Journal of Chromatography B, 879 (2011) 2583–2594

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

The effects of hitchhiker antigens co-eluting with affinity-purified research antibodies

Lilly Mechetner[a], Radhika Sood[a], Van Nguyen[a], Pete Gagnon[b], Missag H. Parseghian[a,*]

[a] Scientific Affairs, Avid Bioservices Inc., 14272 Franklin Avenue, Tustin, CA, USA
[b] Biotechnology Processing Institute, Singapore

ARTICLE INFO

Article history:
Received 14 November 2010
Accepted 10 July 2011
Available online 20 July 2011

Keywords:
Hitchhiker antigens
Antibody purification
Commercial-scale purification
Affinity chromatography
Host cell proteins
Chromatin immunoprecipitation

ABSTRACT

The popularity of Protein G for the purification of antibodies has given rise to an entire industry that supplies scientists with research grade immunoreagents; however, many times the supplied product is contaminated with antigens bound to the antibody's complementarity-determining regions (CDRs). These "hitchhikers" are a category of host cell proteins that are elusive to detect due to their interaction with the antibody in the final product and yet their impact on an experiment or an entire field of study can be far reaching. In an earlier work, the role of hitchhikers on a human anti-histone antibody destined for clinical usage was explored and a stringent purification scheme developed. Here we use a murine monoclonal, which reflects the type of commercial antibody usually purchased for research. We evaluate three purification schemes: a traditional approach using a one-step, low pH elution buffer (pH 2.5); a gentler approach using a pH gradient elution scheme (pH 7 down to pH 2.5); and finally, a more stringent purification patterned on our earlier published method that uses a quaternary amine guard column and a high salt wash during antibody immobilization on the Protein G. We stress that the stringent purification incorporates the pH gradient scheme and is gentler than the low-pH approach. The resulting product from all three purifications is directly compared for binding potency, histone content (using an ELISA based assay) and residual DNA (using quantitative PCR). The results demonstrate that the first two methods are inadequate for hitchhiker removal. The traditional one-step, low pH approach produces a single elution peak containing histone contaminated antibody with picogram quantities of residual DNA, however, the trailing end of the same peak is loaded with antibody complexed to nanogram amounts of DNA, in some cases, over 100 ng. The pH gradient approach provided antibodies accompanied by only picograms of residual DNA and, on average, 1 out of every 10–20 CDRs occupied by a histone antigen. The more stringent approach, using the salt wash prior to elution with the pH gradient, has an average of 1 out of every 75 CDRs contaminated with a histone while the majority of the residual DNA is captured by the quaternary amine column placed in front of the Protein G. The consequences of these contaminants is illustrated by showing how they manifest themselves in unusual antibody potency values ranging from 558% for antibody bound to histone hitchhikers down to 15% for antibody contaminated with DNA hitchhikers. Those samples purified by the recommended stringent approach show potency values between 90 and 101%. Most importantly, we repeatedly demonstrate in a simulated chromatin immunoprecipitation (ChIP) assay the ability to precipitate clean plasmid DNA with histone contaminated antibody that had been purified using the traditional one-step, low pH elution approach. Expectedly, those antibodies stringently purified and showing 100% binding potency were unable to precipitate DNA in the absence of histone hitchhikers.

1. Introduction

Affinity purification of antibodies using Protein A or G has proven to be a powerful technology for rapidly and cost-effectively providing purified antibodies as research reagents to the general scientific community. It might be bold to also state that the technology has provided researchers working with antibodies the false sense of security that using Protein A or G leads to a "pure" antibody product. Commercial suppliers of antibodies destined for

* Corresponding author. Present address: Research & Development, Stonsa Biopharm, Inc., 2453 Impala Drive, Carlsbad, CA, USA. Tel.: +1 818 422 5734.
E-mail addresses: mparseghian@stonsabio.com, missaghp@earthlink.net (M.H. Parseghian).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.07.016

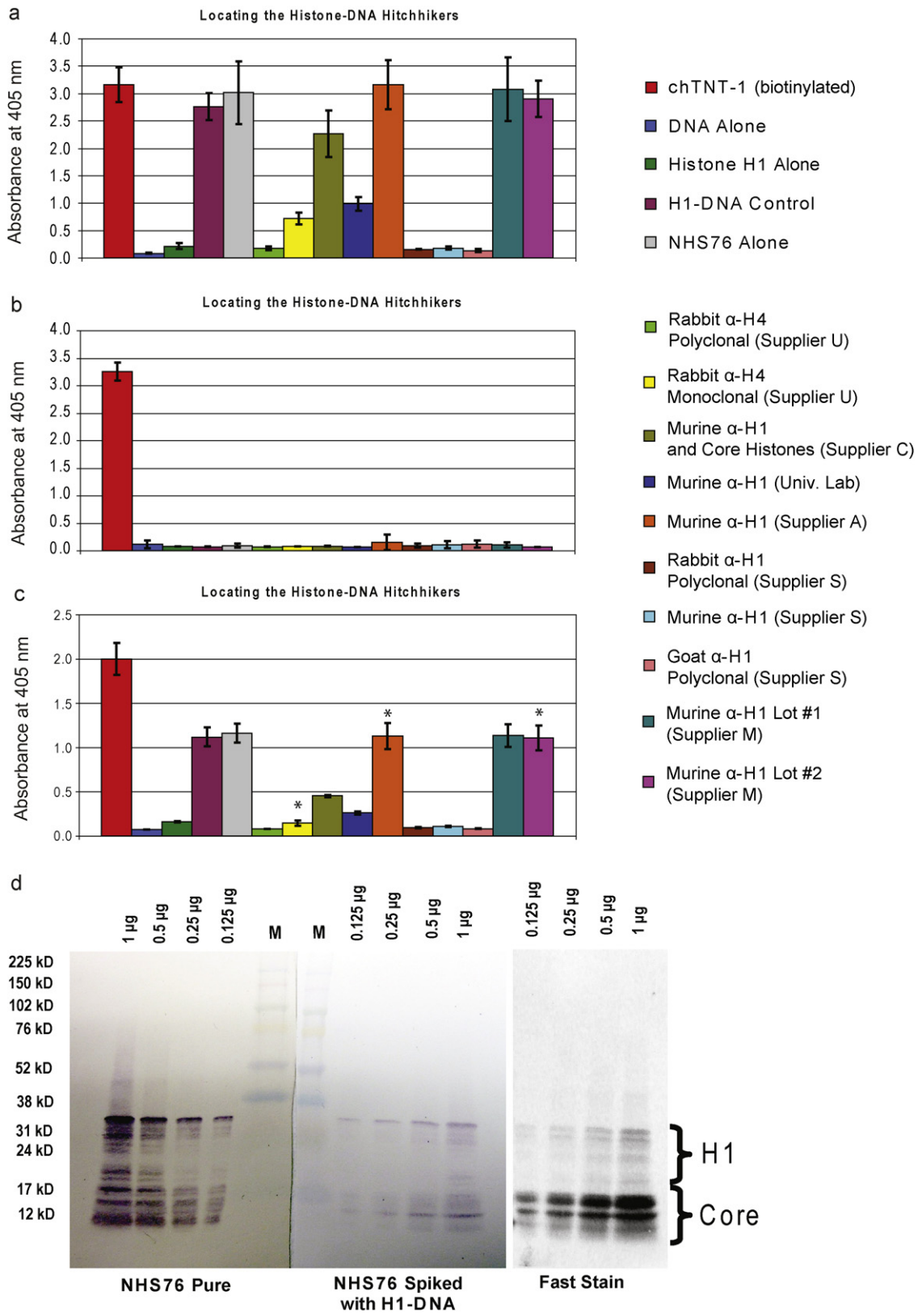

Fig. 1. Surveying commercially available antibodies for histone-DNA hitchhikers. (a) Absorbance values (405 nm) for the detection of H1/DNA were obtained by placing 5 μg of some commercially available anti-H1 or anti-H4 antibodies in wells in triplicate. The presence of histones and DNA were detected with a biotinylated chimeric antibody targeting these antigens; it was purified using a 2 M NaCl wash. Biotin was detected with streptavidin-AP and developed with PNPP substrate for 5 min. (b) A duplicate plate was incubated only with streptavidin-AP followed by a 5 min incubation with substrate to demonstrate lack of cross-reactivity. (c) The same plate in (b) was incubated with the biotinylated detection antibody and then streptavidin-AP followed by a 1 min incubation with substrate. *Three datapoints previously published in [1] are included here for the comprehensiveness of the survey. (d) H1 histones and the closely related core histones were resolved on an SDS-PAGE at quantities ranging from 1 μg to 0.125 μg to illustrate effects on the sensitivity of detection in a Western blot format. M designates the molecular weight marker lane. Suppression of signal can be seen when the blot in the center is incubated with NHS76 at a concentration of 0.5 μg/mL in the presence of only 25 ng/mL H1-DNA hitchhikers.

clinical use have long realized that this is not the case (e.g. [1]) and have implemented methods to measure the amount of process specific impurities that elute with the antibody (e.g. endotoxin, residual host cell DNA, and host cell proteins—which were recently reviewed in [2]). Suppliers of antibodies destined for *research laboratories* have yet to achieve a similar level of testing, as evidenced by recent reports highlighting the frustrations of scientists working with commercially available research antibodies [3–6]. An editor recounts how authors have had to withdraw papers because an antibody against a novel marker is found to stain tissue in knockout animals that lack the actual marker they are targeting [3]. Even the removal of closely related variants to a targeted protein, can still result in the staining of tissue, as documented by researchers who have had to confront the problem in double [7] and triple [8] knockout mice. Further examples are reviewed in [6]. Our purpose here is to highlight the disruptive role played by the targeted antigens that actually hitchhike along during the purification process, bound to an antibody's complementarity-determining regions (CDRs). We first described these hitchhiker antigens for the purification of clinical antibodies [1], however their impact on basic research can be just as severe.

Prior to the widespread use of Proteins A or G, affinity chromatography meant using a column made of a specific antigen that your antibody of interest would selectively interact with. If both arms of an antibody were contaminated with the targeted antigens from a cell culture harvest, those antibodies would not bind the column and would wash away. However, the binding of the Fc region by Protein A or G allows for antibody CDRs to bring along such antigens during purification. Given the growing use among scientists of antibodies for targeting intracellular structures, such reagents contaminated with their own antigens can compromise research; and nowhere can that be more problematic than the targeting of histones—a cornerstone in the widely popular chromatin immunoprecipitation assays used to characterize the transcriptional state of genes [9]. We have demonstrated the phenomenon of hitchhiking antigens using a fully human antibody that binds histones and has a higher affinity for histones complexed with DNA [1]. Here we will use a fully murine version of a similar antibody to simulate the murine monoclonals most often used in the research lab. The goal is twofold. First, illustrate the complications created using research antibodies which may appear to be pure. Second, present an adaptation for murine antibodies to our earlier method removing hitchhikers with a high salt wash when the antibody is immobilized on a Protein G column.

2. Materials and methods

2.1. Reagents

Murine TNT-1 (muTNT-1), a mouse IgG2a monoclonal antibody targeting histone H1 complexed with DNA, and chimeric TNT-1 (chTNT-1), the same variable regions from the murine version grafted onto human IgG1 constant regions, were produced at Peregrine Pharmaceuticals and Avid Bioservices, respectively (Tustin, CA). NHS76, a fully human IgG1 antibody targeting the same antigen complex, was developed by Peregrine Pharmaceuticals (Tustin, CA) and Cambridge Antibody Technology (Cambridge, UK). All antibodies were manufactured at Peregrine and Avid in mammalian cell cultures incubated in a humidified 5% carbon dioxide atmosphere set at 37 °C. Calf histone H1 and herring sperm DNA (both, Roche

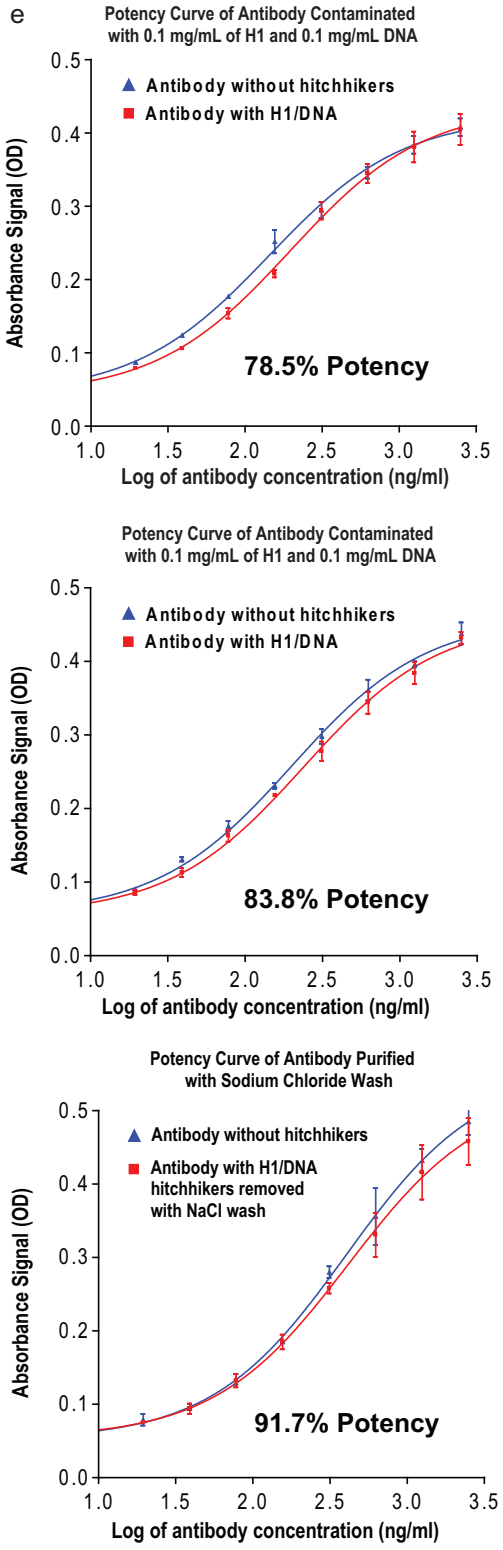

Fig. 1. (*Continued*).

The same blot was stained with a general protein stain to verify the presence of the histones (far right panel). (e) H1 histones and DNA were placed in a 96-well plate and probed with a serial dilution of NHS76 from 2.5 μg/mL down to 9.75 ng/mL (plotted logarithmically here). The absorbance values (450 nm) at each concentration were plotted as a sigmoidal dose–response curve and the linear portion of the curves compared between the two samples. The percentage of relative potency was then determined from an analysis of variance between the two curves. Error bars indicate standard deviations (SD).

Applied Science, Indianapolis, IN) were used for antibody spiking experiments in Fig. 1.

2.2. Antibody purification

Starting material for the purification studies consisted of cell culture supernatant clarified of cell debris by centrifugation at 2000 rpms for 15 min in an Allegra 6R centrifuge (Beckman Coulter, Brea, CA). A starting concentration in the supernatant of 2.4 μg/mL muTNT-1 was estimated using an ELISA-based antibody capture format (see Section 2.12). Approximately 400–500 mL samples were purified during each run on a BioLogic DuoFlow chromatographic system (BioRad, Hercules, CA) using a 5 mL Pharmacia HiTrap Q HP (quaternary amine) column and a 1 mL Pharmacia HiTrap rProtein G HP column placed in tandem (both, GE Healthcare, Uppsala, Sweden). Henceforth, these columns will be referred to as the Q column and the Protein G column, respectively. The flow rate was 1 mL/min throughout the procedure.

Control purification runs simulating standard elution schemes from a Protein G column did not have a Q column attached in front. The control runs included an equilibration step with PBS (150 mM NaCl, pH 7.4) for 35 min (35 Protein G column volumes [CV]) followed by elution with one of three buffer formulations that were evaluated: (i) 20 mM citrate, 150 mM NaCl, pH 2.5; (ii) 20 mM citrate, 20 mM phosphate, 150 mM NaCl, pH 2.5; and (iii) 0.5 M arginine, 1 M NaCl, 20 mM citrate, 20 mM phosphate, pH 2.5. Elution occurred for 10 min (10 CV) prior to re-equilibration with PBS (150 mM NaCl, pH 7.4).

Purification runs designed to remove hitchhikers had the cell culture supernatant adjusted to 400 mM NaCl using PBS containing 2 M NaCl based on a method described in [1]. Purifications were conducted with a Q column present in front of the Protein G column to capture residual DNA. Upon sample loading, the columns were equilibrated with PBS (at 400 mM NaCl, pH 7.4) for 40 min (for the 5 mL Q column that equals 8 CV and for the 1 mL Protein G column that equals 40 CV). The Q column was then removed from the line and the Protein G was subjected to an increase to 2 M NaCl in 2 min (2 CV) using PBS containing 2 M NaCl (pH 7.4), it was rinsed with the 2 M NaCl PBS for 10 min (10 CV), before being adjusted to 1 M NaCl or 150 mM NaCl PBS (pH 7.4) in 1 min (1 CV). After equilibration for 5 min (5 CV), the antibody was eluted into a fraction collector with a gradient of 0.5 M arginine, 20 mM citrate, 20 mM phosphate and NaCl (either 1 M or 150 mM) from pH 7 down to pH 2.5. The arginine, citrate, phosphate and sodium chloride concentrations were kept the same as the gradient elution dropped from pH 7 down to pH 2.5. Our investigations evaluated gradients of 10 and 20 min (10–20 CV). The Protein G column was then adjusted to 400 mM NaCl PBS over 1 min and re-equilibrated for another 5 min.

For the study to be complete, we also evaluated a purification scheme using only the elution gradient of 0.5 M arginine, 20 mM citrate, 20 mM phosphate and NaCl (either 1 M or 150 mM) from pH 7 down to pH 2.5 but lacking a Q column and any NaCl adjustment to 400 mM or washing step at 2 M.

For all three of the purification formats the antibody fractions were neutralized with 1 M Tris (pH 8) upon elution from the Protein G, and then dialyzed against PBS. The concentration of total protein was then determined in each collected fraction using the MicroBCA Protein Assay Kit from Pierce (Cat. # 23235; Thermo Fisher Scientific, Rockford, IL). The kit allows for general estimation of protein quantity using bicinchoninic acid (BCA) in a dye binding assay format (see Section 2.12). Further analyses on the pure fractions, as described in Section 3 and in Sections 2.4–2.11 in the Supplementary Materials and Methods, used the protein concentration values determined with the MicroBCA Protein Assay Kit. This BCA format does not differentiate between antibodies and other cellular proteins present. Therefore, antibody concentration in the starting cell culture supernatant was estimated using an ELISA-based antibody capture format (see Section 2.12).

2.3. Quaternary amine (Q column) elution

When taken off-line from the purification process, the quaternary amine column was still equilibrated in the 400 mM NaCl concentration of the adjusted sample. To elute any bound materials, the column was reattached to the system (*sans* any Protein G column) and further rinsed with the adjusted salt concentration at 4 mL/min for 5 min (4 Q column volumes). Then it was subjected to an increase to 2 M NaCl PBS over 5 min (4 CV), rinsed with 2 M NaCl PBS for 5 min (4 CV), before being equilibrated at 150 mM NaCl PBS (pH 7.4) for 5 min (4 CV). Fractions were collected throughout the process, dialyzed against PBS and the protein concentration values determined with the MicroBCA protein assay kit as described in Section 2.12.

3. Results and discussion

3.1. The deleterious effects of hitchhiking antigens

Antibodies purchased through commercial sources sometimes do not live up to their promise as sensitive detection agents and on other occasions appear to bind non-specifically. A quick analysis of the reagent by SDS-PAGE often reveals nothing but the characteristic banding pattern for an antibody because hitchhiker antigens usually are present in quantities below the level of detection using common protein stains (data not shown). To illustrate the problem, Fig. 1a–c presents a survey of several commercially and privately available anti-histone antibodies screened for hitchhikers using a sandwich assay format first described elsewhere [1]. An ELISA-based approach is more appropriate than evaluating the samples electrophoretically, especially when investigating larger amounts of sample. Different manufacturers stabilize their antibody products with proprietary excipients. Having to concentrate 5 μg of antibody for application to the well of an SDS-PAGE can have the unintended consequence of concentrating the stabilizers also; perhaps, to the point where they can interfere with the proper gel migration of an antibody and its hitchhikers. Applying samples to the wells of an ELISA avoids the complication of concentrating or manipulating the antibody sample and preserves the hitchhiker interaction in its native conformation. Furthermore, the stabilizers are then easily washed out during the rinsing step prior to the experiment.

Using a salt-washed chimeric version of the TNT-1 antibody as the detection reagent, we detected histone-DNA antigen associated with several purchased antibodies. To address concerns that these results are artifactual, the detection reagent was biotinylated and it was detected using streptavidin conjugated to alkaline phosphatase (AP), countering any argument that the signals seen can be attributed to secondary antibody cross-reactivity (Fig. 1b). Two ELISA plates were evaluated to show consistency of the results (Fig. 1a and c). Furthermore, as a control to show the streptavidin-alkaline phosphatase conjugate was working properly, 5 μg of the biotinylated antibody used as the detection reagent was also placed in ELISA wells, in triplicate (red bar; signal > 3 OD at 5 min). The signals from these antibody samples were quantitatively compared to a series of standards which included 2.5 μg H1 complexed with DNA (dark purple bar), 2.5 μg DNA (dark blue bar), and 2.5 μg H1 (green bar), all applied in triplicate, as well as 5 μg of a human anti-histone antibody (NHS76) containing about 2.5 μg of hitchhikers (light gray bar). For three of the commercially available samples being tested, the 5 μg antibody samples appear to be contaminated with about 2.5 μg of histone H1/DNA (compare them to the dark

purple bar). Assuming a molecular weight of 150 kDa for the antibodies and 22 kDa for the H1, every 1 mol of these three antibodies may be contaminated with 3.4 mol of H1. This is conceivable given the complexation of histones and DNA, with one histone bound to the CDR region of an antibody and the remaining histones complexed to the first through their mutual interactions with a long strand of DNA.

Deleterious effects due to the presence of hitchhikers can be demonstrated by comparing Western blots incubated with a pure, fully human, anti-histone antibody (NHS76) or the pure antibody spiked with histone H1 and DNA (Fig. 1d). For these experiments, a solution of NHS76 at 2 mg/mL and an identical solution spiked with 0.1 mg/mL H1 and DNA were diluted to several concentrations. Fig. 1d demonstrates how at one of those dilutions the sensitivity of NHS76 is compromised when 0.5 μg/mL (3.3 nM) of the antibody is contaminated with only 25 ng/mL (1.1 nM) of histone. Similar results were obtained at lower dilutions of 1 and 1.5 μg/mL NHS76 (data not shown). Heterogeneity in the size of the DNA molecules prevented an estimation of its molarity. To ensure a proper comparison, both blots were incubated with a general protein stain (Fast Stain) prior to probing with primary and secondary antibodies to verify equal loading of histones on both membranes, one of which is shown here. After washing out the Fast Stain and probing with the antibodies, both blots were incubated with NBT/BCIP substrate simultaneously for 5 min to detect the goat anti-human λ conjugated to alkaline phosphatase (AP), which should be bound to the NHS76 primary antibody.

The deleterious effects due to hitchhikers are equally complicated when the antigenic target is not just histone proteins immobilized on a membrane, but the entire H1-DNA complex immobilized in a 96-well plate. Such a complex structure is the target for testing NHS76 potency in an ELISA format as described in Section 2.7 of the Supplementary Data and [1]. The problems arising from a 2 mg/mL solution of NHS76 spiked with 0.1 mg/mL H1 and DNA are illustrated in Fig. 1e. Serial dilutions from 2.5 μg/mL down to 9.75 ng/mL result in a decreased potency value when comparing the spiked antibody to its pure counterpart. With dilution of the NHS76 to 2.5 μg/mL, there is only 125 ng/mL of H1 and DNA contaminating the sample, yet binding potency results from two separate experimental runs were 78.5% and 83.8%, illustrating that a minor amount of hitchhikers can cause an apparent decrease in potency and that this phenomenon is repeatable (Fig. 1e, upper two panels). To confirm that hitchhiker antigens are responsible for these results, the 2 mg/mL NHS76 solution spiked with 0.1 mg/mL H1-DNA was subjected to a purification procedure for the removal of histone hitchhikers, which is extensively discussed in [1]. By having the contaminated antibody adjusted to 400 mM NaCl, applied to quaternary amine and Protein A columns and then washed with 2 M NaCl prior to elution with a low pH buffer (20 mM sodium acetate, 150 mM NaCl, pH 3.2), the potency of the sample was restored close to the original pure antibody (91.7%; Fig. 1e, lower panel).

3.2. Anatomy of a peak: a simple low pH elution scheme

While a Protein A purification scheme works well for human antibodies, Protein G is more frequently employed for the purification of mouse and rat monoclonals used in research. We sought to develop a similar technique that would guide research antibody manufacturers in the development of more stringent purification processes. Fig. 2 illustrates why this is necessary. We explored several elution buffers for a histone-DNA murine antibody (muTNT-1) bound to Protein G starting with the common, low-pH elution scheme utilized by commercial research antibody manufacturers. Increasingly sophisticated buffers were investigated: (i) 20 mM citrate, 150 mM NaCl, pH 2.5; (ii) 20 mM citrate, 20 mM phosphate, 150 mM NaCl, pH 2.5; and (iii) 0.5 M arginine, 1 M sodium chloride, 20 mM citrate, 20 mM phosphate, pH 2.5; each of which provided a similar chromatographic pattern dissected in Fig. 2a, a result of elution buffer iii. The addition of arginine and sodium chloride were to promote antibody solubility [10] and antibody release from the Protein G column [11], respectively. Collected fractions were analyzed by SDS-PAGE (Supplementary Fig. 2b) using Coomassie and silver staining, neither of which revealed histone hitchhikers using elution buffer iii, although some were seen in fraction 3 using elution buffers i and ii (data not shown).

SDS-PAGE proved sensitive enough for detection of antibody fragments, however, a sandwich ELISA format was employed for detection of histone H1, using a salt-washed chimeric version of the TNT-1 antibody as the detection reagent, as detailed in Section 2.5 of the Supplementary Data. For this data set only, 40 μg of protein from each fraction were applied to the wells (rather than 50 μg), the results being summarized in Fig. 2c. When compared to control quantities of H1-DNA (gray bars), 40 μg of the main elution peak, fraction 1, has about 1.1 μg of H1-DNA (Fig. 2c, upper panel). If the hitchhikers are solely H1, that would be an antibody to histone molar ratio of 5.37:1, in other words, given the fact that an IgG antibody has two CDRs, nearly one out of every 11 antibody CDR is occupied by a hitchhiking antigen. We used this molar ratio as our measurement of *histone H1 hitchhiker* contamination. The same wells were probed for muTNT-1, revealing that nearly equal quantities of antibody were present in the wells containing fractions 1 and 2 (Fig. 2c, lower panel). Nearly half of the antibodies being purified can be lost if fraction 2 were not collected. All three fractions were also subjected to DNA analysis using a quantitative PCR technique detailed in Section 2.6 of the Supplementary Data. We used this residual DNA analysis as our measurement of *DNA hitchhiker* contamination. Surprisingly, the concentration of DNA in each fraction differed by orders of magnitude, not just with the run illustrated here, but repeatedly in 4 separate runs that were fractionated in the same manner and investigated for residual DNA (see Supplementary Figure 1). In the example here, fraction 2, which comprises the tail of the elution peak, has 145,201.63 pg (~145 ng) of DNA/microgram of antibody (Fig. 2d, left panel). In comparison, the histone contaminated fraction 1 has only 25.62 pg DNA/μg antibody and fraction 3 has 983 pg DNA/μg (Fig. 2d, right panel). Fig. 2e illustrates the dichotomous effects of histone H1 and DNA hitchhikers on the apparent potency of an antibody. The H1 contaminated fraction 1 returns a binding potency value of 557.8% relative to an antibody sample from the stringent purification procedure described in Section 3.4 (Fig. 2e, upper panel). On the other hand, the DNA contaminated fraction 2 returns a binding potency of only 15% relative to the antibody purified in Section 3.4 (Fig. 2e, lower panel).

To prevent losing significant amounts of antibody, researchers often collect a peak's tail in the same tube with the main peak. We wanted to evaluate what might happen if the sample was collected as a single peak encompassing fractions 1 and 2. We did collect five purification runs in this manner and found a deceptive averaging of the effects from those muTNT-1 antibodies heavily contaminated with DNA and those contaminated with H1. The hitchhiking antigens for these five muTNT-1 samples ranged from 0.9 to 10 ng DNA/μg antibody and from 0.27 to 0.62 μg of H1-DNA/50 μg antibody (see the blue and yellow highlighted cells in Table 1 of the Supplementary Data). Calculating the molar ratio of antibody CDR to histones as our measure of H1 contamination, the overall numbers ranged from 23:1 down to 55:1, indicating fewer H1 contaminated CDRs than the elution buffer iii method discussed in Fig. 2 (see the green highlighted cells in Table 1). However, the level of DNA contamination seen for these five purification runs far exceeded that seen with the purified antibody using elution

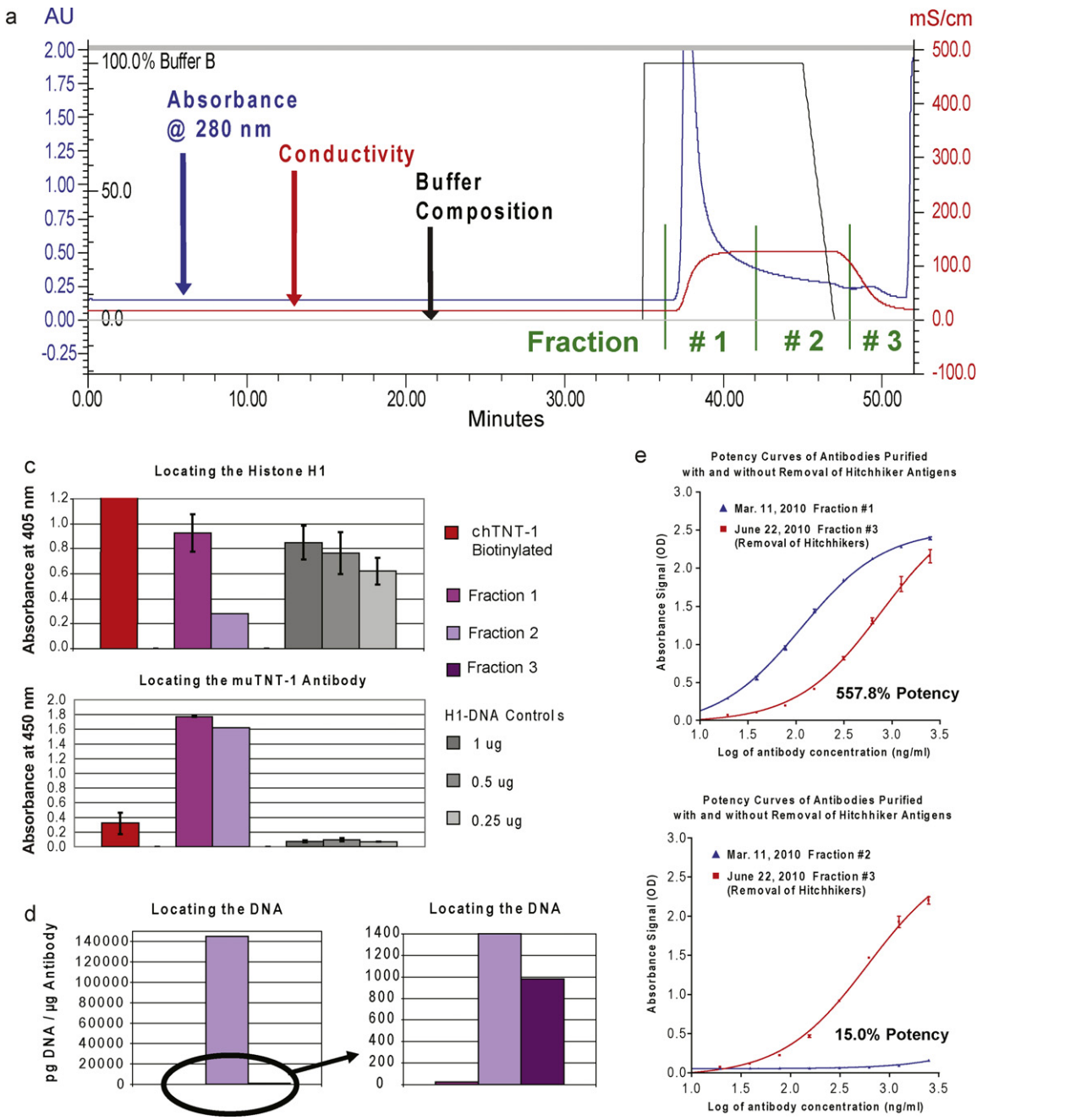

Fig. 2. Anatomy of a peak: a simple low pH elution scheme. (a) Sample chromatogram encompassing three traces, including the buffer composition at each timepoint (black), the actual shift in conductivity in the flow cell as the mobile phase changes (red), and the UV absorbance of the column elution at 280 nm (blue). Collected fractions are delineated in green. This sample chromatogram was generated using elution buffer iii (discussed in Section 3.2). (b) Non-reduced SDS-PAGE of the collected fractions are presented in Fig. 2b of the Supplementary Data. (c) Forty micrograms of protein from fractions 1 and 2 were applied to the wells of an ELISA plate to locate H1 and the muTNT-1 antibody as described in Section 2.5 of the Supplementary Data (upper and lower panels, respectively). Fraction 3 did not have sufficient material for this analysis. As a control, 1 μg of the H1 detection reagent, biotinylated chTNT-1, was also placed onto the ELISA plate in triplicate (red bar). (d) Locating the fractions with DNA involved the use of quantitative PCR as described in Section 2.6 of the Supplementary Data. Due to the magnitude of DNA present in fraction 2, it dominates the bar graph (left panel). An expanded view of the circled portion, reveals the amount of DNA in fractions 1 and 3 as well (right panel). (e) Relative binding potency of the antibody from fraction 1 (upper panel) and fraction 2 (lower panel) was determined as described in Section 2.7 of the Supplementary Data and [1] by comparing the sigmoidal dose–response curve against a curve generated from the purified antibody in Fig. 4 (fraction 3 from June 22, 2010 purification). Error bars indicate SD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

buffer iii and fraction collection (compare DNA levels in Table 1 to fraction #1 in Supplementary Fig. 1). Even a purification run four times greater in scale (Supplementary Fig. 1d—Purification of June 29, 2010) than these five purification runs has less DNA than most of these runs. Such a combination of H1 and DNA hitchhikers influence the results of a binding potency assay, as is the case for one of the five samples tested against the same antibody purified with the method described in Section 3.4. The presence of H1-DNA hitchhikers returned a relative value of only 37.6% (data not shown).

Table 1 summarizes the results in terms of a data "Range" which is a more useful parameter than "Average" or "Standard Deviation" when trying to quantify an often sticky and complex, heterogeneous hitchhiker consisting of discreet histone proteins and DNA of variable size. Because readers expect to see statistical analyses,

average and standard deviation have been included for the record, however, given the physical nature of the hitchhikers, the size of the standard deviations are not unexpected.

3.3. Anatomy of a chromatogram: introducing a gradient elution scheme

Given the presence of multiple hitchhikers co-eluting with muTNT-1, we investigated a gradient elution format using buffer iii (0.5 M arginine, 1 M sodium chloride, 20 mM citrate, 20 mM phosphate) but starting from pH 7 and going down to pH 2.5. Since those antibodies heavily contaminated with DNA appeared in the trailing edge of the elution peak in Fig. 2a (fraction 2), we reasoned that such a gradient could identify the highest and gentlest pH that would elute muTNT-1 without hitchhikers or at least resolve them from antibody with hitchhikers.

Fig. 3a illustrates one of four sample chromatograms, each of which had a significant removal of histone hitchhikers in a pre-gradient elution peak (fraction 1 and yellow bars in Fig. 3c and d). Fractionation of the histones from their antibody carriers occurred regardless of whether the elution buffer had 1 M or only 150 mM sodium chloride (data not shown). SDS-PAGE analysis clearly shows the presence of the two major histone classes in fraction 1 (H1 as well as core histone bands seen in Supplementary Fig. 3b); however, an ELISA analysis of 50 μg from each fraction compared to a 1 μg control quantity of H1-DNA (gray bar) reveals the antibody fractions 3 and 4 still carry about 0.6 and 0.75 μg of hitchhikers, respectively (upper panel Fig. 3c). Probed for muTNT-1, the same wells also reveal a similar loading of the antibody in fractions 3, 4 and 5; however, a significant portion of the 50 μg protein loaded into the fraction 1 wells are histones, not antibodies (lower panel Fig. 3c). We would have expected to have even less antibody present in fraction 1 in the hopes of minimizing antibody loss as much as possible. Fractions 1, 3 and 4 were also subjected to DNA analysis using a quantitative PCR technique detailed in Section 2.6 of the Supplementary Data (Fig. 3d). The goal was to fractionate the hitchhiker containing antibodies from their pure counterparts by gradient elution with the hope of having the pure isoforms elute first. That was not the case, as seen in Fig. 3d (left panel) with the bulk of the DNA located in fraction 1 (6.291 μg/μg antibody). The gradient eluted fractions 3 and 4 have only 102.78 and 61.1 pg of DNA/μg muTNT-1 antibody (Fig. 3d, right panel). To verify these observations, DNA analysis was conducted on another one of the four samples purified with this method and found to have a similar distribution with 1.049 μg of DNA/μg antibody in fraction 1 and only 46.79 and 82.0 pg of DNA/μg muTNT-1 antibody in the remaining two fractions collected during the gradient elution (data not shown).

As Fig. 3a illustrates, the bulk of the antibody eluted at a pH far higher and milder than the pH 2.5 most individuals consider using for a Protein G elution, therefore, we were interested in seeing how this would affect the performance of the muTNT-1 antibody that eluted in the main antibody peak (fraction 3). With 0.6 μg of hitchhiking H1/DNA present in 50 μg of antibody, there would be an antibody to histone molar ratio of 11.66:1. In other words, only 1 out of every 23 muTNT-1 CDR is occupied by a hitchhiking antigen; and yet, despite the removal of significant amounts of hitchhikers, Fig. 3e returns a binding potency value of 558.7% for the purified antibody (fraction 3) compared to an antibody sample from the stringent purification procedure described in Section 3.4. Since the bulk of the DNA contamination was removed in fraction 1, the aberrant potency value is likely due to the histone contaminants. Summarizing the antibody CDR to histone molar ratios for all four sample runs that used this gradient (see Supplementary Table 2), we see a range of results indicating a substantial number of CDRs are still occupied by histones despite their affinity "purification" (see the green highlighted cells in Table 2).

3.4. Anatomy of a purification process: incorporating anion capture and stringent wash steps prior to the gradient elution scheme

Since our gradient elution step demonstrated the removal of a portion of the hitchhikers accompanying muTNT-1, it was logical to then add the steps we first described for purification of a human antibody for clinical use [1]. A quaternary amine guard column was added in front of the Protein G to capture the bulk of the DNA. DNA binding to the support was increased by adjusting the cell culture supernatant to 400 mM NaCl for removal of the histone H1 shielding the negatively charged phosphate backbone. To increase the removal of hitchhikers attached to the antibody's CDRs, the Protein G bound material was subjected to a 2 M NaCl wash prior to elution with the gradient step.

Fig. 4a illustrates the procedure just summarized and detailed further in Section 2.2. The chromatograms for the Q column and the Protein G elution are placed in the left and right panels of Fig. 4a, respectively, to illustrate the amount of material captured on each of the columns placed in tandem. The Q column is taken off-line prior to having the Protein G subjected to a 2 M NaCl wash and then the elution gradient. For the purpose of this study, the Q column is then separately washed with 2 M NaCl for the recovery and analysis of the captured DNA.

By adjusting the supernatant to 400 mM NaCl prior to loading on the columns, much of the H1 histones were removed and washed away in the flow through, as evidenced by the presence of only core histones in the Q column fractions (Supplementary Fig. 4b, fractions Q1 and Q2) and the 2 M NaCl wash step (Supplementary Fig. 4b, fraction 1). Unlike the ELISAs in Figs. 2 and 3, quantitatively comparing 50 μg from each fraction to a 1 μg control of H1-DNA proved insufficient (Fig. 4c, upper panel, dark gray bar). Here, the collected fractions were compared to a control of 0.25 μg H1-DNA as seen in an expanded view of Fig. 4c (middle panel, light gray bar). Results suggest that 50 μg of the main antibody peak (fraction 2) has only 0.1 μg of hitchhikers; an antibody to histone molar ratio of 69.76:1, indicating only one out of every 140 muTNT-1 CDR is occupied by a hitchhiking antigen. The same wells were probed for antibody, revealing that nearly equal quantities of muTNT-1 were present in the Protein G fractions (Fig. 4c, lower panel). Although we hoped less antibody would have been present in the 2 M NaCl wash (fraction 1), those antibodies may be damaged or may never have bound the Protein G column, instead forming a bridge with H1-DNA to link them to other antibodies already bound to the column. Tracking the DNA using quantitative PCR finds the bulk of it in the Q column fractions (2.75 ng and 759.6 ng DNA/μg muTNT-1 for Q1 and Q2, respectively). A large amount also elutes with the 2 M NaCl wash (132.8 ng DNA/μg muTNT-1 for fraction 1), leaving only 38.78 pg and 42.95 pg DNA/μg muTNT-1 for fractions 2 and 3, respectively (Fig. 4d). The relative binding potency for an antibody having undergone such a stringent purification procedure had to be evaluated against another batch purified under the same conditions (Fig. 4e). Such a comparison returned a binding potency of 101.8% and demonstrated the consistency of the purified product from one lot to another.

This purification process for the muTNT-1 antibody was used 11 times prior to publication and included gradients of 10 min, 20 min or no gradient at all, both with elution buffer containing 1 M or only 150 mM NaCl (see Supplementary Table 3). The H1 hitchhiker content for most of the purified antibodies was similar to the run illustrated in Fig. 4, regardless of NaCl concentration in the elution buffer. On average, the H1 hitchhiker content was greater for runs with a 20 min gradient than a 10 min one. The run we conducted

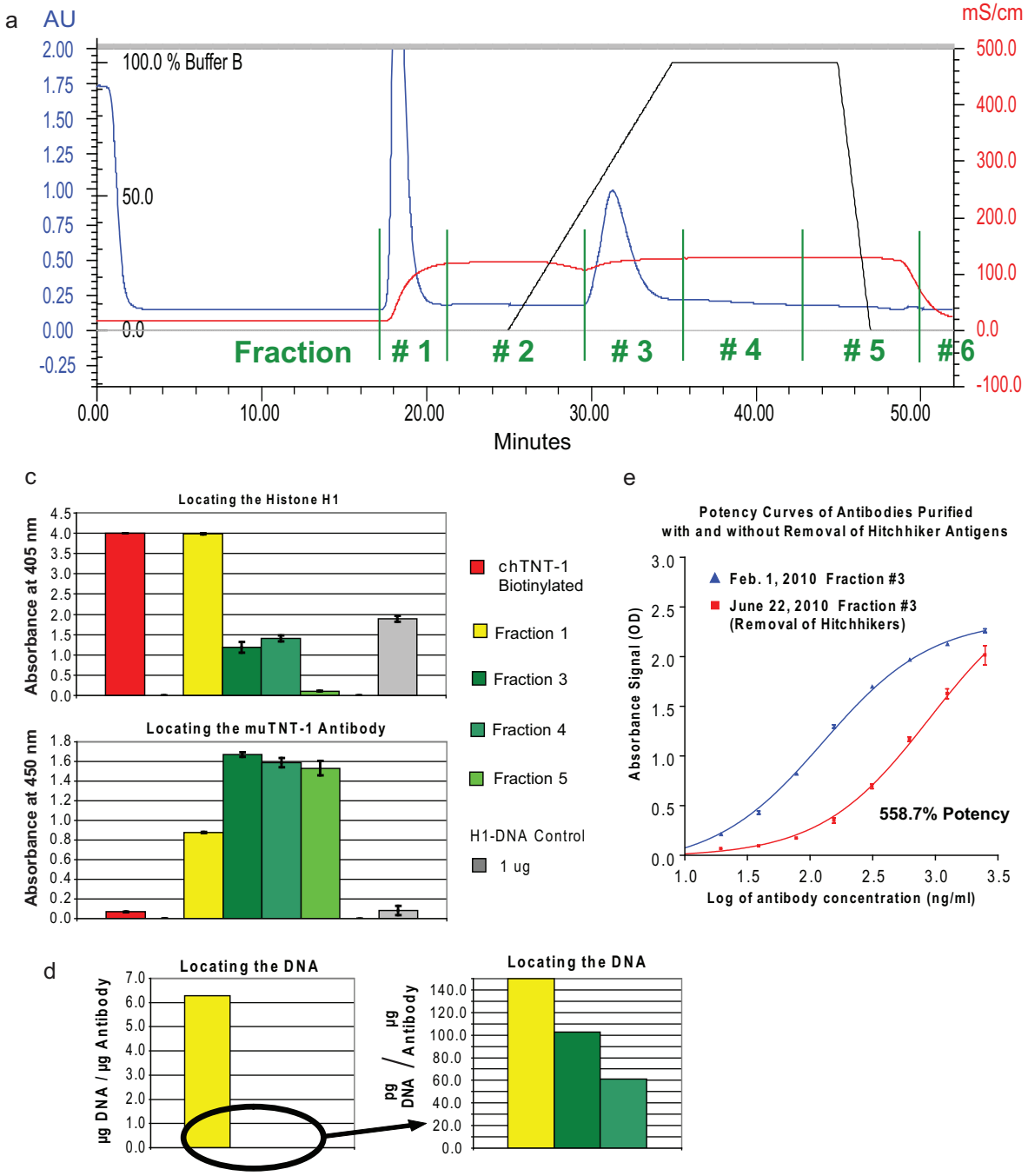

Fig. 3. Anatomy of a chromatogram: introducing a gradient elution scheme. (a) Sample chromatogram with the same markings described in Fig. 2a. (b) Non-reduced SDS-PAGE of the collected fractions are presented in Fig. 3b of the Supplementary Data. (c) Fifty micrograms of protein from fractions 1, 3, 4 and 5 were applied to the wells of an ELISA plate to locate H1 and the muTNT-1 antibody as described in Section 2.5 (upper and lower panels, respectively). Fraction 2 did not have sufficient material for this analysis. As a control, 1 µg of the H1 detection reagent, biotinylated chTNT-1, was also placed onto the ELISA plate in triplicate (red bar). (d) Locating the fractions with DNA involved the use of quantitative PCR as described in Section 2.6 of the Supplementary Data. Due to the magnitude of DNA present in fraction 1, it dominates the bar graph (left panel). An expanded view of the circled portion, reveals the amount of DNA in fractions 3 and 4 as well (right panel). (e) Relative binding potency of the antibody from fraction 3 was determined as described in Fig. 2e. Error bars indicate SD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

with no gradient still benefited from the capture of DNA with the Q-column; however, the antibodies were eluted at the low pH of 2.5 and the quantity of H1 hitchhikers were higher than the 10 min ones. We chose to go with the shorter gradient (10 min) and 1 M NaCl for future purifications.

The utility of incorporating a stringent wash for an antibody at a neutral pH prior to the elution gradient is best summarized in the range of molar ratios obtained from our 11 purification runs (compare the green highlighted cells in Table 3 to those in Table 2). On average, only one out of every 75 muTNT-1 CDR is occupied by a hitchhiking antigen when a stringent salt wash was incorporated, whereas the average number of CDRs contaminated with histones in Table 2 is five times that (compare average molar ratio 75.15 to 14.66).

To demonstrate that such a procedure can be scaled up, we also used the same method in two purification runs starting with

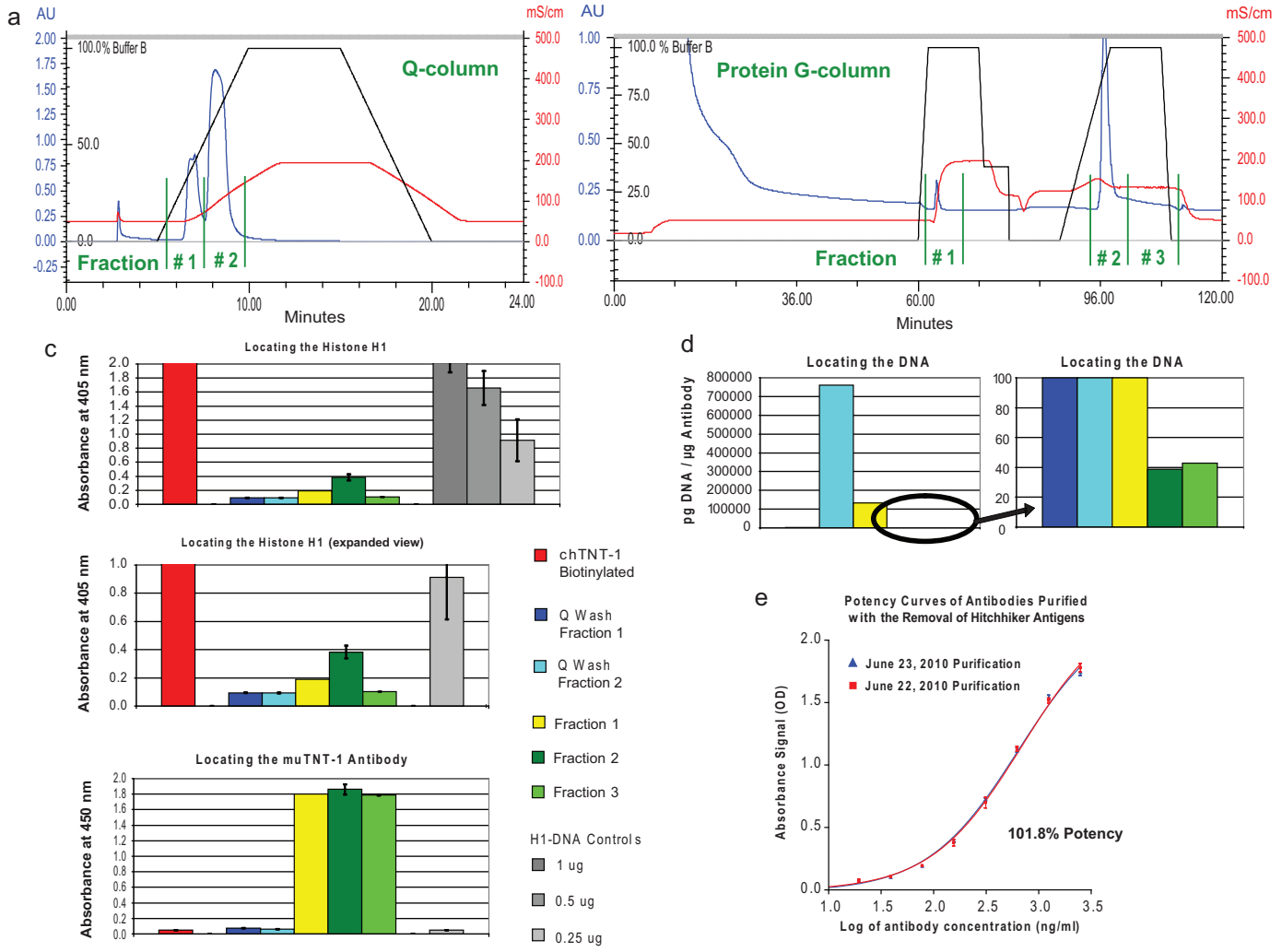

Fig. 4. Anatomy of a purification process: incorporating anion capture and stringent wash steps prior to the gradient elution scheme. (a) Sample chromatograms for the Protein G elution (right panel) and the subsequent elution of captured DNA hitchhikers from the quaternary amine guard column (left panel). The chromatograms have the same markings as those described in Fig. 2a. (b) Non-reduced SDS-PAGE of the collected fractions are presented in Fig. 4b of the Supplementary Data. (c) Fifty micrograms of protein from fractions 1, 2, 3 and Q-column fractions 1 and 2 were applied to the wells of an ELISA plate to locate H1 and the muTNT-1 antibody as described in Section 2.5 (upper and lower panels, respectively). As a control, 62.5 ng of the H1 detection reagent, biotinylated chTNT-1, was also placed onto the ELISA plate in triplicate (red bar). (d) Locating the fractions with DNA involved the use of quantitative PCR as described in Section 2.6 of the Supplementary Data. Due to the magnitude of DNA captured by the Q column and in fraction 1, they dominate the bar graph (left panel). An expanded view of the circled portion, reveals the amount of DNA in fractions 2 and 3 as well (right panel). (e) Relative binding potency of the antibody from fraction 2 was determined as described in Fig. 2e. Error bars indicate SD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

1.6 L each of the same cell culture supernatant. As stated in Section 2.2, the starting material for runs highlighted in Figs. 2–4 were 400–500 mL, therefore, a fourfold scale up required a few minor adjustments to the overall purification procedure. To capture all of the DNA, two Q columns were placed in tandem in front of the Protein G column. To handle the increased antibody load, the Pharmacia HiTrap rProtein G HP column was upgraded from a 1 mL to 5 mL volume. Finally, to maintain similar elution profiles with the smaller scale runs, the flow rate was increased to 5 mL/min throughout the procedure. As Supplementary Fig. 4f illustrates, the chromatogram for the first of two scale up runs is not very different from the one in Fig. 4a. The relative binding potency of the antibodies purified from each of the two scale up runs were tested against the same antibody purified in Fig. 4a. Obtaining potency results of 94.7% for the purification of July 8th and 91.9% for the purification of July 14th demonstrate the lot-to-lot reproducibility of the purified antibodies and, conversely, confirm the suitability of the antibody in Fig. 4a as a reference standard (Suppl. Fig. 4g and h). Regardless of scale, with the incorporation of the pH gradient in this process, the antibodies were stringently washed at a neutral pH and then eluted at the highest pH possible, hence, minimizing exposure of the purified product to an acidic environment.

3.5. The hitchhiker threat to research

Contamination of biopharmaceuticals with host cell proteins (HCPs) has been a recent focus of the industry with major efforts devoted to creating techniques for their detection in the final product [2]. What differentiates hitchhikers from other HCPs is the product specific nature of this subset of proteins; in this case, different antibodies will bring a unique set of hitchhikers along with them during the purification process. This has already been successfully demonstrated not just by our lab [1], but by others who have shown definitively that host cell impurities do not bind avidly to Protein A coated beads without the presence of antibody [12]. Furthermore, different antibodies produced in the same host cell can have different levels of HCPs co-purifying with them even if the antibodies have the same constant regions and only differ in their

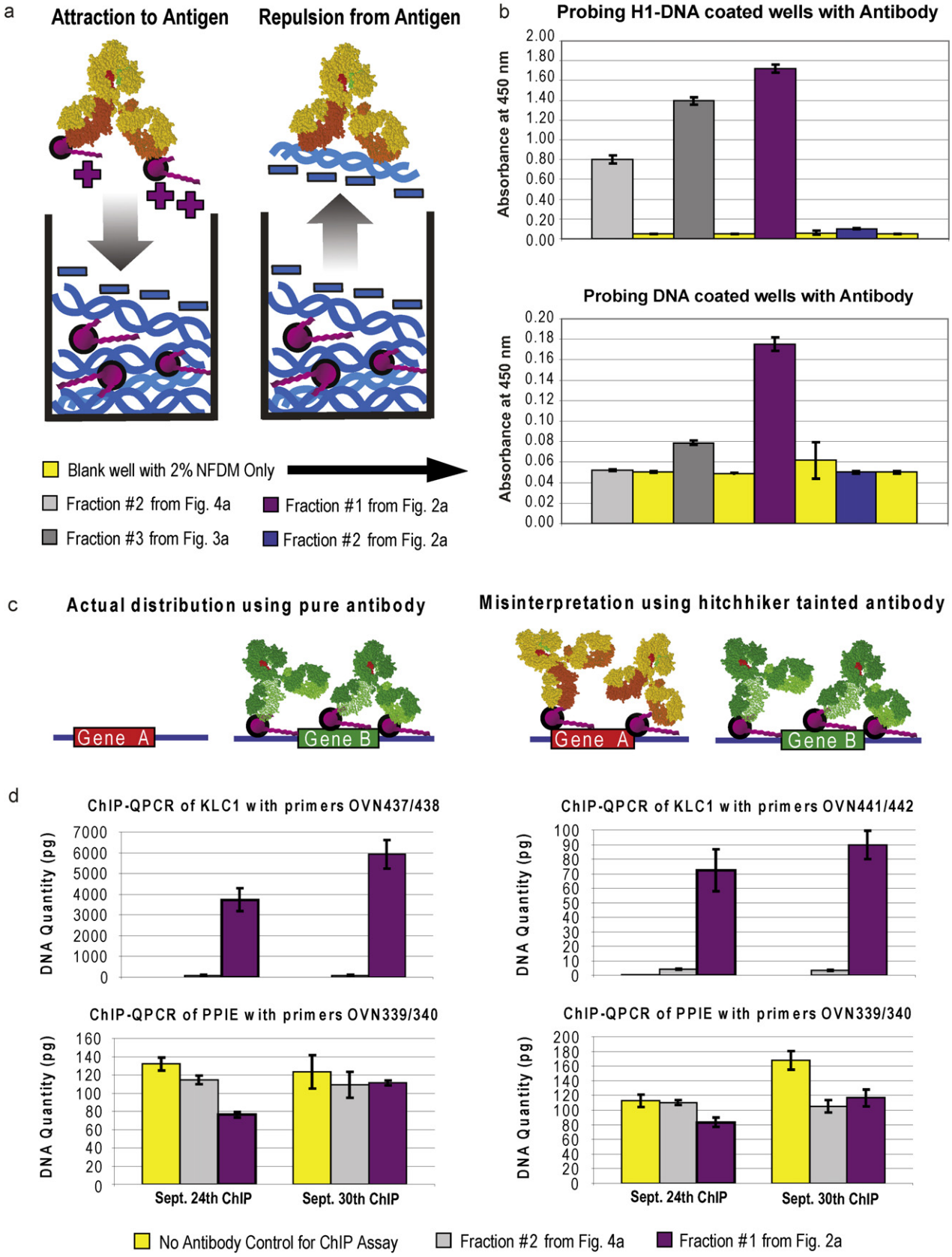

Fig. 5. Histone hitchhikers are a threat to chromatin immunoprecipitation (ChIP) assays. (a) Illustrations explaining the dichotomous results obtained with antibodies bearing histone versus DNA hitchhikers. (b) The main antibody fractions from the purifications illustrated in Figs. 2–4 were directly compared for their DNA binding ability as described in Section 2.10 and Section 3.5. To address any concerns that the signals from the wells were due to non-specific interactions, muTNT-1 binding to wells with 1 μg H1 and 1 μg DNA were compared for each antibody to wells with no antigen (yellow bars to the right of each antibody, upper panel). Similarly, binding to wells coated only with 1 μg DNA were compared for each antibody to corresponding wells with no antigen (lower panel). (c) Truly pure antibodies (green) should help researchers determine which genes carry a particular histone and which do not (left panel); whereas, hitchhiker contaminated ones (yellow) may cause false positive results during a ChIP assay

CDRs (reviewed in [2]). Hitchhiker antigens may also differ from other HCPs by the manner in which they can elude detection with current methods, such as sandwich ELISAs and 2-dimensional (2D) Western blots, both of which employ polyclonal antibodies [13]. Generally, these polyclonals are created by targeting HCPs found in the cell culture supernatant of a host cell culture not expressing the antibody. Since hitchhikers co-elute with the antibody during purification, the situation can arise where the most common hitchhiker epitopes recognized by the polyclonals are inaccessible due to interactions with the CDRs of your antibody of interest. This can lead to under-detection of hitchhikers when using polyclonals in assays such as the aforementioned sandwich ELISA. Similarly, those polyclonals generated using the supernatant from high viability cells may not detect intracellular hitchhikers which happen to be prevalent in the cell culture media in low viability cells [14].

Given the prevalence of protein interactions with other cellular components, it should not be surprising that hitchhikers associated with a particular antibody may comprise more than the targeted antigen, as is the case with our H1-DNA example for muTNT-1. Fig. 2e illustrates the dichotomous results obtained whether muTNT-1 is carrying mostly histone H1 or DNA. The nature of the H1-DNA interaction is such that a single H1 hitchhiker can string along multiple other H1s, all attached to a strand of DNA. Our potency assay, first described in [1], has wells coated with an antigen cocktail of 1 μg H1 and 5 μg DNA. The apparent potency value of 557.8% seen in Fig. 2e (upper panel) is explained when you consider that any well in our potency assay should have approximately 1 μg of histone. The addition of a hitchhiker contaminated antibody into the well introduces more antigenic target allowing a greater number of antibodies to bind that well, hence, resulting in a greater signal than wells containing antibody without histone hitchhikers (Fig. 5a, left panel). While the positively charged H1 hitchhikers would be attracted to the DNA laden wells, those antibodies contaminated mostly with DNA should show repulsion due to the negatively charged phosphate backbones (Fig. 5a, right panel), which explains an apparent 15% potency value seen in Fig. 2e (lower panel). A somewhat equimolar quantity of H1 and DNA contaminants also results in a suppressed potency value, albeit not as severe (Fig. 1e, 78.5% and 83.8% potency values).

To verify these concepts, a multi-well plate was prepared for comparison of antibody binding to wells coated either with 1 μg H1 and 1 μg DNA, with only 1 μg DNA or with no antigen at all (Fig. 5b). The key antibody fractions from Figs. 2–4 were directly compared to each other in H1-DNA coated wells and show an increasing binding signal for those fractions possessing an increasing amount of H1 contaminants (Fig. 5b, upper panel). The muTNT-1 fraction heavily contaminated with DNA had a signal comparable to those wells with no antigen, reinforcing the theory that repulsion is the factor. To counter any argument that the data reflects loss of potency with greater purification stringency rather than an artifactual increase in potency due to hitchhikers, the antibody fractions were also directly compared using DNA coated wells. The antigenic target is H1 complexed with DNA, with no significant binding of the CDRs to DNA alone. As expected, neither the fraction from our stringent purification in Fig. 4, nor the DNA contaminated fraction in Fig. 2, provided signal above those of the blank wells. However, the fractions possessing an increasing amount of hitchhikers still bind to the wells, reinforcing the theory that contaminating H1 promotes the muTNT-1's binding to DNA alone (Fig. 5b, lower panel).

If the antibody does not bind DNA alone, how is it possible for nanogram quantities of DNA to hitchhike with muTNT-1 even when H1 is present in picograms? While some of the DNA is bound to hitchhiking H1, the rest appears to bind to conserved, relatively alkaline regions found on most antibodies. Such non-specific complexation of DNA directly to the antibody structure, independent of CDRs, has been observed on IgGs [15] as well as on IgMs [16].

The presence of hitchhikers could help explain how some antibodies appear to bind their targeted antigen when staining tissue in knockout animals [3]. For anti-histone antibodies, the threat is greater given the popularity of chromatin immunoprecipitation (ChIP) assays for determining the histone content on a particular DNA fragment. Having demonstrated that hitchhiking H1 helps muTNT-1 bind DNA coated wells (Fig. 5b), one must ask whether hitchhiking histones can help antibodies promote immunoprecipitation of DNA where no such histones really exist *in vivo* (Fig. 5c). To verify this possibility, we chose to immunoprecipitate plasmid DNA harvested from a bacterial source, lacking in any DNA binding proteins, using a quantitative PCR technique described in Section 2.11 of the Supplementary Data. Since muTNT-1 does not bind DNA with its CDRs unless H1 is present, we should not see significant quantities of DNA immunoprecipitated with our stringently purified antibody (fraction 2 from Fig. 4a), and this appears to be the case when comparing it to a precipitation experiment with no antibody (Fig. 5d, upper panels). Conversely, using antibody purified in a more traditional manner (fraction 1 from Fig. 2a) resulted in an obvious precipitation of DNA, which could lead to the misinterpretation that the plasmid originally had significant amounts of H1 (see purple bars in Fig. 5d). To demonstrate that this is not a peculiarity of the DNA fragment being studied, two separate regions of plasmid DNA were subjected to the same ChIP analysis and they provided similar results (Fig. 5d, left and right panels). Furthermore, the ChIP assays were run on two separate occasions to demonstrate repeatability. Naturally, a control DNA (PPIE) and relevant primers were spiked into the reaction tubes prior to PCR for verification that the polymerase was functional in all of the reactions (Fig. 5d, lower panels).

Hitchhikers do not just threaten experiments, they can threaten an entire field of research. Appropriate to our histone example are the complicated results now being deciphered by auto-immune researchers, particularly those studying lupus. Anti-histone antibodies are a characteristic of the disease, however, much time has been spent also studying anti-DNA antibodies believed to be generated by those stricken with lupus. It has been almost 20 years since researchers suggested that the binding of "anti-DNA" antibodies to the glomerular basement membrane in kidneys is actually due to histone proteins imparting their antibody counterparts with the ability to bind that negatively charged membrane structure [17]. To clearly demonstrate how histone hitchhikers are the bridge between DNA and what appear to be anti-DNA antibodies, Guth et al. [18] instituted a high salt wash for a popular mouse antibody derived from auto-immune sera. The high salt wash disrupted binding to DNA, which could then be restored upon reconstitution of the antibody with histones [18]. And to illustrate that this is not an esoteric problem relegated to mouse models, Mason et al. [19] similarly demonstrated the phenomenon using an antibody derived from a human patient afflicted with lupus.

(right panel). (d) Immunoprecipitation of plasmid DNA was compared using muTNT-1 from the one-step, low pH elution described in Fig. 2 (fraction 1) and the same antibody from the stringent purification process described in Fig. 4 (fraction 2). Two DNA regions were amplified from a plasmid encoding an irrelevant mouse IgG (pKLC1) on two separate occasions, demonstrating that the phenomenon is not sequence specific or due to a technical error on a particular day. Amplification of the DNA region flanked by primers OVN 437 and 438 (left panel) shows a comparable pattern to the DNA flanked by primers 441 and 442 (right panel). The consistency of the reaction across all tubes was monitored with the use of mouse genomic DNA and primers 339 and 340, designed to amplify the PPIE gene (lower panel). Error bars indicate SD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4. Conclusion

We have presented a purification process that removes a complex of hitchhiker antigens. The process incorporates (i) the capture of antigens on a quaternary amine guard column, (ii) further removal of antigens stringently washed at a neutral pH, and then (iii) elution of the antibody using a pH gradient to minimize the unnecessary exposure to an acidic environment seen with the purification of some commercially available products. These components can be modified accordingly to suit other antigen–antibody pairs. The time has come for those in the business of supplying research antibodies to begin applying some of the more stringent purification techniques now employed in the clinical antibody industry. Furthermore, it is also time for those in research to think about the possibility that an anomalous result from an otherwise potent and functional antibody may be a not so simple case of hitchhiking antigens.

Acknowledgements

We thank Debra Harris, Aparna Roy and Dr. Eugene Mechetner for helpful discussions during the course of the experimentation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.07.016.

References

[1] K.A. Luhrs, D.A. Harris, S. Summers, M.H. Parseghian, J. Chromatogr. B 877 (2009) 1543.
[2] X. Wang, A.K. Hunter, N.M. Mozier, Biotechnol. Bioeng. 103 (2009) 446.
[3] C.B. Saper, J. Comp. Neurol. 493 (2005) 477.
[4] C.B. Saper, J. Histochem. Cytochem. 57 (2009) 1.
[5] J.R. Couchman, J. Histochem. Cytochem. 57 (2009) 7.
[6] J. Bordeaux, A.W. Welsh, S. Agarwal, E. Killiam, M.T. Baquero, J.A. Hanna, Biotechniques 48 (2010) 197.
[7] G. Jositsch, T. Papadakis, R.V. Haberberger, M. Wolff, J. Wess, W. Kummer, Naunyn Schmiedebergs Arch. Pharmacol. 379 (2009) 389.
[8] B.C. Jensen, P.M. Swigart, P.C. Simpson, Naunyn Schmiedebergs Arch. Pharmacol. 379 (2009) 409.
[9] P.M. Das, K. Ramachandran, J. vanWert, R. Singal, Biotechniques 37 (2004) 961.
[10] A.P. Golovanov, G.M. Hautbergue, S.A. Wilson, L.-Y. Lian, J. Am. Chem. Soc. 126 (2004) 8933.
[11] P.S. Gagnon, Purification Tools for Monoclonal Antibodies, Validated Biosystems, Tucson, Arizona, 1996, p. 155 (Ch. 9).
[12] A.A. Shukla, P. Hinckley, Biotechnol. Prog. 24 (2008) 1115.
[13] K.L. Hoffman, BioPharm 13 (2000) 38.
[14] M. Jin, N. Szapiel, J. Zhang, J. Hickey, S. Ghose, Biotechnol. Bioeng. 105 (2010) 306.
[15] P.S. Gagnon, BioProcessing J. 9 (2010) 14.
[16] P.S. Gagnon, F. Hensel, S. Lee, S. Zaidi, J. Chromatogr. A, 2010, doi:10.1016/j.chroma.2010.12.066.
[17] C. Kramers, M.N. Hylkema, M.C.J. van Bruggen, R. van de Lagemaat, H.B.P.M. Dijkman, K.J.M. Assmann, R.J.T. Smeenk, J.H.M. Berden, J. Clin. Invest. 94 (1994) 568.
[18] A.M. Guth, X. Zhang, D. Smith, T. Detanico, L.J. Wysocki, J. Immunol. 171 (2003) 6260.
[19] L.J. Mason, A. Lambrianides, J.D. Haley, J.J. Manson, D.S. Latchman, D.A. Isenberg, A. Rahman, Arthritis Res. Ther. 7 (2005) R971.
[20] M.H. Parseghian, R.L. Newcomb, S.T. Winokur, B.A. Hamkalo, Chromosome Res. 8 (2000) 405.
[21] H. Kohzaki, Y. Murakami, Proteomics 7 (2007) 10.
[22] W. Wray, T. Boulikas, V.P. Wray, R. Hancock, Anal. Biochem. 118 (1981) 197.