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Sandra Wiart-Letort, Laurence Finot. Two recombinant antibodies recognize (cyto)keratin 14 by immunofluorescence in farm animals (cow, goat and swine). 2025, n.p. <10.60675/yfh3-8k03/sn20250923-8/short-notes>. <hal-05285752>

HAL Id: hal-05285752

<https://hal.science/hal-05285752v1>

Submitted on 26 Sep 2025

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Two recombinant antibodies recognize (cyto)keratin 14 by immunofluorescence in farm animals (cow, goat and swine)

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Abstract

As part of the GIS FC3R leading project to replace animal-derived antibodies by recombinant ones, we tested four recombinant antibodies to detect by immunofluorescence, keratin 14, a protein expressed by the basal epithelial cells. Two antibodies, the clones E7W6V and SP53 from respectively, Cell Signaling Tech. and Abcam, were able to recognize the target in the mammary gland of bovine and porcine species. Only the clone E7W6V recognized keratin 14 in the mammary tissue of all three species, including goats.

Category: original study including positive and negative results

Models: *in vitro* and *in vivo*

Species: cow, goat and swine

Keywords : CK14, epithelial cells, 3R



Introduction:

Antibodies of animal origin are currently controversial for both ethical and scientific reasons. In the context of reducing the use of animals in scientific experiments and procedures (Replace-Reduce-Refine or 3R principles), using animals to produce antibodies by immunization campaigns is proving problematic. In addition, the variability from batch to batch of animal-derived antibodies complicates the reproducibility of experiments. In line with this, Scientific Interest Group of the French reference center for 3R (GIS FC3R) has initiated a program to implement recombinant antibodies in France and funded several pilot studies in histology platforms and research laboratories. By joining one of these pilot studies, we tested four recombinant antibodies, more ethical and reproducible, in our histological approaches to replace our currently used animal-derived anti-keratin (KRT) 14 antibody.

In livestock, the mammary gland is a key organ for milk production, which relies on the development of the mammary epithelium implying different types of epithelial cells (Finot et al., 2018; Pal et al., 2017). In our research, we aim to identify and characterize in depth the different constitutive cell types of the mammary epithelium by histology. We focus on mammary epithelia and their cellular characterization using markers as keratins (KRT) targeting specific type (luminal or basal) of epithelial cells. Keratin 14 (KRT14; UniProt # P02533) also known as cytokeratin 14, is an intermediate filament protein belonging to the cytokeratin family, which are proteins constitutive of the epithelial cell cytoskeleton. In the mammary gland, KRT14 is predominantly expressed in the basal cells surrounding the luminal epithelial cell layer (Gieniec & Davis, 2022). In our previous studies, we detected KRT14 in the bovine mammary epithelium with a polyclonal antibody produced in goats (Finot et al., 2019). To replace this animal-derived antibody, we tested four recombinant antibodies to detect KRT14 by immunofluorescence in the mammary gland of three livestock species (bovine, caprine and porcine) of agronomic interest. In parallel, as bovine MAC-T cells express KRT14, these cells served as a positive control in our test (Arevalo Turrubiarte et al., 2016).

Results & Discussion:

In this study, we carried out our staining protocol both on MAC-T cells as the positive sample and on paraffin-embedded mammary tissue of the cow, goat and sow. The recombinant antibodies were selected based on their multiple cross-reactivity or their predicted cross-reactivity in species other than human and mice when mentioned in their datasheet. We adopted this strategy as we experienced that multiple cross-reactivity antibodies are more likely to cross-react with exotic species including farm animals.

The reference for the expected signal of KRT14 was obtained from previous immunofluorescence analysis using the animal-derived (goat polyclonal) antibody (Santa Cruz Biotec.) as shown in Supplementary Figure 1. For this “reference” antibody, all cells of the MAC-T cell line were positively stained (Fig. S1, upper panels) and cells from the outer layer of epithelial structures or alveoli were stained in the mammary tissue of the cow (Fig. S1, lower panels). Actually, this localization is as expected for the basal cells known to surround the luminal cells within the alveoli and to express basal KRT as KRT14 (Rauner & Barash, 2012).

From the four antibodies tested (see supplemental table 1), the antibodies from Absolute antibodies were unable to recognize the KRT14 in the mammary tissue of the three species as seen in the supplemental Figure 2 (for the clone RCK107) and 3 (for the clone 2G4). Surprisingly, some MAC-T cells, not all, were stained by the clone RCK107. Results for the clone E7W6V (Cell Signaling Tech.) in Figure 1 and for the clone SP53 (Abcam) in Figure 2, showed that all cells of the bovine MAC-T cell line were stained by both the recombinant antibodies (upper panels in Figures 1 and 2), consistent with the positive control status of these cells. These data suggest that both clones recognized the bovine KRT14. We then tested these antibodies on the mammary tissue of the three species of agronomic interest. In Figure 1 displaying immunostaining for the clone E7W6V (Cell Signaling Tech.), a signal was localized in cells surrounding a layer of cells, presumably luminal cells in the mammary gland of the cow (second panels), sow (third panels), and goat (fourth panels). Based on these data, we assume that the anti-KRT14 (Clone E7W6V) recombinant antibody cross-reacts with mammary cells of the bovine, caprine and porcine species. In Figure 2 showing KRT14 staining with the Abcam’s recombinant antibody (clone SP53), a similar signal was detected in cells surrounding the luminal cells of alveoli in cow (second panels) and

sow (third panels), but not in goat (fourth panels). These results highlight the ability of the clone SP53 to recognize KRT14 from the bovine and porcine mammary gland.

To conclude, two of the four recombinant antibodies tested were able to detect KRT14 expressed by the MAC-T cells and the mammary basal cells of the cow and sow. Only one (the clone E7W6V from Cell Signaling Tech.) presents the advantage to recognize KRT14 from all the three species. These results provide reliable, reproducible, and immunological ethical tools to study the effects of farming practices on the epithelia involved in milk production.

Acknowledgements:

The authors are grateful to the staff of the dairy experimental farm IEPL (INRAE, UMR PEGASE, Le Rheu, France) and the experimental farm of UE3P (INRAE, Unité Experimentale Physiologie et Phénotypage des Porcs, Saint-Gilles, FRANCE) for their help in providing the samples.

Authors contributions:

Sandra Wiart-Letort : Methodology, Acquisition of data, reviewing of original draft.

Laurence Finot: Conceptualisation, Supervision, Methodology, Acquisition of data and formal analysis, Validation, Writing-original draft.

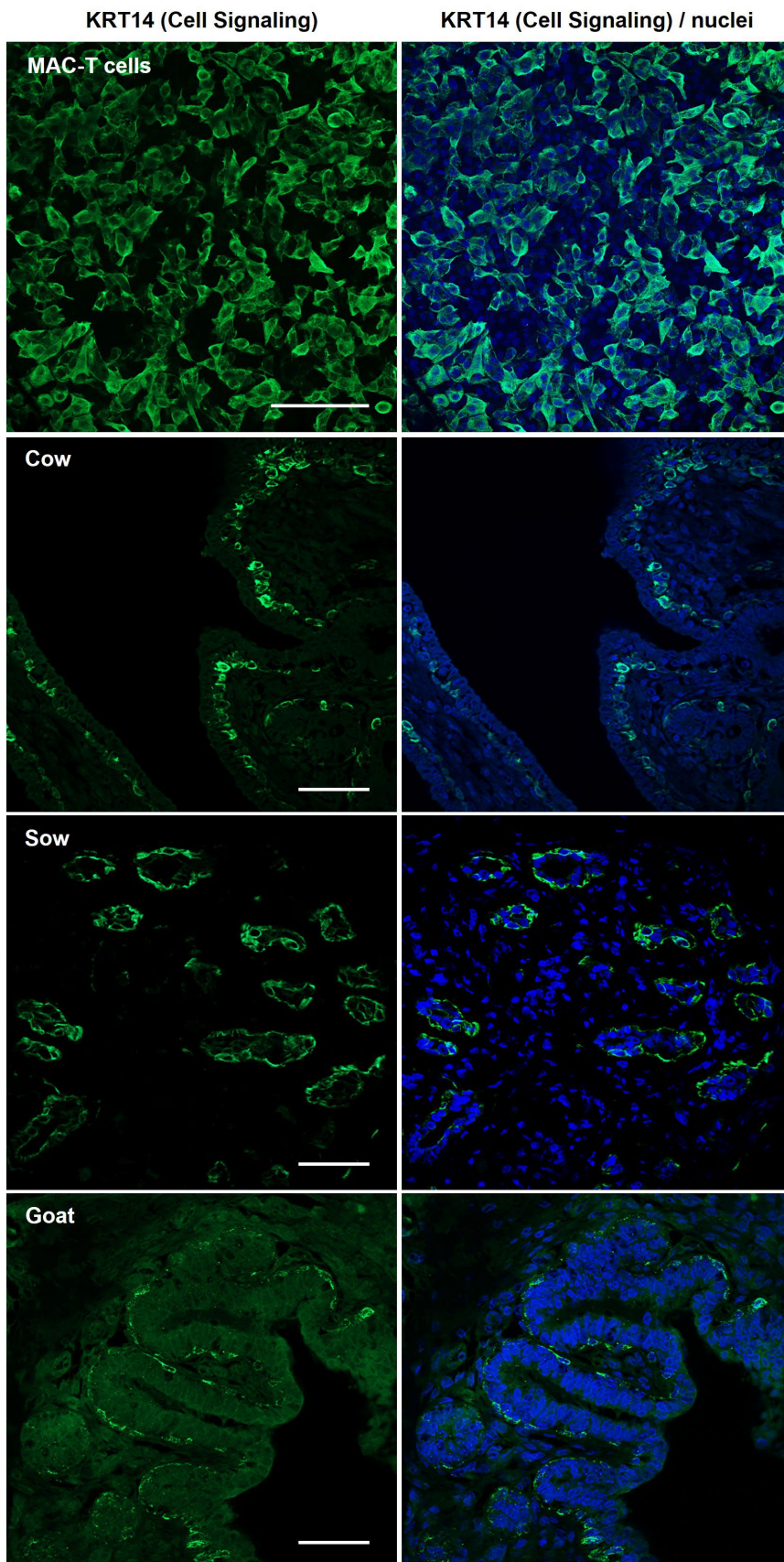


Figure 1. Immunofluorescent staining of the keratin 14 (KRT14, in green, left panels) by the recombinant clone E7W6V (Cell Signaling) antibody in the bovine MAC-T cells and the mammary gland of cow, sow and goat. A merge of the KRT14 staining with nuclei counterstained with Hoechst 33342 is presented in the right panels. Scale bars = 50 μ m (for MAC-T cells) and 100 μ m for cow, goat and sow.

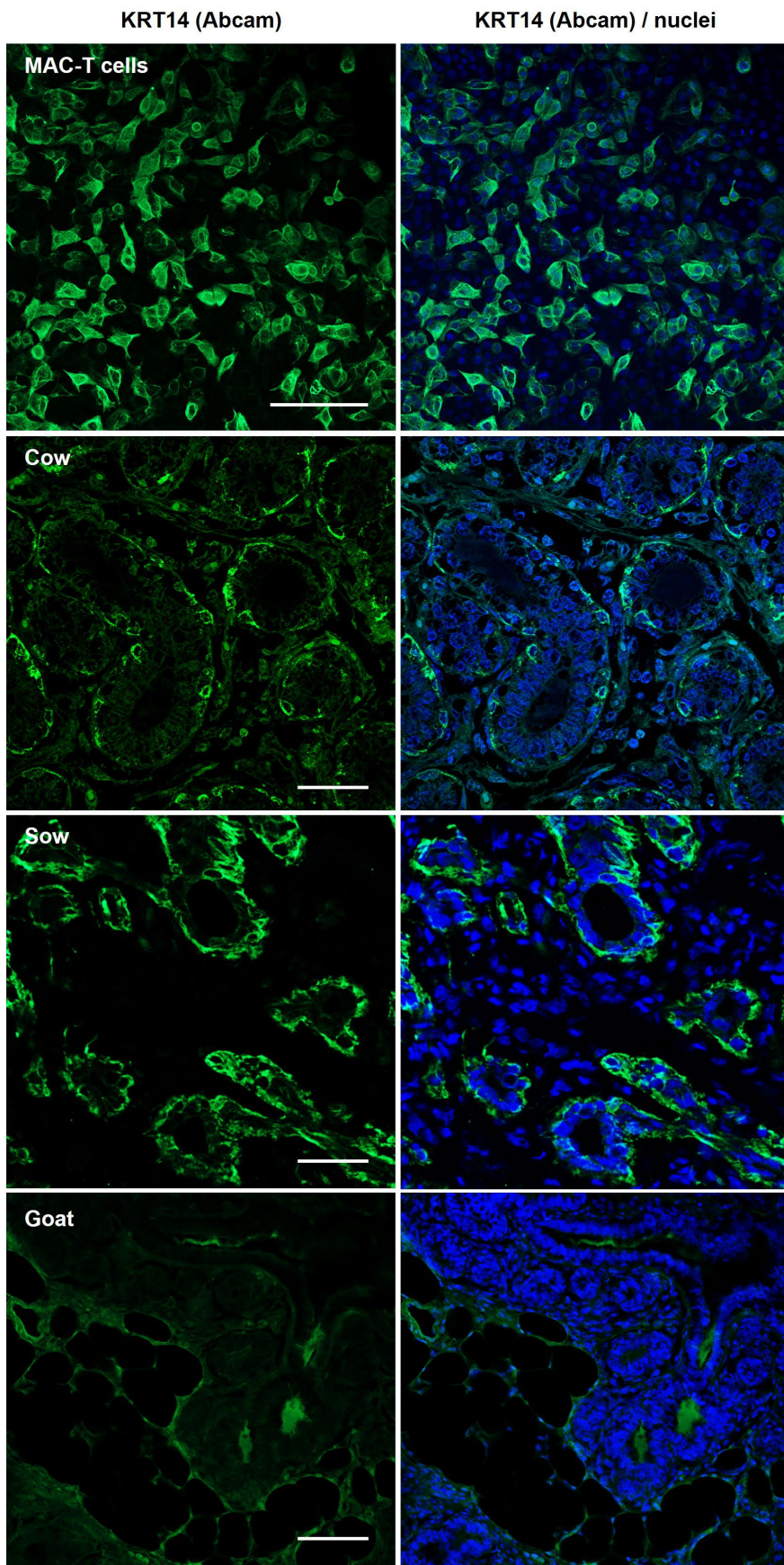


Figure 2. Immunofluorescent staining of the keratin 14 (KRT14, in green, left panels) by the recombinant clone SP53 (Abcam) antibody in the bovine MAC-T cells and the mammary gland of cow, sow and goat. A merge of the KRT14 staining with nuclei counterstained with

Hoechst 33342 is presented in the right panels. Scale bars = 50 µm (for MAC-T cells) and 100 µm for cow, goat and sow.

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Conflict of interest:

This study was supported by the GIS FC3R and the authors declare no conflict of interest.

Data Availability Statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Materials & Methods:

Antibodies: Anti-keratin 14 (clone E7W6V) rabbit recombinant monoclonal antibody (74956, Cell Signaling Technology) (<https://www.cellsignal.com/products/primary-antibodies/keratin-14-e7w6v-rabbit-mab/74956>)
Anti-keratin 14 (clone SP53) rabbit recombinant monoclonal antibody (ab119695, Abcam) (<https://www.abcam.com/en-us/products/primary-antibodies/cytokeratin-14-antibody-sp53-ab119695?srsId=AfmBOop9fem1eJBbMuD-oj-omE3LCDjtBRRVFlxAl57IzWLUjPxi5JMY#>)
Anti-keratin 14 (clone RCK107) rabbit recombinant monoclonal antibody (Ab03338-23.0, Absolute antibody) (https://absoluteantibody.com/product/anti-cytokeratin-14-rck107/Ab03338-23.0_rabbit_igg/) and Keratin 14 (clone 2G4) rabbit recombinant monoclonal antibody (Ab03180-23.0, Absolute antibody) (https://absoluteantibody.com/product/anti-cytokeratin-14-2g4/Ab03180-23.0_rabbit_igg/)
Anti-keratin 14 (clone C-14) goat polyclonal antibody (sc17104, Santa Cruz Biotechnology) (<https://www.scbt.com/fr/p/cytokeratin-14-antibody-c-14?srsId=AfmBOoqFsv22271-4fe7yZLWCbaHh1cJbNOI-NXO0u16ISJg5fuMqZTy>)
Anti-rabbit Alexa Fluor 488 (A11008, Life Technologies, France) as secondary antibody.

Biological materials: Paraffin-embedded sections of cow (17-month-old heifer), goat (prepubertal) and pig (lactating sow) mammary gland were used. The samples used in this study were provided from previous experiments for which additional slides were made. No mammary tissue collection was performed specifically for this study. In addition, the bovine MAC-T cell line of mammary epithelial cells cultured on inserts represented

a positive control as cells express the KRT14 (Arevalo Turrubiarte et al., 2016). Culture of MAC-T cells was described in (Arevalo Turrubiarte et al., 2016)

Methods: For cell staining, cells on inserts were fixed with 4% PFA (FOR0060AF59001, VWR, France) for 30 min at room temperature (1 ml of PFA per compartment), then washed twice for 5 min with PBS. For each step (other than antibody and Hoechst incubation), 1 ml of solution was added to both compartments (basolateral and apical) of the insert. Washing consisted of incubating cells for 5 min in PBS. Cells were permeabilized for 5 min with PBS containing 0.5% Triton X-100 (1.08603.1000, Merck, France), then washed twice. After a 30 min blocking in PBS with 2% BSA (1.12018.0100, Merck) followed by a wash, cells were incubated for 1 h 30 min at 37°C with primary antibody diluted in PBS 0.2% BSA (100 µl per insert), freshly prepared. Before antibody incubation, the insert was placed on the plate lid to ensure a hard surface. The insert was then returned to its well and washed twice.

For mammary gland paraffin-embedded sections, sections were deparaffinized using a Gemini AS automated slide stainer (Thermo Scientific, France) with a dewaxing program (twice 10 min in xylene followed by successive 5 min baths in 100%, 90%, 80% and 70% ethanol. Slides were washed 5 min in distilled water then in two successive baths of PBS). Slides were left overnight at 4°C in PBS before staining. The day of staining, slides were washed twice for 5 min in TBS (20 mM Tris-base, 150 mM NaCl, pH 7.6). Washing consisted in incubating slides for 5 min in TBS. An epitope retrieval step was performed with TRIS 1 mM (pH 8) buffer (for cow samples) or EDTA buffer (immunoDNA retriever EDTA 20X, BSB0033, Bio SB) (for goat and sow samples) using the TintoRetriever (BioSB) device at 110°C (pressure) for 15 min. After two washes, slides were incubated 1 h with blocking buffer (TBS, 10% horse serum, 3% Triton X-100). Subsequently, tissue sections were incubated with primary antibodies diluted (100 µl/section) in blocking buffer for 1 h 30 min at 37°C then washed twice. Dilution of primary antibodies is indicated in the S1 supplemental table.

Following primary antibody incubation, cells or mammary tissue sections were incubated for 45 min at 37°C with the anti-rabbit Alexa Fluor 488 secondary antibody diluted in PBS 0.2% BSA (for cells) or in blocking buffer (for mammary sections). Cells and tissue sections were washed twice, and nuclei were stained with bisbenzimidazole H33342 or Hoechst (14533, Merck) at 5 µg/ml (100 µl per insert) for 3 min, followed by two washes and a final wash in Milli Q water. For cells, the membrane of insert was cut and mounted on a slide whereas tissue sections were dried. Both were then mounted with mounting medium and coverslip. Pictures were acquired using an Apotome™ microscope and the Zen acquisition software (Zeiss France).

Supporting information and files:

S1 supplemental table: Data on primary antibodies used in the study

S1 supplemental figure: Immunofluorescent staining of KRT14 by the goat-derived polyclonal antibody from Santa Cruz Biotec in bovine mammary tissue and cells.

S2 supplemental figure: Immunofluorescent staining of KRT14 by the clone RCK107 from absolute antibody in bovine, caprine and porcine mammary tissue.

S3 supplemental figure: Immunofluorescent staining of KRT14 by the clone 2G4 from absolute antibody in bovine, caprine and porcine mammary tissue.