Development of a direct duplex real-time PCR assay for rapid testing of domestic cat hepadnavirus

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Summary[†]

Domestic cat hepadnavirus (DCH) is a novel hepadnavirus, first identified in 2018. The DCH is generally diagnosed using conventional polymerase chain reaction (PCR) assays, which include a time-consuming agarose gel electrophoresis. In this study, we developed a rapid, sensitive, and specific real-time PCR assay for the detection of the DCH genome. To streamline the procedure, our real-time PCR assay was carried out using blood samples, without deoxyribonucleic acid (DNA) extraction. A primers/probe set was designed based on the nucleotide sequences of the surface gene of the DCH strain Japan/KT116/2021 (Accession# LC668427), which we recently identified from a feline blood sample in Japan. To exclude the possibility that the PCR reaction was blocked by anticoagulants, we also used a primers/probe set for amplifying the housekeeping beta-actin gene. Direct duplex real-time PCR assay had a high sensitivity, with a limit of detection of 10 copies/μL for DCH. We successfully established a rapid and highly sensitive duplex real-time PCR assay for the detection and quantification of DCH. This direct duplex real-time PCR assay is a useful tool for DCH diagnosis and surveillance.

Keywords: domestic cat hepadnavirus, direct duplex real-time PCR assay, rapid, sensitive.

[†] ACTB, Assay detects the housekeeping beta-actin; CV, Coefficient of variation; DCH, Domestic cat hepadnavirus; FeLV, Feline leukemia virus; FIV, Feline immunodeficiency virus; HBV, Hepatitis B virus; LoD, Limit of detection; ORF, Open reading frames

1. Introduction

The *Hepadnaviridae* is a family of small DNA viruses, which are known to cause liver diseases, such as hepatitis, hepatocellular carcinomas, and cirrhosis, in its hosts. Known hosts include humans, apes, and birds. In 2018, a novel virus similar to the hepatitis B virus (HBV) now known as domestic cat hepadnavirus (DCH) was identified in a domestic cat in Australia. This was the first report of hepadnavirus infection in a companion animal (Aghazadeh et al., 2018). A recent survey of canine serum samples discovered hepadnaviral DNA which was genetically close to DCH (Diakoudi et al., 2022). Companion animals play an essential role in the daily life of many people, and maintaining their health is therefore important. There is, therefore, a clear need for a rapid assay for the presence of DCH, both for diagnosis and to monitor the distribution and prevalence of the virus worldwide.

The DCH is a small DNA virus, 42–50 nm in diameter, belonging to the genus *Orthohepadnavirus*, family *Hepadnaviridae*. Viruses belonging to family *Hepadnaviridae* have a broad host range, including mammals, birds, fish, reptiles, and frogs (Magnius et al., 2020). It has a circular DNA genome, which is partially double-stranded and is approximately 3.2 kb in length. Like other hepadnaviruses, the genome contains four overlapping open reading frames encoding the polymerase (L), surface (S), core (C), and X proteins (Aghazadeh et al., 2018; Magnius et al., 2020). The DCH is genetically close to HBV, a prototype hepadnavirus, and like HBV is frequently associated with immunosuppressive infections (Aghazadeh et al., 2018; Anpuanandam et al., 2021; Lanave et al., 2019; Piewbang et al., 2020).

Since DCH is a relatively newly discovered virus, little information about its genetic diversity and evolution is currently available. The DCH has been identified in Italy, Thailand,

Malaysia, United Kingdom, and Japan (Aghazadeh et al., 2018; Anpuanandam et al., 2021; Diakoudi et al., 2022; Jeanes et al., 2022; Lanave et al., 2019; Piewbang et al., 2020; Takahashi et al., 2022), but the distribution and prevalence of DCH in other countries have not been investigated in depth. Most previous studies have used conventional PCR to detect DCH from clinical specimens, and some groups have used real-time PCR (Fruci et al., 2022; Lanave et al., 2019). Since DCH is detected using blood samples, it is necessary to consider whether the anticoagulants present in whole blood or plasma samples interfere with the assays, potentially leading to erroneous negative results. To facilitate the diagnosis and investigation of DCH, it is particularly important to develop a rapid, sensitive, and accurate method for the detection of this virus.

In this study, we developed a probe-based, direct duplex real-time PCR assay for detecting DCH from blood samples. The assay can be used to analyze whole blood samples without time-consuming DNA extraction. To check whether the amplification reaction is blocked by anticoagulants, our assay detects the housekeeping beta-actin (*ACTB*) gene in the same reaction tube. This method is an optimal assay for the rapid and accurate detection of DCH.

2. Materials and Methods

2.1 Design of PCR primers

The complete genome sequence of DCH strain Japan/KT116/2021 (Accession# LC668427) and *Felis catus* Fca126 chromosome E1 (Accession# NC058381) were used as templates for designing primers/probe sets. Two primers/probe sets were designed based on the nucleotide sequences of the surface gene of DCH and the *ACTB* gene of *Felis catus* as a housekeeping gene, using the PrimerQuest tool (https://www.idtdna.com/Primerquest/home/Index; **Table 1**). The target primer region for DCH is illustrated in **Figure 1**. The sequences of the DCH

primers/probe set were aligned with the sequences of 16 DCH strains available in GenBank using CLC Genomics Workbench 22.0.1 in viewing mode (Qiagen) and Blast Primer (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to evaluate the presence of any mismatches.

2.2 Real-time PCR assays

Real-time PCR reactions were conducted using Probe qPCR Mix kits (TaKaRa Bio Inc., Shiga, Japan), which are relatively tolerant to several inhibitors of PCR, according to the company. Each 20 μL reaction contained 500 nM of each primer (forward and reverse), 250 nM of the probe, 10 μL of Probe qPCR Mix, and 0.2 μL of ROX reference dye II, with 1μL of the blood sample as a template. The reaction was performed on a QuantStudio 3 Real-Time System Thermal Cycler (Applied Biosystems, Foster City, CA, USA) at 95°C for 20 sec, followed by 40 cycles at 95°C for 3 sec, and then 60°C for 30 sec. For a positive control for two primer/probe sets (DCH and *ACTB*), a DCH-positive blood sample identified in a previous study was used (Takahashi et al., 2022). A DCH-negative blood sample was used as a negative control for DCH and positive control for *ACTB*. The use of these blood samples was approved by the Animal Experiment Committee of the University of Miyazaki (authorization number: 2021-019). A reaction without template DNA (distilled water) was used as a negative control in all experiments.

2.3 Generation of a DCH DNA standard for real-time PCR

The target region of the direct duplex real-time PCR (736 bp) was amplified using conventional PCR assays, as described previously (Takahashi et al., 2022). The amplicon was purified using QIAquick PCR Purification kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of DNA was determined using a NanoDrop

105 (Thermo Fisher Scientific, Waltham, MA, USA) and the copy number was calculated using
106 the following equation:

107 $$\text{Number of copies (molecules)} = \frac{\text{X ng} \times 6.0221 \times 10^{23}\ \text{molecules/mole}}{\left(\text{N} \times 660 \frac{\text{g}}{\text{mole}}\right) \times 1 \times 10^{9}\ \text{ng/g}}$$

108 where X is the amount of amplicon (ng) and N is the length of the dsDNA amplicon—736 bp
109 in this study—and 660 g/mole is the average mass per 1 bp dsDNA.

110 **2.4 Generation of a standard curve**

111 A ten-fold dilution series of 10^6 to 10^1 DNA copies/μL was prepared using Tris-EDTA buffer
112 (Nippon Gene, Tokyo, Japan) as a diluent, and was used for evaluating the standard curve
113 parameters, especially the slope, from which the amplification efficiency and the coefficient
114 of determination (R^2) were derived. Each dilution was prepared in triplicate and amplified as
115 described above.

116 **2.5 Determination of the limit of detection**

117 The limit of detection (LoD) of DCH was determined using ten-fold serial dilutions of DCH
118 DNA fragments from 10^4 copies/μL to 1 copy/μL, with six replicates for each concentration.
119 The lowest concentration that met the positive detection rate of 95% was considered to be the
120 LoD.

121 **2.6 Specificity of the direct duplex real-time PCR assay**

122 The specificity of the DCH primers/probe set was evaluated using plasmids containing two
123 strains of feline leukemia virus (FeLV) and two strains of the feline immunodeficiency virus
124 (FIV). The FIV plasmids used in this study were FIV-34T FIV strains of F10 (Talbott et al.,
125 1989) and FIV-PPR (Phillips et al., 1990), while the FeLV plasmids were pEECC-FeLV and

p61E-FeLV (Donahue et al., 1988; Overbaugh et al., 1988). These plasmids were obtained through the NIH HIV Reagent Program, Division of AIDS, NIAID, NIH, and used as templates for the direct duplex real-time PCR reactions.

2.7 Reproducibility of the direct duplex real-time PCR assays

To evaluate the reproducibility of the direct duplex real-time PCR assays, seven dilutions of cat blood containing DCH DNA fragments ranging from 10^6 to 10^1 DNA copies/µL were used for real-time PCR reactions. The standard deviation and coefficient of variation (CV) were calculated based on the cycle threshold (Ct) values obtained from the triplicate assay.

2.8 Statistical analysis

The standard curve, LoD 95%, and reproducibility were analyzed using GraphPad Prism v9.1.1 (GraphPad Software, San Diego, California USA, www.graphpad.com).

3. Results

3.1 Design and evaluation of primers and probes

The position of the primers/probe set targeting DCH in this study is illustrated in **Figure 1**. The primers/probe set designed here perfectly matched the sequences of 14 out of 16 DCH strains (87.5%) available on GenBank. Only 2 out of 16 strains (12.4%) had mismatches with the primers/probe set. These two strains were DCH strain Rara, Japan (Accession# LC685967) and CP79H THA/2019, Thailand (Accession# MT506043) (**Figure 2**).

Before testing a duplex PCR, we performed a singleplex PCR to check the amplification of DCH and *ACTB* with the primers/probe set. Singleplex real-time PCR using a DCH-positive blood sample showed fluorescence signals for both DCH and *ACTB*, while a DCH-negative blood sample only showed a fluorescence signal for *ACTB*. The direct duplex real-time PCR

148 for detecting both DCH and *ACTB* in the same tube showed comparable Ct values to those
149 observed in the singleplex real-time PCR. The results of the comparison are summarized in
150 **Table 2**. We visualized the PCR amplicons on an agarose gel after the duplex real-time PCR
151 and found no unspecific bands (**Figure 3**). These results suggested that our direct duplex real-
152 time PCR did not generate unwanted complexes of oligos, such as primer dimers.

153 To compare the linearity and efficiency of the direct duplex real-time PCR, we prepared a
154 standard curve for DCH and performed duplex real-time PCR. The results showed high
155 linearity (R^2 value = 0.9928) and perfect efficiency (103.9%) with a standard curve slope of
156 −3.32, demonstrating a perfect amplification efficiency and linear equation required for DNA
157 quantification (**Figure 4**).

158 **3.2 Sensitivity, reproducibility, and specificity of the direct duplex real-time PCR assay**
159 The sensitivity of the direct duplex real-time PCR was assessed using serially diluted DCH
160 DNA fragments ranging from 10^4 copies/μL to 10^0 copies/μL. **Table 2** shows that the
161 detection rate of 1 copy/μL was less than 95% of replicates, demonstrating that the LoD was
162 10 copies/μL.

163 We tested the specificity of the direct duplex real-time PCR assay using plasmids encoding
164 two FIV strains and two FeLV strains since previous studies suggested an association
165 between infection with these viruses and DCH. The result showed that the direct duplex real-
166 time PCR assay did not amplify these plasmids encoding non-DCH sequences (**Table 4).**

167 To test the reproducibility of the direct duplex real-time PCR assay, we used serially diluted
168 DNA templates ranging from 10^6 copies/μL to 10^1 copies/μL. The direct duplex real-time
169 PCR assay showed good reproducibility, with a CV% of less than 10% (**Table 5**). The

variance analysis also showed that the *P*-value of interassay variation for each concentration gradient of the template was >0.05, indicating high reproducibility of the direct duplex real-time PCR assay.

4. Discussion

In this study, we successfully developed a direct duplex real-time PCR assay for detecting and quantifying DCH from cat blood samples without the need for DNA extraction (**Figure 5**). Our assay specifically detected DCH-positive blood samples using fluorescence signals for both DCH and cat *ACTB* (**Table 2**). In terms of sensitivity, the LoD of this assay was 10 copies/μL, with high linearity (R^2 value = 0.9928) and perfect efficiency (103.9%), presenting results that this assay can be used for a large-scale screening with high sensitivity and reproducibility (**Figure 4**).

Since the direct duplex real-time PCR assay does not involve electrophoresis of the PCR products, this assay is faster than conventional PCR (**Figure 5**). Contamination by DNA from other PCR reactions can affect highly sensitive PCR assays, potentially leading to erroneous positive results (Hu, 2016). Since our assay does not require the opening of PCR tubes after the reaction, the risk of contamination is minimized. In a recent study, we demonstrated that a DNA extraction-free method can be used for detecting DCH in blood samples using a conventional PCR assay (Takahashi et al., 2022). This DNA extraction-free method was used in the direct duplex real-time PCR assay, making this approach faster and cheaper than conventional PCR. Due to the elimination of the DNA extraction and electrophoresis steps, our direct duplex real-time PCR assay can be performed in a much shorter time than conventional PCR, while retaining high sensitivity (**Figure 5**).

We used plasmids encoding two strains of FIV or two strains of FeLV, viruses that are reportedly associated with DCH infection (Aghazadeh et al., 2018; Anpuanandam et al., 2021; Lanave et al., 2019; Piewbang et al., 2020) to test the specificity of the assay. None of the four plasmids tested showed any positive signals in the direct duplex real-time PCR assay, presenting results that our assay was specific for DCH. The CV values obtained by the reproducibility analysis ranged from 0.34% to 3.35% (**Table 5**). These results indicated that the assay was highly stable and reproducible.

This study had some limitations. We included just one DCH-positive sample for the assay. Nevertheless, *in silico* alignment analysis suggested that our assay would be able to detect 87.5% of DCH strains available in GenBank. Alignment analysis showed that our primers/probe set has two (C-T and C-A) mismatches on the forward primer with CP79H THA/2019 (Accession# MT506043), and one mismatch(C-A) on the probe with DCH strain Rara, Japan (Accession# LC685967) (**Figure 2**). Although these mismatches may affect the amplification efficiency and sensitivity, previous studies have demonstrated that C-T and C-A mismatches produced no significant effect on amplification (Stadhouders et al., 2010; Lacouture et al., 2020). Moreover, Ye et al. suggested that a few mismatches in the middle or at the 5′ ends minimally affected PCR amplification (Ye et al., 2012). Further research will be needed to develop a direct duplex real-time PCR assay that can detect all DCH strains. In addition, we need to determine whether our assay can detect DCH strains carrying mismatches with the primers/probe set developed in this study.

In summary, the direct duplex real-time PCR assay developed in this study is a sensitive, time-saving, and cost-effective method for detecting DCH and cat *ACTB*. The assay has

214 considerable potential for routine surveillance, diagnosis, and understanding the kinetics of
215 viral loads, which may be associated with disease severity and transmission.

216

217 Figure legends

218

219 **Figure 1. Schematic illustration of the positions of the primers used in this study.**
220 A primers/probe set for DCH was designed based on the genome of the DCH strain
221 Japan/KT116/2021 (Accession# LC668427).

222

223 **Figure 2. Alignment of the DCH primers/probe set with four DCH strains.**
224 The sequences of the DCH primers/probe set were aligned with the sequences of four
225 representative DCH strains using CLC Genomics Workbench 22.0.1 viewing mode to show
226 mismatches.

227

228 **Figure 3. Amplification of the direct duplex real-time PCR assay and PCR products on**
229 **an agarose gel.**
230 (Left) Amplification plot of the direct duplex real-time PCR assay.
231 (Right) Electrophoresis of PCR products on an agarose gel. Lane M: DNA Ladder; Lanes1–6:
232 PCR products of the direct duplex real-time PCR assay with serially diluted DCH-positive
233 sample; Lane 7: PCR product of the singleplex PCR assay of a DCH-negative blood sample
234 with DCH primers, Lane 8: PCR product of the singleplex PCR assay of a DCH-negative
235 blood sample with the *ACBT* primers.

236

Figure 4. Standard curve of DCH generated from serial dilution of DCH DNA standard (three replicates).

The X-axis represents log 10 copies of the DCH DNA standard, whereas the Y-axis represents the cycle threshold (Ct).

Figure 5. Comparison of PCR-based assays for detecting DCH from cat blood samples.

Conventional PCR requires DNA extraction, PCR, and visualization of the amplification products by electrophoresis on an agarose gel. In contrast, the direct duplex real-time PCR assay can measure the copy number of DCH in cat blood without DNA extraction.

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Authorship contribution statement

Maya Shofa: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization.

Akiho Ohkawa: Validation, Investigation.

Tamaki Okabayashi: Writing – review & editing, Funding acquisition.

Yasuyuki Kaneko: Resources, Visualization.

Akatsuki Saito: Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

All authors contributed to the article, read, and approved the final manuscript.

Declaration of competing interests

The authors declare no competing interests.

Data availability

Data will be made available on request.

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Tables

Table 1. Primers and probes used in this study

Primer/ probe	Sequence (5′-3′)	Nucleotide position	Gene target	Species/ Accession number
Forward	TACCAAACCTCCAAGCACTC	163–183	Surface protein	DCH LC668427
Probe	/56-FAM/ACTTCCTGT/ZEN/CCTCCGACCT GTACT/3IABkFQ/	187–211		
Reverse	GCCAGGAGCAAGAGGTAAAT	224–264		
Forward	TCTCGATCTGTGCAGGGTATTA	2455–2477	*ACTB*	*Felis catus* NC058381
Probe	/5HEX/TGGCAAGAG/ZEN/TCCTGAACCAGT TGT/3IABkFQ/	2516–2540		
Reverse	AGACCGGCAAGACAGAAATG	2540–2560		

353

354 **Table 2. Comparison of singleplex and duplex real-time PCR assays**

Template DNA		Singleplex	Singleplex	Duplex	
DCH-positive	DCH-negative	DCH	Cat*ACTB*	DCH	Cat*ACTB*
-	1	N.D.	25.8	N.D.	25.6
-	1:10	N.D.	31.2	N.D.	30.9
-	1:100	N.D.	32.6	N.D.	33.1
1	-	23.7	29.3	24.8	27.3
1:10	-	27.3	34.7	28.2	32.4
1:100	-	29.5	35.0	30.9	36.9

355 N.D. : Not determined as it is below the detection limit.

356

357 **Table 3. Sensitivity of the direct duplex real-time PCR assay**

Concentration (copies/μL)	CI
10000	96.88%
1000	96.88%
100	96.88%
10	96.88%
1	87.50%

358

359

360 **Table 4. Specificity of primer/probe set used in this study**

Sample	Ct Value	
	DCH	Cat*ACTB*
FIV (Petaluma)	N.D.	N.D.
FIV (PPR)	N.D.	N.D.
FeLV (EECC)	N.D.	N.D.
FeLV (61E)	N.D.	N.D.
DCH DNA (10^3 copies/μL)	31.2	N.D.
DCH-positive blood (×1,000 diluted)	31.7	34.6
DCH-negative blood	N.D.	23.2
Negative control (water)	N.D.	N.D.

361 N.D. : Not determined as it is below the detection limit.

362

363 **Table 5. Reproducibility of the direct duplex real-time PCR assay**

Copy number of DCH	Intra-assay				Interassay			
Standard (copies/uL)	N	Average Ct	*SD*	%CV	*N*	Average Ct	*SD*	%CV
10^6	3	19.4	0.22	1.15	2	19.7	0.39	1.96
10^5	3	22.2	0.35	1.60	2	22.6	0.58	2.57
10^4	3	25.9	0.09	0.34	2	26.3	0.53	2.04
10^3	3	29.9	0.29	0.95	2	30.8	1.03	3.35
10^2	3	33.4	0.31	0.93	2	33.6	0.34	1.02
10^1	3	36.4	0.91	2.50	2	37.3	1.20	3.22

364

365

Figure 1

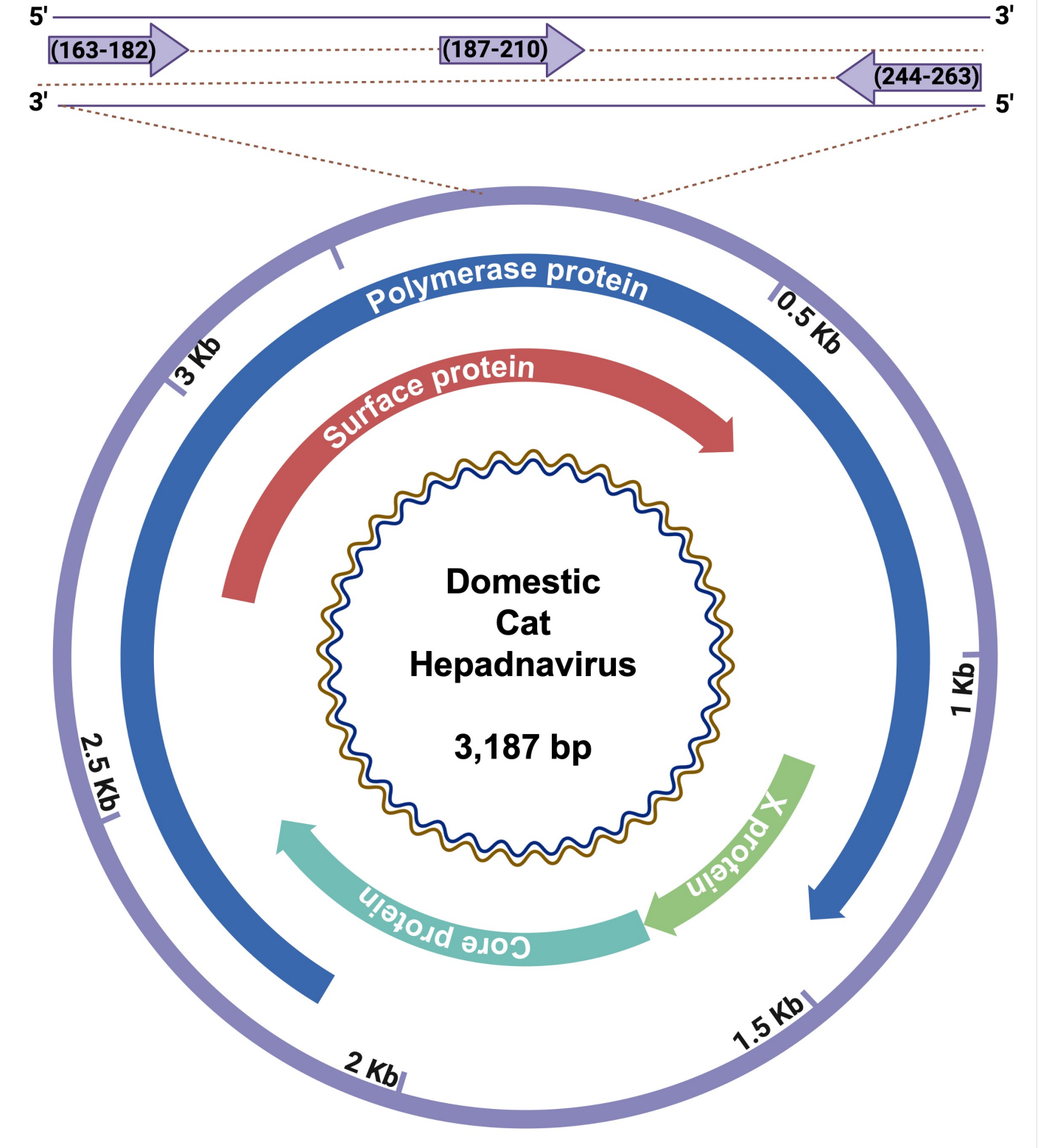

5'
3'
(163-182)
(187-210)
(244-263)
3'
5'
Polymerase protein
Surface protein
Domestic
Cat
Hepadnavirus
3,187 bp
X protein
Core protein
3 Kb
0.5 Kb
1 Kb
1.5 Kb
2 Kb
2.5 Kb

Figure 2

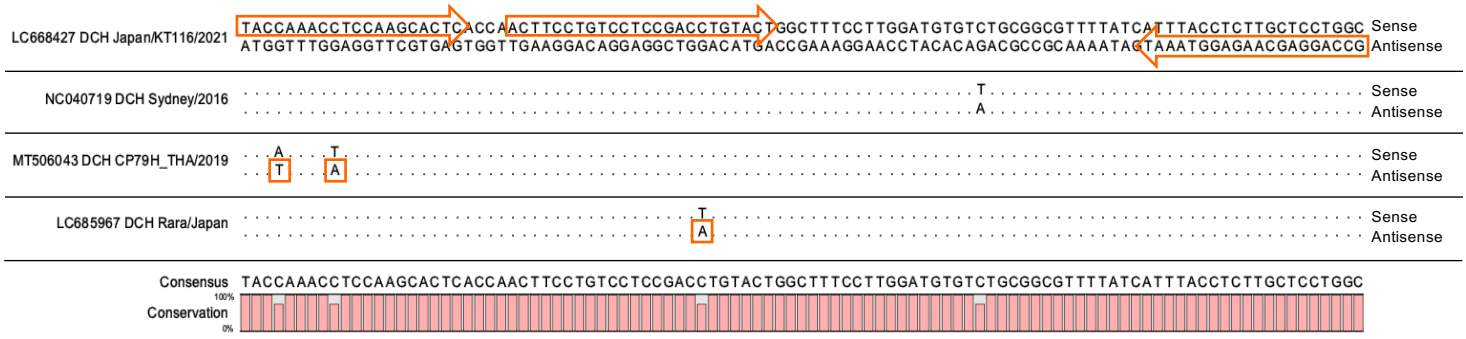

LC668427 DCH Japan/KT116/2021
TACCAAACCTCCAAGCACTCACCAACTTCCTGTCCTCCGACCTGTACTGGCTTTCCTTGGATGTGTCTGCGGCGTTTTATCATTTACCTCTTGCTCCTGGC
ATGGTTTGGAGGTTCGTGAGTGGTTGAAGGACAGGAGGCTGGACATGACCGAAAGGAACCTACACAGACGCCGCAAAATAGTAAATGGAGAACGAGGACCG
Sense
Antisense
NC040719 DCH Sydney/2016
T
A
Sense
Antisense
MT506043 DCH CP79H_THA/2019
A
T
T
A
Sense
Antisense
LC685967 DCH Rara/Japan
T
A
Sense
Antisense
Consensus
TACCAAACCTCCAAGCACTCACCAACTTCCTGTCCTCCGACCTGTACTGGCTTTCCTTGGATGTGTCTGCGGCGTTTTATCATTTACCTCTTGCTCCTGGC
Conservation
100%
0%

Figure 3

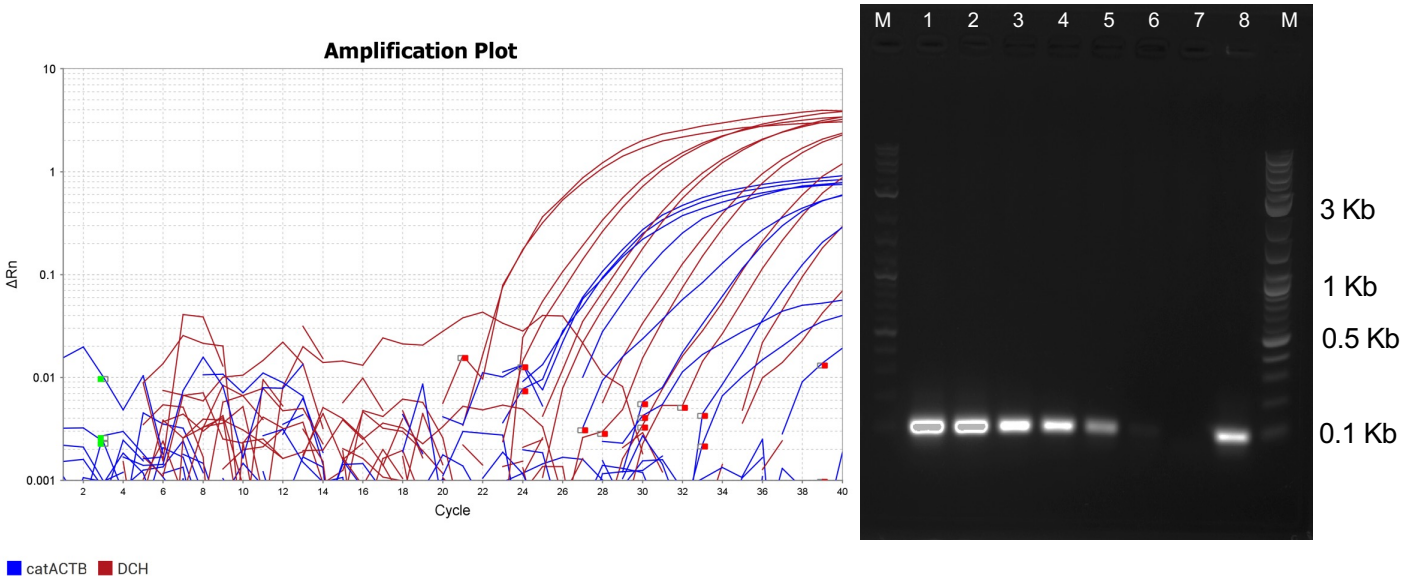

Amplification Plot
ΔRn
Cycle
catACTB
DCH
M 1 2 3 4 5 6 7 8 M
3 Kb
1 Kb
0.5 Kb
0.1 Kb

Figure 4

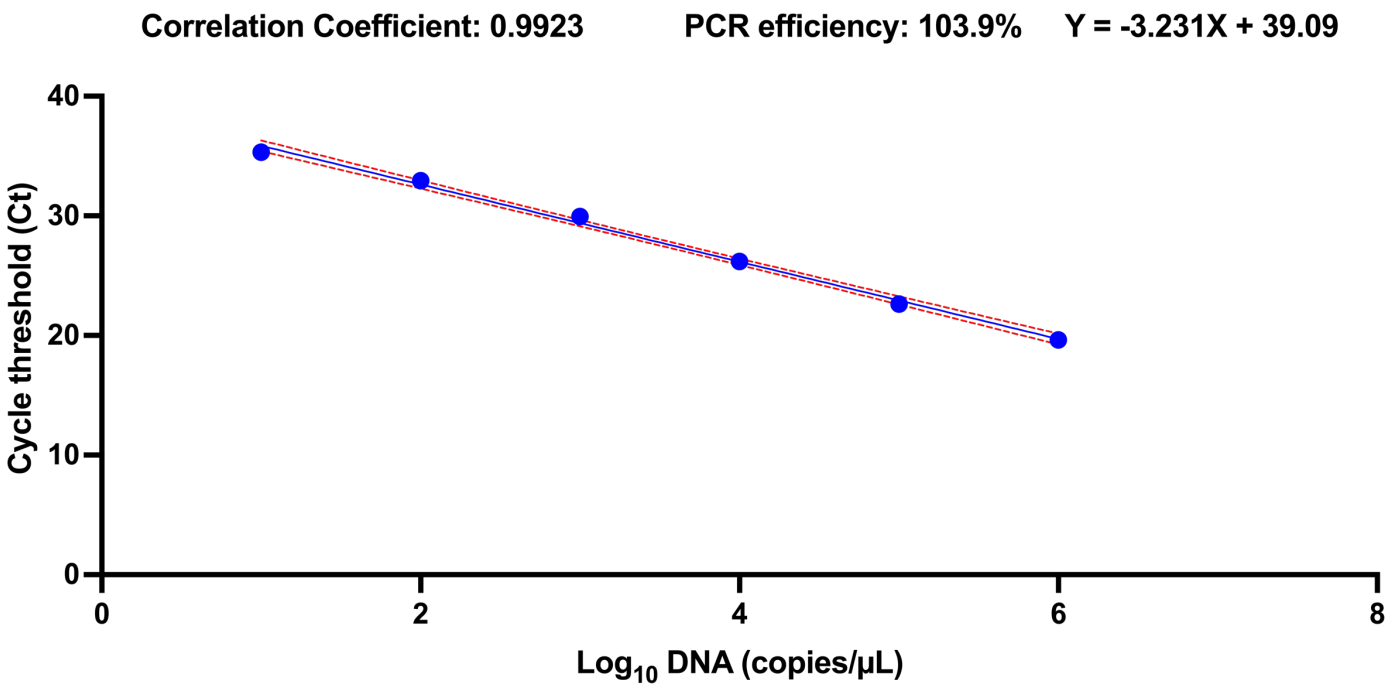
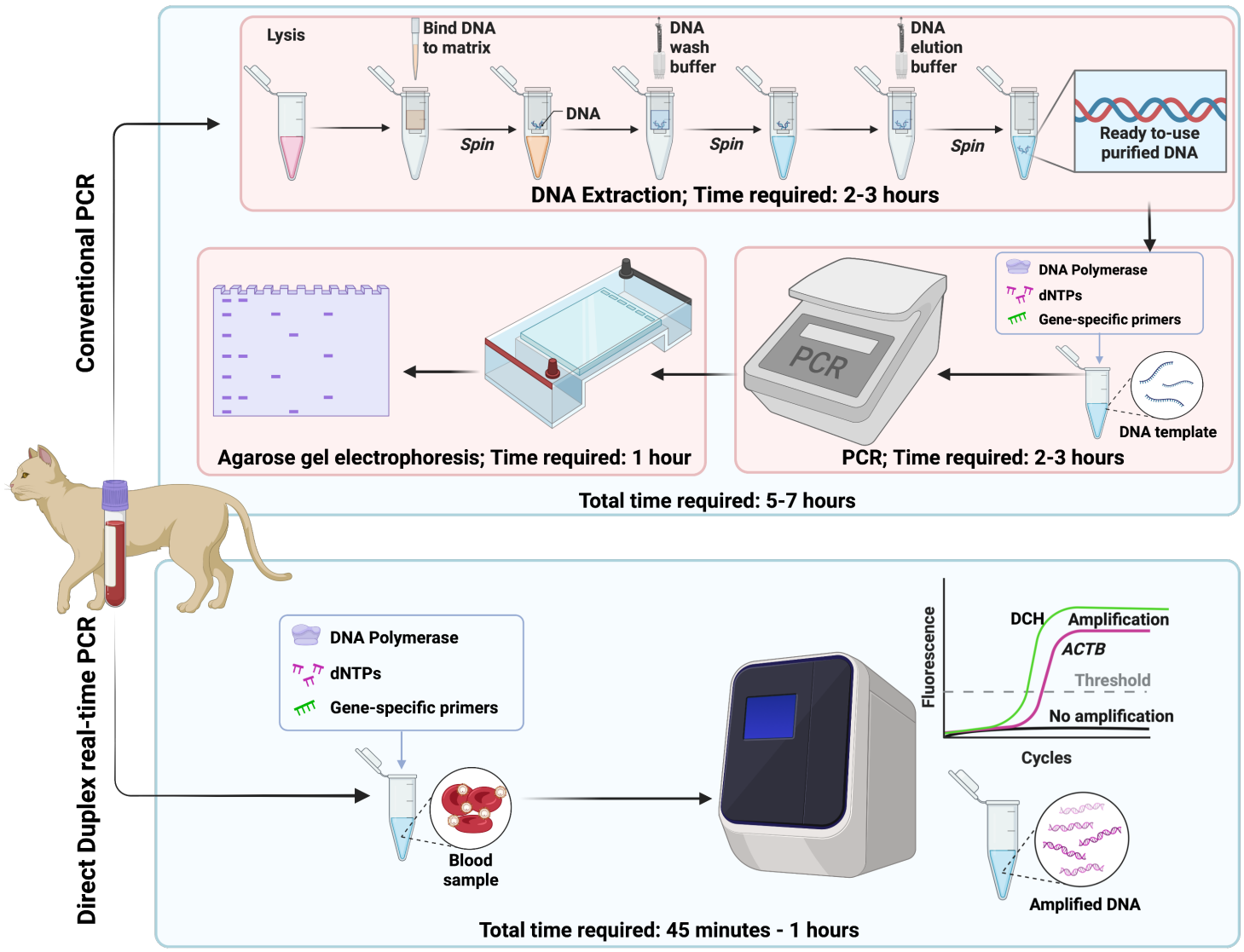

Correlation Coefficient: 0.9923
PCR efficiency: 103.9%
Y = -3.231X + 39.09
Cycle threshold (Ct)
40
30
20
10
0
0
2
4
6
8
Log10 DNA (copies/µL)

Figure 5


Conventional PCR
Lysis
Bind DNA to matrix
Spin
DNA
DNA wash buffer
Spin
DNA elution buffer
Spin
Ready to-use purified DNA
DNA Extraction; Time required: 2-3 hours
DNA Polymerase
dNTPs
Gene-specific primers
PCR
DNA template
PCR; Time required: 2-3 hours
Agarose gel electrophoresis; Time required: 1 hour
Total time required: 5-7 hours
Direct Duplex real-time PCR
DNA Polymerase
dNTPs
Gene-specific primers
Blood sample
Fluorescence
DCH
Amplification
ACTB
Threshold
No amplification
Cycles
Amplified DNA
Total time required: 45 minutes - 1 hours