APPLICATION NOTE

Streamlined nucleic acid extraction from extended bovine and porcine semen samples with the IndiMag® Pathogen Kit

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Introduction

Pathogen screening of bovine and porcine semen is essential for animal health surveillance, reproductive efficiency, and biosecurity [1,2].

Bovine semen can transmit a range of bacterial, viral, and protozoal pathogens, including *Brucella abortus*, *Campylobacter fetus*, bovine herpesvirus-1 (BHV-1), bovine viral diarrhea virus (BVDV), and *Tritrichomonas foetus*. It has also been implicated in the international spread of *Mycoplasma bovis* (*M. bovis)*, including introduction events in New Zealand [3]. The emergence of highly pathogenic avian influenza A virus in U.S. dairy cattle in 2024, together with the detection of influenza A virus (IAV) in bovine samples, has further highlighted the need for robust molecular surveillance approaches for emerging viral targets in bovine germplasm [4].

Porcine semen may harbor important pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus, classical swine fever virus, African swine fever virus, *Brucella suis*, and *Leptospira* spp.

However, semen represents one of the most challenging matrices for molecular diagnostics due to its viscous, inhibitor-rich composition, including proteins, nucleases, polysaccharides, and lipid-rich seminal plasma. These challenges are further compounded by extender components such as egg yolk, milk proteins, soy lecithin, and antibiotics, which can interfere with nucleic acid extraction and PCR amplification [1,5,6].

As a result, robust extraction workflows are required but are often labor-intensive and variable in performance. Here, we present nucleic acid extraction workflows for pathogen detection in extended bovine semen and evaluate analytical sensitivity in bovine and porcine semen. The workflows are designed to improve extraction performance in inhibitor-rich matrices, reduce PCR inhibition, and enable sensitive and reliable molecular detection, supporting veterinary diagnostics for pathogen surveillance and international trade requirements.

Keywords: *bovine semen, porcine semen, pathogen detection, semen diagnostics, nucleic acid extraction, PCR inhibitor removal, extended semen, NAHLN, veterinary molecular diagnostics, biosecurity*

Materials and methods

Samples and extenders

Negative extended bovine semen samples were grouped by extender composition into four categories: egg yolk-based (Yellow) and three milk-based extenders (Green, Pink, and White). To evaluate PCR inhibition and extraction efficiency, 64 PCR-negative bovine semen samples (*n* = 16 per extender) were processed using the IndiMag Pathogen KF96 Cartridge Kit (INDICAL BIOSCIENCE) and an alternative commercial extraction system (Provider T), applying both default and optimized workflows.

For assessment of diagnostic sensitivity, 31 confirmed pathogen-positive bovine semen samples naturally infected with BVDV (n = 3), BHV-1 (*n* = 21), bluetongue virus (BTV; *n* = 1), epizootic hemorrhagic disease virus (EHDV; *n* = 1), or *M. bovis* (*n* = 5) were processed using the optimized extraction protocols.

Analytical sensitivity was evaluated by extraction in triplicate of ten-fold serial dilutions of *M. bovis* (ATCC strain 25523) spiked into white milk-based bovine extended semen and embryo wash fluid and of PRRSV (field strain) spiked into extended porcine semen and Phosphate-buffered saline (PBS). Embryo wash fluid and PBS served as reference matrices for *M. bovis* and PRRSV, respectively.

Nucleic acid extraction

Two NAHLN-approved extraction kits were evaluated: the IndiMag Pathogen KF96 Cartridge Kit and an alternative commercial extraction system, referred to as Provider T. Both kits were run on a KingFisher™ 96 instrument (Thermo Fisher Scientific Inc.) according to each manufacturer's general instructions, with the modifications below to address semen-associated inhibition.

Both kits facilitate lysis, binding, and purification of total nucleic acids (DNA and RNA) in a single run.

Figure 1: The IndiMag Pathogen KF96 Cartridge Kit with ready-to-use prefilled cartridges.

An exogenous extraction-control RNA (intype IC-RNA, INDICAL BIOSCIENCE) was spiked into the lysis solution of every sample and co-extracted with the target nucleic acids. The intype IC-RNA exogenous extraction control is detected by a dedicated primer/probe set built into the IndiMix® JOE master mix (INDICAL BIOSCIENCE), allowing extraction efficiency and PCR inhibition to be monitored on a separate fluorescent channel, independently of the diagnostic target.

For the IndiMag Pathogen KF96 Cartridge Kit, four input strategies were evaluated:

- 200 µL semen, no modification (IMP 200 µL).
- 100 µL semen + 100 µL PBS to maintain the manufacturer-recommended input volume (IMP 100 µL).
- 100 µL semen + 20 µL proteinase K + 90 µL Buffer ATL (QIAGEN), incubated at 56°C for 10 min with shaking (IMP 100 µL + ATL).
- 75 µL semen + 125 µL PBS, no chemical pretreatment (IMP 75 µL), tested as an additional reduced-input variant.

For Provider T, three protocols were tested: the New Zealand semen import requirement 200 µL input [7] (Provider T 200 µL); a reduced 50 µL input (Provider T 50 µL); and a manufacturer recommended 12.5 µL input combined with proteinase K pretreatment at 60°C for 5 min (Provider T 12.5 µL + PK).

PCR amplification

Extracted nucleic acids were tested with the NAHLN Influenza A virus (IAV) matrix primer/probe set in IndiMix JOE master mix following the manufacturer's instructions. Extraction efficiency was monitored via amplification of the intype IC-RNA control, with successful extraction defined as intype IC-RNA $C_t < 35$.

Confirmed positive bovine semen samples were also analyzed by in-house PCR using VetMAX™ Fast reagents (Thermo Fisher Scientific) and IndiMix JOE reagents (INDICAL BIOSCIENCE) to assess the diagnostic sensitivity of the refined extraction protocols.

For analytical sensitivity, *M. bovis*-spiked samples were evaluated using the lab in-house *M. bovis* assay; PRRSV spiked samples were evaluated using virotype® PRRSV PRO primers/probes with IndiMix JOE according to the manufacturer's instructions. The limit of detection (LOD) was defined as the highest dilution at which all three replicates were detected; partial-replicate detection at the next dilution is reported separately. The R^2 and PCR efficiency were calculated from the resulting dilution series.

Representative application results

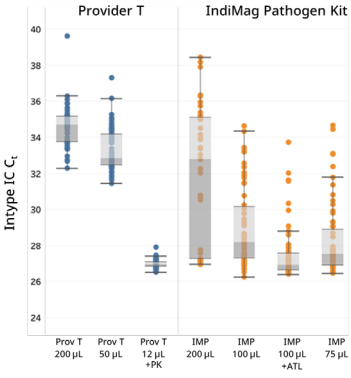
Evaluation of extraction workflows

To evaluate extraction performance, 64 negative milk- or egg-based extended bovine semen samples were spiked with intype IC-RNA exogenous extraction control and processed using standard workflows for Provider T and the IndiMag Pathogen Kit. The initial standard protocols showed limited efficiency in removing PCR inhibitors, with average intype IC-RNA passing rates of 57.8 % for Provider T and 43.8 % for the IndiMag Pathogen Kit. Notably, inhibition varied with the type of semen extender.

For Provider T, satisfactory performance was achieved only after reducing the sample input volume combined with a Proteinase K pre-treatment at 60°C for 5 min. For the IndiMag Pathogen Kit, performance improved when the input volume was reduced to 100 µL. Complete intype IC-RNA recovery (100 %) was achieved either by further reducing the input volume to 75 µL or by applying an ATL buffer pre-treatment at 56°C for 10 min prior to extraction.

Table 1: Passing rates of the intype IC-RNA control in negative extended bovine semen samples across extender types and extraction conditions using Provider T (Prov T) or IndiMag Pathogen Kit (IMP). PBS was used to top up samples to the kit-recommended input volume where required. Graph on the right illustrates C_t variation of intype IC-RNA (IC) between pretreatments

Extender (*n* = 16)	Prov T 200 µL	Prov T 50 µL	Prov T 12.5µL+PK	IMP 200 µL	IMP 100 µL	IMP 100µL +ATL	IMP 75 µL
Milk, Green	56.3 %	100 %	100 %	56.3 %	100 %	100 %	100 %
Milk, Pink	93.8 %	93.8 %	100 %	87.5 %	100 %	100 %	100 %
Milk, White	25.0 %	68.8 %	100 %	0.0 %	75.0 %	100 %	100 %
Egg, Yellow	56.3 %	87.5 %	100 %	31.3 %	100 %	100 %	100 %
Overall (*n* = 64)	57.8 %	87.5 %	100 %	43.8 %	93.8 %	100 %	100 %



Diagnostic sensitivity on naturally infected bovine semen

The optimized extraction workflows were evaluated using 31 semen samples naturally infected with BVDV, BHV-1, BTV, EHDV, or *M. bovis* (Table 2). The IndiMag Pathogen 100 µL workflow detected all pathogen-positive samples, demonstrating 100 % diagnostic sensitivity across the evaluated sample set. In comparison, the optimized Provider T workflow detected 90.3 % of positive samples overall. The intype IC-RNA extraction control passed in 100 % of samples for both workflows, indicating that the observed difference in detection rates was attributable to pathogen recovery rather than residual PCR inhibition. Although the number of BTV- and EHDV-positive samples (*n* = 1 each) was insufficient for target-specific performance assessment, the IndiMag Pathogen 100 µL workflow matched or exceeded the performance of Provider T for the more extensively represented targets, including *M. bovis* and BHV-1. Using 100 µL sample input with the IndiMag Pathogen Kit further reduced C_t values for target pathogens, indicating improved diagnostic sensitivity (Figure 2).

Table 2: Diagnostic sensitivity of naturally infected bovine semen samples using different extraction protocols using Provider T with 12.5 µL of sample and proteinase K treatment and IndiMag Pathogen (IMP) with 100 µL sample. Values indicate the percentage of positive samples correctly detected for each pathogen and the mean detection rate across all tested samples.

Target	*n*	Prov T 12.5 µL + PK	IMP 100 µL
BVDV	3	66.7 %	100 %
BHV-1	21	90.5 %	100 %
BTV	1	100 %	100 %
EHDV	1	100 %	100 %
M. bovis	5	100 %	100 %
Overall	31	90.3 %	100 %

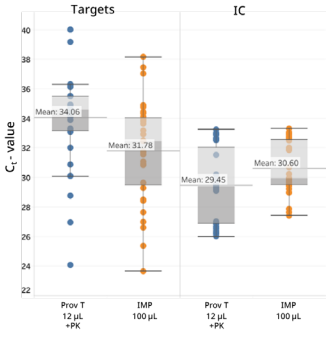


Figure 2: Comparison of extraction performance between Provider T and IndiMag Pathogen protocols. Mean C_t values for targets (34.06 vs 31.78) and intype IC-RNA control (IC; 29.45 vs 30.60), respectively.

Analytical sensitivity

For *M. bovis* ATCC 25523 in white milk–based bovine extender, the IndiMag Pathogen 100 µL workflow achieved an LOD of dilution 6, with two of three replicates remaining positive at dilution 7, effectively matching the dilution 7 endpoint observed in embryo wash fluid. Assay performance was highly precise across both matrices (R^2 = 0.999 – 1.000), with PCR efficiencies ranging from 90.4 % – 94.1 %. Inclusion of the ATL + heat pretreatment reduced analytical sensitivity, shifting the LOD to dilution 5 and lowering PCR efficiency to 71.3 – 92.8 %. These findings indicate that the pretreatment step negatively affected detection of low-abundance spiked *M. bovis* and did not provide a performance advantage under the conditions evaluated.

For PRRSV field strain in extended porcine semen, the IndiMag Pathogen 100 µL workflow achieved an LOD of dilution 6, matching the performance observed in PBS. Excellent assay precision was observed in both matrices (R^2 = 0.999–1.000), with PCR efficiencies ranging from 98.3 % to 107.1 %. These results demonstrate that the semen matrix did not adversely affect PRRSV detection and that the workflow maintained sensitivity and amplification performance comparable to the reference matrices (Table 3).

Table 3: Analytical sensitivity (LOD, R^2, PCR efficiency) for *M. bovis* (bovine semen) and PRRSV (porcine semen) with the IndiMag Pathogen Kit.

Parameter	*M. bovis* embryo wash fluid	*M. bovis* bovine milk extender	*M. bovis* bovine milk extender	PRRSV PBS	PRRSV porcine semen ext.
Protocol	IMP 100 µL	IMP 100 µL	IMP 100µL + ATL	IMP 100 µL	IMP 100 µL
LOD (all reps)	Dilution 7	Dilution 6[1]	Dilution 5	Dilution 6	Dilution 6
R^2	0.996	0.999 – 1.000	0.981 – 0.994	1.000	0.999 – 1.000
Efficiency (%)	90.5	90.4 – 94.1	71.3 – 92.8	98.3	106.1 –107.1

[1] Two of three replicates also amplified at dilution 7; reported as dilution 6 under the all-three-replicates LOD criterion.

Conclusion

Semen is among the most inhibitor-rich matrices in veterinary molecular diagnostics, and standard extraction workflows were not sufficient for the higher input volume of 200 µL without modification. The IndiMag Pathogen Kit improved extraction performance under the evaluated conditions with a minimal adjustment by reducing input volume to 100 µL, resulting in improved diagnostic sensitivity, with 100 % detection observed across 31 naturally infected samples evaluated for both RNA and DNA targets.

An optional Buffer ATL + heat pretreatment further improved inhibitor tolerance but reduced performance for low-abundance bacterial DNA detection (e.g., *M. bovis*), supporting its use as an optional rather than default step. Overall, the IndiMag Pathogen Kit provides a robust, sensitive, and flexible workflow for nucleic acid extraction from milk- and egg-based extended bovine semen, supporting reliable pathogen surveillance and germplasm testing. Independent NAHLN-aligned evaluation is described in the companion study [8].

Data origin and relationship to published work

The data presented in this application note are derived from broader fee-for-service activities between the Wisconsin Veterinary Diagnostic Laboratory, University of Wisconsin–Madison, and INDICAL Inc., and were previously presented at a scientific conference [9]. These independent experiments used the intype IC-RNA internal control.
A separate, larger study from the same collaboration [8] used a different sample set and internal controls, so the results are not directly comparable. The results presented here extend these findings with additional evaluations supporting the described workflow recommendations.

UW–Madison's involvement is limited to the provision of laboratory services and data generation and does not constitute endorsement, recommendation, validation, or certification of any commercial product or manufacturer.

References

1. Givens, M.D. Review: Risks of disease transmission through semen in cattle. Animal 2018, 12, s165–s171. doi:10.1017/S1751731118000708.
2. Haapala, V.; Pohjanvirta, T.; Vähänikkilä, N.; Halkilahti, J.; Simonen, H.; Pelkonen, S.; Soveri, T.; Simojoki, H.; Autio, T. Semen as a source of *Mycoplasma bovis* mastitis in dairy herds. Vet. Microbiol. 2018, 216, 60–66.
3. Jordan, A.; Sadler, R.J.; Sawford, K.; van Andel, M.; Ward, M.; Cowled, B. *Mycoplasma bovis* outbreak in New Zealand cattle: An assessment of transmission trends using surveillance data. Transbound. Emerg. Dis. 2021, 68, 3381–3395.
4. Lim, A.; Poulsen, K.; Caserta, L.C.; Guan, L.; Opgenorth, E.; Beal, M.P.; Eisfeld, A.J.; Kawaoka, Y.; Diel, D.G. Unexpected Detection of Highly Pathogenic Avian Influenza (HPAI) H5N1 virus in bovine semen from a bull used for natural breeding on an affected dairy farm. *bioRxiv* 2025.
5. Samanta, L.; Parida, R.; Dias, T.R.; Agarwal, A. The enigmatic seminal plasma: A proteomics insight from ejaculation to fertilization. Reprod. Biol. Endocrinol. 2018, 16, 41.
6. Gautier, C.; Aurich, C. "Fine feathers make fine birds" — The mammalian sperm plasma membrane lipid composition and effects on assisted reproduction. Anim. Reprod. Sci. 2022, 246, 106884.[AL6.1]
7. Jaramillo, D.; Foxwell, J.; Burrows, L.; Snell, A. *Mycoplasma bovis* testing for the screening of semen imported into New Zealand. N. Z. Vet. J. 2023, 71, 200–208.
8. Zimmerman, A.; Vandenburg-Carroll, A.; Marthaler, D.G.; Lim, A. Optimizing Nucleic Acid Extraction from Extended Bovine Semen for Endemic and High-Consequence Pathogens. Animals 2025, 15, 3411. doi:10.3390/ani15233411.
9. Lim, A.; Marthaler, D. Optimized Nucleic Acid Extraction Protocols for Detecting Avian Influenza A Virus in Extended Bovine Semen. Poster presented at the 21st International Symposium of the World Association of Veterinary Laboratory Diagnosticians (ISWAVLD); June 2025; Calgary, Alberta, Canada

The handbooks and supporting documentation used in this study, including the IndiMag Pathogen Kit Handbook, the intype IC-RNA documentation, the virotype PRRSV PRO Kit documentation, and the IndiMix JOE information, are available from INDICAL BIOSCIENCE. Visit shop.indical.com for these and related protocols, plus a complete overview of our nucleic acid extraction kits, detection assays, and instruments. Your local INDICAL BIOSCIENCE representative is also happy to help.

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