Technical note: Droplet digital PCR precisely and accurately quantifies sex skew in bovine semen

Nicole Cray,¹ Matthias Wagner,² Jami Hauer,¹ and Elon Roti Roti^{1*}

¹GenusPlc, ABS Global, 6308 Pepsi Way, Windsor, WI 53598

²Cellino Biotech Inc., 14 Mellen St., Cambridge, MA 02138

ABSTRACT

Quantifying the relative population of sperm cells bearing the X or Y chromosome in a sexed-semen sample has historically been limited to methods that are either low throughput and sensitive to user subjectivity (e.g., fluorescence in situ hybridization), conterminous (using the same technology to generate and confirm the sex skew), or relatively insensitive (including quantitative PCR with a change detection threshold of 2 $\times$). Customers pay a premium for sexed semen and should have access to reliable sex skew data, generated by an accurate, precise test that is orthogonal to the method used to generate sexed semen. Droplet digital PCR (ddPCR) has the capacity to provide an accurate and precise sex skew quantitation by subdividing a pool of template DNA into nanoliter-scale droplets containing either 1 or 0 copies of template DNA. Then PCR amplification is conducted in the droplets, and the number of copies of the amplicon of interest can be counted as the number of fluorescence-positive droplets based on classic quantitative PCR fluorescent reporters. We have optimized and validated a multiplexed ddPCR assay that uses this copy counting method to quantify the sex skew (ratio of X or Y chromosomes) in frozen-thawed bovine sexed semen. The assay interrogates at least 1,000 cells per sample well, quantifying X and Y chromosome copy numbers along with an autosomal gene, *GAPDH*, used as an internal assay control to confirm total cells counted. The ddPCR sex skew assay achieved a 0.5-percentage-point variance for %X or %Y with a broad linear detection range, from 10 to 95% X, and provided reproducible skew values across a range of 9 to 27 ng of genomic DNA input. This approach overcomes some limitations of other sex skew assays by quantifying absolute X and Y chromosome copy numbers, thus providing a rigorous, independent assessment of sex-skewed semen.

Key words: sexed semen, dairy, droplet digital PCR (ddPCR)

Technical Note

Dairy farmers increase profitability by utilizing X-enriched or “skewed” sexed semen from sires with desired genetic traits to maintain a high-quality milk-producing herd. The utility of sexed semen depends upon the percentage of X- and Y-chromosome-bearing sperm (“female” or “male” cells) present in the product, which in turn depends on the accuracy of both making sexed semen and verifying the final sex skew. To produce commercial sexed semen, sperm are stained with Hoechst 33342, which binds stoichiometrically to DNA (Lalande and Miller, 1979) and emits a fluorescent signal, which allows cytometers to discriminate X- and Y-chromosome-containing sperm based on the ~4% difference in total DNA content between the X and Y chromosomes (Moruzzi, 1979; Johnson, 1995; van Munster et al., 1999). Sperm may then be segregated into separate containers based on sex (Johnson and Welch, 1999) or the cells of the undesired sex are laser-ablated (Faust et al., 2016). Given the inherent challenges of separating 2 cell populations based on a 4% DNA content difference (Seidel, 2014), the maximum achievable sex skew is variable and depends upon how well the sperm are stained, how well they are aligned in the optical detection plane, and on the sperm flow rate through the cytometer (Johnson and Welch, 1999). Field performance indicates that X-skewed sexed-semen inseminations produce 86 to 93% female calves (Cerchiaro et al., 2007; Borchersen and Peacock, 2009; Norman et al., 2010; Healy et al., 2013), likely reflecting the inherent variance of X-skewed sexed semen.

Bovine semen sex skew is commonly determined by requantification using the same cytometers used to manufacture the sexed semen (Seidel, 2014), though available orthogonal methods include fluorescence in situ hybridization (**FISH**) and quantitative PCR (**qPCR**). Each approach has advantages and limitations. Semen-sexing cytometers quantify sex skew using

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*Corresponding author: elon.rotiroti@genusplc.com

the same DNA-binding dye and detection scheme that originally generated the sexed semen, rather than an independent test; the skew data are therefore subject to the same variations or biases as manufacturing the sex-skewed product and are limited by the lack of unique, positive identifiers for the X and Y chromosomes. The FISH and qPCR approaches both specifically identify X and Y chromosomes (Kawarasaki et al., 1998; Rens et al., 2001; Habermann et al., 2005; Parati et al., 2006; Puglisi et al., 2006; Khamlor et al., 2014), but lack quantitative sensitivity and statistical rigor due to sampling (FISH) and sensitivity (qPCR) limitations. In an effort to overcome these challenges, we present methods for a droplet digital PCR (**ddPCR**) sex skew assay that quantifies total X and Y chromosome copies, reporting sex skew as a percentage with a variance of ± 0.5 percentage point. The numbers of X and Y chromosome copies are quantified independently in a multiplex reaction, along with an autosomal amplicon to confirm the total number of cells represented in the assay as an internal assay control. The methods have been filed for patent and can be found at <http://appft.uspto.gov/netacgi/nph-Parser?Sect1=PTO2&Sect2=HITOFF&p=1&u=%2Fnetacgi%2FPTO%2Fsearch-bool.html&r=1&f=G&l=50&co1=AND&d=PG01&s1=%2216%2F121,077%22&OS=%2216/121,077%22&RS=%2216/121,077%22> (Roti Roti et al., 2018).

Assay development began with screening primer combinations via standard PCR, followed by assessing reproducibility in qPCR, and ultimately testing quantitative reproducibility in ddPCR, as summarized in Figure 1. To develop a robust sex skew assay, intron/exon spanning primer pairs were designed based on the Bos_taurus_UMD_3.1.1 Assembly (https://www.ncbi.nlm.nih.gov/assembly/GCF_000003055.6/). Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-17877>) details the primer pairs screened, including amplicons on the X and Y chromosomes, along with several autosomal genes targeted based on published qPCR data. Primer combinations that consistently generated PCR product of the predicted molecular weight across genomic DNA samples from 16 unique sires, including Holstein, Jersey, and Angus breeds (data not shown), were evaluated for efficiency and reproducibility with their respective probes using qPCR. Primer combinations that failed even once to produce robust bands of the predicted molecular weight from all sires tested were eliminated from further assay development, because an assay designed for screening a commercial product must be insensitive to genetic variation inherent in any population. These primer combinations included HMBS, PRLPO, TBP, X₁, X₃, and Y₅.

The next criteria to develop primers and probes for the ddPCR sex skew assay required each primer/probe

set to produce a qPCR cycle threshold (**CT**) value (the number of thermocycles it takes for the fluorescent signal to become greater than the background threshold) between 15 and 35, with a variance of <0.5 CT, as expected for a high-abundance gene. Probes used Taqman chemistry (Heid et al., 1996) with either FAM or HEX fluorescent tags and are listed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-17877>). A 20- μ L qPCR reaction contained 10 μ L of Bullseye TaqProbe qPCR MasterMix (MidSci, St. Louis, MO), 1 μ L of each 10 μ M forward and reverse primer, 1.5 μ L of 2 μ M probe, and 5 μ L of genomic DNA. Samples were thermocycled in a PrimePro48 Real-Time PCR System (Techne/Cole-Parmer, Stone, UK) as follows: 10 min at 95°C and 40 cycles of (15 s at 95°C, 60 s at 60°C). Data were analyzed using Techne Pro and ProStudy (Cole-Parmer). Supplemental Figure S1A (<https://doi.org/10.3168/jds.2019-17877>) plots the mean CT values and Figure 2B plots the standard deviation of the CT values for each candidate autosomal primer/probe combination. Although all candidate amplicons exhibited absolute CT values in the acceptable range (Supplemental Figure S1A), *ACT2B* and *HPRT1* amplicons exhibited variance outside the acceptance criteria (Supplemental Figure S1B, $n = 7$ sires) and were eliminated from further assay development. Similarly, Supplemental Figure S1 panels C and D plot the mean CT values and standard deviations for 2 candidate X- and Y-chromosome amplicons, all of which met requirements for CT value and variance. These data identified candidate primer/probe combinations for ddPCR that exhibited minimal variance across individual genomic DNA templates, and limited further assay development to candidate autosomal amplicons for *GAPDH* and *B2M*, which both met requirements in the qPCR reactions.

To use an autosomal amplicon as a cell counting tool, the amplicon should report the predicted copy number in a 1:1 relationship across a range of DNA input amounts (ng). The reported copy numbers for *GAPDH* and *B2M* were evaluated in singleplex ddPCR reactions to determine which provided a more accurate cell counting tool with tight correlation to the predicted copy number based on bovine genomic molecular weight, input DNA amount (ng), and the haploid nature of sperm cells. Predicted copies per ddPCR reaction was calculated as follows:

$$\begin{aligned} & \text{Amount of genomic template (ng)} \\ & \times \{[\text{Avogadro's number (molecules/mol)}] / \\ & \quad [\text{genomic length (bp)}] \\ & \times \text{MW per base pair (Da)} \times 10^8\}, \end{aligned}$$

using the *Bos taurus* genomic molecular weight obtained from https://uswest.ensembl.org/Bos_taurus/Info/Annotation, which used the UMD 3.1 INSDC Assembly GCA_000003055.3, Nov 2009, referencing a total of 2,649,685,036 bp in the *Bos taurus* genome. Although *GAPDH* copy counts reflected nanograms of input DNA in a 1:1 ratio, *B2M* consistently reported

twice the predicted copy number (data not shown) and was therefore excluded from further assay development.

The sex skew assay was designed to interrogate one target each on the X and Y chromosomes as well as an autosomal target to confirm the total cells counted. The assay was developed using the Bio-Rad Droplet Digital Reader (Bio-Rad Laboratories, Hercules, CA),

Figure 1. Schematic depicting the assay development pipeline and primer/probe screening for the droplet digital PCR (ddPCR) sex skew assay. qPCR = quantitative PCR; CT = cycle threshold.

however, which limits detection to 2 fluorophores and creates a challenge in identifying 3 unique targets. Optimizing a triplex ddPCR assay required maximizing the fluorescent signal separation of the droplet populations, which may contain only individual target amplicons (X, Y, or *GAPDH*), or any combination of the 3 targets (X+Y, X+*GAPDH*, Y+*GAPDH*, and X+Y+*GAPDH*). Two amplicons can be discretely identified, 1 in each of the 2 fluorescent channels, when utilizing HEX and

FAM probes separately for the 2 targets. The third amplicon must be detected using a mix of 2 probes that are identical in sequence but differentially tagged with either the HEX or FAM fluorophore (Figure 2A). Input quantities for template genomic DNA and each primer and probe were iteratively adjusted to achieve maximal separation of the fluorescent droplet populations while assaying the maximal total number of cells. The final validated assay conditions used 900 nM primer with

Figure 2. Optimized droplet digital PCR (ddPCR) sex skew assay chemistry. (A) Schematic representing primer and probe combinations in which the X chromosome is reported by a FAM-labeled probe, Y chromosome by a HEX-labeled probe, and *GAPDH* by a mixture of FAM- and HEX-labeled probes. (B) Scatterplots of fluorescent signal from droplets from the triplex sex skew ddPCR assay interrogating 12 ng of genomic template DNA with 900 nM primer and 250 nM probe for *GAPDH* and the X chromosome, and 1,300 nM primer and 350 nM probe for the Y chromosome. Droplet populations are labeled as follows: X chromosome FAM = red, Y chromosome HEX = purple, *GAPDH* HEX+FAM = yellow, dual-occupancy X + *GAPDH* = tan, X+Y = light blue, Y+*GAPDH* = salmon, triple occupancy X+Y+*GAPDH* = orange, and droplets lacking fluorescent signal (negative) = gray. (C) Boxplot showing the percent sex chromosome distribution in genomic DNA isolated from frozen-thawed conventional semen, calculated as $X/(X+Y)$, $X/GAPDH$, and $Y/GAPDH$, for $n = 40$ unique Holstein sires. All 3 calculations revealed a 50/50 sex chromosome ratio. (D) Scatterplot of *GAPDH* copies counted per reaction (rxn) versus sex chromosome copies per reaction revealed a linear slope of 0.94 ± 0.08 for (X+Y), demonstrating that the autosomal marker tallied the same total number of cells as the additive sum of X + Y copies. The linear slope for *GAPDH* versus X chromosome copies/reaction was 0.44 ± 0.05 , consistent with a predicted 50% X chromosome distribution in conventional semen.

125 nM probe for *GAPDH* (split equally between FAM- and HEX-labeled probes), 900 nM primer with 250 nM FAM probe for the X chromosome, and 1,300 nM primer with 350 nM HEX probe for the Y chromosome in a 24- μ L reaction containing 12 μ L of 2 $\times$ ddPCR Supermix for Probes (Bio-Rad Laboratories), 0.5 μ L of BamHI-HF (high-fidelity preparation of the BamHI restriction enzyme, New England Biolabs, Ipswich, MA), 0.5 μ L of 2 μ M dNTPs, and 12 ng of genomic DNA template. Droplets were generated using an automated droplet generator with automated droplet generation oil (Bio-Rad Laboratories). Thermocycling was performed in a Bio-Rad C100 Touch Thermal cycler as follows: 10 min at 95°C, 40 cycles of (30 s at 94°C and 60 s at 60°C), and then 10 min at 98°C, followed by a 4°C indefinite hold, with a ramp rate of 2°C/s. The QX200 Droplet Reader (Bio-Rad Laboratories) was used to count droplets and quantify fluorescent signal per droplet. Amplicon copies/reaction and copies/microliter were quantified using QuantaSoft and Analysis Pro software (Bio-Rad Laboratories, version 1.7.4.0917). Figure 2B shows an example fluorescent scatterplot from a single assay well run under optimized conditions such that $\geq 1,000$ cells were interrogated; droplet populations identified on the scatterplot demonstrate clear separation. To verify the optimized chemistry, genomic DNA from conventional bovine semen straws was analyzed to determine whether the ddPCR sex skew assay consistently reported the expected $\sim 50/50$ X/Y chromosome ratio. The percentage of each sex chromosome amplicon was quantified as a function of either total X+Y amplicons or total autosomal (*GAPDH*) amplicons from $n = 40$ Holstein sires, with each sample run in triplicate. Figure 2C demonstrates that X and Y chromosome amplicon representation equaled 50% when calculated as $X/(X+Y)$, $X/GAPDH$, or $Y/GAPDH$. Figure 3D plots *GAPDH* copies quantified versus total quantified (X+Y) copies; a linear slope of 0.94 ± 0.08 demonstrates that the total number of sex chromosomes counted was equal to the number of autosomal *GAPDH* copies counted. Figure 2D also plots *GAPDH* copies counted versus X chromosome copies counted, where a slope of 0.44 ± 0.05 corresponds to 50% X chromosome distribution in conventional semen. These data from conventional semen confirmed that the ddPCR sex skew assay reported the expected sex chromosome distribution across all genomic templates (sires) tested, and interrogated sufficient cells to meet statistical rigor requirements.

To validate the cell counting accuracy of the ddPCR assay for genomic DNA from sexed semen, both the 1:1 *GAPDH*:(X+Y) copy ratio and *GAPDH* copy number prediction accuracy were confirmed by assaying genomic DNA isolated from the viable sperm population

in frozen-thawed sex-skewed semen straws. Given the unique nature of IntelliGen X skew sexed semen (ABS Global Inc., DeForest, WI), which contains laser-sliced cells predominantly comprising Y-chromosome-bearing sperm, careful attention was paid to sample cleanup before genomic DNA extraction. If DNA is extracted from a frozen-thawed sexed-semen sample without removing the laser-killed cells, the predicted result is an apparent 50/50 X/Y chromosome representation. This does not, however, reflect the viable, motile population of cells that may ultimately produce a pregnancy. Standard cleanup protocols were used to eliminate nonviable sperm from the sample before genomic DNA extraction as follows. For each individual genomic DNA sample, sperm from 2 frozen-thawed sexed-semen straws or 1 frozen-thawed conventional semen straw (Genus, ABS Global) were centrifuged for 10 min at $300 \times g$ through a Bovipure gradient (0.5 mL of 40% layered over 0.5 mL of 80%; Nidacon International AB, Mölndal, Sweden). Cells from sexed-semen straws generated by laser ablation technology (IntelliGen, Genus, Windsor, WI) were then run through a glass wool column [0.03 g of glass wool borosilicate (112X-475, JohnsManville, Denver, CO) in a 1-mL syringe packed to a volume of 0.1 mL] by gravity drip to remove nonviable cells. Cells were lysed in buffer (50 mM potassium chloride, 10 mM Tris base, pH 8.3, 2.5 mM magnesium chloride, 0.5% Tween-20, 1 M dithiothreitol, 1 μ g/ μ L RNase A, and 40 mg/mL proteinase K) for 18 ± 1 h at 37°C with constant rotation, followed by 1 h at 55°C with constant rotation. Genomic DNA was purified using the Agenecourt DNAdvance Kit (Beckman Coulter, Indianapolis, IN) per the manufacturer's instructions, mixing 200 μ L of lysate with 75 μ L of Bind 1 and 40 μ L of Bind 2 buffer (containing magnetic beads) for 5 min at 750 rpm at 24°C. Samples were placed on the magnetic platform for 4 min to allow the magnetic DNA-binding beads to move to the magnet, and the unbound lysate fraction was removed from the wells. Beads bound to DNA were washed 3 times with 150 μ L of 75% ethanol, shaking 5 min at 850 rpm at 24°C for each wash, followed by 4 min on the magnet before removing wash fraction from the beads. Then DNA was eluted in 25 μ L of supplied elution buffer, with shaking for 10 min at 1,200 rpm at 37°C. Samples were placed on the magnet for 4 min before transferring the eluted DNA to a new tube. The concentration of DNA was quantified on a nanophotometer (Implen GmbH, Munich, Germany). Genomic DNA from frozen-thawed sexed-semen straws representing 220 sires (including Holstein, Jersey, and Angus) was analyzed using the ddPCR sex skew assay and confirmed a 1:1 linear relationship between *GAPDH* and (X+Y) counts per reaction was maintained (Supplemental Figure S2A; <https://doi.org/10.3168/>

Figure 3. Titrating X-skewed and Y-skewed genomic DNA samples demonstrated that the droplet digital PCR (ddPCR) sex skew assay quantified percentage X (%X) with a linear dynamic range from 10 to 95%. The plots show predicted versus calculated %X from $n = 6$ experiments that mixed known high X-skew with known high Y-skew genomic bovine DNA to create a stepwise dose-response curve. DNA samples represented $n = 12$ bulls. Linear fit slope = 1.01 ± 0.04 .

jds.2019-17877). The dynamic range for the *GAPDH* primer/probe set was defined by quantifying the total number of copies counted per triplex ddPCR reaction (X, Y, *GAPDH* primer/probes) across a range from 0.1 to 27 ng of DNA input from frozen-thawed, sexed-semen straws from 4 sires (2 Jersey, 2 Holstein) read in triplicate reactions. Supplemental Figure S2B plots predicted copy number per reaction against *GAPDH* copies counted, revealing a slope of 1 when using a *Bos taurus* genomic molecular weight of 324.22 g/mol (Supplemental Figure S2B, slope = 1.02 ± 0.04) or 349.65 g/mol (slope = 0.95 ± 0.04 , not shown). The linear dynamic range for accurate *GAPDH* copy counts in the sex skew assay was from 0.3 to 27 ng of genomic DNA/20- μ L ddPCR reaction, corresponding to 100 to 9,000 *GAPDH* copies/reaction.

To determine the input DNA range across which the calculated %X exhibited minimal variance and provided a precise value for the calculated %X (or %Y) chromosome, sex skew was quantified as a function of DNA input in nanograms. Supplemental Figure S3A

(<https://doi.org/10.3168/jds.2019-17877>) plots apparent %X as a function of input DNA (ng), and demonstrated that the calculated %X did not change for a template input ranging between 9 and 27 ng of total genomic DNA per reaction, exhibiting a standard error <1% (error propagated from duplicate sexed-semen samples from $n = 4$ unique sires). The apparent %X decreased when input DNA was <3 ng, such that the apparent skew value was statistically different for 0.3 and 0.1 ng of genomic DNA compared with the stable assay range (one-way ANOVA, $P < 0.05$, Bonferroni means comparison). These data demonstrate that the sex skew assay reported reproducible X skew from 9 to 27 ng of input genomic DNA. The X skew was then calculated in repeat measures for genomic template from frozen-thawed sex-skewed straws representing 20 sires. Supplemental Figure S3B plots the calculated %X from quadruplicate assays. The average standard deviation in repeat measures was 0.5 percentage points, demonstrating that under these conditions, the ddPCR sex skew assay reports the X skew $\pm 0.5\%$, making the

assay the most precise measurement of sex skew currently available.

Although repeat measures showed tight variance, we chose to improve the ddPCR sex skew robustness by building the following assay requirements: each replicate assay well must interrogate at least 1,000 cells (where the assay is performed in duplicate) to ensure sufficient sample size; the number of autosomal (*GAPDH*) copies quantified must equal (X+Y) within-assay variance; and the absolute copy number across replicate wells must exhibit <10% variance. Supplemental Figure S4 (<https://doi.org/10.3168/jds.2019-17877>) presents total *GAPDH* counts from duplicate reactions as mean $\pm$ SD for $n = 220$ unique sex-skewed straw batches. Samples that met both the minimum interrogation threshold and variance criteria are shown as filled squares. Samples that met count criteria but exhibited variance outside the acceptable range are shown as open circles, and samples that failed to meet total interrogated cell criteria are shown as filled triangles. This figure demonstrates our ability to flag samples that fail to meet stringent assay requirements so they may be reevaluated.

The sex skew assay demonstrated elegant precision in repeat measures and accuracy when quantifying genomic DNA from conventional (non-sexed) bovine semen straws. To define the precision of the assay across a range of sex skews (varying the expected %X), genomic DNA template from samples of known X or Y skew (where starting skew was $\geq 90\%$ X or Y, respectively) were mixed in a dose-response manner to span 10 to 95% X. Figure 3 plots the predicted versus calculated %X from $n = 5$ dose-response template mixtures (representing a total of 10 sires). These data reveal a linear fit with a slope of 1.01 ± 0.004 (Figure 3), demonstrating that even at very low X amplicon counts (10% X, 90% Y), the assay provides accurate skew quantification.

To determine whether the X-skew data reflect the customer experience, sex skew was quantified from commercially available (ABS Global) sexed semen from 2017 and compared with the historical customer experience of female calves born from sexed-semen inseminations from 2012 to 2017. Figure 4A plots the distribution of female calves born from 2012 to 2017 from inseminations using sexed semen from the Real World Database (mean = 84.0% female calves, $n = 168$ records, where each record represents data from 1 sire from all farms reporting ≥ 100 births for that sire from a total of 294,732 birth records; <https://www.absglobal.com/services/real-world-data/>). Figure 4B plots the histogram of the sex skew from commercial Sexcel (ABS Global) sexed semen as reported by the ddPCR assay (mean = 88.6% X chromosome, $n = 115$,

representing 63 Holstein and 52 Jersey in 629 straw batches dated 17011–17118). Although the data do not represent paired sampling, the histograms demonstrate that the ddPCR sex skew assay of commercially available sexed bovine semen generally reflected the historical customer experience of female calves born.

To date, the ddPCR sex skew assay has been used to quantify the percentage of X-chromosome-containing cells in sexed semen from 24 Angus, 22 Brown Swiss, 5 Hereford, 13 Kobe, 120 Jersey, 616 Holstein, and 15

Figure 4. Droplet digital PCR (ddPCR) sex skew assay correlated with historical field-reported female calves born from sexed semen. (A) Histogram depicts the percentage of female calves born from sexed-semen inseminations from 2012 to 2017, with mean = 84.0% female calves, minimum = 74%, maximum = 91%. Each count represents a data record from at least 100 births/sire per farm. (B) Histogram depicts %X chromosome sperm in commercial sexed-semen straw batches from Julian dates 17011–17118 ($n = 115$ straw batches), with mean = 88.6% X chromosome, minimum = 72.7%, maximum = 96.5%. These data are not paired with those of panel A. These data show that the sex skew assay results correspond with the historical distribution of female calves born from sexed-semen inseminations.

other assorted beef breed sires, demonstrating that the assay is relatively insensitive to background genetic variation and can be successfully applied across *Bos taurus* breeds. This precise, sensitive sex skew assay with a wide dynamic range and robust reproducibility represents an orthogonal testing methodology for independent labs. It should provide customers with confidence in the product they are purchasing, may facilitate market definitions for differential skew products, and may have unique applications for difficult-to-sex species.

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REFERENCES

- Borchersen, S., and M. Peacock. 2009. Danish A.I. field data with sexed semen. *Theriogenology* 71:59–63. <https://doi.org/10.1016/j.theriogenology.2008.09.026>.
- Cerchiario, I., M. Cassandro, R. Dal Zotto, P. Carnier, and L. Gallo. 2007. A field study on fertility and purity of sex-sorted cattle sperm. *J. Dairy Sci.* 90:2538–2542. <https://doi.org/10.3168/jds.2006-694>.
- Faust, M. A., J. Betthausen, A. Storch, and S. Crego. 2016. Effects for fertility of processing steps of a new technology platform for producing sexed sperm. *J. Anim. Sci.* 94(Suppl_5):544.
- Habermann, F. A., A. Winter, I. Olsaker, P. Reichert, and R. Fries. 2005. Validation of sperm sexing in the cattle (*Bos taurus*) by dual colour fluorescence in situ hybridization. *J. Anim. Breed. Genet.* 122(Suppl 1):22–27. <https://doi.org/10.1111/j.1439-0388.2005.00488.x>.
- Healy, A. A., J. K. House, and P. C. Thomson. 2013. Artificial insemination field data on the use of sexed and conventional semen in nulliparous Holstein heifers. *J. Dairy Sci.* 96:1905–1914. <https://doi.org/10.3168/jds.2012-5465>.
- Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams. 1996. Real time quantitative PCR. *Genome Res.* 6:986–994. <https://doi.org/10.1101/gr.6.10.986>.
- Johnson, L. A. 1995. Sex preselection by flow cytometric separation of X and Y chromosome-bearing sperm based on DNA difference: A review. *Reprod. Fertil. Dev.* 7:893–903. <https://doi.org/10.1071/RD9950893>.
- Johnson, L. A., and G. R. Welch. 1999. Sex preselection: High-speed flow cytometric sorting of X and Y sperm for maximum efficiency. *Theriogenology* 52:1323–1341. [https://doi.org/10.1016/S0093-691X\(99\)00220-4](https://doi.org/10.1016/S0093-691X(99)00220-4).
- Kawarasaki, T., G. R. Welch, C. R. Long, M. Yoshida, and L. A. Johnson. 1998. Verification of flow cytometrically-sorted X- and Y-bearing porcine spermatozoa and reanalysis of spermatozoa for DNA content using the fluorescence in situ hybridization (FISH) technique. *Theriogenology* 50:625–635. [https://doi.org/10.1016/S0093-691X\(98\)00167-8](https://doi.org/10.1016/S0093-691X(98)00167-8).
- Khamlor, T., P. Pongpiachan, S. Sangsritavong, and N. Chokesajjawatee. 2014. Determination of sperm sex ratio in bovine semen using multiplex real-time polymerase chain reaction. *Asian-Australas. J. Anim. Sci.* 27:1411–1416. <https://doi.org/10.5713/ajas.2014.14223>.
- Lalande, M. E., and R. G. Miller. 1979. Fluorescence flow analysis of lymphocyte activation using Hoechst 33342 dye. *J. Histochem. Cytochem.* 27:394–397. <https://doi.org/10.1177/27.1.86569>.
- Moruzzi, J. F. 1979. Selecting a mammalian species for the separation of X- and Y-chromosome-bearing spermatozoa. *J. Reprod. Fertil.* 57:319–323. <https://doi.org/10.1530/jrf.0.0570319>.
- Norman, H. D., J. L. Hutchison, and R. H. Miller. 2010. Use of sexed semen and its effect on conception rate, calf sex, dystocia, and stillbirth of Holsteins in the United States. *J. Dairy Sci.* 93:3880–3890. <https://doi.org/10.3168/jds.2009-2781>.
- Parati, K., G. Bongioni, R. Aleandri, and A. Galli. 2006. Sex ratio determination in bovine semen: A new approach by quantitative real time PCR. *Theriogenology* 66:2202–2209. <https://doi.org/10.1016/j.theriogenology.2006.07.007>.
- Puglisi, R., R. Vanni, A. Galli, D. Balduzzi, K. Parati, G. Bongioni, G. Crotti, R. Duchi, C. Galli, G. Lazzari, and R. Aleandri. 2006. In vitro fertilisation with frozen-thawed bovine sperm sexed by flow cytometry and validated for accuracy by real-time PCR. *Reproduction* 132:519–526. <https://doi.org/10.1530/rep.1.01173>.
- Rens, W., F. Yang, G. Welch, S. Revell, P. C. O'Brien, N. Solanky, L. A. Johnson, and M. A. Ferguson Smith. 2001. An X-Y paint set and sperm FISH protocol that can be used for validation of cattle sperm separation procedures. *Reproduction* 121:541–546. <https://doi.org/10.1530/rep.0.1210541>.
- Roti Roti, E., N. Cray, M. Wagner, and M. R. Botts. 2018. Methods and systems for assessing and/or quantifying sperm cell subpopulations bearing a specific genetic signature. Genus PLC, Basingstoke, UK, 20190071725.
- Seidel, G. E. Jr. 2014. Update on sexed semen technology in cattle. *Animal* 8(Suppl 1):160–164. <https://doi.org/10.1017/S1751731114000202>.
- van Munster, E. B., J. Stap, R. A. Hoebe, G. J. te Meerman, and J. A. Aten. 1999. Difference in volume of X- and Y-chromosome-bearing bovine sperm heads matches difference in DNA content. *Cytometry* 35:125–128. [https://doi.org/10.1002/\(SICI\)1097-0320\(19990201\)35:2<125::AID-CYTO3>3.0.CO;2-H](https://doi.org/10.1002/(SICI)1097-0320(19990201)35:2<125::AID-CYTO3>3.0.CO;2-H).

ORCIDS

- Nicole Cray <https://orcid.org/0000-0002-6377-6565>
 Jami Hauer <https://orcid.org/0000-0001-9811-971X>
 Elon Roti Roti <https://orcid.org/0000-0002-0526-9223>