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Research paper

Optimized small molecule antibody labeling efficiency through continuous flow centrifugal diafiltration

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ABSTRACT

Protein immuno-detection encompasses a broad range of analytical methodologies, including western blotting, flow cytometry, and microscope-based applications. These assays which detect, quantify, and/or localize expression for one or more proteins in complex biological samples, are reliant upon fluorescent or enzyme-tagged target-specific antibodies. While small molecule labeling kits are available with a range of detection moieties, the workflow is hampered by a requirement for multiple dialysis-based buffer exchange steps that are both time-consuming and subject to sample loss. In a previous study, we briefly described an alternative method for small-scale protein labeling with small molecule dyes whereby all phases of the conjugation workflow could be performed in a single centrifugal diafiltration device. Here, we expand on this foundational work addressing functionality of the device at each step in the workflow (sample cleanup, labeling, unbound dye removal, and buffer exchange/concentration) and the implications for optimizing labeling efficiency. When compared to other common buffer exchange methodologies, centrifugal diafiltration offered superior performance as measured by four key parameters (process time, desalting capacity, protein recovery, retain functional integrity). Originally designed for resin-based affinity purification, the device also provides a platform for up-front antibody purification or albumin carrier removal. Most significantly, by exploiting the rapid kinetics of NHS-based labeling reactions, the process of continuous diafiltration minimizes reaction time and long exposure to excess dye, guaranteeing maximal target labeling while limiting the risks associated with over-labeling. Overall, the device offers a simplified workflow with reduced processing time and hands-on requirements, without sacrificing labeling efficiency, final yield, or conjugate performance.

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1. Introduction

The use of labeled antibodies (Abs) for target identification is a cornerstone of biological science with application across a wide range of samples including cellular lysates, single cell suspensions (cell lines, clinical samples), culture supernatants or clinical bio-fluids, and tissue sections (Coons et al., 1942; Coons and Kaplan, 1950). While indirect detection strategies are sufficient for single target analyses, multiplexing requires uniquely labeled reagents. When compared to indirect methods, direct detection offers workflow simplification as well as a reduced risk of non-specific binding; such factors decrease assay variability and improve data quality (Johnson, 2006). Direct conjugates are commercially available but these reagents can be costly. Moreover, while the list of detection moieties is vast, Abs are not produced in all formats thus limiting flexibility in the design of multiplex assays. The significance of this final point

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is underscored by the ever-growing use of rare and precious samples where increasing the information content per test, through the use of multiplex assay platforms such as flow cytometry or immunofluorescent microscopy, is paramount to improved understanding of biological processes.

Protein labeling kits fill this gap, providing the means to customize detection panels. For most reactions, the antibody must be pure (preferably >95%) and at a concentration of >0.5 mg/mL. While many commercial Abs meet these criteria, those provided in crude formats require upfront purification. Also, since dilute protein solutions (<1 mg/mL) are prone to degradation or loss through binding to the storage vessel, carriers such as bovine serum albumin (BSA), are commonly added for stabilization. If not removed prior to labeling, such carriers can prove problematic serving both as "sinks" for dye binding and as sources of background staining (Goldstein et al., 1961; Pittman et al., 1967).

Many small molecule labeling kits are N-hydroxysuccinimide (NHS) chemistry-based, and therefore target lysine residues for conjugation; samples must be devoid of ammonium ions and primary amines (Tris, glycine, sodium azide) necessitating buffer exchange. Dialysis, gel filtration, and diafiltration represent the most common methods for buffer exchange. Dialysis is well-established, using size-selective diffusion across a semi-permeable membrane to effect desalting. Dialysis is very gentle; it is the gold-standard for buffer exchange of labile species prone to degradation. However, this method is time-consuming, requires large volumes of buffer (≥500× sample size) with multiple exchanges, and is subject to sample loss. Lengthy dialyses can also promote aggregation of both free dye and labeled probe. Gel filtration relies on molecular sieve chromatography to facilitate exchange. While relatively fast, gel filtration requires collection and assessment of eluted fractions; this can be tedious if performed manually and is subject to sample loss. Both column desalting and dialysis dilute samples thus requiring a final concentration step. By contrast, diafiltration offers simultaneous concentration with buffer exchange combining two preparative steps into a single device. Diafiltration achieves rapid buffer exchange by using external pressure (centrifugation) to drive solutions (both solvent and solute) through porous membranes. However, diafiltration devices may require multiple spins to achieve high exchange efficiency. Multiple rounds of concentration/dilution cycling can have deleterious effects on protein structure potentially reducing yield and/or specific activity.

The standard small molecule-dye labeling workflow involves initial buffer exchange to remove reagents that would interfere with labeling, followed by a static labeling reaction (in a tube), removal of unbound dye, and concentration of the tagged Ab. Due to requirements for buffer exchange prior to and following labeling, the current process is time-consuming and subject to significant protein loss at multiple points of sample transfer. In addition to the basic workflow, optimization of labeling reaction parameters is often required to ensure that performance of the resulting conjugate has not been compromised by over-labeling and is further commensurate with specifications outlined by the analysis platform in which it will be used. Herein, we describe application of the Amicon Pro device, a centrifugal-based affinity purification tool with the capacity for continuous flow diafiltration, to the process workflow for small-scale antibody labeling with small molecule detection moieties.

2. Materials and methods

2.1. Buffer exchange using the Amicon Pro device (EMD Millipore)

To minimize non-specific binding, each device was pre-wet by passage of 0.5 mL TBS-T [1% Tween-20 in Tris buffered saline (TBS)] (1000 $g \times 1$ min). Prior to buffer exchange, an Amicon Ultra 0.5 filter [10 kDa NMWL (nominal molecular weight limit), EMD Millipore] was attached to the base of the device. 0.5 mL (1 mg/mL) affinity-purified GST-LPP (Glutathione-S-transferase lambda protein phosphatase fusion protein) was added to the exchange reservoir and concentrated (4000 $g \times 15$ min). The retentate was buffer exchanged with 1.5 mL appropriate buffer (4000 $g \times 15$ min.). Sample was recovered by reverse spin (inverted Amicon Ultra 0.5 filter) in a microcentrifuge (1000 $g \times 2$ min). On average, samples concentrated to 50 μL (10-fold). All steps were performed at room temperature (RT).

2.2. Buffer exchange by dialysis

3 mL cassettes (10 kDa NMWL, Pierce) were hydrated with appropriate buffer for 2 min. 0.5 mL GST-LPP was injected using a 1 mL syringe and 18-gauge needle. Cassettes were immersed in 500 mL exchange buffer, and dialyzed for 16 h at 4 °C exchanging the buffer 2× during the process. Dialyzed samples were concentrated using Amicon® Ultra-0.5 filters (EMD Millipore).

2.3. Buffer exchange by diafiltration

Two formats were tested: 0.5 mL (10 kDa NMWL, EMD Millipore) and 20 mL (10 kDa NMWL, Sartorius). 0.5 mL device — 500 μL GST-LPP was centrifuged at 14,000 $g \times 15$ min. Samples were buffer exchanged (14,000 $g \times 15$ min.) using 0.5 mL 1× PBS or appropriate buffer. The process was repeated twice more to complete exchange. Samples were recovered by reverse spin. 20 mL device — 2 ml diluted GST-LPP sample (containing 500 μL eluted protein) was placed in the concentrator, 10 mL exchange buffer was added to the diafiltration chamber and centrifuged at 5000 $g \times 5$ min. Samples were recovered from the base by pipeting. All steps were performed at RT.

2.4. Buffer exchange by gravity

Columns (5 kDa NMWL, GE Healthcare) were washed 5× with exchange buffer by gravity. 0.5 mL GST-LPP sample was loaded onto the column and allowed to enter the resin bed (discard flowthrough). Columns were washed extensively, eluted in 4 mL appropriate buffer, and concentrated using 0.5 mL centrifugal filters (10 kDa NMWL). All steps were performed at RT.

2.5. Conductivity assay

0.05 mg/mL BSA (in 1 M Tris, pH 7.5, 1 M NaCl) was buffer exchange as described above, using 1 M Tris pH 7.5. Resulting

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