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Surface Preparation Strategies for Improved Parallelization and Reproducible MALDI-TOF MS Ligand Binding Assays

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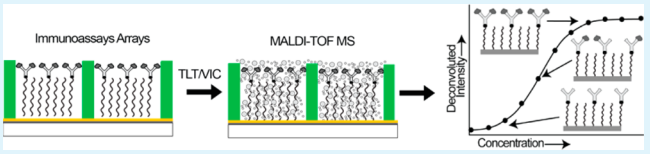
Supporting Information

ABSTRACT: Immunoassays are employed in academia and the healthcare and biotech industries for high-throughput, quantitative screens of biomolecules. We have developed monolayer-based immunoassays for MALDI-TOF MS. To improve parallelization, we adapted the workflow to photolithography-generated arrays. Our work shows Parylene-C coatings provide excellent "solvent pinning" for reagents and biofluids, enabling sensitive MS detection of immobilized components. With a unique MALDI-matrix crystallization technique we show routine interassay RSD <10% at picomolar concentrations and highlight platform compatibility for relative and label-free quantitation applications. Parylene-arrays provide high sample densities and promise screening throughputs exceeding 1000 samples/h with modern liquid-handlers and MALDI-TOF systems.



KEYWORDS: *mass spectrometry, self-assembled monolayers, MALDI-TOF, arrays, immunoassays, biomarker*

INTRODUCTION

Ligand binding assays (LBAs) (e.g., ELISA assays[1] and SPR-arrays[2]) are widely used in the scientific community supporting applications such as quantification of a biomolecule concentration, determination of equilibrium constants (K_d/K_a), discovery/characterization of biomolecule interactions in the "omics" fields, and pharmacokinetics/pharmacodynamics studies on drugs and biologics.[3] Additionally, immunoassay LBAs are a robust platform for validation of candidate biomarkers designed to diagnose presymptomatic patients and stratify potential respondents (or toxicities) to specific treatments. Despite broad appeal, LBAs often have limited specificity and are susceptible to diverse interferences.[4,5] High-throughput screening approaches with improved specificity that better rationalize biological variation (e.g., post-translational modifications or splice events) or cross-reacted species (e.g., small molecule ligands) simultaneously and in the context of complex mixtures (e.g., biofluids, cell lysates, or tissue homogenates/extracts) will have a profound impact on LBA reliability and cost-effectiveness.

LBA detection by mass spectrometry (MS) improves assay selectivity compared to photometric detection. In particular, modern matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) instrumentation provides high sampling capability using lasers with up to 2000 Hz laser repetition rates that do not sacrifice assay sensitivity.[6] Such sampling characteristics have potential for analysis of thousands of samples in a few minutes as demonstrated by recent small molecule screens from high density arrays.[7,8] However, direct macromolecule analysis (e.g., intact proteins) from LBA arrays in large-scale environments has been limited. Challenges associated with improving throughput for MALDI-TOF immunoassays are the incompatibility of many materials with the vacuum requirements, the need for low chemical background signatures, and the lack of robust array-based workflows that enable quantitative processing of enriched biomolecules without the use of internal standards.

We recently developed a robust immunoassay workflow for MALDI-TOF MS.[9−11] The assays were developed on self-assembled monolayers (SAMs) bound to gold surfaces and had a high degree of control over antibody surface density and orientation and demonstrated resistance to fouling by abundant proteins in biofluids.[9,12] The protein-G monolayer also provided an adaptable scaffold for immobilizing IgG antibodies through the antibody Fc domain, which facilitates targeting of a wide variety of antigens (e.g., proteins and small molecules) from biofluids with limited effort required for assay redesign. We subsequently showed that improved sampling reproducibility was possible when monolayer-based immunoassays were combined with the recently reported *t*hin-*l*ayer *t*ransfer (TLT) and *v*apor-sorption *i*nduced co-*c*rystallization (VIC) processes.[11] TLT/VIC minimizes signal "hot-spots" resulting from nonuniform analyte redistribution on the biosensor during matrix deposition and crystallization. The combination of controlled analyte density on the surface with homogeneous matrix/analyte cocrystallization by TLT/VIC enabled assay

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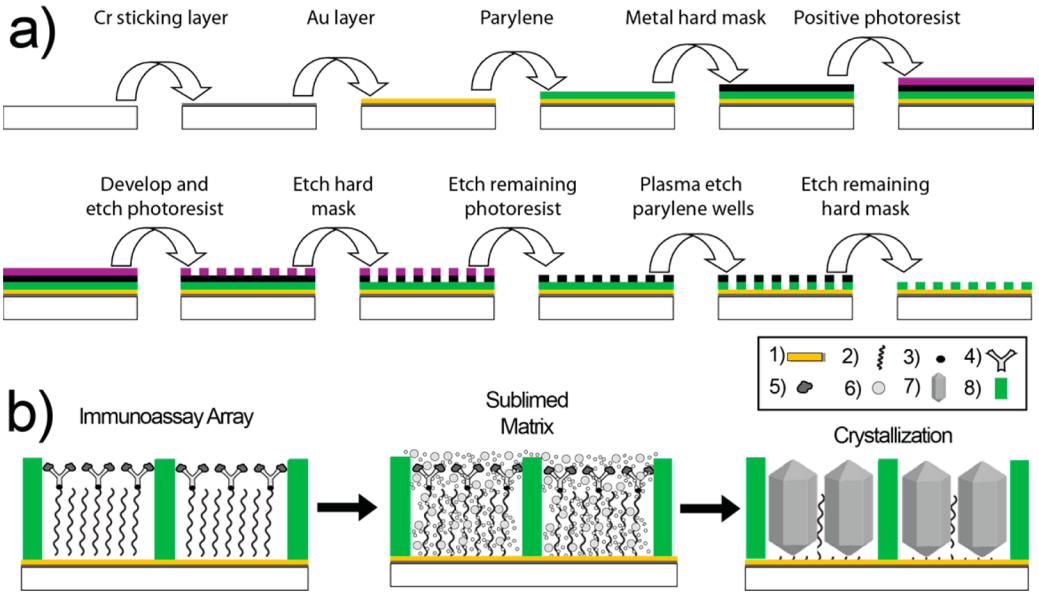

Figure 1. (a) Schematic of the lithographic process used to develop parylene array immunoassay substrates. (b) Schema for MS-immunoassays and TLT/VIC processing leading to uniform matrix/analyte crystallization on arrays. Legend: (1) gold surface, (2) monolayer, (3) protein-G, (4) antibody, (5) antigen, (6) polymorphic matrix, (7) matrix/analyte cocrystal, (8) parylene wall.

precisions similar to those observed for photometric immunoassays and supports label-free quantitation via end-point calibration curves.[10] The previous work also demonstrated quantitative detection of analyte from complex matrices including biofluids. Additionally, combining enrichment of target analyte with antibodies and MALDI-TOF yields key molecular mass information, providing a high degree of assay selectivity for resolving related or complexed species.

Given the ease of generation, versatility, and potential for biomolecule quantitation with our immunoassays, we sought to improve parallelization by adopting a high-density array methodology. Previous reports have shown different methods for generating arrays for MALDI-TOF MS including chemical functionalization of gold or titanium oxide coatings to generate hydrophilic wells.[8,13] Also, work has also shown extension of MALDI-TOF MS to high-density microarrays generated by photolithography in order to screen cellular metabolites and small molecules,[14−17] including work by Lee et al., who described the use of "nanolayered" microspots separated by a hydrophobic coating to screen a library of small molecules.[7] Here we sought a similar lithography-derived strategy for generation of hydrophilic wells for immunoassays on gold surfaces. Given the ubiquitous use of Parylene C in the medical and microfluidic communities and the ease of creating surface structures by lithography,[18] this work sought to test the compatibility of parylene-arrays for SAM-immunoassays and MALDI-TOF MS. We found that lithographic techniques and parylene coatings provided a high degree of adaptability for array size and densities offering excellent "solvent pinning" because of the high contact angle compared to the gold surface. We further demonstrate that high-fidelity surface preparation, including TLT/VIC matrix deposition, enables concentration dependent evaluation of biomolecules from solution with good interassay sampling precisions (<10% RSD).

EXPERIMENTAL PROCEDURE

For array generation (Figure 1a), #3 glass coverslips (VWR) were mechanically cleaned followed by ultrasonication in a 1 g/L Alconox solution. Substrates were rinsed with deionized water followed by ultrasonication in acetone, isopropanol, and then dried under nitrogen. Surfaces were coated with 30 Å chromium and 300 Å gold using a CHA Mark 50 E-gun evaporator (CHA Industries, Fremont, CA), followed by 1 μm of Parylene-C dimer using a SCS PDS 2010 Labcoter 2 (Specialty Coating Systems, Indianapolis, IN). A 1000 Å layer of copper was then deposited by E-gun evaporator to form a sacrificial etch mask. Lastly, 1.1 μm S1813 positive photoresist was deposited with a Cee spin coater (Brewer Sciences, Rolla, MO) and the substrates baked at 115 °C for 90 s. The surface was exposed with a Karl Suss MA6/BA6 contact printer, (Suss MicroTec Group, Garching, Germany), developed with a CPK solvent spinner (CPK Industries, Harleysville, PA), and descumming was performed in a Technics Series-85 Reactive Ion Etcher (Technics, Inc., Pleasanton, CA). The copper hard-mask layer was etched with PC etching solution (Philmore-Datak, Rockford, IL) and photoresist was removed with acetone in a CPK solvent spinner. Exposed parylene well spots were oxygen plasma-etched in a March Asher PX-250 RIE (Nordson March, Westlake, OH). The sacrificial hard-mask was removed with PC etching solution. SAMs and immunoassays were developed on the arrays as discussed previously.[9] Briefly, gold exposed arrays were exposed to an ethanol solution mixture of triethylene glycol (EG3) terminated alkanethiolate and nitrolotriacetic acid (NTA) terminated alkanethiolate to form an inert EG3 surface with active NTA ligand at a surface density of ~1.16×10^{-10} mol NTA/cm^2 (Figure 1b). The surface was then treated with 10 mM $NiSO_4$ to form an NTA-Ni^{2+} complex capable of ligating histidine-tagged ~1.0 μM protein-G from 1× PBS solution. IgG antibodies at ~1.0 μM in 1× PBS with 20 mM EDTA were bound to protein-G and the antigen-containing sample was incubated for ~2 h in a humidified chamber. TLT/VIC was performed as previously described.[10,11] MALDI matrix, α-cyano-4-hydroxycinnamic acid (CHCA), was sublimed at ~25 ng/mm^2 surface density and VIC performed with methanol. Samples were analyzed with a Voyager DE-Pro MALDI-TOF MS (Applied Biosystems, Inc., Foster City, CA). Spectra were averaged from ~50 shots from different locations within a sample spot. Hemoglobin ELISA calibrator (Bethyl Laboratories, Inc., Montgomery, TX) was obtained commercially and von Willebrand substrate preparation and reaction conditions are described in the Supporting Information.

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RESULTS AND DISCUSSION

Initial experiments sought to determine the compatibility of lithographically generated arrays on gold surfaces with our established MALDI-TOF LBA workflow for macromolecule detection, including monolayer generation and controlled MALDI matrix deposition and crystallization on the biosensor surface by TLT/VIC (Figure 1). Preparation of hydrophilic wells in the hydrophobic parylene coating was accomplished through the use of multiple lithographic steps (Figure 1a). Although multiple steps were involved in the array development, the workflow was amenable to batch array generation and array substrates tolerated long-term storage without diminished performance (data not shown). Early work established that lithography techniques permitted generation of complex surface features including spot diameters ranging from 50 μm to 0.5 mm (Figure 2). The arrays provided excellent solvent pinning

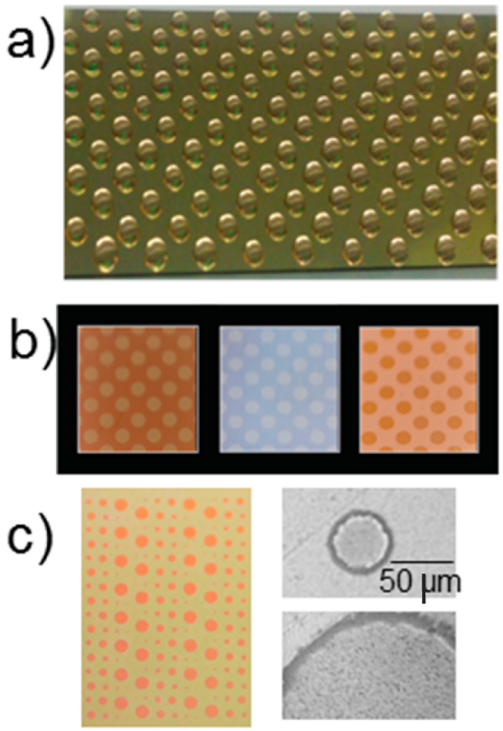

Figure 2. (a) Photographs of parylene arrays demonstrating effective sample pinning. Sample incubating on 2 mm array spots in 384-well format. (b) Images of three equally sized portions of a 2.5 cm × 7.5 cm array with array surface (left), array after TLT of CHCA matrix (center), and array after methanol VIC (right). (c) Multiplex parylene arrays presenting spots from 50 to 500 μm with CHCA matrix deposited by TLT and crystallized by methanol VIC (left). Microscope image of 50 μm spot shows matrix crystals distributed across the well surface (right).

for all incubation steps (Figure 2a) and were compatible with a variety of buffer agents (e.g., PBS or EDTA) although addition of detergents resulted in wetting of the parylene surface.

We recently demonstrated that precise concentration-dependent evaluation of protein species and ligated small molecules from biofluids was possible with MALDI-TOF when using TLT/VIC on mechanically generated biosensors (i.e., nonarrays).[10] In these investigations, we sought to confirm the performance of the array platform for similar quantitative applications. We first examined if MALDI matrix could be uniformly deposited by TLT and crystallized by VIC without redistribution of analyte on the array surface (Figure 1b). CHCA MALDI matrix was deposited at ~25 ng/mm^2 surface density with TLT and crystallized by methanol-VIC in wells down to 50 μm in diameter (Figure 2b, c). Subsequent MS analysis of array wells showed alkanethiolate reagents immobilized on exposed gold with minimal MS background observed (Figure 3a, b). MS spectra of protein components (e.g., protein-G and antigen) associated with antihemoglobin immunoassays show that excellent solvent pinning and uniform matrix deposition and crystallization by TLT/VIC confines species to the individual wells and not the surrounding parylene materials (Figure 3c–f), although it was noted that high concentrations of protein-G leads to minor nonspecific adsorption on parylene from solvent exposed areas immediately adjacent to the well (Figure 3d). We next developed a series of model antihemoglobin immunoassays[10] on an array (2 mm diameter wells) for the capture of hemoglobin antigen spiked into 10 μM BSA. Antigen concentrations ranged from 50 pM to 10 μM. Deconvolution of the resulting $[M + H^+]^+$ and $[M + 2H^+]^{2+}$ for both the α and β hemoglobin subunits in the MS spectra (e.g., Figure 3e) yielded a sigmoidal calibration curve that was fitted to a four-parameter logistic function (Figure 4). A working range >3 log was observed for both subunits and signal was detectable down to ~100 pM from ~1.0 μL of sample (corresponding to ~100 amole per well) with ~2 h incubation periods. Further improvements in detection limits have been demonstrated for SAM LBA with optimized loading kinetics.[10] The observed detection limits are consistent with conventional ELISA assays (Bethyl Laboratories Inc.), while superior dynamic range and specificity is afforded by MALDI-TOF LBA. We also noted that variation in interspot replicate analysis processed proximal to one another on the same array averaged ~4.1% RSD for the α and β subunits. Distal replicates on the 250 pM, 5 nM, and 10 nM samples averaged ~8.0% RSD, which is consistent with monolayer-based immunoassays analyzed by surface plasmon resonance (SPR) (<6% RSD)[19] and with our "non-array" biosensors where intra-assay replicates on small (<1 cm^2) and large surface areas (~1 in^2) were ~4% and ~7% RSD, respectively.[10,11] Initial results from three separate arrays (~45 spots) show that ~12.5% RSD can be achieved across TLT/VIC replicates (not shown). The observations from these model systems suggest that MALDI-TOF LBAs supported by high-fidelity arrays and matrix deposition techniques can achieve precisions necessary to conform to the traditional 4–6–30 and 4–6–20 rules for LBA without the use of internal standards (i.e., label-free quantitation).[20,21]

Finally, we sought to challenge the robustness of the MALDI-TOF LBA workflow for the characterization of clinically relevant samples. As a model system, we used an enzyme activity assay for the plasma metalloprotease, ADAMTS-13.[22] A 9.3 kDa substrate was spiked at ~125 nM into normal pooled plasma diluted 10× in 50 mM MES buffer with 20 mM $CaCl_2$ at pH 6.25. A progress curve was generated from time points obtained over the course of the reaction with each point reflecting the ratios of the 9.3 kDa substrate and 7.6 kDa product relative to the total signal in the MS spectra (Figure 5a and 5b). These data were generated for analyte ions captured from plasma, demonstrating the selectivity of MALDI immunoassays and tolerance to complex matrices. The data shows efficient generation of product over time with the standard error of the curve fit ~1.5%. The average intraassay precision for calculated ratios on each time point was ~2.1%

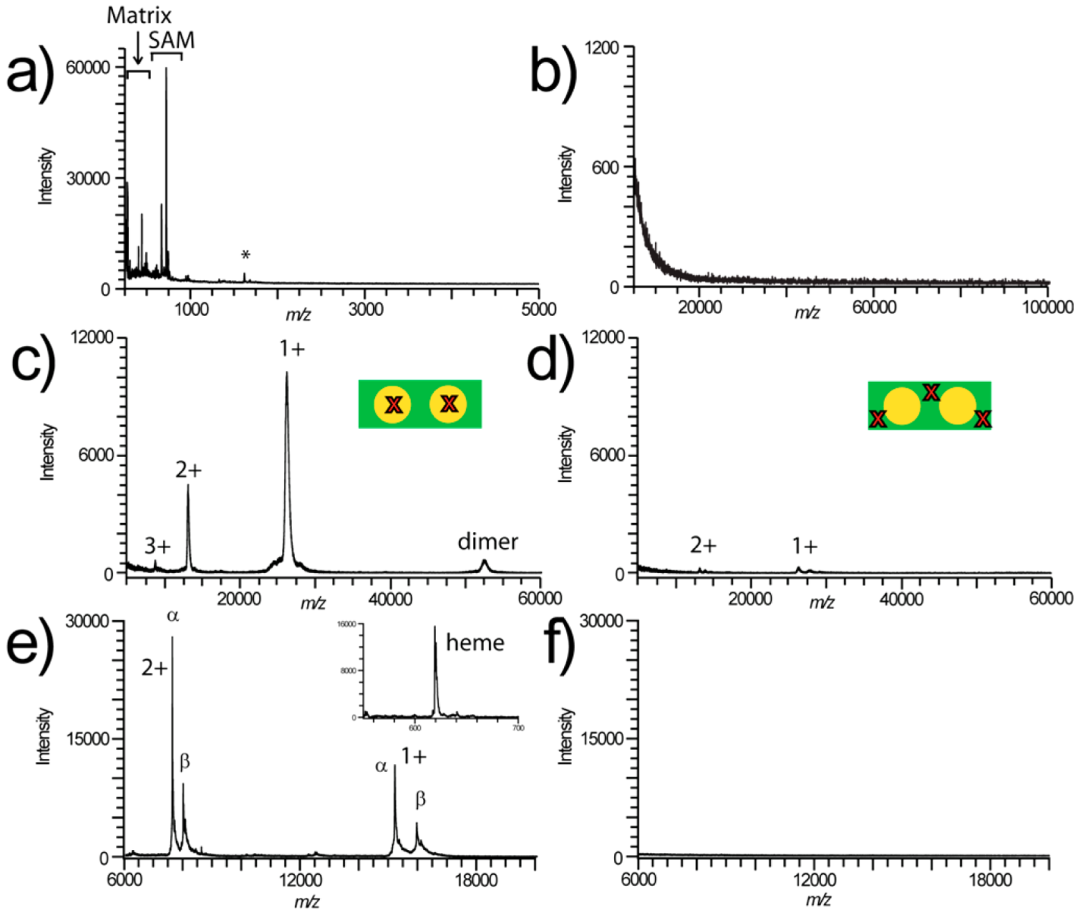

Figure 3. MALDI-TOF MS spectra of a SAM surface generated on exposed well in parylene array shows the (a) low and (b) high m/z ranges. MALDI-TOF MS spectra of array wells as opposed to adjacent parylene for (c, d) immobilized protein G and (e, f) hemoglobin captured with an antihemoglobin immunoassays. The inset in e shows spectrum of the complexed porphyrin−heme. The heme ligand was previously shown to exhibit concentration dependence that paralleled that of the protein subunits (see ref 9).

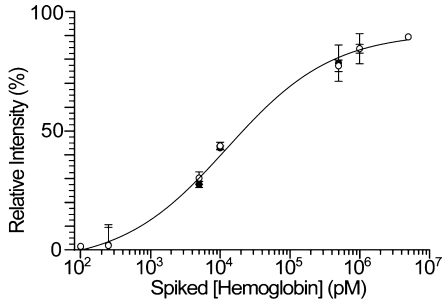

Figure 4. Calibration curves for hemoglobin α (open circles) and β subunits (closed circles) generated from deconvoluted signal from MS data collected from parylene arrays with matrix deposited and crystallized by TLT/VIC. Error bars derive from a minimum of three interspot replicates.

RSD, with interspot error for replicates of absolute signal intensity averaged 6.5% RSD. Similar experiments with dried-droplet matrix deposition on individual biosensors yielded a similar response curve although significant heteroscedastic spreading was noted with dried-droplet deposition (Figure 5c), likely due to >5× decrease in analyte S/N for DD experiments compared to TLT/VIC assays.[11] Indeed with TLT/VIC on arrays at higher starting substrate concentrations (i.e., high S/N) the average error reduced to ∼1% RSD (not shown). The good precision obtained with TLT/VIC for relative ratio measurements are consistent with previous MALDI-TOF MS reports that used internal standards or reference materials to support relative quantitation of related species with MALDI-TOF MS.[8,23,24]

CONCLUSIONS

These preliminary investigations demonstrate implementation of common lithographic technologies in the preparation of SAM-immunoassays for MALDI-TOF MS analysis of macromolecules. The results revealed that parylene provided efficient solvent pinning to contain samples for analysis. Additionally, the steps required for parylene lithography yielded chemically clean surfaces, resulting in minimal MS background, relative to gold-coated glass substrates. Importantly, the arrays were amenable to the recently introduced TLT/VIC process, which resulted in uniform matrix deposition and crystallization without significant analyte redistribution on the biosensor. High-density SAM arrays coupled with modern MALDI-TOF MS systems has the promise to enable throughputs similar to traditional photometric immunoassays with improved information for rapid, high-quality profiling of target molecules from complex biological matrices without sample preparation (i.e., no purification or separations required). The results suggest that the improved sensitivity achieved with TLT/VIC on parylene arrays allowed more precise measurements for both label-free and relative quantitation strategies.

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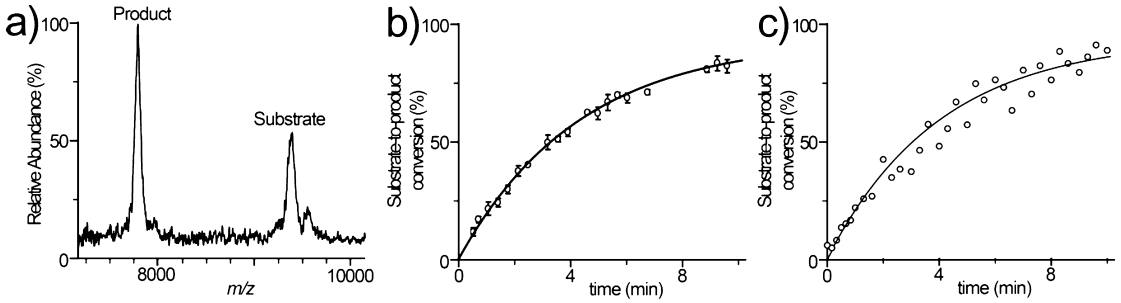

Figure 5. Improved precision for enzyme kinetic MS immunoassay using TLT/VIC. (a) MALDI mass spectra demonstrating simultaneous detection of substrate and product ions in a single measurement for relative quantitation. (b) Product conversion curve generated from MS-immunoassays with TLT/VIC. (c) Product conversion curves generated by MS-immunoassays with dried droplet matrix deposition demonstrates reduced precision compared to Figure 5b. All data points are the average of three replicates collected across the sample spot.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional details on experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Author Contributions

†These authors share equal responsibilities for this work

Notes

The authors declare no competing financial interest.

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