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Accelerated Articles

Multiplexed Gene Expression Analysis Using the Invader RNA Assay with MALDI-TOF Mass Spectrometry Detection

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A mass spectrometric approach for measuring gene expression levels has been developed. This technique utilizes a signal amplification system and analysis by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Signal amplification from the targeted RNA employs a recently developed invasive cleavage assay that does not require prior PCR amplification. The assay uses a set of target-specific probes (oligonucleotides), which hybridize to the RNA being measured to create an overlap structure with a single-stranded flap. This flap is enzymatically cleaved and accumulates linearly in a target-specific manner. The products of the reaction, short DNA oligomers, are well suited for quantitative detection by MALDI-TOF mass spectrometry. Multiplexing is achieved by designing the assays so that reaction products for different mRNA targets have discrete masses that can be resolved in a single mass spectrum. Simultaneous analysis of human cytokine in vitro transcripts IL-1β, TNF-α, and IL-6, with GAPDH as a reference standard, was used as a model system to demonstrate this novel method of gene expression analysis.

The detection and quantitative analysis of specific RNA molecules is important for monitoring gene expression and viral RNA loads. We describe here the use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) for the detection and quantitative analysis of specific RNA targets. Our approach adapts a recently developed signal amplification method, the RNA invasive cleavage assay[1] and herein termed the invader RNA assay, to MALDI-TOF MS detection. The invader assay is an isothermal, highly sequence-specific and structure-specific method for the analysis of nucleic acids.[2] This method detects target RNA or DNA molecules directly and does not require the use of reverse transcription or the polymerase chain reaction (PCR), thus avoiding the commonly encountered limitations of the PCR including cross-contamination issues and difficulties in obtaining reproducible and quantitative results.[3−6]

The recently developed invader RNA assay[1] has been shown to be accurate, sensitive (<100 copies/reaction), specific (discrimination of 1 in >20 000 for 95% homologous sequences), and quantitative (dynamic range over 4 orders of magnitude). Signal from this assay has been detected via a fluorescence resonance energy transfer (FRET)-based system and can be performed in either single (one fluorophore) or biplex (two fluorophores) reaction formats. The approach described here adapts the invader RNA assay for MALDI-TOF mass spectrometry detection. In going from the FRET to the MALDI detection format, overall detection sensitivity is sacrificed to achieve higher level multiplex analysis that is compatible with automation and high-throughput screening, while maintaining great flexibility in assay design.

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Table 1. Oligonucleotides Used in the Invader RNA Assays

human RNA target	probe oligonucleotide[a]	invader oligonucleotide
GAPDH	5′-biotin-**TTG**CTCCTGGAAGATG	5′-CACTTGATTTTGGAGGGATCTCA
IL-1β	5′-biotin-**TTTT**CATCTGTTTAGGGC	5′-GTCCAGGTCCTGGAAGGAGCACTA
TNF-α	5′-biotin-**TTTTT**CTGACTGCCTGG	5′-GTCACTCGGGGTTCGAGAAGATGAA
IL-6	5′-biotin-**TTTTTT**CTCCTCATTGAATC	5′-CCAGTGATGATTTTCACCAGGCAAGA

[a] Boldface, underlined nucleotides plus biotin represent the cleavage product (signal molecule).

MALDI-TOF mass spectrometry has found significant use as a bioanalytical technique in recent years. Advantages of MALDI-TOF MS for bioanalysis[7] include the following: (1) speed, in that ionization, mass separation, and detection are accomplished in milliseconds; (2) accuracy, in that measurements are based on the intrinsic mass-to-charge ratio of the analyte and are not influenced by biopolymer secondary structure; and (3) throughput via automation and multiplexed acquisition. The application of MALDI-TOF MS for high-throughput genetic analysis was recently demonstrated by Bray et al.[8] They implemented a high-throughput, multiplexed MALDI-based approach for genotyping human DNA and compared this method to other genotyping techniques for 989 DNA samples. Three genotyping methods were evaluated: (1) a traditional gel-based technique (RFLP); (2) a fluorescence-based assay (TaqMan); and (3) a MALDI-based approach using the primer extension technique. A cost comparison between the various methods illustrated the substantial savings that were realized by even modest levels of multiplexing the MALDI method. The inherent variability in MALDI signal intensities, which originally limited the technique to qualitative analyses, can be overcome by referencing the analyte to a chemically similar internal standard. With MALDI analysis being a technique particularly well suited for the analysis of large biomolecules, the quantitative detection of peptides, proteins,[9–11] and nucleic acids[12–14] has been reported.

We demonstrate here the simultaneous detection of four different in vitro RNA transcripts: interleukin-1beta (IL-1β), tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[15] in a multiplex format. The linear dynamic range of the MALDI-TOF mass spectrometric analysis was determined using synthetic, short, biotinylated oligonucleotides, which represent the invader assay cleavage products or signal molecules. Quantitative analysis of cytokine IL-1β RNA was achieved by referencing to GAPDH RNA, which is used as an internal standard. These results demonstrate that gene expression analysis by mass spectrometry is a rapid, accurate, and simple technique with the potential for high-throughput, cost-efficient, and quantitative analysis.

MATERIALS AND METHODS

Invader RNA Assay Reactions. The invader assay reactions were prepared by mixing 3 μL of nuclease-free water, 1 μL of 10× reaction buffer (100 mM MOPS pH 7.5, 1 M KCl, 0.5% Tween 20, 0.5% NP-40), 1 μL of 50 mM MgSO$_4$, 1 μL of 5 μM invader oligonucleotide, 1 μL of 8 μM probe oligonucleotide, 2 μL of RNA target that was diluted in a 10 ng/μL solution of transfer RNA (tRNA), and 1 μL of 10 ng/μL cleavase enzyme. The cleavase enzyme, a recombinant polymerase-deficient *Tth* 5′-nuclease, was

cloned, expressed, and purified as described.[16,17] The reaction mix was incubated at 60 °C for 2 h, followed by reaction purification (described below). In vitro transcripts were generated from PCR amplicons using a T7-MEGAshortscript in vitro transcription kit (Ambion, Austin, TX), gel-purified on denaturing polyacrylamide gels, and quantitated by A_{260} measurement. Transcripts were diluted with 10 ng/μL tRNA (Sigma, St. Louis, MO); the tRNA solution also served as the no-target control. Additional information on PCR primer sequences can be found in the Supporting Information. All oligonucleotides were synthesized by the University of Wisconsin Biotechnology Center (Madison, WI); sequences are listed in Table 1. All probe oligonucleotides were purified by PAGE and diluted in 10 mM Tris (pH 7.4). All other oligonucleotides were column purified through Sep-Pak C$_{18}$ reversed-phase purification cartridges (Waters, Milford, MA) and diluted in 10 mM Tris (pH 7.4).

Reaction Purification and MS Sample Preparation. A previously described solid-phase purification method was employed in which the assay products are labeled with biotin and captured with streptavidin-coated magnetic beads.[18] Upon completion of the invader assay, the reaction solution is mixed with 100 μg of Dynabeads M-280 streptavidin-coated magnetic beads (Dynal, Oslo, Norway) contained in 120 μL of immobilization buffer (10 mM Tris-HCl/2 M NaCl, pH 7.0) and incubated at room temperature for 10 min. The beads are collected by application of a magnet to the side of the tube. The solution is drawn off and the beads are washed a total of six times, once in 10 mM ammonium citrate buffer, pH 7.0, with 0.1% SDS, twice in 200 mM ammonium citrate buffer, pH 7.0, and three times in Milli-Q water. The captured cleavage products are released by incubating the

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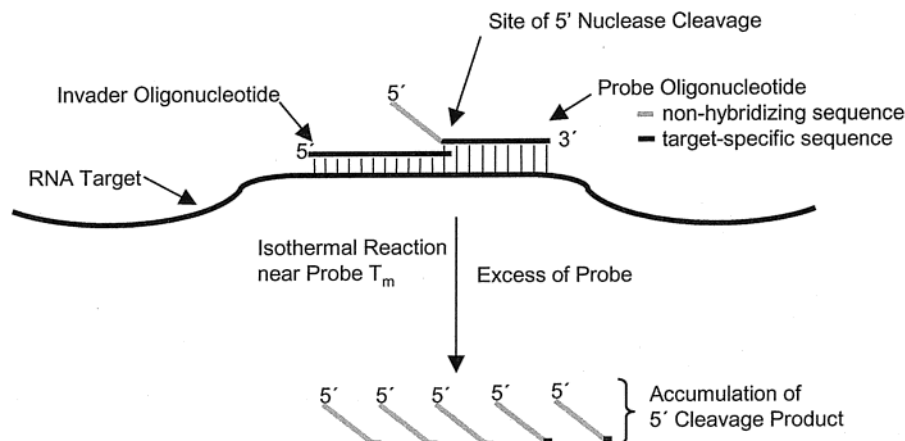
Figure 1. Schematic of the invader RNA assay. Cleaved 5′ flaps include one nucleotide of the target-specific region of the probe oligonucleotide.

beads in 40 μL of a 1:1 solution of concentrated (30%) ammonium hydroxide/methanol at 60 °C for 10 min. The solution is collected and lyophilized to dryness, resuspended in matrix solution, spotted on the MALDI probe, and allowed to crystallize.

MALDI Analysis. MALDI-TOF MS analysis was performed on a Voyager DE-STR mass spectrometer (ABI, Framingham, MA) using a nitrogen laser at 337 nm with an initial accelerating voltage of 20 kV and a delay time of 100 ns. Negative ions were analyzed in both linear and reflector modes. A summation of 64 laser shots was used for each spectrum, and the data were processed with the accompanying Voyager software package (Grams/386, Galactic, Salem, NH). The matrix solution employed was trihydroxyacetophenone (THAP; Aldrich, Milwaukee, WI) consisting of 100 mM 2,4,6-THAP, 50 mM 2,3,4-THAP in 1:1 water/acetonitrile with 55 mM ammonium citrate.[19]

Multiplex Analysis. Each of the four in vitro transcripts (1 fmol/reaction) was added to the same reaction tube. Reaction components, including the invader and probe oligonucleotides, were added for each of the four target RNAs and the reaction was run under standard conditions at a final reaction volume of 40 μL. Purification was performed as described above with four times the amount of magnetic beads and twice the solution volumes for a single purification reaction. The MS sample was resuspended in 2 μL of matrix solution and spotted in 0.5–1-μL aliquots onto the MALDI sample stage.

Quantitative Analysis of Mock Invader Assay Reaction Products. The analyte, a biotinylated deoxythymidine 5-mer (dT$_5$), was serially diluted over a concentration range of 0.05–50 pmol/μL and mixed with a reference standard, biotinylated deoxythymidine 7-mer (dT$_7$), at a fixed 3 pmol/per MS sample. The oligonucleotide mix was combined with matrix, spotted on the MALDI sample stage, and allowed to dry at room temperature. MALDI spectra were acquired from the first five locations on each MS sample spot that produced signal. The peak area was calculated using the integration function included with the Grams/386 (Galactic, Salem, NH) software package. Both peaks were integrated over a 75 amu mass range to include the area of adduct peaks. A baseline value was determined by integrating the baseline immediately preceding the peak of interest over an equivalent mass range and subtracting that value from the peak area.

The ratio of dT$_5$/dT$_7$ peak areas was calculated and averaged for the five spectra from each spot. These peak area ratios were plotted against the concentration of dT$_5$ analyte. Linear regression was performed to find the best-fit line and linear regression coefficient.

Quantitative Analysis of IL-1β RNA. The in vitro transcript of IL-1β was serially diluted over the range of 0.31–10 fmol/reaction and was added to invader assay reactions that also contained the GAPDH in vitro transcript at a fixed 1 fmol/reaction. The in vitro transcripts were diluted with a 10 ng/μL tRNA solution. The reactions, purifications, and MALDI sample preparation were performed as described above. MALDI analysis was performed and five to six spectra were taken from each sample spot. Peak area integrations were performed as described above for the mock cleavage product study.

RESULTS AND DISCUSSION

Mechanism of the Invader RNA Assay. The invader assay is a nucleic acid analysis method that involves the adjacent hybridization of two sequence-specific oligonucleotides, termed the "invader" and the "probe" oligonucleotides, to the nucleic acid target. The probe oligonucleotide is composed of two regions: (1) the target hybridization region and (2) the 5′ flap, which does not hybridize to the target. Additionally, the probe and invader oligonucleotides are designed so that when both are hybridized to the target, the nucleotide on the 3′ end of the invader oligonucleotide overlaps, or "invades", into the hybridized region of the downstream probe by at least one nucleotide as shown in Figure 1. This branched, overlapped structure is recognized as a substrate and cleaved enzymatically by a 5′-nuclease, which results in release of the 5′ flap (signal molecule). Use of thermostable 5′-nuclease variants permits the reaction to be run near the melting temperature (T_m) of the duplex formed between the probe and target, such that cleaved and noncleaved probe oligonucleotides will rapidly cycle on and off the RNA target sequence (probe replacement).[20,21] Operating the reaction in the presence of excess probe oligonucleotide results in the linear accumulation of cleavage product with respect to both time and target concentra-

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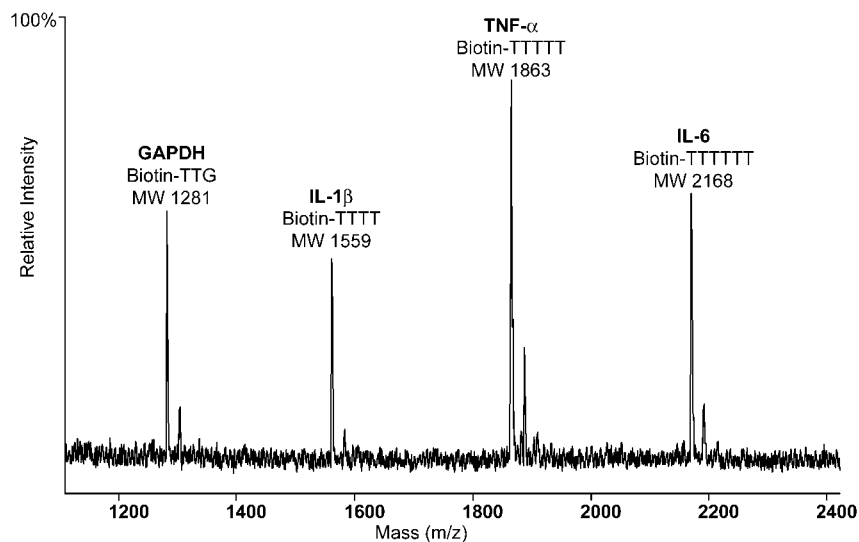
Figure 2. Multiplexed detection of four different RNA targets. The RNA species targeted, the cleavage product, and the molecular weight are listed above each of the four mass spectral peaks. All cleavage products include a 5′-biotin moiety, which is used for post-invader-reaction MS sample preparation (see Materials and Methods).

tion.[1,2] The invader oligonucleotide is designed to have a higher T_m than the reaction temperature and remains hybridized to the target RNA during the reaction. The work presented here utilized an early version of the *Thermus thermophilus (Tth)* enzyme.[17,20,22] Recently, a new *Tth* enzyme variant was engineered that provides enhanced activity on both RNA and DNA targets.[1]

Multiplex Signal Acquisition. An advantage of MALDI-TOF mass spectrometry is the ability to multiplex, i.e., to analyze multiple signals in a single mass spectrum. The invader assay is well suited to this type of multiplex analysis because there is great flexibility in designing the mass of the cleavage product. Since the 5′ flap of the probe oligonucleotide is independent of target sequence, it can be designed to yield a cleavage product of unique mass for its specific RNA target. Figure 2 shows the mass spectrum obtained from simultaneous signal amplification and subsequent MALDI-TOF analysis of four different in vitro transcripts, GAPDH, IL-1β, TNF-α, and IL-6. In the multiplex reactions, approximately equal amounts of the four RNAs were mixed, reaction components were added, and the signal amplification, purification, and MS sample preparation were all performed in the same reaction tube. Specific variations in peak height for different targets evident in Figure 2 are likely due to differences in the rate of signal production, which is dependent on the rate of probe turnover. For a given probe oligonucleotide sequence, the primary factors governing probe turnover are as follows: the reaction temperature, the accessibility of the RNA target for hybridization with the probe, and the probe's concentration. Methods for optimizing reaction temperature and RNA target accessibility for hybridization have been described previously.[1,2,31] The cleavage products (signal molecules) detected for each RNA target are shown in Table 1 (boldface, underlined region of the probe oligonucleotide), and their design is described below.

The design of probe oligonucleotides (specifically, the 5′ flap) for multiplexed mass spectrometry detection was determined by three main criteria. Each cleavage product must (1) be of a unique

and resolvable mass, (2) fall within the optimal mass range of 3−30 nucleotides, and (3) be composed mostly of poly(deoxythymidine) (poly-dT) residues. While the instrument is capable of resolving peaks that differ by one to a few mass units, it is advantageous to have a larger mass difference to allow for the presence of cation adduct peaks. In our design, with each cleavage product separated by the mass of a nucleotide ($\sim$300 Da), there is ample mass difference to accommodate the Na$^+$ and K$^+$ adduct peaks that are commonly found in the mass spectra of nucleic acids. The second criterion deals with mass range limitations. The lower mass range (<600 Da) is dominated by the signal from MALDI matrix ions, which makes analyte signal definition difficult and can suppress signal from less abundant analytes. The upper mass range (>10−15 kDa) is limited by rapidly diminishing signal intensity with increasing mass. In MALDI-TOF MS analysis, signal intensity decreases with increasing length of the nucleic acid analyte ions.[23,24] While MALDI-TOF mass spectrometry has been used to sequence DNA oligomers of up to 100 nucleotides with single-nucleotide resolution,[25] the strongest signal intensities, and thus the most practical mass range, is seen for ions shorter than 25−35 nucleotides. The third criterion dictates that the signal molecules be composed mostly of deoxythymidine nucleotides as such oligonucleotides are less prone to base loss and fragmentation in the MALDI process than oligonucleotides of a mixed-base composition and, therefore, produce stronger, better-resolved signals.[26,27] In addition, each 5′ flap includes a biotin group on the 5′ terminus to facilitate purification.[18]

Quantitative Range in MALDI. Quantitative analysis by this system depends on three principle factors: (1) the invader assay must be quantitative, (2) the instrument must quantitatively detect the products of the invader reaction, and (3) the quantitative

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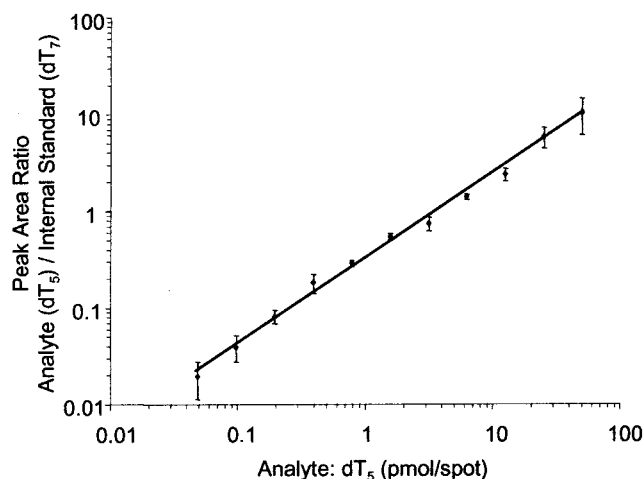
Figure 3. Quantitative MALDI-TOF MS analysis of mock invader assay reaction products. The peak area ratio of mock cleavage product to internal standard (dT_5/dT_7) is plotted against the amount of mock cleavage product (dT_5) to demonstrate the linear dynamic range of the mass spectrometer for invader assay signal molecules. The linear response covers a 3-log concentration range (0.05−50 pmol/sample); the internal standard dT_7 was kept constant at 3 pmol/sample. Each data point is the average of five spectra from different locations of the same sample spot with error bars representing ±1 SD.

response from factors 1 and 2 must be compatible. It was previously shown that the invader assay produces a quantitative and linear response over a 4-log concentration range of target using a fluorescence-based real-time detection system.[1] To investigate the second requirement, the quantitative response of the MALDI instrument was evaluated using mock reaction products. A dT_5 was employed as a mock invader assay reaction product (signal molecule). The concentration of dT_5 was varied and its mass spectral signal referenced to an internal standard, a dT_7. The ratio of mock cleavage product to internal standard (dT_5/dT_7) peak areas was plotted against the concentration of mock cleavage product (dT_5), which resulted in a linear calibration curve over a 3-log concentration range of dT_5, as shown in Figure 3. A biotinylated mock cleavage product was used for this work to most closely mimic the invader assay reaction products. Initially a nonbiotinylated internal standard was employed for the quantitative MALDI analysis, but inconsistent results were obtained over a limited dynamic range. Switching to the biotinylated dT_7 internal standard solved the problem, while maintaining sufficient mass resolution.

We investigated the use of both peak areas and peak heights for quantitative analysis. The peak area plot was linear over a 1000-fold concentration range (0.05−50 pmol/μL) with a linear regression coefficient (R^2) of 0.9982 (Figure 3). Conversely, the plot using peak heights displayed two distinct linear regimes, had greater standard deviations of data points, and extended over a smaller concentration range (0.1−25 pmol/μL) (data not shown). The source of this bimodal response is not well understood but may be caused in part by focusing only on the signal intensity of the primary ion peak and neglecting the contributions from adduct peaks, which are prevalent at higher concentrations of analyte. The peak area integrations were performed over a wide enough mass window to include the majority of adduct peaks into the calculation of total peak area. We also investigated the effect of
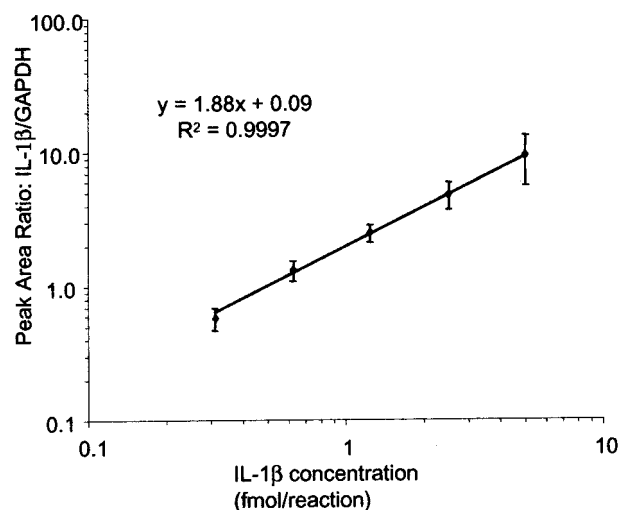

Figure 4. Quantitative MALDI-TOF MS detection of IL-1β in vitro transcript in an invader RNA assay. The IL-1β in vitro transcript was added at varying levels to the assay and referenced to a GAPDH in vitro transcript internal standard. A linear response was observed over a range of IL-1β RNA levels (0.31−5.0 fmol/reaction), with the GAPDH internal standard held constant (1.0 fmol/reaction). Error bars represent ± 1 SD.

acquiring the spectra in linear versus reflector TOF mode. The linearity of response was comparable, $R^2 = 0.9982$ for linear TOF mode and $R^2 = 0.9953$ for reflector TOF mode. The only appreciable difference was a 2-fold better signal detection limit in linear mode, which is consistent with the greater sensitivity often seen in linear mode.[28]

The final component involves the compatibility of the buffered, high-salt, solution-phase conditions required for the enzymatic signal amplification reaction with the high-vacuum, gas-phase conditions of mass spectrometry. Reaction purification is therefore required to bridge the two. We employed a cleanup method that involves capturing the biotinylated cleavage product (generated by cleavage of the probe) with streptavidin-coated magnetic beads (see Materials and Methods for a complete description). Substantial washing is followed by cleavage with the strong base ammonium hydroxide. Ammonium hydroxide is used instead of the manufacturer's recommended sodium hydroxide because ammonium counterions are much more "MALDI-friendly", causing fewer adduct peaks due to their higher degree of volatility. The presence of even small amounts of sodium ions (or other mono- or divalent salts) can completely suppress an otherwise strong DNA signal.

Quantitative Analysis of RNA Samples. The quantitative MALDI analysis technique was tested using the IL-1β in vitro transcript and the GAPDH transcript as an internal control. Both transcripts were diluted in tRNA solution (10 ng/μL) prior to performing the invader assays. The peak area ratios of IL-1β to GAPDH were calculated and averaged for five spectra taken from each sample spot. Figure 4 illustrates a plot of the IL-1β/GAPDH peak area ratio versus the concentration of IL-1β RNA. The GAPDH internal standard was kept at a constant 1 fmol/reaction. An excellent linear response ($R^2 = 0.9997$) was found for an IL-1β RNA transcript range of 0.31−5.0 fmol/reaction. At higher

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amounts of target IL-1β RNA (10 fmol or greater), the assay's linear production of signal molecules declines, presumably due to insufficient probe or enzyme levels. Assay optimizations of both probe and enzyme concentrations would be required to extend the linear dynamic range of this analysis technique.

Sensitivity of the Invader RNA Assay with MALDI Detection. About half of the total mRNA mass per cell represents mRNAs derived from a small number of abundantly expressed genes (10 000−100 000 copies of each RNA/cell). About 15% of the mRNA mass is derived from moderate expression level transcripts (1000−10 000 copies/cell).[29] As shown here, the invader RNA assay coupled with MALDI-TOF mass spectrometry gives reliable signal down to 0.3 fmol ($<2 \times 10^8$ copies) of in vitro transcript per reaction (Figure 4). With this sensitivity, MALDI analysis of invader assays can be readily used to detect moderate to high expression level mRNA transcripts ($\geq$1000 copies/cell) in RNA samples corresponding to ~200 000 cells/assay. The remaining mRNA mass of a cell is derived from more than 10 000 different mRNA species with low expression levels in the range of 1−1000 copies/cell.[29] The mass spectrometric methods described here are not presently suitable for analyzing low levels of RNA targets (<1000 copies/cell), although such detection limits might be attained by using more sensitive invader assay signal amplification methods[18,30] or by designing the invader assay to target accessible regions in the RNA.[31]

CONCLUSION

The use of an invader assay coupled to MALDI-TOF mass spectrometry permits rapid, quantitative analysis of RNA transcript levels. Analysis of RNA molecules by this method benefits from a simple and straightforward design process, requiring only the synthesis of two unlabeled oligonucleotides complementary to the known RNA target sequence. Multiplex analysis is easily accomplished by designing the reaction cleavage products (i.e., signal molecules) to be of unique and sufficiently separated masses. The linearly amplified signal molecules in the invader assay do not serve as templates for subsequent amplification as in the PCR process, thus avoiding problems with cross-contamination (false positive results). Further, detection of signal molecules by MALDI-TOF mass spectrometry precludes the need for fluorescent or radioactive labels, which have limited multiplex capabilities and can be costly.

This RNA analysis method is readily amenable to automation and high-throughput processing. The setup of invader assays uses straightforward pipetting steps that can be performed by liquid-handling robotic systems. The same methodology used to achieve 4-fold multiplexing here could be readily extended to 30 independent signals per MALDI spectrum. This degree of multiplexing, combined with standard MALDI automated acquisition of up to 400 samples in a few hours, would permit 12 000 different RNA targets to be analyzed in a single 8-h day (including setup, reaction time, and MALDI analysis). The primary remaining challenge is the reaction purification step, which requires manual manipulations that are not easily automated. Work is in progress to develop an automated purification system compatible with both the invader assay and MALDI-TOF mass spectrometry.

SUPPORTING INFORMATION AVAILABLE

Included is a table listing primer sequences and transcript lengths for the in vitro transcripts employed in this work. This material is available free of charge via the Internet at http://pubs.ac.org.

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