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Research paper

# MALDI Immunoscreening (MiSCREEN): A method for selection of anti-peptide monoclonal antibodies for use in immunoproteomics

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ABSTRACT

A scalable method for screening and selection of peptide-specific monoclonal antibodies (mAbs) is described. To identify high affinity anti-peptide mAbs in hybridoma supernatants, antibodies were captured by magnetic affinity beads followed by binding of specific peptides from solution. After timed washing steps, the remaining bound peptides were eluted from the beads and detected by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). This allowed measurement of monovalent interactions of peptides with single antigen binding sites on the antibodies, thus reflecting antibody affinity rather than avidity. Antibodies that were able to bind target peptides from solution phase and retain them during washing for a minimum of 10 min were identified by the strength of the appropriate m/z peptide MS signals obtained. This wash time reflects the minimum peptide dissociation time required for use of these antibodies in several current immuno-mass spectrometry assays. Kinetic analysis of antibody-peptide binding by surface plasmon resonance (SPR) showed that the selected antibodies were of high affinity and, most importantly, had low dissociation constants. This method, called MALDI immunoscreening (MiSCREEN), thus enables rapid screening and selection of high affinity anti-peptide antibodies that are useful for a variety of immunoproteomics applications. To demonstrate their functional utility in immuno-mass spectrometry assays, we used the selected, purified RabMAbs to enrich natural (tryptic) peptides from digested human plasma.
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## 1. Introduction

There is currently a shortage of antibodies for use in a variety of proteomics applications. The lack of such reagents presents a serious bottleneck for measuring the large number of different proteins in biological samples including tissues and plasma, the major source of biomarkers used in clinical diagnostics. Several ambitious projects are underway in both Europe (http://www.hupo.org/research/hai/; www.proteomebinders.org) and the USA (http://antibodies.cancer.gov) to make and characterize antibodies for use in immunohistochemical assays and for immuno-enrichment of proteins from complex mixtures. Such antibodies will be useful for expression analysis of proteins in tissues and cells and in “top-down” proteomics methods where

*Abbreviations:* MiSCREEN, MALDI immunoscreening; mAbs, monoclonal antibodies; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; SPR, surface plasmon resonance; SISCAPA, stable isotope standards and capture by anti-peptide antibodies; hPDQ, human proteome detection and quantitation project; MRM, multiple reaction monitoring; HPLC, high performance liquid chromatography; KLH, keyhole limpet hemocyanin; ELISA, enzyme linked immunosorbent assay; RabMAbs, rabbit monoclonal antibodies; PBS, phosphate buffered saline; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; $K_D$, equilibrium dissociation constant; ka, association rate constant; kd, dissociation rate constant.

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intact protein targets are enriched before analysis by methods such as mass spectrometry, a method pioneered by Nelson et al. (1995).

Much less thought and effort has been focused so far on the development of anti-peptide antibodies suitable for quantitation of signature peptide surrogates of proteins from digests of complex biological materials. These kinds of antibody reagents are used in quantitative assays such as immuno-Matrix-Assisted Laser Desorption/Ionization (i-MALDI; Raska et al., 2003; Jiang et al., 2007) or Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA; Anderson et al., 2004). These applications use antibodies for specific enrichment of tryptic peptides from digests that are much more complex (in terms of number of different molecules present) than the protein mixtures from which they are derived. The unique requirements of such assays make it a challenge to derive and select antibodies with the desired characteristics. Essentially, such antibodies must be able to bind the target peptides from complex tryptic digests (typically from human plasma) and retain them through the washing steps prior to peptide elution and mass spectrometric analysis. Thus these antibodies must have high affinities, or more specifically, low off-rates for their peptide analytes. With current SISCAPA assays, the retention time required for effective peptide enrichment is a minimum of 10 min during which time unbound peptides are washed away. The MiSCREEN method described in this manuscript allows selection of such anti-peptide reagents.

A project to make quantitative assays for all human proteins, based on surrogate peptides, anti-peptide antibodies and mass spectrometry, has been proposed by Anderson et al. (2009). This human Proteome Detection and Quantitation Project (hPDQ) would function as an assay resource available worldwide. The project would require both MS-friendly proteotypic tryptic peptides for each target protein and renewable anti-peptide reagents specific for each. Currently, few of such anti-peptide reagents are available and the capacity to produce them is limited. Although some commercial and academic research labs claim to be able to make antibodies (or other affinity reagents) with high throughput, this has not been widely demonstrated for anti-peptide reagents with the performance characteristics required for use in immuno-MS assays.

We previously developed a surface plasmon resonance (SPR) method that allows selection of monoclonal anti-peptide antibodies that are able to bind tryptic peptides in solution phase and that are suitable for immuno-MS assays (Pope et al., 2009). This method measures true antibody affinities (not avidities) and is useful for kinetic analysis of small numbers of mAbs. However, the assay is too slow, cumbersome and expensive for high throughput screening.

We therefore sought to develop a method that would allow selection of high affinity anti-peptide mAbs (more specifically, those with low off-rates) in a more cost effective and high throughput fashion. To do this we have developed a method called MALDI immunoscreening (MiSCREEN) for rapid screening of hybridoma supernatants. The method allows the identification of antibodies that are able to bind specific peptides in solution phase from complex mixtures and that have low dissociation constants (kd) suitable for ultimate use in immuno-MS assays.

## 2. Materials and methods

### 2.1. Peptides

Synthetic tryptic peptides chosen as surrogates of protein biomarkers were used throughout. Peptides that occur in a single protein encoded within the human genome and that yield several, strong multiple reaction monitoring (MRM) transitions in a triple quadrupole mass spectrometer were selected (Anderson et al., 2004). Peptides were synthesized by solid-phase methods by either the Chinese Peptide Company (Hangzhou, China) or by the UVic-Genome BC Proteomics Centre (Victoria, BC) and were tested by the vendors for the correct masses by MALDI-TOF mass spectrometry and for purity by high performance liquid chromatography (HPLC). Stable isotope labeled versions of selected peptides were made by chemical synthesis at the UVic-Genome BC Proteomics Centre. A mass increment was added in each case through use of labeled C-term Arginine (6 or 10 amu) or Lysine (6 amu), providing mass shifts of $m/z = 3$ or 5 for typical doubly-charged peptide ions. All peptides were of greater than 80% purity, were quantified by amino acid analysis (Advanced Protein Technology Centre, The Hospital for Sick Children, Toronto, Ontario) and stored at −20 °C for short periods (2 weeks or less) at 4 °C in solution phase to prevent solubility problems that occur with some peptides after lyophilization. After thawing and/or just before use in MiSCREEN, all peptides were analyzed by MALDI-TOF MS to determine their integrity and to assess the presence of altered forms.

Peptides were first synthesized with C-terminal cysteines to allow thiol coupling to keyhole limpet hemocyanin (KLH) carriers for immunization (Pierce Chemical Co., St Louis, MO). The same peptides synthesized without C-terminal cysteines were used in enzyme linked immunosorbent assays (ELISA; see peptide ELISA below) and in MiSCREEN and SPR assays for measuring antibody-peptide binding without interference from the linker cysteine. Although the peptides for this work were chosen as proteotypic surrogates of a variety of protein biomarkers, any peptide of interest that can be bound by an antibody and detected by MALDI-TOF mass spectrometry can be used. The peptides used in this work are described in Table 1.

### 2.2. Anti-peptide monoclonal antibodies

Rabbit monoclonal antibodies (RabMAbs) were produced by Epitomics Inc. (Burlingame, CA) using a proprietary, stabilized rabbit plasmacytoma cell line derived from the original parental myeloma 240-W (Spieker-Polet et al., 1995) as the fusion partner. To select hybridomas secreting anti-peptide antibodies, 4000 hybridoma supernatants from each fusion were tested by peptide ELISA (see succeeding discussions) using the immunizing peptides (without carrier or added C-terminal cysteine) dried onto ELISA plates. Positive rabbit hybridoma supernatants (usually obtained in small volumes of 400 μL after the initial peptide ELISA) were used for MiSCREEN and SPR assays. All hybridoma supernatants were stored at 4 °C before use to avoid freeze–thaw cycles.

Mouse monoclonal antibodies were also used in MiSCREEN assays. One of these, mAb 2A7 specific for peptide PPI-

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