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# Effect of semen preparation on casa motility results in cryopreserved bull spermatozoa

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**Abstract**

Computer-assisted sperm analyzers (CASA) have become the standard tool for evaluating sperm motility and kinetic patterns because they provide objective data for thousands of sperm tracks. However, these devices are not ready-to-use and standardization of analytical practices is a fundamental requirement. In this study, we evaluated the effects of some settings, such as frame rate and frames per field, chamber and time of analysis, and samples preparations, including thawing temperature, sperm sample concentration, and media used for dilution, on the kinetic results of bovine frozen-thawed semen using a CASA. In Experiment 1, the frame rate (30–60 frame/s) significantly affected motility parameters, whereas the number of frames per field (30 or 45) did not seem to affect sperm kinetics. In Experiment 2, the thawing protocol affects sperm motility and kinetic parameters. Sperm sample concentration significantly limited the opportunity to perform the analysis and the kinetic results. A concentration of 100 and $50 \times 10^6$ sperm/mL limited the device's ability to perform the analysis or gave wrong results, whereas 5, 10, 20, and $30 \times 10^6$ sperm/mL concentrations allowed the analysis to be performed, but with different results (Experiment 3). The medium used for the dilution of the sample, which is fundamental for a correct sperm head detection, affects sperm motility results (Experiment 4). In this study, Makler and Leja chambers were used to perform the semen analysis with CASA devices. The chamber used significantly affected motility results (Experiment 5). The time between chamber loading and analysis affected sperm velocities, regardless of chamber used. Based on results recorded in this study, we propose that the CASA evaluation of motility of bovine frozen-thawed semen using Hamilton-Thorne IVOS 12.3 should be performed using a frame rate of 60 frame/s and 30 frames per field. Semen should be diluted at least at $20 \times 10^6$ sperm/mL using PBS. Furthermore, it is necessary to consider the type of chamber used and perform the analysis within 1 or 2 min, regardless of the chamber used.


*Keywords:* Bull; Frozen-thawed semen; Motility; CASA

## 1. Introduction

Conventional methods for semen analysis, including semen motility evaluation, are subjective. Massal motility and grading of the forward progression of sperm are common in routine semen analysis in most andrology clinics [1]. World Health Organization (WHO) guidelines for human semen analysis suggest four grades of motility: (A) fast progressive; (B), slow progressive; (C), motile but not progressive; (D), immotile [2]. Because such evaluations can be performed anywhere and are less expensive, they have, unfortunately, led to results with wide discrepancies between the lab-

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oratories and the technicians performing them. Variations of 30–60% have been reported in subjective microscopic evaluations of human and animal semen in the same ejaculates [3,4,5,6,7,8]. Despite a close match between subjective and objective evaluations of sperm motility [9,10], subjective estimation of motility is affected by numerous factors [11,12,13].

The development of computer-assisted semen analysis (CASA), using software that analyzes and records every sperm track characteristic, has strongly improved the semen evaluation. The availability of data recorded by CASA facilitates the comparison of results and makes it possible to find subtle differences between males or treatments [11]. Furthermore, CASA systems appear to have high accuracy and repeatability [14,15]. However, CASA are not ready-to-use devices, thus results depend largely on the expertise of the user [16,17] and the technical settings [12,18]. Although CASA systems are based on similar principles, they differ in terms of optics and hardware characteristics, as well as algorithms for sperm identification and trajectory reconstruction [19]. Numerous variables (e.g., the frequency of frame acquisition, the number of fields analyzed, sample concentration and dilution, and analysis chamber) can affect motility results in canine semen evaluation even with the same CASA device [12]. The ESHRE Andrology Special Interest Group provided guidelines for the use of CASA technology in sperm analysis, underlining the need for standardization and quality control [20].

Motility is one of the most important parameters used for sperm quality evaluation in both raw and cryopreserved semen. The evaluation of sperm motility provides important information on the energy status of mammalian sperm [21,22]. Furthermore, the motility function can play an important role once spermatozoa reach the uterotubal junction, which contains mucus [23,24] and might act as barrier to sperm with poor motility [25].

Some studies reported procedures for the evaluation of bovine fresh semen [26,27]. However, despite the economic importance of cryopreserved semen for bovine reproduction, specific CASA settings for bovine frozen-thawed semen were not reported. The aim of this study was to evaluate and quantify the effect of certain CASA settings (frame rate, frames per field, chamber, time of analysis) and semen preparation parameters (thawing temperature, extender and sample concentration) on motility results of cryopreserved bull semen using Hamilton-Thorne IVOS 12.3. A worldwide accepted procedure for frozen-thawed bovine semen evaluation using CASA possibly resulted in the more ready and direct comparison of data, with a remarkable advance in semen cryopreservation techniques. In this study, we suggest some recommendations for frozen-thawed bovine semen evaluation using CASA systems.

## 2. Materials and methods

### 2.1. Animals

This study was performed on 10 Swiss Brown bulls belonging to Superbrown Consorzium Bz/Tn (2–7 yr old) and used in regular artificial insemination (AI) service. The bulls were housed in the Alpenseme AI Center of the Provincial Breeders Federation of Trento (Ton, Trento, Italy).

### 2.2. Semen collection and freezing

Semen was collected using an artificial vagina and evaluated. Volume was read from the graded collection tube soon after collection, concentration was determined using Accucell photometer (IMV Technologie, L'Aigle, France), progressive motility was evaluated subjectively (200 × magnification) at 37°C by phase contrast microscopy, and morphology was evaluated using phase contrast microscopy (magnification: × 1000) after fixation with 0.9% NaCl solution with 3% glutharaldehyde. Ten ejaculates, one for each bull, with progressive motility ≥60% and normal morphology ≥80%, were frozen. Semen was diluted with Bioexcell (IMV Technologies) at $100 \times 10^6$ spermatozoa/mL, packaged in 0.25 mL straws, and frozen with a programmable nitrogen freezer (Digicool 5300, IMV Technologies) [28]; the straws were stored in liquid nitrogen until laboratory evaluations were conducted.

### 2.3. Sperm motility evaluation

Frozen-thawed bovine semen was evaluated for motility parameters using a CASA IVOS 12.3 (Hamilton-Thorne Bioscience, Beverly, MA, USA). To reduce the possible variability made by different technicians, semen evaluation in all experiments was conducted by the same operator, with a specific training on the use of this CASA system. The chamber used in all experiments was a Makler chamber (Sefi Medical Instruments, Haifa, Israel), with the exception of Experiment 4, in which both Makler and Leja 4 chamber slide (Leja, Nieuw-Vennep, The Netherlands) were used. After gentle mixing, 10 μl of diluted semen was dropped on the center of the Makler chamber and the coverslip was