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Molecular Identification of Siberian tiger Target Gene (Cytb gene) Quantitative PCR: A Study in Wildlife Conservation Genetics

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Abstract

Background: The Siberian Tiger (Panthera tigris altaica) is a critically endangered keystone species. Accurate molecular identification of biological traces is essential for effective conservation, anti-poaching enforcement, and ecological monitoring.

Objective: The main objective of the current study was to validate the performance of a cytb-targeted quantitative PCR assay for the molecular identification of Siberian Tiger DNA and to assess the utility of droplet digital PCR as a complementary confirmatory tool.

Methods: The current study validated a standardized quantitative PCR (qPCR) assay targeting the mitochondrial cytochrome *b* (cytb) gene for specific detection of Siberian Tiger DNA. Assay performance was evaluated using the YouSeq Siberian Tiger qPCR Test Kit on known reference samples, non-target species (Arabian sand cat, cheetah), and serial dilutions. Confirmatory analysis was performed using droplet digital PCR (ddPCR) to ensure specificity and absolute quantification.

Results: The qPCR assay demonstrated high sensitivity, with a limit of detection below 100 target copies per reaction, and high specificity, showing no cross-reactivity with non-target species. Siberian Tiger samples produced robust, early amplification (Cq 15.74–18.03). ddPCR provided absolute quantification, confirming target presence and resolving a case of ambiguous qPCR amplification from a White Tiger sample, highlighting ddPCR's utility in eliminating false positives. Serial dilution analysis confirmed excellent dynamic range and reproducibility.

Conclusion: This study validated a sensitive, specific, and reliable cytb-based qPCR/ddPCR assay for the molecular identification of Siberian Tiger DNA. The integrated approach provides a robust tool for wildlife forensics and conservation genetics, enabling accurate detection from trace and degraded samples to support legal enforcement and biodiversity monitoring.

Keywords: Siberian Tiger; ; cytb gene; qPCR; ddPCR; wildlife forensics; conservation genetics; molecular identification.

1 Introduction

The Siberian Tiger (Panthera tigris altaica), is the largest extant felid and a keystone apex predator whose survival is critical for maintaining ecological balance within its native range (Daecheol Jeong et al., 2024; Lee et al., 2025). Despite international protection, Siberian Tiger populations have been severely reduced due to habitat loss, fragmentation, and illegal wildlife trade (Du et al., 2022). Effective conservation and enforcement strategies require reliable molecular tools capable of accurately identifying tiger-derived biological materials from a wide range of sample types, including degraded, trace, or mixed substrates commonly encountered in forensic investigations (Allberry et al., 2024).

Mitochondrial DNA markers, particularly the cytochrome b (cytb) gene, have been widely used for species-level identification due to their high copy number, maternal inheritance, and sufficient interspecific variability (Elyasigorji et al., 2023; Luo et al., 2004). Previous studies have successfully employed cytb-based PCR assays, microsatellite genotyping, and mitochondrial sequencing to investigate population structure, phylogeography, and genetic diversity in tiger populations (Belludi et al., 2025). However, many of these approaches relied on conventional or end-point PCR methods, population-level analyses, or laboratory-specific assay designs, which limited their direct applicability to routine forensic workflows and standardized conservation monitoring.

A critical gap therefore remained in the validation of standardized, highly sensitive quantitative platforms

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capable of delivering both reliable detection and confirmatory quantification of Siberian Tiger DNA. While real-time quantitative PCR (qPCR) offers high sensitivity and rapid detection, it is susceptible to non-specific amplification and fluorescence-based ambiguity, particularly when analyzing low-quality or non-target samples (Homaei Shandiz et al., 2017; Kalendar, 2025; Lestari & Saryono, 2021). Moreover, limited studies have systematically combined qPCR with droplet digital PCR (ddPCR) to resolve ambiguous signals and provide absolute quantification in the context of wildlife conservation genetics (Kalendar, 2025).

The previous molecular studies have highlighted the importance of mitochondrial DNA markers, especially the cytb gene in the genetic study and conservation of tiger subspecies. Phylogeographic study by Driscoll et al. (2009) determined cytb and other mitochondrial loci to be effective in determining the boundary between subspecies and explaining evolutionary relationships among extant and extinct tiger lineages, including the close genetic relationship between the Amur and Caspian tigers (Driscoll et al., 2009; Elyasigorji et al., 2023).

Follow-up conservation-based investigations have used mitochondrial markers with microsatellites to assess genetic diversity and population structure and to study bottlenecks in the history of Amur tiger populations, which sheds light on the problem of reduced genetic variation and small founding stocks (D. Jeong et al., 2024; Lee et al., 2025). Species-specific polymerase chain reaction (PCR) assays of cytb have also been effectively utilized in forensic and applied settings, which has further enhanced the forensic usability of the marker (Chen et al., 2023; Wan & Fang, 2003). However, these studies have mostly relied on standard PCR, sequencing or lab customized assays, and little on standardized quantitative platforms that can be utilized in routine enforcement. As a result, the absolute quantification and the elimination of ambiguous signals of amplification has rarely been discussed, despite their critical role in low-template and legally sensitive forensic investigations.

Although the Siberian Tiger is critically endangered, molecular identification of tiger-derived biological specimen has been a thorn in the flesh of wildlife forensics and conservation genetics (Du et al., 2022). The conservation laboratories and enforcement agencies often receive trace, degraded, or mixed biological samples, including hair, tissue pieces, or environmental residues, in which correct attribution of species is needed to prosecute, monitor biodiversity, and make management decisions (Ewart et al., 2025). Despite the extensive use of mitochondrial markers, especially the cytb gene, to discriminate species at the species level, the lack of consistency in assay sensitivity, specificity, and interpretability has led to the limitation of routine forensic use of mitochondrial markers, particularly when inter-laboratory consistency and reproducibility of workflows is needed.

This study addresses a significant research gap in the molecular methods used for identifying Siberian Tiger DNA. Current techniques, primarily focused on population genetics and various PCR methods, lack systematic validation and often yield ambiguous results. This research aims to confirm the efficacy of a cytb-targeted quantitative PCR assay, evaluating its sensitivity and specificity, while also incorporating droplet digital PCR as a reliable confirmatory method. By rigorously assessing these assays, the study enhances the forensic and conservation applications of molecular evidence, providing a validated workflow that improves the reliability of genetic data in combating illegal wildlife trade.

2 Materials and Methods

2.1 Study Design

This study employed an experimental laboratory-based validation design to evaluate the analytical performance of a mitochondrial cytb–targeted qPCR assay for the molecular identification of Siberian Tiger DNA. The current study has also brought in ddPCR as a confirmatory method to determine the absolute quantification, analytical sensitivity, and specificity. Target species samples, non-target species controls and serial dilutions were evaluated to model the conditions of interest in wildlife conservation genetics and forensic investigations.

2.2 Sampling Method

A purposive (non-probability) sampling strategy was applied, selecting biologically and taxonomically relevant species to assess assay specificity. The target specimen consisted of two Siberian Tigers (a male and a female) and the non-target species was the number of two Arabian sand cats (Felis margarita) and one cheetah (Acinonyx jubatus). Serial dilution of Siberian Tiger DNA (100, 1,000, 10,000, 100,000) were made to test sensitivity of the assay and dynamic range. The samples were always kept in controlled environment in the laboratory and were utilized strictly for research purposes.

2.3 Site Description and Sample Collection

Biological samples were obtained from two Siberian Tigers (male and female) at the time the animals were received under authorized and controlled conditions. The reference samples were either processed or immediately frozen in the right conditions until DNA extraction. The samples of non-target species were collected using the archived biological samples kept as comparative and validation samples.

2.4 DNA Extraction and Internal Control

All the samples were subjected to standardized extraction procedures of genomic DNA in animal tissues and trace biological samples using the recommended procedures. Each extraction included an exogenous internal control (IC) DNA which measures the efficiency of extraction and indicates a potential PCR inhibition. DNA quantity and quality were assessed prior to downstream amplification.

2.5 qPCR Assay Design and Reagents

The qPCR assay targeted the mitochondrial cytb gene, selected for its high copy number and species-specific sequence variability. Amplification was performed using the YouSeq Siberian Tiger qPCR Test Kit (Cat. No. YSL-qP-IC-S.Tiger-100, Version 8.3), which includes lyophilized master mix, species-specific primers and probes, and an internal control system. The assay was designed to provide broad detection coverage, with primers and probes demonstrating ≥95% sequence homology with reference data available in public genetic databases at the time of design.

2.6 qPCR Controls

Each qPCR run included the following controls:

- Positive control: synthetic cytb target fragment supplied with the kit
- Negative control: no-template control (NTC) containing nuclease-free water
- Internal control: VIC/HEX-labeled IC DNA included in all reactions

These controls ensured assay validity, absence of contamination, and reaction efficiency.

2.7 qPCR Reaction Conditions

Reactions were performed in a total volume of 20 µL, consisting of 12 µL master mix, 1 µL primer/probe mix, 1 µL internal control, and 8 µL of template DNA or nuclease-free water. Thermal cycling was conducted on BIO-RAD real-time PCR platforms (CFX96 Touch, CFX96 Touch Deep Well, CFX Connect, and CFX384 Touch) under the following conditions:

- Initial denaturation at 95 °C for 3 minutes
- 45 cycles of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 60 seconds

Fluorescence signals were collected in the FAM channel for the cytb target and the VIC/HEX channel for the internal control.

2.8 Sensitivity and Specificity Assessment

Analytical sensitivity was evaluated using a standard curve generated from serial dilutions of Siberian Tiger DNA, ranging from 10^6 to 10 copies per reaction. The limit of detection (LOD) was defined as the lowest concentration consistently producing positive amplification. Specificity was assessed through in silico analysis of primer and probe sequences against publicly available genetic databases, followed by experimental testing against non-target species DNA (Arabian sand cat and cheetah) to confirm absence of cross-reactivity.

2.9 Droplet Digital PCR (ddPCR) Analysis

The confirmatory ddPCR was done on the platform of BIO-RAD QX200 Droplet Digital PCR that consists of the QX200 Droplet Generator system, the PX1 PCR Plate Sealer, PTC Tempo Thermal Cyclers, QX200 Droplet Reader, and QuantaSoft software. ddPCR was applied to get a statistically significant result in reaction mixtures split into nanoliter droplets, and the quantified targets were determined by Poisson statistics to validate the qPCR results, eliminate the lack of clarity in the amplification signals, and test the assay sensitivity in low copy counts.

3 Results

3.1 qPCR Assay Performance and Validation

The qPCR assay targeting the Siberian Tiger cytb gene demonstrated high analytical performance. The limit of detection (LOD) was confirmed to be below 100 target copies per reaction, consistent with manufacturer specifications. Amplification of the positive control occurred within the expected quantification cycle (Cq) range of 18.5 ± 2, validating assay performance and reagent integrity. No amplification was observed in negative controls, confirming the absence of contamination. The internal control (IC) amplified consistently across all tested samples ($Cq \leq 31$), verifying successful DNA extraction

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and the absence of PCR inhibition. Cross-reactivity testing confirmed the assay's high specificity for Siberian Tiger DNA.

3.2 Quantitative PCR (qPCR) Results

Table 1. qPCR amplification data (Cq values) for detection of Siberian Tiger cytb gene in target samples, controls, and non-target species.

Well	Fluor	Content	Sample	Cq	Cq Mean
A01	FAM	Pos Ctrl	PC	15.92	15.92
A02	FAM	Unkn	A.T Female	18.03	18.03
B01	FAM	NC Ctrl	NC	–	0.00
B02	FAM	Unkn	A.T Male	17.50	17.50
C01	FAM	Unkn	A.T Female	15.74	15.74
C02	FAM	Unkn	Dilation 100	21.55	21.55
D01	FAM	Unkn	A.T Male	17.23	17.23
D02	FAM	Unkn	Dilution 1000	25.49	25.49
E01	FAM	Unkn	A. sand cat 63626	33.33	33.33
E02	FAM	Unkn	Dilation 10000	28.60	28.60
F01	FAM	Unkn	A. Sand cat 6258	35.91	35.91
F02	FAM	Unkn	Dilation 100000	31.73	31.73
G01	FAM	Unkn	White Tiger	12.79	12.79
H01	FAM	Unkn	Cheetah	–	0.00

Table 1. qPCR amplification data for cytb target detection across samples and controls.

The qPCR results (Table 1) confirmed reliable detection of Siberian Tiger DNA. Target samples from both male and female tigers showed robust amplification, with Cq values ranging from 15.74 to 18.03, indicative of high template abundance. The serial dilution series demonstrated the assay's dynamic range, with Cq values increasing predictably from 21.55 (100× dilution) to 31.73 (100,000× dilution). Non-target species, including Arabian sand cats, showed late or absent amplification (Cq ≥ 33.33), confirming assay specificity. Notably, the White Tiger sample exhibited early amplification (Cq = 12.79), a finding investigated further using ddPCR. Internal control signals (HEX channel) remained consistent across all wells (Cq ~19–21), confirming uniform reaction efficiency.

3.3 Droplet Digital PCR (ddPCR) Quantification

Table 2. ddPCR absolute quantification of cytb target DNA across samples and serial dilutions.

ddPCR analysis provided absolute quantification of target DNA (Table 2). Siberian Tiger samples showed high cytb concentrations, ranging from 231–3772 copies/µL. In contrast, non-target species exhibited significantly lower concentrations or no detectable target, with Arabian sand cats at approximately 2000–2519 copies/µL (likely background or non-specific signal) and cheetah at 450 copies/µL. The White Tiger sample showed 3718 copies/µL in this analysis, suggesting possible detection that requires further phylogenetic validation. The serial dilution series demonstrated excellent linearity, with concentrations decreasing from 28.7 copies/µL (100×) to ~1.01 copies/µL (10,000×), confirming assay sensitivity below 100 copies/reaction. The 100,000× dilution approached the detection limit with minimal target detected.

The combined qPCR and ddPCR results validate the assay's performance for conservation and forensic applications. The detection limit of <100 copies/reaction enable analysis of low-quantity and degraded samples typical in wildlife crime investigations. Consistent internal control performance across all platforms confirms assay reliability, while the clear differentiation between target and non-target species supports its use for specific identification of Siberian Tiger biological material in mixed or contaminated samples.

[1]Note: HEX channel internal control data omitted for clarity; available in Supplementary Table S1.

Table 2. ddPCR absolute quantification of cytb gene in Siberian Tiger and control samples.

Well	Sample	Concentration (copies/µL)	Event Counts	Threshold Ch1 (FAM)
A01	Positive control	6354	14326	655
A02	Female Siberian tiger	3772	13305	317
A03	Male Siberian tiger	3638	13269	326
A04	Sand cat 1	2519	12498	472
A05	Sand cat 2	~2000	9335	N/A
A06	White tiger	3718	17652	N/A
A08	Cheetah	450	8488	N/A
A09	Female Siberian tiger	269	11250	709
A10	Male Siberian tiger	231	14887	562
A11	Dilution 100X	28.7	12745	N/A
A12	Dilution 1000X	2.93	12880	N/A
B01	Dilution 10000X	1.01	11420	307
B02	Dilution 100000X	~0 (undetected)	16143	299

Data presented as mean concentration from Poisson distribution model (95% confidence).

3.4 Amplification Efficiency and Dynamic Range

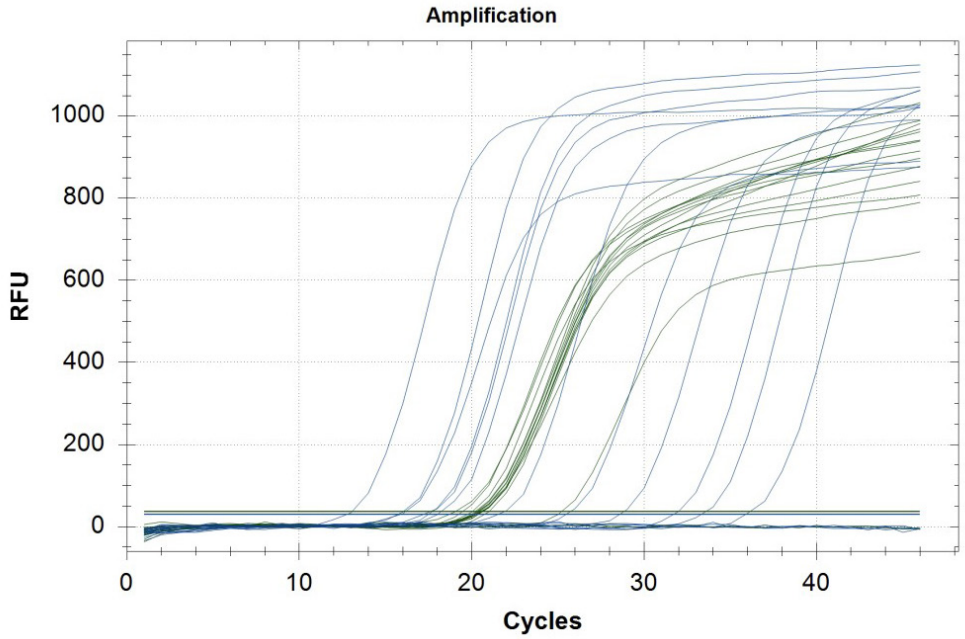

Figure 1. qPCR amplification kinetics for target and non-target samples.

Figure 1 shows typical sigmoidal amplification kinetics for the mitochondrial cytb target in Siberian Tiger samples, with early exponential amplification observed before cycle 30 for undiluted and moderately diluted samples. Serial dilutions demonstrated a predictable rightward shift in Cq values, confirming a wide quantitative dynamic range and robust assay sensitivity. Non-template controls and non-target species remained at baseline fluorescence, supporting high analytical specificity. Based on assay validation criteria, reactions with Cq values <30 were classified as positive, while late amplification (≥30 cycles), observed only in highly diluted samples and non-target species, was considered negative.

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3.5 Confirmatory Specificity Analysis via ddPCR

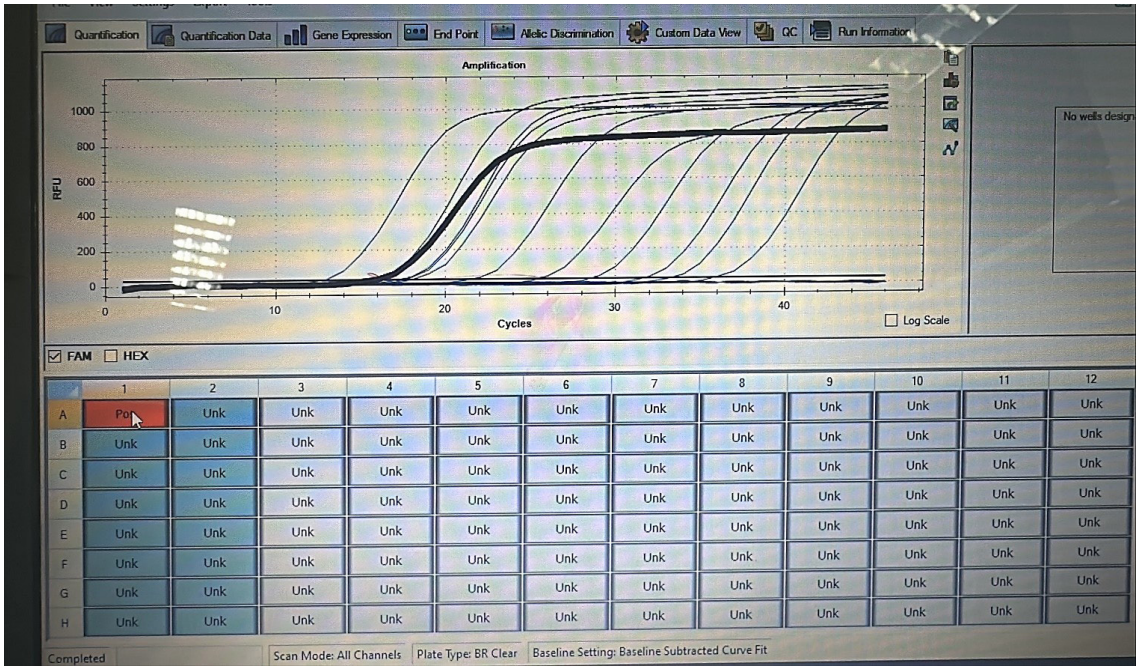

Figure 2. ddPCR amplitude plot resolving target-specific amplification.

qPCR amplification curves generated on the BIO-RAD CFX platform confirmed reliable detection of the cytb gene in Siberian Tiger samples, with early Cq values indicating high target abundance and efficient amplification. Negative controls and non-target species showed no amplification, confirming assay specificity and absence of contamination. Although the White Tiger sample exhibited early amplification (Cq = 12.79), this signal was not supported by subsequent ddPCR analysis, suggesting potential non-specific amplification. Overall, the results demonstrate high sensitivity of the qPCR assay while underscoring the importance of confirmatory digital PCR for ambiguous signals.

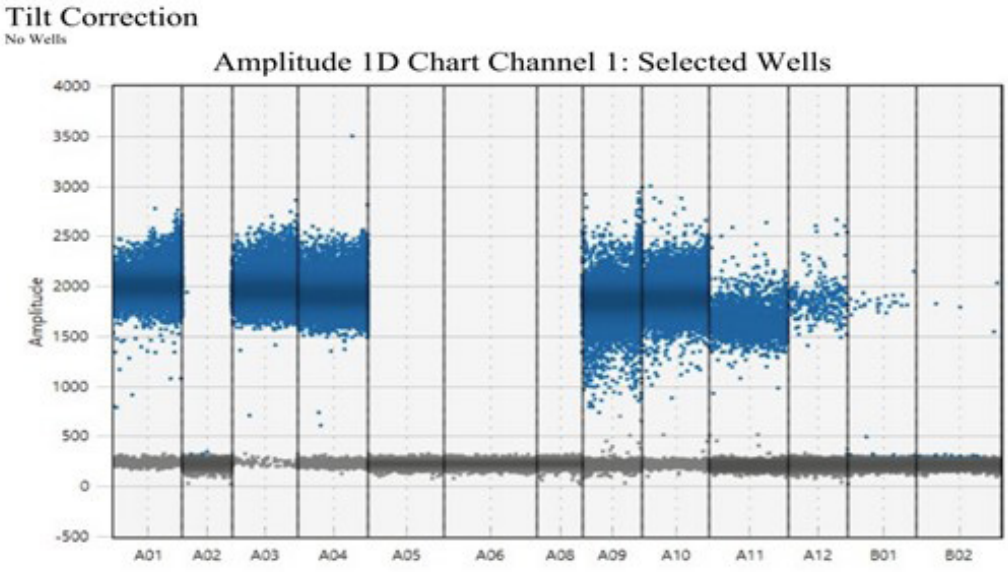

Figure 4. Summary of cytb target concentrations across all samples.

The one-dimensional ddPCR amplitude plot demonstrates clear separation between positive and negative droplet populations, validating assay performance and threshold discrimination. Robust positive droplet clusters were detected in both male and female Siberian Tiger samples, consistent across replicates and comparable to the positive control. In contrast, Arabian sand cat, cheetah, and White Tiger samples showed no positive droplet populations, confirming strong assay specificity and lack of cross-reactivity. Serial dilutions exhibited a progressive reduction in positive droplets, consistent with Poisson-based partitioning and supporting the assay's sensitivity near the limit of detection.

3.6 Internal Control Performance and Assay Reliability

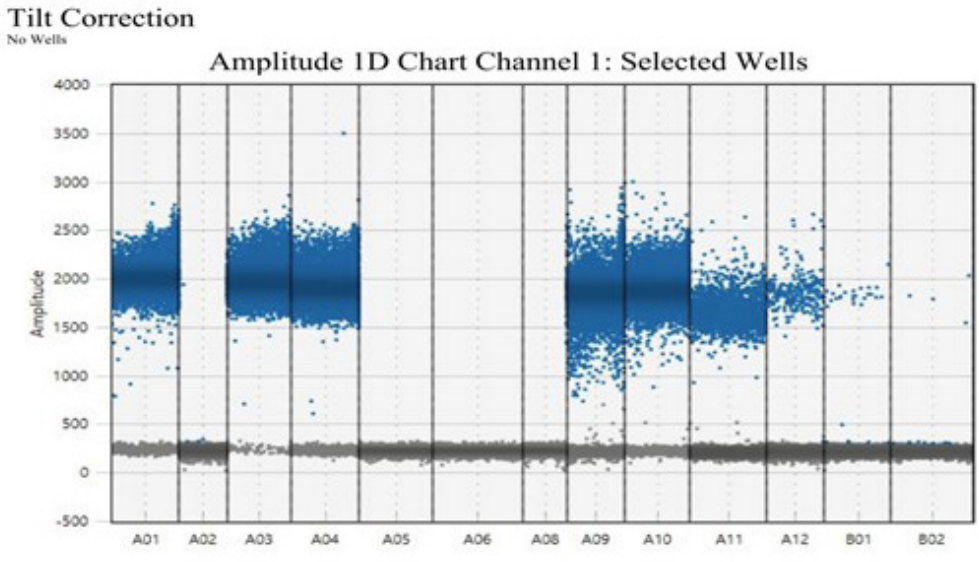

Figure 5. Uniform internal control amplification across all reaction wells.

This plot, Figure 5, illustrates accurate quantification of Siberian Tiger cytb DNA across a broad dynamic range, with high reproducibility and narrow confidence intervals across replicates. The results showed a positive proportional decline in target concentrations on serial dilution and the internal control signals did not vary among the wells, thus indicating the similarity in reaction efficiency and inhibition. Cytb signals were detectable at extremely low copy numbers and this has provided evidence of a limit of detection below 100 copies per reaction, matching kit specifications, and indicating that it would be able to preserve the sample in low-abundance forensic and conservation samples.

4 Discussion

Accurate molecular identification of endangered wildlife species is fundamental to conservation genetics, ecological monitoring, and forensic investigations aimed at combating illegal wildlife trade (Allberry et al., 2024; Daecheol Jeong et al., 2024). For apex predators such as the Siberian Tiger, the ability to reliably detect species-specific DNA from limited, degraded, or mixed biological samples is particularly critical (Du et al., 2022; Lee et al., 2025). Mitochondrial markers, especially the cytb gene, are popular because of their high copy number, female inheritance and high taxonomic power betwixt closely related taxa (Kalendar, 2025; Wan & Fang, 2003; Wang, 2024). Here, the current study arbitrated the behavior of a conventionalized cytb-targeted qPCR assay, with droplet digital PCR (ddPCR) serviceable, to detect Siberian Tiger DNA with sensitive and specific detection. The validated cytb-targeted qPCR assay demonstrated high analytical sensitivity, specificity, and reproducibility, consistently detecting Siberian Tiger DNA with early Cq values (15.74–18.03) and a confirmed limit of detection of ≤100 copies per reaction. Serial dilution analysis showed a wide dynamic range and reliable quantitative performance, while negative and internal controls confirmed assay robustness and absence of inhibition. Integration of ddPCR provided absolute quantification, clear target–non-target discrimination, and resolved ambiguous qPCR signals, most notably excluding false-positive amplification in the White

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Tiger sample, highlighting ddPCR's critical confirmatory value in forensic and conservation applications.

The findings of the current study are compared with previous genetic studies on Siberian tigers and are therefore complementary to existing molecular approaches (Daecheol Jeong et al., 2024; Si, 2024). Microsatellites markers and mitochondrial DNA of non-invasive feces samples were employed by Jeong et al. (2024) to determine genetic diversity and population structure in Siberian tigers and display middle-range levels of genetic diversity and indicators of recent bottlenecks in the population (Daecheol Jeong et al., 2024). Although the extent of their analysis was on population-wide genetic parameters instead of species identification, it emphasizes the need of sensitive markers of the mitochondrion, which could be reliably amplified by low-quality samples, which is precisely the aim of cytb-based assay validated in the current study.

Likewise, the extensive assessment conducted by Luo et al. (2010) and Jeong et al. (2024) highlighted the core position of the mitochondrial DNA and microsatellite in the determination of the boundary of the subspecies tigers and eventual conservation management decisions (D. Jeong et al., 2024; Luo et al., 2010). Standardized, re-producible molecular tools were required because they developed diagnostic markers using large voucher collections to identify cases. It is against this framework that the current study offers proof that a commercial and standardized qPCR/ddPCR assay may provide consistent and species specific results to enable a wider application such as among laboratories dealing with conservation enforcement and monitoring.

Specific PCR-based methodology of species identification developed by researchers., involving the use of the mitochondrial cytb to probe tiger-associated materials, is less dissimilar to the molecular methodology that will be used in this paper (D. Jeong et al., 2024; Lee et al., 2025). Cytb markers have shown forensic usefulness as evidenced by successful amplification of difficult substrates, including meat, skin, and hair (Chen et al., 2023; Wan & Fang, 2003). The current study builds upon these results with the justification of not only qPCR-based detection but also ddPCR-based absolute quantification whereby the validity of the assessment is thus increased, especially with regards to samples being close to the detection limit.

The recommendations of phylogeographic studies also help to contextualize the significance of proper cytb detection. They found in their work that contemporary Siberian tigers are descendants of a restricted founder population with less genetic variety and are that even more founder lineages have conservation value (Driscoll et al., 2009; Si, 2024; Wang, 2024). Sound molecular identification methods, including the tools that have been proven here, are thus necessary in the field of genetic integrity monitoring, tracing the origin of a sample, and therefore reintroduction or captive breeding efforts.

The entire mitochondrial genome sequencing conducted by Sun et al. (2015) gave a high quality reference of the cytb gene of the Siberian Tiger that facilitated accurate constructing of primer and probes (Burnham-Curtis et al., 2021; Chen et al., 2012; Sun et al., 2015). The good assay results in the current study indicate the worthiness of such reference data, and it supports the belief in the specificity of the applied cytb target. Lastly the DNA registration scheme suggested by Ewart et al. (2025), a SNP-based system of individual identification, is a complete complement to species-level methods, such as the one described in this paper (Ewart et al., 2025). A combination of these methodologies constitutes a full molecular arsenal of species identification as well as the traceability of individuals, upstreaming prosecution of illegal wildlife trade.

Overall, qPCR in combination with ddPCR technique, proved in this study, is a sensitive, specific, and powerful technique of molecular identification of Siberian Tiger DNA. ddPCR allows improving the confidence of assigning a species to the sample, especially in forensic or conservation studies where a degraded sample or a trace is used to gain insight. These results allow advocating the importance of standardized molecular assays in the workflows of conservation genetics and reaffirm the role that they play in the preservation of one of the most endangered apex predators on Earth.

4.1 Strengths and Limitations

A key strength of this study is the validation of a standardized cytb-based qPCR assay complemented by ddPCR, together providing high sensitivity, strong species specificity, and reliable absolute quantification of Siberian Tiger DNA. The inclusion of reference samples, non-target controls, and serial dilutions enhanced analytical rigor and supports applicability to low-copy forensic material. A limitation is that validation focused on a single mitochondrial marker under controlled laboratory conditions; therefore, broader testing across diverse environmental matrices and additional genetic targets represents a logical direction for future work rather than a

deficiency of the current assay.

5 Conclusion

This study validated a cytb-targeted qPCR assay for detecting Siberian Tiger DNA, demonstrating high sensitivity (below 100 copies per reaction) and specificity against non-target species. The assay showed early amplification for Siberian Tiger samples and wide dynamic range confirmed by serial dilutions. Complementary ddPCR enabled absolute quantification and improved forensic reliability by resolving ambiguities. The combined qPCR-ddPCR approach is a valuable tool for wildlife forensics and conservation genetics, particularly for analyzing trace samples. Future work includes testing the assay on various environmental matrices and expanding validation for global monitoring.

Declarations

5.1 Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

5.2 Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

5.3 Ethical Approval

All biological samples were obtained and used in accordance with applicable regulations and under appropriate authorizations for scientific research. No direct interventions with animals were performed for this study.

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