# *ABCD_RC012 recognizes Dictyostelium discoideum AplA protein by ELISA and western blot*

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## Abstract

Four recombinant antibodies ABCD_RC012 to ABCD_RC015 were shown to detect the purified *Dictyostelium discoideum* AplA protein by ELISA. In addition, the ABCD_RC012 antibody recognizes AplA by western blot in non-reducing conditions in *D. discoideum* cells overexpressing AplA.

## Introduction

AplA (Amoeba Saposin A, DDB_G0284043, UniProt #Q54Q68) is a member of the saposin family of proteins (IPR008373). Saposins play a crucial role in activating hydrolase enzymes involved in sphingolipid degradation (Rorman *et al*., 1989). In this study, we describe the ability of four recombinant antibodies (ABCD_RC012–RC015) to detect, via ELISA, a purified Twin Strep-tagged AplA protein from *D. discoideum*. Moreover, we show that ABCD_RC012 specifically recognizes AplA by western blot in *D. discoideum* cells overexpressing AplA, but not at the endogenous levels in wild-type DH1 cells.

## Materials & Methods

**Antibodies:** ABCD_RC012 to ABCD_RC015 antibodies (ABCD nomenclature, http://web.expasy.org/abcd/, referred to collectively as RC012-RC015) were discovered by the Geneva Antibody Facility (http://unige.ch/medecine/antibodies/). Briefly, a synthetic VHH phage display library was panned against a Twin Strep-tagged AplA protein (see antigen section). After three rounds of panning, selected phage vectors were isolated using a plasmid preparation kit (Qiagen), and the VHH inserts were subcloned into custom-made expression vectors and sequenced. The selected antibodies were produced as minibodies with the antigen-binding VHH fused to a rabbit IgG Fc. HEK293 suspension cells growing in HEK TF medium (Sartorius #861-0001), supplemented with 0.1% Pluronic F68 (Sigma #P1300) were transiently transfected with the vectors coding for each VHH-Fc. Supernatants were collected after 3 days. Estimated production yields were approximately 30-53 mg/L for all antibodies.

**Antigen:** We used a fusion protein composed of a human IL2 signal sequence for insertion in the ER followed by the full coding sequence of the *D. discoideum* AplA protein without sequence signal (amino acids 22 to 522) and fused at its C-terminus to a Twin-Strep-Tag® (IBA Lifesciences, AplA-TST). The fusion protein was produced in transiently transfected HEK293 cells and purified using MagStrep Streptactin XT beads according to the manufacturer instruction (IBA Lifesciences #2-5090-002). A TST tagged cprD protein from *D. discoideum* (cprD-TST, UniProt #P54639, residues 111-442) was produced the same way and used as a negative control.

**ELISA:** The whole ELISA procedure was carried out at room temperature. Purified TST tagged proteins were coated directly on Maxisorp Elisa plates (Nunc #44-2404-21) at 3 µg/ml in PBS for 45 min. As a saturation agent, 50 µL of PBS-BSA 3% (w/v) was added, followed by 20 minutes of incubation. Each well was then rinsed three times with 100 µL of washing buffer (PBS + 0.5% (w/v) BSA + 0.05% (w/v) Tween20), then incubated for 1 hour with 50 µL of RC antibody-containing supernatant diluted in washing buffer (Fig. 1). After rinsing 3 times with 100 µL of washing buffer, wells were incubated with horseradish peroxidase-coupled goat anti-rabbit IgG (Invitrogen #A16110, dilution 1:1000, 50 µL per well) for 30 min. After 3 rinses, Tetramethylbenzidine (TMB) substrate (Sigma #T5569) was added (50 µL per well). The reaction was stopped by the addition of 25 µL of 2 M H2SO4. The absorbance (OD) was measured at 450 nm, and the absorbance at 570 nm was subtracted for background correction.

***D. discoideum* cell lines:** Cells were cultured in HL5 medium at 21°C (Froquet *et al*., 2009) in 10 mL Petri dishes. The AplA knock-out (AplA KO) line was generated in a DH1-10 wild type background (Cornillon *et al*., 2000) by deleting 521 nucleotides of the genomic sequence and replacing them with a blasticidin resistance cassette via homologous recombination (Ayadi *et al*., 2024). To generate AplA KO cells overexpressing AplA (AplA KO + AplA), the full-length AplA coding sequence was cloned into the G418-resistant prepSC3 vector and transfected into AplA KO cells, as described previously (Froquet *et al*., 2012). Clones were selected with 15 mg/L G418.

**Western blot:** $6x10^6$, $12x10^6$ or $24x10^6$ *D. discoideum* DH1 cells were pelleted and resuspended in 240 µl of sample buffer (20.6% (w/v) sucrose, 100 mM Tris pH 6.8,

10 mM EDTA, 0.1% (w/v) bromophenol blue, 4% (w/v) SDS; 6% (v/v)). For reducing conditions, 3% (v/v) ß-mercaptoethanol was added. 20 µl of each sample (corresponding to $5x10^5$, $1x10^6$ or $2x10^6$ cells per well) were loaded and electrophoresed (200V, 35 min) on a 4-20% acrylamide gel (Genscript, SurePAGE Bis-Tris, #M00657), then transferred onto a nitrocellulose membrane using a dry transfer system for 7 minutes (iBlot 2 Gel Transfer Device, Invitrogen #IB21001). Membranes were blocked for 60 min in PBS containing 0.1% (v/v) Tween 20 and 7% (w/v) milk, and incubated overnight at 4 °C with the primary antibody (5 µg/ml in PBS-Tween + 7% milk). After three washes (15 + 15 + 10 min) in PBS-Tween, membranes were incubated for 1 hour with horseradish peroxidase–conjugated goat anti-rabbit IgG (Invitrogen #A16110, 1:3000 dilution), then washed five times (5 min each) in PBS-Tween. The signal was detected by enhanced chemiluminescence (ECL) (Immobilon® Classico, #WBLUC0500) using a Fusion FX gel imaging system (Vilber).

## Results & Discussion

By ELISA, antibodies RC012 to RC015 bound to AplA-TST in a concentration-dependent manner, but not to the negative control CprD-TST. RC012 showed a weak background signal at the highest concentration (Fig. 1).

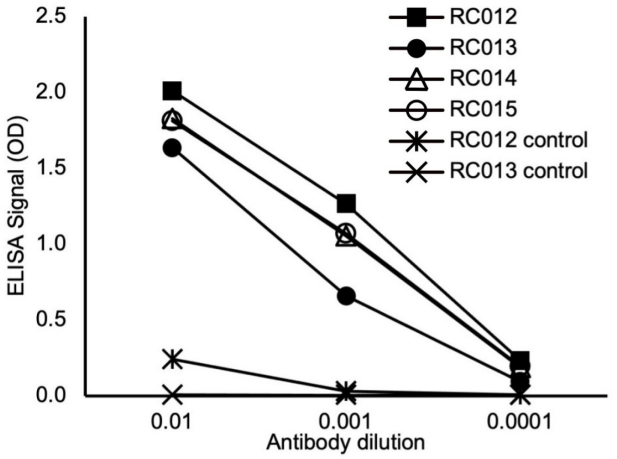


**Fig. 1.** Specific binding of antibodies RC012 to RC015 to the target AplA-TST protein, but not to the negative control (no antigen; shown only for RC012 and RC013; background curves for the other antibodies are superimposed with the RC013 control), as detected by ELISA.

Following ELISA validation, the ability of the four antibodies to recognize the full-length protein was assessed by western blot analysis (Fig. 2). Western blotting was performed using lysates from *D. discoideum* cells. AplA KO cells served as a negative control, whereas AplA KO cells overexpressing the AplA protein were used as a positive control. Under non-reducing conditions, RC012 detected a distinct band of approximately 60 kDa, consistent with the predicted molecular weight of the AplA protein (57 kDa). However, under reducing conditions, antibodies RC012 to RC015 detected non-specific background signals. This suggests that epitope recognition by RC102 is likely dependent on the native or partially folded conformation of AplA and is disrupted upon reduction, consistent with the known structural properties of saposin-like proteins, which are stabilized by disulfide bonds (Vaccaro *et al*., 1999).

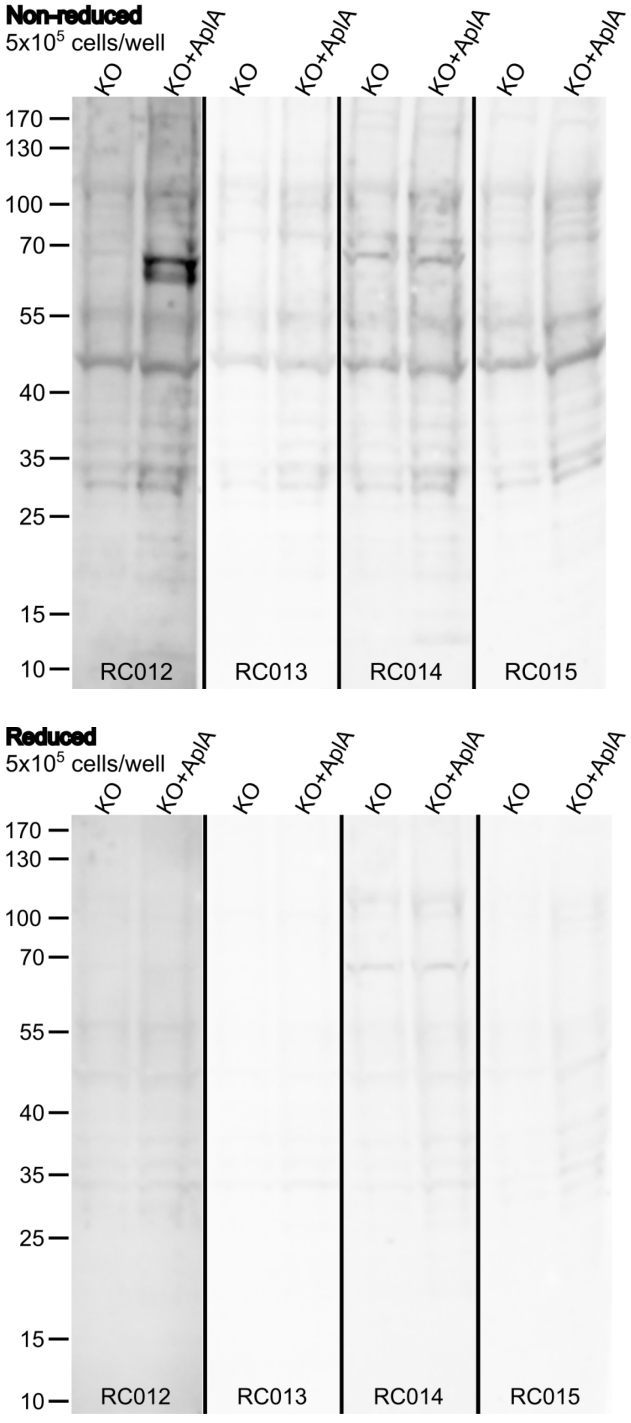


**Fig. 2.** Western blot of *D. discoideum* lysates from AplA KO cells and AplA KO cells overexpressing AplA, under non-reducing or reducing conditions. RC012 detected a specific band at ~60 kDa under non-reducing conditions.

Finally, RC012 was tested for its ability to detect endogenous levels of AplA by western blot in DH1-10 wild-type cells. AplA KO cells were included as a negative control. Under these conditions, RC012 failed to detect

endogenous AplA in wild-type cells (Fig. 3). This suggests that endogenous AplA is either expressed at levels below the detection limit of the antibody under the conditions tested, or that its native cellular context limits epitope accessibility. AplA belongs to the saposin family of proteins, which share conserved structural features and sequence similarities. Further analyses would be required to assess potential cross-reactivity of RC102 with other members of this protein family.

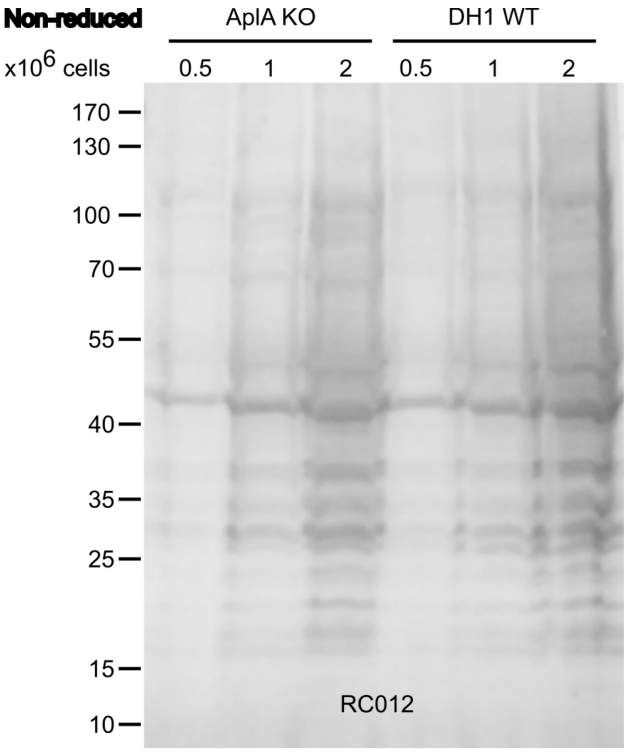


**Fig. 3.** Western blot analysis of *D. discoideum* lysates from wild-type (WT) and AplA knockout (AplA KO) cells using antibody RC012 under non-reducing conditions. RC012 failed to detect endogenous levels of AplA in wild-type cells.

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## Conflict of interest

Philippe Hammel is a cofounder and shareholder of ABCD antibodies SA.

## Data Availability Statement

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