Monoclonal Antibodies Against Transcription Factors Preliminary Characterization for Use in Affinity Capture

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Introduction

Cellular functionality is chiefly mediated through protein-protein interaction (PPI) networks, the study of which enables understanding of the molecular biology of the cell. Moreover, diseases can frequently be traced to changes in the PPIs formed by proteins within the cell. In our project we decided to make a preliminary attempt to purify protein complexes associated with transcription factors (TFs) using affinity capture.

This decision was motivated by the fact that TFs are essential for gene expression, and (save for very few well studied factors) are known to have poorly described PPIs. Understanding what proteins cooperate with TF's will provide us with a picture of how PPIs coordinate to control gene expression, which could result in a breakthrough in our understanding of TF biology and biomedicine.

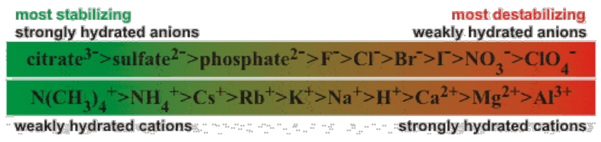
Example: **TP53**, among the most well studied TFs, acts as a *tumor suppressor*. Such proteins commonly exert influences on cell division, *preventing cells from growing and dividing too fast* or in an *uncontrolled way*, as well as on DNA repair, ensuring errors and breaks in the genome are properly resolved. Tumor suppressor proteins may also provide signals that trigger cells to undergo a programmed death (apoptosis) when cell division and/or DNA repair problems cannot be adequately resolved.

We tested antibodies against the transcription factors TP53, STAT3 and SMAD7 for use in affinity capture of their associated protein complexes.

Methods

1. Breaking Cells

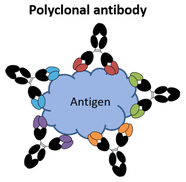
In our experiments we used a **Planetary Ball Mill** to produce a cell powder. The **cells are broken in the solid state at liquid nitrogen temperature** so that no additional solutions are needed. These can affect downstream experiments. The principle is that the balls in the jar are subjected to planetary motion (orbiting a central point and also spinning on an axis), producing frictional and impact forces. This procedure reduces cells to a **fine powder of roughly micron-scale**; which enhances the extraction of cell components.

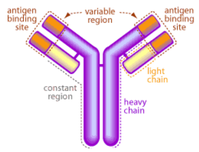


Described at the right →

2. Antibodies

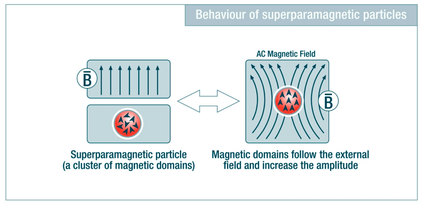
An **antibody** is a **Y-shaped** protein that is used by the immune system to identify and neutralize pathogens by specifically binding to a target foreign molecule with high affinity. Because of this property, antibodies (Abs) are used by researchers as reagents to identifying and/or capture target proteins and protein complexes. Abs consist of **four chains** (two heavy, and two light) and contain variable domains at the N-terminus of the protein (**paratope**) that allow making contacts with antigens (**epitope**). This characteristic is used by a vast range of biochemical analyses, including western blots and affinity capture.

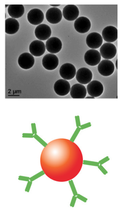




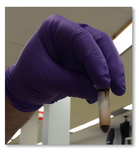
In nature there also exists a variant of conventional Abs which is composed of only the heavy chain (HCAb). The variable region that recognizes the antigen on **HCAb** can be chopped of and used as smaller an affinity reagent much smaller than a conventional antibody and which can be produced in bacteria. This is called a nanobody.

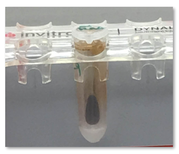
3. Superparamagnetic beads





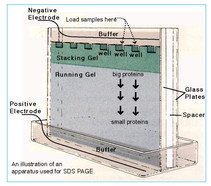
We used *superparamagnetic beads* (**Dynabeads M-270 Epoxy**) as the solid medium to immobilize our antibodies. These beads have many properties that make them excellent material for affinity capture sample preparation: **1.** The **small spherical size** of the beads (*2.8 µm diameter*) provides a large relative surface area, permitting high density antibody coupling and **fast surface binding kinetics** as well as **rapid magnetic separation** from solutions – so they can be washed and handled very easily. **2.** The surface exhibits **low nonspecific binding**, reducing the need for additional blocking agents. Epoxy reactive groups on the beads primarily **react with sulfhydryl and amino groups** in proteins.

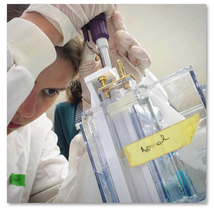




3. Extraction Solutions

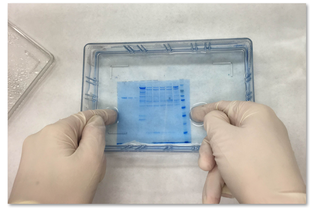
The solution used to extract protein complexes from cells should separate them from other components of the cell and maintain the integrity of the interactions within the discrete complexes. Depending of the chemical character of the solution used, the solubility and/or stability of different protein complexes may change. Some factors that **affect** the **stability of protein interactions** are the **pH** of the solution, the **detergent**, and the **salt** used. The capacity of salts to make protein precipitate or dissolve is catalogued into the **Hofmeister series** (aka the lyotropic series). In the diagram (**LEFT**), some stabilizing and destabilizing ions are displayed in order. Hofmeister also discovered that anions have larger effect than cations.





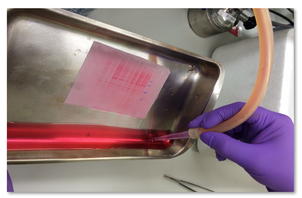
4. SDS-PAGE

sodium dodecyl sulfate-polyacrylamide gel electrophoresis is used to separate proteins on the basis of their molecular mass. The SDS detergent binds proteins strongly and charges them negatively. Proteins loaded onto a gel migrate because of and electric field from the cathode to the anode. The result is that proteins are separated by their mass across the gel.



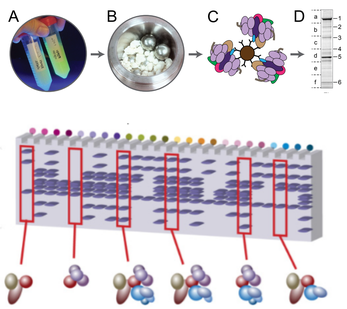
5. Detection of Proteins

Detection may be achieved in many ways including general protein staining, e.g. with **Coomassie Brilliant Blue (CBB)** – shown above. **CBB** typically has a *lower detection limit* (or sensitivity) in the tens of nanogroams range (compare that with another general protein staining, **silver stain**, which has a sensitivity *in the nanogram range*). We used a **CBB** formulation known as "blue silver," because its sensitivity is more comparable to **silver staining** than traditional **CBB** formulations. **Western blot** is an analytical technique used in molecular biology to detect specific proteins in a sample. After SDS-PAGE the proteins are then electrophoretically transferred to a membrane and they can can be probed with antibodies to reveal the presence of specific targets. To detect proteins we used **Chemiluminiscent detection**.



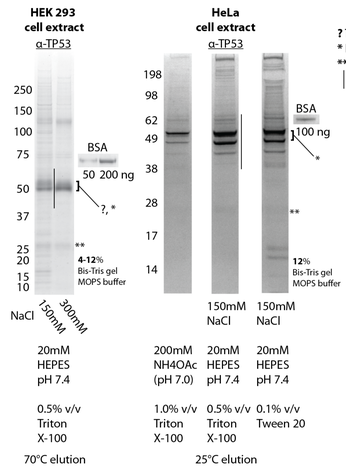
Ponceau stain may be used for Western Blot to reveal some major bands or to confirm that proteins have been successfully transferred in the membrane. It is reversible and easily removed by water

6. Overall Workflow



Cells are milled at cryogenic temperature to produce a powder. The **cell powder is extracted** in many ways in an attempt to preserve protein complexes during **affinity capture** using antibody coupled magnetic beads. **Different conditions** will yield distinctive results. Numerous methods exist to **detect and visuals proteins and protein complexes.**

Initial Results



LEGEND
? TP53 ~44kDa
* IgG HC ~50kDa
** IgG LC ~25kDa
Region of Interest to be analyzed by MS

(**LEFT**) The presence of TP53 is obscured by the 50kDa antibody bands but an extra density is observed in HEK cell extracts. A comparable additional density was not observed in HeLa cell extracts. Several prominent bands will be excised and analyzed by mass spectrometry to verify their identities.

LEGEND
& STAT3 ~88kDa
% SMAD7 ~46kDa
* IgG HC ~50kDa
** IgG LC ~25kDa
Region of Interest to be analyzed by MS

(**LEFT**) The presence of STAT3 is observed at the appropriate molecular mass in both HEK and HeLa cell extracts. An extra density may also correspond to SMAD7, but is obscured by IgG HC – the density is distinct from the suspected TP53 density.

Summary

Although no apparently stiochiometric interactors were observed in the conditions tested, additional bands were observed. These may correspond to physiological cofactors of the TFs being studied. After verification of the suspected TF bands by MS, additional MS of the other proteins will be carried out. These results may also support potential differences in TF abundance in HEK and HeLa cells.