Materials and Methods

Experimental Strategy

The histology platform from Inrae St Gilles evaluated four recombinant Fc Rabbit anti-Occludin antibodies on formalin-fixed cells and paraffin-embedded (FFPE) tissue sections from pig and cow.

As no other antibody worked for these species (no positive control), all antibodies were tested on mouse tissue (internal & biological positive control).

Antibody performance was assessed based on staining intensity, specificity, and background signal. Validation criteria in tissues included a clear intense staining localized at the tight junctions.

Biological Material

All formalin-fixed paraffin-embedded (FFPE) samples were already available on the platform. No animal has been sacrificed for these tests.

- **Intestinal porcine epithelial cells-jejunum 2 (IPEC-J2)**
- **Tissues**:
    - Paraffin-embedded sections (6 µm thick) of:
        - Mouse jejunum (from experiment "Candy S36 ileon").
        - Piglet jejunum (samples P22001, P117).
        - Heifer mammary gland (sample G4297).
- **Sample preparation**:
    - Fixed in neutral buffered formalin, dehydrated, and embedded in paraffin.
    - Sections mounted on slides and stored at 4°C until use.

Antibody Testing

- Reference antibody : Invitrogen 33-1500 anti-occludin monoclonal (mouse, clone OC-3F10)
- Abcam Ab216327 anti-Occludin (rabbit recombinant monoclonal Occludin antibody)
- Absolute Antibody AB 04036 AbAb 1-3 anti-Occludin (rabbit) recombinant
- Absolute Antibody AB 04037 AbAb 37-5 anti-Occludin (rabbit) recombinant
- Cell Signaling Technology #91131 E6B4R anti-Occludin (Rabbit) recombinant

- **Secondary Antibody**

Goat anti-rabbit IgG Alexa Fluor 488 (Life Technologies, #A11008), diluted 1:500 in blocking buffer.

- **Staining protocol (FFPE samples)**

The recombinant anti-Occludin antibodies were tested under these conditions:

1. **Deparaffinization**:
    - Automated process using the Gemini staining system:

        - Xylene (10 min × 2) → Ethanol series (100%, 90%, 80%, 70% – 5 min each) → Distilled H_2O (5 min) → PBS 1X (5 min × 2).
    - Slides stored overnight in PBS 1X at 4°C.
2. **Epitope Retrieval**:
    - Pressure cooker (Diagomics "cocotte") with preheated EDTA buffer (commercial, Diagomics).
    - Program: 110°C, 15 min (low-pressure mode).
3. **Blocking**:
    - 1 h incubation with blocking buffer: PBS 1X + 10% horse serum + 3% Triton X-100.
4. **Primary Antibody Incubation**:
    - 50 µl/section, 1.5 h at 37°C.
    - Two dilutions tested for each antibody: 1:200 and 1:400 (prepared in blocking buffer).
5. **Secondary Antibody Incubation**:
    - 50 µl/section, 30 min at room temperature.
6. **Nuclear Counterstaining**:
    - Mounting medium with DAPI (Gold Antifade Mountant with DAPI, 13 µl/section).
7. **Reagent Details**

Blocking buffer: PBS 1X (20 mM Tris-base, 150 mM NaCl, pH 7.6) + 10% horse serum + 3% Triton X-100.

Antibody diluent: Blocking buffer for primaries; PBS 1X + 0.2% BSA for secondaries.

Mounting medium: ProLong Gold Antifade with DAPI (Thermo Fisher, #P36935).

- **Staining protocol (cells)**

Pre-Fixation of plates with 4% PAF for 30 minutes. Then rinse them twice for 10 minutes with PBS before adding PBS until the day of immunostaining.

The day of immunostaining

1. **Fixation:**
   10 min with Methanol (1ml in the insert + 1 ml in the well)
2. **Permeabilization**
   5 min with permeabilization solution : PBS 1X + 0.5% Triton X100 (1ml in the insert + 1 ml in the well)

3. **Primary Antibody Incubation**:
    a. 100 µl/insert, 1.5 h at 37°C.
    b. Two dilutions tested for each antibody: 1:200 and 1:400
4. **Secondary Antibody Incubation**:
    a. 100 µl/insert, 1 h at 37°C.
5. Cutting the insert
6. **Nuclear Counterstaining**:
    a. Mounting medium with DAPI (Gold Antifade Mountant with DAPI, 13 µl/insert).
7. **Reagent Details**

Permeabilization buffer: PBS 1X (20 mM Tris-base, 150 mM NaCl, pH 7.6) + 0.5 % Triton X-100.

Antibody diluent: PBS 1X + 0.2% BSA for primaries and secondaries.

Mounting medium: ProLong Gold Antifade with DAPI (Thermo Fisher, #P36935)

Image Acquisition

- **Microscope**: Zeiss ApoTome Imager 2 with Zen acquisition software.
- **Settings**:
  - Objective: 40x oil immersion.
  - Fluorescence filters: Alexa Fluor 488 (ex/em 495/519 nm) and DAPI (ex/em 358/461 nm).
  - Z-stack imaging with Apotome optical sectioning to reduce background noise.
- **Analysis**:
  - Three slides per tissue type (mouse, piglet, heifer) imaged.
  - Staining intensity and localization assessed for Occludin at tight junctions.