ISSN: 3048-8397

Bio Vet Innovator Magazine
(Fueling The Future of Science…)
Volume 3 (Issue 8) AUGUST 2026

OPEN ACCESS | World Mosquito Day – 20th August 2026 | Popular Article

TLR 7/8 Mediated Sperm Sorting Beyond Conventional Sexed Semen

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DOI: https://doi.org/10.5281/zenodo.22230685

Received: August 24, 2026
Published: August 27, 2026

Abstract:

Sexed semen technology is an important reproductive tool for improving the efficiency, profitability, and sustainability of dairy production by increasing the probability of producing calves of the desired sex. Conventional methods, including density gradients, swim-up, albumin gradients, electrophoresis, and flow cytometry, have shown varying degrees of success but are often constrained by low sorting efficiency, high cost, specialized equipment, and reduced fertility. Recent advances in molecular approaches have identified Toll-like receptors 7 and 8 (TLR7/8) as potential markers for X-bearing spermatozoa. Activation of these receptors using Resiquimod (R848) can selectively reduce sperm motility and enrich specific sperm populations while maintaining sperm viability and acrosomal integrity. Recent studies in cattle and other species demonstrate promising sex-enrichment outcomes, including approximately 78% Y-bearing sperm in Sahiwal bulls. Resiquimod-based sperm enrichment therefore represents a simple, economical, and potentially practical alternative to conventional sex-sorting methods for controlled breeding, in vitro fertilization, and embryo transfer.

Keywords: TLR7/8, sex-sorted semen, Sahiwal, Resiquimod, Immunofluorescence

Introduction:

The sex of a calf has a major influence on the profitability and sustainability of dairy farming. Female calves are highly valued because they become future milk-producing cows and serve as replacements for expanding and improving the herd, whereas male dairy calves often have lower economic value (De Vries et al., 2008). Conventional artificial insemination produces nearly equal numbers of male and female calves, offering no control over the offspring's sex. Sex-sorted semen addresses this limitation by increasing the probability of producing the desired sex, with an accuracy of about 90%.

The targeted use of sex-sorted semen in genetically superior females accelerates genetic

improvement, increases the availability of replacement heifers, and enables farmers to breed genetically inferior cows with beef semen to produce higher-value crossbred calves (Murphy et al., 2016). Producing more female calves through the use of sex-sorted semen offers several important advantages (Fig. 1) beyond herd replacement. Female calves are generally smaller at birth than male calves, reducing the risk of difficult calving (dystocia), particularly in heifers. Lower incidences of dystocia improve the health and welfare of both the cow and the newborn calf by decreasing the occurrence of retained placenta, uterine infections, delayed return to estrus, and calf mortality (Norman et al., 2010). Furthermore, reducing the number of low-value male dairy calves addresses important animal welfare concerns and minimizes ethical issues associated with their disposal or poor management.

Figure 1: Advantages of Sex sorted semen

Conventional Methods of Semen Sex Sorting:

Several conventional methods have been investigated for enriching X- or Y-chromosome-bearing spermatozoa based on differences in density, size, motility, surface charge, antigen expression, and DNA content.

- **Percoll Density Gradient:** This method separates sperm based on differences in their density. X-bearing sperm contain slightly more DNA, making them slightly denser than Y-bearing sperm, so they tend to move deeper into the Percoll gradient. However, the level of X- or Y-sperm enrichment was generally low. Percoll separation markedly enhanced progressive motility and sperm viability while reducing morphological abnormalities compared with the unsorted semen sample (Biswas et al., 2026)
- **Volumetric Difference:** The method based on the small difference in the size of sperm heads. X-bearing sperm have slightly more DNA, so their heads are slightly larger. Interference or DIC microscopy can detect this difference. The method could theoretically achieve only about 80% sorting efficiency (Van Munster, 2002).

- **Free-Flow Electrophoresis:** Separation is based on proposed differences in sperm surface electrical charge. X- and Y-bearing sperm are expected to move differently when exposed to an electric field. However, the results have not been consistent, which has limited the practical use of this method (Singh et al., 2019).
- **Swim-Up:** The Y-bearing sperm move faster than X-bearing sperm. During the procedure, the faster sperm are expected to swim into the upper layer of the medium. About 81% male offspring were reported using this method (Check et al., 1989). However, later studies found no clear difference in the movement of X- and Y-bearing sperm.
- **Albumin Gradient:** In this method, sperm move through a bovine serum albumin (BSA) gradient. Y-bearing sperm were thought to move faster and reach the lower part of the gradient more quickly than X-bearing sperm. The reported success rate was about 75%, but different studies showed inconsistent results (Beernink et al., 1993).
- **H-Y Antigen Method:** This method uses antibodies against the H-Y antigen to identify and separate Y-bearing sperm through affinity chromatography or magnetic beads. Although >90% efficacy was reported, H-Y antigen occurs on both X- and Y-bearing sperm, making the method unreliable for sex sorting (Blecher et al., 1999).
- **Flow Cytometry:** The method uses Hoechst 33342 dye to stain sperm DNA and detect the small difference in DNA content between X- and Y-bearing sperm. The sperm are then separated based on their fluorescence signals, producing enriched X- or Y-sperm populations. It is considered the most reliable conventional method, but it requires specialized equipment and may affect sperm quality (Garner, 2009; Vishwanath & Moreno, 2018).

Advantages and Limitations of Sexed Semen:

Sexed semen offers several advantages in livestock production by enabling the production of calves of the desired sex, thereby improving herd management and accelerating genetic progress. It facilitates rapid herd expansion with reduced disease risk, increases selection intensity by allowing the use of genetically superior females, and supports the production of superior replacement heifers. It can also reduce dystocia, particularly by limiting the birth of male calves in dairy herds, and may lower the costs associated with progeny testing and selective culling. However, sexed semen has certain limitations, including higher cost, lower sperm concentration, reduced conception rates, and greater susceptibility of sperm to damage during the sorting process. Additional limitations include low sorting efficiency and speed, the need for standardized protocols, limited availability of elite indigenous bulls, and the requirement for highly skilled technicians for successful artificial insemination.

TLR 7/8 Mediated Sperm Sorting:

Recent studies have demonstrated that Toll-like receptors 7 and 8 (TLR7/8) can be selectively

detected in X-bearing spermatozoa, with TLR7 predominantly localized in the sperm tail and TLR8 in the midpiece. In contrast, these receptors are absent from Y-bearing sperm, providing a potential basis for the selective separation of X- and Y-spermatozoa (Umehara et al., 2019, 2020).

Activation of TLR7/8 using agonists such as Resiquimod (R848) has been shown to differentially affect X- and Y-bearing spermatozoa. R848 treatment reduced sperm motility and resulted in relative enrichment of X-sperm. TLR7/8 expression has been detected in epididymal and testicular sperm cells as well as Leydig cells, whereas only weak expression was observed in Sertoli cells. Following treatment with R848, the proportion of X-sperm in the swim-up fraction decreased, while the proportion of Y-sperm increased, without compromising sperm viability or acrosomal integrity. These findings suggest that TLR7/8-mediated sperm selection may provide an effective approach for separating X- and Y-bearing sperm in species such as goats (Ren et al., 2021), mice (Umehara et al., 2019) and cattle (Wan & Lenardo, 2010) while preserving sperm quality and viability.

The decrease in sperm motility induced by R848, is associated with the inhibition of hexokinase activity and mitochondrial function in spermatozoa. Notably, this reduction in motility is reversible once R848 is removed from the culture medium, indicating that the effects of TLR7/8 activation are transient (Umehara et al., 2019).

Immunofluorescence analysis confirmed the expression of TLR7/8 receptors in *Sahiwal* bull spermatozoa, with their localization observed in the anterior region of the sperm head, acrosome, and tail. Following the separation procedure, the Y-enriched fraction contained approximately 78.18% Y-bearing spermatozoa, demonstrating the effectiveness of the method in altering the sperm sex ratio toward males (Nistane et al., 2026).

Conclusion:

Semen sexing technology enables the production of calves of the desired sex, helping reduce unwanted male calves and improving breeding efficiency. Although conventional sorting methods are limited by cost, processing speed, and reduced fertility. Resiquimod-based sperm enrichment offers a simpler and more economical alternative. It requires minimal technical expertise, fewer personnel, and less processing time, while providing satisfactory enrichment of Y-bearing spermatozoa for controlled breeding, IVF, and embryo transfer.

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