A follow-up investigation of the reported high herd prevalence of bovine viral diarrhea in dairy farms in Georgia and Florida

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Abstract

Bovine viral diarrhea virus (BVDV) causes economically significant disease in the dairy industry worldwide. Testing and removing persistently infected (PI) animals is an essential part of controlling BVD in infected herds. A previous surveillance study using bulk-tank milk testing by real-time RT-PCR revealed a herd prevalence of 38.4% in Georgia and Florida. The high herd prevalence of BVD in these dairy farms prompted subsequent investigation to update the prevalence and reveal the subtype of the circulating BVDV in the region. Ninety bulk milk samples from 37 dairy farms were tested during 2021-2022. A survey related to management practices was also conducted. Overall, 10.0% of the bulk milk samples tested positive (9/90) and 10.8% of the herds tested positive (4/37). The sequences of 5'- untranslated and E1-E2 regions of the viral genome from positive samples all matched to the subtype of BVDV-1b. Phylogenetic analysis indicates these BVDVs are regional circulating strains. Most farms routinely use vaccines in calves (83.8%) and cows (89.2%) to reduce virus transmission and prevent clinical diseases and PI animals. However, only 24.3% and 27.0% of the farms test for BVD in replacement heifers and newborn calves, respectively. And none of the farms quarantine newly purchased animals. Despite high vaccination rate, BVD can still be an issue for dairy farms in the southeast region of the United States, exemplifying the importance of PI animals' removal and biosecurity procedures implementation to efficiently control BVD in dairy herds.

Key words: BVD, bulk milk, prevalence, RT-PCR, survey

Introduction

Bovine viral diarrhea virus (BVDV) belongs to the family Flaviviridae, genus Pestivirus and is a small, enveloped virus. The genome of BVDV is composed of a positive single-stranded RNA. The virus has 2 biotypes demonstrated in cell culture: cytopathic (causes cell death) and non-cytopathic (does not cause cell death). While some strains exhibit cell killing ability, most BVDVs are non-cytopathic. This phenotype difference is caused by the NS2/3 protein sequence at the cleavage site and is not related to viral virulence.[1] Based on antigenic and genetic differences, BVDV is divided into 2 genotypes, type 1 (BVDV-1) and type 2 (BVDV-2).[2,3] Currently, 21 subtypes of BVDV-1 (1a-1u) and 4 subtypes of BVDV-2 (2a-2d) have been identified.[4] However, only genotypes 1a, 1b, 2a, 2b and 2c have been reported in the United States.[5–9] Although phylogenetic analysis of 5'-UTR sequences generally provides correct allocation in genotyping, limitation has been reported.[10–12] Inclusion of multiple genomic regions such as N^{pro} and E2 regions is recommended for phylogenies and genotyping of BVDV isolates.[4,13,14]

Infections of BVDV lead to significant economic loss to the dairy industry.[15,16] Diseases observed in infected herds include reproductive failure (failure to conceive, embryonic loss, abortion, congenital defects and stillborn or weak, unthrifty calves) and respiratory disease and diarrhea in young calves.[17,18] Some cattle infected with BVDV do not show clinical signs of disease. However, the virus suppresses the immune system which can make them more susceptible to other diseases or they can be a potential source of infection for other animals. Acutely infected cattle usually recover from the disease, although a severe form with hemorrhagic lesions had been reported.[2,17] These transiently infected (TI) animals can shed BVDV in their bodily secretions such as nasal, ocular discharges and in semen as well.[17,19] When infections happen during gestation, the effect of BVDV via transplacental infection on the developing fetus varies depending on the stage of pregnancy. Infection early in pregnancy will usually result in embryonic loss or abortion. Exposure of the fetus to the non-cytopathic BVDV biotype between 30 to 125 days of gestation can result in the development of a permanently infected (PI) calf.[20,21] The PI calf's immune system does not recognize the BVDV as foreign, so the immune system does not attack it.

Although many PI calves are born unthrifty and die or are culled within the first 6 months of life, some go on to become seemingly healthy adults. PI calves shed large numbers of the virus in all body secretions including nasal discharge, saliva, ocular discharge, milk, feces, urine and semen throughout their lifetime and continually expose other cattle to the virus. Transmission from PIs to susceptible members of the herd can be by direct contact, through contaminated veterinary

and farm equipment or facilities.[2,22] Due to being immunotolerant to the virus, PI calves will not seroconvert to the infecting BVDV. However, they could still generate antibody responses to a heterologous BVDV genotype or subtype from field infection or modified live vaccines.[23] Hence, serology should not be the only method to determine calf PI status.

Management practices to control BVD include vaccination of all members of the herd, biosecurity procedures to prevent introduction, biocontainment to interrupt the spread of the virus and laboratory testing of samples for the presence of the virus in a herd, with the final goal of identifying and removing PI animals from the herd.[24] PI animals shed large quantity of BVDV in the milk, hence for dairy operations, a sensitive and economical method to screen PI animals among lactating cows is to test bulk-tank milk by RT-PCR.[25–27] The detection limits for the dilution factor have been reported to be in the range of 1:600 to 1:1600 when the milk of a PI animal was diluted with BVDV-negative milk.[26,28,29]

While the dairy herd prevalence of 17 western and eastern states was as low as 1.7%,[30] higher regional herd prevalence of BVD has been reported (9%, 12.4% and 15% in intermountain west, northeast states, and Michigan, respectively).[27,28,31] However, a surveillance study in 2015-2016 revealed the cumulative incidence of BVD as 38.4% across dairy farms in Georgia and Florida (at least one positive test from bulk milk samples during the 6-month period).[32] The high percentage of BVDV-positive farms in this region is concerning and prompted us to carry out a follow-up surveillance study. The objectives of this study were to update the prevalence of BVD and determine the circulating BVDV subtype in this region. Questionnaires were also collected from the participating dairy farms regarding general information and farm management practices including vaccination, PI testing, biosecurity maintenance and degree of veterinary involvement.

Materials and methods

Study participation

Dairy managers/producers were contacted by a variety of methods including direct mailing, dairy co-operative e-newsletter, direct emails, phone calls and in-person visits. Participants in the study were also asked to complete a questionnaire regarding farm information and management practices. The project was approved by the University of Georgia, College of Veterinary Medicine Clinical Review Committee (CR-596) and participants were required to complete an informed consent and waiver of liability to be included in the study. Bulk tank samples were collected by milk co-operative testing laboratory staff or field representatives, dairy personnel or on-site collection by the authors (1-2 tube per bulk tank). Samples were submitted to Tifton Veterinary Diagnostic and Investigational Laboratory (TVDIL) for testing BVDV by real-time RT-PCR (detailed described below).

BVDV RNA extraction from milk pellet

Milk pellets of somatic cells from bulk tank samples were prepared as previously described.[28] The RNA from the pellet was extracted with MagMax™ Total Nucleic Acid Isolation Kit[a] by resuspending the pellet with 200 µl of sterile PBS and then by following the kit instructions for extracting liquid samples. Extractions were performed on the Qiagen BioSprint 96 workstation[b].

BVDV real-time RT-PCR

Real-time RT-PCR was performed using VetMax™-Gold BVDV PI Detection Kit[c] on the Applied Biosystems ABI 7500 Fast[d] following kit instructions. A Ct value less than 40 is interpreted as BVDV-positive in the pool sample of bulk-tank milk.

5'-UTR and E1-E2 sequencing of BVDV-positive samples

cDNA from milk pellet RNA was synthesized by SuperScript VILO cDNA Synthesis Kit[e], followed by PCR targeting 5'-UTR (287 bp) and E1-E2 regions (606 bp) of BVDV with primers BE & B2 and B11 & B32, respectively.[33,34] PCR was performed with GoTaq G2 Hot Start Master Mix[f] (Promega) with a final concentration of 0.4 µM for each primer using cycling condition: 94 °C 2 min, 35 cycles of 94 °C 20 sec, 52 °C 20 sec, 72 °C 25 sec and hold at 72 °C for 5 min for 5'-UTR; 94 °C 2 min, 35 cycles of 94 °C 30 sec, 60 °C 60 sec, 72 °C 60 sec and hold at 72 °C for 7 min for E1-E2. PCR product sizes were examined in 1.5% agarose gel. After confirmation of single band, PCR products were cleaned up with ExoSAP-IT reagent[g] before submitting to Eurofins Genomics LLC for Sanger Sequencing with both the forward and reverse primers.

Sequence analysis for BVDV-positive samples

The sequence analysis for 5'-UTR and E1-E2 regions was performed with Geneious Prime software[h]. For each pair of primers, the full sequence was obtained by executing "Trim ends" and "De Novo assembly". The assembled sequence was blasted against NCBI databases for nucleotide collection.

Phylogenetic analysis of BVDV

Phylogenetic analysis for 5'-UTR and E1-E2 regions was performed with Geneious Prime software[h]. All BVDV sequences were trimmed off the primer sequences and aligned using Clustral Omega. Reference sequences were retrieved from NCBI nucleotide databases except for "Tifton PI-calf" which was a clinical case submitted to TVDIL in 2022. The reference strain 5912c (BVDV-2a) was used as outgroup for tree building. Trees were constructed by Neighbor-Joining method with Tamuri-Nei distance model. Bootstrap resampling (1,000 replicates) and "Create Consensus Tree" were checked when building the trees.

Statistical analysis for the survey data

The answers to the questionnaire were categorized by BVD-positive or -negative farms in Excel. Further statistical analysis was performed in GraphPad Prism 9[i] using Fisher's exact test for 2 × 2 table data sets or Mann-Whitney test for numeric data comparison between the two groups.

Results

BVD bulk tank testing results

A total of 37 dairy farms participated in the study during the time frame of 2021-2022 (28 dairy farms from Georgia and 9 farms from Florida). Farms at separate locations owned by the same producer were counted as different farms if the cows were not able to mingle. Based on the survey data, about 57,600 milking cows were screened from these 37 farms with a total of 90 bulk tank samples screened. Overall, 10.0% of samples (9/90) tested positive for BVDV with 5 samples from

Georgia (6.76%, 5/74) and 4 samples from Florida (25.0%, 4/16) (Table 1). About 10.8% of farms (4/37) tested positive for BVDV with 2 farms in Georgia (7.1%, 2/28) and 2 farms in Florida (22.2%, 2/9) (Table 2). No BVDV was detected in herds less than 500 cows (Table 2). In contrast, dairy farms larger than 1,000 cows had the highest BVD prevalence (17.6%) among all the herds (Table 2). However, due to many large farms also tested negative for BVDV, herd size was not significantly different between positive and negative farms ($P > 0.05$).

Samples from the 4 positive farms were coded by state and a numeric number for further analysis (Table 3). Each BVD-positive farm either had a positive sample rate of 50% or 100%. The milking cow number per tank ranged from 200 to 1,200 and the Ct values ranges from 23.00 to 36.64 (Table 3). Due to the high Ct values, farm D collected a set of string samples for follow-up testing after 17 days of the first sample collection. However, none of the 6 string samples tested positive for BVDV.

BVDV subtype

The sequence of 5'-UTR and E1-E2 regions were amplified by PCR from the cDNA of BVD positive samples. Among the 9 positive samples, 3 samples (FL 2-4) did not produce adequate amplicon for sequencing due to low viral load (real-time RT-PCR with Ct values > 35). The remaining 6 samples produced a single PCR product for each target and were successfully sequenced. Blasted by either 5'-UTR or E1-E2 nucleotide sequences, the subtype was identified as BVDV-1b from all 6 samples. When comparing to the known vaccine strain NY-1, the BVDV-1b strains identified in our study are different at the 5'-UTR region (Figure 1). There is no published sequence for another BVDV-1b vaccine strain 6309.

Phylogenic analysis of 5'-UTR and E1-E2

The phylogenetic analysis of 5'-UTR and E1-E2 sequences grouped all 6 samples with other BVDV-1b strains, confirming the blast results. Similar phylogenetic trees were generated when the phylogenetic analysis was performed using 5'-UTR (246 bp) or E1-E2 regions (555 bp). Despite clustering with different reference strains, the topology of GA 1-4 and GA 5 & FL 1 remained in separate clades (Figure 1 and Figure 2). Closely or identical relationships for 5'-UTR or E1-E2 were observed between samples of farm A (GA 1-4) with similar results for the sample of farm B (GA 5) and the Tifton PI calf_2022 (Figure 1 and Figure 2). FL-1 is in a different clade than those of the GA samples which indicates the viruses were circulating within a small area, including within the same farm.

Table 1: Positive rate by sample number.

State	Sample #	Total
Georgia	5/74 (6.76%)	**9/90 (10.0%)**
Florida	4/16 (25.0%)	

Survey data analysis

Approximately 16% (6/37) and 76% (28/37) of the dairy managers/producers have 11-20 years and more than 20 years of experience in the dairy industry, respectively. A majority of farms (92%, 34/37) of farms reported having a regular veterinarian visiting daily, weekly, monthly, or on an as needed basis (32.4%, 14.7%, 8.8% and 44.1%, respectively). For biosecurity procedures, while 89.2% (33/37) of the respondents reported they have a sick cow pen for treating diseased animals, only 21.6% (8/37) of farms test purchased cows and none of the farms (0.0%) keep un-tested cows from the rest of the herd – no quarantine procedure was implemented in all participating farms.

Most participants (81.1%, 30/37) reported at least some knowledge about BVD and 40.5% (15/37) reported they have previous BVD diagnosis on the farm. However, only 24.3% (9/37) and 27.0% (10/37) of the farms test BVD for replacement heifers/cows and newborn calves, respectively. Most of the farms reported routine use of BVD vaccines in their calves (83.8%, 31/37) and cows (89.2%, 33/37). While 71.0% (22/31, 6 left blank) of farms use modified-live virus (MLV) BVD vaccines and 22.6% (7/31, 6 left blank) use a combination of MLV plus killed vaccines in calves, 45.5% (15/33, 4 left blank) use MLV vaccines and 18.2% (6/33, 4 left blank) use MLV plus killed vaccines in cows.

Analysis of survey data showed that herd size, purchasing or raising replacement heifers, using artificial insemination, testing for BVD in newborn calves and replacements, or previous BVD diagnosis were not significantly different between farms with positive and negative BVD results. However, positive BVD farms have significantly higher number of dry cows than BVD negative farms with median of 284 cows (270.5-298.3 interquartile range) and 124 cows (29.5-240 interquartile range), respectively ($P = 0.038$).

Discussion

Our study revealed that the BVD herd prevalence among the dairy farms in Georgia and Florida is similar to those in other regions of the United States falling within 9-15% range.[27,28,31] The incidence of BVD in this region was found to be 38.4% in 2015-2016. Compared to the current method, the previous incidence was cumulative: bulk tank samples were tested at least twice for each farm in a 6-month period. The high herd prevalence of 38.4% collected for the same region could be due to the accumulative format of data collection and/or the dairy herds with lower production efficiency may have been closed over the past 6 years. Indeed, the dairy operation number has been in decline in the region in recent years.

Table 2: Positive rate by herd size (milking + dry cows).

	< 200	200-500	501-1000	> 1000	Total
Georgia	0/8	0/2	0/4	2/14	2/28 (7.1%)
Florida	0/0	0/1	1/5	1/3	2/9 (22.2%)
Total	0/8 (0%)	0/3 (0%)	1/9 (11.1%)	3/17 (17.6%)	**4/37 (10.8%)**

Table 3: Bulk milk sample information from bovine viral diarrhea virus (BVDV)-positive herds.

Farm code	Milking cow #	Bulk milk sample #	Positive sample #	Cow # per bulk tank	Sample code and Ct. value	BVDV subtype
A farm	3000	4	4 (100%)	750	GA 1: 29.39 GA 2: 26.39 GA 3: 25.99 GA 4: 27.79	1b 1b 1b 1b
B farm	2400	2	1 (50%)	1200	GA 5: 34.76	1b
C farm	400	2	1 (50%)	200	FL 1: 23.00	1b
D farm	1650	3	3 (100%)	550	FL 2: 35.10 FL 3: 35.90 FL 4: 36.64	NA* NA* NA*

* NA: None answer, samples (FL 2-4) did not produce adequate amplicon for sequencing due to low viral load (real-time RT-PCR with Ct. values > 35).

Figure 1: Phylogenetic tree for 5'-UTR (246 bp). Each reference virus is labeled with strain name and Genbank ID number except for "Tifton PI-calf" which was a clinical case submitted to the Tifton Lab in 2022. BVDV-2a 5912c is set as the outgroup.

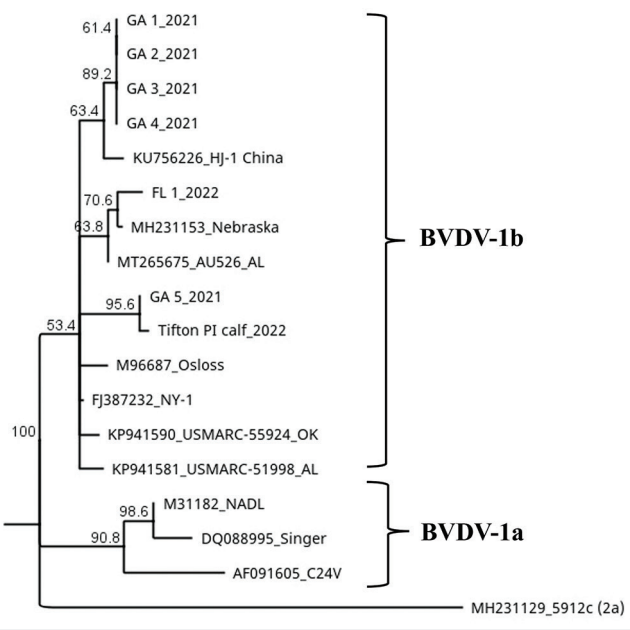
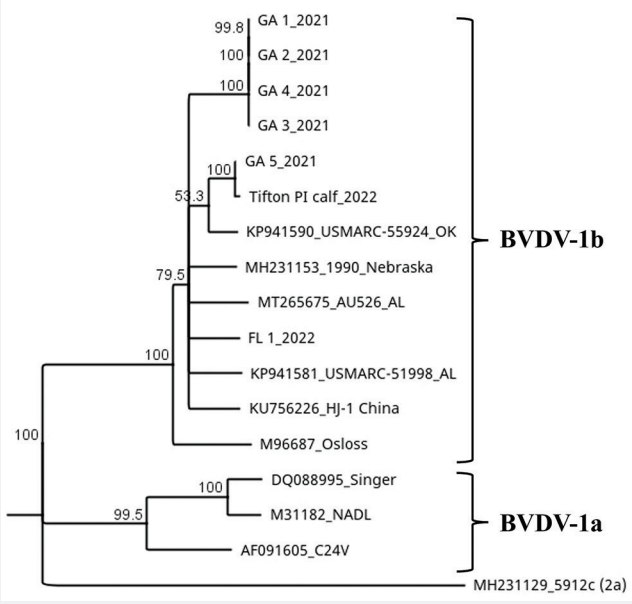


Figure 2: Phylogenetic tree for E1-E2 region (555bp). Each reference virus is labeled with strain name and Genbank ID number except for "Tifton PI-calf" which was a clinical case submitted to the Tifton Lab in 2022. BVDV-2a 5912c is set as the outgroup.



We found all positive samples were from herds larger than 500 cows (Table 2). However, the herd size was not significantly different between BVD positive and negative farms due to many large farms also tested negative in our study. Nonetheless, larger herd size has been reported to be associated with higher BVD prevalence.[35] The discrepancy of our result can be attributed to the small number of BVD positive herds resulting in low statistical power. This limitation is also reflected in the analysis of the survey data for possible risk factors. To correct the caveat of low statistical power, we would need more BVD positive herds in the study. However, to have at least 10 positive farms in the study, we would need to recruit more than 93 dairy farms based on the current herd prevalence of 10.8%. Despite various attempts through different channels, we could only recruit 37 dairy farms from the region within the 2-year time frame. Most dairy producers were reluctant to participate in this surveillance study. It would be extremely challenging to enlist 93 dairy farms without the initiative and strong support from the dairy co-operative organizations and Dairy Checkoff programs. Unlike the current study, the 2015-2016 surveillance project was fully supported by the Florida Milk Checkoff Program and the bulk tank samples were all collected directly by the milk co-op laboratory. With the high-level support from both partners in the previous study, the total number of participants was 78 dairy farms.

The only statistical significance from the survey data was the high dry cow numbers in BVD-positive farms. However, this could be a random finding, or it could be attributed to the correlation of large herd size simply having more dry cows. Herd size was significantly correlated with dry cow numbers across all 37 farms by Spearman correlation ($P < 0.0001$).

The milking cow numbers per tank in the positive samples in our study were in the range of 200-1200 (Table 3). Assuming there is only one PI animal in each sample and every milking cow contributed equal volume of milk, the detection limit is well under the reported dilution factor of 1:1600.[29] However, all 3 samples of farm D showed high Ct. values (>35) indicating low viral load and were unable to produce amplicons for sequencing. Upon re-testing after 17 days, none of the milk samples from farm D tested positive, suggesting the viral RNA detected from the first round was from transient infection, not from a PI animal. It also demonstrates the high sensitivity of this screening method among milking cows. However, no other dairy producers followed up with TVDIL to perform more tests to identify the PI animals on the farm.

For the past 20 years, subtype BVDV-1b has been reported as the most prevalent subtype in beef herds and feedlots in the United States.[6,36–38] However, only limited studies were done for genotyping BVDV isolated from dairy operations. One study reported about 40% of the dairy isolates were BVDV-2 while 49% to be BVDV-1b and 11% to be BVDV-1a for viruses isolated between 1998-2001 from 16 states.[5] Our study revealed the current predominant subtype in dairy herds was also BVDV-1b in the southeast region of the United States. The increased dominance of BVDV-1b over time in both beef and dairy cattle populations could be partially explained by the strain antigenic difference in the vaccines.[14,39] The BVD vaccines on the market are mainly multivalent vaccines and contain BVDV-1a and BVDV-2a subtypes, especially for MLV vaccines. Despite concerns about the cross-neutralization ability for BVDV-1a against BVDV-1b due to the antigenic differences,[36,37,40,41] earlier studies showed the MLV containing BVDV-1a and BVDV-2a could provide fetal protection from BVDV-1b.[42–44] At present, no scientific data support the cause-effect of the vaccine subtypes to the dominance of BVDV-1b.[14] Nonetheless, BVDV-1b subtype has been included in the new generation of MLV vaccines.

Another consideration of vaccinating cows is whether maternal antibody protection transferred to calves may interfere with the immune responses of the calves when receiving their first BVD vaccine, regardless of vaccine type.[45–48] Strategies to overcome maternal antibody interference include delaying the age of vaccination, repeating vaccination, alternate routes of immunization, or use of adjuvants in the vaccines.[47,49] More studies are warranted to determine how to better protect cattle from BVD via vaccination. Options to consider include adding a BVDV-1b subtype and/or adjuvants into vaccines. Furthermore, strategies such as testing and removing PI animals, movement restrictions and implementing strict biosecurity procedures are all necessary for an effective BVD control program. Vaccination alone has not proven to be effective in BVD control in part due to the complexity of BVDV subtypes and PI animals. In fact, several countries have successfully implemented BVD control programs without using vaccination including Sweden, Norway, Finland, Austria and Switzerland.[50]

Funding

This work was supported by USDA NIFA Animal Health Capacity Grant, titled “Investigation of the high herd prevalence of bovine viral diarrhea (BVD) in dairy farms in the southeast region of the United States”. Grant #NI19AHDRXXXXG013.

Author contributions

Study conception and design: Jones and Mosley; acquisition of data: McDaniel, Jones, Whittington, Malphus, Wells, Mosley; analysis and interpretation of data: Mosley; drafting the manuscript or revising it critically for important intellectual content: Malphus, Wells, Whittington, McDaniel, Jones, Mosley; and approval of the final version to be published: Malphus, Wells, Whittington, McDaniel, Jones, Mosley.

Acknowledgements

We would like to thank Lucy Ray, Kim Goins, Angie Norris and Kristie Goins for their help in recruitment of participants; Daniel Christensen, Alison Moore, Ray Egan, Amy Lehr and Anna Plair for their assistance in obtaining the bulk milk samples. We also thank Robert Mims for his assistance in organizing the survey data.

End notes

[a] MagMax Pathogen RNA/DNA Kit, ThermoFisher Scientific, Waltham, MA

[b] Qiagen BioSprint 96 Magnetic Particle Processor, ThermoFisher Scientific, Waltham, MA

[c] VetMax™-Gold BVDV PI Detection Kit, ThermoFisher Scientific, Waltham, MA

[d] Applied Biosystems ABI 7500 Fast, ThermoFisher Scientific, Waltham, MA

[e] SuperScript VILO cDNA Synthesis Kit, ThermoFisher Scientific, Waltham, MA

[f] GoTaq G2 Hot Start Master Mix, Promega, Madison, WI

[g] ExoSAP-IT PCR Product Cleanup Reagent, ThermoFisher Scientific, Waltham, MA

[h] Geneious Prime software, Dotmatics, Boston, MA

[i] GraphPad Prism 9 software, Dotmatics, Boston, MA

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