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Replication competent retrovirus testing (RCR) in the National Gene Vector Biorepository: No evidence of RCR in 1,595 post-treatment peripheral blood samples obtained from 60 clinical trials

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The clinical impact of any therapy requires the product be safe and effective. Gammaretroviral vectors pose several unique risks, including inadvertent exposure to replication competent retrovirus (RCR) that can arise during vector manufacture. The US FDA has required patient monitoring for RCR, and the National Gene Vector Biorepository is an NIH resource that has assisted eligible investigators in meeting this requirement. To date, we have found no evidence of RCR in 338 pre-treatment and 1,595 post-treatment blood samples from 737 patients associated with 60 clinical trials. Most samples (75%) were obtained within 1 year of treatment, and samples as far out as 9 years after treatment were analyzed. The majority of trials (93%) were cancer immunotherapy, and 90% of the trials used vector products produced with the PG13 packaging cell line. The data presented here provide further evidence that current manufacturing methods generate RCR-free products and support the overall safety profile of retroviral gene therapy.

INTRODUCTION

Retroviral vectors derived from murine gammaretroviruses have been an important tool for advancing gene therapy, particularly in the setting of cancer immunotherapy. Most retroviral vectors are engineered to be replication defective by separating the transgene of interest from the viral structural genes (gag and pol) and glycoprotein envelope required for vector particle formation. Early packaging systems can recombine and restore replicative function,[1,2] and vector-associated replication competent retrovirus (RCR) may cause malignancy in mice and non-human primates.[3,4] Therefore, inadvertent exposure to RCR is considered a serious risk factor when using

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this class of vectors (see Cornetta et al. for a review of RCR risk as it relates to insertional oncogenesis).[5] While RCR exposure in humans has not been reported to date, the finding of vector-related malignancy in a subset of patients treated with replication defective vectors targeting hematopoietic stem cells[6–9] has further prompted regulatory agencies to continue to recommend post-trial monitoring for RCR exposure.[10]

The National Gene Vector Biorepository (NGVB, www.NGVBCC.org),[11] a program funded by the US National Heart, Lung and Blood Institute (NHLBI), has assisted in monitoring for RCR using biologic assays[12] and quantitative PCR (qPCR). In this paper, we report no evidence of RCR in peripheral blood from patients tested at a variety of time points post-infusion of vector-transduced cells. The predominant method of manufacture has been packaging cell lines using the Gibbon Ape Leukemia Virus (GALV) envelope. The data provide further evidence of the safety of gene therapy using current retroviral vectors and packaging systems.

RESULTS

The NGVB uses qPCR to detect the envelope used in pseudotyping viral vectors as a surrogate test for RCR. This is in line with US Food and Drug Administration (FDA) guidelines for post-treatment RCR monitoring.[10] In 2018, investigators who submitted samples to the NGVB received a questionnaire requesting information about the clinical trial, vector manufacture, *ex vivo* cell target, and whether the vector lot used in generating the *ex vivo* cell product was screened for RCR. All trials in this study utilized gammaretroviral vectors to transduce *ex vivo* cell targets. As shown in Table 1, most studies employed the GALV envelope, of which 87% utilized the PG13 packaging cell line in vector production.[13] Two studies used vector pseudotyped with the amphotropic retrovirus AM-MLV using PA317 cells.[14] Only a minority employed transient transfection methods. The majority of trials prepared genetically modified T cells for use in cancer immunotherapy trials. In two trials, vector was used to modify neuroblastoma cell lines. Three trials, at different sites, transduced CD34+ hematopoietic stem cells (HSCs) using the same vector construct that contained a self-inactivating LTR (Long Terminal Repeat). The disease target was X-linked severe combined immunodeficiency (SCID). Two additional trials used CD34+ HSC, one for treatment of adenosine deaminase deficiency and another conferred a drug resistance gene.

Investigators were asked to provide the time from cell product transfusion to sample collection (Table 2). The earliest samples described in this report were evaluated on March 9, 2011. For patients who received multiple transfusions (16.7%), the time of RCR testing was calculated from the first administration of a transduced cell product. A total of 737 patients had at least one sample tested. The distribution of timing is shown in Figure 1. Pre-treatment samples accounted for approximately 17.5% of all the samples tested. Not all patients had pre-treatment samples submitted, and a few patients screened did not go on to get transduced cells or received them after the data collection time points in this study. Most samples were collected between 90 and 364 days, and approximately 25% post-treatment samples were collected 1 or more years after treatment. All samples analyzed were negative for RCR.

We also reviewed qPCR assay performance. All testing was performed under current good manufacturing practice (cGMP) guidelines. In addition to the samples reported in Table 2, we received 15 samples that had insufficient DNA and were not analyzed. An additional 11 samples were tested and were negative for envelope but had insufficient DNA to meet the assay requirements of 0.2 ug of DNA per reaction, so these results are reported as inconclusive. In four samples, the assay detected GALV sequence, and an out-of-specification investigation was initiated. Working with the investigator, it was determined the samples were contaminated with GALV-containing plasmid at the submitting investigator's site. Additional sample material for the subjects tested negative for RCR, and the initial samples were deemed false positives.

DISCUSSION

Retroviral vectors have been in use for the past 30 years, and the FDA requirements for RCR testing have changed over time. In 1998, the "Guidance for Human Somatic Cell Therapy and Gene Therapy"[15] was published and stated, "Patients given retrovirus-related products should be monitored for RCR exposure. Please consult CBER for guidance." At that time, some investigators were asked to perform monitoring annually. In 2006, the FDA published a specific guidance for RCR testing that recommended "pre-treatment, 3 months, 6 months, 1 year after treatment, and yearly thereafter. If all post-treatment assays are negative during the first year, the yearly samples should be archived."[16] In 2020, this guidance was replaced by "Testing of Retroviral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up. Guidance for Industry," which kept the testing schedule the same except archiving is not required if all samples in year 1 were negative.[10] Given that the first sample in our study was analyzed in 2011, the changing expectations are reflected in a variation in the length of time over which samples were collected.

In this study we compiled results of blood monitoring for RCR in 60 clinical trials. Cell lines of both human and murine origin were utilized in generating vector products, and the majority of studies utilized vectors with intact LTRs, a vector component with a higher risk of RCR than self-inactivating LTRs. While we have some samples out as far as 9 years, the majority were collected within the first 2 years after exposure to retroviral vector. All 338 pre-treatment samples were negative for RCR sequences, suggesting humans have a low incidence of sequences with homology to GALV envelope. All 1,595 samples collected post-infusion of genetically modified cells were negative for RCR using qPCR testing. These findings are consistent with NGVB analysis of gene therapy products using a sensitive RCR biological assay.[12] In that study, RCR was undetectable in 282 *ex vivo* products from 14 clinical trials. This outcome is in line with earlier results noting a lack of RCR in *ex vivo* transduced products.[17,18]

Table 1. Studies evaluating replication competent retrovirus (RCR) exposure after infusion of gene modified cells

IU master agreement number	Investigator	Institution	NCT number	Indication	Method	Pseudotype	Target cell	Vector product negative for RCR	SIN LTR
M2	Ramos	Baylor College of Medicine	NCT00881920	cancer	PG13	GALV	T cell	yes	
M4	Williams	Boston Children's Hospital	NCT01129544	X-linked SCID	transient	GALV	CD34+ HSC	yes	X
M5	Ramos	Baylor College of Medicine	NCT00709033	cancer	PG13	GALV	T cell	yes	
M6	Brenner	Baylor College of Medicine	NCT01192555	cancer	PA317	Ampho	neuroblastoma cell line	yes	
M7	Brenner	Baylor College of Medicine	NCT00710892	cancer	PG13	GALV	T cell	yes	
M8	Brenner	Baylor College of Medicine	NCT00703222	cancer	PA317	Ampho	neuroblastoma cell line	yes	
M9	Ramos	Baylor College of Medicine	NCT00586391	cancer	PG13	GALV	T cell	yes	
M10	Heslop	Baylor College of Medicine	NCT00889954	cancer	PG13	GALV	T cell	yes	
M11	Brenner	Baylor College of Medicine	NCT00902044	cancer	PG13	GALV	T cell	yes	
M12	Kochenderfer	NIH Surgery Branch	NCT01087294	cancer	PG13	GALV	T cell	yes	
M16	Heslop	Baylor College of Medicine	NCT00368082	cancer	PG13	GALV	T cell	yes	
M18	Ramos	Baylor College of Medicine	NCT00840853	cancer	PG13	GALV	T cell	yes	
M20	Brenner	Baylor College of Medicine	NCT01109095	cancer	PG13	GALV	T cell	yes	
M22	Heslop	Baylor College of Medicine	NCT01192464	cancer	PG13	GALV	T cell	yes	
M23	Shah	NIH Pediatric Oncology Branch	NCT01593696	cancer	PG13	GALV	T cell	yes	
M27	Brenner	Baylor College of Medicine	NCT01494103	cancer	PG13	GALV	T cell	yes	
M31	Kohn	University of CA Los Angeles	NCT01129544	X-linked SCID	transient	GALV	CD34+ HSC	yes	X
M40	Rosenberg	NIH Surgery Branch	NCT01273181	cancer	PG13	GALV	T cell	yes	
M41	Rosenberg	NIH Surgery Branch	NCT00670748	cancer	PG13	GALV	T cell	yes	
M42	Rosenberg	NIH Surgery Branch	NCT01236573	cancer	PG13	GALV	T cell	yes	
M43	Rosenberg	NIH Surgery Branch	NCT01218867	cancer	PG13	GALV	T cell	yes	
M44	Rosenberg	NIH Surgery Branch	NCT01454596	cancer	PG13	GALV	T cell	yes	
M45	Rosenberg	NIH Surgery Branch	NCT01583686	cancer	PG13	GALV	T cell	yes	
M46	Heslop	Baylor College of Medicine	NCT01316146	cancer	PG13	GALV	T cell	yes	
M48	Brenner	Baylor College of Medicine	NCT01460901	cancer	PG13	GALV	T cell	yes	
M49	Kohn	University of CA Los Angeles	NCT00794508	ADA SCID	PG13	GALV	CD34+ HSC	yes	
M56	Sauter	Memorial Sloan Kettering	NCT01840566	cancer	PG13	GALV	T cell	yes	
M57	J. Park	Memorial Sloan Kettering	NCT03085173	cancer	PG13	GALV	T cell	yes	
M58	J. Park	Memorial Sloan Kettering	NCT01416974	cancer	PG13	GALV	T cell	yes	
M59	Curran	Memorial Sloan Kettering	NCT01860937	cancer	PG13	GALV	T cell	yes	
M60	Rosenberg	NIH Surgery Branch	NCT00509288	cancer	PG13	GALV	T cell	yes	
M61	Rosenberg	NIH Surgery Branch	NCT00273910	cancer	PG13	GALV	T cell	yes	
M62	Rosenberg	NIH Surgery Branch	NCT00706992	cancer	PG13	GALV	T cell	yes	
M66	Kaplan	NIH Pediatric Oncology Branch	NCT02107963	cancer	PG13	GALV	T cell	yes	
M70	Kiem	Fred Hutchison Cancer Center	NCT00669669	cancer	transient	GALV	CD34+ HSC	yes	

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Table 1. *Continued*

IU master agreement number	Investigator	Institution	NCT number	Indication	Method	Pseudotype	Target cell	Vector product negative for RCR	SIN LTR
M76	Booth/Thrasher	Great Ormond Street Hospital	NCT01175239	X-linked SCID	transient	GALV	CD34+ HSC	yes	X
M77	Brenner	Baylor College of Medicine	NCT01822652	cancer	PG13	GALV	T cell	yes	
M83	Rosenberg	NIH Surgery Branch	NCT02111850	cancer	PG13	GALV	T cell	yes	
M85	Rosenberg	NIH Surgery Branch	NCT01967823	cancer	PG13	GALV	T cell	yes	
M95	Ramos	Baylor College of Medicine	NCT02050347	cancer	PG13	GALV	T cell	yes	
M96	Ramos	Baylor College of Medicine	NCT01853631	cancer	PG13	GALV	T cell	yes	
M102	Heslop	Baylor College of Medicine	NCT01953900	cancer	PG13	GALV	T cell	yes	
M105	Heslop	Baylor College of Medicine	NCT02065362	cancer	PG13	GALV	T cell	yes	
M106	Norberg/Hinrichs	NIH Center for Cancer Research	NCT02280811	cancer	PG13	GALV	T cell	yes	
M107	J. Park	Memorial Sloan Kettering	NCT00466351	cancer	PG13	GALV	T cell	yes	
M112	Kochenderfer	NIH Surgery Branch	NCT02215967	cancer	PG13	GALV	T cell	yes	
M113	Slovin	Memorial Sloan Kettering	NCT01140373	cancer	PG13	GALV	T cell	yes	
M116	Curran	Memorial Sloan Kettering	NCT01430390	cancer	PG13	GALV	T cell	yes	
M122	Ribas	University of CA Los Angeles	NCT01697527	cancer	PG13	GALV	T cell	yes	
M123	Adusumilli	Memorial Sloan Kettering	NCT02414269	cancer	PG13	GALV	T cell	yes	
M141	Ribas	University of CA Los Angeles	NCT02070406	cancer	PG13	GALV	T cell	yes	
M142	O'Cearbhaill	Memorial Sloan Kettering	NCT02498912	cancer	PG13	GALV	T cell	yes	
M143	Rosenberg	NIH Surgery Branch	NCT02153905	cancer	PG13	GALV	T cell	yes	
M144	Rosenberg	NIH Surgery Branch	NCT02062359	cancer	PG13	GALV	T cell	yes	
M152	J. Park	Memorial Sloan Kettering	NCT02792114	cancer	PG13	GALV	T cell	yes	
M161	Mailankody	Memorial Sloan Kettering	NCT03070327	cancer	PG13	GALV	T cell	yes	
M168	J. Park	Memorial Sloan Kettering	NCT03085173	cancer	PG13	GALV	T cell	yes	
M178	Ramos	Baylor College of Medicine	NCT02379520	cancer	PG13	GALV	T cell	yes	
M226	Ribas	University of CA Los Angeles	NCT03240861	cancer	PG13	GALV	T cell	yes	
M228	Ribas	University of CA Los Angeles	NCT02775292	cancer	PG13	GALV	T cell	yes	

ADA = adenosine deaminase deficiency, SCID = severe combined immunodeficiency, PG13 = PG13 packaging cell line, GALV = Gibbon ape leukemia virus, SIN = self-inactivating LTR.

Table 2. Timing of replication competent retrovirus assessment by study

IU master agreement number	Number of unique patients	Number of patients with multiple infusions	Pre-treatment	<90 days	90–179 days	180–364 days	year 1	year 2	year 3	year 4	year 5 or greater
M2	14	7	2	4	16	17	15	4	0	0	0
M4	4	0	4	0	4	4	7	3	4	3	2
M5	2	0	1	0	2	2	2	0	0	0	0
M6	9	0	0	0	4	6	3	0	0	0	0
M7	10	4	10	0	11	10	10	0	0	0	0
M8	5	0	0	0	3	4	4	1	0	0	0
M9	12	3	9	3	12	13	9	0	0	0	0
M10	10	7	3	8	12	10	7	2	1	0	0
M11	21	9	9	8	18	23	17	2	0	0	0
M12	28	4	32	2	23	23	15	0	0	0	0
M16	8	6	6	9	12	12	17	2	0	0	0
M18	17	5	6	4	16	13	15	2	0	0	0
M20	10	6	1	5	9	12	8	2	0	0	0
M22	3	1	1	0	3	3	4	0	0	0	0
M23	51	2	47	0	38	25	19	3	0	0	0
M27	8	3	0	0	8	8	9	0	0	0	0
M31	3	0	1	1	2	3	1	1	0	1	0
M40	8	0	0	2	6	5	0	0	0	0	0
M41	39	8	0	18	26	25	6	1	0	1	2
M42	31	0	0	8	33	11	9	1	2	3	3
M43	15	0	0	8	10	1	4	0	0	0	0
M44	8	0	0	4	6	2	1	0	0	0	0
M45	9	0	0	7	4	2	1	0	0	0	0
M46	9	5	0	4	9	11	9	2	0	0	0
M48	2	2	0	0	2	2	0	0	0	0	0
M49	9	0	1	0	2	4	3	0	0	0	0
M56	11	0	13	0	11	4	6	3	0	0	0
M57	56	14	49	10	30	20	9	2	0	0	0
M58	8	0	5	0	6	6	3	1	1	1	0
M59	21	2	21	1	13	10	8	0	0	0	0
M60	18	0	0	7	15	9	6	0	0	0	0
M61	13	0	0	5	11	6	2	1	1	0	0
M62	42	0	0	15	22	37	18	2	3	3	5
M66	14	0	14	0	10	6	2	0	0	0	0
M70	5	0	0	0	4	3	2	0	0	0	0

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Table 2. *Continued*

IU master agreement number	Number of unique patients	Number of patients with multiple infusions	Pre-treatment	<90 days	90–179 days	180–364 days	year 1	year 2	year 3	year 4	year 5 or greater
M76	1	0	1	0	1	1	0	0	1	1	1
M77	11	5	0	3	9	10	6	0	0	0	0
M83	17	0	0	13	6	9	2	2	1	0	0
M85	10	0	0	6	7	9	3	0	0	0	0
M95	5	0	0	1	4	3	2	0	0	0	0
M96	12	4	0	1	12	14	7	0	0	0	0
M102	6	3	0	0	6	8	3	0	0	0	0
M105	8	1	0	1	6	6	5	0	0	0	0
M106	9	2	0	6	2	7	5	0	0	0	0
M107	24	0	21	0	21	13	11	0	0	2	1
M112	20	3	17	0	18	14	10	0	0	0	0
M113	7	0	3	0	4	4	2	0	0	0	0
M116	6	3	4	4	2	4	2	0	0	0	0
M122	7	0	0	1	1	4	0	1	0	0	0
M123	12	0	12	0	3	2	0	0	0	0	0
M141	6	0	0	0	3	2	1	0	0	0	0
M142	13	0	14	0	10	7	2	0	0	0	0
M143	2	0	0	1	1	0	0	0	0	0	0
M144	1	0	0	1	0	0	0	0	0	0	0
M152	5	0	5	0	1	1	0	0	0	0	0
M161	11	6	11	3	6	2	0	0	0	0	0
M168	5	2	6	0	5	3	0	0	0	0	0
M178	3	3	0	0	2	5	0	0	0	0	0
M226	2	2	0	0	2	2	0	0	0	0	0
M228	1	1	0	0	1	0	0	0	0	0	0
Totals	737	123	329	174	546	482	312	38	14	15	14

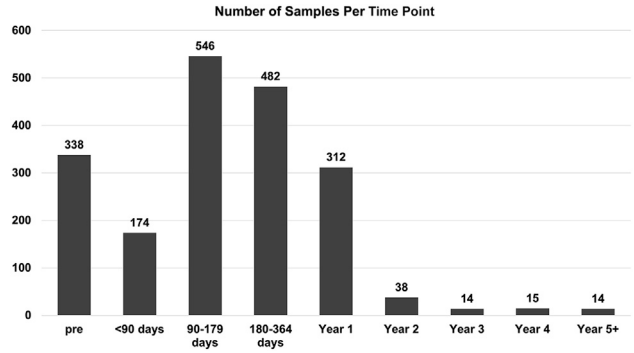

Figure 1. Number of samples analyzed using qPCR targeting vector envelope pseudotype

X axis is the time from first exposure to vector-transduced cells. Pre = pre-treatment sample.

Limitations of the study include the small number of vector products generated by transient transfection, as most studies utilized the stable PG13 cell line for vector manufacture. The FDA has stated a preference for stable packaging cell lines given their potential to limit lot-to-lot variability that can be seen with transient transfection methods.[10] Nevertheless, transient transfection methods are being increasingly employed, as they do not require the extended time for clone selection, generation of master cell banks, and expansion for vector production. The extensive cell expansion associated with packaging cell lines is also predicted to increase likelihood of recombination and RCR generation. From a safety perspective, our findings add to data from prior studies documenting the generation of RCR-free vector by PG13 cells.[12,17,18] It is unclear whether this finding can be extrapolated to other packaging cell lines. Part of the success of PG13 may be in the use of the xenotropic GALV envelope in a murine cell line (NIH3T3). Murine cells lack the receptor for the GALV envelope, so if a GALV pseudotype RCR developed, it would not be able to propagate in culture.

Another limitation is the relatively small number of patients in several of the studies. This issue arises because our study design looks at the time frame when samples were tested. Nonetheless, the inclusion of studies with smaller number of accrued patients allows us to survey more vector products, as the majority of studies used distinct vector products specific to each clinical trial.

The majority of clinical trials in our study were related to cancer immunotherapy. This finding is expected given the time frame of samples collection. The first report of malignancy related to retroviral vectors occurred in 2003 in a trial aimed at treating patients with X-linked SCID.[19] This was followed by reports of similar adverse events in trials for chronic granulomatous disease,[8] Wiskott-Aldrich syndrome,[9] and most recently adenosine deaminase deficiency.[20] All these trials targeted HSCs, and our study received samples when most investigators targeting HSCs had moved away from gammaretroviral vectors. To date, malignancy has not been reported when retroviral vectors are used to transduce in T cells, a practice that continues in many cancer trials.

In conclusion, the qPCR testing presented here provides further evidence that patients treated with retroviral vectors have a low risk of RCR exposure. The data also support the safety of current manufacturing methods and release testing requirements for retroviral vector products. This indicates that limited testing of *ex vivo* products and patient monitoring are sufficient when using established manufacturing systems, although the risk of RCR development will need to be assessed experimentally for new vector production methods, new pseudotypes, and novel packaging cell lines.

MATERIALS AND METHODS

Collection of study data

For this survey, investigators were provided an Excel spreadsheet that contained the NGVB ID number of the samples tested, the lab completion date, and the results. Investigators were requested to provide the following data: (1) ClinicalTrials.gov number, (2) the general indication of the trial (e.g., CAR T cell immunotherapy, genetic disease, etc.), (3) the cell type transduced (e.g., T cell, CD4, CD8, hematopoietic stem cell), (4) production method (transient transfection or packaging cell line), (5) the specific cell line used, if a packaging cell line was used, (6) were the vector supernatant and end of production cells tested and found to be negative for RCR? (Yes/No), and (7) the time from infusion of gene transduced cells to sample collection and whether patients received multiple infusions of transduced cells.

Sample processing

Investigators are requested to submit de-identified samples to the NGVB as DNA, buffy coat cells, isolated cells, or blood. Samples are isolated using either the Qiagen Puregene DNA Isolation Kit or the QIAamp DNA Blood Mini Kit (both available Qiagen USA, Germantown, Maryland). Isolations are performed under cGMP guidelines. DNA is evaluated for purity (OD 260/280), and concentration to ensure sufficient material is present to perform triplicate reactions with 0.2 ug of DNA per reaction.

qPCR reaction

Detection of target sequences was performed with the ABI 7500 (Applied Biosystems, Foster City, CA) using multiplex reactions with primers and probe for the target sequence and a second set of primers and probe for human ApoB. The ApoB determination was used to confirm that a reaction contained a minimum of 0.2 ug of DNA. Negative controls include water (NTC) and target-negative human DNA. Positive control samples were generated from plasmid DNA containing the target sequence diluted in the target-negative control DNA. Positive controls over a 5-log range (from 10 to 10^5 copies per 0.2 ug DNA) were included in each run to determine the standard curve. Probe and primer sets were qualified by evaluating slope, intercept, and R^2 for the standard curve. The NGVB has set the range of amplification efficiency of 85%–105%, which corresponds to a slope of −3.74 to −3.21. R^2 limits were values between 0.981 and 1. The Intercept was determined for each probe and primer set, and the acceptable criterion is +2 standard deviations from nine or more runs. The intercept for GALV is 37.74–41.05 and for amphotropic envelope is 38.34–39.13. For the assays to be acceptable, (1) the

amplification plots of the NTC must not cross the threshold, (2) the amplification plots of the negative-amplification control must not cross the threshold, and (3) the acceptance criteria for the specific probe and primer set must be met. The ApoB acceptability criteria are determined for each run by calculating the mean and standard deviation Ct value for all samples in the assay. The acceptable range for test articles is less than the mean + 2 standard deviations.

A sample is considered positive if any of the triplicate reactions are above the limits of detection for the envelope (10 copies per 0.2 μg of DNA), regardless of the total DNA content in the reaction. For a sample to be considered negative, all replicates must be less than 10 copies per reaction, and at least two of the three reactions must meet the criteria of >0.2 ug of DNA. If only one or none of the reactions meet the 0.2 ug of DNA requirement, the sample is retested (if sufficient DNA is available). If the repeat remains negative for envelope sequence and fails to show sufficient DNA to meet acceptance criteria, the samples are reported as inconclusive.

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AUTHOR CONTRIBUTIONS

K.C., J.Y., K.H., L.D., and T-Y.L. designed the methods, qualified assays, and wrote the article; the remaining authors provided data regarding the manufacturing method and timing of sample collection. All authors contributed to review and editing of the manuscript.

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