



Research paper

A novel method for reproducible fluorescent labeling of small amounts of antibodies on solid phase

Emma Lundberg, Mårten Sundberg, Torbjörn Gräslund,
Mathias Uhlén, Helene Andersson Svahn*

Department of Biotechnology, Royal Institute of Technology, Roslagstullsbacken 21, SE-106 91 Stockholm, Sweden

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Abstract

Fluorescently labeled antibodies are very important tools in cell biology, providing for specific and quantitative detection of antigens. To date, fluorophore labeling of antibodies has been performed in solution and has been limited by low-throughput methods requiring a substantial amount of pure antibody sample at a high concentration. We have developed a novel solid-phase labeling protocol for small amounts (i.e. micrograms) of antibodies with fluorescent dyes. Protein A affinity medium was used as solid support in a micropipette tip format. This solid-phase approach, including the advantage of the strong and specific interaction between Protein A and antibodies, allows for simultaneous purification, labeling and concentration of the antibody sample, making it possible to start with unpure antibody samples at low concentrations. We have optimized the protocol with regard to reaction pH, time, temperature and amount of amine reactive dye. In addition, we have evaluated the stability and activity of the labeled antibodies. To evaluate the reproducibility and robustness of this method we labeled eight antibodies with amine reactive fluorescent dyes followed by evaluation of antibody specificity on protein arrays. Interestingly, this gave an extremely high conformity in the degree of labeling, showing the robustness of the method. The solid-phase method also gave predictable and reproducible results and by varying the amount of reactive dye, the desired degree of labeling can easily be achieved. Antibodies labeled using this solid-phase method were similar in stability and activity to antibodies labeled in solution. This novel solid-phase antibody labeling method may also be applicable for other conjugation chemistries and labels, and has potential for high-throughput applications.

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1. Introduction

Many applications in cell biology require the use of antibodies for specific interaction and the antibody to be labeled with detector molecules for visualization.

Depending on the application, these detector molecules can be bioluminescent markers, enzymes or fluorophores. Fluorescently labeled antibodies are a very important tool in this field to provide for the specific detection of antigens. Fluorescently labeled antibodies are commonly used in immunohistochemistry, fluorescent microscopy, protein arrays, flow cytometry, western blots, etc., and are the reagents of choice for

* Corresponding author. Tel.: +46 8 5537 8322; fax: +46 8 5537 8481.
E-mail address: helene.andersson@biotech.kth.se (H.A. Svahn).

quantification of antigen in all of these applications. There are two methods for fluorescent detection of antigens: either the primary antibody can be conjugated to a fluorophore or else a secondary antibody can be conjugated to a fluorophore. An advantage of using labeled primary antibodies is the reduced staining time and multiplexing possibilities. Labeled primary antibodies allow simultaneous visualization of several antibodies from the same animal species in the same system (for example in the same protein array, cell or tissue) without risking cross-reactivity.

According to the literature, today's methods for fluorescent labeling of antibodies are all based on labeling in solution (Mujumdar et al., 1993; Panchuk-Voloshina et al., 1999; Gruber et al., 2000). The fluorescent labels are usually coupled to the lysine residues in antibodies using amine reactive reagents but other coupling chemistries, such as maleimide coupling to sulfhydryl groups, are also commonly used (Haugland, 2005). No matter what coupling chemistry is used a prerequisite of a successful labeling reaction is a pure antibody sample (free from albumin) at a high concentration, ranging from 2 to 10 mg ml⁻¹. The labeling reaction in solution is difficult to control and the resulting degree of labeling is hard to predict. The manufacturers of fluorescent dyes therefore often recommend doing several labelings simultaneously with different amounts of fluorophores to ensure at least one successful labeling reaction. When the labeling reaction is finished, unreacted fluorophore must be removed by dialysis or desalting columns, adding one extra time-consuming step; the disadvantages include dilution and loss of antibody during the post-reaction purification step as well as incomplete removal of free dye (Haugland, 2005).

There is a great need for primary labeled antibodies but the problems with reproducibility, recovery of small amounts of antibody and the need for pure samples at high concentrations often makes it impossible using existing methods. To meet these needs, research efforts have been made; for example, a solid-phase biotinylation protocol has been developed for biotinylation of small amounts (0.1–10 mg) of antibody on an IMAC support (Strachan et al., 2004). Here they successfully biotinylate antibodies on both amine and sulfhydryl groups. This protocol is faster than the conventional solution-phase protocols but still requires a pure antibody sample. So the need for a truly miniaturized protocol that is applicable for both biotinylation and fluorescent labeling of antibodies still remains.

To further push the limits towards a miniaturized high-throughput method for labeling of antibodies, we

have developed a novel method for solid-phase fluorescent labeling of small amounts of antibodies. By using a solid support throughout the labeling, excess reagents are easily washed away while the antibody remains bound to the solid support. Protein A affinity medium was chosen as the solid support. Taking advantage of the strong and specific interaction between protein A and antibodies, compared to the less specific interaction with IMAC support, allows simultaneous purification, concentration and labeling of antibody samples. By doing the labeling in a micropipette tip format, it was possible to reproducibly label as little as micrograms of antibodies starting with 100-fold lower antibody concentrations than is required for labeling in solution. This novel solid-phase labeling method offers equivalent levels of antigen binding activity and stability plus the added advantages of shortened protocol time, the ability to start with unpure samples, combined with maximal recovery of antibody following labeling.

One area where the need for labeled antibodies is great is in proteomics. There are several large-scale projects involving systematic use of antibodies for high-throughput studies of proteomes (Warford et al., 2004; Uhlen and Ponten, 2005). One example of such a project is the Human Protein Atlas (HPA) Program where mono-specific polyclonal antibodies are generated for all non-redundant human proteins. (Nilsson et al., 2005; Uhlen et al., 2005) These antibodies are produced in duplicate by duplicate immunizations (i.e. in several rabbits) and before considering them as equal, an extensive quality assurance of the specificity is needed. To investigate the reproducibility of this novel solid-phase labeling method, we chose four pairs of antibody duplicates and labeled each part of a pair with different amine reactive Alexa dyes (Panchuk-Voloshina et al., 1999; Berlier et al., 2003). This allowed simultaneous analysis of both duplicates and hence simultaneous quality assurance. The specificity and quality of these labeled antibody duplicates were analyzed on protein arrays and the results compared to immunohistochemical staining. In the HPA Program as well as in proteomics research in general, high-throughput analysis is an important factor. The classical labeling of antibodies in solution is not very well suited for high-throughput applications. This success of this novel solid-phase labeling method is due to miniaturization in terms of the amount of antibodies required and the reagents more suitable for this. Since the labeling procedure is performed in micropipette tips, automation to a 96-well format might be a possibility. This method may therefore function as a platform for the future

development of systems for high-throughput fluorescent labeling of antibodies.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma unless otherwise noted. The following Alexa Fluor dyes were supplied by Molecular Probes: Alexa Fluor® 488 carboxylic acid, succinimidyl ester “mixed isomers”, Alexa Fluor® 555 carboxylic acid, succinimidyl ester, Alexa Fluor® 647 carboxylic acid, succinimidyl ester. One milligram was dissolved in DMSO to a final concentration of $40 \mu\text{g } \mu\text{l}^{-1}$ and stored at -80°C . The mono-specific polyclonal rabbit antibodies were obtained from the Human Protein Atlas Program; the antibodies used were α -his₆-ABP, α -C1 tetrahydrofolate synthase, α -ornithine carbamoyltransferase, α -programmed cell death 4 isoform 1 and α -activator of 90 kDa heat shock protein ATPase homolog 1 (AHA1). Alexa Fluor 555 rabbit-anti-mouse IgG was obtained from Molecular Probes. Slide-A-Lyzer dialysis cassettes were obtained from Pierce Biotechnology and NAP-5 desalting columns from GE Healthcare. Micropipette tips (TopTip 10–200 μl) filled with protein A affinity medium were obtained from Glycen Corp.

2.2. Covalent labeling of antibodies with Alexa Fluor dyes in solution

All procedures with Alexa Fluor dyes were carried out in dim light. Rabbit α -his₆-ABP IgG was purified on a protein A column to remove albumin before labeling with Alexa Fluor 555 in solution according to the manufacturer's instructions. To obtain a proper pH for labeling, 10 μl of 1 M sodium bicarbonate buffer (pH 8.3) was added to 90 μl of antibody diluted in PBS giving a final antibody concentration of 6 mg ml^{-1} . A 10-fold molar excess of Alexa Fluor 555 dye was added to the antibody sample and conjugation was allowed to proceed for 1 h at room temperature before free dye was removed with a NAP-5 desalting column. To ensure that all free dye was properly removed this was repeated with a new NAP-5 column. The antibody and Alexa Fluor dye concentrations were measured as described below.

2.3. Covalent labeling of antibodies with Alexa Fluor dyes on solid phase

All procedures with Alexa Fluor dyes were carried out in dim light. Protein A micropipette tips were used

as mini-spin columns with centrifuge adaptors holding them in place in Eppendorf tubes (according to the manufacturer's instructions). To force liquid through the micropipette tips, centrifugation at $400 \times g$ for 10–60 s, depending on the volume, was performed.

Protein A micropipette tips were conditioned with 100 μl of wash buffer (15 mM NaH_2PO_4 , 500 mM NaCl, pH 8.0) before 5–20 μg of antibody were added and mixed with the protein A matrix. The antibody was allowed to bind for 1 min before the liquid was forced through the column and the procedure was repeated 2–5 times for maximal binding. Albumin and other non-IgG proteins were washed out twice with 150 μl of wash buffer before equilibration with 100 μl of labeling buffer. Alexa Fluor dye (from the DMSO stock) was mixed with labeling buffer (100 mM NaCl, 35 mM H_3BO_3 , pH 8.3 unless otherwise noted) to a final volume of 15 μl . Unless otherwise noted, 8 nmol of dye was used. The dye mixture was added to the micropipette tips and mixed with the matrix/protein A–antibody complexes and the conjugation was allowed to proceed for 1–3 h at room temperature or at 4°C overnight. Unreacted dye was removed by five washings with wash buffer. The Alexa Fluor dye labeled antibody was eluted with 50–500 μl of elution buffer (0.2 M HAc, pH 3.3) depending on the required antibody concentration. The pH of the eluate was raised immediately by addition of one-quarter of the elution volume of 0.5 M Tris (pH 8.5). Dye and antibody concentrations were measured before the addition of BSA to a final concentration of 0.1% (w/v) for stabilization of the antibodies. The buffer was changed to PBS with either dialysis or dilution.

2.4. Measurement of antibody and dye concentrations

The Alexa Fluor dye concentrations were determined from the visible light absorption spectrum, according to the manufacturer's instructions, using published extinction coefficients ($\epsilon_{495}=71,000 \text{ M}^{-1} \text{ cm}^{-1}$ for Alexa Fluor 488, $\epsilon_{555}=150,000 \text{ M}^{-1} \text{ cm}^{-1}$ for Alexa Fluor 555, $\epsilon_{650}=239,000 \text{ M}^{-1} \text{ cm}^{-1}$ for Alexa Fluor 647). The concentration of labeled antibodies was determined from the UV–Vis absorption spectrum ($\epsilon_{280}=170,000 \text{ M}^{-1} \text{ cm}^{-1}$) taking into account the absorbance contribution from the covalently bound Alexa Fluor dye at 280 nm ($\text{CF}_{280}=0.11$ for Alexa Fluor 488, $\text{CF}_{280}=0.08$ for Alexa Fluor 555, $\text{CF}_{280}=0.03$ for Alexa Fluor 647) according to the manufacturer's instructions. All absorbance measurements were performed with a Cary 50 UV–Visible spectrophotometer (Varian).

2.5. Fluorescence measurements of free Alexa Fluor dyes and Alexa Fluor-conjugated antibodies

Unconjugated Alexa Fluor dyes 488, 555 and 647 were diluted to a final concentration of 1 μ M in the following buffers: PBS (pH 7.2), 0.1 M sodium citrate (pH 2.5), 0.1 M sodium citrate (pH 3.0), 0.1 M sodium citrate (pH 3.5), 0.1 M sodium citrate (pH 4.0), 0.1 M sodium citrate (pH 4.5) and 0.2 M acetic acid (pH 3.3) and incubated for 1 h before pH was raised by the addition of one-quarter volume of Tris (pH 8.5) before the fluorescence was read.

A rabbit- α -his₆-ABP antibody labeled with Alexa Fluor 555 using the solid-phase protocol, with a resulting average incorporation of 2.4 mol dyes per mol antibody and a commercial rabbit- α -mouse conjugated with Alexa Fluor 555 was diluted to a final concentration of 0.1 μ M in PBS before the fluorescence was read.

All fluorescence measurements were performed at 25 °C using a Perkin Elmer luminescence spectrometer LS50B. The dyes were excited at λ_{max} (λ_{max} =495 for Alexa Fluor 488, λ_{max} =555 for Alexa Fluor 555, λ_{max} =650 for Alexa Fluor 647) and the fluorescence was read at the emission maxima (E_{max} =519 for Alexa Fluor 488, E_{max} =565 for Alexa Fluor 555, E_{max} =665 for Alexa Fluor 647) all according to the manufacturer's instructions.

2.6. Protein arrays

The PrEST antigens were diluted to approximately 40 μ g ml⁻¹ in 0.1 M urea and 1 $\times$ PBS (pH 7.4) and 50 μ l were transferred to a 96-well plate. The HPA antigens (PrESTs) were printed and immobilized onto epoxy slides (Epoxide, Corning) using a pin-and-ring arrayer (Affymetrix 417). The slides were blocked with Super-Block (Pierce) for 30 min. An adhesive 16-well silicone mask (Schleicher & Schuell BioSciences) was applied to the glass before addition of the labeled mAb diluted in 1 $\times$ PBST (1 $\times$ PBS supplemented with 0.1% Tween 20, pH 7.4) to a final concentration of approximately 50–75 ng ml⁻¹. The glass slides were incubated on a shaker for 60 min before washing twice with 1 $\times$ PBST and once with 1 $\times$ PBS. The slides were spun dry and scanned using a G2565BA array scanner (Agilent Technologies) and images were quantified using the image analysis software GenePix 5.1 (Axon Instruments).

2.7. Immunohistochemistry

Unlabeled antibodies were used to stain the cell microarrays. The immunohistochemistry and imaging at

20 $\times$ magnification were performed as described earlier (Uhlen et al., 2005).

3. Results and discussion

We present the development of a novel solid-phase labeling method for reproducible labeling of small amounts (micrograms) of antibodies with fluorescent dyes. Protein A affinity medium in a micropipette format was used as solid support throughout the labeling allowing simultaneous purification, labeling and concentration of the antibody sample.

For optimization and evaluation of this novel method we used amine reactive Alexa Fluor dyes. A low pH is required for elution of bound antibodies from protein A. We therefore started by determining if the Alexa Fluor dyes could withstand the required elution conditions and how large an elution volume was needed for effective elution of bound antibodies. We continued by optimizing the pH of the labeling buffer, followed by conjugation time and temperature. By adding different amounts of reactive dye, the desired degree of labeling could easily be achieved in a predictable manner. The reproducibility of the protocol and the fluorescence of the labeled antibodies were evaluated. The functionality of the labeled antibodies was evaluated on protein arrays and this experiment was repeated after 3 months to evaluate the stability of the labeled antibodies.

3.1. Effect of low pH on Alexa Fluor dye fluorescence

According to the manufacturer, Alexa Fluor dyes are stable between pH 4 and 10 (Haugland, 2005). A lower pH (around 3) is typically used to effectively elute the labeled antibodies from a protein A matrix (Langone, 1982). To test whether the Alexa Fluor dyes could withstand prolonged exposure in a low pH environment, Alexa Fluor 488, Alexa Fluor 555 and Alexa Fluor 647 were diluted in buffers at different pHs, ranging from 2.5 to 7, to a final concentration of 1 μ M and incubated for 1 h before the pH was raised by the addition of one-quarter volume of Tris. The fluorescence was read and there were no significant difference between the fluorescence of any of the fluorophores in the different buffers compared to PBS (data not shown). This shows that elution of labeled antibodies with 0.2 M acetic acid (pH 3.3) does not lower the fluorescence of the conjugated dye and can thus be used for efficient elution of labeled antibody from the protein A affinity medium.

3.2. Elution of antibody

To evaluate the volume needed for efficient elution of labeled antibody from the micropipette tips containing protein A affinity medium, 14 μg of antibody was allowed to bind to the protein A before the albumin was washed away and the antibodies were eluted in 50 μl fractions. Eighty-seven percent of the eluted antibodies were collected in the first fraction (Fig. 1). This shows that it is possible to elute the labeled antibody in a small volume and obtain a more concentrated sample than the start sample. It should also be possible to further lower the elution volume by passing the eluate through the micropipette tip several times.

3.3. Effect of pH of labeling buffer during solid-phase labeling

To evaluate how the pH of the labeling buffer affects the conjugation of fluorescent dye to antibodies, 14 μg of rabbit α -his-ABP IgG was labeled with Alexa Fluor 555 on solid phase in protein A micropipette tips in four different labeling buffers at different pHs. The buffers were: (A) 50 mM phosphate, pH 7.3; (B) 100 mM NaCl, 35 mM H_3BO_3 , pH 8.3; (C) 100 mM Na_2CO_3 , pH 9.3; (D) 100 mM glycine–NaOH, pH 9.3. The results (Fig. 2) show that for a 2 h incubation time, labeling using the buffer containing 100 mM NaCl and 35 mM H_3BO_3 at pH 8.3 was superior to the other buffers giving an average of 2.5 fluorophores incorporated per antibody molecule, compared to a degree of labeling lower than 2 fluorophores per antibody for the other buffers. This correlates well with the manufacturer's

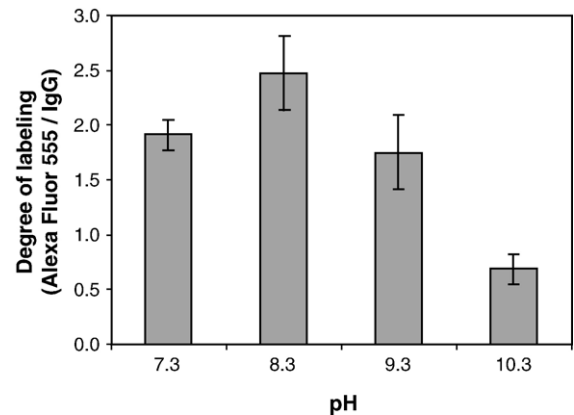


Fig. 2. The degree of labeling obtained after conjugating Alexa Fluor 555 to rabbit- α -his-ABP on solid phase (i.e. protein A) in different labeling buffers at different pH values. The x-axis shows the pH of the different labeling buffers. The y-axis corresponds to the degree of labeling obtained in terms of moles of Alexa Fluor 555 dye per mole of antibody.

recommendations of using 0.1–0.2 M sodium bicarbonate buffer (pH 8.3) for conjugation with succinimide esters. All subsequent solid-phase labelings were performed with a labeling buffer containing 100 mM NaCl and 35 mM H_3BO_3 at pH 8.3.

3.4. Effect of labeling time and temperature

The next step was evaluation of the incubation time and temperature giving the maximal number of incorporated fluorophores per antibody. Rabbit α -his-ABP IgG was labeled with Alexa Fluor 555 using the solid-phase labeling protocol and the reactions were allowed to proceed for 1, 2 or 3 h at room temperature or overnight at 4 $^{\circ}\text{C}$. The results (Fig. 3) show that after 1 h, an average of 1.6 fluorophores were incorporated per molecule of antibody and after 2 h that number increased to 2.4. Continuing with the labeling for 3 h at room temperature does not give a higher degree of labeling. This indicates that 1 h incubation was not sufficient to complete the conjugation reaction, but after 2 h the reaction was complete. Labeling overnight at 4 $^{\circ}\text{C}$ gives a degree of labeling comparable to that after 2 h at room temperature and may be a good option when labeling unstable or sensitive antibodies. In all further solid-phase labelings the conjugation was allowed to proceed for 2 h at room temperature.

3.5. Effect of amount dye for labeling

To evaluate the effect of dye concentration, we labeled 14 μg of rabbit α -his-ABP IgG in protein A micropipette tips with different amounts of Alexa Fluor

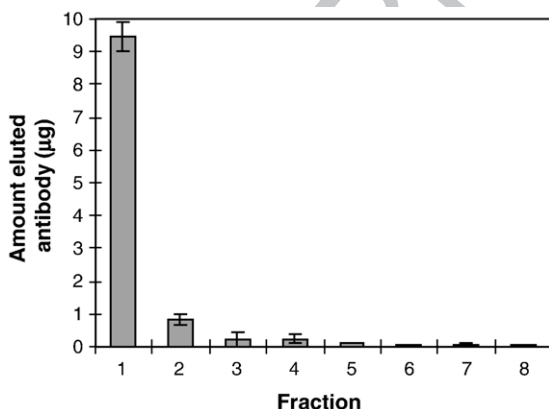


Fig. 1. The amount of antibody eluted in each 50 μl fraction from a Protein A, micropipette tip is shown; the antibody was eluted using 0.2 M HAc (pH 3.3). The x-axis corresponds to the eight different fractions where 1 is the first fraction and 8 is the last. The y-axis corresponds to the amount of antibody eluted on a microgram scale.

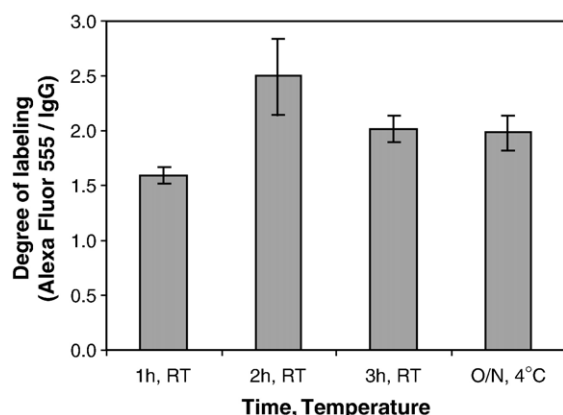


Fig. 3. The degree of labeling obtained after conjugating Alexa Fluor 555 to rabbit- α -his₆-ABP on solid phase (i.e. protein A) with different incubation times for the conjugating reaction. The x-axis shows the incubation time and temperature during labeling. The y-axis corresponds to the degree of labeling obtained in terms of moles of Alexa Fluor 555 dye per mole of antibody.

555 dye (Fig. 4). When labeling on solid phase, both protein A on the affinity medium and the bound antibody will be labeled; the molar excess of dye must therefore be compared to the molar amount of antibody plus protein A. According to the manufacturer, one micropipette tip contains about 30–50 μ g of protein A and together with 14 μ g of antibody the total moles of protein is approximately 0.8–1.3 nmol. Starting with 0.4 nmol of Alexa Fluor 555 yielded degrees of labeling (DOL, the number of moles of dye incorporated per mole of antibody) of approximately 0.26; 64 nmol of dye gave a DOL of 15. It is clear that by adding more dye, a higher degree of labeling was achieved. The DOL could be reproduced several times showing the robustness of the method. These results show that by adjusting the amount of added fluorophore, predictable degrees of labeling can easily be achieved using the novel solid-phase method. For some applications one incorporated fluorophore is optimal, while in other cases a maximal DOL may be required. To achieve a reproducible DOL high enough to produce bright signals, yet low enough to avoid interference with antigen binding activity and use as small amount of dye as possible, the addition of 8 nmol dye (corresponding to approximately 6–10 times molar excess) was chosen for all further solid-phase labelings.

3.6. Fluorescence of solid-phase fluorescent labeled antibodies

To evaluate the fluorescent quality of the antibodies labeled using the solid-phase labeling protocol, we

wanted to compare it with the same antibody labeled by the soluble protocol recommended by the manufacturer and a commercial Alexa Fluor 555 conjugated antibody. Rabbit α -his-ABP IgG was labeled in solution with Alexa Fluor 555 according to the manufacturer's instructions. After removal of albumin using a protein A affinity column, a 10-fold molar excess of dye was used to label the antibody at a concentration of 6 mg ml⁻¹ of antibody. This resulted in an average of 2.5 fluorophores incorporated per antibody, which corresponds well to the DOL obtained using the solid-phase labeling protocol with a 6–10-fold molar excess of dye. Antibodies labeled with Alexa Fluor 555 in solution, on solid-phase and a commercial Alexa Fluor 555-rabbit-anti-chicken IgG were diluted to 17 μ g ml⁻¹ in PBS before the fluorescence was read. There were no significant differences in the fluorescence intensity between the three antibodies (data not shown). These results show that the two labeling methods were comparable in terms of labeling efficiency given a certain molar excess of dye and that the resulting labeled antibodies showed a fluorescence intensity comparable to that of a commercial fluorescently labeled antibody.

3.7. Reproducibility of the solid-phase labeling protocol

To evaluate the reproducibility of the solid-phase labeling method, eight antibodies were labeled. Half of the antibodies were labeled with Alexa Fluor 555 and half with Alexa Fluor 647 using the optimized solid-phase labeling protocol: 10 μ g of each antibody were mixed

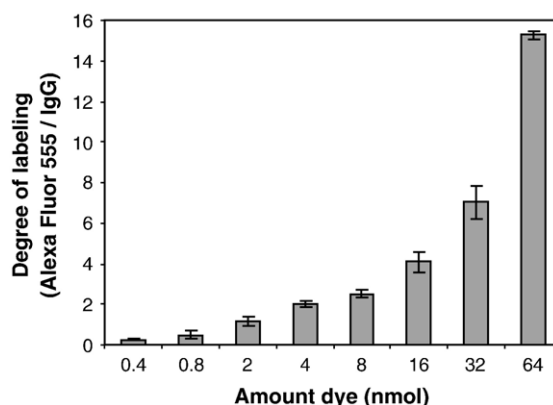


Fig. 4. The degree of labeling obtained after conjugating Alexa Fluor 555 to 14 μ g rabbit- α -his₆-ABP on solid phase (i.e. protein A) varying the different amounts of added dye. The x-axis shows the amount of added Alexa Fluor 555 dye in nanomoles. The y-axis corresponds to the degree of labeling obtained in terms of moles of Alexa Fluor 555 dye per mole of antibody.

with 8 nmol of dye (approximately 6–10 times molar excess) in the labeling buffer with pH 8.3 (100 mM NaCl, 35 mM H₃BO₃) and incubated for 2 h at room temperature. Labeling with Alexa Fluor 555 generated an average DOL of 2.1 ± 0.08 while labeling with Alexa Fluor 647 generated an average DOL of 4.1 ± 0.4 . Thus, this labeling method is applicable for labeling different antibodies at different concentrations straight from a

mixture of antibody and albumin in less than 3 h, generating reproducible results with a precise DOL. The fact that the average DOL obtained when labeling with Alexa Fluor 647 is higher than when labeling with Alexa Fluor 555 shows that the amount of dye needed to generate antibodies with a specific DOL might vary between different fluorophores and have to be adjusted for each fluorophore.

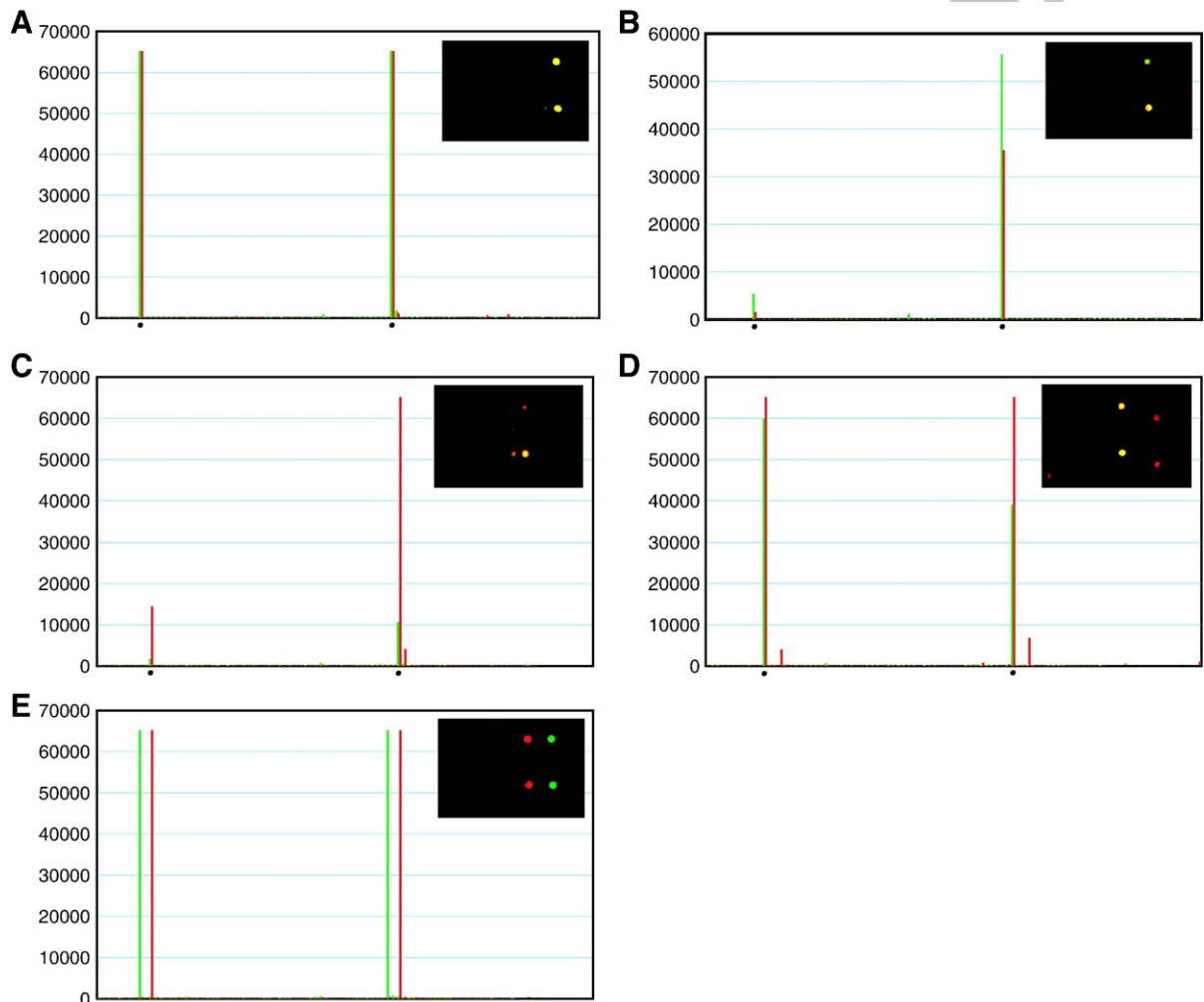


Fig. 5. Results for staining of the PrEST array containing 48 different PrEST fusions spotted in duplicate. Four antibody pairs were used for staining the slide, where each antibody part in the pairs was labeled with Alexa Fluor 555 (shown in green) or Alexa Fluor 647 (shown in red). The x-axis corresponds to different PrEST spots on the array. The y-axis corresponds to the intensity of the fluorescent signals, with a maximal signal intensity of 65,000 units. The antigens which the antibodies were generated towards are marked with a filled black circle. (A) The first antibody pair where both antibodies bind specifically to the correct PrEST fragment corresponding to C-1 tetrahydrofolate synthase. (B) The second antibody pair where both antibodies bind specifically to the correct PrEST fragment corresponding to ornithine carbamoyltransferase, a mitochondrial precursor. (C) The third antibody pair of which one (in red) binds specifically to the correct PrEST fragment corresponding to programmed cell death 4 isoform 1, while the other antibody (in green) shows very weak specific binding. (D) The fourth antibody pair of which one (in green) binds specifically to the correct PrEST fragment corresponding to the activator of 90 kDa heat shock protein ATPase homolog1 (AHA1); the other antibody (in red) also shows binding to another PrEST fragment. (E) Shows two antibodies binding to different PrESTs. This shows that there is no bleed-through or unwanted overlap between the primary labeled antibodies.

3.8. Protein arrays for evaluation of the labeled antibodies

To evaluate the functionality of the labeled antibodies and to demonstrate the possible usage of a fast reproducible method for labeling small amounts of antibodies, we use the application of protein arrays. In the HPA project, protein array assays are used for quality assurance of the specificity of the mono-specific antibodies generated (Nilsson et al., 2005). Carefully designed protein fragments, termed PrEST, are used as antigens for generation of these antibodies. Eight HPA-generated antibodies were chosen. These antibodies constitute four pairs where each pair was generated to bind to the same PrEST fragments. Each part of an antibody pair was labeled with Alexa Fluor 555 or Alexa Fluor 647 (see Section 3.7) allowing simultaneous analysis of the antibodies (i.e. on the same protein array at the same time). Using labeled secondary antibodies for detection in this case would be impossible since the antibodies are from the same species (rabbit) and would cause cross-reactivity.

We spotted 48 different PrEST fusions (including the antigen corresponding to the antibodies analyzed) in duplicate onto glass slides. The specificity of the solid-phase labeled antibodies was evaluated by incubating

the antibodies pairwise on the slide; duplicate evaluations were performed. The results are presented in Fig. 5. The signal from the antibody conjugated with Alexa Fluor 555 is shown in green and that from antibodies conjugated with Alexa Fluor 647 is shown in red. These examples show strong, specific fluorescent signals with high S/N ratios.

Fig. 5A and B shows the result for the first and second antibody pairs. They are both examples of antibody duplicates of good quality. They showed strong fluorescent signals in the spots corresponding to the correct antigen, indicating high specificity. The fact that one spot in Fig. 5B has low intensity was due to technical problems during the printing resulting in one bad spot with a lower protein content, which is normal in microarray preparations.

Fig. 5C and D shows the results for the third and fourth antibody pairs. In both examples, one of the duplicate antibodies does not reach the desired specificity and quality. In Fig. 5C one of the antibodies (shown in green) gives much weaker fluorescent signals, indicating an antibody that lacks target specific recognition. In Fig. 5D one of the antibodies (shown in red) shows cross-reactivity with other PrEST spots while its duplicate antibody shows a strong specific staining to the correct antigen. Fig. 5E shows staining with two

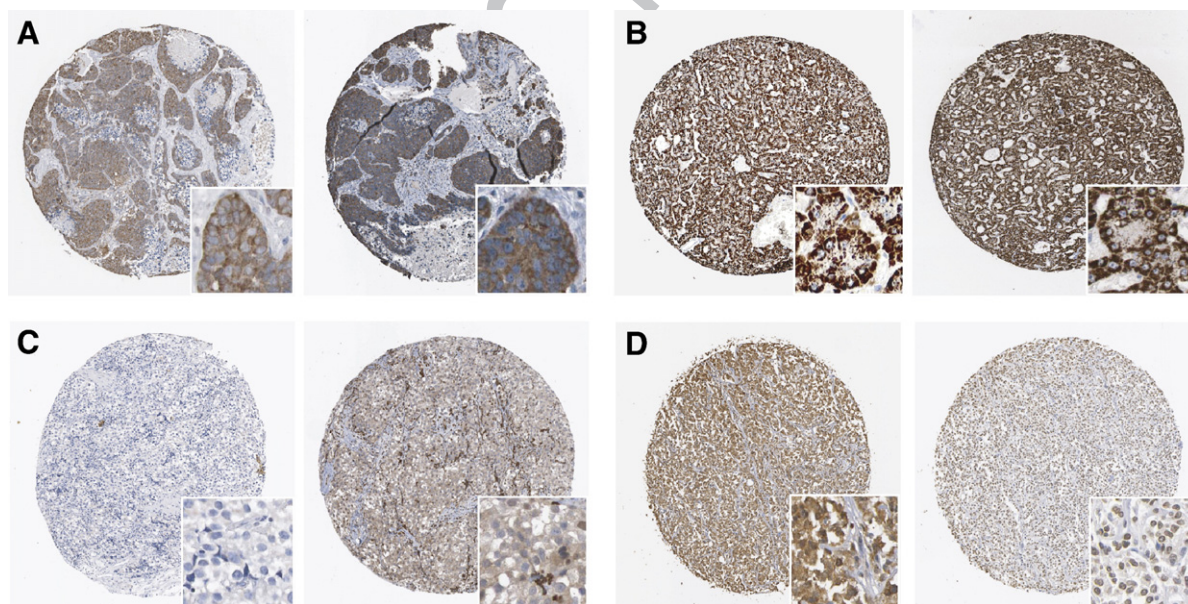


Fig. 6. Images showing immunohistochemical staining of different cancer tissues using the four antibody pairs. (A) The antibodies in the first pair staining lung cancer tissue. The antibodies give an identical staining pattern. (B) The antibodies in the second pair staining lung cancer tissue. The antibodies give an identical staining pattern. (C) The antibodies in the third pair staining testicular cancer tissue. One antibody shows strong staining while the other shows no staining, indicating a difference in antibody specificity. (D) The antibodies in the fourth pair staining malignant lymphoma. One antibody shows strong cytoplasmic and nuclear staining while the other only shows strong nuclear staining, indicating a difference in antibody specificity.

antibodies generated towards different antigens; it is clear that there is no unwanted bleed-through or cross-talk between the two antibodies and it is possible to analyze two antibodies from the same species at the same time using the solid-phase labeling method.

To show the importance of the results from the protein array assays, one can compare the results with the results from immunohistochemical staining of tissue microarrays using the same antibodies (Fig. 6). Fig. 6A and B shows staining of lung and liver cancer tissue with the first and second antibody pair (corresponding to Fig. 5A and B), respectively. They both show an identical staining pattern, supporting the results from the protein array and confirming the equal specificity of the antibody pairs. Fig. 6C shows staining of testicular cancer tissue with the third antibody pair (corresponding to Fig. 5C). Here, one antibody shows strong staining while the other is negative; in this case, the antibody that showed a strong specific signal in the protein array assay shows strong histochemical staining. For another tissue type, e.g. liver (data not shown), this correlation is the other way around; the first antibody shows a strong negative staining and the other one gives no staining. This difference in specificity of these antibodies correlates well with the results from the protein arrays. Fig. 6D shows the fourth antibody pair staining malignant lymphoma (corresponding to Fig. 5D). Here one antibody shows a nuclear and cytoplasmic staining pattern while the other shows only a nuclear staining pattern indicating a difference in specificity. In this case, the antibody that passed the protein array quality control shows the cytoplasmic staining pattern. For more details about the tissue microarrays see Uhlen et al. (2005). The immunohistochemical stainings for the last two antibody pairs (Fig. 6C and D) also supports the results from the protein arrays, indicating that one of the duplicates in each pair is of good quality with the required specificity while the other is not.

This shows that the primary labeled antibodies have good antigen binding activity and produce bright fluorescent signals even though the concentration during staining was as low as 50 ng ml^{-1} . This also shows the advantage of using primary labeled antibodies for simultaneous quality assurance of duplicate antibodies. By doing so the duplicates are tested under identical conditions, making the evaluation easier, and the number of protein arrays needed is reduced to half, an important factor in high-throughput projects.

3.9. Antibody activity and stability

The labeled antibodies retained their good antigen binding activity after storage for 3 months in PBS

supplemented with 1% (w/v) bovine serum albumin. This was confirmed by repeating the protein array experiments (data not shown).

4. Conclusions

This paper describes the development of a novel solid-phase labeling method for reproducible labeling of small amounts of antibodies with fluorescent dyes. We used protein A affinity medium as solid support in a micropipette tip format. When using a solid support throughout the labeling, excess reagents are easily washed away, while the antibody remains bound to the solid support. Using a solid phase also adds the advantage of simultaneous purification and concentration of the antibody sample, making it possible to start with unpure antibody samples at low concentrations (down to $20 \mu\text{g ml}^{-1}$ compared to the required 2 mg ml^{-1} of pure antibody when labeling in solution). By doing the labeling in a micropipette tip format it is possible to reproducibly label as little as micrograms of antibodies and easily handle multiple samples simultaneously.

To prove the applicability and reproducibility of the method, we labeled antibody duplicates generated by the HPA Program with two different fluorescent dyes followed by pairwise quality assurance of the antibody specificity on protein arrays. The solid-phase method turned out to be very robust, giving predictable and reproducible results. By varying the amount of reactive dye a desired degree of labeling was easily achieved. The solid-phase labeled antibodies were found to fluoresce as well as commercially available labeled antibodies and to be equivalent in stability and activity as antibodies labeled in solution.

To date, fluorophore labeling of antibodies has been limited by low-throughput methods requiring a substantial amount of pure antibody sample at a high concentration. This novel solid-phase labeling method is due to the miniaturization in terms of the amount of antibodies required and reagents more suitable for high-throughput applications. Since the labeling procedure is performed in micropipette tips, it might be possible to modify this technology to work automatically in 96-well format.

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