96-WELL PLATE COMPATIBLE ASSAY FORMAT FOR SCREENING ANKYRIN REPEAT PROTEINS ON FILTER PAPER

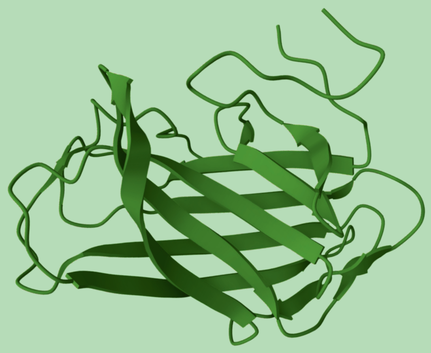

Figure 1 Protein structure of cellulose binding domain. PDB: 4JO5

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Introduction

The project is divided into two different aims:

1. **Assess cellulose binding domains (CBM) to bind different filter papers**
2. **To control capillary flow of the paper between separate wells**

Plastic single use products are an everyday sight in the lab. This is a heavy burden to the nature and petroleum based plastic is not environmental choice. This has led us to seek alternative options. Our solution for the problem is paper based immunoassay.

In this study cellulose binding domain (CBM) (**Figure 1**) is from *Clostridium thermocellum* and it's fused to anti-eGFP protein. The binding towards cellulose paper occurs via ten polar amino acids that binds on the surface of cellulose.

Binding of the complex is detected with eGFP which works as model antigen. Different assay parameters were determined such as dynamic range for the CBM complex, optimal antigen molarity, and dissociation of the complex with increased wash steps.

In the future the assay may be optimized to replace plastic 96 well plates in recombinant protein screening assay.

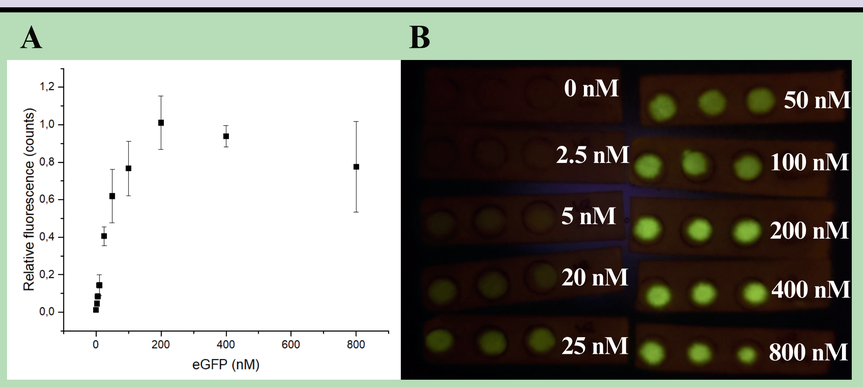


Figure 3 GFP saturation binding assay on anti-GFP-CBM fusion fusion coated filter. A) Optimal GFP content in the assay was determined via assay that contains 200 µg/ml CBM-clamp as triplicates and in range from zero to 800 nM of eGFP was added. Saturation initiates after 200 nM and the optimal GFP concentration for the paper assays is 400 nM. measured volumes of eGFP, fluorescence measured with BioRad volume tool. B) Dark reader image with different molarities of eGFP.

Results

The main results in the thesis -project was to proof that it is possible to make an immunoassay on top of filter paper with CBM-fusion protein. Dynamic range for the detection of anti-GFP-CBM fusion protein in the presence of 400 nM GFP was 25 µg/ml to 800 µg/ml (**Figure 2B**). The sensitivity between different filter papers was calculated as well and the best sensitivity was detected in Whatman grade 0903 with clamp-CBM. Nitrocellulose had lowest eGFP signals and the highest clamp signal which means that the nitrocellulose binds proteins unspecific. The optimal GFP molarity was determined to be 200 nM (**Figure 3A**). We used 400 nM in the assays to determine that GFP label is not the limiting factor.

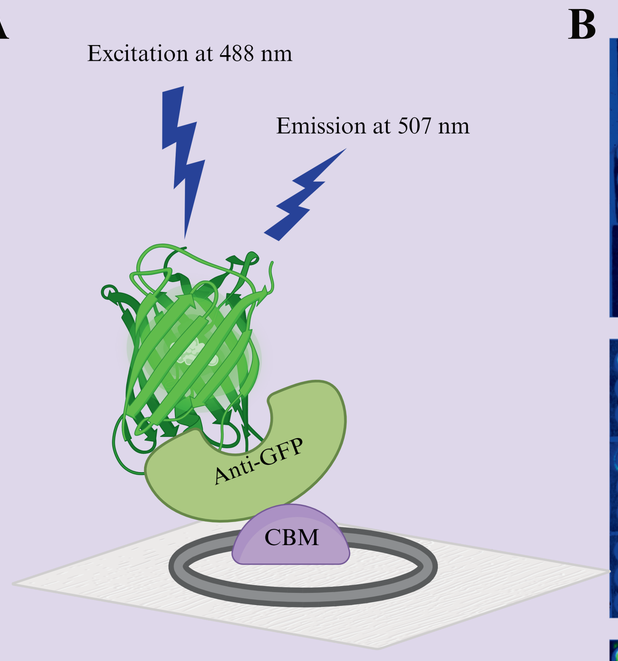
Conclusions

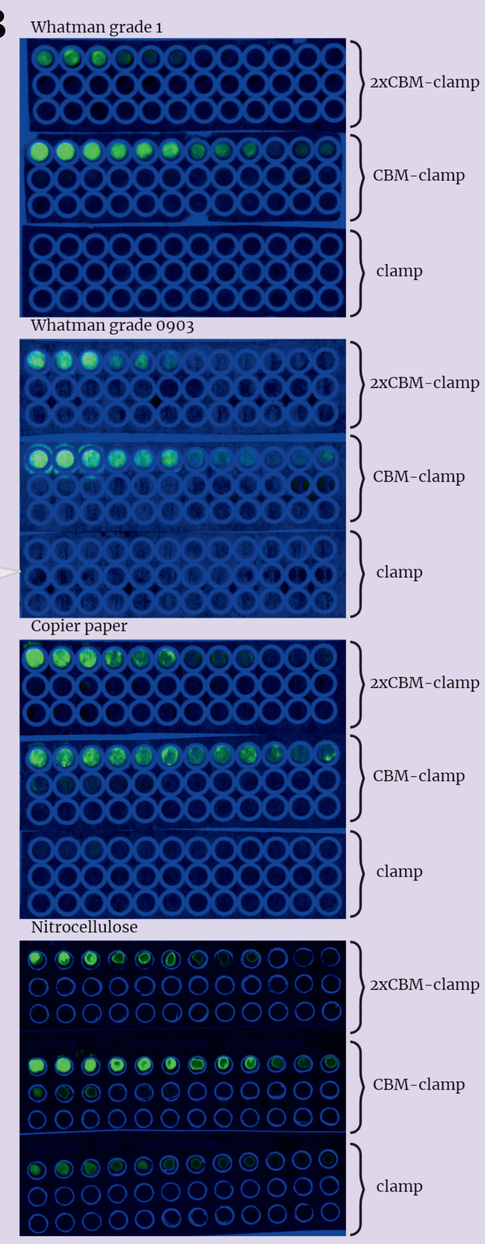
First aim in the project was to proof that CBM is needed in the immunoassay complex which we demonstrated in **Figure 2B**.

Overall we did not reach the maximum binding capacity or saturation of the Whatman filters due our produced proteins which stock concentration reached up to 0.91 mg/ml.

Detection limit was determined to be 25 µg/ml which can be improved by changing imaging method and/or label, or increasing the sample volume from 5 µl. For the proof-of concept study the limit is enough.

CBM binding was studied with increased washes which revealed that CBM binds tightly to the filters without significant reduce in the eGFP signal.





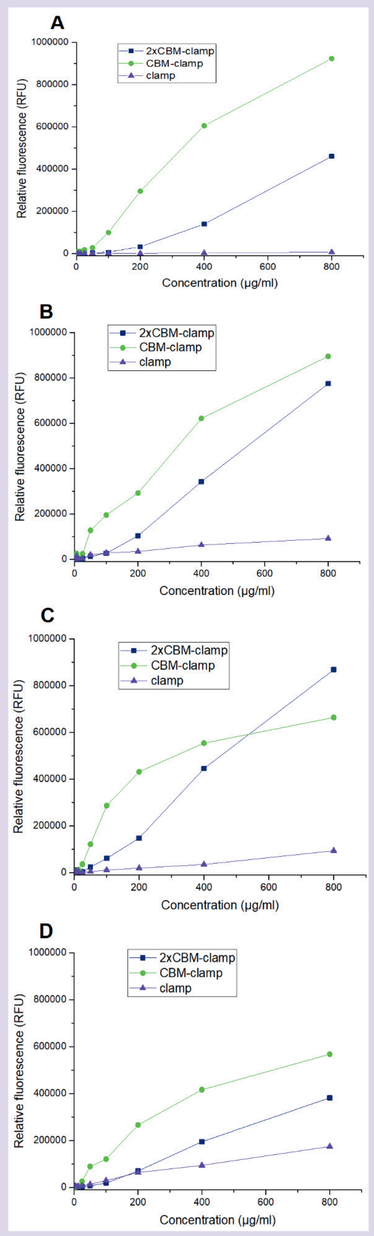


Figure 2 A) Illustration of the complex build on the filter paper with 3D printed well. Clamp-CBM -fusion binds to the filter paper with printed PLA well and antigen eGFP binds tightly to the complex. This image was prepared with BioRender. B) Comparison of different papers in the maximum dynamic range imaged with BioRad (in the left) and RGB signal determined with python script; BA Whatman grade 1, BB Whatman grade 0903 BC copier paper and BD nitrocellulose.

Materials and methods

Wells for the assays were printed straight on top of filters (Whatman grades 1 & 0903, copy paper, nitrocellulose) with poly lactic acid (PLA) with standard 3D printing settings (Prusa MK4, bed 60 °C, extruder 215 °C, 0.4 mm nozzle). In the protocol 5 µl of clamp-CBM or clamp-2xCBM samples was added to drawn or 3D printed well. Rest of the paper was coated with 5 % w/v milk powder in PBST0.05 and 400 nM eGFP label was added to the blocking solution after 30 min. After one hour incubation, unbound eGFP was washed three times with PBST0.1 and the filter paper was imaged with Bio-Rad imager and dark reader, and for the washing assay Hidex imager. Clamp was used as a binder control. Images were analyzed with python script or Bio-Rad imager volume tool and figures drawn with Origin 2016. All used constructs for the assays were produced in E. coli under IPTG-inducible promoter overnight and purified with Ni-NTA followed by SEC. Purity of the constructs were verified with SDS-page.

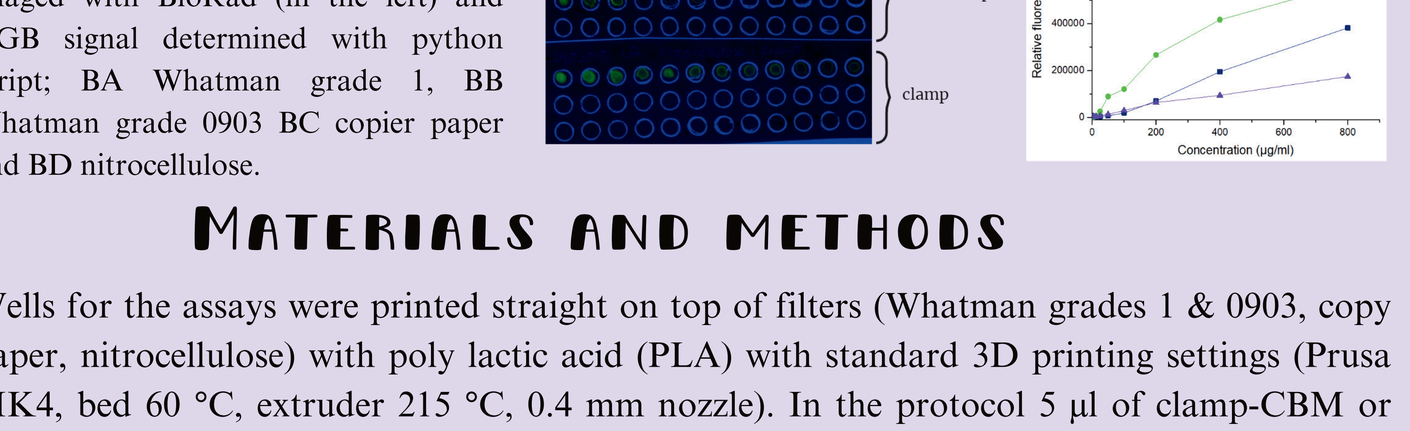


Figure 4 The binding of CBM towards cellulose was studied with increasing amount of washes of the complex. The studied constructs were clamp-CBM-CBM, clamp-CBM**,** and clamp as control. The study revealed that CBM binds extremely tightly and there is no significant decrease in the e GFP signal. The filter paper assay was imaged with BioRad Chemidoc Imager (excitation 488 nm, emission filter 530/28, exposure 0.05 s)(A) and quantitated using a python script that automatically identifies wells and sums up the green pixel values from the imported jpg-image. B) The filter paper assay was quantitated with Hidex for comparison (excitation 488 mm, modified z-plane to 1 mm) C) raw image from BioRad before signal detection.

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