NANOBODIES: promising reagents in basic research and biomedicine

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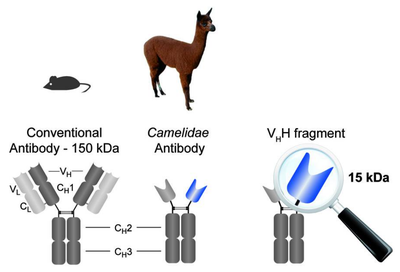
Introduction

Conventional antibodies vs Heavy chain antibodies.

Antibodies (Abs) are proteins produced by the immune system to capture antigen **epitopes**. There are two types of antibodies: **conventional (eg IgG)** and **heavy chain Abs** (HCAb). Conventional antibodies consist of heavy and light chains and interact with antigens *via* both variable domains. HCAb is a type of antibody comprising only of heavy-chain domains. They were found in 1990's in the family *Camilidiae* and cartilaginous fishes. The *VHH* domain on the HCAb is referred to as the nanobody.

Антитела - это белки, вырабатываемые иммунной системой для захвата антигенов. Существует два типа антител: 1 - состоящие из тяжелых и легких цепей , 2 – состоящие только из тяжелых цепей (HCAb). HCAb были обнаружены у сем. Верблюдовые и у хрящевых рыб в 1990-х. VHH домен HCAb называют наноантителами.



Conventional antibody	Heavy-chain antibody
Will denature at higher temperature and pH	Resistant to heat and pH
Large	Small in size
Has two HC and two LC	Has variable HC domain
Complex protein	Simple protein
outsourced	Can make in lab

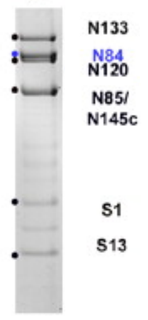
Nup84 complex

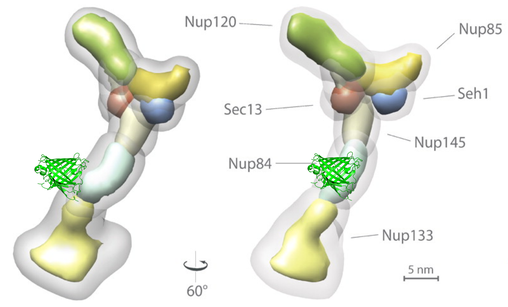
The Nup84 complex constitutes a key building block in the nuclear pore complex (NPC). This complex is composed of 7 different proteins. The main functions of Nup84 complex are related to nucleocytoplasmic transport and NPC biogenesis. It also plays roles in several processes that may require localization of genes or chromosomes at the nuclear periphery, including double-strand break repair, transcription and chromatin silencing.

Комплекс Nup84 представляет собой ключевой строительный блок в ядерном поровом комплексе (NPC). Этот комплекс состоит из 7 различных белков. Основные функции Nup84 - это осуществление нуклеоцитоплазматического транспорта и формирование NPC. Он также участвует в некоторых процессах, необходимых для локализации генов или хромосом в ядре, включая репарацию двунитевых разрывов, транскрипцию и сайленсинг хроматина.

MW of Nups in Nup84 complex
- Nup133 = 129 kDa
- Nup120 = 120 kDa
- Nup84**+GFP** = ~112 kDa
- Nup145c = ~82 kDa
- Nup85 = ~80 kDa
- Seh1 = 39 kDa
- Sec13 = 33 kDa

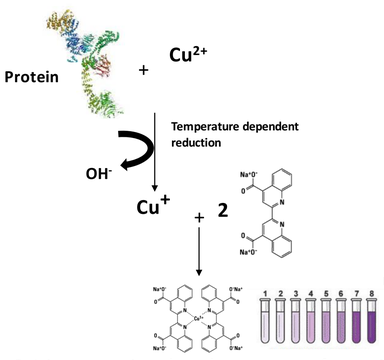
Fernandez-Martinez et al.; 2012





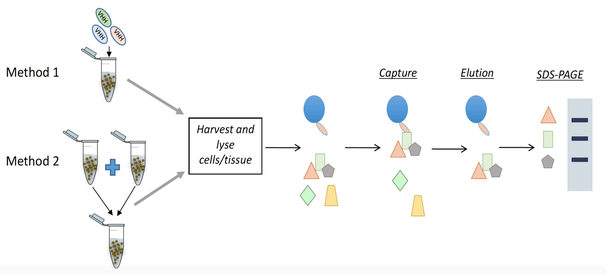
Methods

Bicinchoninic acid (BCA) assay



Cu^+ is proportional to the amount of protein
Cu^++BCA_2 is proportional to the amount of protein
$Abs562_{nm}$ is proportional to the amount of protein

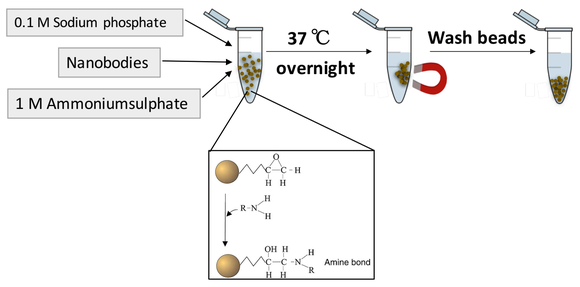
Affinity capture experiments:



Method 1: *mixing different nanobodies, then conjugating mixture to beads*

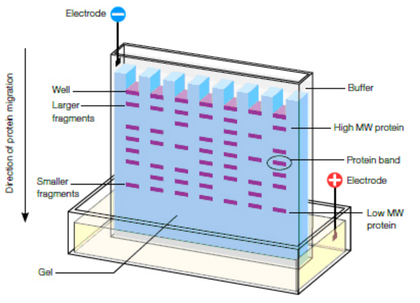
Method 2*: mixing different beads conjugated with single nanobodies*

Dynabeads conjugation with nanobodies



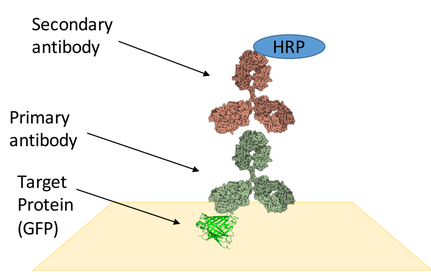
*Dynabeads are paramagnetic beads that carry an **epoxy group**. These epoxy groups covalently bind to amine groups on the proteins.*

SDS-PAGE electrophoresis gel



In order to separate proteins according to their molecular weight we used SDS-PAGE.

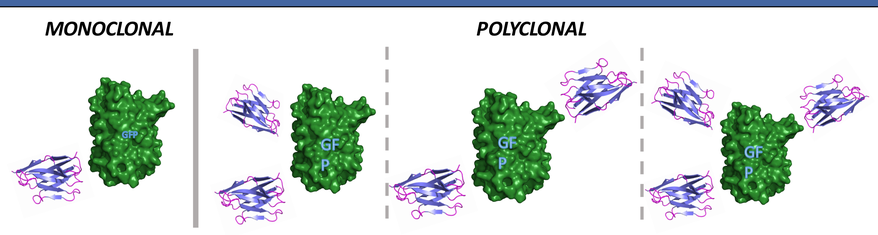
Western-blot experiments



Target protein containing GFP binds to anti-GPF primary antibody. Secondary antibody with HRP allows us to visualize protein bands.

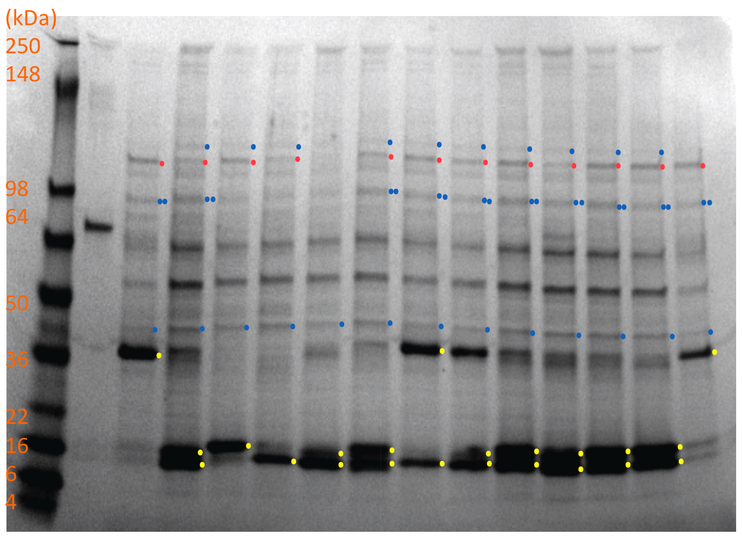
Project 1: Monoclonal and polyclonal nanobodies for capturing Nup84 complex

Antibodies can be ***monoclonal***, where they are specific only for one epitope on an antigen and ***polyclonal***, where there are mixtures of antibodies that can bind an antigen on many different epitopes.
The main aim of this project is to estimate whether monoclonal or polyclonal antibodies allows us to capture our antigen (Nup84). Thus, we proceed to produce mixtures of individual nanobodies and compare their efficiency in affinity capture experiments to monoclonal antibodies.



RESULTS

Monoclonal *Vs* Polyclonal affinity capture (AC)



LOADING LIST
1. MW Marker
2. BSA 20 ug/mL
3. LaG 1
4. LaG 2
5. LaG 3
6. LaG 4
7. LaG m18
8. LaG 1+ LaG 4
9. Lag 1+ JaG m18
10. LaG 2+ LaG 3+ LaG 4
11. LaG 2+ LaG 3+ LaG m18
12. LaG m18+ LaG 2
13. LaG 3+LaG 2
14. LaG 3+ LaG m18
15. Control LaG 1

Nup84-GFP
Associated proteins
Nanobodies

Figure 1 SDS-PAGE gel of different affinity capture experiments comparing *monoclonal* nanobodies to *polyclonal* nanobodies.

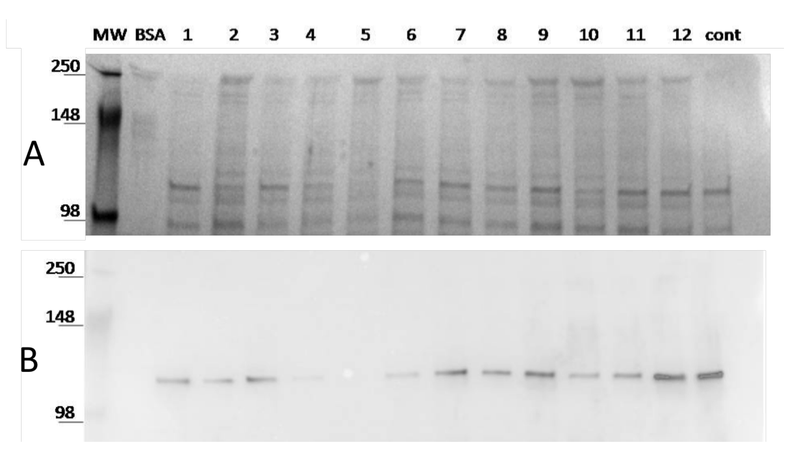


Figure 2 (A)- SDS-PAGE Blue Silver R250 staining and (B) corresponding Western Blot with α-GFP antibody

DISCUSSION AND CONCLUSIONS

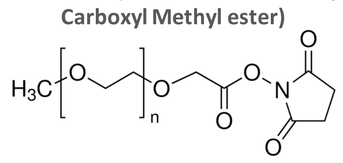
From the SDS-PAGE of affinity capture experiments we cannot definitively identify the protein Nup84-GFP and its associated proteins for a few reasons:
- Due to unusual molecular weight standard mobility compared to BSA control
- Due to the poor resolution in the AC experiments.

Thus we performed a Western blot to identify target Nup84-GFP protein localization using anti-GFP antibody. The results allowed us to determine that Nup84-GFP was indeed captured.
We observed that the bands differ in intensity and that most of the polyclonal combinations resulted in a higher intensity band. Thus we can interpret a highest affinity when mixing monoclonal nanobodies in order to obtain polyclonal nanobodies. The most effective combinations results from LaG 3 and m18. This combination performs the same affinity of the control. Nevertheless, we can underline that m18 by it self does not show any band.

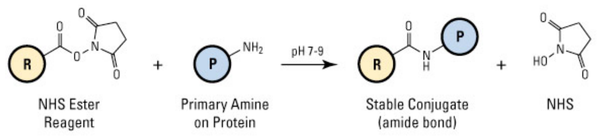
Project 2: Varying PEG types and concentrations for capturing Nup84 complex

Polyethylene glycol (PEG) is a polymer that is commonly used to assist potential drug targets being recognized by the immune system.
The purpose of this project is to optimize the nanobody-pegylation reaction by varying different parameters in the reaction mixture. Parameters we aim at changing are (i) concentration of nanobody; (ii) concentration of PEG reagent and (iii) incubation time. We will perform the reactions using two PEGs with two different molecular weights, being 5 kDa and 20 kDa.

mPEG-SCM (PEG-NHS: Succinimidyl Carboxyl Methyl ester)



Pegylation reaction



RESULTS

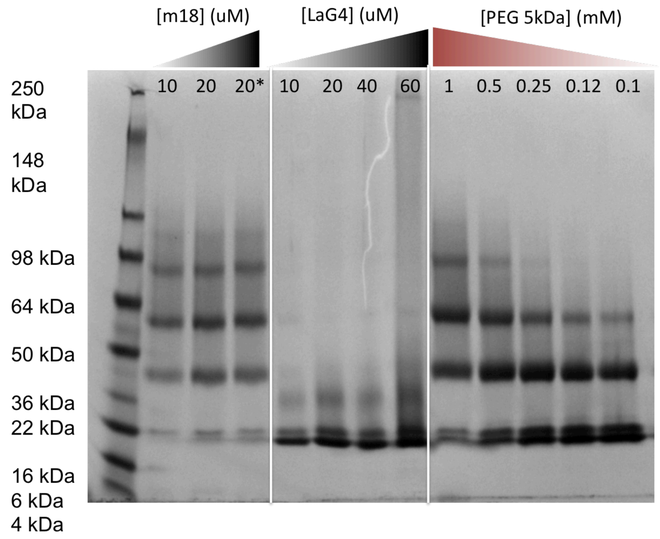


Figure 3 SDS-PAGE protein gel showing results of varying different parameters in the pegylation reaction

DISCUSSION & CONCLUSIONS

From the SDS-PAGE in Figure 3, we see that increasing the concentration of m18 whilst keeping the concentration of PEG constant, higher concentrations of m18 in the reaction shows more un-pegylated m18.
We see that the reactions with 20 kDa PEG did not modify as many lysines as 5 kDa PEG.
Wee see that decreasing the concentration of 5 kDa PEG results in less modified lysines.
Because of this, we conclude that a larger ration of [PEG]:[protein] is necessary to favor more pegylated lysines.