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Assessing incomplete deprotection of microarray oligonucleotides *in situ*

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ABSTRACT

En masse **analysis of gene structure and function by array technologies will have a lasting and profound effect on biology and medicine. This impact can be compromised by low quality of probes within arrays, which we show can be caused by incomplete removal of chemical protecting groups. To solve this quality control problem, we present a sensitive, specific and facile method to detect these groups *in situ* on arrays using monoclonal antibodies and existing instrumentation. Screening of microarrays with these monoclonal antibodies should guide the consideration given to data derived from these and should enhance the accuracy of the results obtained.**

INTRODUCTION

During the chemical synthesis of DNA and RNA, branching is prevented by ensuring that only one chemically reactive group is present in the growing oligonucleotide and one in the nucleoside 3′-phosphoramidite. This is achieved by blocking other reactive groups within the sugars and bases (e.g. exocyclic amines) with protecting groups. The removal of the protecting groups that block exocyclic amines, commonly known as deprotection, occurs only after synthesis. Additionally, the 5′-OH capping group in a newly incorporated nucleotide, usually 4,4′-dimethoxytrityl (DMT), must be removed prior to the addition of the next residue and at the completion of synthesis. Although decapping and deprotection are efficient reactions, the yield is not nearly 100%. Indeed, using monoclonal antibodies to specific protecting groups we previously showed that oligonucleotides from commercial sources claiming deprotection retained significant levels of protecting groups [see Refs (1,2); Barley-Maloney and Agris, in press]. This antibody-based method was previous validated using high-performance liquid chromatography (HPLC) (1). Whereas this level of contamination may be adequate for routine procedures, it is clearly not acceptable for diagnostic and prognostic uses of oligonucleotides, such as arrays. These considerations led us to ask two questions: (i) can we detect remaining protecting groups in oligonucleotides printed on arrays and (ii) do protecting groups alter array results?

MATERIALS AND METHODS

Detection of DMT groups in situ. Corning UltraGapglass slides were spotted with the Operon Human Genome Array-Ready Oligo Set (Version 2.0) and custom design oligonucleotides on an Omnigrid 100 (GeneMachines) arrayer. The arrays were washed in a pre-hybridization buffer (5× SSC, 0.1% SDS, 1% BSA) for 1 h at 42°C. Arrays were washed in deionized water and then probed with a 1:100 dilution [in wash buffer (sodium phosphate, 0.9% NaCl, 0.5% Tween-20, 1% BSA)] of anti-DMT (Ab 1-30-26) monoclonal hybridoma supernatant (1) and incubated for 2 h at room temperature (RT). The arrays were then washed three times with wash buffer. The secondary antibody, goat anti-mouse conjugated 660 AlexaFluor (Molecular Probes) was diluted in wash buffer (1:100 dilution) and incubated on the array for 1 h at RT. Arrays were washed according to protocols found at the Duke Microarray Facility web site (http://microarray.genome.duke.edu/spotFolder/protocolFolder/) and scanned on the Axon GenePix 4000B scanner (Molecular Devices).

Labeling and hybridization

Labeling and hybridization protocols are further detailed at the Duke Microarray Facility web site (http://microarray.genome.duke.edu/spotFolder/protocolFolder/).

RESULTS AND DISCUSSION

The presence of protecting groups on arrays was tested on glass slides spotted with the Operon Human Genome Array-Ready Oligo Set (Version 2.0). On the same array we spotted mixtures of decapped and deprotected β4-thymosin and ribosomal protein L9 oligonucleotides, and

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capped and protected β4-thymosin and ribosomal protein L9 oligonucleotides. The decapping and deprotection, which meant the DMT cap and the protecting benzoyl- (Bz), isobutiryl- (Ibu) and isopropropylphenoxyacetyl- (IprPAC) groups had been removed, followed our previously published procedure that was validated by HPLC (1). Near complete deprotection of the ß4-thymosin and ribosomal protein L9 oligonucleotides was observed by gel electrophoresis and the dot-blot antibody-based assay described previously (1) (data not shown). Mixing of fully protected and completely deprotected oligonucleotides insured accurate determination of the percentage of protecting groups in any mixture and percentages from 2 to 100% were evaluated for detection with a monoclonal antibody to the DMT group (1).

The DMT group in the β4-thymosin and ribosomal protein L9 oligonucleotides was easily visualized by an anti-DMT monoclonal antibody (Figure 1A, left panel) (see figure legend for methods). DMT could be detected even with only ~10% of the DMT groups remaining (data not shown). Whereas this signal was not easy to quantify in the Cy3 or Cy5 channel it was obvious upon inspection of the spot morphology in the Cy5 channel. The anti-DMT signal was characterized by a yellow/green spot surrounded by a red halo while the background signal observed with fully deprotected oligonucleotides was detected as a low intensity green signal on the spots (Figure 1A, anti-DMT panels). The data above led us to conclude that we could identify DMT groups remaining on spotted oligonucleotides *in situ*.

Using this criterion, we detected the DMT signal, which we defined as spots containing a red halo, in only very few spots (~3 in 21 000) among oligonucleotides from the Operon set. We surmise that these represent rare cases where the supplied oligonucleotide was poorly deprotected (Figure 1A, middle panel).

In order to ascertain the effect of incomplete protection on array function, we analyzed the hybridization signals obtained with two total RNA samples, which were labeled and hybridized using standard direct labeling protocols. First we evaluated spot morphology and this was found to be normal for the majority of the oligonucleotides in the Operon set (e.g. open arrow, Figure 1A, right panel). There were no signs of spot spreading or poor spot morphology due to printing techniques, thus we do not believe that the halos observed above with anti-DMT antibody staining are a result of poor printing quality. The same was true for β4-thymosin and ribosomal protein L9 oligonucleotides that were fully deprotected (0% protection in Figure 1B, hybridization panels); however, the protected oligonucleotides resulted in aberrant spot morphology (closed arrow, Figure 1A and B, hybridization panels). Anomalous spot morphology is evident in spots that contain 20% protected oligonucleotides and becomes extreme with higher levels of protection (Figure 1C). We also noticed that the spots with aberrant morphology yielded an altered ratio of medians, thus giving a false indicator of expression (data not shown). Clearly even modest levels of protecting groups led to aberrant spot morphology and potentially altered expression data.

The alteration of medians suggested that protecting groups would interfere with microarray performance. In order to further test this, we measured the mean signal intensity for the same mixtures of oligonucleotide probes (β4-thymosin and ribosomal protein L9). The presence of protected oligonucleotides clearly reduced the sum of the mean signal intensity for both probes and a significant reduction could be seen at low levels of protection (Figure 2A and B). The reduction in signal intensity correlated with aberrations in spot morphology. Whereas we cannot conclude that the DMT capping group was responsible for the observed reduction of signal and change in morphology, we can deduce that incomplete removal of DMT, Bz, Ibu or IprPAC leads to suboptimal microarray performance.

Whereas the method of mixing protected and deprotected oligonucleotides provides a very precise measure of the percentage of protecting groups in a spot, it can only supply an estimate of the untoward effects of protecting groups on probe performance. In one spot where each oligonucleotide is 10% protected hybridization of each oligonucleotide within the spot is likely to be affected and therefore we argue that the negative effect on hybridization will be greater than effects for a spot where 10% of the oligonucleotides are

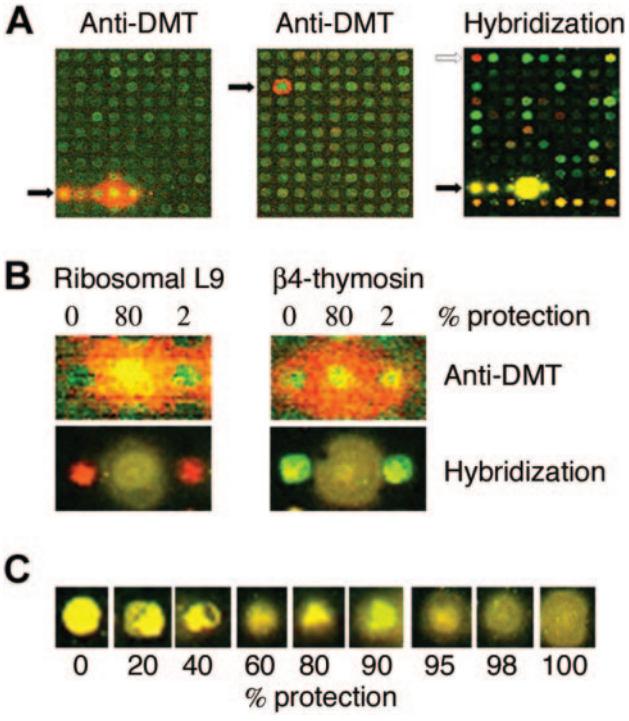

Figure 1. Detection of DMT groups in spotted oligonucleotides. (**A**) Left panel: shown is a section of an spotted array probed with anti-DMT (Ab 1-30-26) monoclonal hybridoma supernatant (1). Middle panel: a section of the Human Operon v2.0 oligonucleotide array that contains an oligonucleotide that displayed a red halo when probed for DMT groups as described for the left panel. Right panel: hybridization of a cDNA probe to the Human Operon oligonucleotide v2.0 array is shown. An open arrow points to the normal hybridization of a cDNA probe to an oligonucleotide of unknown protection status. Closed arrow represents the location of a series of oligonucleotides, which possess varying amounts of DMT, Bz, Ibu, and IprPAC groups (0–100%). (**B**) Top panels represents sections of the arrays with ribosomal protein L9 and β4-thymosin oligonucleotides that contain varying amounts of DMT, Bz, Ibu and IprPAC groups (0, 80 and 2%) and were probed with anti-DMT monoclonal antibody (see text). Bottom panels represent hybridization of a cDNA probe to the same region of the array. Antibody probing, and cDNA probe labeling and hybridization protocols were described above. (**C**) Spot morphology of a series of oligonucleotides with 0–100% protection. cDNA probe labeling and hybridization protocols were described above.

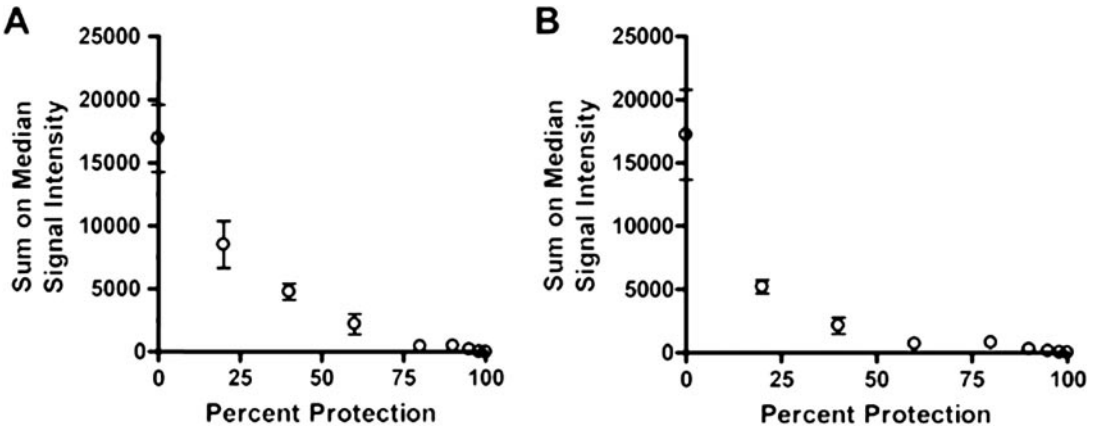

Figure 2. Protecting groups interfere with array function. (**A**) A regression analysis of the sum of median signal intensity from differentially protected (0–100% DMT, Bz, Ibu and IprPAC) β4-thymosin oligonucleotides that were hybridized with a cDNA probe. Data points represent triplicate experiments; variation within signal intensities is represented by error bars. (**B**) A regression analysis of the sum of median signal intensity from differentially protected (0–100% DMT, Bz, Ibu and IprPAC) ribosomal protein L9 oligonucleotides that were hybridized with a cDNA probe. Data points represent triplicate experiments; variation within signal intensities is represented by error bars.

fully protected. Hence we believe that the data in Figure 2 provide an underestimate of the negative effects of incomplete deprotection. This also means, however, that at this time we cannot give a strict criterion for rejection of a probe other than visual inspection and exclusion of any spot that produces a characteristic halo as seen in Figure 1.

The wide use and the formidable potential of microarray technologies has led to appropriate concern with microarray quality control (3,4). Issues of statistical power and biological variation have been carefully considered and the overall value of microarray data has been well validated. Nonetheless, the standards for use in therapy and diagnosis will have to be developed and will likely be more rigorous than current best practices (3). The quality of the oligonucleotides, whether pre-made and spotted, or synthesized *in situ* have begun to be analyzed. Pre-screening with DNA-binding fluorescent dyes can be used to interrogate arrays for spot morphology and for some level of quality and quantity per spot (5,6). Statistical methods (7,8) and *ab initio* calculations (9,10) can be used to identify poorly performing oligonucleotides or even regions of arrays. To date, however, there has been little discussion of incomplete deprotection in microarrays. In this report we show that incomplete deprotection could lead to poor array performance and, more importantly, we provide a methodology to detect it. While we limit our conclusions to DNA oligonucleotide spotted arrays, the potential for incomplete deprotection can also exist in arrays where oligonucleotide synthesis is carried out on the solid support. In fact, the significant negative functional consequences of incomplete deprotection may be exacerbated in these microarrays because of the shorter length of the oligonucleotides.

We suggest that screening of microarrays with monoclonal antibodies to identify protecting groups should guide the consideration given to data derived from individual features and should enhance the accuracy of the results obtained.

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Conflict of interest statement. I am a founder and co-owner of Veri-Q, Inc., which has licensed and is commercializing the use of reagents and methods described in this communication. This, I believe, must be declared as a conflict of interest. Two other authors (HKD and PFA) also declare competing interests related to ownership of Veri-Q, Inc stock or stock options.

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