

TITLE

REAL TIME PCR METHOD TO DETECT BOVINE PARVOVIRUS 3

This application claims the benefit of U.S. Provisional Application No. 63/126,939, filed on
5 December 17, 2020, and U.S. Provisional Application No. 63/211,607, filed on June 17, 2021 which are
hereby incorporated by reference in its entirety and for all purposes as if fully set forth herein

The present application is being filed along with a Sequence Listing in electronic format. The Sequence
Listing is provided as a file entitled A-2741-WO-PCT_ST25.txt, created December 9, 2021, which is 6KB in
10 size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in its
entirety.

FIELD OF DISCLOSURE

The present invention relates to the field of biopharmaceutical manufacturing. In particular, the
invention provides a PCR assay for the qualitative or quantitative detection of *Bovine parvovirus 3* (BPV-3)
15 genomic DNA contamination in the extracted DNA of a test sample with an optional internal control.

BACKGROUND

Manufacturing therapeutic biological drugs using cell culture processes carries an inherent risk of
transmitting viral contaminants. Such contaminants can come from many sources, including materials and
equipment, the use of reagents of animal origin during manufacture, and through contamination of the
20 manufacturing system due to failures in the GMP process. Animal-derived raw materials, such as bovine serum
(FBS), are sometimes used as a component of cell culture-based manufacturing processes and are one of the
main sources for bovine-derived viral contamination in a biomanufacturing process. To meet the requirements
of regulatory health to ensure absence of adventitious agents in biotherapeutics derived from mammalian cells,
biomanufacturers need to take steps to detect, remove, and/or inactivate viral contamination. Raw materials
25 can be tested for viral contamination prior to use and remedial action taken. Depending on the biologic
molecule being manufactured, dedicated virus inactivation and removal steps can be added to the downstream
purification process to ensure viral safety of biotherapeutics.

The *Parvoviridae* family includes single stranded DNA (ssDNA) viruses with small, non-enveloped
capsids with T=1 icosahedral symmetry, known collectively as parvoviruses. Their diameter ranges between
30 18-26 nm. The viral capsid encompasses a ~5kb genome that encodes two main proteins, a non-structural (NS)
protein, and a structural capsid (VP) protein. Virus in the *Parvoviridae* family infect a wide range of hosts and

are divided into two subfamilies: the *Parvovirinae* and the *Densovirinae*, which infect vertebrate and arthropod hosts, respectively.

Bovine parvovirus 3 (BPV-3) belongs to the subfamily *Parvovirinae*, genus of *Erythroparvovirus* (species of *Ungulate erythroparvovirus 1*). While it is known that BPV-3 infect bovines, its pathogenesis and clinical manifestation remains unclear.

Per the requirements for testing in the Code of Federal Regulations (9 CFR113), the presence or absence of certain viruses in ingredients of animal origin used in production of biologics are required to be evaluated by incubating the animal-derived raw materials on specific indicator cells and which are subsequently observed for virus induced cytopathic effects and tested by hemadsorption or antibody fluorescence. Among the bovine parvoviruses, BPV-1 is capable of replicating in at least one of the selected indicator cell lines and can be detected by 9 CFR 113 test.

However, a permissive cell line to support BPV-3 replication has not been identified to date and, therefore, it cannot be detected by routine 9 CFR 113 testing. Although BPV-3 was detected by next-generation sequencing (NGS) a few years ago, it remains an emerging virus with limited information available. Traditional cell-culture based virus testing is not capable of supporting replication for BPV-3, thus, molecular biology assays are an essential alternative method to detect BPV-3 genomic DNA. The impact of BPV-3 in cell culture-based manufacturing remains unclear. There is a need for a rapid and specific method to detect the presence or absence of BPV-3 genomic DNA in raw materials such as fetal bovine serum (FBS) and/or unprocessed non-GMP bulk harvest samples containing FBS from cell culture-based manufacturing. The invention described herein meets this need.

BRIEF SUMMARY OF THE INVENTION

The invention provides a composition comprising oligonucleotides selected from the groups consisting of a) oligonucleotides having the nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3; b) oligonucleotides having the nucleic acid sequence of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6; c) oligonucleotides having the nucleic acid sequence of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; d) oligonucleotides having the nucleic acid sequence of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12; and e) oligonucleotides having the nucleic acid sequence of SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15; f) oligonucleotides having the nucleic acid sequence of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22; g) oligonucleotides having the nucleic acid sequence of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25; and h) oligonucleotides having the nucleic acid sequence of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28. In one embodiment the composition optionally includes a second composition comprising oligonucleotides having the sequence of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18. In one embodiment the

composition comprises oligonucleotides having the nucleic acid sequence of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9. In one embodiment the composition comprises a first composition comprises oligonucleotides having the nucleic acid sequence of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9 and a second composition comprises oligonucleotides having the sequence of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18.

5 The invention provides a reagent for detecting DNA encoding *Bovine parvovirus 3* (BPV-3) genomic DNA in the extracted DNA of a test sample selected from the group consisting of a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3; b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6; c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; d)
10 a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12; and e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15; f) a primer probe combination having the nucleic acid sequences of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22; g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25; and h) a primer probe combination having the nucleic acid
15 sequences of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28.

In one embodiment SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:22, SEQ ID NO:25; or SEQ ID NO:28 have a fluorescent reporter dye and/or a non-fluorescent quencher. In a related embodiment one or more of SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:22, SEQ ID NO:25; or SEQ ID NO:28 have a fluorescent reporter dye 6-
20 carboxyfluorescein (FAM) at the 5' end and/or either a Minor Groove Binder non-fluorescence quencher (MGB-NFQ) or ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end. In one embodiment the primer probe combination detects DNA encoding the structural capsid (VP) protein and/or the non-structural (NS) protein of *Bovine parvovirus 3* in a test sample. In one embodiment the reagent in combination with an internal positive control primer combination. In one embodiment the reagent comprises SEQ ID NO:7, SEQ
25 ID NO:8, and SEQ ID NO:9.

The invention provides a reagent for use as an internal positive control primer probe combination in an assay for detecting *Bovine parvovirus 3* (BPV-3) genomic DNA in the extracted DNA of a test sample comprising a primer probe combination. In a related embodiment the internal positive control primer probe combination has the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18. In one
30 embodiment SEQ ID NO: 18 has a fluorescent reporter dye and/or a non-fluorescent quencher. In a related embodiment SEQ ID NO: 18 has a fluorescent reporter dye, 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment the reagent comprises SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9.

The invention provides a primer probe combination for detecting *Bovine parvovirus 3* genomic DNA in the extracted DNA of a test sample in combination with an internal positive control primer probe combination for detecting *Bovine parvovirus 3* (BPV-3) genomic DNA. In one embodiment the primer probe combination is selected from the groups consisting of a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3; b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6; c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; d) a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12; e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15; f) a primer probe combination having the nucleic acid sequences of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22; g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25; and h) a primer probe combination having the nucleic acid sequences of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28. In one embodiment SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, or SEQ ID NO:15 have a fluorescent reporter dye and/or a non-fluorescent quencher. In one embodiment one or more of SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:22, SEQ ID NO:25; or SEQ ID NO:28 have a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and/or either a Minor Groove Binder non-fluorescence quencher (MGB-NFQ) or ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end. In one embodiment the primer probe combination detects DNA encoding the structural capsid (VP) protein and/or the non-structural (NS) protein of *Bovine parvovirus 3* in a test sample. In one embodiment an internal positive control primer probe combination having the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18 is used in combination with a primer probe combination used for detecting *Bovine parvovirus 3* (BPV-3) genomic DNA in a test sample as described above. In a related embodiment SEQ ID NO: 20 has a fluorescent reporter dye and/or a non-fluorescent quencher. In a related embodiment SEQ ID NO: 18 has a fluorescent reporter dye, 2'-chloro-7-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end.

The invention provides a primer probe combination for detecting DNA encoding *Bovine parvovirus 3* genomic DNA in the extracted DNA of a test sample comprising SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9 in combination with an internal positive control primer probe combination comprising SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18, wherein SEQ ID NO: 9 has a fluorescent reporter dye 6-carboxyfluorescein at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) non-fluorescence quencher at the 3' end and SEQ ID NO:18 has a fluorescent reporter dye, 2'-chloro-7-phenyl-1,4-dichloro-6-carboxy-fluorescein at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end.

The invention provides a kit for detecting *Bovine parvovirus 3* (BPV-3) genomic DNA contamination in the extracted DNA of a test sample comprising primer probe combination that detects DNA encoding BPV-3 selected from the group consisting of a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3; b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6; c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; d) a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12; and e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15; f) a primer probe combination having the nucleic acid sequences of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22; g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25; and h) a primer probe combination having the nucleic acid sequences of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28; and optionally an internal positive control primer probe combination having the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18. In one embodiment the kit comprises a primer probe combination that detects DNA encoding BPV-3 selected from the group consisting of a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3; b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6; c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; d) a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12; and e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15; f) a primer probe combination having the nucleic acid sequences of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22; g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25; and h) a primer probe combination having the nucleic acid sequences of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28; and an internal positive control primer probe combination having the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18.

The invention provides a method for determining the presence or absence of *Bovine parvovirus 3* genomic DNA in the extracted DNA of a test sample comprising 1) a reaction mixture comprising a test sample, a positive control, a BPV-3_IPC positive control plasmid DNA, nucleic acid amplification reagents, an internal positive control primer probe combination having the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18, wherein SEQ ID NO:18 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end, and a primer probe combination selective for a DNA sequence of *Bovine parvovirus 3*, selected from the group consisting of a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3, wherein SEQ ID NO:3 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6, wherein SEQ ID NO:6 has a

fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9, wherein SEQ ID NO:9 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; d) a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12, wherein SEQ ID NO:12 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15, wherein SEQ ID NO:15 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25, wherein SEQ ID NO:25 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; and h) a primer probe combination having the nucleic acid sequences of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28, wherein SEQ ID NO:28 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; 2) subjecting the reaction mixture to a quantitative PCR technique to obtain copies of the target sequence, 2) subjecting the reaction mixture to a quantitative PCR technique to obtain copies of the target sequence, 3) measuring any increase in fluorescence signal, wherein an increase in fluorescence signal indicates the presence of *Bovine parvovirus 3* genomic DNA in the test sample. In one embodiment the fluorescent reporter dye is 6-carboxyfluorescein (FAM) and the non-fluorescence quencher is a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) or ZEN-IB and Iowa Black Fluorescence quencher (IBFQ). In one embodiment the invention further comprises one or more negative extraction control, no template control, positive extraction control, positive control, and/or inhibition control. In one embodiment the sensitivity or analytical limit of detection is 22 genome copies per reaction. In one embodiment the sample limit of detection is 25 genome copies per reaction. In one embodiment the primer probe combination selective for a DNA sequence of *Bovine parvovirus 3* detects genomic DNA encoding the non-structural (NS) protein and/or structural capsid (VP) protein of *Bovine parvovirus 3*. In one embodiment the primer probe combination selective for a DNA sequence of *Bovine parvovirus 3* amplifies a 144 bp fragment. In one embodiment the primer probe combination selective for a DNA sequence of *Bovine parvovirus 3* comprises the combination of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9, wherein SEQ ID NO:9 has a 6-carboxyfluorescein (FAM) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment the method further comprises an internal positive control primer probe combination. In a related embodiment the primer probe combination has the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18. In a related embodiment SEQ ID NO: 18 has a fluorescent reporter dye and/or a non-fluorescent quencher. In a related embodiment SEQ ID NO: 18 has a fluorescent reporter dye, 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end.

The invention provides a method for the quantification of 1E3 to 1E8 genome copies of *Bovine parvovirus 3* genomic DNA in a PCR reaction comprising 1) a reaction mixture comprising the extracted DNA

of a test sample, a positive control, a BPV-3_IPC positive control plasmid DNA, nucleic acid amplification reagents, an internal positive control primer probe combination having the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18, wherein SEQ ID NO:18 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end, and a primer probe combination selective for a DNA sequence of *Bovine parvovirus 3*, selected from the group consisting of a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3, wherein SEQ ID NO:3 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6, wherein SEQ ID NO:6 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9, wherein SEQ ID NO:9 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; d) a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12, wherein SEQ ID NO:12 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15, wherein SEQ ID NO:15 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; f) a primer probe combination having the nucleic acid sequences of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22, wherein SEQ ID NO:22 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25, wherein SEQ ID NO:25 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; and h) a primer probe combination having the nucleic acid sequences of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28, wherein SEQ ID NO:28 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; 2) subjecting the reaction mixture to a quantitative PCR technique to obtain copies of the target sequence, and 3) measuring any increase in fluorescence signal. In one embodiment the limit of detection (LOD_{95%}) of the method is 27 genome copies of *Bovine parvovirus 3* genomic DNA per reaction with a 95% confidence interval of 22 and 34 genome copies per reaction. In one embodiment the linearity of the method has a correlation coefficient (R^2) ≥ 0.98 and a PCR amplification efficiency within 90-110%. In one embodiment the method has a repeatability value that is a %CV of quantity equal or less than 25%. In one embodiment the method has an intermediate precision value that is %CV of quantity equal or less than 30%. In one embodiment the method has an accuracy value within $\pm 30\%$ of the accepted reference value (ST) across the whole dynamic range of the assay. In one embodiment the method has a limit of quantitation that is the %CV of quantity for repeatability at $\leq 25\%$, intermediate precision at $\leq 30\%$ and acceptance criterion for the accuracy within $\pm 30\%$ of the expected standard reference value. In another embodiment the method has a robustness that has a percent CV of quantity for repeatability of $\leq 25\%$, an intermediate precision of $\leq 30\%$, and an accuracy of the mean of quantity of the combination matrix condition tested of $\pm 30\%$ of the mean of quantity of the optimized

condition. In another embodiment the method includes one or more of a no template control, a positive control, a negative extraction control, a positive extraction control, an inhibition control, an internal positive control, and a standard.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1. The Probit analysis used for determination of ALOD in BPV-3 qPCR with 95% of probability (LOD95%).

Figure 2. POD graph and LOD95% analysis results.

DETAILED DESCRIPTION OF THE INVENTION

As described herein, a qualitative real-time PRC method has been developed to detect potential *Bovine*
10 *parvovirus 3* (BPV-3) genomic DNA contamination in the extracted of test samples. Qualification parameters, including specificity, limit of detection (LOD), robustness and repeatability were used to test and confirm the performance of the assay.

The assay makes use of optimized primer probe combinations for detecting *Bovine parvovirus 3* (BPV-3) genomic DNA in the extracted DNA of a test sample. The BPV-3 PCR primers amplify conserved regions
15 in the genes encoding the BPV-3 non-structural (NS) protein and/or structural capsid (VP) protein in the BPV-3 genome. “Method Validation of U.S. Environmental Protection Agency (EPA) Microbiological Methods of Analysis”, REVISION: December 21, 2016, was consulted and considered in the design and development of the assay.

The invention provides oligonucleotide primer probe combinations that can be used in detection
20 methods, such as in quantitative polymerase chain reaction (qPCR) techniques, to detect DNA encoding *Bovine parvovirus 3* genomic (BPV-3) and for use in methods for determining the presence or absence of BPV-3 in the extracted DNA of a test sample.

As used herein, “oligonucleotides” are short single stranded synthetic DNA or RNA molecules, less than 200 nucleotides in length, typically in the range of 13-25 nucleotides in length. Oligonucleotides bind to
25 their complement oligonucleotide to form a duplex. As a result, oligonucleotides are commonly used in a variety of applications where detection of the presence or absence of specific DNA or RNA sequences is desired. In particular, oligonucleotides are useful as primers for use in polymerase chain reactions (PCR). Methods to synthesize oligonucleotides are known in the art, equipment to produce oligonucleotides are commercially available and there are service providers who will make custom oligonucleotides on demand.
30 Oligonucleotides can also be obtained from the breakdown of larger nucleic acid molecules, or naturally occurring oligonucleotides, such as micro RNA.

The invention provides a composition comprising oligonucleotides selected from the groups consisting of a) oligonucleotides having the nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3; b) oligonucleotides having the nucleic acid sequence of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6; c) oligonucleotides having the nucleic acid sequence of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; d) oligonucleotides having the nucleic acid sequence of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12; e) oligonucleotides having the nucleic acid sequence of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15; f) oligonucleotides having the nucleic acid sequence of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22; g) oligonucleotides having the nucleic acid sequence of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25; and h) oligonucleotides having the nucleic acid sequence of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28.

In one embodiment the composition comprises oligonucleotides having the nucleic acid sequence of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9.

In one embodiment the composition optionally comprises a second composition comprising oligonucleotides having the sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18 in combination with one or more of the primer probe combination mentioned above. In one embodiment the composition comprises oligonucleotides having the nucleic acid sequence of SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18. In one embodiment the composition comprises a first composition comprises oligonucleotides having the nucleic acid sequence of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9 and a second composition comprises oligonucleotides having the sequence of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18.

In one embodiment one or more of SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:18, SEQ ID NO: 22, SEQ ID NO: 25, and SEQ ID NO: 28, may include at least one fluorescent reporter dye at the 5' end and/or at least one non-fluorescence quencher at the 3' end. In one embodiment one or more of SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:18, SEQ ID NO: 22, SEQ ID NO: 25, and SEQ ID NO: 28, have at least one fluorescent reporter dye at the 5' end and at least one non-fluorescence quencher at the 3' end. In one embodiment one or more of SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:18, SEQ ID NO: 22, SEQ ID NO: 25, and SEQ ID NO: 28, have a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end.

In one embodiment one or more of SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:18, SEQ ID NO: 22, SEQ ID NO: 25, and SEQ ID NO: 28, have a fluorescent reporter dye selected from 6-carboxyfluorescein (FAM) or 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end. In one embodiment SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO: 22, SEQ ID NO: 25, and SEQ ID NO: 28, have a fluorescent reporter dye 6-

carboxyfluorescein (FAM) at the 5' end. In one embodiment, SEQ ID NO:18 has the fluorescent reporter dye 2'-chloro-7'phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end.

In one embodiment one or more of SEQ ID NO:3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO: 18, SEQ ID NO: 22, SEQ ID NO: 25, and SEQ ID NO: 28, have a non-fluorescent quencher at the 3' end. In one embodiment the quencher is selected from a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) and ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end. In one embodiment one or more of SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, and SEQ ID NO: 18 have a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment one or more of SEQ ID NO: 3, SEQ ID NO: 22, SEQ ID NO: 25, and SEQ ID NO: 28, has ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end.

In one embodiment SEQ ID NO: 3 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end. In one embodiment SEQ ID NO: 6 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment SEQ ID NO: 9 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment SEQ ID NO: 12 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment SEQ ID NO: 15 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment SEQ ID NO: 18 has a fluorescent reporter dye 2'-chloro-7'phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment SEQ ID NO: 22 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end. In one embodiment SEQ ID NO: 25 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end. In one embodiment SEQ ID NO: 28 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end.

The invention provides a reagent for use in detecting *Bovine parvovirus 3* (BPV-3) genomic DNA. This reagent can be used for determining the presence or absence of *Bovine parvovirus 3* genomic DNA in the extracted DNA of a test sample.

As used herein, "test sample" means any sample of extracted DNA for which a determination of the presence or absence of BPV-3 is desired. The test sample may be known or suspected to contain BPV-3. Test samples may come from raw materials, such as those used in the manufacture of biotherapeutics. Raw materials include those known or suspected of having an animal origin or contain components from an animal origin, particularly a bovine origin or known or suspected of having been in contact with other materials that

have an animal, particularly a bovine origin, including such raw materials as fetal bovine serum and fetal calf serum. Test samples may also come from cell culture media, particularly cell culture media containing fetal bovine or fetal calf serum. The media can be obtained prior to or during cell culture, or after harvest of cell culture media from a cell culture operation. Periodic samples may be taken during cell culture, this may be multiple times a day, daily, at critical points during the culture, particularly at the start and harvest of the culture. Test samples may also come from cell lines used in the manufacture of biotherapeutics, including cells and cell lines of a bovine origin. Test samples may also come from samples taken during downstream processing, for example from the eluate from downstream purification steps, samples can be taken from the drug substance, and from the drug product.

The invention provides a reagent for detecting *Bovine parvovirus 3* (BPV-3) genomic DNA in the extracted DNA of a test sample selected from the group consisting of a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3; b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6; c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; d) a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12; and e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15. In one embodiment the primer probe combination comprises the nucleic acid sequence of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9.

In one embodiment, the reagent optionally includes an internal positive control primer probe combination. In one embodiment, the reagent further comprises an internal positive control primer probe combination. In one embodiment the internal positive control primer probe combination comprises the nucleic acid of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18. In one embodiment the reagent a primer probe combination having the nucleic acid sequence of SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and a primer probe combination having the nucleic acid sequence of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18.

In one embodiment one or more of SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, or SEQ ID NO:18 may include at least one fluorescent reporter dye at the 5' end and/or at least one non-fluorescence quencher at the 3' end. In a related embodiment one or more of SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15 or SEQ ID NO:18 have at least one fluorescent reporter dye at the 5' end and at least one non-fluorescence quencher at the 3' end. In a related embodiment one or more of SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, and SEQ ID NO:18 have a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end.

In one embodiment one or more of SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, and SEQ ID NO:18 have a fluorescent reporter dye selected from 6-carboxyfluorescein (FAM) or 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end. In one embodiment SEQ ID NO: 3,

SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, and SEQ ID NO:15 have a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end. In one embodiment, SEQ ID NO:18 has the fluorescent reporter dye 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end.

5 In one embodiment one or more of SEQ ID NO:3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, or SEQ ID NO: 18 have a non-fluorescent quencher at the 3' end. In one embodiment the quencher is selected from a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) and ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end. In one embodiment one or more of SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, and SEQ ID NO: 18 have a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment SEQ ID NO: 3 has ZEN-IB and Iowa Black Fluorescence
10 quencher (IBFQ) at the 3' end.

In one embodiment SEQ ID NO: 3 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end. In one embodiment SEQ ID NO: 6 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment SEQ ID NO: 9 has a fluorescent
15 reporter dye 6-carboxyfluorescein (FAM) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment SEQ ID NO: 12 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment SEQ ID NO: 15 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment
20 SEQ ID NO: 18 has a fluorescent reporter dye 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end.

The invention provides primer probe combinations that targets the structural capsid (VP) protein of BPV-3. In one embodiment, the primer probe combination comprises a forward primer that targets oligo position 3535-3554 of the BPV-3 isolate having the GenBank accession number AF406967, a reverse primer
25 that targets oligo position 3667-3643 of the BPV-3 isolate having the GenBank accession number AF406967, and a probe that targets oligo position 3571-3588 of the BPV-3 isolate having the GenBank accession number AF406967. In one embodiment, the primer probe combination the forward primer has the nucleic acid sequence of SEQ ID NO:1, the reverse primer has the nucleic acid sequence of SEQ ID NO:2, and the probe has the nucleic acid sequence of SEQ ID NO:3. In one embodiment, the primer probe combination comprises a forward
30 primer that targets oligo position 1734-1716 of the BPV-3 isolate having the GenBank accession number AF406967, a reverse primer that targets oligo position 1618-1645 of the BPV-3 isolate having the GenBank accession number AF406967, and a probe that targets oligo position 1665-1678 of the BPV-3 isolate having the GenBank accession number AF406967. In one embodiment, the primer probe combination the forward primer has the nucleic acid sequence of SEQ ID NO:13, the reverse primer has the nucleic acid sequence of

SEQ ID NO:14, and the probe has the nucleic acid sequence of SEQ ID NO:15. In one embodiment, the primer probe combination comprises a forward primer that targets oligo position 2862-2878 of the BPV-3 isolate having the GenBank accession number AF406967, a reverse primer that targets oligo position 2963-2938 of the BPV-3 isolate having the GenBank accession number AF406967, and a probe that targets oligo position 2902-2930 of the BPV-3 isolate having the GenBank accession number AF406967. In one embodiment, the primer probe combination the forward primer has the nucleic acid sequence of SEQ ID NO:20, the reverse primer has the nucleic acid sequence of SEQ ID NO:21, and the probe has the nucleic acid sequence of SEQ ID NO:22. In one embodiment, the primer probe combination comprises a forward primer that targets oligo position 3051-3068 of the BPV-3 isolate having the GenBank accession number AF406967, a reverse primer that targets oligo position 3134-3114 of the BPV-3 isolate having the GenBank accession number AF406967, and a probe that targets oligo position 3079-3102 of the BPV-3 isolate having the GenBank accession number AF406967. In one embodiment, the primer probe combination the forward primer has the nucleic acid sequence of SEQ ID NO:23, the reverse primer has the nucleic acid sequence of SEQ ID NO:24, and the probe has the nucleic acid sequence of SEQ ID NO:25. In one embodiment, the primer probe combination comprises a forward primer that targets oligo position 3061-3079 of the BPV-3 isolate having the GenBank accession number AF406967, a reverse primer that targets oligo position 3140-3122 of the BPV-3 isolate having the GenBank accession number AF406967, and a probe that targets oligo position 3081-3103 of the BPV-3 isolate having the GenBank accession number AF406967. In one embodiment, the primer probe combination the forward primer has the nucleic acid sequence of SEQ ID NO:26, the reverse primer has the nucleic acid sequence of SEQ ID NO:27, and the probe has the nucleic acid sequence of SEQ ID NO:28.

The invention provides primer probe combinations that targets the non-structural (NS) protein and structural capsid (VP) protein of BPV-3. In one embodiment, the primer probe combination comprises a forward primer that targets oligo position 2190-2209 of the BPV-3 isolate having the GenBank accession number AF406967, a reverse primer that targets oligo position 2256-2236 of the BPV-3 isolate having the GenBank accession number AF406967, and a probe that targets oligo position 2213-2226 of the BPV-3 isolate having the GenBank accession number AF406967. In one embodiment the forward primer has the nucleic acid sequence of SEQ ID NO:4, the reverse primer has the nucleic acid sequence of SEQ ID NO:5, and the probe has the nucleic acid sequence of SEQ ID NO:6.

The invention provides primer probe combinations that targets the non-structural (NS) protein of BPV-3. In one embodiment, the primer probe combination comprises a forward primer that targets oligo position 1331-1352 of the BPV-3 isolate having the GenBank accession number AF406967, a reverse primer that targets oligo position 1474-1453 of the BPV-3 isolate having the GenBank accession number AF406967, and a probe that targets oligo position 1377-1391 of the BPV-3 isolate having the GenBank accession number AF406967. In one embodiment the forward primer has the nucleic acid sequence of SEQ ID NO:7, the reverse primer has

the nucleic acid sequence of SEQ ID NO:8, and the probe has the nucleic acid sequence of SEQ ID NO:9. In one embodiment, the primer probe combination comprises a forward primer that targets oligo position 1453-1474 of the BPV-3 isolate having the GenBank accession number AF406967, a reverse primer that targets oligo position 1562-1539 of the BPV-3 isolate having the GenBank accession number AF406967, and a probe that targets oligo position 1507-1522 of the BPV-3 isolate having the GenBank accession number AF406967. In one embodiment the forward primer has the nucleic acid sequence of SEQ ID NO:10, the reverse primer has the nucleic acid sequence of SEQ ID NO:11, and the probe has the nucleic acid sequence of SEQ ID NO:12.

In one embodiment, the reagent for detecting BPV-3 genomic DNA in a sample is used in combination with an internal positive control primer combination. The invention provides a primer probe combination for detecting DNA encoding *Bovine parvovirus 3* genomic DNA in the extracted DNA of a test sample in combination with an internal positive control primer probe combination comprising SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18. The invention provides a primer probe combination for detecting DNA encoding *Bovine parvovirus 3* genomic DNA in the extracted DNA of a test sample comprising SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9 in combination with an internal positive control primer probe combination comprising SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18, wherein SEQ ID NO: 9 has a fluorescent reporter dye 6-carboxyfluorescein at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) non-fluorescence quencher at the 3' end and SEQ ID NO:18 has a fluorescent reporter dye, 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment SEQ ID NO:18 has a fluorescent reporter dye and/or a non-fluorescent quencher. In one embodiment SEQ ID NO:18 has a fluorescent reporter dye 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end.

The invention provides a primer probe combination for detecting *Bovine parvovirus 3* genomic DNA in the extracted DNA of a test sample in combination with an internal positive control primer probe combination for detecting *Bovine parvovirus 3* (BPV-3) genomic DNA. In one embodiment the primer probe combination for detecting *Bovine parvovirus 3* genomic DNA as described above wherein the primer probe combination is selected from the groups consisting of a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3; b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6; c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; d) a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12; e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15; f) a primer probe combination having the nucleic acid sequences of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22; g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:23, SEQ ID NO:24,

and SEQ ID NO:25; and h) a primer probe combination having the nucleic acid sequences of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28. In one embodiment the primer probe combination wherein SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:22, SEQ ID NO:25, and SEQ ID NO:28 have a fluorescent reporter dye and/or a non-fluorescent quencher. In one embodiment, the primer probe combination wherein one or more of SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:22, SEQ ID NO:25, and SEQ ID NO:28 have a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and/or either a Minor Groove Binder non-fluorescence quencher (MGB-NFQ) or ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end. In one embodiment the primer probe combination detects DNA encoding the structural capsid (VP) protein and/or the non-structural (NS) protein of *Bovine parvovirus 3* in the test sample.

In one embodiment an internal positive control primer probe combination for detecting *Bovine parvovirus 3* (BPV-3) genomic DNA in the extracted DNA of a test sample in combination with a primer probe combination for detecting *Bovine parvovirus 3* genomic DNA, wherein the primer probe combination has the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18. In one embodiment SEQ ID NO: 18 has a fluorescent reporter dye and/or a non-fluorescent quencher. In one embodiment SEQ ID NO: 18 has a fluorescent reporter dye, 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end.

Also provided is a primer probe combination for detecting *Bovine parvovirus 3* genomic DNA in the extracted DNA of a test sample comprising SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9 in combination with an internal positive control primer probe combination comprising SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18, wherein SEQ ID NO: 9 has a fluorescent reporter dye 6-carboxyfluorescein at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) non-fluorescence quencher at the 3' end and SEQ ID NO:18 has a fluorescent reporter dye, 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end.

The invention provides a method for determining the presence or absence of *Bovine parvovirus 3* genomic DNA in the extracted DNA of a test sample. The method includes a reaction mixture comprising the extracted DNA of a test sample, nucleic acid amplification reagents, a primer probe combination selective for *Bovine parvovirus 3* genomic DNA, and optionally an internal positive control primer probe combination; subjecting the reaction mixture to a real-time PCR technique (qPCR) to obtain copies of the target sequence, measuring any increase in fluorescence signal, wherein an increase in fluorescence signal indicates the presence of *Bovine parvovirus 3* genomic DNA in the test sample. The reagents described herein can also be used for quantitative of the BPV-3 genome copy number in a test sample, for example, to determine the level of positivity, the viral load, in terms of viral genome quantity per ml of test sample.

The reaction mixture comprises the components to needed perform the quantitative PCR technique. Standard master mixes, component mixes and the like are commercially available, 2X TAQMAN[®] Universal PCR Master Mix (Applied Biosystems). The components typically include dNTPs (dATP, dCTP, dGTP, dTTP or dUTP), magnesium, TAQ DNA polymerase, buffers, and loading dyes if required by the PCR thermocycler being used. Others include Quantabio, PerfeCTa qPCR SuperMix, Low ROX (Quantabio, Beverly, MA). There are also a variety of commercially available thermocyclers. One of skill in the art would be able to determine which met their needs.

Real time PCR or qPCR is a technique that requires relatively small amounts of DNA, cDNA or RNA that can be quantified and facilitates monitoring the progress of a PCR in real time, as the reaction progresses. This PCR technique makes use of combinations of oligonucleotide primers and dual-labeled oligonucleotide probes. The probes act as a reporter, if amplified they accumulate with each cycle of the PCR reaction.

Specific detection of amplified product can be performed using one or more oligonucleotide probes that are labeled with a reporter fluorescent dye and a quencher dye. Such probes are known to those skilled in the art and are commercially available, including molecular beacons, dual-labeled probes, FRET (fluorescence resonance energy transfer) probes, and Scorpion[®] probes. Oligonucleotide probes can be labeled with a reporter fluorescent dye and one or more quencher dyes. Examples of fluorescent reporter dyes include 6-carboxyfluorescein (FAM or 6-FAM), 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC), TET[™], HEX[™], JOE, Cy[®] 3, CY[®] 5, CY[®] 5.5, TAMRA, ROX[™], LC Red 610, Texas Red[®], LC640, SUN[™], MAX[™], ATTO[™] 550, ATTO 647[™], Cal Fluor Gold 540 and Orange 560, TxRd (Sulforhodamine 101-X), Quasar 570 and 670. Examples of fluorescent quenchers include a Minor Groove Binder (MGB-NFQ), ZEN-IB, Black Hole Quencher[®] (BHQ 1, 2 and 3), TAMRA, Iowa Black[®] FQ and RQ.

For example, dual labeled probes may be labeled with one or more fluorescent reporter dyes at the 5' end which fluoresce in presence of a complementary target and one or more non-fluorescence quenchers at the 3' end. The dual-labeled probes are designed to hybridize to the template between the two primers and are used in conjunction with a DNA polymerase enzyme that has inherent 5' to 3' endonuclease activity. When the probe is intact, the fluorescence of the reporter dye is quenched by the proximity of the quencher. During the extension phase of each PCR cycle, the 5' exonuclease activity of DNA polymerase enzyme cleaves the annealed probe, releasing the reporter dye from the probe, resulting in an increase in fluorescence. This increase in fluorescence is directly proportional to the amount of amplified target DNA present in the reaction. The fluorescence is continually monitored throughout the PCR reaction. During the early cycles of the PCR reaction, the amount of fluorescence is below the detection threshold of the instrument. Monitoring for fluorescence signal continues, the first PCR cycle in which fluorescence is detected is noted. The more target

DNA present in the sample at the outset of the reaction, the earlier fluorescence is detected, which reversely correlates with the sample target DNA quantity.

In one embodiment the primer probe combination selective for a DNA sequence of *Bovine parvovirus* 3 amplifies a 144 bp fragment. In one embodiment, the primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9 amplifies a 144 bp fragment.

The reaction mixture also includes a primer probe combination selective for a DNA sequence of *Bovine parvovirus* 3. In one embodiment the primer probe combination is selected from the group consisting of a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3, wherein SEQ ID NO:3 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6, wherein SEQ ID NO:6 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9, wherein SEQ ID NO:9 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; d) a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12, wherein SEQ ID NO:12 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15, wherein SEQ ID NO:15 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; f) a primer probe combination having the nucleic acid sequences of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22, wherein SEQ ID NO:22 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25, wherein SEQ ID NO:25 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; and h) a primer probe combination having the nucleic acid sequences of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28, wherein SEQ ID NO:28 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end.

In one embodiment, the reaction mixture optionally includes an internal positive control primer probe combination. In one embodiment the mixture includes an internal positive control primer probe combination. In one embodiment the internal positive control primer probe combination has the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18, wherein SEQ ID NO:18 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end.

In one embodiment the method further comprising an internal positive control primer probe combination. In one embodiment the primer probe combination has the nucleic acid sequences of SEQ ID NO:

16, SEQ ID NO:17, and SEQ ID NO:18. In one embodiment SEQ ID NO: 18 has a fluorescent reporter dye and/or a non-fluorescent quencher. In one embodiment SEQ ID NO: 18 has a fluorescent reporter dye, 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end.

5 In one embodiment one or more of SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:18, SEQ ID NO: 22, SEQ ID NO: 25, and SEQ ID NO: 28 have a fluorescent reporter dye selected from 6-carboxyfluorescein (FAM) or 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end. In one embodiment SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO: 22, SEQ ID NO: 25, and SEQ ID NO: 28 have a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end. In one embodiment, SEQ ID NO:18 has the fluorescent reporter dye 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end.

In one embodiment one or more of SEQ ID NO:3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO: 18, SEQ ID NO: 22, SEQ ID NO: 25, and SEQ ID NO: 28 have a non-fluorescent quencher at the 3' end. In one embodiment the quencher is selected from a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) and ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end. In one embodiment one or more of SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, and SEQ ID NO: 18 have a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment one or more of SEQ ID NO: 3, SEQ ID NO: 22, SEQ ID NO: 25, and SEQ ID NO: 28 has ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end.

20 In one embodiment SEQ ID NO: 3 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end. In one embodiment SEQ ID NO: 6 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment SEQ ID NO: 9 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment SEQ ID NO: 12 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment SEQ ID NO: 15 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment SEQ ID NO: 18 has a fluorescent reporter dye 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end. In one embodiment SEQ ID NO: 22 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end. In one embodiment SEQ ID NO: 25 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and ZEN-IB and Iowa Black Fluorescence quencher

(IBFQ) at the 3' end. In one embodiment SEQ ID NO: 28 has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end.

In one embodiment the primer probe combination targets the non-structural (NS) protein of BPV-3. In one embodiment the primer probe combinations target the non-structural (NS) protein and structural capsid (VP) protein of BPV-3. In one embodiment the primer probe combinations target the structural capsid (VP) protein of BPV-3.

In one embodiment the primer probe combinations target the structural capsid (VP) protein of BPV-3. In one embodiment, the primer probe combination comprises a forward primer that targets oligo position 3535-3554 of the BPV-3 isolate having the GenBank accession number AF406967, a reverse primer that targets oligo position 3667-3643 of the BPV-3 isolate having the GenBank accession number AF406967, and a probe that targets oligo position 3571-3588 of the BPV-3 isolate having the GenBank accession number AF406967. In one embodiment, in the primer probe combination the forward primer has the nucleic acid sequence of SEQ ID NO:1, the reverse primer has the nucleic acid sequence of SEQ ID NO:2, and the probe has the nucleic acid sequence of SEQ ID NO:3. In one embodiment, in the primer probe combination comprises a forward primer that targets oligo position 1734-1716 of the BPV-3 isolate having the GenBank accession number AF406967, a reverse primer that targets oligo position 1618-1645 of the BPV-3 isolate having the GenBank accession number AF406967, and a probe that targets oligo position 1665-1678 of the BPV-3 isolate having the GenBank accession number AF406967. In one embodiment, in the primer probe combination the forward primer has the nucleic acid sequence of SEQ ID NO:13, the reverse primer has the nucleic acid sequence of SEQ ID NO:14, and the probe has the nucleic acid sequence of SEQ ID NO:15. In one embodiment, in the primer probe combination comprises a forward primer that targets oligo position 2862-2878 of the BPV-3 isolate having the GenBank accession number AF406967, a reverse primer that targets oligo position 2963-2938 of the BPV-3 isolate having the GenBank accession number AF406967, and a probe that targets oligo position 2902-2930 of the BPV-3 isolate having the GenBank accession number AF406967. In one embodiment, in the primer probe combination the forward primer has the nucleic acid sequence of SEQ ID NO:20, the reverse primer has the nucleic acid sequence of SEQ ID NO:21, and the probe has the nucleic acid sequence of SEQ ID NO:22. In one embodiment, in the primer probe combination comprises a forward primer that targets oligo position 3051-3068 of the BPV-3 isolate having the GenBank accession number AF406967, a reverse primer that targets oligo position 3134-3114 of the BPV-3 isolate having the GenBank accession number AF406967, and a probe that targets oligo position 3079-3102 of the BPV-3 isolate having the GenBank accession number AF406967. In one embodiment, in the primer probe combination the forward primer has the nucleic acid sequence of SEQ ID NO:24, the reverse primer has the nucleic acid sequence of SEQ ID NO:25, and the probe has the nucleic acid sequence of SEQ ID NO:26. In one embodiment, in the primer probe combination comprises a forward primer that targets oligo position 3061-3079 of the BPV-3 isolate having the GenBank accession number AF406967,

a reverse primer that targets oligo position 3140-3122 of the BPV-3 isolate having the GenBank accession number AF406967, and a probe that targets oligo position 3081-3103 of the BPV-3 isolate having the GenBank accession number AF406967. In one embodiment, in the primer probe combination the forward primer has the nucleic acid sequence of SEQ ID NO:26, the reverse primer has the nucleic acid sequence of SEQ ID NO:27,
5 and the probe has the nucleic acid sequence of SEQ ID NO:28.

In one embodiment the primer probe combinations target the structural capsid (VP) protein and the non-structural (NS) protein of BPV-3. In one embodiment, the primer probe combination comprises a forward primer that targets oligo position 2190-2209 of the BPV-3 isolate having the GenBank accession number AF406967, a reverse primer that targets oligo position 2256-2236 of the BPV-3 isolate having the GenBank
10 accession number AF406967, and a probe that targets oligo position 2213-2226 of the BPV-3 isolate having the GenBank accession number AF406967. In one embodiment the forward primer has the nucleic acid sequence of SEQ ID NO:4, the reverse primer has the nucleic acid sequence of SEQ ID NO:5, and the probe has the nucleic acid sequence of SEQ ID NO:6.

In one embodiment the primer probe combination targets the non-structural (NS) protein of BPV-3. In
15 one embodiment, the primer probe combination comprises a forward primer that targets oligo position 1331-1352 of the BPV-3 isolate having the GenBank accession number AF406967, a reverse primer that targets oligo position 1474-1453 of the BPV-3 isolate having the GenBank accession number AF406967, and a probe that targets oligo position 1377-1391 of the BPV-3 isolate having the GenBank accession number AF406967. In one embodiment the forward primer has the nucleic acid sequence of SEQ ID NO:7, the reverse primer has the
20 nucleic acid sequence of SEQ ID NO:8, and the probe has the nucleic acid sequence of SEQ ID NO:9. In one embodiment, the primer probe combination comprises a forward primer that targets oligo position 1453-1474 of the BPV-3 isolate having the GenBank accession number AF406967, a reverse primer that targets oligo position 1562-1539 of the BPV-3 isolate having the GenBank accession number AF406967, and a probe that targets oligo position 1507-1522 of the BPV-3 isolate having the GenBank accession number AF406967. In
25 one embodiment the forward primer has the nucleic acid sequence of SEQ ID NO:10, the reverse primer has the nucleic acid sequence of SEQ ID NO:11, and the probe has the nucleic acid sequence of SEQ ID NO:12.

In one embodiment the primer probe combination comprises SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9 in combination with an internal positive control primer probe combination comprising SEQ ID NO:16, SEQ ID NO:17, and SEQ ID NO:18, wherein SEQ ID NO: 9 has a fluorescent reporter dye 6-carboxyfluorescein at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) non-fluorescence quencher at the 3' end and SEQ ID NO:18 has a fluorescent reporter dye, 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end.
30

The reaction mixtures used in the methods described herein may further comprise one or more controls. These controls include one or more of a no template control that contains all the PCR reagents except for the DNA template; a negative extraction control to monitor cross contamination during nucleic acid extraction and verify that the assay does not produce false positive results with non-specific background DNA; a positive
5 extraction control to evaluate DNA extraction performance during test sample preparation and verify that the assay does not produce false negative results; a positive control to verify that the qPCR components work accurately and give a PCR amplification signal specific to the target sequence; an inhibition control to
10 determined sample interference/inhibition level, a standard to determine the PCR efficiency, linear range and to quantify the absolute copy number of target sequence in the extracted DN of the test sample/control, and/or internal positive control to monitor cross contamination of positive control plasmid in the test sample that is used during sample preparation and assay execution.

A suitable level of precision, accuracy and linearity of the assay is preferentially demonstrated within the dynamic range of the analytical assays described herein, particularly for quantitative assays. The precision of the assay is based on repeatability (intra-assay precision) and intermediate precision (within-laboratory
15 precision). Repeatability is the coefficient of variation (CV) of the results obtained with the same method by the same analyst, in the same laboratory, with the same equipment, on the same samples over a short period of time. To determine the repeatability of the assay the mean and standard deviation (StdDev) of quantity (genome copy of target sequence per reaction, GC/rxn) and %CV of quantity is calculated from each set of PCR reactions at respected concentration. Intermediate precision accounts for the inherent variability, such as different
20 analysts, different detection systems, different times. To determine intermediate precision of the assay the mean and StdDev of quantity and %CV of quantity is calculated from all sets of PCR reactions of the all respected concentrations of the independent experiments over the time.

Accuracy (also referred to as trueness) compares the obtained value from a series of samples (such as a positive control plasmid with defined concentrations) to the actual or reference value, called the standard
25 (ST). To determine accuracy of the assay the mean of quantity (genome copy of target sequence per reaction, GC/rxn) is calculated from each set of PCR reactions at the respected concentration.

The limit of quantification (LOQ) of a quantitative qPCR assay is the lowest amount of target sequence in a sample which can be quantitatively determined with suitable precision (repeatability and intermediate precision) and accuracy. To determine the LOQ, the lowest, highest, and some number of middle ranges are
30 tested. The lowest, middle and upper range concentration of positive control plasmid is prepared and spiked into an exogenous extracted DNA and quantified in an appropriate number of replicates in the presence of standard curve preferably using different analysts performing the assay on different dates.

The limit of detection (LOD) or sensitivity of the assay is the lowest amount of target sequence which can be detected by the assay, but not necessarily quantitated as an exact value.

The robustness of the assay may be determined according to the method of Youden and Steiner (Youden, Steiner, Statistical Manual of the Association of Official Analytical Chemists, Association of Official Analytical Chemists ed., Arlington 1975, pps. 33-36, 70-71, 82083). For example, changes to certain critical reagent concentrations may be evaluated. Certain critical PCR factors may be selected and subjected to slight changes. The acceptance criterion would require that the response obtained for any robustness condition with respect to the applied small changes should meet established assay acceptance criteria.

The invention provides an assay for the quantification of *Bovine parvovirus 3* genomic DNA in the extracted DNA of a test sample. In one embodiment is provided an assay for the quantification of 1E3 to 1E8 genome copies of *Bovine parvovirus 3* genomic DNA in a PCR reaction.

The invention provides a method for the quantification of 1E3 to 1E8 genome copies of *Bovine parvovirus 3* genomic DNA in PCR reaction comprising 1) a reaction mixture comprising the extracted DNA of a test sample, a positive control, a BPV-3_IPC positive control plasmid DNA, nucleic acid amplification reagents, an internal positive control primer probe combination having the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18, wherein SEQ ID NO:18 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end, and a primer probe combination selective for a DNA sequence of *Bovine parvovirus 3*, selected from the group consisting of a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3, wherein SEQ ID NO:3 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6, wherein SEQ ID NO:6 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9, wherein SEQ ID NO:9 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; d) a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12, wherein SEQ ID NO:12 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15, wherein SEQ ID NO:15 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; f) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:20, and SEQ ID NO:21, wherein SEQ ID NO:21 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25, wherein SEQ ID NO:25 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; and h) a primer probe combination having the nucleic acid sequences of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28, wherein SEQ ID NO:28 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; 2)

subjecting the reaction mixture to a quantitative PCR technique to obtain copies of the target sequence, and 3) measuring any increase in fluorescence signal.

In one embodiment the dynamic range is 1E3 to 1E8 GC/rxn. In one embodiment the lower limit of quantification is 1E3 GC/rxn. In one embodiment the upper limit of quantification is 1E8 GC/rxn. In one embodiment the limit of detection (LOD95%) of the quantification assay is 27 genome copies per reaction with a 95% confidence interval of 22 and 34 genome copies per reaction. In one embodiment the assay has linearity having a correlation coefficient (R^2) ≥ 0.98 and a PCR amplification efficiency within 90-110%. In one embodiment the assay has a repeatability value that is a %CV of quantity equal or less than 25%. In one embodiment the assay has an intermediate precision value that is %CV of quantity equal or less than 30%. In one embodiment the assay has an accuracy value within $\pm 30\%$ of the accepted reference value (ST) across the whole dynamic range of the assay. In one embodiment the assay has a limit of quantitation that is the %CV of quantity for repeatability at $\leq 25\%$, intermediate precision at $\leq 30\%$ and acceptance criterion for the accuracy (the value for lowest, middle and upper ranges) within $\pm 30\%$ of the expected standard (ST) reference value. In one embodiment the assay has a robustness that has a percent CV of quantity for repeatability of $\leq 25\%$, intermediate precision of $\leq 30\%$ and accuracy of the mean of quantity of the combination matrix condition tested of $\pm 30\%$ of the mean of quantity of the optimized condition.

The invention provides a kit for use in detecting *Bovine parvovirus 3* (BPV-3) genomic DNA contamination in the extracted DNA of a test sample comprising a primer probe combination that detects DNA encoding BPV-3 selected from the group consisting of a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3; b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6; c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; d) a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12; e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15; f) a primer probe combination having the nucleic acid sequences of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22; g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25; and g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28; and optionally an internal positive control primer probe combination having the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18.

While the terminology used in this application is standard within the art, definitions of certain terms are provided herein to assure clarity and definiteness to the meaning of the claims. Units, prefixes, and symbols may be denoted in their International System of Units (SI) accepted form. Numeric ranges recited herein are inclusive of the numbers defining the range and include and are supportive of each integer within the defined range. The methods and techniques described herein are generally performed according to conventional

methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. All documents, or portions of documents, cited in this application, including but not limited to patents, patent applications, articles, books, and treatises, are hereby expressly incorporated by reference.

5 The present invention is not to be limited in scope by the specific embodiments described herein that are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the
10 appended claims. What is described in an aspect or embodiment of the invention can be combined with other aspects and/or embodiments of the invention.

The following examples, including the experiments conducted and the results achieved, are provided for illustrative purposes only and are not to be construed as limiting the scope of the appended claims.

EXAMPLES

15 Example 1 BPV-3 primer/probe set development

To locate conserved regions on the BPV-3 genome, all published full-length genome sequence of BPV-3 isolates were retrieved from GenBank (Table 1).

Table 1. GenBank Accession numbers.

Genus	Species	Isolate	GenBank Accession #
Erythroparvovirus	Ungulate erythroparvovirus 1	Bovine parvovirus 3	AF406967
			MG745680
			NC_037053
			MG026727
			MG026728

20 Multiple alignments were performed and different conserved regions in the genes encoding the BPV-3 non-structural (NS) protein and structural capsid (VP) protein were selected and used to design eight different primer/probe sets (BPV-3 v1.0 – BPV-3 v8.0). The probes were labeled with a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and either a Minor Groove Binder, non-fluorescence

quencher (MGB-NFQ) or ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end, (Applied Biosystems, Carlsbad, CA or Integrated DNA Technologies, Inc., Coralville, IA). Table 2 provides the oligo positions and sequences of the probes and primer sets. The primer/probe sets were synthesized and tested using five-log concentration range of positive control plasmid (1e7 to 1e3) with 6 replicates.

5 Table 2. Prime and probe sequence used for BPV-3 v3.0. Oligonucleotide position for the probe/primers is based on BPV-3 isolate GenBank accession number AF406967.

Probe Primer Target	Oligo position	Oligo function	Sequence (5' → 3')
BPV-3 v1.0 Gene: VP	3535-3554	Forward Primer	CATCGGCTATAAAACCCCAT SEQ ID NO: 1
	3667-3643	Reverse Primer	GGACAGTATTATTTCCATGCTCTCT SEQ ID NO: 2
	3571-3588	Probe	CAACGCCATCAATCGCCA SEQ ID NO: 3 Reporter Dye: FAM Quencher Dye: ZEN AND IBFQ
BPV-3 v2.0 Gene: NS-VP	2190-2209	Forward Primer	GGGTTAGAAACGCTGCAATC SEQ ID NO: 4
	2256-2236	Reverse Primer	AGCAAGCGAGAGGACATAGTC SEQ ID NO: 5
	2213-2226	Probe	TTAGGTATGGCGTT SEQ ID NO: 6 Reporter Dye: FAM Quencher Dye: MGB-NFQ
BPV-3 v3.0 Gene: NS	1331-1352	Forward Primer	GCATAAATGTGTCTTGGTGTGG SEQ ID NO: 7
	1474-1453	Reverse Primer	ATTAATACGGGAGTGGGAGACA SEQ ID NO: 8
	1377-1391	Probe	ATTGTTGAGGCTGTA

			SEQ ID NO: 9 Reporter Dye: FAM Quencher Dye: MGB-NFQ
BPV-3 v4.0 Gene: NS	1453-1474	Forward Primer	TGTCTCCCACTCCCGTATTAAT SEQ ID NO: 10
	1562-1539	Reverse Primer	AATGACCATCCTCTCACTTAAAGC SEQ ID NO: 11
	1507-1522	Probe	ATGGAAACATTGTTAC SEQ ID NO: 12 Reporter Dye: FAM Quencher Dye: MGB-NFQ
BPV-3 v5.0 Gene: VP	1618-1645	Forward Primer	TTGTTGATTGGCTAAACTATGTAAAGTC SEQ ID NO: 13
	1734-1716	Reverse Primer	GGGCTTTCCGCTTTATCTC SEQ ID NO: 14
	1665-1678	Probe	GCTGATACCGTTCA SEQ ID NO: 15 Reporter Dye: FAM Quencher Dye: MGB-NFQ
BPV-3 v6.0 Gene: VP	2862-2878	Forward Primer	ATGCCTCCGAGCAACAC SEQ ID NO: 20
	2963-2938	Reverse Primer	CCTCTCTAGACTCTTCTCTAGATTCA SEQ ID NO: 21
	2902-2930	Probe	CCCAGATATCACATCTTCTGTCTCGGAAACA SEQ ID NO: 22 Reporter Dye: FAM Quencher Dye: ZEN AND IBFQ

BPV-3 v7.0 Gene: VP	3051-3068	Forward Primer	CGGTGGCCACAGCATATC SEQ ID NO: 23
	3134-3114	Reverse Primer	GGGACCCACTAGGATACATTG SEQ ID NO: 24
	3079-3102	Probe	TCAGTTCTTGCGGGACTACTTGGC SEQ ID NO: 25 Reporter Dye: FAM Quencher Dye: ZEN AND IBFQ
BPV-3 v8.0 Gene: VP	3061-3079	Forward Primer	AGCATATCCTCGGCCTGAT SEQ ID NO: 26
	3140-3122	Reverse Primer	GATACCGGGACCCACTAGG SEQ ID NO: 27
	3081-3103	Probe	AGTTCTTGCGGGACTACTTGGCC SEQ ID NO: 28 Reporter Dye: FAM Quencher Dye: ZEN AND IBFQ

Based on LOD, dynamic range, PCR efficiency and R^2 value, BPV-3 v3.0 was selected for use in assay development and qualification. The primers amplified a 144-bp fragment of BPV 3. The probe has a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and a Minor Groove Binder, non-fluorescence quencher (MGB-NFQ) at the 3' end, (Applied Biosystems, Carlsbad, CA). The designed BPV-3 v3.0 primer/probe sequences fully matched with the sequences of all five of the *Parvovirinae* subfamily sequences by *in silico* analysis. Multiple alignments with other parvoviruses did not produce a match indicating the sequences of the primer probe combination were specific for BPV-3.

Internal Positive Control primer/probe set and BPV-3_IPC positive control plasmid DNA

An internal positive control (IPC) was designed to monitor the extraction efficiency and any accidental cross-contamination between spiked positive control plasmids and the extracted DNA of the test sample. The IPC probe (IPC v2.0) was labeled with a fluorescent reporter dye, 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC), at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end (Applied Biosystems, Carlsbad, CA). The IPC primer/probe set amplified a 67-bp fragment, to facilitate distinguishing it from the BPV-3 primer/probe. Table 3 provides the oligo function and sequences of the probes and primer. This probe/primer set was used for further BPV-3 assay development and qualification.

Table 3. Prime and probe sequence used for IPC v2.0.

Probe Primer Target	Oligo function	Sequence (5' → 3')
IPC v2.0	Forward Primer	AACTTGGTCAGAGATCGAGGAGG SEQ ID NO: 16
	Reverse Primer	CCGCCAGTGTGCTGGAAT SEQ ID NO: 17
	Probe	CGCCCTTCGAAGCAC SEQ ID NO: 18 Reporter Dye: VIC-MGB Quencher Dye: MGB-FNQ

To generate BPV-3_IPC positive control plasmid DNA, the target sequences of the BPV-3 and IPC were synthesized and cloned into a pUC57 vector. The 5' termini of the positive control sequence carried the BPV-3 target genome and 3' termini and the positive control sequence carried IPC target sequence. Therefore, the positive control plasmid carries both BPV-3 and IPC sequence, which served as an BPV-3 positive control as well as internal positive control to monitor extraction efficiency and any accidental cross contamination. The sequence of the positive control DNA is shown in Table 4.

Table 4. BPV-3_IPC positive control plasmid DNA.

5' AATGAAAATTTCCCTTCAATGATGCGCCGCATAAATGTGTCTTGGTGTGGGACGAAGG CCGAATTACGGCCAAAATTGTTGAGGCTGTAAAGAGCATTCTGGGGGGTCAGGCGGTGCG GGTGGATCAGAAATGCAAGGGCTCTGTAAGCTTGTCTCCCACTCCCGTATTAATCACATCT AATGCTGATATTCGATATGTGCGTGATGGAAACATTGTTACTGGGGATCATGTAAAAGCT TTAAGTGAGAGGATGGTCATTGTACATTTCTCTACTCCGTGCCCCGCCAATTTTGGGCTTT TGAAAGCGGAGGAAATTGTTGATTGGCTAAACTATGTAAAGTCATGCCCGGGGAGTATCA CTGCTGATACCGTTCAGGCCACGTGGGGAACACGCTCCGCCCCCAATTTATTTGAGATAA AGCGGAAAGCCCCACAGCCCGCCAGTCCCATTGAACCTCAGACGGAGGAACAAGAAGAA GCAGCCGCATATCGCTGTCAGAGCCCACTTGGTCAGAGATCGAGGAGGATTTGAGAGCG TGCTTCGAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTGCGCGCACATTCACCAAG 3' SEQ ID NO: 19
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Example 2 BPV-3 Qualitative Assay

Sample preparation

For test samples that contained cells, the samples were subjected to a low speed centrifugation (LSC) before DNA extraction, 320xg to 1000xg, for 10 minutes at room temperature. The supernatant was collected for DNA extraction. Cell free samples were processed directly.

5 To a 250µL of a test sample was added, 10µL of an RNase Cocktail™ Enzyme Mix (Thermo Fisher, Carlsbad, CA) and 0.5M EDTA, pH 8.0 (9µL:1µL) mixture. An AutoMate Express™ Nucleic Acid Extraction System (ThermoFisher) was used to extract the DNA from the test sample. On the AutoMate, the PrepSEQ (PS) Express 1-2-3 protocol was selected for the DNA extraction procedure with following parameters: 1-hour proteinase K (PK) digestion and 100µL elution.

10 Extracted DNA was visually inspected and if there were any residual magnetic beads in the eluted DNA, a DynaMag™-2 Magnet benchtop workstation (ThermoFisher) was used to remove any remaining beads in the eluted DNA. The DNA was used immediately or stored at -30C.

Alongside the DNA extracted from the test samples, a BPV-3_IPC (PEC) plasmid control and a negative extraction control (NEC) were also prepared and run as described above. For the positive extraction control, 250µL sterile 1X phosphate buffered saline (PBS) was spiked with 62,000 genome copies (GC) BPV-3_IPC plasmid DNA. The positive extraction control was used to evaluate DNA extraction efficiency during sample preparation. Assuming 100% efficiency of DNA extraction, final eluted DNA should contain 500 GC/µL. The negative extraction control contained 250µL sterile 1x PBS. The negative extraction control was used to monitor any accidental cross contamination that may happen during nucleic acid extraction.

20 Preparation of additional BPV-3 detection assay controls

No template control (NTC): The no template control contained all the PCR reagents except for the DNA template. This was used as a negative control to verify qPCR raw materials were free of any DNA contamination.

25 Positive control (PC): the positive control contained 2000 GC/reaction of BPV-3_IPC recombinant plasmid DNA. The positive control was used to verify the accuracy of the assay.

Inhibition control (IHN): The inhibition control is used to monitor PCR amplification and the level of interference or inhibition imposed from the test sample matrix. The final extracted DNA from the test sample was spiked (+S) with same amount of BPV-3_IPC plasmid (2000 GC/reaction) as the positive control. The CT (cycle threshold) is the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). The mean cycle threshold (CT) value of the inhibition control (+S) was compared with the 30 mean CT value of the positive control as a measure of interference or inhibition. The ΔCT value should be less

than 3.32 CT (equal to one log of DNA concentration). The inhibition or interference was calculated as $\Delta CT = |PC (\text{mean CT}) - IHN (\text{mean CT})|$.

BPV-3 qPCR and IPC qPCR detection assay setups

Two different master mixes are prepared, a BPV-3 qPCR master mix for the BPV_3 pPCR assay and an IPC qPCR master mix and the IPC qPCR assay.

The BPV-3 qPCR detection assay setup contained the BPV-3 v3.0 prime/probe set to detect BPV-3 DNA in the DNA extracted from a test sample. The BPV-3 qPCR master mix comprised the BPV-3 v3.0 Primer-Probe set, water, and 2X TAQMAN[®] Universal PCR Master Mix (Applied Biosystems). The 2X TAQMAN[®] Universal PCR Master Mix contained the essential components for the qPCR reaction including optimized buffers to amplify G/C-rich sequence, dNTPs with dUTP, AmpliTaq Gold[™] DNA polymerase, ROX dye (as a passive internal reference) and AmpErase[™] UNG (Uracil-DNA Glycosylase enzyme to eliminate carry-over contamination PCR products containing dU).

Table 5 provides the amount of each reaction component for each sample tested in the BPV-3 qPCR reaction: the DNA extracted from the test sample and the five controls: negative extraction control (NEC), no template control (NTC), positive extraction control (PEC), inhibition control (IHN), and positive control (PC) at two different concentrations, an above detection limit (ADL) amount and a detection limit (DL) amount.

Table 5 With inhibition positive controls tested at and above the detection limit

	Water (μL)	2X TAQMAN [®] Universal PCR Master Mix (μL)	BPV-3 v3.0 Primer-Probe 60x (μL)	Extracted DNA (μL) from test sample	BPV_IPC PC Plasmid (GC/ μL)	
					125	12.5
Test Sample	4.5	15	0.5	10	n/a	n/a
Controls						
NEC	4.5	15	0.5	10	n/a	n/a
NTC	14.5	15	0.5	n/a	n/a	n/a
PEC	4.5	15	0.5	10	n/a	n/a
IHN_ADL (250 GC/RXN)	2.5	15	0.5	10	2	n/a
IHN_DL (25 GC/RXN)	2.5	15	0.5	10	n/a	2
PC_ADL	12.5	15	0.5	n/a	2	n/a

(250 GC/RXN)						
PC_DL (25 GC/RXN)	12.5	15	0.5	n/a	n/a	2

For the IPC qPCR reaction an IPC prime/probe master mix was prepared that comprised the IPC Primer-Probe, water, and 2X TAQMAN[®] Universal PCR Master Mix (Applied Biosystems). The amount of each reaction component for the test sample as well as for the four controls, negative extraction control, no template control, positive extraction control, and positive control at two different concentrations, an above detection limit (ADL) amount and a detection limit (DL) amount are provided in Table 6.

Table 6 IPC qPCR reaction component (qualitative assay)

	Water (μL)	2X TAQMAN [®] Universal PCR Master Mix (μL)	IPC v2.0 Primer-Probe 60x (μL)	Extracted DNA (μL) from test sample	BPV_IPC PC Plasmid (GC/μL)	
					125	12.5
Test Sample	4.5	15	0.5	10	n/a	n/a
Controls						
NEC	4.5	15	0.5	10	n/a	n/a
NTC	14.5	15	0.5	n/a	n/a	n/a
PEC	4.5	15	0.5	10	n/a	n/a
PC_ADL (250 GC/RXN)	12.5	15	0.5	n/a	2	n/a
PC_DL (25 GC/RXN)	12.5	15	0.5	n/a	n/a	2

Both the BPV_3 qPCR reaction and the IPC qPCR reaction were executed at least in triplicate (3 PCR reaction wells) for each sample and control in a 96 well reaction plate. The reaction components could be set up either manually or using a liquid handler robot, such as the QIAgility HEPA/UV liquid handler (QIAGEN, Inc., Germantown, MD). A MicroAmp Optical 96-well plate (Applied Biosystems) was used to accommodate the qPCR reaction mixtures and covered with MicroAmp Optical Adhesive Film (Applied Biosystems). The amount of PCR reagent components, samples, and controls in each PCR reaction is listed in Tables 6 and 7. The sealed plate was then loaded into a QuantStudio[™] 7 Flex Real-Time PCR System (Applied Biosystems).

A QuantStudio[™] 7 Flex Real-Time PCR System (Applied Biosystems) was used to perform the qPCR reactions. The thermal cycling program was set for the following reaction times and temperatures: 50°C 2 min,

95°C 10 min, and 40 cycle of 95°C 15 sec, 60°C 1 min (data collection). Table 7 provides the experimental properties that were selected.

Table 7 QuantStudio™ software experimental properties for BPV-3 qPCR and IPC qPCR.

5	Criteria for a valid test and evaluation of the test sample	Experimental property name:	Experimental property selected:
		Instrument	QuantStudio™ 7 Flex System
		Block	96-Well (0.2mL)
		Experiment	Standard Curve
		Reagent	TAQMAN® Reagents
		Run Properties	Standard
		Reporter	- FAM (for BPV-3 v3.0 primer/probe set) - VIC (for IPC v2.0 primer/probe set)
		Quencher	MGB-NFQ
		Passive Dye	ROX
		Threshold	- ΔR_n 0.1 for BPV-3 assay - ΔR_n 0.2 for IPC assay
		Baseline	Automatic
10			
15	Before test	Reaction volume	30 μ L

samples are evaluated, the assay must have met system suitability and assay acceptance criteria to be considered a valid assay. If the assay acceptance criteria are not met, the assay must be repeated. If the test sample was valid and positive, the assay should be repeated to confirm the achieved positive result. The criteria for a valid assay and the evaluation of the test results for the BPV-3 qualitative assay is outlined in Table 8. If the test sample presents a positive amplification signal, the IHN and ΔCT controls are irrelevant and should not be considered as a part of system suitability and assay acceptance criteria.

Table 8 System suitability and assay acceptance criteria.

Target BPV-3 and IPC for NTC and NEC	At least 2 out of 3 reactions of the NTC and NEC must show no amplification signal (CT = 40.00).
Target BPV-3 for PC, PEC, and IHN	The PC, PEC and IHN must produce amplification signal in at least 2 out of 3 reactions (CT less than 40.00).

Target IPC for PC and PEC	The PC and PEC must produce amplification in at least 2 out of 3 reactions (CT less than 40.00).
Target BPV-3 for Δ CT	Calculate interference/inhibition per Δ CT = PC (mean CT) – IHN (mean CT) equation. If the Δ CT is equal and greater than 3.32, the assay is invalid.

Test sample results acceptance criteria

If the assay meets the system suitability and assay acceptance criteria, Table 9 provides the test sample acceptance criteria. The results of the qualitative assay are reported as “positive” or “negative”.

5

Table 9 Test sample acceptance criteria.

Positive test sample criteria	A test sample is reported as “positive” if at least 2 out of 3 reactions present a positive amplification signal (CT less than 40.00) for target BPV-3; and at least 2 out of 3 replicates present no amplification signal (CT = 40.00) for target IPC.
Negative test sample criteria	A test sample is reported as “negative” if at least 2 out of 3 replicates show no amplification signal (CT = 40.00) for both targets BPV-3 and IPC.

Repeating testing strategy

The assay may be repeated using 1:2 dilution of extracted DNA if Δ CT does not meet the acceptance criteria using neat DNA sample. The dilution will reduce sample matrix interference. In this case all samples and control DNA must be diluted as 1:2 with molecular biology grade water before repeating the assay.

10

If a test sample shows positive amplification signal for the target IPC, this indicates cross contamination of positive control plasmid DNA with test sample. In this case the assay is invalid and after implementation of decontamination per a site applicable procedure, the assay needs to be repeated.

15

Example 3 Testing the BPV3 qualitative assay.

Specificity: Sample matrix effect (to test for the absence of false positives)

Three sample matrices (Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher) and two fetal bovine serum (FBS) (SAFC St. Louis, MO, and Hyclone Logan, UT) were tested to demonstrate the absence of false positive results between media components and the BPV-3 v3.0 and IPC v2.0 primer/probe sets. The assay procedures and set up was as described in Example 2.

5 Table 10 shows the tested samples and the results of qPCR testing with the BPV-3 and IPC assays. The acceptance criterion for this procedure required that all tested samples show a negative result with both primer/probe sets. Results from valid experiments when all implemented controls and system suitability met acceptance criteria revealed that all tested samples were negative and showed no amplification signal (CT = 40.00).

10 Table 10 Results from the sample matrices.

Sample matrix	PCR Rep.	BPV-3 v3.0 Mean $\pm$ SD CT	IPC v2.0 Mean $\pm$ SD CT	Results
DMEM	6	40.00 $\pm$ 0.00	40.00 $\pm$ 0.00	NEG/Pass
DMEM	6	40.00 $\pm$ 0.00	40.00 $\pm$ 0.00	NEG/Pass
FBS	6	40.00 $\pm$ 0.00	40.00 $\pm$ 0.00	NEG/Pass
FBS	6	40.00 $\pm$ 0.00	40.00 $\pm$ 0.00	NEG/Pass
NTC	3	40.00 $\pm$ 0.00	40.00 $\pm$ 0.00	NEG/Pass
PC	3	29.21 $\pm$ 0.26	28.67 $\pm$ 0.04	POS/Pass
PEC	3	28.57 $\pm$ 0.13	28.10 $\pm$ 0.21	POS/Pass

BPV-3 detection (to test the absence of false negatives)

15 Due to lack of a commercially available BPV-3 virus stock or BPV-3 genomic DNA, the BPV-3_IPC positive control plasmid was used to test the specificity of the BPV-3 v3.0 primer/probe set. The BPV-3_IPC plasmid was sequenced, and the accuracy of the BPV-3 NS gene was confirmed by BLAST analysis.

The assay as described in Example 2 was used. As part of specificity testing, two fetal bovine serum samples and an unprocessed non-GMP bulk harvest sample containing FBS which were confirmed to be positive for BPV-3 DNA (using Bioreliance (BREL) qPCR Bovine Parvo Panel, Bioreliance, Rockville, MD),
 20 served as true positive samples. The positive control (PC) was confirmed by sequencing.

The acceptance criteria for this procedure required that all tested samples show a positive result using the BPV-3 assay, and that the assay did not generate a false negative result on a previously confirmed positive BPV-3 sample.

25 Table 11 shows the results of the BPV-3 detection in PC, which all met the acceptance criterion. Of note, the IPC v2.0 primer/probe were negative in the positive FBS and the unprocessed non-GMP bulk harvest samples containing FBS as expected, and only generated positive results when positive control plasmid (BPV-3_IPC) was used. The results of IPC qPCR reactions were as expected and met the acceptance criterion.

Table 11 Results of true positive samples testing.

Sample	Reps	BPV-3 v3.0 Mean $\pm$ SD CT	IPC v2.0 Mean $\pm$ SD CT	Results
FBS #1	4	21.78 $\pm$ 0.21	40.00 $\pm$ 0.00	POS/Pass
FBS #2	4	25.35 $\pm$ 0.12	40.00 $\pm$ 0.00	POS/Pass
Unprocessed non-GMP bulk harvest containing FBS	6	29.55 $\pm$ 0.29	40.00 $\pm$ 0.00	POS/Pass
PC	3	29.21 $\pm$ 0.26	28.67 $\pm$ 0.04	POS/Pass
NTC	3	40.00 $\pm$ 0.00	40.00 $\pm$ 0.00	NEG/Pass

Cross reactivity

5 To test the effect of cross reactivity of the BPV-3 v3.0 and IPC v2.0 primer/probe sets, extracted nucleic acids derived from various viruses and cell lines were evaluated using the assay as described in Example 2. The closest commercially available parvoviruses to BPV-3 are *Bovine parvovirus 1* (BPV-1) (ATCC® VR-767™) and *Mouse minute virus* (MMV, rodent parvovirus), were included to test for cross reactivity. Several other DNA viruses were included to test cross reactivity: *Porcine circovirus 2* (PCV 2), *Herpes simplex virus* 1 (HSV 1), *Pseudorabies virus* (Prv). DNA from four cell lines were also tested: African green monkey kidney (VERO), baby hamster kidney cells (BHK), Chinese hamster ovary (CHO), MSV-transformed cat brain (PG 4). DNA was extracted as described in Example 2.

The accuracy of extracted nucleic acid from all viruses was confirmed using in-house specific qPCR or next generation sequencing (NGS) method.

15 The acceptance criterion for this procedure required that all tested species show a negative result (CT = 40.00) using BPV-3 v3.0 and IPC v2.0 primer/probe sets. Table 12 shows that cross reactivity testing results revealed that all tested species were negative for BPV-3 and met the acceptance criterion.

Table 12 Results of cross reactivity testing.

Species	gene	Repl icates	BPV-3 v3.0 Mean $\pm$ SD CT	IPC v2.0 Mean $\pm$ SD CT	Results
<i>Bovine parvovirus 1</i>	ssDNA	6	40.00 $\pm$ 0.00	40.00 $\pm$ 0.00	NEG/Pass
<i>Mouse minute virus</i>	ssDNA	6	40.00 $\pm$ 0.00	40.00 $\pm$ 0.00	NEG/Pass
<i>Porcine circovirus 2</i>	ssDNA	6	40.00 $\pm$ 0.00	40.00 $\pm$ 0.00	NEG/Pass
<i>Herpes simplex virus 1</i>	dsDNA	6	40.00 $\pm$ 0.00	40.00 $\pm$ 0.00	NEG/Pass

<i>Pseudorabies virus</i>	dsDNA	6	40.00 ± 0.00	40.00 ± 0.00	NEG/Pass
African green monkey kidney	dsDNA	6	40.00 ± 0.00	40.00± 0.00	NEG/Pass
Baby hamster kidney	dsDNA	6	40.00 ± 0.00	40.00± 0.00	NEG/Pass
Chinese hamster ovary	dsDNA	6	40.00 ± 0.00	40.00± 0.00	NEG/Pass
MSV-transformed cat brain	dsDNA	6	40.00 ± 0.00	40.00± 0.00	NEG/Pass
NTC	n/a	6	40.00 ± 0.00	40.00 ± 0.00	NEG/Pass

Limit of detection (LOD)

The BPV-3 qPCR method is intended to obtain a qualitative result, proof of linearity is not required. In practice, the LOD is determined as the cut-off point in the form of the minimum number of amplified target sequences by positively ratio detected in 95% of the sample series, which is referred to as LOD95%.

To determine the sensitivity or analytical limit of detection (ALOD) of the method, BPV-3_IPC plasmid DNA was diluted at different concentrations (1, 5, 10, 20, 25, 50, 75, 100, 250, 500 GC/PCR reaction). The ALOD is a concentration of the target DNA at which an amplification product is detected with a probability of at least 0.95 (LOD95%). To test this, twelve PCR replicate measurements from each plasmid DNA concentration were evaluated in the absence of sample matrix, using the assay as described in Example 2. The concentration level with the lowest number of genome copies for which all 12 replicates were positive was considered an approximate value for LOD95%.

Probit analysis was employed with associated probability of 95% to calculate ALOD of BPV-3 qPCR assay. Results of analysis demonstrated that the ALOD of the BPV-3 qPCR assay is about 22 genome copies per reaction (Figure 1).

The sample limit of detection (SLOD) is the minimum amount of target sequence that can be detected with a given level of confidence (LOD95%) in presence of sample matrix. Experiments to determine the SLOD were performed in the same manner as the ALOD experiments; with the exception that positive control plasmid DNA at different level of concentrations (25, 50, 75 and 100 GC/reaction) were spiked into the extracted DNA from the different sample matrices rather than molecular biology grade water (MBGW).

In this study, different sample matrices were used, see Table 13. Each sample was spiked with 25, 50, 75 and 100 genome copies (BPV-3_IPC positive control plasmid) per reaction using a QIAgility liquid handler (QIAGEN, Inc.) and tested using the BPV-3 qPCR detection assay described in Example 2. All samples were evaluated in 12 replicates. Table 14 and 15 present the results of the SLOD. Results revealed that the SLOD of

the BPV-3 qPCR assay was 25 genome copies per PCR reaction. Similar results were achieved for the IPC qPCR assay (Table 16).

Table 13 Matrix samples used for SLOD determination.

Sample	DNA amount
FBS	10µL DNA/qPCR reaction
Vero	30ng/10µL DNA/qPCR reaction
BHK	30ng/10µL DNA/qPCR reaction
CHO	50ng/10µL DNA/qPCR reaction

Table 14 BPV-3 assay SLOD results using FBS and Vero cells.

Sample matrix	FBS spiked with PC				Vero spiked with PC			
Replicates and GC/rxn	25	50	75	100	25	50	75	100
rep 1	34.35	34.15	32.41	32.65	34.83	33.82	33.54	31.94
rep 2	34.90	34.68	32.77	32.47	34.71	34.45	33.23	32.97
rep 3	34.35	33.80	32.52	32.35	34.91	33.70	33.45	33.08
rep 4	34.32	34.31	33.04	32.71	34.75	34.01	33.73	33.11
rep 5	32.96	33.87	32.67	32.57	35.38	34.22	32.85	33.00
rep 6	35.13	33.39	33.20	32.97	34.04	33.86	32.97	33.16
rep 7	34.63	34.08	33.24	33.05	35.96	34.32	33.43	32.97
rep 8	35.78	34.57	33.21	33.39	34.44	33.71	33.40	33.10
rep 9	33.06	34.33	32.80	33.30	36.55	33.43	33.46	33.30
rep 10	35.04	33.61	33.39	32.96	34.78	34.12	33.54	32.96
rep 11	34.78	33.70	33.41	32.97	34.29	33.69	33.18	32.95
rep 12	34.45	34.56	33.38	33.17	36.01	34.01	33.31	33.62
Mean	34.48	34.09	33.00	32.88	35.05	33.95	33.34	33.01
Positive signal ratio	12/12	12/12	12/12	12/12	12/12	12/12	12/12	12/12
Positive signal ratio %	100	100	100	100	100	100	100	100

5

Table 15 BPV-3 assay SLOD results using BHK and CHO cells.

Sample matrix	BHK spiked with PC	CHO spiked with PC

Replicates and GC/rxn	25	50	75	100	25	50	75	100
rep 1	34.84	34.23	33.05	32.39	34.55	34.07	32.58	33.31
rep 2	34.22	33.94	33.20	32.14	34.17	33.46	32.16	32.74
rep 3	35.22	33.70	32.97	32.96	34.37	33.61	33.06	32.77
rep 4	34.50	33.96	33.26	32.96	33.44	33.60	32.54	31.99
rep 5	34.09	34.29	33.12	32.96	34.24	32.51	32.94	33.35
rep 6	34.29	33.54	32.99	32.55	33.79	33.43	33.11	33.47
rep 7	34.62	34.19	33.43	32.81	34.18	33.36	33.58	33.34
rep 8	34.18	33.67	33.31	32.95	34.47	33.50	32.68	33.15
rep 9	33.96	34.10	33.11	32.16	33.93	33.53	33.68	32.10
rep 10	34.78	33.44	32.78	32.52	34.44	33.51	33.36	32.60
rep 11	34.20	33.91	33.12	32.63	34.25	34.09	33.05	32.85
rep 12	34.25	33.45	32.87	32.99	35.01	33.51	32.29	32.94
Mean	34.43	33.87	33.10	32.67	34.24	33.51	32.92	32.88
Positive signal ratio	12/12	12/12	12/12	12/12	12/12	12/12	12/12	12/12
% of positive signal	100	100	100	100	100	100	100	100

Table 16 IPC assay SLOD results using FBS and Vero cells.

Sample matrix	FBS spiked with PC				Vero spiked with PC			
Replicates and GC/rxn	25	50	75	100	25	50	75	100
rep 1	34.22	33.23	32.28	32.32	35.02	33.97	32.13	32.09
rep 2	33.69	32.76	31.83	31.86	33.88	34.19	31.95	32.19
rep 3	34.08	34.20	32.19	31.48	34.61	33.82	32.17	30.96
rep 4	34.54	33.74	32.49	32.15	33.54	33.77	32.39	32.17
rep 5	33.75	33.70	32.17	32.21	33.81	33.79	32.01	31.77
rep 6	33.88	33.78	32.19	32.37	34.46	33.45	32.08	32.47
rep 7	35.36	33.88	32.36	31.94	34.37	33.26	31.98	31.98
rep 8	34.34	33.83	32.06	32.34	34.25	34.24	32.08	31.69
rep 9	31.13	33.54	32.22	32.26	34.26	33.81	31.80	31.99
rep 10	33.79	33.45	30.79	29.74	34.51	33.51	32.45	31.30
rep 11	35.83	33.90	31.97	31.98	35.57	33.70	32.35	32.00
rep 12	35.17	33.31	32.57	31.38	34.64	33.25	32.11	31.55
Mean	34.15	33.61	32.09	31.84	34.41	33.73	32.12	31.85

Positive signal ratio	12/12	12/12	12/12	12/12	12/12	12/12	12/12	12/12
% of positive signal	100	100	100	100	100	100	100	100

Robustness

To evaluate the robustness of the BPV-3 qPCR detection assay, unprocessed non-GMP bulk harvest samples containing FBS, and FBS samples were tested (Table 17). The acceptance criterion would be to meet the proposed system suitability and assay acceptance criteria and sample acceptance criteria as described in Example 2. The samples were previously confirmed to be negative or positive for BPV-3 DNA by alternative methods such as NGS, sanger DNA sequencing or Bioreliance (BREL) Bovine Parvo Panel qPCR assay.

Samples were subjected to DNA extraction as described in Example 2. All controls were prepared, and qPCR executed in parallel with the test sample and then subjected to the BPV-3 qPCR detection assay as described in Example 2. The results of BPV-3 qPCR assay were interpreted as described in Example 2 and reported as “positive” or “negative”.

Table 17 Test samples used for BPV-3 assay robustness evaluation.

Sample	BPV-3 status
FBS	negative
irradiated FBS #1	negative
characterized FBS	negative
unprocessed non-GMP bulk harvest sample containing FBS # 1	positive
unprocessed non-GMP bulk harvest sample containing FBS # 2	positive
irradiated FBS #2	positive
irradiated FBS #3	positive

The results of negative test samples from Table 17 are shown in Tables 18-20. The validity of the assay was demonstrated with results of assay controls including negative results in NTC and NEC for BPV-3 and IPC assays in 3 out of 3 PCR reaction replicates; detection of target amplification signal in PC with BPV-3 and IPC assays in 3 out of 3 PCR reaction replicates; detection of amplification signal with BPV-3 and IPC assays for PEC in 3 out of 3 PCR reaction replicates indicating an accurate extraction recovery; detection of BPV-3 in spiked test sample (+S) in 3 out of 3 PCR reaction replicates and Δ CT results indicating no sample matrix interference. Test samples were negative with the IPC assay in 3 out of 3 PCR reaction replicates indicating

the absence of cross contamination between positive control and sample, also the test samples were negative with BPV-3 assay in 3 out of 3 PCR reaction replicates indicating absence of BPV-3 DNA. Altogether, BPV-3 assay results indicate that assay and sample acceptance criteria were met and passed, and test samples were valid and “negative” for BPV-3.

5 Table 18 Results for FBS Sample

qPCR reaction controls / samples	BPV-3 assay CT			IPC assay CT			Results BPV-3/IPC
No template control (NTC)	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
Negative extraction control (NEC)	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
Positive extraction control (PEC)	28.84	28.89	28.72	28.04	28.22	28.33	POS/POS
Positive control (PC)	28.18	29.12	29.33	28.34	28.43	28.78	POS/POS
Inhibition control (IHN)	29.96	29.97	30.16	n/a			POS
Sample matrix interference (Δ CT)	1.15			n/a			met
Test sample	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
System suitability and assay acceptance criteria			Passed				
Sample results acceptance criteria			Passed				
Test sample result			Valid and Negative				

Table 19 Results for irradiated FBS #1

qPCR reaction controls / samples	BPV-3 assay CT			IPC assay CT			Results BPV-3/IPC
No template control (NTC)	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
Negative extraction control (NEC)	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
Positive extraction control (PEC)	28.00	28.06	27.87	27.16	27.16	27.41	POS/POS
positive control (PC)	27.70	28.72	28.78	28.10	28.39	28.55	POS/POS
Inhibition control (IHN)	28.42	28.87	29.00	n/a			POS
Sample matrix interference (Δ CT)	0.36			n/a			met
Test sample	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
System suitability and assay acceptance criteria			Passed				
Sample results acceptance criteria			Passed				
Test sample result			Valid and Negative				

Table 20 Results for characterized FBS

qPCR reaction controls / samples	BPV-3 assay CT			IPC assay CT			Results BPV-3/IPC
No template control (NTC)	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
Negative extraction control (NEC)	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
Positive extraction control (PEC)	28.48	28.72	28.57	28.01	28.33	27.95	POS/POS
positive control (PC)	28.95	29.46	29.24	28.67	28.71	28.63	POS/POS
Inhibition control (IHN)	29.03	28.74	29.29	n/a			POS
Sample matrix interference (Δ CT)	0.19			n/a			met
Test sample	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
System suitability and assay acceptance criteria			Passed				
Sample results acceptance criteria			Passed				
Test sample result			Valid and Negative				

The BPV-3 assay results for the positive test samples (two unprocessed non-GMP bulk harvest samples containing FBS and two FBS samples (FBS#2 and FBS#3) are shown in Tables 21-24. The validity of the assay was demonstrated, the results of the assay controls including the expected with negative results for NTC and NEC for BPV-3 and IPC assays in 3 out of 3 PCR reaction replicates; and detection of target amplification signal in PC with BPV-3 assay in 3 out of 3 PCR reaction replicates.

When a test sample showed a positive amplification signal and detection of BPV-3, evaluation of the spiked test sample (IHC) and Δ CT became irrelevant and did not need to be considered as a part of assay acceptance criteria. Detection of amplification signal with BPV-3 assay and IPC assays for PEC in 3 out of 3 PCR reaction replicates indicated an accurate extraction recovery; test sample negative signal with IPC assay in 3 out of 3 PCR reaction replicates indicated the absence of cross contamination between positive control and sample. Test sample presented a positive amplification signal with BPV-3 assay in 3 out of 3 PCR reaction replicates indicating the presence of BPV-3 DNA. Altogether, results indicated that assay and sample acceptance criteria were met and passed, and the test sample was valid and positive for BPV-3.

Table 21 Results for unprocessed non-GMP bulk harvest sample 1

qPCR reaction controls / samples	BPV-3 assay CT			IPC assay CT			Results BPV-3/IPC
No template control (NTC)	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
Negative extraction control (NEC)	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
Positive extraction control (PEC)	28.39	28.46	28.14	27.81	27.91	27.68	POS/POS

positive control (PC)	28.49	28.42	28.35	28.15	28.02	28.06	POS/POS
Inhibition control (IHN)	n/a			n/a			n/a
Sample matrix interference (Δ CT)	n/a			n/a			n/a
Test sample	29.36	29.21	29.52	40.00	40.00	40.00	POS/NEG
System suitability and assay acceptance criteria			Passed				
Sample results acceptance criteria			Passed				
Test sample result			Valid and Positive				

Table 22 Results for unprocessed non-GMP bulk harvest sample 2

qPCR reaction controls / samples	BPV-3 assay CT			IPC assay CT			Results BPV-3/IPC
No template control (NTC)	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
Negative extraction control (NEC)	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
Positive extraction control (PEC)	28.61	28.57	28.42	27.99	28.13	27.81	POS/POS
positive control (PC)	28.53	28.38	28.37	27.98	27.92	27.94	POS/POS
Inhibition control (IHN)	n/a			n/a			n/a
Sample matrix interference (Δ CT)	n/a			n/a			n/a
Test sample	28.76	28.79	28.88	40.00	40.00	40.00	POS/NEG
System suitability and assay acceptance criteria			Passed				
Sample results acceptance criteria			Passed				
Test sample result			Valid and Positive				

Table 23 Results for FBS #2

qPCR reaction controls / samples	BPV-3 assay CT			IPC assay CT			Results BPV-3/IPC
No template control (NTC)	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
Negative extraction control (NEC)	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
Positive extraction control (PEC)	28.39	28.43	28.52	27.87	27.93	28.12	POS/POS
positive control (PC)	28.21	28.25	28.44	28.13	27.91	27.95	POS/POS
Inhibition control (IHN)	n/a			n/a			n/a
Sample matrix interference (Δ CT)	n/a			n/a			n/a

Test sample	21.31	21.43	21.53	40.00	40.00	40.00	POS/NEG
System suitability and assay acceptance criteria			Passed				
Sample results acceptance criteria			Passed				
Test sample result			Valid and Positive				

Table 24 Results for FBS #3

qPCR reaction controls / samples	BPV-3 assay CT			IPC assay CT			Results BPV-3/IPC
No template control (NTC)	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
Negative extraction control (NEC)	40.00	40.00	40.00	40.00	40.00	40.00	NEG/NEG
Positive extraction control (PEC)	28.47	28.58	28.61	28.08	28.10	28.29	POS/POS
positive control (PC)	28.45	28.44	28.40	27.83	27.89	27.69	POS/POS
Inhibition control (IHN)	n/a			n/a			n/a
Sample matrix interference (Δ CT)	n/a			n/a			n/a
Test sample	24.98	25.09	25.22	40.00	40.00	40.00	POS/NEG
System suitability and assay acceptance criteria			Passed				
Sample results acceptance criteria			Passed				
Test sample result			Valid and Positive				

Repeatability

To test the repeatability, the extracted DNA from BHK and Vero cells (30ng/PCR reaction) were prepared either as unspiked or spiked with 25 genome copies of BPV-3_IPC positive control plasmid (spiked at the SLOD level). The BPV-3 detection assay (as described in Example 2) was performed in 12 PCR reaction replicates on different days.

The acceptance criterion for this procedure required that at least 11 out of the 12 replicates show no amplification signal for unspiked samples by BPV-3 and IPC qPCR assays and at least 11 out of 12 samples showed a positive amplification signal for spiked samples by BPV-3 and IPC qPCR assays. The results of the repeatability assay revealed that 12 out of 12 unspiked (Table 25) and spiked (Table 26) met the acceptance criteria demonstrating the repeatability of the BPV-3 detection assay.

Table 25 Repeatability of BPV-3 qPCR on unspiked test samples.

Sample	PCR Reaction Replicates	BPV-3 assay CT	IPC assay CT	Negative signal ratio	Negative signal %	BPV-3 and IPC qPCR assay results
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Vero	12	40.00	40.00	12 out of 12	100	NEG/Passed
BHK	12	40.00	40.00	12 out of 12	100	NEG/Passed

Table 26 Intra- and inter-assay repeatability of BPV-3 qPCR results on spiked test sample at two different days.

Spiked (25 GC/PCR reaction)						
Assay	BPV-3 CT				IPC CT	
Sample matrix	BHK		Vero		BHK	Vero
PCR Reaction replicate & repeat	Day 1	Day 2	Day 1	Day 2	One run	
rep 1	34.30	34.84	34.63	34.83	33.76	34.44
rep 2	34.59	34.22	35.17	34.71	34.17	34.95
rep 3	34.47	35.22	35.14	34.91	34.65	34.18
rep 4	34.97	34.50	34.68	34.75	32.81	34.32
rep 5	34.21	34.09	34.86	35.38	34.24	34.30
rep 6	33.23	34.29	35.95	34.04	33.71	33.96
rep 7	34.85	34.62	35.69	35.96	32.91	34.44
rep 8	33.66	34.18	35.03	34.44	33.99	34.60
rep 9	34.28	33.96	34.72	36.55	33.92	34.05
rep 10	34.41	34.78	34.17	34.78	34.17	34.19
rep 11	34.47	34.20	34.14	34.29	33.83	34.32
rep 12	34.94	34.25	34.46	36.01	33.68	34.16
Positive signal ratio	12/12	12/12	12/12	12/12	12/12	12/12
% of positive signal	100	100	100	100	100	100
Results	POS/ Passed	POS/ Passed	POS/ Passed	POS/ Passed	POS/ Passed	POS/ Passed
Mean CT	34.37	34.43	34.89	35.05	33.82	34.33
StdDev CT	0.51	0.37	0.55	0.76	0.53	0.26
CV%	1.48	1.07	1.58	2.18	1.55	0.77

In summary, all tested samples met required qualification specifications for a qualitative assay including specificity, limit of detection, robustness, and repeatability. The qualified BPV-3 detection assay

allows for robust detection of 25 BPV-3 genome copies per PCR reaction. All tested samples met the proposed system suitability and assay acceptance criteria as well as test sample acceptance criteria. Thus, the qualified BPV-3 real-time PCR assay allowed the detection of BPV-3 DNA in test sample.

5 Example 4 Bovine parvovirus 3 (BPV-3) real-time quantitative polymerase chain reaction assay

Sample preparation

For test samples that contained cells, the cells were subjected to a low speed centrifugation (LSC) before DNA extraction, 320xg, for 10 minutes at room temperature. The supernatant was collected for DNA extraction. Cell free samples were processed directly.

10 To 250µL of a test sample was added 10µL of an RNase Cocktail™ Enzyme Mix (Thermo Fisher, Carlsbad, CA) and 0.5M EDTA, pH 8.0 (9µL:1µL) mixture. An AutoMate Express™ Nucleic Acid Extraction System (ThermoFisher) was used to extract 200 µL of the test sample. On the AutoMate, the PrepSEQ (PS) Express 1-2-3 protocol was selected for the DNA extraction procedure with the following parameters: 1-hour proteinase K (PK) digestion and 100µL elution. Extracted DNA was visually inspected and if there were any
15 residual magnetic beads in the eluted DNA, a DynaMag™-2 Magnet benchtop workstation (ThermoFisher) was used to remove any remaining beads in the eluted DNA. The DNA was used immediately or stored at -20C.

Alongside the test samples, a BPV-3_IPC (PEC) plasmid control and a negative extraction control (NEC) were also prepared and run as described above. For the positive extraction control, 250µL sterile 1X
20 phosphate buffered saline (PBS) was spiked with 62,000 genome copies (GC) BPV-3_IPC plasmid DNA. This was used to evaluate DNA extraction performance during test sample preparation and verified that assay does not produce false negative results. Assuming 100% efficiency of DNA extraction, final eluted DNA should contain 500 GC/µL. For the negative extraction control, ~300ng human genomic DNA in 1X PBS was used. The NEC was used to monitor cross contamination during nucleic acid extraction and verifies that assay did
25 not produce false positive results with non-specific background DNA.

Preparation of other assay controls

No template control (NTC): Contained all the PCR reagents except for the DNA template. This was used as a negative control to verify qPCR raw materials were free of any DNA contamination.

Positive control (PC): Three PC concentrations were included, 2E4, 2E5, and 2E7 GC/reaction of BPV-
30 3_IPC positive control plasmid DNA. This was used to verify that qPCR components work accurately and should have PCR amplification signal specific to target sequence.

Positive extraction control (PEC): The ~400ng human genomic DNA (~3μL) added to 250 μL of 1x PBS and is also spiked with 62,500 copies of BPV-3_IPC positive control plasmid DNA (6.25 μL) followed by extraction.

Inhibition control (IHN): Contains extracted DNA from the test sample which is spiked with BPV-3_IPC plasmid DNA at 2E4 GC/reaction. This is used to determine sample interference/inhibition level.

Standard (ST): A 10-fold dilution of BPV-3_IPC plasmid DNA (1E8, 1E7, 1E6, 1E5, 1E4, 1E3 GC/reaction) used to determine PCR efficiency, linear range, and to quantify the absolute copy number of target sequence in the test sample/control.

BPV-3 qPCR and IPC qPCR detection assay setups

The BPV-3 qPCR detection assay setup contained the BPV-3 v3.0 primer/probe set to detect BPV-3 DNA in test sample and the IPC v2.0 primer/probe set to monitor any accidental cross contamination between positive samples and negative samples. Two different master mixes were prepared, a BPV-3 qPCR master mix and an IPC qPCR master mix.

The BPV-3 v3.0 primer/probe master mix comprised the BPV-3 v3.0 Primer-Probe, water, and 2X TAQMAN® Universal PCR Master Mix (Applied Biosystems). The TAQMAN® master mix contained the essential components for the qPCR reaction including optimized buffers to amplify G/C-rich sequence, dNTPs with dUTP, AmpliTaq Gold™ DNA polymerase, ROX dye (as a passive internal reference) and AmpErase™ UNG (Uracil-DNA Glycosylase enzyme to eliminate carry-over contamination PCR products containing dU). Table 27 provides the amount of each reaction component for each sample tested, the test sample and the five controls: negative extraction control (NEC), no template control (NTC), positive extraction control (PEC), inhibition control (IHN), and positive control (PC).

Table 27 Reaction components for BPV_3 v3.0 quantitative assay

Reagent	Stock concentration	Reaction concentration
BPV-3 v3.0 primer/probe set, Assay ID: AP47Y2C	60X	1X
IPC v2.0 primer/probe set, Assay ID: APGZJVP	60X	1X
ABI Universal TaqMan PCR master mix 2X	2X	1X
Extracted DNA from test sample	n/a	10μL
Human genomic DNA	Variable	~300ng
Standard/Controls	n/a	variable
Water	n/a	up to 30μL

Each reaction for samples and controls was performed in triplicate (3 wells) in a 96 well reaction plate. The qPCR reaction set up could be performed manually or using a liquid handler robot, such as QIAgility HEPA/UV liquid handler (Qiagen, Inc., Germantown, MD). A MicroAmp Optical 96-well plate (Applied Biosystems) was used to accommodate the qPCR reaction mixtures and then covered with MicroAmp Optical Adhesive Film (Applied Biosystems). The amount of PCR reagent components, sample, and controls in each PCR reaction is listed in Table 27 and 28. The sample layout on 96-well plate can be set up per analyst, and the sealed plate loaded into the a QuantStudio™ 7 Flex Real-Time PCR System (Applied Bioscience).

A QuantStudio™ 7 Flex Real-Time PCR System (Applied Bioscience) was used to perform the qPCR reactions. The thermal cycling program was set for the following reaction times and temperatures: 50°C 2 min (AmpErase™ UNG activation), 95°C 10 min (AmpliTaQ Gold™ DNA polymerase activation/denaturation), and 40 cycle of 95°C 15 sec, 60°C 1 min (annealing/extension/data collection). Table 28 provides the experimental properties that were selected.

Table 28 QuantStudio™ software experimental properties for BPV-3 and IPC qPCR.

Experimental property name:	Experimental property selected:
Instrument	QuantStudio™ 7 Flex System
Block	96-Well (0.2mL)
Experiment	Standard Curve
Reagent	TAQMAN® Reagents
Run Properties	Standard
Reporter	- FAM (for BPV-3 v3.0 primer/probe set) - VIC (for IPC v2.0 primer/probe set)
Quencher	MGB-NFQ
Passive Dye	ROX
Threshold	- ΔRn 0.1 for BPV-3 assay - ΔRn 0.2 for IPC assay
Baseline	Automatic
Reaction volume	30μL

The assay utilizes the QS7 real-time PCR system (ThermoFisher Scientific, Waltham, MA) in combination with BPV_3 and IPC primers and probes described herein.

The assay must meet the following assay acceptance criteria to be considered a valid assay, see Table 29. If any of the assay acceptance criteria are not met, the assay should be repeated.

Table 29. Valid assay acceptance criteria

BPV_3	
Control	Valid assay acceptance criteria
No Template Control	A minimum of 2 out of 3 reactions must show no amplification signal, CT = 40.00 (undetermined) and the obtained mean of GC quantity per reaction (if any) shall be less than the assay LOD95% (27 GC/reaction).
Negative Extraction Control	Any CT value reported as “Undetermined” represents the absence of DNA and can be converted to 40.00 for statistical analysis.
Positive Extraction Control	A minimum of 2 out of 3 reactions must show an amplification signal of CT < 40.00, and the obtained mean GC of quantity per reaction greater than assay LOQ (1E3 GC/rxn).
Positive control PC 2E4 PC 2E5 PC 2E7	A minimum of 2 out of 3 reactions must show amplification signal of CT < 40.00, and the obtained mean GC of quantity per reaction must meet the established assay accuracy criteria, $\pm 30\%$ of PC reference value: PC_2E7 acceptance accuracy range: 1.4E7 to 2.6E8 GC/rxn PC_2E5 acceptance accuracy range: 1.4E5 to 2.6E6 GC/rxn PC_2E4 acceptance accuracy range: 1.4E4 to 2.6E4 GC/rxn
Inhibition Control (IHN or +S)	A minimum of 2 out of 3 reactions must show amplification signal of CT < 40.00, and the obtained mean GC of quantity per reaction shall be within one magnitude of the original (2E4) spiked concentration level; the INH mean GC of quantity should be within 2E3 to 2E5 GC/reaction. INH control is irrelevant and not applicable if test sample is positive with BPV-3 with mean GC of quantity of equal or greater than 2E4 per reaction.
Standard	- Correlation coefficient (R^2) ≥ 0.98 - PCR amplification efficiency within 90 to 110%.
IPC	
Control	Valid assay acceptance criteria
Negative Extraction Control	A minimum of 2 out of 3 reactions must show no amplification signal, CT = 40.00 (undetermined).
Positive Extraction Control	A minimum of 2 out of 3 reactions must show amplification signal of CT < 40.00, when the IPC is the target sequence.
Test Sample	A minimum of 2 out of 3 reactions must show no amplification signal, CT = 40.00 (undetermined), when the IPC is the target sequence.

Statistical analysis of the data was carried out with Excel software (Microsoft, Redmond, WA) and/or TIBCO SPOTFIRE® software (Palo Alto, CA). Any CT value reported as “Undetermined” represented the absence of target DNA and was converted to 40.00 for further statistical analysis. The sample, standard,

and controls were tested in 3 replicates. The outlier rule based on Grubbs' test or Dixon's Q test may be applied to the CT values from samples, controls or standards. An outlier analysis test may be applied to the triplicates data points to determine if one of the results could be omitted from the analysis as an outlier. If outlier exclusion is statistically significant (significance level: $\alpha = 0.05$) and is allowed, repeat data analysis with valid duplicate results. Results based on duplicate data points must meet all acceptance criteria for the controls and standard to be valid. Determination of outlier (two-sided test with significant level of alpha 0.05; may be tested using online or offline resources known to those of skill in the art. Examples of reporting and interpretation of data is provided in Table 30.

Table 30. Reporting and interpretation of data.

Test sample (BPV-3 GC/rxn)	Report	Interpretation
BPV-3 CT value was Undetermined (CT = 40.00) indicating the absence of BPV-3 or the mean of the BPV-3 GC/rxn was equal or less than assay LOD95% (27 GC/rxn)	BPV-3 Not Detected	Target sequence not detected or detected at a level below the LOD95% of the assay.
Mean GC/rxn of BPV-3 was less than assay LOQ (1E3) and greater than assay LOD95% (27 GC/rxn)	BPV-3 Detected (quantity as Log10 of mean $\pm$ StdDev per mL provided)	Target sequence was detected, but at a level below the LOQ of the assay; not accurately quantifiable.
Mean of BPV-3 GC/rxn was within the dynamic range of the assay (1E3 to 1E8 GC/rxn)	BPV-3 Detected (quantity as Log10 of mean $\pm$ StdDev per mL provided)	Target sequence was detected, and quantification was accurate and within the linear range of the assay.
Mean GC/rxn of BPV-3 was greater than upper set point (1E8) of assay dynamic range	BPV-3 Detected (quantity as Log10 of mean $\pm$ StdDev per mL provided) Alternatively, the quantity may be reported as greater than the upper level of the dynamic range of the assay.	Target sequence was detected and was greater than the upper dynamic range of the assay. The assay can be repeated with different dilution factors to allow for quantification within operational range.

Example 5 Quantitation assay qualification

Test sample results acceptance criteria

Qualification of the quantitative assay was performed. The following parameters were qualified: specificity, limit of detection (LOD), linearity, dynamic range, precision including repeatability (intra-assay specificity) and intermediate precision (within-laboratory precision), accuracy, limit of quantification (LOQ), and robustness and assay verification was determined.

Specificity: Sample matrix effect (to test the absence of false positives)

To demonstrate that the assay did not generate false positive results (an error in which qPCR incorrectly results in a positive in the absence of the target sequence) the following sample matrixes were tested.

5 Cell lines tested: PG4 (Cat brain Moloney sarcoma virus-transformed), Vero (African green monkey kidney), 324K (SV40-transformed human newborn kidney), L929 (Mouse fibroblast cell line), CHO (Chinese hamster ovary), HEK293 (human embryonic kidney), MDBK (Madin-Darby bovine kidney), NRK (Normal Rat kidney), human white blood cells (Promega, San Luis Obispo, CA). Media tested: DMEM, McCoy 9A, HAM F12. Animal serum tested: fetal bovine serum and horse serum.

10 Acceptance criterion required that all tested samples show a negative result in at least 5 out of 6 PCR replicates. No false positives were detected in 6 out of 6 replicates.

Specificity: BPV-3 detection (to test the absence of false negatives)

To demonstrate that the assay did not generate false negative results (an error in which qPCR incorrectly shows negative in the presence of the target sequence) the above-mentioned cell lines, media, and
15 animal serums were spiked with a BPV-3 positive control plasmid (50,000 genome copies / 200µL of sample) followed by DNA extraction and BPV-3 qPCR assay as described in Example 4. The acceptance criteria required that at least 5 out of 6 replicates show a positive result. All of the tested samples showed 6 out of 6 replicates detecting BVP-3. No false negatives were detected.

Specificity: Cross reactivity

20 To determine the cross reactivity of the assay, closely related viral species/isolates were evaluated for exclusivity and inclusivity.

Exclusivity demonstrated that the assay did not detect non-target viral species/isolates which are closely related to the target sequence. BPV-1 (*Bovine Parvovirus 1*, VR-767™ ATCC®, Manassas, VA), BPV-2 (*Bovine Parvovirus 2*) and MMVp (*Mouse Minute Virus* prototype) viruses were extracted as described above.
25 Since BPV-2 is not commercially available, full length non-structural (NS) gene sequence of BPV-2 (GenBank accession number: NC_006259) was synthesized and cloned in a plasmid and verified by sequencing. The plasmid was used as a BPV-2 reference isolate with concentration of 1E6 GC per PCR reaction.

The viral species/isolates were used in the BPV-3 qPCR assay as described in Example 4. The acceptance criterion required that all tested samples show a negative result in at least 5 out of 6 replicates. The
30 results of the BPV-3 qPCR did not show any cross reactivity with these closely related species and meet the acceptance criteria with 6 out of 6 replicates not detecting the non-target species/isolates.

Inclusivity demonstrates that the assay can specifically detect BPV-3 target sequence species/isolates. A previously confirmed BPV-3 positive fetal bovine serum sample (confirmation was done by full-length

genome sequencing of BPV-3) was extracted followed by BPV-3 qPCR assay. Acceptance criterion for this procedure required that the tested sample show a positive result in at least 5 out of 6 replicates. The results of the BPV-3 qPCR assay did specifically detect BPV-3 DNA and met the acceptance criteria with 6 out of 6 replicates detecting the target isolates.

5 Limit of Detection (LOD)

Limit of detection (LOD) is the lowest amount of target sequence which can be detected, but not necessarily quantitated as an exact value. Probit analysis with LOD95% detection limit approach was employed to determine the assay LOD. LOD95% is the number of copies of the target DNA sequence required to ensure 95% probability of detection (POD) in the qPCR assay.

- 10 BPV-3 positive control DNA (1000, 100, 50, 25, 20, 10, 5, and 1 genome copies/reaction) was spiked into an exogenous extracted DNA (~300ng human genomic DNA) and was tested using the BPV-3 qPCR assay as described in Example 4. Each concentration was tested in 12 PCR replicates to obtain a statistically reliable POD curve from which the LOD95% with 95% confidence interval (CI) was derived. The experiment was repeated by 3 different analysts. Any CT value ≤ 39.99 was considered a positive signal, and any undetermined
- 15 CT value (CT = 40.00) was considered as a negative signal. The 36 set points for each concentration (gathered from all analysts) were obtained for Probit analysis to achieve a statistically reliable POD curve from which the LOD95% with 95% confidence interval (CI) was derived, See Table 31 and Figure 2. The Probit analysis results showed that the BPV-3 qPCR assay LOD95% was 27 GC/rxn with a 95% confidence interval of 22 and 34 GC/rxn.

20 Table 31. Result of LOD95% from all experiments

DNA concentration (GC/rxn)	# of PCR replicates from all experiments	# positive PCR signal CT ≤ 39.99
1	36	4
5	36	14
10	36	26
20	36	34 25
25	36	33
50	36	35
100	36	36
1000	36	36

Dynamic Range and Linearity

A suitable level of precision, accuracy and linearity must be demonstrated within the dynamic range of the analytical procedure. To evaluate linearity, the minimum and maximum dynamic (operational) range of the standard curve, a preliminary standard curve was generated and evaluated with concentrations spanning 8 orders of magnitude with the defined copy number of positive control plasmid. Three replicates at each of 1E8, 1E7, 1E6, 1E5, 1E4, 1E3, 1E2, and 1E1 genome copies/reaction, were used in the BVP-3 assay.

A minimum of 6 magnitudes were selected for operational range not only per assay application and expected use but also per acceptable standard curve performance parameters derived from the preliminary testing results. For this procedure, the linearity of the standard curve within 6 chosen magnitudes (including maximum and minimum set points) must meet the acceptance criteria of (i) correlation coefficient (R^2) $\geq$ 0.98 and (ii) PCR amplification efficiency within 90-110%.

Although the dynamic range at seven set points (1E2 to 1E8 genome copies/reaction) met assay acceptance criteria, it was anticipated that using seven set points from 1E2 to 1E8 as the minimum and maximum standard range would not be robust. Therefore, an upper magnitude (1E3) for low limit of quantification (LOQ) was selected and the minimum and maximum dynamic range of standard curve was designated to be six data set points between 1E3 to 1E8 genome copies/reaction, Table 32.

Table 32. Standard curve dynamic range and performance

Experiment	Dynamic Range	Correlation coefficient (R^2)	PCR amplification efficiency (%)	Acceptance Criteria $R^2 \geq 0.98$ PCR efficiency: 90-110%
1	1E1 to 1E8	0.997	91.328	Met
	1E2 to 1E8	0.999	95.572	Met
	1E3 to 1E8	0.999	94.312	Met
2	1E1 to 1E8	0.997	94.957	Met
	1E2 to 1E8	0.998	97.441	Met
	1E3 to 1E8	1.000	93.731	Met
3	1E1 to 1E8	0.995	85.425	Not Met
	1E2 to 1E8	0.998	90.827	Met
	1E3 to 1E8	1.000	94.630	Met

The accuracy (trueness) and the precision (repeatability and intermediate precision) of the assay were determined. To determine accuracy and precision two separate sets of positive control plasmids with the defined range and genome copy number for BPV-3 (1E8, 1E7, 1E6, 1E5, 1E4, and 1E3 genome copies/reaction) were prepared and tested in parallel in one 96-well PCR reaction plate performing the BVP-3 assay. One set served as standard reference set points and the other set was used for assay evaluation. This procedure was tested by three different analysts on different days.

For repeatability (intra-assay precision), the mean and standard deviation of the quantity (genome copy of the target sequence/reaction) and %CV (coefficient of variation) of quantity was calculated for each triplicate at each concentration. Acceptance criterion for this procedure was that the %CV of quantity was $\leq 25\%$.

For intermediate precision (within-laboratory precision), the mean and standard deviation of quantity and %CV were calculated from all triplicate PCR reactions at all concentrations of the three independent experiments. The acceptance criterion was that the %CV of quantity was $\leq 30\%$.

For accuracy, (accounted for the inherent variability in different analysts performing the assay, different detection systems, and the assay being performed on different times/times) the mean quantity (genome copy of target sequence/reaction) was calculated from each triplicate PCR reaction at each concentration (1E8, 1E7, 1E6, 1E5, 1E4, and 1E3). The acceptance criterion was that the %CV of quantity, was calculated for each sample concentration described above. The acceptance criterion was that the mean quantification was within $\pm 30\%$ of the reference value across the entire dynamic range of the assay. Acceptance criterion for this procedure also required that the linearity for each independent experiment must show the correlation coefficient (R^2) ≥ 0.98 and PCR amplification efficiency within 90-110%.

The results of the 3 independent runs showed that the assay met the established acceptance criteria for linearity, See Tables 33-36. Repeatability was $\leq 25\%$, intermediate precision was $\leq 25\%$ and accuracy was within $\pm 30\%$. The Linearity also was within the acceptance criteria with a correlation coefficient (R^2) ≥ 0.98 and PCR amplification efficiency within 90-110%.

Table 33. Run #1: Repeatability and accuracy of the first experiment met acceptance criteria.

Sample Name	CT	Quantity GC/rxn	Accuracy	StdDev of quantity	Repeatability	Acceptance Criteria Accuracy: $\pm 30\%$ of standard Repeatability: %CV $\leq 25\%$ $R^2 \geq 0.98$ PCR efficiency: 90-110%
			Mean of Quantity (GC/rxn)		%CV of Quantity	
PC_1E8	12.465	92,025,368	93,049,523	1,502,632	1.61	Met
	12.421	94,774,544				
	12.460	92,348,656				

PC_1E7	15.510	12,117,075	10,908,287	1,047,926	9.61	Met
	15.760	10,256,219				
	15.746	10,351,568				
PC_1E6	19.432	889,522	895,233	7,440	0.83	Met
	19.408	903,646				
	19.427	892,530				
PC_1E5	22.919	87,213	88,386	1,252	1.42	Met
	22.877	89,704				
	22.902	88,239				
PC_1E4	26.323	9,039	9,318	287	3.08	Met
	26.280	9,302				
	26.231	9,613				
PC_1E3	30.033	764	715	47	6.64	Met
	30.141	711				
	30.232	669				
Linearity	Correlation coefficient (R ²): 0.999 PCR amplification efficiency: 94.312%					Met

Table 34. Run #2: Repeatability and accuracy of the second experiment met acceptance criteria.

Sample Name	CT	Quantity GC/rxn	Accuracy	StdDev of quantity	Repeatability	Acceptance Criteria Accuracy: $\pm 30\%$ of standard Repeatability: $\%CV \leq 25\%$ $R^2 \geq 0.98$ PCR efficiency: 90-110%
			Mean of Quantity (GC/rxn)		%CV of Quantity	
PC_1E8	12.051	95,262,496	94,595,301	917,893	0.97	Met
	12.079	93,548,496				
	12.056	94,974,912				
PC_1E7	15.515	9,642,861	9,573,740	64,503	0.67	Met
	15.535	9,515,152				
	15.527	9,563,208				
PC_1E6	18.915	1,017,622	1,031,438	16,246	1.58	Met
	18.869	1,049,335				
	18.901	1,027,357				
PC_1E5	22.457	97,816	94,600	2,821	2.98	Met
	22.541	92,543				
	22.526	93,441				
PC_1E4	25.229	15,640*	9,572	20	0.21	Met
	25.970	9,586				
	25.974	9,558				
PC_1E3	29.679	825	822	4	0.45	Met

	29.691	818				
	29.680	824				
Linearity	Correlation coefficient (R^2): 1.000 PCR amplification efficiency: 93.731%					Met

* This value was an outlier per Dixon's Q test and excluded from data analysis.

5 Table 35. Run #3: Repeatability and accuracy of third experiment met acceptance criteria.

Table 55: Run #5: Repeatability and accuracy of data experiment met acceptance criteria.						
Sample Name	CT	Quantity GC/rxn	Accuracy	StdDev of quantity	Repeatability	Acceptance Criteria
			Mean of Quantity (GC/rxn)		%CV of Quantity	Accuracy: $\pm 30\%$ of standard Repeatability: $\%CV \leq 25\%$ $R^2 \geq 0.98$ PCR efficiency: 90-110%
PC_1E8	11.818	117,211,200	115,519,208	2,229,747	1.93	Met
	11.873	112,992,544				
	11.829	116,353,880				
PC_1E7	15.668	9,084,705	12,202,317	2,713,306	22.24	Met
	15.073	13,492,047				
	15.014	14,030,200				
PC_1E6	18.758	1,166,552	1,279,299	119,641	9.35	Met
	18.634	1,266,533				
	18.478	1,404,811				
PC_1E5	22.277	112,593	110,984	6,333	5.71	Met
	22.397	104,002				
	22.228	116,357				
PC_1E4	25.871	10,344	10,570	1,468	13.89	Met
	26.043	9,227				
	25.630	12,138				
PC_1E3	29.156	1,167	922	213	23.07	Met
	29.686	820				
	29.763	780				
Linearity	Correlation coefficient (R^2): 0.999 PCR amplification efficiency: 94.312%					Met

Table 36. Results of intermediate precision based on 3 separate runs by different analysts (Runs # 1, 2 and 3).

Sample Name	Mean of Quantity (GC/rxn)	StdDev of Quantity	Intermediate precision	Acceptance Criteria
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			%CV of quantity	Intermediate Precision: %CV ≤ 30%
PC_1E8 Run # 1, 2 and 3	101,054,677	10,961,469	10.85	Met
PC_1E7 Run # 1, 2 and 3	10,894,782	1,847,080	16.95	Met
PC_1E6 Run # 1, 2 and 3	1,068,657	179,151	16.76	Met
PC_1E5 Run # 1, 2 and 3	97,990	10,706	10.93	Met
PC_1E4 Run # 1, 2 and 3	9,851	1,003	10.18	Met
PC_1E3	820	141	17.23	Met

Limit of Quantification

The limit of quantification (LOQ) of the quantitative qPCR assay is the lowest amount of target sequence in a sample which can be quantitatively determined with suitable precision and accuracy. To determine the LOQ, the lowest set point (1E3 genome copies/reaction), the mid set points (1E4 and 1E6 genome copies/reaction) and upper set point (1E8 genome copies/reaction) of dynamic range were tested. Positive control samples with a concentration at the lowest, middle and upper set points of the dynamic range were prepared and spiked into an exogenous extracted DNA sample (~300ng human genomic DNA per reaction, to serve as background sample matrix DNA) and evaluated in 12 replicates performing the BVP-3 assay in the presence of a standard curve. The assay was performed by three analysts on different dates. Precision (repeatability and intermediate precision) and accuracy were determined as described above with the acceptance criteria as described above.

Results of the three independent LOQ experiments were within the established acceptance criteria for repeatability, intermediate precision, and accuracy (Tables 37-39), and linearity (Table 40).

Table 37. Run #1: Linearity, repeatability and accuracy of the first LOQ experiment met acceptance criteria.

Sample Name	CT	Quantity GC/rxn	Accuracy	StdDev of quantity	Repeatability	Acceptance Criteria Accuracy: ±30% of standard
			Mean of Quantity (GC/rxn)		%CV of Quantity	

						Repeatability: %CV $\leq$ 25% $R^2 \geq 0.98$ PCR efficiency: 90-110%
PC_1E8	12.088	99,773,728	102,123,087	5355285	5.24	Met
	12.071	100,869,600				
	12.092	99,482,992				
	12.105	98,636,408				
	12.126	97,306,984				
	12.128	97,162,088				
	11.979	107,288,808				
	12.072	100,809,184				
	12.067	101,182,688				
	12.029	103,763,872				
	12.046	102,613,232				
	11.854	116,587,456				
PC_1E6	19.376	781,088	995,748	109618	11.01	Met
	19.132	918,892				
	19.104	936,234				
	19.074	954,863				
	19.156	904,037				
	19.144	911,503				
	18.916	1,060,829				
	18.893	1,076,882				
	18.879	1,086,972				
	18.803	1,143,163				
	18.837	1,117,681				
	18.921	1,056,834				
PC_1E4	25.953	9,815	10,475	2076	19.82	Met
	25.947	9,850				
	26.233	8,147				
	26.162	8,539				
	26.029	9,330				
	25.283	15,321				
	25.849	10,513				
	25.888	10,248				
	26.029	9,329				
	26.034	9,297				
	25.521	13,078				
	25.621	12,237				
PC_1E3	29.313	1,049	1,162	274	23.56	Met
	28.819	1,457				
	29.434	968				
	29.798	760				
	29.571	883				

	29.050	1,249				
	29.420	977				
	28.775	1,500				
	28.688	1,590				
	28.847	1,430				
	29.250	1,094				
	29.407	985				
Linearity	Correlation coefficient (R^2): 0.999 PCR amplification efficiency: 94.544%					Met

Table 38. Run #2: Linearity, repeatability and accuracy of the second LOQ experiment met acceptance criteria.

Sample Name	CT	Quantity GC/rxn	Accuracy	StdDev of quantity	Repeatability	Acceptance Criteria Accuracy: $\pm 30\%$ of standard Repeatability: $\%CV \leq 25\%$ $R^2 \geq 0.98$ PCR efficiency: 90-110%
			Mean of Quantity (GC/rxn)		%CV of Quantity	
PC_1E8	12.095	95,058,592	93,650,034	1,438,673	1.54	Met
	12.120	93,524,168				
	12.115	93,828,104				
	12.118	93,629,304				
	12.112	94,045,632				
	12.154	91,458,488				
	12.109	94,209,456				
	12.100	94,746,560				
	12.094	95,136,672				
	12.135	92,587,520				
	12.168	90,615,920				
	12.097	94,959,992				
PC_1E6	19.256	838,090	854,259	24,034	2.81	Met
	19.177	882,874				
	19.225	855,340				
	19.217	859,674				
	19.237	848,636				
	19.230	852,521				
	19.295	816,498				
	19.207	865,467				
	19.261	835,148				
	19.132	909,394				
	19.253	839,838				
	19.239	847,628				
PC_1E4	26.141	8,864	8,691	430	4.95	

	26.052	9,400				Met
	26.174	8,670				
	26.226	8,378				
	26.154	8,787				
	26.174	8,672				
	26.194	8,560				
	26.314	7,907				
	26.197	8,543				
	26.265	8,168				
	26.083	9,209				
	26.095	9,138				
PC_1E3	29.738	823	828	75	9.03	Met
	29.680	855				
	29.774	804				
	29.703	842				
	29.747	818				
	29.647	874				
	29.528	946				
	29.952	715				
	29.871	754				
	29.895	742				
	29.503	961				
	29.770	806				
Linearity	Correlation coefficient (R^2): 1.000 PCR amplification efficiency: 93.621%					Met

Table 39. Run #3: Linearity, repeatability and accuracy of the third LOQ experiment met acceptance criteria.

Sample Name	CT	Quantity GC/rxn	Accuracy	StdDev of quantity	Repeatability	Acceptance Criteria Accuracy: $\pm 30\%$ of standard Repeatability: $\%CV \leq 25\%$ $R^2 \geq 0.98$ PCR efficiency: 90-110%
			Mean of Quantity (GC/rxn)		$\%CV$ of Quantity	
PC_1E8	11.987	87,553,632	86,941,879	1,185,070	1.36	Met
	12.009	86,286,488				
	12.016	85,904,016				
	11.983	87,763,088				
	11.996	87,037,040				
	11.987	87,556,344				
	11.976	88,172,408				
	12.015	85,993,864				
	11.958	89,230,456				

	12.034	84,890,344				
	12.012	86,159,392				
	12.001	86,755,472				
PC_1E6	19.003	919,457	869,476	39,354	4.53	Met
	19.096	865,214				
	19.106	859,918				
	19.088	869,696				
	19.140	841,225				
	19.095	866,096				
	19.091	868,491				
	19.138	842,210				
	19.174	822,775				
	18.935	960,591				
	19.168	826,054				
	19.049	891,982				
PC_1E4	26.388	7,597	9,014	1148	12.73	Met
	26.298	8,053				
	26.240	8,365				
	26.272	8,188				
	26.242	8,350				
	25.987	9,854				
	25.865	10,671				
	24.184	31,781*				
	26.077	9,298				
	25.823	10,964				
	25.211	16,316*				
	26.162	8,795				
PC_1E3	30.058	701	732	39	5.33	Met
	29.949	752				
	30.100	682				
	30.149	660				
	29.909	772				
	29.909	772				
	29.079	1,323*				
	29.114	1,294*				
	30.011	722				
	29.948	753				
	29.958	748				
	29.935	759				
Linearity	Correlation coefficient (R ²): 1.000 PCR amplification efficiency: 91.443%					Met

* Values were outliers per IQR test and excluded from data analysis.

Table 40. Results of intermediate precision based on 3 independent LOQ experiments/runs met acceptance criteria.

Sample Name	Mean of Quantity (GC/rxn)	StdDev of Quantity	Intermediate precision	Acceptance Criteria
			%CV of Quantity	Intermediate Precision: %CV $\leq$ 30%
PC_1E8 Run # 1, 2 and 3	94,238,333	7056365	7.49	Met
PC_1E6 Run # 1, 2 and 3	906,494	92636	10.22	Met
PC_1E4 Run # 1, 2 and 3	9,416	1583	16.81	Met
PC_1E3 Run # 1, 2 and 3	918	250	27.19	Met

Robustness

To determine the robustness of the assay, changes in critical reagent concentrations of up to 20% were evaluated according to the method of Youden and Steiner (supra). Several critical qPCR factors were selected and subjected to slight changes, Table 41. A BPV-3 positive fetal bovine serum test sample was extracted and tested in 12 replicates using the optimized and changed conditions in the BPV-3 assay in parallel with a standard curve.

Table 41 qPCR factors tested

PCR Factor	Combination matrix condition #				
	Optimized condition	1	2	3	4
Applied Biosystems qPCR Master Mix	Lot A	Lot A	Lot A	Lot B	Lot B

BPV-3 primer/probe	Lot A	Lot A	Lot B	Lot B	Lot A
BPV-3 primer/probe concentration	1x	1.2x	0.8x	1.2x	0.8x
PCR Vol. (μL)	30	25	25	35	35

The acceptance criterion for this procedure required that the response obtained from any robustness condition with respect to the applied small changes meet the established assay acceptance criteria. Acceptance criterion for this procedure included precision, the %CV of quantity for repeatability and intermediate precision was $\leq 25\%$ as described above. For accuracy, the mean of quantity of the combination matrix conditions (conditions 1 to 4) was $\pm 30\%$ of the mean of quantity of the optimized condition.

Results of robustness study showed that slight changes in the qPCR critical reagents were tolerable and the robustness results met the precision and accuracy ($118,100 \pm 35,430$) acceptance criteria when compared to the optimized condition, Table 42.

10 Table 42 Repeatability and accuracy of the robustness experiment met acceptance criteria

Sample Name	CT	Quantity GC/rxn	Accuracy	StdDev of quantity	Repeatability	Acceptance Criteria Accuracy: $\pm 30\%$ of optimized cond. Repeatability: $\%CV \leq 25\%$ $R^2 \geq 0.98$ PCR efficiency: 90-110%
			Mean of Quantity (GC/rxn)		%CV of Quantity	
Optimized condition	21.342	119,550	118,100	3359	2.84	Met
	21.347	119,099				
	21.345	119,303				
	21.400	114,637				
	21.357	118,231				
	21.402	114,449				
	21.388	115,630				
	21.413	113,586				
	21.381	116,243				
	21.288	124,286				
	21.301	123,106				
	21.347	119,083				
Robustness condition (1)	21.198	132,632	129,070	3071	2.38	Met
	21.229	129,739				
	21.261	126,719				
	21.248	127,949				
	21.265	126,411				
	21.283	124,755				
	21.274	125,541				
	21.247	128,075				

	21.216	130,993				
	21.204	132,088				
	21.177	134,715				
	21.234	129,221				
Robustness condition (2)	21.582	100,455	97,559	4251	4.36	Met
	21.633	96,803				
	21.600	99,194				
	21.630	97,070				
	21.594	99,617				
	21.697	92,446				
	21.725	90,595				
	21.697	92,449				
	21.651	95,582				
	21.596	99,439				
	21.524	104,787				
	21.558	102,275				
Robustness condition (3)	21.230	129,609	124,259	5180	4.17	Met
	21.248	127,920				
	21.283	124,744				
	21.278	125,207				
	21.369	117,245				
	21.324	121,089				
	21.351	118,724				
	21.349	118,902				
	21.357	118,260				
	21.246	128,132				
	21.227	129,892				
	21.211	131,381				
Robustness condition (4)	21.513	105,590	101,792	4841	4.76	Met
	21.547	103,024				
	21.526	104,609				
	21.575	100,966				
	21.618	97,889				
	21.633	96,866				
	21.648	95,779				
	21.626	97,330				
	21.621	97,667				
	21.544	103,268				
	21.490	107,378				
	21.443	111,135				
Standard	Correlation coefficient (R ²): 0.987 PCR amplification efficiency: 106.209%					Met

Verification

Assay system suitability and assay acceptance criteria were defined to verify assay performance per qualification protocol, see Table 44. To demonstrate that the assay performs as expected a test sample containing target sequence (Sample A) and a test sample with no target sequence (Sample B) were subjected to DNA extraction followed by BPV-3 qPCR. The verification was tested 3 times by different analysts on different days. Assay controls, system suitability and assay acceptance criteria and reporting and interpretation of test results are shown in Tables 43-44.

Table 43 Defined system suitability and assay acceptance criteria for BPV-3 qPCR assay

BPV-3 Target Sequence	Control	Valid assay acceptance criteria
BPV-3	NTC NEC	A minimum of 2 out of 3 reactions must show no amplification signal, CT = 40.00 (undetermined) and the obtained mean of GC quantity per reaction (if any) shall be less than the assay LOD95% (27 GC/reaction).
BPV-3	PEC	A minimum of 2 out of 3 reactions must show amplification signal of CT < 40.00, and the obtained mean GC of quantity per reaction shall be greater than assay LOQ (1E3 GC/rxn).
BPV-3	PC 2E4 PC 2E5 PC 2E7	A minimum of 2 out of 3 reactions must show amplification signal of CT < 40.00, and the obtained mean GC of quantity per reaction must meet the established assay accuracy criteria, $\pm 30\%$ of PC reference value: PC_2E7 acceptance accuracy range: 1.4E7 to 2.6E8 GC/rxn PC_2E5 acceptance accuracy range: 1.4E5 to 2.6E6 GC/rxn PC_2E4 acceptance accuracy range: 1.4E4 to 2.6E4 GC/rxn
BPV-3	INH or (+S)	A minimum of 2 out of 3 reactions must show amplification signal of CT < 40.00, and the obtained mean GC of quantity per reaction shall be within one magnitude of the original (2E4) spiked concentration level; the INH mean GC of quantity should be within 2E3 to 2E5 GC/reaction. NOTE: INH control is irrelevant and not applicable if test sample is positive with BPV-3 with mean GC of quantity of equal or greater than 2E4 per reaction.
BPV-3	ST	- Correlation coefficient (R^2) ≥ 0.98 - PCR amplification efficiency within 90 to 110%.
IPC Target Sequence	Control	Valid assay acceptance criteria
IPC	NEC	A minimum of 2 out of 3 reactions must show no amplification signal, CT = 40.00 (undetermined).
IPC	PEC	A minimum of 2 out of 3 reactions must show amplification signal of CT < 40.00.

IPC	Test Sample	A minimum of 2 out of 3 reactions must show no amplification signal, CT = 40.00 (undetermined).
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Table 44 Reporting and interpretation of data.

Test sample (BPV-3 GC/rxn)	Report	Interpretation
BPV-3 CT value is Undetermined (CT = 40.00) indicating the absence of BPV-3 or the mean of the BPV-3 GC/rxn is equal or less than assay LOD95% (27 GC/rxn)	BPV-3 Not Detected	Target sequence not detected or detected at a level below the LOD95% of the assay.
Mean GC/rxn of BPV-3 is less than assay LOQ (1E3) and greater than assay LOD95% (27 GC/rxn)	BPV-3 Detected (provide quantity as Log10 of mean $\pm$ StdDev per mL)	Target sequence is detected, but at a level below the LOQ of the assay; not accurately quantifiable.
Mean of BPV-3 GC/rxn is within the dynamic range of the assay (1E3 to 1E8 GC/rxn)	BPV-3 Detected (provide quantity as Log10 of mean $\pm$ StdDev per mL)	Target sequence is detected, and quantification is accurate and within the linear range of the assay.
Mean GC/rxn of BPV-3 is greater than upper set point (1E8) of assay dynamic range	BPV-3 Detected (provide quantity as Log10 of mean $\pm$ StdDev per mL) Alternatively, the quantity may be reported as greater than the upper level of the dynamic range of the assay.	Target sequence is detected and is greater than the upper dynamic range of the assay. The assay can be repeated with different dilution factors to allow for quantification within operational range.

5 Results of the verification study

Tables 45, 47 and 49 present the results of assay system suitability and assay controls for the three independent experiments. All three independent experiments/runs met the established system suitability and assay control acceptance criteria as defined herein. Tables 45, 47 and 49 present test samples results (Sample A: previously confirmed positive sample, and Sample B: previously confirmed negative sample). Results of verification study demonstrated that BPV-3 was not detected in the negative sample (Sample B) among all three independent experiments/runs and BPV-3 could be detected and quantified in the positive sample (Sample A) through all three independent experiments/runs.

Table 45 Verification Run #1 met acceptance criteria for assay system suitability and assay controls.

Target	Control	CT	Quantity GC/rxn	Mean of quantity (GC/rxn)	StdDev of quantity	Acceptance Criteria
BPV-3 v3 PP	PC_2E7	14.31	18,326,098	18,401,424	67,871	Met
		14.30	18,420,354			
		14.29	18,457,820			
	PC_2E5	21.49	168,383	167,435	3,214	Met
		21.53	163,853			
		21.47	170,069			
	PC_2E4	24.77	19,824	19,485	359	Met
		24.79	19,521			
		24.82	19,109			
	Sample B +S	24.97	17,393	17,452	53	Met
		24.96	17,468			
		24.96	17,495			
	Sample A +S	20.99	232,852	225,642	6,254	N/A
		21.07	221,700			
		21.06	222,372			
	PEC	27.67	2,981	2,935	124	Met
		27.65	3,029			
		27.77	2,794			
	NEC	40.00	0	0	0	Met
		40.00	0	0	0	
		40.00	0	0	0	
	NTC	40.00	0	0	0	Met
		40.00	0	0	0	
		40.00	0	0	0	
IPC v2 PP	Sample B	40.00	N/A			Met
		40.00				
		40.00				
	Sample A	40.00				Met
		40.00				
		40.00				
	PEC	27.40				Met
		27.35				
		27.42				
	NEC	40.00				Met
		40.00				
		40.00				
BPV-3 v3 PP	Standard	Correlation coefficient (R ²): 1.000 PCR amplification efficiency: 92.08%				Met

Table 46 Verification Run #1 sample results demonstrated that Sample B was negative for BPV-3, and Sample A was positive for BPV-3 and the quantity within the dynamic range of the assay

5

Target	Test Sample	CT	Quantity GC/rxn	Mean of quantity (GC/rxn)	StdDev of quantity	BPV-3 detected?	Quantity Log10 GC/mL (Mean ± StdDev)
	Sample B	40.00	0	0	0	No	N/A

BPV-3 v3 PP		40.00	0				
		40.00	0				
		21.21	202,610				
	Sample A	21.15	210,154	206,757	3,828	Yes	7.01 ± 0.01
		21.17	207,507				

BPV-3 was detected in Sample A at concentration of Log₁₀ 7.01 ± 0.01 genome copies/mL (Mean ± StdDev). No BPV-3 was detected in Sample B.

5 Table 47 Verification Run #2 met acceptance criteria for assay system suitability and assay controls

Target	Control	CT	Quantity GC/rxn	Mean of quantity (GC/rxn)	StdDev of quantity	Acceptance Criteria Refer to Table 19
BPV-3 v3 PP	PC_2E7	14.12	20,329,452	20,227,899	112,064	Met
		14.12	20,246,576			
		14.13	20,107,670			
	PC_2E5	21.12	214,577	208,974	11,572	Met
		21.26	195,666			
		21.11	216,678			
	PC_2E4	24.72	20,728	20,763	497	Met
		24.75	20,285			
		24.68	21,277			
	Sample B +S	24.86	18,896	18,752	1,277	Met
		24.78	19,951			
		24.99	17,409			
	Sample A +S	21.13	214,134	213,831	263	N/A
		21.13	213,692			
		21.13	213,667			
	PEC	27.67	3,044	2,874	148	Met
		27.81	2,777			
		27.80	2,799			
	NEC	40.00	0	0	0	Met
		40.00	0			
		40.00	0			
	NTC	40.00	0	0	0	Met
		40.00	0			
		40.00	0			
IPC v2 PP	Sample B	40.00	N/A			Met
		40.00				
		40.00				
	Sample A	40.00				Met
		40.00				
		40.00				
	PEC	27.52				Met
		27.66				
		27.57				
	NEC	40.00				Met

		40.00		
		40.00		
BPV-3 v3 PP	Standard	Correlation coefficient (R ²): 1.000 PCR amplification efficiency: 91.50%		Met

Table 48 Verification Run #2 sample results demonstrated that Sample B was negative for BPV-3, and Sample A was positive for BPV-3 and the quantity within the dynamic range of the assay

5

Target	Test Sample	CT	Quantity GC/rxn	Mean of quantity (GC/rxn)	StdDev of quantity	BPV-3 detected?	Quantity Log ₁₀ GC/mL (Mean ± StdDev)
BPV-3 v3 PP	Sample B	40.00	0	0	0	No	N/A
		40.00	0				
		40.00	0				
	Sample A	21.15	210,494	218,073	6,591	Yes	7.04 ± 0.01
		21.07	222,461				
		21.08	221,264				

BPV-3 was detected in Sample A at concentration of Log₁₀ 7.04 ± 0.01 genome copies/mL (Mean ± StdDev). No BPV-3 was detected in Sample B.

10 Table 49 Verification Run #3 met acceptance criteria for assay system suitability and assay controls.

Target	Control	CT	Quantity GC/rxn	Mean of quantity (GC/rxn)	StdDev of quantity	Acceptance Criteria Refer to Table 19
BPV-3 v3 PP	PC_2E7	13.92	23,374,386	24,090,493	835,483	Met
		13.82	25,008,390			
		13.89	23,888,704			
	PC_2E5	21.05	223,478	226,814	5,914	Met
		21.05	223,321			
		20.98	233,642			
	PC_2E4	24.79	19,401	19,309	213	Met
		24.82	19,065			
		24.79	19,459			
	Sample B +S	24.69	20,650	20,570	213	Met
		24.69	20,732			
		24.72	20,329			
	Sample A +S	21.12	212,975	211,790	2,873	N/A
		21.15	208,515			
		21.11	213,881			
	PEC	27.46	3,390	3,292	140	Met
		27.48	3,355			
		27.58	3,132			
	NEC	40.00	0	0	0	Met
		40.00	0	0	0	
		40.00	0	0	0	

	NTC	40.00	0	0	0	Met
		40.00	0	0	0	
		40.00	0	0	0	
IPC v2 PP	Sample B	40.00	N/A			Met
		40.00				
		40.00				
	Sample A	40.00				Met
		40.00				
		40.00				
	PEC	27.51				Met
		27.52				
		27.57				
	NEC	40.00				Met
		40.00				
		40.00				
BPV-3 v3 PP	Standard	Correlation coefficient (R ²): 1.000 PCR amplification efficiency: 92.114			Met	

Table 50 Verification Run #3 sample results demonstrated that Sample B is negative for BPV-3, and Sample A is positive for BPV-3 and the quantity within the dynamic range of the assay

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Target	Test Sample	CT	Quantity GC/rxn	Mean of quantity (GC/rxn)	StdDev of quantity	BPV-3 detected?	Quantity Log ₁₀ GC/mL (Mean ± StdDev)
BPV-3 v3 PP	Sample B	40.00	0	0	0	No	N/A
		40.00	0				
		40.00	0				
	Sample A	21.09	217,541	220,903	4,909	Yes	7.04± 0.01
		21.03	226,536				
		21.08	218,632				

BPV-3 is detected in Sample A at concentration of Log₁₀ 7.04 ± 0.01 genome copies/mL (Mean ± StdDev). No BPV-3 is detected in Sample B.

Table 51 presents the established BPV-3 assay qualification criteria. The qualified quantitative BPV-3 assay allows for quantification of 1E3 to 1E8 BPV-3 genome copies per PCR reaction.

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Table 51 BPV-3 assay qualified criteria

Parameter	Qualified Criteria
Dynamic Range	1E3 to 1E8 GC/rxn
Lower limit of quantification	1E3 GC/rxn
Upper limit of quantification	1E8 GC/rxn
Limit of detection (LOD95%)	27 GC/rxn with a 95% confidence interval of 22 and 34 GC/rxn

Tested Samples A and B met required qualification specifications for a quantitative assay including specificity, limit of detection, limit of quantification, linearity, precision, accuracy and robustness.

All tested samples (Samples A and B) met the proposed system suitability and assay acceptance criteria. The quantification level between three independent runs showed similar results (Tables 45, 47 and 49) indicating the accurate, precise and robust performance of the qualified assay. The quantitative BPV-3 assay and associated qualification data demonstrated that the BPV-3 quantitative real-time PCR assay allowed detection and quantification of BPV-3 DNA in test sample.

What is claimed is:

1. A composition comprising oligonucleotides selected from the groups consisting of
 - a) oligonucleotides having the nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3;
 - b) oligonucleotides having the nucleic acid sequence of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6;
 - c) oligonucleotides having the nucleic acid sequence of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9;
 - d) oligonucleotides having the nucleic acid sequence of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12;
 - e) oligonucleotides having the nucleic acid sequence of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15;
 - f) oligonucleotides having the nucleic acid sequence of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22;
 - g) oligonucleotides having the nucleic acid sequence of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25; and
 - h) oligonucleotides having the nucleic acid sequence of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28.
2. A composition according to claim 1, wherein the composition optionally includes a second composition comprises oligonucleotides having the sequence of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18.
3. A composition according to claim 1, wherein the composition comprises oligonucleotides having the nucleic acid sequence of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9.
4. A composition according to claim 1, wherein the composition comprises a first composition comprises oligonucleotides having the nucleic acid sequence of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9 and a second composition comprises oligonucleotides having the sequence of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18.
5. A reagent for detecting *Bovine parvovirus 3* (BPV-3) genomic DNA in the extracted DNA of a test sample selected from the group consisting of

a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3;

b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6;

5 c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9;

d) a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12;

10 e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15;

f) a primer probe combination having the nucleic acid sequences of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22;

g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25; and

15 h) a primer probe combination having the nucleic acid sequences of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28.

6. The reagent according to claim 5, wherein SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:22, SEQ ID NO:25; or SEQ ID NO:28 have a fluorescent reporter dye and/or a non-fluorescent quencher.

20 7. The reagent according to claim 5, wherein one or more of SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:22, SEQ ID NO:25; or SEQ ID NO:28 have a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and/or either a Minor Groove Binder non-fluorescence quencher (MGB-NFQ) or ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end.

8. The reagent according to claim 5, wherein the primer probe combination detects DNA encoding the structural capsid (VP) protein and/or the non-structural (NS) protein of *Bovine parvovirus 3* in the test sample.

9. The reagent according to claim 5 in combination with an internal positive control primer combination.

10. The reagent according to claim 5, comprising SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9.

11. A reagent for use as an internal positive control primer probe combination in an assay for detecting *Bovine parvovirus 3* (BPV-3) genomic DNA in the extracted DNA of a test sample comprising a primer probe combination.

12. The reagent according to claim 11, wherein the primer probe combination has the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18.
13. The reagent according to claim 11, wherein SEQ ID NO: 18 has a fluorescent reporter dye and/or a non-fluorescent quencher.
- 5 14. The reagent according to claim 13, wherein SEQ ID NO: 18 has a fluorescent reporter dye, 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end.
15. A primer probe combination for detecting *Bovine parvovirus 3* genomic DNA in the extracted DNA of a test sample in combination with an internal positive control primer probe combination for detecting *Bovine*
10 *parvovirus 3* (BPV-3) genomic DNA.
16. A primer probe combination for detecting *Bovine parvovirus 3* genomic DNA according to claim 15 wherein the primer probe combination is selected from the groups consisting of
- a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3;
 - 15 b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6;
 - c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9;
 - d) a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11,
20 and SEQ ID NO:12;
 - e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15;
 - f) a primer probe combination having the nucleic acid sequences of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22;
 - 25 g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25; and
 - h) a primer probe combination having the nucleic acid sequences of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28.
17. The primer probe combination according to claim 16, wherein SEQ ID NO: 3, SEQ ID NO:6, SEQ ID
30 NO:9, SEQ ID NO:12, SEQ ID NO:15 have a fluorescent reporter dye and/or a non-fluorescent quencher.

18. The primer probe combination according to claim 16, wherein one or more of SEQ ID NO: 3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:22, SEQ ID NO:25; or SEQ ID NO:28 have a fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and/or either a Minor Groove Binder non-fluorescence quencher (MGB-NFQ) or ZEN-IB and Iowa Black Fluorescence quencher (IBFQ) at the 3' end.
19. The primer probe combination according to claim 15, wherein the primer probe combination detects DNA encoding the structural capsid (VP) protein and/or the non-structural (NS) protein of *Bovine parvovirus 3* in a test sample.
20. An internal positive control primer probe combination for detecting *Bovine parvovirus 3* (BPV-3) genomic DNA in a test sample according to claim 15, wherein the primer probe combination has the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18.
21. The internal positive control primer probe combination according to claim 20, wherein SEQ ID NO: 18 has a fluorescent reporter dye and/or a non-fluorescent quencher.
22. The primer probe combination according to claim 20, wherein SEQ ID NO: 18 has a fluorescent reporter dye, 2'-chloro-7'phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end.
23. A primer probe combination for detecting *Bovine parvovirus 3* genomic DNA in the extracted DNA of a test sample comprising SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9 in combination with an internal positive control primer probe combination comprising SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18, wherein SEQ ID NO: 9 has a fluorescent reporter dye 6-carboxyfluorescein at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) non-fluorescence quencher at the 3' end and SEQ ID NO:18 has a fluorescent reporter dye, 2'-chloro-7'phenyl-1,4-dichloro-6-carboxy-fluorescein at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end.
24. A kit for detecting *Bovine parvovirus 3* (BPV-3) genomic DNA contamination in the extracted DNA of a test sample comprising
- primer probe combination that detects DNA encoding BPV-3 selected from the group consisting of
- a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3;
- b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6;

c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9;

d) a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12;

5 e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15;

f) a primer probe combination having the nucleic acid sequences of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22;

10 g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25; and

h) a primer probe combination having the nucleic acid sequences of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28; and

optionally an internal positive control primer probe combination having the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18.

15 25. A method for determining the presence or absence of *Bovine parvovirus 3* genomic DNA in the extracted DNA of a test sample comprising

1) a reaction mixture comprising a test sample, a positive control, a BPV-3_IPC positive control plasmid DNA, nucleic acid amplification reagents, an internal positive control primer probe combination having the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18, wherein SEQ ID
20 NO:18 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end, and a primer probe combination selective for a DNA sequence of *Bovine parvovirus 3*, selected from the group consisting of

a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3, wherein SEQ ID NO:3 has a fluorescent reporter dye at the 5' end and a non-fluorescence
25 quencher at the 3' end;

b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6, wherein SEQ ID NO:6 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end;

c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and
30 SEQ ID NO:9, wherein SEQ ID NO:9 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end;

d) a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12, wherein SEQ ID NO:12 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end;

5 e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15, wherein SEQ ID NO:15 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end;

f) a primer probe combination having the nucleic acid sequences of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22, wherein SEQ ID NO:22 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end;

10 g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25, wherein SEQ ID NO:25 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; and

h) a primer probe combination having the nucleic acid sequences of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28, wherein SEQ ID NO:28 has a fluorescent reporter dye at the 5' end and a non-fluorescence
15 quencher at the 3' end;

2) subjecting the reaction mixture to a quantitative PCR technique to obtain copies of the target sequence,

3) measuring any increase in fluorescence signal,

wherein an increase in fluorescence signal indicates the presence of *Bovine parvovirus 3* genomic DNA
20 in the test sample.

26. The method according to claim 25, wherein the fluorescent reporter dye is 6-carboxyfluorescein (FAM) and the non-fluorescence quencher is a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) or ZEN-IB and Iowa Black Fluorescence quencher (IBFQ).

27. The method according to claim 25, further comprising one or more negative extraction control, no
25 template control, positive extraction control, positive control, and/or inhibition control.

28. The method according to claim 25, wherein the sensitivity or analytical limit of detection is 22 genome copies per reaction.

29. The method according to claim 25, wherein the sample limit of detection is 25 genome copies per reaction.

30. The method according to claim 25, wherein the primer probe combination selective for a DNA sequence of *Bovine parvovirus 3* detects DNA encoding the non-structural (NS) protein and/or structural capsid (VP) protein of *Bovine parvovirus 3*.
31. The method according to claim 25, wherein the primer probe combination selective for a DNA
5 sequence of *Bovine parvovirus 3* amplifies a 144 bp fragment.
32. The method according to claim 25, wherein the primer probe combination selective for a DNA sequence of *Bovine parvovirus 3* comprises the combination of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9, wherein SEQ ID NO:9 has a 6-carboxyfluorescein (FAM) at the 5' end and a Minor Groove Binder (MGB-NFQ) at the 3' end.
- 10 33. The method according to claim 25 further comprising an internal positive control primer probe combination.
34. The method according to claim 33, wherein the primer probe combination has the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18.
35. The method according to claim 34, wherein SEQ ID NO: 18 has a fluorescent reporter dye and/or a
15 non-fluorescent quencher.
36. The method according to claim 34, wherein SEQ ID NO: 18 has a fluorescent reporter dye, 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) at the 5' end and a Minor Groove Binder non-fluorescent quencher (MGB-NFQ) at the 3' end.
37. A method for the quantification of 1E3 to 1E8 genome copies of *Bovine parvovirus 3* genomic DNA
20 in a PCR reaction comprising
- 1) a reaction mixture comprising the extracted DNA of a test sample, a positive control, a BPV-3_IPC positive control plasmid DNA, nucleic acid amplification reagents, an internal positive control primer probe combination having the nucleic acid sequences of SEQ ID NO: 16, SEQ ID NO:17, and SEQ ID NO:18, wherein SEQ ID NO:18 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3'
25 end, and a primer probe combination selective for a DNA sequence of *Bovine parvovirus 3*, selected from the group consisting of
- a) a primer probe combination having the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3, wherein SEQ ID NO:3 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end;

b) a primer probe combination having the nucleic acid sequences of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6, wherein SEQ ID NO:6 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end;

5 c) a primer probe combination having the nucleic acid sequences of SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9, wherein SEQ ID NO:9 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end;

d) a primer probe combination having the nucleic acid sequences of SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12, wherein SEQ ID NO:12 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end;

10 e) a primer probe combination having the nucleic acid sequences of SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15, wherein SEQ ID NO:15 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; f) a primer probe combination having the nucleic acid sequences of SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22, wherein SEQ ID NO:22 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end;

15 g) a primer probe combination having the nucleic acid sequences of SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25, wherein SEQ ID NO:25 has a fluorescent reporter dye at the 5' end and a non-fluorescence quencher at the 3' end; and

h) a primer probe combination having the nucleic acid sequences of SEQ ID NO:26, SEQ ID NO:27, and SEQ ID NO:28, wherein SEQ ID NO:28 has a fluorescent reporter dye at the 5' end and a non-fluorescence
20 quencher at the 3' end;

2) subjecting the reaction mixture to a quantitative PCR technique to obtain copies of the target sequence, and

3) measuring any increase in fluorescence signal.

38. The method according to claim 37, wherein the limit of detection (LOD95%) of the method is 27 genome
25 copies of *Bovine parvovirus* 3 genomic DNA per reaction with a 95% confidence interval of 22 and 34 genome copies per reaction.

39. The method according to claim 37, wherein the linearity of the method has a correlation coefficient (R^2) ≥ 0.98 and a PCR amplification efficiency within 90-110%.

40. The method according to claim 37, wherein the method has a repeatability value that is a %CV of quantity
30 equal or less than 25%.

41. The method according to claim 37, wherein the method has an intermediate precision value that is %CV of quantity equal or less than 30%.
42. The method according to claim 37, wherein the method has an accuracy value within $\pm 30\%$ of the accepted reference value (ST) across the whole dynamic range of the assay.
- 5 43. The method according to claim 37, wherein the method has a limit of quantitation that is the %CV of quantity for repeatability at $\leq 25\%$, intermediate precision at $\leq 30\%$ and acceptance criterion for the accuracy within $\pm 30\%$ of the expected standard reference value.
44. The method according to claim 37, wherein the method has a robustness that has a percent CV of quantity for repeatability of $\leq 25\%$, an intermediate precision of $\leq 30\%$, and an accuracy of the mean of quantity of the
10 combination matrix condition tested of $\pm 30\%$ of the mean of quantity of the optimized condition.
45. The method according to claim 37, wherein the method includes one or more of a no template control, a positive control, a negative extraction control, a positive extraction control, an inhibition control, an internal positive control, and a standard.