Goncalves et al., 2016 –Revision April 22, 2017:
Supplementary Files

Supplementary File S1: Microbiological Procedures:

Creamy, grayish-white, or golden-yellow pigmented colonies, mannitol fermenting, coagulase and catalase positive gram positive cocci that exhibited complete, incomplete, or both complete and incomplete hemolysis were identified as *Staphylococcus aureus* as described by Anderson and Lyman (2006). All catalase positive *Staphylococcus* non-*aureus* or coagulase negative staphylococci (**CNS**) were speciated using the API Staph. identification system (bioMérieux SA, Marcy-L'Etoile, France). Gram positive, catalase negative cocci were identified as streptococci, enterococci, or aerococci and were speciated using the CAMP test and growth on bile-esculin and inulin agars. If necessary, API-20 Strep strips (bioMérieux SA, Marcy-L'Etoile, France) were used to speciate. Gram negative rods were identified using API-20E strips (bioMérieux SA, Marcy-L'Etoile, France). Yeast and *Nocardia* sp. identifications were based on morphology and Gram stain and *Prototheca* spp. by appearance after staining with lactophenol cotton blue. White-gray or yellowish color colonies with a slightly raised, dry and/or flaky, and nonhemolytic appearance (small and circular colonies approximately 1 mm in diameter) composed of gram-positive bacteria on Gram Stain that appeared at about 48 h of incubation were identified as *Corynebacterium* spp. as described by Gonçalves *et al.* 2014. Milk samples with more than two morphologically different colonies were considered contaminated.

In 11 mammary quarters, two pathogens were isolated. This included five with environmental streptococci and CNS, two with environmental streptococci and *Staphylococcus aureus*, one with environmental *Streptococcus* and *Corynebacterium* spp., one with *Corynebacterium* spp. and CNS, one with *Enterococcus* spp. and CNS, and one with *S. chromogenes* and *S. hyicus*. Those cases were designated as follows: (1) samples with a major and a minor pathogen were designated as being caused by the major pathogen; (2) samples with both *Staphylococcus aureus* and streptococci were designated as being infected by *Staphylococcus aureus*; and (3) samples with both *Corynebacterium* spp. and CNS were designated as being due to CNS.

Anderson KL & Lyman RL 2006 Long-term persistence of specific genetic types of mastitis-causing Staphylococcus aureus on three dairies. *Journal of Dairy Science* **89**(12) 4551-4556.

Goncalves JL, Tomazi T, Barreiro JR, Braga PA, Ferreira CR, Araujo Junior JP, Eberlin MN & dos Santos MV 2014 Identification of Corynebacterium spp. isolated from bovine intramammary infections by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Veterinary Microbiology* **173**(1-2) 147-151.

Supplementary File S2: Milk Leucocyte Differential Instrumentation:

Instrument description: Milk from each quarter was collected into independent chambers of a collection and transfer device (Calderwood *et al.*, 2014, US patent #D720,468) that allows for milk to move into a test slide without the need for a pipette. Capillary action draws milk into the slide and a dried fluorescent stain reagent mixes into solution as the sample flows through the slide (Wardlaw *et al.*, 1999, US patent #5,948,686). Stain uptake occurs quickly and the slide may be read automatically by the reader, which includes a fluorescent microscope (QScout Farm Lab, Advanced Animal Diagnostics, Inc., Durham, NC). Collected images are processed with patented software that identifies and distinguishes immune cells into three classes: neutrophil, lymphocyte, and macrophage, utilizing fluorescence emission of the cell, as described by Wardlaw *et al.* (2002) US patent #6,350,613, supplemented by analysis morphological characteristics. Specific indices are available for early lactation, mid-lactation/hospital, and dryoff. A user selects the index that corresponds with the sample type being processed. Samples may be processed in either a rapid mode (<4 min/cow) or a research mode (approximately 15 min/cow). The research mode collects a much larger and standardized number of images for each quarter. The reader (QScout Farm Lab, Advanced Animal Diagnostics, Inc., Durham, NC) has programmable threshold levels within each index that may be selected by the user. By changing thresholds, a user can weight results towards higher sensitivity or higher sensitivity. Threshold settings for early lactation index range from 1-18, for mid-lactation range from 1-12, and for dryoff index range from 1-12.

Instrument validation: Performance of the MLD test was validated by comparing MLD test results to reference methods. Total leukocyte counts were compared to DeLaval DCC results, with an Intra-Class Correlation (ICC) of 0 .91. Differentials were evaluated by comparing percentages of neutrophils, lymphocytes, and macrophages from the MLD to the same values determined when Wright stained smears of milk were read by two independent, trained cytologists blinded to MLD results. Calculated ICC were 0.87, 0.60, and 0.80 for % neutrophil, % lymphocyte, and % macrophage, respectively. Concentrations of each cell type was evaluated by comparing the total neutrophil, leukocyte, and macrophage counts

(cells/ml) as determined by QScout MLD to the total neutrophil, lymphocyte and macrophage counts calculated by multiplying the percentage of each cell type as determined by Wright Stain by the total SCC reported by the DeLaval DCC. The ICC were 0.86, 0.65, and 0.90 for neutrophils/ml, lymphocytes/ml, and macrophage/ml, respectively. The level of agreement in such tests is referred to as an Inter-rater reliability (IRR), of which the ICC is one example (Hallgren, 2012; Cicchetti, 1994). Values for ICC have been classified as "excellent" when between 0.75 and 1, "good" between 0.60 and 0.74, "fair" between 0.40 and 0.59, and poor if less than 0.40 (Hallgren, 2012; Cicchetti, 1994). Using these values, QScout MLD ICC performance was found to be good to excellent.

Calderwood D, Drach JP, Paul C, Rodriguez R, Hockett M, Marcuson R, Miggels SG, Mack HJ, Young D, Pollard JN, inventors; Advanced Animal Diagnostics, Inc., assignee. Sample collection device assembly. United States Patent 720,468. 2014 Dec 30.

Cicchetti DV. Guidelines, criteria, and rules of thumb for evaluating normed and standardized assessment instruments in psychology. Psychological Assessment. 1994;6(4):284–290.

Hallgren, KA. Computing Inter-Rater Reliabiity for Observational Data: An Overview and Tutorial. Tutor Quant Methods Psychol. 2012 (8): 23-34.

Wardlaw SC, inventor; Robert AL, assignee. Method for performing blood cell counts. United States Patent 5,948,686. 1999 Mar 4.

Wardlaw SC, Levine RA, Rodriguez RR, inventors; Becton Dickinson & Co., assignee. Determination of white blood cell differential and reticulocyte counts. United States Patent 6,350,613. 2002 Feb 26.

Supplementary File S3: Statistical Analysis, Including Model:

The following statistical model was used:

$$Y_{ijklmno} = \mu + H_i + COW_j + Q_k + MY_l + DIM_m + P_n + B_o + IMI_p + e_{ijklmno}$$

where: $Y_{ijklmnop}$ was the dependent variable; μ was the overall mean, H_i and COW_j were the random effects of herd i (i = 1 to 2) and cow j (j = 1 to 78), Q_k was the fixed effect of quarter position k (k = 1 to 4); MY_l was the fixed effect of milk yield Kg/yr l [l = 1 to 3; MY_1 = high milk yield (≥ 10,675 Kg/yr is equal to >35 Kg/d in 305 days in milk), MY_2 = medium milk yield (6,100 to 10,674 Kg/yr is equal to 20 to 34.9 Kg/d in 305 d) and MY_3 = low milk yield (< 6,100 Kg/yr is equal to < 20 Kg/d in 305 d); DIM_m was the fixed effect of days in milk m

(m = 1 to 3; DIM_1 = 4 to 100, DIM_2 = 101 to 200 and DIM_3 = 201 to 431); P_n was the number of parity n (n = 1 to 3;); B_o is the fixed effect of breed o (o = 1 to 3; B_1 = Holstein, B_2 = Jersey and B_3 = cross bred cows); IMI_P was the intramammary infection at quarter level focusing in the proposed objectives p [p = 1 to 2; IMI_1 = mastitis definition (healthy, latent infection, non-specific subclinical mastitis and specific subclinical mastitis); IMI_2 = specific groups of pathogens causing subclinical mastitis (healthy, minor, environmental, contagious and miscellaneous)]; and $e_{ijklmno}$ was the random error term.