TECHNICAL BRIEF

Analysis conditions for proteomic profiling of mammalian tissue and cell extracts with antibody microarrays

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Antibody microarrays are a developing tool for global proteomic profiling. A protocol was established that permits robust analyses of protein extracts from mammalian tissues and cells rather than body fluids. The factors optimized were buffer composition for surface blocking, blocking duration, protein handling and processing, labeling parameters like type of dye, molar ratio of label *versus* protein, and dye removal, as well as incubation parameters such as duration, temperature, buffer, and sample agitation.

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In the last few years, antibody microarrays have had a significant impact on proteomic research [1, 2]. The format owes its success to the capacity to analyze proteomes globally in high throughput. However, in contrast to the growing need for studies of clinically relevant tissues, the platform still finds its major biomedical applications in the analysis of conditioned cell culture media, serum, and plasma samples as well as other body fluids like urine, cerebrospinal fluid, saliva, and tears (Supporting Information Table 1S). In our experience and that of others [3], the analysis of cellular proteins using current standard protocols, which were optimized for plasma or serum samples, failed to produce results of adequate quality. To date, there were only relatively few reports about analyses of cellular proteomes from tissue homogenates and cell lysates (Supporting Information Table 2S). Most of these used commercial antibody arrays, which display variable performances [2]. Furthermore, many protocols are time-consuming and involve the use of up to ten different buffers. This study reports on a thorough evaluation and concomitant optimization of the parameters for proteomic analysis of tissue protein extracts on antibody microarrays.

For analyses, the following standard protocol was established; more experimental detail is provided in the Supporting Information. Proteins were extracted from four pancreatic cancer cell lines and 18 human pancreatic cancer tissues as recently described [4]. Extracted protein was labeled with fluorescence dye DY-549 or DY-649 at a dye to protein (D/P) molar ratio of 18, with the assumption that 60 kDa is the average molecular weight of a protein. The protein concentration was adjusted to 2 mg/mL. Labeling occurred in the dark in 0.1 M carbonate buffer, pH 8.5, at 4°C for 2 h. Unreacted dye was quenched with 10% glycine for 30 min at 4°C in the dark. Labeled samples were stored at −20°C until use. Antibodies were spotted on epoxysilane-coated slides (Nexterion-E; Schott, Jena, Germany) using the contact printer MicroGrid-2 (BioRobotics, Cambridge, UK) and SMP3B pins (Telechem, Sunnyvale, USA) at a humidity of 40–45%. The printing buffer was composed of 0.1 M carbonate buffer (pH 8.5) containing 0.01% Tween-20, 0.05% sodium azide, 0.5% dextran, 5 mM magnesium chloride, 137 mM sodium chloride, and 1 mg/mL of the respective antibody. After printing, the slides equilibrated at a humidity of 40–45% overnight and were stored in dry and dark conditions at 4°C until use.

Printed slides were washed once for 5 min followed by another wash for 15 min with PBS (137 mM sodium

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Abbreviations: D/P, dye to protein; **NHS-ester,** *N*-hydroxysuccinimide ester; **PBST80,** PBS plus Tween-80; **SNR,** signal-to-noise ratio

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chloride, 2.7 mM potassium chloride, 10.0 mM disodium hydrogen phosphate, 1.76 mM sodium dihydrogen phosphate, pH 7.4) containing Tween-80 at a final concentration of 0.05% (PBST80). The slides were blocked with 5 mL of 10% non-fat dry milk (Biorad, Munich, Germany) in PBST80 for 3 h at room temperature using Quadriperm chambers (Greiner Bio-One, Frickenhausen, Germany) on an orbital shaker. Blocked slides were incubated in Quadriperm chambers with 50 µg labeled sample in 5 mL of 10% milk in PBST80 overnight in the dark at 4°C. The slides were then washed four times for 5 min in large volumes of PBST80, rinsed several times with deionized water, and dried in a ventilated oven at 22°C. Scanning of slides was performed with a ScanArray-4000XL (Perkin Elmer, Waltham, USA) at constant laser power and PMT. The images were analyzed with the software GenePix Pro 6.0

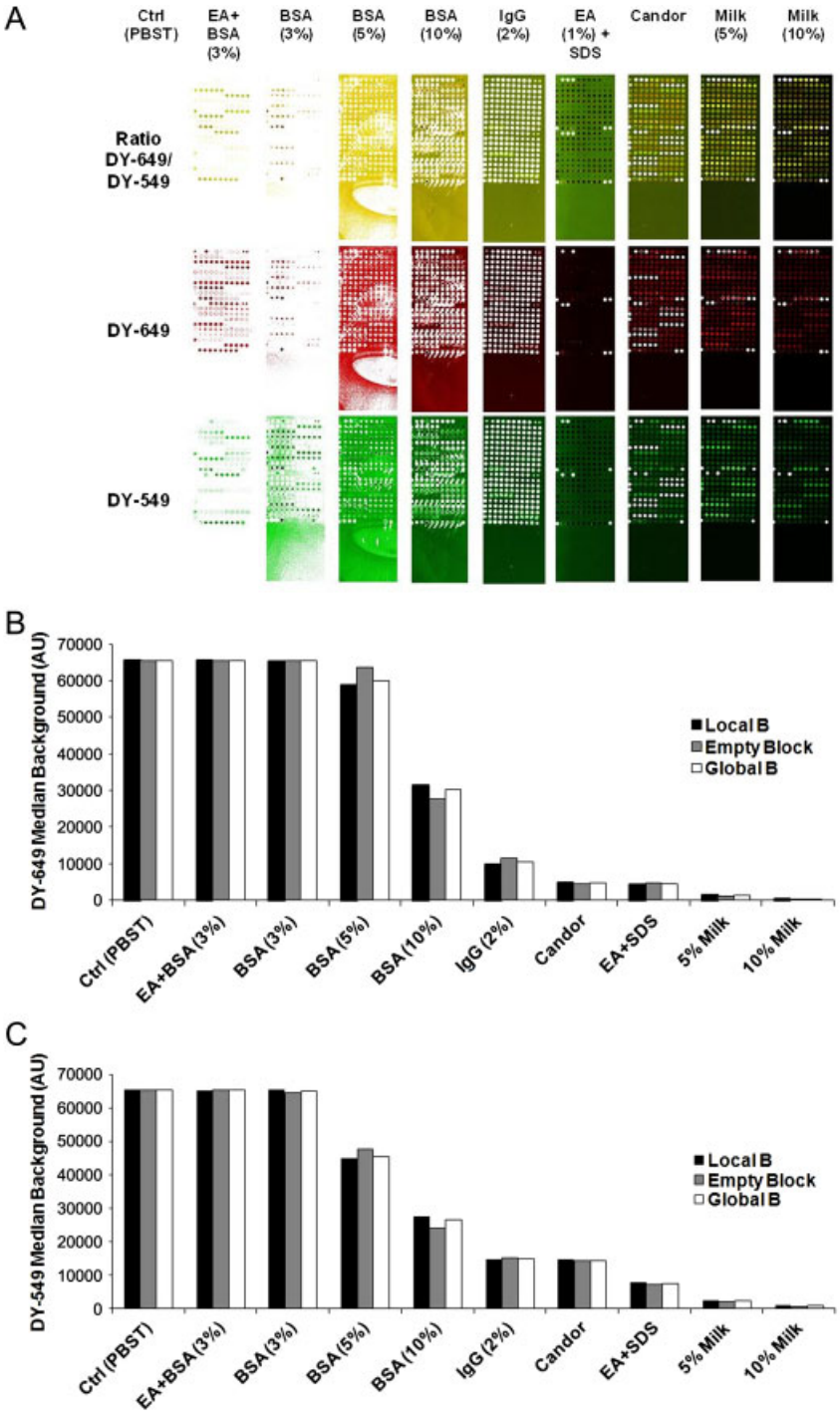

Figure 1. The effect of blocking buffers on background signal. In (A), the signals obtained with red and green dyes and the superimposed images are presented. All slides had been blocked for 3 h at room temperature followed by incubation with BxPC-3 lysate in Quadriperm chambers at 4°C overnight. The median background signal intensities from three slides for each buffer are shown for red (B) and green (C) fluorophors. Intensities were obtained at identical laser power and PMT for all slides.

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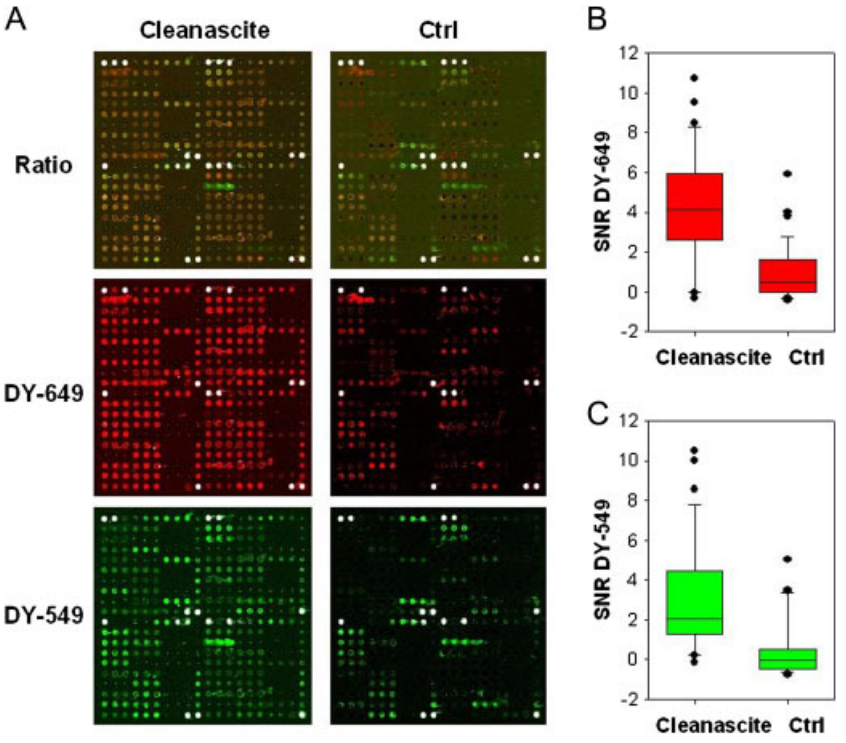

Figure 2. Lipid removal. Seven pancreatic cancer tissue samples with high lipid content were split into half. One half of each was treated with Cleanascite prior to labeling. The other half was not treated with Cleanascite. Treated and untreated samples were pooled separately, labeled, and analyzed in triplicate as described in the Supporting Information. In (A), the slide incubation results from the red and green channel scans and the superimposed image are shown. In (B) and (C) the respective SNRs of the feature intensities are presented.

(Molecular Devices, Sunnyvale, USA) and the software Acuity 4.0 (Molecular Devices).

The above protocol produced identical results for protein extracts from liquid samples as well as cells and tissues. While larger structures in serum or other body fluids are predominantly proteins, cells are generally more complex in their biomolecule content. The presence of nucleic acids, lipids, and metabolites drastically affects data quality. Improvements have been made by adapting protein preparation [4]. Still, there are additionally intrinsic differences in complexity and dynamic mass. To date, no optimization for cellular proteomes had been performed. In order to make the methodology amenable to the analysis of protein extracts from mammalian tissues, several steps were studied.

For blocking, ten commercial and home-made buffers were compared (see Supporting Information data for details). Earlier studies with tissues provided only limited information with regard to blocking (Supporting Information Table 2S). In most of them, undisclosed recipes were used or there was no mentioning of this essential step. In none of them, quality control measures were provided. We found that 10% milk in PBST80 produced best results (Fig. 1). Also, a time-dependent decrease in local and global background intensity was observed (Supporting Information Fig. 1S). However, blocking for more than 3 h increased tenfold the percentage of spots flagged as absent by the analysis software. Since the majority of antibodies used in our study were developed in rabbits, we also applied 2% IgG globulins from rabbit in PBS as blocking buffer. Although the background was significantly higher than with 10% milk, the slides nevertheless exhibited a slightly better signal-to-noise ratio (SNR). However, the use of IgG globulins is not feasible for economical reasons.

The incubation conditions have immediate consequences on quality and sensitivity and have been a focus of our work [5]. Here, the effects of buffer type, incubation time, temperature, and sample agitation method were evaluated. Quality was assessed in terms of spot signal uniformity and SNR. Knezevic *et al.* [6] used 1% BSA for incubation of tissue lysates for 8–12 h at 4°C. In another study [7], 5% BSA was used for incubation of tissue and cell lysates. Others provided no information. We found BSA associated with lower quality, while superior results were obtained with 10% milk-PBST80 (Supporting Information Fig. 2S).

Proteins are usually kept at low temperature to preserve their integrity. Microarray quality was significantly lower in overnight incubations at room temperature compared with 4°C (data not shown). However, incubations at 4°C overnight or at room temperature for 1 h produced similar quality. This conforms with the proposition that for antibody–antigen complexes, which fit to a 1:1 Langmuir association model, the dissociation rate constant is more temperature-dependent than the association rate constant [8]. Besides temperature, sample agitation is also critical for array performance [5]. We compared mechanical agitation (Quadriperm) and surface acoustic wave stimulation (Slidebooster; see Supporting Information). The former produced a higher SNR (Supporting Information Fig. 3S). As little as 10 μg of protein generated signals of sufficient quality (Supporting Information Fig. 4S).

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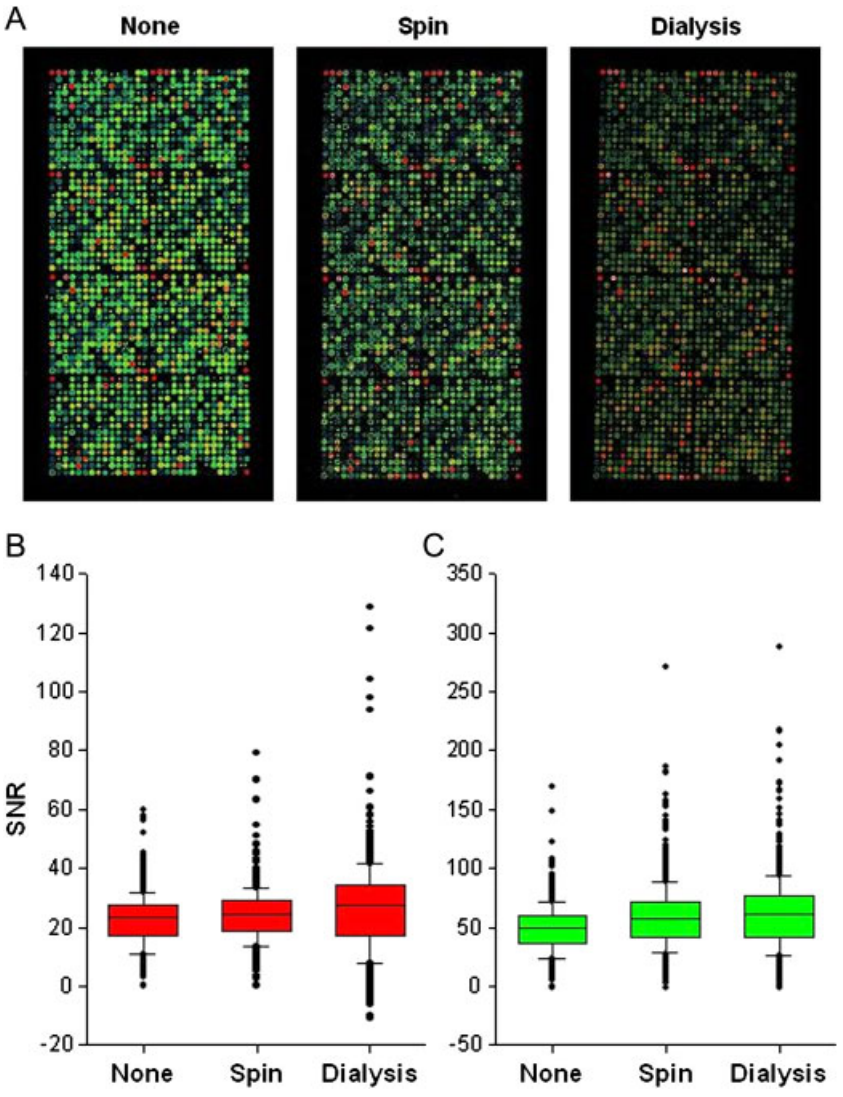

Figure 3. Removal of unincorporated dye molecules. Image scans of large antibody microarrays with some 1800 features produced from 810 cancer-associated antibodies are shown. Suit-007 and Suit-028 protein extracts were labeled with Cy3 and Cy5, respectively. Prior to incubation, the dye had not been removed (None) or removal occurred by spin column or dialysis (A). The quality of the microarrays is presented in terms of SNR (mean from triplicate experiments) in the red (B) and green (C) channels.

Protein processing also contributed to assay quality and reproducibility. Extraction under native conditions resulted in a better SNR than extraction with protein denaturation (Supporting Information Fig. 5S). Also, the removal of lipids from the sample was tested. Lipids like phosphatidyl-ethanolamine may undergo labeling and bind to the hydrophobic slide surface producing background signal. Sreekumar *et al.* [9] reported previously the use of Triton-X114 and ExtriGel beads to remove lipids from LoVo cells. However, no experimental details were provided. We tested sample delipidation with Cleanascite reagent and observed a substantial improvement of the array quality results (Fig. 2). Lipid removal was only necessary for tissues homogenates, however, and mainly in those with higher lipid content.

Protein labeling was investigated intensively before. Also, it has been shown that direct labeling using a two-color approach can substantially improve microarray performance in terms of reproducibility and discriminative power [10]. Here, we extend this issue by analyzing additional dye-pairs and assessing the D/P molar ratio. Five fluorescent dye-pairs were tested (Supporting Information). Dye bias was less pronounced with increasing polarity of the dyes (Supporting Information Fig. 6S). Cy3 and Cy5 were second to DY-549 and DY-649 in water solubility but performed slightly better with tissue homogenates. Maximal labeling efficiency was achieved at a molar ratio of 14–22 D/P (Supporting Information Fig. 7). Gel electrophoresis, on the other hand, showed a continuous increase in the fluorescence intensities of protein bands even at high D/P ratios (Supporting Information Fig. 8S). In a study with cell lysates, Kopf *et al.* [11] suggested that increasing the D/P molar ratio is beneficial for sensitivity. We found, however, that higher ratios induced a negative effect, presumably due to masking of the antigenic sites by excessive amounts of the dye.

We also evaluated the impact of dye removal after labeling. Usually, dialysis or gel filtration is applied. Fluorescent dyes or haptens like biotin in the form of NHS-esters are the

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most commonly used conjugates, taking advantage of the abundance of lysine in proteins. It was shown that upon the attachment of unlabeled biotin, followed by an addition of fluorescence-labeled strepavidin, the removal of remaining biotin is unnecessary [12]. However, there is a substantial difference between a biotin-strepavidin system and direct protein labeling with NHS-ester fluorescence dyes. Fluorphores may interact non-covalently and unspecifically with hydrophobic proteins or the hydrophobic array surfaces and thus deteriorate image quality. Unlike most fluorescent dyes, biotin is highly soluble in water and removed during washing. We found that the removal step is superfluous (Fig. 3). Un-reacted NHS-ester moieties undergo spontaneous hydrolysis in the aqueous extraction medium, even if not quenched by glycine. Our experiment also precludes an effect of hydrogen-bonding, van-der-Waals, or other weak interaction forces. The ionic strength of PBS along with the amphiphilicity of Tween eliminates traces of the inactive dye during the washing steps (Supporting Information Fig. 9S). Besides cutting expenses, avoiding dye removal has other advantages, too, such as shortening the time required for the assay, minimizing technical complexity, and – most importantly – avoiding a loss of small proteins or peptides, which may occur during dialysis or gel filtration.

In conclusion, a combination of measures, modifications and adaptations was introduced to the process of protein analysis by antibody microarrays, which led to a substantial improvement in data quality of studies of complex protein samples from tissues and cell cultures. The entire procedure makes use of only one buffer (PBST80) throughout. The intra- and inter-array coefficient of variance for replicate spot intensities was less than 10 and 20%, respectively. We employ the protocol in ongoing studies on samples from pancreas and bladder cancer tissues and performed experiments on cell lines of different origin. In all experiments, the results obtained are in agreement with the data shown here (Supporting Information Fig. 10S), confirming the benefit of the refinements in a large number of samples.

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