FAST-LV: CatDX Feline Leukemia Virus (FeLV) Saliva-Based Test - Sensitivity, Specificity and Concordance with Point of Care Detection

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Introduction

Feline Leukemia Virus (FeLV) diagnosis can significantly affect the cat's health and longevity, leading, in some cases, to premature euthanasia. Therefore the detection of FeLV by new and reliable assays may impact overall feline health, adaptability, and long-term survival. Immune-based assays capable of detecting FeLV p27 antigen at the point of care (POC) have long been used as the standard of care for the rapid detection of FeLV. Despite good sensitivity/specificity, these methods occasionally produce false positive or negative results, prompting alternative methods for detecting the virus. Polymerase chain reaction (PCR), used to amplify and detect viral oligonucleotides, has been proposed as a sensitive and specific method for detecting FeLV in domestic cats (1,2). The European Advisory Board on Cat Disease (ABCD) has previously identified saliva as a suitable biological matrix for detecting viral products, including oligonucleotides (3). Here we report developing a sensitive and specific saliva-based PCR reaction for FeLV mRNA using a reverse transcriptase step coupled with FeLV specific primers and probes. RNA integrity and quality were validated by amplifying an independent feline housekeeping gene (HKG). CatDX saliva-based test, commercial name FAST-LV, is designed to provide direct-to-consumer (DTC) access to FeLV detection. CatDX FAST-LV assay was tested on a cohort of positive FeLV cats (IDEXX SNAP Test) as part of a single-arm diagnostic trial. Subsequent testing of random cats with confirmed FeLV from US households showed a 97.5% concordance with the previous results.

Assay Limit of Detection (LOD)

Primers and probes were designed against the FeLV mRNA using the NCBI database. Primer annealing and melting temperatures were determined using IDT tools (4). Synthetic template DNA corresponding to the FeLV region of interest was synthesized and serially diluted to determine assay LOD. PCR amplification of 40 cycles was performed with cycle numbers equal to or higher than 36 designated as negative results. **Figure 1A** depicts a representative PCR run of $9x10^3$, $9x10^2$, 90, and 0 copies of the FeLV template. All PCR reactions were performed in triplicates. **Table I** shows a summary of the results. Mean and SEM demonstrated a high degree of intra-assay reproducibility, even at a very low copy number (9 copies). Based on these results, FeLV assay LOD using primer and probe combination was set at a minimum of 90 copies of FeLV template. The addition of the non-FeLV templates, including FIV, did not produce detectable copy numbers using FeLV-specific primers.

Detection of Native FeLV mRNA

The detection of RNA from FeLV was confirmed using heat-inactivated FeLV virus. Here, the FeLV virus was lysed and RNA extracted. Purified RNA was subjected to reverse transcription. The resulting cDNA was used as

an input template for FeLV PCR reaction. **Figure 1B** demonstrates the detection of native FeLV RNA using CatDX FeLV-specific primers and probes. Standard curve calculation of total copy numbers showed $9x10^6$ mRNA copies in both virus preps.

Testing of CatDX Saliva FAST-LV Test on FeLV Positive Cats

Next, we sought to test CatDX Saliva FAST-LV PCR assay on previously diagnosed FeLV cats. Cat samples were collected from a single cohort housed in a rescue shelter (Missouri, USA). A total of 10 FeLV-positive cats, previously diagnosed by IDEXX SNAP POC testing, were analyzed using CatDX PCR assay. Saliva was collected using a sterilized cotton swab at the shelter. Once saliva was collected, samples were returned to CatDX laboratory via courier and mRNA extracted. cDNA was synthesized using extracted RNA and amplified using FeLV-specific primers and probes. **Table 2** shows a summary of animal demographics, including lapsed time duration from diagnosis. 9 out of 10 FeLV-positive cats produced ΔCt values equal to or less than 36 cycles. Cat age, gender, or breed did not affect result distribution. A separate PCR reaction designed to detect feline housekeeping genes was also done to ensure RNA integrity. All samples showed a high degree of RNA integrity with ΔCt values of 30 cycles or less. Samples 7 through 10 were stored at 4◦C for 60 days to examine the effect of long-term storage on RNA integrity. Amplification of feline HKG produced ΔCt lower than 31.7 cycles, well below the 36 ΔCt cutoff, indicating a high degree of RNA stability following prolonged storage.

Evaluating CatDX Saliva FAST-LV Test on Previously Tested Felines Located from US-households

We examine the concordance of the CatDX Saliva FAST-LV PCR test with previously confirmed positive or negative cats across the USA. Saliva samples from 41 previously tested cats were analyzed using the CatDX FAST-LV PCR test. **Table 3** summarizes the results of the FAST-LV test against predicate POC testing done at local veterinaries. A total of 10 confirmed positive and 31 confirmed negative cats were analyzed. CatDX FAST-LV detected FeLV RNA in all ten positive cats regardless of gender, age, or disease duration. Of the confirmed negative FeLV cats, 30/31 cats tested negative using the FAST-LV PCR test, while one cat (1/31) tested positive. Sensitivity and specificity calculation using a disease prevalence of 3.1% (2) showed at 100% (95% CI 69-100%) and 96.9% (95% CI 83-99%), respectively, supporting the use of test in house cats.

Summary

This report describes the development and use of saliva-based FeLV testing using CatDX Saliva FAST-LV PCR test. LOD, detection of native FeLV mRNA and preliminary testing in FeLV positive cats demonstrate good assay performance and reproducibility. Analysis of previously FeLV tested cats in households across the US further demonstrated the utility of CatDX FeLV test.

References

1) Levy, J., C. Crawford, et al. (2008). 2008 american association of Feline Practitioners' feline retrovirus management guidelines. Journal of Feline medicine & surgery 10 (3): 300-316.
2) Levy JK, scott hm, Lachtara JL, Crawford PC (2006b) seroprevalenceof feline leukemia virus and feline immunodeficiency virus infectionamong cats in North america and risk factors for seropositivity. Journal of the american veterinary medical association 228, 371-376.
3) http://www.abcdcatsvets.org/feline-leukaemia-virus-infection/
4) https://www.idtdna.com/pages/tools/oligoanalyzer

Figure 1: FeLV LOD Assay. A. ΔCT values of serial dilution of FeLV template (9000-9) and no-template control (NTC). B. Amplification of FeLV native mRNA from FeLV virus preparation. Two independent extraction of FeLV mRNA were prepared and cDNA generated by reverse transcription. cDNA was amplified using FeLV specific-primers and probes. VP1- virus preparation 1. VP2- virus preparation 2.

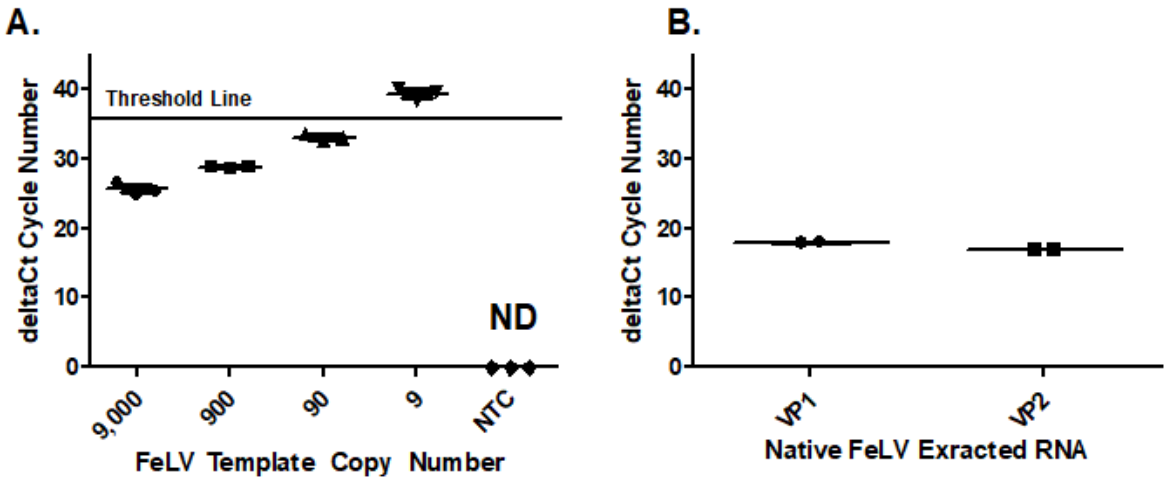

Table 1: FeLV LOD assay. FeLV DNA was synthesized and serially diluted to produce 9000-9 copies and no template control (NTC). FeLV specific-primers and probes were added to the well tougher with amplification buffer. PCR signal was produced on a real-time PCR machine (Bio-Rad). All serial dilutions were done in triplicates. Mean and SEM represent intra-assay results.

	FeLV Gene Copy Number				
	9,000	900	90	9	NTC
Mean	25.6	28.77	33.1	39.4	0
Std. Error	0.4726	0.08819	0.3215	0.5686	0
Lower 95% CI of mean	23.57	28.39	31.72	36.95	0
Upper 95% CI of mean	27.63	29.15	34.48	41.85	0

Table 2: FeLV Detection in Saliva of FeLV positive cats. CatDX saliva-based FeLV FAST-LV test was performed on 10 confirmed FeLV positive animals. Initial diagnosis was performed using IDEXX SNAP POC test. RNA quality was assessed by PCR amplification of feline housekeeping gene. Animal number 4 tested negative using CatDX assay. *samples stored at 4◦C for 60 days. DSH- domestic short hair. SF- Scottish fold

Animal Number	Age (Years)	Gender	Breed	Time since last test (Months)	CatDX ΔCt
1	4	F	DSH	60	23.2
2	2	F	DSH	24	25.6
3	2	F	DSH	15	25.6
4	2	M	DSH	15	**38.1**
5	2	F	DSH	19	22.6
6	4	F	DSH	42	34.5
7*	1.5	F	DSH	12	35
8*	0.5	F	SF	3	25
9*	0.5	M	SF	3	20.6
10*	0.5	F	SF	3	24

Table 3: CatDX Fast-FV Assay Sensitivity and Specificity. CatDX saliva-based FeLV FAST-LV test was performed on 41 confirmed FeLV positive or negative animals. Initial diagnosis was performed at the by a local veterinarian. RNA quality was assessed by PCR amplification of feline housekeeping gene. For sensitivity/specificity analysis a FeLV disease prevalence of 3.1% was assumed based on previous publications (2).

	Reference Test	CatDX FAST-LV	Sensitivity (95% CI)	Specificity (95% CI)
Number of negative cats	31	30	**100% (69-100%)**	**96.88% (83-99%)**
Number of positive cats	10	11		