Development, Optimization, and Validation of Multiplex Real-time RT-PCR Assay for the Simultaneous Detection of BVDV, BTV, and BLV in Cattle.

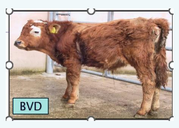
[1]Ditsaporn Attawet, ,, [1]Kiitpong Jaroonkulkij , [1]Nantaporn Wandee [2]Naruebeth Nernthong

1Virology Section, National Institute of Animal Health, Department of Livestock Development
2Animal health management Section, National Institute of Animal Health, Department of Livestock Development

*Corresponding author: Ditsaporn Attawet; E-mail; Ditsaporn1611@gmail.com

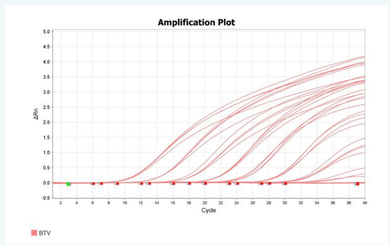
Background and Problem Statement

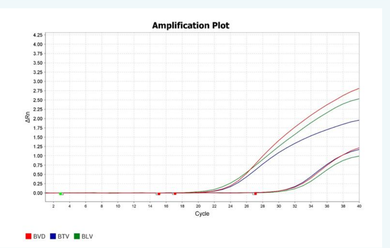
- The National Institute of Animal Health (NIAH) is responsible for safeguarding Thailand's national biosecurity by screening a massive volume of imported live cattle annually—a process crucial for breed development, livestock production, and driving the rural economy. Under the Animal Epidemics Act B.E. 2558 (2015),
- Bovine Viral Diarrhea Virus (BVDV), Bluetongue Virus (BTV), and Bovine Leukemia Virus (BLV) are classified as high-risk transboundary and emerging diseases by the World Organisation for Animal Health (WOAH). Consequently, mandatory official health screening and certification are strictly required prior to any international movement into the country.
- However, the NIAH laboratory currently relies on singleplex real-time PCR and ELISA methods, which require separate testing protocols for each individual pathogen.
- This traditional approach is highly resource-intensive, drives up operational costs, and extends turnaround times. Such inefficiencies create a significant operational bottleneck, hindering the rapid diagnostic screening necessary to prevent immediate biosecurity threats during urgent or high-volume cattle importations.

The Solution: Multiplex Real-time RT-PCR

- **The Solution:** Shifting to a newly developed Multiplex Real-time RT-PCR Assay for simultaneous detection of BVDV, BTV, and BLV within a single reaction.
- **Key Advantages**: Drastically reduces turnaround time for high-throughput screening while cutting laboratory operational costs and saving resources.
- **Standard Compliance:** Validated in strict accordance with the World Organisation for Animal Health (WOAH) standards.
- **Evaluated Parameters:** Assessed for core diagnostic features, including Analytical/Diagnostic Sensitivity and Specificity to ensure strong accuracy and agreement with reference methods.

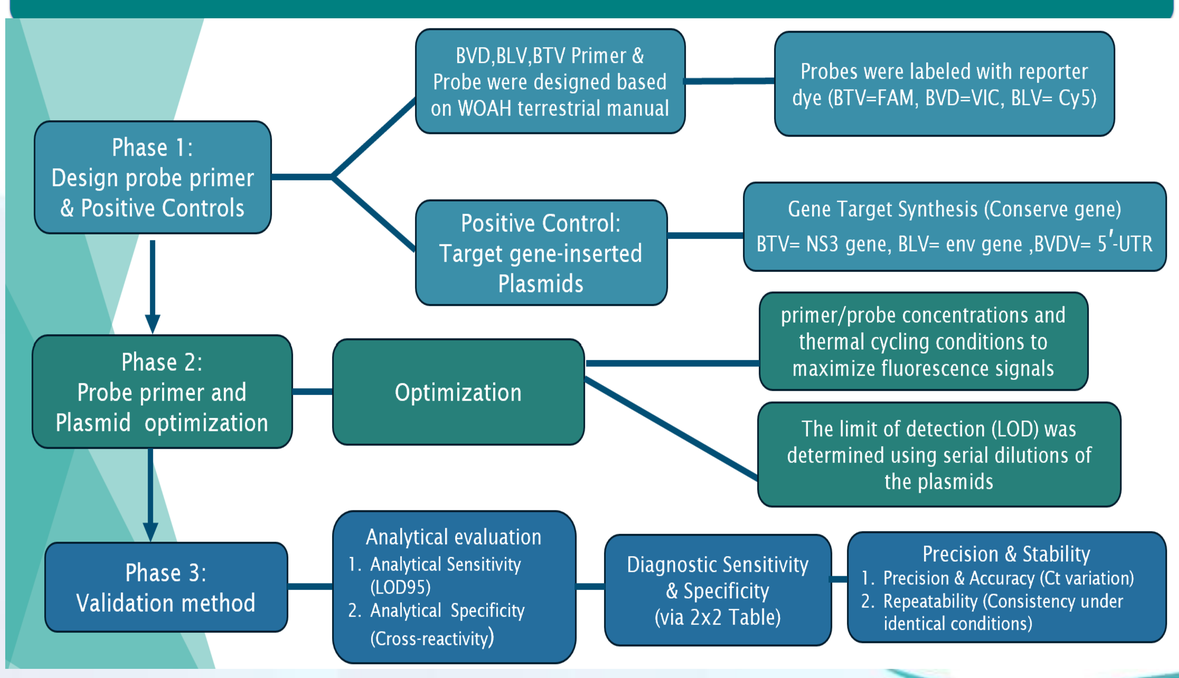




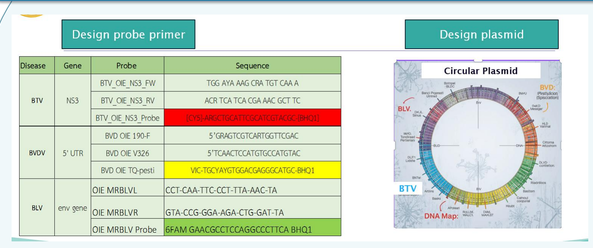
Study objective

This study aimed to develop, optimization and validation a multiplex Real-time RT-PCR assay for the simultaneous detection of BVDV, BTV, and BLV in a single reaction to enhance the efficiency and speed of diagnostic services for imported cattle.

Material and Methods



Results and Discussion



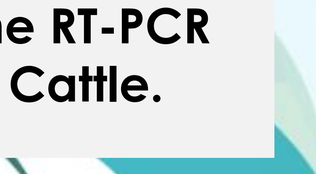
Disease	Gene	Probe	Sequence
BTV	NS3	BTV_OIE_NS3_FW	TGG AYA AAG CPA TGT CAA A
		BTV_OIE_NS3_RV	ACR TCA TCA CGA AAC GCT TC
		BTV_OIE_NS3_Probe	[CY5]-ARCCTGCATTCGCATCGTTCCGC-[BHQ3]
BVDV	5' UTR	BVD OIE 190-F	5'GRAGTCGTCARTGGTTCGAC
		BVD OIE V326	5'TCAACTCCATGTGCCATGTAC
		BVD OIE TQ-pesti	VIC-TGCYAYGTGGACGAGGGCATGC-BHQ1
BLV	env gene	OIE MRBLVL	CCT-CAA-TTC-CCT-TTA-AAC-TA
		OIE MRBLVR	GTA-CCG-GGA-AGA-CTG-GAT-TA
		OIE MRBLV Probe	6FAM GAACGCCTCCAGGGCCCTTCA BHQ1

Optimization

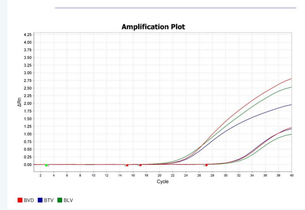
- **Reagent Selection:** Utilized a **qScript ™ XLT One-Step RT-qPCR Master Mix ToughMix®)** for multiplex detection of BVDV (RNA), BTV (RNA), and BLV (DNA).
- **Reverse Transcription Phase:** Converts BVDV and BTV RNA into cDNA during the initial setup.
- **PCR Amplification Phase:** Simultaneously amplifies the co-extracted BLV DNA alongside the newly synthesized cDNA at higher temperatures within a single-tube reaction.

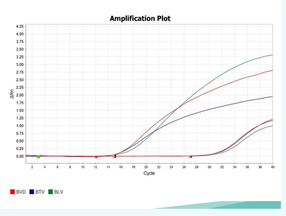
Step	Stage	Cycles	Temperature (C)	Time
Reverse transcription	1	1	50 °C	10 minutes
Pre-amplification	2	1	95 °C	10 minutes
Amplification	3	40	95 °C	15 Seconds
			60 °C	45 Seconds

Amplification Plot of Single Plex



Amplification Plot of Multiplex Real time RT PCR





Limit of detection (LOD)

Limit of Detection (LOD) Determination:
- Standard plasmids of **BVDV, BLV, and BTV** with an initial concentration of 3.206 10^{11} copies/µL were prepared.
- A **10-fold serial dilution** was performed ranging from 10^{-1} to 10^{-10}
- The LOD was defined as the lowest plasmid concentration that still yielded a detectable signal and reported a Cycle Threshold (Ct) value. The LOD obtained was 3.21x 10^5 copies/reaction."

Dilution	Copy number (Copies/reaction)	Magmax Pathogen Ct value			Magmax Microbiome Ct value		
		BVD	BTV	BLV	BVD	BTV	BLV
10^{0}	3.206+10^{11}	10.748	11.135	11.327	10.624	10.573	10.955
10^{-1}	3.206+10^{10}	14.862	15.724	14.258	14.868	14.822	14.321
10^{-2}	3.206+10^{9}	18.372	20.797	18.258	17.735	18.389	18.913
10^{-3}	3.206+10^{8}	22.924	23.299	23.522	21.266	22.288	22.871
10^{-4}	3.206+10^{7}	25.049	27.043	25.345	24.629	25.206	25.145
10^{-5}	3.206+10^{6}	29.346	30.71	29.601	27.666	28.903	28.217
10^{-6}	3.206+10^{5}	33.018	34.618	33.148	32.451	33.012	34.161
10^{-7}	3.206+10^{4}	UND.	UND.	UND.	UND.	UND.	UND.
10^{-8}	3.206+10^{3}	UND.	UND.	UND.	UND.	UND.	UND.
10^{-9}	3.206+10^{2}	UND.	UND.	UND.	UND.	UND.	UND.
10^{-10}	3.206+10^{1}	UND.	UND.	UND.	UND.	UND.	UND.

Validation Sample Panel

A total of 80 samples referenced from the National Institute of Animal Health (NIAH) were included in the evaluation, which consisted of:
- **BVDV reference field strains:** 20 samples
- **BTV reference field strains:** 20 samples
- **BLV reference field strains:** 20 samples
- **Negative control samples :** 20 samples

Nucleic acid extraction was performed using two commercial kits: MagMAX Pathogen and MagMAX Microbiome. The resulting numbers of positive and negative detections were then used to calculate diagnostic parameters according to the following formulas.

		Known samples	
		Positive	Negative
Test result	Positive	True positive (TP)	False positive (FP)
	Negative	False negative (FN)	True negative (TN)

Diagnostic sensitivity (%) = [TP/(TP + FN)] x 100
Diagnostic specificity (%) = [TN/(TN + FP)] x 100
Accuracy (%) = [(TP+TN)/(TP+FP+FN+TN)] x100

Diagnostic sensitivity

Pathogen Group	Matrix	N	MagMag Pathogen	MagMag Microbiome	%Diagnostic sensitivity
BVD (Bovine Viral Diarrhea)	Blood	20	20/20 detected	20/20 detected	100%
BTV (Bluetongue Virus)	Blood	20	20/20 detected	20/20 detected	100%
BLV (Bovine Leukemia Virus)	Blood	20	20/20 detected	20/20 detected	100%
Negative samples	Blood	20	20/20 Not detected	20/20 Not detected	100%
TOTAL SAMPLES	-	80	60/60	60/60	100%

Diagnostic specificity

Pathogen Group	Matrix	N	MagMag Pathogen	MagMag Microbiome	%Diagnostic specificity
Bovine herpesvirus 1 (BoHV-1),	Tissue suspension	10	10/10 Not detected	10/10 Not detected	100%
Bovine Respiratory Syncytial Virus (BRSV)	Tissue suspension	10	10/10 Not detected	10/10 Not detected	100%
Bovine Parainfluenza-3 (PI-3)	Tissue suspension	10	10/10 Not detected	10/10 Not detected	100%
TOTAL SAMPLES	-	30	30/30	30/30	100%

Repeatability and Reproducibility

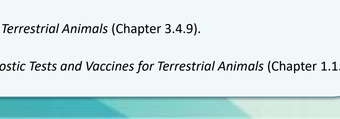
The objective of this test is to compare the within-group repeatability and the inter-operator reproducibility among three participants. Reference Field Strains (BVD, BTV, and BLV—3 samples each, totaling 9 replicates) at a concentration of 10^{-4} were processed using the MagMAX Pathogen and MagMAX Microbiome extraction kits. The test results are detailed in the table below."

Target Strain	Concentration	Mean Ct (Op 1)	Mean Ct (Op 2)	Mean Ct (Op 3)	% CV
BVD (Bovine Viral Diarrhea)	10^{-4}	32.45	32.51	32.48	0.09%
BTV (Bluetongue Virus)	10^{-4}	34.12	34.20	34.15	0.12%
BLV (Bovine Leukemia Virus)	10^{-4}	33.88	33.92	33.85	0.10%

Target Strain	Concentration	Mean Ct (Op 1)	Mean Ct (Op 2)	Mean Ct (Op 3)	% CV
BVD (Bovine Viral Diarrhea)	10^{-4}	33.10	33.25	33.18	0.22%
BTV (Bluetongue Virus)	10^{-4}	35.05	35.12	35.08	0.18%
BLV (Bovine Leukemia Virus)	10^{-4}	34.60	34.75	34.68	0.21%

Conclusion and Next steps

- The validated Multiplex Real-time RT-PCR provides a rapid, cost-effective, and highly reliable diagnostic tool.
- It significantly strengthens the biosecurity measures of NIAH by accelerating the health certification process for imported live cattle and ensuring the effective prevention of transboundary disease outbreaks into Thailand.



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References

- Nilnont, S., CTC, S., Sthitmatee, N., Tsuboi, T., & Sekiguchi, S. (2016). Seroprevalence of bovine viral diarrhea virus and factors associated with the serological status in dairy cattle in western region of Thailand. *Indian Journal of Animal Research*, 50(2), 232–236. https://doi.org/10.18805/ijar.9299
- Rattanapanaddaa, S., Kuo, S. T., & Chang, H. W. (2023). Molecular characterization and phylogenetic analysis of bovine leukemia virus (BLV) in dairy cattle from Thailand. *Veterinary Sciences*, 10(4), 275. https://doi.org/10.3390/vetsci10040275 .
- Thongwatsai, S., et al. (2019). Serotype distribution and genetic diversity of bluetongue virus isolated from cattle and Culicoides biting midges in Thailand. *Acta Tropica*, 191, 108–115. https://doi.org/10.1016/j.actatropica.2018.12.032 .
- World Organisation for Animal Health [WOAH]. (2021). Bluetongue (Infection with bluetongue virus). In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (Chapter 3.1.3). https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.01.03_BLUETONGUE.pdf.
- World Organisation for Animal Health [WOAH]. (2022). Bovine viral diarrhoea. In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (Chapter 3.4.7). https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.04.07_BVD.pdf.
- World Organisation for Animal Health [WOAH]. (2023). Enzootic bovine leukosis. In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (Chapter 3.4.9). https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.04.09_EBL.pdf.
- World Organisation for Animal Health [WOAH]. (2024). Validation of diagnostic tests for infectious diseases. In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (Chapter 1.1.6). https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/1.01.06_VALIDATION.pdf.