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Development and Evaluation of a TaqMan Real-Time PCR Assay for the Rapid Detection of Cross-Contamination of RD (Human) and L20B (Mouse) Cell Lines Used in Poliovirus Surveillance

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Abstract

Background: The cross-contamination of cell lines in culture is a persistent problem. Genetically modified L20B (Mouse) and RD (Human Rhabdomyosarcoma) cell lines are commonly used in poliovirus research, surveillance, and diagnostics. Cross-contamination between these cell lines leads to unreproducible results and unreliable surveillance data, negatively affecting public health. The gold standard method for cell authentication is Short Tandem Repeats analysis, which is time-consuming and expensive. The disadvantage of STR is limited detection of interspecies contamination.

Methods: This assay targets the mitochondrial cytochrome c oxidase subunit I (MTCO1) gene, a highly conserved and emergent DNA barcode region for detection of cross-contamination in RD and L20B cell lines. The MagNA Pure Compact instrument and ABI 7500 Fast Dx Real-time PCR systems were used for DNA extraction and to perform real-time PCR respectively.

Results—The newly developed assay is very sensitive with a limit of detection of 100 RD cells/1 million L20B/mL. The amplification efficiency and R^2-value were 102.26% and 0.9969

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Conflict of interest:
The authors declare that they have no conflict of interest.

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respectively. We evaluated specificity of the assay with five human and four mouse cell lines, as well as monkey and rat cell lines. The assay showed no cross-reactivity with genomic DNA from human, mouse, rat, or monkey cell lines. The analytical sensitivity was also evaluated by spiking varying amounts of RD cells (0.001% - 10%) into L20B cells. There was no difference in C_T values when running single-plex or duplex PCR reactions with similar experimental conditions.

Conclusions: We have developed and validated a TaqMan real-time PCR assay, a sensitive method for the detection of cross-contamination of RD and L20B cell lines.

Keywords

Cell lines cross-contamination; RD cells; L20B cells; TaqMan Real-time PCR

1. INTRODUCTION:

The Global Polio Eradication Program of the World Health Organization (WHO) is the largest public health initiative [1]. Type 2 and 3 wild polioviruses were declared eradicated in 2015 and 2019 respectively, and only type 1 wild poliovirus remains [2]. Poliovirus surveillance is critical for the containment of wild poliovirus. Two cell lines, RD cells derived from a human rhabdomyosarcoma, and L20B cells, a genetically engineered mouse cell line expressing the human poliovirus receptor CD155 are commonly used for isolation, molecular typing, and accurate poliovirus surveillance [1, 3–5].

Cross-contamination of cell lines is a common problem, with frequencies ranging from 16 to 35% [6, 7] including both intra-species (e.g., one human cell type by another human cell type) or inter-species (e.g., human cell type by mouse cell type) contamination. This problem has persisted for more than 50 years with limited quality control strategies to quickly and accurately identify contamination [8, 9]. Cell line cross-contamination has a significant impact on public health, causing unreliable surveillance data, with inaccurate and unreproducible results [8, 10–12].

There are various methods available for cell line authentication including DNA barcode, Karyotype, STR (Short Tandem Repeat) profiling, SNP (Single Nucleotide Polymorphism) array, PCR (species-specific primers), and WGS (Whole Genome Sequencing) [13–20]. The gold standard method for identification of contamination is Short Tandem Repeats (STR) analysis, which is time-consuming and expensive. The disadvantage of STR is limited detection of interspecies contamination. A recent study showed that STR profiling alone is insufficient to exclude inter-species cross-contamination of human cell lines [21]. PCR based assays could be used for the detection of inter-species contamination [16, 22]. The real-time PCR method for cell line authentication is more specific, highly sensitive, less expensive, as well as less time consuming compared with most of the currently used methods such as STR profiling and SNP based methods and whole genome sequencing. Two available real-time PCR methods for identification of cross-contamination of RD and L20B cell lines are based on SYBR Green chemistry and DNA Barcoding [3, 4] which is less sensitivity and less expensive than TaqMan chemistry methods. DNA barcodes show very small differences among same species (<1–2%) but closely related species show more than 2% difference in DNA barcodes [3, 14]. Dunn, (2016) targeted the mitochondrial

Cytochrome c oxidase subunit I [3] whereas Nejati et a., (2017) targeted mitochondrial Cytochrome b [4] for species identification. The specificity, sensitivity, and reproducibility of the TaqMan assay is higher than the SYBR Green assay. To our knowledge, there is no TaqMan assay available for the identification of cross-contamination of RD and L20B cell lines. Our aim was to develop and validate a TaqMan real-time PCR assay for more sensitive and more specific identification of cross-contaminations of RD and L20B cell lines. This assay will help in the generation of accurate, reproducible, and reliable surveillance data for Poliovirus research.

2. METHODS AND MATERIALS:

2.1 Cell Lines and Culture:

A total of 13 cell lines from four different species (Human, Monkey, Mouse, and Rat) were used in this study (Table 1). All cell lines were obtained from the Centers for Disease Control and Prevention (CDC) cell repository. The authentication of the cell lines used for routine production at CDC was carried out by Reagents, Cell Lines, and Media Laboratory (RDSB, DSR). The cell lines were maintained in the appropriate conditions and cell medium as recommended by American Type Culture Collection (ATCC). The L20B cells used for this study were authenticated by STR profiling at ATCC for cross-contamination of RD cells. (https://www.atcc.org). Seventeen short tandem repeat (STR) loci and the gender determining locus, Amelogenin, were included in the analysis.

The cells were harvested using standard procedures. Briefly, cells were treated with 0.01M Trypsin-EDTA (Cat # 25300–054, ThermoFisher Scientific, Waltham, MA; USA) incubated at 37°C for 5 minutes, and washed with 1X PBS (Cat # 10010031, ThermoFisher Scientific, Waltham, MA; USA).

2.2 DNA Extraction:

DNA extractions were performed with the MagNA Pure Compact Instrument (Roche Diagnostic, Indianapolis, IN; USA), using the MagNA Pure Compact Nucleic Acid Isolation Kit I (Cat # 03730964001, Roche Diagnostic, Indianapolis, IN; USA) according to the manufacturers' instructions. Total 1×10^6 cells were resuspended in 200μL 1X PBS (Cat # 10010031, ThermoFisher Scientific, Waltham, MA; USA) for extraction TheTotal_NA_Plasma_100_400_V3_2 protocol was used with an elution volume of 100μL. The concentration of DNA was determined using the NanoDrop spectrophotometer (Cal # ND-2000C, ThermoFisher Scientific, Waltham, MA; USA).

2.3 Primer and probe design:

The mitochondrial cytochrome c oxidase subunit I (MTCOI) sequences of Human (Accession no.: NC_012920.1), Monkey (Accession no: NC_007009.1) Mouse (Accession no: NC_005089.1), and Rat (Accession no: MK410392.1) were obtained from NCBI (https://www.ncbi.nlm.nih.gov/). All sequences were aligned using the MultAlin Tool [23] (Supplementary Figure 1a and 1b) to find a semi-conserved regions for primer and probe designing. The Human and Mouse specific primers and probes (Table 2) were designed using the PrimerQuest Tool (https://www.idtdna.com/Primerquest/Home/Index).

The OligoAnalyzer Tool (https://www.idtdna.com/calc/analyzer) was used to analyze the sequences for hairpin, self-dimerization, and hetero dimerization. The primer and probe sequences were checked for overlap with DNA sequences of other species using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The HPLC purified primers and probes were obtained from the Biotechnology Core Facility Branch, CDC (Atlanta, GA; USA).

2.4 Real-Time PCR Assay:

All performance characteristics were determined by TaqMan real-time PCR assay using the ABI 7500 Fast Dx Real-time PCR system (Applied Biosystems Inc., Foster City, CA; USA.). Each sample was analyzed in three independent runs and in triplicate PCR reactions of 20μL. We used TaqMan® Fast Advanced Master Mix (Cat # 4444556, ThermoFisher Scientific, Waltham, MA; USA) and followed the manufacturer's instructions. Briefly, each PCR reaction mixture contained 1X Master Mix and 5μL of template DNA. The final concentration of each primer and probe was 500nM and 100nM respectively. Each real-time PCR run also included two positive controls (RD and L20B cell lines; 50pg/μL) and a no template control (NTC). The PCR amplification conditions were 50°C for 2 minutes (for the activation of uracil-N-glycosylase (UNG) in the TaqMan master mix) and 95°C for 2 minutes, followed by 45 cycles of 95°C for 3 seconds and 60°C for 30 seconds. We followed a strict uni-directional workflow to prevent any cross-contamination by using separate rooms for the preparations of the sample, master mix, and PCR amplification.

2.5 Analytical Sensitivity and Specificity:

To measure the analytical sensitivity and specificity, an equal number of RD and L20B cells were mixed. Cell counts were performed using an automated Cellometer K2 cell counter (Nexcelom Bioscience, Lawrence, MA; USA). Nucleic acid was extracted, and concentration determined as described above. Serial dilutions of the extracted DNA (10,000pg/μL to 1pg/μL) were prepared and analyzed by the real-time PCR assay. To determine analytical sensitivity, five different concentrations of RD cells;10, 100, 1000, 10, 000 and 100,000 RD cells (0.001%, 0.01%, 0.1%, 1.0%, and 10%) were combined with L20B cells; 0.9–1.0 million/mL L20B cells (90% to 100%) and DNA was extracted and analyzed by the real-time PCR assay (100% L20B cells equal to 10^4 cells/PCR reaction). Reproducibility was evaluated by performing three independent DNA extractions and subsequent real-time PCR analysis. The analytical specificity was evaluated using limited number of different types of cell lines (n=13) from Human, Monkey, Mouse, and Rat (Table 1). For all analyses, each sample was analyzed in triplicate, in three independent runs.

3. RESULTS

3.1 Assay Optimization (Analytical Sensitivity and Specificity):

We have standardized the duplex real-time PCR assay for the detection of cross-contamination of Human (RD cells) and Mouse (L20B cells). We evaluated the analytical sensitivity and specificity (assay optimization) for the real-time PCR assay with 10-fold serially diluted DNA from RD and L20B mixed cell lines (equal number of cells). With the RD cells, the instrument showed a 3.56-fold (Standard deviation: 0.32) increase in C_T value with every 10-fold dilution. The amplification efficiency and R^2-value were

90.84%and 0.9983 respectively (Figure 1a). With the L20B cells, the instrument showed a 3.60-fold (Standard deviation: 0.26) increase in C_T value with every 10-fold dilution. The amplification efficiency and R^2-value were 89.57% and 0.9989 respectively (Figure 1b). The reproducibility study showed a Co-efficient of variance (Cv) for RD cells and L20B cells of 0.34% - 0.95% and 1.15% - 1.59% respectively.

3.2 Evaluation of Analytical Sensitivity:

For Analytical sensitivity, five different amounts of RD cells (0.001% to 10%) were mixed with L20B cells (90% to 100%). The TaqMan real-time PCR assay was reproducible and sensitive with a cross-contamination detection limit of 100 RD cells/1million L20B cells/mL (0.01% RD:100% L20B mixed cells). The amplification efficiency and R^2-value were 102.26% and 0.9969 respectively (Figure 2). The reproducibility study for RD cells and L20B cells showed a Co-efficient of variance (Cv) of 0.50% – 3.94% and 2.08% – 3.14% respectively.

3.3 Evaluation of Specificity:

The analytical specificity was evaluated with limited number of different types of cell lines (n=13) from Human, Monkey, Mouse, and Rat (Table 1) and no cross-reactivity observed between any cell lines from any species.

4. DISCUSSION:

The real-time PCR method for cell line authentication is a very sensitive, inexpensive and less time-consuming method compared with the gold standard STR and other available methods. We have developed and validated a TaqMan real-time assay for the detection of cross-contamination of cell lines used in Poliovirus laboratories. Accurate poliovirus surveillance is critical for the containment of poliovirus. Genetically modified L20B and RD cell lines are commonly used in poliovirus laboratories, and good quality control of these two cell lines is essential for effective poliovirus surveillance. Misidentification and cross-contamination of cell lines is a serious problem with frequencies ranging up to 35% [6, 7]. Cross-contamination in the cell lines leads to unreliable surveillance data and inaccurate results [8, 10–12]. Adherence to the Good laboratory practice (GLP) for tissue culture is important for prevention of the cross-contamination.

STR profiling is considered the gold standard for the detection of cross-contamination, but it is occasionally insufficient if human cells that are partially contaminated by animal cells always shows correct STR profiles. [21]. The two other existing methods for detection of RD and L20B cross-contamination are based on SYBR Green chemistry and DNA barcode [3, 4]. The SYBR Green is a low-cost method, but non-specific PCR products and primer-dimer formation could significantly decrease the detection sensitivity of the assay. [24–26]. We developed and evaluated a highly sensitive and more specific TaqMan real-time PCR assay than SYBR green assay, targeting frequently used DNA barcode gene MTCO1, for detection of cross-contamination of RD and L20B cell lines (Supplementary Figure 1a and 1b). The limit of detection (LoD) of cross-contamination was 100 RD cells/1million L20B cells/mL (0.01% RD:100% L20B mixed cells). This is significantly improved from the

SYBR Green assay LoD reported by Nejati et a., (2017) of 1.0% targeting the mitochondrial Cytochrome b gene, not the MTCO1 gene. We also measured detection in the mixture of L20B cells containing 0.001% RD i.e., 10 RD cells/1million L20B cells/mL but data showed high variability in the LoD (Figure 2). The assay was reproducible with the Co-efficient of variance (Cv) <4.0%.

We have evaluated multiple passages (passage # 15 to 33) of L20B cells in active production, for the detection of cross-contamination with RD cells. Each passage of L20B cells was evaluated twice a week (Monday and Friday). A total of 1×10^6 cells from each passage was taken for DNA extraction and real-time PCR analysis, as described in the methods and materials section. The analysis did not detect contamination with RD cells in any passage of L20B cells. Four of these same passages of L20B cells were verified by STR profiling at ATCC (https://www.atcc.org) and no contamination of RD cells were identified.

The analytical specificity data from limited number of different cell lines (n=13) showed no cross-reactivity between any cell lines from any species (Table 1

5. Conclusions:

The assay developed and validated in this study provides a reliable and reproducible method for the detection of cross-contamination of RD and L20B cell lines used in Poliovirus surveillance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Cross-contamination between cell lines leads to unreproducible results and unreliable data
- Developed a TaqMan real-time PCR assay for detection of cross-contamination of cell lines
- Assay is specific, no cross-reactivity with human, mouse, rat, or monkey cell lines

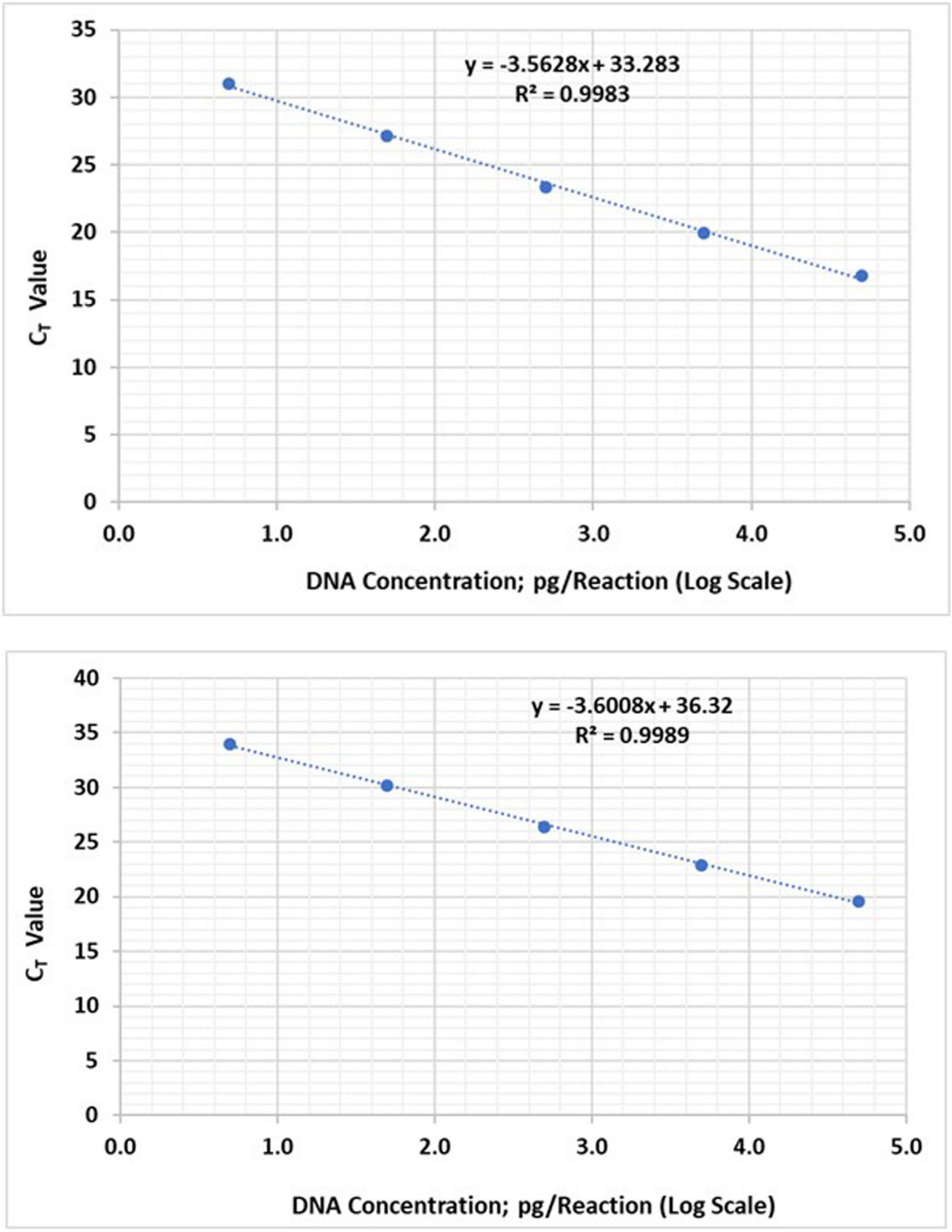

Figure 1:

Evaluation of Analytical Sensitivity and Specificity TaqMan real-time PCR assay. The equal number of RD and L20B cells were mixed and DNA was extracted. Five dilutions (From 10,000pg/μL to 1pg/μL) were prepared for the evaluation of the instrument response. The

real-time PCR assay amplification curve and linear regression data for RD cells are shown in Figure 1a. With RD cells, the instrument showed a 3.56-fold (Standard deviation: 0.32) increase in C_T value with every 10-fold dilution. The real-time PCR assay amplification curve and linear regression data for L20B cells are shown in Figure 1b. With L20B cells, the instrument showed a 3.60-fold (Standard deviation: 0.26 increase in C_T value with every 10-fold dilution.

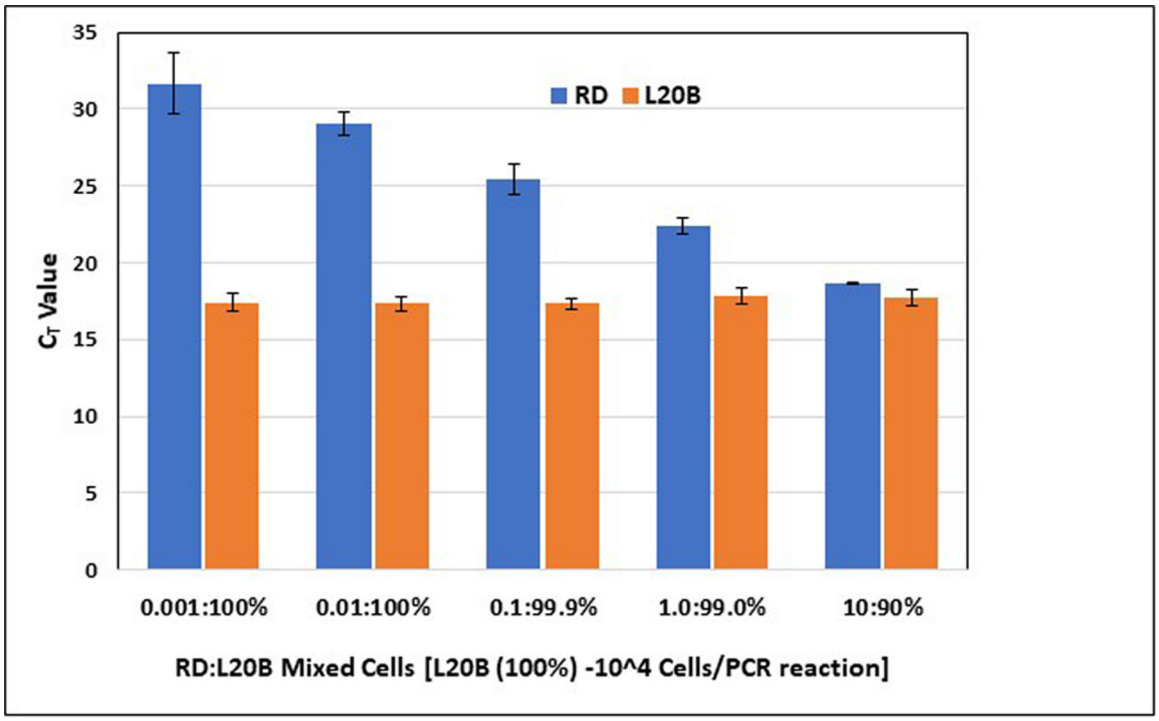

Figure 2:

Evaluation of analytical sensitivity of TaqMan real-time PCR assay. For Analytical sensitivity, five different amount of RD cells (0.001%, 0.01%, 0.1%, 1.0% to 10%) were mixed with different L20B cells (90% to 100%) and DNA was extracted (100% L20B cells equal to 10^4 cells/PCR reaction). The limit of detection of cross-contamination was 100 RD cells/1million L20B cells/mL (0.01% RD:100% L20B mixed cells).

Table 1:

List of species and cell lines used for the analysis of the specificity of the assay.

Species	Cell Line
Human	HL-60 (RRID:CVCL_0002), HuT-78 (RRID:CVCL_0337), RD (RRID:CVCL_1649), A549 (RRID:CVCL_0023), and CEM (RRID:CVCL_0207)
Mouse	L20B, L929 (RRID:CVCL_0462), McCOY (CVCL_3742) and 5E2:1A1 (Mouse Hybridoma Clone)
Monkey	CV-1 (RRID:CVCL_0229) and BGM (RRID:CVCL_4125)
Rat	NRK-49F (RRID:CVCL_2144) and IEC-18 (RRID:CVCL_0342)

Table 2:

List of Mouse and Human Primer and Probe targeting the mitochondrial cytochrome c oxidase subunit I (MTCOI) gene.

Species	Primer and Probe Sequence	Tm (°C)	GC (%)
Mouse	Mo-Forward: 5'-TCG TTG ATT ATT CTC AAC CAA TCA C-3'	62.0	36.0
	Mo-Reverse: 3'-GCA CCT GGT TGA CCT AAT TCT-5'	62.0	47.6
	Mo-Probe: 5'-Quas570-CTA TTC GGA GCC TGA GCG GGA ATA GT-IRQ-3'	68.6	53.8
Human	Hu-Forward: 5'-CTT CGT CTG ATC CGT CCT AAT C-3'	62.0	50.0
	Hu-Reverse: 3'-TCG AAG AAG GTG GTG TTG AG-5'	62.0	50.0
	Hu-Probe: 5'-FAM-TCC TAC TTC TCC TAT CTC TCC CAG TCC T-BHQ1-3'	68.0	50.0