Calprotectin Antibodies With Different Binding Specificities Can Be Used as Tools to Detect Multiple Calprotectin Forms

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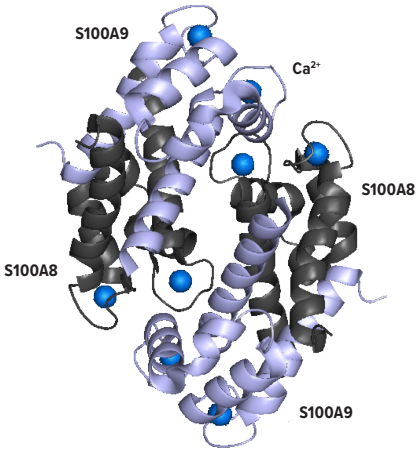
Calprotectin — A Pro-Inflammatory Protein

Calprotectin (leucocyte L1-protein) is a pro-inflammatory protein primarily secreted by neutrophils, macrophages and monocytes at the site of inflammation[1–4]. Neutrophils accumulate in mucosa, where calprotectin is released and easily detectable[4].

Calprotectin is comprised of two calcium-binding monomers, a 93-amino-acid **S100A8** (MRP-8) and a 114-amino-acid **S100A9** (MRP-14). Dimers pair non-covalently with each other, forming heterotetramers. S100A8 and S100A9 both contain two EF-hand type Ca^{2+} binding sites (Figure 1).

Elevated serum or fecal calprotectin levels are indicative of several inflammatory diseases, including rheumatoid arthritis[5], multiple sclerosis[6], cystic fibrosis[6] and inflammatory bowel diseases (IBD)[7]. Fecal calprotectin is an important factor in the follow-up of IBD development and monitoring of treatment response[8].

Figure 1. *The structure of human calprotectin heterotetramer. S100A8 monomer in dark grey, S100A9 monomer in violet and eight Ca2+ ions shown in blue. The model was generated using PyMol software from a publicly available calprotectin crystal structure (PDB ID: 1XK4).*



Materials & Methods

We have developed five mouse monoclonal antibodies (mAbs) against human calprotectin: **3403** (#100460), **3404** (#100468), **3405** (#100469), **3406** (#100470) and **3407** (#100618). Binding specificities of the antibodies were studied in fluorescent immunoassays (FIA) using purified recombinant monomeric calprotectin subunits S100A8 (#710018; Medix Biochemica) and S100A9 (#710019; Medix Biochemica), and the S100A8/A9 complex (#610061; Medix Biochemica) as antigens.

Antigens were coated onto a microtiter plate (#473709; NUNC®) at 50 ng/well, blocked for 1h at room temperature, and antibodies added at concentrations 31–1,000 ng/mL. Bound antibodies were detected using an europium (Eu)-labeled DELFIA® Eu-N1 Rabbit Anti-Mouse IgG antibody (#AD0207; PerkinElmer) as described previously[9].

The specificities of calprotectin antibody pairs were studied in sandwich FIA. Capture antibodies (150 ng/well) were incubated with the S100A8/A9 complex at concentrations 0.15–1,000 ng/mL. Biotin-conjugated [#704-0030, Lightning-Link® Biotin Conjugation Kit (Type A); Innova Biosciences] antibodies and Eu-labeled streptavidin were used to detect the bound antigens. A widely studied calprotectin antibody 27E10 (#T-1023; BMA Biomedicals), that recognizes the S100A8/A9 dimer but not individual monomers[10], was included as a reference antibody. Data were recorded on EnVision® Xcite Multilabel Plate Reader (PerkinElmer).

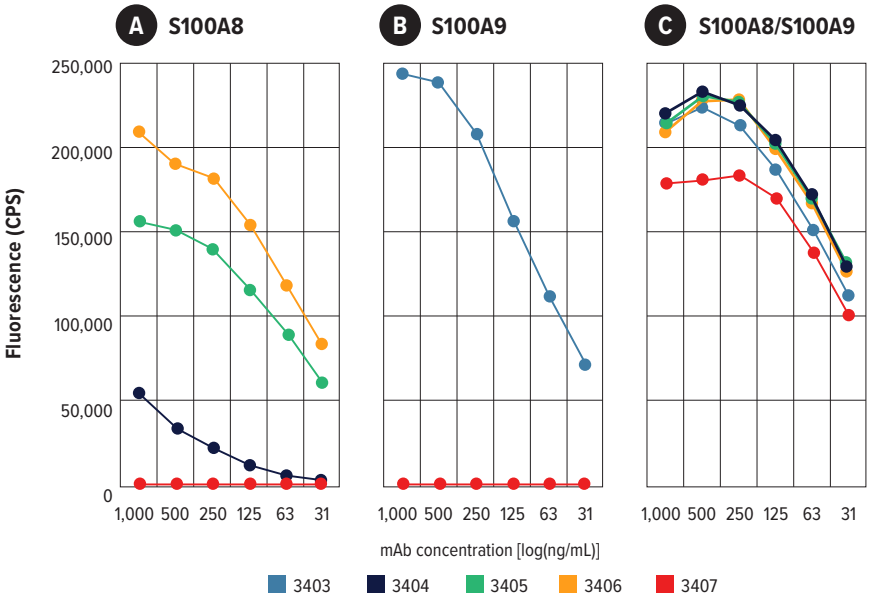
Binding Specificities of Calprotectin Antibodies

Direct FIA results with purified recombinant S100A8, S100A9, and S100A8/A9 complex proteins indicated that the antibodies were clustered in three groups:

1. Antibodies 3404, 3405 & 3406 that specifically recognized the subunit S100A8 (Figure 2A)
2. Antibody 3403 that specifically bound to subunit S100A9 (Figure 2B)
3. Antibody 3407 that did not bind to either of the isolated calprotectin monomers but specifically recognized the S100A8/S100A9 complex (Figure 2C)

All antibodies recognized the S100A8/A9 complex (Figure 2C).

Figure 2. *Binding of antibodies to calprotectin subunits. Detection of calprotectin subunits S100A8 (A) or S100A9 (B), and S100A8/A9 heterocomplex (C) by calprotectin mAbs 3403, 3404, 3405, 3406 and 3407.*



Calprotectin Antibody Pairing

Sandwich FIA results varied significantly between different antibody pairs. Antibody 3407 yielded similar results as the reference antibody 27E10 when used as a pair with itself (Figure 3A).

Significantly higher signal-to-noise ratios were obtained across the concentration range tested when 3407 was combined with an antibody recognizing a specific subunit, such as antibody 3406 or 3404 (Figure 3B).

Antibodies 3407 and 3403 did not function as a pair, indicating that their epitopes may be partially overlapping, despite the differences in calprotectin subunit recognition.

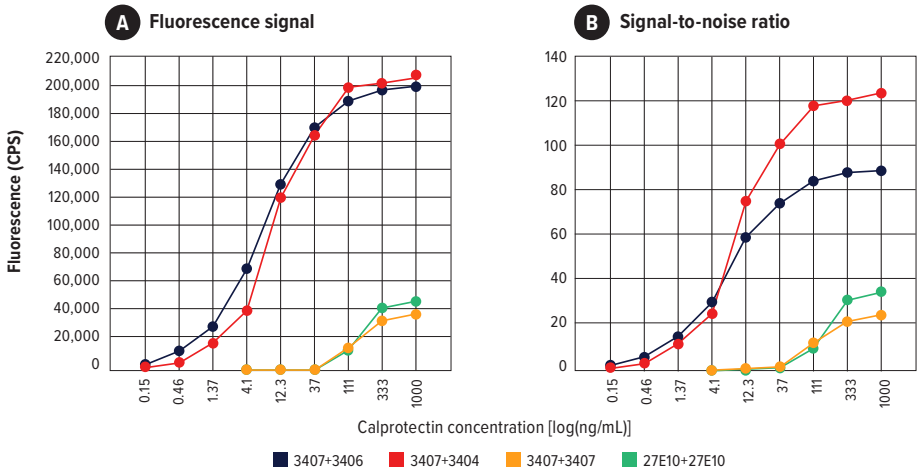
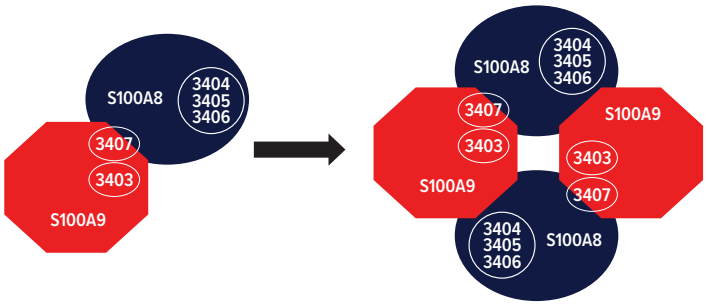


Figure 3. *Calprotectin antibody pairing. Fluorescence signals (A) and signal-to-noise ratios (B) of sandwich FIA assays using calprotectin mAbs 3404, 3406, 3407, and 27E10. Capture antibody is listed first in each pair.*

The Binding Profile of Calprotectin Antibodies

The present study demonstrated that each calprotectin antibody has a distinct binding profile for the calprotectin monomers and/or the heterodimeric/tetrameric calprotectin complex (Figure 4).

Figure 4. *A schematic representation of calprotectin antibody binding sites. The binding regions of calprotectin antibodies 3403, 3404, 3405, 3406, and 3407 within the calprotectin subunits S100A8 and S100A9. It is to be noted that this representation is purely schematic, as the exact amino acid residues of antibody-binding epitopes are yet to be determined.*



Heterodimeric calprotectin complex *Tetrameric calprotectin complex*

Conclusions

This study demonstrates that the developed calprotectin mAbs have variable specificities towards the calprotectin subunits, S100A8 and S100A9, and that the choice of mAbs has a significant effect on the assay outcome. The multimerization tendency of S100A8/A9 has been suggested to be a major reason for the observed substantial differences across commercial calprotectin assays[11]. Depending on the specificity of the mAbs used in each assay, different multimeric forms may be detected from patient samples.

Scientific interest towards the use of calprotectin as a biomarker for subclinical or clinical inflammation has increased substantially during the past years. In addition to IBD and rheumatic diseases, expression of the S100A8/A9 heterodimer has been found elevated in various cancer types[12] and cardiovascular diseases[13], where it has also been suggested to have potential prognostic value. However, the relative proportions of monomeric, dimeric and tetrameric forms of S100A8/A9 in different diseases, and their correlation with disease status remain to be elucidated.

The highly specific calprotectin mAbs described in this study may be used for the development of new diagnostic assays for various inflammatory diseases. Owing to their well-characterized binding specificities, these calprotectin mAbs have vast potential for utilization across various clinical applications that require accurate distinction between the monomeric and multimeric calprotectin forms.

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