Received: 3 November 2022 | Revised: 13 April 2023 | Accepted: 24 April 2023

DOI: 10.1002/cyto.a.24737

TECHNICAL NOTE

ISAC CYTOMETRY Journal of Quantitative Cell Science PART A

Validation of multiparametric panels for bovine mesenchymal stromal cell phenotyping

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Funding information
CUSTOMEAT, Grant/Award Number: S002821N

Abstract

Bovine mesenchymal stromal cells (MSCs) display important features that render them valuable for cell therapy and tissue engineering strategies, such as self-renewal, multi-lineage differentiation, as well as immunomodulatory properties. These cells are also promising candidates to produce cultured meat. For all these applications, it is imperative to unequivocally identify this cell population. The isolation and in vitro tri-lineage differentiation of bovine MSCs is already described, but data on their immunophenotypic characterization is not yet complete. The currently limited availability of monoclonal antibodies (mAbs) specific for bovine MSC markers strongly hampers this research. Following the minimal criteria defined for human MSCs, bovine MSCs should express CD73, CD90, and CD105 and lack expression of CD14 or CD11b, CD34, CD45, CD79α, or CD19, and MHC-II. Additional surface proteins which have been reported to be expressed include CD29, CD44, and CD106. In this study, we aimed to immunophenotype bovine adipose tissue (AT)-derived MSCs using multi-color flow cytometry. To this end, 13 commercial Abs were screened for recognizing bovine epitopes using the appropriate positive controls. Using flow cytometry and immunofluorescence microscopy, cross-reactivity was confirmed for CD34, CD73, CD79α, and CD90. Unfortunately, none of the evaluated CD105 and CD106 Abs cross-reacted with bovine cells. Subsequently, AT-derived bovine MSCs were characterized using multi-color flow cytometry based on their expression of nine markers. Bovine MSCs clearly expressed CD29 and CD44, and lacked expression of CD14, CD45, CD73, CD79α, and MHCII, while a variable expression was observed for CD34 and CD90. In addition, the mRNA transcription level of different markers was analyzed using reverse transcription quantitative polymerase chain reaction. Using these panels, bovine MSCs can be properly immunophenotyped which allows a better characterization of this heterogenous cell population.

KEYWORDS
cattle, cross-reactivity, heterogeneity, MSC characterization, multi-color flow cytometry

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1 | INTRODUCTION

Healthy and sustainable food provision for a continuously growing human population is one of the major challenges for the next decades. Within the context of alternative food production methods, cultured meat is a promising avenue for increasing the total supply of high-quality protein and, at the same time, responding to a societal demand for reduced animal slaughtering [1–3]. Traditionally, myosatellite cells are used to produce cultured meat as these skeletal muscle stem cells serve in vivo as a robust cell source for skeletal muscle repair [4]. Their proliferation and differentiation potential in vitro, however, is limited.

Mesenchymal stromal cells (MSCs) are considered as a promising alternative cell source due to their abundance, their role during muscle development and their ability to differentiate into myocytes, adipocytes, fibroblasts, neuronal and endothelial cells [5–8]. Indeed, MSCs are easily isolated from a variety of sources, such as bone marrow, adipose tissue, and neonatal tissues [9, 10]. Human MSCs are commonly characterized following the guidelines of the International Society for Cellular Therapy (ISCT) [11, 12]: they must be [1] plastic adherent under standard culture conditions; [2] be capable of differentiating toward the osteogenic, adipogenic and chondrogenic lineage; and [3] express surface markers such as CD73, CD90, and CD105 but lack expression of hematopoietic or endothelial markers such as CD11b or CD14, CD34, CD45, CD79α, or CD19 and MHC class II. However, this definition by cell surface marker expression is constantly evolving. For example, the ISCT has recently stated that CD34 negativity is not as definitive [12], as subpopulations of human adipose tissue-derived MSCs have been shown to express CD34 [13, 14].

The isolation, in vitro differentiation and immunophenotype of bovine MSCs has already been reported (Table 1). As shown in Table 1, it is clear that bovine MSCs represent a heterogeneous cell population with differences originating from donor age, tissue of origin, genetic background, passage, and culture conditions [10, 51, 52]. For example, Kato et al. showed that bone marrow (BM)-derived bovine MSCs lacked CD90 expression, while other researchers showed a variable expression of CD90 (Table 1) [53]. Like human MSCs, Rossi et al. confirmed a moderate CD34 expression in amniotic fluid-derived bovine MSCs, while others showed only a low expression (Table 1) [54]. Such heterogeneity may reflect differences in proliferation and differentiation potential, for example, predisposition for dedicated lineages, which is important to identify when considering bovine MSCs as a cell source to produce cultured meat [51, 55].

The expression of MSC markers is often analyzed on the mRNA level using gel electrophoresis or reverse transcription quantitative polymerase chain reaction (RT-qPCR) and/or protein level using single-color flow cytometry. A multi-color flow cytometry protocol enables to simultaneously evaluate the expression of different markers and as such to identify MSC heterogeneity [56, 57]. However, there are currently no specific criteria to immunophenotype bovine MSCs, partly due to the limited availability of bovine-specific monoclonal antibodies (mAbs) or validated cross-reactive mAbs [9], as illustrated by the study of Naraoka et al. Indeed, half of the tested 246 Abs showed cross-reactivity toward bovine epitopes, but 5%–10% was the highest percentage of cross-reactivity reported (for 97 Abs) while only 1%–5% cross-reactivity was reported for 17 Abs [58].

In this study, 13 commercially available Abs were first evaluated for their cross-reactivity using bovine leukocytes (LEU) or bovine umbilical cord endothelial cells (UVECs) as appropriate positive controls. For all evaluated antibodies, flow cytometric results were confirmed using immunofluorescent microscopy. Subsequently, bovine AT-derived MSCs were characterized by tri-lineage differentiation and multi-color flow cytometry using two selected panels consisting of nine Abs in total. Additionally, marker expression was analyzed on the mRNA level by RT-qPCR.

2 | MATERIALS AND METHODS

2.1 | Isolation methods

Bovine umbilical cord veins and blood waste products were obtained from one routine health screening and two adult cows during caesarean section, provided by the Department of Internal Medicine, Reproduction and Population of the Faculty of Veterinary Medicine. Bovine MSCs were isolated from subcutaneous adipose tissue (AT) from seven healthy calves, as part of an external research project, approved by the institutional ethical committee of the Flanders Research Institute for Agriculture, Fisheries and Food (EC 2018/313).

After collection of approximately 5 mL venous blood using EDTA-coated tubes, red blood cells were lysed with a NH_4Cl lysing solution to isolate bovine LEUs. Briefly, upon incubating for 5 min at room temperature (RT), cells were centrifuged for 5 min at 300*g* at RT. The cell pellet was washed in phosphate buffered saline (PBS) without Ca^{2+}/Mg^{2+} (Gibco), centrifuged for 5 min at 300*g* and finally resuspended in 1 mL low glucose Dulbecco's Modified Eagle Medium (DMEM-LG, Invitrogen) containing 1% fetal bovine serum (FBS, Sigma) [59].

To isolate primary bovine UVECs, tissue was digested using 1 mg/mL collagenase IA (Sigma) and 1.2 mg/mL dispase II (Sigma) for 30 min in a humidified incubator at 38.5°C and 5% CO_2, as previously described [60]. The enzymatic reaction was blocked by flushing the vein with prewarmed PBS + 10% FBS. The solution was then centrifuged for 5 min at 250*g* at 4°C, resuspended in PBS + 10% FBS and filtered over a 70 μm cell strainer. The cell suspension was centrifuged for 5 min at 250*g* at 4°C and the pellet was resuspended in EGM-2 endothelial growth medium (Lonza). The medium was refreshed twice weekly until 70–80% confluency was reached. Cells were passaged until passage 3 prior to use as positive control cells for Ab screening.

Bovine MSCs were isolated from subcutaneous AT ($n = 7$) using an enzymatic digestion method containing 1 mg/mL liberase (Sigma), as previously described [61]. The isolated cells were seeded in culture medium consisting of DMEM-LG, 30% FBS, 10^{-11} M dexamethasone (Sigma), 1% antibiotic-antimycotic solution (Sigma) and 1% L-glutamine (Invitrogen) in a 25 cm^2 culture flask and cultured at 38.5°C in a humidified atmosphere containing 5% CO_2. After 24 h, non-

TABLE 1 Overview of immunophenotypic profile on mRNA and protein level of bovine mesenchymal stromal cells.

	Expression on			
Cellular marker	**mRNA level**		**Protein level**	
CD13	+	[15]	+	[16]
CD29	+	[15, 17–31]	+	[15, 17, 19, 21, 24, 26–28, 30–35]
CD44	+	[15, 18–21, 23–27, 29–31, 36]	+	[15, 16, 19, 21, 24–27, 30, 31, 34, 37–39]
			–	[17, 28, 40]
CD49d		ND	+	[16]
CD71		ND	+	[19]
CD73	+	[15, 17–21, 23–28, 30, 31, 41–44]	+	[17, 25, 27, 28, 33, 35, 40, 45–50]
CD90	+	[15, 17, 22–25, 28, 30, 31, 41, 43]	+	[16, 17, 28, 35, 40, 45–50]
			±	[15]
CD105	+	[15, 17, 22, 23, 28, 29, 41, 43]	+	[16, 17, 28, 29, 32, 35, 40, 45–47, 49, 50]
	–	[18]	–	ND
CD106	+	[24, 25]		ND
	–	[15, 30]		
CD166	+	[15, 19, 20, 24–26, 29, 31]	+	[29]
	–	[30]		
MHCI	+	[15, 18, 29]	+	[15]
CD11b	+	[42]	–	[15]
	–	[15]		
CD14	–	[15, 18, 29]	–	ND
CD31	–	[15]	–	ND
CD34	+	[42]	±	[29, 33, 34, 40, 48]
	–	[15, 18–21, 23–27, 29–31, 41, 43]	–	[16, 17, 19, 21, 24, 27, 28, 32, 35, 38, 39, 47, 50]
CD45	+	[41–43]	±	[33, 48]
	–	[15, 19–21, 23–26, 31, 41]	–	[15, 17, 28, 32, 35, 40, 45, 47, 50]
CD79α		ND	–	[40, 47, 50]
CD117	–	[15]	–	ND
MHCII	–	[15, 18, 24, 26, 29, 31]	±	[29]
			–	[15, 34, 38, 39]

Note: At the mRNA level, + is defined as presence and – is defined as absence of mRNA (as determined by PCR agarose gel images and/or RT-qPCR). At the protein level, + is defined as presence (> 10%) and – is defined as absence of protein (<2%), ± is defined as relative marker expression between 2% and 10% (as determined by immunofluorescence and western blot images). Percentages are linked to relative marker expression, at the protein level, analyzed using quantitative methods such as flow cytometry.
Abbreviation: ND, not done.

adherent cells were removed by replacing the culture medium. Subsequently, culture medium was replaced twice weekly and cells were passaged as soon as 70%–80% confluency was observed using 2.5 mg/mL trypsin (Sigma)-0.2 mg/mL EDTA (Sigma) in expansion medium (identical to the culture medium without dexamethasone). Cells were passaged until passage 4 prior to use for multi-color flow cytometry.

2.2 | Tri-lineage differentiation of bovine AT-MSCs

To confirm their MSC identity, undifferentiated bovine MSCs of the 3rd passage were induced toward the adipogenic, chondrogenic, and osteogenic lineage, respectively, as previously described [61]. Non-induced MSCs cultured in expansion medium were used as appropriate negative controls. Adipogenic differentiation was assessed using Oil Red O histological staining with a Mayer's modified hematoxylin (Abcam) counterstaining after cycles of 72 h culturing in adipogenic induction medium (DMEM-LG supplemented with 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 10 µg/mL rh-insuline (Sigma), 0.2 mM indomethacin (Sigma), 15% rabbit serum (Sigma), 50 µg/mL gentamycin (Gibco) and 1% antibiotic-antimycotic solution) and 24 h of culturing in adipogenic maintenance medium (identical to the adipogenic induction medium except for the omission of dexamethasone, indomethacin and 3-isobutyl-1-methylxanthine). Chondrogenic differentiation was evaluated after 21 days of culture

in chondrogenic medium (based on the basal differentiation medium (Lonza), supplemented with 10 ng/mL Transforming Growth Factor-β3 (Lonza)) by Alcian blue (Sigma) histological staining, with 0.1% Nuclear Fast Red (Sigma) counterstaining. Osteogenic differentiation was evaluated after 21 days of culture in osteogenic medium (DMEM-LG supplemented with 10% FBS, 0.05 mM L-ascorbic acid-2-phosphate (Sigma), 100 nM dexamethasone, 10 mM β-glycerophosphate (Sigma), 50 µg/mL gentamycin and 1% antibiotic-antimycotic solution) using the Alizarin Red S histological staining (Sigma), according to the manufacturer's instructions [59].

2.3 | Single-color flow cytometry

An overview of the primary monoclonal and polyclonal Abs evaluated in this study is given in Table 2. Secondary antibodies included fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Ig)G (BioRad), AlexaFluor488 (AF488)-conjugated goat anti-rabbit IgG (Invitrogen), allophycocyanin-cyanine 7 (APC-Cy7)-conjugated goat anti-rabbit IgG (AAT Bioquest), R-phycoerythrin-cyanine 5 (PE-Cy5)-conjugated goat anti-rabbit IgG (ThermoFisher). Additionally, a secondary streptavidin peridinin chlorophyll protein-cyanine5.5 (PerCP-Cy5.5) label (Invitrogen) was used. The isotype controls in this study included mouse IgG_1-APC, IgG_1-FITC and IgG_1-PE-Cy7 (BioLegend).

To screen for Ab reactivity, 500,000 positive control cells (either LEU or UVECs, Table 2) were centrifuged in staining buffer (DMEM +1% FBS) for 5 min at 400*g* at 4°C and incubated for 30 min at 4°C in the dark with each of the primary Abs (Table 2). After two washing steps for 5 min at 400 g at 4°C, a FITC-conjugated goat anti-mouse secondary Ab or an AF488-conjugated goat anti-rabbit Ab was added to the cells for 20 min at 4°C in the dark. After one washing step, cell pellets were resuspended in 100 µL PBS with the viability dye Sytox Blue (0.001 mM; ThermoFisher), following the manufacturer's guidelines. For intracellular antigen detection (CD79α), cells were fixed and permeabilized using BD Cytofix/Cytoperm™ (BD Biosciences) for 20 min at 4°C prior to primary Ab incubation. At least 10,000 viable single cells were analyzed using a CytoFLEX V2-B4-R3 flow cytometer (Beckman Coulter) equipped with a 405 nm, a 488 nm and a 638 nm laser, and data was subsequently analyzed in the CytExpert software. All data were corrected for autofluorescence as well as for unspecific binding using either secondary Abs alone or isotype-matched negative controls.

2.4 | Immunofluorescence microscopy

Binding of the Abs to the bovine positive control cells was confirmed by immunofluorescence microscopy, as routinely performed [62] Briefly, cells (either LEU or UVECs) were stained as described for the single-color flow cytometry, and subsequently fixed using 4% paraformaldehyde (PFA) for 20 min at 4°C in the dark. After centrifugation, cells were incubated with 0.01 mg/mL Hoechst33258 (Sigma) to visualize the nuclei, for 20 min at 4°C in the dark. Following centrifugation and a washing step, the stained cells were imaged with a magnification of 63x using fluorescence microscopy (Leica DMi8).

TABLE 2 Overview of the primary antibodies evaluated in this study.

Host	Target species	Antibodies	Isotype	Clone	Company, product number	Positive controls	Cross-reactivity
Ra	Hu, Mo, Rt, Pi	CD34	IgG	Polyclonal	Bioss Antibodies, bs-8996R	UVEC	+
Mo	Hu	CD73-PC7	IgG_1	AD2	BioLegend, 344,009	LEU	–
Mo	Hu	CD73	IgG_{2b}	45M4F9	Novus Biologicals, NBP2-25237SS	LEU	–
Ra	Hu, Mo, Rt, Sh, Ca	CD73	IgG	Polyclonal	Bioss Antibodies, bs-4834R	LEU	+
Mo	Hu	CD79α	IgG_1	HM57	BioRad, MCA2538GA	LEU	+
Mo	Hu	CD90-APC	IgG_1	5E10	BioLegend, 328133	LEU	–
Mo	Hu	CD90	IgG_1	AF-9	Novus Biologicals, NBP2-45230	LEU	–
Ra	Hu, Mo, Rt, Ca, Sh, others	CD90	IgG	Polyclonal	Bioss Antibodies, bs-0778R	LEU	+
Mo	Hu	CD105	IgG_1	SN6h	Invitrogen, MA5-11854	UVEC	–
Rt	Mo, Rt, Hu	CD105	IgG_{2a}	2Q1707	Santa Cruz Biotechnology, sc-71042	UVEC	–
Mo	Hu	CD105-PC7	IgG_1	SN6	ThermoFisher, 25-1057-42	UVEC	–
Ra	Hu, Mo, Rt	CD105	IgG	Polyclonal	Bioss Antibodies, bs-057R	UVEC	–
Mo	Hu	CD106-FITC	IgG_1	1.G11B1	BioRad, MCA907F	UVEC	–

Note: Each antibody was tested using the appropriate bovine positive control cells.
Abbreviations: APC, allophycocyanin; Ca, canine; FITC, fluorescein isothiocyanate; Hu, human; LEU, leukocytes; Mo, mouse; PC7, PE-Cyanine7; Pi, pig; Ra, rabbit; Rt, rat; Sh, sheep; UVEC, umbilical cord vein endothelial cells.

2.5 | Multi-color flow cytometry

For multi-color flow cytometry, bovine MSCs of 7 donors were incubated with two panels of both bovine-specific (CD14, CD44, CD45 and MHC-II) and cross-reacting Abs (CD29, CD34, CD73, CD79α and CD90), as described in Table 3.

Approximately 500,000 cells per tube were centrifuged for 5 min at 400*g* at 4°C in staining buffer. For panel 1, cells were first incubated for 30 min at 4°C in the dark with a CD73-specific Ab. After blocking and washing with the staining buffer for 5 min at 400*g* at 4°C, cells were incubated with the appropriate secondary Ab for 20 min at 4°C in the dark. The cells were washed and subsequently blocked for 15 min using 10% rabbit serum in PBS, to block free binding spots of the indirectly labeled primary rabbit CD73-specific Ab.

Next, cells were incubated for 30 min at 4°C in the dark with a biotinylated CD90-specific Ab. After washing with the staining buffer, cells were incubated with a secondary Streptavidin PerCP-Cy5.5 label, together with the directly labeled primary mAbs (CD29-APC, CD45-FITC and MHCII-(R)PE) for 20 min at 4°C in the dark. After a washing step, cell pellets were finally resuspended in 100 µL PBS with 0.001 mM Sytox Blue.

In panel 2, cells were first stained with a fixable live/dead violet stain (ThermoFisher) for 30 min at 4°C in the dark. After one washing step with PBS, cells were incubated for 30 min at 4°C in the dark with the CD34-specific Ab. After blocking and washing with the staining buffer, cells were incubated with an appropriate secondary Ab together with the directly labeled primary mAbs (CD14-(R)PE and CD44-FITC) for 20 min at 4°C in the dark. Subsequently, cells were fixed and permeabilized using BD Cytofix/Cytoperm™ for 20 min at 4°C. After blocking and washing with 1X Perm/Wash washing buffer, cells were incubated with the CD79α-AF700 Ab for 20 min at 4°C in the dark. After a washing step, cell pellets were finally resuspended in 100 µL PBS.

For all panels, at least 10,000 cells were analyzed using a CytoFLEX and CytExpert software. All data were corrected for autofluorescence, compensated and compared to specific fluorescence minus one (FMO) controls. Compensation for spectral overlap between fluorophores was performed using an automatic calibration technique and subsequently evaluated individually with a compensation matrix.

2.6 | RT-qPCR

In order to investigate if the protein levels of the different markers, as analyzed in panel 1 and 2 via multi-color flow cytometry, are reflected in their mRNA levels, RT-qPCR was performed. Approximately 500,000 cells were trypsinized, washed with PBS and stored as cell pellets at −80°C until RNA extraction. The cell pellets were thawed in 1 mL TRIR (ABgene) at RT for 5 min and total RNA was isolated in 30 µL using the Aurum Total RNA Mini Kit (Bio-Rad), according to the manufacturer's instructions and including an on-column DNase treatment of 20 min at RT. RNA purity and concentration was measured via Nanodrop analysis (Thermo Scientific) and DNA contamination was checked via minus-RT PCR with the TBP assay. Up to 1 µg of DNA-free RNA was converted into cDNA with random hexamers and oligo-dT using the ImProm-II Reverse Transcription System (Promega) [63]. The integrity and the PCR amplificabillity of the cDNA (1/10 dilution) was confirmed via the UBC integrity assay [63].

qPCR reactions were performed on 2 µL 1/10 diluted cDNA with the KAPA SYBR FAST qPCR Master Mix (KAPA Bio-systems) and 500 nM primers (Table S1) in a final volume of 10 µL. The reactions were performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Initial denaturation at 95°C for 3 min to activate the DNA polymerase was followed by 40 cycles of denaturation at 95°C for 20 s and a combined annealing/extension/signal detection step at the primer annealing temperature for 40 s (Table S1). Finally, a melt

TABLE 3 Overview of the panels of the selected primary antibodies to immunophenotype viable bovine MSCs using multicolor flow cytometry.

Antibody	Clone	Isotype	Dilution	Fluorochrome	Manufacturer, product number	Reactivity
Panel 1						
Mo CD29-APC	TS2/16	IgG_1	1:50		BioLegend, 303,007	Cross-reactive
Mo CD45-FITC	CC1	IgG_1	1:10		BioRad, MCA832GA	Bovine-specific
Mo MHCII-(R)PE	CC158	IgG_{2a}	1:20		BioRad, MCA5655	Bovine-specific
Ra CD73		IgG	1:50	IgG-APC-Cy7	AAT Bioquest, 16873	Cross-reactive
Ra CD90-biotin		IgG	1:50	Streptavidin PerCP-Cy5.5	Invitrogen, 45-4317-80	Cross-reactive
Panel 2						
Mo CD14-(R)PE	CC-G33	IgG_1	1:100		BioRad, MCA2678GA	Bovine-specific
Mo CD44-FITC	IL-A118	IgG_1	1:10		BioRad, MCA2433F	Bovine-specific
Mo CD79α-AF700	HM57	IgG_1	1:50			Cross-reactive
Ra CD34		IgG	1:100	IgG-PE-Cy5	ThermoFisher, L43018	Cross-reactive

Note: When appropriate, the relevant secondary labels are also provided.
Abbreviations: (R)PE, R-phycoerythrin; AF700, Alexa Fluor 700; APC, allophycocyanin; APC-Cy7, APC-cyanine 7; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; Mo, mouse; PE-Cy5, PE-Cyanine5; PerCP-Cy5.5, peridinin chlorophyll protein-cyanine5.5; Ra, rabbit.

curve analysis was performed (from 70°C to 95°C with 0.5°C increments of 5 min) to confirm that the detected signals came from the intended amplicons. All reactions were performed in duplicate and a no template control (NTC) was included in each run. A four-fold dilution series of one reference cDNA sample was additionally included for each gene to acquire PCR efficiency based on relative standard curves. Calculation of the Cq-values (quantification cycle), PCR efficiencies, correlation coefficients and analysis of the melting curves was performed by means of the CFX Maestro Software.

After duplicate RT-qPCR experiments, inter-run calibration was performed. When inter-sample variability exceeded a Cq-value of 0.5, a third run was performed. The mean Cq-value of the two or three replicates was used for further data processing. To this end, mean Cq values were transformed, using the exact PCR amplification efficiency, into corrected Cq-values assuming a uniform PCR amplification efficiency of 100% across all samples (Equation 1):

$$Cq_{corrected} = Log_2\left(E_{exact}{}^{Cqmean}\right). \quad (1)$$

In a preliminary experiment, the expression stability of six commonly used reference genes was determined in the MSC samples [64, 65]. After identifying the most efficient reference genes (ACTB, SDHA, TBP, and HPRT1), NormFinder for Microsoft Excel was applied. Applying this software program, ACTB and TBP were indicated as the most stable genes in MSC samples, since the average stability values of these genes was 0.212. Relative quantification was calculated based on the delta Cq-method, with ACTB and TBP used as reference genes as described [66].

2.7 | Data analysis

Quantitative data are presented as mean ± standard deviation (SD) from seven replicates and visualized using GraphPad Prism 8.

3 | RESULTS

3.1 | Evaluation of cross-reactive antibodies

Using bovine UVECs as appropriate positive controls, cross-reactivity against bovine epitopes was identified for the commercially available Abs directed against CD34 (3.37%), whereas none of the four CD105 Abs and the CD106 Ab tested showed cross-reactivity (Figure 1). Furthermore, cross-reactivity against bovine epitopes was identified for Abs directed against CD73 (17.83%), CD79α (27.41%), and CD90 (16.09%), using bovine LEU as appropriate positive controls (Figure 1). Additionally, binding of the above-mentioned Abs to the bovine positive control cells was confirmed with immunofluorescence microscopy (Figure 1).

3.2 | Tri-lineage differentiation potential of bovine AT-derived MSCs

To confirm the MSC identity of the isolated cells from AT, cells were differentiated toward adipocytes, osteocytes, and chondrocytes, respectively. Tri-lineage differentiation potential was confirmed for all donors (Figure S1).

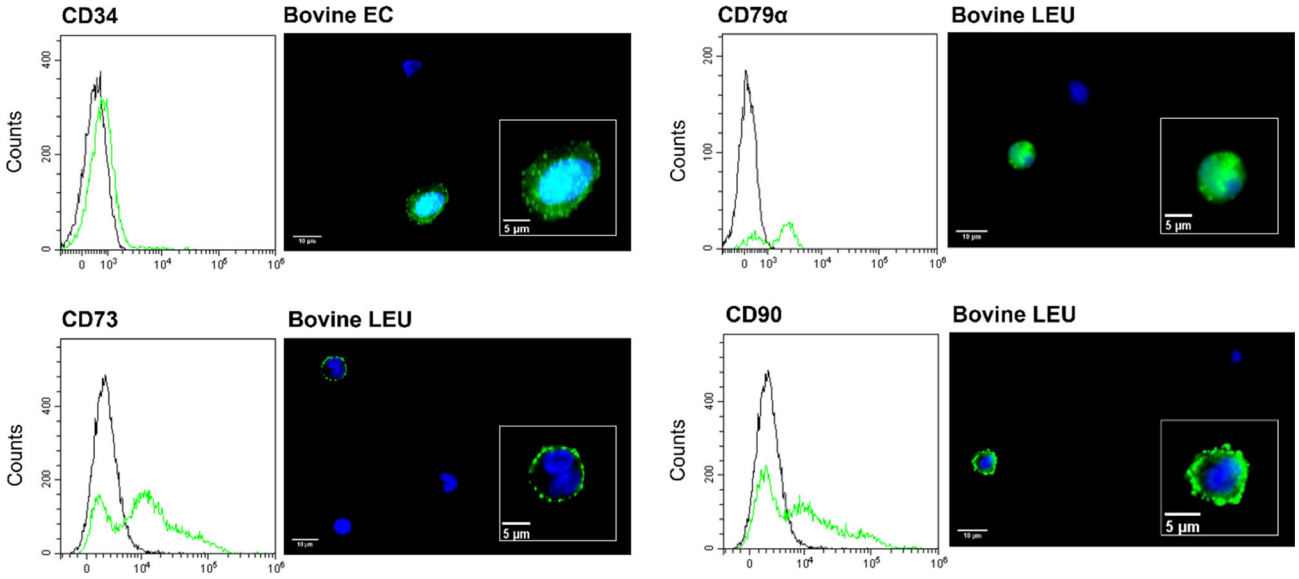

FIGURE 1 Evaluation of cross-reactive antibodies against mesenchymal stromal cell markers using the appropriate bovine positive control cells. The black histogram represents the negative control, while the green histogram represents the cells stained with the selected antibodies. Immunofluorescence microscopy was used to confirm specific cellular binding. Nuclei were visualized using Hoechst. Scale bar = 10 micron; scale bar inset = 5 micron. EC, endothelial cells; LEU, leukocytes. [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | Immunophenotyping of bovine AT-derived MSCs

Based on the commercially available bovine-specific and the identified cross-reacting Abs, two panels were developed to adequately immunophenotype bovine AT-derived MSCs using multi-color flow cytometry. The gating strategy to assess marker expression of bovine MSCs for panel 1 and 2 is presented in Figure 2. As shown in Figure 3A, bovine AT-derived MSCs uniformly expressed CD29 (99.95% ± 0.04%) and CD44 (93.93% ± 4.60%), showed a variable expression of CD34 (76.15% ± 19.74%) and CD90 (7.56% ± 6.31%), and lacked expression of CD14 (0.30% ± 0.19%), CD45 (0.13% ± 0.24%), CD73 (0.23% ± 0.36%), CD79α (0.05% ± 0.03%) and MHCII (0.18% ± 0.13%). In panel 1, bovine MSCs are positive for both CD29 and CD90, and negative for CD45, CD73 and MHCII. As all cells are expressing CD29, the same variation in percentage $CD90^+$ cells is observed when focusing either on the total cell population or only on the $CD29^+$ cells. In panel 2, bovine MSCs are positive for CD34 and CD44, and negative for CD14 and CD79α. Most cells in panel 2 co-express both CD34 and CD44 (76.78 ± 18.26), while some cells lack expression of both markers (2.90 ± 2.64) or express only CD34 (4.46 ± 2.69) or only CD44 (15.85 ± 16.87) (Figure 3B).

To evaluate marker expression on transcript level, mRNA expression of MSC markers was assessed by RT-qPCR. RNA yield concentrations ranged between 70 and 121 ng/μL and RNA samples gave a 260/280 ratio between 1.9 and 2.1. Bovine AT-MSCs showed a higher mRNA expression of CD29, CD44 and CD90 ($-\Delta Cq > 0$), a moderate expression of CD34 and CD105 ($-5 \leq -\Delta Cq \leq 0$), and a low expression of CD14, CD73, CD79α and MHCII ($-\Delta Cq < -5$), when compared to the reference genes (Figure 4). CD45 was not expressed above the detection limit of the RT-qPCR assay (Figure 4).

4 | DISCUSSION

In general, the immunophenotypic characterization of bovine MSCs is hampered by the lack of bovine-specific or validated cross-reactive mAbs. In this study, 13 commercially available Abs were screened to compose two panels of bovine-specific and/or cross-reactive Abs. Cross-reactivity with bovine epitopes was identified for Abs directed against CD34, CD73, CD79α and CD90. Unfortunately, no cross-reactive Abs were identified against CD105 and CD106. Although the Abs directed against CD29 (TS2/16) and CD79α (HM57) were already used to identify bovine cells [67–70], they were not validated yet using the appropriate positive control cells. This validation is important as it turned out that three of the four tested anti-CD105 clones (SN6, SN6h, and 2Q1707) were already used to characterize bovine MSCs [40, 46, 67], while, in our hands, these antibodies did not bind to both bovine adipose tissue-derived MSCs and UVECs. The tissue of origin of the MSCs might explain the difference in CD105 expression by MSCs reported in these studies and in our study [40, 46, 71]. On the other hand, the polyclonal CD34 Ab was evaluated in this study, although only a low percentage of positive cells was observed in the positive control, most likely because the primary endothelial cells were not purified. This is in line with other reports where human and bovine primary endothelial cells were reported to show variable CD34 expression [72, 73].

Based on the evaluated cross-reactive and bovine-specific Abs, two panels were developed to adequately immunophenotype bovine MSCs. The use of multi-color flow cytometry enhances the characterization of the heterogenous MSC population [74]. Similar to human MSCs, bovine MSCs highly expressed CD29 and CD44, and lacked expression of CD14, CD45, CD79α, and MHCII. However, some remarkable differences in marker expression between human and bovine MSCs were observed. Bovine AT-derived MSCs showed a variable expression of the hematopoietic stem and progenitor marker CD34, only a low expression of CD90, and lacked expression of CD73. These results were largely similar on the mRNA level.

As already mentioned, a variable CD34 expression is described for both human and bovine MSCs, depending on sources and passages [13, 40, 54, 75, 76]. It is hypothesized that MSCs originate from the perivascular cell population as they share markers and functionality with perivascular and mural cells [12, 77]. This might explain the CD34 positivity of certain MSC subpopulations. Traktuev et al. (2008), for example, identified a subpopulation of human AT-derived stem cells that is, $CD34^+/CD31^-/CD144^-$ cells, which simultaneously expressed mesenchymal, pericytic and smooth muscle markers [77]. For bovine MSCs, mostly CD34 negativity is reported at the mRNA and protein level (Table 1). As proper Ab evaluation and/or quantitative data on CD34 expression, in case of immunofluorescent staining of bovine MSCs, is often lacking [16, 19, 27, 47, 49, 50], defining bovine MSCs as CD34 negative cells remains a point of discussion [12]. Furthermore, CD34 positivity has been reported for bovine MSCs isolated from placental tissues [40, 54].

Interestingly, CD73, known to be expressed by human MSCs, was negative in the bovine AT-derived MSC population, as confirmed on both the mRNA and protein level. In general, CD73 positivity has been reported for bovine MSCs (Table 1). In parallel with CD34 expression, proper Ab evaluation and quantitative data at the protein level is often lacking [15, 26, 41]. In one bovine study, however, lung-derived MSCs showed low CD73 mRNA levels in passage 3 and 9, with an increased expression observed in passage 17 and 25 [26]. Low CD73 expression was also observed for equine MSCs isolated from umbilical cord blood, Whartons Jelly and peripheral blood [78]. Recently, two subpopulations based on the presence or absence of CD73 expression were identified in murine MSCs, isolated from pericardial AT [79]. Additionally, these researchers observed a heterogenous CD73 expression (ranging from 19% to 84%) in MSCs from other murine sources as well, such as bone marrow, subcutaneous AT and umbilical cord [79].

Regarding CD90 expression, a discrepancy between mRNA and protein level was observed in this study, with a high mRNA level but a rather low expression on the protein level. This finding confirms that the absence of the corresponding proteins is preferably evaluated rather than their mRNA levels [80]. Low expression of CD90 was reported as well in two other bovine MSC studies. In the study of

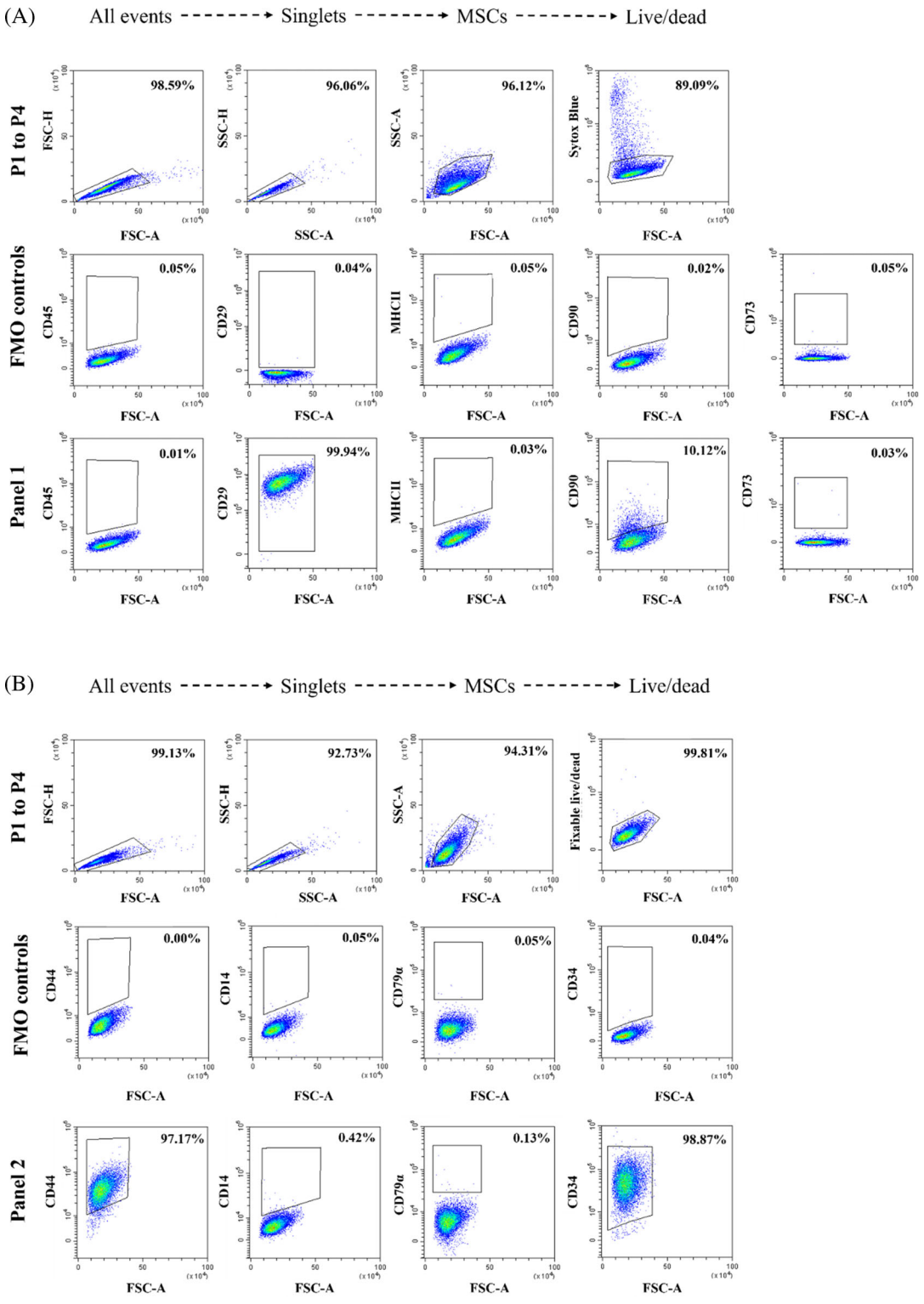

FIGURE 2 Gating strategy for bovine mesenchymal stromal cells, (A) for panel 1 and (B) panel 2. First, singlets were selected in FSC-A/FSC-H (P1) and SSC-A/SSC-H (P2) dot plots. The population of interest (P) was identified based on side scatter area versus forward scatter area (SSC-A/FSC-A) characteristics. The viable single cell population (P4) was then identified by Sytox Blue (panel 1) or fixable violet blue (panel 2) staining. The final gate for analysis (P5) was identified by defining unspecific binding to cells in P4 using fluorescence minus one (FMO) controls. Marker expression of a representative donor for panel 1 and panel 2 is shown. [Color figure can be viewed at wileyonlinelibrary.com]

Pipino et al. (2018), only a fraction (30%–40%) of bovine MSCs isolated from milk was $CD90^{+}$ [46], while Xiong et al. observed low mRNA expression of CD90 in MSC from bovine umbilical cord [31].

In conclusion, a multi-color flow cytometry protocol was developed to immunophenotype bovine MSCs after properly evaluating cross-reacting Abs. In a recent ISCT communication, it is stated that

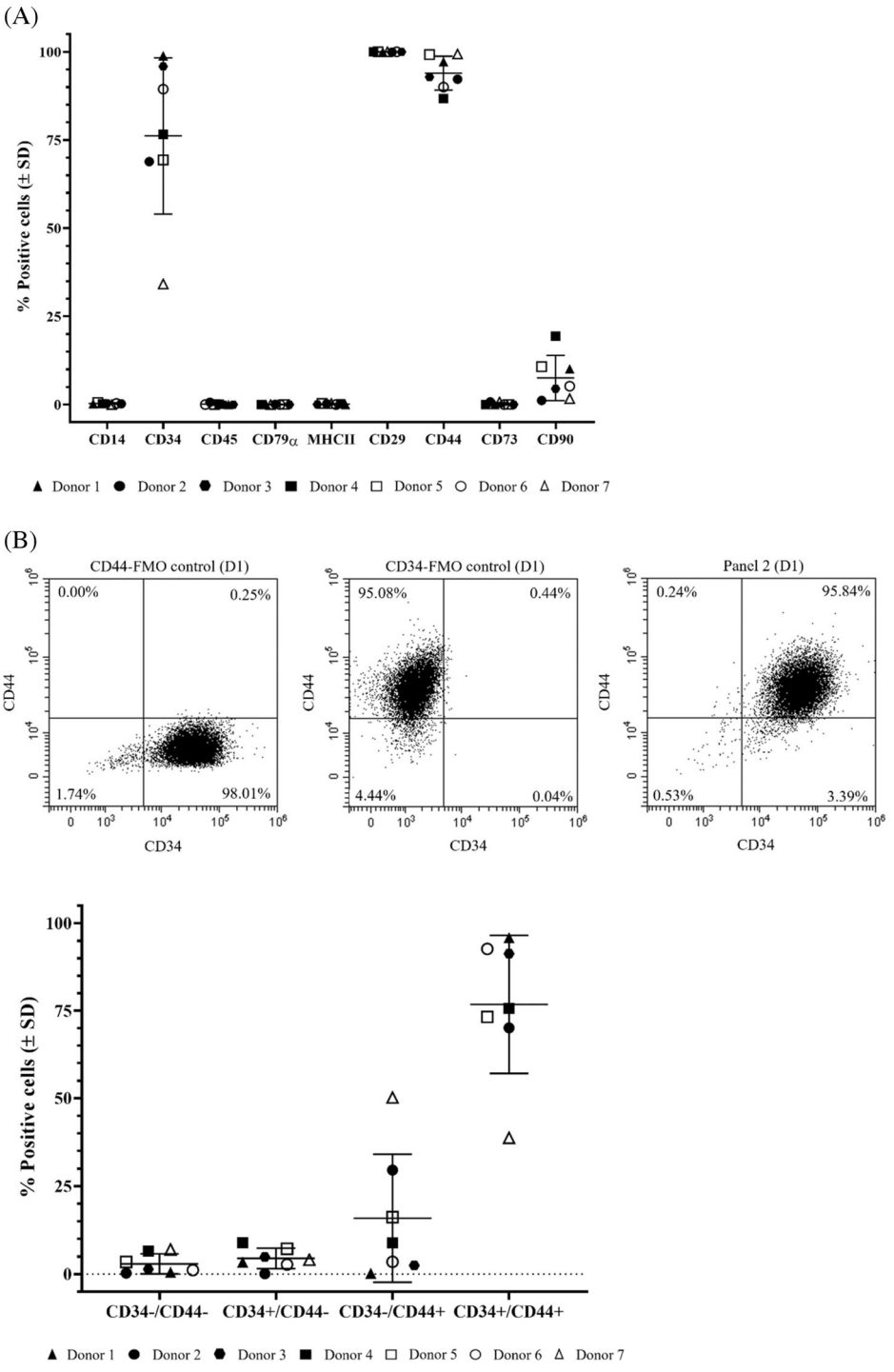

FIGURE 3 Immunophenotypic profile of bovine AT-mesenchymal stromal cells (MSCs) as assessed by multi-color flow cytometry. The expression of nine MSC markers was evaluated on bovine AT-derived MSCs ($n = 7$). (A) The horizontal line shows the mean % positive cells and the error bars the standard deviation. The dotted line shows the 2% positive cells, i.e. the upper limit set by the ISCT to determine 'lack of expression' (≤2%).
(B) Representative image of co-expression of CD44 and CD34, i.e. the only positive markers in panel 2.

the characterization of MSCs, as defined by cell surface marker expression, is evolving as new insights are gained [12]. It is also clear that several subpopulations are present within the MSC population [79]. Such heterogeneity may also reflect differences in proliferation and differentiation potential. Suga et al. (2009), for example, showed that human AT-derived $CD34^+$ MSCs exhibited a higher proliferative potential, while $CD34^-$ MSCs showed a greater ability for adipogenic and osteogenic differentiation [14]. When considering bovine MSCs as alternative cell source to produce cultured meat, it is important to identify those MSC subpopulations which are able to proliferate on the one hand (in order to generate large amounts of cultured meat) and to differentiate myogenically on the other hand. The immunophenotypic characterization protocol described in this study provides an interesting tool to rapidly identify different MSC subpopulations. In future research, the proliferation and myogenic differentiation potential of the different MSC subpopulations should be evaluated after sorting, to identify the most appropriate MSC subpopulation to produce cultured meat. Furthermore, our results suggest that species-specific immunophenotypic profiles should be formulated when characterizing MSCs, as it is clear from this study and other studies that the expression of the commonly used panel of MSC markers is highly variable and

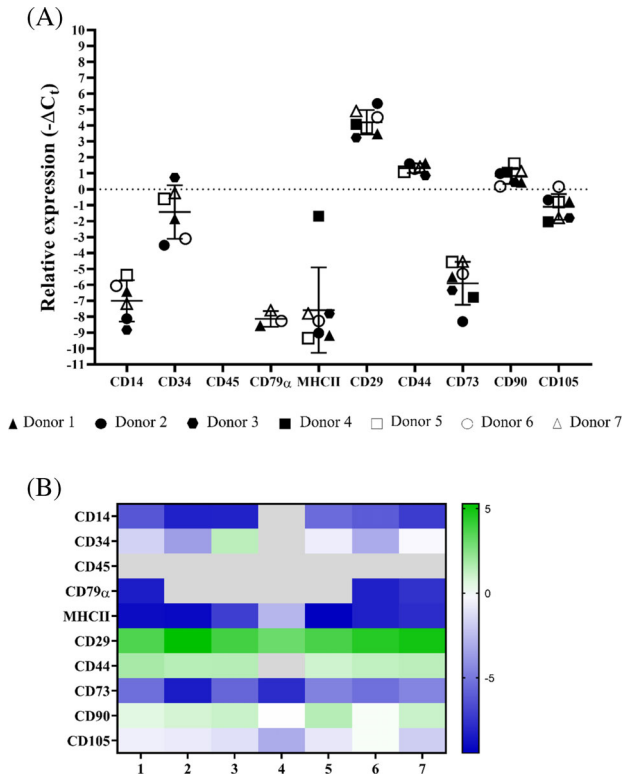

FIGURE 4 mRNA expression profile of bovine AT-MSCs as assessed by RT-qPCR. Relative expression ($-\Delta$Cq) compared to reference genes of the markers for bovine AT-derived-MSCs ($n = 7$). High mRNA expression indicated as $-\Delta$Cq >0, moderate mRNA expression indicated as $-5 \leq -\Delta$ Cq ≤0, low mRNA expression indicated as $-\Delta$Cq < -5 when compared to reference genes (A); Heatmap representing the relative expression. Dark green shades indicate higher expression and dark blue shades indicate lower expression; white shades indicate $-\Delta$Cq values = 0; gray shades indicate missing values, marker gene expression was not expressed above the detection limit of the RT-qPCR assay (B). [Color figure can be viewed at wileyonlinelibrary.com]

depends on the species, tissue of origin, donors, passages, and culture conditions.

AUTHOR CONTRIBUTIONS

Emma Heyman was involved in conception and design, execution of experiments and manuscript writing. M. Meeremans was involved in RT-qPCR experiments and data analysis. M. Van Poucke and L. Peelman were involved in RT-qPCR experiments and manuscript writing. Catharina De Schauwer and B. Devriendt were involved in conception and design, data analysis and manuscript writing.

ACKNOWLEDGMENTS

We gratefully acknowledge Joachim Christiaens and Delphine Ameye (Department of Pathobiology, Pharmacology and Zoological Medicine, Faculty of Veterinary Medicine, Ghent University) for their excellent technical assistance.

FUNDING INFORMATION

This study was partially funded by the CUSTOMEAT project (FWO-SBO project S002821N).

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

An example of the multi-color flow cytometry results (FCS-files) was uploaded to the Flow Repository website: http://flowrepository.org/id/FR-FCM-Z64A. Additional data files presented in this study are available upon request, please contact the corresponding author Emma Heyman.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Heyman E, Meeremans M, Van Poucke M, Peelman L, Devriendt B, De Schauwer C. Validation of multiparametric panels for bovine mesenchymal stromal cell phenotyping. Cytometry. 2023. https://doi.org/10.1002/cyto.a.24737