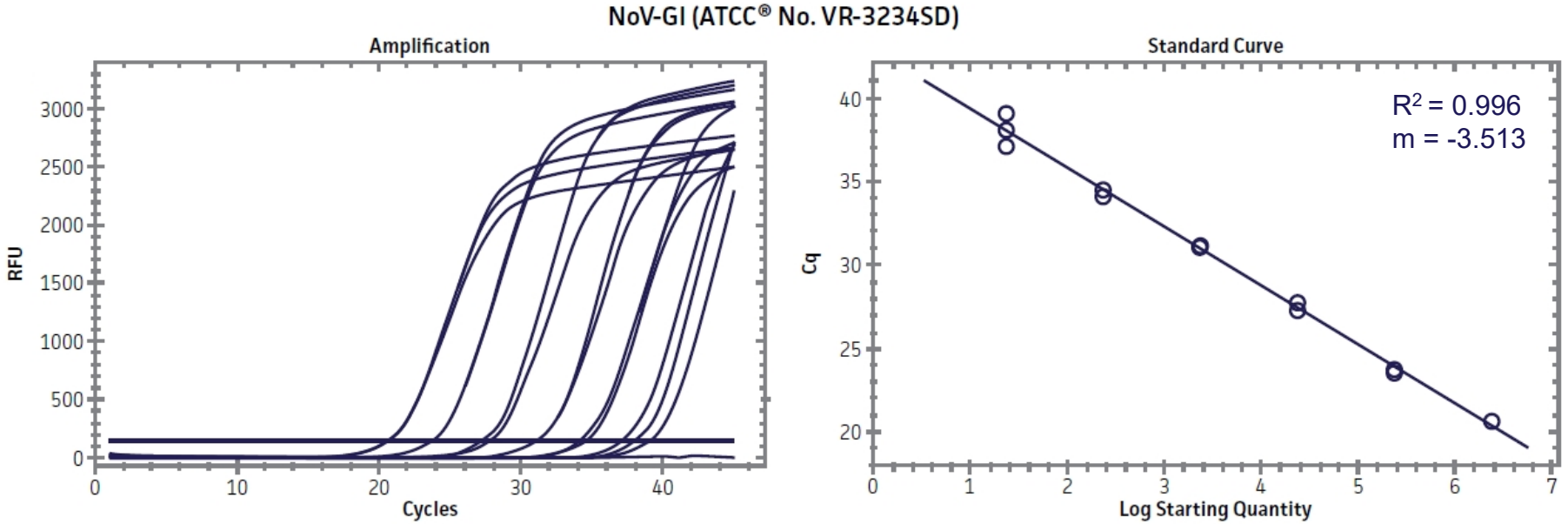
Versatility

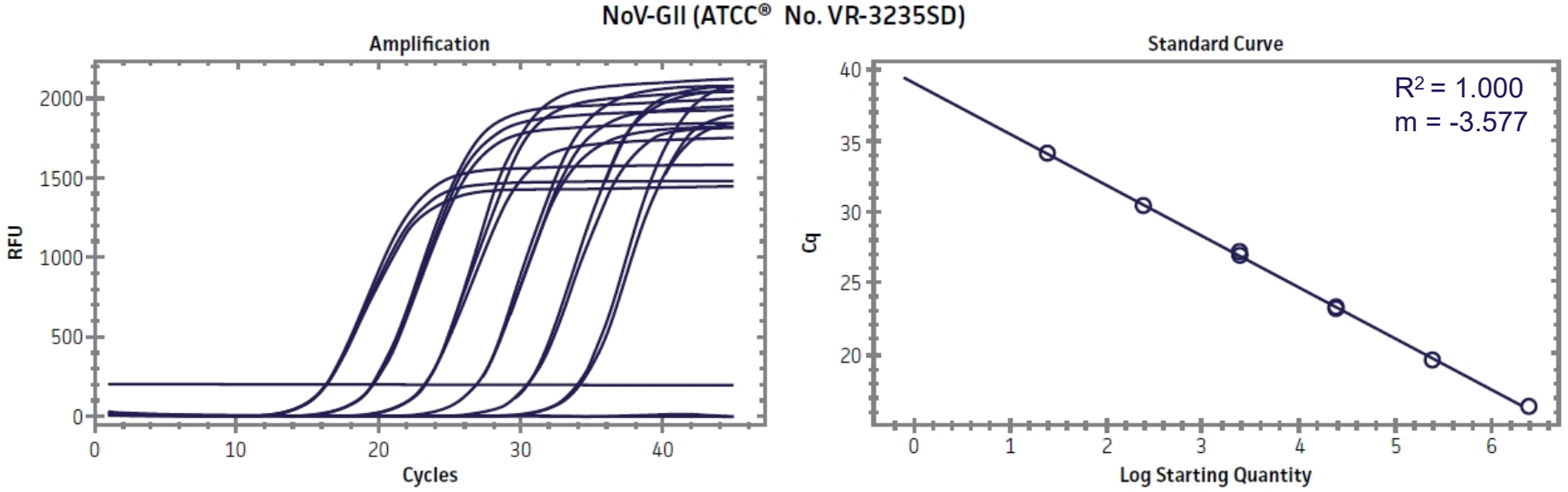
Added RdRp fragments to construct

ATCC

Validating the next generation of standards

www.atcc.org/2015posters



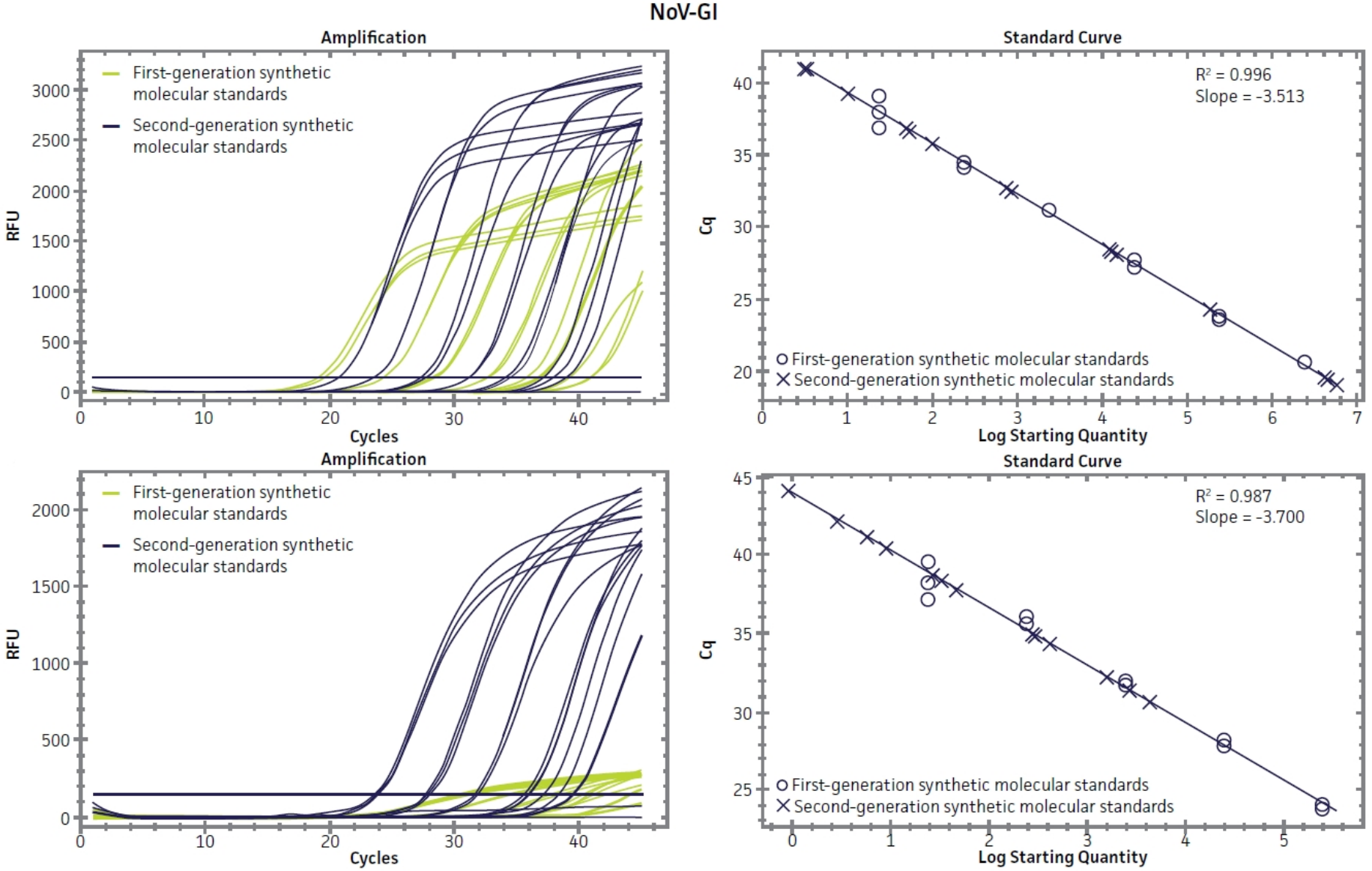


Pictured: Standard curves generated with CaliciNet primer and probe set.

Also tested: ECS working group primer and probe set.

- VR-3234SD™
  - R^2 = 0.987
  - m = -3.692
- VR-3235SD™
  - R^2 = 0.998
  - m = -3.625

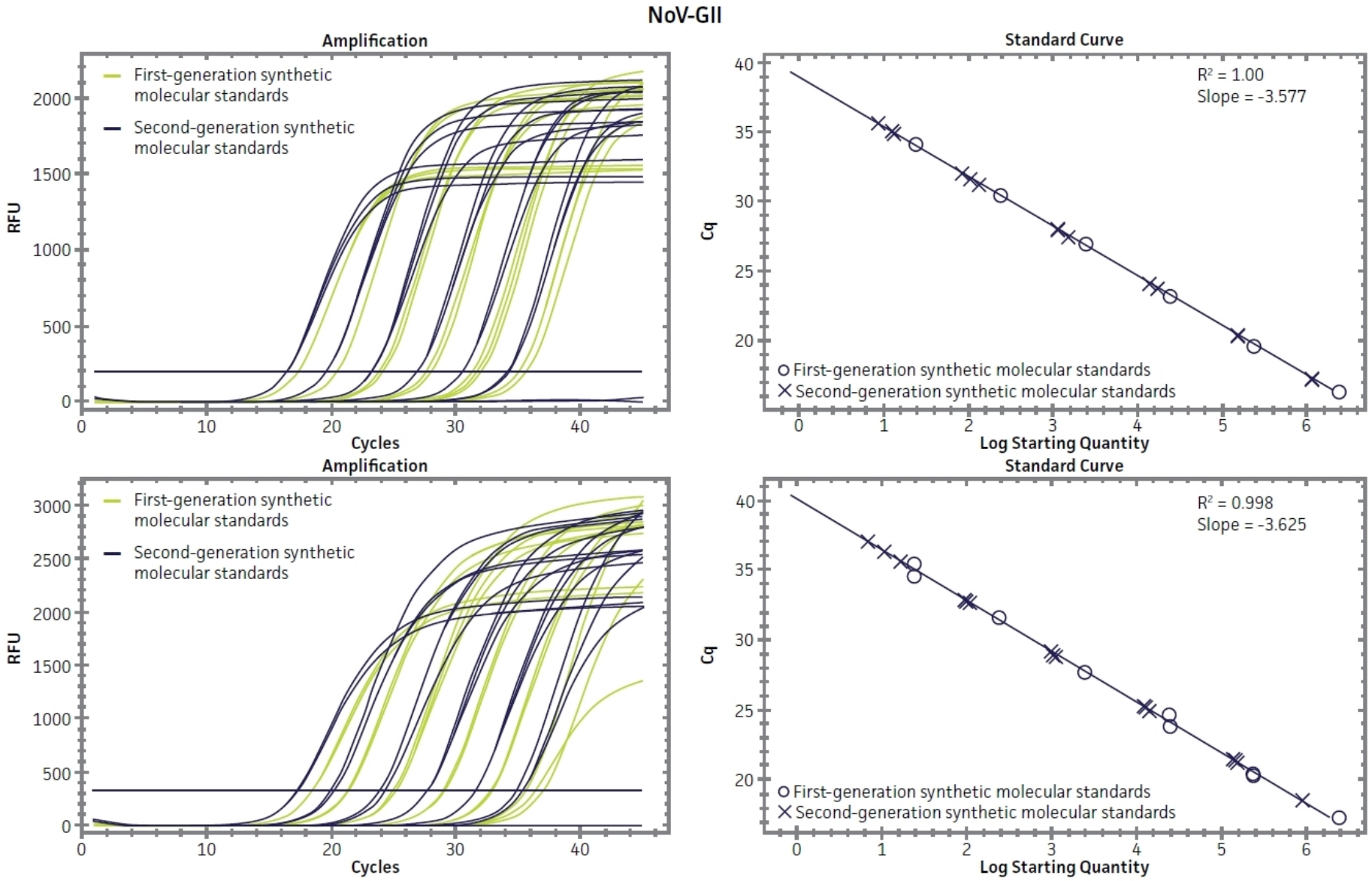
Old vs. new standards, Genogroup 1



CaliciNet primers & probe

ECS working group primers & probe

Old vs. new standards, Genogroup 2

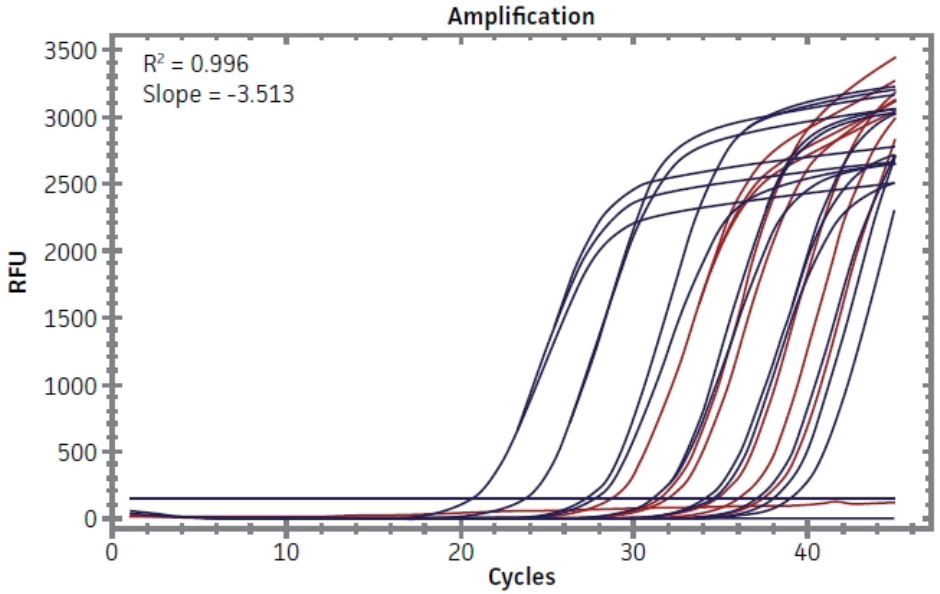


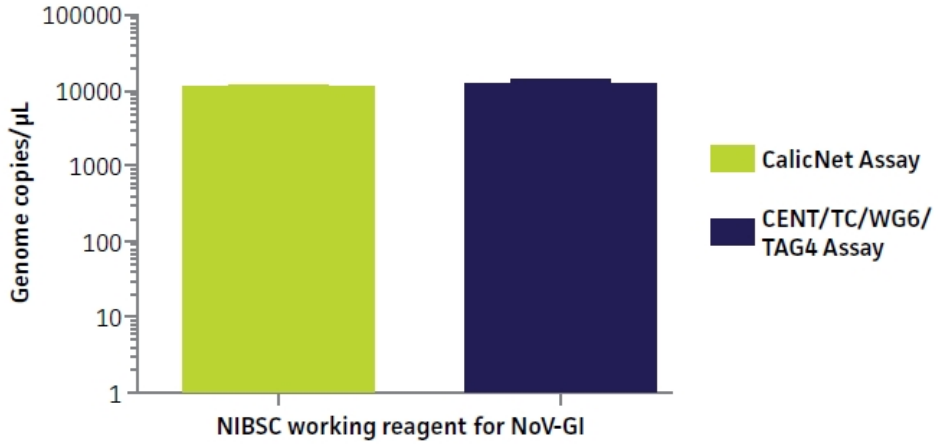
CaliciNet
primers & probe

ECS
primers & probe

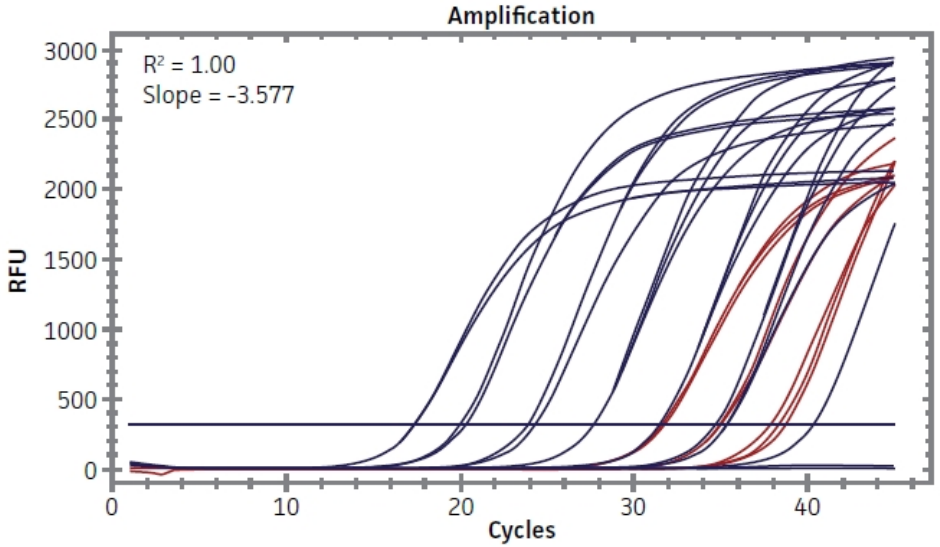
A test drive with NIBSC working reagents

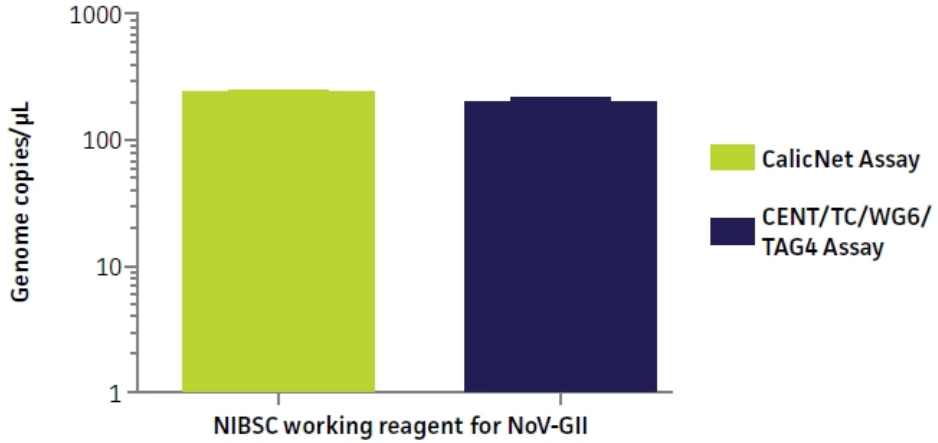
NoV-GI





NoV-GII





Design approach following design control

Data collection

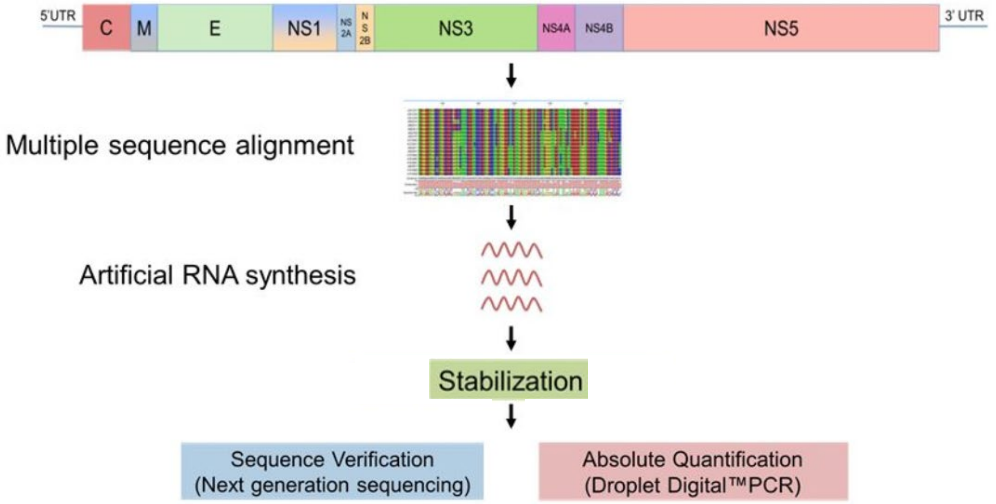
- Literature review
- Bioinformatics sequence data mining
- Technical review

Construct design

- Construct design
- Assay selection and optimization
- Technical review

Validation

- Verify construct specificity
- Technical review
- Quality control testing



Design approach – synthetic standards

Construct

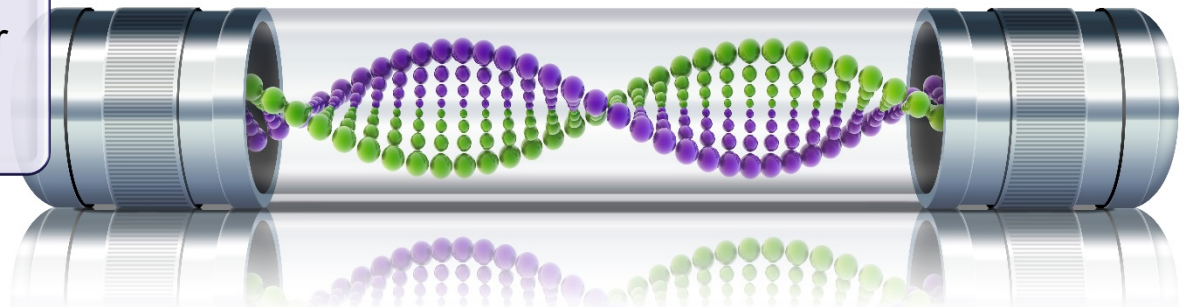
- Genome fragments targeted for assays design
- Nucleic acid matches organism

Assays

- qPCR reported on ATCC website
- Each gene fragment reported on website
- References indicate other compatible assays

Data

- Sequence is proprietary
- For design support, ATCC will confirm whether primers bind



Validation of Molecular Standards

ATCC®

Validation of synthetic standards for hepatitis viruses

Hepatitis B virus

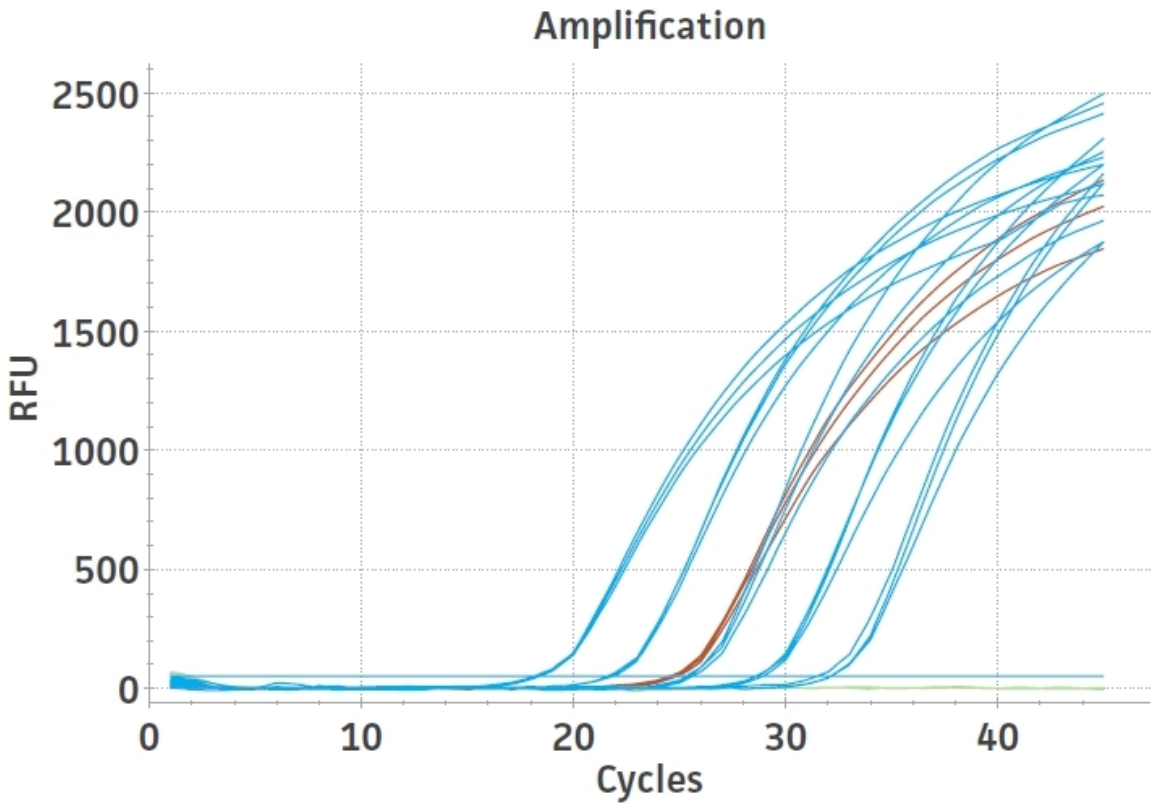
- ATCC catalog VR-3232SD™
- Hepadnaviridae, Orthohepadnavirus
- DNA construct
- Portions of precure, core, P, S, and X regions

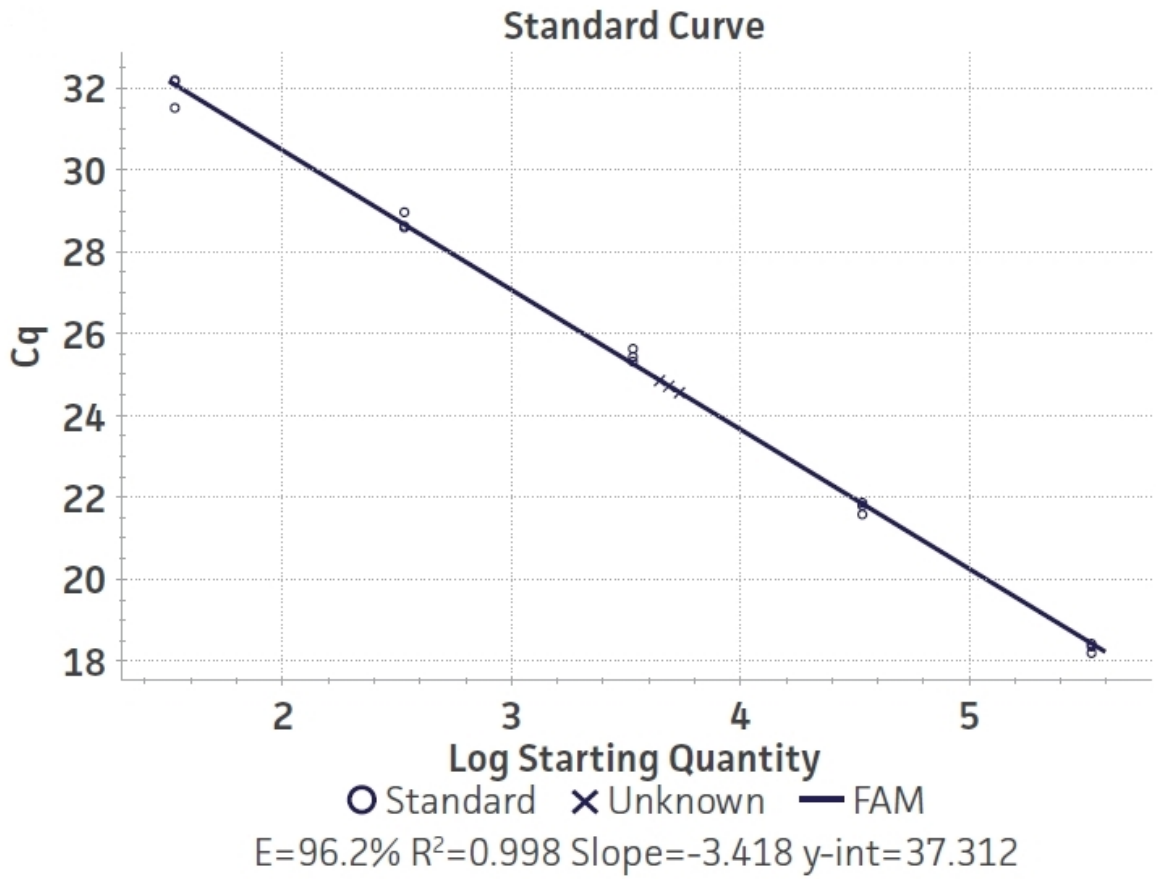
Hepatitis C virus

- ATCC catalog VR-3233SD™
- Flaviviridae, Hepacivirus, Hepacivirus C
- RNA construct
- Portions of 5’ UTR, and X-tail region (3’ UTR)

ATCC has also designed synthetic constructs for Hepatitis A virus (VR-3257SD™) and Hepatitis E virus (VR-3258SD™), and ATCC maintains a number of Hepatitis A viral stocks in its collection.

Hepatitis B virus



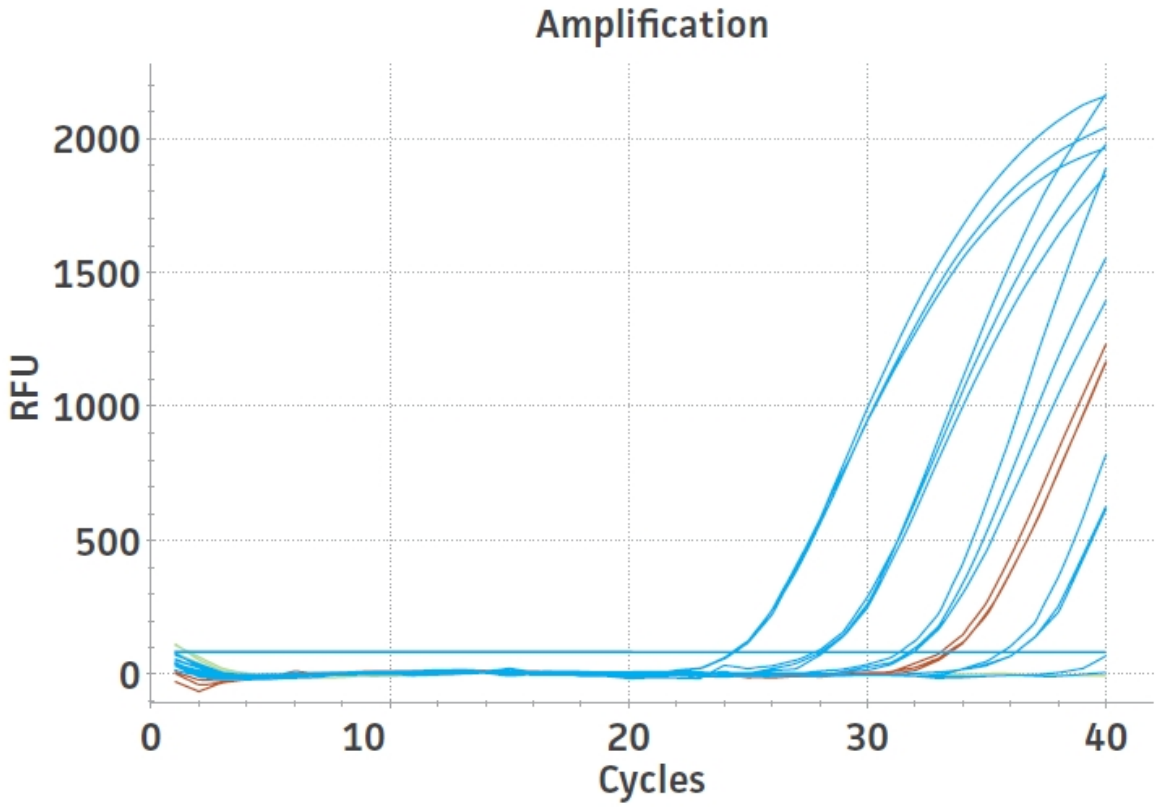


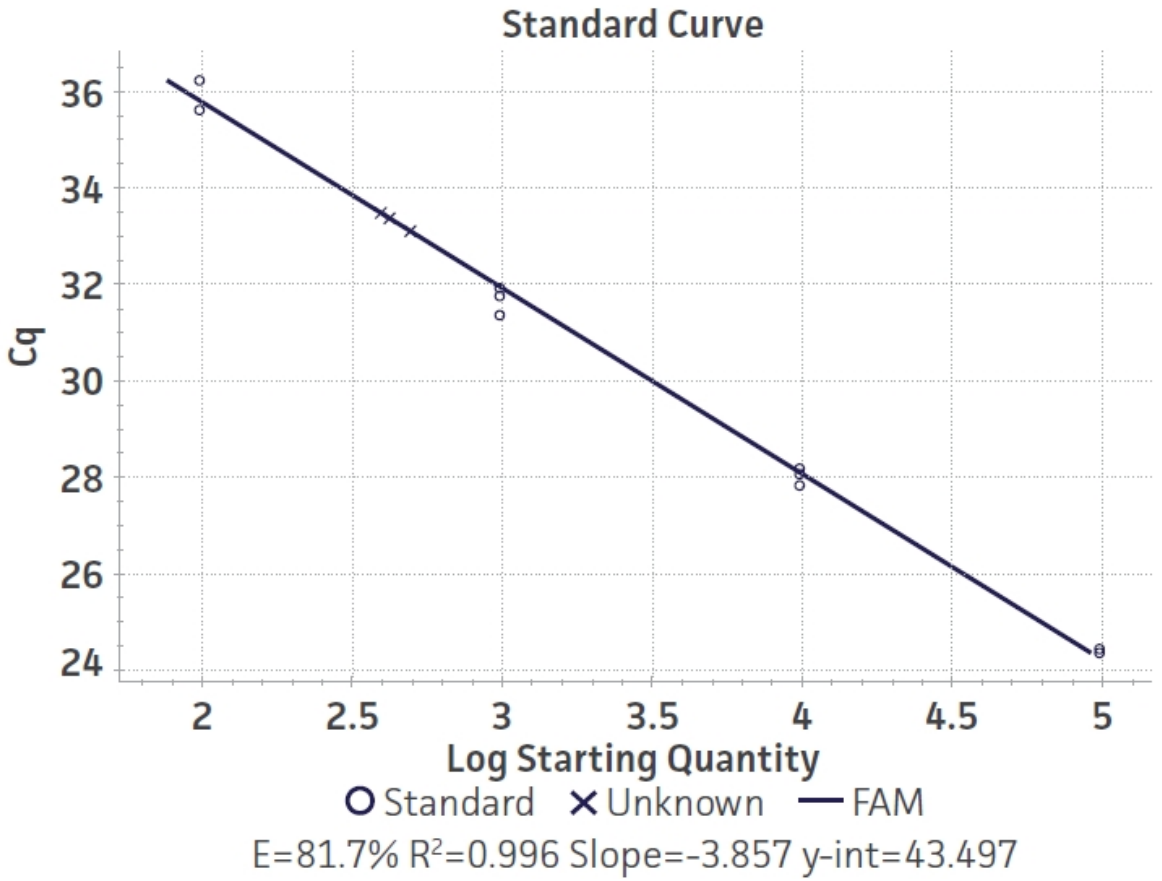
Blue = VR-3232SD™

Red = NIBSC code 10/264 (3rd WHO international working reagent for HBV)

Sun S, *et al*. Development of a new duplex real-time polymerase chain reaction assay for hepatitis B viral DNA detection. Virol. J. 8: 227, 2011. PubMed: 21569595

Hepatitis C virus





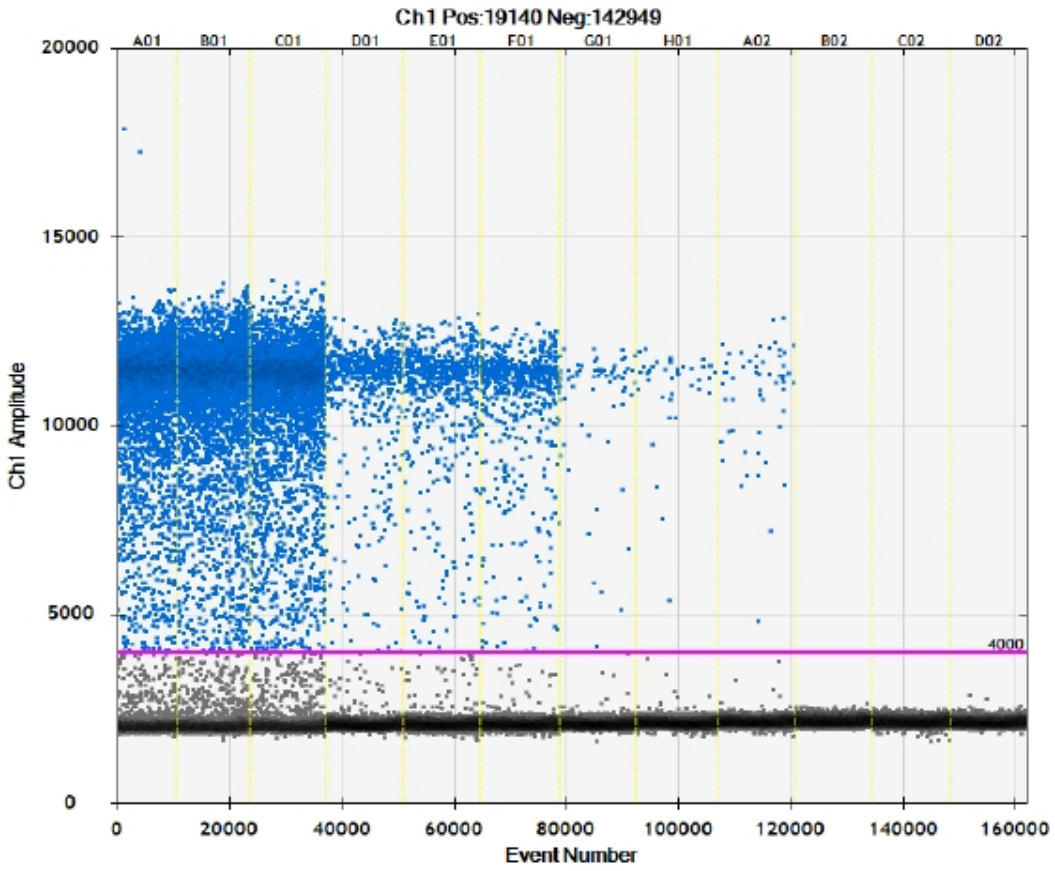
Blue = VR-3233SD™

Red = NIBSC code 06/102 (4th WHO international standard for HCV)

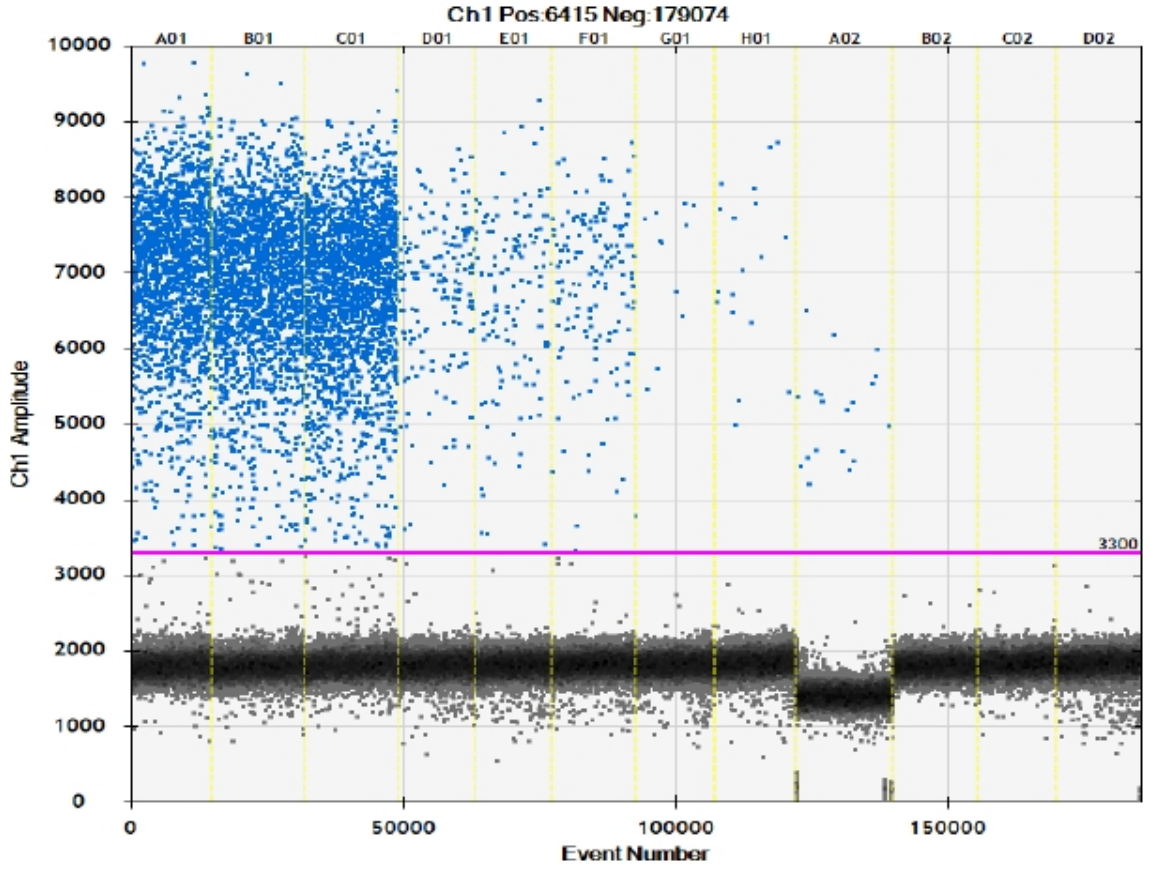
Lee SC, *et al*. Improved version 2.0 qualitative and quantitative AMPLICOR reverse transcription-PCR tests for hepatitis C virus RNA: calibration to international units, enhanced genotype reactivity, and performance characteristics. J. Clin. Microbiol. 38(11): 4171-4179, 2000. PubMed: 11060086

Hepatitis viruses

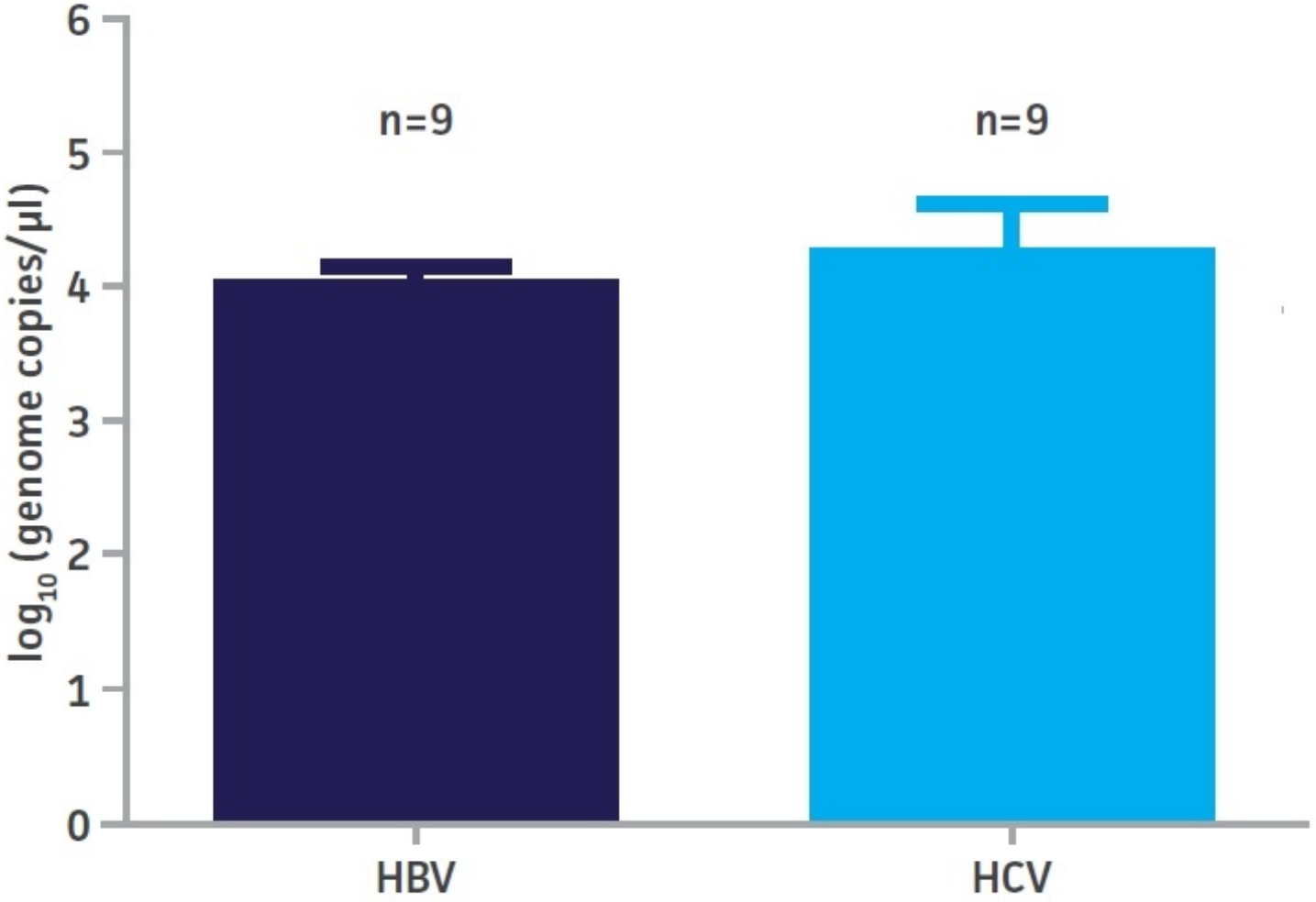
VR-3232SD™ (HBV synthetic standard)



VR-3233SD™ (HCV synthetic standard)



Quantitation of NIBSC Hepatitis standards



As determined by the WHO:

- HBV standard = 8.5 x 10^5 IU/mL
- HCV standard = 2.6 x 10^5 IU/mL

qRT-PCR and qPCR quantitation at ATCC:

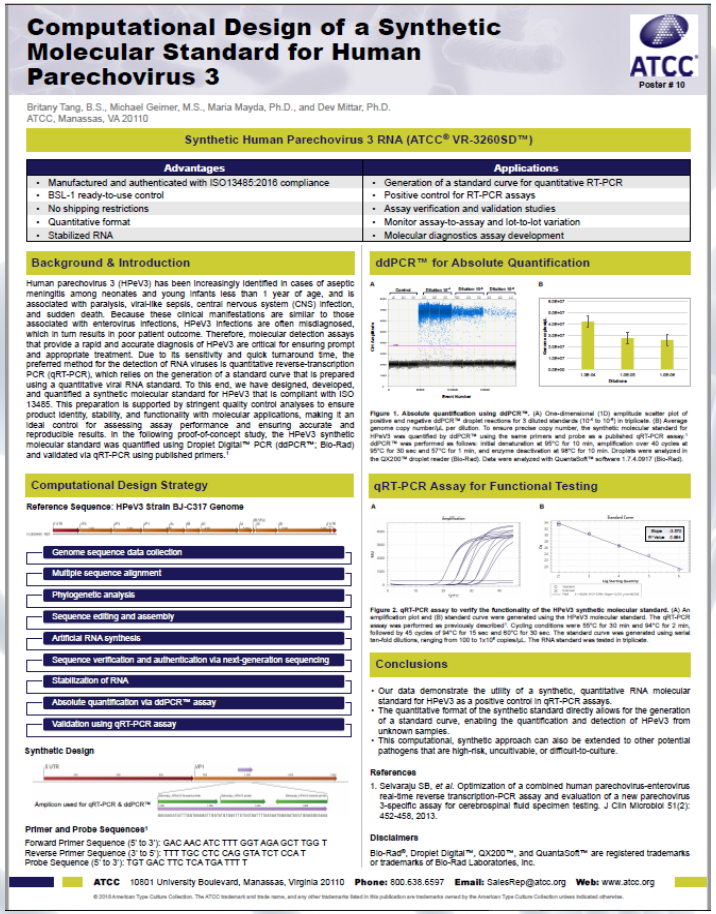
- HBV: 9.7 x 10^6 genome copies/mL
- HCV: 1.6 x 10^7 genome copies/mL

Conversion ratio as quantified at ATCC:

- HBV: 1 IU/mL = 11.4 genome copies
- HCV: 1 IU/mL = 61.5 genome copies

ATCC

Other application data and posters



Computational Design of a Synthetic Molecular Standard for Human Parechovirus 3

ATCC Poster # 10

Britany Tang, B.S., Michael Geimer, M.S., Maria Mayda, Ph.D., and Dev Mittar, Ph.D.
ATCC, Manassas, VA 20110

Synthetic Human Parechovirus 3 RNA (ATCC® VR-3260SD™)

Advantages	Applications
• Manufactured and authenticated with ISO13485:2016 compliance • BSL-1 ready-to-use control • No shipping restrictions • Quantitative format • Stabilized RNA	• Generation of a standard curve for quantitative RT-PCR • Positive control for RT-PCR assays • Assay verification and validation studies • Monitor assay-to-assay and lot-to-lot variation • Molecular diagnostics assay development

Background & Introduction

Human parechovirus 3 (HPeV3) has been increasingly identified in cases of aseptic meningitis among neonates and young infants less than 1 year of age, and is associated with paralysis, viral-like sepsis, central nervous system (CNS) infection, and sudden death. Because these clinical manifestations are similar to those associated with enterovirus infections, HPeV3 infections are often misdiagnosed, which in turn results in poor patient outcome. Therefore, molecular detection assays that provide a rapid and accurate diagnosis of HPeV3 are critical for ensuring prompt and appropriate treatment. Due to its sensitivity and quick turnaround time, the preferred method for the detection of RNA viruses is quantitative reverse-transcription PCR (qRT-PCR), which relies on the generation of a standard curve that is prepared using a quantitative viral RNA standard. To this end, we have designed, developed, and quantified a synthetic molecular standard for HPeV3 that is compliant with ISO 13485. This preparation is supported by stringent quality control analyses to ensure product identity, stability, and functionality with molecular applications, making it an ideal control for assessing assay performance and ensuring accurate and reproducible results. In the following proof-of-concept study, the HPeV3 synthetic molecular standard was quantified using Droplet Digital™ PCR (ddPCR™; Bio-Rad) and validated via qRT-PCR using published primers.[1]

ddPCR™ for Absolute Quantification

Figure 1. Absolute quantification using ddPCR™. (A) One-dimensional (1D) amplitude scatter plot of positive and negative ddPCR™ droplet reactions for 5 diluted standards (10^4 to 10^8) in triplicate. (B) Average genome copy number/µL per dilution. To ensure precise copy number, the synthetic molecular standard for HPeV3 was quantified by ddPCR™ using the same primers and probe as a published qRT-PCR assay.[1] ddPCR™ was performed as follows: initial denaturation at 95°C for 10 min, amplification over 40 cycles at 95°C for 30 sec and 57°C for 1 min, and enzyme deactivation at 98°C for 10 min. Droplets were analyzed in the QX200™ droplet reader (Bio-Rad). Data were analyzed with QuantaSoft™ software 1.7.4.0917 (Bio-Rad).

Computational Design Strategy

Reference Sequence: HPeV3 Strain BJ-C317 Genome

- Genome sequence data collection
- Multiple sequence alignment
- Phylogenetic analysis
- Sequence editing and assembly
- Artificial RNA synthesis
- Sequence verification and authentication via next-generation sequencing
- Stabilization of RNA
- Absolute quantification via ddPCR™ assay
- Validation using qRT-PCR assay

Synthetic Design

Primer and Probe Sequences[1]

Forward Primer Sequence (5' to 3'): GAC AAC ATC TTT GGT AGA GCT TGG T
Reverse Primer Sequence (3' to 5'): TTT TGC CTC CAG GTA TCT CCA T
Probe Sequence (5' to 3'): TGT GAC TTC TCA TGA TTT T

qRT-PCR Assay for Functional Testing

Figure 2. qRT-PCR assay to verify the functionality of the HPeV3 synthetic molecular standard. (A) An amplification plot and (B) standard curve were generated using the HPeV3 molecular standard. The qRT-PCR assay was performed as previously described[1]. Cycling conditions were 55°C for 30 min and 94°C for 2 min, followed by 45 cycles of 94°C for 15 sec and 60°C for 30 sec. The standard curve was generated using serial ten-fold dilutions, ranging from 100 to $1x10^6$ copies/µL. The RNA standard was tested in triplicate.

Conclusions

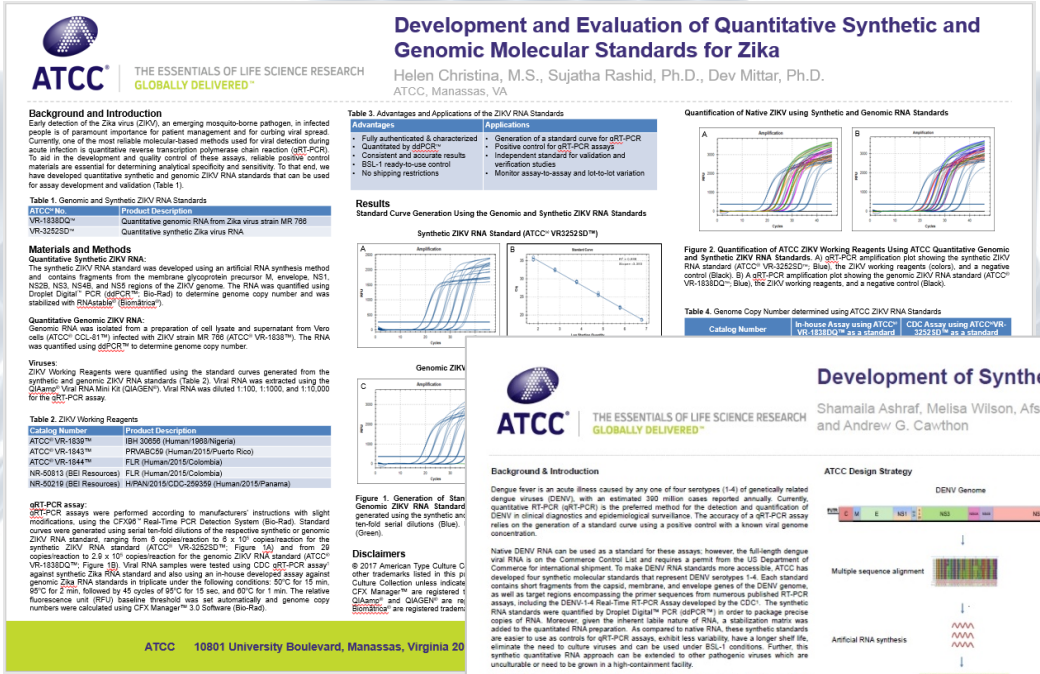
- Our data demonstrate the utility of a synthetic, quantitative RNA molecular standard for HPeV3 as a positive control in qRT-PCR assays.
- The quantitative format of the synthetic standard directly allows for the generation of a standard curve, enabling the quantification and detection of HPeV3 from unknown samples.
- This computational, synthetic approach can also be extended to other potential pathogens that are high-risk, uncultivable, or difficult-to-culture.

References

1. Selvarajju SB, *et al.* Optimization of a combined human parechovirus-enterovirus real-time reverse transcription-PCR assay and evaluation of a new parechovirus 3-specific assay for cerebrospinal fluid specimen testing. J Clin Microbiol 51(2): 452-458, 2013.

Disclaimers

ATCC 10801 University Boulevard, Manassas, Virginia 20110 **Phone:** 800.638.6597 **Email:** SalesRep@atcc.org **Web:** www.atcc.org



Development and Evaluation of Quantitative Synthetic and Genomic Molecular Standards for Zika

ATCC® THE ESSENTIALS OF LIFE SCIENCE RESEARCH GLOBALLY DELIVERED™

Helen Christina, M.S., Sujatha Rashid, Ph.D., Dev Mittar, Ph.D.
ATCC, Manassas, VA

Background and Introduction

Early detection of the Zika virus (ZIKV), an emerging mosquito-borne pathogen, in infected people is of paramount importance for patient management and for curbing viral spread. Currently, one of the most reliable molecular-based methods used for viral detection during acute infection is quantitative reverse transcription polymerase chain reaction (qRT-PCR). To aid in the development and quality control of these assays, reliable positive control materials are essential for determining analytical specificity and sensitivity. To that end, we have developed quantitative synthetic and genomic ZIKV RNA standards that can be used for assay development and validation (Table 1).

Table 1. Genomic and Synthetic ZIKV RNA Standards

ATCC® No.	Product Description
VR-1838DQ™	Quantitative genomic RNA from Zika virus strain MR 766
VR-32525D™	Quantitative synthetic Zika virus RNA

Materials and Methods

Quantitative Synthetic ZIKV RNA:
The synthetic ZIKV RNA standard was developed using an artificial RNA synthesis method and contains fragments from the membrane glycoprotein precursor M, envelope, NS1, NS2B, NS3, NS4B, and NS5 regions of the ZIKV genome. The RNA was quantified using Droplet Digital™ PCR (ddPCR™; Bio-Rad) to determine genome copy number and was stabilized with RNAstable® (Biomatrica®).

Quantitative Genomic ZIKV RNA:
Genomic RNA was isolated from a preparation of cell lysate and supernatant from Vero cells (ATCC® CCL-81™) infected with ZIKV strain MR 766 (ATCC® VR-1838™). The RNA was quantified using ddPCR™ to determine genome copy number.

Viruses:
ZIKV Working Reagents were quantified using the standard curves generated from the synthetic and genomic ZIKV RNA standards (Table 2). Viral RNA was extracted using the QIAamp® Viral RNA Mini Kit (QIAGEN®). Viral RNA was diluted 1:100, 1:1000, and 1:10,000 for the qRT-PCR assay.

Table 2. ZIKV Working Reagents

Catalog Number	Product Description
ATCC® VR-1839™	IBH 36656 (Human/1968/Nigeria)
ATCC® VR-1843™	PRVABC59 (Human/2015/Puerto Rico)
ATCC® VR-1844™	FLR (Human/2015/Colombia)
NR-50913 (BEI Resources)	FLR (Human/2015/Colombia)
NR-50219 (BEI Resources)	H/PAN/2015/CDC-259359 (Human/2015/Panama)

qRT-PCR assay:
qRT-PCR assays were performed according to manufacturers' instructions with slight modifications, using the CFX96™ Real-Time PCR Detection System (Bio-Rad). Standard curves were generated using serial ten-fold dilutions of the respective synthetic or genomic ZIKV RNA standard, ranging from 6 copies/reaction to 6×10^5 copies/reaction for the synthetic ZIKV RNA standard (ATCC® VR-32525D™; Figure 1A) and from 29 copies/reaction to 2.9×10^5 copies/reaction for the genomic ZIKV RNA standard (ATCC® VR-1838DQ™; Figure 1B). Viral RNA samples were tested using CDC qRT-PCR assay[1] against synthetic Zika RNA standard and also using an in-house developed assay against genomic Zika RNA standards in triplicate under the following conditions: 50°C for 15 min, 95°C for 2 min, followed by 45 cycles of 95°C for 15 sec, and 60°C for 1 min. The relative fluorescence unit (RFU) baseline threshold was set automatically and genome copy numbers were calculated using CFX Manager™ 3.0 Software (Bio-Rad).

Table 3. Advantages and Applications of the ZIKV RNA Standards

Advantages	Applications
• Fully authenticated & characterized • Quantitated by ddPCR™ • Consistent and accurate results • BSL-1 ready-to-use control • No shipping restrictions	• Generation of a standard curve for qRT-PCR • Positive control for qRT-PCR assays • Independent standard for validation and verification studies • Monitor assay-to-assay and lot-to-lot variation

Results

Standard Curve Generation Using the Genomic and Synthetic ZIKV RNA Standards

Synthetic ZIKV RNA Standard (ATCC® VR32525D™)

Genomic ZIKV

Figure 1. Generation of Stan
Genomic ZIKV RNA Standard
generated using the synthetic an
ten-fold serial dilutions (Blue).
(Green).

Quantification of Native ZIKV using Synthetic and Genomic RNA Standards

Figure 2. Quantification of ATCC ZIKV Working Reagents Using ATCC Quantitative Genomic and Synthetic ZIKV RNA Standards. A) qRT-PCR amplification plot showing the synthetic ZIKV RNA standard (ATCC® VR-32525D™; Blue), the ZIKV working reagents (colors), and a negative control (Black). B) A qRT-PCR amplification plot showing the genomic ZIKV RNA standard (ATCC® VR-1838DQ™; Blue), the ZIKV working reagents, and a negative control (Black).

Table 4. Genome Copy Number determined using ATCC ZIKV RNA Standards

Catalog Number	In-house Assay using ATCC® VR-1838DQ™ as a standard	CDC Assay using ATCC®VR-32525D™ as a standard

ATCC 10801 University Boulevard, Manassas, Virginia 20

Poster # 1272

Development of Synthetic Molecular Standards for Dengue Virus

ATCC® THE ESSENTIALS OF LIFE SCIENCE RESEARCH GLOBALLY DELIVERED™

Shamaila Ashraf, Melisa Wilson, Afshin Sohrabi, Stephen King, Brian Chase, Dev Mittar, Kurt Langenbach and Andrew G. Cawthon

Background & Introduction

Dengue fever is an acute illness caused by any one of four serotypes (1-4) of genetically related dengue viruses (DENV), with an estimated 390 million cases reported annually. Currently, quantitative RT-PCR (qRT-PCR) is the preferred method for the detection and quantification of DENV in clinical diagnostics and epidemiological surveillance. The accuracy of a qRT-PCR assay relies on the generation of a standard curve using a positive control with a known viral genome concentration.

Native DENV RNA can be used as a standard for these assays; however, the full-length dengue viral RNA is on the Commerce Control List and requires a permit from the US Department of Commerce for international shipment. To make DENV RNA standards more accessible, ATCC has developed four synthetic molecular standards that represent DENV serotypes 1-4. Each standard contains short fragments from the capsid, membrane, and envelope genes of the DENV genome, as well as target regions encompassing the primer sequences from numerous published RT-PCR assays, including the DENV-1-4 Real-Time RT-PCR Assay developed by the CDC[1]. The synthetic RNA standards were quantified by Droplet Digital™ PCR (ddPCR™) in order to package precise copies of RNA. Moreover, given the inherent labile nature of RNA, a stabilization matrix was added to the quantitated RNA preparation. As compared to native RNA, these synthetic standards are easier to use as controls for qRT-PCR assays, exhibit less variability, have a longer shelf life, eliminate the need to culture viruses and can be used under BSL-1 conditions. Further, this synthetic quantitative RNA approach can be extended to other pathogenic viruses which are uncultivable or need to be grown in a high-containment facility.

In the following proof-of-concept study, we amplified the synthetic molecular standards with the published primers from the CDC assay[1] and Waggoner *et al*[2], and used the generated standard curves to quantify viral RNA extracted from various DENV strains.

Materials and Methods

Viruses:
The following DENV strains, representing DENV serotypes 1, 2 and 4 were quantified using the standard curves generated from the DENV 1, 2 and 4 synthetic molecular standards, respectively:

Dengue virus 1 = TH-S-man (ATCC® VR-1586™)
Dengue virus 2 = New Guinea C (NR-84, BEI Resources)
Dengue virus 4 = H241 (ATCC® VR-1257™)

Viral RNA was extracted using the QIAamp® Viral RNA Mini Kit (QIAGEN®). Viral RNA was diluted 1:100, 1:1000, and 1:10,000 for the qRT-PCR assay. Uninfected Vero (ATCC® CCL-81™) and LLC-MK2 Derivative (ATCC® CCL-7.1™) cell lines were used as negative controls in the qRT-PCR assays.

qRT-PCR assay:
qRT-PCR assays were performed according to instructions provided[1], with slight modifications, using the CFX96™ Real-Time PCR Detection System (Bio-Rad®). Two different primer sets were employed to examine each individual Dengue serotype using the same conditions for both: the commercial primer set included in the CDC Real-Time RT-PCR assay[1] and the primer set published by Waggoner *et al*[2]. Cycling conditions for all primer sets were 50°C for 15 min and 95°C for 2 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 30 sec. The exception was for the DENV-4 published primer set; the annealing temperature was 55°C for 30 sec. Standard curves were generated using serial ten-fold dilutions of the synthetic RNA standards, ranging from 1 copy to 1×10^6 copies/µL. RNA samples and standards were tested in triplicate. The relative fluorescence unit (RFU) baseline threshold was calculated using CFX Manager™ 3.0 Software (Bio-Rad).

ATCC Design Strategy

ATCC Synthetic Molecular Standards	
DENV-1	ATCC® VR-3228SD™
DENV-2	ATCC® VR-3229SD™
DENV-3	ATCC® VR-3230SD™
DENV-4	ATCC® VR-3231SD™

Advantages	Applications
• ATCC fully authenticated & characterized Genome Nucleics • Consistent and accurate results • BSL-1 ready-to-use control • No shipping restrictions • Quantitative format • Stabilized RNA	• Generation of a standard curve for quantitative RT-PCR (qRT-PCR) • Positive control for RT-PCR assays • Independent standard for validation and verification studies • Monitor assay-to-assay and lot-to-lot variation

Generation of Standard Curves from DENV Synthetic Molecular Standards

Primer and Probe		DENV-1	DENV-2	DENV-4
CDC Assay[1]	Slope	-3.244	-3.277	-3.642
	R^2	0.99	0.996	0.996
Published Assay[2]	Slope	-3.536	-3.535	-3.775
	R^2	0.991	0.997	0.996

Figure 1. An example of A) amplification plot and B) standard curve generated using the DENV-4 molecular standard (ATCC® VR-32315D™) in conjunction with the CDC primer and probe set[1]. C) The slope and R^2 values generated using DENV 1, 2 and 4 molecular standards with the primer and probe sets from the CDC DENV-1-4 Real-Time RT-PCR Assay[1] and Waggoner *et al.* assay[2].

Quantification of Native DENV-1, 2 and 4 Using Synthetic Molecular Standards

Figure 2. A) An example of qRT-PCR amplification plot showing DENV-4 synthetic molecular standards (blue) and the native sample of unknown concentration (pink). Unknown samples were serial diluted ten-fold and run in triplicate wells. B) Copy numbers of DENV-1, 2 and 4 samples as determined by the qRT-PCR standard curves generated by using DENV (1, 2 and 4) molecular standards respectively.

References

1. CDC. DENV-1-4 Real-Time RT-PCR Assay for Detection and Serotype Identification of Dengue virus. Instructions for Use Package Insert, http://www.cdc.gov/dengue/resources/rt_pcrCDCPackageInsert.pdf
2. Waggoner JJ, *et al.* Single-reaction, multiplex, real-time RT-PCR for the detection, quantitation, and serotyping of dengue viruses. PLoS Negl Trop Dis 7(4): e2116, 2013.

Disclaimers

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www.atcc.org/resources

Summary

- ATCC Molecular Standards are a fast, easy, reliable control for assay development and validation & control.
- Genomic standards eliminate the costs of growth, extraction, and quantitation.
- Synthetic standards provide controls for organisms that are difficult to culture or extract.
- Over 230 standards currently in the portfolio.
  - Over 170 genomic standards
  - Over 60 synthetic standards
  - Standards for pathogens, microbiome, & food safety
  - And more to come!

Thank you to the project team!

ATCC R&D, Technical Transfer, and Marketing Teams

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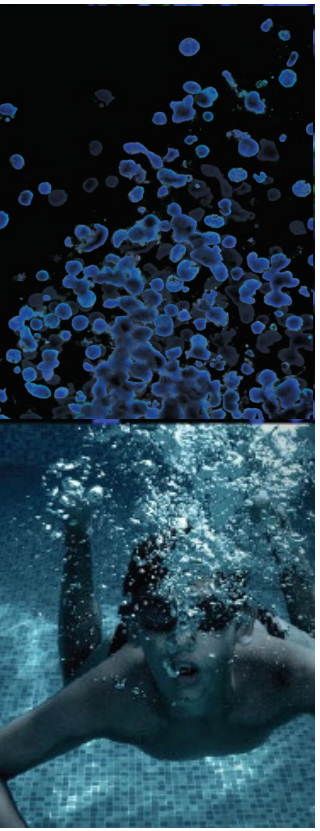

Questions?

Credible Leads to Incredible™