Tackling Sample Loss and Lot-to-Lot Variability During Protein Labeling

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INTRODUCTION

One of the first steps in the development of a ligand-binding assay consists of ensuring the availability of appropriately labeled detection and capture reagents. While widely used labeled proteins can be obtained from commercial sources, reagents specific to the target analyte often have to be labeled for a single purpose. Protein labeling is often done routinely and may be standardized and optimized to work for a variety of proteins, including antibodies and recombinant peptides. Besides the actual labeling reaction, typical protocols includes protein buffer exchange before and after labeling to remove interfering chemicals and excess label, respectively. Nevertheless, in some cases, low protein recovery or variability between batches can be observed and may affect assay performance and reproducibility. Protein loss during labeling can also present a critical issue, if the amount of starting material is limited.

Here, we present an example where loss of protein was observed after labeling, specifically during the buffer exchange steps. We compared different buffer exchange methods regarding yield and consistency.

THE CHEMISTRY OF NHS ESTERS

N-hydroxysuccinimide (NHS) esters readily react with primary amines in neutral to slightly alkaline conditions (pH 7.2 to 9) to form stable amide bonds (figure 1). This property is widely exploited in order to covalently label proteins with a variety of compounds such as fluorescent dyes biotin and PEG and even entire proteins such as horseradish peroxidase.

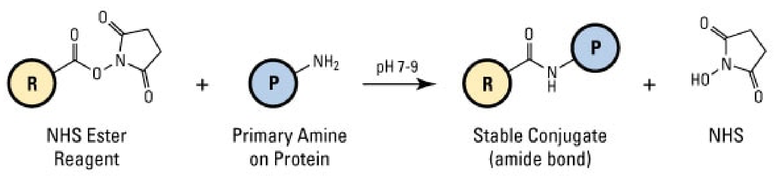

Figure 1. Reaction scheme of an NHS-ester reagent (R) with a primary amine of a protein (P).

As NHS esters are prone to hydrolysis, the water in aqueous reaction buffers competes with the reaction on the primary amine. The efficiency of the labeling reaction will therefore decrease in less-concentrated protein solutions. Additionally, buffer compounds or preservative agents containing primary amines such as tris or sodium azide are incompatible, as they will compete with the primary amine groups of the protein.

TECHNIQUES FOR BUFFER EXCHANGE

Since the conditions during the labeling reaction dictate the outcome, it is imperative to remove interfering compounds, adjust the pH and – if possible – concentrate the protein solution. All of the above objectives can be reached with various buffer exchange procedures, each of them possessing advantages and disadvantages over each other.

Diafiltration

Diafiltration relies on a semipermeable membrane with a defined molecular weight cut-off (MWCO) through which the sample is passed by applying pressure. While the membrane retains molecules with a size greater than the MWCO, smaller compounds, such as buffer components will pass through and therefore be removed (figure 2).

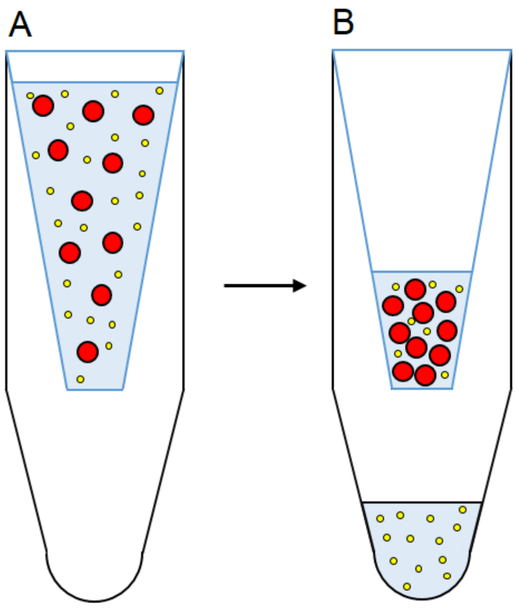

Figure 2. Schematic representation of buffer exchange and sample concentration by diafiltration spin columns. Sample containing protein (red) and buffer components (yellow) is loaded into the upper chamber (A). After centrifugation, buffer components have passed through the membrane while proteins are retained and concentrated. Multiple concentration-dilution cycles allow for near complete buffer exchange.

Spin column-based diafiltration devices are commonly employed, such as the Amicon Ultra Centrifugal Filter Units. Unlike other buffer exchange procedures, diafiltration allows sample concentration, which can potentially enhance the labeling efficiency. While many antibodies and a variety of other proteins tolerate such high concentrations, it can lead to precipitation and thus sample loss in some cases.

Size-exclusion chromatography

Size-exclusion chromatography is a buffer exchange technique, which relies on resin beads with a defined pore size. Similar to diafiltration, components in a solution are separated based on their size. Small molecules such as buffer components will enter the bead pores and therefore take longer to pass through the chromatography columns. Larger molecules, such as the protein of interest will not enter the beads and therefore quickly pass through the column, effectively being separated from the contaminants (figure 3).

Unlike diafiltration, size-exclusion chromatography does not concentrate the sample, which therefore makes it the method of choice for proteins, which were shown to be sensitive to precipitation at high concentration.

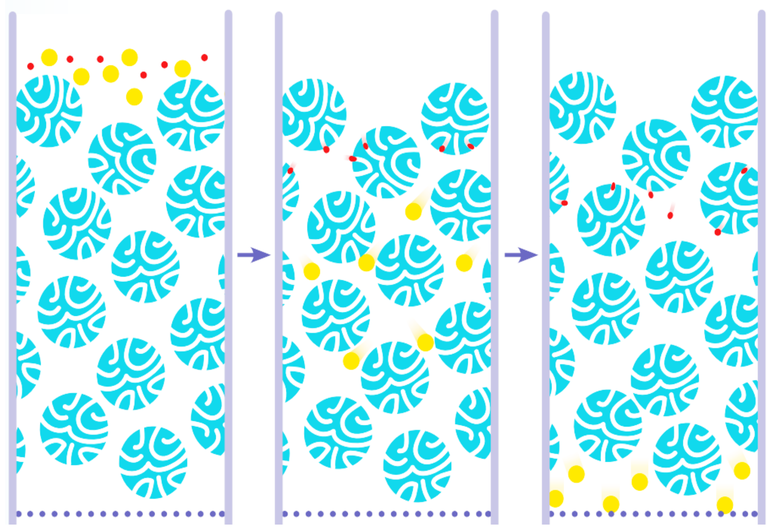

Figure 3. Schematic representation of buffer exchange by size-exclusion chromatography. Buffer components to be removed are depicted in red, the protein of interest in yellow.

Dialysis

Dialysis – similar to diafiltration – relies on a semi-permeable membrane with a defined MWCO. In contrast to diafiltration, the driving force is not pressure but diffusion. Buffer exchange is achieved by submerging the dialysis chamber containing the sample into a reservoir of the target buffer. The protein of interest will be retained in the chamber while the buffer components can pass into the reservoir. Over time, an equilibrium between the dialysis chamber and the reservoir is reached (figure 4).

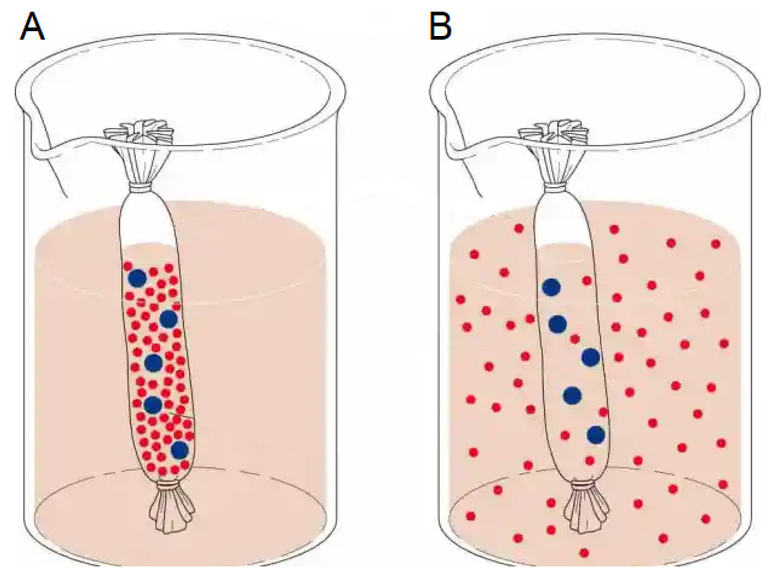

Figure 4. Schematic representation of buffer exchange by dialysis with proteins represented as blue dots and buffer components illustrated as red dots. Over time (A vs. B), buffer components diffuse through the dialysis membrane, drastically reducing their concentration in the protein solution.

Dialysis is considered a very gentle buffer exchange procedure and therfore the method of choice for sensitive proteins. Additionally, the sample will not be concentrated. However, it takes longer to proceed and requires considerably more buffer than column-based procedures.

CASE STUDY: SULFOTAG LABELING

The goal was to develop a electrochemilumniescence-based immunoassay (ECLIA to detect the drug, an antibody against a peptide hormone in serum for a series of pre-clinical and clinical studies in a variety of species. The detection reagent was generated in house by labeling an anti-drug antibody using SulfoTag-NHS-ester in-house.

Initial labeling attempts were performed using Amicon Ultra Centrifugal Filter Units during the buffer exchange steps and led a large loss of protein, with only 58 and 53% of protein recovered after labeling (figure 5). Most likely, this was due to precipitation during the buffer exchange, as the protein concentration rises significantly during buffer exchange by diafiltration.

Switching to Zeba Spin Desalting columns improved the yield, however, recovery rates were inconsistent, ranging from 72 – 92%. This consequently caused lot-dependent variations in the assay performance (figure 6A).

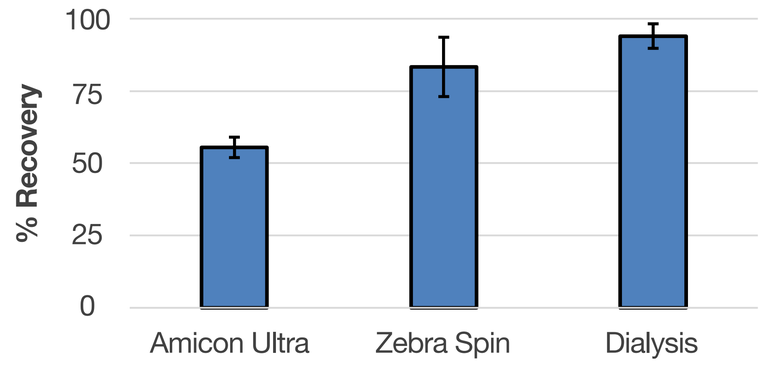

Figure 5. Recovery rates and variability between individual labeling attempts, according to buffer exchange method employed. Yield improved when buffer exchange is performed by size-exclusion chromatography (Zeba Spin) or dialyisis, however, only the latter consistently resulted in yields above 90%.

Finally, using dialysis as buffer exchange method, we managed to increase protein recovery to an average of 94% (ranging from 91 – 97%). Individual lots were compared and found equivalent regarding assay performance (figure 6B).

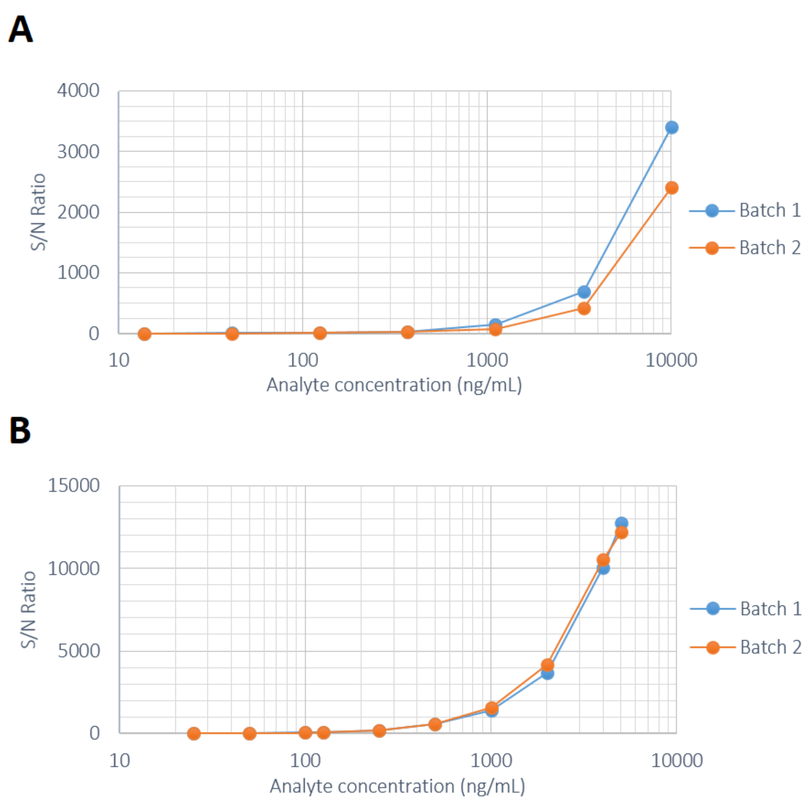

Figure 6. Standard curves using multiple batches of SulfoTag-labeled detection antibody using Zeba Spin desalting columns (A) or dialysis (B) as buffer exchange method. Buffer exchange by dialysis results in more consistent yields which translate to minimized lot-to-lot variability in assay performance.

DISCUSSION & CONCLUSIONS

While labeling of antibodies and proteins represents an essential step in the development of a ligand-binding assay, its impact on assay performance is often overlooked. Ideally, the labeling process should proceed without significant loss of proteins. Especially in the case of larger studies, where multiple batches of the same protein will be labeled, minimizing lot-to-lot variation is another factor that should be considiered. As most of the protein loss occurs during the buffer exchange steps, protein recovery should be monitored and other buffer exchange methods should be considered in case of low recovery.

In the presented example, initial sample loss could be mitigated by switching to size exclusion chromatography-based buffer exchange, at the cost of higher lot-to-lot variability. Finally, dialysis was established as the buffer exchange method of choice for this specific protein, as it reproducibly led to high yields. However, dialysis comes with significant disadvantages: Due to multiple overnight incubations, it prolongs the entire labeling procedure to three days. Additionally, buffer exchange by dialyisis consumes an order of magnitude more buffer than column-based procedures.

These results highlight the limitations of a standardized labeling approach as well as the importance of adapting the labeling conditions to individual proteins if expectations regarding yield and lot-to-lot variability are not met.

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Figure 3:
Zeba Desalting Handbook, ThermoFisher Scientific.

Figure 4:
Adapted from:
https://masteringcollegebiochemistry.wordpress.com/2019/01/25/protein-purification-dialysis/